Published in final edited form as:

Hum Gene Ther. 2005 April ; 16(4): 457–472.

Transfer of a TCR Gene Derived from a Patient with a Marked Antitumor Response Conveys Highly Active T-Cell Effector Functions

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Abstract

The genes for the α and β chains of a highly reactive anti-MART-1 T-cell receptor were isolated from T-lymphocytes that mediated *in vivo* regression of tumor in a patient with metastatic melanoma. These genes were cloned and inserted into MSCV-based retroviral vectors. After transduction, greater than 50% gene transfer efficiency was demonstrated in primary T-lymphocytes stimulated by an anti-CD3 antibody. The specificity and biologic activity of TCR gene-transduced T-cells was determined by cytokine production after coculture of T-cells with stimulator cells pulsed with MART-1 peptide. The production of interferon- γ and granulocyte macrophage-colony stimulating factor (GM-CSF) was comparable to highly active MART-1 specific peripheral blood lymphocytes (PBL) in the amount of cytokine produced and transduced cells recognized peptide pulsed cells at dilutions similar to cytotoxic T lymphocyte (CTL) clones. Human leukocyte antigen (HLA) class I restricted recognition was demonstrated by mobilization of degranulation marker CD107a, by cell lysis, by cytokine production, and by proliferation in the presence of HLA-A2-positive but not HLA-A2-negative melanoma cell lines. Similar data was obtained when tumor-infiltrating lymphocytes (TIL) were transduced with the TCR genes, converting previously nonreactive cells to tumor reactive cells. TCR-transduced T-cells are thus attractive candidates for evaluation in cell transfer therapies of patients with cancer.

OVERVIEW SUMMARY

A novel retroviral vector was used to encode the α and β chains of a highly active anti-MART-1 T-cell receptor that previously conferred antitumor reactivity *in vivo*. This receptor was used to transduce both primary human lymphocytes and tumor infiltrating lymphocytes to confer reactivity to antigen and tumor cell lines *in vitro*. Transduction efficiencies were greater than 50% in primary lymphocytes activated with OKT3. The T-cell receptor was shown to be functional by the release of cytokines in tumor cell coculture assays and was human leukocyte antigen (HLA) class I restricted. Tumor-infiltrating lymphocytes, previously nonreactive, were converted via transduction with this retrovirus to reactive, lytic lymphocytes capable of tumor lysis. The transduced cells also had the ability to proliferate when grown in the presence of the appropriate antigen expressing tumor cell line. These manipulations are designed to improve and make adoptive immunotherapy widely applicable.

INTRODUCTION

ATTEMPTS AT THE IMMUNOTHERAPY of patients with metastatic melanoma by direct immunization with tumor-associated antigens (TAA) have elicited both humoral and cellular responses but tumor regression has only rarely been observed (Marchand et al., 1995; Rosenberg et al., 1998; Jager et al., 2000; Rosenberg et al., 2004). In contrast, adoptive immunotherapy, which uses the transfer of lymphocytes with highly reactive antitumor activity, can mediate tumor regression (Rosenberg et al., 1988; Papadopoulos et al., 1994; Mackinnon et al., 1995; Walter et al., 1995; Dudley and Rosenberg, 2003; Dudley et al., 2003). Tumor-infiltrating lymphocytes (TIL) have been used in these cell transfer therapies and have been shown to recognize a variety of melanoma TAA (Kawakami et al., 2000, 2001; Harada et al., 2001; Huang et al., 2004). In melanoma, the most commonly recognized TAA is the MART-1, melanoma melanocyte differentiation antigen, which is expressed in approximately 90% of melanomas (Cormier et al., 1998; Berset et al., 2001). In a recent trial of adoptive immunotherapy after nonmyeloablative lymphodepleting chemotherapy, 46% of patients with metastatic melanoma exhibited an objective regression (Dudley et al., 2002a). In two of these patients repopulation of lymphocytes was associated with a distinct oligoclonal T-cell expansion (as detected by TCR V β analysis) of MART-1-reactive cells and near-total regression of their tumor burdens. We have now cloned the genes encoding one of the MART-1 T-cell receptors from one of these patient's TIL, inserted them into retroviral vectors, and studied the antitumor properties of human lymphocytes and TIL transduced with these retroviruses.

MATERIALS AND METHODS

Cell lines

The cell lines used in experiments include the PG13 gibbon ape leukemia virus packaging cell line (ATCC CRL 10,686), the human ecotropic packaging cell line, Phoenix Eco (kindly provided by Dr. Gary Nolan, Stanford University, Stanford, CA), and the human lymphoid cell line Sup T1 (ATTC CRL1942). HLA-A2-positive (Mel 526 and Mel 624) and HLAA2-negative (Mel 888 and Mel 938) human melanoma cell lines were established in the Surgery Branch, National Cancer Institute (NCI) from resected tumors (Topalian et al., 1989). T2 cells are a lymphoblastoid cell line deficient in TAP function whose HLA class I protein can be easily loaded with exogenous peptides (Salter et al., 1985). The cell lines JB2F4 and RC612 are cytotoxic T lymphocyte clones that are HLA-A2-restricted and specific for the MART 1:27-35 peptide or the gp100:209-217 peptide, respectively (Dudley et al., 2001). All cells were cultured in R10 media consisting of RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS; Biofluids, Rockville, MD), gentamicin (180 μ g/ml) (Gibco, Grand Island, NY), and were maintained in a 37°C and 5% CO₂ incubator. In some assays, peripheral blood lymphocytes (PBL) from a patient with metastatic melanoma were stimulated *in vitro* with MART-1 peptide and used as positive controls (Liu and Rosenberg, 2001). Lymphocytes were cultured in AIM-V medium (Gibco) supplemented with 5% human AB serum (Valley Biomedical, Winchester, VA) and 300 IU/ml interleukin-2 (IL-2) at 37°C and 5% CO₂.

Construction of retroviral vectors

RNA isolated from the MART-1-reactive T-cell clone (M1F12) was subjected to RACE (rapid amplification of cDNA ends) polymerase chain reaction (PCR) and DNA sequence analysis in order to determine TCR α and β chain usage. In accordance with IMGT nomenclature, the M1F12 clone TCR usage is: α chain TRAV35/TRAJ49/TRAC and β chain TRBV10-3/TRBD1/TRBJ1-5/TRBC1. This information was used to design PCR primers for cloning of the individual chain full-length cDNAs. Briefly, polyA⁺ RNA was isolated from 1×10^7 M1F2 T cells using the Poly (A) Pure mRNA purification kit (Ambion, Austin, TX). Reverse

transcription-polymerase chain reaction (RT-PCR) was performed using the Titan One Tube RT-PCR kit (Roche, Indianapolis, IN) according to the manufacturer's suggestions with the following pairs of oligonucleotide primers: forward primer cccgcggacatgttgcttgaacattattaataatcttggtggtgcagc and reverse primer gtttaactagttcagctggaccacagccgcagc (for the rearranged α chain), forward primer cccatgggcacaaggtgttcttctatgtggc and reverse primer cgggttaactagttcagaatcctttcttgcacatggc (for the rearranged β chain). The amplified products were gel purified and cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and subsequently confirmed by sequencing.

The retroviral vector backbone used in this study, pMSGV1, is a derivative of the vector pMSGV (MSCV-based splice-gag vector) that utilizes a murine stem cell virus (MSCV) long terminal repeat (LTR; Hawley et al., 1994), and contains the extended gag region and env splice site from vector SFGtcLuc+ITE4 (Lindemann et al., 1997). Vector pMSGV was generated from pMINV (Hawley et al., 1996) by substituting a 756-bp *SpeI/XhoI* fragment with a 798-bp *SpeI/XhoI* fragment from SFGtcLuc+ITE4 and by replacing a 1955-bp *XhoI/BamHI* fragment containing a PGK-IRES-NEO cassette with a 47-bp *XhoI/BamHI* polylinker containing unique *XhoI*, *EcoRI*, *SalI*, *SacII*, and *BamHI* sites. Vector pMSGV1 was derived from pMSGV by replacing a 43-bp *PmlI/XhoI* fragment of pMSGV with a 76-bp *PmlI/XhoI* fragment from the vector Gcsap (Onodera et al., 1998). The latter modification incorporates a naturally occurring Kozak sequence to enhance translational efficiency.

