Human CTLs to Wild-Type and Enhanced Epitopes of a Novel Prostate and Breast Tumor-Associated Protein, TARP, Lyse Human Breast Cancer Cells

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ABSTRACT

Vaccine therapy for prostate and breast cancer may have potential for treating these major causes of death in males and females, respectively. Critical to the development of tumor-specific vaccines is finding and characterizing novel antigens to be recognized by CD8+ T cells. To define new CD8+ T-cell tumor antigens, we determined two wild-type HLA-A2 epitopes from a recently found tumor-associated protein, TARP (T-cell receptor γ alternate reading frame protein), expressed in prostate and breast cancer cells. We were also able to engineer epitope-enhanced peptides by sequence modifications. Both wild-type and enhanced epitopes induced peptide-specific CD8+ T-cell responses in A2Kb transgenic mice. In vitro restimulation of human CD8+ T cells from a prostate cancer patient resulted in CD8+ T cells reactive to the peptide epitopes that could lyse HLA-A2+ human breast cancer cells (MCF-7) expressing TARP. Epitope-specific human CD8+ T cells were also enumerated in patients’ peripheral blood by tetramer staining. Our data suggest that HLA-A2-binding TARP epitopes and enhanced epitopes discovered in this study could be incorporated into a potential vaccine for both breast and prostate cancer.

INTRODUCTION

Up to 30% of 180,000 United States patients with potentially curable early-stage breast and prostate cancer will fail standard surgical or radiotherapy in 2004. In addition, patients with metastatic prostate cancer and the majority of patients with metastatic breast cancer enjoy limited benefit of standard chemotherapy and hormone-based therapies. Immunotherapy may have great potential to improve on these results, combining the tumor specificity of cell-mediated immunity with freedom from toxic chemotherapies.

Recent immunotherapy of cancer patients has built on the finding that CD8+ CTLs recognize and kill tumor cells displaying peptides from tumor-associated antigens presented by MHC class I molecules. Several tumor antigens and HLA allele-specific peptides from prostate cancer-associated antigens have been identified as CD8+ T-cell epitopes, including HLA-A2-binding peptides derived from prostate-specific antigen (1), prostate-specific membrane antigen (2), prostate stem cell antigen (3), and prostate acid phosphatase (4), which are all now components of current vaccine trials (5–8).

Finding additional, more tumor-specific antigens, however, will be critical to the success of effective immunotherapy. In this regard, several new genes specifically expressed in human prostate and breast cancer have been identified by analysis of the expressed sequence tag database (9–11). One of these, TARP [T-cell receptor (TCR) γ alternate reading frame protein], is expressed in both prostate and breast cancer cells (12, 13). TARP was originally discovered because there were unusually large numbers of expressed sequence tags in prostate cancer samples that contained TCRγ sequences but not TCRδ sequences as might be expected if the prostate cancer samples used to make the expressed sequence tags contained many TCRδ T cells. Subsequently, it was shown that both normal prostate and prostate cancer cell line LNCaP express large amounts of a transcript that hybridizes with a TCRγ cDNA probe but that the mRNA was smaller than mRNAs encoding TCRγ present in lymph nodes and other organs (12). Cloning and sequencing of this smaller mRNA showed that it is initiated within the Jγ1 exon of the TCRγ gene and that it encodes a 58-amino acid protein produced from a reading frame different from that of TCRγ. In the past several years, three groups of investigators have used cDNA arrays to analyze gene expression patterns in prostate cancer and found that prostate cancers contain RNA transcripts hybridizing with probes containing TCRγ sequences but not TCRδ transcripts and that these transcripts are elevated in prostate cancer (14–16). The implication is that these transcripts in prostate cancers encode TARP and not TCRγ. Furthermore, we have used in situ hybridization to determine the cellular localization of TARP in human prostate cancer samples and shown that TARP is expressed in >90% of cancer specimens examined.7

In this study, we first determined human HLA-A2-presented epitopes derived from TARP, and then their immunogenicities were tested in A2Kb transgenic mice that have a chimeric MHC class I molecules composed of α1 and α2 domains from HLA-A2 and α3 domain from Kb (17). Next, we enhanced the immunogenicity of the peptides by increasing their binding affinities to HLA-A2 molecules. Epitope enhancement can potentially increase the efficacy of a vaccine because natural tumor epitopes are not necessarily optimized for immunogenicity (18–23). Because it takes a stronger signal to activate a response than to be the target of a response (24), the natural epitope may be sufficient to allow killing of the tumor by T cells raised with the enhanced epitope. Furthermore, we have detected CD8+ T cells reactive to the wild-type and enhanced epitopes in prostate and breast cancer patients. By in vitro restimulation of peripheral blood mononuclear cells from prostate cancer patients with peptide-loaded dendritic cells (DCs), we could detect and expand peptide-specific CD8+ T cells that recognized peptide/MHC complexes, mediating lysis of a human breast cancer cell line. In addition, HLA-A2 tetramers containing individual peptides made in this study allowed us to detect the presence of peptide-specific CD8+ T cells in both breast and prostate cancer patients. These studies provide the preclinical basis for clinical trials of TARP-directed vaccine immunotherapy of prostate and breast cancer.

