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Selective Targeting of Melanoma and APCs Using a Recombinant Antibody with TCR-Like Specificity Directed Toward a Melanoma Differentiation Antigen

Galit Denkberg,* Avital Lev,* Lea Eisenbach,† Itai Benhar,‡ and Yoram Reiter2*†

Tumor-associated, MHC-restricted peptides, recognized by tumor-specific CD8+ lymphocytes, are desirable targets for novel approaches in immunotherapy because of their highly restricted fine specificity. Abs that recognize these tumor-associated MHC-peptide complexes, with the same specificity as TCR, would therefore be valuable reagents for studying Ag presentation by tumor cells, for visualizing MHC-peptide complexes on cells, and eventually for developing new targeting agents for cancer immunotherapy. To generate molecules with such a unique, fine specificity, we immunized HLA-A2 transgenic mice with a single-chain HLA-A2, complexed with a common antigenic T cell HLA-A2-restricted epitope derived from the melanoma differentiation Ag gp100. Using a phage display approach, we isolated a recombinant scFv Ab that exhibits a characteristic TCR-like binding specificity, yet, unlike TCRs, it did so with a high affinity in the nanomolar range. The TCR-like Ab can recognize the native MHC-peptide complex expressed on the surface of APCs, and on peptide-pulsed or native melanoma cells. Moreover, when fused to a very potent cytotoxic effector molecule in the form of a truncated bacterial toxin, it was able to specifically kill APCs in a peptide-dependent manner. These results demonstrate the utility of high affinity TCR-like scFv recombinant Abs directed toward human cancer T cell epitopes. Such TCR-like Abs may prove to be very useful for monitoring and visualizing the expression of specific MHC-peptide complexes on the surface of tumor cells, APCs, and lymphoid tissues, as well as for developing a new family of targeting agents for immunotherapy. The Journal of Immunology, 2003, 171: 2197–2207.

The expression of specific peptides in a complex with MHC class I molecules on cells was shown to be associated with cancer and autoimmune disorders (1–3). In cancer, the discovery of these peptides emerged from the now well-established observation that human tumor cells often express Ags that are recognized by CTLs derived from patients (1–5).

Moreover, it has been demonstrated that the immune response against the tumor is insufficient to cause tumor regression and that tumor cells can develop effective mechanisms to escape such an immune attack (6–9). Therefore, numerous approaches are being developed in the field of tumor vaccination in an attempt to augment the antitumor immune responses, including cancer peptide vaccines, autologous cancer vaccines, and the cancer-dendritic cell hybrid vaccine (7, 10, 11). Because the specificity of the immune response is regulated and dictated by these class I MHC-peptide complexes, it should be possible to use these very specific and unique molecular cell surface markers as targets to eliminate the cancer cells, while sparing the normal cells. Thus, it would be very desirable to devise new molecules in a soluble form that will mimic the fine, unique specificity of the TCR to the cancer-associated MHC-peptide complexes. One promising approach is to generate recombinant Abs that will bind the MHC-peptide complex expressed on the cancer cell surface with the same specificity as the TCR. These unique Abs can subsequently be armed with an effector cytotoxic moiety such as a radioisotope, a cytotoxic drug, or a toxin. For example, Abs that target cancer cells are genetically fused to powerful toxins originating from both plants and bacteria, thus generating molecules termed recombinant immunotoxins (12).

Abs with the MHC-restricted specificity of T cells are rare and have been difficult to generate by conventional hybridoma techniques because B cells are not educated to be self-MHC restricted (13–16). The advantages of Ab phage-displayed technology make it possible to also select large Ab repertoires for unique and rare Abs against very defined epitopes. This has been demonstrated by the ability to isolate by phage display a TCR-like restricted Ab to a murine class I MHC H-2Kb complexed with a viral epitope (17), and more recently against the melanoma Ag MAGE-A1 in a complex with HLA-A1; however, this Ab exhibited a low affinity and could be used to detect the specific complexes on the surface of APCs only when expressed in a multimeric form on a phage and not as a soluble Ab (18).

In this work, we have immunized HLA-A2 transgenic mice with a single-chain HLA-A2 (scHLA-A2)3 molecule complexed with a peptide derived from the melanoma differentiation Ag gp100 (19–22). Using phage display, we isolated from the immune murine Ab library repertoire a soluble high affinity recombinant single-chain Ab with the Ag-specific MHC-restricted specificity of T cells directed toward a gp100-derived cancer epitope in complex with HLA-A2.

