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Targeting of Human p53-overexpressing Tumor Cells by an HLA A*0201-restricted Murine T-Cell Receptor Expressed in Jurkat T Cells[1]

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ABSTRACT

A potent anti-human (hu) p53 $CD8^+$ CTL response develops in HLA A*0201 transgenic (Tg) mice after immunization with peptides corresponding to HLA A*0201 motifs from hu p53. Mice immunized with the hu $p53_{149–157}$ peptide develop a CTL response that is of moderately high affinity and is capable of recognizing hu tumor cells expressing mutated p53. In this report, the mRNAs encoding the predominantly expressed T-cell receptor (TCR) sequences were molecularly cloned from a murine (mu) CTL clone derived from immunized Tg mice, which recognized endogenously processed hu p53 restricted by HLA A*0201. The separate A and B chain TCR cDNAs were transfected in the corresponding TCR A^- and B^- Jurkat-$CD3^-$ mutant T-cell lines, and each rescued CD3 surface expression. Both TCR chains were simultaneously introduced into Jurkat-$CD3^+$ cells, and the transfected Jurkat cells recognized hu T2 cells sensitized with the $p53_{149–157}$ CTL epitope but not T2 cells sensitized with a nonspecific CTL epitope. Breast, pancreatic, and sarcoma tumor cell lines, which overexpress endogenous mutated p53, were recognized in the presence of anti-CD28 costimulation, only if they also expressed HLA A*0201. Normal hu fibroblasts established from skin cultures were not recognized. These results represent the first time that a p53-specific TCR capable of recognizing hu cancer cells was heterologously expressed in a naive recipient cell, converting that cell to one recognizing hu tumor cells with mutated p53. This TCR represents a candidate molecule for a genetic strategy in combating hu cancer by an adoptive immunotherapy approach, which uses the strong xenorecognition of hu p53 in mice.

INTRODUCTION

p53 acts as a checkpoint for cell division in most eukaryotic cells and is normally expressed at low levels with rapid decay kinetics. Mutations in p53 characterize a large proportion of hu[3] solid tumors. These mutations, which abrogate the function of p53 as a suppresser of cell division (1), are associated with a prolonged half life and much higher nuclear and cytoplasmic concentration of the p53 protein (2). The mutations are generally single-base missense (3), and the remainder of the overexpressed p53 molecule is wt. Unfortunately, the immune response to most tumors that aberrantly express p53 is not adequate to prevent their unrestricted growth. Either tolerance or other immune-based selective mechanisms may be responsible for inadequate host defense against these tumors.

Because the p53 gene product is overexpressed in a large proportion of all solid tumors, it provides an ideal target for enhancement of the T lymphocyte anticancer immune response. What makes wt p53 epitopes such an attractive target for an adaptive immune response is that the intracellular concentration of p53 is normally very low (4), and cells expressing normal p53 at low levels will most likely escape an enhanced immune response to overexpressed p53.

mu experimental models have clearly demonstrated the ability to target overexpressed p53 as a means of achieving tumor rejection (5, 6). Furthermore, they have established that tumor rejection can be achieved without autoreactivity to cells that express normal levels of p53 (7). It remains a challenge to translate these findings into an effective immunotherapy strategy for hu malignancy because of the relatively poor hu immune response to p53-overexpressing tumors (8). By contrast, mice generate a vigorous response to both mutant and nonmutant p53 epitopes (7, 9, 10). Consequently, it is a realistic goal to overcome the weak hu immune response to p53 by adapting to hu T cells the favorable characteristics of the powerful mu immune response to p53.

The TCR is the surface molecule on T cells that recognizes processed antigen, either self or allo forms. The A/B TCR has been recognized as the main form of the TCR that recognizes alloantigen, in contrast to the G/D form, and it initiates a cascade in which cells expressing the recognized antigen are cytolytically attacked or growth restricted by the actions of T cell-secreted lymphokines. We and others have shown that adoptive immunotherapy using A/B TCR expressing cytotoxic T cells will augment the eradication of tumor in a SCID model (11, 12). The capability of CTLs to cause tumor regression is thought to be far greater than a humoral response or a $CD4^+$-mediated Th infiltration of the tumor. Harnessing of the powerful antitumor property of CTLs has been elusive over the past 10 years; however, using gene transfer strategies with chimeric single-chain, TCRs provides a new approach that might overcome some of the earlier difficulties (13).

Previous studies in our laboratory (14) and by others (15) have focused on immunizing Tg mice that express the hu transplantation antigen HLA A*0201 with immunogenic epitopes derived from wt hu p53. A potent CTL response was generated against one of these epitopes (14). Isolated clonal CTLs selectively lyse/kill p53-overexpressing hu tumor cells *in vitro* and in *in vivo* tumor models in SCID mice (11). In the present report, we demonstrate molecular cloning of both chains of the mu TCR and transfer and functional expressions of the p53-specific TCR in hu Jurkat T cells. The functional expression of the mu TCR results in the recognition of endogenously processed hu p53 expressed in tumor cells. The recognition of p53-expressing tumors by the Jurkat TCR transfectants succeeded in the presence of CD28 costimulation, which provides a powerful activation stimulus for Th cell function (16–18). This suggests the potential for an anticancer immunotherapy by the adoptive transfer of *TCR* gene-modified autologous T cells.

MATERIALS AND METHODS

Cell Lines. Previously described cell lines used in these experiments included T2 (19), Saos (20), Panc-1 (21), AsPC-1 (22), MDA-MB-231 (23), SK-BR-3 (24), Jurkat (25), JRT.3T.1 (26), and J.RT3-T3.5 (26) and were obtained from the American Type Culture Collection. The HLA status of each antigen-presenting cell line was determined by PCR on genomic DNA as well as by indirect immunofluorescence as described (14). p53 expression in these

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[3] The abbreviations used are: hu, human; mu, murine; TCR, T-cell receptor; SCID, severe combined immunodeficient; Tg, transgenic; IVS, *in vitro* stimulation; TNF, tumor necrosis factor; APC, antigen-presenting cells; CRA, chromium release assay; dsDNA, double-stranded cDNA; RT-PCR, reverse transcription-PCR; wt, wild type; mAb, monoclonal antibody; IL, interleukin; CMV, cytomegalovirus.

cell lines was confirmed by immunohistochemistry (Table 2) as described (27). Briefly, cell lines were pelleted, mixed with agarose gel, fixed in 10% buffered formalin, and embedded in paraffin. Cell blocks sections were cut to 5-μm thickness, mounted on Probe-on slides (Ventana Biotech System, Tucson, AZ), and baked at 42°C overnight and then for 1 h at 56°C. The slides were deparaffinized in xylene and hydrated in distilled water. Antigen retrieval was by steaming (Black & Decker Handy Steamer Plus, Hampstead, MD) with 0.01 M EDTA-Tris buffer (pH 8.0) for 20 min, followed by cooling for 20 min, and rinsing in distilled water (27). Slides were stained using a Biotech Techmate 1000 Immunostainer (Ventana Biotech System). Sections were incubated for 25 min with a 1:1000, 1:2500, and 1: 5000 dilution of the D07 mAb specific for hu p53 (NovoCastra Laboratories, Newcastle, England). Sections incubated with PBS served as a negative control. Controls were embedded in the same blocks as the tumor tissue. The antibody-antigen complex was detected by a modified ABC method (Ventana Biotech System), per the manufacturer's directions, with the chromagen 3′,3-diaminobenzidine to develop a brown color. The slides were graded by an individual blinded to the sample notation, using a scale of 0 to 4+, based on the intensity of staining of the majority(>75%) of cells. Cells with no staining were graded as 0, and cells with maximal staining with respect to a laboratory control were graded as 4+.

