

Kimberlyn F. Card · Shari A. Price-Schiavi · Bai Liu  
Elizabeth Thomson · Esperanza Nieves  
Heather Belmont · Janette Builes · Jin-an Jiao  
Javier Hernandez · Jon Weidanz · Linda Sherman  
John L. Francis · Ali Amirkhosravi · Hing C. Wong

## A soluble single-chain T-cell receptor IL-2 fusion protein retains MHC-restricted peptide specificity and IL-2 bioactivity

Received: 25 February 2003 / Accepted: 18 August 2003 / Published online: 11 November 2003  
© Springer-Verlag 2003

**Abstract** Antibody-based targeted immunotherapy has shown promise as an approach to treat cancer. However, many known tumor-associated antigens are not expressed as integral membrane proteins and cannot be utilized as targets for antibody-based therapeutics. In order to expand the limited target range of antibodies, we have constructed a soluble single-chain T-cell receptor (TCR) fusion protein designated 264scTCR/IL-2. This fusion protein is comprised of a three-domain HLA-A2-restricted TCR specific for a peptide epitope of the human p53 tumor suppressor protein, which is overexpressed in a broad range of human malignancies. The 264scTCR/IL-2 fusion protein has been expressed at high levels in mammalian cells, and milligram quantities have been purified. MHC-restricted antigen-specific binding properties are maintained in the single-chain, three-domain TCR portion of the fusion protein, and the IL-2 portion retains bioactivity similar to that of free recombinant IL-2. Moreover, this fusion protein is capable of conjugating target and effector cells, remains intact in the blood and substantially increases the half

life of the IL-2 portion of the molecule. Finally, the 264scTCR/IL-2 fusion protein can be used to stain tumor cells and is capable of reducing lung metastases in an experimental model of metastasis. Thus, TCR-based fusion proteins may provide a novel class of targeted immunotherapeutics for cancer.

**Keywords** T cell receptors · Tumor immunity · Molecular biology · Cytokines

### Introduction

There is increasing evidence that immunotherapy is a promising approach to treat cancer. One strategy that has received attention is treatment with cytokines such as IL-2 to enhance anti-tumor immunity. IL-2 has stimulatory effects on a number of immune cell types including T and B cells, monocytes, macrophages, lymphokine activated killer cells (LAK) and NK cells [10, 41]. Thus, systemic administration of IL-2 has been used to treat patients with metastatic melanoma and renal cell carcinoma [42]. Although treatment with cytokines has shown some promise, the toxicity associated with these treatments makes it difficult to achieve an effective dose at the site of the tumor [31, 33, 40, 43, 44, 52]. For example, systemic treatment with IL-2 at tolerated doses induces lymphoid activation in virtually all treated patients, but only a minority of these achieves antitumor responses [42, 46, 47, 55]. Thus, there is some interest in devising strategies for concentrating cytokines at the site of the tumor to increase efficacy and reduce toxicity.

Strategies that have been used to concentrate cytokines at the tumor site include direct injection of cytokine into the tumor, transfer of the cytokine gene into the tumor cells and targeted delivery of the cytokine by fusing it to a tumor antigen-specific antibody [20]. Direct injection and transfer of cytokine genes may be effective on a small scale, but have major drawbacks in that direct

K. F. Card · S. A. Price-Schiavi · B. Liu · E. Thomson  
H. Belmont · J. Builes · H. C. Wong (✉)  
Altor BioScience Corporation, 2810 N. Commerce Parkway,  
Miramar, FL 33025, USA  
E-mail: hingwong@altorbioscience.com  
Tel.: +1-954-443-8600/801  
Fax: +1-954-8610

E. Nieves  
Dade-Behring Corporation, Newark, DE 19702, USA

J. Hernandez · L. Sherman  
The Scripps Research Institute, La Jolla, CA 92037, USA

J. Weidanz  
Department of Pharmaceutical Sciences, School of Pharmacy,  
Texas Tech University, Amarillo, TX 79106, USA

J. L. Francis · A. Amirkhosravi  
Florida Hospital Cancer Institute, Orlando, FL 32804, USA

J. Jiao  
Hematech LLC, Sioux Falls, SD 57106, USA

injection into micrometastases is impossible and gene transfer is labor-intensive with limited success, making treatment of large patient populations impractical. Antibodies directed against tumor-specific antigens have been fused to cytokines and used as anticancer therapies in animal models. For example, fusion of IL-2 to antibodies that recognize ganglioside GD2 or the Id of a murine lymphoma has shown some promise for stimulating specific tumor responses in animal models [4, 16, 17, 27, 28, 36]. Further, fusion proteins consisting of IL-12 or IL-2 fused to anti-HER2/neu antibodies can mediate killing of HER2/neu positive tumor cells [29, 35]. One potential problem with antibody-based tumor targeting is that antibodies only recognize antigens expressed on the cell surface as integral membrane protein; however, there are many known tumor antigens that are not displayed in this way. Moreover, since many tumor-specific antigens are derived from aberrant expression of cell type specific proteins, they may exist in only a small number of tumor types. Thus, they have limited value as targets for antibody-based therapy. Therefore, it is highly desirable to identify a tumor antigen that is expressed in a broad range of tumors that may be recognized by molecules other than antibodies.

p53 is an intracellular tumor suppressor protein that acts to arrest abnormal cells at the G1/S phase of the cell cycle. When p53 is mutated, it loses its function as a tumor suppressor, thus allowing abnormal cells to continue to proliferate [49]. Mutated p53 has been shown to have a longer half-life than the wild type protein, and this longer half-life allows for accumulation and thus overexpression in a wide range of human malignancies [19, 22, 49]. Furthermore, overexpression of p53 in breast cancer cells has been correlated with aggressiveness of the tumor, such that individuals bearing p53 overexpressing tumors have shorter disease-free time and lower overall survival rates [30, 48]. Hence, p53 overexpression appears to be a significant tumor marker for a large number of human malignancies and represents a good target for broad spectrum targeted tumor immunotherapy. However, p53 cannot be used as a target for antibody-based therapies because it is not displayed independently on the cell surface. Instead the p53 gene product is processed and certain peptide epitopes are displayed on the cell surface in the context of MHC, which are recognized by TCRs [49]. As a result, we are investigating the use of TCRs rather than antibodies in targeting p53 for the creation of tumor immunotherapies.

In this report we describe the construction and characterization of a novel fusion protein comprising a soluble single chain HLA-A2.1-restricted TCR that recognizes an unmutated p53 peptide spanning p53 amino acid residues 264–272 genetically linked to human IL-2. We investigate the peptide-specific binding of the TCR portion of the molecule to peptide-loaded HLA-A2 as well as the specific IL-2 receptor binding capability and bioactivity of the IL-2 portion of the molecule. Our results suggest that these types of TCR-based fusion proteins could serve as an alternative to antibody-based

targeted tumor therapies or as an addition to other targeted tumor therapies such as antibody based immunocytokines. Separate and distinct approaches to targeting a tumor may demonstrate additive or synergistic antitumor effects.

## Materials and methods

### Materials

A2.1 264 CTL clone no. 5 was derived by limiting dilution cloning [50] from a CTL line specific for the human p53 264–272 peptide generated in HLA-A2.1 transgenic mice [49]. The expression vectors for producing peptide-loaded HLA-A2 tetramers were a generous gift from Dr. John Altman at Emory University in Atlanta, Ga. CHO.K1 Chinese hamster ovary, Jurkat human T lymphocyte, CTLL-2 mouse cytotoxic T lymphocyte, T2 human lymphoblast, A375 human melanoma, H57–597 hybridoma and BB7.2 hybridoma cell lines were obtained from American Type Culture Collection (Rockville, Md.). The T2 human lymphoblast cells are positive for HLA-A2.1, but deficient in TAP 1 and 2 proteins, which allows them to display empty MHC molecules that can then be loaded with exogenous peptide [2]. The A375 human melanoma cell line was tested in our laboratory for both HLA-A2.1 and p53 and was found to be positive for both antigens (data not shown). The H57–597 hybridoma produces a monoclonal antibody that recognizes an epitope in the murine TCR  $\beta$  constant region, and the BB7.2 hybridoma produces the BB7.2 monoclonal antibody that specifically recognizes an epitope on the alpha 2 domain of HLA-A2. The highly metastatic subclone of the human melanoma cell line A375, A375-C15N, which was used only for in vivo metastasis studies, was maintained by Dr. John Francis at the Florida Hospital Cancer Research Institute [53]. Recombinant human IL-2 and biotinylated anti-human IL-2 polyclonal antibodies used for the ELISA in the pharmacokinetic study were purchased from R&D Systems, Inc. (Minneapolis, Minn.). Anti-TCR  $C\beta$  mAb H57–597, anti-murine TCR  $V\beta 3$  mAb, anti-murine CD3 $\epsilon$  mAb, anti-human IL-2 mAb, anti-human CD25 blocking antibody and isotype control antibody, and FITC labeled goat anti-mouse IgG were obtained from Pharmingen (San Diego, Calif.). All cell culture media and additives were purchased from CellGro (Herndon, Va.), and all cell culture materials were purchased from Nunc (Rochester, N.Y.) unless otherwise noted. All mice were purchased from Harlan Labs (Indianapolis, Ind.).

### Cell culture

All cell lines were maintained in complete culture medium comprised of IMDM supplemented with 10% heat inactivated FBS, 2 mM L-glutamine and 1 mg/ml G418 (for transfected CHO cells only) at 37°C and 5% CO<sub>2</sub>. CTLL-2 cells were maintained in the same medium with the addition of 9 U/ml recombinant human IL-2. A375-C15N cells were maintained in RPMI-1640 with 10% heat inactivated FBS, penicillin and streptomycin (Life Technologies).