The isolated Ab binds with high affinity, but TCR-like specificity to the recombinant gp100-derived MHC-peptide complex and can detect the specific HLA-A2-peptide complex on the surface of...
APCs as well as melanoma cells. The Ab was used to directly visualize in situ the specific gp100-derived epitope on intact melanoma cells by immunohistochemistry. Moreover, we have fused the Ab gene to a truncated form of Pseudomonas exotoxin A (12) to form a recombinant immunotoxin and tested its ability to specifically target APCs. In this study, we show, for the first time, the ability of such a soluble fusion protein to kill, in a TCR-like restricted manner, those target cells that express a particular human cancer-associated MHC-peptide complex.

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 previously (23, 24). Biotinylation was performed using the BirA enzyme (Avidity, Denver, CO), as previously described (25).

Mice immunization

We used the D-/- × β2-microglobulin (β2m) null mice transgenic for a hHLA-A2/1D/β2m single chain (HHD) mice (26) to immobilize with an emulsion containing purified protein-derived peptide of tuberculin (PPD) covalently coupled with HLA-A2/G9-209 complexes, as described previously (17). Briefly, mice were initially immunized subdermally and subsequently s.c. for 2–3 wk for a period of 3–5 mo with 20–30 μg/mice of the antigenic mixture in IFA. Spleens were collected 2 wk after the last immunization.

Library construction and selection of phage Abs on biotinylated complexes

Total RNA was isolated from immunized mice, and an Ab scFv library was constructed by RT-PCR from the mRNA, as described (27). The scFv repertoire was cloned as an 8kl-NotI fragment into the pCANTAB5e or pCC-CBD phagemid vectors (28). The complexity of both libraries was 1 × 10^10 independent clones. For panning, biotinylated scHLA-A2/G9-209 M complexes (29) were incubated with streptavidin-conjugated magnetic beads (2 × 10^8), washed, and incubated with 10^11 CFU of the libraries (1 h, room temperature (RT)). Starting with the second round, panning was performed in the presence of an excess (5 μg) of scHLA-A2/G9-209V complexes. Beads were washed extensively 10–12 times with 2% skimmed milk in PBS + 0.1% Tween. Bound phages were eluted by using 1 ml of triethylamine (100 mM, pH 12) for 5 min at RT, followed by neutralization with 0.1 ml of 1 M Tris-HCl, pH 7.4. Eluted phages were expanded in exponentially growing E. coli TG1 cells that were later superinfected with M13KO7 helper phage, as described (28).

Expression and purification of soluble recombinant scFv and scFv-P38 fusion protein

The G1 scFv gene was rescued from the phage clone by PCR and was subcloned into the phagemid vector pCANTAB6 by using the 8kl-NotI cloning sites. A Myc and hexahistidine tags were fused to the C terminus of the scFv gene. The scFv Ab was expressed in BL21 HI9261 cells by using the phagemid vector pCANTAB6 by using the 8kl-NotI cloning sites. A Myc and hexahistidine tags were fused to the C terminus of the scFv gene. The scFv Ab was expressed in BL21 HI9261 cells, as previously described (29), and purified from the periplasmic fraction by metal-ion affinity chromatography. For the expression of the G1scFv-P38 fusion protein, the scFv gene was subcloned as an NcoI-NotI fragment into the plasmid pBB-NN, which encodes the translocation and ADP-riboseylation domains of PE38 (30). Expression in BL21 HI9263 cells, refolding from inclusion bodies, and purification of G1scFv-P38 were performed, as previously described (30).

ELISA with phage clones and purified scFv or scFv-P38

Binding specificity studies were performed by ELISA using biotinylated scMHC-peptide complexes. Briefly, ELISA plates (Falcon) were coated overnight with BSA biotin (1 μg/well), washed, incubated (1 h, RT) with streptavidin (1 μg/well), again washed extensively, and further incubated (1 h, RT) with 0.5 μg of MHC/peptide complexes. Plates were blocked with PBS/2% milk (30 min, RT), incubated with phage clones (~10^8 phag-es/well, 1 h, RT) or 0.5–1 μg of soluble scFv or scFv-P38, and afterward washed with 1:1000 HRP-conjugated/anti-M13, anti-myc Ab, or anti-PE Ab, respectively. The HLA-A2-restricted peptides used for specificity studies are gp100 (154), KTWGQIQWQP, gp100 (209), IMDQVPFSV, gp100 (280), YLEPGPGVT-MUC1, LLLTVLTLV, HTLV-1 (TAX), LLFQYPVVYY, ITERT (540), IILKFLHWL, ITERT (865), RLVDDEFILV.

