The HLA A*0201–restricted hTERT$_{540–548}$ peptide is not detected on tumor cells by a CTL clone or a high-affinity T-cell receptor

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Abstract

Tumor-associated human telomerase reverse transcriptase (hTERT) is expressed in >85% of human tumors but not in most normal cells. As a result, this antigen has received considerable attention from those interested in cancer immunotherapy. Specifically, there has been strong interest in MHC class I–associated peptides derived from hTERT because these are expressed on the cell surface and thus may enable the targeting of tumor cells. Much of this interest has focused on peptide 540–548, ILAKFLHWL, which was predicted to exhibit the strongest binding to the common HLA A*0201 presenting molecule. The hTERT$_{540–548}$ peptide is currently being assessed in therapeutic vaccination trials; however, there is controversy surrounding whether it is naturally processed and presented on the surface of neoplastic cells. Here, we generate two highly sensitive reagents to assess the presentation of hTERT$_{540–548}$ on tumor cells: (*a*) a CD8$^+$ CTL clone, and (*b*) a recombinant T-cell receptor (TCR) that binds with picomolar affinity and a half-life exceeding 14 h. This TCR enables the identification of individual HLA A2-hTERT$_{540–548}$ complexes on the cell surface. The use of both this TCR and the highly antigen-sensitive CTL clone shows that the hTERT$_{540–548}$ peptide cannot be detected on the surface of tumor cells, indicating that this peptide is not a naturally presented epitope. We propose that, in future, rigorous methods must be applied for the validation of peptide epitopes used for clinical applications. [Mol Cancer Ther 2007;6(7):2081–91]

Introduction

Human tumor-associated antigens (TAA) that are either absent in normal tissue or expressed at low levels only in a few tissues represent potential targets for anticancer therapies. Peptides derived from such TAAs (TAAP) are presented on the surface of tumor cells in association with MHC class I (MHC-I) complexes. These peptide-MHC-I (pMHC-I) complexes can potentially be recognized by the T-cell antigen receptor (TCR) and so represent targets for eliciting therapeutically beneficial antitumor T-cell responses. To date, the most well-characterized MHC-restricted tumor antigen epitopes derive from TAAs that are overexpressed in malignant melanoma, including the MAGE family of proteins, the cancer-testis antigen NY-ESO, MART-1/melan-A, gp100, and the tyrosinase-related proteins. More recently, new TAAP candidates have emerged that seem to be presented on a wide variety of tumor types; these include peptides derived from p53, carcinoembryonic antigen, survivin, P450 CYP1-B1, OFA-iLRP, and telomerase (reviewed in refs. 1, 2).

Human telomerase reverse transcriptase (hTERT) catalyzes the RNA-dependent synthesis of telomeric DNA, controlling the rate-limiting step in assembly of the telomerase complex (3). The majority of human cancers have telomerase activity and express hTERT, whereas most normal human tissue cells, which do not maintain their telomeres, do not express hTERT. The ribonucleoprotein telomerase has been linked to the replicative potential of tumor cells (4, 5) and inhibition of telomerase activity has been shown to lead to cell death (6, 7). Therefore, if an effective immune response against telomerase can be generated, it is possible that any hTERT-antigen-loss variants that may arise *in vivo* under such selection pressure may be less viable. Telomerase has therefore been suggested as one of the most therapeutically attractive TAAs. hTERT as well as potential MHC-presented peptides derived from it is currently under evaluation in tumor vaccination clinical trials (8–10).

To date, identification of new MHC-restricted peptide epitopes from TAAs has most commonly been achieved

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through the use of computer algorithms which predict potential MHC-binding peptides according to the location of MHC peptide anchor residues (11–17). A significant, and frequently overlooked, problem with this approach is that the peptides that are predicted to have the highest affinity for MHC are often not presented on the cell surface. In many cases, the peptides that are actually efficiently processed and dominate presentation on the cell surface have low affinities for MHC and therefore are not conspicuous in the output from computer algorithm predictions (18–22). Validation of predicted peptide epitopes is therefore of the utmost importance; this is generally achieved by either of two methods. Presentation of antigens can be confirmed by isolation of monoclonal T-cell populations recognizing a specific pMHC from the blood of cancer patients or normal donors, which are able to lyse tumor cells. Alternatively, mass spectrometry can be used to confirm the presence of peptides eluted from the cell surface or from purified MHC antigens. More recently, we have described a phage-display method for producing soluble recombinant TCRs with enhanced affinities for their cognate pMHCs; these high-affinity TCRs can be used to visualize cell-surface presentation of specific pMHCs. For example, a TCR specific for the HLA-A*0201–restricted NY-ESO-1$_{157-165}$ epitope was affinity enhanced from K_D 24 μmol/L to 26 pmol/L and was used to quantify cell-surface pMHC-I on tumor cell lines (23).

With respect to telomerase, attention has focused on a putative HLA A2*0201–restricted peptide epitope, hTERT$_{540-548}$ [ILAKFLHWL] (hereafter HLA A2-hTERT$_{540-548}$), which was originally identified by *in silico* prediction (16). Three groups (16, 24, 25) reported that polyclonal CTL lines specific for this pMHC can lyse tumor cells exhibiting telomerase activity. However, two subsequent studies using monoclonal CTL populations could not reproduce these observations (26, 27). Furthermore, Ayyoub et al. presented data showing that precursors of the hTERT$_{540-548}$ peptide are cleaved by the proteosome (26). Despite the controversy, this peptide is currently being used in tumor vaccination clinical trials (8, 10). Here, we use a CTL clone and a high-affinity TCR to investigate the presentation of the hTERT$_{540-548}$ epitope on a range of tumor cell lines.

Materials and Methods

T-Cell Cloning

Peripheral blood mononuclear cells from a healthy HLA-A2$^+$ donor were stimulated by autologous antigenic presentation using the hTERT$_{540-548}$ peptide at 10 μmol/L in the presence of interleukin (IL)-7. Three days after initial antigen exposure, IL-2 was gradually added to the culture up to 100 units/mL. Similar rounds of restimulation were repeated every 12 to 14 days. Following successful expansion, antigen-specific cells were sorted by flow cytometry on the basis of expression of the activation markers CD25 and CD69. Briefly, 24 h after antigenic stimulation, the CTL line was stained for the activation markers CD25 and CD69. Double-positive cells were sorted (1,000 per well) and grown for 2 to 3 weeks before limiting dilution cloning of the line in a 96 well-plate at an average of 0.3 cell per well. Cells were initially grown in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (R10 medium) containing 10% T-STIM (Becton Dickinson) final volume and 100 units/mL IL-2 in the presence of mixed irradiated allogeneic feeders from three unrelated donors. The antigenic specificity of the clone ILA1 was then tested by pMHC-I multimer staining and IFN-γ ELISpot assays.

