Gene therapy with human and mouse T cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen

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Running title: TCR gene therapy causes melanocyte destruction

Abbreviations used: ACT, adoptive cell transfer; TCR, T cell receptor; TIL, tumor-infiltrating lymphocytes; PBL, peripheral blood lymphocytes.
Abstract

Gene therapy of human cancer using genetically engineered autologous lymphocytes is dependent on the identification of highly reactive T cell receptors (TCR) with anti-tumor activity. We immunized HLA transgenic mice and also conducted high-throughput screening of human lymphocytes to generate TCR highly reactive to melanoma/melanocyte antigens. Genes encoding these TCR were engineered into retroviral vectors and used to transduce autologous peripheral lymphocytes administered to 36 patients with metastatic melanoma. Transduced patient lymphocytes were CD45RA⁻ and CD45RO⁺ following ex-vivo expansion. After transfer in vivo the persisting transduced cells displayed a CD45RA⁺ and CD45RO⁻ phenotype. Gene engineered cells persisted at high levels in the peripheral blood of all patients at one month after treatment, with responding patients demonstrating higher ex-vivo anti-tumor reactivity than non-responders. Objective cancer regressions were seen in 30% and 19% of patients who received the human or mouse TCR respectively. However, patients exhibited destruction of normal melanocytes in the skin, eye and ear, and sometimes required local steroid administration to successfully treat uveitis and hearing loss. Thus T cells expressing highly reactive TCR mediate cancer regression in humans and target rare cognate-antigen containing cells throughout the body, a finding that has important implications for the gene therapy of cancer. These studies were registered at www.clinicaltrials.gov under NCI-07-C-0174 and NCI-07-C-0175.
**Introduction**

Tumor associated antigens have been identified on a wide variety of human cancers. Many of these antigens are normal, non-mutated self proteins selectively expressed or overexpressed on cancers\(^1\). Antigens such as MART-1 and gp100 are expressed on melanomas and normal melanocytes in the skin, eye and ear\(^2,3\). Other cancer-associated antigens such as carcinoembryonic antigen (CEA), Her2/neu and Muc-1 are expressed at low levels on some normal tissues whereas antigens such as NY-ESO-1 and the MAGE family of proteins are expressed on fetal tissue and the adult testes but not on other normal adult tissues\(^4\). The presence of these normal proteins during fetal development leads to central tolerance based on negative selection in the thymus of lymphocyte clones bearing high-affinity self-reactive T cell receptors (TCR). Occasionally, lymphocytes bearing high-affinity TCR escape thymic deletion, and in these instances mechanisms of peripheral tolerance can suppress their activity\(^5\).

Cell transfer therapies have emerged as a tool to overcome the limitations imposed by both central and peripheral tolerance\(^6-9\). Transfer of anti-tumor T cells to lymphodepleted mice can mediate the rejection of large, vascularized tumors\(^10\) and the administration of naturally occurring anti-melanoma tumor infiltrating lymphocytes (TIL) can mediate objective cancer regressions in 51-72\(^\circ\) of lymphodepleted patients with metastatic melanoma\(^7,8,11\).

A major obstacle to the widespread application of cell transfer therapies is the difficulty in identifying human T cells with anti-tumor recognition. Only about half of melanomas
reproducibly give rise to anti-tumor TIL\textsuperscript{12}, and other cancer types only rarely contain identifiable tumor-reactive lymphocytes. An alternative to finding these natural tumor reactive cells for every patient is the transfer to normal lymphocytes of tumor reactive TCR genes recognizing shared tumor antigens.

In a prior study we identified a TCR (MSGV1AIB, here referred to as DMF4) that recognized the MART-1 melanoma-melanocyte antigen cloned from the TIL of a resected melanoma lesion\textsuperscript{13}. We treated 31 patients with autologous PBL transduced with genes encoding this receptor (17 were previously reported)\textsuperscript{14} and four patients (13\%) experienced an objective regression of metastatic melanoma. None of the 31 patients exhibited skin rash or normal melanocyte toxicity in the eye or ear. The DMF4 receptor had only moderate ability to recognize limiting amounts of antigen and we hypothesized that a more highly reactive TCR might be clinically more effective in recognizing malignant cells that expressed the target antigen.

We thus generated a high avidity TCR from a human T cell that recognized the MART-1:27-35 epitope (here referred to as DMF5)\textsuperscript{15}. The gp100:154-162 epitope from the gp100 melanoma-melanocyte antigen is the most highly expressed peptide from this protein, displayed on the cell surface. Attempts to generate a high avidity human TCR against this epitope were unsuccessful. We were able however, to generate a highly avid TCR against this epitope in HLA-A2 transgenic mice. These anti-MART-1 and anti-gp100 TCRs were used to treat patients with metastatic melanoma.
Materials and Methods

Patients

Thirty-six patients with metastatic melanoma were treated at the Surgery Branch, NCI, Bethesda MD, between July 2007 and March 2008, in protocols approved by the IRB and FDA, 20 with TCR recognizing the HLA-A*02-restricted melanoma antigen MART-1 (DMF5) \(^{15}\), and 16 with TCR recognizing the HLA-A*02-restricted melanoma antigen gp100(154) \(^{16}\). All patients gave informed consent for treatment in accordance with the Declaration of Helsinki. Patients were HLA-A*02\(^+\), 18 years of age or older, had measurable metastatic melanoma; ECOG status 0 or 1. All patients had progressed after prior treatment with IL-2/Aldesleukin and had tumors that expressed the appropriate antigen (MART-1/Melan-A or gp100/HMB45). Contraindications were: concurrent major medical illnesses; any form of primary or secondary immunodeficiency; severe hypersensitivity to any of the agents used in this study; contraindications for high-dose IL-2 administration; systemic steroid treatment within 30 days prior to treatment; untreated intracranial metastases >1.0 cm in diameter.

