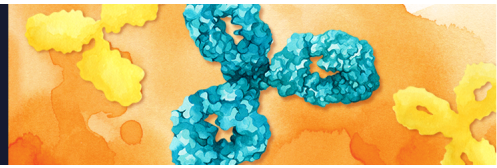


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# High Efficiency TCR Gene Transfer into Primary Human Lymphocytes Affords Avid Recognition of Melanoma Tumor Antigen Glycoprotein 100 and Does Not Alter the Recognition of Autologous Melanoma Antigens

Richard A. Morgan,<sup>1</sup> Mark E. Dudley, Yik Y. L. Yu, Zhili Zheng, Paul F. Robbins, Marc R. Theoret, John R. Wunderlich, Marybeth S. Hughes, Nicholas P. Restifo, and Steven A. Rosenberg

The  $\alpha$ - and  $\beta$ -chains of the TCR from a highly avid anti-gp100 CTL clone were isolated and used to construct retroviral vectors that can mediate high efficiency gene transfer into primary human lymphocytes. Expression of this TCR gene was confirmed by Western blot analysis, immunocytometric analysis, and HLA Ag tetramer staining. Gene transfer efficiencies of >50% into primary lymphocytes were obtained without selection for transduced cells using a method of prebinding retroviral vectors to cell culture vessels before the addition of lymphocytes. The biological activity of transduced cells was confirmed by cytokine production following coculture with stimulator cells pulsed with gp100 peptides, but not with unrelated peptides. The ability of this anti-gp100 TCR gene to transfer high avidity Ag recognition to engineered lymphocytes was confirmed in comparison with highly avid antimelanoma lymphocytes by the high levels of cytokine production (>200,000 pg/ml IFN- $\gamma$ ), by recognition of low levels of peptide (<200 pM), and by HLA class I-restricted recognition and lysis of melanoma tumor cell lines. CD4<sup>+</sup> T cells engineered with this anti-gp100 TCR gene were Ag reactive, suggesting CD8-independent activity of the expressed TCR. Finally, nonmelanoma-reactive tumor-infiltrating lymphocyte cultures developed antimelanoma activity following anti-gp100 TCR gene transfer. In addition, tumor-infiltrating lymphocytes with reactivity against non-gp100 melanoma Ags acquired gp100 reactivity and did not lose the recognition of autologous melanoma Ags following gp100 TCR gene transfer. These results suggest that lymphocytes genetically engineered to express anti-gp100 TCR may be of value in the adoptive immunotherapy of patients with melanoma. *The Journal of Immunology*, 2003, 171: 3287–3295.

**T**umor immunotherapy can be performed by direct immunization with tumor-associated Ags (TAA)<sup>2</sup> or by the transfer of immune-reactive cells, termed adoptive immunotherapy. Although immunization of cancer patients (using any number of different routes) has been demonstrated to elicit both humoral and cellular immune responses, tumor regression is rare and sporadic (1–3). In contrast, significant clinical benefit has been observed in a number of adoptive immunotherapy trials (4–7). Adoptive immunotherapy using tumor-reactive T cells in the setting of nonmyeloablative lymphodepleting chemotherapy was recently demonstrated to mediate the regression of established tumors in ~50% of malignant melanoma patients (8). Tumor regression is believed to be mediated by the recognition of melanoma TAA in the context of MHC class I molecules by T cells with the appropriate TCR genes. Melanoma tumor-infiltrating lymphocytes (TIL) have been shown to recognize a variety of TAA,

with melanoma Ag recognized by T cells-1 (MART-1) and gp100 being among the most frequently observed targets. The gp100 Ag is widely expressed in melanomas. In one study, reactivity with Ab HMB-45 (reactive with gp100) was present on 100% of non-spindle cell type melanomas and on 62 of 67 total melanomas (9). In another study, 32 of 35 melanoma studies (91%) expressed gp100 (10), and in a third study, 60 of 62 (97%) melanomas expressed gp100 (11). In addition to being widely expressed in melanoma cells, recognition of gp100 by HLA-A2-restricted TIL significantly correlated with clinical response to adoptive immunotherapy (12).

It is often not possible to resect sufficient tumor samples from melanoma patients to generate TIL. When tumor is available, it is not always possible to generate melanoma-reactive TIL cultures. As a potential alternative to the requirement to establish TIL cultures from melanoma patients, we sought methods that could be used to easily obtain a polyclonal population of T cells with anti-TAA properties. Transfer of Ag-specific TCR genes to PBL has recently been described as a potential method of generating large numbers of Ag-reactive T cells (reviewed in Refs. 13 and 14). In a murine model of this approach, T cells retrovirally transduced with gene-encoding TCRs could expand *in vivo* upon viral challenge, efficiently homed to effector sites, and mediated the rejection of Ag-expressing tumors (15).

We previously reported that retroviral vector-mediated transfer of anti-MART-1 TCR genes could endow human PBL with the ability to recognize MART-1-expressing cells in an HLA-A201-restricted manner (16). We have now extended those observations

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<sup>2</sup> Abbreviations used in this paper: TAA, tumor-associated Ag; MART-1, melanoma Ag recognized by T cells-1; IRES, internal ribosome entry site; LTR, long terminal repeat; PGK, phosphoglycerol kinase; TIL, tumor-infiltrating lymphocyte.

by showing that the transfer of the TCR genes encoding a highly avid anti-gp100 TCR could engineer a bulk population of PBL to recognize the gp100 Ag as evidenced by specific cytokine release and cell lysis. Furthermore, gene transfer into CD4<sup>+</sup> T cells demonstrated that this activity can occur in a CD8-independent fashion. Finally, anti-gp100 TCR gene transfer into TIL from a patient with non-gp100 autologous tumor reactivity could endow these cells with recognition of gp100 and not interfere with its native antitumor recognition. These results have significant potential impact on the ability to treat cancer patients using adoptive immunotherapy.

## Materials and Methods

### Cell lines

The cell lines used include: PG13 gibbon ape leukemia virus-packaging cell line (ATCC CRL 10,686), the human lymphoid cell line SupT1 (ATCC CRL-1942), and the human ecotropic packaging cell line, Phoenix Eco (kindly provided by G. Nolan, Stanford University, Stanford, CA). Cells were cultured at 37°C, 5% CO<sub>2</sub> in RPMI 1640 (Life Technologies-BRL, Gaithersburg, MD), in the presence of 10% FCS (Life Technologies). T2 is a lymphoblastoid cell line deficient in TAP function, whose HLA class I proteins can be easily loaded with exogenous peptides (17). HLA-A2-positive and A2-negative human melanoma cell lines expressing MART-1 and gp100 were established in the Surgery Branch from resected tumor lesions, as previously described (18). The 526 Mel (HLA-A2<sup>+</sup>), 624 Mel (HLA-A2<sup>+</sup>), 888 Mel (HLA-A2<sup>-</sup>), and 938 (HLA-A2<sup>-</sup>) lines were maintained in complete medium consisting of RPMI 1640 medium supplemented with heat-inactivated 10% FBS (Biofluids, Rockville, MD), penicillin (100 U/ml)-streptomycin (100 μg/ml), and L-glutamine (2.92 mg/ml) (Life Technologies). Cells were cultured at 37°C in a 5% CO<sub>2</sub> humidified incubator.

