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Isolation and Characterization of Human Recombinant Antibodies Endowed with the Antigen-specific, Major Histocompatibility Complex-restricted Specificity of T Cells Directed toward the Widely Expressed Tumor T-cell Epitopes of the Telomerase Catalytic Subunit[1]

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ABSTRACT

The recent characterization of MHC-displayed tumor-associated antigens that recognize effector cells of the immune system has created new perspectives for cancer therapy. Antibodies that recognize these tumor-associated MHC-peptide complexes with the same specificity as the T-cell antigen receptor will therefore be valuable tools for immunotherapy as well as for studying antigen presentation in human cancers. Most tumor-associated antigens are expressed in only one or a few tumor types; however, recently specific T-cell epitopes derived from the telomerase catalytic subunit (hTERT) that are widely expressed in many cancers were identified and shown to be recognized by CTLs derived from cancer patients. We selected a large nonimmune repertoire of phage Fab antibodies on recombinant human class I HLA-A2 complexes displaying two distinct antigenic T-cell epitopes derived from hTERT. We isolated a surprisingly large panel of high-affinity human recombinant Fab antibodies that exhibited peptide-specific, MHC-restricted binding characteristics of T cells. The analyzed Fabs not only recognize the cognate MHC-peptide complex in a recombinant soluble form but also the native complex as displayed on the surface of antigen-presenting cells and hTERT-expressing tumor cells. These findings demonstrate for the first time the ability to transform the unique fine specificity but low intrinsic affinity of TCRs on T cells into high-affinity soluble antibody molecules endowed with a T-cell antigen receptor-like specificity. These molecules may prove to be very important and widely applicable for monitoring the expression of specific MHC-peptide complexes on the surface of tumor and immune cells, for structure-function studies of TCR-peptide-MHC interactions, as well as for developing new targeting agents for immunotherapy.

INTRODUCTION

The design and development of strategies to augment active, specific immunotherapies in patients with a malignant disease has been greatly influenced by and benefited from the progress made in better understanding the mechanisms that lead to an immune response. This is attributable mainly to the progress made in the availability of well-characterized TAAs[3] and to the advent of methodology developed to monitor immune responses (1–5). Consequently, antitumor immune responses can now be correlated with clinical responses in patients immunized with well-defined TAAs. Especially with melanoma, it is now well established that human melanoma cells and other types of tumor cells express antigens that are recognized by CTLs derived from cancer patients (1–3). Exciting clinical trials are therefore now in progress to target these TAAs using various strategies such as vaccination with the cancer peptides or dendritic cells and adoptive cell therapy to generate more effective antitumor immune responses in cancer patients (6–8). The presence of tumor-specific MHC-peptide complexes on the surface of tumor cells may also represent a unique and specific target for an antibody-based therapeutic approach. To develop such a strategy, new targeting moieties must be isolated such as recombinant antibodies that will recognize specific peptide-MHC complexes. In addition to being used as targeting agents, such antibodies would serve as a valuable tool for obtaining precise information about the presence, expression pattern, and distribution of the target tumor antigen, *i.e.*, the MHC-peptide complex, on the tumor's cell surface, on tumor metastases, in lymphoid organs, and on professional antigen-presenting cells. Such unique antibodies with T-cell receptor-like specificity will, for the first time, enable measurement of the antigen presentation capabilities of tumor cells by direct visualization of the specific MHC-peptide complex on the tumor cell surface. Attempts to use soluble T-cell receptors for this purpose have proven difficult because of the inherent low affinity for their target and their instability as recombinant-engineered molecules (9).

Antibodies that specifically recognize class I peptide-MHC complexes have already been used in murine systems to study antigen presentation, to localize and quantify antigen-presenting cells displaying a T-cell epitope, or as a targeting tool in a mouse model (10–19). Antibodies with such exquisite specificity have proven difficult to make using immunization strategies, even in combination with *in vitro* selection from phage display libraries. The direct selection of such antibodies from very large nonimmune phage antibody libraries was demonstrated only recently (20). Using this route, a phage display-derived recombinant Fab antibody was isolated that recognizes the melanoma antigen MAGE-A1 in complex with the human HLA-A1 MHC molecule (20).

In this study, we attempted to isolate human recombinant antibodies directed to T-cell epitopes derived from the telomerase catalytic subunit (hTERT). Interestingly, the ribonucleoprotein telomerase is expressed by >85% of human cancers (21–24). Telomerase maintains the telomeric ends of linear chromosomes, protecting them from degradation and end-to-end fusion (21–25). Most human cells do not express telomerase and lose telomeric DNA with each cell division (26, 27). In contrast, most human tumors exhibit strong telomerase activity and maintain the length of their telomeres (28–30). Recent studies have demonstrated that peptides derived from the telomerase catalytic subunit can be naturally processed by tumor cells; they are presented in an HLA-A2-restricted manner and serve as a target for antigen-specific CTLs (31, 32). Cytotoxicity was achieved against target cells from a wide variety of tumors including carcinoma,

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[3] The abbreviations used are: TAA, tumor-associated antigen; hTERT, human telomerase reserve transcriptase; scMHC, single-chain MHC; TCR, T-cell receptor; APC, antigen-presenting cell; TRAP, telomerase repeat amplification protocol; NHF, normal human fibroblast.

sarcoma, melanoma, leukemia, and lymphoma (31–34). These findings, together with the identification of telomerase activity in the vast majority of human cancers, suggest that hTERT represents the most widely expressed TAA described thus far. Therefore, we have screened a large nonimmune phage antibody library (35) on recombinant-engineered single-chain MHC-peptide complexes displaying two distinct hTERT-derived epitopes.

We describe the isolation of a panel of human antibodies with antigen-specific, MHC-restricted specificity of T cells binding with high affinity HLA-A2 complexes that display the specific hTERT-derived peptide. These antibodies have been used to directly visualize, by flow cytometry, the specific HLA-A2/hTERT epitopes on antigen-presenting cells as well as on the surface of tumor cells.

MATERIALS AND METHODS

Production of Biotinylated scMHC/Peptide Complexes. scMHC/peptide complexes were produced by *in vitro* refolding of inclusion bodies produced in *Escherichia coli* as described (36). Briefly, a single-chain β_2-microglobulin-HLA/A2 (scMHC) construct, in which the *β2m* and *HLA-A2* genes are connected to each other by a flexible peptide linker, was engineered to contain the BirA recognition sequence for site specific biotinylation at the COOH terminus (scMHC-BirA). This construct is expressed in *E. coli* and upon induction with isopropyl-1-thio-β-D-galactopyranoside, intracellular inclusion bodies that contain large quantities of the recombinant protein accumulate. Inclusion bodies are purified, reduced, and subsequently refolded in a redox-shuffling buffer system (0.1 M Tris, 0.5 M arginine, and 0.09 mM oxidized glutathione, pH 8.0) in the presence of a 5–10 molar excess of the antigenic peptides. Correctly folded MHC/peptide complexes were then isolated and purified by anion exchange Q-Sepharose chromatography (Pharmacia). Filtration using Centricon-30 units (Centricon) was used to exchange the elution buffer with Tris-HCl (10 mM, pH 8.0) and concentrate the scMHC-peptide complex to 1 mg/ml for specific biotinylation using the BirA enzyme (Avidity, Denver, CO) as described previously (4, 36). Excess biotin was removed from biotinylated complexes using a G-25 desalting column. The homogeneity and purity of the scMHC-peptide complexes was analyzed by various biochemical means including SDS-PAGE, size exclusion chromatography, and ELISA assays as described previously (36).

Selection of Phage-Antibodies on Biotinylated Complexes. A large human Fab library containing 3.7×10^{10} different Fab clones was used for the selection (35). Phages (10^{13}) were first preincubated for 1 h at room temperature in PBS containing 2% nonfat dry milk with streptavidin-coated paramagnetic beads (200 μl; Dynal, Oslo, Norway,) to deplete the streptavidin binders. Streptavidin-coated paramagnetic beads (200 ml; Dynal) were also incubated in PBS + 2% milk for 1 h at room temperature. The remaining phages were subsequently incubated for 1 h with decreasing amounts of biotinylated scMHC-peptide complexes (500 nM for the first round and 100 nM for the following rounds). Streptavidin magnetic beads were added, and the mixture was incubated for 15 min with continuous rotation. A magnetic force was applied to pull down phages bound to biotinylated complexes. After 10 washes of the streptavidin-bound complexes with PBS/0.1% Tween and 2 washes with PBS, bound phages were eluted by incubation for 5 min with 1 ml of triethylamine (100 mM). The elusion mixture was neutralized by the addition of 100 μl of Tris-HCl (1 M, pH 7.4) and used to infect *E. coli* TG1 cells (A_{600}, 0.5) for 30 min at 37°C. Bacteria were grown overnight at 30°C on 2YT plates containing 100 μg/ml ampicillin and 2% glucose (2YT/A/G).