TCR recognizing shared melanoma antigens

The generation of the DMF5 TCR has been previously described \(^{15}\). The patient from whom the MART-1 reactive TCR was derived experienced vitiligo but no eye or ear toxicity. To generate a high avidity TCR against the gp100:154-162 epitope, HLA-A*0201 (A2.1) mice were immunized twice with the human gp100:154-162 peptide and the I-A\(^b\)-binding synthetic T helper peptide representing residues 128-140 of the hepatitis B virus core protein (HBV-Core) emulsified in incomplete Freund’s adjuvant. Seven days
after the second immunization, splenocytes were stimulated in vitro with equal numbers of irradiated, LPS activated, A2.1 transgenic mouse splenocytes pulsed with 0.01 μg/ml of human gp100:154-162 peptide and 10 μg/ml of human β2-microglobulin in media containing 5 IU IL-2. Eight days after a third stimulation, T cells were cloned by limiting dilution in presence of irradiated T2 cells pulsed with 0.01 μg/ml of human gp100:154-162 peptide and irradiated C57Bl/6 splenocytes. 5’ RACE TCR isolation and RNA electroporation into donor PBL was as previously described. The gp100(154) TCR conferring the highest anti-tumor avidity to donor PBL functioned independently of CD4 or CD8 co-receptor, and was selected for clinical use.

Retroviral gene therapy vectors

pMSGV1 is derived from pMSGV murine stem cell virus (MSCV) long terminal repeat containing an extended gag region and Kozak sequence. Vector pMSGV1 gp100(154)-AIB was produced by linking the TCRα via an IRES element followed by insertion of the TCRβ chain. Vector pMSGV DMF5 f2A was generated by introducing DMF5 TCRα cDNA followed by a furin T2A cleavage sequence and DMF5 TCRβ. Clinical grade cGMP-quality retroviral supernatants were produced by the National Gene Vector Laboratories at Indiana University. Patient PBL were stimulated with anti-CD3 mAb OKT-3 2 days prior to transduction using Retronectin (Takara Corp, Japan) coated plates, per manufacturer’s recommendations.
Preparation of PBL transduced with TCR

5-10 x10^8 patient PBL were obtained by leukapheresis and stimulated in vitro at 1 x10^6/mL with 50 ng/mL anti-CD3 mAb OKT-3, in complete AIMV media (Invitrogen Life Technologies) supplemented with 5% human serum (Surgery Branch, NCI), and 300 IU IL-2 (TCR media). Two days later, TCR-encoding retroviral supernatant was rapid-thawed, diluted 1:1 in TCR media, and added to plates that had been coated overnight with 10 ug/mL retronectin (Takara Bio Inc., Japan). Supernatant was spin-loaded onto plates by centrifuging 2 hours at 2000xG at 32 ºC. The stimulated PBL were washed and resuspended at 0.25-0.5 x10^6/mL in TCR media and 1-2 x10^6 PBL were added per well to the retrovirus-loaded plates. Plates were spun at 1000xG at 32 ºC, for 10 minutes, and incubated overnight at 37 ºC, 5% CO₂. The next day (day 3) PBL were transferred to newly prepared retroviral-coated 6-well plates as on day 2. The following day transduced PBL were washed, resuspended in fresh TCR media and transferred to flasks at 37 ºC, 5% CO₂. On days 9 to 12, cells were expanded or not an additional 9-14 days in 6000 IU IL-2 with 50 ng/mL anti-CD3 mAb OKT-3 and 100-fold excess 5 Gy irradiated allogeneic PBL feeder cells. Prior to treatment, TCR-transduced PBL from all patients were evaluated for expression of the appropriate TCR by tetramer staining and flow cytometric analysis, and cell function was evaluated by overnight co-culture with cognate antigen-bearing target cells (1x10^5:1x10^5) and ELISA measurement (Pierce Endogen) of IFNγ produced in the culture supernatant. Treatment cells were washed in saline prior to infusion into patients intravenously (i.v.).
Clinical protocol

The clinical trial registration numbers and approved registry names are as follows:
NCI-07-C-0175, Phase II Study of Metastatic Melanoma Using Lymphodepleting Conditioning Followed by Infusion of Anti-MART-1 F5 TCR-Gene Engineered Lymphocytes; NCI-07-C-0174, Phase II Study of Metastatic Melanoma Using Lymphodepleting Conditioning Followed by Infusion of Anti-gp100:154-162 TCR-Gene Engineered Lymphocytes.

Prior to receiving treatment with transduced PBL, patients were transiently lymphoablated using a non-myeloablative lymphodepleting regimen as previously described, by i.v. administration of cyclophosphamide 60 mg/Kg for two days followed by fludarabine 25 mg/m² for five days. One day following completion of their lymphodepleting regimen, patients received transduced lymphocytes infused i.v. followed by high-dose (720,000 U/kg) IL-2 (Aldesleukin, Chiron Corp) every 8h, to tolerance. Five DMF5 patients (#7-11), and four gp100(154) patients (#7-10) received TCR transduced cells on days 10 to 12 after stimulation. The remaining 15 DMF5 patients and 12 gp100(154) patients received a larger number of TCR transduced cells which were grown for an additional 9-14 days after a second OKT-3 stimulation.

