Cancer Immunol Immunother (2002) 51: 565–573
DOI 10.1007/s00262-002-0312-4

ORIGINAL ARTICLE

Malka Epel · Joshua D. Ellenhorn
Don J. Diamond · Yoram Reiter

A functional recombinant single-chain T cell receptor fragment capable of selectively targeting antigen-presenting cells

Received: 9 May 2002 / Accepted: 11 July 2002 / Published online: 13 September 2002

Abstract Specificity in the immune system is dictated and regulated by specific recognition of peptide/major histocompatibility complexes (MHC) by the T cell receptor (TCR). Such peptide/MHC complexes are a desirable target for novel approaches in immunotherapy because of their highly restricted fine specificity. Recently a potent anti-human p53 $CD8^+$ cytotoxic T lymphocyte (CTL) response has been developed in HLA-A2 transgenic mice after immunization with peptides corresponding to HLA-A2 motifs from human p53. An α/β T-cell receptor was cloned from such CTL which exhibited a moderately high affinity to the human $p53_{149–157}$ peptide. In this report, we investigated the possibility of using a recombinant tumor-specific TCR for antigen-specific elimination of cells that express the specific MHC-peptide complex. To this end, we constructed a functional single-chain Fv fragment from the cloned TCR and fused it to a very potent cytotoxic molecule, a truncated form of *Pseudomonas* exotoxin A (PE38). The p53 TCR scFv-P38 fusion protein was generated by in vitro refolding from bacterially-expressed inclusion bodies, and was found to be functional by its ability to bind antigen-presenting cells (APC) which express the specific p53-derived peptide. Moreover, we have shown that the p53-specific TCR scFv-PE38 molecule specifically kills APC in a peptide-dependent manner. These results represent the first time that a TCR-derived recombinant single-chain Fv fragment has been used as a targeting moiety to deliver a cytotoxic effector molecule to cells and has been able to mediate the efficient killing of the particular cell population that expresses the specific MHC/peptide complex. Similarly to antibody-based targeting approaches, TCR with tumor cell specificity represent attractive candidates for generating new, very specific targeting moieties for various modes of cancer immunotherapy.

Keywords Cancer-specific targeting · Single-chain Fv · T-cell receptor

M. Epel · Y. Reiter (✉)
Faculty of Biology,
Technion-Israel Institute of Technology,
Technion City, Room 333, Haifa 32000, Israel
E-mail: reiter@tx.technion.ac.il
Tel.: +972-4-8292785
Fax: +972-4-8225153

J.D. Ellenhorn
Division of General and Oncologic Surgery,
Beckman Research Institute of the City of Hope
and City of Hope National Medical Center,
Duarte, California 91010, USA

D.J. Diamond
Laboratory of Vaccine Research,
Beckman Research Institute of the City of Hope
and City of Hope National Medical Center,
Duarte, California 91010, USA

Introduction

The biological activity of the T cell receptor (TCR) involves specific recognition of peptide-major histocompatibility complex (pMHC) antigens in cell-mediated immunity. The TCR is a membrane-bound receptor. There are two types of TCR: one is a heterodimer consisting of two disulphide-linked polypeptides (α and β); the other is structurally similar, but consists of the γ and δ polypeptides [6, 10, 25]. Sequence analysis of TCR demonstrates that their framework is closely related to that of immunoglobulins [9, 31]. Moreover, structural studies have revealed multiple points of similarity between antibodies and TCR, for instance, the V domains of the α/β TCR associate similarly to the VH/VL domains of antibody molecules [9, 12, 14, 15, 29, 31]. Recent structural studies indeed indicate that the three-dimensional structure of TCR is very similar to that of antibodies [12, 14, 15]. Despite these structural similarities, the functional properties of TCR and antibodies differ: (a) the interactions between the peptide and the CDR3 regions of the TCR are highly specific in comparison to those of antibodies; (b) the affinity of the TCR-pMHC interactions is quite low compared to the

high affinity interactions of antibodies; and (c) antibodies function as membrane-bound and soluble proteins that bind to soluble antigens, whereas TCR function only as membrane-bound molecules that bind to cell-associated peptide/MHC antigens [6, 10, 25]. Due to these differences it was difficult to determine the three-dimensional structure and the affinity of TCR α/β heterodimers, in part because crystallography and affinity determination require the production of relatively large amounts of soluble functional molecules. In an attempt to overcome these difficulties, soluble TCR molecules were produced in *Escherichia coli* in a form similar to that of "single-chain" (sc) antibodies, in which the Vα and Vβ domains are connected to each other directly via a linker polypeptide [34]. Several examples of such functional sc TCR have been reported [8, 16, 18, 30, 41]. Using scFv antibody fragments is a very common strategy today for constructing targeting moieties for the specific elimination of cells. Many scFv antibodies recognizing tumor-related antigens are utilized for the specific targeting of cytotoxic effector mechanisms such as toxins (generating immunotoxins) [33, 35], radioisotopes [5], cytokines (generating immunocytokines) [28] and cells (via bi-specific scFv antibodies) [40]. TCR are capable of recognizing a very unique target on cells, the pMHC. It is well established today that tumors can induce anti-tumor immunity by the generation of tumor-specific cytotoxic T lymphocytes (CTL) that recognize tumor-specific pMHC [3, 37]. In recent years, many cancer-associated MHC-restricted peptides have been isolated and because of their highly restricted fine specificity, they are desirable targets for novel approaches in immunotherapy. Since these tumor-specific pMHC are recognized by CTL TCR, it would be desirable to isolate and clone these TCR with the aim of constructing, similarly to antibodies, an scFv fragment that would be able to target a cytotoxic drug or effector mechanism to the tumor target cells expressing a particular tumor-related pMHC. This approach has not been explored until now because of the difficulties, compared to antibody scFv, in generating functional TCR scFv with sufficient affinity to serve as a targeting moiety for a tumor antigen.