Colonies were collected from the plates in 2YT/A/G and diluted 1:100 in 50 ml of medium. Cells were grown to $A_{600\ nm} = 0.5$, and M13KO7 helper phage (5×10^{11} colony-forming units) was added to 5 ml of the culture. After having been incubated at 37°C for 30 min, the cells were centrifuged and resuspended in 25 ml of 2YT/ampicillin (100 μg/ml)/kanamycin (50 μg/ml) and grown overnight at 30°C. Phages were collected from culture supernatants and purified for the next round of panning by polyethylene glycol precipitation as described previously. The diversity of the selected antibodies was determined by DNA fingerprinting. The Fab DNA of different clones was PCR amplified using the primers pUC-reverse (5′-AGCGGATAACAATTTCACACAGG-3′) and fd-tet-seq24 (5′-TTTGTCGTCTTTCCAGACGTTAGT-3′). The resulting PCR fragments were digested with *Bst*NI (NEB; 2 h; 37°C) and analyzed by agarose gel electrophoresis.

Expression and Purification of Soluble Recombinant Fab Antibodies. An overnight starter culture of Fab-specific clones was grown at 30°C. Cells were diluted 1:100 into 500 ml of 2YT/A/G, grown to $A_{600\ nm} = 0.8–1.0$ and induced to express the recombinant Fab antibody by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3–4 h at 30°C. Cells were centrifuged, and the pellet was resuspended in 5 ml of a B-PER solution (Pierce) to release the periplasmatic content. After 30 min of rotated incubation at room temperature, the solution was centrifuged (15,000 rpm for 15 min), and the supernatant was incubated with 0.5 ml of prewashed TALON bead suspension (Clontech) for 45 min at room temperature. The solution was applied onto a Bio-Rad disposable column, and after sedimentation, the beads were washed three times with 10 ml of PBS/0.1% Tween 20 (pH 8.0). The bound Fabs were eluted using 0.5 ml of 100 mM imidazole in PBS. The eluted Fabs were dialyzed twice against PBS (overnight at 4°C) to remove residual imidazole. The homogeneity and purity of the purified Fabs was determined by analysis on nonreduced and reduced SDS-PAGE.

ELISA with Phage Clones and Purified Fab Antibodies. Binding specificity of individual phage clones and soluble Fab fragments were determined by ELISA using biotinylated scMHC-peptide complexes. ELISA plates (Falcon) were coated overnight with BSA-biotin (1 μg/well). After having been washed, the plates were incubated (1 h at room temperature) with streptavidin (1 μg/well), washed extensively, and further incubated (1 h at room temperature) with 0.5 μg of MHC/peptide complexes. The plates were blocked for 30 min at room temperature with PBS/2% skim milk and subsequently were incubated for 1 h at room temperature with phage clones (~10^9 phages/well) or various concentrations of soluble purified Fab. After washing, plates were incubated with horseradish peroxidase-conjugated/anti-*myc* antibody (for soluble Fabs) or horseradish peroxidase-conjugated anti-M13 phage (for phage-displayed Fabs). Detection was performed using TMB reagent (Sigma). The HLA-A2-restricted peptides used for specificity studies of the Fab phage clones or purified Fab antibodies are listed in Table 1.

Table 1 *HLA-A2-restricted peptides used to determine Fab antibody specificity*

	Sequence	Protein	Position	Score[a]
1	ILAKFLHWL	hTERT	540–548	1745
2	RLVDDFLLV	hTERT	865–873	541
3	IMDQVPFSV	Gp100	209–217	198
4	YLEPGPVTV	Gp100	280–288	20
5	KTWGQVWQV	Gp100	154–162	484
6	LLLTVLTVV	MUC1	13–21	412
7	NLVPMVATV	CMV	495–503	159
8	SLFPGKLEV	Flightless 1 homologue	1010–18	257
9	SLSEEKTVLL	CD59	106–114	88
10	KIADFGWSV	Serine/threonine kinase	271–279	3911
11	SLLSHVEQL	Mad2B protein	114–122	89
12	GLIEKNIEL	DNA methyl transferase (MTDM)	425–433	87
13	GLYPGLIWL	Interferon regulatory factor-6	21–29	865
14	FLAMLKNTV	Cancer/testis antigen CT7	1087–1096	320
15	LMVDHVTEV	Steroid receptor RNA activator	183–92	805
16	ALSDHHIYL	Fructose bisphosphate aldolase	216–224	482
17	NLAEDIMRL	Vimentin	177–185	201
18	GVYDGEEHSV	MAGE-B2	231–240	80
19	TLWVDPYEV	B-cell translocation gene (*BTG1*)	103–111	577
20	FTWEGLYNV	UHX1 protein	353–41	348
21	FLFDGSPTYV	Fatty acid synthase (FAS)	292–301	26664
22	FLFDGSPTYVL	Fatty acid synthase (FAS)	292–302	611
23	ALWDIETGQQTV	Guanine nucleotide-binding Regulatory protein-β-2 subunit	167–178	2366
24	GLLGTLVQL	β-Catenin	400–408	182
25	ALFGALFLA	Phospholipid transfer protein	2–10	245
26	SLLGGDVVSV	Hypothetical protein	22–32	592
27	NLTISDVSV	MUC1	130–138	70
28	SLWGQPAEA	Human collagen type IV	18–25	42
29	SLIGHLQTL	Protein tyrosine phosphatase	336–344	49
30	MLAALNGLSV	Protein tyrosine phosphate	866–875	118
31	SVRDRLARL	EBV	596–604	1.34
32	EAAGIGILTV	MART	26–35	0.17
33	STPPPGTRV	P53	149–157	0.97
34	LLFGYPVYV	TAX	11–19	2406

[a] Relative binding affinity to HLA-A2 (estimate of half time of disassociation of molecule containing this subsequence) calculated using the HLA Peptide Binding Predictions computer program generated by the Bioinformatics and Molecular Analysis Section, DCRT, NIH (http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html).

Flow Cytometry. The B-cell line RMAS-HHD, which is transfected with a single-chain *β2M-HLA-A2* gene (37), the EBV-transformed HLA-A2$^+$ JY cells, human tumor cells, or telomerase-transfected human foreskin fibroblasts were used to determine the reactivity of the recombinant Fab antibodies with cell surface-expressed HLA-A2/peptide complexes. About 10^6 RMAS-HHD or JY cells were washed twice with serum-free RPMI 1640 and incubated overnight at 26°C or 37°C, respectively, in medium containing 100 μM of the peptide. The RMAS-HHD cells were subsequently incubated at 37°C for 2–3 h to stabilize cell surface expression of MHC-peptide complexes. Next, the cells were incubated for 60–90 min at 4°C with recombinant Fab antibodies (20 μg/ml) in 100 μl. After three washes, the cells were incubated with 1 μg of antihuman Fab (Jackson). After another three washes, the cells were resuspended in ice-cold PBS.

When RMAS-HHD cells were loaded with a pool of HLA-A2-restricted peptides, a mixture of peptides at 1:1 ratio was used. The final concentration of each peptide in the mixture was 3 μM. Melanoma and prostate carcinoma cells were harvested by trypsinization and resuspended in cold RPMI. All subsequent washes and incubations were performed in ice-cold conditions as described above for RMAS-HHD or JY peptide-loaded cells. Analysis of the cells was performed by a FACStar flow cytometer (Becton Dickinson), and the results were analyzed with the WinMDI program.[4]

Competition Binding Assays. Flexible ELISA plates were coated with BSA-biotin and scMHC-peptide complexes (10 μg in 100 μl) were immobilized as described above. Binding of soluble purified Fabs was performed by competitive binding analysis, which examined the ability of purified Fab to inhibit the binding of ^{125}I-labeled Fab to the specific immobilized scMHC-peptide complex. The recombinant Fab antibodies were labeled with ^{125}I using the Bolton-Hunter reagent. The labeled Fab was added to wells as a tracer ($3–5 \times 10^5$ cpm/well) in the presence of increasing concentrations of the cold Fab fragments as competitor. The binding assays were performed at room temperature for 1 h in PBS. The plates were washed (five times) with PBS, and the bound radioactivity was determined in a gamma counter. The apparent affinity of the Fabs was determined by extrapolating the concentration of competitor necessary to achieve 50% inhibition of ^{125}I-labeled Fab binding to the immobilized scMHC-peptide complex. Nonspecific binding was determined by the addition of a 20–40-fold excess of unlabeled Fab.