MATERIALS AND METHODS

Animals. A2Kb transgenic mice expressing a chimeric HLA-A2.1 transgene with the α1 and α2 domains from HLA-A2.1 and the α3 domain from Kb, to allow binding to mouse CD8, on a C57BL/6 background were developed at The Scripps Clinic by Linda Sherman (17). These mice were bred

7 A. Bhattacharyya, T. K. Bera, and I. Pastan, unpublished data.
and housed in appropriate animal care facilities. All procedures with animals were conducted in accordance with the institutionally approved protocols.

**Peptides.** HLA-A2-binding peptides were synthesized on a Model Symphony Peptide Synthesizer (Perkin-Elmer, Boston, MA) using conventional fluorenylmethoxycarbonyl (f-MOC) chemistry and cleaved from the resin by trifluoroacetic acid. The purity and molar concentration were analyzed by reverse-phase high-performance liquid chromatography on a C18 column using a gradient of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile and further purified by preparative reverse-phase high-performance liquid chromatography using a similar gradient. Some peptides were purchased from Multiple Peptide Systems (San Diego, CA) at >95% purity and were single peaks by reverse-phase high-performance liquid chromatography.

**Cell Lines.** The T2 cell line is deficient in TAP1 and TAP2 transporter proteins and expresses low levels of HLA-A2. The C1R-A2.1 cell line is derived from human B lymphoblastoid cell line HMYC1R transfected with HLA-A2.1. Cells were maintained in complete medium (RPMI 1640 supplemented with 10% FCS, 100 IU of penicillin, and 10 μg/ml streptomycin). RPMI 1640 and other supplements were purchased from Cellgro (Gaithersburg, MD). For C1R-A2.1 cells, 200 μg/ml Geneticin (Sigma, St. Louis, MO) was added into the medium. LNCap, PC3, MCF-7, and DU145 cells were maintained in the complete media. For PC3-TARP cells (25), 200 μg/ml hygromycin B (Invitrogen, Carlsbad, CA) was added into the medium.

**T2 Binding Assay.** Peptide binding capacity to HLA-A2 molecules was measured by using the T2 cell line according to a protocol described previously (26). T2 cells (3 × 10^5 cells/well) were incubated overnight in 96-well plates with culture medium (1:1 mixture of RPMI 1640/Eagle-Hank’s amino acid containing 2.5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) with 10 μg/ml β2-microglobulin (Sigma) and different concentrations of peptides. Cells were washed twice with cold PBS containing 2% fetal bovine serum and incubated for 30 min at 4°C with anti-HLA-A2.1 BB7.2 monoclonal antibody (1:80 dilution from hydridoma supernatant). After washing, cells were stained with FITC-labeled goat antimouse immunoglobulin (PharMingen, San Diego, CA), and the level of HLA-A2 expression was measured by flow cytometry. HLA-A2 expression was quantified as fluorescence index according to the following formula: fluorescence index = (mean fluorescence intensity with peptide – mean fluorescence intensity without peptide)/mean fluorescence intensity without peptide.

**Immunizations.** A2K transgenic mice were immunized with syngeneic peptide-loaded DCs, plasmid DNA expressing TARP, or a mixture of peptide and cytokine in incomplete Freund’s adjuvant. For DC immunization, DCs were pulsed with 10 μg peptide in serum-free RPMI 1640 for 2 h at 37°C, and then mice were immunized s.c. on days 1–3 × 10^6 DCs without washing. DCs were prepared from bone marrow as described previously (27). Alternatively, mice were immunized i.m. with 100 μg of plasmid DNA, pcDNA5/FRT/TARP, generated by inserting the TARP DNA into the pcDNA5/FRT (Invitrogen) vector. Mice were also immunized s.c. in the base of the tail with 100 μl of an emulsion containing 1:1 incomplete Freund’s adjuvant and PBS with antigens and cytokines (50 nmol of CTL epitope, 50 nmol of Hepatitis B virus core 128–140 helper epitope, 3 μg of interleukin 12, and 5 μg of granulocyte macrophage colony-stimulating factor. Incomplete Freund’s adjuvant and cytokines were purchased from Sigma and Peprotech (Rocky Hill, NJ), respectively.