Mouse splenocytes were isolated by pressing spleens aseptically dissected from BALB/c mice through a nylon mesh screen and washing with culture medium. Red blood cells were lysed with Gey's solution for 2 min followed by addition of culture medium to stop the lysis. Single cell pellets were washed twice, resuspended at  $2.5 \times 10^6$  cells per ml in culture medium and cultured in complete culture medium containing 50  $\mu$ M 2-ME, 100 IU/ml recombinant human IL-2, and 50 ng/ml anti-murine CD3 $\epsilon$  mAb.

### Primers

Oligonucleotide primers were synthesized from sequences matching or complementing the mouse T cell receptor and human IL-2 genes:

KC228: 5'-GAGGTGGCCAGCCGGCCATGGCCAGTCAG TGACGCAGC-3'; KC229: 5'-GAGGTGACTAGTGTCTGG CTTTATAATTAG-3'; PRIB4: 5'-GGGGGCTCGAGCAAT TCAAAGTCATTAGACTC-3'; KC176: 5'-GAGGTGGAGC CCGGGTCTGCTCGGCCAGGC-3'; ET-TCRF1: 5'-CCC ACCGGTCAGTCAGTCAGCCAGCC-3'; KC-170: 5'-GTG GAGTTCGAAAAGGTGACTTACGTTTGTCTGCTCGGCC CAG-3'; KC231: 5'-CGATAAGTGTACTTACGTTTTTATT ATTCCATCGGCATGTACTCTTCTTCTCTCG-3'; KC208: 5'-GTGGAGATCGATAAGTGTACTTACGTTTTTATTATCG CGATCCGGAGTTAACGTCTGCTCGGCCAG-3'; KC327B: 5'-TAGGTGTCCGGAGCACCTACTTCAAGTTCTAC-3'; KC 328B: 5'-TAGGTGTCCGGAAGTTAGTGTGAGATGATG-3'; AP2: 5'-ACTCACTATAGGGCTCGAGCGGC-3'; C $\alpha$  HYB: 5'-GCTGTCTGAGACCGAGGATCTTTAACTG-3'; C $\beta$  HYB: 5'-TTGTTTGTTCGAATCTGTGCTTTTGATGG-3'.

## Constructs

The TCR gene was cloned from the T cell clone A2.1 264#5. We designate the single-chain TCR derived from this T cell clone 264scTCR. Poly(A)<sup>+</sup> RNA was extracted from the cells using a MicroFast Track kit (Invitrogen, Carlsbad, Calif.), and double-stranded cDNA was prepared and ligated to a double-stranded adaptor oligonucleotide using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, Calif.). To identify the V $\alpha$  and V $\beta$  segments, 5'-RACE PCR was performed using the A2.1 264#5 cDNA preparation and primers AP2 (specific for the adaptor DNA) and C $\alpha$  HYB (specific for the constant domain of the  $\alpha$  chain) or C $\beta$  HYB (specific for the constant domain of the  $\beta$  chain). PCR fragments were cloned into the pCR2.1 vector using the TA cloning kit (Invitrogen), and the sequence was determined using M13 forward and reverse primers. The T cell receptor V $\alpha$  chain was amplified using primers KC228 and KC229 to produce an *SfiI/SpeI* fragment, and the V $\beta$ C $\beta$  chain was amplified using primers PRIB4 and KC176 to generate an *XhoI/XmaI* fragment. The C $\beta$  chain was truncated just before the cysteine residue at amino acid 127 of the full length C $\beta$  chain. The *SfiI/SpeI* V $\alpha$  chain fragment was subcloned into *SfiI/SpeI* digested pKC60, an *E. coli* expression vector that encodes an irrelevant TCR, replacing the original TCR insert. The *XhoI/XmaI* V $\beta$ C $\beta$  fragment was then ligated into an *XhoI/XmaI* digest of this vector, yielding a vector encoding a soluble three-domain 264scTCR. The three-domain T cell receptor from this construct was amplified using primers ET-TCRF1 and KC170 to generate an *AgeI/Clal* DNA fragment, which was then used as a template for PCR with primers KC231 and KC208 to produce an *AgeI/HpaI* fragment.

The human IL-2 coding sequence was cloned by RT-PCR from total RNA isolated from Jurkat cells using a Mini Total RNA Kit (Qiagen, Valencia, Calif.) and Qiashtredder (Qiagen, Valencia, Calif.). Reverse transcription was carried out using primer KC328B, and PCR was carried out using primers KC327B and KC328B to produce a *BspEI/NruI* human IL-2 fragment. The *BspEI/NruI* IL-2 fragment was cloned into *BspEI/NruI* digested p149B1SP, a cloning vector encoding an irrelevant TCR/antibody fusion protein, replacing the antibody portion of the fusion protein. The IL-2 modified vector was digested with *AgeI* and *HpaI*, and the *AgeI/HpaI* 264scTCR fragment described above was ligated into it. Finally, an *AgeI/Clal* 264scTCR/IL-2 fusion protein fragment was cloned into *AgeI/BstBI* digested pSUN27, a scTCR/mouse kappa fusion vector, replacing the irrelevant TCR originally cloned in the vector, yielding the 264scTCR/IL-2 fusion protein expression vector, pSUN38. The 264scTCR/kappa fusion used as a negative control for some of the flow cytometry analyses was generated by cloning an *AgeI/BstBI* 264scTCR fragment into *AgeI/BstBI* digested pSUN27, replacing the original TCR.

For production of fusion protein in mammalian cells, CHO.K1 cells were electroporated using a Bio-Rad Gene Pulser, followed by limiting dilution cloning and selection in medium containing 1 mg/ml G418.

## Protein purification

264scTCR/IL-2 was purified from cell culture supernatant fluid by immunoaffinity chromatography using the monoclonal anti-murine TCR antibody H57-597, which recognizes an epitope in the constant region of the TCR  $\beta$  chain, coupled to a Sepharose 4B column (Amersham Pharmacia, Piscataway, N.J.). The purified sample was then concentrated and buffer-exchanged into PBS using an Ultrafree-15 centrifugal filter with a 30 kDa molecular weight cutoff membrane (Millipore, Bedford, Mass.). The TCR fusion protein samples were stored at 2–8°C (short term) or at –80°C (long term) for biochemical and functional analysis.

## SDS-PAGE

SDS-PAGE was performed under either reducing or non-reducing conditions using 4–12% Nu-PAGE polyacrylamide gels (Novex, San Diego, Calif.) and the Novex EX-Cell II system. SDS-PAGE gels were stained with Coomassie blue.

## ELISA

All ELISAs were performed using Maxisorb 96-well plates (Nunc, Rochester, N.Y.) coated with 100–200 ng/well anti-human IL-2 mAb or anti-murine TCR V $\beta$ 3 mAb. Fusion protein was detected with biotinylated anti-murine TCR H57 mAb, anti-murine TCR V $\beta$ 3 mAb or anti-IL-2 polyclonal Ab followed by streptavidin-HRP (Kirkegaard and Perry Laboratories, Gaithersburg, Md.), TMB substrate, and 0.18 M H<sub>2</sub>SO<sub>4</sub> to quench the reaction (BioFX, Owings Mills, Md.). Absorbance was measured at 450 nm using a 96-well plate reader (Bio-Tek Instruments, Inc., Winooski, Vt.).

## Cell staining with TCR fusion proteins

T2 cells pulsed with either p53 (aa 149–157) or p53 (aa 264–272) peptide were incubated with 0.5  $\mu$ g of 264scTCR/IL-2 fusion protein in 1% FBS in PBS for 30 min at room temperature. The cells were then incubated with 0.5  $\mu$ g anti-IL-2 Ab or 0.5  $\mu$ g biotinylated anti-TCR H57–597 mAb for 30 min at room temperature followed by 1  $\mu$ g anti-murine kappa-PE or 5 ng streptavidin-PE, respectively (both from Becton Dickinson, Franklin Lakes, N.J.). Samples were washed with 1% FBS in PBS before FACScan analysis (Becton Dickinson, Franklin Lakes, N.J.). To determine if both p53 peptides bound to HLA-A2 similarly, peptide-loaded cells were stained with BB7.2 for 30 min at room temperature followed by FITC-labeled goat anti-mouse IgG and analyzed on a FACScan instrument.

CTLL-2 cells were incubated with 0.5  $\mu$ g of fusion protein for 30 min at room temperature. To detect the bound fusion protein, 0.5  $\mu$ g biotinylated anti-TCR V $\beta$ 3 mAb was added and incubated for 30 min at room temperature followed by incubation with 5 ng streptavidin-PE, or the protein was detected using 0.5  $\mu$ g PE-labeled HLA-A2.1 p53 (aa 264–272) tetramer for 30 min. Conjugated HLA-A2 tetramers loaded with p53 peptides were produced as described previously [1]. Samples were washed with 1% FBS in PBS before FACScan analysis. For IL-2 receptor blocking experiments, CTLL-2 cells were incubated with  $\alpha$ -human CD25 blocking antibody or isotype control antibody for 30 min before incubation with 264scTCR/IL-2 or 264scTCR/kappa fusion protein. For staining of BALB/c mouse splenocytes, staining was carried out as described for the CTLL-2 cells using HLA-A2.1 p53 (aa 264–272) tetramers to detect bound fusion protein.

A375 cells were harvested with enzyme-free cell dissociation buffer (Sigma, St. Louis, Mo.). Samples of 5 $\times$ 10<sup>5</sup> cells were washed with 1% FBS in PBS and incubated with no fusion protein, 5  $\mu$ g 3C8 (an irrelevant TCR/IL-2 fusion protein), or 5  $\mu$ g 264scTCR/IL-2 for 30 min at room temperature followed by incubation with 1  $\mu$ g biotinylated H57–597 mAb. Cells were then incubated with

PE-labeled streptavidin for 15 min at room temperature, washed, and analyzed by FACScan.