HLA A*0201$^+$ fibroblast cell lines were a kind gift of S. J. Forman (City of Hope). All hu cell lines were maintained in RPMI (supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and fresh glutamine). Saos-p53 was derived from transfection of the p53-deficient parent cell line with mutated p53 (arginine to histidine at position 175), as described previously (14).

Derivation of mu hu p53-specific CTL Clone. p53-specific CTL clones were generated by immunizing HLA A*0201 Tg mice (a kind gift of V. H. Engelhard, University of Virginia, Charlottesville, VA) with the immunodominant hu $p53_{149-157}$ CTL epitope and PADRE (28) Th peptide emulsified in incomplete Freund's adiuvant as described (14). Spleen cell suspensions were prepared from immunized animals, further stimulated with syngeneic lipopolysaccharide-treated splenic blasts, and loaded with the priming peptide as described (29). Additional IVSs (3) were performed every 7 days using citric acid-treated and peptide-pulsed Jurkat T cells transfected with HLA A*0201 as APCs (14). Long-term CTL lines were established by restimulating peptide-primed CTLs at weekly intervals with the addition of RAT T-stim with Con A (Collaborative Biomedical Products, Bedford, MA). After three rounds of IVS, $p53_{149-157}$-reactive CTL lines were cloned by limiting dilution in 96-well, flat-bottomed microtiter plates. The cells were plated under IVS conditions as described above and were refed 7 days later with identical fresh medium. Two to three weeks later, clones were expanded by restimulation in 24-well plates. Several clones were evaluated for recognition of p53-sensitized T2 cells and were further expanded for additional studies, including the demonstration of recognition of endogenously processed p53 on transfected cells and tumor cells lines. Several of the clones exhibited equivalent cytotoxicity and were further evaluated for TCR usage.

Chromium Release Assay Using mu CTL 3A3/3C9. To assess peptide-specific lysis, T2 cells were labeled with 150 μCi (ICN, Costa Mesa, CA) ^{51}Cr and peptide pulsed (1.0 μM) for 90 min. For cytotoxic assays involving cell lines without peptide sensitization, cells were pretreated for 24 h with 20 ng/ml hu IFN-γ and 3 ng/ml hu TNF-α and labeled with ^{51}Cr for 90 min. Labeled target cells and diluted effector cells were coincubated for 4 h. Supernatants were harvested (Skatron Instruments, Lier, Norway) and counted using a gamma counter (Packard Instrument Company, Meriden, CT). Specific lysis was determined as follows:

$$\%\ \text{specific release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

Cloning and Characterization of A/B TCR cDNAs Using RT-PCR and Inverse PCR. mu $p53_{149-157}$-specific CTL clone 3A3/3C9 was grown after stimulation with mAb 2C11 without feeder cells. RNA was prepared using a modification of the method of Chomczynski and Sacchi (30) using the Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). One μg of total RNA was then annealed to 20 ng of oligo$(dT)_{12-18}$ (Pharmacia, Uppsala, Sweden) at 65°C for 10 min. The first-strand cDNA was synthesized in a 20-μl volume using five units of avian myeloblastosis virus reverse transcriptase at 45°C for 60 min. The second-strand of the cDNA was synthesized by addition of 15 units of *Escherichia coli* DNA ligase, 18 units of *E. coli* DNA polymerase, and 1.75 units of *E. coli* RNase H (New England Biolabs, Beverley, MA), according to the manufacturer's recommendations. After incubation for 120 min at 16°C, 1 unit of T4 DNA polymerase was added for blunt end formation. The dsDNA was circularized by incubation with 1 unit of T4 DNA ligase (Life Technologies). TCR cDNA fragments were amplified from the circles using PCR with Taq DNA polymerase and the recommended buffer (Perkin-Perkin-Elmer Corp., Norwalk, CT) for 35 cycles using the GeneAmp 9600 (ABI-Perkin-Perkin-Elmer Corp., Norwalk, CT). The amplification cycle consisted of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s. Fifteen pmol of primers CA or CB (sense and antisense) were added to the reaction (Table 1). The products of the PCR reaction were separated on a 1% agarose gel, and DNA fragments of the expected sizes were removed from the gels and purified with the help of glass beads (GeneClean II; BIO 101, La Jolla, CA). The DNA fragments were cloned into the pGEM-T vector (Promega Corp., Madison, WI). Resultant recombinants were screened by Southern blot hybridization analysis using TCR-specific primers CAM and CBM (Table 1). DNA sequencing was carried out on 5–10 candidate *TCR A* or *B* genes with complementary primers for the Sp6 or T7 promoter sites flanking the cloning sites.

Table 1 *Oligonucleotides used to detect and clone mu TCR A and B genes*

Sequences of oligonucleotides were used to amplify or detect mu *TCR* gene segments that were cloned from the murine CD8$^+$ CTL, 3A3/3C9. As described in "Materials and Methods," inverse PCR was conducted with RNA, after conversion to dsDNA and circle formation. *TCR* gene segments were amplified using CA-S and CA-AS primers as shown in Fig. 2*A*. These primers amplify a fragment of ~800 bp. The CAM primer was used, as shown in Fig. 2*B*, as a probe to detect *TCR A* gene fragments amplified using the CA (S + AS) primers. The full-length murine TCR cDNA was amplified from cellular RNA using primers VA16-5′ and CA-3′ with added restriction sites as shown. Similarly, *TCR B* cDNAs were cloned using the CB-AS and CB-S primers from the inverse PCR-generated circles as described in "Materials and Methods." The CBM probe was used to detect *TCR B* genes using Southern hybridization. The full-length *TCR B* gene was amplified from cellular RNA using VB-5′ and CB-3′ primers with added restriction sites as shown. Numbering for all sequences is relative to the initiating ATG codon (+1) of the TCR cDNA sequences.

Primer name	*TCR A* sequence	Nucleotide location
CA-AS	CATAGCTTTCATGTCCAGCACAG	570–548
CA-S	CTCCTGCTACTGTGTGTATTGAGC	1259–1282
CAM	GTGCTGTCCTGAGACCGAGGATC	467–445
VA16-5′ (*Xba*I)	TCTCTAGATGCTGATTCTAAGCCT	−7 to 17
CA-3′ (*Sal*I)	TGGTCGACTCAACTGGACCACAG	827–805
	TCR B sequence	
CB-AS	GACCTCCTTGCCATTCAC	542–526
CB-S	ACCATCCTCTATGAGATCC	811–829
CBM	GACCTTGGGTGGAGTCACATTTCTCAGATC	420–391
VB-5′ (*Xba*I)	ACTCTAGATGGGCTCCAGACTC	−7 to 15
CB-3′ (*Sal*I)	CAGTCGACATAAAAGTTTGTCTCAGG	937–912

Reamplification of cellular RNA using a combination of specific sense *V* gene segment primers from the identified *VA* or *VB* genes (Table 1) along with either CA-3′ or CB-3′ antisense primers were used to make mu *A* and *B* cDNA fragments into full-length genes. Recovered PCR DNA fragments were then digested with *Xba*I and *Sal*I restriction endonucleases for cloning into expression vectors. The whole cDNA for each gene was resequenced, and examples were found without PCR-induced errors. Both *A* and *B* TCR cDNAs were subcloned separately into the expression vector, pCI-neo (Promega) containing the neomycin phosphotransferase gene.