Four different retroviral vectors were constructed each expressing both chains of the TCR. Vector APB was assembled by combining the TCR α (*AflIII/SpeI* fragment) plus PGK promoter (*XbaI/XhoI* fragment) into the *NcoI/XhoI* sites of pMSGV1, followed by insertion of the TCR α (as an *EcoRI* fragment). Vector AIB was produced by linking the TCR α (*AflIII/SpeI* fragment) via an internal ribosome entry site (IRES) element (Morgan et al., 1992) (as an *XbaI/XhoI* fragment) into the *NcoI/XhoI* sites of pMSGV1, followed by insertion of the TCR α (as an *EcoRI* fragment). Vector BPA was assembled by combining the TCR β (*NcoI/SpeI* fragment) plus PGK promoter (*XbaI/XhoI* fragment) into the *NcoI/XhoI* sites of pMSGV1, followed by insertion of the TCR β (as an *EcoRI* fragment). Vector BIA was produced by linking the TCR β (*NcoI/SpeI* fragment) via an IRES element (Morgan et al., 1992) (as an *XbaI/XhoI* fragment) into the *NcoI/XhoI* sites of pMSGV1, followed by insertion of the TCR α (as an *EcoRI* fragment).

To generate PG13 packaging cell clones, a coculture of PG13 and Phoenix Eco cells was transfected with 8 μ g of DNA for each construct (AIB, BIA, APB, BPA) using the Gene Porter reagent (Gene Therapy Systems, San Diego, CA). After 14 days of coculture, the Phoenix Eco cells were removed using magnetic antibody coated beads (anti-CD8 Lyt2 from Dynal, Lake Success, NY) and PG13 clones were generated by limiting dilution. The lack of a selectable marker gene necessitated an initial screening of PG13 clones for high physical titer using an RNA dot blot procedure as previously described (Onodera et al., 1997). Production of biologically active virus by clones with the highest relative physical titer was tested by retroviral vector transduction of SupT1 cells and analysis by fluorescence-activated cell sorter (FACS) staining for CD3 or anti-V β 12. Southern blot analysis was used to confirm vector integration and copy number in the PG13 clone AIB 18. Its genomic DNA was extracted using Easy-DNA Kit (Invitrogen, Carlsbad, CA).

MSGIN (murine stem cell virus-based splice vector green fluorescent protein-internal ribosomal entry site-neo) vector was used to express green fluorescent protein (GFP). Another retroviral vector encoding the anti-gp100 TCR (APB9) (Morgan et al., 2003) was used as a control to transduce aliquots of PBL or TIL.

Transduction of PBL and TIL

PBL were obtained by leukapheresis from two HLA-A2-positive melanoma patients. One of the patients had received multiple vaccinations with gp100: 209-217(210M) and tyrosinase: 386-376(370D) peptides in incomplete Freund's adjuvant and was leukapheresed 1 year after completing vaccination. The other patient had no prior treatment or vaccinations. Lymphocytes were purified by centrifugation on a Ficoll/Hypaque cushion, washed in Hanks' balanced saline solution (HBSS) and resuspended in AIM-V medium supplemented with 50 ng/ml of OKT3, 300 IU/ml IL-2 and 5% human AB serum at a concentration of 1×10^6 cells per milliliter. The lymphocytes were cultured in 24-well plates (Costar, Cambridge, MA) for 48 hr prior to transduction.

Nontissue culture-treated 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) were treated with 25 μ g/ml of recombinant fibronectin fragment as directed by the manufacturer (RetroNectin, Takara, Otsu, Japan). Retroviral vector supernatant (4-6 ml) was added and the plates were incubated at 32°C for 2-4 hr after storage at 4°C overnight. Plates were warmed to room temperature, supernatant was removed and 10^6 stimulated PBL per milliliter were added to each well with 3-5 ml per well. These plates were incubated overnight and the transduction process was repeated the following day. After transduction, the cells were grown in the above media without OKT3 and the cultures split to maintain a density between $0.5-3 \times 10^6$ cells per milliliter.

TIL were obtained from surgical specimens as described previously (Kawakami et al., 1988). TIL were selected that were nonreactive to common antigens but were reactive to the patient's autologous tumor. Cryopreserved cells were thawed, washed in media with serum and cultured in AIM-V media with 5% human AB serum and 1000 IU/ml IL-2 at $0.5-4 \times 10^6$ cells per milliliter. After 5 days in culture, the cells were actively dividing and therefore were transduced twice using the same methods used for PBL.

FACS analysis

Cell surface expression of TCRV β 12, TCRV β 8, CD3, CD4, CD8, and CD107a molecules on PBL or TIL were measured using fluorescein isothiocyanate (FITC), phycoerythrin (PE) or cyanogens (Cy)-conjugated antibodies. Anti-TCR V β 8 and V β 12 were supplied by Immunotech (Westbrook, ME) and the other antibodies were from Becton Dickinson (San Jose, CA). Immunofluorescence, analyzed as the relative log fluorescence of live cells, was measured using a FACScan flow cytometer (Becton Dickinson). A combination of forward angle light scatter and propidium iodide staining was used to gate out dead cells. Approximately 1×10^5 cells were analyzed. For tetramer staining an existing anti-gp100 and anti-MART product was used (iTAg MHC Tetramer, Beckman Coulter, Fullerton, CA). Cells were stained in a FACS buffer made of phosphate-buffered saline (PBS; Bio Whittaker, Walkersville, MD) and 1% fetal calf serum (FCS). All intracellular FACS staining was done with the Cytofix/Cytoperm Plus Kit (BD Biosciences Pharmingen, San Diego, CA).

Measurement of lymphocyte reactivity to antigen

T2 cells pulsed with peptides (using concentrations as indicated in the figure legends) were incubated with cells for 3 hr at 37°C in AIM-V and 5% human serum with the appropriate IL-2 doses depending on the cell type (PBL versus TIL). T2 cells pulsed with either HLA-A2-restricted influenza peptide (GILGFVFTL), MART-1:27-35 peptide or gp100:209217(210M) were incubated with TIL or PBL in AIM-V and 5% human serum. If IL-2 was the cytokine measured, the cells were washed with media without IL-2 prior to co-culture. For all of these assays, the responder cells (PBL or TIL) and the stimulators (T2 pulsed) were co-cultured in a ratio of 1:1 with 100,000 cells each in 96-well U-bottom plate (Costar, Corning, NY) with a total volume of 0.2 ml for 24 hr except tumor necrosis factor (TNF)- α , which was harvested

at 6 hr. Cytokine secretion was measured via enzyme-linked immunosorbent assay (ELISA; Endogen, Cambridge, MA). The cell surface mobilization of the CD107a antigen was determined as a measure of degranulation (Rubio et al., 2003). In brief, 500,000 melanoma cells were plated into one well of a 24-well plate and incubated overnight. The next day, 500,000 gene-transduced PBL were added to wells containing the tumor cell lines and cocultured for 2 hr. PBL were removed and stained with antibodies for V β 12, CD3, CD107a, and analyzed by FACS. The ability of the transduced cells (PBL or TIL) to lyse HLA-A2-positive melanoma targets were measured using a 4-hr ^{51}Cr release assay as described previously (Topalian et al., 1989).