#### Cell conjugation

T2 cells pulsed with either p53 (aa 264–272) peptide or p53 (aa 149–157) peptide were labeled with 7.88 ng/ml dihydroethidium (HE) (Molecular Probes, Inc., Eugene, Ore.), and CTLL-2 cells were labeled with 50 ng/ml calcein AM (Molecular Probes, Inc., Eugene, Ore.). After washing, the two populations of labeled cells were mixed together at a 1:1 ratio in the presence or absence of 2  $\mu$ g 264scTCR/IL-2 fusion protein for 20 min at room temperature. Cells were then analyzed by FACScan.

#### Bioassay

CTLL-2 cells were seeded at  $4 \times 10^3$  cells/well in 100  $\mu$ l culture medium containing various concentrations of either recombinant IL-2 or 264scTCR/IL-2 and incubated for 21 h at 37°C and 5% CO<sub>2</sub>. As a control for specificity CTLL-2 cells were incubated with 264scTCR/IL-2 in the presence or absence of 5 or 50  $\mu$ g anti-human CD25 blocking antibody or isotype control antibody and incubated for 21 h at 37°C and 5% CO<sub>2</sub>. Cell proliferation reagent WST-1 (Roche Inc., Indianapolis, Ind.) was added at 20  $\mu$ l/well and incubated for 4 h at 37°C and 5% CO<sub>2</sub>. Absorbance was read at 450 nm on a 96-well plate reader.

#### Pharmacokinetics in mice

For all experiments involving animals, principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed, as well as specific national laws where applicable. Female BALB/c mice were injected intravenously via the lateral tail vein with 32  $\mu$ g 264scTCR/IL-2 fusion protein diluted with PBS to a total volume of 100  $\mu$ l. Serum was collected from one group of mice not injected with 264scTCR/IL-2 to establish background levels. Serum was collected by tail bleed from the injected groups at 15 and 30 min, 1, 2, 4, 8 and 24 h. Blood samples were centrifuged at  $14,000 \times g$  at 4°C for 10 min, and serum was collected and stored at –80°C until use. 264scTCR/IL-2 concentrations were determined by ELISA using anti-TCR V $\beta$ 3 or anti-IL-2 monoclonal antibodies for capture and either biotinylated anti-TCR H57 monoclonal or anti-IL-2 polyclonal antibodies followed by streptavidin HRP for detection.

#### In vivo studies

Female athymic nude mice (nu/nu) were injected with  $5.0 \times 10^5$  A375-C15N cells via the lateral tail vein. Animals were injected with varying doses of either 264scTCR/IL-2 (32, 10, 3, 1 or 0.1  $\mu$ g in 100  $\mu$ l total volume) or recombinant human IL-2 (8, 2.5, 0.75, 0.25, or 0.025  $\mu$ g in 100  $\mu$ l total volume) on days 1, 2, 3, 4, 7, 10, 14, 17, 21, 28 and 35 post-tumor cell injection. Forty-two days after tumor cell injection, all animals were humanely killed, the lungs were removed and fixed in Bouin's solution, and surface pulmonary tumor nodules were counted. Tumor nodules on each lung were counted by two observers and the average counts were recorded.

## Results

### Generation of TCR fusion protein constructs

We have constructed a fusion protein comprising a three-domain, HLA-A2.1 restricted mouse TCR specific

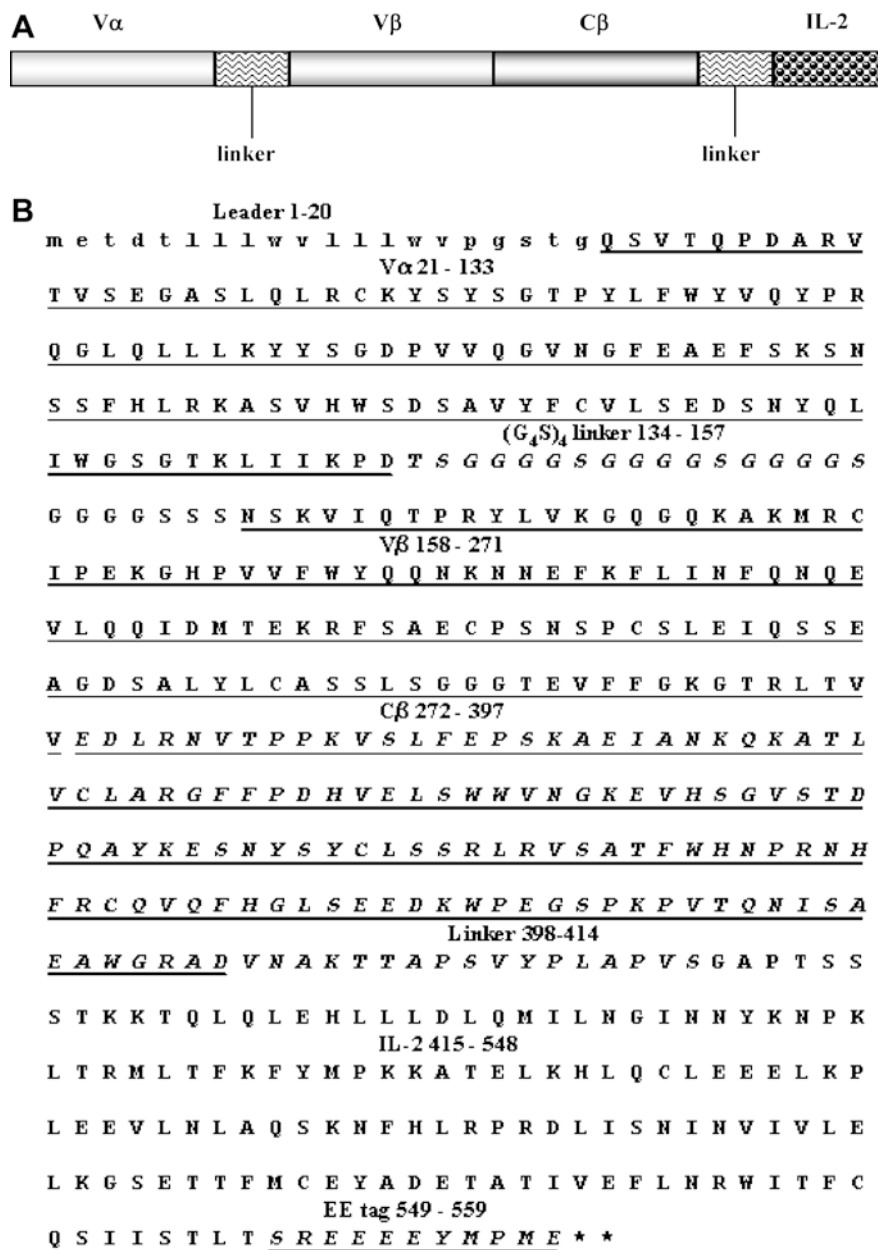
for a p53 peptide antigen fused to human IL-2. For this TCR fusion protein construct, the V $\alpha$  and V $\beta$ /C $\beta$  regions were generated by RT-PCR from RNA isolated from a mouse T cell clone that produces TCRs specific for human p53 (aa 264–272) peptide. The carboxyl-terminal end of the variable region of the TCR $\alpha$  chain (V $\alpha$ 3) was fused via a flexible linker (G<sub>4</sub>S)<sub>4</sub> [21] to the N-terminus of the V $\beta$  (V $\beta$ 3) to generate the antigen binding portion of the TCR. The C $\beta$  domain, which is directly linked to the V $\beta$  domain, was truncated at the amino acid residue just prior to the final cysteine, removing the transmembrane and cytoplasmic domains, to generate a soluble single-chain TCR molecule (Fig. 1A and B). Human IL-2 was fused to the TCR portion via a short linker (amino acid sequence VNAKTTAPSVYPLAPV). An EE tag (amino acid sequence EEEEEYM-PME) [11] was inserted just downstream of the IL-2 portion of the fusion molecule to allow for detection of the TCR/IL-2 fusion protein by an anti-EE tag mAb [11] if desired. Mammalian cell expression is driven by a CMV promoter, secretion is directed by an antibody light chain leader, and selection is carried out by G418 resistance.

### Expression of TCR/IL-2 fusion protein in mammalian cells

To characterize the 264scTCR/IL-2 fusion protein, the 264scTCR/IL-2 construct was stably transfected into CHO-K1 cells. Stable transfectants secreting 264scTCR/IL-2 fusion protein were selected using ELISA assays as described in Materials and Methods. Positive signals for these ELISAs indicate that the transfected cells secrete 264scTCR/IL-2 fusion protein that is recognized by both anti-murine TCR and anti-human IL-2 antibodies, suggesting that the secreted 264scTCR/IL-2 is properly assembled and folded in the transfected cells and that it remains intact when it is secreted from the cells.

264scTCR/IL-2 fusion protein was purified from cell supernates by immunoaffinity chromatography with a yield of approximately 1.8 mg/l of supernate. Purified fusion protein was subjected to SDS-PAGE and Coomassie staining. Under either reducing or non-reducing conditions, the predominant stained band migrated at approximately 60 kDa (Fig. 2), which is consistent with the predicted molecular mass for this protein and indicates that the fusion protein remains intact with no unexpected intramolecular disulfide bonds when it is secreted from the cells. The larger band in the non-reducing gel may be a dimer form of the fusion protein. This conclusion is based on the observation that the larger band has the apparent molecular mass approximately twice that of the fusion protein and this band is reduced to the size of the fusion protein under reducing conditions. Taken together, these data indicate that the transfected CHO cells produce 264scTCR/IL-2 fusion protein of the expected molecular mass and that it is properly folded, assembled and secreted as a soluble fusion protein.