Patients received baseline CT and/or MRI prior to treatment, and underwent pre- and post-treatment ocular and audiology examinations. Tumor size was evaluated monthly by CT, MRI, or documented with photography for cutaneous/subcutaneous lesions. Tumor measurements and patient responses were determined according to RECIST. Samples of patient PBL and serum were taken following transduced PBL infusion. Serum cytokine levels were measured by Pierce Endogen ELISA assay.
**Evaluation of cell activity and persistence**

Skin and tumor biopsies were obtained and immunohistochemically stained for the presence of CD4 and CD8 T cells. In one patient with uveitis, ocular fluid was evaluated for the presence of transduced anti-tumor T cells by flow cytometry. Cell activity was evaluated by co-culturing patient PBL with cognate antigen on T2 target cells, or HLA-matched and mismatched melanomas mel526, mel624 (HLA-A*02\(^+\)) or mel888 and mel938 (non-HLA-A*02). IL-2 and IFN\(\gamma\) were measured by ELISA (Pierce Endogen), ELISPOT (reagents from MAbtech Inc., OH; Millipore Corp., MA; Invitrogen, IL; BD Pharmingen, NJ; Kirkegaard & Perry, MD), or intracellular staining (mAbs from E-Biosciences, CA) and flow cytometry. Lysis was evaluated by target cell\(^{51}\) Chromium-release assay. Transferred cell persistence in blood was followed by tetramer staining with HLA-A2/MART-1:27-35 or HLA-A2/gp100:154-162 tetramer (Beckman Coulter Immunotech).

Statistical significance was evaluated using the paired T-test.
Results
DMF5 and gp100(154) are highly reactive TCR that confer melanoma tumor reactivity to donor PBL

We have used two highly reactive TCR capable of recognizing the MART-1 or gp100 melanocyte antigens overexpressed on melanomas. To overcome the problem of central deletional tolerance of lymphocytes expressing high affinity anti-tumor TCR we raised a highly reactive TCR called gp100(154) against the human melanocyte gp100:154-162 epitope by immunizing HLA-A*0201 transgenic mice with this peptide that differs from the mouse sequence by a single amino acid. In addition, extensive screening of over 600 clones in TIL from multiple patients revealed a lymphocyte clone called DMF5 with far greater reactivity than the previously identified DMF4 against the MART-1:27-35 peptide epitope. The TCR genes from the mouse cells and from the human DMF5 clone were isolated and equivalent amounts of RNA were generated in vitro and transferred into both Jurkat and donor PBL. After transferring the same amount of exogenous TCR (evaluated by CD3 surface expression in Jurkat cells), the higher avidity DMF5 and gp100(154) TCR conferred higher reactivity to donor PBL than the previously identified DMF4 in recognizing tumor antigen. (Fig. 1A). The genes encoding the alpha and beta chains from these two receptors, gp100(154) and DMF5, were each cloned into bicistronic gamma-retroviral vectors utilizing an internal ribosome entry site (IRES) or a furin 2-A picornavirus-like cleavage sequence (f2A) respectively, to drive expression of the second gene (Fig. 1B). These retroviral vectors were used to transduce normal human peripheral lymphocytes (Fig. 2). It has been demonstrated that DMF4 binds tetramer weakly, under-representing the amount of surface TCR, a result also
observed here (Fig. 2A). Using similarly prepared retroviral supernatants in the same
donor cell transduction procedure, DMF4 on the surface of transduced CD8\(^+\) PBL only
bound 2\(^\%\) of MART-1 tetramer and transduced CD4\(^+\) cells bound no tetramer. In contrast,
30-60\(^\%\) of both CD4\(^+\) and CD8\(^+\) lymphocytes transduced with the improved gene
constructs encoding the highly reactive DMF5 or gp100(154) TCR bound tetramer
efficiently (Fig. 2A). These new TCR constructs conferred high expression of TCR which
was co-receptor independent on donor PBL. A comparison of the functional reactivity of
donor lymphocytes transduced with the respective gamma-retroviruses encoding these
DMF4, DMF5 or gp100(154) TCR are shown in Figure 2B,C. Cells expressing either the
highly reactive DMF5 or gp100(154) TCR recognized 100-fold lower peptide
concentrations, produced more IFN\(\gamma\) and lysed melanoma targets more effectively than
those expressing DMF4.

**Administration of TCR transduced autologous PBL to patients with metastatic
melanoma**

To investigate the in vivo activity of autologous cells transduced with these highly
reactive TCR, 36 patients with heavily pretreated, progressive metastatic melanoma
received transduced cells (20 patients with DMF5 and 16 with gp100(154)), following a
lymphodepleting preparative regimen to deplete endogenous circulating lymphocytes \(^8\).
Patient demographics and treatment details are presented in Tables 1 and 2. Intravenous
IL-2 was administered to patients starting 8 hours after adoptive cell transfer, and
continued every 8 hours for up to three days. All of the patients were refractory to prior
treatment with IL-2 and 42% and 33% had progressed through prior chemotherapy and radiotherapy respectively.

Based on tetramer staining, the mean transduction efficiencies for cells administered to these 36 patients were 71% and 82% for DMF5 and gp100(154) TCR respectively (Tables 1,2). All treatment cells showed high levels of specific reactivity against cognate antigen-bearing tumor targets as assessed by intracellular cytokine staining and ELISPOT analysis (both interferon-gamma (IFNγ) and IL-2) (Tables 1,2) and for IFNγ release (Table 3) and target cell lysis (data not shown).

Adoptively transferred highly reactive anti-tumor cells persisted at high levels in patients

In prior trials of patients receiving unmodified TIL, in vivo cell persistence of the transferred cells highly correlated with anti-tumor response 18. We thus evaluated the persistence of the human DMF5 and the murine gp100(154) TCR transduced cells using tetramer binding and ELISPOT assays. All patients had measurable levels (≥1%) of tetramer-positive T cells in their circulation at 1 month post-treatment (Fig. 3A,B). There was no difference in the persistence of cells bearing the human DMF5 TCR (22 +/- 6%) or the murine gp100(154) TCR (22 +/- 5%; P = 0.4) (Fig. 3B). To measure functional recognition of tumor cells, post-treatment PBL were co-cultured with MHC matched or mismatched melanomas and cell reactivity was assessed using ELISPOT and intracellular FACS assays (Fig. 3). Interferon-gamma ELISPOT assay showed persistence (>20 specific spots/100,000 cells) of tumor-reactive transduced cells in 11/20 DMF5 and 7/16
gp100(154) patients (Fig. 3B). Similarly, 11 and 7 of the DMF5 and gp100(154) patients, respectively exhibited persistence of active cells via IL-2 ELISPOT assays of PBL at one month post treatment (Fig. 3A). At this same one month time point specific intracellular IFNγ staining was seen in 12 of the 20 DMF5 patients (Fig. 3B). Thus, as determined in multiple assays, both murine- and human-derived TCR gene-modified cells persisted in the circulation at one month in the majority of patients. Comparing between responding and non-responding patients, responding patients all had highly persistent tumor-reactive cells at ≥ 10% tetramer positive T cells in the blood, one month after treatment. However, some non-responding patients also had high levels of active persistent cells, suggesting that persistence may be necessary, but not sufficient to cause tumor regression in patients.