The CTL clones R6C12, CK3H6, and D4F12 are HLA-A2 restricted and gp100:209–217 specific. Their derivation has been described elsewhere (19). Briefly, PBLs from patients who had been previously vaccinated with the gp100:209–217 (210M) peptide were sensitized *in vitro*, then cloned by limiting dilution. The clones were expanded using a modification of the rapid expansion protocol of Riddell and Greenberg (20) with OKT3, IL-2, and irradiated allogeneic feeder cells.

### Construction of TCR-containing retroviral vectors

Isolation of poly(A)<sup>+</sup> RNA from R6C12 cells was accomplished using poly(A) pure mRNA purification kit (Ambion, Austin, TX). RT-PCR was used to isolate the individual α and β cDNAs while simultaneously introducing restriction enzyme sites to facilitate subsequent molecular cloning. Oligonucleotide primers used for vector APB were: (to amplify α-chain) ccaacagcgccctgtgagcttctgc + cagaggcaagactagctgaagagct and (to amplify β-chain) ttgctctagaagctccacatctgc + catcccaggtcgacctatcc. Oligonucleotide primers used for vector BIA were: (to amplify α-chain) aacagacctgatcgagctctctgc + cagaggcaagtgatgctgaagagct and (to amplify β-chain) ttgctctagaagctccacatctgc + catcccaggtcgacctatcc. The individual PCR products were inserted into the pCR2.1 vector using the TA cloning method (Invitrogen, Carlsbad, CA) to produce plasmids pCR2.1 TCR α and pCR2.1 TCR β. Following DNA sequence analysis, the individual TCR genes (TRAV19 and TRBV12–3) were inserted into two different retroviral vectors to produce GCsamgp100APB and GCsappgp100BIA (Fig. 1A), as follows. The β-chain was first combined with the human phosphoglycerol kinase (PGK) promoter (in plasmid pBS-PGK) to yield pBS-PGK TCR β. The DNA fragments containing the α and the PGK-TCR β1 sequences were then inserted into retroviral vector pGCsam (21) to yield the final vector pGCsamgp100APB (APB). To produce retroviral vector BIA, the α- and β-chains were first inserted into internal ribosome entry site (IRES) vector pPBS (22) to yield the product pPBS TCR β-IRES-α. The DNA fragment containing TCR β-IRES-α was then inserted into the retroviral vector of GCsap (23) to yield the product of pGCsappgp100BIA. Generation of PG13-packaging cell clones was initiated by starting a coculture of PG13 and Phoenix Eco cells. This coculture was then transfected with 50 μg of DNA for each construct, GCsamgp100APB and GCsappgp100BIA, using the GenePorter reagent (Gene Therapy Systems, San Diego, CA). After 14 days of coculture, Phoenix Eco cells were removed from PG13 cells using magnetic bead negative selection (with anti-LYT-2; Dynal, Lake Success, NY) and PG13 cell clones obtained by limiting dilution. Clones were expanded and high titer clones were selected by the dot-blot titration method (24). As estimated by physical titer determination using RNA dot blot, both vectors had approximately the same phys-

ical titer (relative to a known titer control vector) and were estimated by this method to be ~1 × 10<sup>6</sup> CFU/ml. Clones were determined to be producing biologically active retrovirus vector by transduction of SupT1 cells and analysis of FACS data (using anti-Vβ8). Southern blot analysis was used to confirm vector integration and copy number.

### Transduction of PBL

PBL were collected by leukopheresis, and lymphocytes were separated by centrifugation on a Ficoll/Hypaque cushion, washed in HBSS, then resuspended at a concentration of 1 × 10<sup>6</sup>/ml in AIM-V medium (Life Technologies) supplemented with 50 ng/ml OKT3, 300 IU/ml IL-2, and 5% human AB serum (Valley Biomedical, Winchester, VA). The lymphocytes were then plated at 1 × 10<sup>6</sup> cells/ml in 24-well plates (Costar, Cambridge, MA). The lymphocytes were cultured *in vitro* for 48 h before transduction. Following stimulation, lymphocytes were transduced with retroviral vectors by transfer to culture dishes that had been precoated with retroviral vectors. To coat culture plates with vector, nontissue culture-treated six-well plates (BD Labware, Franklin Lakes, NJ) were first treated with 25 μg/ml recombinant fibronectin fragment (RetroNectin; Takara, Otsu, Japan). To these plates was then added retroviral vector supernatant, and the plates were incubated at 32°C for 2 h, followed by overnight incubation at 4°C. The following day, plates were allowed to warm to room temperature, the supernatant was removed, and stimulated PBL was added to each well at 1 × 10<sup>6</sup> cells/ml, 4–6 ml/well. Cells were then incubated overnight at 32°C, and the procedure was repeated the following day (total of two transductions), after which time cells were expanded at 37°C in a 5% CO<sub>2</sub> incubator and split as necessary to maintain cell density between 0.5 and 4 × 10<sup>6</sup> cells/ml.

### Cytokine release assays

PBL cultures and TIL were tested for reactivity in cytokine release assays using commercially available ELISA kits (IFN-γ, GM-CSF, IL-2 IL-4, IL-10, and TNF-α; Endogen, Cambridge, MA). Cytokine release was measured with T2 cells either alone or pulsed with peptide (2 μg/ml, or as described in figure legends) in complete medium. Peptides were incubated with T2 cells for 3 h at 37°C, followed by washing (three times) before initiation of cocultures. For these assays, 100,000 responder cells (PBL) and 100,000 stimulator cells (T2) were incubated in a 0.2-ml culture volume in individual wells of 92-well plates. In experiments in which melanoma cells served as stimulator cells, 50,000 cells were used in the same volume. Stimulator cells and responder cells were cocultured for 24 h for all cytokines, except TNF-α, which was measured after 6 h of incubation. Cytokine secretion was measured in culture supernatants diluted as to be in the linear range of the assay.

### FACS analysis

Cell surface expression of TCRBV8, CD3, CD4, and CD8 molecules on PBL or TIL was measured by immunofluorescence using FITC- or PE-conjugated Abs, as directed by the supplier of anti-TCR BV8 (Immunotech, Westbrook, ME) and all others (BD Biosciences, San Jose, CA). For analysis, the relative log fluorescence of live cells was measured using a FACScan flow cytometer (BD Biosciences). For tetramer analysis, a commercially available anti-gp100 product was used (iTag MHC Tetramer; Beckman Coulter, Fullerton, CA). Intracellular cytokine staining was performed using BD FastImmune intracellular cytokine detection kits, as directed by the manufacturer (BD Biosciences).