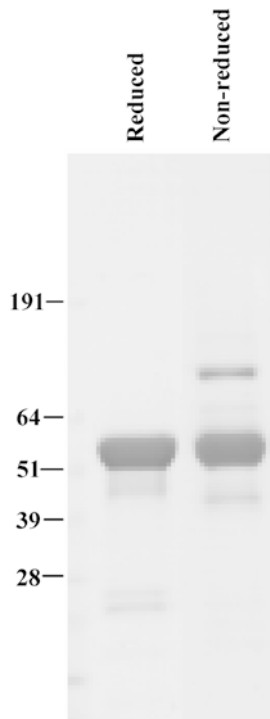
**Fig. 1** The 264scTCR/IL-2 fusion protein. **A** Schematic representation of the domain structure of the 264scTCR/IL-2 fusion protein. **B** Amino acid sequence of 264scTCR/IL-2 fusion protein. Amino acid numbers for each domain of the fusion protein are indicated in the figure



#### MHC/peptide binding ability of the TCR portion of the 264scTCR/IL-2 fusion protein

The ability of the 264scTCR/IL-2 fusion protein to bind to peptide-loaded MHC was determined by flow cytometry. T2 cells were loaded with p53 (aa 264–272) or p53 (aa 149–157) (control) peptide and stained with 264scTCR/IL-2 fusion protein. Cells loaded with p53 (aa 264–272) stained positively with 264scTCR/IL-2 when detected with either the anti-TCR Cβ mAb or the anti-IL-2 detection antibody (Fig. 3A and B). Cells loaded with p53 (aa 149–157) control peptide did not stain with either the anti-TCR Cβ mAb or the anti-IL-2 detection antibodies. To demonstrate that the lack of staining of p53 (aa 149–157) loaded T2 cells is not due to an inability to of the p53 (aa 149–157) peptide to

bind to HLA-A2, T2 cells loaded with no peptide, p53 (149–157), or p53 (264–272) peptide were stained with BB7.2 α-HLA-A2 monoclonal antibody. Cells loaded with either p53 peptide stained stronger than cells loaded with no peptide, suggesting that both peptides are capable of binding to HLA-A2 molecules (Fig. 3C). T2 cells were also stained for IL-2 receptor and were found not to express IL-2 receptor (data not shown); thus, these data indicate that binding of the 264scTCR/IL-2 fusion protein is mediated by the TCR component of the fusion protein. The lack of staining by the fusion protein when T2 cells were loaded with the control peptide also indicates that staining is mediated by the TCR component and that the staining is specific for the appropriate peptide. These data indicate that the TCR portion of the 264scTCR/IL-2 fusion protein is capable

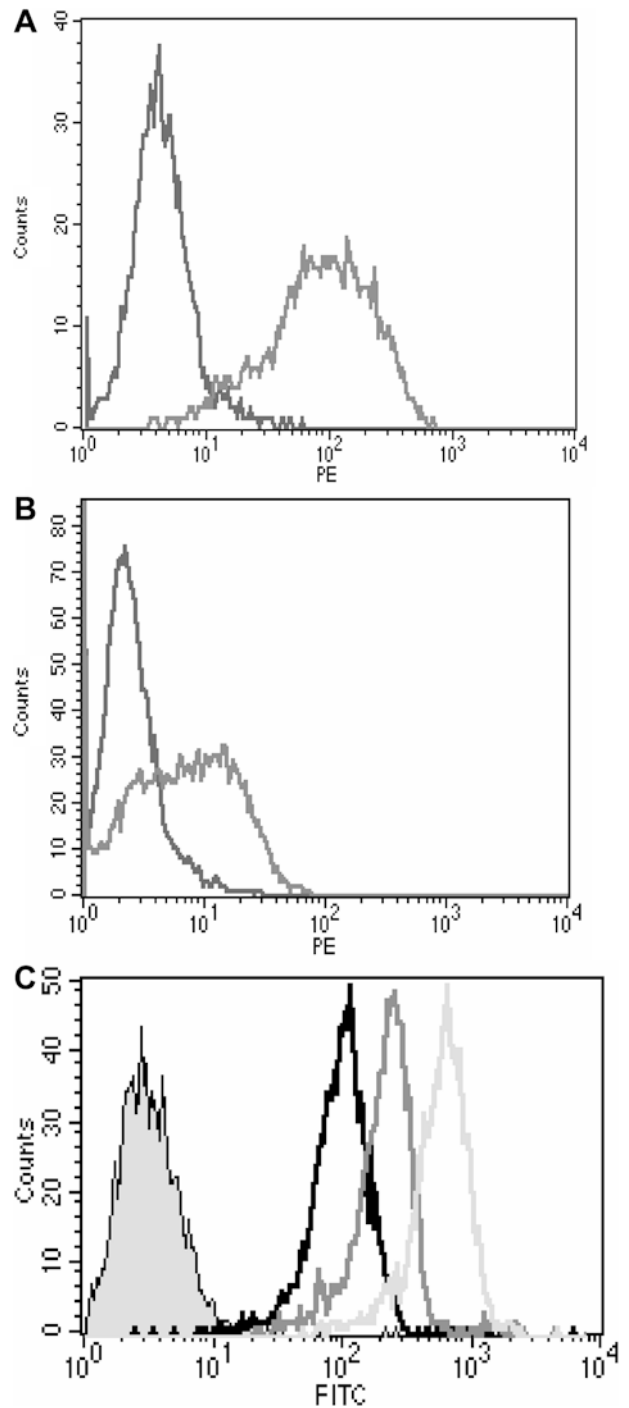


**Fig. 2** Production of 264scTCR/IL-2 fusion protein in transfected CHO cells. CHO cells were stably transfected with the 264scTCR/IL-2 expression vector. The secreted fusion protein was purified by immunoaffinity chromatography and subjected to SDS-PAGE under either reducing or non-reducing conditions as indicated at the top of the figure. SDS-PAGE gels were stained with Coomassie brilliant blue

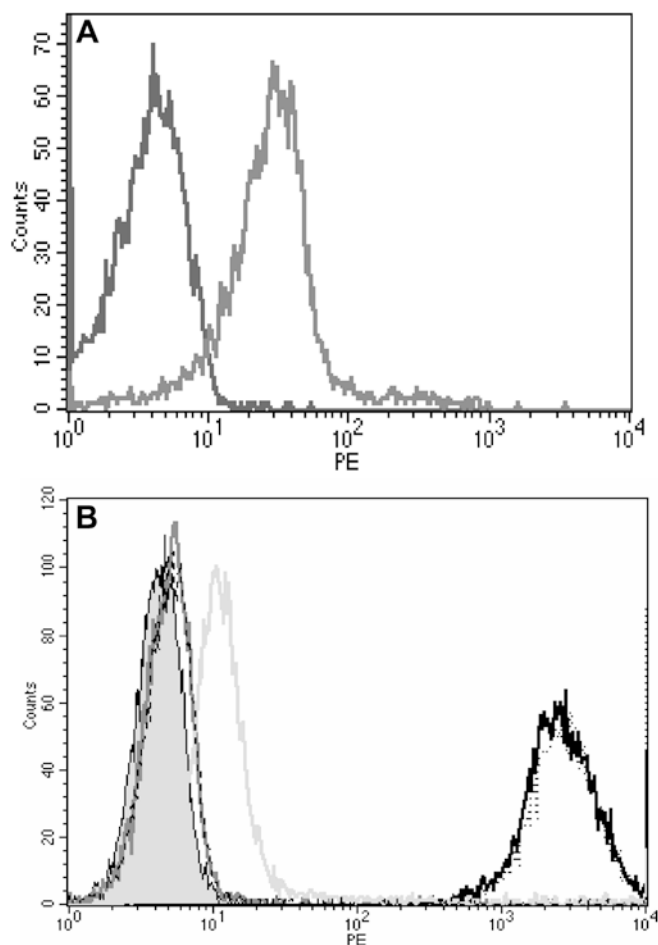
of recognizing its specific peptide in the context of HLA-A2.

#### IL-2 receptor binding ability of the IL-2 portion of the 264scTCR/IL-2 fusion protein

The IL-2 receptor binding capability of the IL-2 portion of the 264scTCR/IL-2 fusion protein was studied by flow cytometry. Primary mouse splenocytes were isolated and stimulated with rIL-2 and anti-CD3 to generate T cell blasts. Stimulated splenocytes that express IL-2 receptor stained positively with p53 (aa 264–272) loaded HLA-A2 tetramers only in the presence of 264scTCR/IL-2 fusion protein (Fig. 4A). Likewise, CTLL-2 mouse cytotoxic T lymphocytes, which constitutively express IL-2 receptor, stained positively with the 264scTCR/IL-2 fusion protein, but not with a 264scTCR/kappa fusion protein (Fig. 4B). When CTLL-2 cells were incubated with  $\alpha$ -human CD25 blocking antibody or isotype control antibody followed by 264scTCR/IL-2, staining was substantially reduced when the cells were incubated with the blocking antibody, but not with isotype control antibody. The lack of signal from the CTLL-2 cells incubated with a 264scTCR/mouse kappa chain fusion protein or with IL-2 receptor blocking antibody indicates that staining of these cells is mediated by the IL-2 portion of the



**Fig. 3** MHC/peptide binding ability of the TCR portion of 264scTCR/IL-2 fusion protein. T2 cells were loaded with p53 (aa 264–272) peptide (*gray line*) or p53 (aa 149–157) peptide (*black line*), and stained with either (A) 264scTCR/IL-2 fusion protein and anti-TCR C $\beta$  mAb or (B) 264scTCR/IL-2 fusion protein and anti-IL-2 mAb. (C) T2 cells loaded with p53 (aa 264–272) peptide (*dark grey line*), p53 (aa 149–157) peptide (*light grey line*), or no peptide (*black line*) were stained with anti-HLA-A2 BB7.2 mAb followed by FITC labeled goat anti-mouse IgG. The *shaded peak* is unstained T2 cells

