Human CTL Epitopes Prostatic Acid Phosphatase-3 and Six-Transmembrane Epithelial Antigen of Prostate-3 as Candidates for Prostate Cancer Immunotherapy

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Abstract

Specific immunotherapy of prostate cancer may be an alternative or be complementary to other approaches for treatment of recurrent or metastasized disease. This study aims at identifying and characterizing prostate cancer–associated peptides capable of eliciting specific CTL responses in vivo. Evaluation of peptide-induced CTL activity in vitro was done following immunization of HLA-A2 transgenic (HHID) mice. An in vivo tumor rejection was tested by adoptive transfer of HHD immune lymphocytes to nude mice bearing human tumors. To confirm the existence of peptide-specific CTL precursors in human, lymphocytes from healthy and prostate cancer individuals were stimulated in vitro in the presence of these peptides and CTL activities were assayed. Two novel immunogenic peptides derived from overexpressed prostate antigens, prostatic acid phosphatase (PAP) and six-transmembrane epithelial antigen of prostate (STEAP), were identified; these peptides were designated PAP-3 and STEAP-3. Peptide-specific CTLs lysed HLA-A2.1+ LNCaP cells and inhibited tumor growth on adoptive immunotherapy. Furthermore, peptide–primed human lymphocytes derived from healthy and prostate cancer individuals lysed peptide-pulsed T2 cells and HLA-A2.1+ LNCaP cells. Based on the results presented herein, PAP-3 and STEAP-3 are naturally processed CTL epitopes possessing anti–prostate cancer reactivity in vivo and therefore may constitute vaccine candidates to be investigated in clinical trials. (Cancer Res 2005; 65(14): 6435-42)

Introduction

Prostate cancer is the most diagnosed noncutaneous cancer in the western male population and the second leading cause of cancer-related death in this population (1). Yet, treatment of hormone-refractory metastatic prostate cancer remains ineffective (1). Alternative approaches based on immunotherapy have used recent advances in identification of tumor-specific and tumor-associated antigens (TAA) and have shown therapeutic promise (2). Tumor-specific antigens come from mutated proteins and from viral proteins in virally associated tumors. TAAs are embryonal reexpressed or overexpressed proteins and differentiation antigens expressing PSMA (10). There is evidence of CD8 T-cell response to induce effective CTL responses against prostate tumor cells when cultured in peripheral blood mononuclear cells (PBMC) of HLA-A2 or HLA-A24 donors (7, 8). In a phase II trial, dendritic cell vaccines pulsed with HLA-A2+-restricted PSMA peptides resulted in CTL induction of 2 of 33 patients not accompanied by clinical manifestations (9). In accordance, both peptides failed to induce effective CTL responses against prostate tumor cells expressing PSMA (10). There is evidence of CD8 T-cell response against PAP (11, 12). Yet, to the best of our knowledge, there are no reports demonstrating in vivo antitumor reactivity of PAP-derived peptide vaccines. Recent studies describe differentially expressed prostate-specific genes, which may be a potential source of candidates for peptide-based vaccination. These include six-transmembrane epithelial antigen of prostate (STEAP; ref. 13), prostate stem cell antigen (PSCA; ref. 14), prostate carcinoma tumor antigen-1 (PCTA-1; ref. 15), prostate tumor-inducing gene-1 (PTI-1; ref. 16), prostate-specific gene with homology to G protein–coupled receptor (17), and prostate, an androgen-regulated serine protease (18).

Direct and reverse strategies are used for identification of TAA epitopes. The direct approach depends on patient-derived established CTL lines and genetic or biochemical screening. However, it is difficult to establish human carcinoma lines as well as carcinoma-associated CTL lines. Moreover, conceptually CTL lines from cancer patients may partially represent the repertoire of the anergized immune system. Furthermore, the in vitro propagation of CTL lines may enhance emergence of sporadic clones rather than tumor-specific clones. Alternatively, the modified reverse approach involves prediction of putative TAA-derived epitopes with amino acid anchor motifs specific for the defined human HLA allele (often A2.1, a prevalent allele in the population). Next, the predicted peptides are screened in HLA transgenic mice to identify immunodominant epitopes. In the last few years, several studies have determined concordance between the CTL repertoires of HLA-A2.1-restricted peptides in human PBMC and the CTLs (19).