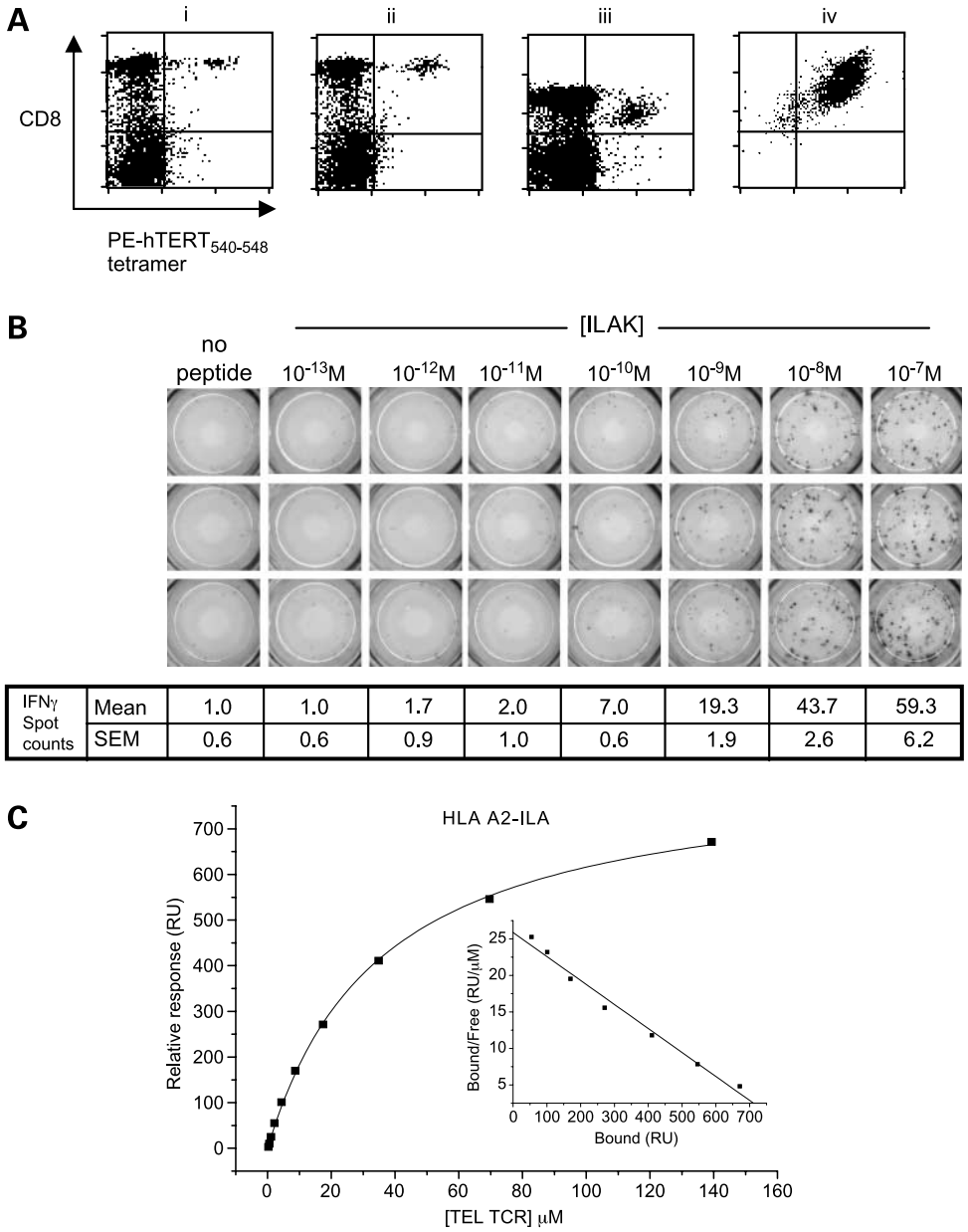
cDNA Production and Isolation of TCR Chains

cDNA was produced as previously described (28). Briefly, T cells from clone ILA1 were resuspended in Tri-reagent (Sigma) and RNA was dissolved in RNase-free water following isolation according to the manufacturer's instructions. Reverse transcription was carried out using an oligo (dT)^{15} primer (Promega) and an Omniscript RT kit (Invitrogen) to produce cDNA.

TCR chains were amplified by PCR cloning using forward primers containing an ATG start codon and a reverse primer designed to anneal to a site within the TCR chain constant domain. Each forward primer was specific for a separate TCR variable gene. α-Chain forward primers were designed to incorporate a *Cla*I restriction site and the reverse α-chain constant primer contained a *Sal*I site. β-Chain forward primers included an *Ase*I restriction site and the reverse β-chain constant primer contained an *Age*I site. *Cla*I/*Sal*I α-chain fragments were ligated into pEX954, a pGMT7 bacterial expression vector construct containing the remaining α-chain constant domain with the introduction of a mutant cysteine codon at position 160 (29) to obtain the plasmid pG020. *Ase*I/*Age*I β-chain fragments were ligated into pEX821 to produce the pGMT7-derived expression plasmid pG019. The pEX821 construct contains a mutant cysteine codon at position 169 within the β-chain constant domain and an unpaired cysteine at position 187 mutated to an alanine to prevent incorrect disulfide bonding occurring during refolding (29).

Soluble Protein Production and Affinity Measurement

The binding properties of TCRs specific for the HLA-A*0201–restricted hTERT epitope ILAKFLHWL (hTERT$_{540-548}$) were determined at 25°C by surface plasmon resonance analysis using a BIAcore 3000 (BIAcore AB). Soluble disulfide-linked heterodimeric TCRs were prepared as previously described (29). Biotinylated peptide-HLA complexes were produced as previously described (30). Biotinylated HLA A2-hTERT$_{540-548}$ monomers were immobilized to streptavidin-coated flow cells (150RU for analysis of the high-affinity TELa13b1 TCR). Kinetic analysis of wild-type and high-affinity TCRs was carried out as previously described (31). Serial dilutions of wild-type TCR were flowed over the chip surface and equilibrium binding constants were calculated using BIAevaluation software (BIAcore). The high-affinity TELa13b1 TCR was diluted to 100 nmol/L to produce dissociation phase data, which were collected for at least 60 min to establish accurate K_{off} rates.



IFNγ Spot counts								
	Mean	1.0	1.0	1.7	2.0	7.0	19.3	43.7	59.3
	SEM	0.6	0.6	0.9	1.0	0.6	1.9	2.6	6.2

Figure 1. **A,** amplification and isolation of CTL specific for $hTERT_{540-548}$. For each panel, $CD8^+$ cells staining with an anti-CD8 antibody are shown on the *Y* axis and cells staining with a phycoerythrin-labeled HLA-A2-$hTERT_{540-548}$ tetramer on the *X* axis. **i** to **iii,** expansion of CTLs from the peripheral blood mononuclear cells of a healthy donor by successive *in vitro* restimulations. Monitoring of the specific CTL population by tetramer staining was typically done at days 10 to 12 after each stimulation. **iv,** staining of the ILA1 CTL clone obtained by limiting dilution cloning of a line enriched for antigen-specific cells by sorting of CD25/CD69 – positive cells. **B,** IFNγ ELISpot assay for $hTERT_{540-548}$ reactivity. The ILA1 clone was incubated with PP cells and varying concentrations of ILAKFLHWL for 24 h (peptide was present throughout the assay). Five hundred T cells were added to 5×10^4 PP cells per well. **C,** BIAcore surface plasmon resonance binding of wild-type ILA1 TEL TCR to HLA A2-$hTERT_{540-548}$. The ILA1 TEL TCR was titrated (*X* axis) against a fixed concentration of HLA to determine the equilibrium K_D value.