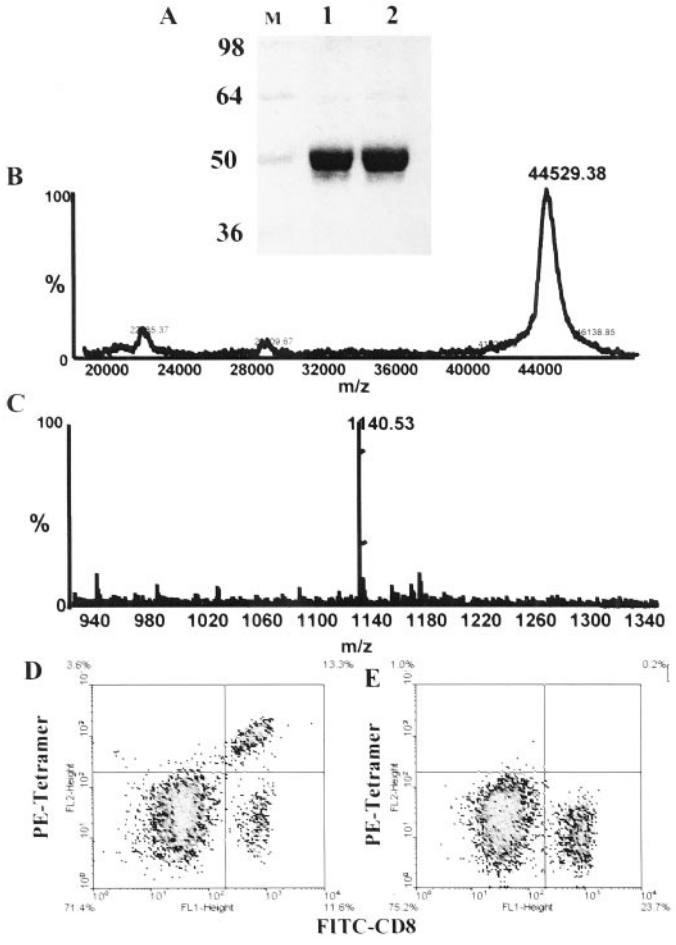


Fig. 1. Biochemical characterization of scMHC-hTERT complexes. *A,* SDS-PAGE analysis of scHLA-A2/T540 (*Lane 1*) and scHLA-A2/T865 (*Lane 2*) telomerase-derived complexes after ion-exchange Q-Sepharose chromatography (see "Materials and Methods" for experimental details). *B* and *C,* mass spectrometry analysis of scMHC-peptide complex containing the T540 telomerase peptide. *B,* scMHC protein profile. *C,* peptide profile. *D* and *E,* functional analysis of scHLA-A2/T540-derived complexes showing the ability of tetramers to stain CTLs, T1-H12, specific for the telomerase-derived peptide T540 in complex with HLA-A2 (*D*). Staining of 13% of the cell population is observed in comparison with control tetramers containing the T865 telomerase-derived peptide (*E*). The cells were double-stained with PE-labeled tetramers and FITC-labeled anti-CD8 antibody.

RESULTS

Recombinant Single-Chain MHC-Peptide Complexes with Two hTERT-derived HLA-A2-restricted Peptides. Two major T-cell epitopes were identified in hTERT that were recognized by HLA-A2-restricted CTLs derived from different patients (31, 32): peptide 540 (ILAKFLHWL; T540) and peptide 865 (RLVDDFLLV; T865). Recombinant MHC-peptide complexes that present the two hTERT-derived epitopes were generated by using a scMHC construct that was described previously (36, 38). In this construct, the extracellular domains of HLA-A2 are connected into a single-chain molecule with β_2-microglobulin using a 15-amino acid flexible linker. The scMHC-peptide complexes were produced by *in vitro* refolding of inclusion bodies from bacterial cultures transformed with the scMHC construct. Refolding was performed in the presence of the two hTERT-derived peptides followed by a purification protocol using ion-exchange chromatography. The refolded hTERT-derived peptide-MHC complexes were very pure, homogeneous and monomeric, as shown by analysis on SDS-PAGE and size-exclusion chromatography (Fig. 1*A*). Recombinant scMHC-peptide complexes generated by this strategy have been characterized previously in detail for their biochemical, biophysical, and biological properties and were found to be correctly folded and functional (36, 38).

To clearly demonstrate that the scMHC complex is folded correctly and contains peptide, we performed mass spectrometry analysis. The MHC-peptide complexes were deposited on a metal target as cocrystals with α-xyano-4-hydroxycinnamic acid (for the peptide identification) and separately as cocrystals with sinapinic acid (for the protein identification). The mass spectrometry analysis was done using Matrix-assisted laser-desorption time-of-flight in the positive ion mode. As shown in Fig. 1*C*, the peptide was easily detected, with the expected mass of 1140 daltons corresponding to the mass of the T540 peptide used for the refolding of the scMHC-peptide complex. This was the only peptide detected indicating that the refolded complex is a homogeneous population of molecules containing a single specific peptide. The profile of the scMHC protein revealed a single peak with a mass of 44.5 kDa corresponding to the expected molecular weight of the scMHC protein (Fig. 1*B*). As shown above for the peptide, this was the only identified protein peak in the analyzed spectrum indicating that the protein consists of a very homogeneous population of folded complexes.

To further demonstrate that the refolded telomerase-derived MHC-peptide complexes are functional, we tested their ability to stain telomerase-derived T540-specific CTLs. To this end, we generated scMHC-T540 tetramers. To date, this is a well-established strategy for overcoming the low affinity of the MHC-peptide-TCR interactions (4, 5). The scMHC- T540 tetramers could specifically stain T540-

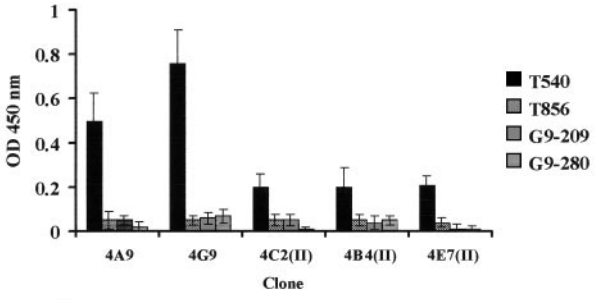
[4] J. Trotter, http://facs.scripps.edu/.

A

Selection of Recombinant Fab Antibodies with TCR-like Specificity

Cycle No.	Phage Input	Phage Output	Ration (O/I)	Enrichment	MHC-peptide binders	TCR-like Binders	Finger Pattern
A. T540							
1	$6x10^{13}$	$1x10^{7}$	$2x10^{-7}$	-			
2	$5x10^{12}$	$1x10^{7}$	$3x10^{-6}$	-	23/94 (24%)	13/94 (14%)	2
3	$1x10^{13}$	$1x10^{10}$	$9x10^{-4}$	1200	60/94 (64%)	41/94 (44%)	3
B. T865							
1	$6x10^{13}$	$2x10^{7}$	$3x10^{-7}$	-			
2	$8x10^{12}$	$1x10^{7}$	$2x10^{-6}$		17/94 (18%)	5/94 (5%)	3
3	$4x10^{12}$	$6x10^{9}$	$2x10^{-3}$	600	58/94 (62%)	21/94 (22%)	3

B



C

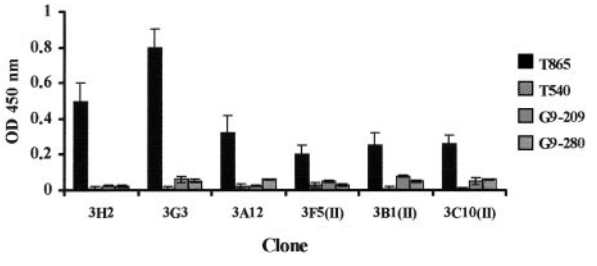


Fig. 2. Frequency (*A*) and specificity (*B* and *C*) of recombinant Fab antibodies selected on telomerase-derived HLA-A2-restricted peptides. ELISA with phage particles was performed on immobilized scHLA-A2/peptide complexes as described in "Materials and Methods." *A*, summary of panning against hTERT T-cell epitopes T540 and T865 in complex with scHLA-A2. *B*, phage ELISA of clones selected against scHLA-A2/T540 complex. [Clones 4C2(II), 4B4(II), and 4E7(II) are from the second round of panning, and clones 4A9 and 4G9 are from the third round.] Statistical significance, $P < 0.01$. *C*, phage ELISA of clones selected against scHLA-A2/T865. [Clones 3F5(II), 3B1(II), and 3C10(II) are from the second round of panning, and clones 3H2, 3G3, and 3A12 are from the third round.] The background reactivity of a control negative clone was $A_{450\ nm} = 0.05$. Statistical significance, $P < 0.01$. *Bars*, SE.

restricted CTL T1-H12 (Fig. 1*D*). However, a T865 epitope-containing tetramer did not bind to these cells (Fig. 1*E*) nor to tetramers containing the melanoma gp100-derived, HLA-A2-restricted epitope G9-209 (not shown). These results demonstrate that the recombinant scMHC complexes are functional and retain the conformation of the native MHC-peptide complex.