### <sup>51</sup>Cr release assay

The ability of transduced PBL to lyse HLA-A2<sup>+</sup>, melanoma target cells was measured in <sup>51</sup>Cr release assays, as described (18). Briefly, 10<sup>6</sup> melanoma cells were labeled for 1 h at 37°C with 200 mCi of <sup>51</sup>Cr (Amersham, Arlington Heights, IL). Labeled target cells (5 × 10<sup>3</sup>) were incubated with effector cells at the ratios indicated in the text for 4 h at 37°C in 0.2 ml of complete medium. Supernatants were harvested and counted using a Wallac 1470 Wizard automatic gamma counter (Wallac, Gaithersburg, MD). Total and spontaneous <sup>51</sup>Cr release by each target was determined by incubating 5 × 10<sup>3</sup> labeled target cells in 2% SDS or medium, respectively, for 4 h at 37°C. Each point represented the average of quadruplicate wells, and percentage of specific lysis was calculated as follows:

$$\text{specific lysis} = \frac{\text{specific } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{total } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100$$

**Results**

*Vector construction and transduction of target cell line*

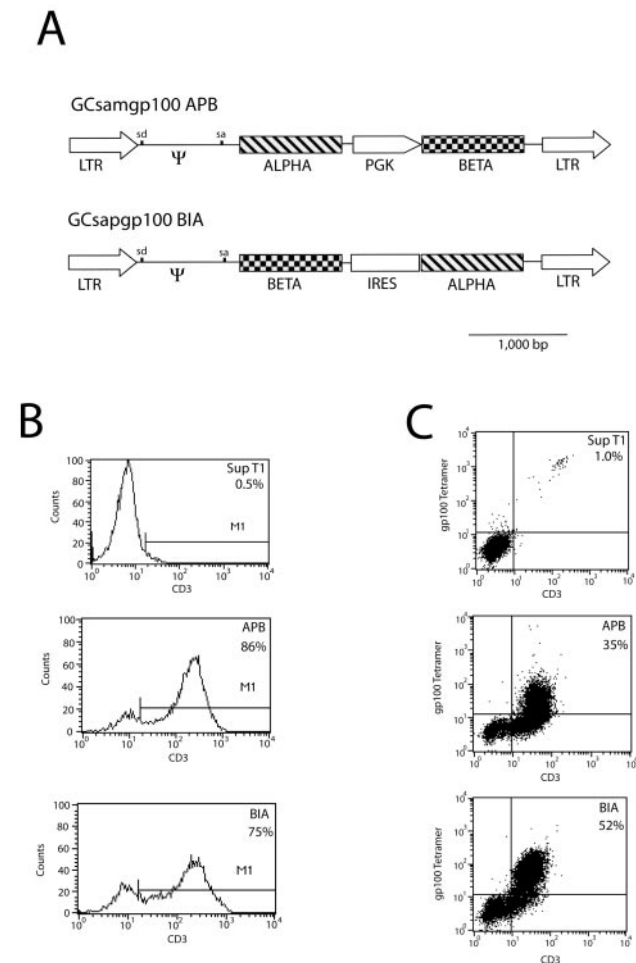
CTL line R6C12 is a highly avid CTL clone derived from the PBL of a melanoma patient who had been vaccinated with gp100 peptide (gp100:209–217 (210M)). To obtain the optimum expression of the TCR from this clone, two different retroviral vector designs were produced (Fig. 1A). In construct GCsamgp100APB (APB), the  $\alpha$ -chain cDNA expression is mediated by transcription from the retroviral long terminal repeat (LTR), while the  $\beta$ -chain is driven off an internal promoter derived from the human PGK gene. Construct Gcsapgp100BIA (BIA) uses the LTR to express a bicistronic RNA in which the expression of the second gene (in this case the  $\alpha$ -chain) is governed by an IRES.

As an initial test of the TCR vectors, we transduced the human T cell line SupT1, a human T cell leukemia cell line with chro-

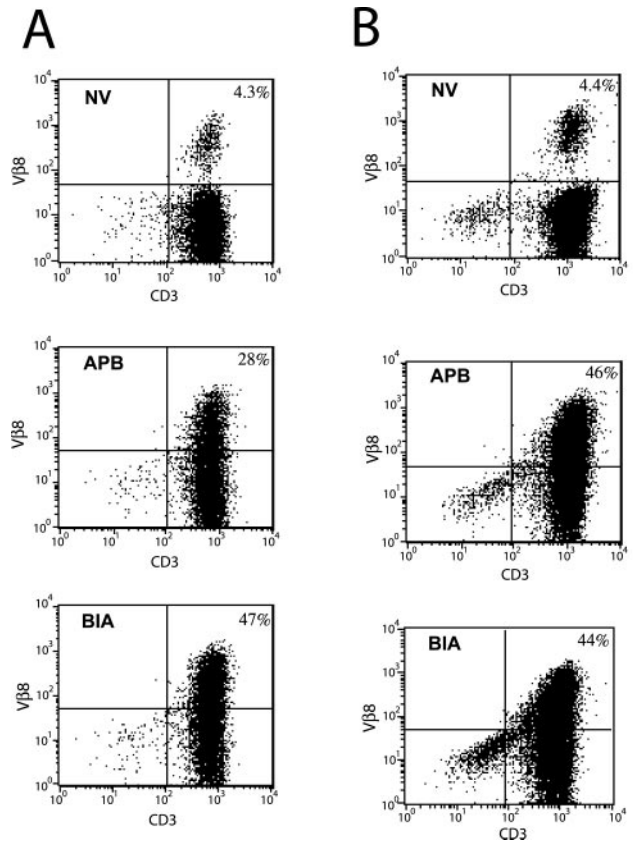
somal translocations involving both the  $\alpha$  and  $\beta$  TCR genes that prevents surface expression of the endogenous TCR complex. SupT1 cells were transduced with vectors APB and BIA and tested by Western blot analysis for expression of the  $V\beta 8$  protein, and both vector-transduced cell populations produced an appropriately sized protein (data not shown). Transduced cells were next assayed for cell surface expression of the CD3 protein. As expected from the lack of TCR  $\alpha$  and  $\beta$  expression by Sup T1, untransduced cells did not stain for CD3 (Fig. 1B). Significant CD3 expression was detected in vector-transduced cells (86 and 75% for APB- and BIA-engineered cells, respectively), suggesting successful assembly of the full TCR complex. To determine whether the transduced cells had the potential to recognize the gp100 peptide that was recognized by the native TCR in R6C12 cells, HLA-A2/peptide tetramer staining was performed (Fig. 1C). Cells positive for both CD3 and the gp100-specific tetramer were readily detected by FACS analysis of transduced cells.

*Transduction of PBL*

To engineer PBL with the anti-gp100 TCR vector, we used a modification of a procedure (25) that involves precoating tissue culture plates with Retronectin (a recombinant fibronectin molecule). This method allows for high efficiency retroviral vector-mediated gene transfer, while permitting the cultured T cells to remain in the appropriate growth medium because vector supernatant is removed before transduction. PBL were obtained from two patients, one from a fresh leukopheresis (patient 1) and the other from cryopreserved cells (patient 2). T cell cultures were initiated with IL-2 plus



**FIGURE 1.** TCR expressing retroviral vectors. *A*, Diagrams of the two retroviral vectors used to transfer and express the TCR gene from CTL clone R6C12. In vector GCsamgp100APB (APB), expression of the  $\alpha$ -chain is mediated by the vector LTR, while the  $\beta$ -chain expression is driven by an internal PGK gene promoter. In vector Gcsapgp100BIA (BIA), expression of both chains is mediated by the LTR, with translation coupled by the use of an IRES element. Location of retroviral vector-packaging signal ( $\psi$ ) and splice donor (sd) and splice acceptor (sa) sites is as indicated. *B*, FACS histogram of CD3-reactive cells from Sup T1 cells, or Sup T1 cells transduced with the APB or BIA vectors. The percentage of positive staining cells was as indicated. *C*, FACS profile of Sup T1 cells double stained with anti-CD3 Ab plus gp100 tetramer. Profile of Sup T1 cells, or Sup T1 cells transduced with the APB or BIA vectors is shown with the corresponding percentage of positive cells, as indicated.