Construction of TCR Phage Display Constructs

The TCR V genes were amplified from two expression vectors, pG020 for α-chain and pG019 for β-chain, and the details are described as follows. The Vα was amplified with forward primer YOL241 (GCCCAGCCGGCCATGGCCGGAATACAAGTGGAGCAGAGTC) and reverse primer YOL237 (GAGTCTCTCAGCTGGTACACG); the Vβ was amplified with forward primer YOL242 (CTTTCTATTCTCACAGCGCGCAGAATGCTGGTGTCACTCAGAC) and reverse primer YOL240 (CAGTGTGGCCTTTTGGGTGTG). To clone the V genes into a three-cistron TCR phage display vector (31), a Cα gene together with the second Shine-Dalgarno sequence and geneIII leader were amplified from the TCR phage display vector pEX952 with primers of YOL236 (CGTGTACCAGCTGAGAGACTC; forward) and YOL238 (GCGCGCTGTGAGAATAGAAAG; reverse); a Cβ gene together with part of geneIII was amplified from pEX952 with primers YOL239 (CACACCCAAAAGGCCACACTG; forward) and YOL22 (CATTTTCAGGGATAGCAAGC; reverse). The PCR was done with Expand high fidelity Taq DNA polymerase (Roche) according to the manufacturer's instructions, with a program of 95°C for 2 min followed by 30 cycles of 94°C for 20 s, 55°C for 10 s, and 72°C for 45 s. The four fragments, Vα, Cα (including a Shine-Dalgarno and geneIII), Vβ, and Cβ (including part of geneIII), were assembled into one fragment with primers of YOL241

(forward) and YOL22 (reverse), using PCR conditions of 96°C for 2 min followed by 30 cycles of 94°C for 20 s, 53°C for 30 s, and 72°C for 1 min and 45 s. The final PCR product was purified with a Qiagen gel purification kit, digested with *Nco*I and *Not*I, and then cloned into pEX922. The clones were screened by a phage ELISA assay and confirmed by DNA sequencing.

Affinity Maturation of the Wild-Type TCR Isolated from Clone ILA1

The third complementarity determining regions (CDR3) of the α and β chains were targeted for affinity maturation. The methods for introducing mutations were previously described (31), but with the following modifications. For construction of α-chain CDR3 libraries, the first two PCR fragments were generated with primers YOL13 (TCACACAGGAAACAGCTATG; forward) and YOL290 (GGTAGCAGAGTCCACAGCAC; reverse), and YOL295 (ACAAAACAGAATGGAAGATTAAG; forward) and YOL238 (GCGCGCTGTGAGAATAGAAAG; reverse). The assembly PCR was done using the first two PCR fragments and the mutation primer YOL291 (GTTTATTTCTGTGCTGTGGACTCTGCTACCNNKNNKNNKNNKNNKTACATCTTTGGAACAGGCAC) as the template, with YOL13 (forward) and YOL238 (reverse) as primers. For construction of β-chain CDR3 libraries, the first two PCR fragments were generated with primers YOL299 (CACAGACAAAACTGTGCTAGAC; forward) and YOL247 (ACAGAAGTACACAGATGTCTG; reverse), and YOL249 (GGCACTGAAGCTTTCTTTGGAC; forward) and YOL22 (reverse). The assembly PCR was carried out using the first two PCR fragments and the mutation primer YOL248 (CAGACATCTGTGTACTTCTGTNNKNNKNNKNNKNNKGGCACTGAAGCTTTCTTTGGAC) as the template, with YOL299 (forward) and YOL22 (reverse) as primers. The fragments were cloned back into pEX922:ILA. The methods for isolation of high-affinity binders were previously described (32).

Cell Lines

J82, MCF-7, and T2 cell lines were obtained from the American Type Culture Collection. The Mel-624 cell line