In this study, we constructed a TCR scFv molecule from an α/β TCR cDNA that was isolated from a murine CTL produced in human HLA-A2 transgenic mice. The TCR specificity is directed toward the p53-derived, HLA-A2 restricted-peptide $p53_{149–157}$. This pMHC is presented on tumor cells having mutations in p53 [26].

We demonstrate here, for the first time, that a recombinant TCR Vα/Vβ scFv fragment is capable of specifically targeting antigen-presenting cells (APC) and killing them in an antigen (peptide)-specific manner. This was achieved by constructing a TCR scFv-toxin fusion protein in which the TCR scFv fragment was fused to a truncated form of a very potent bacterial toxin, *Pseudomonas* exotoxin (PE) [1, 21]. The truncated toxin contains the functional domains for its toxic biological activity, but lacks the cell-binding domain which is replaced by the TCR scFv fragment. Our results suggest that with proper engineering, recombinant TCR fragments can serve as targeting moieties for a large variety of immunological applications including the specific elimination of cell populations that express a particular, and in some cases, a disease-related pMHC.

Materials and methods

Peptides

Peptides were synthesized by a standard fluorenylmethoxy-carbonyl procedure and purified to >95% by reverse phage-HPLC. The cancer-associated peptides used were: hu $p53_{149–157}$ (STPPPGTRV), and G9–209–2M (IMDQVPFSV), which is derived from the gp100 melanoma antigen [32].

Plasmid constructs

The human p53-specific TCR α and β cDNA were cloned previously by an inverse polymerase chain reaction (PCR) cloning approach from a murine CTL clone, 3A3/3C9, derived from HLA-A*0201 transgenic mice [26]. The Vα and Vβ TCR variable domains were PCR-amplified from the corresponding cDNA. The Vα domain was amplified by using the primers TCR p53 scFv-5: 5'-ttaagcgttggcgcatATGGCCCAGAAGGTA-3'; and TCR p53 Linker alfa-3: 5'-agatccgccaccaccggatccgcctccgccAGGGAGAA CATGAAG-3'. The 5' primer introduced an *Nde*I cloning site, and the 3' primer encoded two-thirds of the peptide linker sequence $(Gly_4Ser)_3$ connecting the Vα and the Vβ domains.

The Vβ domain was amplified by PCR using the primers: TCR p53-3: 5'-cagtaaaagcTTTTAAAACCGTGAGCCTGGT-3'; and TCR p53 Linker beta-5: 5'-ggtggtggcggatctggaggtggcggaagc GAGGCTGCAGTCACC-3'. The 5' primer inserted two-thirds of the linker sequence and the 3' primer a *Hin*dIII cloning site.

The resulting Vα and Vβ PCR products were used in a second PCR overlap extension reaction to assemble the scFv gene using the TCR p53 scFv 5' primer: 5'-ttaagcgttggcgcatATGGCCCA-GAAGGTA-3'and theTCR p53 3 primer: 5'-cagtaaaagcTTTTA-AAACCGTGAGCCTGGT-3'. The resulting PCR fragment encoding the TCR scFv gene was cloned into the pCRII vector and sequenced.

The nucleotide and amino acid sequence of the scFv TCR is shown in Fig. 1.

To construct the expression plasmid for the TCR scFv-PE38 fusion protein, the TCR scFv gene was subcloned into the plasmid pRB98-Amp using the *Nde*I and *Hin*dIII cloning sites. pRB98-Amp encodes the translocation (domain II) and ADP-ribosylation (domain III) domains of whole PE, but lacks the cell-binding domain which is replaced by the TCR scFv gene and is fused to the N-terminus of PE38. The resulting expression plasmid, pMA-02, encodes the TCR scFv-PE38 fusion protein under the control of the T7 promoter.

Expression, refolding and purification of TCR scFv-PE38

The TCR scFv-PE38 fusion protein was produced in *E. coli* BL21 (λDE3) cells containing the plasmid pMA-02. Upon induction with IPTG, large amounts of recombinant protein accumulated in intracellular inclusion bodies (IB). The IB were isolated, solubilized in guanidine (6 M guanidine-HCl, 0.1 M Tris, pH 7.4, 2 mM EDTA), reduced with dithioerythritol (DTE; 65 mM) and refolded in a redox-shuffling buffer containing 0.1 M Tris, pH 8.0, 0.5 M L-arginine, 0.9 mM oxidized glutathione (GSSG) and 2 mM EDTA, as described previously [4, 7]. Properly folded TCR scFv-PE38 fusion protein was separated from contaminating proteins and aggregates of improperly folded molecules by sequential ion-

