

# Identification of MHC Class II-restricted T-cell Epitopes in Prostate-specific Membrane Antigen<sup>1</sup>

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## ABSTRACT

An effective tumor vaccine may be required to induce both CTLs and T-helper (Th) responses against tumor-associated antigens. CD4<sup>+</sup> Th cells that recognize MHC class II-restricted epitopes play a central role in the initiation and maintenance of antitumor immune responses. Prostate-specific membrane antigen (PSMA) is highly expressed in prostate cancer and thus is a potential target for prostate cancer immunotherapy. In this study, we attempted to identify Th epitopes derived from PSMA for enhancing prostate cancer vaccine by eliciting PSMA-specific Th responses. We first screened a panel of six epitope peptide candidates selected with the TEPITOPE program and found that all six peptides induced peptide-specific T-cell proliferation from one or more donors with estimated T-cell precursor frequencies of 0–4.17 × 10<sup>-6</sup>. We then established peptide-specific T-cell clones for five of these six peptides and demonstrated that the T-cell clone specific for the PSMA<sub>459</sub> epitope (NYTLRVDTPLMYSL) can recognize processed antigens from recombinant PSMA proteins. The PSMA<sub>459</sub> peptide was found to induce CD4<sup>+</sup> T-cell responses in healthy individuals and prostate cancer patients with different HLA-DR alleles. To test the potential clinical application, human HLA-DR4 transgenic mice were immunized with PSMA<sub>459</sub> peptide and we found that PSMA<sub>459</sub> peptide immunization activated T cells that specifically responded to antigenic peptides derived from PSMA proteins and PSMA-positive tumor. Thus, the naturally processed Th epitope

PSMA<sub>459</sub> could be included in prostate tumor vaccines to enhance PSMA-specific CTL responses.

## INTRODUCTION

PSMA<sup>4</sup> is a type II integral membrane protein with a short NH<sub>2</sub>-terminal intracellular domain, a membrane-spanning domain, and a large COOH-terminal extracellular domain (1, 2). PSMA, a folate hydrolase/carboxypeptidase, is very highly expressed in prostate cancer tissues and normal prostate tissues, although it is also expressed in breast and other tissues at lower levels. It has also been found that PSMA is expressed in tumor-associated neovasculature, but not in normal vasculature (3, 4). A potential problem in activating an immune response against tissue-specific antigens is that this may induce an undesirable autoimmune response. However, from numerous tumor vaccination trials with tumor antigens shared by normal tissues, no apparent autoimmunity was observed (5–7). Thus, PSMA is a potential target for prostate cancer immunotherapy.

Accumulating evidence indicates that CD4<sup>+</sup> Th cells play critical roles in initiating, regulating, and maintaining antitumor immune responses (8, 9). Such Th cells exert helper activity for the induction and maintenance of CD8<sup>+</sup> CTLs (8, 10). They also have an effector function against tumors via macrophage activation, cytokine production, or direct killing of MHC class II-positive tumors (8, 11–15). Dissection of cellular interactions reveals that Th cells must recognize antigens on the same APC that cross-presents the CTL epitopes in a cognate manner, indicating the requirement for epitope linkage between Th and CTL epitopes for induction of potent antitumor immune responses (16–19). Combined application of class I and class II epitopes derived from the same tumor antigen such as PSMA may be essential to induce potent and long-term antitumor responses.

Despite the importance of CD4<sup>+</sup> Th cells in the induction of antitumor immunity, MHC class II-restricted Th epitopes in PSMA remain to be identified. We hypothesized that Th epitopes could exist in PSMA, which may induce PSMA-specific Th responses. In this study, we combined TEPITOPE peptide prediction (20–22) with *in vitro* T-cell biological analysis to identify natively processed class II-restricted epitopes in PSMA. The results demonstrate that the PSMA<sub>459</sub> epitope (NYTLRVDTPLMYSL) is a naturally processed class II epitope in PSMA that could be used to enhance the potency of pros-

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<sup>4</sup> The abbreviations used are: PSMA, prostate-specific membrane antigen; APC, antigen-presenting cell; CFA, complete Freund's adjuvant; DC, dendritic cell; PBMC, peripheral blood mononuclear cell; SI, stimulation index; TAA, tumor-associated antigen; Th, T-helper; ATCC, American Type Culture Collection; FBS, fetal bovine serum; rhIL, recombinant human interleukin; mAb, monoclonal antibody; PE, phycoerythrin; hTERT, human telomerase reverse transcriptase.