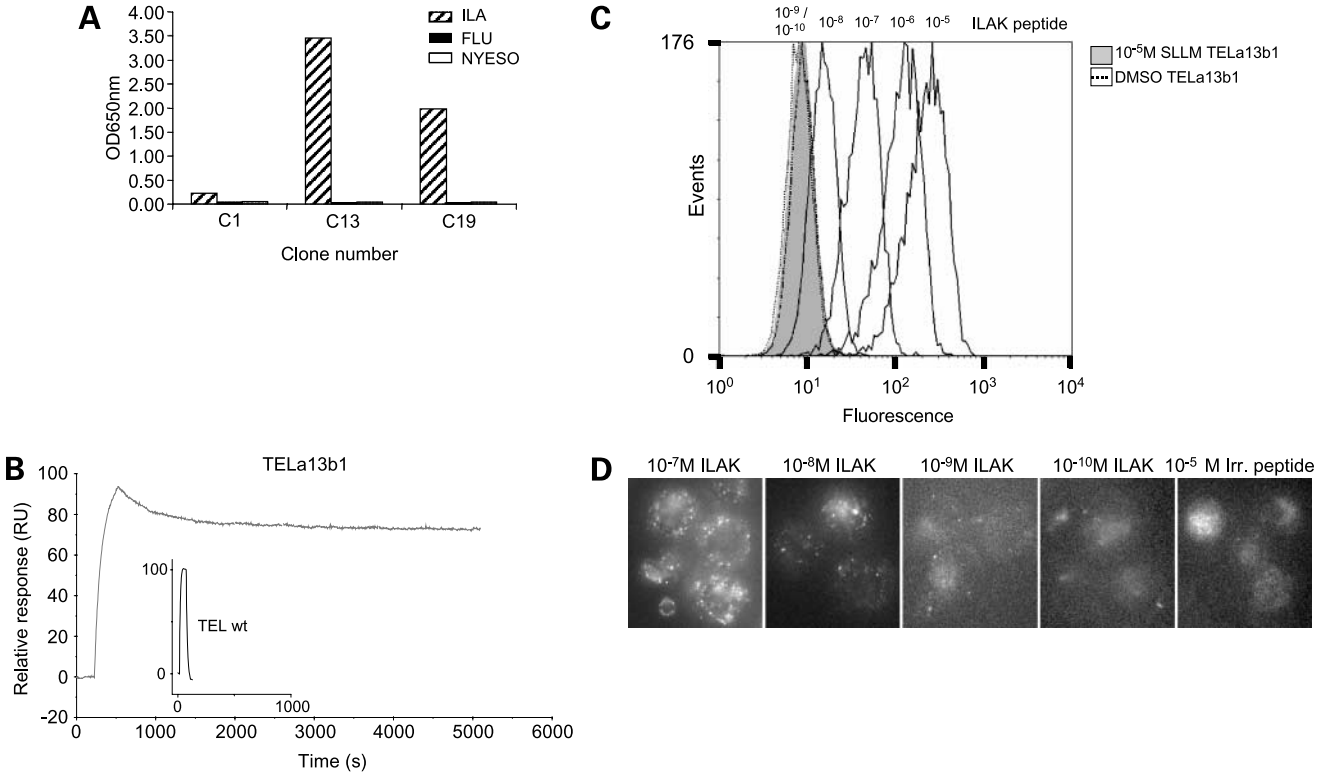


Figure 2. **A,** ELISA analysis of the first generation high-affinity binders. The specificities of three isolated clones were assayed by immobilizing pHLA A2 in complex with hTERT$_{540-548}$, influenza MP$_{58-66}$, or NY-ESO$_{157-165}$, followed by binding of phage-TCR particles. Bound phage particles were detected with rabbit anti-fd antibody as the primary antibody, and mouse anti-rabbit monoclonal antibody alkaline phosphatase conjugate as the secondary antibody. The signals were read after adding Blue-phose alkaline phosphatase substrate. **B,** BIAcore surface plasmon resonance binding of a high-affinity hTERT$_{540-548}$-specific TCR. The K_{off} and K_{on} rates were determined from a single injection of TCR using the BIAevaluation program. TELa13b1 binds to HLA-A*0201-hTERT$_{540-548}$ with a K_D of 115 pmol/L and a $t_{1/2}$ of 876 min. **C** and **D,** binding of high-affinity TELa13b1 mTCR to peptide-pulsed HLA-A2 T2 cells. T2 cells were pulsed with either a titration of hTERT$_{540-548}$ (ILAKFLHWL) peptide, 0.25% DMSO, or NY-ESO-1$_{157-165}$ (SLLMWITQC) at the concentrations indicated and then stained with TELa13b1-Bio/streptavidin-phycoerythrin and analyzed by flow cytometry (**C**) or three-dimensional fluorescence microscopy (**D**). **D,** images are three-dimensional reconstructions of all the *z*-stacks illustrated as a single plane, and the brightness/contrast of individual images was adjusted to optimize epitope visualization; the 10^{-10} mol/L ILAK peptide and 10^{-5} mol/L control peptide images were adjusted equally to allow direct comparison.

Table 1. Sequences of TCRs isolated from the affinity maturation process (first and second generation high-affinity binders) and data for BIAcore affinity determinations

Clone no.	α3	β3	K_{off} (sec^{-1})	K_{on} (mol/L^{-1} sec^{-1})	K_D (pmol/L)	$t_{1/2}$ (min)
WT	AVDSATSGTYKYI	ASSYQGTEAF			35,000,000	0.11
C1	–––––––––––––	GIQP––––––	4.39e−04	6.63e+04	6,600	26
C13	––––––ALP–G––	––––––––––	1.26e−04	2.07e+04	6,100	9
C19	––––––SPP–G––	––––––––––	nd	nd	nd	nd
a19b1	––––––SPP–G––	GIQP––––––	2.27e−05	6.86e+04	331	534
a13b1	––––––ALP–G––	GIQP––––––	1.30e−05	1.13e+05	115	888

NOTE: Table shows the sequences of the α and β CDR3 regions for the wild type TCR and for selected variants isolated following affinity maturation (dashes indicate amino acids which remain unchanged in the wild-type TCR and higher-affinity variants). C1, C13, and C19 are TCR phage clones isolated directly from phage libraries (first generation of high-affinity binders). a19b1 and a13b1 are TCRs combining the variant α and β chains from the first generation of high-affinity mutants (second generation of high-affinity TCRs). K_{off}, K_{on}, K_D, and $t_{1/2}$ values from surface plasmon resonance (BIAcore) measurements are given for the soluble TCRs (nd, not determined).

was provided by Thymed; JY cells were provided by Prof. M. Zanetti (University of California, San Diego, CA); and the PP cell line was provided by Scott Burrows (Queensland Institute of Medical Research, Herston, Queensland, Australia). Cells were maintained in R10 medium. Where indicated, cells were treated with 1,000 units/mL human IFNγ (Peprotech) or 40 μmol/L calpain inhibitor I (ALLN) and 1.5 μmol/L epoxomicin (Sigma-Aldrich) for 24 h at 37°C and 5% CO_2. ILA1 CTL were cultured in R10 medium plus 25 ng/mL recombinant human IL-15 (Peprotech).

ILAKFLHWL Minigene Transfectants (ILAK Minigene)

The ILAKFLHWL epitope was expressed as a COOH-terminal fusion to the third ubiquitin subunit of human polyubiquitin. The ubiquitin subunit was amplified by PCR from cDNA derived from bladder carcinoma cell line TCCSUP (American Type Culture Collection). The Telomerase $hTERT_{540-548}$ epitope was fused to ubiquitin by PCR, with the 3′ primer used in this amplification lacking the ubiquitin COOH-terminal cysteine and stop codons, but encoding the (human-codon optimized) epitope sequence followed by a TAG stop codon. The fusion construct was ligated into the first multiple cloning site of pIRES (Clontech) and the blasticidin resistance gene from vector pEM7/bsd (Invitrogen) was inserted into the second multiple cloning site of pIRES. The vector was purified using an endotoxin-free maxiprep kit (Qiagen), and cells were transfected using a standard calcium phosphate method. Transfectants were selected in the presence of 10 μg/mL blasticidin (Invitrogen).

Cell Staining by Flow Cytometry and Microscopy

For staining with high-affinity TCR, 10^6 T2 cells were pulsed with peptide for 90 min at 37°C and washed twice with PBS. Cells were stained with 5 μg/mL monomeric TCR-bio in 0.5% bovine serum albumin/PBS for 30 min at room temperature, followed by two washes in 10-mL PBS. Secondary staining was done in 0.5% bovine serum albumin/PBS for 30 min at room temperature with 5 μg/mL streptavidin-phycoerythrin (PharMingen). Cells were washed twice in PBS and analyzed by FC500 (Beckman Coulter). Cell-surface HLA-A2 levels were determined by flow cytometry using FITC-linked mouse anti–HLA-A2 monoclonal antibody (Serotech).