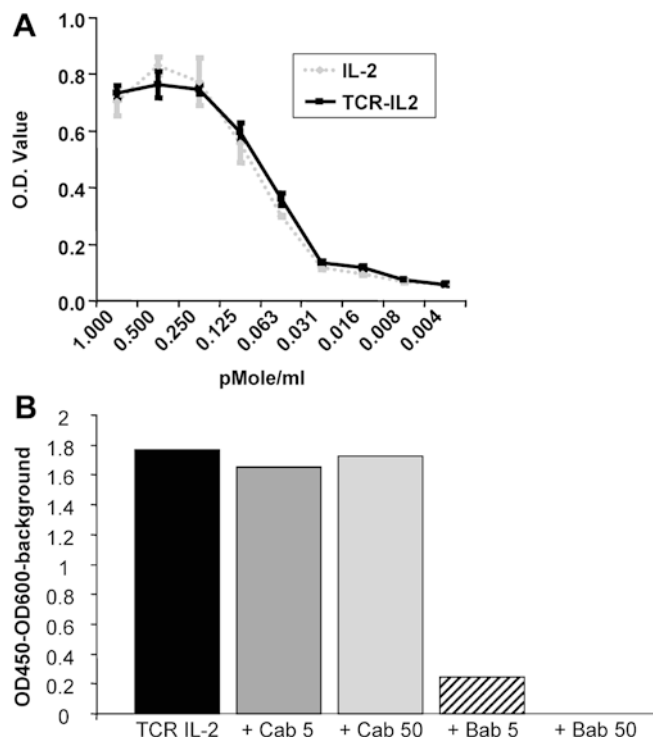


**Fig. 4** IL-2 receptor binding ability of the IL-2 portion of 264scTCR/IL-2 fusion protein. **A** Mouse splenocytes were stimulated with IL-2 and anti-CD3 $\epsilon$  mAb and then incubated in the presence (gray line) or absence (black line) of 264scTCR/IL-2 fusion protein. Bound fusion protein was detected with PE labeled HLA-A2 p53 (aa 264–272) tetramers. **B** CTLL-2 cells were incubated with  $\alpha$ -human CD25 blocking antibody or isotype control antibody followed by 264scTCR/IL-2 or 264scTCR/kappa fusion protein. Bound fusion protein was detected with PE labeled  $\alpha$ -TCR-V $\beta$  antibody. The shaded peak is unstained CTLL-2 cells. Black line: CTLL-2 cells stained with 264scTCR/IL-2 only. Gray dotted line: CTLL-2 cells incubated with control antibody followed by 264scTCR/IL-2. Light gray line: CTLL-2 cells incubated with  $\alpha$ -human CD25 blocking antibody followed by 264scTCR/IL-2. Dark gray line: CTLL-2 cells stained with 264scTCR/kappa fusion protein. Black dashed line: CTLL-2 cells stained with  $\alpha$ -TCR-V $\beta$

264scTCR/IL-2 fusion protein. These data suggest that the IL-2 portion of the 264scTCR/IL-2 fusion protein is capable of binding to the IL-2 receptor.

#### Biological activity of 264scTCR/IL-2 fusion protein

To demonstrate biological activity of the IL-2 portion of the 264scTCR/IL-2 fusion protein, IL-2 dependent CTLL-2 cells were cultured with either 264scTCR/IL-2 or recombinant IL-2 at various concentrations, and cell viability was assessed using WST-1. As shown in Fig. 5A, the ability of rIL-2 or 264scTCR/IL-2 to



**Fig. 5** Biological activity of 264scTCR/IL-2 fusion protein. **A** CTLL-2 cells were cultured with 264scTCR/IL-2 (solid line) or recombinant IL-2 (dotted line) at various concentrations as indicated at the bottom of the figure. **B** CTLL-2 cells were incubated with 264scTCR/IL-2 and  $\alpha$ -human CD25 blocking antibody or isotype control antibody as indicated at the bottom of the figure. Cell viability was measured by incubation with WST-1 and absorbance was read at 450 nm. Cab + 5: 5  $\mu$ g control antibody; Cab + 50: 50  $\mu$ g control antibody; Bab + 5: 5  $\mu$ g blocking antibody; Bab + 50: 50  $\mu$ g blocking antibody

support the growth of CTLL-2 cells was dose dependent, wherein there was more cell proliferation at higher doses of either recombinant IL-2 or 264scTCR/IL-2. Further, there were similar levels of cell proliferation when equivalent molar amounts of either recombinant IL-2 or 264scTCR/IL-2 were used. As a further control for specificity, CTLL-2 cells were incubated with 264scTCR/IL-2 with  $\alpha$ -human CD25 blocking antibody or isotype control. When the blocking antibody was included in the culture, proliferation was substantially decreased with both concentrations of blocking antibody, but proliferation of the CTLL-2 cells was unaffected by either concentration of control antibody (Fig. 5B). Taken together these data indicate that the IL-2 portion of 264scTCR/IL-2 has similar biological activity to recombinant IL-2 in vitro and that the proliferation activity of the fusion protein is dependent on the IL-2 portion of the molecule.

#### Conjugation of cells mediated by 264scTCR/IL-2 fusion protein

In order for the 264scTCR/IL-2 fusion protein to be useful as a therapeutic agent, it must be able to bring

together target and effector cells through its TCR and cytokine portions, respectively. To demonstrate that the 264scTCR/IL-2 fusion protein can effectively conjugate cells, T2 cells were loaded with either p53 (aa 264–272) or p53 (aa 149–157) peptides and then labeled with dihydroethidium (HE). CTLL-2 cells were labeled with calcein AM, and the two labeled cell populations were mixed and incubated in the presence or absence of 264scTCR/IL-2 fusion protein. Samples were analyzed by flow cytometry. When the two cell populations were incubated in the absence of the 264scTCR/IL-2 fusion protein (Fig. 6A and B) or when the T2 cells were loaded with control peptide and incubated with the CTLL-2 cells in the presence of 264scTCR/IL-2 fusion protein (Fig. 6C), the cells remained as two distinct populations on the flow cytometry histograms representing approximately 45% of the total population each (Fig. 6A, B and C, regions 1 and 3), with only approximately 0.46% of the total population falling in the double-stained cell window (Fig. 6A, B and C, region 2). However, when the T2 cells were loaded with p53 (aa 264–272) peptide and incubated with the CTLL-2 cells in the presence of the 264scTCR/IL-2 fusion protein (Fig. 6D), a double staining population of cells appears, representing 4.1% of the total population (Fig. 6D, region 2, conjugated cells), suggesting that T2 cells were conjugated to CTLL-2 cells via the 264scTCR/IL-2 fusion protein.

#### Pharmacokinetics of 264scTCR/IL-2 in mice

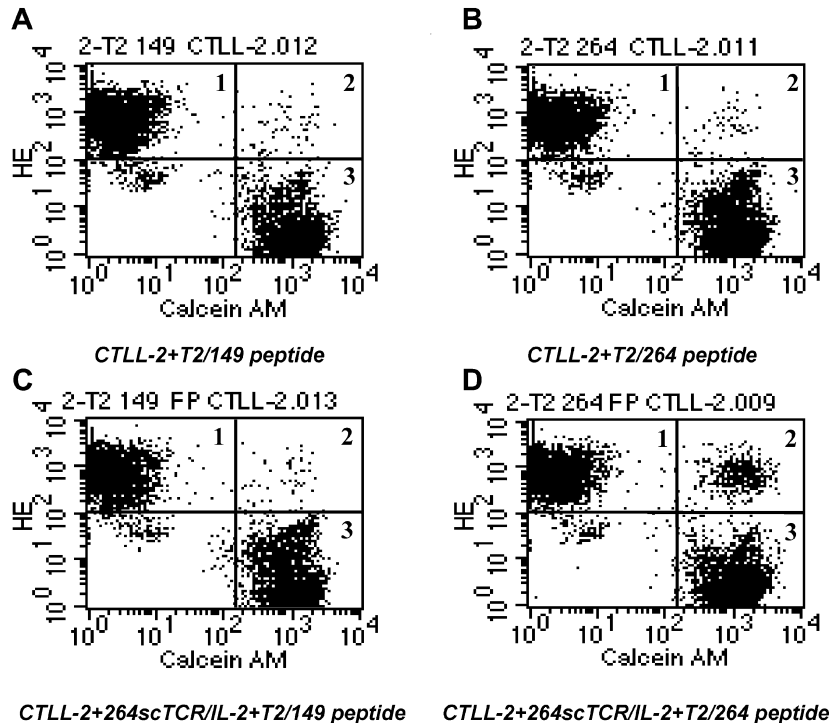
The pharmacokinetics of the 264scTCR/IL-2 fusion protein was measured in BALB/c mice. Mice were injected intravenously, and serum samples were collected

at various time points. The serum levels of 264scTCR/IL-2 fusion protein were measured using ELISA. The ELISA detection was performed using anti-TCR mAb capture/anti-IL-2 Ab detection (Fig. 7A), anti-TCR mAb capture/anti-TCR mAb detection (Fig. 7B) or anti-IL-2 mAb capture/anti-IL-2 polyclonal Ab detection (Fig. 7C) to determine whether the fusion protein is modified or cleaved *in vivo*. Mice injected with 264scTCR/IL-2 fusion protein showed no obvious signs of toxicity. In these assays we detected a maximum concentration of 0.75 to 2.5  $\mu\text{g/ml}$  of 264scTCR/IL-2 with an apparent serum half-life of 1.6 to 3.0 h, depending on the ELISA format used (Fig. 7). Since the reported serum half-life of free IL-2 is only  $\sim 5$  min [6], these data suggest that the fusion protein is not cleaved *in vivo*, but instead remains intact for a relatively long period of time in the blood. The small variability in the half-life of 264scTCR/IL-2 determined in these studies is most likely due to the differences in the sensitivity of the ELISA assays.