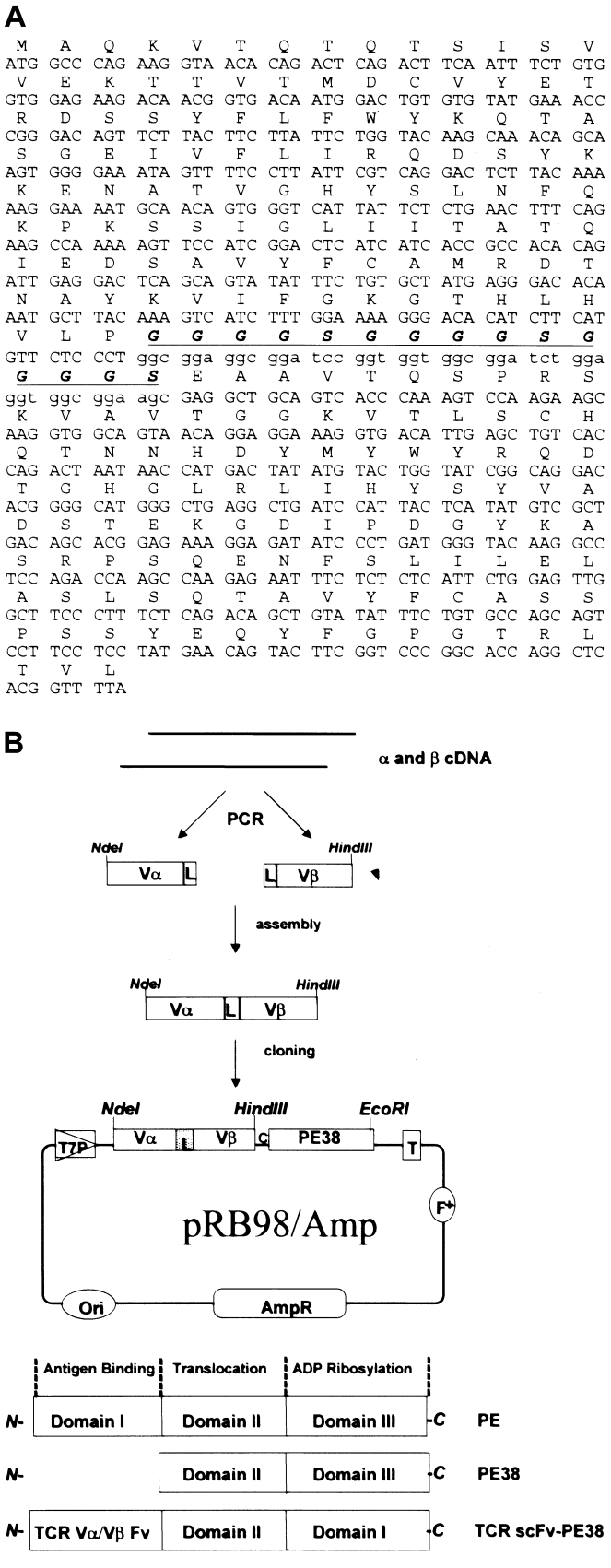

Fig. 1. A, B Nucleotide and deduced amino-acid sequence of $p53_{149–157}$ peptide-specific TCR single-chain Fv and construction of the TCR scFv-PE38 fusion protein. **A** The p53-specific TCR scFv fragment was cloned by PCR as described in Materials and methods. The peptide linker connecting the Vα and Vβ domains is shown in *bold*. **B** *Pseudomonas* exotoxin (PE) is a single-chain 66-kDa molecule secreted by *Pseudomonas aeruginosa* that irreversibly ADP-ribosylates EF2, and as a consequence protein synthesis is inhibited and cell death occurs. PE is composed of three major domains: the N-terminal domain I, aa 1–252 mediates binding to the alpha-2 macroglobulin receptor. Domain II, aa 253–364 mediates the translocation of domain III aa 400–613, the carboxyl-terminal ADP-ribosylating domain, into the cytosol of target cells. Currently, almost all PE-derived recombinant immunotoxins are constructed with PE38 (m.w.: 38 kDa), a PE derivative in which the cell binding domain (domain I) has been deleted and replaced by a targeting Fv molecule. Here we have fused the TCR scFv fragment to the N-terminus of the truncated PE to yield the TCR scFv-PE38 construct

exchange chromatography on Sepharose and MonoQ as previously described [4]. A final purification and buffer exchange was performed by TSK3000 size-exclusion chromatography. The purity of the TCR scFv-PE38 protein was determined by SDS-PAGE analysis on a non-reducing 10% gel.

Peptide-loading of APC and FACS analysis

RMA-S HHD cells (10^6) were washed twice with serum-free RPMI, and incubated overnight at 26°C in serum-free medium containing 100 μM of the $p53_{149–157}$ or G9–209–2M melanoma peptide. The cells were then exposed to 37°C for 2–3 h to stabilize cell surface expression of MHC-peptide complexes and were subsequently incubated for 1 h on ice with the W6/32 conformational monoclonal antibody (mAb), washed twice with phosphate-buffered saline (PBS) and incubated for 45–60 min with FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Inc.). Cells were then washed and analyzed by FACStar$^+$ flow cytometry (Beckton-Dickinson). For binding of the TCR scFv-pE38 fusion protein to peptide-loaded cells, 10^6 RMA-S HHD cells were loaded with the appropriate peptide as described above, followed by incubation for 1 h on ice with 10–50 μg/ml of TCR scFv-PE38. Detection of binding was carried out with rabbit anti-PE polyclonal antibody (45–60 min on ice) followed by washing twice with PBS and incubating for 45–60 min with FITC-labeled anti-rabbit IgG. Cells were washed again and analyzed by FACStar$^+$ flow cytometry.

Cytotoxicity assays with TCR scFv-PE38

Cytotoxicity assays were performed by measuring the incorporation of [^{3}H]-leucine into cell proteins as previously described [4]. Briefly, cells were loaded with the appropriate peptide as described above for 12 h at 26°C (for RMA-S HHD cells) or at 37°C (for T2 cells). Cells were plated in 96-well microtiter plates (1.4×10^4/well) and incubated with increasing concentrations of TCR scFv-PE38 for 48 h at 37°C. Control cells were incubated in medium without recombinant protein. IC_{50} was defined as the concentration of recombinant fusion protein causing 50% inhibition of protein synthesis.

Results

Construction of the p53-specific TCR scFv fragment and TCR scFv-toxin fusion

The human p53-specific TCR α and β cDNA were cloned previously by an inverse PCR cloning approach

from a murine CTL clone, 3A3/3C9, derived from HLA-A*0201 transgenic mice [26]. It has been demonstrated that a potent anti-human p53 $CD8^+$ CTL response develops in these HLA-A2 transgenic mice after immunization with peptides corresponding to HLA-A2 motifs from human p53. Mice immunized with the human $p53_{149–157}$ peptide develop a CTL response that is of moderately high affinity and is capable of recognizing human tumor cells expressing mutated p53 [26]. To construct the scFv fragment of this TCR gene, the variable regions of the α and β chains (Vα and Vβ) were PCR-amplified from the cDNA. Next, two complementary oligonucleotides were designed which assembled and linked the Vα and Vβ domains into a single gene by introducing a 15-amino acid peptide linker [$(Gly_4Ser)_3$] by a PCR overlap extension reaction. The nucleotide and deduced amino-acid sequence of the p53 TCR scFv is shown in Fig. 1A. The final PCR product was cloned into pCRII and sequenced.