**In Vitro Human CD8+ T-Cell Priming with DCs.** Elutriated monocytes and lymphocytes were obtained from HLA-A2+ patients before they underwent surgery or chemotherapy. HLA typing was performed by the Clinical Center laboratory (NIH), which uses a PCR-based low-resolution molecular technique. To prepare DCs to make an autologous peptide-specific CTL line, monocytes from prostate cancer patient 1 (in Fig. 8) were cultured at 10^6 cells/ml in complete medium containing human interleukin 4 (1000 units/ml) and human granulocyte macrophage colony-stimulating factor (1000 units/ml). On days 2 and 4, half of the media was exchanged. Cells were harvested on day 7 and then pulsed with 10 μg peptides for 2 h before γ-irradiation. Peptide-pulsed DCs (1 × 10^6) and autologous CD8+ T cells (2 × 10^6) were mixed and cultured in 24-well plates. Interleukin 2 (10 units/ml) was added on day 2 during restimulation. Cells were restimulated every 7–9 days for approximately four to seven cycles.

**CTL Assay.** For murine CTLs, CD8+ T cells from the immunized mice were restimulated with peptide-loaded splenocytes for 1 week as described previously (28) and then tested in 5-h °Cr release assays. Target cells were labeled with °Cr first and then washed twice. Cells were then pulsed with peptides for 2 h and washed again before further washing. To measure human CTL activity against peptide-loaded target cells, target cells were labeled with °Cr first and then loaded with peptides. In the CTL assay against human tumor cells expressing naturally processed epitopes, target cells were incubated without any peptide addition in complete medium containing 1000 units/ml IFN-γ for 72 h before CTL assay. The mean of triplicate samples was calculated, and the percentage of specific lysis was determined using the following formula: percentage of specific lysis = 100 × (experimental °Cr release – control °Cr release)/(maximum °Cr release – control °Cr release). The maximum release refers to counts from targets in 2.5% Triton X-100.

**HLA-A2 Tetramers.** Tetrameric MHC class I/peptide complexes were synthesized as described previously (29, 30) using a single-chain HLA-A2 construct. Briefly, purified single-chain HLA molecule in which the heavy chain and β2-microglobulin are covalently linked using a flexible peptide linker were synthesized by means of a prokaryotic expression system (pET, R&D Systems, Minneapolis, MN). The heavy chain was modified by deletion of the transmembrane and cytosolic tail and COOH-terminal addition of a sequence containing the Bir-A enzymatic biotinylation site. Single-chain heavy chain-β2-microglobulin and peptide were refolded by dilution into a redox-shuffling buffer system. The refolded product was isolated by ion-exchange chromatography and then biotinylated by Bir-A in the presence of biotin ligase (Avidity, Denver, CO). Streptavidin-Phycoerythrin conjugate (Jackson ImmunoResearch, West Grove, PA) was added in a 1:4 molar ratio.

**Antibodies and Flow Cytometry.** FITC-labeled antitumor CD8 (53-6.7), CD11c, CD80 (B7-1), CD54 (ICAM-1), antihuman CD8 (RPA-T8), CD14 (M5E2), CD80 (B7-1), CD86 (B7-2), Vβ3 TCR (JOV1.3), Vβ5 TCR (MH3-2), Vβ8 TCR (JR2), Vβ12 TCR (5511), and Vβ23 TCR (AHUT7) were used for staining of cell surface molecules. Cell association of IFN-γ-staining followed the manufacturer’s protocol. All antibodies and reagents were purchased from PharMingen. For flow cytometric analysis of cell surface, 5 × 10^6 cells were washed and resuspended in PBS containing 0.2% BSA and 0.1% sodium azide. Cells were incubated on ice with the appropriate antibody for 30 min and then washed. Samples were analyzed on a FACScan (BD Biosciences, Mountain View, CA). Background staining was assessed by use of an isotype control antibody. For tetramer staining, cells were incubated with tetramers for 20 min and then stained with FITC-labeled anti-CD8.