**FIGURE 2.** TCR vector-transduced primary human PBL. FACS profiles of PBL from mock transduced (no vector, NV) or transduced with TCR vectors APB or BIA. Cells were double stained with anti- $V\beta 8$  plus anti-CD3, percentage of positive cells, as indicated. *A*, Patient 1. *B*, Patient 2.

Table I. Cytokine production of TCR-transduced PBL<sup>a</sup>

Responder Cells	Stimulators (pg/ml IFN- $\gamma$ released)				
	T2 alone	T2 + Flu	T2 + MART-1	T2 + gp100 (209)	T2 + gp100 (209-2M)
Patient 1					
No vector	7.5 (3.5)	10 (0)	12.5 (10.6)	0	0
YFP	28.8 (7.5)	106 (100)	62.5 (15.5)	10 (10)	13.8 (7.5)
APB	86.3 (15.5)	104 (17)	136 (35)	78,525 (5,610)	204,700 (12,757)
BIA	47.5 (19.4)	52.5 (34.8)	78.8 (14.9)	58,800 (24,528)	158,200 (109,419)
R6C12	0	0	0	41,390 (6,820)	67,040 (9,010)
Patient 2					
Stimulators (pg/ml GM-CSF released)					
No vector	3,875 (1,732)	4,000 (565)	3,400 (989)	4,275 (1,166)	5,350 (424)
YFP	4,750 (1,750)	6,550 (1,000)	6,750 (1,933)	5,025 (1,485)	6,764 (757)
APB	8,000 (1,156)	7,188 (826)	7,613 (1,780)	30,438 (3,996)	46,850 (3,513)
BIA	6,025 (2,514)	5,663 (1,251)	5,688 (1,083)	33,763 (9,080)	49,113 (4,655)
TIL	5,500 (919)	1,900 (1,060)	45,550 (9,687)	35,200 (4,808)	51,375 (1,450)

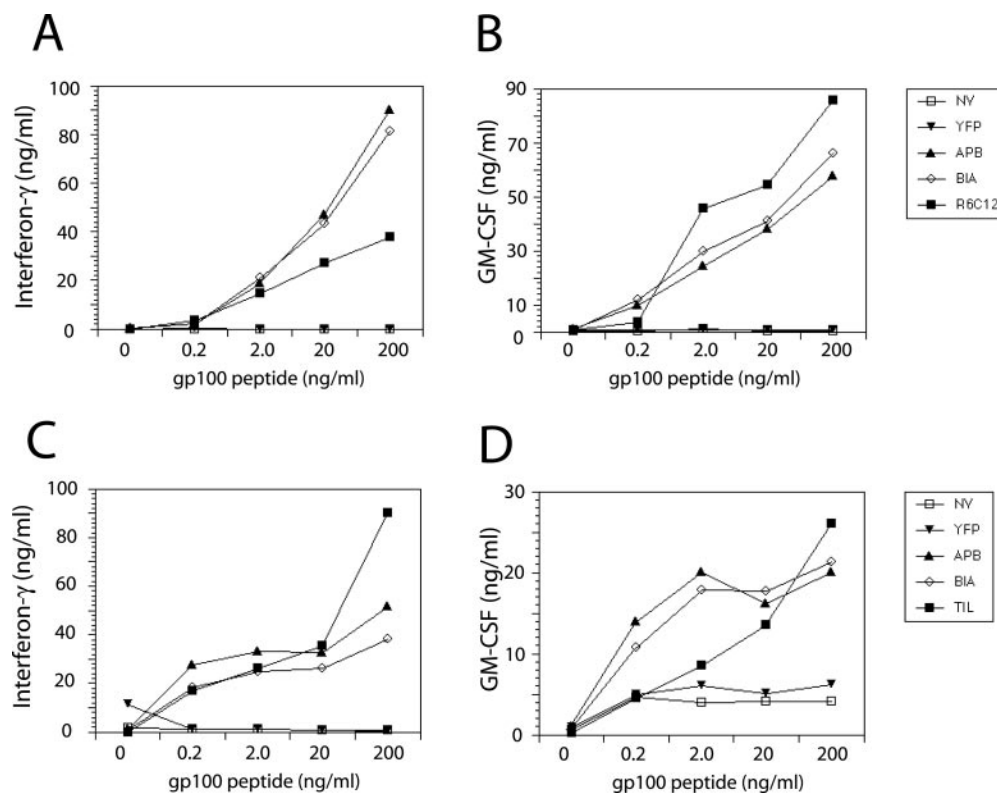
<sup>a</sup> Cytokine secretion of TCR vector-transduced PBL (responders) was exposed to T2 cells (stimulators) pulsed with HLA-A2-specific peptides for influenza virus (Flu) melanoma MART-1 (MART), gp100:209–217, or gp100:209–217 (210M). Data from PBL from patients 1 and 2 are the mean values of quadruplicate samples, with SD in parentheses. Control responder cells were CTL line R6C12 and a melanoma-reactive TIL culture from patient 2.

OKT3 Ab stimulation. Transduction was performed at day 2 post-stimulation, and 2 days later (4 day poststimulation), cells were stained for CD3 and V $\beta$ 8 and subjected to FACS analysis (Fig. 2). The percentage of V $\beta$ 8-positive cells in the transduced cell populations ranged from 40 to 60% (background staining for V $\beta$ 8 was ~4%). Gene transfer occurred equally in both CD4- and CD8-positive cells (data not shown). As a control for vector transduction, we transduced cells with a high titer YFP-expressing retroviral vector, and between 70 and 80% gene transfer was obtained (data not shown). The overall cell surface phenotype to the trans-

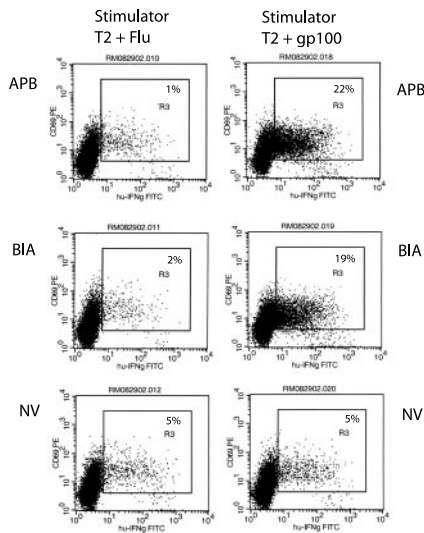
duced cell populations at 2–3 wk poststimulation is consistent with an Ag-experienced population (CD45RO<sup>+</sup>) of an intermediate effector-like phenotype (data not shown).