CFSE labeling of transduced PBL for proliferation analysis

To study the proliferation of transduced PBL, they were labeled with carboxy-fluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Inc. Eugene, OR) (Coligan et al., 2001) and cultured in R10 media. Two million CFSE-labeled PBL (mock-transduced or AIB 18-transduced) were added to 10^6 melanoma cells in 6-well tissue culture plates in a total volume of 3 ml with either no IL-2 or 1 IU/ml or 10 IU/ml of IL2. Viable cells were counted on days 2 and day 4. The cells were stained with anti-CD3 antibody to gate on T cells and the immunofluorescent peak of CFSE was examined at 488-nm wavelength with a 525-nm bandpass filter using a Becton Dickinson FACscan on days 0, 2, and 4.

RESULTS

TCR vector construction and analysis of constructs

We recently reported that 46% of patients treated by adoptive immunotherapy after nonmyeloablative lymphodepleting chemotherapy exhibited objective tumor regression (Dudley et al., 2002a). Patient 9 in that trial exhibited a near-complete regression of tumor associated with the oligoclonal expansion of an administered V β 12+, CD8 $^{+}$ MART-reactive clone. Several different T-cell clones present in the infused TIL were MART1-reactive. The V β 12+ T cells accounted for 14% of infused cells and 55% of the total PBL 7 days after infusion. Four months after cell transfer (d124) 51% of the PBL were MART1-reactive, and this pool almost completely comprised the V β 12 expressing clone.

TCR V β 12 containing clone M1F12 is a highly reactive T-cell clone derived from patient 9 via limiting dilution of post-treatment PBL and was confirmed by DNA sequence, to be the clone infused into the patient (data not shown). The α and the β chain TCR cDNAs were isolated from clone M1F12 and were used to construct four different gene transfer vectors. All constructs used the retroviral MSCV LTR to drive expression of either α or β chains with the second chain either promoted by an internal PGK promoter or the two genes were linked by an IRES. The combinations were thus AIB or BIA (α IRES β or β IRES α) and BPA or APB (β PGK α or α PGK β) (Fig. 1A). The plasmid DNA vectors were transfected into PG13 packaging cells lines, which were tested by intracellular staining with anti-V β 12 antibody (Fig. 1B). Constructs AIB, BIA, and BPA yielded 23% to 53% of cells expressing V β 12 staining while construct APB yielded only 6% positive cells and thus APB was not analyzed further.

We first tested the activity of these vector producer cell populations by transduction of the human T-cell leukemia cell line, Sup T1 (this line contains chromosomal translocations involving both α and β TCR genes that prevents surface expression of the endogenous TCR complex including CD3). Transduced cells were thus assayed for cell surface expression of the CD3 protein to assess the expression of the TCR α and β chains. After transduction with constructs AIB and BIA using supernatants from the PG13 producer populations, 44% and 21% of Sup T1 cells expressed CD3 (Fig. 2A) suggesting that these cloned anti-MART-1 α and β chain genes can participate in the formation of a functional TCR complex.

To isolate high-titer vector producing cells, PG13 vector producer cell clones were obtained by limiting dilution and were retested by transduction of SupT1. Sup T1 cells transduced with supernatant from PG13 clones ranged from 15% to 89% MART-1 tetramer positive. Representative data from two of the most active clones (AIB18 and AIB54) is shown in Figure 2B. Vector 18 transduced cells exhibited 69% tetramer staining and AIB 54 engineered cells had 89% tetramer staining. Producer cell clones (AIB 18, AIB 54, BIA 71, and BPA 34) with both high titer and tetramer staining were chosen for further study (data not shown).

Transduction of PBL

In order to engineer PBL with the anti-MART-1 TCR vector, we used a modification of a procedure (Hanenberg et al., 1996) that involved precoating tissue culture plates with Retronectin (Takara Bio, Inc., Shiga, Japan), a recombinant fibronectin molecule, which colocalized the retrovirus with the target cell. This method allowed for high efficiency retroviral vector mediated gene transfer and optimized PBL culture conditions since the vector supernatant is removed prior to adding the target cells (PBL).

Transduced T cells were stained for V β 12 and MART-1 tetramer 48 hr after the final transduction and analyzed via FACS. The percentage of V β 12 positive cells in the transduced cell populations varied from 43%-86% (background staining for V β 12 was from 1-3%). Examples of two transductions, AIB18 and AIB54 are shown in Figure 3A. These transductants, which contained 86% and 75% V β 12-positive cells also exhibit 37% and 29% of cells, respectively, stained with MART-1 tetramer (Fig. 3B). Both CD4 and CD8-positive cells in the engineered population stained for V β 12 (data not shown).

Functional tests of the TCR transductants

To determine if TCR-transduced PBL could mediate the release of effector cytokines when exposed to appropriate antigen, coculture experiments were performed. The coculture consisted of transductants and T2 cells pulsed with various peptide antigens (Flu, gp100 and MART-1). PBL were either not transduced (NV), transduced with control vectors encoding GFP (MSGIN), or gp100 specific TCR (APB9) (Morgan et al., 2003), and various MART-1 TCR retroviral vectors (AIB 18, BPA 34, BIA 71). Positive controls for these assays were the MART-1-reactive T lymphocyte clone JB2F4 or PBL from a patient with metastatic melanoma derived by multiple *in vitro* stimulations with MART-1 peptide (PBL-MART).

In these assays, the transduced PBL specifically secreted cytokines when exposed to the relevant peptide stimulus (Table 1). Transduced PBL secreted between 48,375 and 75,546 pg/ml of interferon (IFN)- γ compared to control transduced cells that produced 409 pg/ml. The variation in IFN- γ secretion was dependent on which TCR vector clone was used for transduction. For GM-CSF, MART-1 TCR-transduced PBL secreted 5100 pg/ml compared to control transduced cells that secreted 786 pg/ml. The transduced PBL when cocultured with relevant peptide made low but detectable amounts of IL-2 compared to control PBL, which made no detectable IL-2. The PBL modified with the anti-MART-1 TCR vectors (AIB 18 and AIB 54) secreted 2552 and 1941 pg/ml of TNF- α , respectively, compared to 9 pg/ml for the mock-transduced population. One measure of the relative reactivity of a particular TCR is the ability to react to cells pulsed with limiting dilutions of peptides. The antiMART-1 TCR gene-modified PBL assayed in this experiment recognized T2 cells pulsed with as little as 0.1 ng/ml MART-1 peptide, comparable to the CTL clone JB3F4.

Recognition of melanoma cell lines by transduced lymphocytes

To assess the recognition of melanoma cells, the modified lymphocytes (transduced with AIB 18, BIA 71, BPA34 and MSGIN) were cocultured with two HLA-A2 cell lines (526, 624) and two non-HLA-A2 cell lines (888, 938). Specific release of IFN- γ and GM-CSF, was seen when

the gene modified PBL were cocultured with HLA-A2-positive but not the HLA-A2-negative melanoma cell lines (Fig. 4). IFN γ secretion by transduced PBL ranged from 66-318 pg/ml when exposed to MEL 526 compared to control secretion of only 12 pg/ml. For MEL 624, transduced PBL secreted between 77-235 pg/ml while control PBL only secreted 1 pg/ml. Combinations of PBL with non-HLA-A2 melanoma lines had insignificant IFN γ . Similarly for GM-CSF, anti-MART-1 TCR-transduced PBL secreted between 195-449 pg/ml when exposed to A2+ melanoma compared to less than 94 pg/ml for A2- melanoma lines.

To determine the functional reactivity of transduced T-cells, we performed a CD107a mobilization assay (Fig. 5). In this experiment PBL were transduced with the AIB 18 vector and then the population cocultured with HLA-A2-negative/MART-1-positive melanoma cell line 888 or HLA-A2+/MART-1 + melanoma cell line 624. After coculture, transduced T cells (i.e., V β 12-positive cells) were analyzed for CD3 and CD107a antigen expression. Data in Figure 5 demonstrate that while few (1.6%) of transduced cells mobilized CD107a upon coculture with melanoma line 888, 29% of the TCR vector-transduced cells became positive for CD107a expression upon coculture with melanoma line 624.