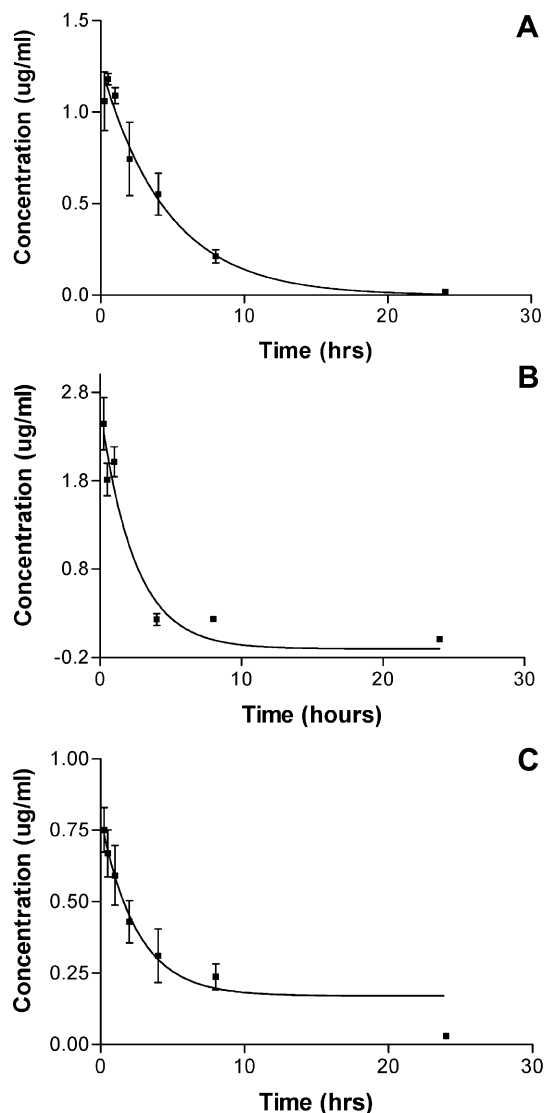
#### Tumor cell staining with 264scTCR/IL-2

In order for the 264scTCR/IL-2 fusion protein to be useful as a therapeutic agent, it must be able to recognize and bind to its target tumor cells. To test whether the 264scTCR/IL-2 is capable of binding to tumor cells, A375 human melanoma cells, which express both HLA-A2.1 and p53, were stained with either 264scTCR/IL-2 or 3C8, an irrelevant TCR/IL-2 fusion protein. Cells not incubated with fusion protein and cells incubated with 3C8 did not stain with the H57–597 detection antibody, while cells incubated with 264scTCR/IL-2 stained

**Fig. 6** Conjugation of CTLL-2 cells with peptide-loaded T2 cells mediated by 264scTCR/IL-2 fusion protein. T2 cells were loaded with either p53 (aa 264–272) (B and D) or p53 (aa 149–157) (control) peptides (A and C) and then labeled with HE. CTLL-2 cells were labeled with calcein AM. Labeled cells were mixed and incubated in the presence (C and D) or absence (A and B) of 264scTCR/IL-2 fusion protein, and the samples were analyzed by flow cytometry. Assay conditions including loading peptide used and presence or absence of fusion protein are indicated *beneath* each histogram. Single-stained regions are marked 1 and 3 and the double-stained cell population is marked 2





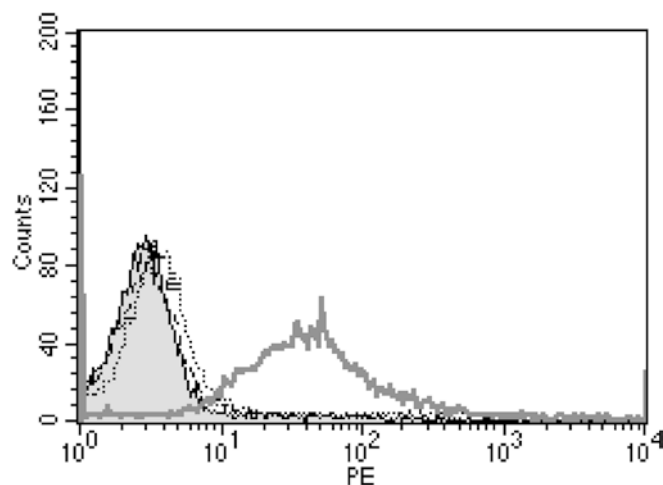


**Fig. 7** Serum half-life of 264scTCR/IL-2 fusion protein. BALB/c mice were injected with 264scTCR/IL-2 fusion protein, and serum samples were collected at 15 and 30 min, 1, 4, 8 and 24 h post injection. Serum concentrations of 264scTCR/IL-2 were determined by ELISA using the following formats: (A) anti-TCR mAb capture/anti-IL-2 Ab detection, (B) anti-TCR mAb capture/anti-TCR mAb detection and (C) anti-IL-2 mAb capture/anti-IL-2 Ab detection

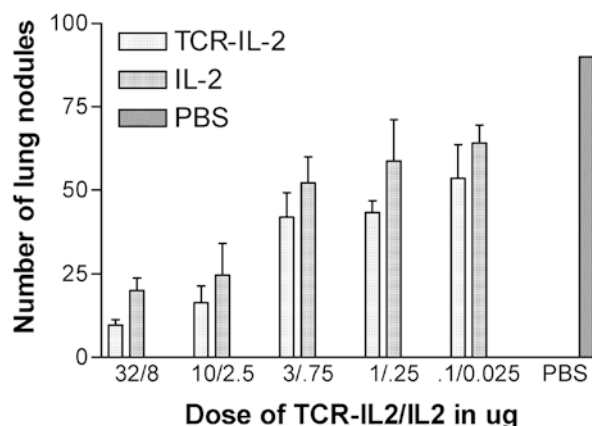
positively with the detection antibody (Fig. 8). This result suggests that the 264scTCR/IL-2 fusion protein is capable of recognizing and binding to its target tumor cell and may be useful as an anti-cancer therapeutic in vivo.

#### Anti-tumor effects of 264scTCR/IL-2 fusion protein

To determine if the 264scTCR/IL-2 fusion protein has anti-tumor activity in vivo, an experimental metastasis assay was performed. Female athymic nude mice were injected with the highly metastatic A375 human melanoma subclone, A375-C15N and treated with varying



**Fig. 8** Tumor cell staining with 264scTCR/IL-2. A375 human melanoma cells were incubated with no fusion protein (dashed black line), 5 μg 3C8 TCR/IL-2 fusion protein (control) (dotted line), or 5 μg 264scTCR/IL-2 fusion protein (solid black line) followed by staining with H57-597 mAb. Unstained cells are represented by the shaded area



**Fig. 9** Anti-tumor effect of 264scTCR/IL-2 fusion protein. Female athymic nude mice were injected with highly metastatic A375-C15N cells and treated with 264scTCR/IL-2, recombinant IL-2 or PBS. Forty-two days after tumor cell injection, the lungs were removed, lung nodules were counted, and the mean number of lung nodules relative to the PBS-treated control group was plotted

doses of either 264scTCR/IL-2 or recombinant IL-2. Forty-two days after tumor cell injection, lung nodules were counted. Both 264scTCR/IL-2 and recombinant IL-2 reduced lung metastasis in a dose-dependent manner (Fig. 9). However, at all doses lung metastasis was reduced to a greater degree with the 264scTCR/IL-2 fusion protein, suggesting that targeting the cytokine to the tumor may provide greater efficacy as a cancer therapeutic.

#### Discussion

In these studies we describe the construction and expression of a soluble three-domain mouse scTCR that

recognizes human p53 peptide (aa 264–272) in the context of HLA-A2.1. The three-domain scTCR is fused to human IL-2 yielding a soluble 264scTCR/IL-2 fusion protein that is expressed at high levels and secreted from mammalian cells. The TCR portion of the 264scTCR/IL-2 fusion protein retains its MHC-restricted, peptide-specific antigen-binding properties, and the IL-2 portion binds to IL-2 receptor and is biologically active. Moreover, these studies further demonstrate that this fusion protein is capable of conjugating target and effector cells, exhibits favorable pharmacokinetics in mice, can bind to target tumor cells and has anti-tumor effects. Therefore, soluble scTCR fusion proteins may provide access to another repertoire of antigens for targeted immunotherapy that are not recognizable by antibody-based immunotherapies. TCR-based therapies may serve as an alternative to antibody-based treatments or as a useful addition to other targeted tumor therapies. Treating tumors using multiple distinct approaches may have additive or synergistic antitumor effects and hence greater success in the clinical setting.

There has been some difficulty expressing soluble recombinant TCRs that are stable and retain antigen binding properties. Several attempts have been made at expressing recombinant TCRs as membrane-associated fusion proteins or as inclusion bodies in *E. coli*. Each of these methods was hindered by low or unstable expression or dimerization and also by the need for proteolytic cleavage and/or refolding to generate soluble TCRs [5, 8, 9, 18, 24, 26, 54]. Further, the activity of soluble TCRs made by these methods tends to be low. Our scTCRs are produced in mammalian cells as soluble fusion proteins without any membrane association, eliminating the extra step of enzymatic cleavage or refolding, which could affect fusion protein function. As the data presented here demonstrate, these TCRs fold correctly and are secreted intact from transfected eukaryotic cells. Moreover, fusion of a TCR in this format to a cytokine via a short linker does not interfere with MHC-restricted, peptide-specific antigen-binding or specific cytokine activity. No enzymatic modification or protein refolding is necessary to produce soluble active TCRs, and the effector molecule, in this case IL-2, is covalently bound, eliminating the need for chemical crosslinking, which can be unstable. Thus, this is the first report of the production of a truly soluble recombinant TCR that retains its MHC-restricted, peptide-specific antigen-binding properties.