**Adoptively transferred T cell phenotype reverts from CD45RA⁻, CD45RO⁺ to CD45RA⁺, CD45RO⁻ in vivo**

We evaluated the phenotype of the DMF5 MART-1 reactive TCR treated patients’ cells pre, during, and post treatment by flow cytometry for the costimulatory molecules CD27 and CD28, and the cell differentiation markers CD45RA and CD45RO. Prior to TCR gene modification, on average (+/- SEM), patients’ peripheral T lymphocytes consisted of 53 +/- 5% CD27⁺, 62 +/- 4% CD28⁺, 42 +/- 3% CD45RA⁺, 53 +/- 3% CD45RO⁺ cells (Fig. 4). After expansion ex vivo, the tetramer positive infusion cells displayed less CD27 (36 +/- 6%), similar CD28 (59 +/- 6%), almost complete loss of CD45RA (3.5 +/- 1.2%), and a gain of CD45RO to 94 +/-2% (Fig. 4). Evaluating tetramer positive cells in the blood one month after treatment showed levels of CD27 similar to infusion (30 +/- 5%), reduced levels of CD28 (36 +/- 5%), and intriguingly, levels of CD45RA increased and CD45RO
decreased, reverting back to levels similar to those seen in the pre-treatment PBL samples (27 +/- 4% CD45RA+, 66 +/- 4% CD45RO+) (Fig. 4). This suggests that either the few tetramer positive CD45RA+ cells present at infusion persisted and expanded, or, in agreement with a previous published clinical report of a patient receiving a gene-marked allogeneic cell transfer to treat leukemia 19, that cells that had been CD45RA- and CD45RO+ reverted to CD45RA+ and CD45RO- in vivo. There were no substantial differences in the cell phenotype between responding and non-responding patients.

**Clinical course of patients receiving TCR transduced cells: reactivity against normal tissues**

The clinical course of patients receiving the highly tumor-reactive DMF5 and gp100(154) TCR transduced cells was quite different from that of our prior study with the DMF4 receptor 14. In the current trial, increased levels of IFNγ were detected in the serum of patients, peaking around day 3-5 post treatment (Fig. 5A). The mean peak IFNγ serum values for patients receiving the improved DMF5 and gp100(154) gene constructs were 128 pg/mL and 210 pg/mL, respectively, compared with 14 pg/mL for the previous DMF4 patients (P = 0.03) (Fig. 5B). As IFNγ is an effector cytokine produced by activated T lymphocytes, and patients remained depleted of their endogenous lymphocytes during the first week following the preparative lymphodepletion, it is presumed that the transferred cells were the source of this cytokine production.

At the time of this cytokine surge, 29 of the 36 patients in the current trial exhibited a widespread erythematous skin rash that on biopsy showed prominent epidermal
spongiosis and necrotic epidermal keratinocytes, with a dense infiltrate of CD3+ T lymphocytes (predominantly CD8+) (Fig. 6A). Surprisingly, there was destruction of epidermal melanocytes in all biopsies performed on 14 DMF5 patients and 13 gp100(154) patients, starting as early as day 5 post-treatment (Fig. 6A), although rare remaining melanocytes were sometimes observed around hair follicles. This loss of melanocytes coincided with the dermal and epidermal infiltration of lymphocytes as well as evidence of vitiligo in patients upon later follow-up. This rash gradually subsided over several days without treatment in all patients. To elucidate the factors inciting the rash and the lymphocyte infiltration into skin, we studied two patients who presented with pre-existing patchy vitiligo. These two patients developed a rash only in pigmented and not in vitiliginous skin (Fig. 6B). Biopsies following treatment revealed diffuse infiltrates of CD8+ cells in areas of pigmented skin with little or no lymphocytic infiltrate in vitiliginous skin (Fig. 6B). These findings, along with the evidence of melanocyte destruction at a time when endogenous lymphocytes have been depleted, strongly suggested that the epidermal melanocytes were the targets of immune attack by the gene engineered cells.

Because melanocytic cells expressing MART-1 and gp100 exist in the eye, all patients underwent ophthalmologic examination prior to and after treatment. None of the patients in the prior DMF4 TCR trial developed uveitis (cellular infiltrate into the eye). In contrast, 11/20 patients (55%) receiving the more reactive DMF5 TCR cells and 4/16 (25%) receiving the gp100(154) TCR cells developed an anterior uveitis, two of which were asymptomatic and thirteen required the transient administration of steroid eyedrops.
Two patients developed synechiae of the iris that were asymptomatic (Fig. 6C). In all patients, ocular findings reverted to normal. Sampling of the eye anterior chamber fluid in one patient with uveitis who received the DMF5 transduced cells revealed a predominance of MART-1 tetramer-positive CD3+ T-cells by FACS analysis (Fig. 6D), indicating trafficking of gene-modified cells into the eye.

