Identification of Naturally Processed Helper T-Cell Epitopes from Prostate-Specific Membrane Antigen Using Peptide-Based *in Vitro* Stimulation

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ABSTRACT

Purpose: There is growing evidence that CD4+ helper T lymphocytes (HTLs) play an essential role in the induction and long-term maintenance of antitumor CTL responses. Thus, approaches to develop effective T-cell-based immunotherapy should focus in the stimulation of both CTLs and HTLs reactive against tumor-associated antigens. The present studies were performed with the purpose of identifying HTL epitopes for prostate-specific membrane antigen (PSMA) for the optimization of vaccines for prostate cancer.

Experimental Design: Synthetic peptides from regions of the PSMA sequence that were predicted to serve as HTL epitopes were prepared with use of computer-based algorithms and tested for their capacity to trigger *in vitro* HTL responses in lymphocytes from normal volunteers.

Results: Our results show that 4 peptides from PSMA were effective in eliciting HTL responses. Moreover, HTL reactive to 3 of the 4 peptides were capable of reacting with naturally processed antigen in the form of freeze/thaw lysates or apoptotic cells produced from PSMA-positive LNCaP tumor cell lines.

Conclusions: Human HTLs are capable of effectively recognizing epitopes derived from PSMA. The information presented here should facilitate the design of improved vaccination strategies for prostate cancer.

INTRODUCTION

Cancer of the prostate is a serious health problem both in the United States and worldwide (1, 2). Most significantly, prostate adenocarcinoma is the most commonly diagnosed cancer and the second leading cause of cancer deaths in American men. Although surgery and radiation remain the treatments of choice for the early (localized) stage of prostate cancer, there is no clear effective treatment for patients who develop recurrences or those who have metastatic disease of the time of diagnosis. Therefore, there is an urgent need for new types of treatments such as T-cell-based immunotherapy and vaccine-based treatments for this cancer.

Immunotherapy targeting PSMA,[2] a M_r 100,000 type II membrane glycoprotein bearing some homology with the transferrin receptor (3, 4), has gained great interest for its possible use as a novel noninvasive treatment to prevent metastatic spread or delay recurrences. Normal prostate epithelial cells produce PSMA, and its expression is increased in most prostate tumor cells, particularly in undifferentiated, metastatic, and hormone-resistant carcinomas. Thus it would be predicted that PSMA could be an ideal TAA for immunotherapies against prostate cancer.

The design and implementation of T-cell-based immunotherapy for cancer relies heavily in the identification of immunogenic T-cell peptide epitopes from TAAs capable of eliciting antitumor responses. Because MHC class I-restricted CTLs can directly kill tumor cells, most efforts have been devoted toward the identification of peptide epitopes that elicit these responses. As a consequence, CTL epitopes from PSMA restricted by the HLA-A2 and HLA-A24 alleles were described recently (5, 6), opening the door to the development of an epitope-based immunotherapy for prostate cancer.

Because HTLs play an important role both in the induction and maintenance of CTL responses (7–10), antitumor vaccines that activate both CTLs and HTLs should be more effective that vaccines that target only CTL responses (11). Ample evidence exists in animal models demonstrating the critical role that antigen-specific HTLs play in the antitumor effects of CTLs (12). Furthermore, we have reported that the presence of HTLs was critical for the complete elimination of tumor cells by CTLs in an *in vitro* model system (13). Additionally, a direct role for HTLs in tumor rejection has been demonstrated; HTLs have been shown to kill tumor cells directly and to produce lymphokines such as IFN-γ, and GM-CSF.

[2] The abbreviations used are: PSMA, prostate-specific membrane antigen; TAA, tumor-associated antigen; HTL, helper T lymphocyte; IFN-γ, interferon-γ; GM-CSF, granulocyte macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; APC, antigen-presenting cell; mAb, monoclonal antibody.

In view of this, one obvious way to improve vaccines designed to induce antitumor CTLs is to include in these vaccines MHC class II-restricted epitopes that would trigger HTL responses to TAAs.

Because our goal is to design more effective T-cell-based immunotherapies for prostate cancer using PSMA as the primary target, the present study was aimed at examining whether this TAA was capable of eliciting MHC class II-restricted CD4+ T-cell responses and to identify the corresponding peptide epitopes. We used a computer-based algorithm to identify peptides from PSMA bearing potential MHC class II binding motifs that would be capable of stimulating *in vitro* CD4$^+$ T cells from healthy volunteers. The results showed that several of the predicted epitopes were able to trigger HTL responses in individuals expressing diverse and commonly found *HLA-DR* alleles. Most importantly, some of the peptide-generated HTLs were also shown to be capable of recognizing naturally processed antigen in the form of cell lysates or apoptotic cells prepared from the PSMA-expressing LNCaP prostate tumor cell line. The present findings should hold some value for the development of epitope-based vaccines designed to elicit CTL and HTL responses against prostate cancer.

MATERIALS AND METHODS

Cell Lines. Mouse fibroblast cell lines (L-cells) transfected and expressing individual human MHC class II molecules were kindly provided by Dr. R. W. Karr (Pfeizer Global R&D, New London, CT) and by Dr. T. Sasazuki (Tokyo, Japan). The prostate cancer cell line LNCaP and the Jurkat T-cell lymphoma were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in tissue culture as recommended by the supplier. The melanoma cell line 624mel was kindly provided by Dr. S. A. Rosenberg (National Cancer Institute, NIH, Bethesda, MD).