As an additional test of T-cell effector cell function, we next determined the ability of the transduced PBL to lyse melanoma tumor cell lines. TCR-transduced PBL were tested against melanoma cell lines in a 4-hr ^{51}Cr release assay (Fig. 6) using anti-MART-1 CTL clone JB2F4 as positive control. The TCR-engineered cells (using vector AIB 18) readily lysed HLA-A2-positive melanoma cell lines (526, 624) but not the HLA-A2-negative 938 melanoma, while mock transduced PBL (NV) had little to no lytic capability.

Conversion of non-reactive TIL cultures to MART-1 reactivity

To be effective in adoptive immunotherapy, TIL cultures must be reactive to shared tumor antigens or the patient's autologous tumor. We next determined whether nonreactive TIL could be converted, via transduction with the anti-MART-1 TCR, to antigen-reactive TIL. Approximately 17-24% of TIL after transduction were V β 12-positive compared to less than 1% for the mock-transduced TIL controls (data not shown). Patient's TIL was transduced with either the AIB18 anti-MART1 TCR vector or the APB9 anti-gp100 TCR vector and then analyzed for CD8 expression and appropriate tetramer binding. The cultures were almost entirely CD8 $^{+}$ (96%) and each had 7-8% tetramer staining versus less than 1% for controls.

TIL transduced with the AIB 18 anti-MART-1 TCR vector, the anti-gp100 TCR vector, no vector, or GFP exhibited specific release of IFN γ and GM-CSF as shown in Table 2. The anti-MART-1 TCR-transduced cells secreted 14,695 pg/mL of IFN γ when the TIL were exposed to T2 cells pulsed with the MART-1 peptide (versus <500 pg/ml in control-engineered cultures). Similarly, the transduced TIL specifically secreted large amounts of the two other cytokines GM-CSF (79,969 pg/ml) and IFN α (1568 pg/ml).

The TCR-engineered TIL also recognized MART-1 antigens processed on HLA-A2-positive melanoma cells but not HLA-A2-negative lines assessed both by cytokine release and in four-hour ^{51}Cr release assays (Table 3 and Fig. 7). The MART-1 TCR vector-transduced cells produced 1674 and 2139 pg/ml of IFN γ in the presence of A2+ melanoma cell lines (526 and 624) compared to 18 and 14 pg/ml in the presence of A2- cell lines. Similar results were observed for GM-CSF (Table 3). The ^{51}Cr release assay demonstrated lysis of tumor by the MART1 TCR transduced TIL compared to the near-complete lack of lysis in the mock transduced TIL in HLA-A2-positive melanoma cell lines. The anti-MART-1 TCR transduced TIL lysed between 50-70% of the targets at the highest effector to target ratio. In addition to the lysis of the two established melanoma cell lines (526 and 624), the transduced TIL also specifically recognized an HLA-A2-positive primary fresh melanoma digest (Fig. 7).

Antigen-specific proliferation of TCR-engineered lymphocytes

Last, we sought to examine whether these engineered cells would proliferate *in vitro* when stimulated by an appropriate antigen. Mock-transduced PBL and anti-MART-1 TCR-transduced PBL were labeled with CFSE dye and then were cocultured with MEL 526 (A2+) or MEL 888 (A2-) in concentrations of IL-2 ranging from 0-10 IU/ml. Four days after stimulation, the proliferation of CD3⁺ lymphocytes was determined by FACS analysis (Fig. 8), where dilution of the CFSE peak was indicative of cell proliferation. FACS analysis of melanoma/lymphocyte cocultures in the presence of no exogenous IL-2, demonstrated 25% of the cells had undergone cell division versus 2-5% of control cultures. When minimal amounts of exogenous IL-2 were added (1-10 IU/ml), up to 55% of the lymphocytes in the MEL 526 coculture had demonstrable proliferation, compared to 7-13% of control cultures.

DISCUSSION

Introduction of anti-TAA TCR genes has been proposed as a method to produce antitumor lymphocytes for the immunotherapy of cancer (Schumacher, 2002; Sadelain et al., 2003; Willemsen et al., 2003). This may provide a means to bypass tolerance to tumor-associated antigens as has been demonstrated in murine models (Stanislawski et al., 2001; Fujio et al., 2004). The data presented here, and in our previous reports (Clay et al., 1999; Morgan et al., 2003) suggest that this approach is reproducible for a variety of antigens and can have immediate clinical applications.

There are potential safety concerns regarding the infusion of large numbers of MART-1-reactive, but many of these theoretical concerns have, by and large, been addressed in previous clinical studies. The gene used in this report is a naturally occurring anti-MART-1 TCR derived from the TIL of patient 9 in the report by Dudley et al. (2002a). This exact gene, in the context of TIL, was administered to this patient (1.2×10^{10} cells) along with high-dose IL-2 after nonmyeloablative but lymphocyte-depleting chemotherapy. No toxicities associated with the infusions of this highly reactive T cell were observed in this patient (other than vitiligo). In addition, we have treated 15 patients using infusions of large numbers of antigen100 T-cell clones in the setting of nonmyeloablative chemotherapy with no side effects (Dudley et al., 2002b). While autoimmunity (e.g., vitiligo or uveitis) may be a possible consequence of anti-MART-1-reactive cell infusions, this response may correlate with antitumor responses (Dudley et al., 2002a; Phan et al., 2003). If severe autoimmunity is observed, it can be controlled by chemotherapy or steroid treatment.

A second potential safety concern is the induction of novel or diverse functional activity and the possibility of the selective expansion of novel reactivity driven by the tumor antigen and its potential consequences. The expansion of tumor reactive cells is a desirable outcome following the infusion of antigen-reactive T cells and any associated autoimmunity (or other novel reactivity) can be managed if it becomes medically necessary. We have administered over 3×10^{11} TIL with widely heterogeneous reactivity including CD4, CD8, and natural killer (NK) cells without difficulty. Finally, in regard to the malignant potential of these cells, we do not believe this is a significant risk for this patient population (i.e., patients with malignant melanoma). While the risk of insertional mutagenesis is a known possibility using retroviral vectors (Hacein-Bey-Abina et al., 2003) this has only been observed in the setting of infants treated for X-linked severe combined immunodeficiency (X-SCID) using retroviral vector-mediated gene transfer in to CD34⁺ bone marrow cells. In the case of retroviral vector-mediated gene transfer into mature T cells, there has been no evidence of long-term toxicities associated with these procedures since the first National Cancer Institute sponsored gene transfer study in 1989. Although continued follow-up of all gene therapy patients will be required, data suggest that the introduction of retroviral vector transduced mature T cells is an acceptable risk for appropriately informed adult patients with metastatic cancer.

As the source of the genes encoding the anti-TAA TCR, we selected T-cell clone M1F12, which possessed a highly reactive MART-1 TCR associated with an objective antimelanoma response in one of our adoptive immunotherapy patients (Dudley et al., 2002a). At the height of this patient's antitumor response, this particular V β 12 clone comprised 50% of the patient's peripheral lymphocytes and could be detected in tumor biopsy samples. We chose as retroviral vector backbone to express these genes, the retroviral vector MSGV1. The design of MSGV1 incorporates two key elements that were intended to optimize TCR gene expression. First, the LTR promoter derives from the MSCV retrovirus that we had previously shown to promote high levels of gene expression in a variety of cell types, including primary hematopoietic cells (Cheng et al., 1998). Next, the inclusion of the naturally occurring MLV envelope gene splicing acceptor site, as exemplified in the MFG-class of retroviral vectors, was used to facilitate mRNA splicing and translation via a Kozak consensus translation initiation signal (Onodera et al., 1998). The vector BPA had the α and β chains expressed from independent promoters while the AIB and BIA vectors had the expression of the chains coupled by an IRES. In multiple FACS analysis of transduced Sup T1, PBL, and TIL, staining with MART-1 tetramer was comparable, suggesting that any of these vector designs could mediate high levels of gene expression and transfer a biologically active TCR gene into transduced human cells.