Melanocytic cells also exist in the striae vascularis of the inner ear. Though none of the patients in the prior DMF4 trial exhibited hearing loss, audiometric exams revealed evidence of hearing loss in 10/20 of the DMF5 patients starting about one week after treatment (Table 1 and Fig. 6E), 7 of whom received intratympanic steroid injections. All patients improved, and the grade 3 hearing losses resolved or improved to a grade 1 or 2 with the exception of two patients who died of progressive metastatic melanoma before they were retested. Five of the sixteen gp100(154) patients developed mild hearing loss (Table 2), only one of whom required treatment and continues to have mild residual changes. Nine of the 36 patients experienced inner-ear related dizziness that responded to treatment. This constellation of symptoms is similar to that observed in patients with Vogt-Koyanagi-Harada disease, thought to result from autoimmune destruction of melanocytes located in the eye, the inner ear, skin, and hair. There were no off-target autoimmune effects observed in any patients.

DMF4 and DMF5 TCR recognize the same MART-1:27-35 epitope and were derived from the same melanoma patient. The improved ability of transduced cells expressing the DMF5 or gp100(154) TCR to recognize target antigens in the skin, eye and ear and to
persist in patients at levels up to 80% of circulating peripheral T-lymphocytes for several months appears due to the higher reactivity conferred by these improved TCR constructs compared with the prior DMF4 TCR.

**Anti-tumor impact of the TCR transduced cells**

One patient who received DMF5 transduced cells underwent a series of sequential biopsies of subcutaneous metastatic lesions before and after treatment. Beginning at about five days after treatment the biopsies revealed an increasing infiltration of CD8+ T lymphocytes throughout the tumor that coincided with the progressive necrosis and partial regression of his tumors (Fig. 7A,B). Growth of lymphocytes from resected lesions at days 9, 15 and 26 revealed > 97% MART-1 tetramer positive lymphocytes (Fig. 7C). Although initially the majority of this patient’s disease regressed, numerous new lesions developed after 2 months and as such, this patient was a non-responder.

Six of the 20 patients (30%) treated with DMF5 TCR and 3 of 16 (19%) treated with gp100(154) TCR experienced an objective anti-tumor response, as defined by RECIST criteria (Tables 1,2). Tumors regressed in multiple organs including the brain, lung, liver, lymph nodes and subcutaneous sites (Fig. 7D-F). There were no treatment related deaths and all patients recovered from treatment.

In analyzing the 36 patients there was no correlation between the number of cells administered and the likelihood of a clinical response, with some responding patients receiving a log fewer cells than others (Table 1). There was also no correlation between
clinical response and the duration the cells were grown ex-vivo, or whether they received one or two OKT-3 stimulations. There was a correlation with clinical response and the persistence of administered cells at one month as assessed by ELISPOT analysis of both specific IFNγ and IL-2 release (both P = 0.02) (Fig. 3B). Although all responding patients had ≥ 10% tetramer positive cells persisting in blood at one month, this parameter did not correlate with response (P = 0.4) (Fig. 3B).
Discussion

The ability of genetically modified normal peripheral lymphocytes bearing highly avid TCR to destroy isolated antigen expressing cells, such as individual melanocytes in the ear, the basal layer of the epidermis, and in immunoprivileged sites such as the eye, as well as melanoma metastases in visceral organs and the brain, has important implications for the development of cancer immunotherapy. This approach bypasses the need to identify and isolate anti-tumor effector cells from each patient. These gene modified cells targeted only specific shared differentiation antigens and thus these findings validate the use of differentiation antigens as targets of cancer immunotherapy. It is of interest that cells expressing the murine gp100(154) TCR persisted at the same levels as those expressing the human DMF5 TCR, and also mediated cancer regression, suggesting that an immune reaction against the mouse TCR sequences may not be a limiting factor in TCR gene therapy in humans. The ability to utilize HLA transgenic mice to raise TCR against human cancer antigens represents a valuable approach to bypass the tolerance of patients to self antigens and thus enable the generation of highly reactive antitumor TCR

It was surprising that the percentage of PBL staining positive for tetramer was always higher than the proportion of cells displaying anti-tumor activity by ELISPOT or intracellular cytokine staining (Tables 1, 2 and Fig. 3). Part of this difference may be accounted for by the inclusion of all mononuclear cells in patient PBL functional assays, compared with the ability to gate on the CD3 positive T lymphocyte population by flow cytometry. However, this difference is small compared with the logs of difference between tetramer positive cells by FACS (approaching 100% in treatment samples, and up
to 80% in post-treatment PBL), and in ELISPOT results (generally less than 1% of cells).

It is also possible that while tetramer staining identifies all lymphocytes expressing the tumor-reactive TCR, not all TCR+ cells have equal anti-tumor function. As T lymphocytes can have an anti-tumor effector phenotype, they can also be anergic, or have a suppressive phenotype, such as regulatory T cells. In the case of these latter phenotypes, anti-tumor functional response could be diminished or abrogated. Another potential explanation is the different sensitivities of direct tetramer staining for flow cytometry versus ELISPOT or intracellular cytokine staining.

The recognition of normal quiescent cells expressing the targeted cancer antigen raises obvious questions concerning the toxicity of this gene therapy approach. These results paralleled our findings in a murine melanoma model which showed an association of ocular toxicity and anti-tumor activity using gp100 as the target antigen 22, although clinically the local application of steroids attenuated normal tissue toxicity for patients treated with these melanocyte specific TCR. These findings emphasize the importance of the targeted tumor antigen. It may be highly effective to target differentiation antigens on cancers that arise in non-essential organs such as the prostate, ovary, breast, and thyroid. Other cancers have higher expression of differentiation antigens such as CEA and Muc-1 than are expressed in normal tissues and this may create a therapeutic window to be exploited. The cancer-testes class of antigens not expressed on normal adult tissues may be ideal targets for this approach. In any case, the risks versus benefits of infusing highly reactive self-antigen specific TCRs must be carefully considered.
Although the low numbers in these clinical trials preclude statistical conclusions, the 30% and 19% objective cancer response rates seen with the DMF5 and gp100(154) TCR were lower than the 51-72% response rates seen with the use of autologous TIL screened and grown individually from each patient’s resected tumor. Anterior uveitis and ototoxicity were seen in six of 93 (6.5%) and only one of 93 (1.1%) melanoma patients treated with TIL, respectively, in contrast with the 41.7% incidence of both toxicities seen in the current trial (P < 0.0001), sometimes in the absence of tumor regression, implying that the shared melanoma/melanocyte antigens may not be the predominant targets of therapeutic TIL. Although many TIL do recognize the MART-1 and gp100 antigens, T cell clones have been isolated from many clinically effective TIL that recognize mutated or unidentified antigens as well and these may also be responsible for the anti-tumor effects of TIL transfer. Alternatively, differences in homing molecules or other phenotypic differences between blood and tumor-derived lymphocytes may account for this finding.