The TCR scFv gene was used to construct a plasmid in which a truncated form of *Pseudomonas* exotoxin A (PE38) was fused to to the C-terminus of the TCR scFv gene (Fig. 1B). This was performed by introducing the *Nde*I and *Hin*dIII cloning sites into the TCR scFv sequence by PCR and subsequent subcloning into the plasmid pRB98-Amp which contains the truncated form of PE, termed PE38, encoding the translocation and ADP-ribosylation domains of whole PE required for its biological activity, but which lack the cell-binding domain which is replaced by the TCR scFv gene [21].

The resulting expression plasmid, pMA-02, encodes the recombinant TCR scFv-PE38 fusion protein. In this plasmid, expression is driven by the T7 promoter.

Expression, refolding, and purification of the TCR scFv-toxin fusion protein

To express the TCR scFv-PE38 fusion protein, *E coli* BL21 (λDE3) cultures harboring the plasmid were induced with IPTG, and consequently large amounts of recombinant protein accumulated as intracellular inclusion bodies (1.5–2.0 g wet weight inclusion bodies per liter of culture in shaker flasks induced at an optical density of 2.5 at 600 nm). SDS-PAGE showed that inclusion bodies from cultures expressing TCR p53 scFv-PE38 contained more than 90% recombinant protein (Fig. 2A). A major band with an apparent molecular weight of 63 kDa was observed by SDS-PAGE analysis of solubilized proteins from inclusion bodies isolated from cells induced to express the TCR scFv-PE38 protein (Fig. 2A). The inclusion bodies were isolated, denatured in 6 M guanidine-HCl, and reduced with DTE. Solubilized inclusion bodies were refolded in a redox-shuffling refolding buffer, as described in Materials and methods. After renaturation, properly folded TCR scFv-PE38 molecules were first purified by ion-exchange chromatography on Q-Sepharose and MonoQ columns

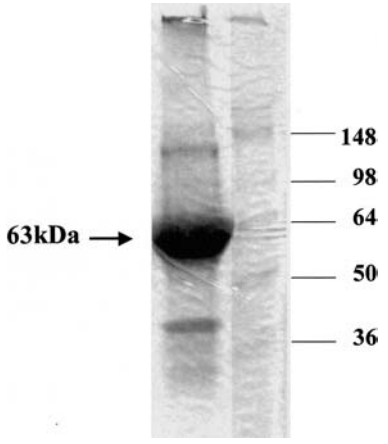

Fig. 2. Expression and purification of the TCR scFv-PE38 proteinSDS-PAGE of purified TCRscFv-PE38

and then by size-exclusion chromatography using a TSK3000 column. A highly purified TCR scFv-PE38 fusion protein with the expected size of 63 kDa was obtained in fractions from MonoQ chromatography analyzed by SDS-PAGE under non-reducing conditions (Fig. 2B).

The molecular profile of the scFv-immunotoxin was analyzed by size-exclusion chromatography and revealed a single protein peak in a monomeric form with an expected molecular mass of 63 kDa (not shown).

The yield of the refolded scFv-immunotoxin was ~4%; thus, 4 mg of highly pure protein could be routinely obtained from the refolding of 100 mg protein derived from inclusion bodies containing 80–90% recombinant protein. This yield is similar to that of previously reported scFv-immunotoxins which are well expressed, and were produced using a similar expression and refolding system [35].

Antigen-specific binding of recombinant TCR p53 scFv to APC

To demonstrate that the recombinant TCR scFv can bind the specific pMHC in the native form as expressed on the cell surface, we utilized a mutant APC system which consisted of murine TAP2-deficient RMA-S cells transfected with the human HLA-A2 gene in a single-chain format (HLA-A2.1/Db-β_2m single chain; RMA-S-HHD cells) [27]. The $p53_{149–157}$-derived peptide and control peptide derived from the melanoma antigen gp100 were loaded on the RMA-S-HHD cells and the ability of the TCR scFv-PE38 fusion protein to bind to peptide-loaded cells was monitored by flow cytometry. Initially, we tested peptide-induced MHC stabilization of the TAP2 mutant RMA-S-HHD cells, as determined by analyzing the reactivity of anti HLA antibodies with-peptide loaded and unloaded cells using FACS (Fig. 3). The conformation-specific anti-HLA antibody w6/32,which binds to the pMHC only when folded

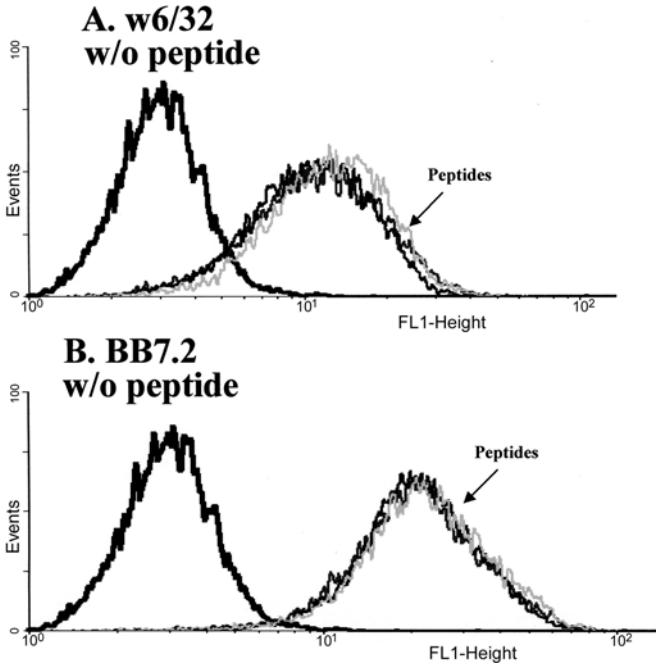

Fig. 3. Peptide-induced stabilization of HLA-A2-peptide expression on RMAS-HHD cells. RMAS-HHD cells were loaded with the HLA-A2-restricted $p53_{149–157}$ and control peptides derived from melanoma gp100 (G9–209 and G9–280) and telomerase (T865). Expression of stable HLA-A2-peptide complexes was monitored with the mAb w6/32 (**A**) and BB7.2 (**B**) using flow cytometry