Transfection of A and B TCR cDNAs into Jurkat CD3$^+$/$^-$ T Cells. The mu TCR A chain was introduced into the JRT-T3.1 cell line that lacks the TCR A chain under the selective control of the antibiotic G418. Similarly, the mu TCR B cDNA was introduced into the JRT-T3.5 cell line that lacks the TCR B chain. Both TCR A and B chain cDNAs were introduced into the Jurkat T cell positive for both *TCR A/B* genes under the selective control of the antibiotic G-418. This approach was taken based upon our previous work, showing more balanced expression of both TCR chains in non-TCR mutant cell lines.[4]

Gene transfer was accomplished through the use of standard liposome transfection procedures (Lipofectin; Life Technologies). Briefly, 2×10^6 cells were resuspended in 0.8 ml of serum-free growth medium and put into one well of a six-well plate. 0.2 ml of the Lipofectin Reagent-DNA complexes

[4] Unpublished observations.

containing 5 μg of plasmid DNA and 20 μl of Lipofectin were added to the cell suspensions with gentle mixing. The cultures were then incubated at 37°C in a 5% CO_2 incubator for 5 h, and 4 ml of growth medium supplemented with 10% FCS was added. After 2 days, the selective antibiotic was added. The medium was changed weekly until resistant cells grew to adequate numbers. Transfectants were cloned by limiting dilution at 0.3 cell/well in 96-well flat-bottomed plates. Positive colonies were scored when there was substantial growth, usually after 2 weeks. Colonies were expanded into successively larger flasks, and when there were enough cells, further analysis was performed.

Flow Cytometry Analysis of mu TCR Chains Transfected into Jurkat-CD3$^+$ and Jurkat-CD3$^-$ Cells. TCR mutants JRT T3.1 and J.RT3-T3.5 do not express a cell surface TCR because they lack expression of either TCR A or B chains; as a consequence, they are both negative for surface CD3 (31). Transfection of *TCR A* or *B* chain genes restores surface expression of CD3, as detected by mAb OKT3 (31). Previous work showed that a combination of hu and mu TCR chains could rescue surface CD3 expression on Jurkat mutant cells (32). The transfectants were examined by flow cytometry after staining with mAb OKT3 and a goat antimouse IgG phycoerythrin-conjugated secondary antibody. In addition, the mu Vβ8-specific mAb F23.1 (33) was used to detect surface expression of the mu p53-specific CTL clone *TCR B* chain gene. Matched isotype controls were used in each experiment for background fluorescence. Events (10,000) were collected for each histogram on the MOFLO flow cytometer (Cytomation Instruments).

RT-PCR Analysis of mu TCR RNA in Jurkat Transfectants. Molecular analysis of the expression of the transfectant RNA in JRT.3T.1 and J.RT3-T3.5 cells was performed by standard RT-PCR methods (34). mu-specific TCR primers (VA or VB and CA or CB) were used to amplify either the *TCR A* or *B* genes in the respective transfectants (Table 1). In the doubly transfected Jurkat cell, both mu A and B TCR chains were amplified at the same time to examine the equivalence of expression in various transfectant cell lines.

Proliferation Assays Using Jurkat mu TCR Transfectants. For recognition of p53 epitope, T2 cells (APC) were preincubated with 50 μM peptide for 4 h. Then, 1×10^5 cells/well in a 96-well, round-bottomed plates were incubated with 1×10^5 Jurkat transfectants at 37°C in a 5% CO_2 incubator. For recognition of endogenously processed hu p53 by TCR transfectants, 1×10^5 transfected cells were incubated in the presence of a mAb (mAb 9.3) recognizing CD28 (31) with 1×10^5 HLA A*0201-positive or HLA A*0201-negative tumor cells or fibroblasts pretreated with 20 ng/ml IFN-γ and 3 ng/ml TNF-α at 37°C in a 5% CO_2 incubator. The medium was harvested after 24 h and IL-2 production was assessed in a bioassay using an IL-2-dependent cell line (CTLL; Ref. 35). Briefly, triplicates of 5×10^3 CTLL/well in a 96-well, flat-bottomed plate were incubated with 50 μl of recombinant IL-2 standard or medium at 37°C in a 5% CO_2 incubator for 24 h. The cultures were harvested after 4 h after pulsing with 1 μCi of [^{3}H]thymidine. [^{3}H]Thymidine incorporation was measured by liquid scintillation counting. All results are expressed as the mean of triplicate cpm.

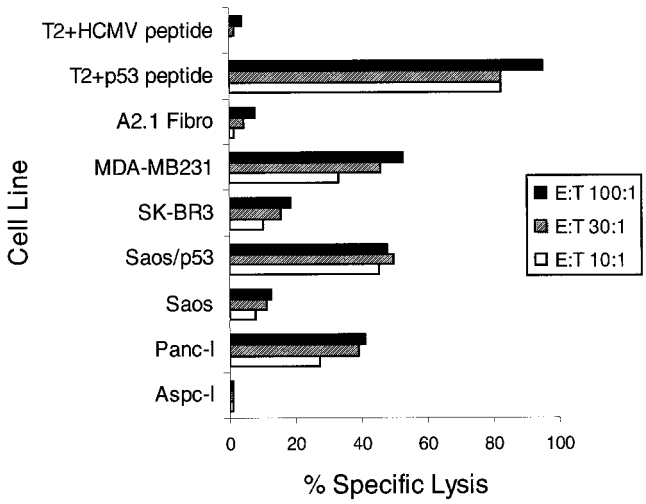

Fig. 1. Cytotoxic activity of $p53_{149-157}$-specific CTL effector cells using hu cell line targets in a 4-h CRA. E:T ratios were 100:1, 30:1, and 10:1. The following cell lines were used as targets: T2 pulsed with $pp65_{495-503}$ epitope (*T2+HCMV peptide*), T2 pulsed with $p53_{149-157}$ epitope (*T2+p53 peptide*), HLA A*0201 fibroblasts (*A2.1 Fibro*), breast cancer cell lines MDA-MB231 and SK-BR3, osteosarcoma line Saos/p53 transfected and native cells, and pancreatic cancer cell lines Panc-1 and AsPC-1 (see Table 2). SEs of triplicate cultures were always <5% of the mean.

Table 2 *APCs used in studies of TCR specificity*

Cell lines as shown were used in the study as targets either by peptide sensitization (T2 cells) or after transfection of mutant p53 (Saos/p53) compared with the parental p53$^-$ cell line (Saos). The remaining cell lines are positive for p53 expression, as detected by immunohistochemistry, and differ in their expression of the HLA A*0201 antigen. HLA-A*0201 expression was determined by immunofluorescence as described (14). p53 expression as determined by immunohistochemistry (see "Materials and Methods") was graded on a scale of 0 to 4$^+$, based on the intensity of staining of the majority (>75%) of cells.