#### Cytokine release

To determine whether TCR vector-transduced PBL could mediate the release of effector cytokines IFN- $\gamma$  and GM-CSF, coculture experiments were performed. T2 cells were pulsed with HLA-A2-specific peptides derived from the influenza virus (Flu), the



**FIGURE 3.** Sensitivity of cytokine secretion to dilutions of gp100 peptide. Shown is the resultant cytokine secretion of TCR vector-transduced PBL exposed to T2 cells pulsed with dilutions of gp100 peptide. *A* and *B*, Data from PBL from patient 1. *C* and *D*, Data for patient 2. Samples were: no vector-transduced cells (NV), control YFP vector-transduced cells (YFP), anti-gp100 TCR vector-transduced cells APB and BIA, anti-gp100 CTL (R6C12), and antimelanoma TIL (TIL).



**FIGURE 4.** Intracellular cytokine staining of transduced CD8 cells. Shown are the resultant FACS histograms for control PBL from patient 1, or PBL transduced with TCR vectors APB or BIA. Cells were cocultured with T2 cells pulsed with either HLA-A2-specific influenza virus peptide (Flu) or gp100 peptide 209–217 (210M). Cells were gated for CD3+ plus CD8+ cells and then analyzed for CD69 plus intracellular IFN- $\gamma$  expression. The percentage of positive cells in the gated boxes was as shown.

MART-1:27–35 peptide, the native gp100:209–217 peptide recognized by R6C12 (gp100), or the anchor residue-modified peptide gp100:209–217 (210M). PBL cultures serving as responder cells were mock-transduced cells (no vector), control vector-transduced cells (YFP), or cultures of transduced with the anti-gp100 TCR vectors APB and BIA. As positive controls for cytokine release assay, the CTL clone R6C12 as well as a gp100-reactive TIL culture derived from melanoma patient 2 were used (Table I).

The TCR-engineered T cell cultures from patient 1 (both APB- and BIA-transduced populations) secreted high levels of the IFN- $\gamma$  in response to gp100 peptide-pulsed T2 cells (78,525 and 58,800 pg/ml for APB- and BIA-transduced cells, respectively) that were comparable to the R6C12 CTL clone. The amount of IFN- $\gamma$  produced following exposure to T2 cells pulsed with the anchor-modified peptide was between 158,200 and 204,700 pg/ml in this assay. Similar results were obtained by analysis of GM-CSF production using PBL from melanoma patient 2 (Table I). Although this patient’s cells had a higher background level of GM-CSF production, a 6-fold increase in GM-CSF production was demonstrated when TCR-engineered cells were incubated with gp100 peptide-pulsed T2 cells (~30,000 pg/ml of GM-CSF was

produced by the TCR-engineered cells). GM-CSF production of both TCR vector-engineered cell populations was comparable to a nontransduced melanoma-reactive TIL culture (this TIL also reacted to MART-1 peptide-pulsed cells). No significant differences in cytokine production were observed between mock-transduced and control vector-transduced cell populations. Analysis of other cytokines produced by the engineered PBL revealed no detectable expression of IL-4 or IL-10, with peptide-inducible low level expression of IL-2, IL-8, IL-13, RANTES, and TNF- $\alpha$  (data not shown). The relative avidity of the transduced PBL populations was determined by coculturing transduced cells with T2 cells pulsed with serial dilutions of the gp100 peptide (Fig. 3). The engineered PBL populations were capable of releasing cytokines at peptide concentrations as low as 0.2 ng/ml, and in the case of GM-CSF, released more cytokine at the lowest dilution than did the R6C12 and melanoma TIL cultures.

*Distribution of cytokine-producing cells in the TCR-engineered PBL population*

The data presented on cytokine release by the bulk engineered PBL populations cannot distinguish between cytokine expression evenly distributed throughout the transduced cell population or whether cytokine release is favored in a small percentage of engineered cells. We used intracellular cytokine staining followed by FACS analysis to determine the relative distribution of IFN- $\gamma$  expression in the transduced cell populations (Fig. 4). Transduced and control-engineered cell populations were cocultured overnight with gp100 peptide-pulsed T2 cells. Cells within the lymphocyte gate of the FACS were further gated for CD3+ and CD8+ cells, which were then analyzed for the expression of the T cell activation marker CD69 plus intracellular expression of IFN- $\gamma$ . Between 19 and 22% anti-gp100 TCR-engineered cells were double positive for CD69 and intracellular IFN- $\gamma$  compared with 1–2% of the control cocultures (transduced cell plus T2 pulsed with no peptide or the control Flu peptide). The distribution of staining for IFN- $\gamma$  was uniform, suggesting that IFN- $\gamma$  expression in the TCR gene-transduced cell populations was evenly distributed.

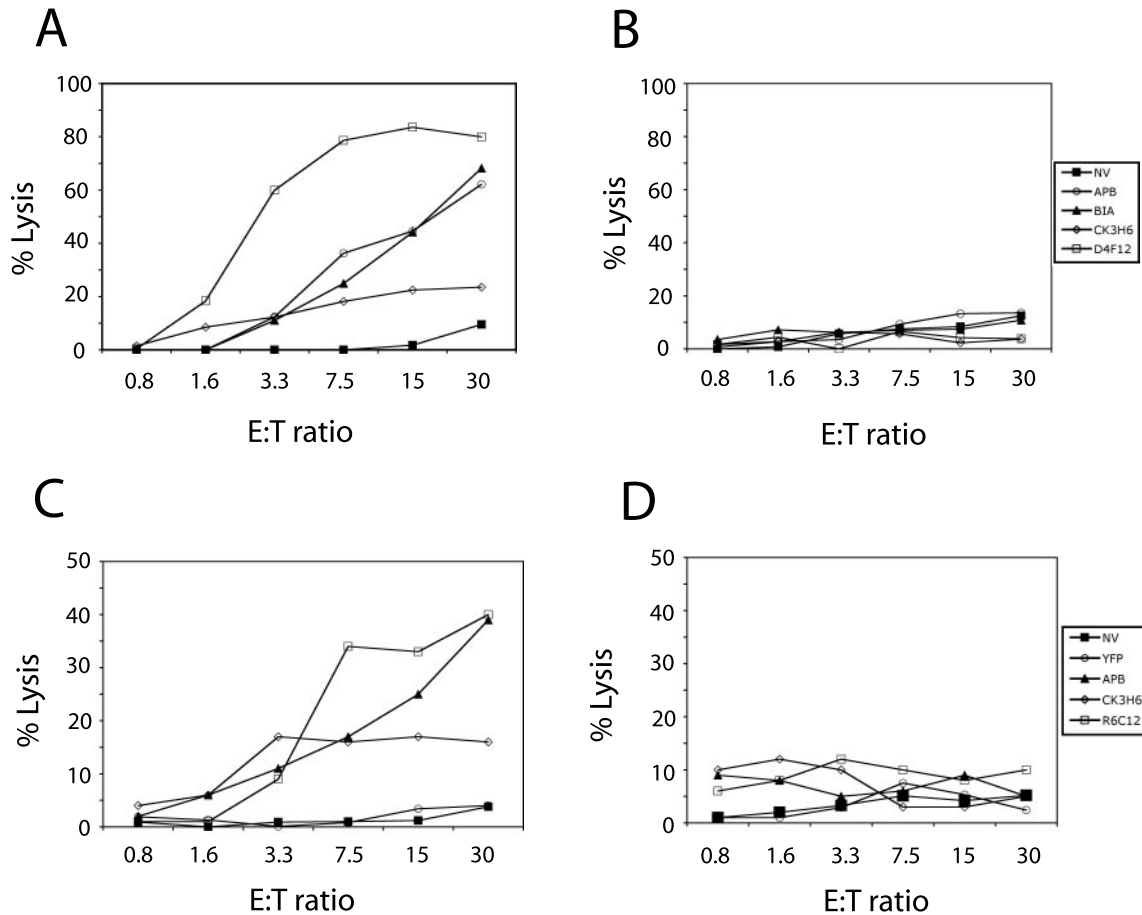
*Recognition of melanoma cells by TCR-transduced lymphocytes*