The results reported here demonstrate significantly enhanced antimelanoma activity from anti-MART-1 TCR gene-transduced T cells than our previous observations with anti-MART1 TCR gene transfer (Clay et al., 1999). The main difference in these two reports were different retroviral vector backbone designs and the specific anti-MART-1 TCR used. While we believe that our new vector designs contributed to the enhanced antitumor activity in our current report, the main determinant of this superior activity is likely the specific TCR used herein. While many factors contribute to the overall reactivity of the TCR gene-transduced T lymphocytes, the specific TCR used has been demonstrated to be the major contributor to antitumor activity (Roszkowski et al., 2003; Rubinstein et al., 2003; Schaft et al., 2003). Our previous anti-MART-1 TCR was obtained from a tumor-reactive CTL derived *in vitro* from a TIL culture by limiting dilution (Clay et al., 1999). While the *in vivo* activity of the previous anti-MART-1 clone is unknown, the current anti-MART-1 TCR was derived directly from a CTL clone associated with a pronounced antitumor response derived from *in vivo* samples obtained from a patient effectively treated with adoptive cell therapy (Dudley et al., 2002a).

The transfer of these highly reactive TCR genes into primary lymphocytes had efficiencies always greater than 30% and occasionally as high as 75-80% via V β 12 staining (Fig. 3A). The anti-MART-1 engineered primary lymphocytes secreted large amounts of proinflammatory cytokines, IFN- α , GM-CSF, and IFN γ in an antigen-specific manner. These transduced PBL exhibited activity comparable to MART-1-specific PBL (Table 1) and while they do not produce the same magnitude of cytokine production as do control CTL clones (Tables 2 and 3), their affinity for peptide observed in limiting dilutions experiments (Table 1) appears to be equivalent to CTL clones.

The engineered cells were effective at detecting antigen and releasing effector cytokines in response to MART-1 in the context of melanoma cell lines (Fig. 4 and Table 3). In addition, the anti-MART-1 TCR-engineered PBL were able to mobilize CD107a and effectively lyse melanoma cell lines in an HLA2-restricted manner (Figs. 5 and 6). Together these data suggest that TCR gene-transduced T cells can be an alternative to highly active CTL clones in adoptive immunotherapy. The engineering of polyclonal T-lymphocyte populations such as PBL or TIL has a practical advantage over CTL clones, in that, these populations can generally be expanded more than 1000-fold *in vitro* while it is difficult to expand CTL clones by greater than 200-fold *in vitro*. In addition, while infusion of large numbers of CTL have not been associated with tumor regression (Dudley et al., 2001), polyclonal TIL populations can mediate the regression of large established tumors in melanoma patients (Dudley et al., 2002a).

Given the random assortment of TCR α and β chains in the transduced PBL, (likely reflected by the lower percentage of tetramer-positive cells compared to V β 12-positive cells, Fig. 3) it was possible that insufficient anti-MART-1 activity could have been observed. Our results demonstrate that despite lower percentages of TCR-transduced cells in the engineered populations, and a Gaussian distribution of TCR V β expression (Fig. 3), activity comparable to that of highly active CTL clones was observed (Tables 1 and 2, and Fig. 4). The ability of our TCR gene-transduced polyclonal T cells to exhibit antitumor reactivity is likely a function of starting with a highly reactive TCR gene and the fact that only a few TCR-antigen/major histocompatibility contacts are required for full effector cell function (Padovan et al., 1993; Labrecque et al., 2001). Using a native TCR with high reactivity (such as the TCR from patient 9's CTL clone M1F12) may be the critical determinant for successful transfer of antitumor properties to nontumor-reactive lymphocytes, because the TCR is the main determinant of avidity, and high avidity is correlated with *in vivo* antitumor activity (Zeh et al., 1999).

Adoptive transfer of tumor-reactive TIL has been shown to mediate cancer regression *in vivo* in several clinical trials (Rosenberg et al., 1988; Papadopoulos et al., 1994; Mackinnon et al., 1995; Walter et al., 1995; Dudley et al., 2002a). Sometimes it has not been possible to obtain TIL from patients with metastatic melanoma, either because of the inability to resect tumor, or even with sufficient tumor available, some cultures do not yield tumor-reactive TIL. In our hands, approximately 39% of TIL cultures are not reactive to either shared melanoma antigens or autologous tumor (Dudley et al., 2003). These nonreactive TIL may retain the cell surface molecules for homing but lack the anti-TAA activity, which is necessary for tumor lysis. In our experiments, we studied TIL that were nonreactive. The efficiency of transduction of TIL was not as high as was shown for PBL, which we speculate was because of the slower replication of the TIL. Effector cytokines IFN γ and GM-CSF were secreted in large quantities compared to untransduced TIL when exposed to peptide-pulsed targets (Table 2). Also, effector cytokines could be generated in a specific manner when TCR-engineered TIL was exposed to HLA-A2-positive melanoma cell lines (Table 3). Transduced TIL had improved cytolytic activity versus HLA-A2-positive melanoma cell lines, and primary cultures of A2-positive tumor were lysed effectively by TCR-transduced but not untransduced TIL (Fig. 7). This is the first time that lysis of a primary human tumor has been reported by an anti-MART-1 TCR gene-transduced human T lymphocytes. Clinically, it may be possible to apply this transfer of TCR genes to TIL that lack tumor reactivity, potentially increasing the number of patients with metastatic melanoma who can be treated by immunotherapy.

Last, *in vitro* transduced PBL showed the ability to proliferate in response to MART-1 in the context of a melanoma cell line (Fig. 8). Growth occurred only when the antigen was present in the context of the HLA-A2 molecule. Also, growth occurred despite suboptimal levels of IL-2 in the culture media. This was consistent with the ability of these T cells to synthesize IL-2 after coculture with MART-1-expressing targets (Table 1). The ability of TCR gene-transduced human T cells to proliferate in response to tumor antigens has not been previously reported. In a mouse model of this approach (Kessels et al., 2001), antigen-driven expansion of TCR gene-modified T-lymphocytes was essential to the antitumor response. In future clinical application of this technology, expansion of transduced cells following antigen exposure *in vivo* will be critical as proliferation of adoptively transferred TIL appears to be associated with the ability of TIL to mediate tumor regression (Dudley et al., 2002a).

ACKNOWLEDGMENTS

We acknowledge the excellent help of Arnold Mixon and Shawn Farid for FACS analysis as well as the members of the TIL laboratory who generated TIL for these experiments.