A Zeiss 200M/Universal Imaging system with a 63× objective was used for single-molecule widefield fluorescence microscopy and data analysis as described (23, 33, 34). Eight-well chambered coverslips (Nunc) with minimal imaging medium (R10 lacking phenol red) were used for imaging. Data were analyzed as described (23, 33). For antigen staining with high-affinity TCR, 5×10^4 cancer cells were allowed to adhere to chambered coverslips overnight. Cells were washed twice with PBS supplemented with 400 nmol/L $MgCl_2$ and 400 nmol/L $CaCl_2$ ($PBS_{Mg/Ca}$) and then incubated for 30 min at room temperature with 5 μg/mL TCR-bio in 0.5% bovine serum albumin/$PBS_{Mg/Ca}$ to prevent cellular detachment from the chamber slides. Cells were washed thrice with $PBS_{Mg/Ca}$ and incubated for 20 min at room temperature with 5 μg/mL streptavidin-phycoerythrin in 0.5% bovine serum albumin/$PBS_{Mg/Ca}$. Cells were washed five times and chamber wells filled with 400 μL of imaging media. For washed, peptide-pulsed T2 cells, staining with TCR-bio and streptavidin-phycoerythrin was essentially as for adherent cells above, except that cells were processed in 10-mL Falcon tubes, washed once with 10-mL PBS between the primary and secondary stains, and twice with 10-mL PBS following the secondary stain. These cells were allowed to settle on chambered coverslips before analysis. Phycoerythrin fluorescence was detected using a 535/50 excitation, 610/75 emission, and 565LP dichroic filter set (Chroma). As staining of cell surface–bound biotinylated complexes with an excess of steptavidin-phycoerythrin has been shown to result in monomeric association of streptavidin-phycoerythrin with target protein (33, 34), a single detected phycoerythrin signal corresponds to a single TCR/peptide/HLA complex. To cover the entire three-dimensional surface of the cell, *z*-stack fluorescent images were taken (21 individual planes, 1 μm apart). Data analysis was previously described (33).

IFNγ ELISpot Assay

Target cells (J82, MCF-7, PP) were treated with 1,000 units/mL recombinant human IFNγ (Peprotech) or proteosome inhibitors (1.5 μmol/L epoxomicin, 40 μmol/L calpain I inhibitor; Sigma) for 20 h, or pulsed with varying concentrations of ILAKFLHWL peptide for 1 h before use. 500 ILA1 clone cells were added to 5×10^4 target cells per well. Plates were incubated overnight at 37°C/5% CO_2 and developed according to the manufacturer's instructions (human IFNγ ELISpot kit, Diaclone). Spots were evaluated using an automated ELISpot reader (Autoimmun Diagnostika).

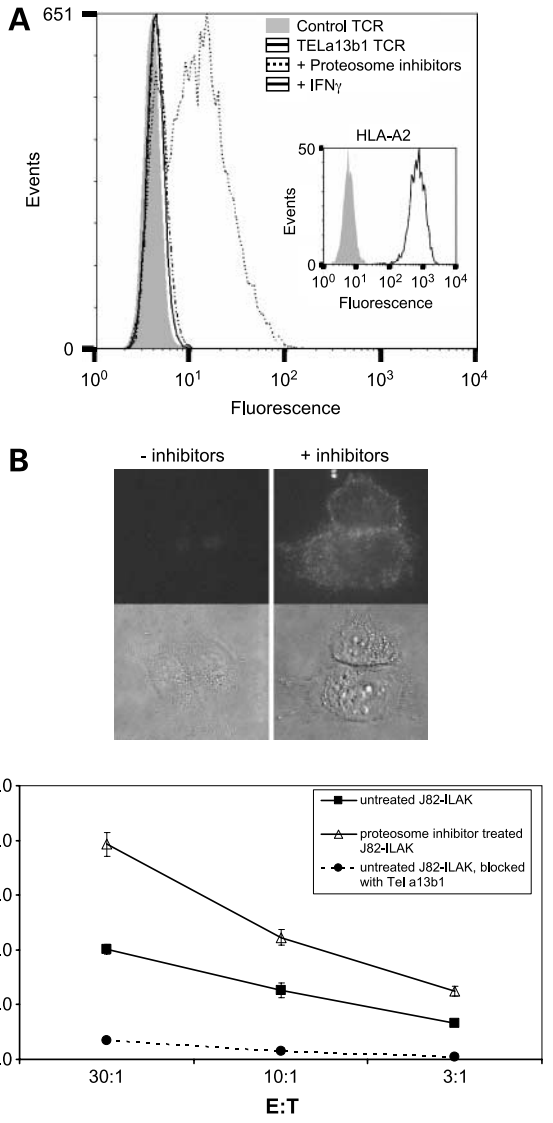


Figure 3. The effect of IFNγ and proteosome inhibitor treatment on the cell-surface presentation of hTERT$_{540-548}$ (ILAKFLHWL) peptide on J82-ILAK minigene–transfected cells. **A,** J82-ILAK minigene–transfected cells were treated overnight with either calpain inhibitor I (ALLN) and epoxomicin, IFNγ, or left untreated. The cells were stained with TELa13b1-Bio/streptavidin-phycoerythrin and analyzed by flow cytometry. The control TCR was NYE(113), specific for an HLA A2 NY-ESO epitope. *Inset,* HLA-A*0201 expression on the surface of these cells, using an anti–HLA-A2-FITC antibody. **B,** J82-ILAK minigene–transfected cells were treated overnight with calpain inhibitor I (ALLN) and epoxomicin or left untreated. The cells were stained with TELa13b1-Bio/streptavidin-phycoerythrin and analyzed by three-dimensional fluorescence microscopy. *Top row,* fluorescent image as a single plane through the *z*-stack; *bottom row,* the equivalent bright-field image. **C,** hTERT$_{540-548}$-specific killing assay: the ILA1 clone recognizes and lyses untreated J82-ILAK minigene–transfected target cells (■) or target cells pretreated with proteosome inhibitors (△) in a 2-h killing assay. Inclusion of 100 nmol/L TELa13b1 TCR (●) blocks the recognition of the target cells by the clone and inhibits killing.

CTL Killing Assay

J82 target cells (T) were treated with or without proteosome inhibitors as above. Targets were loaded with BATDA as described by Delfia cytotoxicity assay kit instructions (Perkin-Elmer). ILA1 clone effector cells (E) were incubated with 5,000 target cells per well at differing effector-to-target ratios at 37°C/5% CO_2 for 2 h. Samples of supernatant were added to Europium solution (Perkin-Elmer) and analyzed by time-resolved fluorometry. The percent target cell lysis was calculated according to the manufacturer's instructions.

Cytokine Bead Assay

Ten thousand ILA1 CTL were added to each well of a 96-well flat-bottomed plate containing confluent monolayers of the adherent tumor cell lines J82, J82-ILAK, and MCF-7, or to each well of a 96-well U-bottomed plate containing 30,000 target cells per well for the nonadherent cell line JY. The following assays were set up in a total volume of 200 μL: targets alone +/− 10^{-8} mol/L peptide, ILA1 CTL alone +/− 10^{-8} mol/L peptide, and ILA1 CTL + targets +/− 10^{-8} mol/L peptide. Plates were incubated overnight at 37°C. IFNγ in the supernatant was determined by cytometric bead array using IFNγ multiplex beads (Becton Dickinson).

Results

Generation of a CTL Clone Specific for hTERT$_{540-548}$

To evaluate hTERT$_{540-548}$ as a target for tumor immunotherapy, our rationale was to isolate a T-cell clone specific for the epitope and affinity mature its TCR to picomolar K_D. High-affinity TCRs can be used to quantify the number of antigens per cell and to target specific pMHC complexes on the surface of tumor cells.