Selection of Recombinant Antibodies with TCR-like Specificity to HLA-A2-restricted T-Cell Epitopes of hTERT. To enable efficient selection, scMHC-peptide complexes were biotinylated using a BirA sequence tag that was engineered at the COOH terminus of the *HLA-A2* gene for site-specific biotinylation as described previously (4, 36). The phage display large repertoire of 3.7×10^{10} human recombinant Fab fragments (35) was incubated first with streptavidin-coated beads to avoid the selection of anti-streptavidin antibodies. A magnetic field was applied to precipitate the beads, and the supernatant containing the library depleted of streptavidin binders was used for the subsequent panning in solution on soluble recombinant MHC-peptide complexes containing the two hTERT-derived T-cell epitopes. After incubation of the library with soluble complexes, binding phages were collected using streptavidin-coated magnetic beads, followed by elution with triethylamine. A 600-1200-fold enrichment in phage titer was observed after three rounds of panning using the two different hTERT-derived peptide-MHC complexes (Fig. 2*A*). An ELISA with phage particles was performed on biotinylated recombinant scMHC-peptide complexes immobilized on streptavidin-coated immunoplates to determine antibody specificity. The fine specificity of the selected phage antibodies was determined by a differential ELISA on wells coated with scMHC HLA-A2 complexes containing either the specific hTERT-derived peptide or control complexes containing other HLA-A2-restricted peptides. Phage clones analyzed after the third round of selection exhibited two types of binding pattern toward the MHC-peptide complex. One class of antibodies were pan-MHC binders which cannot differentiate between the various MHC-peptide complexes; the second type were antibodies that bound the MHC-peptide complex in a peptide-specific manner. The ELISA screen revealed that 62–64% of randomly selected clones from the third round of panning appeared to be binding to the HLA-A2/peptide complex. Twenty % (for the T540 epitope) and 40% (for the T865) bound to four to five of five different peptide/MHC complexes tested. However, a surprisingly high percentage of antibodies were fully specific for the peptide/MHC used in selection when tested as phage antibodies in ELISA on different peptide/MHC complexes. As shown in Fig. 2*A*, 22 and 44% of the clones directed toward the T865 and T540 epitopes, respectively, exhibited antigen-specific, MHC-restricted binding characteristics of T cells. Thus, they bound only to the MHC peptide complex containing the specific T540 or T865 (Fig. 2*A*) hTERT-derived peptides and did not bind to control complexes containing other HLA-A2-restricted peptides. These apparent MHC/

peptide-specific positive clones remained specific in a secondary screening on more complexes (data not shown). We examined the diversity pattern of these peptide-specific clones by DNA fingerprint analysis and found five to six different restriction patterns (from round two and three) for each hTERT-derived complex, indicating the selection of several different antibodies with TCR-like specificity. DNA sequencing analysis confirmed these observations.

Fig. 2 shows a representative analysis of five TCR-like Fab clones of each of the two selections. The five different T540-specific clones tested reacted only with scMHC-T540 complexes and not with MHC-peptide complexes displaying the hTERT-derived T865 epitope or two melanoma gp100-derived epitopes, G9-209 and G9-280 (Fig. 2*B*). Similar results were observed in phage ELISA assays that determined the specificity of six phage clones isolated against the hTERT-derived T865 epitope (Fig. 2*C*).

Characterization of Recombinant Soluble Fab Antibodies with TCR-like Specificity. We produced soluble Fab fragments from the phage clones (analyzed above, Fig. 2, *B* and *C*) that exhibited the specific binding pattern to the different hTERT-derived HLA-A2-peptide complexes in *E. coli* BL21 cells.

These were purified by metal affinity chromatography from the periplasm by use of the hexahistidine tag fused to the CH1 domain of the Fabs. SDS-PAGE analysis of the affinity-purified material revealed homogeneous, very pure Fab antibodies with the expected molecular weight (Fig. 3*A*). Approximately 0.5–2 mg of pure material could be obtained from 1 liter of bacterial culture.

We determined the fine specificity of the soluble molecules by ELISA on biotinylated MHC-peptide complexes that were immobilized to BSA-streptavidin-coated wells. The BSA-streptavidin-biotin spacer enables the correct folding of the complexes, which can be distorted by direct binding to plastic. To determine the correct folding of the bound complexes and their stability during the binding assays, we monitored their ability to react with the conformational specific monoclonal antibody w6/32, which recognizes HLA complexes only when folded correctly and when containing peptide. Fig. 3*B* shows a representative analysis of five soluble Fab antibodies directed to HLA-A2/T540 complexes. All five antibodies react specifically with the T540-containing HLA-A2 complexes but not with control complexes containing the T865 hTERT-derived MHC-peptide complex nor with HLA-A2 complexes containing the two melanoma gp100-derived epitopes, G9-209 and G9-280. Similarly, soluble purified Fab fragment antibodies from the antibody clones isolated against the T865 epitope bound to the specific HLA-A2/T865 complexes but not to control T540 hTERT-derived complexes nor to the melanoma gp100-derived HLA-A2/G9–209 and HLA-A2/G9–280 complexes (Fig. 3*C*). As shown in Fig. 3*D*, these binding studies were extended to 10 different HLA-A2-restricted peptides. The purified Fabs 4A9 or 4G9 and 3H2 or 3G3 specific for the T540 or T865 telomerase-derived epitopes, respectively, reacted only with the specific complex but not with any of the other nine control HLA-A2-peptide complexes used in the assay. Thus, these peptide-specific and MHC-restricted Fab fragments exhibit the binding characteristics and fine specificity of a TCR-like molecule. The Fab antibodies did not recognize the peptide alone when immobilized on the plate neither streptavidin or other protein antigens (such as BSA, IgG, RNase, and chymotrypsin; data not shown).

Further proof for the specificity of the TCR-like Fab antibodies isolated in this study was obtained in a T-cell stimulation/inhibition assay (Fig. 4). The HLA-A2-restricted, T540-specific CTL line T1-H12 was stimulated in the presence of APCs loaded with the T540 peptide but not with control HLA-A2-restricted peptides (Fig. 4*A*). In the inhibition assays, the T540-specific Fab 4G2 was able to inhibit the release of IFN-γ from T540-specific CTL T1-H12, whereas a control T865-specific Fab 3H2 did not inhibit peptide-specific CTL stimulation (Fig. 4).

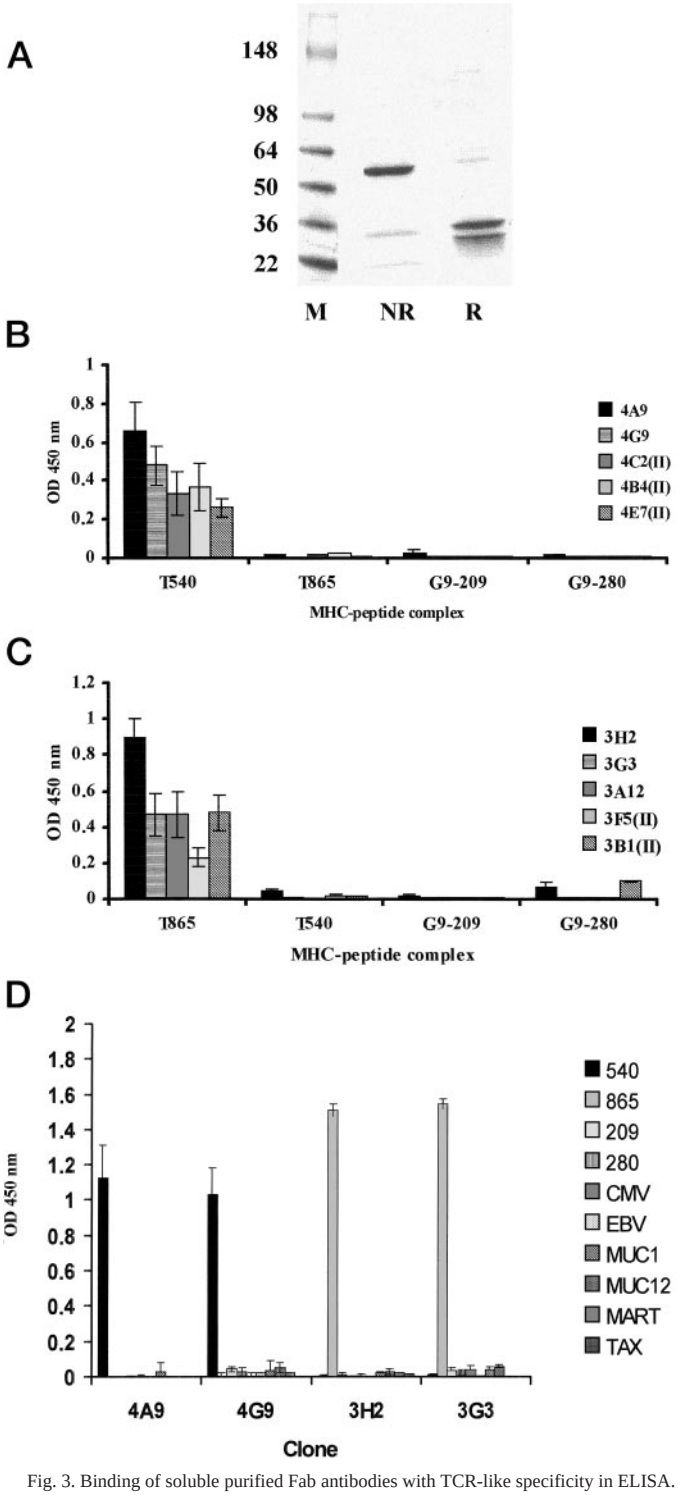


Fig. 3. Binding of soluble purified Fab antibodies with TCR-like specificity in ELISA. *A*, SDS-PAGE analysis of reduced and nonreduced purified Fab protein after metal affinity chromatography. *B* and *C*, binding of soluble Fabs to immobilized MHC-peptide complexes containing various HLA-A2-restricted peptides. In *B*, Fab clones selected against scHLA-A2/T540 complexes. In *C*, Fab clones selected against scHLA-A2/T865 complexes. $P < 0.005$. *D*, binding of the T540/HLA-A2-specific Fabs 4A9 or 4G9 and the T865/HLA-A2-specific Fabs 3H2 or 3G3 to 10 HLA-A2/peptide complexes. $P < 0.0005$. *Bars*, SE.