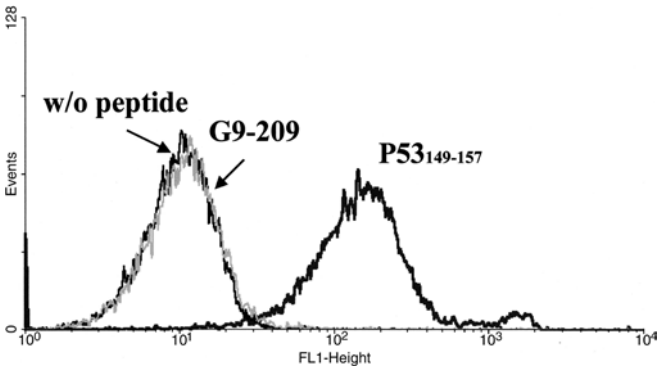

Fig. 4. Peptide-specific binding of the p53-specific TCR scFv to APC. RMAS-HHD cells were loaded with the $p53_{149–157}$ and control G9–209 peptides as described in Materials and methods. The specific binding of the TCR scFv-PE38 protein to peptide-loaded cells was performed by flow cytometry using anti-PE antibodies

correctly and contains peptide, reacted with $p53_{149–157}$-loaded RMAS-HHD cells, as well as with cells loaded with other HLA-A2-restricted peptides derived from melanoma gp100 (G9–209 and G9–280) or the telomerase-derived peptide, T865 (Fig. 3A). Similarly, mAb BB7.2 specific to HLA-A2 binds to RMAS-HHD cells loaded with the $p53_{149–157}$ peptide, as well as to cells loaded with the indicated control HLA-A2-restricted peptides (Fig. 3B). Next, we tested the reactivity of peptide-loaded RMAS-HHD cells with the p53 TCR scFv-PE38 fusion protein as shown in Fig. 4. The recombinant TCR scFv-PE38 protein binds quite intensely only to $p53_{149–157}$ peptide-loaded RMAS-HHD cells, but not to cells loaded with the control HLA-A2-restricted 209 M peptide derived from gp100 [32].

As shown in Fig. 3, the loading efficiency and expression levels on RMAS-HHD cells with the $p53_{149–157}$ and control peptides are similar, indicating that the differential peptide-specific binding of the TCR scFv fragment to $p53_{149–157}$-loaded cells but not to control cells is due to specific recognition by the TCR scFv and not because of differences in expression levels of either peptide on the surface of peptide-loaded cells. These results suggest that the TCR scFv fragment is biologically functional, thus retaining its fine specificity and recognizing the specific pMHC expressed in the native form on the cell surface.

Cytotoxic activity of TCR scFv-PE38 toward APC

To test the ability of TCR p53 scFv to serve as a targeting moiety for a toxic effector function such as the truncated PE38, we examined the cytotoxic activity of the TCR scFv-PE38 fusion protein on the murine and human mutant APC RMAS-HHD and T2. As described, the temperature-sensitive TAP mutant mouse cell line RMAS, transfected with the single-chain β_2 microglobulin-HLA-A2 gene (RMAS-HHD), when cultured at low temperature expresses MHC class I molecules that do not contain endogenously-derived peptides. These empty MHC class I molecules can be stabilized by the addition of MHC-binding peptides. T2 cells are the human T-B hybrid cell analog of the RMAS system; however, the TAP mutation is not temperature-sensitive.

RMAS-HHD and T2 cells were loaded with the $p53_{149–157}$-derived peptide and the control 209 M HLA-A2-restricted peptide derived from gp100. FACS analysis confirmed the surface expression of these pMHC on peptide-loaded RMAS-HHD cells (Fig. 3). Peptide-loaded RMAS-HHD and T2 cells were incubated with the recombinant p53 TCRscFv-PE38 fusion protein for 20 h at 37°C. As shown in Fig. 5, cytotoxicity induced by the TCR p53 scFv-PE38 was observed only on RMAS-HHD (Fig. 5A) and T2 (Fig. 5B) cells loaded with the $p53_{149–157}$-derived peptide with an IC_{50} of 10–50 ng/ml. No significant cytotoxic activity was observed on RMAS-HHD or T2 cells loaded with the control 209 M gp100-derived peptide, nor on RMAS-HHD or T2 cells that were not incubated with peptide (not shown). The decrease in protein synthesis observed on these cells loaded with the 209 M control peptide at high doses of 1,000 ng/ml was due to the non-specific killing of PE, as also previously shown using other cell types [4, 35].

We also used a second type of APC, the Epstein-Barr virus (EBV)-transformed B lymphoblast JY cells, which