Flow cytometry

The B cell line RMAS-HHD transfected with a single-chain β2m-HLA-A2 gene (26) or the EBV-transformed B-lymphoblast JY cells (106 cells) were washed twice with serum-free RPMI and incubated overnight at 26°C or 37°C, respectively, in medium containing 100 μM of the peptide. The APCs were subsequently incubated at 37°C for 2–3 h to stabilize cell surface expression of MHC-peptide complexes, followed by incubation with recombinant scFv (10–50 μg/ml, 60–90 min, 4°C) in 100 μl. The cells were then washed, incubated with FITC-labeled anti-Myc Ab (30–45 min, 4°C), and finally washed and analyzed by a FACStar flow cytometer (BD Biosciences, San Jose, CA).

Melanoma cells were pulsed at 37°C with 1–10 μM of peptide and then stained with the scFv, as described above.

Competition-binding assays

Flexible ELISA plates were coated with BSA biotin, and scMHC peptide complexes (10 μg in 100 μl) were immobilized, as previously described. The recombinant G1scFv-P38 was labeled with 125I using the Bolton-Hunter reagent. Labeled protein was added to wells as a tracer (3–5 × 10^3 cpm/well) in the presence of increasing concentrations of the cold G1scFv-P38 as a competitor and incubated at RT for h in PBS. The plates were washed thoroughly with PBS, and the bound radioactivity was determined by a gamma counter. The apparent affinity of the G1scFv-P38 was determined by extrapolating the concentration of a competitor necessary to achieve 50% inhibition of 125I-labeled G1scFv-P38 binding to the immobilized scMHC-peptide complex. Nonpecific binding was determined by adding a 20- to 40-fold excess of unlabeled Fab.

Immunohistochemistry

Melanoma and control cells were incubated with 20–30 μg G1 scFv for 1 h at room temperature in RPMI containing 10% FCS, followed by incubation with HRP-anti-Myc Ab (45 min, on ice). The cell suspension was applied onto glass slides precoated with 0.1% poly(l-lysine) (Sigma-Aldrich, St. Louis, MO). Cells were then incubated for 1 h at room temperature. Slides were washed three times with PBS, and incubated with a diaminobenzidine + solution (Dako, Glostrup, Denmark) for 1 min, followed by washing with PBS to remove excess of staining reagent. Cell nuclei were stained with hematoxylin (Sigma-Aldrich).
control scHLA-A2-peptide complexes were >95% pure, homogenous, and monomeric, as shown by analysis on SDS-PAGE and gel filtration chromatography (data not shown). The G9-209 M-containing scHLA-A2 complexes have been previously shown to be functional, by their ability to stimulate specific CTL lines and clones and stain G9-209 M-specific T cells in the form of tetramers (23, 24).

Construction of an Ab scFv phage library and selection of a phage that binds HLA-A2/G9-209 M complexes with TCR-like specificity

For immunization purposes, we coupled PPD to the purified complex and immunized the D6/¼ × β2m null mice transgenic for a rHLA-A2.1/D6-β2m single chain (HHD mice) (26). These mice combine classical HLA transgenesis with selective destruction of murine class I H-2. Hence, unlike the classical HLA transgenics, these mice showed only HLA-A2.1-restricted responses with multiple epitopes such as intact viruses. Moreover, we presume that these mice are a useful tool for immunization with HLA-A2-peptide complexes because they should be largely tolerant to HLA-A2 as a B cell immunogen, and thus may favor the generation of an Ab response directed against the MHC-restricted epitope when in complex with HLA-A2 (the specific tumor-associated peptide). PPD was used for conjugation because it is a highly reactive T cell immunogen (17).

Total spleen mRNA was isolated from immunized mice and reverse transcribed to cDNA. Specific sets of degenerated primers were used to PCR amplify the cDNA segments corresponding to the Ig H and L chain V domains. The VH and VL PCR pools were assembled into a scFv repertoire by a PCR overlap extension reaction and subsequently cloned into the pCANTAB5E phagemid vector or to the phagemid vector pCC-GalF (Fv) in which the scFv is expressed as an in-frame fusion protein with a cellulose-binding domain (CBD) (28). The resulting libraries were transduced into E. coli TG1 cells by electroporation and expressed as fusion with the minor phage coat protein pIII after rescue with a helper phage. The library complexity consisted of 1 × 108 independent clones using both types of vectors.