Cell lines	Cell type	HLA-*0201 expression	p53 accumulation
T2	B and T lymphoblast hybrid	+	−
Saos	Osteosarcoma	+	−
Saos/p53	Osteosarcoma	+	++++
AsPC-1	Pancreatic adenocarcinoma	−	−−−−
Panc-1	Pancreatic adenocarcinoma	+	++++
SK-BR-3	Breast carcinoma	−	++++
MDA-MB-231	Breast carcinoma	+	++++

RESULTS

mu CTLs from HLA A*0201 Tg Mice Specifically Recognized p53$^+$/HLA A*0201-expressing Hu Cells. We showed previously that immunization of HLA A*0201 Tg mice in the C57 BL/6 background with $p53_{149-157}$ resulted in the generation of HLA A*0201-restricted CTLs, which could cause the recognition and lysis of adoptively transferred hu tumor cells in SCID mice (11). One of these clones (3A3/3C9) was further examined for the strength of its recognition of the hu $p53_{149-157}$ HLA A*0201-restricted epitope using a CRA. We reconfirmed that the hu TAP (transporter for antigen presentation) transporter mutant cell line T2, when loaded with the $p53_{149-157}$ CTL epitope, was well recognized at all E:T ratios examined (Fig. 1). This recognition was specific, because an HLA A*0201 binding, CMV-pp65 CTL epitope (36) is not recognized by this clone in *in vitro* assays. Clone 3A3/3C9 was further evaluated for its specificity of recognition of hu tumor. APC used are described in Table 2 and reflect examples of hu fibroblasts or tumors expressing p53 or HLA A*0201, or both, or neither. CTL clone 3A3/3C9 at varying E:T ratios was incubated with a series of hu tumors, and the results shown in Fig. 1 were obtained. Cell lines that were not HLA A*0201$^+$ (ASPC-1 and SKBR3) were not lysed efficiently. Cell lines that did not express hu mutant p53 (Saos; A2.1 Fibro) were not lysed unless pulsed with the $p53_{149-157}$ peptide (data not shown). Tumor lines that were HLA A*0201$^+$ and p53$^+$ were efficiently lysed by the mu CTLs (PANC-1, Saos/p53, and MDA-MB231). The recognition properties of the mu CTL clone demonstrate that it is able to distinguish between hu tumors, which express the appropriate HLA type and mutant overexpressed forms of p53, from those that do not express both molecules (Table 2).

The amount of p53 overexpression required for recognition is difficult to quantify. Cells containing wt p53 are generally negative by immunohistochemistry and are not recognized (Table 2). In contrast, all of the cell lines we have examined with mutated p53 stain intensely for p53 by immunohistochemistry and are recognized (14, 37, 38). It is likely that the mu CTLs recognize the wt form of the $p53_{149-157}$ epitope, because the mutations in these cell lines are known to be at a different location of the p53 DNA sequence (39). Overexpression of p53, caused by specific mutations outside the $p53_{149-157}$ epitope location, is essential for recognition by the mu CTLs. These data also confirmed the specificity of the CTL 3A3/3C9. Because this clone was used earlier in *in vivo* experiments, which demonstrated that the

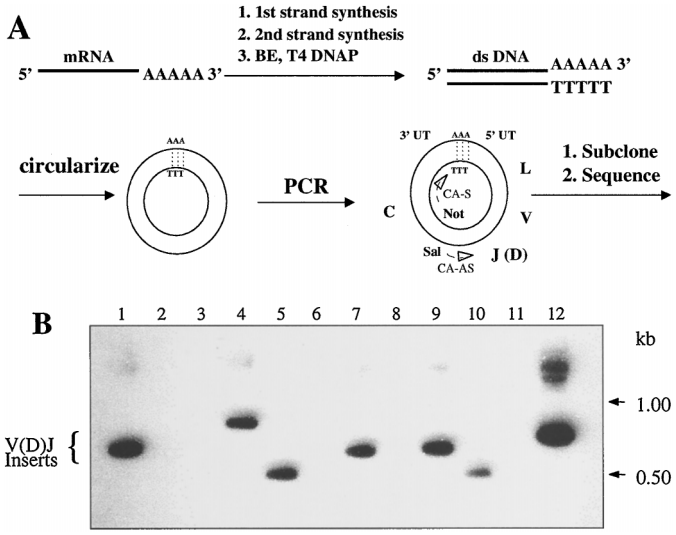

Fig. 2. Inverse PCR analysis of TCR usage. *A*, mRNA is converted to cDNA and then dsDNA and finally enzymatically blunt ended in steps 1–3. T4 DNA ligase is used to circularize dsDNA. Sense and antisense primers that are complementary to *CA* or *CB* gene segments that end with *Sal*I and *Not*I restriction sites are used to synthesize linear DNA using PCR (Table 1). These fragments are subcloned into plasmids by standard methods. *B*, the PCR fragments prepared as above were cloned into the pGEM-T vector. Plasmid DNA was isolated from individual bacterial colonies, and a portion was digested with *Sal*I + *Not*I. The digested DNA was separated on a 1% agarose gel, ethidium bromide stained, and then transferred to a nylon filter by capillary action (Southern blot). The membrane-bound DNA was hybridized with a ^{32}P-labeled oligonucleotide (CBM or CAM), the sequence of which was identical to both mu *CB* genes and located within the fragment generated by the CA or CB primer sets (Table 1). Plasmid DNA from several positive lanes was further analyzed by manual dideoxy sequence analysis.

particular TCR was capable of rejecting hu tumors in a mu SCID model, the data for both *in vivo* and *in vitro* studies confirmed the appropriateness of using this TCR for gene transfer studies (11).

Molecular Cloning of hu p53-specific TCR. Using a novel technique of molecular cloning referred to as "inverse PCR" (40, 41) without the formal use of DNA libraries, the TCR A and B chain cDNAs were identified (Fig. 2). In Fig. 2*A*, the method referred to as inverse PCR shows how the RNA from the CD8$^+$ CTLs was converted to circular dsDNA and subsequently amplified using conventional PCR into linear molecules containing specific TCR sequences. TCR-specific primers, derived from the constant region of either the TCR *A* or *B* genes, were used in amplifying TCR-specific cDNA into amounts that could easily be subcloned into standard plasmid vectors. This amplification step increased the number of TCR-specific colonies after transfer to bacterial plates. Single colonies were picked, minipreps were made, and the size of inserts was examined using agarose gels and then transferred to nylon membranes. Inserts $>$0.7 kb will contain sequence from VA-JA and CA or VB-DB-JB and CB DNA segments. The nylon membranes were hybridized with oligonucleotides derived from the CB region (Table 1), and those inserts that are TCR specific were visualized using ^{32}P radioactivity after end labeling (Fig. 2*B*). Colonies that contained inserts $>$0.7 kb were then sequenced in their entirety to identify the usage of particular VB-DB-JB segments (Table 3). Similar methodology was used to identify and sequence the CA and VA-JA segments.