Synthetic Peptides. Potential HLA-DR-promiscuous CD4$^+$ T-cell epitopes were selected from the amino acid sequence of the PSMA by use of algorithm tables for three *HLA-DR* alleles (*DRB1*0101*, *DRB1*0401*, and *DRB1*0701*) described by Southwood *et al.* (14). Peptides that displayed high algorithm scores were synthesized and purified as described previously (5, 15). The purities (>95%) and identities of peptides were determined by high-performance liquid chromatography and mass spectrometry, respectively.

***In Vitro* Induction of Antigen-Specific HTL Lines with Synthetic Peptide.** The procedure selected for the generation of tumor antigen-reactive HTL lines by use of peptide-stimulated PBMCs has been described in detail elsewhere (15–17). Briefly, DCs were produced in tissue culture from adherent monocytes that were cultured for 7 days at 37°C in a humidified CO$_2$ (5%) incubator in the presence of 50 ng/ml GM-CSF and 1000 IU/ml interleukin-4. Peptide-pulsed DCs (3 μg/ml for 2 h at room temperature) were irradiated (4200 rad) and cocultured with autologous purified CD4$^+$ T cells (using antibody-coated magnetic microbeads from Miltenyi Biotech, Auburn, CA) in 96-round-bottomed-well culture plates. After 7 days, the CD4$^+$ T cells were restimulated with peptide-pulsed irradiated autologous PBMCs, and 2 days later, human recombinant interleukin-2 was added at a final concentration of 10 IU/ml. One week later, the T cells were tested for their proliferative responses to

peptide as described below. Those cultures exhibiting a proliferative response to peptide (at least 2.5-fold over background) were expanded in 24- or 48-well plates by weekly restimulation with peptides and irradiated autologous PBMCs. Where T-cell clones were used (as mentioned in the "Results"), these were produced by limiting dilution as described elsewhere (15–17). Complete culture medium for all procedures consisted of RPMI 1640 supplemented with 5% human male AB serum, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin. The Institutional Review Board on Human Subjects (Mayo Foundation) approved this research, and informed consent for blood donation was obtained from all volunteers.

Measurement of Antigen-Specific Responses. CD4$^+$ T cells (3 × 10^4/well) were mixed with irradiated APCs in the presence of various concentrations of antigen (peptides, tumor lysates, or UV-irradiated tumor cells), in 96-well culture plates. APCs consisted of PBMCs (1 × 10^5/well), HLA-DR-expressing L-cells (3 × 10^4/well), or DCs (5 × 10^3/well). Tumor cell lysates were prepared by three freeze-thaw cycles of 1 × 10^8 tumor cells resuspended in 1 ml of serum-free RPMI 1640. Lysates were used as a source of antigen at 5 × 10^5 cell equivalents/ml. Culture supernatants were collected after 48 h for measuring antigen-induced lymphokine (GM-CSF) production by the HTLs as measured by ELISAs (BD PharMingen, San Diego, CA). The amount of lymphokine constitutively secreted by the T cells incubated alone (without APCs and antigen) was subtracted from all experimental determinations. Cell proliferation assays were incubated at 37°C for 72 h, and during the final 16 h each well was pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ). The radioactivity incorporated into DNA, which correlates with cell proliferation, was measured in a liquid scintillation counter after the cell cultures were harvested on glass fiber filters. To determine MHC restriction molecules involved in antigen presentation, blocking of the antigen-induced proliferative response was investigated by adding anti-HLA-DR mAb L243 (IgG2a, prepared from supernatants of the hybridoma HB-55 obtained from the ATCC; Ref 18) or anti-HLA-DQ mAb SPV-L3 (IgG2a, Beckman Coulter, Inc., Fullerton, CA). All antibodies were used at a final concentration of 10 μg/ml throughout the 48-h incubation period. All assessments of proliferative responses or ELISAs were performed at least in triplicate, and results correspond to the mean (SD) values.