Next, we tested the affinity binding properties of two of the TCR-like soluble Fabs, using a saturation ELISA assay in which biotinylated complexes were bound to streptavidin-coated plates and to

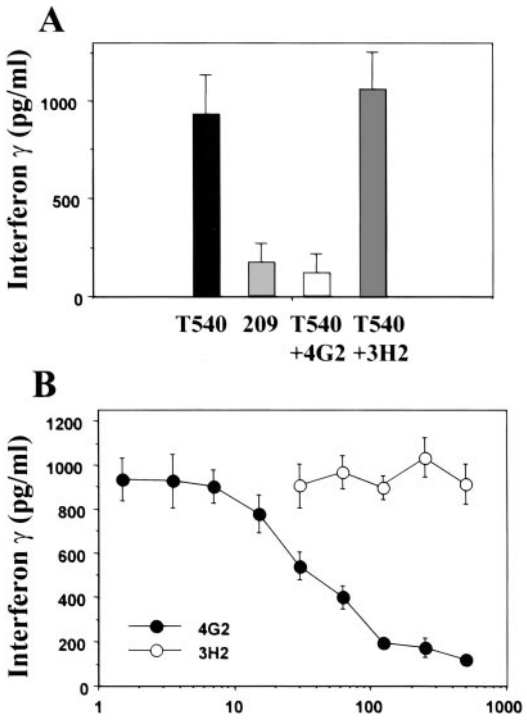


Fig. 4. Specific inhibition of peptide-specific, MHC-restricted T-cell activation with TCR-like Fab. T2 cells were pulsed with peptide as indicated and incubated with the T540-specific HLA-A-restricted CTL line T1-H12 in the presence of various concentrations of Fab 4G2 or control Fabs as indicated. T-cell stimulation was measured by the release of IFN-γ to the culture medium. IFN-γ was determined by a double sandwich ELISA assay. *A*, stimulation of T1-H12 CTLs with the T540 peptide but not control peptides and inhibition of stimulation by Fab 4G2 but not 3H2. The stimulator:responder ratio was 1:1 (10^5 cells). $P < 0.002$. *B*, inhibition of T-cell response using Fab 4G2. Fab 3H2 specific for the T865 peptide was used as a control. *Bars*, SE.

which increasing amounts of Fab antibody were added. As shown in Fig. 5, *A* and *B*, the binding of two specific Fabs (4A9 and 3H2) was dose dependent and saturable. Extrapolating the 50% binding signal of either antibody revealed that their affinity is in the nanomolar range.

Finally, we determined the apparent binding affinity of the TCR-like Fab fragments to their cognate MHC-peptide complex by a competition binding assay in which the binding of ^{125}I-labeled Fab was competed with increasing concentrations of unlabeled Fab fragment. These binding studies (Fig. 5, *C* and *D*) revealed an apparent binding affinity of ~5 nM for the 4A9 antibody specific for the T540 hTERT epitope and 10–15 nM for the 3G3 antibody specific for the T865 epitope.

Binding of Fab Fragments to APCs Displaying the hTERT-derived Epitopes. To demonstrate that the isolated Fab fragments can bind the specific MHC-peptide complex not only in the recombinant soluble form but also in the native form as expressed on the cell surface, we used murine TAP2-deficient RMA-S cells transfected with the human *HLA-A2* gene in a single-chain format (Ref. 37; HLA-A2.1/Db-β2m single chain; RMA-S-HHD cells). The hTERT-derived and control peptides were loaded on RMA-S-HHD cells, and the ability of the selected Fab antibodies to bind to peptide-loaded cells was monitored by fluorescence-activated cell sorting. Peptide-induced MHC stabilization of the TAP2 mutant RMA-S-HHD cells was demonstrated by the reactivity of monoclonal antibodies w6/32 (HLA conformation-dependent) and BB7.2 (HLA-A2-specific) with peptide-loaded but not unloaded cells (see Fig. 7*A*). Fabs 4A9 and 4G9, which recognize the T540-containing HLA-A2 complexes, reacted only with T540-loaded RMA-S-HHD cells but not with cells loaded with the gp100-derived G9–209 peptide or the gp100-derived G9–280 peptide, respectively (Fig. 6, *A* and *B*, and *D* and *E*, respectively). Similarly the T865-HLA-A2-specific Fab antibodies 3G3 and 3H2 recognized only T865-loaded RMA-S-HHD cells (Fig. 6, *G* and *J*) and did not recognize cells loaded with the gp100-derived peptides at all (Fig. 6, *H* and *K*).

We have also used the TAP+ EBV-transformed B-lymphoblast HLA-A2+ JY cells as APCs. They have normal TAP, and consequently peptide loading is facilitated by the exchange of endogenously derived peptides with HLA-A2-restricted peptides supplied externally by incubation of the cells with the desired peptides. We incubated these cells first with the T540, T865 telomerase-derived, and control

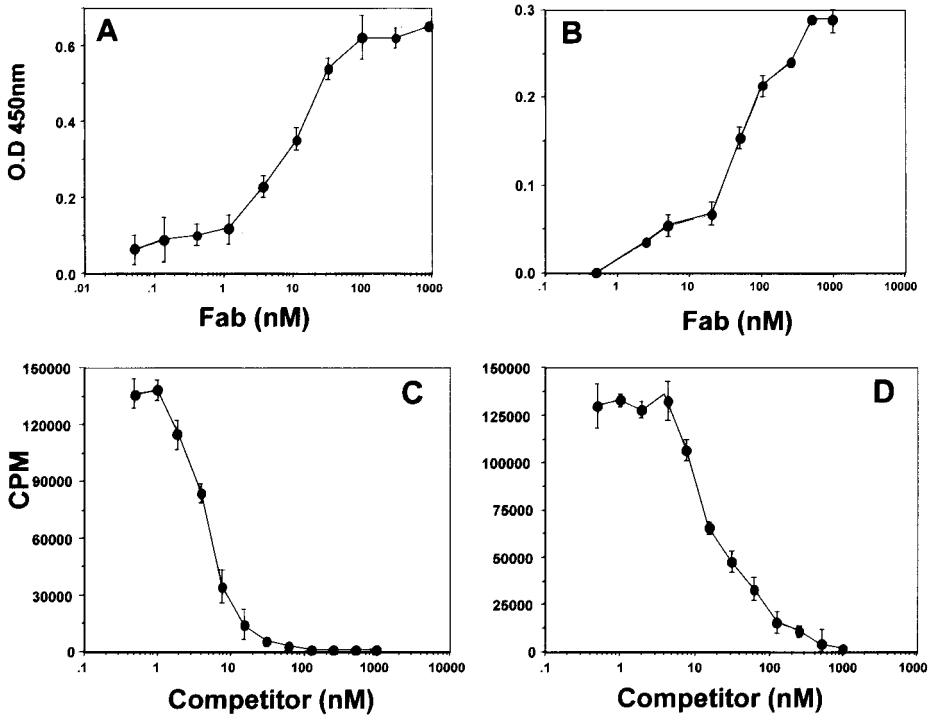


Fig. 5. Binding characteristics of two recombinant TCR-like Fab antibodies. *A* and *B*, titration ELISA of purified soluble Fab antibodies 4A9 (*A*) and 3H2 (*B*) directed to scHLA-A2/T540 and scHLA-A2/T865, respectively. Wells were coated with the corresponding MHC-peptide complexes as described in "Materials and Methods." *C* and *D*, competitive binding analysis of the ability of purified Fab 4G9 (*C*) or 3G3 (*D*) to inhibit the binding of ^{125}I-labeled Fab to the corresponding HLA-A2-peptide complex. The apparent binding affinity of the recombinant Fab was determined as the concentration of competitor (soluble purified Fab) required for 50% inhibition of the binding of the ^{125}I-labeled tracer. *Bars*, SE.