In potential clinical applications, it is essential that the TCR-engineered PBL recognize the naturally processed gp100 peptide as it is expressed on melanoma cells. To determine melanoma reactivity, two HLA-A2-positive melanoma cell lines and two non-HLA-A2 melanoma cell lines were cocultured with TCR and control vector-transduced PBL cultures from melanoma patient 2. Table II presents data on cytokine release from these T cell populations. HLA-A2-restricted IFN- $\gamma$  release (in the range of 866–2528 pg/ml) was demonstrated in both APB- and BIA-engineered PBL populations, with no specific production of IFN- $\gamma$  observed in

Table II. Melanoma reactivity of TCR-transduced PBL<sup>a</sup>

Responder Cells	Stimulators (pg/ml IFN- $\gamma$ released)				
	None	526 Mel (A2 <sup>+</sup> )	624 Mel (A2 <sup>+</sup> )	888 Mel (A2 <sup>-</sup> )	938 Mel (A2 <sup>-</sup> )
No vector	9 (12)	30 (1.4)	101 (91)	91 (22)	28 (40)
YFP	8 (9.7)	35 (12)	56 (18)	150 (75)	80 (98)
APB	80 (62)	2,528 (305)	1,614 (298)	245 (100)	63 (72)
BIA	146 (51)	1,437 (187)	866 (89)	247 (82)	103 (134)
TIL	56 (20)	2,713 (717)	2,240 (235)	10 (14)	21 (30)

<sup>a</sup> Cytokine secretion of TCR vector-transduced PBL from melanoma patient 2 (responders) was cocultured with the HLA-A2-positive cell lines 526 and 624 or HLA-A2-negative cell lines 888 and 938. Following coculture, IFN- $\gamma$  levels were determined in culture medium from no vector PBL, control YFP vector-transduced PBL, or TCR vector-transduced PBL (APB and BIA). Melanoma-reactive TIL from patient 2 were used as control responder cells. Data are the mean values of quadruplicate samples, with SD in parentheses.



**FIGURE 5.** Lysis of melanoma cell lines. PBL from patient 1 (A and B) or 2 (C and D) were tested in a  $^{51}\text{Cr}$  release assay. Transduced or control PBL (see legend) were cocultured with HLA-A2-positive melanoma cell line 526 (A and C) or non-HLA-A2 888 melanoma line (B and D) that had been labeled with  $^{51}\text{Cr}$ . Cells were incubated at the indicated E:T ratios for 4 h, after which the percentage of lysis of the target cells was calculated. The indicated CTL lines served as controls; see text for details. Samples were: no vector-transduced cells (NV), control YFP vector-transduced cells (YFP), anti-gp100 TCR vector-transduced cells APB and BIA, and anti-gp100 CTLs (R6C12, CK3H6, and D4F12).

the coculture with the gp100-expressing, HLA-A2-negative melanoma cell lines. The levels of cytokine production were comparable to control TIL cultures that produced 2240–2703 pg/ml of IFN- $\gamma$ . Two additional effector cytokines, GM-CSF and TNF- $\alpha$ , were also specifically released by TCR-engineered PBL (data not shown).

We next measured lysis of melanoma cell lines by the engineered PBL from both patients in a 4-h  $^{51}\text{Cr}$  release assay (Fig. 5). The TCR-transduced cells readily lysed the HLA-A2-positive cell lines with similar reactivity to CTL clones. There was little or no lysis observed in the non-HLA-A2 melanoma lines, and mock-transduced or control vector-transduced cell populations were nonreactive.

#### CD8-independent Ag recognition

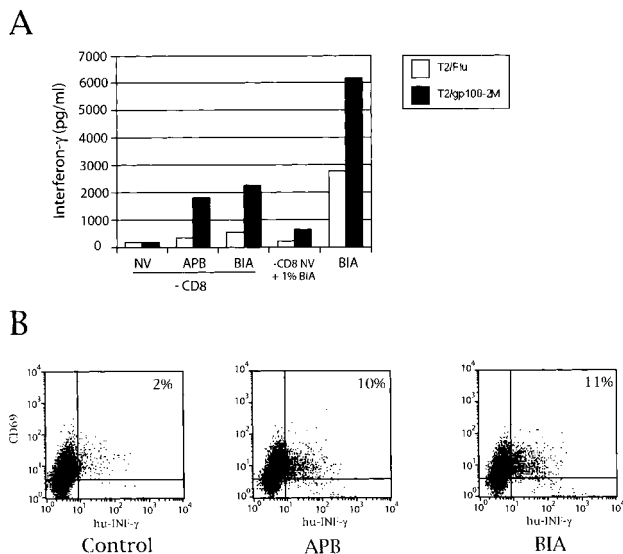
Because TCR gene transfer is evenly distributed into CD8 and CD4 cells, we investigated whether transfer of the anti-gp100 TCR into CD4 $^{+}$  T cells could impart gp100 peptide-mediated reactivity to these cells. Bulk populations of transduced PBL from patient 1 were depleted of CD8 cells using magnetic beads. Cultures that had >60% CD8 $^{+}$  T cells before depletion had less than 1% remaining CD8 $^{+}$  cells using this method. The CD4 $^{+}$ /CD8 $^{-}$  cultures were then cocultured with peptide-pulsed T2 cells, and IFN- $\gamma$  levels were determined following 24-h incubation (Fig. 6A). Peptide-specific cytokine release from the CD4 $^{+}$ /CD8 $^{-}$  lymphocytes was evident in the TCR-engineered cells (APB and BIA) at a level of

1800–2245 pg/ml, with 10-fold less cytokine release observed in the control-engineered cells. These CD4 $^{+}$  cell populations had 0.3% CD8 $^{+}$  T cells at the time of assay. As a control for the potential IFN- $\gamma$  production by a small number of CD8 $^{+}$  cells, 1% CD8 $^{+}$  BIA-engineered cells were spiked into mock-transduced (no vector) CD4 $^{+}$  cells and assayed. The amount of IFN- $\gamma$  produced by this control population (containing 3-fold more CD8 $^{+}$  cells than the TCR-transduced CD4 $^{+}$  cells) was 3 times less (648 pg/ml) than the TCR-engineered CD4 $^{+}$  cell cultures. This result suggested that the TCR-engineered PBL could recognize gp100 peptide in a CD8-independent fashion.