RESULTS

Prediction and Selection of Potential HTL Epitopes for PSMA. Because our set goal was to identify promiscuous MHC class II HTL epitopes for PSMA, we first examined the amino acid sequence of this TAA for the presence of peptides containing binding motifs for *HLA-DRB1*0101*, *-DRB1*0401*, and *-DRB1*0701* (from here on referred simply as *HLA-DR1*, *-DR4*, and *-DR7*), using the algorithm described by Southwood *et al.* (14). This approach has been very successful in our hands, allowing the identification several promiscuous HTL epitopes from diverse TAAs such as HER2/*neu*, gp100, MAGE-A3, EBNA2, and carcinoembryonic antigen (15–17, 19, 20). Interestingly, in some circumstances T-cell responses induced by
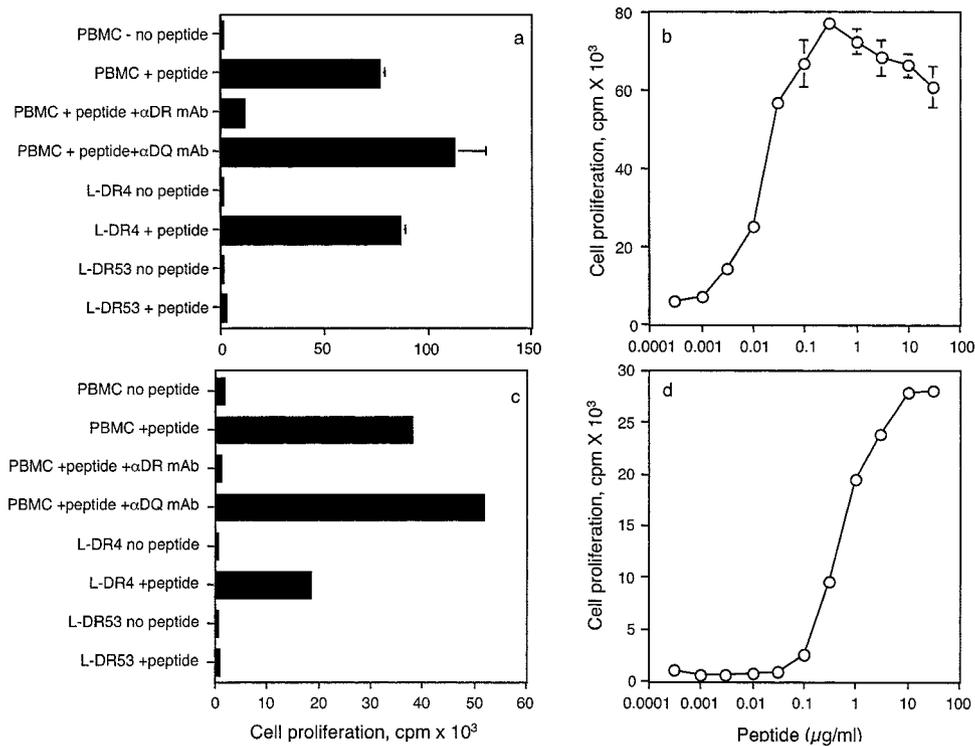
Fig. 1 HLA-DR4-restricted T-cell responses to two PSMA peptides. Two individual HTL clones were selected from a HLA-DR4/DR15 normal male individual by weekly stimulation of CD4$^+$ T cells with peptide PSMA$_{334-348}$ (*a* and *b*) and peptide PSMA$_{567-581}$ (*c* and *d*) as described in "Materials and Methods." *a* and *c*, when autologous PBMCs were used as APCs, proliferation of the HTLs to their corresponding peptides (3 μg/ml) was inhibited by anti-HLA-DR mAb L243 at 10 μg/ml but not by anti-HLA-DQ mAb SPV-L3 at 10 μg/ml. When mouse L-cell fibroblasts transfected with human *HLA-DR* genes were used as APCs, the HTLs recognized their respective peptides in the context of HLA-DR4 molecules. Peptide dose–response curves were constructed to estimate the overall avidity of the two HTLs for peptides PSMA$_{334-348}$ (*b*) and PSMA$_{567-581}$ (*d*) with autologous PBMCs as APCs. Values shown are triplicate determinations; *error bars*, SD. *Columns* and *symbols without error bars* had SD <10% the value of the mean.

peptides predicted by this approach were restricted by MHC class II alleles other than *HLA-DR1, -DR4,* and *-DR7,* such as *-DR9, -DR16, -DR52, -DR53, -DQ2,* and *-DQ6.* For the present analysis, a total of 11 peptide sequences from PSMA (containing a total of 750 residues) were identified as probable binders to HLA-DR1, -DR4, and -DR7 and thus were potentially promiscuous MHC class II-restricted T-cell epitopes (data not shown). From the 11 peptide sequences of PSMA predicted by this algorithm, we randomly selected 6 peptides to determine their ability to trigger HTL responses *in vitro* using PBMCs from normal volunteers.

T-Cell Responses to Peptides from PSMA. Peptides PSMA$_{154-168}$, PSMA$_{334-348}$, PSMA$_{567-581}$, PSMA$_{660-674}$, PSMA$_{687-701}$, and PSMA$_{730-744}$ were selected and tested for their ability to stimulate CD4$^+$ T-cell responses in healthy, MHC-typed, male individuals (HLA-DR1/13, HLA-DR4/15, HLA-DR4/9, HLA-DR7/17, and HLA-DR8/9) on the basis of primary *in vitro* lymphocyte cultures stimulated with peptide-pulsed autologous DCs as APCs. Four of the six peptides (PSMA$_{334-348}$, PSMA$_{567-581}$, PSMA$_{687-701}$, and PSMA$_{730-744}$) were effective in inducing proliferative T-cell responses in our hands. Peptides PSMA$_{334-348}$ and PSMA$_{567-581}$ induced antigen-specific responses in CD4$^+$ T cells that were restricted by the *HLA-DR4* allele (Fig. 1). The responses of both T-cell clones

were shown to be restricted by HLA-DR4 molecules by the capacity of DR4-transfected L-cells to present peptide to the T cells and by the ability of anti-DR antibodies (but not anti-DQ antibodies) to block the T-cell proliferative responses (Fig. 1, *a* and *c*). In both cases, the T cells responded to peptide presented by autologous PBMCs in a dose-dependent manner (Fig. 1, *b* and *d*). However, the HTLs responding to PSMA$_{334-348}$ displayed higher affinity for the antigen (~30-fold) than the HTL responding to PSMA$_{567-581}$, as assessed by the amount of peptide required to attain the maximum proliferative response (compare *panels b* and *d* in Fig. 1). These results indicate that PSMA contains at least two HLA-DR4-restricted epitopes for HTLs.