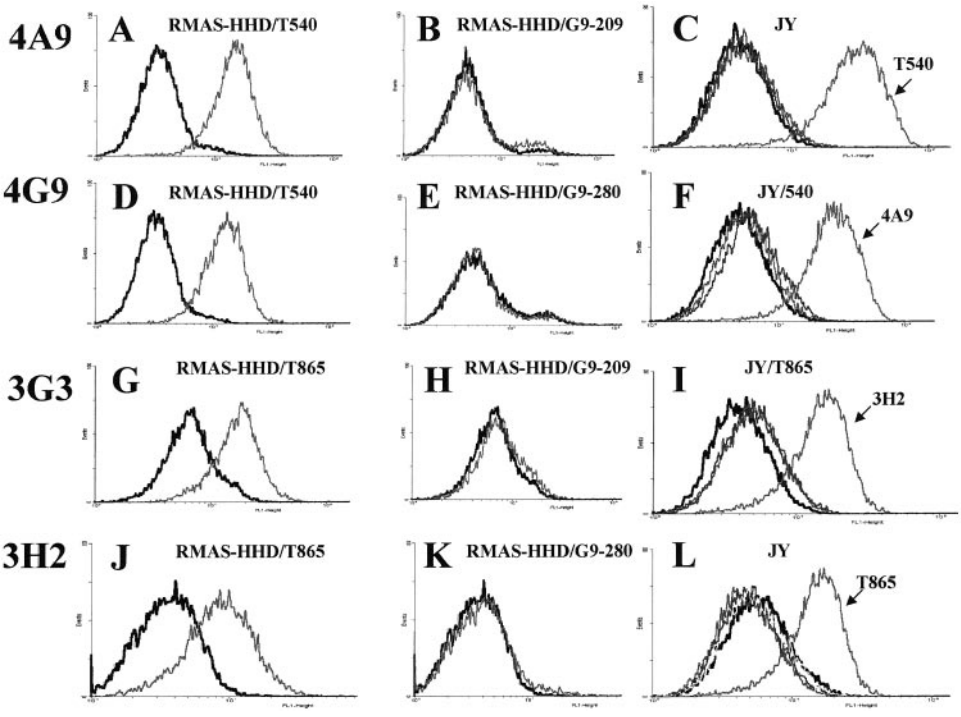


Fig. 6. Detection of MHC-peptide complexes on the surface of APCs. RMAS-HHD or JY cells were loaded with telomerase T540 peptide (*A* and *D*) or control melanoma gp100-derived peptides G9-209 or G9-280 (*B* and *E*). Peptide-loaded cells were then incubated with the HLA-A2/T540-specific soluble purified Fab antibodies 4A9 and 4G9 as shown. Specific staining of the T540-loaded cells but not the control cells is shown. In *G* and *J*, RMAS-HHD cells were loaded with telomerase T865 peptide or control G9-209 or G9-280 (*H* and *K*). The reactivity with T856-specific Fab antibodies 3G3 and 3H2 is shown. In *C*, JY cells were loaded with peptides T540 (marked), T865, G9-209, or unloaded as controls and reacted with Fab 4A9. In *F*, JY cells were loaded with T540 and reacted with Fab 4A9 (marked), 3H2, or Fab directed against the melanoma gp100-derived peptide G9-209. Controls are unloaded cells. In *I*, JY cells loaded with T865 were reacted with Fab 3H2 (marked), 4A9, or control Fab as described above. In *L*, JY cells were loaded with peptides T865 (marked), or T540, G9-209, and G9-280 as controls and reacted with the T865-specific Fab 3H2.

HLA-A2-restricted peptides and then washed the cells, followed by incubation with Fab antibodies 4A9 and 3H2, respectively. These Fab fragments recognize only JY cells incubated with the specific telomerase peptide to which they were selected but not control HLA-A2-restricted peptides, including the other telomerase epitope (Fig. 6, *C* and *L*). We also tested the cross-reactivity of Fabs 4A9 and 3H2 on JY cells loaded with T865 and T540, respectively. JY cells loaded with T540 were only recognized by Fab 4A9 but not by Fab 3H2 nor by control Fabs recognizing a melanoma-derived gp100 epitope (Fig. 6*F*). Similarly, T865-loaded JY cells were recognized by Fab 3H2 specific for T865 in complex with HLA-A2 but not by Fab 4A9 nor by other gp100-specific Fabs (Fig. 6*I*). As a control, we used peptide-loaded HLA-A2−/HLA-A1+ APD B cells. No binding of the Fab antibodies to these cells was detected (data not shown).

To further demonstrate the specificity of the Fab antibodies, we extended the binding studies to peptide-pulsed APCs and used a large panel of naturally occurring HLA-A2-restricted peptides (Table 1) that were isolated by biochemical means from cells transfected with a soluble *HLA-A2* gene and were analyzed by mass spectrometry (39). The binding affinity of these peptides to HLA-A2 was variable with a wide range of stability (affinity) scores from very high through medium to low affinity binders. The binding of these peptides to RMAS-HHD cells was demonstrated by the reactivity of BB7.2 (HLA-A2-specific) with peptide-loaded but not unloaded cells (Fig. 7*A*). Representative analysis of seven peptides that exhibit variable HLA-A2 stability scores (Table 1) shows that all of them induced MHC stabilization on the surface of the TAP mutant RMAS-HHD cells in comparison with unloaded cells. Next, we used each individual peptide listed in Table 1 for loading onto RMAS-HHD cells and tested their reactivity with Fabs 4A9 (Fig. 7*B*) and 3H2 (Fig. 7*C*) specific for the T540 and T865 epitopes, respectively. As shown, Fab 4A9 and 3H2 stained RMAS-HHD cells only when loaded with the specific T540 or T865 peptides, respectively. They did not exhibit staining cross-reactivity with any of the 33 peptides used as control (Table 1). A similar staining specificity pattern was observed when the JY APCs were used for peptide loading. Similar experiments were performed on RMAS-HHD cells loaded with a pool containing all of the control HLA-A2-restricted peptides in the presence or absence of the T540 telomerase-derived epitope. As shown in Fig. 8*A*, when RMAS-HHD cells were loaded with a pool of 33 HLA-A2-restricted peptides (each peptide at 1:1 ratio) without the T540 telomerase-derived epitope, no binding of Fab 4A9 or 4G9 was observed. However, when the T540 peptide was added to the pool of peptides used for loading Fabs 4A9 and 4G9, specific for the T540/HLA-A2 complex, reacted with the peptide-pulsed cells (Fig. 8*B*). Control Fabs 3H2 and 3G3 did not react with these cells (Fig. 8*B*). Similarly, when the T865 epitope was added to the pool, the T865-specific Fabs 3H2 and 3G3 but not T540-specific Fabs reacted with the peptide-pulsed RMAS-HHD cells.

These results demonstrate that the Fab antibodies exhibit a TCR-like fine specificity and can recognize the corresponding native HLA-A2 complexes *in situ* on the surface of cells.

Binding of TCR-like Fab Antibody to Telomerase-expressing Tumor Cells. To confirm that the telomerase-specific TCR-like Fab antibodies can bind endogenously derived MHC-peptide complexes on the surface of tumor cells, we performed flow cytometry analysis on various tumor cells that express hTERT and HLA-A2. These cells represent the normal situation in which MHC-peptide complexes are expected to be present on tumor cells at a much lower density on the cell surface compared with the peptide-loaded APCs. The T540-specific Fab antibody 4A9 and T865-specific Fab 3H2 reacted with the HLA-A2+ FM3D melanoma, LnCap prostate carcinoma, and HeLa epithelial carcinoma tumor cells (Fig. 9, *A–C*) but not with the HLA-A2-prostate carcinoma PC3 cells that express hTERT (Fig. 9*D*). Telomerase activity in these cells was measured by a TRAP assay using total cellular extracts (Fig. 9*G*). FM3D, LnCap, HeLa, and PC3 cells exhibit moderate to high telomerase activity. In these experiments, we observed a moderate shift in fluorescence intensity in most of the cell population. However, a subpopulation (20–30%) of the cells exhibited a substantial shift in staining intensity, indicating increased expression of telomerase T540- and T865-specific MHC-peptide complexes. These observations may reflect the antigenic