Several patients exhibited melanocyte toxicity without experiencing tumor rejection. Treating patients with low (DMF4) and high (DMF5) avidity TCR recognizing the same MART-1 antigen resulted in 13% (4/34) and 30% (6/20) objective tumor responses, respectively, suggesting that increased TCR avidity may improve tumor rejection. However, due to the small sample numbers, it is possible that the avidity of TCR in vivo are not a major determinant of tumor rejection. It is likely that factors such as lymphocyte homing to normal versus tumor tissue, heterogeneity of antigen expression by tumor, T cell exhaustion, suppression in the tumor microenvironment or the need for polyvalent T
cell populations may all need to be considered to improve tumor rejection in patients. Murine tumor models predict that intensifying the lymphodepletion prior to cell transfer and administering a vaccine to stimulate the transferred cells in vivo can improve the anti-tumor efficacy of this approach \(^{10,23}\) and we are currently implementing a clinical trial combining the DMF5 and gp100(154) TCR, with increased patient immunodepletion, followed by administration of a peptide vaccine.

ACT therapy using gene-modified lymphocytes has also been shown to be effective in treating malignancies other than melanoma. In pre-clinical models, lymphocytes have been genetically modified to express costimulatory molecules designed to increase anti-tumor activity and T cell survival such as CD80 or 4-1BB ligand, or to abrogate the effects of inhibitory signals\(^{24}\). Genetically modified human lymphocytes expressing chimeric antigen receptors (CAR) \(^{25}\), combining the antigen-recognition of an antibody with T cell signaling motifs, were able to eradicate human B cell lymphoma by targeting CD19 \(^{26}\), and human prostate tumors by targeting ErbB2/Her2 \(^{27}\) in xenogeneic SCID mouse models. Using Epstein-Barr virus (EBV)-specific human T cells transduced with CAR recognizing the Hodgkin lymphoma-specific CD30 antigen has also been shown to be effective in a xenogeneic mouse model \(^{28}\). Clinically, EBV-specific cells expressing CAR that recognize the neuroblastoma tumor-specific diasialoganglioside GD2 have also recently been used to successfully treat patients with neuroblastoma \(^{29}\).

In an attempt to expand this TCR therapy to treat cancers other than just melanoma, we have now cloned the genes encoding high avidity TCR that recognize a variety of
antigenic epitopes such as NY-ESO-1:157-165 \(^{30}\), p53:264-272 \(^{21,31}\) and CEA:691-699 \(^{32}\) expressed on many common epithelial cancers \(^9\). To target tumors in a non-MHC restricted fashion we have also engineered lymphocytes to express chimeric receptors incorporating the antigen combining site of anti-Her2 and anti-CD19 mAb \(^{26}\), which may target tumor cells over-expressing these determinants on the cell surface.

Possible toxicities resulting from the expression of tumor-associated antigens on normal tissues need to be considered in the application of this approach. Our results however support the hypothesis that the administration, to cancer patients, of T cells transduced with highly reactive human or murine TCRs can mediate in vivo destruction of tissues that express the target antigen and suggest that cell transfer therapies can be a valuable adjunct to the treatment of patients with metastatic cancer.
Acknowledgements

We would like to thank Dr. Yangbing Zhao, Dr. Cyrille Cohen, and Zhili Zheng for their work in constructing the clinical retroviral vectors. Thanks to Dr. Ken Cornetta and the Indiana University Vector Production Facility for providing the cGMP retroviral supernatants, and Takara Bio Inc., Otsu Japan for providing retronectin. Thank you also to Dr. Franz Smith, who conducted assays on patient infusion samples. We also thank the nursing staff on the 3NW ward and the Intensive Care Unit in the Clinical Center, NIH who provided these patients with outstanding care.

Author Contributions

L.A.J. prepared the DMF5 patient treatment cells, conducted the laboratory experiments and analysis and wrote the paper; R.A.M. prepared the gp100(154) patient treatment cells, and conducted experiments; M.E.D., A.P.C. and J.R.W. prepared the patient treatment cells; S.L.S. did experiments; L.C. and N.P.R. identified and isolated the gp100(154) TCR; J.C.Y., M.S.H., U.S.K, R.E.R., R.M.S., J.L.D., A.M., R.T.R. and S.A.R. were physicians and D.A.N. was a Research Nurse on the clinical protocols; R.A.M. constructed the retroviral TCR vectors; C.R.L. conducted immunohistological staining of patient biopsies; R.J.B. conducted patient ocular evaluations; H.J.K, C.C.B., S.F.R., and C.V.W. conducted patient auditory evaluations. C.M.L. provided support in clinical protocol preparation and reporting; S.A.R. supervised the clinical trials and contributed to data analysis and writing of the paper. R.J.B. provided information regarding patient ophthalmological evaluations; H.J.K, C.C.B., S.F.R. and C.V.W.
provided information regarding patient otolaryngological evaluations. L.A.J. and S.A.R.
analyzed the data, to which all authors have access.

**Conflict-of-interest Disclosure**

The authors declare no competing financial interests.
References


### Table 1. DMF5 patient cell treatment characteristics

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<th>% TET</th>
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<th>ELISPOT IFNγ</th>
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* R = Radiation therapy, S = Surgery, C = Chemotherapy, I = Immunotherapy (high-dose IL-2).