REFERENCES

- BERSET M, CEROTTINI JP, GUGGISBERG D, ROMERO P, BURRI F, RIMOLDI D, PANIZZON RG. Expression of Melan-A/MART-1 antigen as a prognostic factor in primary cutaneous melanoma. *Int. J. Cancer* 2001;95:73–77. [PubMed: 11241315]
- CHENG L, DU C, LAVAU C, CHEN S, TONG J, CHEN BP, SCOLLAY R, HAWLEY RG, HILL B. Sustained gene expression in retrovirally transduced, engrafting human hematopoietic stem cells and their lympho-myeloid progeny. *Blood* 1998;92:83–92. [PubMed: 9639503]
- CLAY TM, CUSTER MC, SACHS J, HWU P, ROSENBERG SA, NISHIMURA MI. Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. *J. Immunol* 1999;163:507–513. [PubMed: 10384155]
- COLIGAN, JK.; KRUISBEEK, AM.; MARGULIES, DH.; SHEVACH, EM.; STROBER, W. *Current Protocols in Immunology*. John Wiley & Sons, Inc.; 2001.
- CORMIER JN, ABATI A, FETSCH P, HIJAZI YM, ROSENBERG SA, MARINCOLA FM, TOPALIAN SL. Comparative analysis of the in vivo expression of tyrosinase, MART1/Melan-A, and gp100 in metastatic melanoma lesions: implications for immunotherapy. *J. Immunother* 1998;21:27–31. [PubMed: 9456433]
- DUDLEY ME, ROSENBERG SA. Adoptive-cell-transfer therapy for the treatment of patients with cancer. *Nat. Rev. Cancer* 2003;3:666–675. [PubMed: 12951585]
- DUDLEY ME, WUNDERLICH J, NISHIMURA MI, YU D, YANG JC, TOPALIAN SL, SCHWARTZENTRUBER DJ, HWU P, MARINCOLA FM, SHERRY R, LEITMAN SF, ROSENBERG SA. Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. *J. Immunother* 2001;24:363–373. [PubMed: 11565838]
- DUDLEY ME, WUNDERLICH JR, ROBBINS PF, YANG JC, HWU P, SCHWARTZENTRUBER DJ, TOPALIAN SL, SHERRY R, RESTIFO NP, HUBICKI AM, ROBINSON MR, RAFFELD M, DURAY P, SEIPP CA, ROGERS-FREEZER L, MORTON KE, MAVROUKAKIS SA, WHITE DE, ROSENBERG SA. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002a;298:850–854. [PubMed: 12242449]
- DUDLEY ME, WUNDERLICH JR, YANG JC, HWU P, SCHWARTZENTRUBER DJ, TOPALIAN SL, SHERRY RM, MARINCOLA FM, LEITMAN SF, SEIPP CA, ROGERSFREEZER L, MORTON KE, NAHVI A, MAVROUKAKIS SA, WHITE DE, ROSENBERG SA. A phase I study of nonmyeloablative chemotherapy and adoptive transfer of autologous tumor antigen-specific T lymphocytes in patients with metastatic melanoma. *J. Immunother* 2002b;25:243–251. [PubMed: 12000866]
- DUDLEY ME, WUNDERLICH JR, SHELTON TE, EVEN J, ROSENBERG SA. Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. *J. Immunother* 2003;26:332–342. [PubMed: 12843795]
- FUJIO K, OKAMOTO A, TAHARA H, ABE M, JIANG Y, KITAMURA T, HIROSE S, YAMAMOTO K. Nucleosome-specific regulatory T cells engineered by triple gene transfer suppress a systemic autoimmune disease. *J. Immunol* 2004;173:2118–2125. [PubMed: 15265948]
- HACEIN-BEY-ABINA S, VON KALLE C, SCHMIDT M, LE DEIST F, WULFFRAAT N, MCINTYRE E, RADFORD I, VILLEVAL JL, FRASER CC, CAVAZZANA-CALVO M, FISCHER A. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med* 2003;348:255–256. [PubMed: 12529469]
- HANENBERG H, XIAO XL, DILLOO D, HASHINO K, KATO I, WILLIAMS DA. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat. Med* 1996;2:876–882. [PubMed: 8705856]
- HARADA M, LI YF, EL-GAMIL M, OHNMACHT GA, ROSENBERG SA, ROBBINS PF. Melanoma-Reactive CD8+ T cells recognize a novel tumor antigen expressed in a wide variety of tumor types. *J. Immunother* 2001;24:323–333. [PubMed: 11565834]
- HAWLEY RG, LIEU FH, FONG AZ, HAWLEY TS. Versatile retroviral vectors for potential use in gene therapy. *Gene Ther* 1994;1:136–138. [PubMed: 7584069]
- HAWLEY RG, LIEU FH, FONG AZ, GOLDMAN SJ, LEONARD JP, HAWLEY TS. Retroviral vectors for production of interleukin-12 in the bone marrow to induce a graft-versus-leukemia effect. *Ann. N.Y. Acad. Sci* 1996;795:341–345. [PubMed: 8958949]

- HUANG J, EL-GAMIL M, DUDLEY ME, LI YF, ROSENBERG SA, ROBBINS PF. T cells associated with tumor regression recognize frameshifted products of the CDKN2A tumor suppressor gene locus and a mutated HLA class I gene product. *J. Immunol* 2004;172:6057–6064. [PubMed: 15128789]
- JAGER E, GNJATIC S, NAGATA Y, STOCKERT E, JAGER D, KARBACH J, NEUMANN A, RIECKENBERG J, CHEN YT, RITTER G, HOFFMAN E, ARAND M, OLD LJ, KNUTH A. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1 + cancers. *Proc. Natl. Acad. Sci. U.S.A* 2000;97:12198–12203. [PubMed: 11027314]
- KAWAKAMI Y, ROSENBERG SA, LOTZE MT. Interleukin 4 promotes the growth of tumor-infiltrating lymphocytes cytotoxic for human autologous melanoma. *J. Exp. Med* 1988;168:2183–2191. [PubMed: 3264324]
- KAWAKAMI Y, DANG N, WANG X, TUPESIS J, ROBBINS PF, WANG RF, WUNDERLICH JR, YANNELLI JR, ROSENBERG SA. Recognition of shared melanoma antigens in association with major HLA-A alleles by tumor infiltrating T lymphocytes from 123 patients with melanoma. *J. Immunother* 2000;23:17–27. [PubMed: 10687134]
- KAWAKAMI Y, WANG X, SHOFUDA T, SUMIMOTO H, TUPESIS J, FITZGERALD E, ROSENBERG S. Isolation of a new melanoma antigen, MART-2, containing a mutated epitope recognized by autologous tumor-infiltrating T lymphocytes. *J. Immunol* 2001;166:2871–2877. [PubMed: 11160356]
- KESSELS HW, WOLKERS MC, VAN DEN BOOM MD, VAN DER VALK MA, SCHUMACHER TN. Immunotherapy through TCR gene transfer. *Nat. Immunol* 2001;2:957–961. [PubMed: 11577349]
- LABRECQUE N, WHITFIELD LS, OBST R, WALTZINGER C, BENOIST C, MATHIS D. How much TCR does a T cell need. *Immunity* 2001;15:71–82. [PubMed: 11485739]
- LINDEMANN D, PATRIQUIN E, FENG S, MULLIGAN RC. Versatile retrovirus vector systems for regulated gene expression in vitro and in vivo. *Mol. Med* 1997;3:466–476. [PubMed: 9260158]
- LIU K, ROSENBERG SA. Transduction of an IL-2 gene into human melanoma-reactive lymphocytes results in their continued growth in the absence of exogenous IL-2 and maintenance of specific antitumor activity. *J. Immunol* 2001;167:6356–6365. [PubMed: 11714800]
- MACKINNON S, PAPADOPOULOS EB, CARABASI MH, REICH L, COLLINS NH, BOULAD F, CASTRO-MALASPINA H, CHILDS BH, GILLIO AP, KERNAN NA, et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood* 1995;86:1261–1268. [PubMed: 7632930]
- MARCHAND M, WEYNANTS P, RANKIN E, ARIENTI F, BELLI F, PARMIANI G, CASCINELLI N, BOURLOND A, VANWIJCK R, HUMBLET Y, et al. Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3. *Int. J. Cancer* 1995;63:883–885. [PubMed: 8847150]
- MORGAN RA, COUTURE L, ELROY-STEIN O, RAGHEB J, MOSS B, ANDERSON WF. Retroviral vectors containing putative internal ribosome entry sites: Development of a polycistronic gene transfer system and applications to human gene therapy. *Nucleic Acids Res* 1992;20:1293–1299. [PubMed: 1313966]
- MORGAN RA, DUDLEY ME, YU YY, ZHENG Z, ROBBINS PF, THEORET MR, WUNDERLICH JR, HUGHES MS, RESTIFO NP, ROSENBERG SA. High efficiency TCR gene transfer into primary human lymphocytes affords avid recognition of melanoma tumor antigen glycoprotein 100 and does not alter the recognition of autologous melanoma antigens. *J. Immunol* 2003;171:3287–3295. [PubMed: 12960359]
- ONODERA M, YACHIE A, NELSON DM, WELCHLIN H, MORGAN RA, BLAESE RM. A simple and reliable method for screening retroviral producer clones without selectable markers. *Hum. Gene Ther* 1997;8:1189–1194. [PubMed: 9215736]
- ONODERA M, NELSON DM, YACHIE A, JAGADEESH GJ, BUNNELL BA, MORGAN RA, BLAESE RM. Development of improved adenosine deaminase retroviral vectors. *J. Virol* 1998;72:1769–1774. [PubMed: 9499026]