Two distinct T-cell lines reactive with peptide PSMA$_{687-701}$ isolated from two normal individuals were studied in detail for their responses to antigen and MHC restriction patterns. The data presented in *panels a* and *c* of Fig. 2 indicate that both of the T-cell lines recognized this peptide presented by autologous APCs and that antibodies to HLA-DR but not to HLA-DQ inhibited this reactivity. Interestingly, studies performed to determine the HLA restriction elements for these T-cell lines indicated that in one case the response to peptide PSMA$_{687-701}$ was restricted by HLA-DR9 (Fig. 2*b*), whereas in the other case, the response to peptide PSMA$_{687-701}$ was restricted by HLA-
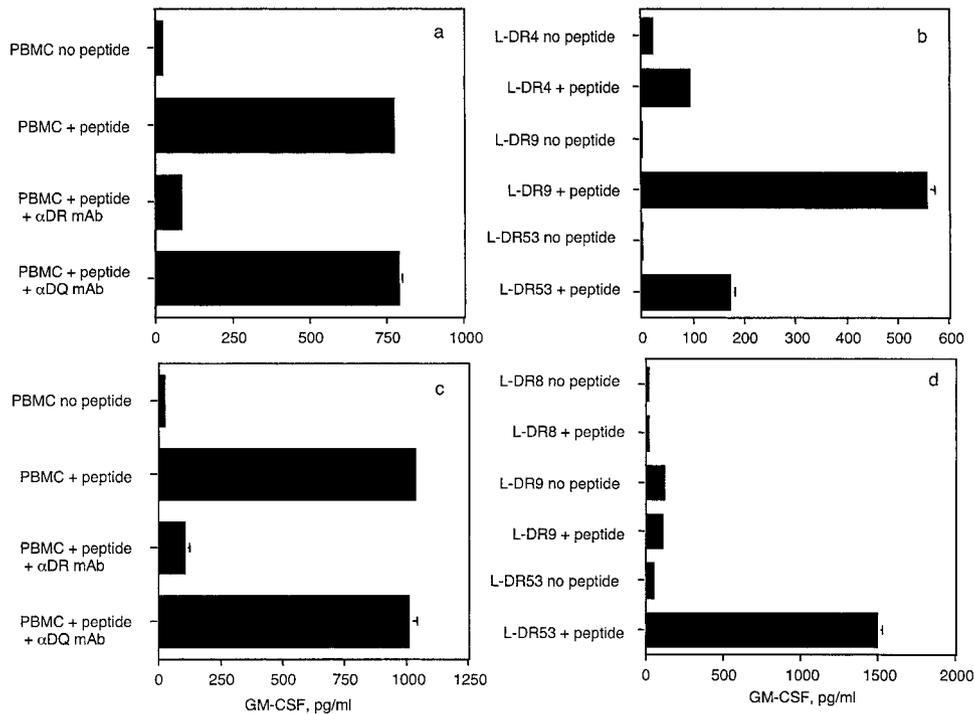
Fig. 2 HTL responses to peptide PSMA$_{687-701}$. HTL lines were selected from a HLA-DR4/DR9, -DR53 normal male individual (*a* and *b*) and from a HLA-DR8/DR9, -DR53 normal male individual (*c* and *d*) by weekly stimulation of CD4$^+$ T cells with peptide as described in "Materials and Methods." When autologous PBMCs were used as APCs, both HTL responses to peptide PSMA$_{687-701}$ were inhibited by anti-HLA-DR mAb L243 at 10 μg/ml but not by anti-HLA-DQ mAb SPV-L3 at 10 μg/ml (*a* and *c*). When mouse L-cell fibroblasts transfected with human *HLA-DR* genes were used as APCs, one of the HTLs recognized peptide PSMA$_{687-701}$ (3 μg/ml) in the context of HLA-DR9 molecules (*b*), and the other HTL recognized the peptide in the context of HLA-DR-53 (*d*). Values shown are triplicate determinations; *error bars*, SD. *Columns* and *symbols without error bars* had SD <10% the value of the mean.
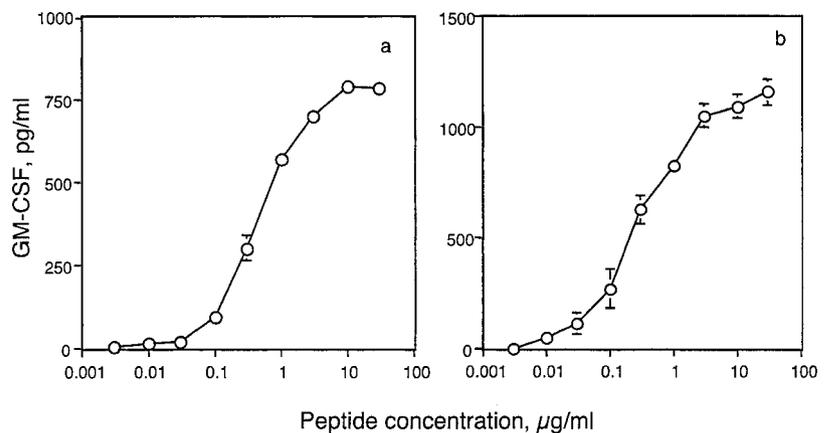

Fig. 3 Both HTL lines reactive to PSMA$_{687-701}$ have similar affinities for antigen. Peptide dose–response curves were constructed to estimate the overall avidity of the HLA-DR9- (*a*) and the HLA-DR53-restricted (*b*) HTLs for peptide PSMA$_{687-701}$, with autologous PBMCs used as APCs. Values shown are triplicate determinations; *error bars*, SD. *Symbols* without *error bars* had SD <10% the value of the mean.