The criterion for selection of a TCR cDNA sequence as being the correct RNA transcript corresponding to the expressed form of the receptor from the CD8$^+$ T cell clone is its frequency of usage. After cloning of individual TCR transcripts in plasmid vectors, it is still important to test whether the predominantly expressed transcript represented the expressed RNA transcript from the original T-cell clone. Sequence analysis of inserts from plasmids revealed that five of seven candidate TCR cDNAs had the identical sequence, whereas two differed from the other five and from each other (data not shown). Presumably, the two nonidentical sequences arose from residual contaminating feeder cells. We then confirmed using RT-PCR that the clonal TCR A and B cDNAs were identical to the expressed transcript in the original mu CD8$^+$ CTLs (data not shown). An oligonucleotide primer was derived from the nucleotide sequence of each unique *V* gene from both clonal TCR A and B cDNAs, together with an antisense primer to the constant gene segment of both A (CA-3′) and B (CB-3′) TCRs (Table 1). Total cellular RNA was amplified with the oligonucleotide pairs, using RT-PCR, and then individual cDNA molecules were molecularly cloned in plasmid vectors as described earlier (see "Materials and Methods"). The complete cDNAs for both TCR *A* and *B* genes were sequenced from five separate plasmid clones. The sequences were aligned and compared and found to be identical for both the TCR *A* and *B* genes. The deduced amino acid sequences at the junctions of gene segments comprising both TCR cDNA chains are shown, and both nucleic acid sequences have been deposited in the EMBL database (Table 3). Subsequently, both TCR A and B cDNAs were transferred to plasmid expression vectors for use in expression studies in eukaryotic cells.

Transfection of A and B TCR cDNAs into Jurkat T-Cell Lines. Jurkat A$^-$ or B$^-$ TCR mutants do not express a TCR:CD3 complex on the cell surface (31). Earlier investigations showed that in the mutants developed by the Weiss Laboratory, that transfection with cDNA encoding single TCR chains could restore cell surface TCR:CD3 expression (42, 43). It was shown previously that mu TCR subunit cDNAs could restore surface TCR:CD3 expression in similar mutants, as a result of the sequence and functional homology of mu and hu TCR chains. Restored expression of the complex could be detected and quantitated using the mAb OKT3 (31). We used those properties of the mutant TCR cell lines to examine the functional expression of the individual mu TCR cDNA chains that had been transfected (Fig. 3). The data shown in Fig. 3*A* shows that both the mu TCR A or B chain could individually rescue TCR:CD3 expression from the cognate TCR mutant cell line. Because the cell surface expression of the TCR:CD3 complex depends upon the newly introduced TCR chain, we can conclude that both the mu TCR A and B chains were expressed appropriately. In addition, these were able to integrate into a complex with the endogenous hu chains to form the cell surface form of the TCR:CD3 complex detectable with OKT3 (Fig. 3*A*).

Because both mu TCR chains were functionally expressed, it prompted us to determine whether they would interact with each other as well as the other hu CD3 chains to form a mu TCR:hu CD3 complex in the Jurkat T-cell mutants. Because we have already shown that both mu and hu TCR chains can independently assemble into a cell surface TCR:CD3 complex, it was logical to use the mutant Jurkat

Table 3 *Deduced amino acid sequence of gene segments and their junctions for TCR A and B cDNA from p53-specific CTL clone 3A3/3C9*

The *TCR* gene sequences were determined as described in the text, after sequence analysis of the complete mu cDNA. The nucleotide sequence was translated, and the corresponding open reading frame for the TCR amino acid sequence was determined. Identification of gene segments that correspond to the variable region (AV16) and junctional region (AJTA13) are shown, as well as the NH_2 terminus of the AC protein region. Correspondingly, the gene segments for the *TCR B* cDNA are identified for the variable region (VB8.1), diversity region (BD2), junctional region (BJ2.6), and the constant region gene used for this cDNA (BC2). Nucleotide sequences corresponding to these mu cDNAs have been deposited in the EMBL database.

	AV16	AJTA13		AC
TCR A	VYFCAMR	DTNAYKVIFGKGTHLHVLP		NIQNPEP
	BV8.1	BD2	BJ2.6	BC2
TCR B	AVYFCASS	PSS	YEQYFGPGIRLTVL	EDLRNVTPP

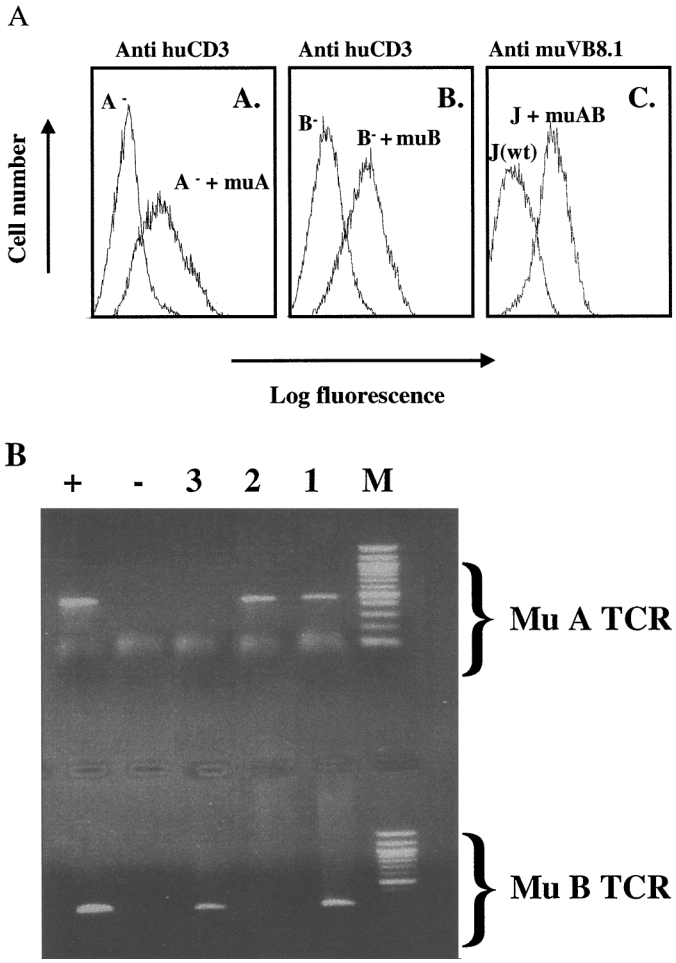

Fig. 3. Expression of mu A and B TCR chains in transfected Jurkat cell lines. *A*, flow cytometry analysis was performed on the A^- parental Jurkat JRT.T3.1 line and the JRT.T3.1 transfected with the mu TCRA. The parental B^- JRT.T3.5 line was evaluated before and after transfection with mu B TCR cDNA. OKT3 was used to evaluate CD3 expression on both cell lines and transfectants. wt and mu A/B TCR transfected Jurkat cells were evaluated for mu TCR expression using mAb F23.1 (mu VB8 specific). Expression was determined by flow cytometry following a phycoerythrin-conjugated goat antimouse immunoglobulin second step. Isotype control antibody staining of the Jurkat lines and transfectants was similar to parental line staining for the three positive cell lines. *B*, Jurkat cells that were transfected with mu A and B TCR cDNAs were evaluated for mu TCR expression by RT-PCR using CA- and CB-specific primers (Table 1). Numerous cell lines expressing only A or B mu TCR cDNAs were generated (*Lanes 2* and *3*). Double transfectants (*Lane 1*) express both mu TCR chains. The mu p53-specific CTL line was used as a positive control, and the parental Jurkat cell line was used as a negative control. Duplicate lanes were deleted from the figure.