One potential drawback of using recombinant TCR fusion proteins as targeted immunotherapies is the weak binding affinity of the TCR for its cognate MHC/peptide. We have determined the dissociation constant of the 264scTCR for its cognate MHC/peptide to be approximately  $10^{-7}$  M at physiological conditions using surface plasmon resonance detection (unpublished results). This high affinity for TCR interaction with MHC/peptide complex is likely due to its CD8 independence as the TCR was generated in HLA-A2.1 transgenic mice [49] and mouse CD8 has been shown not to interact with human HLA [45]. The affinity of the 264scTCR is

sufficient to bind to unmanipulated tumor cells and effectively conjugate target and effector cells, both of which we have shown in these studies. Another potential problem with this type of therapy is that the use of TCR-based cancer therapies may be limited by the HLA type of the patient, which may be confounded by the down-regulation of HLA expression in some tumors. Therefore, it will be necessary to screen patients before treatment with a TCR-based therapy to determine if they would benefit from this type of therapy in much the same way that breast cancer patients are screened for ErbB-2 expression before treatment with herceptin. We are currently developing tumor screening methods using another 264scTCR fusion protein so that tumor histoarrays may be stained to determine the proportion and types of tumors that may be treated with TCR-based therapies. Development of TCR-based fusion proteins targeting other HLA types should broaden the treatable patient population.

With these data in mind, we performed preliminary studies using the 264scTCR/IL-2 fusion protein as an anti-tumor agent against A375-C15N human melanoma cells in an experimental lung metastasis model in nude mice. Mice treated with either 264scTCR/IL-2 or recombinant IL-2 showed no obvious signs of toxicity. Both treatments resulted in reduction of lung metastasis; however, at all doses treatment with 264scTCR/IL-2 was more effective than recombinant IL-2. At this point it is unclear whether the improved anti-tumor efficacy of the 264scTCR/IL-2 fusion protein is due to the increase in the half-life of IL-2 or to the concentration of the cytokine at the site of the tumor. These studies were performed in athymic nude mice in the absence of T-cells; thus, the effector cells in these studies were those of the innate system such as natural killer cells and macrophages that can be stimulated by IL-2 [10, 41]. IL-2 has a profound effect on T-cells, which this model system does not address. Thus, we are currently performing T-cell reconstitution studies in this model to determine if the fusion protein has a stronger effect than free IL-2 in the presence of T-cells. In addition, we are performing imaging studies to determine the degree of 264scTCR/IL-2 fusion protein localization at the site of the tumor.

The main problems with systemic administration of cytokines to treat tumors are their short serum half lives and toxicity. To circumvent this problem cytokines have been delivered over a period of time as infusions; however, high systemic doses of cytokines have widely reported toxic side effects, and delivery by infusion is time consuming and inconvenient for the patient and has its own undesirable side effects [7, 31, 34, 44]. Delivery of cytokines such as interferon alpha-2a in pegylated forms is being tested, and although the pegylated cytokine has a longer half life, the side effects are similar to that of the unpegylated form [32, 39]. Targeting delivery via antibody-cytokine fusion proteins is thought to take advantage of the paracrine nature of IL-2, and because these molecules concentrate cytokine at the site of the tumor, they may allow the cytokine to be used at lower,

less toxic doses. Antibody-cytokine fusion proteins such as the anti-disialoganglioside ch14.18/IL-2 fusion protein (ch14.18-IL-2) have been shown to have serum half lives of  $\sim 4$  h, which significantly increases the serum half life of IL-2 [23]. However, it was reported that this fusion protein is cleaved *in vivo* and that the IL-2 portion is then cleared with kinetics similar to those of free IL-2 [23]. Our 264scTCR/IL-2 fusion protein has an apparent serum half-life of  $\sim 3$  h and appears to remain intact in the blood. Thus, the 264scTCR/IL-2 fusion protein effectively increases the half-life of IL-2 and survives intact in the blood, suggesting that it has potential as a new agent for immunomodulatory cancer therapy. As reported with other biologics, at higher doses the serum half life of our fusion protein may increase [3, 25, 37, 38]. If this is the case for 264scTCR/IL-2, it should further improve the efficacy of the molecule against tumors. Escalating dose studies to further investigate toxicity and pharmacokinetic parameters are underway.

Besides T-cells, IL-2 has been shown to have stimulatory effects on a broad range of immune cell types including B cells, monocytes, macrophages, LAK cells and NK cells [10, 41]. For this reason, IL-2 concentrated at the tumor site should activate local T-cells as well as other IL-2 responsive cells, thereby recruiting effector cells to the site of the tumor. In addition, recirculation of these effector cells may be useful for eradication of distant micrometastases, which may be heterogeneous with respect to expression of the targeted tumor antigen. Straten et al. reported that targeted delivery of IL-2 via anti-ganglioside ch14.18-IL-2 fusion protein to murine melanoma resulted in clonal T-cell expansion [51]. Moreover, both target antigen positive and negative tumors implanted in the same mouse regressed as a result of this treatment, indicating that T cell clones activated locally by targeted therapy recirculated and mediated eradication of the distant tumor that was not targeted by the therapy [51]. The anti-GD<sub>2</sub> ch14.18-IL-2 fusion protein has also been shown to be effective in the eradication of liver and bone marrow metastases in a murine neuroblastoma model. However, in this case the anti-tumor effect was shown to be NK cell mediated [28]. The activation of other types of immune effector cells by IL-2 for cancer therapy may be useful because many cancer patients have severe T-cell dysfunction either from the cancer itself or from the damaging effects of the radiotherapy or chemotherapy used to treat the cancer [14, 56]. However, most patients retain the ability to expand and activate NK cells after IL-2 treatment [12, 13, 15]. Thus, by concentrating IL-2 at the site of a tumor our 264scTCR/IL-2 fusion protein may potentiate a wide ranging immune response including activation and proliferation of a variety of T-cell clones as well as activation of NK cells or other members of the innate immune system. Such a multifaceted anti-tumor response may be more effective for the eradication of primary tumors as well as distant metastases.

In summary, we demonstrate that it is possible to construct a biologically active bi-functional molecule comprised of a TCR and a cytokine. This fusion protein is capable of binding to tumor cells, mediating the conjugation of target and effector cells, and appears to have reasonable pharmacokinetic properties in mice. Although this report restricts its discussion to p53 as a target, other gene products that are upregulated and presented in the context of MHC on tumor or virally infected cells may be used as targets for TCR-based immunotherapies. In addition, other immunomodulatory molecules such as GM-CSF, IFN $\gamma$  or IFN- $\alpha$  could be linked to the TCR to activate other effector cells for an anti-tumor or anti-viral response. Thus, we are also constructing and characterizing additional TCR fusion proteins that target other cancer and viral antigens containing different effector molecules. These types of novel molecules have the potential of forming a new class of immunotherapeutics for the treatment of cancer and viral infection.

**Acknowledgements** This work was supported in part by grant no. 1R43CA88615-01 from the National Institutes of Health.

## References

1. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM (1996) Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94
2. Anderson KS, Alexander J, Wei M, Cresswell P (1993) Intracellular transport of class I MHC molecules in antigen processing mutant cell lines. *J Immunol* 151:3407
3. Bauer RJ, Dedrick RL, White ML, Murray MJ, Garovoy MR (1999) Population pharmacokinetics and pharmacodynamics of the anti-CD11a antibody hu1124 in human subjects with psoriasis. *J Pharmacokinetics Biopharm* 27:397
4. Becker JC, Varki N, Gillies SD, Furukawa K, Reisfeld RA (1996) Long-lived and transferable tumor immunity in mice after targeted interleukin-2 therapy. *J Clin Invest* 98:2801
5. Chung S, Wucherpfennig KW, Friedman SM, Hafler DA, Strominger JL (1994) Functional three-domain single-chain T-cell receptors. *Proc Natl Acad Sci USA* 91:12654
6. Donohue JH, Rosenberg SA (1983) The fate of interleukin-2 after *in vivo* administration. *J Immunol* 130:2203
7. Dummer R, Gore ME, Hancock BW, Guillou PJ, Grobben HC, Becker JC, Oskam R, Dieleman JP, Burg G (1995) A multicenter phase II clinical trial using dacarbazine and continuous infusion interleukin-2 for metastatic melanoma. *Clinical data and immunomonitoring. Cancer* 75:1038
8. Engel I, Ottenhoff TH, Klausner RD (1992) High-efficiency expression and solubilization of functional T cell antigen receptor heterodimers. *Science* 256:1318
9. Gregoire C, Rebai N, Schweisguth F, Necker A, Mazza G, Auphan N, Millward A, Schmitt-Verhulst AM, Malissen B (1991) Engineered secreted T-cell receptor alpha beta heterodimers. *Proc Natl Acad Sci USA* 88:8077
10. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA (1982) Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J Exp Med* 155:1823
11. Grussenmeyer T, Scheidtmann KH, Hutchinson MA, Eckhart W, Walter G (1985) Complexes of polyoma virus medium T antigen and cellular proteins. *Proc Natl Acad Sci USA* 82:7952