The library was subjected to three to four panning cycles, followed by elution of bound phages and reamplification in E. coli. To enhance the efficiency of our selection, we used biotinylated scMHC-peptide complexes. A BirA sequence tag for site-specific biotinylation was engineered at the C terminus of the HLA-A2 gene, as previously described (25). Several selection strategies were used, the most successful of which resulted in the isolation of specific binders consisting of panning protocols with a negative depletion step starting from the second round of panning. The specific HLA-A2/G9-209 M-biotinylated complexes were immobilized onto streptavidin-coated magnetic beads, and the library was incubated with the immobilized complex in the presence of a large excess of HLA-2 complexes that displayed a different gp100-derived epitope, the G9-280V peptide. When this strategy is used, G9-209 M-specific phage will bind to streptavidin-biotin-immobilized complexes that are captured by a magnetic force, whereas pan-MHC binders that are not specific to the G9-209 M peptide in the complex will bind to the nonspecific complex in the solution, and thus can be separated and removed from the specific phage. As shown in Table I, a progressive, marked enrichment for phage that binds the immobilized complexes was observed after three to four rounds of panning, two of which were performed with the negative depletion strategy. Polyclonal phage ELISA was performed to determine phage specificity on biotinylated recombinant scMHC-peptide complexes immobilized to BSA-biotin-streptavidin-coated immunoplates. The BSA-biotin-streptavidin spacer enables the correct presentation and folding of the complexes, which can be distorted by direct binding to plastic. Phage analyzed already after the second and, more dramatically, after the third round of panning revealed a unique specificity pattern only directed toward the specific G9-209 M-containing HLA-A2 complexes (Fig. 1, A and B).

Table I. Phage selection on scHLA-A2/G9-209 M complexes

<table>
<thead>
<tr>
<th>Library</th>
<th>Cycle</th>
<th>Input</th>
<th>Output</th>
<th>Enrichment</th>
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<tr>
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<td>3</td>
<td>5 × 10^11</td>
<td>1 × 10^6</td>
<td>10,000</td>
</tr>
<tr>
<td>scFv-CBD</td>
<td>1</td>
<td>5 × 10^9</td>
<td>1 × 10^6</td>
<td>10</td>
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A fourth round of selection resulted with similar enrichments, as observed in round 3.

Individual monoclonal phage clones were isolated from the population of phages from the last round of panning (no further enrichment observed after a fourth round) and rescreened for specificity by phage ELISA (Fig. 1, C and D). Of the 93 clones tested, 85 (91%) reacted with the HLA-A2/G9-209 M complex (Fig. 1C). A total of 77 of the 85 reactive clones (90%) reacted specifically with the specific HLA-A2/G9-209 M complex, but not with the control G9-280V-containing complex (Fig. 1D). Only a small percentage of the clones (5 of 93; 5%) did not exhibit peptide specificity (Fig. 1C). Thus, the panning procedure yielded a successful enrichment of phage Abs with TCR-like specificity toward the HLA-A2/G9-209 M complex. Fingerprint analysis by means of multicutter restriction enzyme digestion revealed that 50 positive, HLA-A2/G9-209 M-specific clones had a similar digestion pattern, indicating that all are similar (data not shown). Similar results were obtained with the two libraries. Because they were constructed from the same genetic material (the same pool of mRNA), we further characterized phage clones derived from the pCANTAB5E scFv library.

DNA sequencing of VH and VL V domains from 10 clones revealed that all were identical (data not shown), suggesting that they were all derived from a single productive Ab VH/VL combinatorial event.

Characterization of the soluble recombinant scFv Ab with TCR-like specificity

DNA sequencing revealed that the Ab VH sequence belongs to the mouse H chains subgroup III (D), and the VL sequence to mouse κ L chains group IV (according to Kabbat). The nucleotide sequence and deduced amino acid sequence were submitted to GenBank, accession number bankit533067. To further characterize the binding specificity and the biological properties of the selected scFv Ab, termed G1, we used two expression systems: for the first, the scFv was subcloned into the phagemid vector pCANTAB6 in which a Myc and a hexahistidine tag are fused to the C terminus of the scFv gene; the second was a T7 promotor-driven expression system in which the scFv gene is fused to a truncated form of Pseudomonas exotoxin A (PE38) to generate a scFv immunotoxin (12). This truncated form of PE contains the translocation and ADP-ribosylation domains of whole PE, but lacks the cell-binding domain, which is replaced by the scFv fragment fused at the N terminus of the truncated toxin. The G1 scFv was produced in E.