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The dominant murine H-2-restricted responses that take place upon immunization with multiepitope proteins have been overcome in second-generation HLA transgenic mice [i.e., HHD mice (Db−/− × β2-microglobulin (β2m)−/− null mice transgenic for a recombinant HLA-A2.1/Dβ1/β2m single chain (20)]. Recently, novel breast tumor–associated MUC1 and BA46-derived peptides were characterized (21, 22). Noticeably, consistent with their immunogenicity in the HHD mice, BA46-derived peptides stimulated cytotoxic activity in PBMC from HLA-A2.1-positive breast carcinoma patients (22). Thus, the HHD model is an effective means for screening of candidate TAA peptides.

In the current study, we used the HHD mouse system to screen candidate prostate-associated TAA peptides for immunogenic HLA-A2.1-restricted CTL epitopes. The results show that CTLs against PAP-3 (ILLWQPPIPV) and STEAP-3 (LLLGTIHAL) lyse HLA-A2.1 and antigen-positive tumor cells in vitro and inhibit tumor growth in vivo. Furthermore, PAP-3 and STEAP-3 prime antitumor CTLs in human PBMC from healthy and prostate cancer donors.

Materials and Methods

Mice. The derivation of HLA-A2.1/Dβ1/β2m monochain, transgenic, H-2Db−/− × β2m−/− double-knockout mice (HHD mice) was described previously (20). Male CD1nu/nu mice (8-12 weeks old) were bred in Weizmann Institute of Science (Rehovot, Israel). All experiments were conducted in accordance with Weizmann Institute of Science Animal Facility and NIH guidelines.

Cell lines. LNCap-1740 (HLA-A2nu), LNCap-10995 (HLA-A2nu), and PC-3 and DU 145 (both HLA-A2−) are human prostate carcinoma cell lines obtained from the American Type Culture Collection (Manassas, VA). The PC-3-HHD clone is a HLA-A2.1/β2m single-chain (HHD) transfectant of PC-3 cells. E41-HHD and RMA-S-HHD clones are HHD transfectants of the murine T lymphoma EL4 and TAP-2-deficient RMA-S cells, respectively. The RMA-S-HHD-B7.1 clone is a HHD transfectant expressing the murine B7.1 costimulatory molecule. T2 is a TAP-2-deficient lymphoblastoid line of HLA-A2 genotype. PC-3, DU 145, and T2 cells were maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% FCS (Life Technologies, Rockville, MD) and combined antibiotics (complete medium). EL4-HHD and RMA-S-HHD-B7.1 cells were maintained in complete medium supplemented with 500 μg/ml genetin (Life Technologies). LNCap and PC-3-HHD cells were maintained in complete medium supplemented with dehydrotestosterone at 10−8 mol/L and puromycin at 1 μg/ml (both from Sigma), respectively.