† Abbreviations: ln, lymph node; lu, liver; in, lung; br, brain; sp, spleen; sc, subcutaneous; ln, lung; bo, bone; hi, kidney; p, intraperitoneal; ad, adrenal; lar, larynx; im, intramuscular; pan, pancreas.

‡ Treatment cell intracellular IFNγ detected by flow cytometry following 18 hr co-culture with mel20- tumor cells (gated on CD3+).

§ Number of positive IL-2 ELISPOTs per 100,000 injection PBM following 4 hr co-culture with mel20- tumor cells.

∥ Number of positive IFNγ ELISPOTs per 10,000 injection PBM following 4 hr co-culture with mel20- tumor cells.

+ Skin toxicity: Grade 1 = erythema, Grade 2 = desquamation <50%, Grade 3 = desquamation >50%. Eye toxicity (treatment): Grade 1 = asymptomatic, Grade 2 = anterior, sterile eye drops, Grade 3 = pan uveitis, surgery. Ear (treatment): Grade 1 = hearing loss 15-25 dB at 2 frequencies, Grade 2 = >25-40 dB at 2 frequencies, Grade 3 = >40 dB at 2 frequencies (per CTCAE v3.0).

*PR = partial responder, NR = non-responder, by RECIST criteria; (1) = Response duration in months post treatment, + = ongoing.

** 1st treatment.

nd = not done
### Table 2. gp100(154) patient cell treatment characteristics

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<th>% Tet ELISPOT IL2(^‡)</th>
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<th>Doses IL-2</th>
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\(^*\) R = Radiation therapy, S = Surgery, C = Chemotherapy, I = Immunotherapy (high-dose IL-2).

\(^†\) Abbreviations: in, lymph node; il, ileum; lu, lung; br, brain; sp, spleen; sc, subcutaneous; bo, bone; ki, kidney; ip, intraperitoneal; ad, adrenal; lar, larynx; im, intramuscular; pan, pancreas; st, stomach; int, intestine; gb, gallbladder.

\(^‡\) Number of positive IL-2 ELISPOTs per 100,000 infusion PBMC following 4th co-culture with melanA1524 tumor cells.

\(^§\) Number of positive IFN\(\gamma\) ELISPOTs per 10,000 infusion PBMC following 4th co-culture with melanA1524 tumor cells.

\(^‖\) Skin toxicity Grade 1 = erythema; Grade 2 = desquamation <50%; Grade 3 = desquamation 50% - 80%; Grade 4 = desquamation >80%. Eye toxicity Grade 1 = asymptomatic; Grade 2 = anterior, sterile eye drops; Grade 3 = pain, ophthalmic surgery; Ear (hearing) Grade 1 = hearing loss 15-25 dB at 2 frequencies; Grade 2 = >25 dB at 2 frequencies; Grade 3 = >25 dB at 3 frequencies (per CTCAE v3.0).

\(^‖\) CR = complete responder; PR = partial responder, NR = non-responder, by RECIST criteria; ( ) = Response duration in months post treatment; * = ongoing.
Table 3. Interferon-gamma production by TCR transduced infusion cells

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**pg/mL**: interferon-gamma measured by ELISA. 1×10^5:1×10^7 effecter target cell 1:hour co-culture

Bold = responding patients

n/a = not available

*Peptides = MART-1 27-35 and gp100 154-162 (\(+ve\) = cognate peptide, \(-ve\) = noncognate peptide)
Figure legends

Figure 1. Tumor-reactive DMF5 or gp100(154) alpha and beta TCR chain RNA electroporated into PBL confer high reactivity to melanoma tumor antigens. (A) 10-day anti-CD3 stimulated donor PBL were electroporated with in vitro transcribed RNA encoding paired DMF4, DMF5 or gp100(154) TCR alpha and beta chains, or GFP control. Cells were co-cultured for 18h with T2 cells pulsed with peptide, HLA-A*02+ melanomas mel624+ or mel526+, or HLA-A*02– melanomas mel888– or mel938–. IFNγ in the supernatant was detected by ELISA. (B) Structure of the MSGV-based gamma-retroviral vectors, DMF4 and gp100(154) incorporating an internal ribosomal entry site (IRES) and DMF5 with a furin 2A ribosomal skip sequence, allowing for dual gene expression.

Figure 2. DMF5 and gp100(154) TCR retroviral constructs conferred greater anti-tumor reactivity to donor PBL than the original DMF4 receptor. (A) Donor PBL were stimulated with anti-CD3 mAb OKT-3 and separated into CD4 and CD8 populations prior to retroviral transduction with DMF4, DMF5, or gp100(154) TCR constructs. TCR expression was analyzed 7 days later by tetramer staining and flow cytometry. (B) Donor PBL transduced with retroviral TCR constructs were co-cultured with T2 cells pulsed with MART-1:27-35 or gp100:154-162 peptide, and IFNγ secretion was measured by ELISA. (C) Transduced PBL were co-cultured with mel624+ melanomas and tumor target lysis was evaluated by ⁵¹Cr-release assay. Cells did not lyse HLA-mismatched tumors (data not shown).
Figure 3. TCR transduced cells from responding patients persisted and showed anti-tumor activity ex vivo. Blood samples were taken from patients’ cells pre and post TCR-transduced cell infusion. PBMC were evaluated for persistence of infused cells in peripheral blood post treatment by specific tetramer staining, and were also used directly in co-culture assays with mel624 tumors (MART1+, gp100+, HLA-A2+). Anti-tumor activity was evaluated by IFNγ and IL-2 ELISPOT, and also by intracellular staining for IFNγ production. (A) Persistence and activity of DMF5 patient treatment cells prior to treatment (pre), 2 weeks post, and 1 month post infusion. Responding (PR) and non-responding (NR) patients are denoted by solid and broken lines, respectively. (B) Comparison of PBMC from patients treated with either DMF5 or gp100(154) TCR transduced cells. Tetramer staining and ELISPOT analysis of IFNγ production from non-responding (NR) and responding (PR) patients at 1 month post treatment. All samples had <10 ELISPOTs (per 100,000 PBMC) and <1% IFNγ positive cells respectively against the HLA-A*02 negative mel888 tumor (data not shown). Patients with objective clinical responses (PR) had higher numbers of anti-tumor IFNγ (P = 0.02) and IL-2 (P = 0.02) secreting cells than non-responders (NR).