## A PSMA

1 MWNLLHETDS AVATARRPRW LCAGALVLAG GFFLLGFLFG WFIKSSNEAT NITPKHNMKK  
**PSMA<sub>17</sub>**

61 FLDELKAENI KKFLHNTQI PHLAGTEQNF QLAQIQSQW KEFGLDSVEL AHYDVLLSYP  
**PSMA<sub>100</sub>**

121 NKTHPNYISI INEDGNEIFN TSLFEP PPPG YENVSDIVPP FSAFSPQGMPEGLDVVNYA

181 RTEDFFKLER DMKINCSGKI VIARYGKVF GNKVNAQLA GAKGVILYSYD PADYFAPGVK  
**PSMA<sub>206</sub>**

241 SYPDGWNLP GGVQRGNILN LNGAGDPLTP GYPANEYAYR RGIAEAVGLP SIPVHPIGYY

301 DAQKLEKMG GSAPPDSSWR GSKVPYNVG PGFTGNFSTQ KVKMHIHSTN EVTRIYNVIG

361 TLRGAVEPDR YVILGGHRDS WVFGIDPQS GAAVVHEIVR SFGTLKKEGW RPRRTLFS

421 WDAEEFLLG STEWAEENSR LLQERGVAYI NADSSIEGNY TLRVDCTPLM YSLVHNLTKE  
**PSMA<sub>459</sub>**

481 LKSPDEGFEG KSLYESWTKK SPSPEFSGMP RISKLGSGND FEVFFQRLGI ASGRARYTKN

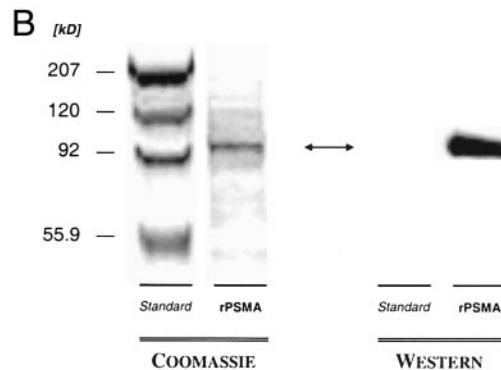
541 WETNKFSGYL LYHSVYETYE LVEKFDYPMF KYHLTVQVR GGMVFELANS IVLPFDCDRY  
**PSMA<sub>576</sub>**

601 AVVLRKYADK IYSISMKHPQ EMKTVSVSPD SLFSAVKNFT EIASKFSERL QDFDKSNPIV

661 LRMNDQLMF LERAFIDPLG LPDRPFYRHY IYAPSSHNY AGESFPGIYD ALFDIESKVD

721 PSKAWGEVKR QIYVAAFTVQ AAAETLSEVA  
**PSMA<sub>730</sub>**

*Fig. 1* A, schematic representation of predicted MHC class II epitopes within PSMA. The MHC class II-restricted epitopes in PSMA predicted with the TEPITOPE program at the 1% threshold are depicted within the complete amino acid sequence of human PSMA. B, production and analysis of recombinant PSMA protein. SF9 insect cells were infected with recombinant PSMA baculoviruses (24). After lysis of whole cells, the histidine-tagged PSMA protein was purified by nickel-chelating affinity chromatography. The purified protein was analyzed on a 10% SDS-PAGE gel by Coomassie Blue staining. A Western blot using an antibody against the histidine tag showed a single band of approximately  $M_r$  100,000, as described previously (24).



tate tumor vaccines by eliciting PSMA-specific CD4+ Th responses.

## MATERIALS AND METHODS

**Epitope Prediction and Peptide Synthesis.** TEPITOPE software was used to predict potential HLA-DR-binding peptides with promiscuous binding characteristics (21, 23). The prediction threshold was set at 1%, and peptides were selected on the basis of their ability to bind to at least three of the following eight HLA-DR molecules: DRB1\*0101; DRB1\*0301; DRB1\*0401; DRB1\*0701; DRB1\*0801; DRB1\*1101; DRB1\*1501; and DRB5\*0101. Based on the prediction, six peptides [PSMA<sub>17</sub> (RPRWLCAGALVLAGGFFLLGF), PSMA<sub>100</sub> (WKEFGLDSVELAHYD), PSMA<sub>206</sub> (GKVFGRGNKVNAQLA), PSMA<sub>459</sub> (NYTLRVDCTPLMYSL), PSMA<sub>576</sub> (VAQVRGGMVFELANSIVLPFD), and PSMA<sub>730</sub> (RQIYVAAFTVQAAAE)] from human PSMA (Fig. 1A) were synthesized. EBNA<sub>482</sub> (AEGLRALLARSHVER), an irrelevant peptide from the EBV protein EBNA-1, was also synthesized. Peptides at a purity of >90% were produced by

Genemed Synthesis (San Francisco, CA) and dissolved in 100% DMSO at a concentration of 10 mg/ml.

**Recombinant Protein, mAbs, and Tissue Culture Reagents.** Recombinant PSMA proteins with an NH<sub>2</sub>-terminal histidine tag were produced from a baculovirus/insect cell expression system and purified using nickel-chelating affinity chromatography as described previously (24). The purified PSMA protein was analyzed by Coomassie Blue staining and Western blotting, which showed a single band of approximately  $M_r$  100,000 (Fig. 1B). The following hybridomas were used to produce mAbs: HB55 (L243; antihuman HLA-DR; ATCC); HB95 (W6/32; antihuman MHC class I; ATCC); HB103 (Genox3.53; antihuman HLA-DQ; ATCC); HB180 (9.3F10; antihuman MHC class II; ATCC); and 2D6 (antihuman HLA-DR and HLA-DQ monomorphic). Antihuman CD4 (RPA-T4; FITC labeled), antihuman CD4 (PE labeled), antihuman CD8 (HIT8a; PE labeled), antihuman HLA-DR (FITC labeled), and antimouse CD4 (FITC labeled) were all purchased from BD PharMingen (San Diego, CA). A monoclonal mouse antibody against His<sub>6</sub> tags (clone, 6-His) was purchased from BabCo

(Richmond, CA). Media used for cell culture were AIM-V serum-free medium (Life Technologies, Inc., Grand Island, NY), RPMI 1640 supplemented with 10% FBS (Life Technologies, Inc.) and L-glutamine/penicillin/streptomycin, and CellGenix DC serum-free medium (CellGenix). rhIL-2 was purchased from Boehringer Roche (Indianapolis, IN).

**Blood Donors and HLA Typing.** Heparinized blood was collected from healthy volunteers and prostate cancer patients in disease remission. PBMCs were isolated by Ficoll (Lymphoprep; Nycomed, Oslo, Norway) gradient separation. HLA typing was performed by PCR-sequence-specific primers DNA-based procedures (The Methodist Hospital, Houston, TX). The Institutional Review Board on Human Subjects (Baylor College of Medicine) approved this research.

**T-cell Proliferation Assay and Generation of T-cell Clones.** Donor's PBMCs were plated in U-bottomed 96-well plates (Costar) at 200,000 cells/well in AIM-V media. Peptides were added into each well at a concentration of 20  $\mu\text{g/ml}$ . A total of 48 wells were prepared for analysis of each peptide. After a week of incubation, the culture medium was removed, and the cells were resuspended in AIM-V media and tested for specific proliferative responses to corresponding peptides (20  $\mu\text{g/ml}$ ) in the presence of  $10^5$  autologous irradiated (6000 rad) PBMCs as a source of APCs. In cell proliferation assays, cells were incubated at 37°C in a 5%  $\text{CO}_2$  incubator for 72 h, and cultures were pulsed with 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine/well during the last 16 h. The incorporation of radioactivity into DNA, which correlates with cell proliferation, was measured in a beta scintillation counter (TopCount NXT; Packard) after automated cell harvesting (Packard). The results were presented as either cpm or SIs [the mean cpm for peptide-pulsed PBMCs/the mean cpm for PBMCs not exposed to peptides (25, 26)]. A T-cell line/well was considered to be positively reactive to peptide if the cpm were greater than 1000 and  $\text{SI} > 3$  (27, 28). The frequency of peptide-specific T cells was determined by dividing the number of positive wells by the total number of PBMCs seeded in the initial culture (28). Overall positive events were defined if there was a significant difference at a 95% confidence level between the number of positive wells of the peptide-containing wells compared with that of the non-peptide-containing wells. Because these low-frequency events were in accordance with Poisson distribution, a value of  $\geq 3$  of 48 peptide-containing wells was defined as a positive event ( $P < 0.05$ ).

PSMA-specific T-cell lines were cloned by limiting dilution at 0.3 cell/well in the presence of  $10^5$  irradiated allogeneic PBMCs as accessory cells and 5  $\mu\text{g/ml}$  phytohemagglutinin protein (PHA-P; Sigma). Cultures were refed with fresh RPMI 1640 containing 10 IU/ml rhIL-2 every 3–4 days. After approximately 12–14 days, growth-positive wells became visible and were tested for specific responses to PSMA peptides in a proliferation assay as described above.

**Preparation of PBMC-derived Human DCs.** Human DCs were prepared as described recently (29). Briefly, PBMCs were resuspended in serum-free DC medium (CellGenix) and incubated at 37°C in humidified 5%  $\text{CO}_2$ . The cell fraction adherent to plastic was cultured in serum-free DC medium with 1000 IU/ml recombinant human granulocyte macrophage colony-stimulating factor (R&D Systems) and 1000 IU/ml rhIL-4 (R&D Systems). On day 5, DCs were matured by stim-

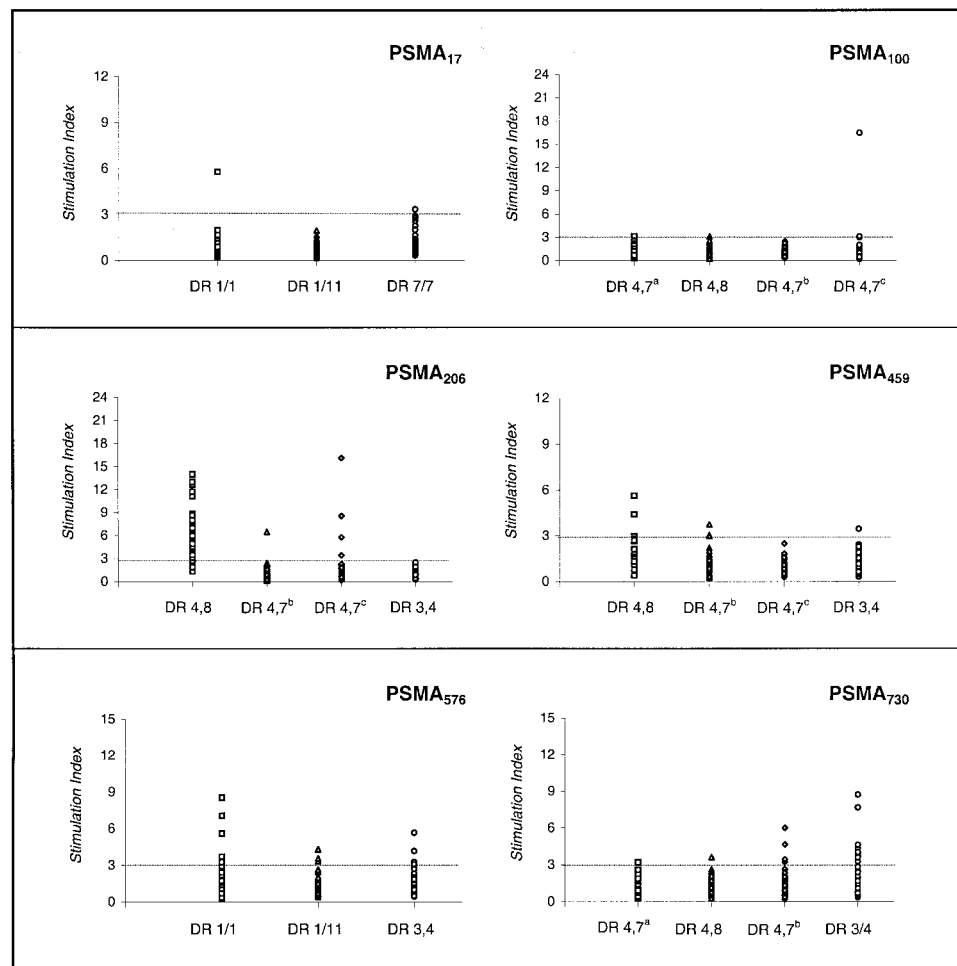
ulating with a cytokine mixture consisting of recombinant human tumor necrosis factor  $\alpha$  (10 ng/ml; R&D Systems), rhIL-1 $\beta$  (1000 ng/ml; R&D Systems), rhIL-6 (10 ng/ml; R&D Systems), and prostaglandin  $\text{E}_2$  (1  $\mu\text{g/ml}$ ; Sigma), as described previously (30).

**Analysis of Antigen-specific Proliferative Responses of T-cell Clones.** T-cells ( $2\text{--}3 \times 10^4$  cells/well) were cocultured with irradiated (4000 rad) DCs ( $1\text{--}1.5 \times 10^3$  cells/well) in complete RPMI 1640 in the presence of various concentrations of antigen (peptides and recombinant protein) in round-bottomed 96-well plates. In some cases, recombinant proteins were pulsed on DCs at day 4 during DC culture 24 h before the addition of the DC maturation mixture. To identify the MHC restriction molecules involved in antigen presentation, inhibition of antigen-induced T-cell proliferation was analyzed by the addition of various antibodies against MHC class I and MHC class II molecules at a final concentration of 20  $\mu\text{g/ml}$ . Antigen-specific T-cell responses were measured by [ $^3\text{H}$ ]thymidine incorporation during the last 16 h of a 72-h culture.

**Peptide Immunization of HLA-DR4 Transgenic Mice.** Human HLA-DR4 transgenic mice (HLA-DRB1\*0401), which are murine class II deficient and transduced with the human CD4 molecule, were generated by Dr. Grete Sonderstrup in the Department of Microbiology and Immunology of Stanford University (31–33). These transgenic mice have been successfully used to identify human class II-restricted epitopes and study immune responses (31–35). Founders of the transgenic mouse line were kindly provided by Dr. Sonderstrup and maintained and bred in a barrier facility at Baylor College of Medicine. HLA-DR4 expression in the transgenic mice was analyzed by flow cytometry. Male 6–10-week-old DR4 transgenic mice were used for the experiments. The transgenic mice were immunized twice at a 1-week interval with 100  $\mu\text{g}$  of PSMA<sub>459</sub> peptide emulsified in CFA (final volume, 100  $\mu\text{l}$ ) and administered s.c. into the rear back. Control group mice were injected with PBS emulsified in CFA.

**Evaluation of T-cell Responses by IFN- $\gamma$  ELISPOT Assay.** A high-resolution ELISPOT assay for IFN- $\gamma$  has been used to analyze peptide-specific T-cell responses by determining the frequency of T-cell precursors specific for a peptide. Mice were sacrificed 10 days after the last immunization, and splenocytes were obtained for assessing IFN- $\gamma$  production. Briefly, 96-well MultiScreen-IP plates (Millipore Corp., Bedford, MA) were coated with 100  $\mu\text{l/well}$  capture mAb against mouse IFN- $\gamma$  (AN-18; Mabtech Inc., Cincinnati, OH) at a concentration of 10  $\mu\text{g/ml}$  and incubated overnight at 4°C. The plates were washed four times with PBS and then blocked with RPMI 1640 plus 10% FBS for 2 h at 37°C. After washing, freshly isolated splenocytes were plated at  $4 \times 10^5$  cells/well in RPMI 1640 with 10% FBS, in the presence or absence of peptide PSMA<sub>459</sub> (20  $\mu\text{g/ml}$ ), recombinant PSMA proteins (20  $\mu\text{g/ml}$ ), or PSMA-positive LNCaP (ATCC) tumor lysate [50  $\mu\text{l/well}$  (36, 37)]. Tumor cell lysates were prepared by three freeze-thaw cycles of  $5 \times 10^7$  tumor cells resuspended in 5 ml of RPMI 1640 with 10% FBS. Then the cells were centrifuged at  $15,000 \times g$  for 30 min at 4°C. Supernatant was recovered, aliquoted, and stored at  $-80^\circ\text{C}$  for later use, as described previously (27). After 20 h of cell culture in the incubator, the cells were removed by washing three times with PBS and four

**Fig. 2** Proliferative T-cell responses to PSMA-derived peptides. PBMCs ( $2 \times 10^5$ ) from healthy donors were cultured with one of six different PSMA peptides (PSMA<sub>17</sub>, PSMA<sub>100</sub>, PSMA<sub>206</sub>, PSMA<sub>459</sub>, PSMA<sub>576</sub>, and PSMA<sub>730</sub>) at a concentration of 20  $\mu\text{g}/\text{ml}$  for 7 days in 96-well plates. A total of 48 wells were seeded per donor and per peptide. [<sup>3</sup>H]Thymidine incorporation of the primed T cells was measured after restimulation with autologous PBMCs ( $1 \times 10^5$ ) and corresponding peptides. Wells were scored positive if the cpm of T cells stimulated with peptides was greater than 1000 and exceeded the reference cpm (without peptides) by at least 3 times (SI > 3; Refs. 25–28). The results are reported as SIs of each tested well of different donors.



times with PBS/Tween20 (0.05%). Biotinylated antimouse IFN- $\gamma$  antibody (R4-6A2; Mabtech Inc.), diluted to 1  $\mu\text{g}/\text{ml}$  in PBS/Tween 20 containing 0.5% BSA, was added and incubated for 2 h at 37°C. The plates were then washed six times with PBS/Tween 20 (0.05%), and subsequently, avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) was added and incubated for 1 h at room temperature before being removed by washing three times with PBS and PBS/Tween 20 (0.05%). The color of the plates was developed by adding horseradish peroxidase substrate 3-amino-9-ethylcarbazole (Sigma). The plates were then washed with tap water and air dried in the dark. The plates were evaluated using an automated ELISPOT reader (Zellnet Consulting Inc., New York, NY).

**Statistical Analysis.** Differences in T-cell proliferation and ELISPOT assay among different groups were analyzed using the two-sided Student's *t* test. The overall significance level was set at 5%.

## RESULTS

**Initial Screening of T-cell Responses to Predicted Peptides from PSMA.** We first used TEPITOPE, a T-cell epitope prediction program (20–22), to analyze potential HLA-DR

binding motifs in the human PSMA protein sequence. At the most stringent prediction threshold (1%), six sequence motifs were predicted to contain promiscuous binding motifs for HLA-DR, including DR1 and DR4 (Fig. 1A). Peptides corresponding to these six epitope candidate sequences were synthesized and purified. To initially screen human CD4<sup>+</sup> T-cell responses to these peptides, PBMCs from DR1-, DR4-, DR7- or DR11-positive healthy donors were stimulated with each peptide in 96-well plates for 7 days. T-cell proliferation was assessed by measuring [<sup>3</sup>H]thymidine incorporation after restimulation with the corresponding peptides and autologous PBMCs as APCs. As shown in Fig. 2, all six peptides elicited proliferative responses in some or all of the donors tested at various degrees. However, according to the statistical analysis described in "Materials and Methods," only PSMA<sub>206</sub>, PSMA<sub>459</sub>, PSMA<sub>576</sub>, and PSMA<sub>730</sub> induced positive proliferative responses in some donors tested, suggesting that they are Th epitope candidates.