DR53 (Fig. 2*d*). Nonetheless, the "HLA-DR9-restricted" HTLs were also able to recognize to some extent the PSMA$_{687-701}$ peptide in the context of HLA-DR53 and -DR4 molecules (Fig. 2*b*), indicating some level of degeneracy of the T-cell receptor of this HTL for its antigen. The peptide dose–response curves for both HTLs revealed similar degrees of affinity for the antigens, which required ~10 μg/ml to attain a maximum response (Fig. 3).

Peptide PSMA$_{730-744}$ was able to stimulate a T-cell response in the HLA-DR4/9 individual, which was restricted by HLA-DR53 (Fig. 4, *a* and *b*). In addition, as shown in Fig. 4*c*, the dose–response curve to peptide PSMA$_{730-744}$ indicated that the affinity of this cell for antigen was similar to the above-described HTLs requiring ~10 μg/ml peptide to obtain the maximum response. Lastly, peptides PSMA$_{154-168}$ and PSMA$_{660-674}$ were unable to trigger T-cell responses in any of
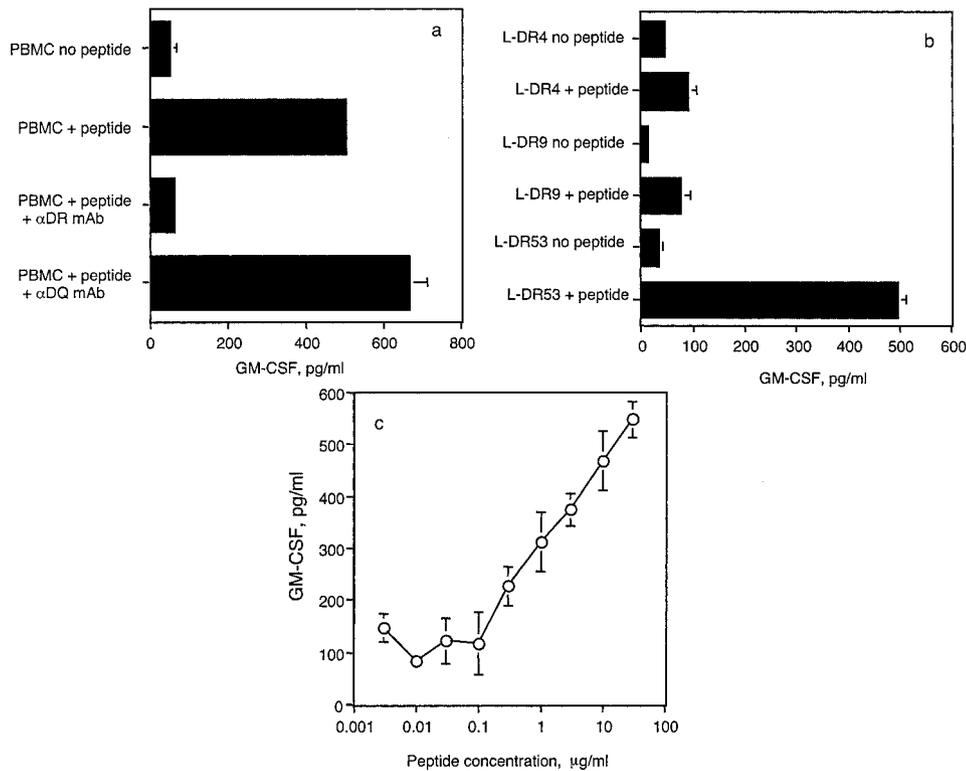
Fig. 4 HLA-DR53-restricted T-cell responses to peptide PSMA$_{730-744}$. A HTL line was selected from a HLA-DR4/DR9, -DR53 normal male individual by weekly stimulation of CD4$^+$ T cells with peptide as described in "Materials and Methods." When autologous PBMCs were used as APCs (a), the T-cell responses to peptide PSMA$_{730-744}$ were inhibited by anti-HLA-DR mAb (L243 at 10 μg/ml) but not by anti-HLA-DQ mAb (SPV-L3 at 10 μg/ml). When mouse L-cell fibroblasts transfected with human HLA-DR genes were used as APCs (b), the HTLs recognized peptide PSMA$_{730-744}$ (at 3 μg/ml) in the context of HLA-DR53 molecules. Peptide dose–response curves were constructed to estimate the overall avidity of the HTLs for peptide PSMA$_{730-744}$ when autologous PBMCs were used as APCs (c). Values shown are triplicate determinations; error bars, SD. Columns and symbols without error bars had SD <10% the value of the mean.

the MHC class II-typed individuals (data not shown), suggesting that these peptides may not be as immunogenic as the other peptides described above.