An enriched T-cell line was generated from the peripheral blood mononuclear cells of a healthy HLA $A2^+$ donor. Clones were isolated by limiting dilution and identified by pMHC-I tetramer staining; Fig. 1A shows staining data for the clone ILA1 and the CTL line from which the clone was isolated. Figure 1B shows IFNγ ELISpot data for ILA1, stimulated by peptide-pulsed cells; the clone is activated by peptide concentrations down to 10^{-10} mol/L.

Isolation of a High-Affinity TCR Specific for hTERT$_{540-548}$

TCR α- and β-chain genes were isolated from ILA1 cDNA and engineered to produce a disulfide-linked, soluble version of the TCR [a monoclonal TCR (mTCR)] as described (29). Surface plasmon resonance measurements show that this native mTCR has an affinity of 33 μmol/L as determined by equilibrium binding (Fig. 1C).

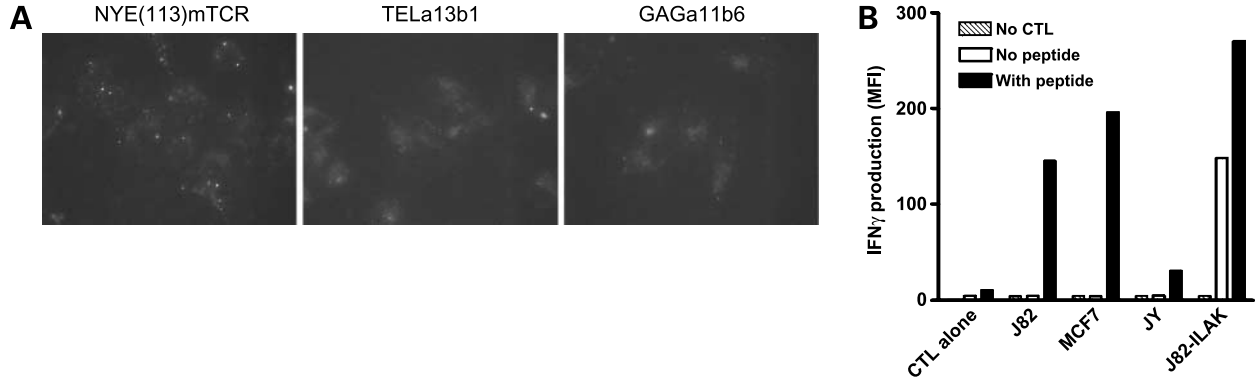


Figure 4. **A,** binding of high-affinity TELa13b1 and NYE(113) mTCRs to the HLA A2–expressing Mel-624 melanoma cell line. Telomerase and LACE-1/NY-ESO-1–positive Mel-624 cells were stained with TELa13b1, NYE(113), and a negative control TCR specific for an HLA A2 HIV epitope, GAGa11b6; streptavidin-phycoerythrin was used to visualize the bound TCRs by three-dimensional epifluorescent microscopy. Images are three-dimensional reconstructions of all the *z*-stacks illustrated as a single plane. **B,** cytokine bead assay with the ILA1 clone and tumor cell lines as targets. Confluent layers of adherent tumor cell lines J82 (bladder cancer) and MCF-7 (breast cancer) and 30,000 cells of nonadherent cell line JY (B-cell lymphoma) were presented to the ILAK-specific CTL clone ILA1 (10,000 cells per well) in an overnight assay. IFNγ release was measured using an anti-IFNγ bead-based assay, with fluorescent detection by flow cytometry. J82-ILAK minigene cell line served as a positive control for cell-surface presentation of $hTERT_{540-548}$ (compare *white column* for J82-ILAK to *white columns* for unmodified cell lines). Externally added $hTERT_{540-548}$ peptide (at 10 nmol/L final concentration) was successfully presented by the tumor lines (*black columns*).

TCR α and β chains were subjected to random mutagenesis in their CDR2 or CDR3 regions, and displayed on the surface of phage as described (31). Each phage-displayed TCR variant contained one wild-type and one mutated chain. Variants with increased affinity were isolated from the TCR libraries by selective binding to HLA A2-$hTERT_{540-548}$; relative affinities for HLA A2-$hTERT_{540-548}$ were estimated using an ELISA competition assay. The three most promising phage clones all bound preferentially to HLA A2-$hTERT_{540-548}$ compared with two other HLA A2-peptide complexes (Fig. 2A). CDR sequences of these variants are listed in Table 1; clones with improved affinity have mutations in the α CDR3 (c13 and c19) or β CDR3 (c1).

Our experience with other TCRs is that affinity can be increased further by combining mutations in several CDRs. Therefore, combinations of wild type, variant α (c13 and c19), and variant β (c1) chains were expressed as soluble mTCRs and their affinities determined by surface plasmon resonance. The data in Table 1 show that the TCR TELa13b1 (combining the α CDR3 mutations from c13 and the β CDR3 mutations from c1) has the highest affinity of the mTCRs analyzed, with a K_D of 115 pmol/L and a $t_{1/2}$ of nearly 15 h (Fig. 2B).

TELa13b1 Binds Specifically to HLA A2-$hTERT_{540-548}$ on the Surface of Cells

Binding of TELa13b1 to T2 cells pulsed with varying concentrations of $hTERT_{540-548}$ was analyzed by flow cytometry (Fig. 2C). Binding can be detected at a concentration of 10^{-8} mol/L peptide or higher, whereas there is no detectable binding to cells pulsed with 10^{-5} mol/L control peptide. TELa13b1 was used to visualize the HLA A2-$hTERT_{540-548}$ antigen on the surface of peptide-pulsed T2 cells by fluorescence microscopy (Fig. 2D). This technique allowed visualization of epitopes on the surface of cells pulsed with peptide concentrations down to 10^{-10} mol/L peptide, in agreement with the activation threshold of the ILA1 clone in an IFNγ ELISpot assay (Fig. 1B).

$hTERT_{540-548}$ Presentation in a Telomerase Minigene–Transfected Cell Can Be Increased by Inhibiting the Proteosome

To confirm that TELa13b1 is able to bind intracellularly processed $hTERT_{540-548}$, we transfected the HLA $A2^+$ bladder carcinoma cell line J82 with a minigene construct expressing $hTERT_{540-548}$ linked to ubiquitin (J82-ILAK minigene cells). Cells that present the epitope and bind to TELa13b1 were sorted by flow cytometry. However, subsequent analysis showed that <5% of the sorted population continue to present the $hTERT_{540-548}$ peptide, and that further rounds of sorting highly expressing cells could not produce a uniform population of epitope positive cells (data not shown).