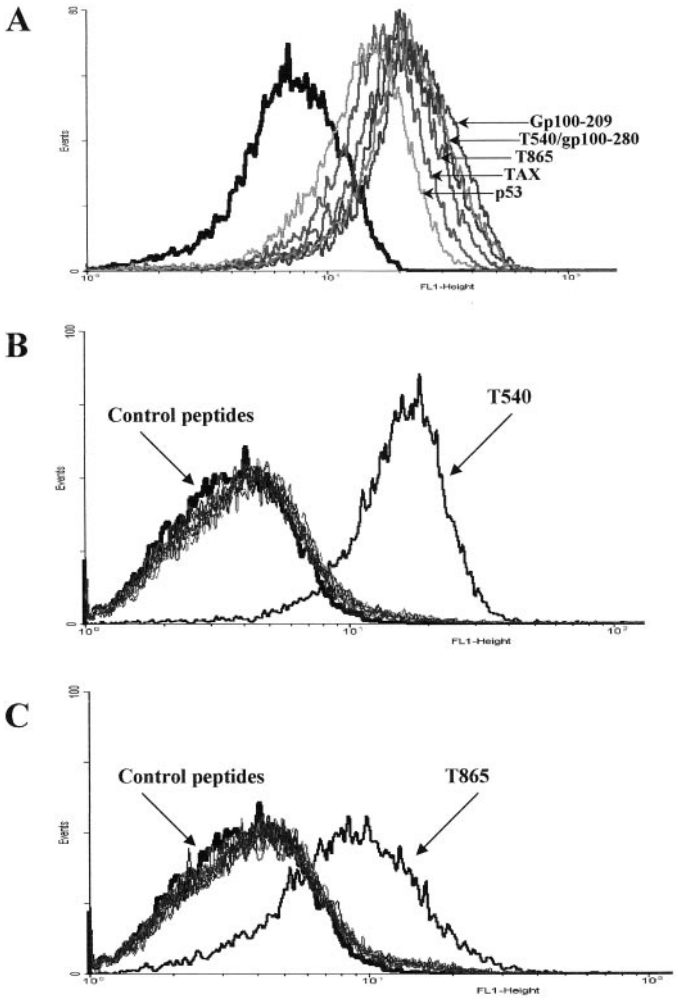


Fig. 7. Binding specificity of telomerase TCR-like Fabs. *A*, peptide-induced HLA-A2 stabilization on pulsed mutant RMAS-HHD cells. RMAS-HHD cells were loaded with various HLA-A2-restricted peptides as indicated, and stabilization was monitored by reactivity of the HLA-A2-specific monoclonal antibody BB7.2. T HLA-A2 stability score is indicated in parentheses (see also Table 1). *B* and *C*, RMAS-HHD cells were pulsed with 34 separate HLA-A2-restricted peptides described in Table 1. The reactivity of Fab 4A9 (*B*) or 3H2 (*C*) with T540- or T865-pulsed cells, respectively, but not with 33 control HLA-A2-restricted peptides is shown. The 33 control peptides are overlayed *versus* unpulsed cells.

variations in expression levels of MHC-peptide complexes expected to occur on the surface of tumor cells. In addition, we tested the reactivity of Fabs 4A9 and 3H2 with HLA-A2-positive human foreskin fibroblasts that were transfected with hTERT and control non-transfected cells (Fig. 9, *E* and *F*). The telomerase-specific Fabs reacted only with the transfected cells but not with the control NHFs, which represent control HLA-A2+ cells that do not express hTERT. TRAP activity assays revealed high telomerase activity in the transfected but not in control NHF fibroblasts (Fig. 9*G*). In addition to the negative TRAP assay, the control HLA-A2+ NHFs show no telomerase activity after transient transfection of hTERT promoter-luciferase reporter (40). In addition, reverse transcription-PCR using hTERT-specific primers show no product when using these fibroblasts RNA (40). These results therefore demonstrate the ability of these high-affinity TCR-like antibodies to detect MHC-peptide complexes on the surface of tumor cells. This occurs although the Fab antibodies are monovalent. Thus, these TCR-like antibodies can bind to cells that express the specific MHC-peptide complex at a density most likely to be found on tumor cells, APCs such as dendritic cells, and other cells involved in tumor antigen presentation to the immune system.

DISCUSSION

This study demonstrates our ability to select from a large nonimmune repertoire of human Fab fragments displayed on phage a panel of antibodies directed against two HLA-A2-restricted T-cell epitopes of the most widely expressed tumor-associated antigen identified thus far, the hTERT. These antibodies can bind soluble HLA-A2 molecules complexed with the cognate peptides with high affinity in an antigen-specific, MHC-restricted manner. Moreover, they can detect and visualize peptide/MHC complexes on the surface of cells. Hence, these are recombinant antibodies with the T-cell antigen receptor-like specificity of T cells. In contrast to the inherently low affinity of TCRs to MHC-peptide complexes, these molecules display the high affinity binding characteristics of antibodies, yet they retain TCR-like fine specificity. Most importantly, unlike recombinant TCRs, these recombinant antibodies recognize the corresponding native MHC-peptide complexes on cells.

We have selected the antibodies against one of the most interesting TAAs isolated thus far, the human telomerase catalytic subunit. It has been shown recently that a CTL repertoire for hTERT is preserved in normal individuals as well as, most importantly, in cancer patients (31–34). Two observations may contribute to the suggested importance of hTERT as a TAA; (*a*) telomerase is expressed and active in >85% of human cancers but not in most normal human somatic cells (21–24); and (*b*) peptides derived from the telomerase catalytic subunit can be naturally processed by tumor cells, presented in an HLA-A2-restricted fashion, and then serve as a target for antigen-specific CTLs (31–34). Moreover, the finding that CTLs specific for telomerase-derived epitopes isolated from a prostate cancer patient

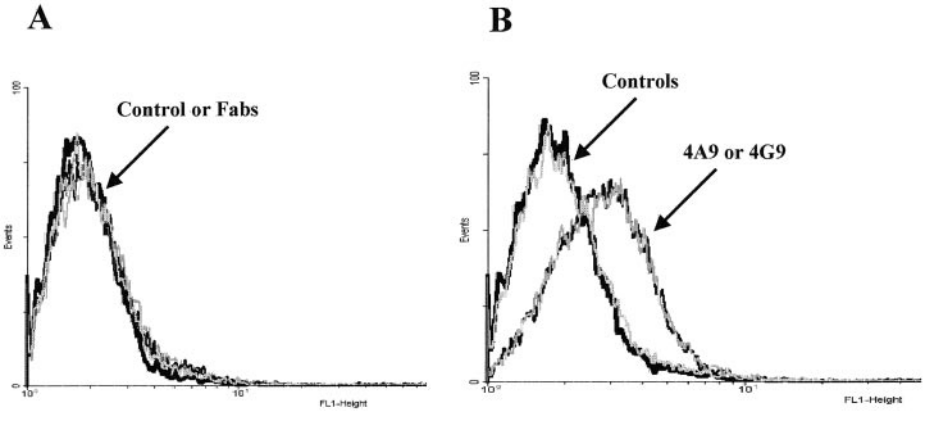


Fig. 8. Binding specificity of telomerase-specific, TCR-like Fabs. RMAS-HHD cells were loaded with a pool containing 33 HLA-A2-restricted peptides as described in Table 1 in the absence (*A*) or presence (*B*) of the telomerase-derived T540 peptide epitope. Fabs 4A9 or 4G9 were able to stain peptide-pulsed RMAS-HHD cells only when the specific T540 peptide was added to the pool of peptides. Controls are unpulsed cells or cells loaded with the peptide pool and reacted with Fab 3H2 specific for the T865/HLA-A2 complex.