Recognition of Processed Tumor Antigens by PSMA Peptide-Reactive HTLs. The data presented above demonstrate that the peptides PSMA$_{334-348}$, PSMA$_{567-581}$, PSMA$_{687-701}$, and PSMA$_{730-744}$ were indeed capable of inducing CD4$^+$ T-cell responses. However, one of the hallmarks of antitumor T-cell responses is the capacity of the T cells to recognize TAAs that are naturally processed by APCs. Thus it is critical to assess whether APCs that capture TAAs in the form of cell lysates or apoptotic/dead cells derived from PSMA-expressing tumor cells are able to process this antigen appropriately to stimulate the peptide-reactive HTLs. Only under these circumstances can one be certain that the predicted T-cell epitopes will be biologically relevant for developing immunotherapy against PSMA-expressing prostate cancer. Consequently, we tested the capacity of the HTL lines and clones that were induced with synthetic peptides from PSMA to recognize naturally processed antigen in the form of tumor cell lysates (freeze/thaw) and apoptotic (irradiated) tumor cells. As shown in Fig. 5a, the HLA-DR4-restricted, PSMA$_{334-348}$-reactive T-cell clone responded well to autologous DCs that were pulsed with cell lysates prepared from the PSMA-expressing LNCaP tumor but not with DCs pulsed with lysates from the PSMA-negative Jurkat T-cell line. Moreover, anti-HLA-DR antibodies (but not anti-HLA-DQ antibodies) were able to block to a large extent the recognition of LNCaP lysates by the HTLs, indicating that the response was mediated through the recognition of antigen by the T-cell receptor. These results demonstrate that the epitope

represented by peptide PSMA$_{334-348}$ is produced by the antigen-processing MHC class II pathway occurring on APCs. We also evaluated the PSMA$_{334-348}$-reactive HTL clone for its capacity to recognize naturally processed tumor antigen, using DCs that were fed with intact apoptotic PSMA-expressing tumor cells (in this case, UV-irradiated LNCaP tumor cells). The data presented in Fig. 5b illustrate that the HLA-DR4-restricted, PSMA$_{334-348}$-specific T-cell clone was also effective in recognizing dead/apoptotic PSMA-positive LNCaP cells but not PSMA-negative Jurkat T-cell lymphoma cells when autologous DCs were used as APCs. A similar analysis was performed with the HLA-DR4-restricted, PSMA$_{567-581}$-reactive HTL clone, but in this case, T-cell responses to APCs pulsed with either tumor cell lysates or apoptotic tumor cells could not be detected (data not shown).

We next determined whether the two HTL lines that were induced with peptide PSMA$_{687-701}$ could also recognize naturally processed antigen, using autologous DCs that were fed with freeze/thaw tumor cell lysates. The data presented in Fig. 6 demonstrate that the both the HLA-DR9- (Fig. 6a) and the HLA-DR53-restricted (Fig. 6c) PSMA$_{687-701}$-peptide-reactive HTLs were quite effective in responding in a dose-dependent manner to DCs fed with cell lysates from PSMA-positive LNCaP cells but not PSMA-negative melanoma 624mel. Moreover, the recognition of naturally processed PSMA in the form of tumor lysates by these HTLs was inhibited to a great extent by anti-HLA-DR antibodies (Fig. 6, b and d), indicating that, as expected, the presentation of the naturally occurring peptide is mediated by MHC class II molecules.

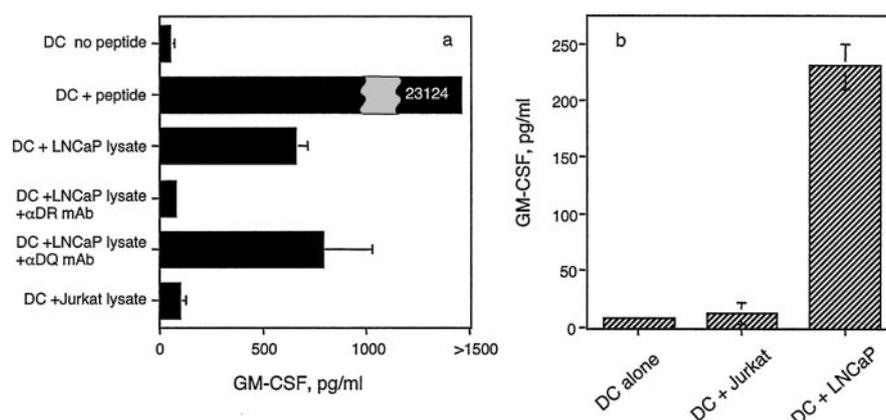Lastly, similar experiments demonstrated that the HLA-

Fig. 5 HLA-DR4-restricted, PSMA$_{334-348}$-specific HTL line recognizes naturally processed PSMA. With autologous DCs used as APCs, the peptide-induced CD4$^+$ HTLs were tested for their capacity to react with antigen in the form of freeze/thaw tumor lysates (*a*). This reactivity was inhibited by anti-HLA-DR mAb L243 at 10 μg/ml but not by anti-HLA-DQ mAb SPV-L3 at 10 μg/ml. The HTLs can recognize UV-irradiated PSMA-positive LNCaP tumor cells via antigen cross-presentation by autologous DCs (*b*). In contrast, DCs incubated with the PSMA-negative Jurkat T-cell lymphoma (*b*) or DC alone (*b*) were not able to stimulate the HTLs. Autologous DCs were incubated with UV-irradiated tumor cells at a 1:1 ratio for 48 h. Various number of antigen-pulsed DCs were then mixed with HTL, and 2 days later, culture supernatants were collected and assayed for the presence of GM-CSF. Values shown are triplicate determinations; *error bars*, SD. *Columns without error bars had SD* <10% the value of the mean.