Reverse transcription-PCR analysis. Total cytoplasmic RNA was isolated from logarithmically growing cell cultures. Tissue samples were obtained from patients who underwent radical prostatectomy at Barzilai Medical Center (Ashkelon, Israel). All tissues were histologically confirmed as benign prostate hyperplasia (BPH) or carcinoma of the prostate. Tissue samples were frozen in liquid nitrogen, and RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. Total RNA (4 μg) was reverse transcribed into cDNA with 200 units SuperScript II RNase H− reverse transcriptase (Life Technologies). The cDNA quality was assured by testing the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). cDNA corresponding to 500 ng total RNA was subjected to PCR amplification (PTC-100; MJ Research, Inc., Watertown, MA) for 40 cycles as follows: 1 minute at 92°C, 1 minute at 55°C (GAPDH, PAP, PSA, PSMA, PCTA, and PT-1), 60°C (PSA), 61°C (prostate), or 62°C (STEAP), and 1 minute at 72°C. Amplification was followed by 10-minute incubation at 72°C. The following primer sequences (5′−3′) were used: PAP (forward) GACGGAGTCCATTGACAC and (reverse) AACATCTAGCCGCTCTTGAG; PSA, (forward) TGCTGCTGCTCTGATGTCGAC and (reverse) CCAGAGCAGCAGCCAGTGCTGCA; PSA, (forward) TACACCTGACCTGACAGA and (reverse) CTTGAGACACACATTACA; PT-1, (forward) ACCGGAGGCCAGTGAATA and (reverse) TGCAGCGATCCATCTACAG; PCTA, (forward) AAGCTGACGCTATTGGA and (reverse) AACACCAAGTGGAACGGGT; PSMA, (forward) GGCCTGTTATCCTGGTGGAG and (reverse) TAGGGACCTTGGAGAGTCCCTC; STEAP, (forward) ACTTTGGTTGATGACCAAGATGGAA and (reverse) CAGAGCTAGCCACCAACGAAAC; and prostates, (forward) GTGGCAAGATCGCTGCTC and (reverse) ACGTGTGCGAGTGTTCCTGG. Products were electrophoresed on 1% or 2% agarose gels and monitored under UV.

Peptide synthesis. Peptides were synthesized on an Abimed AMS 422 peptide synthesizer (Abimed, Switzerland) using Fmoc chemistry and purified by reverse-phase high-performance liquid chromatography. Composition of the products was determined by amino acid analysis and the molecular weight was ascertained by mass spectrometry.

Peptide loading and measurement of peptide binding by stabilization of cell surface MHC. Peptide binding to HHD single chain was measured by stabilization of HHD on RMA-S-HHD cells using fluorescence-activated cell sorting (FACS) analysis. Cells (5 × 105 per sample) were incubated with 0.1 to 100 μmol/L synthetic peptide for 2 hours at 26°C followed by 3-hour incubation at 37°C and incubated in the presence of B-9-12, W6/32 (anti-HLA-A, -B, and -C), 28-14-8 (anti-H-2Db α3 domain), and BB7.2 (anti-HLA-A2) and washed with PBS supplemented with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide. Goat anti-mouse FITC (The Jackson Laboratory, Bar Harbor, ME) was applied for 30 minutes at 4°C. Following washing, the samples were analyzed by FACSAn and CellQuest software (Becton Dickinson, San Jose, CA).

Generation of dendritic cells from murine bone marrow cells. Generation of murine bone marrow–derived dendritic cells was described by Lutz et al. (23) and used with minor modifications. Briefly, bone marrow cells from femurs and tibiae of 4- to 6-week-old HHD male mice were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids 25 mmol/L HEPES (pH 7.4), 50 μmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, combined anti-biotics, and 300 units/ml recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF; Prospek, Rehovot, Israel). On day 8, nonadherent cells were harvested and further cultivated in fresh medium containing 100 units/ml GM-CSF for 24 hours. Dendritic cells were matured by addition of 1 μg/ml lipopolysaccharide (Sigma) for another 24 hours. Nonadherent cells were analyzed by FACS and found to express typical characteristics of mature dendritic cells (CD11c+, CD86+, and MHC class II+).

Vaccination. HHD mice were immunized i.p. thrice at 7-day intervals with 2 × 104 irradiated (7,000 rad) peptide-pulsed RMA-S-HHD-B7.1 or 104 peptide-pulsed (100 μmol/L) for 2 hours at 37°C) dendritic cells. In mixed synthetic vaccines, RMA-S-HHD-B7.1 cells were loaded separately with each peptide and admixed before vaccination.