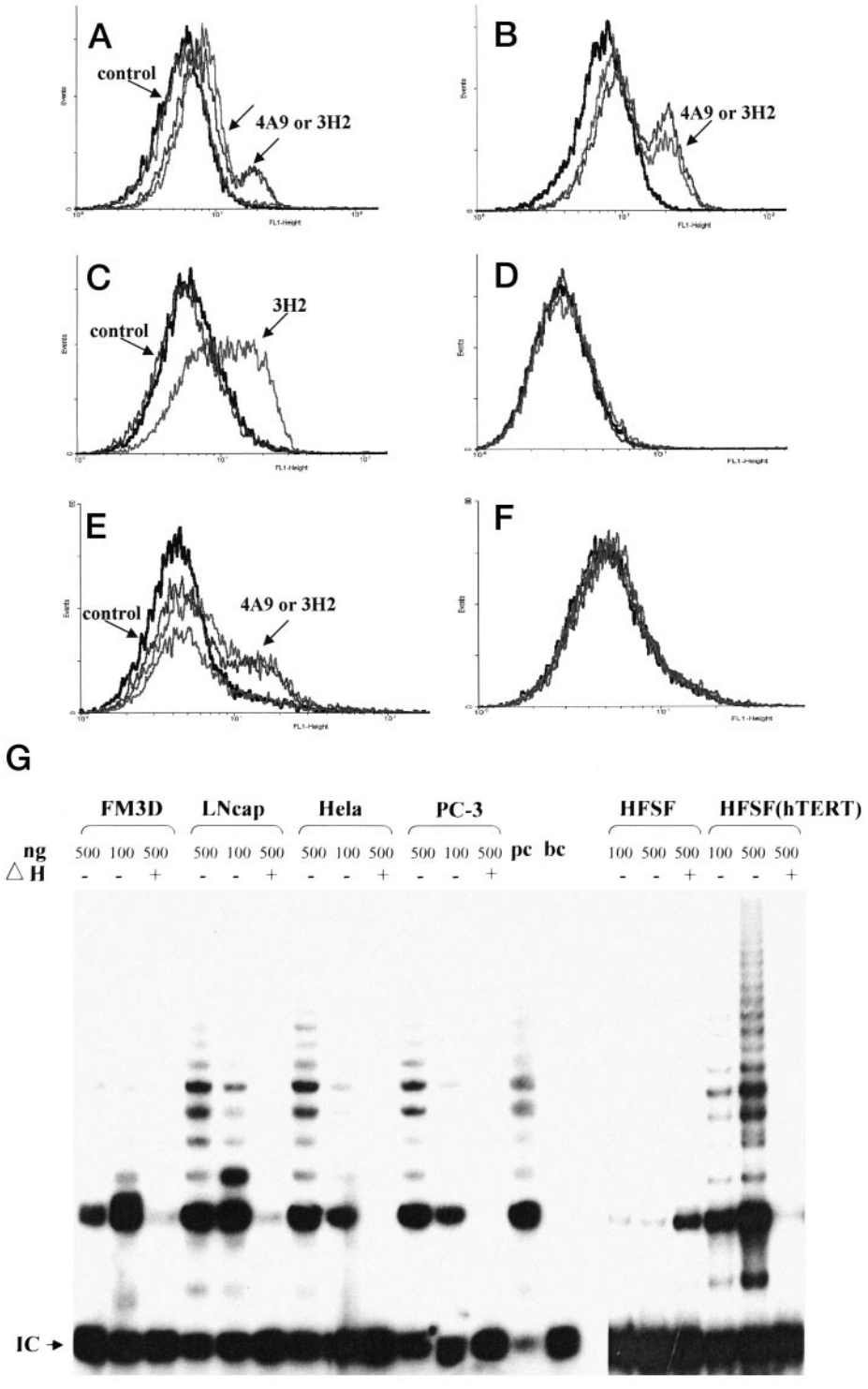


Fig. 9. Detection of HLA-A2/telomerase-derived peptide complexes on tumor cells. HLA-A2-positive FM3D melanoma, LnCap prostate carcinoma, HeLa epithelial carcinoma cells, or hTERT-transfected human foreskin fibroblasts and control nontransfected cells (10^6) expressing telomerase were incubated with Fab antibodies 4A9 and 3H2 specific for the HLA-A2/T540 and HLA-A2/T865 complexes, respectively. Binding was detected using FITC-labeled antihuman Fab. The HLA-A2-negative but hTERT-positive prostate carcinoma PC3 cells are used as control. *A*, FM3D cells stained with 4A9, 3H2, and control Fab directed against a mucin peptide in complex with HLA-A2. Cells stained with secondary FITC-labeled antihuman Fab were used as controls. *B*, LnCap cells stained with 4A9 or 3H2. *C*, HeLa cells stained with 3H2 or control Fab directed to a melanoma gp100-derived peptide in complex with HLA-A2. *D*, PC3 cells stained with 4A9 or 3H2. *E*, hTERT-transfected human fibroblasts stained with 4A9, 3H2, or a control melanoma-specific Fab. *F*, control nontransfected fibroblasts stained with 4A9, 3H2, or control Fab. *G*, telomerase activity assay. TRAP assay was performed using cell extracts, buffer control (*bc*), and of telomerase-positive cells (*pc*). The results shown were obtained using 100 or 500 ng of each extract with and without heat inactivation (15 min at 85°C). A 36-bp internal control for amplification efficiency and quantitative analysis was run for each reaction, as indicated by the *arrowhead*. The reaction products were separated on 10% nondenaturing polyacrylamide gel.

mediate efficient lysis of a variety of HLA-A2+ cancer cells such as prostate, breast, colon, lung, and melanoma is unprecedented (31, 32). Thus, we think that these cancer cells are equally effective in processing and presenting the same endogenous hTERT peptides. Therefore, similar hTERT peptides are expressed and complexed with MHC class I molecules on a variety of cancer cells of different histological origins and types. This suggests that hTERT represents the most widely expressed TAA described thus far and renders telomerase-expressing tumor cells susceptible to destruction by CTLs. Furthermore, this underscores the potential advantages that hTERT may have in controlling primary tumors and metastases in a large variety of cancer types in humans. Thus, hTERT-derived MHC-peptide complexes may turn out to be a very attractive target for cancer immunotherapy.

Our study demonstrates the power of the phage display approach for selecting antibodies with unusual and unique fine specificity. Until now, antibodies with TCR-like specificity have been generated against murine MHC-peptide complexes using various strategies of immunization (10–13, 15, 17, 18, 20). By using the same phage-displayed Fab library, a recombinant Fab antibody was isolated that

recognizes the melanoma antigen MAGE-A1 in complex with the human HLA-A1 MHC molecule. The affinity of this antibody was quite low (250 nM); therefore, it could be used to detect HLA-A1-MAGE-A1 complexes only when displayed in multiple copies on a phage (20). The fact that high-affinity antibodies with such unique fine specificity targeting a rather difficult antigen were readily obtained in this study and that they were in some cases with low nanomolar affinity underscores the power of the display technology for this application, as well as add proof to the quality of the human nonimmune antibody library used in the selections. The observation that 20–40% of the MHC-peptide binding antibodies had the fine specificity of a TCR-like molecule is nevertheless surprising, especially because they were selected from a nonimmune repertoire considered not to be biased toward such specificity. More recently, we have been able to isolate recombinant Fab antibodies against a large variety of MHC-peptide complexes containing other cancer-associated or viral HLA-A2-restricted peptides,[5] indicating that this behavior is not telomerase peptides related. The unexpected high frequency of these antibodies and our ability to isolate several different antibodies directed to either complex is even more surprising in view of previous reports in which the use of immunized or naive phage libraries resulted in only a single antibody clone (10, 11, 20).

It would have been possible that one particular antibody family or antibody V-gene segment would have an intrinsic propensity to bind HLA-A2 molecules, and that the high frequency could be explained by a high abundance of such antibodies in the nonimmune library. However, the sequences of the selected clones are derived from many different antibody families and germ-line segments, without any biases visible in the CDRs either (data not shown). The high frequency and high affinities for some of the antibodies isolated here suggest that these molecules may well be present at a high frequency in the antibody repertoires from the B-cell donors of the phage library, but a role for such antibodies remains unclear.

Recombinant antibodies with TCR-like specificity, such as we have selected and characterized herein, also represent an innovative and valuable tool in molecular immunology. These antibodies may now be used to detect and visualize the presence of specific MHC-restricted T-cell epitopes by standard methods of flow cytometry and possibly immunohistochemistry. As such they should be very useful for the study and analysis of antigen presentation on tumor cells by determining the expression of specific tumor-related MHC-peptide complexes on the surface of tumor cells, metastases, APCs, and lymphoid cells. These antibodies can be used to analyze immunotherapy-based approaches by determining the alterations in MHC-peptide complex expression on APCs before, during, and after vaccination protocols with peptides or with APCs loaded with tumor cell extracts or dendritic-tumor cell hybrid vaccinations (6–8). For immunotherapeutic applications, this approach presents new opportunities for using these specific molecules, which recognize very specific and unique human tumor antigens as candidates to serve as targeting moieties for antibody-based immunotherapies. Such approaches could include the construction of recombinant immunotoxins (41), fusion with cytokine molecules (42) or bispecific antibody therapy (43). This is particularly important for the molecules described herein because they target T-cell epitopes of the hTERT, which as noted above, represents a very widely expressed TAA displayed on cancer cell types of widely various cellular origins.

These antibodies also represent a valuable tool for structural and functional studies of TCR-peptide-MHC interactions. As shown previously for a murine system, TCR-like antibodies were used to define fine specificities of TCR interactions (44). A striking similarity between the specificity of the T cells and that of the murine TCR-like antibody was found, and most of the peptide residues, which could be recognized by the T cells, could also be recognized by the antibody.

The open questions with respect to immunotherapy and indeed many research applications relate to the expected low density (and turnover rate) of these specific epitopes on the target cell surface and the specificity of the antibody. With regard to the density, we have shown previously in a murine model that to achieve efficient killing with a TCR-like immunotoxin molecule, a density of several thousand specific MHC-peptide complexes is required for selective elimination of APCs (19).

It remains to be determined what the density of the telomerase complexes on the cancer cells tested is. The other important issue to consider is the fine specificity of the antibody. The antibodies characterized in this study were specific for their particular peptide in the HLA-A2 context, in two tests, ELISA and flow cytometry, with a panel of 30 other unrelated peptides tested as controls. It is clear from structural studies with MHC-peptide specific antibodies that related peptides with one or a few mutations in the peptide may also be recognized. It therefore remains to be seen that the specificity of the antibodies will be in the context of a true natural repertoire of peptides displayed in the MHC. New data on the use of such antibodies for retargeting T cells to tumor cells are highly encouraging in this respect. A recent study with Fab G8, an antibody that targets the HLA-A1 complexed to MAGE-A1 (20), shows that expression of the Fab genes on the surface of transfected primary human T lymphocytes retargets these cells specifically to MAGE-A1-expressing tumor cells and in a manner indistinguishable from a T-cell receptor with similar specificity (45).

To improve the targeting capabilities of these TCR-like antibody molecules two antibody engineering approaches can be used: (*a*) increasing the affinity of the parental antibody by affinity maturation strategies without alteration of its TCR-like fine specificity (46); and (*b*) increasing the avidity of these recombinant monovalent molecules by rendering them bi- or multivalent. Indeed, we have been able recently to improve the affinity of the G8 mentioned above (20) with a factor of 18, to 14 nM, without affecting the peptide fine-specificity of the antibody.[6] The combination of these affinity maturation strategies and avidity engineering may well result in second-generation, improved antibodies that can recognize levels of MHC-peptide complexes with sufficient sensitivity for their eventual immunotherapeutic use.

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