To further explore the possibility that TCR-engineered CD4 cells were capable of CD8-like effector functions, we used intracellular cytokine staining for IFN- $\gamma$  expression. TCR-transduced PBL gated of CD4 $^{+}$  cells and stimulated with peptide-pulsed T2 cells were then analyzed for expression of CD69 and for the intracellular expression of IFN- $\gamma$ . Data obtained in this experiment (Fig. 6B) demonstrate that between 10 and 11% of the APB and BIA TCR vector-engineered CD4 $^{+}$  T cells specifically produced IFN- $\gamma$  in this assay compared with a background level of 2%.

#### Rescue of nonreactive TIL cultures

To determine whether TCR gene transfer can be used to engineer Ag-nonreactive TIL to recognize melanoma targets, we transduced three nonreactive TIL cultures with the gp100 vector and tested



**FIGURE 6.** Cytokine production by TCR-transduced CD4 cells. *A*, PBL from patient 1 were depleted for CD8 cells using magnetic beads and CD4<sup>+</sup> cells cocultured with T2 cells pulsed with influenza peptide (Flu) or gp100 peptide 209–217 (210M). Twenty-four hours later, IFN- $\gamma$  production was determined. *B*, FACS profile of PBL from patient 2 following exposure to T2 cells pulsed with gp100:209–217 (210M). Untransduced cells (control) or TCR vector-transduced cells (APB and BIA) were gated for CD3 plus CD4-positive cells and then analyzed for CD69 plus intracellular IFN- $\gamma$  expression. The percentage of positive cells in the gated boxes was as shown.

them in 24-h coculture assays (Fig. 7). In each case, control-engineered TIL remained nonreactive to gp100 peptide-pulsed T2 cells, while the gp100-transduced cells from these three different nonreactive TIL cultures produced IFN- $\gamma$ , GM-CSF, and TNF- $\alpha$  when cocultured with appropriately peptide-pulsed T2 cells.

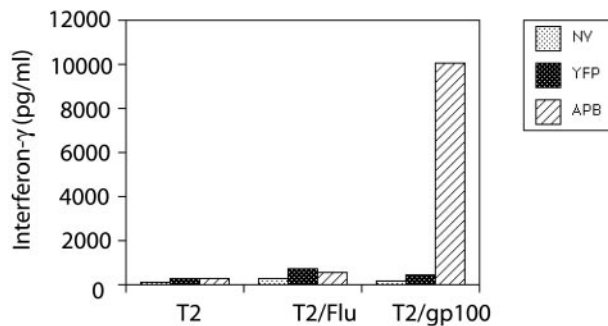
We next determined whether gp100 TCR gene transfer into TIL with native autologous tumor reactivity would negatively affect the autologous TIL reactivity. As seen in Table III, autologous tumor reactivity was not altered (as measured by cytokine release) by anti-gp100 TCR vector transduction. These gp100 TCR gene-transduced TIL now recognized HLA-A2-matched (gp100-expressing) melanoma tumor cell lines (526 and 624), but not non-HLA-A2-matched melanoma lines (888 and 938). These data suggest that it is possible to engineer TIL to be reactive with shared melanoma Ags while not affecting pre-existing recognition of additional tumor Ags.

## Discussion

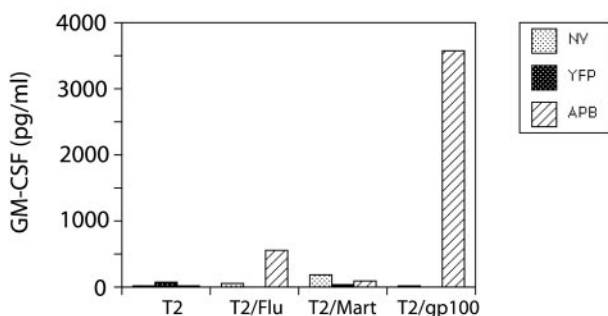
TCR genetic engineering has been suggested as a way to bypass tolerance to TAA. To be effective in the clinical setting, this approach must be reproducible, specific, scalable, and safe. Recent concerns about the safety of retroviral vector-mediated gene transfer have been raised following the report of two insertional mutation events associated with the development of leukemia in children 3 years posttreatment for X-SCID (26). Any future clinical proposal to pursue the approaches described in this study would take place in an adult patient population with a limited life span (the 5-year survival of patients with metastatic melanoma is less than 2%) in which the risk of insertional mutagenesis may be acceptable to properly informed patients.

Although many cellular components are potentially responsible for the avid recognition of TAA, the TCR is the main component of this activity (27, 28). We thus chose as starting material a CTL

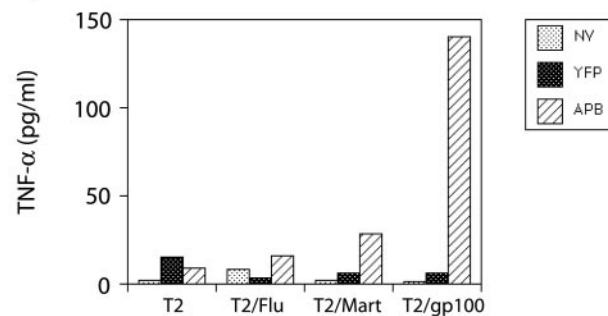
## TIL patient 3



## TIL patient 4



## TIL patient 5



**FIGURE 7.** Rescue of nonreactive TIL cultures. TIL cultures from three melanoma patients were tested and determined to be nonreactive to HLA-matched melanoma cell lines. TIL were transduced with control YFP vector or TCR vector APB and then cocultured with peptide-pulsed T2 cells. Shown are representative cytokine productions for each patient following coculture. Peptides used were HLA-A2-specific peptides for influenza virus (Flu) melanoma Ag MART-1 (MART) or gp100:209–217.

clone R6C12 with highly avid antimelanoma reactivity. As a vector platform to transfer the TCR genes, we chose retroviral vectors GCSam and GCSap, which we had previously reported mediated high levels of expression of a variety of genes in human primary PBL (21, 23). Two different arrangements of the individual TCR chains were investigated in the two vector designs. In vector APB, the  $\alpha$ - and  $\beta$ -chains were expressed from independent promoters, while in the vector BIA, the expression of the two chains is coupled by an IRES. In multiple FACS analysis of transduced Sup T1, PBL, and TIL, expression of the V $\beta$ 8 was comparable using the two different vectors, suggesting that either vector design was capable of both high level gene transfer and expression of biologically active TCR genes in transduced human cells. We obtained comparable results that additional vectors designed to express an anti-MART-1 TCR gene (data not shown).



Table III. *Lack of interference with native melanoma reactivity*

Responder TIL	Stimulators (pg/ml IFN- $\gamma$ released)				
	Auto Mel	526 Mel	624 Mel	888 Mel	938 Mel
No vector	3,509 (129)	380 (33)	550 (50)	641 (13)	561 (48)
YFP	3,269 (178)	209 (31)	316 (8)	411 (206)	336 (65)
APB	3,369 (132)	3,930 (257)	2,579 (193)	310 (33)	282 (23)
Responder TIL	Stimulators (pg/ml GM-CSF released)				
	Auto Mel	526 Mel	624 Mel	888 Mel	938 Mel
No vector	1,190 (102)	19 (3)	66 (13)	120 (9)	67 (15)
YFP	1,325 (143)	15 (14)	54 (10)	88 (12)	45 (1)
APB	1,795 (192)	995 (86)	841 (186)	31 (14)	8 (6)

<sup>a</sup> TIL from a melanoma patient 5 that were reactive with autologous tumor, but not with HLA-A2-matched melanoma cell lines were transduced with TCR vector APB and tested for reactivity. Responder TIL were cocultured with the HLA-A2-positive autologous tumor cell line (Auto Mel), or HLA-A2-positive cell lines 526 and 624, or HLA-A2-negative cell lines 888 and 938. Following coculture, IFN- $\gamma$  and GM-CSF levels were determined in culture medium from no vector TIL, control YFP vector-transduced TIL, or TCR vector-transduced TIL (APB). Data are the mean values of quadruplicate samples, with SD in parentheses.

Anti-gp100 TCR-engineered primary lymphocytes secreted large amounts of effector cytokines IFN- $\gamma$  and GM-CSF in an Ag-specific fashion (Table I). They exhibited an affinity for Ag comparable to R6C12 CTL or a highly avid TIL culture based on assays using limiting concentration of stimulating peptide (Fig. 3). The anti-gp100 TCR-engineered lymphocytes were also effective at recognizing the gp100 protein expressed and processed in melanoma cell lines, as demonstrated by both cytokine production and lysis of tumor cells (Table II; Fig. 5). As a final potential measure of avidity, we were able to demonstrate specific cytokine production by CD4<sup>+</sup> T cells engineered with the anti-gp100 TCR gene (Fig. 6). Although recognition of peptide-pulsed T2 cells may not reflect potential *in vivo* situations, this result does suggest that engineered CD4<sup>+</sup> T cells can recognize the appropriate gp100 peptide presented by class I HLA-A2 molecules in a CD8-independent fashion, and is another measure of the high avidity afforded by transfer of this TCR gene.

The retroviral vector-mediated gene transfer method used in this study involved preattachment of viral vector particles to the culture vessel. The use of recombinant fibronectin fragments required to precoat vector to culture vessels is available as a GMP quality reagent and has been used in human gene therapy clinical trials (29, 30). Using this method, we routinely achieved transduction efficiencies of >50% of the T cells in a bulk population of PBL. This high level of transduction is essential for the ability to scale these approaches for potential clinical applications and has additional benefits. T cell expansion can be compromised by the exposure of T cells to large amounts of retroviral vector supernatant, which is the long-term culture medium from the packaging cell lines and has both been depleted of essential growth factors and contains cellular waste products that inhibit T cell growth. In addition, retroviral vector supernatant generally is produced in cell lines cultured in the presence of calf serum, and exposure of human cells to bovine serum proteins has been demonstrated to elicit an antibody immune response in patients receiving cells cultured in the presence of these products (31). The ability to remove excess retroviral vector supernatant from the T cell culture medium lessens the possibility of unintended immune responses to components found in the retroviral vector supernatant and enables the continued culture of lymphocytes in optimal growth medium.

Although adoptive transfer of tumor-reactive TIL has been shown to mediate cancer regression in several clinical trials, it is not possible to isolate sufficient tumor samples from all potential melanoma patients, and even when tumor is available, it is not always possible to obtain melanoma-reactive TIL cultures. It has

been our experience that ~50% of TIL cultures were not reactive against common tumor targets and/or against autologous tumors. Although these tumor-derived cells may retain the cell surface molecules required for tumor targeting, they often lack anti-TAA activity. In this work, we demonstrate, for the first time, that non-melanoma-reactive TIL can be rescued (for tumor reactivity) by anti-gp100 TCR gene transfer (Fig. 7; Table III).

The transfer of an additional copy of a TCR gene into a T cell with an existing Ag-specific TCR could potentially influence the ability of the native TCR to recognize its Ag. If the individual TCR chains are free to randomly associate with one another, then mixed TCR complexes would most likely form. Such a possibility may not cause significant problems in Ag recognition, as multiple  $\alpha$ -chains can naturally be found in T cells and only a few productive TCR-Ag interactions are required to elicit an effector response (32, 33). To address this possible consequence in the setting of an anti-TAA T cell response, we engineered a TIL cell line with autologous tumor Ag reactivity with the anti-gp100 TCR vector. In this experiment, the engineered cells gained anti-gp100 reactivity, and there was no change in the ability of these engineered cells to produce effector cytokine IFN- $\gamma$  or GM-CSF in response to coculture with autologous melanoma cells (Table III). By use of anti-gp100 TCR gene transfer, we thus have demonstrated that the non-gp100-reactive TIL can be rescued, and were able to produce effector cytokines in response to stimulator cells displaying the shared melanoma Ag gp100. Furthermore, the ability to target multiple TAA could potentially avoid tumor immune escape mutants that may potentially compromise the effectiveness of adoptive immunotherapy (34).

There have now been several reports concerning anti-TAA receptor gene transfer using either native TCRs (including gp100) (16, 28, 35–37) or chimeric receptors (e.g., single-chain Abs fused to the CD3 $\zeta$  protein) (38–40). In comparison with chimeric receptor approaches, the transfer of native TCRs may have distinct advantages. In the chimeric receptor approach, the high affinity of single-chain Abs for Ag will most likely result in qualitative and quantitative differences in T cell activation that may not be optimal, and moreover, it is unclear whether chimeric receptor Ag engagement results in immunological synapse formation. Furthermore, from the standpoint of clinical applications, chimeric receptor approaches could be significantly limited by potential immune responses to novel peptide domains found in the chimeric molecules. The success of any of these approaches will most likely require the reconstitution of a highly avid T cell response against the target Ag. Using a native TCR with high avidity (such as the

TCR from CTL clone R6C12) may be the critical determinant for successful transfer of antitumor properties to nontumor-reactive lymphocytes, as the TCR is the main determinant of avidity, and high avidity is correlated with *in vivo* antitumor activity (41).

As a potential alternative to the requirement to establish TIL cultures from melanoma patients, we sought methods that could be used to easily obtain a polyclonal population of T cells (such as PBL) with anti-TAA properties. The use of retroviral vector-mediated gene transfer of anti-TAA TCR genes, similar to results reported in this work, can potentially have immediate clinical applications in adoptive immunotherapy trials. Furthermore, if the appropriate TCRs can be identified for more common tumor Ags, such as NY-ESO-1, this approach may be applicable to a variety of common epithelial malignancies.

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