In vitro cytotoxicity assay. HHD mice were immunized as described above. In all vaccination modes, spleens were removed on day 10 after the last immunization and cell suspensions were prepared. One third of the splenocytes were pulsed with 100 μmol/L synthetic peptide for 2 hours at 37°C and then added to the rest of the splenocytes. Cultures were incubated for 5 days in lymphocyte medium [RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids 25 mmol/L HEPES (pH 7.4), 50 μmol/L β-mercaptoethanol, and combined antibiotics]. Viable cells (effector cells) were separated by Lymphocyte-M (Cedarlane Laboratories Ltd., Hornby, British Columbia, Canada) gradient centrifugation, washed, resuspended in lymphocyte medium, and admixed at different ratios with 5,000 [3H]methionine-labeled target cells. CTL assays were carried out as described previously (22) in four effector-to-target (E:T) ratios ranging from 100:1 to 12:1. Percentage of specific lysis was calculated as follows: % lysis = (cpm in experimental well − cpm spontaneous release) / (cpm maximal release − cpm spontaneous release) × 100.

Adaptive immunotherapy of CD11c+nu/nu mice bearing human prostate carcinoma. HHD male mice were immunized i.p. six times at 3-day intervals with 2 × 109 peptide-loaded syngeneic dendritic cells. Spleens were removed 10 days after the last immunization and stimulated as above. Lymphocyte-M gradient purified cells were seeded in 24-well plates
from BD PharMingen, San Diego, CA). Male CD1
CD8, and CD45R was detected by FACScan (all antibodies were purchased
lymphocyte medium with hIL-2 for an additional 4 days. Expression of CD4,
measured in two perpendicular dimensions thrice weekly. Mice were
administration of 1,000 units hIL-2 twice daily for 5 days. Tumor size was
the indicated number of HHD-derived activated T cells followed by local s.c.
proposed). Following 3 days of incubation, the cells were diluted to 105/mL in
supplemented with recombinant human interleukin (hIL)-2 (100 units/mL,
(Ci) n5 %CO2. The cells were harvested
screening system using transgenic
3.5% and 37

Characterization of Human CTL Epitopes In vivo

In vitro cytotoxicity assays of human peripheral blood mononuclear
cells. HLA-A2.1-positive healthy volunteers and patients (after informed
consent) with localized prostate carcinoma, after surgery, were recruited for
in vitro generation of human CTLs. Leukapheresed PBMCs (Barzilai Medical
Center) were isolated by centrifugation on Ficoll-Plaque Plus gradients
(Amersham, Uppsala, Sweden). Mature autologous dendritic cells were prepared from adherent PBMC by incubation in the presence of IL-4 (1,000
units/mL, R&D, Minneapolis, MN) and GM-CSF (1,000 units/mL; R&D)
followed by maturation with a cocktail of IL-1β, IL-6, tumor necrosis factor-
α (each at 10 ng/mL, R&D), and prostaglandin E2 (1 µg/mL, Alexis, Carlsbad, CA). Dendritic cells were pulsed with synthetic peptides and then
overlaid with PBMC in the presence of IL-7 (20 ng/mL; R&D). Two days
later, hIL-2 (12 units/mL, Prospect) was added and renewed every 2 days.
Two additional restimulations were done every 7 days over peptide-pulsed monocytes. Seven days later, lymphocytes were harvested and cytolytic
assays were done using peptide-pulsed T2 cells or human prostate carcinoma cells as targets.

Results

Expression patterns of candidate tumor-associated antigens in prostate cancer. Candidate targets for immunotherapy include prostate selective antigens overexpressed in prostate cancer. To evaluate the expression profile of such genes, we used reverse transcription-PCR (RT-PCR) analysis. We examined the expression patterns of PSA, PSMA, PAP, STEAP, PSCA, PCTA, PTI-1, and prostate in human prostate cancer cell lines LNCaP, DU 145, and PC-3, in five prostate cancer specimens and in six BPH samples. PAP, STEAP, PSA, and PSMA were overexpressed in most prostate cancer samples compared with BPH (Fig. 1; data not shown). In our hands, PTI-1, PCTA-1, and prostate were not overexpressed in most prostate cancer specimens (data not shown). Thus, PAP, STEAP, PSA, and PSMA are a possible source for immunogenic peptides.

Screening for HLA-A2.1-restricted peptides. The four selected antigens were screened for potential TAA peptides predicted to bind HLA-A2.1 molecules and scored according to their ability to stabilize MHC using “HLA-binding predictions” software (24). This software scores every possible peptide along the protein sequence for MHC binding and every amino acid in the evaluated peptide is scored according to its contribution to the binding (http://www.bimas.dcrt.nih.gov/molbio/hla_bind/index.html). For each candidate TAA, we synthesized five peptides with the highest binding score (Supplementary Table S1).

Screening for immunogenic HLA-A2.1-restricted peptides: CTL responses in HHD mice. To test the immunogenicity of the candidate peptides (i.e., their potential to induce T-cell responses), we established a two-step in vivo screening system using transgenic HHD mice. HHD mice were immunized with syngeneic dendritic cells pulsed with pools of two to four synthetic peptides derived from each examined protein followed by in vitro restimulation of splenocytes with the same peptides used for priming. We evaluated the lysis patterns of each of the individual peptides loaded on target cells. CTL results showed specific lysis of RMA-S-HHD-B7.1 target cells loaded either with PAP-derived peptide-3 (PAP-3, LLWQIPPV) or with STEAP-derived peptide-3 (STEAP-3, LLGTVHAL). Other peptides showed either background lysis (PAP-, PSA-, and PSMA-derived peptides) or less effective lysis (STEAP-derived peptides; Fig. 2A and B; data not shown). Subsequent immunization of HHD mice with dendritic cells prepulsed with individual PAP-3 or STEAP-3 showed dose-dependent lysis of EL-4-HHD target cells loaded with either PAP-3 or STEAP-3 (Fig. 2C and D). CTL responses were peptide specific. Interestingly, immunization of HHD mice with homologous murine peptides (mPAP-3, RLLWQIPPV; mSTEAP-3, LLLGTVHAL) induced also peptide-specific CTLs comparable with human counterparts. Anti-mPAP-3 CTLs lysed 55 ± 3.2% and 38 ± 2.9% targets at 100:1 and 25:1 E:T ratio, respectively; anti-mSTEAP-3 CTLs lysed 48 ± 3.5% and 37 ± 2.4% targets at 100:1 and 25:1 E:T, respectively.

Confirmation of predicted peptide binding by stabilization of cell surface MHC. To examine the actual peptide binding to HLA-A2.1 molecules, we used TAP-2-deficient, HLA-A2.1-transfected RMA-S cells (RMA-S-HHD), which express low levels of peptide-free and therefore unstable class I MHC molecules. External loading of RMA-S-HHD cells with selected synthetic peptides at 0.1 to 100 µmol/L stabilized HLA-A2.1 molecules at a dose-dependent manner as determined by FACS analysis. MHC binding at 100 µmol/L gave the highest level of stabilization that was comparable with that of reference tyrosinase-derived, HLA-
A2.1-restricted peptide (Fig. 3). A high correlation between predicted and actual binding has been confirmed (22).

HLA-A2.1-restricted lysis of human prostate carcinoma cell lines by CTL induced against prostate acid phosphatase-3 and six-transmembrane epithelial antigen of prostate-3. Although PAP-3 and STEAP-3 induce an immune response, it is crucial to test whether these peptides are indeed presented on tumor cell surface

Figure 1. Profile of PAP and STEAP expression in tissue samples of BPH and prostate cancer and in cell lines. mRNA expression of PAP and STEAP was examined at level of mRNA transcripts by RT-PCR using specific primers on cDNAs. Equal amounts of total RNA from surgical specimens of BPH (BPH-A-BPH-F) and prostate carcinoma (Cpa-A-Cpa-E) and from logarithmically growing prostate cancer-derived cell cultures (LNCaP, PC-3, and DU 145) were reverse transcribed into cDNAs that were amplified by PCR. The 748- and 260-bp PCR products for PAP and STEAP, respectively, were electrophoresed in 1.5% agarose gels.
by MHC class I molecules and therefore may serve as tumor rejection antigens. We assessed CTL responses against relevant prostate cancer cell lines as targets using the HLA-A2.1-positive LNCaP cells that were shown to express PAP and STEAP proteins as targets for CTLs raised against the peptides of interest (refs. 13, 25; Fig. 4C and D). To this end, HHD mice were immunized with RMA-S-HHD-B7.1 cells loaded with either PAP-3 or STEAP-3, and lysis of LNCaP cells was monitored (Fig. 4A). CTLs induced against PAP-3 or STEAP-3 showed similar and specific response profiles against target LNCaP cells. Furthermore, the lysis of HLA-A2.1-negative PC-3 cells by either PAP-3- or STEAP-3-specific CTLs was negligible, suggesting MHC-restricted lysis (Fig. 4A). Expression of PAP in PC-3 and PC-3-HHD transfectant was confirmed by immunoprecipitation (Fig. 4C). In contrast to previous reports describing PC-3 cells as negative for PAP (26, 27), we found that both lines express PAP (Fig. 4D). HHD-derived anti-PAP-3 CTLs lysed efficiently and in a dose-dependent manner PC-3-HHD but not parental PC-3 cells (Fig. 4B). Taken together, these data indicate that both PAP and STEAP proteins undergo intracellular processing in LNCaP cells.

Adoptive immunotherapy of CD11b/nu mice bearing human prostate carcinoma. We next tested the in vivo antitumor reactivity of PAP-3- and STEAP-3-induced lymphocytes. Because neither LNCaP nor PC-3-HHD cells grow progressively in HHD mice, we established a model of adoptive transfer of HHD-derived lymphocytes into CD11b/nu mice bearing prostate carcinoma. PC-3-HHD cells at 5 × 10⁶ admixed with Matrigel but not LNCaP cells formed homogeneous s.c. tumors in 100% of the CD11b/nu mice. To induce peptide-specific CTLs for adoptive transfer, we vaccinated HHD mice with PAP-3, STEAP-3, and HLA-A2.1-restricted, tyrosinase-derived peptide as an irrelevant control, all loaded on syngeneic dendritic cells. CD11b/nu mice were inoculated s.c. with the PC-3-HHD, and 7 days later, after formation of visible tumors, CTLs were injected around the tumors as described. We observed that 11 × 10⁶ per mouse of anti-PAP-3 CTLs completely prevented progressive growth of PC-3-HHD tumors in CD11b/nu mice, whereas the same amount of irrelevant peptide-induced CTLs failed to inhibit tumor development (Fig. 5A). The CTLs induced by STEAP-3 (3 × 10⁶ per mouse) were potent in decreasing the tumor volumes compared...
with the control groups (Fig. 5B). Thus, using adoptive immunotherapy of human prostate carcinoma as an experimental model, we confirmed a strong *in vivo* antitumor reactivity of PAP-3- and STEAP-3-specific CTLs.

**Activation of prostatic acid phosphatase-3–specific and six-transmembrane epithelial antigen of prostate-3–specific CTL precursors in peripheral blood mononuclear cells derived from healthy volunteers and prostate cancer patients.** We then asked whether PAP-3- and STEAP-3-specific CTL precursors are found in PBMC of healthy volunteers and more importantly of prostate cancer patients. To this end, bulk lymphocytes from three healthy and two prostate cancer donors were *in vitro* primed over autologous dendritic cells pulsed with the synthetic peptides followed by two additional cycles of restimulation as described in Materials and Methods. Activated CTLs lysed the TAP-2-deficient HLA-A2.1* T2 target cells loaded with PAP-3 or STEAP-3 and not unloaded cells (Fig. 6A). Moreover, peptide-specific CTLs lysed the HLA-A2.1-positive LNCAp variant (ATCC CRL-1740) but not the HLA-A2.1-negative LNCAp variant (ATCC CRL-10995) and PC-3 cells (Figs. 4C and 6B and C). Based on these data, one may conclude that there are PAP-3- and STEAP-3-specific and potent CTL precursors in peripheral blood of healthy and prostate cancer donors.

**Discussion**

Two approaches govern active anticancer vaccination. The first uses vaccines directed against unidentified antigens as irradiated tumor cells, either intact or genetically modified, total tumor RNA, or cell lysates (28). An alternative strategy, allowing both monitoring of immune response and rational systematic improvement of vaccine design, is based on use of antigen-specific cancer vaccines that induce immune responses against defined antigens. Indeed, active vaccination with a single TAA epitope has been shown to protect from tumor challenge and to palliate established tumors *in vivo* (29). Relatively few prostate cancer–derived epitopes have been identified and examined in clinical trials thus far. Contrary to melanomas, prostate cancer–associated epitopes,
such as PSMA derived epitopes, were largely ineffective in specific CTL and clinical responses (9, 10).

In the current study, we identified two HLA-A2.1-restricted CTL epitopes, PAP-3 and STEAP-3, which induce antitumor immunity in vitro and in vivo. Identification of TAA peptides by the reverse approach is based on recognition of candidate TAAs, in silico prediction of HLA-binding epitopes, and testing the peptides for their ability to induce an immune response in human PBMC. This strategy is hard to reproduce and it is highly dependent on the individual heterogeneity of the population. To overcome these problems, we used the HHD mouse model for initial screening of immunogenic peptides derived from candidate TAAs. The HHD mouse combines classic HLA transgenesis (HLA-A2.1/D b-2m single chain) with selective destruction of murine H-2 (double knock out deletion of the murine H-2m and D b genes) that restricts the whole class I–derived T-cell repertoire to HLA-A2.1. Others and we have identified several HLA-A2.1-restricted epitopes from TAAs, such as MUC1 (21), BA46 (22), PTH-rP (30), HER-2/neu and hTERT (31), and MAGE-A8 (32). PAP, PSA, PSMA, and STEAP were found to be overexpressed in prostate cancer versus BPH samples (Fig. 1). Twenty HLA-A2.1-restricted peptides derived from the protein sequences were selected according to their calculated MHC-binding affinities (Supplementary Data). Positive correlation between calculated binding affinities and immunogenicity of CTL epitopes has been shown (24, 33). Using a two-step screening in HHD mice, we compared in the same experiment the immunogenic potential of each peptide with that of others. Two peptides, PAP-3 and STEAP-3, were highly immunogenic in HHD mice (Fig. 2). One possible explanation for the immunogenicity of PAP-3 and STEAP-3 could be that they represent sequences with minimal similarity to the murine homologues and therefore induce xenogenic responses. Sequence alignment between PAP or STEAP and their murine homologues displays 81% identity in protein sequences in both pairs. Moreover, the PAP-3 and STEAP-3 murine counterparts show conservation of eight/nine amino acids (I/L-LWQPIPV→RLLWQPIPV and LLLGTIHAL→YLLGTIHAL, respectively), keeping the anchor motifs in positions 2 and 9 (34).

To test the immunotherapeutic potential of PAP-3 and STEAP-3, we established an adoptive transfer model in which PC-3-HHD tumor-bearing nude mice were treated by peptide-specific lymphocytes transferred to the tumor vicinity from immunized HHD mice. In immunoprotection experiments on TRAMP mice, memory lymphocytes specific for T antigen have been transferred from mice immunized with SV40-transformed fibroblasts (35). Our system, we achieved a strong therapeutic effect in palpable tumor-bearing mice using peptide-induced lymphocytes (Fig. 5). PC-3-HHD cells are lysed in vitro by anti-PAP-3 (Fig. 4B) but not by anti-STEAP-3 CTLs (data not shown). Two possible mechanisms could account for the lack of lysis of PC-3-HHD by anti-STEAP-3 CTLs; low expression of STEAP by PC-3-HHD cells or low affinity of STEAP-3 to HLA-A2.1 that decreases the effective concentration of the presented peptide. We observed, however, that on adoptive immunotherapy of PC-3-HHD-bearing mice STEAP-3-induced CTL (3 × 10^5/mouse) impaired growth of the tumors (Fig. 5B). We have shown, we believe for the first time, that a CD8-mediated CTL response to STEAP can be induced in vivo.