T-cell lines as a host for simultaneous transfection of both mu TCR chains. Nonetheless, we observed that dual transfection of both mu TCR chains, no matter how varied the relative starting concentrations of expression plasmid, ultimately resulted in imbalanced expression of either the mu TCR A or B chain in the respective Jurkat T-cell mutant (data not shown). For this reason, expression and function of the mu TCR A and B chains was examined in wt-Jurkat. We initially assessed the surface expression of the mu TCR B chain in the doubly transfected Jurkat T cell, using the only available mu TCR-specific mAb that would selectively detect the mu TCR chain associated with hu TCR:CD3 complex (Fig. 3*A*). Cell surface staining with mAb F23.1 did not address the expression level of the mu TCR A chain. However, no high-affinity mu mAb is available with which to detect the mu TCR A chain in the TCR:CD3 complex with either mu or hu TCR chains. Furthermore, a mAb that will detect the mu-specific heterodimer was not available to us, making it difficult to determine whether the mu A/B heterodimer was assembled on the cell surface (data not shown). Instead, we used a PCR method to simultaneously evaluate the mRNA expression for each of the transfected mu TCR chains.

mu TCR mRNA Transcripts Are Equally Expressed in Nonmutant Jurkat T-Cell Lines. Expression of mu TCR transcripts was evaluated by PCR in the double mu TCR Jurkat transfectants, as shown in Fig. 3*B*. When we examined the expression of both mu TCR A and B transcripts in wt-Jurkat T cells, we found Jurkat clones with imbalance (Fig. 3*B*, *Lanes 2–3*) and others with an equivalent level of mu TCR A and B expression (Fig. 3*B*, *Lane 1*). Several different clones derived from the wt-Jurkat T cells exhibited a balanced expression of the mu TCR A and B chains that was stable over a 6-month period. Examples of these T-cell transfectant clones were further examined in functional analysis as described below.

Functional Activation of IL-2 Expression in mu TCR Jurkat Transfectants. To determine whether doubly mu TCR-transfected Jurkat T cells would still recognize the HLA A*0201-restricted T-cell epitope from hu p53, the transfectants were incubated with T2 cells that were sensitized with a saturating concentration of hu $p53_{149-157}$ peptide. To distinguish between specific and nonspecific recognition, an HLA A*0201 epitope derived from CMV pp65 (36), which is not recognized by the parental mu CTL 3A3/3C9, was also used to sensitize T2 cells (Fig. 4*A*). To quantitate the extent of activation caused by recognition of the peptide epitope, we used the release of IL-2 by the transfectants. The results show specific recognition by the Jurkat T-cell transfectant of the hu $p53_{149-157}$ CTL epitope bound to T2 cells with a vigorous response, as measured by $[^3H]$thymidine incorporation in CTLL-2 cells. A further demonstration of the specificity of the recognition is the results of the parallel analysis of the untransfected parental T-cell line incubated with T2 cells sensitized by the same peptides. No significant IL-2 production was measured, in contrast with the mu TCR transfectant. Because T2 cells efficiently present exogenous peptide, the question arose whether the sensitivity of the transfectant to epitope concentration was similar to the parental 3A3/3C9 mu $CD8^+$ CTL clone (Fig. 4*B*). Therefore, a titration of the hu $p53_{149-157}$ T-cell epitope was conducted using a T-cell transfectant clone compared with parental Jurkat T cells. The half-maximal concentration of activation of the clone is ~500 nM peptide (Fig. 4*C*). This concentration of peptide for half-maximal stimulation is ~250-fold greater than what is needed to activate cytolysis by the parental mu $CD8^+$ CTLs (Fig. 4*B*). The difference in level of peptide sensitivity is what might be expected of a transfectant line that contained multiple different TCR heterodimers as a result of independent association of all four expressed hu and mu TCR chains. It seems that the degree of activation is sufficient that the transfectant may still recognize lesser amounts of hu $p53_{149-157}$, as is found in cell lines that express a diverse repertoire of endogenous peptides, in contrast to T2 cells. However, it would require a direct test of recognition of the endogenously processed hu p53 to determine whether smaller amounts of epitope could be recognized by the transfectant and cause its activation and IL-2 production.

Endogenous Processing of Hu p53 Causes the Activation of mu TCR Transfected Jurkat T Cells. Earlier reports had shown that a transfected TCR, either mu or hu, would still be capable of recognition of endogenously processed epitope in transfected APCs (44). In terms of hu tumor antigens, examples exist in which peptide epitope-sensitized APCs were recognized by a TCR transfectant (45). Nonetheless, there is a only a single example of recognition of an endogenously processed hu tumor antigen using a hu-transfected TCR and none as of yet for mu-transfected TCR (46). The recognition of endogenously processed hu p53 represents a testable model to determine whether a mu TCR specific for a wt epitope of hu p53 (hu $p53_{149-157}$) would recognize a hu tumor cell that overexpressed p53.