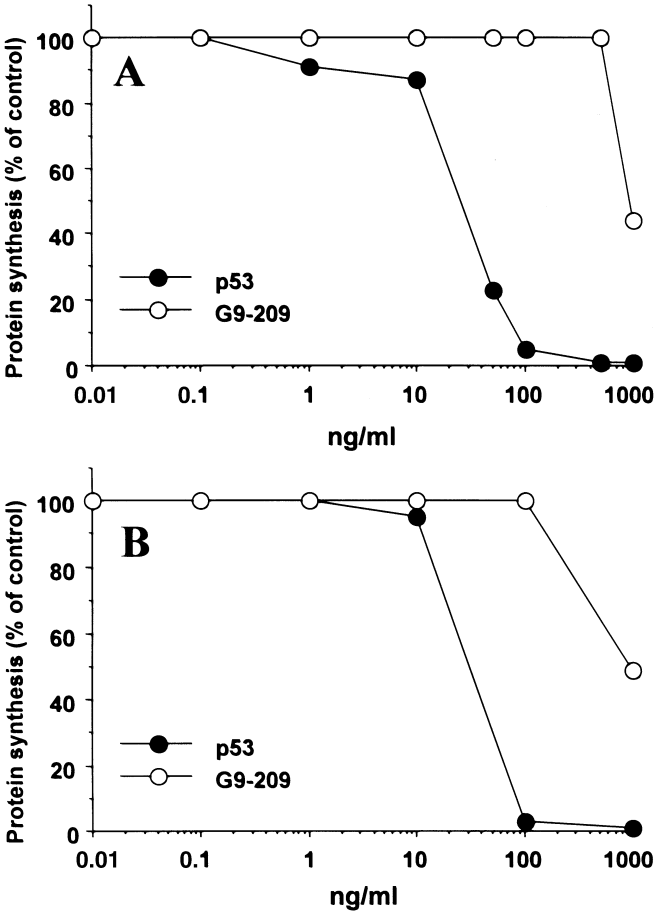

Fig. 5. Peptide-specific killing of RMAS-HHD and T2 mutant APC with the TCR scFv-PE38 protein. RMAS-HHD (**A**) or T2 (**B**) cells were loaded with the HLA-A2-restricted peptides as indicated, followed by incubation with increasing concentrations of the TCR scFv-PE38 protein. Protein synthesis was determined by incorporation of [^{3}H]-leucine into cellular proteins

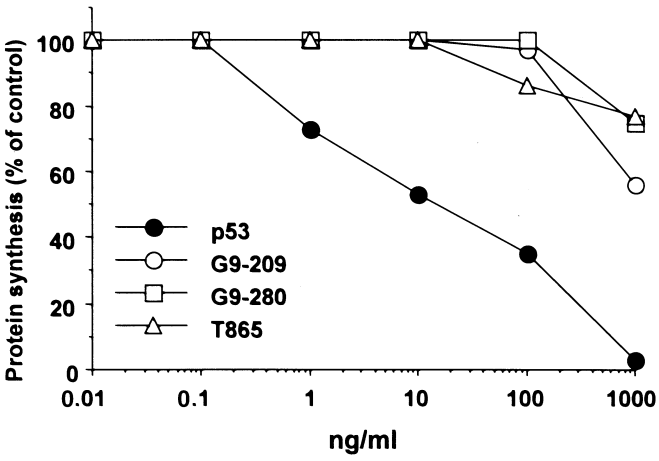

Fig. 6. Peptide-specific killing of JY APC with the TCR scFv-PE38 protein. JY cells were loaded with the HLA-A2-restricted peptides as indicated, followed by incubation with increasing concentrations of TCR scFv-PE38. Protein synthesis was determined by incorporation of [^{3}H]-leucine into cellular proteins

express HLA-A2; they are TAP$^+$, and consequently the display of the exogenously-supplied peptide is facilitated by peptide exchange. Using this strategy, we observed similar results; thus, the TCR scFv-PE38 protein was cytotoxic only to JY cells loaded with the p53-derived peptide, but not to cells loaded with melanoma gp100-derived peptides G9–209 and G9–280 or telomerase-derived peptide T865, all of which are HLA-A2-restricted (Fig. 6).These results demonstrate that the TCR scFv-PE38 fusion protein binds specifically, and kills only cells that express the specific p53 peptide in complex with class I HLA-A2 MHC molecules. Thus, we show that a TCR scFv fragment is capable of specifically targeting an effector function for antigen-specific elimination of a particular cell population.

Discussion

Recombinant scFv antibody fragments have been applied successfully to the design of novel approaches in immunotherapy [33, 35]. These recombinant fragments are capable of targeting various cytotoxic effector mechanisms to the desired cell population which express a unique antigen that is recognized by the scFv antibody fragment, thus achieving specific targeting and elimination of cells [33, 35].

We have shown herein that a similar approach can be utilized for a recombinant scFv fragment derived not from an antibody but rather from a TCR.

We have demonstrated, for the first time, that a TCR scFv fragment when fused to a truncated toxin can be used efficiently to deliver the cytotoxic moiety (a drug or toxin) to APC that express the specific pMHC. These results demonstrate the feasibility of applying recombinant TCR fragments for drug delivery, a strategy that has only been applied until now to recombinant fragments generated from antibodies.

TCR ScFv fragments have been constructed previously [8, 16, 18, 30, 41); however, not so frequently as scFv produced from antibodies (cloned mAb or phage display-derived molecules). The major reason lies in the different nature and biological properties of antibodies and TCR [6, 10, 25]. Although very similar in structure, TCR are membrane-bound molecules which recognize their ligand, the pMHC, with a considerably lower affinity compared to antibodies which are soluble molecules and recognize their antigen with high affinity. TCR scFv not only have lower affinities compared to the antibody counterpart, but they are also less stable [6, 9, 10, 12, 14, 15, 25, 31, 41]. Despite these difficulties, TCR possess a very unique specificity, in that they can recognize with fine specificity pMHC that are displayed by various cells, which is especially important when they are displayed on diseased cells such as tumor cells. In such cases, recombinant TCR fragments can be a desirable tool for use as a targeting moiety to specifically deliver a toxic molecule, a cytokine, or other effector

mechanisms with the final goal of eliminating the diseased cell population.

The design and development of such strategies to augment active, specific immunotherapies in patients with a malignant disease has been greatly influenced by and benefited from the improved understanding of the mechanisms that lead to an immune response. This is due mainly to the progress made in the availability of well-characterized tumor-associated antigens (TAA) and to the introduction of methodology developed to monitor immune responses [3, 37]. Consequently, anti-tumor immune responses can now be correlated with clinical responses in patients immunized with well-defined TAA. Especially with melanoma, it is now well established that human melanoma cells and other types of tumor cells express antigens that are recognized by CTL derived from cancer patients [3, 37]. Exciting clinical trials are therefore now in progress to target these TAA using various strategies such as vaccination with the cancer peptides or dendritic cells (DC) and adoptive cell therapy in order to generate more effective anti-tumor immune responses in cancer patients [13, 24]. The presence of tumor-specific pMHC on the surface of tumor cells may also represent a unique and specific target for an antibody-based therapeutic approach; or as shown in this work, the development of recombinant TCR-based targeting approaches. In addition to being used as targeting agents, such recombinant TCR could also serve as a valuable tool for obtaining precise information about the presence, expression pattern, and distribution of the target tumor antigen, i.e. the pMHC, on the tumor cell surface, on tumor metastases, in lymphoid organs, and on professional APC. However, the major obstacle to achieving these goals is the relatively low affinity of TCR.