Fig. 6 Recognition of naturally processed antigen by the HLA-DR9-restricted, PSMA$_{687-701}$-specific HTL line (*a* and *b*) and the HLA-DR53-restricted, PSMA$_{687-701}$-specific-HTL line (*c* and *d*). When autologous DCs were used as APCs, the peptide-reactive HTLs were able to respond to various amounts of cell lysates derived from LNCaP tumor cells expressing PSMA but not to PSMA-negative 624 melanoma (*624mel*; *a* and *c*). The recognition of tumor lysate via autologous DCs was inhibited by anti-HLA-DR mAb L243 at 10 μg/ml but not by anti-HLA-DQ mAb SPV-L3 at 10 μg/ml (*b* and *d*). Values shown are triplicate determinations; *error bars*, SD. *Columns and symbols without error bars* had SD <10% the value of the mean.
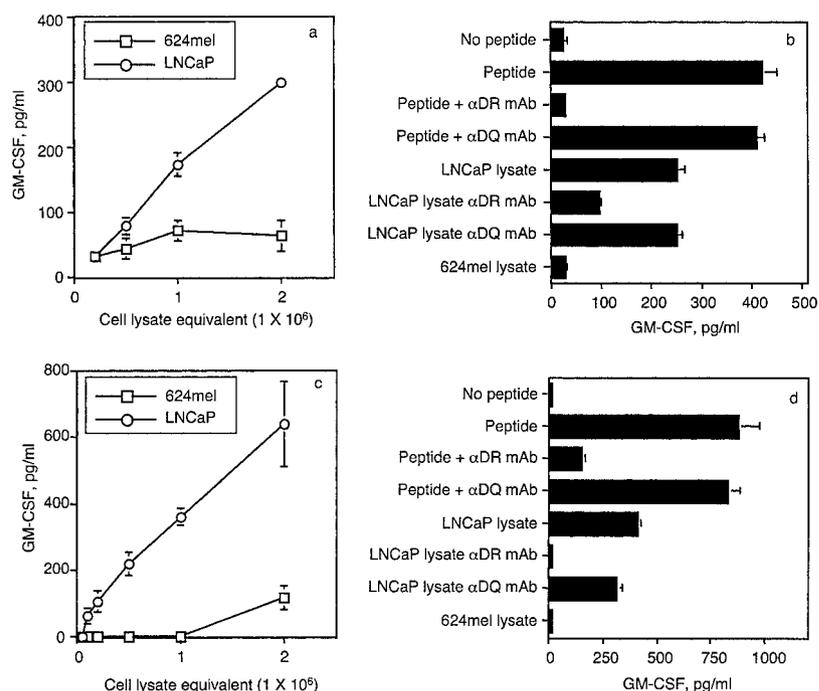
DR53-restricted, T-cell line that was induced with peptide PSMA$_{730-744}$ could also recognize naturally processed antigen in the form of tumor cell lysates presented by DCs (Fig. 7*a*). The capacity of these HTLs to recognize naturally processed PSMA was inhibited by anti-HLA-DR antibodies (but not anti-HLA-DQ antibodies), confirming that the processed T-cell epitope represented by PSMA$_{730-744}$ was presented in the context of MHC class II molecules (Fig. 7*b*).

DISCUSSION

We report here the identification of several peptide epitopes from PSMA capable of triggering HTL responses. Although PSMA CTL epitopes restricted by HLA-A2 and -A24 have been described (5, 6), previous to this report no HTL epitopes for this TAA had been identified. To our knowledge, the only other HTL epitopes for a prostate TAA reported to date have been against human kallikrein 4 (21).
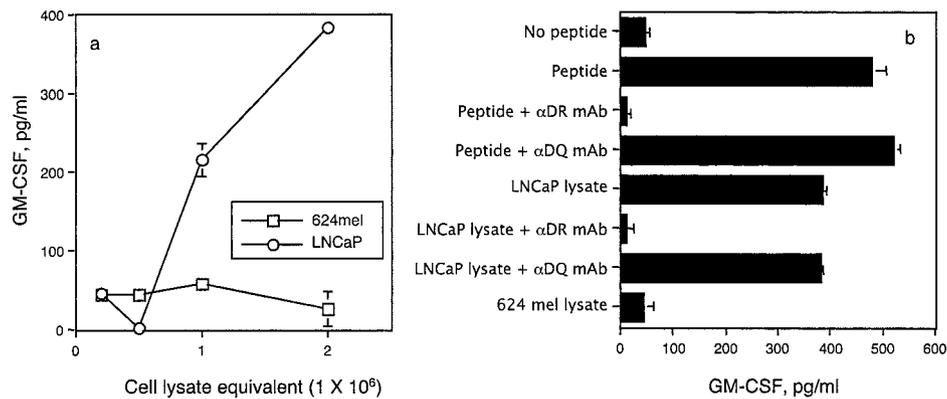
Fig. 7 Recognition of naturally processed antigen by the HLA-DR53-restricted, PSMA$_{730-744}$-specific HTL line. When autologous DCs were used as APCs, the peptide-reactive HTLs were able to respond to various amounts of cell lysates derived from LNCaP tumor cells expressing PSMA but not to PSMA-negative 624 melanoma (*624mel; a*). The recognition of tumor lysate via autologous DCs was inhibited by anti-HLA-DR mAb L243 at 10 μg/ml but not by anti-HLA-DQ mAb SPV-L3 at 10 μg/ml (*b*). Values shown are triplicate determinations; *error bars*, SD. *Columns* and *symbols without error bars* had SD <10% the value of the mean.