Ayyoub et al. (26) reported that the $hTERT_{540-548}$ peptide contains a proteosome cleavage site, which may account for the loss of presentation in the minigene-transfected cells. To test this hypothesis, we treated the J82-ILAK minigene cells with the proteosome inhibitors epoxomycin and calpain inhibitor I. Before treatment, a small minority of the cells stained brightly with TCR by microscopy (not shown). After treatment, the majority of the cells express the epitope, as detected by both flow cytometry (Fig. 3A) and fluorescence microscopy (Fig. 3B). This effect was not seen with a different HLA-A2–restricted, tumor-associated epitope: J82 cells expressing the $NY\text{-}ESO_{157-165}$ peptide from a minigene construct expressed high levels of epitope in the absence of proteosome inhibitors and, after treatment, we observed only small changes in cell-surface presentation (data not shown).

In accordance with the observation that a minority of minigene-transfected J82 cells present the hTERT$_{540-548}$ epitope, CTL activation showed that the ILA1 CTL clone could recognize presentation even in the absence of proteosome inhibitors (Fig. 3C). However, ~40% of the target cells were lysed at an effector-to-target ratio of 30:1, which was significantly higher than expected from the staining analysis with TELa13b1. The higher degree of lysis may reflect temporal leakage of the hTERT$_{540-548}$ epitope during the course of the lysis assay and/or the ability of the ILA1 cells to lyse targets that present even a single epitope. Bystander killing mediated by a small minority of target cells presenting the peptide may also contribute to the higher than expected lysis, particularly at high effector-to-target ratios. The lysis was specific for the hTERT$_{540-548}$ epitope as it was almost completely inhibited by TELa13b1 mTCR. The level of lysis was doubled when the J82-ILAK minigene expressing cells were treated with proteosome inhibitors. Consistent with previous findings (26), these results indicate that the hTERT$_{540-548}$ epitope is destroyed by proteosomal activity.

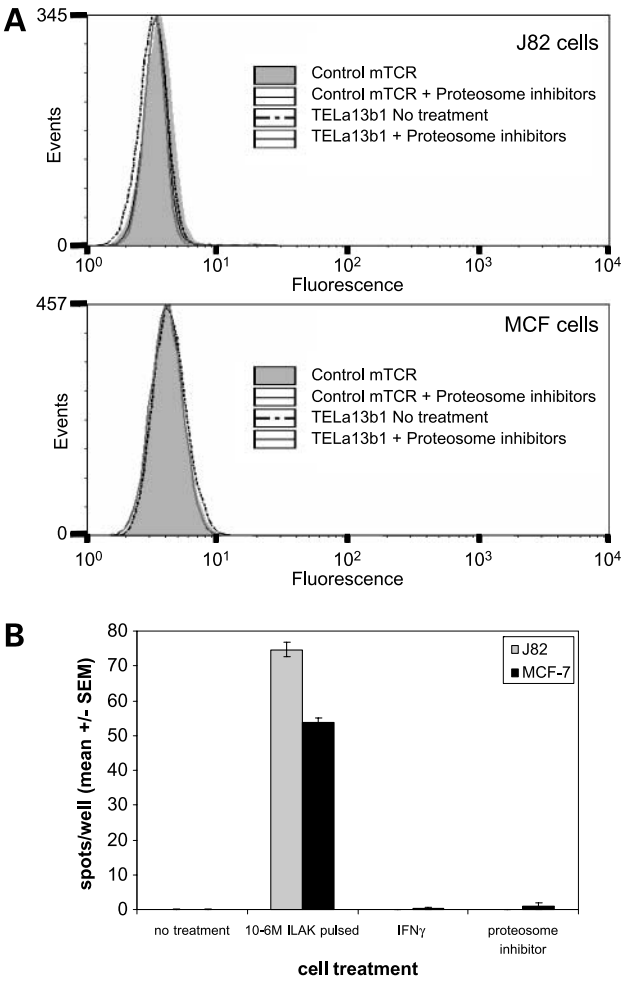


Figure 5. **A,** the effect of IFNγ and proteosome inhibitor treatment on the cell-surface presentation of telomerase hTERT$_{540-548}$ (ILAKFLHWL) peptide on telomerase hTERT–positive J82 and MCF-7 tumor cell lines. J82 and MCF-7 cells were treated overnight with either calpain inhibitor I (ALLN) and epoxomicin, IFNγ, or left untreated. The cells were stained with TELa13b1-Bio or NYE(113) control mTCR/streptavidin-phycoerythrin and analyzed by flow cytometry. **B,** hTERT$_{540-548}$–specific ELISpot assay. The ILA1 clone was incubated with J82 target cells (*gray columns*) or MCF-7 target cells (*black columns*). Target cells were untreated, peptide pulsed (1 μmol/L ILAKFLHWL for 1 h), pretreated with 1,000 units/mL IFNγ, or pretreated with proteosome inhibitors.

HLA A2$^+$ hTERT-Expressing Tumor Cell Lines Do Not Present Detectable Levels of the hTERT$_{540-548}$ Epitope

Flow cytometry is a relatively insensitive technique, requiring at least 75 to 200 phycoerythrin molecules per cell for reliable signal detection above background (35), depending on the specifications of the instrument used. In contrast, three-dimensional fluorescence microscopy allows the detection of individual antigens and therefore enumeration of extremely low antigen densities (23), although photobleaching effects cause some loss of signal leading to an up to 50% underestimation of epitope number. The tumor cell lines Mel-624 (melanoma), J82 (bladder), and MCF-7 (breast) express both HLA-A2 and hTERT (data not shown); however, none of these cell lines bound detectable levels of TELa13b1 by fluorescence microscopy (Fig. 4A and data not shown). Mel-624 cells also express the NY-ESO-1/LAGE-1 tumor antigen but not the HIV Gag antigen; this was confirmed by microscopy using high-affinity mTCRs specific for HLA-A2–restricted peptides derived from these antigens. The NY-ESO-1/LAGE-1 antigen was detected by the HLA A2-NY-ESO$_{157-165}$ specific TCR, but no signal could be detected for the hTERT$_{540-548}$ and HIV Gag$_{77-85}$ epitopes (Fig. 4A). The absence of the hTERT$_{540-548}$ epitope on the surface of tumor cells was further confirmed using several HLA A2$^+$, hTERT$^+$ cell lines as targets for the ILA1 clone (Fig. 4B).

Proteosome Inhibitors Do Not Induce Presentation of hTERT$_{540-548}$ on Tumor Cells

If endogenously derived hTERT$_{540-548}$ epitope is cleaved by the proteosome, it is possible that presentation on tumor cells could be induced by proteosome inhibitors. However, neither J82 nor MCF-7 cells stained with TELa13b1 mTCR after treatment with proteosome inhibitors (Fig. 5A). Furthermore, with J82 and MCF-7 cell lines as targets, the ILA1 clone failed to activate in IFNγ ELISpot assays (Fig. 5B), even when the target cells were treated with the proteosome inhibitors that enhanced cell-surface presentation of hTERT$_{540-548}$ epitope derived from the minigene construct. Treatment with IFNγ, which increases MHC class I levels and induces expression of alternative proteosomal subunits (36, 37), also failed to recover hTERT$_{540-548}$ epitope presentation.