12. Hank JA, Albertini MR, Schiller J, Sondel PM (1993) Activation of multiple effector mechanisms to enhance tumor immunotherapy. *J Immunother* 14:329
13. Hank JA, Robinson RR, Surfus J, Mueller BM, Reisfeld RA, Cheung NK, Sondel PM (1990a) Augmentation of antibody dependent cell mediated cytotoxicity following in vivo therapy with recombinant interleukin 2. *Cancer Res* 50:5234
14. Hank JA, Sosman JA, Kohler PC, Bechhofer R, Storer B, Sondel PM (1990b) Depressed in vitro T cell responses concomitant with augmented interleukin-2 responses by lymphocytes from cancer patients following in vivo treatment with interleukin-2. *J Biol Response Mod* 9:5
15. Hank JA, Surfus J, Gan J, Chew TL, Hong R, Tans K, Reisfeld R, Seeger RC, Reynolds CP, Bauer M, et al (1994) Treatment of neuroblastoma patients with antiganglioside GD2 antibody plus interleukin-2 induces antibody-dependent cellular cytotoxicity against neuroblastoma detected in vitro. *J Immunother* 15:29
16. Harvill ET, Morrison SL (1995) An IgG3-IL2 fusion protein activates complement, binds Fc gamma RI, generates LAK activity and shows enhanced binding to the high affinity IL-2R. *Immunotechnology* 1:95
17. Harvill ET, Fleming JM, Morrison SL (1996) In vivo properties of an IgG3-IL-2 fusion protein. A general strategy for immune potentiation. *J Immunol* 157:3165
18. Hilyard KL, Reyburn H, Chung S, Bell JI, Strominger JL (1994) Binding of soluble natural ligands to a soluble human T-cell receptor fragment produced in *Escherichia coli*. *Proc Natl Acad Sci USA* 91:9057
19. Hinds PW, Finlay CA, Quartin RS, Baker SJ, Fearon ER., Vogelstein B, Levine AJ (1990) Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes. *Cell Growth Differ* 1:571
20. Hurford RK Jr, Dranoff G, Mulligan RC, Tepper RI (1995) Gene therapy of metastatic cancer by in vivo retroviral gene targeting. *Nat Genet* 10:430
21. Huston JS, Levinson D, Mudgett-Hunter M, Tai MS, Novotny J, Margolies MN, Ridge RJ, Brucoleri RE, Haber E, Crea R, et al (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc Natl Acad Sci USA* 85:5879
22. Iggo R, Gatter K, Bartek J, Lane D, Harris AL (1990) Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet* 335:675
23. Kendra K, Gan J, Ricci M, Surfus J, Shaker A, Super M, Frost JD, Rakhmievich A, Hank JA, Gillies SD, Sondel PM (1999) Pharmacokinetics and stability of the ch14.18-interleukin-2 fusion protein in mice. *Cancer Immunol Immunother* 48:219
24. Klausner RD, Lippincott-Schwartz J, Bonifacino JS (1990) The T cell antigen receptor: insights into organelle biology. *Annu Rev Cell Biol* 6:403
25. Lewis LD, Cole BF, Wallace PK, Fisher JL, Waugh M, Guyre PM, Fanger MW, Curnow RT, Kaufman PA, Ernstoff MS (2001) Pharmacokinetic-pharmacodynamic relationships of the bispecific antibody MDX-H210 when administered in combination with interferon gamma: a multiple-dose phase-I study in patients with advanced cancer which overexpresses HER-2/neu. *J Immunol Methods* 248:149
26. Lin AY, Devaux B, Green A, Sagerstrom C, Elliott JF, Davis MM (1990) Expression of T cell antigen receptor heterodimers in a lipid-linked form. *Science* 249:677
27. Lode HN, Xiang R, Varki NM, Dolman CS, Gillies SD, Reisfeld RA (1997) Targeted interleukin-2 therapy for spontaneous neuroblastoma metastases to bone marrow. *J Natl Cancer Inst* 89:1586
28. Lode HN, Xiang R, Dreier T, Varki NM, Gillies SD, Reisfeld RA (1998) Natural killer cell-mediated eradication of neuroblastoma metastases to bone marrow by targeted interleukin-2 therapy. *Blood* 91:1706
29. Lustgarten J, Marks J, Sherman LA (1999) Redirecting effector T cells through their IL-2 receptors. *J Immunol* 162:359
30. McLaughlin R, O'Hanlon D, McHale T, Connolly CE, Given HF (2001) Prognostic implications of p53 and bcl-2 expression in 108 women with stage two breast cancer. *Ir J Med Sci* 170:11
31. Motzer RJ, Rakhit A, Schwartz LH, Olencki T, Malone TM, Sandstrom K, Nadeau R, Parmar H, Bukowski R (1998) Phase I trial of subcutaneous recombinant human interleukin-12 in patients with advanced renal cell carcinoma. *Clin Cancer Res* 4:1183
32. Motzer RJ, Rakhit A, Ginsberg M, Rittweger K, Vuky J, Yu R, Fettner S, Hoofman L (2001) Phase I trial of 40-kd branched pegylated interferon alfa-2a for patients with advanced renal cell carcinoma. *J Clin Oncol* 19:1312
33. Nastala CL, Edington HD, McKinney TG, Tahara H, Nalesnik MA, Brunda MJ, Gately MK, Wolf SF, Schreiber RD, Storkus WJ, et al (1994) Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. *J Immunol* 153:1697
34. Pardoll DM (1995) Paracrine cytokine adjuvants in cancer immunotherapy. *Annu Rev Immunol* 13:399
35. Peng LS, Penichet ML, Morrison SL (1999) A single-chain IL-12 IgG3 antibody fusion protein retains antibody specificity and IL-12 bioactivity and demonstrates antitumor activity. *J Immunol* 163:250
36. Penichet ML, Harvill ET, Morrison SL (1997) Antibody-IL-2 fusion proteins: a novel strategy for immune protection. *Hum Antibodies* 8:106
37. Posey JA, Raspet R, Verma U, Deo YM, Keller T, Marshall JL, Hodgson J, Mazumder A, Hawkins MJ (1999) A pilot trial of GM-CSF and MDX-H210 in patients with erbB-2-positive advanced malignancies. *J Immunother* 22:371
38. Pullarkat V, Deo Y, Link J, Spears L, Marty V, Curnow R, Groshen S, Gee C, Weber JS (1999) A phase I study of a HER2/neu bispecific antibody with granulocyte-colony-stimulating factor in patients with metastatic breast cancer that overexpresses HER2/neu. *Cancer Immunol Immunother* 48:9
39. Reddy KR, Wright TL, Pockros PJ, Shiffman M, Everson G, Reindollar R, Fried MW, Purdum PP 3rd, Jensen D, Smith C, et al (2001) Efficacy and safety of pegylated (40-kd) interferon alpha-2a compared with interferon alpha-2a in noncirrhotic patients with chronic hepatitis C. *Hepatology* 33:433
40. Rosenberg SA, Spiess PJ, Schwarz S (1983) In vivo administration of interleukin-2 enhances specific alloimmune responses. *Transplantation* 35:631
41. Rosenberg SA, Lotze MT, Muul LM, Chang AE, Avis FP, Leitman S, Linehan WM, Robertson CN, Lee RE, Rubin JT, et al (1987) A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med* 316:889
42. Rosenberg SA, Lotze MT, Yang JC, Aebersold PM, Linehan WM, Seipp CA, White DE (1989) Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. *Ann Surg* 210:474
43. Rosenberg SA, Yang JC, White DE, Steinberg SM (1998) Durability of complete responses in patients with metastatic cancer treated with high-dose interleukin-2: identification of the antigens mediating response. *Ann Surg* 228:307
44. Royal RE, Steinberg SM, Krouse RS, Heywood G, White DE, Hwu P, Marincola FM, Parkinson DR, Schwartzentruber DJ, Topalian SL, et al. (1996) Correlates of Response to IL-2 Therapy in Patients Treated for Metastatic Renal Cancer and Melanoma. *Cancer J Sci Am* 2:91
45. Sherman LA, Hesse SV, Irwin MJ, La Face D, Peterson P (1992) Selecting T cell receptors with high affinity for self-MHC by decreasing the contribution of CD8. *Science* 258:815
46. Sondel PM, Kohler PC, Hank JA, Moore KH, Rosenthal NS, Sosman JA, Bechhofer R, Storer B (1988) Clinical and immunological effects of recombinant interleukin 2 given by repetitive weekly cycles to patients with cancer. *Cancer Res* 48:2561

47. Sosman JA, Hank JA, Moore KH, Borchert A, Schell K, Kohler PC, Goldstein D, Bechhofer R, Storer B, Albertini MR, et al. (1991) Prolonged interleukin-2 (IL-2) treatment can augment immune activation without enhancing antitumor activity in renal cell carcinoma. *Cancer Invest* 9:35
48. Temmim L, Baker H, Sinowatz F (2001) Immunohistochemical detection of p53 protein expression in breast cancer in young Kuwaiti women. *Anticancer Res* 21:743
49. Theobald M, Biggs J, Dittmer D, Levine AJ, Sherman LA (1995) Targeting p53 as a general tumor antigen. *Proc Natl Acad Sci USA* 92:11993
50. Theobald M, Biggs J, Hernandez J, Lustgarten J, Labadie C, Sherman LA (1997) Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. *J Exp Med* 185:833
51. Thor Straten P, Guldberg P, Schrama D, Andersen MA, Moerch U, Seremet T, Siedel C, Reisfeld RA, Becker JC (2001) In situ cytokine therapy: redistribution of clonally expanded T cells. *Eur J Immunol* 31:250
52. Tsung K, Meko JB, Peplinski GR, Tsung YL, Norton JA (1997) IL-12 induces T helper 1-directed antitumor response. *J Immunol* 158:3359
53. van Golen KL, Risin S, Staroselsky A, Berger D, Tainsky MA, Pathak S, Price JE (1996) Predominance of the metastatic phenotype in hybrids formed by fusion of mouse and human melanoma clones. *Clin Exp Metastasis* 14:95
54. Weber S, Traunecker A, Oliveri F, Gerhard W, Karjalainen K (1992) Specific low-affinity recognition of major histocompatibility complex plus peptide by soluble T-cell receptor. *Nature* 356:793
55. Weil-Hillman G, Voss SD, Fisch P, Schell K, Hank JA, Sosman JA, Sugamura K, Sondel PM (1990) Natural killer cells activated by interleukin 2 treatment in vivo respond to interleukin 2 primarily through the p75 receptor and maintain the p55 (TAC) negative phenotype. *Cancer Res* 50:2683
56. Wiebke EA, Rosenberg SA, Lotze MT (1988) Acute immunologic effects of interleukin-2 therapy in cancer patients: decreased delayed type hypersensitivity response and decreased proliferative response to soluble antigens. *J Clin Oncol* 6:1440