In our study, we have used as a source for the TCR CTL that were developed in HLA-A2 transgenic mice after immunization with peptides corresponding to HLA-A2 binding motifs from p53. The CTL response that was observed was of moderately high affinity, and was capable of recognizing human tumor cells expressing mutated p53 [26]. We therefore suspect that this TCR possesses a moderate binding affinity that contributed significantly to our ability to generate a functional TCR scFv fragment that, as shown here, was capable of specifically staining APC and killing them in a peptide-dependent manner when generated in the form of a TCR scFv-toxin fusion protein. The approach of isolating CTL from transgenic mice immunized with putative cancer peptides that are predicted to be HLA-A2- or other MHC allele-restricted may serve as a first step toward the specific selection of functional moderate affinity CTL from which functional TCR scFv fragments can be cloned and constructed. Another approach to improving the affinity of TCR and TCR scFv fragments is by using affinity maturations strategies. It has been recently shown that the affinity of recombinant TCR scFv fragments can be improved by molecular engineering approaches, thus suggesting that there is not an inherent genetic or structural limitation on TCR affinity. Higher affinity TCR variants were generated in the absence of in vivo selective pressure by using yeast display and selection from a library of Vα CDR3 mutants [17, 23]. Selected mutants had a greater than 100-fold higher affinity (in the low nM range) for the pMHC ligand while retaining a high degree of specificity [17, 38]. Directed evolution and yeast display were used also to construct a single-chain TCR scaffold with improved stability and soluble expression efficiency [38]. These biochemical and biological properties of the TCR scFv are comparable to those observed for stable single-chain antibodies, and are a marked improvement over existing soluble single-chain TCR [17, 23, 38]. Thus, such strategies can be used to develop TCR scFv with improved binding properties to various pMHC ligands. Recently, high affinity single-chain TCR from yeast display libraries were able to block T cell activation by superantigens [22]. Mutants with inproved affinity were selected, which increased the affinity of the TCR 1,000-fold ($K_d = 7$ nM), and were able to inhibit staphylococcal enterotoxin C3-mediated T cell activation, suggesting that these engineered single-chain TCR may be useful as antagonists [22].

The availability of these engineering strategies together with the major advances in recent years in making large amounts of recombinant functional pMHC in vitro [2, 11] enable us to devise such improved single-chain TCR that can be used in targeting specific pMHC for diagnostic and therapeutic purposes. An important advance would also be the ability to construct phage or yeast display libraries with large repertoires of TCR variable domains similarly to the way in which novel antibodies are being isolated today using large antibody phage display libraries [19, 20].

Our ability to generate a wide variety and large quantities of recombinant pMHC as the ligands for various planning strategies to select specific TCR scFv is the first step toward this goal. As described above, the ability to display single-chain TCR on yeast and also on phage has been recently demonstrated [17, 23, 38, 39].

The open questions with respect to immunotherapy and many research applications of such single-chain TCR molecules relates to the expected low density (and turnover rate) of these specific epitopes (pMHC) on the target cell surface. We have previously shown in a murine model that, to achieve efficient killing with a TCR-like immunotoxin molecule in which a TCR-like antibody is being used, a density of several thousand specific pMHC is required for selective elimination of APC [36].

In the present study, we have shown that the density of specific pMHC on peptide-loaded APC is sufficient to mediate efficient elimination of cells with a recombinant TCR scFv as a targeting moiety.

It remains to be determined what the density is of these complexes on the surface of cancer cells, and wether this density is sufficient to induce effective killing. To improve the targeting capabilities of such recombinant single-chain TCR, we can not only increase the

binding affinity as discussed, but also increase the avidity of these recombinant monovalent molecules by rendering them bi- or multivalent. This has already been shown to be very effective for phenotypic analysis of T cell populations by the generation of pMHC tetramers, thus increasing their avidity to the TCR [2].

If combined, these approaches can lead to the isolation of new recombinant single-chain TCR molecules that may be used for a large variety of immunological applications as well as in the development of new approaches for immunotherapy of various pathological conditions such as cancer, autoimmunity, and other diseases in which specific pMHC ligands are involved.