Discussion

MHC-presented peptide epitopes hold great promise as targets for cancer therapy because they can be derived from intracellular parent antigens that have essential roles for maintenance of the immortalized state of tumor cells. Huge efforts have therefore been devoted to the identification of

peptides that are presented on a wide range of tumor types. However, a degree of uncertainty surrounds the presentation of many tumor-associated peptide antigens. This is mainly due to the difficulties associated with confirming the presence of such epitopes on the cell surface. Until now, only two approaches have been used that can firmly establish the presence of a particular peptide antigen on tumor cells. First, mass spectrometry can provide positive identification of peptides eluted from the cell surface; however, the technique is only sensitive enough to detect peptides that are presented in abundance and it does not provide any insight into how presentation may vary between individual cells. Furthermore, the technology is rarely applicable to noncancerous cells and so the absence of the peptide in normal tissues cannot be confirmed; this is a crucial consideration if the antigen is to be used as a therapeutic target for directed cell killing. Second, the recognition of tumor cells by an antigen-specific T-cell clone provides very strong evidence for the presentation of the epitope. However, for many TAAs, T-cell clones are elusive and, if obtained, can be difficult to maintain in culture. In addition, T-cell clones raised against peptide-pulsed target cells are often of insufficient sensitivity to be activated by the low levels of antigen presented by tumor cells. Other approaches, for example tumor recognition by polyclonal T-cell lines, can be misleading because the reactivity spectrum of the effector cells is unknown. *In silico* prediction of epitopes, which is the most widely used approach to novel peptide epitope identification, is based mainly on predictions of peptide anchoring affinity for the relevant MHC molecule. In several cases, the dominant peptide epitopes from a given antigen have turned out to be weak binders (reviewed in ref. 20). Monoclonal antibodies, raised against peptide-MHC combinations, have also been used to study peptide presentation, mainly by flow cytometry. However, in some cases, antibodies give fluorescence shifts that indicate extremely large numbers of epitopes per cell, conflicting with estimates obtained with other quantitative techniques such as mass spectrometry (38). In other cases, shifts are more consistent with levels of antigen detected by other methods (39, 40).

The most attractive tumor antigen targets would be expressed in a wide variety of tumor types and fulfill an essential role in the maintenance of the immortalized state of the tumor cell. Of such antigen candidates, none has generated more interest than hTERT, which is overexpressed in almost all solid tumors regardless of origin and only expressed in very few normal cell types, predominantly activated T and B cells (5, 41–43). In the absence of telomerase (i.e., in most normal human cells), telomeric DNA erodes progressively with each round of cell division as cells approach replicative senescence. Loss of telomeres leads to increased chromosomal fusions and genetic recombinations, eventually resulting in cell death through apoptosis. Thus, telomerase is important in maintaining telomere length and is therefore essential to immortalize most cancer cells. Indeed, inhibition of telomerase activity has been shown to lead to tumor cell death (6, 7).

Two peptides from hTERT, predicted by *in silico* studies to have the best potential for binding in the peptide groove of one of the dominant MHC class I molecules, HLA A*0201, are residues 540–548 (ILAKFLHWL) and 865–873 (RLVDDFLLV). Tetramer-positive T cells specific for the $hTERT_{540-548}$ peptide have been detected in the peripheral blood of cancer patients (44), and this putative epitope has become the main focus for T-cell therapies. Several groups have grown polyclonal T-cell lines which have been reported to kill HLA $A2^+$, $telomerase^+$ tumor cell lines and primary tumors (16, 24, 25). However, two other groups have reported that T-cell clones to this epitope do not kill tumor cells (26, 27).

Here, we also have generated a T-cell clone, ILA1, to the $hTERT_{540-548}$ peptide (Fig. 1A) and confirmed that the TCR from this clone binds to HLA A2 presenting the $hTERT_{540-548}$ peptide with an affinity and off rate within the reference range expected for T-cell agonists (Fig. 1C). The ILA1 CTL clone recognizes a standard antigen-presenting cell line pulsed with as little as 10^{-10} mol/L peptide (Fig. 1B). It also recognizes three HLA $A2^+$, $hTERT^+$ tumor cell lines, J82, MCF-7, and JY, when the $hTERT_{540-548}$ peptide is applied exogenously, and J82 cells expressing an $hTERT_{540-548}$ minigene (Fig. 4B). However, ILA1 does not recognize any of the three tumor cell lines in the absence of exogenously applied peptide (Fig. 4B). Thus, all data obtained with monoclonal T-cell populations indicate that tumor cells do not present the $hTERT_{540-548}$ peptide in sufficient abundance for T-cell recognition (refs. 26, 27 and this study).

To examine the possible presentation of the $hTERT_{540-548}$ peptide on individual cells and, importantly, with a sensitivity level that allows the detection of single antigen molecules, we generated a soluble, high-affinity mTCR based on the TCR expressed by ILA1. One of these TCRs, TELa13b1, binds to the HLA A2-$hTERT_{540-548}$ antigen complex with a K_D of 115 pmol/L and a half-life of >14 h (Table 1). When labeled with phycoerythrin, TELa13b1 can be used to visualize individual antigen complexes on the cell surface by fluorescence microscopy (Figs. 2–4). With TELa13b1, the antigen can be detected on peptide-pulsed cells (Fig. 2D) but not on any of the HLA $A2^+$, $hTERT^+$ tumor cell lines (Fig. 4A and data not shown); in contrast, the HLA A2–presented NY-ESO-1 peptide was detected on Mel-624 cells using a high-affinity mTCR specific for this antigen complex (Fig. 4A). The failure to detect presentation of the $hTERT_{540-548}$ antigen on the surface of tumor cells is consistent with the observations obtained with ILA1 and with previously reported CTL clones (26, 27). Conflicting evidence has been reported with a monoclonal antibody specific for the HLA A2-$hTERT_{540-548}$ peptide complex (27, 45).

A previous study (26) reported that the $hTERT_{540-548}$ peptide contains a proteosome cleavage site. Here we find that, when the $hTERT_{540-548}$ peptide is expressed from a ubiquitin minigene fusion, presentation could be dramatically increased by treating cells with proteosome inhibitors, enhancing detection by both the high-affinity TCR TELa13b1 and the ILA1 CTL clone (Fig. 3). Finally,

hTERT$_{540-548}$ presentation could not be rescued from endogenously expressed full-length hTERT in untransfected J82 and MCF-7 cells by treatment with proteosome inhibitors (Fig. 5).

In summary, two extremely antigen-sensitive reagents have failed to detect the presence of the hTERT$_{540-548}$ peptide on telomerase-expressing tumor cells, including two lines, MCF-7 and Mel-624, which have previously been reported to activate polyclonal hTERT$_{540-548}$ reactive CTL (25). We conclude that this peptide is not generally processed and presented by tumor cells, lending strong support to the conclusions drawn by some previous studies (26, 27, 46). Taken together, these results highlight the necessity for rigorous validation of peptide epitopes using methods that allow unambiguous demonstration of antigen presentation.

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The HLA A*0201–restricted hTERT$_{540–548}$ peptide is not detected on tumor cells by a CTL clone or a high-affinity T-cell receptor

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