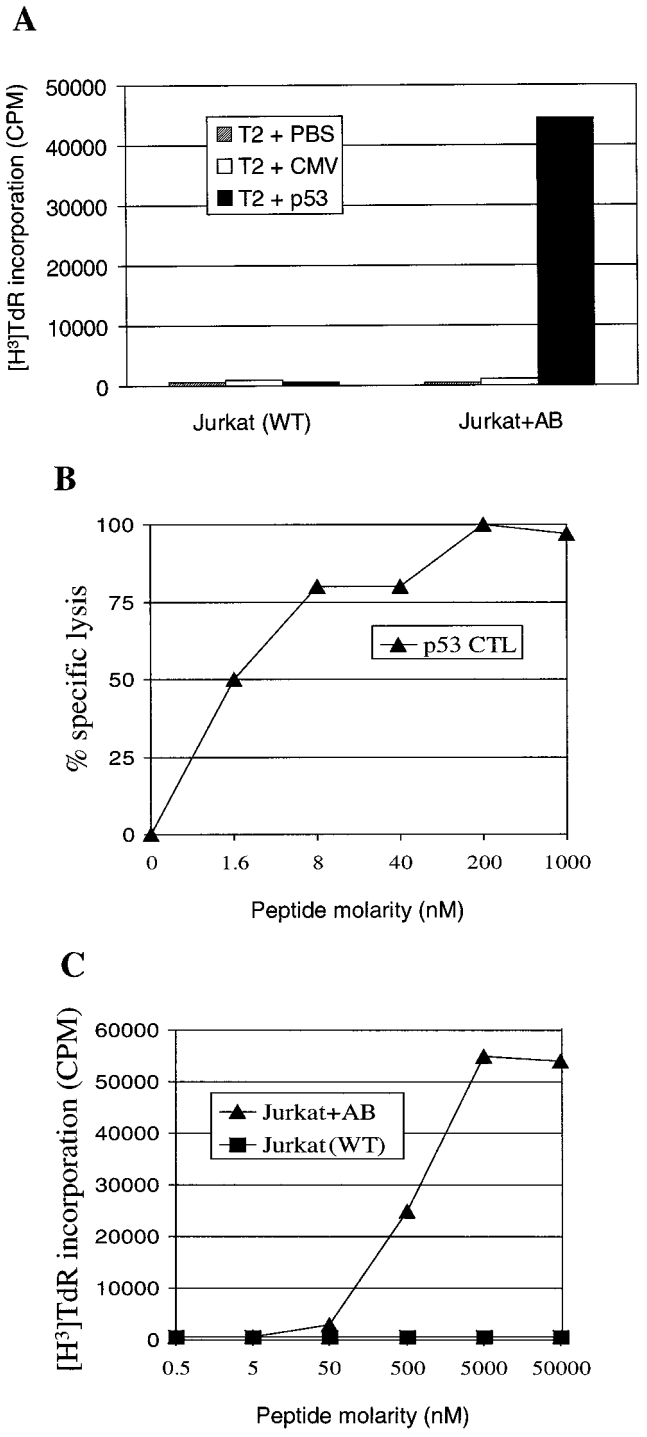

Fig. 4. Function of the mu TCR A/B transfected Jurkat cells. *A*, supernatants were collected after incubation of the mu TCR A/B transfected Jurkat cells with PBS, $pp65_{495-503}$ or $p53_{149-157}$ pulsed T2 cells. IL-2 concentration was measured as ^3H incorporation by CTLL-2 cells, as described in "Materials and Methods." *B*, the efficiency of peptide recognition by the parental mu CTL clone 3A3/3C9 is shown. T2 cells were pulsed with $p53_{149-157}$ peptide at the indicated concentrations prior to use as target cells in a 4-h CRA. The assay was conducted at an E:T of 10:1. *C*, the efficiency of peptide recognition by the mu TCR A/B transfected Jurkat cells is shown. T2 cells were pulsed with $p53_{149-157}$ peptide at the indicated concentrations prior to use as target cells in a 4-h CRA. The assay was conducted as in *A*.

We have shown previously that the osteosarcoma cell line Saos transfected with a hu p53 vector expressing a mutant hu p53 molecule is well recognized by the muCTL clone 3A3/3C9 (37). We posed the question as to whether the mu TCR-transfected Jurkat T cell would also recognize the Saos/p53 and undergo activation and IL-2 production. Therefore, we conducted experiments almost identical to the previous T2 epitope sensitization experiments, this time using the endogenous processing of p53 as a means to generate sensitizing epitope.

We knew from previous work, that the intensity of recognition of the endogenously processed hu p53 may be far less than that using the synthetic CTL epitope (39, 47). Nonetheless, the transfected Saos cell line served as a convenient starting point in our dissection of the level of recognition of the transfected Jurkat T cell. Our initial results with coincubation of the Saos/p53 cell line and Jurkat AB TCR transfectant were negative, despite the addition of IFN-γ + TNF-α, which were used to activate MHC class I on the APCs (data not shown). Our assumption was that because the Jurkat T cell is likely derived from a $CD4^+$ thymocyte and does not coexpress CD8, it might be defective in its adhesion properties when engaging class I-expressing APCs. As a result, the degree of activation may be far less than would be expected if the cell were a CTL that expressed CD8 and could better bind to the APCs (48).

To compensate for inherent difficulties in APC recognition by the ABTCR transfectants, we used the mAb 9.3 as a means to activate CD28 in the hope of enhancing the sensitivity of the T-cell transfectant toward endogenously processed p53 (49). This was especially important because all of the tumor cell lines used in these studies, with the exception of T2, were negative for B7.1 by flow cytometry (data not shown). This strategy worked well using the parental Saos cell line as compared with the Saos cell line transfected with mutant hu p53 (Fig. 5). Incubation of the Jurkat T-cell transfectant with the Saos cell lines resulted in the differential recognition of only the cell lines that expressed hu p53 (Fig. 5). The data show that the presence of hu p53, presumably in processed form, resulted in the activation of the coincubated transfectant to produce IL-2. Because our objective is to evaluate whether this mu TCR will effectively recognize endog-

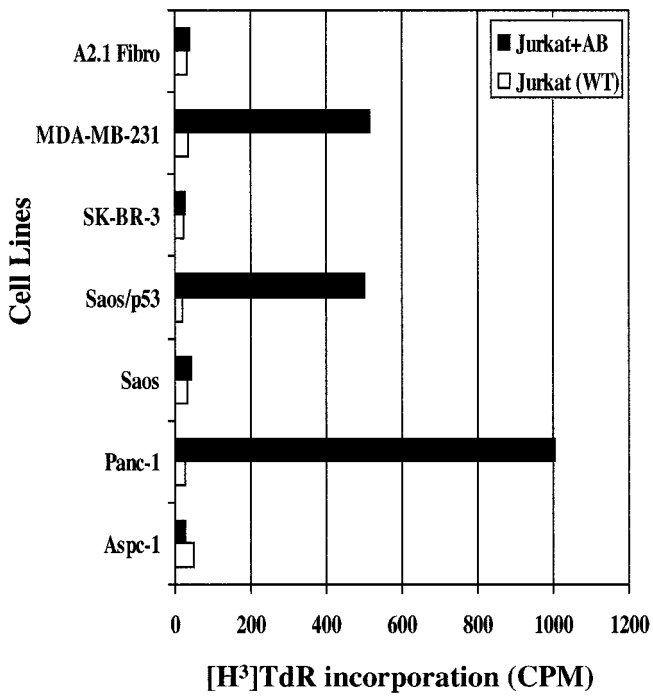

Fig. 5. Specific recognition of endogenously processed p53 by TCR transfected Jurkat cells. The following cell lines were used as targets: HLA A*0201 fibroblasts, breast cancer cell lines MDA-MB231 and SK-BR3, osteosarcoma line Saos/p53 transfected and native cells, and pancreatic cancer cell lines Panc-1 and AsPC-1. IL-2 concentration was measured as ^3H incorporation by CTLL-2 cells as described in "Materials and Methods."

enously processed mutant p53 in tumor cells, we surveyed a series of hu tumors expressing HLA A*0201 and mutant forms of hu p53, as well as their counterparts, which did not express one or both of these molecules (Fig. 5). A similar assay was performed; using as a readout, the production of IL-2 was determined by quantitation of [^{3}H]thymidine incorporation by the CTLL-2 cell line. We used a series of hu tumors described in Table 2 and examined the correlation of production of IL-2 *versus* the expression of HLA A*0201 and hu p53. Two cells lines that expressed mutant forms of hu p53 were able to activate the transfectants (PANC-1 and MDA-MB-231), whereas cell lines that either were p53$^-$ (Saos) or did not express HLA A*0201 (ASPC-1 and SK-BR3) were not able to activate the Jurkat T-cell transfectant to produce IL-2. These data unequivocally demonstrate that the Jurkat T-cell transfectant has been endowed with the property of recognition of wt p53 epitopes generated from endogenously processed hu p53 in the context of the HLA A*0201 molecule.