References

1. Allured VS, Collier RJ, Carroll SF, McKay DB (1986) Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-Angstrom resolution. Proc Natl Acad Sci USA 83:1320
2. 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 [published erratum appears in Science (1998) 280(5371):1821]. Science 274:94
3. Boon T, van der BP (1996) Human tumor antigens recognized by T lymphocytes. J Exp Med 183:725
4. Brinkmann U, Pai LH, FitzGerald DJ, Willingham M, Pastan I (1991) B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. Proc Natl Acad Sci USA 88:8616
5. Britton KE (1997)Towards the goal of cancer-specific imaging and therapy Nucl Med Commun 18:992
6. Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML (2001) The immunological synapse. Annu Rev Immunol 19:375
7. Buchner J, Pastan I, Brinkmann U (1992) A method for increasing the yield of properly folded recombinant fusion proteins: single-chain immunotoxins from renaturation of bacterial inclusion bodies. Anal Biochem 205:263
8. Burrows GG, Chang JW, Bachinger HP, Bourdette DN, Offner H, Vandenbark AA (1999) Design, engineering and production of functional single-chain T cell receptor ligands. Protein Eng 12:771
9. Claverie JM, Prochnicka-Chalufour A, Bougueleret L (1989) Implications of a Fab-like structure for the T-cell receptor. Immunol Today 10:10
10. Davis MM, Boniface JJ, Reich Z, Lyons D, Hampl J, Arden B, Chien Y (1998) Ligand recognition by alpha beta T cell receptors. Annu Rev Immunol 16:523
11. Denkberg G, Cohen CJ, Segal D, Kirkin AF, Reiter Y (2000) Recombinant human single-chain MHC-peptide complexes made from *E. coli* by in vitro refolding: functional single-chain MHC-peptide complexes and tetramers with tumor-associated antigens. Eur J Immunol 30:3522
12. Eisen HN (2001) Specificity and degeneracy in antigen recognition: yin and yang in the immune system. Annu Rev Immunol 19:1
13. Esche C, Shurin MR, Lotze MT (1999) The use of dendritic cells for cancer vaccination. Curr Opin Mol Ther 1:72
14. Garcia KC, Degano M, Speir JA, Wilson IA (1999) Emerging principles for T cell receptor recognition of antigen in cellular immunity. Rev Immunogenet 1:75
15. Hennecke J, Wiley DC (2001) T cell receptor-MHC interactions up close. Cell 104:1
16. 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
17. Holler PD, Holman PO, Shusta EV, O'Herrin S, Wittrup KD, Kranz DM (2000) In vitro evolution of a T cell receptor with high affinity for peptide/MHC. Proc Natl Acad Sci USA 97:5387
18. Hoo WF, Lacy MJ, Denzin LK, Voss EW Jr, Hardman KD, Kranz DM (1992) Characterization of a single-chain T-cell receptor expressed in *Escherichia coli*. Proc Natl Acad Sci USA 89:4759
19. Hoogenboom HR, de Bruine AP, Hufton SE, Hoet RM, Arends JW, Roovers RC (1998) Antibody phage display technology and its applications. Immunotechnology 4:1
20. Hoogenboom HR, Henderikx P, de Haard H (1998) Creating and engineering human antibodies for immunotherapy. Adv Drug Deliv Rev 31:51
21. Hwang J, Fitzgerald DJ, Adhya S, Pastan I (1987) Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *E. coli*. Cell 48:129
22. Kieke MC, Sundberg E, Shusta EV, Mariuzza RA, Wittrup KD, Kranz DM (2001) High affinity T cell receptors from yeast display libraries block T cell activation by superantigens. J Mol Biol 307:1305
23. Kieke MC, Shusta EV, Boder ET, Teyton L, Wittrup KD, Kranz DM (1999) Selection of functional T cell receptor mutants from a yeast surface- display library. Proc Natl Acad Sci USA 96:5651
24. Kugler A, Stuhler G, Walden P, Zoller G, Zobywalski A, Brossart P, Trefzer U, Ullrich S, Muller CA, Becker V, Gross AJ, Hemmerlein B, Kanz L, Muller GA, Ringert RH (2000) Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids [see comments]. Nat Med 6:332
25. Lanzavecchia A (1999) Three signals and a master switch in the regulation of T-cell immunity. Cold Spring Harb Symp Quant Biol 64:253
26. Liu X, Peralta EA, Ellenhorn JD, Diamond DJ (2000) Targeting of human p53-overexpressing tumor cells by an HLA A*0201-restricted murine T-cell receptor expressed in Jurkat T cells. Cancer Res 60:693
27. Ljunggren HG, Stam NJ, Ohlen C, Neefjes JJ, Hoglund P, Heemels MT, Bastin J, Schumacher TN, Townsend A, Karre K (1990) Empty MHC class I molecules come out in the cold. Nature 346:476
28. Lode HN, Reisfeld RA (2000) Targeted cytokines for cancer immunotherapy. Immunol Res 12: 279
29. Manning TC, Schlueter CJ, Brodnicki TC, Parke EA, Speir JA, Garcia KC, Teyton L, Wilson IA, Kranz DM (1998) Alanine scanning mutagenesis of an alpha beta T cell receptor: mapping the energy of antigen recognition. Immunity 8:413
30. Novotny J, Ganju RK, Smiley ST, Hussey RE, Luther MA, Recny MA, Siliciano RF, Reinherz EL (1991) A soluble, single-chain T-cell receptor fragment endowed with antigen-combining properties. Proc Natl Acad Sci USA 88:8646
31. Novotny J, Tonegawa S, Saito H, Kranz DM, Eisen HN (1986) Secondary, tertiary, and quaternary structure of T-cell-specific immunoglobulin-like polypeptide chains. Proc Natl Acad Sci USA 83:742
32. Parkhurst MR, Salgaller ML, Southwood S, Robbins PF, Sette A, Rosenberg SA, Kawakami Y (1996) Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J Immunol 157:2539
33. Pastan I (1997) Targeted therapy of cancer with recombinant immunotoxins. Biochim Biophys Acta 1333:C1
34. Raag R, Whitlow M (1995) Single-chain Fvs. FASEB J 9:73
35. Reiter Y (2001) Recombinant immunotoxins for targeted cancer cell therapy. Adv Cancer Res 81: 93
36. ReiterY, Di Carlo A, Fugger L, Engberg J, Pastan I (1997) Peptide-specific killing of antigen-presenting cells by a recombinant antibody-toxin fusion protein targeted to major

histocompatibility complex/peptide class I complexes with T cell receptor-like specificity. Proc Natl Acad Sci USA 94: 4631
37. Rosenberg SA (2001) Progress in human tumour immunology and immunotherapy. Nature 411:380
38. Shusta EV, Holler PD, Kieke MC, Kranz DM, Wittrup KD (2000) Directed evolution of a stable scaffold for T-cell receptor engineering. Nat Biotechnol 18:754
39. Weidanz JA, Card KF, Edwards A, Perlstein E, Wong HC (1998) Display of functional alpha beta single-chain T-cell receptor molecules on the surface of bacteriophage. J Immunol Methods 221:59
40. Withoff S, Helfrich W, de Leij LF, Molema G (2001) Bi-specific antibody therapy for the treatment of cancer. Curr Opin Mol Ther 3:53
41. Wulfing C, Pluckthun A (1994) Correctly folded T-cell receptor fragments in the periplasm of *Escherichia coli*. Influence of folding catalysts. J Mol Biol 242: 655