**RESULTS**

HLA-A2-Restricted Epitope Prediction and Wild-Type Peptide Binding Affinity to HLA-A2 Molecules. Fifty-eight amino acid residues in TARP were characterized previously (Ref. 13; Fig. 1A). To determine HLA-A2 epitopes from TARP, four different wild-type peptide pools were first predicted based on the anchor residues (Fig. 1B), and their binding affinities to HLA-A2 molecules were measured by
Amino acid substitutions in the wild-type peptides result in increased binding affinity to HLA-A2 molecules (epitope enhancement). Binding affinity of peptide to MHC class I molecules is a major factor determining the immunogenicity of peptide epitopes. To enhance the binding affinity of wild-type epitopes (TARP29–37 and TARP27–35), amino acids in the peptides were replaced. For TARP29–37, Arg at position 3 and Leu at position 9 were substituted with Ala (TARP29–37-3A) and Val (TARP29–37-9V), respectively (Fig. 3A). As shown in Fig. 3C, substitution at position 3 with Ala in TARP29–37 resulted in the greatest increase in the binding affinity of the peptide. The binding affinity of TARP29–37-3A was not less than that of the positive control peptide, flu matrix peptide (32). Although TARP29–37-9V showed a lower binding affinity to HLA-A2 than TARP29–37-3A did, substitution of Leu at position 9 with Val did enhance the binding affinity compared with the wild-type peptide, TARP29–37. In addition to TARP29–37, we tried to improve the binding affinity of TARP27–35 to HLA-A2 molecules by substitution of amino acids in position 2, 3, and 9 with Leu, Ala, and Val, respectively, but there was no significant enhancement in binding (Fig. 3B).

In contrast to the situation with TARP29–37, substitution with Ala at position 3 in TARP27–35 resulted in no binding of the peptide to HLA-A2 molecules, suggesting that the peptide binding affinity to MHC molecules was not simply determined by a single amino acid but influenced by other amino acids in the epitope. Two other substitutions at position 2 with Leu (TARP27–35-2L) and at position 9 with Val (TARP27–35-9V) did not improve the binding affinity compared with the wild-type peptide, TARP27–35.

Immunogenicity of the Enhanced Epitopes and CD8+ T-Cell Responses to the Wild-Type Peptides. Both TARP29–37-3A and TARP27–37-9V showed better binding affinity to HLA-A2 molecules than the wild-type, TARP29–37 (Figs. 1 and 3). To test the immunogenicities and recognition of the wild-type peptide by CD8+ T cells induced with the enhanced epitopes, mice were immunized with those peptides. As shown in Fig. 4A, both TARP29–37-3A and TARP29–37-9V induced over 2-fold higher frequencies of CD8+ T cells specific for the wild-type peptide, TARP27–35.
individual peptides compared with the wild-type, TARP\textsubscript{29–37}. Another wild-type epitope, TARP\textsubscript{27–35} also induced a higher frequency of CD8\textsuperscript{+} CTLs specific for the immunogen TARP\textsubscript{29–35}, but these CD8\textsuperscript{+} CTLs did not recognize TARP\textsubscript{29–35} and the epitope-enhanced peptides (Fig. 4B). In a cross-reactivity analysis (Fig. 4, B and C), CD8\textsuperscript{+} CTLs induced with both enhanced epitopes lysed target cells pulsed with the wild-type TARP\textsubscript{29–37}, suggesting that the TCRs of those CTLs recognize the wild-type peptide (TARP\textsubscript{29–37})/MHC class I complex to some extent. The immunogenicity of the enhanced versus wild-type TARP\textsubscript{29–37} epitope shown in Fig. 4A correlated with the peptide affinity for HLA-A2.1 (shown in Fig. 3), as compared graphically in Fig. 4D. However, CD8\textsuperscript{+} CTLs induced with TARP\textsubscript{29–37-3A} recognized TARP\textsubscript{29–37-3A}/MHC complex better than other peptide/MHC class I complexes. When compared quantitatively (Fig. 4C), TARP\textsubscript{29–37-9V} was the most immunogenic of the peptides in inducing CTLs specific for the wild-type sequence. Thus, TARP\textsubscript{29–37-9V} may be the best immunogen for inducing CTLs against tumor cells expressing the natural antigen. Interestingly, CD8\textsuperscript{+} CTLs induced with TARP\textsubscript{29–37-9V} could also kill target cells pulsed with TARP\textsubscript{27–35}. In contrast, CTLs induced with TARP\textsubscript{29–37-3A} did not kill target cells pulsed with TARP\textsubscript{27–35}, suggesting that CD8\textsuperscript{+} T cells induced with TARP\textsubscript{29–37-9V} have broader cross-reactivity to wild-type peptide/MHC complexes than CD8\textsuperscript{+} T cells induced with TARP\textsubscript{29–37-3A}. Therefore, TARP\textsubscript{29–37-9V} may be a good candidate vaccine to target tumor cells expressing TARP.