DISCUSSION

Molecular genetics has shown that mutations in the *p53* gene, which are generally single-base missense mutations (50, 51), contribute to the development of up to 50% of all cancers (3) and often correlate with a greater extent of disease and a worse outcome (52). Mutations of *p53*, which nullify its function as a suppresser of cell division (1, 53), are associated with a much higher nuclear and cytoplasmic concentration of the p53 protein (54). What makes the *p53* gene product such an attractive target for an adaptive immune response is that the intracellular concentration of nonmutated p53 is normally very low (4, 55), and cells expressing normal p53 at low levels would most likely escape an enhanced immune response to overexpressed mutant p53.

There are reports both describing isolation of CTLs using *in vitro* stimulation with HLA-restricted peptide motifs from p53 and from hu peripheral blood with HLA A*0201 binding peptides of p53 (56, 57). In fact, the precursor frequency for p53 wt epitope-specific CTLs in normal volunteer peripheral blood is as high as 1:27,000 (56). Yet, it is difficult to generate p53 peptide-specific CTLs from hu peripheral blood that are capable of killing p53-expressing tumor cells (58). In fact, there are only limited reports describing hu CTL clones that recognize endogenously processed p53, and these only recognize a small subset of p53 overexpressing hu tumors (59–61). Taken together, these data point to either thymic or peripheral tolerance as the cause of the deletion or inactivation of hu CTLs with high enough affinity to kill tumor cells that overexpress endogenous p53. There is substantial evidence that p53 is expressed throughout development, suggesting that it may play a role in neonatal thymic deletion of p53-specific T cells (62). Therefore, it is unlikely that a direct immunization approach in cancer patients using p53 sequences, either peptides or DNA, will stimulate a high-affinity immune response if the appropriate T cells are deleted or nonfunctional. By contrast, appropriately immunized mice generate a vigorous response to both mutant and wt hu p53 epitopes.

Adapting the vigorous immune response of mouse CTLs to hu CTLs would need to occur to exploit p53 recognition to benefit cancer patients. This can be accomplished by transferring immunoglobulin or TCR specificity to hu T cells. Several groups have been investigating an approach involving the generation and transfer of chimeric TCRs consisting of mAb single-chain Fv linked to the intracellular signaling domain of CD3ζ or FcγRIII (63). By transferring chimeric receptors to mu or hu effector cells, it has been possible to generate CTLs with antibody specificity, which secrete cytokine and mediate lysis of APCs. The ability of these modified cells to mediate tumor rejection in *in vivo* models has been more difficult to demonstrate (64). This may result from the relatively high affinity immunoglobulin/antigen interaction and a resulting inadequate recycling mechanism between single CTLs and multiple tumor cells. In addition, CTLs with immunoglobulin specificity may lack the ability to recognize intracellularly processed antigens presented in the peptide-binding groove of cell surface MHC molecules. Such would be the case for p53, which is exclusively expressed intracellularly.

The approach in the present report takes advantage of recent progress in cellular immunology and involves the transfection of hu T cells with a TCR from cloned tumor-associated antigen-specific CTLs. Chimeric receptors, introduced into T cells, that use the CD3 ξ chain cytoplasmic domain and external ligand-binding domain from the V(D)J region of the A/B TCR are able to directly activate the transfected cells without CD3 expression (65). This can result in lymphokine production (Th2 cell) or lytic function (CTLs; Ref. 65). As a preliminary step to the generation of a single-chain TCR construct for immunogene therapy, it is necessary to demonstrate the function of the TCR of interest in a readily transfected hu T-cell line. This same strategy was used recently by Cole *et al.* (45), who transfected Jurkat cells with a cloned TCR from MART-1-specific hu CTLs. The resulting transfectants were able to recognize and secrete cytokine in response to challenge with the peptide transporter mutant cell, T2 pulsed with the appropriate peptide epitope. However, unlike our own data with the p53-specific TCRs, when the MART-1-specific transfectants were challenged with bona fide melanoma cells expressing the same antigen, they were unable to direct the T cells to secrete cytokine or lyse them. This unexpected result may occur because MART-1, like p53, is expressed during normal hu development, and normal tolerance mechanisms in the thymus may cause the elimination of T cells expressing TCRs that recognize the antigen with high affinity (negative selection). These tolerance mechanisms may be overcome through the xenogeneic transfer of antigen-specific TCRs from mu to hu T cells.

In this report, we show that p53-specific mu TCRs will function in the hu CD4$^+$ T cell line, Jurkat. The transfer of functional mu TCRs not only results in the rescue of hu CD3 expression in TCR mutant forms of Jurkat but transfers antigen recognition from the mu TCR to the hu line. Peptide recognition and secretion of cytokine by the TCR-modified Jurkat in response to peptide-pulsed T2 cells were vigorous. Demonstrating that the TCR-modified Jurkat recognized endogenously processed antigen on p53 overexpressing cells was more difficult. The Jurkat line was used in these studies because it is one of the few stable hu T cell lines that can be readily transfected. However, it may not be the best model to study high-density TCR expression because of the presence of an endogenous and functional TCR heterodimer, and the significantly lower expression of TCR chains as compared with normal T cells (43). These deficits were overcome by optimizing antigen presentation in the tumor cell targets through the preincubation of target cells with IFN-γ and TNF-α.

Recognition of tumor cells by the TCR-modified Jurkat required interaction between the modified CD4$^+$ T helper line and processed p53 presented by MHC class I on the hu tumor cells. This occurred without the added stabilizing interaction of the MHC class I heavy chain domain, α3 on the tumor cells, with the T cell coreceptor, CD8 (66), which is absent in Jurkat. Generating A and B TCR Jurkat transfectants that also express CD8 would require a triple transfection that might be difficult because of the relative instability of the mu A/B TCR Jurkat line. To overcome the inherent inadequacies of Jurkat cells as functional effectors after mu A/B TCR transfer, additional costimulation was provided by preincubating the TCR-modified Jurkat cells with a mAb recognizing CD28 (mAb 9.3; Ref. 67). This particular mAb was chosen because of its known agonist activity on CD28$^+$ Jurkat T cells. In the well-controlled experiments described

here, the CD28-stimulated Jurkat secrete IL-2 only in response to appropriate HLA A*0201$^+$, p53-overexpressing APCs (Fig. 5).

The strategy of *ex vivo* expansion and adoptive immunotherapy using antigen-specific CTLs has been under investigation for several years with mixed results. Initial attempts at adoptive CTL transfer were hindered by technical difficulty in generating large numbers of CTLs with defined specificity. These difficulties have been largely overcome. The efficacy of adoptive T-cell therapy has been successfully demonstrated recently in clinical trials using the adoptive transfer of large numbers of EBV (68) or CMV (69) specific CTLs. Translation of these promising results to the adoptive immunotherapy of malignancy will be difficult, because unlike viral antigens, most tumor-associated antigens are expressed on normal cells and are subject to tolerance. The current demonstration that high-affinity tumor p53 specificity can be transferred from mu to hu T cells should encourage efforts to apply xenogeneic TCR transfer for the immunotherapy of poorly immunogenic tumors.

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