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coli BL21 (ADE3) cells by secretion and was purified from periplasmic fractions by metal affinity chromatography using the hexahistidine tag fused to the C terminus (Fig. 2B). The G1 scFv-PE38 was expressed in BL21 cells, and, upon induction with isopropyl-β-D-thiogalactoside, large amounts of recombinant protein accumulated as intracellular inclusion bodies. SDS-PAGE showed that inclusion bodies from cultures expressing G1 scFv-PE38 contained >90% recombinant protein (data not shown). Using established renaturation protocols, we succeeded in refolding G1 scFv-PE38 from solubilized inclusion bodies in a redox-shuffling refolding buffer and purified it by ion-exchange chromatography on Q-Sepharose and MonoQ columns, and later by size-exclusion chromatography. A highly purified G1 scFv immunotoxin with the expected size of 63 kDa was obtained, as analyzed by SDS-PAGE under nonreducing conditions (Fig. 2A). The molecular profile of the G1 scFv and G1 scFv immunotoxin was analyzed by size-exclusion chromatography and revealed a single protein peak in a monomeric form with an expected molecular mass of 26 and 63 kDa, respectively (data not shown). The yield of the refolded G1 scFv immunotoxin was ~2%; thus, 2 mg of highly pure protein could be routinely obtained from the refolding of 100 mg of protein derived from inclusion bodies containing 80–90% of recombinant protein. This yield is similar to previously reported scFv immunotoxins that expressed well and were produced using a similar expression and refolding system (30). The yield of the G1 scFv was 3 mg from a 1-L bacterial culture.

The binding specificity of the soluble purified G1 scFv Ab and G1 scFv-PE38 fusion protein was determined by ELISA on biotinylated MHC-peptide complexes immobilized to wells through BSA-biotin-streptavidin to ensure correct folding of the complexes. The correct folding of the bound complexes and their stability during the binding assays were determined by their ability to react with the conformational, specific mAb w6/32, which binds HLA complexes only when folded correctly and when it contains peptide (data not shown). When we used the soluble purified G1 scFv or G1 scFv-PE38 protein, the ELISA revealed a very specific recognition pattern corresponding to the hallmarks of MHC-restricted T cell specificity (Fig. 2C). The G1 scFv selected to bind the G9-209 M-containing HLA-A2 complex reacted only with the specific complex and not with complexes displaying the G9-280 and G9-154 gp100-derived MHC-peptide complexes nor to other control complexes containing HLA-A2-restricted telomerase-derived epitopes 540 and 865 (32), a MUC1-derived peptide (33), or the HTLV-1-derived TAX peptide (34) (Fig. 2C). The G1 scFv, which was selected on HLA-A2 complexes containing the G9-209 M-modified peptide, in which position 2 of the peptide was altered to methionine to improve its binding affinity to the MHC, also recognized the native peptide G9-209 in complex with HLA-A2
In these assays, the binding was detected with an anti-PE38 Ab. Similar results were obtained when using the unfused G1 scFv Ab in which detection was performed with anti-Myc tag Ab (data not shown). Thus, this Ag-specific scFv fragment exhibits binding characteristics and the fine specificity of a TCR-like molecule. The G1 scFv or G1 scFv-PE38 did not recognize the peptide alone nor empty HLA-A2 molecules (which are difficult to produce because they are unstable in the absence of a peptide), neither streptavidin nor other protein Ags (data not shown).

Next, the binding properties of the TCR-like soluble purified G1 scFv-PE38 were determined using a saturation ELISA in which biotinylated complexes were bound to BSA-biotin-streptavidin-coated plates to which increasing amounts of G1 scFv-PE38 were added. The binding of G1 scFv or G1 scFv-PE38 to the specific gp100-derived HLA-A2/G9-209 M complex was dose dependent and saturable (Fig. 3A). Extrapolating the 50% binding signal revealed that this Ab possessed high affinity, with a binding affinity in the nanomolar range. To determine the apparent binding affinity of the TCR-like scFv fragments to its cognate MHC-peptide complex, we performed a competition-binding assay in which the binding of 125I-labeled G1 scFv-PE38 was competed with increasing concentrations of unlabeled protein. These binding studies revealed an apparent binding affinity in the low nanomolar range of 5 nM (Fig. 3B). Importantly, these results underscore our success in isolating a high affinity scFv Ab with TCR-like specificity from the phage-displayed Ab repertoire of immunized HLA-A2 transgenic mice.

**Binding of G1 scFv to APCs displaying the gp100-derived epitope**

To demonstrate that the isolated soluble G1 scFv can bind the specific MHC-peptide complex, not only in its recombinant soluble form, but also in the native form, as expressed on the cell surface, we used two APC systems. One consists of the murine TAP2-deficient RMA-S cells that were transfected with the human HLA-A2 gene in a single-chain format (26) (HLA-A2.1/D3β2, single chain) (RMA-S-HHD cells). The gp100-derived peptide and control peptides were loaded on the RMA-S-HHD cells, and the ability of the selected G1 scFv Ab to bind to peptide-loaded cells was monitored by FACS (Fig. 4). Peptide-induced MHC stabilization of the TAP2 mutant RMA-S-HHD cells was determined by analyzing the reactivity of the conformational anti-HLA Ab w6/32 and the anti-HLA-A2 mAb BB7.2 with peptide-loaded and unloaded cells (Fig. 4, A and B). The G1 scFv, which recognized the G9-209 M-containing HLA-A2 complex, reacted only with RMA-S-HHD cells loaded with the G9-209 M peptide, but not with cells loaded with the G9-280 peptide (Fig. 4C) or control cells not loaded with peptide. The G1 scFv did not bind to cells loaded with other HLA-A2-restricted control peptides such as TAX, MUC1, or telomerase-derived peptides used for the specificity analysis (see Fig. 2C).