Figure 4. Phenotype of patient treatment cells pre and post infusion. Patient PBMC were stained by tetramers for TCR recognizing gp100:154-162, or MART1:27-35, and by mAB for CD3 and the activation and differentiation markers CD27, CD28, CD45RA, and CD45RO. Cell phenotype was evaluated by flow cytometry, gated on CD3+ cells pre treatment, and on CD3+tetramer+ cells for infusion (Rx) and 1 month post treatment (1
Figure 5. Patients treated with the highly reactive DMF5 or gp100(154), but not DMF4 TCR had increased serum IFN\(\gamma\) levels following treatment. Patient IFN\(\gamma\) serum levels were measured daily pre, during and post cell infusion by ELISA. (A) Serum IFN\(\gamma\) levels increased from baseline to a peak at 3-6 days post cell infusion (3 representative DMF5 patients are shown). (B) Peak IFN\(\gamma\) levels in serum post treatment for patients treated with DMF5, gp100(154) and DMF4 TCR transduced cells. Only patients treated with the highly reactive DMF5 or gp100(154) TCR demonstrated increased IFN\(\gamma\) in serum (\(P = 0.001\) and \(P = 0.0001\) respectively, compared with \(P = 0.09\) for DMF4 TCR).

Figure 6. Tissue trafficking and target cell destruction in patients following infusion of TCR transduced cells. (A) Biopsy of inflamed skin from DMF5 patient 2, day 6 following treatment, demonstrating spongiotic vesicles and necrotic/dyskeratotic keratinocytes (arrows), stained with H&E, and immunohistochemically stained for CD8\(^+\) cells or the Melan-A antibody recognizing MART-1 antigen. Bottom left shows positive control staining for the anti-MART-1 Melan-A antibody showing normal epidermal melanocytes and a subcutaneous melanoma deposit (20x objective). (B) 1 week post-treatment biopsies from gp100(154) patient with pre-existent patchy vitiligo, demonstrating CD8\(^+\) cellular infiltrate into the epidermis of pigmented, but not vitiliginous skin (20x objective). (C) Slit lamp ophthalmologic evaluation of DMF5
patient 5 eye, two weeks post treatment, demonstrating cloudy cellular anterior chamber infiltrate (above) and (below) with induced iris dilation six months post TCR treatment and steroid eye drop administration, demonstrating posterior synechiae (asymptomatic). (D) Cells present in ocular fluid from (C) above, analyzed directly by staining and flow cytometry. (E) Audiologic examination of DMF5 patient 2 pre-TCR treatment, day 13 post-treatment showing hearing loss, and 11 months post-treatment, showing hearing recovery following intratympanic steroid treatment.

Figure 7. Highly reactive transferred cells traffic to and destroy melanoma tumors in patients. (A) Sequential biopsies of subcutaneous tumors from DMF5 patient 4 pre (d0) and post treatment, stained with H&E (left), or anti-CD8 (right); (20x objective). (B) DMF5 patient 4 thigh covered with multiple subcutaneous melanoma lesions pre-treatment, and following partial tumor regression 5 weeks post-treatment. (C) Flow cytometry of TIL grown from DMF5 patient 4 subcutaneous tumor resected 4 weeks after treatment. (D-F) Pre- and post-treatment CT scans of (D) DMF5 patient 2 lungs, (E) DMF5 patient 8 brain (upper) and axilla (lower), and (F) gp100(154) patient 14 lungs (upper) and liver (lower). Arrows indicate location of melanoma metastases.
Figure 1

A

[Graph showing IFN-γ production levels for different targets and conditions.

1.0 uM Mart
1.0 μM gp154
mel526+
mel624+
mel888-
mel938-

Target

pg/mL IFN-γ

B

DMF4
DMF5
gp100(154)

5' LTR TCRα IRES TCRβ 3' LTR

5' LTR TCRα IRES TCRβ 3' LTR

5' LTR TCRα IRES TCRβ 3' LTR
Figure 3

A

B

Patient PBL sample

% Tetramer+ (of CD3+)

% Tetramer+ (CD3 gated)

Patient PBL sample

IFNγ ELISPOTs per 10^5 PBMC

IL-2 ELISPOTs per 10^5 PBMC

pre 2 wk post 1 mo post

PR NR PR NR

DMF5 gp100(154)

>300

1

>300

1

PR NR PR NR

DMF5 gp100(154)
Figure 4
Figure 5

**A**

- Graph showing pg/mL IFNg over days with different lines labeled DMF5 TCR.
- Days range from -10 to 20.
- Cell infusion indicated.

**B**

- Graph showing pg/mL IFNg with pre, peak, and post periods.
- Lines for DMF5, gp100(154), and DMF4 with P-values provided:
  - DMF5: P = 0.001
  - gp100(154): P = 0.0001
  - DMF4: P = 0.09
- Values >600 indicated in specific periods.
Figure 6

A. H&E and CD8 staining in skin samples from a control and a patient with melan-A.

B. CD8 staining in vitiliginous and pigmented skin from a patient.

C. An image showing the eye of a patient.

D. A graph showing the relationship between MART1 tetramer and CD3 expression.

E. Audiograms before and after treatment, showing changes in hearing levels across different frequencies.
Figure 7

A

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B

Pre

5 wk post

C

![Image](image9)

D

Pre

Post

E

Pre

Post

F

Pre

Post

For personal use only.on April 1, 2017. By guest.
Gene therapy with human and mouse T cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen