Recognition of Fresh Human Tumor by Human Peripheral Blood Lymphocytes Transduced with a Bicistronic Retroviral Vector Encoding a Murine Anti-p53 TCR

Cyrille J. Cohen,* Zhili Zheng,* Regina Bray,* Yangbing Zhao,* Linda A. Sherman,† Steven A. Rosenberg,* and Richard A. Morgan1* The p53 protein is markedly up-regulated in a high proportion of human malignancies. Using an HLA-A2 transgenic mouse model, it was possible to isolate high-avidity murine CTLs that recognize class I-restricted human p53 epitopes. We isolated the α- and β-chain of a TCR from a highly avid murine CTL clone that recognized the human p53264–272 epitope. These genes were cloned into a retroviral vector that mediated high efficiency gene transfer into primary human lymphocytes. Efficiencies of >90% for gene transfer into lymphocytes were obtained without selection for transduced cells. The p53 TCR-transduced lymphocytes were able to specifically recognize with high-avidity, peptide-pulsed APCs as well as HLA-A2.1+ cells transfected with either wild-type or mutant p53 protein. p53 TCR-transduced cells demonstrated recognition and killing of a broad spectrum of human tumor cell lines as well as recognition of fresh human tumor cells. Interestingly, both CD8+ and CD4+ subsets were capable of recognizing and killing target cells, stressing the potential application of such a CD8-independent TCR molecule that can mediate both helper and cytotoxic responses. These results suggest that lymphocytes genetically engineered to express anti-p53 TCR may be of value for the adoptive immunotherapy of patients with a variety of common malignancies. The Journal of Immunology, 2005, 175: 5799–5808.

The p53 protein has been extensively studied as a potential target for cancer therapy (1) because it is mutated in approximately half of all human cancers, leading to a prolonged protein half-life and subsequent overexpression (2). Additionally, mutant p53 is thought to have a dominant transforming function in vivo, resulting in selection pressure to maintain its expression throughout tumor growth (3, 4).

Immunogenicity of p53 was first suggested to occur in certain cancer patients for which the presence of Abs to p53 correlated with p53 mutations in lung cancer (5). Cellular immune responses specific to p53 have been reported in both cancer patients and healthy individuals (6–10). Immune responses against p53 were generated in colorectal cancer patients after vaccination with recombinant vaccinia virus engineered to express the p53 protein (11). In addition, CTLs recognizing the wild-type (wt) p53264–272 epitope could be generated using in vitro peptide-stimulated PMBCs from both healthy donors and cancer patients (12, 13). Despite these observations, it has not been possible to isolate highly avid cytotoxic human CTLs to p53 epitopes, probably due to tolerance to self-Ags (14, 15).

As an alternative approach, two groups have demonstrated that it is possible to circumvent this issue and generate potent CTL responses against human p53 epitopes by using a transgenic mouse model that expresses the human class I HLA-A2 molecule (16–18). One major advantage of this approach is the possibility to isolate highly avid lymphocytes that recognize their target in a CD8-independent manner because the binding contribution of murine CD8 to the α3-chain of the HLA-A2 molecule is minimal (19). Murine CTLs isolated in such transgenic hosts could recognize and kill tumor cell lines.

Cellular adoptive immunotherapy has been shown to mediate the regression of large solid tumors in patients with metastatic melanoma (20, 21). However, this strategy is limited by the need to isolate and expand antitumor reactive lymphocytes that pre-exist in the patient (22). Gene modification of T lymphocytes (23, 24) may overcome the requirement for pre-existing tumor-specific immunity. We and others (25–28) showed that it is possible to efficiently transduce human PBLs with retroviral vectors that encode both chains of a specific TCR, thus conferring to the transduced lymphocytes a novel antitumor specificity. In the present work, we demonstrate that primary human T lymphocytes engineered to express a murine anti-human p53 TCR can recognize both tumor lines and fresh human tumors and are able to kill p53-expressing human tumors, suggesting the potential for the direct clinical application of this approach.

Materials and Methods

Patient PMBCs and cell lines

All of the PMBCs used in this study were from metastatic melanoma patients treated at the Surgery Branch, National Cancer Institute (NCI, National Institutes of Health, Bethesda, MD). The cell lines used in experiments include the PG13 gibbon ape leukemia virus packaging cell line (CRL-10686; American Type Culture Collection (ATCC)), the human ecotropic packaging cell line Phoenix Eco provided by Dr. G. Nolan (Stanford University, Stanford, CA), and the human lymphoid cell line Sup T1 (CRL-1942; ATCC). HLA-A2.1/p53+ cell lines 526 and 624 were generated at the Surgery Branch (NCI) as previously described (29); 6647 and BIC-1 cell lines were a gift from Dr. H. Khong (Surgery Branch, NCI); BE-3 is a gift from Dr. D. Schrump (Surgery Branch, NCI); H2087 (CRL-5922; ATCC); HepG2

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p53 TCR GENE TRANSFER

The retroviral backbone used in this study, pMSGV1, is a derivative of the MSCV-based splice-gag vector (pMSGV), which uses a murine stem cell virus (MSCV) long terminal repeat (34) and has been previously described (16).

TCR-α and β-chain cDNAs were amplified by PCR using the following pairs of oligonucleotide primers: α primer forward 5'-TAAGCCTGTTGC GACTAGTATTACCGGCTCCCCTCCAGTGC3'- and reverse 5'-TAAC GCCTCAGTGAAAAGGAGCTGCATGCTG-3'; β primer forward 5'-CCAGATTTGCTGTCAGAAGGTCATCTGTAATGCATC-3' and reverse 5'- TGGCAAGAATTCATCATATCTTCTCTTCTTGGATACGCATG-3' to introduce appropriate restriction enzyme sites for cloning. Vector was assembled by ligation of four DNA fragments: pMSGV1 (Ncol/HindIII), TCR-α cDNA (AluIII/XbaI), internal ribosomal entry site (IRE5) (35) (XbaI/ClaI), and TCR-β cDNA (ClaI/HindIII). Resulting vector (MSGp53AB) construction was confirmed by DNA sequencing. pMSGIN (GIN), is derived from the pMSGV vector and contains the GFP-IRE5-Neo genes (25).

Generation of PG13 packaging cell clones was initiated by transfection of Phoenix Eco cells with 10 μg of DNA for MSGp53AB construct using the Lipofectamin reagent (Invitrogen Life Technologies). At 48 h after transfection, supernatant of the Phoenix Eco cell culture was used to infect PG13 cells. At 48 h after the infection, the transgene expression was tested by intracellular staining with anti-tCR Ab (H57-597; BD Biosciences). PG13 cell clones were obtained by limiting dilution. Clones were expanded and high titer clones were selected by the dot-blot titration method (36). Clones were determined to be producing biologically active retrovirus vector by transduction of Sup T1 cells and analysis of FACS data (using anti-mouse TCR and anti-CD3). Southern blot analysis of packaging cell clones used to confirm proper vector integration and copy number.

The wt and mutant (H143, H175, H273) p53 encoding expression plasmids were a gift of Dr. C. Harris (NCI, Bethesda, MD).

Transduction of PBL

PBLs were collected by leukopheresis, and lymphocytes were separated by centrifugation on a Ficoll/Hypaque cushion, washed in HBBS, then resuspended at a concentration of 1 x 10^7 cells/ml in AIM-V medium supplemented with 5% human serum, 50 ng/ml OKT3, 300 IU/ml IL-2, and plated at 1 x 10^6 cells/ml in 24-well plates (Costar). 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 vector. Supernatant of the Phoenix Eco culture was used to infect PBLs by transduction of PBLs with vector, nontissue culture-treated 6-well plates (BD Biosciences). PG13 cell clones were obtained by limiting dilution. Clones were expanded and high titer clones were selected by the dot-blot titration method (37). To coat culture plates with vector, nonnissure culture-treated 6-well plates (BD Labware) were first treated with 25 μl of recombinant fibronectin fragment (RetroNectin; Takara Shuzo). To these plates was 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 PBLs were added to each well at 1 x 10^5 cells/ml. 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% CO2 incubator and split as necessary to maintain cell density between 0.5 and 3 x 10^6 cells/ml.

FACS analysis

Cell surface expression of mouse TCR, human CD3, CD4, CD8, CD107a, and HLA-A2.1 molecules on PBL was determined by Ab staining (FITC, PE, Cy5, or allophycocyanin-conjugated Abs; BD Biosciences). p53 accumulation was determined using an PE-labeled Ab for intracellular staining (BD Biosciences). All intracellular FACS staining was done with the Cytofix/Cytoperm Plus kit (BD Pharmingen). Immunofluorescence, analyzed as the relative log fluorescence of live cells, was measured using a FACSScan flow cytometer (BD Biosciences). A combination of forward angle light scatter and propidium iodide staining was used to gate out dead cells. Approximately 1 x 10^6 cells were analyzed. Cell death was determined in a FACs buffer made of PBS (BioWhittaker) and 0.5% BSA.

Cytokine release assays

PBL cultures were tested for reactivity in cytokine release assays using commercially available ELISA kits (IFN-γ, IL-2, GM-CSF; Endogen). T2 cells were pulsed with peptide (1 μg/ml or as described with figures) in medium for 3 h at 37°C, followed by washing (three times) before initiation of coculture. For these assays, 1 x 10^5 responder cells (PBL) and 1 x 10^5 stimulator cells (T2, tumor lines or fresh human tumor digest) were incubated in a 0.2ml culture volume in individual wells of 96-well plates. Stimulator cells and responder cells were cocultured for 24 h. Cytokine

Electroporation of PBLs

In vitro-transcribed RNA for both α and β TCR chains was generated using mMESSAGE mMACHINE ULTRA (Ambion) and purified using the RNAeasy mini kit (Qiagen). PBLs were collected by leukopheresis, and lymphocytes were separated by centrifugation on a Ficoll/Hypaque cushion, washed in HBBS, then resuspended at a concentration of 1 x 10^7/ml in AIM-V medium supplemented with 5% human serum, 50 ng/ml OKT3, 300 IU/ml IL-2, and plated at 1 x 10^6 cells/ml in 24-well plates (Costar). The lymphocytes were then cultured for at least 1 wk with the addition of new medium (without OKT3) as needed to maintain cell density of 10^6 cells/ml. Electroporation was performed as follow: the lymphocytes were washed in 45 ml of OPTI-MEM (Invitrogen Life Technologies) and resuspended at 2.5 x 10^7 cells/ml. Cells were transferred in 2-mm cuvettes (50–200 μl of cell suspension), chilled on ice, and then electroporated at 300 V/500 μF with a Square Wave Pulse Source ECM 830 (BTX). The amount of in vitro-transcribed RNA for each chain was 2 μg per 1 ml PBMC. Following electroporation, cells were transferred to 6-well plates containing fresh medium and cultured at 37°C.

Cloning of murine p53-specific, HLA-A2-restricted TCR-α and TCR-β cDNA

Total RNA was extracted with TRIzol Total RNA Isolation Reagent (Invitrogen Life Technologies) from CTL clone p53/264.15. One microgram of total RNA was used to clone the TCR cDNAs by a RACE method (GeneRacer kit; Invitrogen Life Technologies). Before synthesizing the single strand cDNA, the RNA was dephosphorylated, decapped, and ligated with an RNA oligonucleotide according to the instruction manual of the 5'-RACE GeneRacer kit. SuperScript III RT and GeneRacer Oligo(dT) were used for reverse-transcribing the RNA oligonucleotide-ligated mRNA to single strand cDNAs. 5'-RACE was performed using the 5'-GeneRacer primer and 3'-primer of gene-specific primer TCR-CαRev (5'-ACTG GACCAAGCAGGCTGCCCAGTTC3'); TCR-βRev (5'-TGATATTCTT GTTTTGACTGACATCCATGC3'); or TCR-Cβ2Rev (5'-GGAAATT TTTCTGACCACTGCGGAG3') as 3' primers for murine TCR α-, β-, or β2-chain, respectively. The PCR products were cloned into pCR2.1 TOPO GeneRacer (Invitrogen Life Technologies). Plasmid DNA were prepared from 32 individual clones, 16 clones from TCR-α chain cDNA, and 16 clones from TCR-β chain cDNA. Full-length assembly of all 32 plasmids was confirmed by sequencing.

Construction of retroviral vectors

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secretion was measured in culture supernatants diluted as to be in the linear range of the assay. Additional cytokine (IL-4, IL-6, IL-10, IL-12p70, TNF-α) and chemokine (RANTES, MIP-1α) release was determined by Ab array-based analysis (SearchLight; Pierce Biotechnology).

**CD107a mobilization**

The cell surface mobilization of the CD107a Ag was determined as a measure of degranulation and functional reactivity (38). In brief, 50,000 tumor 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 2–4 h. PBL were removed and stained with Abs for mouse TCR, CD107a and analyzed by FACS.

**51Cr release assay**

The ability of the transduced PBL to lyse HLA-A2+/p53+ targets was measured using a 51Cr release assay as described in detail in prior publications (29). Briefly, 1 × 106 tumor cells were labeled for 1 h at 37°C with 50 μCi of 51Cr (Amersham Biosciences). Labeled target cells (5 × 105) 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. Harvested supernatants were counted using a Wallac 1420 Wizard automatic gamma counter. Total and spontaneous 51Cr release were determined by incubating 5 × 105 labeled targets in either 2% SDS or medium for 4 h at 37°C, respectively. Each data point was done as an average of quadruplicate wells. The percentage of specific lysis was calculated as: (specific 51Cr release – spontaneous 51Cr release)/total 51Cr release × 100.

**CFSE labeling of transduced PBL for proliferation analysis**

To study the proliferation of transduced PBL, cells were labeled with CFSE as described by the manufacturer (Molecular Probes) and cultured in RPMI 1640/FCS media. One-half million CFSE-labeled PBLs (mock-transduced or p53 TCR-transduced) were added to 105 melanoma cells in a 24-well plate. CFSE was examined at 488 nm wavelength with a 525 nm band-pass filter using a BD Biosciences FACSscan.

**CD4/CD8 separation**

CD4+ and CD8+ populations were separated using a magnetic beads-based approach for both negative and positive selection of those subsets (Dynal Biotech).

**Results**

**Cloning of functional p53-specific TCR, retroviral vector construction and analysis**

We have previously reported that murine anti-human p53 CTL were generated by vaccination of HLA-A2 transgenic mice with human p53-specific peptides (16). The α- and β-chain encoding a p53-specific TCR from the murine clone p53/264.15 were isolated using a 5’-RACE approach. The function of the TCR αβ pair was tested by RNA electroporation of stimulated primary PBL. Over 95% gene expression was achieved when in vitro-transcribed TCR α- and β-chain RNAs were electroporated into PBL (Fig. 1A). To test the function of the TCR RNA transfected PBLs, T2 cells were pulsed with peptide and cocultured with p53 TCR, RNA electroporated PBLs. Ag-specific recognition was detected by IFN-γ secretion (Fig. 1B). These functional HLA-A2-restricted and p53-specific TCR α- and β-chain cDNAs were used for retroviral vector construction.

A bicistronic retroviral vector design was chosen to coexpress both α- and β-chain in a single gene transfer vector (25, 26). The murine stem cell virus-based vector MSGV1 was used as a backbone to drive the expression of the α gene with the β gene governed by an IRES (Fig. 1C). The organization of vector MSGp53AIB was confirmed by DNA sequencing. Six PG13 retroviral vector packaging cell clones that express p53 TCR encoding retroviral particles were isolated based on physical titer and were used to transduce the human T cell line Sup T1. CD3 and mouse TCR expression were determined by FACS analysis and ranged from 35 to 90% (data not shown). Clone PG-MSGp53AIB/B10, which exhibited both high physical titer and high transduction efficiency, was chosen for further study.

To engineer PBL with the anti-p53 TCR vector, we used a method of preloading vector onto culture plates followed by the addition of stimulated T cells. Transduction was started at day 2–3

**FIGURE 1.** Functional analysis of p53 TCR. A, RNA electroporation of stimulated PBL. PBLs stimulated with OKT3 Ab plus IL-2 were electroporated with in vitro-transcribed RNA at 2 μg/μl × 105 cells. Twenty hours after electroporation, murine TCR expression was determined by FACS analysis for PBLs electroporated with RNA encoding p53 TCR that were stained with anti-murine TCR (1), with isotype control (2), and for non-electroporated cells stained with anti-murine TCR (3). B, PBLs were electroporated with p53 TCR RNA and cocultured with peptide-pulsed T2 cells. Peptides used were HLA-A2-restricted p53264–272 and NY-ESO-1157–165V. C, Schematic representation of the retroviral vector encoding the p53 TCR. LTR, Long terminal repeat.
poststimulation with anti-CD3 Ab, and 2 days after the last transduction cells were harvested, stained for mouse TCR, and subjected to FACS analysis. A representative analysis is shown in Fig. 2 (background staining for mock-transduced PBL using antimouse TCR was <2%). High transduction efficiencies, ranging from 70% to 97% without any selection were observed in PBLs from four different donors (Fig. 2). TCR expression was maintained in culture for most of the donors for up to 2 mo (data not shown) without the need of further selection or specific stimulation.

**Recognition of peptide-pulsed and p53-transfected cells by TCR-transduced PBLs**

To determine whether TCR-transduced PBL could mediate the release of effector cytokines, PBLs were transduced with retroviral vector particles produced by the packaging cell clone PG-MSGp53AI/B10. p53 TCR-transduced lymphocytes were then cocultured with T2 cells that were pulsed with different concentrations of specific (p53<sub>264–272</sub>) or control peptides (NY-ESO-1, gp100, MART-1, HBV) to assess the avidity of the transduced PBLs (Fig. 3A). Ag-specific IFN-γ release (up to 81,500 pg/ml) was detected in cocultures with T2 cells pulsed with the specific p53 epitope but not in cocultures with control peptides (at 1 µM) or no peptide (the average background for those control cocultures was 480 pg/ml). The engineered PBL populations were capable of releasing IFN-γ 2-fold above the background at peptide concentration as low as 1 pM.

To determine the relative reactivity of transduced PBL to endogenously processed p53, we transfected CosA2 cells with wt and mutant p53 encoding vectors. Twenty-four hours after transfection, p53-expressing cells were cocultured with p53 TCR or mock-transduced PBLs. IFN-γ concentrations 3-fold above the background were demonstrated in cocultures with p53-transfected cells (Fig. 3B). p53 mutant 273 showed minimal IFN-γ production (relative to background), possibly due to the fact that mutation in the 273 residue of p53 (which is adjacent to recognized epitope 264–272) has been shown to alter recognition of this particular epitope (39). The relatively high background observed in untransfected or GFP-transfected CosA2 cells may be due to the fact that CosA2 naturally express a high level of p53 and that the endogenous pri-mate p53<sub>264–272</sub> epitope is identical with the human one.

**Recognition of tumor cell lines by TCR-transduced PBLs**

It has been reported that 50% of all tumors express high levels of p53 (2). We screened a panel of different tumors for HLA-A2 and p53 expression (data not shown) and used these tumor cell lines in

![FIGURE 3](image-url)
coculture assays with p53 TCR-transduced PBLs or control mock-transduced PBLs. HLA-A2 and p53 double positive tumors including melanoma, hepatoma, sarcoma, small-cell lung cancer, and esophageal and breast tumors specifically stimulated TCR-transduced T cells to secrete cytokines IFN-γ/H9253 and IL-2 (Fig. 5).

However, no significant secretion was observed in control cocultures with either HLA-A2/H11001/p53/H11002 or HLA-A2/H11002/p53/H11001 cell lines. The recognition was HLA-A2-specific as confirmed by blocking experiments using an anti-HLA-A2 Ab (data not shown). Additional secretion of cytokines (IL-4, IL-10, GM-CSF, TNF-α) and chemokines (RANTES, MIP-1α) was also observed in cocultures with HLA-A2+/p53+ tumor lines (data not shown).

Ag-specific proliferation of TCR-engineered PBLs

As previously described, we observed relatively high levels of IL-2 secretion by p53 TCR-transduced lymphocytes following coculture with specific tumor cell lines. To examine whether TCR-transduced cells proliferated in vitro upon stimulation by an appropriate Ag, we labeled p53 TCR- or mock-transduced PBLs with CFSE dye and cultured these cells in the presence of p53+ tumor cell lines. The proliferation of the lymphocytes was determined after 3 and 6 days by FACS analysis, in which dilution of CFSE (measured as decrease of cell fluorescence) was indicative of cell proliferation.

FACS analysis (Fig. 6) of TCR-engineered PBLs cocultured with HLA-A2+/p53+ cell lines (H2087 and MDA-MB-231) demonstrated Ag-specific proliferation after days 3 and 6 (Fig. 6, C and D, respectively), where 69 and 32% (respectively) of the lymphocytes underwent cell division by 6 days of culture (Fig. 6D) vs 5% in coculture with Saos 2 cells (HLA-A2+/p53−). These results were compared with a control p53 TCR-engineered PBL cocultured with no targets. In parallel, no significant proliferation was observed in cocultures of mock-transduced PBLs (Fig. 6, A and B). These results demonstrated that the TCR-engineered PBLs were able to proliferate in vitro in response to p53+ tumor cell lines.

**FIGURE 6.** In vitro proliferation of TCR-engineered PBLs. Human PBLs expressing the p53 TCR were labeled with CFSE and cocultured with tumor cell lines. Ag-specific proliferation was measured after 3 (A and C) and 6 (B and D) days as shown by decreasing CFSE fluorescence of mock-transduced (A and B) or p53 TCR-expressing (C and D) PBLs cocultured with Saos 2, H2087, MDA-MB-231, or no target.

**FIGURE 7.** Functional reactivity of p53 TCR-transduced lymphocytes. Expression of CD107a (degranulation marker) was detected by FACS analysis on the surface of human PBLs expressing the p53 TCR after 2 h coculture with the indicated tumor cell line (control: A–D; p53+/HLA-A2+: E–K). The percentage of p53 TCR+/CD107a+ cells was as shown.
CD107a mobilization and cell-mediated cytotoxicity assays

To determine the functional reactivity of transduced T cells, we performed a CD107a mobilization assay (Fig. 7). p53 TCR-transduced lymphocytes were incubated with different tumor cell lines for 2–4 h. After the coculture, the lymphocytes were analyzed for mouse TCR and CD107a Ag expression. Significant anti-CD107a staining (ranging from 10 to 53% of the mouse TCR gated population) was observed on the p53 TCR-transduced PBLs after coculture with HLA-A2+/p53− tumor lines. In parallel, no significant staining was seen for transduced PBLs that were cocultured with control tumor cell lines.

We also measured lysis of tumor cell lines by the engineered PBL in a 4-h 51Cr release assay (Fig. 8). p53 TCR-transduced, but not mock-transduced PBL could efficiently kill HLA-A2.1/p53− expressing tumors (H2087, MDA-MB-231, Saos 2/#143, BE-3). There was little or no lysis of HLA-A2+/p53− (MDA 386) or HLA-A2−/p53− (Saos 2) tumor cell lines (Fig. 8A). In addition, we also tested for specific lysis of normal tissues by p53 TCR-transduced lymphocytes. There was little or no lysis of the normal renal cells ELW91, resting or activated normal PBLs compared with control HLA-A2−/p53− H2087 tumor (Fig. 8B).

Recognition of fresh human tumor cells

The potential application of p53 TCR-engineered PBLs in clinical settings requires recognition of p53 expressed by primary human tumors. Briefly, nine HLA-A2+ and four HLA-A2− fresh melanoma tumor samples were cocultured with p53 TCR-transduced lymphocytes for 24 h. Six of the HLA-A2+ samples (Fig. 9) stimulated the secretion of IFN-γ by the p53-engineered PBLs, whereas three HLA-A2+ as well as all four HLA-A2− (control) samples did not elicit any cytokine production. This experiment underscores the clinical relevance and application of p53 TCR-engineered PBLs.

p53 TCR-transduced CD4 lymphocytes recognize and kill tumor lines

This murine anti-p53 TCR is able to act in a CD8-independent manner (16, 19). To determine the biological activity of lymphocyte subsets, p53 TCR-transduced human PBLs were sorted into CD4+ and CD8− population. After coculture with different target tumor lines and fresh human tumor cells, significant IFN-γ secretion (Fig. 10A) as well as other cytokines (IL-4, IL-10, GM-CSF, TNF-α, data not shown) was demonstrated. Furthermore, p53 TCR-transduced CD4+ and CD8− cells were able to efficiently lyse tumor cell lines that were positive for HLA-A2 and p53 but not control cell lines (Fig. 10, B–E). These results demonstrated that it was possible to engineer CD4+ lymphocytes with a CD8− independent TCR to recognize and kill human p53+ tumor lines. Specific IFN-γ secretion was usually lower for CD4+ than for CD8− subset (Fig. 10A) possibly related to metabolic differences between those two subsets (CD8 and CD4) rather than to poorer target recognition. Indeed, we observed increased secretion of IL-4 and IL-10 by CD4+ cells compared with CD8+ cells (data not shown).

Discussion

In this work, we demonstrate the ability to engineer human PBLs to express a murine TCR that recognizes an HLA-A2.1-restricted epitope derived from the p53 protein. We constructed a single retroviral vector that contains both α- and β-chain and can mediate genetic transfer of this TCR with high efficacy (>90%) without the need to perform any selection. This work confirms and extends a previous report using a similar TCR that used two separate vectors encoding the α- and β-chains of the TCR and that required multiple-vector transductions as well as extensive antibiotic selection process to produce similarly engineered cells (40). Extensive ex
vivo T lymphocyte culture and antibiotic selection is known to impair T cell function and alter TCR repertoire (41, 42). Furthermore, human and nonhuman lymphocytes engineered to express foreign proteins can be eliminated by the in vivo induction of CTL directed against these non-self Ags (43, 44). Our simplified vector system has considerable advantages for clinical application where minimal culture times and the lack of selection schemes will be essential to minimize these potential complications. The use of a single vector also decreases the potential for insertional mutagenesis (45).

We observed higher transduction efficiencies with this retroviral vector compared with previous studies in which human TCRs were gene-transferred into PBLs (25–28). This can be due to the fact that we selected a high-titer producer cell clone. Moreover, we speculate that transduced murine TCR receptors may be expressed more efficiently in human lymphocytes than transduced human

**FIGURE 10.** Recognition and killing of tumor by transduced CD4+ cells. A, Different subsets (CD8+, CD4+, or bulk population) of human PBLs expressing the p53 TCR were cocultured for 16 h with the indicated peptide-pulsed APCs, tumor cell lines, or fresh human tumor samples (patient no. 7, HLA-A2+ and patient no. 12, HLA-A2+). The concentration of IFN-γ secreted in the medium was measured by ELISA. B–E, Different T cell subsets (CD8+, CD4+, or bulk population) of human PBLs expressing the p53 TCR were cocultured for 6 h with different tumor cell lines previously labeled with 51Cr. Specific lysis of Saos 2 (B), H2087 (C), MDA-MB-231 (D), JY (E) was measured at the E:T ratio indicated using: \([(\text{specific release} - \text{spontaneous release})/\text{total release} - \text{spontaneous release}]\).
TCR because of the potentially restricted pairing between the murine constant regions compared with their human counterparts. Another possible explanation might be a better stabilization of the murine TCR-human CD3 complex through noncovalent interactions (46).

Human PBLs expressing this anti-p53 TCR recognized with high affinity the p53264–272 epitope presented by peptide-pulsed T2 cells and p53-transfected cells. The ability of these p53 TCR-transduced T cells to recognize peptide at 1 pM is an indication of the high avidity of this receptor and is significantly better than the results recently reported by Kuball et al. (40). In addition, p53 TCR-engineered cells were able to specifically recognize and kill a broad spectrum of p53-expressing tumor cell lines. T cell recognition did not require pretreatment of the target cells with TNF or IFN-γ, in contrast with previous reports (16, 17) that used cytokine incubation to enhance recognition or killing of the tumor cells. We have also shown the ability of these p53 TCR-expressing PBLs to mobilize CD107α (degranulation marker) demonstrating the functional reactivity of the transduced T cells (38). p53 TCR-transduced PBL showed the ability to proliferate in the context of p53264/HLA-A2+ tumor cell lines. This was consistent with the ability of these T cells to synthesize IL-2 following coculture with p53-expressing targets (Fig. 5). The ability of TCR gene-transduced human T cells to proliferate in response to tumor Ags may be essential to future clinical applications. In a mouse model of TCR gene therapy (47), Ag-driven expansion of TCR gene-modified T lymphocytes was essential to the antitumor response. In vivo expansion of transduced cells following Ag exposure in patients may be critical as proliferation of adoptively transferred tumor-infiltrating lymphocytes (TIL) appears to be associated with the ability of TIL to mediate tumor regression (20).

Because the 264–272 targeted sequence is a wt p53 epitope and as p53 message is widely expressed in most tissues of the body (48), there may be the potential for autoimmune manifestations caused by the adoptive transfer of anti-p53 T cells. This potential may be considered unlikely because wt p53 is expressed at very low levels in normal tissues (48–50), and in several normal tissues tested (PBMCs and fibroblasts, Fig. 4; normal renal cells, activated or resting PBLs, Fig. 8D), we did not observe significant reactivity of p53 TCR-engineered cells. Furthermore, in the few clinical reports in which anti-p53 responses were noted, autoimmune manifestations were not reported (11, 51). We have targeted a wt p53 epitope because the same p53 mutations are not common to different types of tumors (52, 53). Overexpression of wt p53 epitopes on cancer cells provides an ideal opportunity for differential recognition of malignant vs normal tissues. Data from different groups reveal that CTL immunity already exists at tumor sites (10) or can be raised in human T cell cultures against the same epitope (p53264–272) used in the present study (7, 12, 39, 54–56) and that CTL immunity constitutes a naturally processed epitope. Furthermore, an inverse correlation was observed when comparing the presence of CTLs that recognize the p53264–272 epitope with p53 level of expression by the tumor in patients who have squamous cell carcinoma of the head and neck, suggesting that lymphocytes endowed with that specificity may play an in vivo role in the eradication of tumor cells expressing this epitope (9, 12).

The present study is the first to demonstrate the p53-mediated recognition of fresh human tumor samples from melanoma patients by transduced lymphocytes. p53 expression in melanocytic skin lesions varies considerably in different studies from a small percentage of positively stained cells (57) to >50% of tumor cells (58, 59). However, different studies support the notion that p53 alterations may play a role in later stages of melanoma development and progression (60, 61). This idea could explain the relatively high percentage of recognition (60%) of fresh human tumor samples we have seen because most of the fresh tumors examined in this study come from patients in the later stages of their disease (Fig. 9).

Interestingly, both CD8+ and CD4+ subsets were capable of recognizing and killing target cells, stressing the potential application of such a CD8-independent TCR molecule that can mediate not only helper, but also cytotoxic responses (Fig. 10). This result is in contrast to a previous study that used a comparable model and failed to show direct evidence of CD4+ T cells cytolysis (40). Although both this report and the previous study presumably used a similar TCR, we were able to demonstrate not only CD4 killing, but also reactivity at 1 pM of peptide, proliferation in response to Ag exposure, and recognition of primary human tumor explants. The increased reactivity of our vector system may relate to the fact that we used an enhanced single-vector design that expresses both α- and β-chain TCR. This system affords high levels of gene transfer, which do not require prolonged selection of the transduced lymphocytes that could have impaired the biological activity in their studies.

The T cell reactivity by the transduced CD4+ cells may be especially important because CD4 helper responses can be essential for mediating efficient antitumor activity in general (62–66) and more specifically in a p53 model (8). In addition to their helper functions, CD4+ lymphocytes have also been shown to exhibit cytotoxic activity in several experimental systems (67–69) and more recently in vivo (70, 71). The use of a CD8-independent TCR for gene transfer into lymphocytes was also useful in a recent study in which a TCR from a CD4+ TIL specific for a class I melanoma epitope (33) was expressed in lymphocytes, conferring to both CD8+ and CD4+ subsets the capability to recognize melanomas (27). Another approach to generate such molecules that could mediate specific MHC-peptide complex recognition in a CD8-dependent manner would be to use Ab fragments with TCR-like specificity. Such targeting moieties were recently isolated (72) and shown to trigger recognition of target cells when expressed in lymphocytes (73).

Our recent clinical studies have shown that the adoptive transfer of autologous TIL with antitumor activity could mediate objective cancer regressions in 50% of patients with metastatic melanoma refractory to other treatments (20–22). The results obtained with the p53-restricted TCR discussed in this study represent an opportunity to extend cell transfer therapy to patients with common epithelial malignancies.

Disclosures
The authors have no financial conflict of interest.

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