We are convinced that in any vaccine strategy, the induction of tumor-reactive HTLs will be critical for the development of effective antitumor properties, even if these are mediated solely by CTLs (11). HTLs can participate in the induction and maintenance of CTL responses at various time points of the immune response. During the induction of CTLs, which occurs in secondary lymphoid organs (lymph nodes and spleen), the interaction HTLs with APCs results in the further activation/maturation of the APCs necessary for the effective presentation of antigen to CTL precursors (22–24). This process, which is known as "APC licensing," occurs mainly through CD40/CD40L interactions between APCs and HTLs and consequently can take place with HTLs that become activated by surrogate (non-tumor-related) antigens such as tetanus toxoid and keyhole limpet hemocyanin. For this reason, some peptide-based vaccines have included surrogate HTL epitopes to increase the immunogenicity of CTL tumor epitopes. It has recently been reported that antigen priming of CTLs in the presence of HTLs can lead to the generation of effective CTL memory responses (7–10), which in the case of antitumor immunity would reduce the likelihood of tumor recurrences. Thus, this would be another benefit of stimulating HTL responses in vaccines that are designed to elicit antitumor CTLs.

Another important function of HTLs takes place at a later time point at the tumor site during the effector phase of the immune response. Here, HTLs can be stimulated by either MHC class II-positive tumors or by tumor-resident APCs that capture, process, and present tumor-derived antigens in the context of MHC class II. As a result of activation at the tumor site, the HTLs will produce lymphokines such as IFN-γ that will increase MHC class I expression on the tumor cells, making these cells better targets for CTL lysis. In addition, other lymphokines, such as tumor necrosis factor, produced by locally activated HTLs could display a direct antitumor effect. Activated HTLs at the tumor site also enhance CTL function by allowing these effector cells to persist and to proliferate through the production of growth factors and direct costimulation (13). It becomes evident that HTLs specific for surrogate antigens

would not be able to perform these functions at the tumor site because there would be no reason for these antigens to be present at this site. Thus, because only tumor-reactive HTLs will be able to enhance CTL function at the tumor site, it would be preferable to use tumor-derived HTL epitopes in vaccines designed to elicit antitumor CTL immunity.

Our data demonstrate that several of the peptide-stimulated HTLs were effective in recognizing naturally processed PSMA in the form of tumor cell lysates or apoptotic tumor cells that were presented by DCs. The ability of HTLs to recognize naturally processed antigen depends on numerous factors. First and perhaps most importantly, is the issue of whether the peptide representing the predicted HTL epitope is generated by the antigen-processing machinery of the APCs. TAAs such as PSMA that are ingested by APCs in the form of tumor lysates or apoptotic cells undergo proteolytic degradation in the endosomal compartments of the APCs, resulting in the generation of a multitude of peptide fragments that may or may not bind to MHC class II molecules, depending on the presence of anchor residues on the peptides that allow them to interact with complementary pockets on the MHC peptide-binding groove. It is conceivable that a peptide that is predicted to function as a HTL epitope based on the presence of such MHC-binding anchor residues will not be generated as such by the APCs, *e.g.,* because it becomes completely degraded by the proteolytic machinery of the APCs before it gets a chance to bind to MHC. These types of epitopes, referred to as "cryptic epitopes," would not be of much use for inducing HTLs that are expected to augment CTL activity at the tumor site.

Other factors that determine whether a peptide-induced HTL can recognize naturally processed antigen are those related to the overall binding affinity (or avidity) of the T cell to the APC, which in turn will be influenced by the density of specific MHC/peptide complexes on the APC, the affinity of the T-cell receptor for these complexes, and the presence of adhesion molecules on both T cells and APCs, which help stabilize the binding between these cells. Our results clearly demonstrate that HTLs induced with peptides PSMA$_{334-348}$, PSMA$_{687-701}$, and

PSMA$_{730-744}$ were quite effective in recognizing naturally processed PSMA, indicating that these peptide epitopes are not cryptic and that the overall affinity interactions between the T cells and APCs is sufficiently strong to allow activation of the HTLs. On the other hand, the observation that the HLA-DR4-restricted HTL reactive with peptide PSMA$_{567-581}$ failed to recognize naturally processed antigen could indicate that either this epitope is cryptic or that the HTL lacked sufficient overall affinity to respond under these circumstances. However, these results are based on a single T-cell line, and further work will be required to assess whether PSMA$_{567-581}$ is a naturally processed HTL epitope.

In summary, we report here the identification three novel HTL epitopes from PSMA that are restricted by MHC class II alleles expressed in a large proportion of the population. Inclusion of these HTL epitopes in CTL-based immunotherapy for prostate cancer may enhance the effectiveness of this approach to treat patients or to prevent recurrences of their disease.

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