We also used a second type of APC, namely the EBV-transformed B lymphoblast JY cells, which express HLA-A2; these cells were incubated with the gp100-derived or control peptides. They are TAP−/H11001−, and consequently, the displaying of the exogenously supplied peptide is facilitated by peptide exchange. Using this strategy, we observed a similar binding specificity with the G1 scFv Ab (data not shown). These results demonstrate that the scFv Ab can specifically recognize its corresponding native HLA-A2 complex on the surface of cells.

**Detection of complexes formed by active intracellular processing**

To examine the ability of the TCR-like G1 scFv to detect HLA-A2/G9-209 complexes produced by physiological Ag processing, we transfected the gp100 gene into the EBV-transformed B cell
HLA-A2-positive Ag-presenting JY cells and into HLA-A2-negative APD cells as controls. Twenty-four hours after transfection, we tested the reactivity of the HLA-A2/G9-209-specific Ab G1 scFv by flow cytometry. As shown in Fig. 5A, significant staining above control could be clearly seen only with HLA-A2-positive JY cells transfected with the g100 gene, but not with control non-transfected JY cells (Fig. 5B) or control HLA-A2-negative cells transfected or not transfected with the g100 gene (Fig. 5, C and D). The peptide-specific, MHC-restricted pattern of reactivity by G1 scFv is not due to differences in transfection efficiency or HLA expression of JY and APD cells because the percentage of transfected cells was similar, as determined in control experiments transfecting green fluorescence protein into these cells (Fig. 5E), and the staining intensity of these cells with w6/32, a pan-MHC mAb, was similar (data not shown). These results indicate that the TCR-like scFv G1 Ab is capable of detecting the specific MHC-peptide complex after active and naturally occurring endogenous intracellular processing.

**Binding of G1 scFv to melanoma cells**

To demonstrate that the G1 scFv can bind the specific MHC-peptide complex displayed on the surface of tumor cells, we used HLA-A2+ melanoma FM3D cells (Fig. 4D) that were pulsed with the specific gp100-derived G9-209 peptide or control peptide. Similarly to the JY APCs, pulsing the melanoma cells enables the display of the exogenously supplied peptide facilitated by peptide exchange. Using this strategy, we obtained a mixture of exog-
TCR-like Ab to detect specific peptide-MHC complexes in situ on cells and potentially in tissue sections after naturally occurring active intracellular processing. To our knowledge, this is the first demonstration of in situ detection of a tumor-derived T cell epitope using TCR-like scFv recombinant Abs.

**Cytotoxic activity of G₃scFv-PE38 toward APCs**

To determine the ability of the G₁ scFv Ab to serve as a targeting moiety for T cell-like specific elimination of APCs, we constructed, as described, the G₃scFv-PE38 molecule in which the very potent truncated form of *Pseudomonas* exotoxin A is fused to the C terminus of the scFv gene and tested its ability to kill peptide-loaded APCs. RMSA-HHD or JY cells were loaded with the gp100-derived epitopes G9-209 M and G9-280V as well as with other control HLA-A2-restricted peptides. FACS analysis with anti-HLA-A2 Ab revealed a similar expression pattern of HLA-A2 molecules with G9-209 M, G9-280V, and other control peptide-loaded cells (Fig. 4B). As shown in Fig. 4A, cytotoxicity by G₃scFv-PE38 was observed only on RMSA-HHD cells loaded with the G9-209 peptide with an IC₅₀ of 10–20 ng/ml. No cytotoxic activity was observed on RMSA-HHD cells that were loaded with the gp100-derived G9-280V epitope or with other control HLA-A2-restricted peptides or cells that were not loaded with peptide. G9-209 M-loaded RMSA-HHD cells were not killed with an irrelevant immunotoxin in which an anti-human Lewis Y scFv Ab is fused to PE38 (B3(Fv)-PE38) (Fig. 4A). In the EBV-transformed JY cells, which express normal TAP, the display of the exogenously supplied peptide is facilitated by peptide exchange. Using this strategy, we observed similar sp. act. in which G₁scFv-PE38 kills only cells loaded with the G9-209 M peptide (Fig. 4B). Additional proof for specificity is demonstrated in competition experiments in which excess specific and control soluble scHLA-A2-peptide complex was present in solution, to compete for binding and inhibit cytotoxicity by G₁scFv-PE38. An example of this type of assay is shown in Fig. 4C, in which excess soluble G9-209 M-containing HLA-A2, but not the G9-280V/HLA-A2 complex competed and inhibited the cytotoxic activity of G₁scFv-PE38 toward G9-209 M-loaded JY cells.