- PADOVAN E, CASORATI G, DELLABONA P, MEYER S, BROCKHAUS M, LANZAVECCHIA A. Expression of two T cell receptor alpha chains: Dual receptor T cells. *Science* 1993;262:422–424. [PubMed: 8211163]
- PAPADOPOULOS EB, LADANYI M, EMANUEL D, MACKINNON S, BOULAD F, CARABASI MH, CASTROMALASPINA H, CHILDS BH, GILLIO AP, SMALL TN, et al. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N. Engl. J. Med* 1994;330:1185–1191. [PubMed: 8093146]
- PHAN GQ, YANG JC, SHERRY RM, HWU P, TOPALIAN SL, SCHWARTZENTRUBER DJ, RESTIFO NP, HAWORTH LR, SEIPP CA, FREEZER LJ, MORTON KE, MAVROUKAKIS SA, DURAY PH, STEINBERG SM, ALLISON JP, DAVIS TA, ROSENBERG SA. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. U.S.A* 2003;100:8372–8377. [PubMed: 12826605]
- ROSENBERG SA, PACKARD BS, AEBERSOLD PM, SOLOMON D, TOPALIAN SL, TOY ST, SIMON P, LOTZE MT, YANG JC, SEIPP CA, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N. Engl. J. Med* 1988;319:1676–1680. [PubMed: 3264384]
- ROSENBERG SA, YANG JC, SCHWARTZENTRUBER DJ, HWU P, MARINCOLA FM, TOPALIAN SL, RESTIFO NP, DUDLEY ME, SCHWARZ SL, SPIESS PJ, WUNDERLICH JR, PARKHURST MR, KAWAKAMI Y, SEIPP CA, EINHORN JH, WHITE DE. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med* 1998;4:321–327. [PubMed: 9500606]
- ROSENBERG SA, YANG JC, RESTIFO NP. Cancer immunotherapy: moving beyond current vaccines. *Nat. Med* 2004;10:909–915. [PubMed: 15340416]
- ROSZKOWSKI JJ, YU DC, RUBINSTEIN MP, MCKEE MD, COLE DJ, NISHIMURA MI. CD8-independent tumor cell recognition is a property of the T cell receptor and not the T cell. *J. Immunol* 2003;170:2582–2589. [PubMed: 12594285]
- RUBINSTEIN MP, KADIMA AN, SALEM ML, NGUYEN CL, GILLANDERS WE, NISHIMURA MI, COLE DJ. Transfer of TCR genes into mature T cells is accompanied by the maintenance of parental T cell avidity. *J. Immunol* 2003;170:1209–1217. [PubMed: 12538678]
- RUBIO V, STUGE TB, SINGH N, BETTS MR, WEBER JS, ROEDERER M, LEE PP. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. *Nat. Med* 2003;9:1377–1382. [PubMed: 14528297]
- SADELAIN M, RIVIERE I, BRENTJENS R. Targeting tumours with genetically enhanced T lymphocytes. *Nat. Rev. Cancer* 2003;3:35–45. [PubMed: 12509765]
- SALTER RD, HOWELL DN, CRESSWELL P. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics* 1985;21:235–246. [PubMed: 3872841]
- SCHAFT N, WILLEMSSEN RA, DE VRIES J, LANKIEWICZ B, ESSERS BW, GRATAMA JW, FIGDOR CG, BOLHUIS RL, DEBETS R, ADEMA GJ. Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR alpha beta genes into primary human T lymphocytes. *J. Immunol* 2003;170:2186–2194. [PubMed: 12574392]
- SCHUMACHER TN. T-cell-receptor gene therapy. *Nat. Rev. Immunol* 2002;2:512–519. [PubMed: 12094225]
- STANISLAWSKI T, VOSS RH, LOTZ C, SADOVNIKOVA E, WILLEMSSEN RA, KUBALL J, RUPPERT T, BOLHUIS RL, MELIEF CJ, HUBER C, STAUSS HJ, THEOBALD M. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. *Nat. Immunol* 2001;2:962–970. [PubMed: 11577350]
- TOPALIAN SL, SOLOMON D, ROSENBERG SA. Tumor-specific cytolysis by lymphocytes infiltrating human melanomas. *J. Immunol* 1989;142:3714–3725. [PubMed: 2785562]
- WALTER EA, GREENBERG PD, GILBERT MJ, FINCH RJ, WATANABE KS, THOMAS ED, RIDDELL SR. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N. Engl. J. Med* 1995;333:1038–1044. [PubMed: 7675046]
- WILLEMSSEN RA, DEBETS R, CHAMES P, BOLHUIS RL. Genetic engineering of T cell specificity for immunotherapy of cancer. *Hum. Immunol* 2003;64:56–68. [PubMed: 12507815]

ZEH HJ 3rd, PERRY-LALLEY D, DUDLEY ME, ROSENBERG SA, YANG JC. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. *J. Immunol* 1999;162:989–994. [PubMed: 9916724]

FIG. 1.

TCR expressing retroviral vectors. **A:** Diagrams of four bicistronic retroviral vectors used to transfer and express the TCR gene from cytotoxic T lymphocyte (CTL) clone M1F2. The first gene in each vector was driven by the vector long-terminal repeat (LTR), while the second gene was preceded by an I internal ribosome entry site (IRES) sequence or a PGK promoter. **B:** FACS profile of PG-13 packaging cells intracellularly stained with anti-Vβ-12 antibody. The percent positive staining was as indicated in parenthesis using antibody isotype controls for gating.

FIG. 2.

Transduction of human SupT1 T cell line. **A:** Fluorescence-activated cell sorter (FACS) histogram of CD3⁺ cells after transduction of Sup T1 cells by AIB or BIA retroviral vectors using supernatant from producer cell populations. The percentage positive cells were as indicated. **B:** FACS profile of MART-1 tetramer staining of Sup T1 cells transduced with no vector (NV), or supernatants from PG13 producer cell clones AIB 18 and AIB 54. The corresponding percent positive cells were as shown in parenthesis. Data are representative of multiple (> 10) analyses of MART-1 TCR vector-transduced Sup T1 cell populations.

FIG. 3. Transduction of primary human lymphocytes. **A:** Fluorescence-activated cell sorter (FACS) histogram of peripheral blood lymphocytes (PBL) transduced with either AIB 18 or AIB 54 retroviral vectors or untransduced and stained for V β 12 48 hr posttransduction. Percent positive cells were as indicated. **B:** FACS histogram of the same transduced cells but stained with MART-1 tetramer. Data are representative of four independent patient transductions.

FIG. 4.

Recognition of melanoma cell lines. Shown are cytokine production values (interferon [IFN]- γ top graph, granulocyte macrophage-colony stimulating factor [GM-CSF] bottom graph) of transduced peripheral blood lymphocytes (PBL) after coculture with melanoma cell lines. Vectors used for transduction of PBL were MSGIN (a control green fluorescent protein [GFP] vector), anti-MART-1 TCR vectors (AIB 18, BIA 71 and BPA 34), or a vector containing an anti-gp100 TCR (gp100). JB2F4 is a cytotoxic T lymphocyte clone that is highly MART-1-reactive. MEL 526 and 624 are HLA-A2-positive melanoma cell lines and 888 and 938 are HLA-A2-negative. Data are representative of four independent coculture experiments.

FIG. 5.