**Human CD8\textsuperscript{+} T Cells Raised in Vitro Lysed Peptide-Pulsed Target Cells.** To test for the presence of peptide-specific CD8\textsuperscript{+} T cells in a HLA-A2\textsuperscript{+} prostate cancer patient, CD8\textsuperscript{+} T cells from the leukapheresis of the patient donor were restimulated with peptide-pulsed autologous DCs over several cycles. During the in vitro restimulation, peptide-specific CD8\textsuperscript{+} T cells were detected after only four cycles of restimulation with TARP\textsubscript{29–37-3A}-pulsed DCs, whereas TARP\textsubscript{29–37-9V}-specific CD8\textsuperscript{+} T cells required at least five cycles. For both wild-type peptides (TARP\textsubscript{29–37} and TARP\textsubscript{27–35}), CD8\textsuperscript{+} CTLs required at least six cycles of in vitro restimulation to be detected. Cytolytic activity of those CD8\textsuperscript{+} CTLs raised with individual peptides was tested against peptide-pulsed C1R-A2.1 target cells. All four CTL lines were CD8\textsuperscript{+} and could lyse peptide-pulsed target cells specifically, as shown in Fig. 5. However, CD8\textsuperscript{+} CTLs raised with TARP\textsubscript{29–37-3A} and TARP\textsubscript{27–35} resulted in higher levels of cytolytic activity against the corresponding peptides compared with CD8\textsuperscript{+} CTLs raised with TARP\textsubscript{29–37} and TARP\textsubscript{29–37-9V}.

**Human CD8\textsuperscript{+} T Cells Raised against TARP\textsubscript{29–37-9V} but not TARP\textsubscript{29–37-3A} Recognize the MHC Complex with the Wild-Type Peptide, TARP\textsubscript{29–37.** We have shown that murine CD8\textsuperscript{+} CTLs induced by the enhanced epitopes lysed wild-type peptide-pulsed target cells more efficiently than TARP\textsubscript{29–37-3A} and TARP\textsubscript{29–37-9V} lysed with the wild-type TARP\textsubscript{29–37} peptide. Therefore, TARP\textsubscript{29–37-9V} may be a good candidate vaccine to target tumor cells expressing TARP. 

**Fig. 4. Immunogenicity of the enhanced epitopes in A2K\textsuperscript{b} transgenic mice.** A2K\textsuperscript{b} transgenic mice were immunized s.c. with a mixture of peptide and cytokine in adjuvant as described in "Materials and Methods." A, 2 weeks after the second boost, pooled spleen CD8\textsuperscript{+} T cells from three to four mice were restimulated with 1.0 \mu M each peptide. During restimulation, CD8\textsuperscript{+} T cells were treated with brefeldin A for 5 h. Cells were stained with anti-CD8-FITC and IFN-\gamma-PE as described in the manufacturer's protocol (PharMingen). B, cross-reactivity on each peptide (E:T = 100:1). Two weeks after the second boost, pooled spleen CD8\textsuperscript{+} T cells from three to four mice were restimulated with irradiated splenocytes in medium containing 1.0 \mu M each peptide for 7 days. In a 5-h 51 Cr release assay, Jurkat cells transinfected with HLA-A2 were labeled with \textsuperscript{51}Cr and then pulsed with peptides. Without washing, target cells were mixed with different numbers of effector cells and then cultured for 5 h before harvesting. C, CTLs raised against the individual peptides indicated were tested on target cells pulsed with either wild-type TARP\textsubscript{29–37} (closed symbols) or no peptide (open symbols). Lytic activity is shown as a function of E:T ratio. Bars represent SE in triplicate assay, and bars smaller than the symbols are not shown. D, relationship between peptide binding affinity and immunogenicity of each peptide. Peptide concentrations for fluorescence index value = 0.5 (F\textsubscript{50}) from Fig. 3 were plotted versus the numbers of IFN-\gamma-producing cells (A). Data were reproducible in two repeated experiments with similar results.
important to know the avidity of CD8⁺ CTLs to naturally processed peptide. However, CD8⁺ CTLs raised by TARP₂⁹₋₃⁷₋₃₅ could not recognize the wild-type peptide efficiently, and the immunogenicity of the wild-type peptide, TARP₂⁹₋₃⁷, was not strong enough (Fig. 4). In this study, therefore, we measured the avidity of CD8⁺ CTLs specific for TARP₂⁹₋₃⁷₋₉₉ to different peptides (Fig. 6B). CTLs for TARP₂⁹₋₃⁷₋₉₉ recognized TARP₂⁹₋₃⁷₋₉₉ at the 0.001 μM level. Although the avidity of these CTLs for wild-type TARP₂⁹₋₃₇ was lower, they could recognize the wild type as well as TARP₂⁹₋₃⁷₋₃₅ with only slightly lower avidity. However, these CTLs did not recognize TARP₂⁷₋₃₅ at <10 μM concentration.

**Human CD8⁺ T Cells Raised against TARP₂⁹₋₃⁷₋₉₉ and TARP₂⁷₋₃₅ Kill TARP-Expressing Tumor Cells.** To test whether these CTLs could kill human tumor cells that endogenously express TARP, a CTL assay was performed against tumor cell lines that express both HLA-A2 and TARP (Fig. 7A). At a 50:1 E:T ratio, all CD8⁺ T cells could kill the breast cancer line MCF-7 but showed marginal (10–12%) lytic activity against the prostate cancer cell line LNCaP. Before CTL assay, all target cells were cultured in medium containing IFN-γ, and the expression levels of HLA-A2 before and after IFN-γ treatment were measured (Fig. 7B). As expected from the CTL assay, LNCaP cells express an extremely low level of HLA-A2 that was not much increased by IFN-γ. In contrast, the level of

cells (Fig. 4) and that human CD8⁺ CTLs raised against individual peptides lysed target cells pulsed with the corresponding peptides (Fig. 5). However, it is important to know whether human CD8⁺ CTLs raised against enhanced epitopes could lyse target cells pulsed with wild-type peptide expected to be presented on tumor cells. To address this question, cytolytic activity of individual CD8⁺ CTLs was measured against target cells pulsed with different peptides. As shown in Fig. 6A, human CD8⁺ CTLs raised with TARP₂⁹₋₃⁷ could recognize and lyse target cells pulsed with the wild type as well as the two enhanced epitopes (TARP₂⁹₋₃⁷₋₃₅ and TARP₂⁹₋₃⁷₋₉₉). However, TARP₂⁹₋₃⁷₋₃₅-specific CD8⁺ T cells recognized TARP₂⁹₋₃⁷₋₃₅ very well but recognized the wild-type and TARP₂⁹₋₃⁷₋₉₉ comparatively less well. In contrast, CD8⁺ T cells specific for TARP₂⁹₋₃⁷₋₉₉ could recognize both wild type and TARP₂⁹₋₃⁷₋₃₅, comparatively. These data confirm that CD8⁺ T cells raised with TARP₂⁹₋₃⁷₋₉₉ could recognize both wild type and enhanced epitope/MHC complexes, whereas those raised against TARP₂⁹₋₃⁷₋₃₅ cross-reacted relatively poorly. CD8⁺ CTLs induced with TARP₂⁷₋₃₅ recognized TARP₂⁷₋₃₅/MHC complex but recognized other peptide/MHC complexes tested less well. Our data also showed that all three CTLs raised with TARP₂⁹₋₃⁷₋₃₅, TARP₂⁹₋₃⁷₋₃₅, and TARP₂⁹₋₃⁷₋₉₉ could recognize TARP₂⁷₋₃₅ to some extent, possibly due to the 7-residue overlap between them. In terms of vaccine development for prostate and breast cancers, it is

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**Fig. 5. Human CD8⁺ CTLs raised by in vitro stimulation kill peptide-loaded target cells.** CD8⁺ T cells from a prostate cancer patient were restimulated with 10 μM peptide-pulsed autologous dendritic cells (DCs). DCs were derived from the culture of autologous monocytes in granulocyte macrophage colony-stimulating factor and interleukin 4. To mature the DCs, CD40 ligand (2 μg/ml) was added on day 4, and then cells were further cultured for 2–3 days before loading peptides. After several cycles of in vitro restimulation, CD8⁺ T cells were used as effector cells. A, cells were stained with anti-CD8α or isotype control antibody. B, in 5-h ⁵¹Cr release assay, C1R-A2.1 cells were labeled with ⁵¹Cr and then pulsed with peptides. Closed and open circles represent target cells pulsed with and without peptides, respectively. Data are representative of two repeated experiments with similar results.

**Fig. 6. Cross-reactivity of human CD8⁺ CTLs to different HLA-A2 peptide epitopes.** Human CD8⁺ CTLs were raised as described in the Fig. 5 legend. A, in a 5-h ⁵¹Cr release assay, C1R-A2.01 cells were labeled with ⁵¹Cr and then pulsed with 10 μM peptides. B, specificity and avidity of CD8⁺ T cells specific for TARP₂⁹₋₃⁷₋₉₉ to other peptides were tested by using target cells pulsed with different concentrations of the indicated peptides. Data are representative of two repeated experiments with similar results.
DISCUSSION

TARP is a newly found protein expressed in both breast and prostate cancer specimens tested. In this study, we first report that human CD8+ T cells from a prostate cancer patient specific for peptides derived from TARP kill a human breast cancer cell line (13, 25). Therefore, TARP could be used as a target protein for vaccine immunotherapy for both prostate and breast cancer, and TARP peptides described here may serve as an effective cancer vaccine in the nearly half of the population that is HLA-A2.