Several studies have reported that higher frequencies of survivin (36), HER-2/neu, and carcinoembryonic antigen (37) specific CTL precursors exist in patients suffering from melanoma and colorectal cancer, respectively, than in healthy subjects. On the other hand, cancer patient–derived CTLs that were successfully activated against defined TAA epitope presented by antigen-presenting cells have shown inability to lyse cancer cells presenting the same epitope (38). In our study, PAP-3 and STEAP-3 induced human CTLs in blood lymphocytes from healthy and prostate cancer donors. Importantly, peptide-specific CTLs lysed not only peptide-loaded T2 cells but also MHC-positive LNCaP cells that naturally express the TAA of interest (Fig. 6). Yet, HLA-A2low cells are not lysed. Thus, low MHC–expressing tumors might be resistant to peptide-based immunotherapy.

Figure 5. Adoptive immunotherapy of PC-3-HHD tumor-bearing CD1nu/nu mice. CTLs specific to either PAP-3 (A, ▲, 1 × 10^6/mouse), STEAP-3 (B, ●, 3 × 10^6/mouse), or irrelevant peptide (A and B, ◆, 11 × 10^6/mouse) were obtained on vaccination of HHD mice, restimulated in vitro, and injected around the 7-day-old PC-3-HHD tumors in CD1nu/nu mice (n = 10/group) as described in Materials and Methods. All mice (n = 40) had palpable tumor at the time of adoptive transfer. Points, mean tumor size (in mm^3) of the treatment groups plotted against the days after tumor challenge; bars, SD. Tumor growth was followed for 40 days. CTLs from mice immunized with irrelevant tyrosinase-derived peptide or PBS-injected (No treatment) CD1nu/nu tumor-bearing mice are the negative controls in the experiment. Adoptive transfer of specific CTLs significantly completely inhibited (A, PAP-3, P < 0.01, unpaired Student’s t test) or slowed down (B, STEAP-3, P < 0.01, unpaired Student’s t test) tumor growth.
Characterization of Human CTL Epitopes In vivo

In conclusion, this study presents a novel approach for in vivo two-step screening of candidate HLA-A2.1-resticted CTL epitopes for cancer immunotherapy. Using the HHD mouse as a model for in vivo evaluation of immunogenic and antigenic potential of putative TAA epitopes, PAP-3 and STEAP-3 have been identified as good vaccine candidates for immunotherapy of prostate cancer.

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Figure 6. Specific antitumor reactivity of peptide-stimulated CTLs derived from healthy and prostate cancer donors. Leukapheresed nonadherent PBMCs from HLA-A2+ donors were primed in vitro with peptide-pulsed autologous dendritic cells. PBMC were supplemented with HIL-7, and 2 days later, HIL-2 was added and renewed every 3 days. Additional two cycles of restimulation were done weekly over peptide-pulsed monocytes. CTL assays were done using peptide-pulsed T2 cells (A) or prostate cancer–derived cells (B and C) as targets. A, lymphocytes derived from healthy donor were primed and restimulated with either PAP-3 (top) or STEAP3 (bottom) pulsed dendritic cells/monocytes. Relevant (●) for PAP-3 and (●) for STEAP-3 or irrelevant (●) for tyrosinase peptide-pulsed T2 cells were used as targets. Representative of three independent experiments with leukapheresis samples derived from three healthy and two prostate cancer donors. Lymphocytes derived from a healthy volunteer (B) or a prostate cancer donor (C) were primed and restimulated with PAP-3 (top) or STEAP3 (bottom) pulsed dendritic cells/monocytes. Human prostate carcinoma LNCaP (HLA-A2top and HLA-A2bottom variants) and HLA-A2 PC-3 cells were used as targets. Data obtained at E:T ratio of 10:1, 5:1, and 2.5:1 (A) and 20:1, 10:1, 5:1, and 2.5:1 (B and C). The range of triplicates was <5% of the mean of the triplicates in these experiments.

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Human CTL Epitopes Prostatic Acid Phosphatase-3 and Six-Transmembrane Epithelial Antigen of Prostate-3 as Candidates for Prostate Cancer Immunotherapy

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