CD107a mobilization by TCR transduced peripheral blood lymphocytes (PBL). Fluorescence-activated cell sorter (FACS) analysis for degranulation marker protein CD107a was determined after coculture of AIB 18 vector-transduced PBL with no melanoma cells (No Mel), HLA-A2-negative melanoma line 888 (Mel 888), or HLA-A2-positive melanoma line 624 (Mel 624). Transduced cells were analyzed by gating for Vβ12 (**left panels**) followed by staining for CD3 and CD107a. The percentage of cell staining for both CD3 and CD107a were as indicated.

FIG. 6.

Lysis of melanoma cells by transduced peripheral blood lymphocytes (PBL). ^{51}Cr release assay was performed on PBL either transduced with AIB18 (anti-MART-1 TCR) or a no vector (NV) control population. After 4-hr incubation with ^{51}Cr -labeled melanoma cell lines the percent lysis was determined. MEL 526 and 624 are HLA-A2-positive and 938 is HLA-A2-negative. The effector to target ratio (E:T ratio) was as depicted on the x-axis. JB2F4 is a highly MART-1 reactive A2 restricted cytotoxic T lymphocyte clone. Data are representative of three independent experiments.

FIG. 7.

Lysis of melanoma cells by transduced tumor-infiltrating lymphocytes (TIL). TIL transduced with the anti-MART-1 AIB vector or mock transduced (NV) were tested for their ability to lyse ^{51}Cr -labeled melanoma cells. After a 4-hr incubation with ^{51}Cr -labeled melanoma cell lines the percent lysis was determined. JB2F4 is a highly MART-1-reactive cytotoxic T lymphocyte. Effector to target ratio (E:T ratio) was as depicted on the x-axis. MEL 526 and 624 are HLA-A2-positive and 938 is an HLA-A2-negative melanoma cell line. Transduced TIL were also tested for their ability to lyse a fresh (not cultured) HLA-A2-positive melanoma tumor explant (fresh A2+ tumor).

FIG. 8.

Proliferation of TCR-transduced peripheral blood lymphocytes (PBL) in melanoma cocultures. Shown are resultant fluorescence-activated cell sorter (FACS) histograms of carboxy-fluorescein diacetate, succinimidyl ester (CFSE)-labeled PBL cocultured with melanoma lines. PBL were either not transduced (NV) or transduced with MART-1 TCR (AIB) and then cocultured with melanoma lines 526 (A2+) or MEL 888 (A2-). Histograms of CFSE-stained and -unstained populations at time zero were as shown (**top**). Below are three columns each containing histograms from cocultures after 4 days in varying amounts of interleukin (IL)-2. Percent of cell proliferation was as indicated. AIB-transduced cells proliferated only in the presence of an HLA-A2-positive melanoma line.

TABLE 1

TRANSDUCED PBL SPECIFICITY

Stimulators						
Responder cells	Vector	T2 alone	T2+FLU	T2+gp100	T2+MART	
PBL	MSGIN	464	pg/ml IFN- γ 001	10,856	409	
APB 9	gp100	532	659	44,065	591	
AIB 18	MART	209	178	403	48,375	
BPA 34	MART	515	509	687	52,706	
BIA 71	MART	628	519	15,457	75,546	
PBL-MART	NONE	0	0	428	49,376	
PBL			pg/ml GM-CSF			
PBL	MSGIN	437	573	722	786	
APB 9	gp100	498	901	1200	715	
AIB 18	MART	567	702	738	5,100	
PBL-MART	NONE	1	3	45	2,200	
PBL	MSGIN	0	pg/ml IL-2 0	0	0	
APB9	gp100	0	0	0	0	
AIB 18	MART	0	0	0	72	
BPA 34	MART	0	0	0	78	
BIA 71	MART	0	18	0	189	
PBL-MART	NONE	0	0	25	122	
PBL	NONE	0	pg/ml TNF- α 0	0	9	
PBL	MSGIN	0	0	0	0	
APB9	gp100	0	0	287	0	
AIB 18	MART	0	0	0	2,552	
AIB 54	MART	0	0	0	1,941	
T2+MART	100 ng/ml	10 ng/ml	Stimulators (pg/ml IFN- α) 1 ng/ml	0.1 ng/ml	0.01 ng/ml	T2 alone
AIB 18	115	746	Responder 570	73	0	0
JB2F4	800	511	380	48	0	0

Cytokine production of transduced peripheral blood lymphocytes (PBL) in coculture with peptide pulsed T2 cells. MSGIN is a control vector, which contains green fluorescent protein. APB9 is a retroviral vector encoding the anti-gp100 TCR. AIB 18, BPA 34, BIA 71, and AIB 54 are anti-MART-1 TCR containing retroviruses. PBL-MART is a PBL that was stimulated in vitro with MART-1 peptide and JB2F4 is a MART-1-specific cytotoxic T lymphocyte; both used as positive controls.

IFN- γ , interferon- γ ; TNF- α , tumor necrosis factor- α ; IL, Interleukin-2; GM-CSF, granulocyte macrophagecolony stimulating factor.

TABLE 2
TRANSDUCED TIL SPECIFICITY

Responder cells	Vector	Stimulators			
		T2 alone	T2+FLU	T2+gp100	T2+MART
			pg/ml IFN- γ		
TIL	none	73	59	64	58
TIL	MSGIN	451	242	309	301
APB 9	gp100	95	63	11,345	100
AIB 18	MART	119	90	95	14,695
JB2F4	none	1	0	50	20,800
RC612	none	0	12	28,350	15
			pg/ml GM-CSF		
TIL	none	122	97	98	104
TIL	MSGIN	1820	1464	1833	1526
APB 9	gp100	502	409	36,329	532
AIB 18	MART	333	242	297	79,960
JB2F4	none	14	13	162	8042
RC612	none	25	170	1400	226
			pg/ml TNF- α		
TIL	none	15	7	9	10
TIL	MSGIN	21	27	21	16
AIB18	MART	6	306	518	1,568
JB2F4	none	3	508	896	2,835

Cytokine production from coculture of transduced TIL and peptide pulsed T2 cells. MSGIN is a control vector containing GFP. APB 9 is a retroviral vector encoding the anti-gp100 TCR. AIB 18 encodes the anti-MART-1 TCR. JB2F4 and RC612 are cytotoxic T lymphocytes, which are highly MART-1 and gp100 reactive, respectively.

TIL, tumor-infiltrating lymphocytes; IFN- γ , interferon- γ ; TNF- α , tumor necrosis factor- α ; IL; GM-CSF, granulocyte macrophage-colony stimulating factor.

TABLE 3
TRANSDUCED TIL MELANOMA REACTIVITY

Responder	Vector	Stimulators			
		MEL 526	MEL 624	MEL 888	MEL 938
TIL	none	22	pg/ml IFN- γ 22	24	42
TIL	MSGIN	14	13	58	13
AIB 18	MART	2139	1674	18	14
APB9	gp100	364	176	8	0
JB2F4	none	4900	8100	0	1
RC612	none	11,285	6650	0	0
TIL	none	34	pg/ml GM-CSF 32	44	84
TIL	MSGIN	29	35	437	122
AIB 18	MART	2802	3125	51	105
APB 9	gp100	2850	1057	101	136
JB2F4	none	4365	3961	3	86
RC612	none	1448	1464	8	84

Rescue of nonreactive TIL cultures. Cytokine production from coculture of transduced TIL and melanoma cell lines. Untransduced TIL or TIL transduced with GFP control vector (MSGIN), anti-MART-1 TCR vector (AIB18), or anti-gp100 TCR vector (APB9) were cocultured with the indicated cell lines (stimulators). MEL 526 and 624 are HLA-A2-positive melanoma lines while MEL 888 and 938 are A2-negative. JB2F4 and RC612 are cytotoxic T lymphocytes, which are MART-1 and gp 100 reactive, respectively.

TIL, tumor infiltrating lymphocytes; GFP, green fluorescent protein; IFN- γ , interferon- γ ; GM-CSF, granulocyte macrophage-colony stimulating factor.