In this study, we have examined the potential of this new protein as an antigen for the immunotherapy of prostate or breast cancer patients and defined peptide immunogens that were enhanced by sequence modification. A first step for developing a vaccine that could induce CD8+ T cell-mediated immunity, a critical immune arm for cancer immunotherapy, is to identify CD8+ T-cell epitopes that can induce immune responses and serve as targets for lysis of tumor cells. To find HLA-A2 epitopes from the TARP protein, 8- or 9-mer peptides were predicted based on amino acid anchor residues that determine binding to HLA-A2 molecules (33–35). TARP is composed of 58 amino acid residues and contains several hydrophobic amino acids including five leucines, but data from the T2 binding assay showed that only 2 wild-type peptides (TARP29–37 and TARP27–35) had a measurable binding affinity to HLA-A2 molecules. Seven of nine amino acids in both wild-type peptides overlap. Moreover, they both share two amino acids at corresponding positions, Phe at position 1 and Leu at position 9, although the residue at position 1 is not a primary anchor residue. However, TARP27–35 showed a better binding affinity than TARP29–37, probably because of other residues in nonanchor positions, such as Phe at position 3 in TARP29–37. Although the other two wild-type peptides, TARP29–37 and TARP27–35, possess Leu at position 9 and Met or Leu at position 23, respectively, neither showed a measurable binding affinity to HLA-A2 molecules.

In a phenotype analysis, about 40–60% and 19–40% of peptide-specific CD8+ T cells in the patients expressed CD45RA and CD45RO, respectively. However, <2% of CD8+ T cells express CCR7, and the majority of cells were CD62Llow, suggesting that most of the peptide-specific CD8+ T cells are activated forms and that the majority of the memory cells are not central memory CD8+ T cells.
serve as a good target for CTLs raised against the enhanced peptide, such an enhanced peptide would not be useful.

When the wild-type peptide does bind, but perhaps not optimally, one strategy to improve the usage of self-peptides such as TARP29–37 and TARP27–35 is to develop enhanced epitopes that are potentially more immunogenic. Substitution of Arg at position 3 with Ala in TARP29–37 greatly improved the peptide binding affinity to the HLA-A2 molecules. This could be explained by an adverse effect of Arg at position 3 reducing the affinity. Substitution of Leu at position 9 with Val in TARP29–37 also resulted in increased binding affinity, and this is consistent with other reports on HLA-A2-binding peptides (33, 35). We have also tried to define enhanced epitopes for TARP27–35 by substitutions for Val at position 2, Phe at position 3, and Leu at position 9 with Leu, Ala, and Val, respectively, but those substitutions did not improve the affinity. Amino acid residues associated with poor binding to HLA-A2 are Asp, Glu, and Pro at position 1; Asp and Glu at position 3; Arg, Lys, His, and Ala at position 4; Pro at position 5; Arg, Lys, and His at position 7; Asp, Glu, Arg, Lys, and His at position 8; and Arg, Lys, and His at position 9 (33, 35). Both TARP27–35 and TARP29–37 do not have any residue known to be associated with poor HLA-A2 binding at the secondary anchor positions. In contrast, as noted, the low binding affinity of the wild-type peptides, TARP29–37 and TARP27–35, is probably due to the Pro at position 5 and Glu at position 3, respectively.

Immunogenicities of the wild-type and enhanced peptides were compared by using A2Kb transgenic mice. The immunogenicity of each peptide was largely dependent on its affinity for HLA-A2 molecules (Fig. 4D). Our data from the DNA immunization experiments indicate that the murine antigen processing system is not a limitation for production of peptides presented by human class I molecules. Moreover, data from our laboratory and others suggest that such HLA-transgenic mice can be used for the study of peptides recognized by CD8 T cells specific for HLA-A2 molecules (36–40). Another concern for using transgenic mice is that peptide/HLA-A2 complexes recognized by murine T cells may be different from those recognized by human T cells, explaining why the cross-reactivity of CD8 T cells from mouse and human was not exactly the same. It is possible that those species differences may be due to different TCR repertoires. However, our data showed that both wild-type and enhanced epitopes resulted in the induction of peptide-specific CD8 T cells in HLA-A2 transgenic mice, and therefore these mice can be used as good predictors of human T-cell epitopes (36).
CD8+ CTLs typically express clonally distributed TCRs that possess exquisite specificity for a particular MHC/peptide complex. In both mice and humans, however, CD8+ T cells raised with individual peptides can recognize a range of cross-reactive peptide/MHC complexes, depending on the individual CD8+ CTLs and peptides. The cross-reactivity observed in this study not only among variants of one peptide but between the two wild-type peptides may be explained by the fact that the two wild-type peptides overlap by seven amino acid residues. A number of recent studies have shown degenerate recognition of MHC/peptide complexes by individual TCRs: examples range from T-cell recognition of dissimilar peptides presented by the same MHC molecules to recognition of identical peptides bound to different MHC molecules (41–45). For example, CD8+ T cells specific for one peptide of polyoma virus recognize another epitope that has no sequence homology (46). However, those CD8+ T cells require a much higher concentration of the alternative peptide for recognition.