Next, we attempted to test the activity of G₁scFv-PE38 on melanoma cells that are HLA-A2 positive and express gp100. As shown in Fig. 8D, G₁scFv-PE38 exhibited specific cytotoxicity on gp100-expressing FM3-29, FM3-D, and HA141 melanoma cells, but not on the HLA-A2-negative, gp100-positive G43 cells or on HLA-A2-positive breast carcinoma MDB-MB-231 cells. A low degree of activity was also observed on ZA (HLA-A2/gp100⁻) melanoma cells (Fig. 8D). These results further demonstrate the fine and unique specificity of the G₁ scFv Ab and its ability to serve as a targeting moiety to deliver a cytotoxic effector molecule with Ag (peptide)-specific, MHC-restricted specificity of T cells directed toward a human tumor T cell epitope.

**Discussion**

In recent years, the advent of the application of recombinant class I MHC-peptide complexes and their tetrameric arrays now enables us to detect and study rare populations of Ag-specific T cells (25, 35, 36). However, fundamental questions in immunology in general, and in tumor immunology in particular, regarding Ag presentation are still open because of the lack of reagents that will enable phenotypic analysis of Ag (MHC-peptide) presentation, the other side of the coin to MHC-peptide-TCR interactions. One way to generate such reagents is by making TCR-like Abs; however, only a few publications have reported the generation of self MHC-restricted Abs by conventional means such as the hybridoma technology (13–16). The major reason for these past difficulties may be...
found in the molecular nature and the resolved structures of MHC-peptide complexes. More specifically, the peptides are deeply buried inside the MHC-binding groove, and therefore they are presented as extended mosaics of peptide residues intermingled with the MHC residues. It has been shown that no more than 100–300 Å² of class I MHC-bound peptide faces outward and thus is available for direct recognition, whereas Abs recognizing protein molecules engage ~800 Å² of their ligand (17). Thus, when generating TCR-like Abs, these molecules will presumably recognize the peptide, but will also have to be dominated by the MHC.

Until now, Abs with TCR-like specificity have been generated against murine MHC-peptide complexes using various strategies of immunization (17). Recently, a large human Fab library was used to select for HLA-A1-MAGE-A1-specific binding Abs (18). One specific clone, G8, was selected that exhibited TCR-like specificity, but revealed a relatively low affinity of 250 nM.

In this study, we have demonstrated the ability to select from an immune repertoire of murine scFv fragments, a high affinity Ab directed toward a human T cell epitope derived from a cancer Ag, the melanoma-associated Ag gp100.

G₁ scFv exhibits a very specific and special binding pattern; it can bind in a peptide-specific manner to HLA-A2 complexes. Hence, this is a recombinant Ab with TCR-like specificity. In contrast to the inherent low affinity of TCRs, this molecule displays the high affinity binding characteristics of Abs, while retaining TCR specificity. Our study strikingly demonstrates the power of the phage display approach and its ability to select especially fine specificities from a large repertoire of different Abs.

Our ability to select high affinity TCR-like Abs, despite the fact that such peptide-specific binders are thought to be rare and difficult to isolate, may result from the following considerations. One is the mode of immunization and selection: the fact that we use a transgenic animal for immunization combined with the power of various selection strategies used by phage display. We think that using HLA-A2 transgenic mice is an advantage because they are usually tolerant to HLA-A2 complexes, unless a new foreign peptide is presented on the complex. The ability to isolate TCR-like Ab molecules may represent a situation in which lymphocytes that were tolerant to HLA-A2 are now exposed to new epitopes contributed by the melanoma gp100-derived peptide presented on HLA-A2. The panning procedure that combined an excess of non-specific complex in solution significantly contributed to the selection process and allowed us to isolate a rare Ab clone (1 of 10⁸ clones). Another important issue relates to the state of the Ag used in the selection process. The conformation of the Ag has to be as natural as possible, especially when produced in a recombinant form. We have found that in vitro refolding from inclusion bodies produced in E. coli of a scMHC molecule complexed with various peptides yields large amounts of correctly folded and functional protein. The fact that G₁ scFv was isolated from a relatively small library of ~10⁸ clones, yet is highly specific with an affinity in the nanomolar range, strongly indicates that the HLA-A2 transgenic mice we used for immunization indeed developed high affinity Abs to the HLA-A2/G9-209 complexes. The observation that only a single anti-HLA-A2/G9-209 Ab was isolated may reflect that only one such specificity exists or that other specificities were not generated during the immune response because such a response could not be easily generated and tolerated by the HLA-A2 transgenic mice. Quite astonishing is the fact that similar results were reported in the past for a murine MHC-peptide system, in which, using phage display, a recombinant TCR-like Ab directed toward a class I murine H-2Kk molecule in complex with the influenza hemagglutinin peptide HA 255–262 was isolated (17). Similarly to the results presented in this work, of the 50 clones tested, 7 reacted specifically with the H-2Kk/HA 255–262 complexes only, and not with other H-2Kk/peptide complexes. Interestingly, the DNA sequences of these specific clones were determined and found to be identical (17). Similar observations in two independent studies using different strains of mice and different class I MHC-peptide complexes indicate that these Abs are quite rare.