In the cross-reactivity test for human and CD8+ T cells, CD8+ T cells specific for TARP29-37 could recognize all four peptide/MHC complexes. However, CD8+ T cells specific for TARP27-35 or TARP29-37.3A did not recognize other peptides as much as they did the immunogens. Of the enhanced peptides, only CTLs specific for TARP29-37.9V could recognize the wild-type peptide, TARP29-37, to a similar degree to the immunogen. In consideration of choosing peptides for immunotherapy, either TARP29-37.9V or TARP27-35 will likely be more useful than the other two peptides. Although TARP29-37.3A showed the highest binding affinity and resulted in high CD8+ T-cell responses in transgenic mice (Fig. 4D), CD8+ T cells specific for this peptide did not recognize wild-type peptide very well in the human and showed weak cross-reactivity to wild-type peptide in the mice. Most importantly for tumor killing assay, CD8+ T cells specific for TARP29-37.3A showed a significant range of specific lysis only at a 100:1 E:T ratio (data not shown). At a 50:1 E:T ratio, CD8+ T cells specific for TARP29-37.3A showed much lower lytic activity against tumor cells than CTLs to TARP29-37.9V. In contrast, TARP29-37.9V induced a higher level of CD8+ T-cell responses than TARP29-37 and CD8+ T cells specific for TARP29-37.9V could recognize the wild-type peptide, TARP29-37, and could kill human tumor cells. CD8+ T cells specific for TARP27-35 could kill human tumor cells as well.

Data from tetramer staining experiments support the presence of peptide-specific CD8+ T cells in prostate cancer patients. The frequency of tetramer-positive CD8+ T cells detected in this study could be influenced by the binding affinity of peptides, and thus the number in Fig. 8 may represent a lower limit on the frequency of peptide-specific CD8+ T cells. However, those data consistently show that a higher number of peptide-specific CD8+ T cells is present in the prostate and breast cancer patients than in normal donors studied concurrently. Interestingly, the frequency of tetramer-positive CD8+ T cells in prostate cancer patient 1 (Fig. 8) was surprisingly high. It is not clear whether these high levels are related to the stage of tumor progression or to some other mechanism. Data from phenotype analysis of tetramer-positive CD8+ T cells also indicated that most CD8+ T cells were highly activated. It would be interesting to test the frequency of tetramer-positive CD8+ T cells, their functional activities, and phenotype changes over the course of the disease in the future.

Besides vaccines, another approach for immunotherapy using the TARP epitopes is the generation of recombinant antibodies that can recognize TARP/HLA-A2 complexes in a peptide-specific, MHC-restricted manner. This approach was applied recently to a number of tumor- and viral-specific epitopes (30, 47–51). Such antibodies bind with high affinity to the MHC-peptide complex in a TCR-like restricted manner and thus can be used as a targeting moiety to deliver potent toxins or drugs to eliminate a specific cell population that expresses the particular MHC/peptide complex. These antibodies were also used to visualize and quantitate the specific MHC/peptide complex on the surface of tumor cells as well as antigen-presenting cells (47–51).

We conclude that the peptides TARP27-35 and TARP29-37.9V can potentially be used for several immunotherapeutic strategies, including immunotoxins and vaccines, whether in adjuvant or as peptide-pulsed DCs, for prostate and breast cancer patients who are positive for the HLA-A2 allele. Given that HLA-A2 is present in nearly half of the population of North America, as well as much of the world, and that the expression of TARP is common in prostate and breast cancers, a vaccine containing or expressing these peptides may be effective in a sizable fraction of prostate and breast cancer patients. If such an approach is successful in HLA-A2+ patients as prototype, it will provide motivation to define TARP epitopes presented by other common HLA class I alleles that could broaden the coverage. Such a vaccine may also be used in combination with other antigens for prostate or breast cancer to enhance the efficacy of vaccine therapy. To enhance CD8+ T-cell-mediated immune responses, recombinant vectors including adenovirus or vaccinia virus expressing those antigens can also be used. Definition of novel tumor antigens such as TARP may provide new opportunities to harness the exquisite specificity of the immune system to fight cancer.

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Human CTLs to Wild-Type and Enhanced Epitopes of a Novel Prostate and Breast Tumor-Associated Protein, TARP, Lyse Human Breast Cancer Cells

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