Despite the fact that these Abs are rare, this and our study demonstrate the power of the phage display approach, which can be applied in a generic form to isolate recombinant Abs with TCR-like specificity to a variety of MHC-peptide complexes related to various pathological conditions such as cancer, viral infections, and autoimmune diseases.
Recombinant Abs with TCR-like specificity represent a new, valuable tool for future research in two major areas of tumor immunology. First, these Abs may now be used to detect and directly visualize the presence of specific T cell epitopes or MHC-peptide complexes by standard methods of flow cytometry and immunohistochemistry. They should be very useful for the study and analysis of Ag presentation in cancer by determining the expression of specific tumor-related MHC-peptide complexes on the surface of tumor cells, metastasis, APCs, and lymphoid cells. Moreover, such Abs 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 (7–11). Thus, questions relating to how and where certain events occur during Ag presentation may be directly addressed, for the first time, and the expression of T cell epitopes on the APC may be visualized and quantitated. Second, Abs with such exquisitely fine specificity directed toward a very specific and unique human tumor Ag present new opportunities for use as targeting moieties for various Ab-based immunotherapeutic approaches. This includes using such Abs to construct recombinant immunotoxins (12), for fusion with cytokine molecules (37), or for bispecific Ab therapy (38). The open question with respect to these applications relates to the low density of the specific epitope on the surface of the target cell. It has been previously demonstrated, using the murine H-2Kk/influenza hemagglutinin peptide complex and a similar Ag-presenting system, as was used in this study, that to achieve efficient killing with a TCR-like immunotoxin molecule, a density of several thousand particular MHC-peptide complexes is required for the selective elimination of APCs (39). The results of this study support these findings, achieving a similar cytotoxic potential of a T cell-like immunotoxin.

Thus, a major application of TCR-like Abs is for the development of a new class of Ab-based tumor-targeting agents. In this
approach, small recombinant fragments of Abs are being used to specifically target a cytotoxic drug or a toxin to cancer cells. The Fv targeting moiety of the Ab is composed of the H and L chain V regions (VH and VL), which are held together by a peptide linker, thereby forming an scFv molecule. scFv immunotoxins are being developed against a wide variety of cancer-specific targets and are being tested in various clinical trials, some of which already present promising anticancer activity (12). scFvs and scFv immunotoxins have many advantages over conventional immunoconjugates in which the whole Ab is chemically conjugated to the toxin. 1) The former is produced directly in a single-chain form in bacteria, thereby avoiding complex chemical modification steps. 2) They can be produced in large quantities and are chemically homogenous as opposed to conventional immunoconjugates. 3) The most important advantage of scFvs and scFv immunotoxins is their small size, which allows them to penetrate into tumors more efficiently. ScFv fragments are also being developed as tumor-imaging agents. Further preclinical characterization is required to determine whether the G1scFv-PE38 molecule can be used to specifically eliminate melanoma cells that express the gp100-derived MHC-peptide complex. Our results indicate that recombinant Abs can be used to selectively target tumor cells that have been shown to express a particular MHC-peptide complex. Thus, the number of specific MHC-peptide complexes on the surface of human cancer cells could be sufficient to serve as a target for such a recombinant cytotoxic molecule. An obvious advantage arising from the use of these targets, such as gp100-derived epitopes, as an Ag for the specific targeting of drugs and toxins to tumor cells is the fact that gp100 is a differentiation Ag that is selectively expressed only on melanoma cells (or on normal melanocytes), but not on other essential tissues of a different histological origin. As such, these are considered very specific and unique targets for immunotherapy. To improve the targeting and detection capabilities of these TCR-like Ab molecules, two Ab engineering approaches can be used: 1) to increase
References