**Specificity and MHC Restriction of PSMA Peptide-reactive T-cell Clones.** To characterize these Th epitope candidates further, we set out to generate peptide-specific T-cell clones from each of these epitopes. Individual T-cell clones specific for five of the six peptides tested were generated from

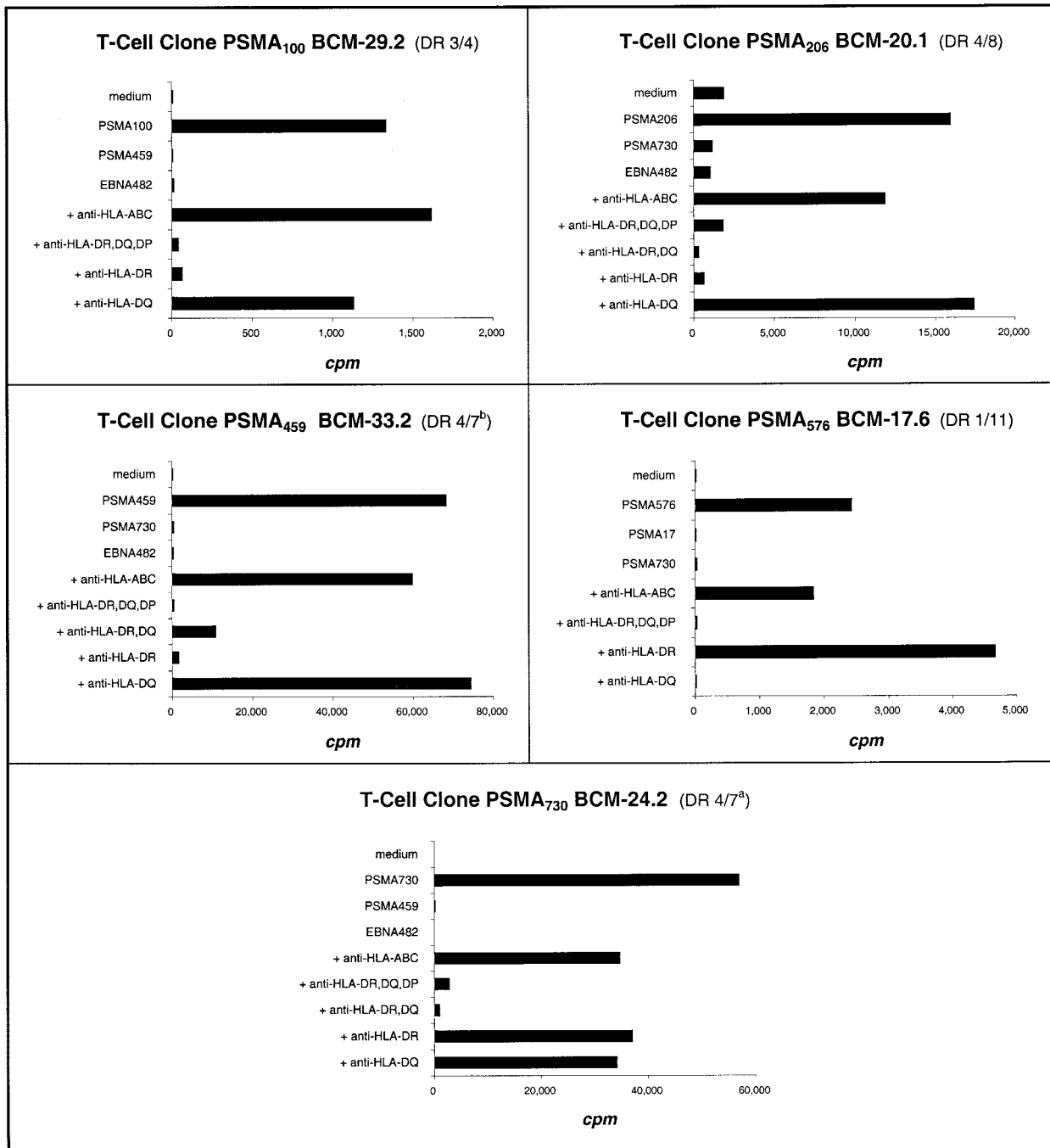
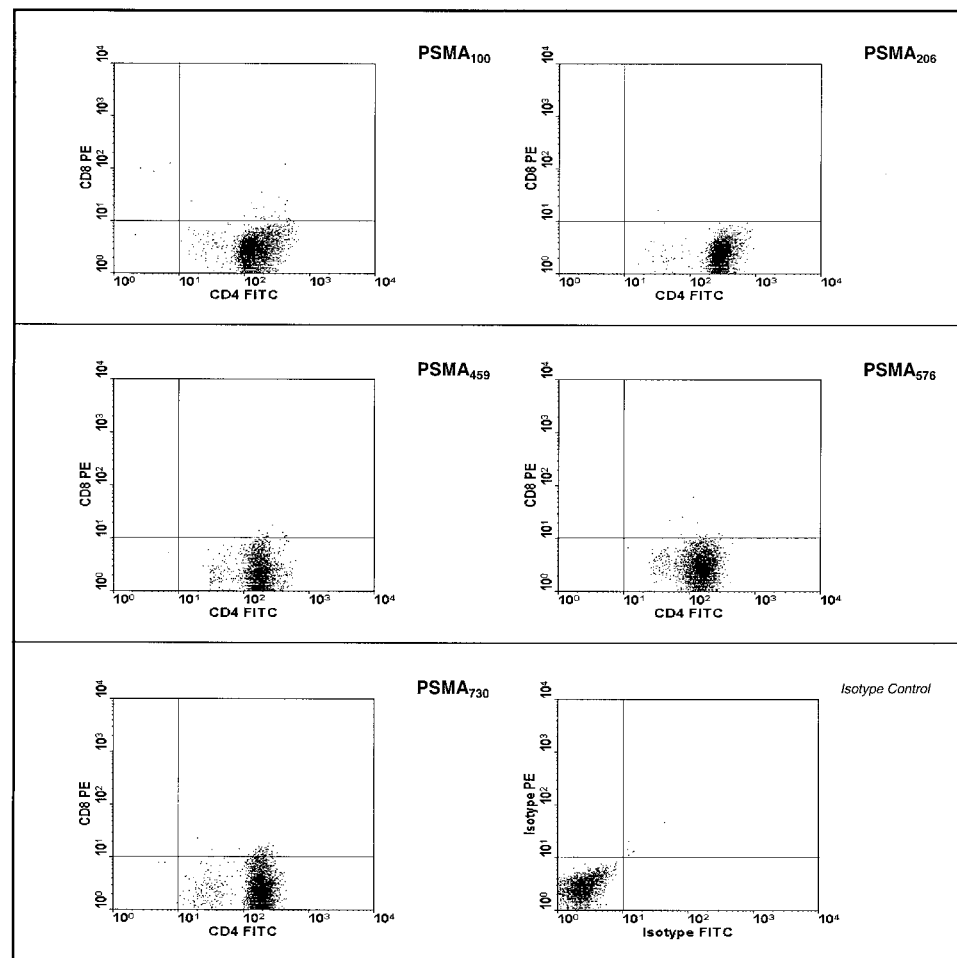


Fig. 3 Specificity and MHC restriction of T-cell clones. T-cell clones for each PSMA-derived peptide ( $3 \times 10^4$ /well) were restimulated with autologous PBMC-derived DCs ( $1.5 \times 10^3$ /well) in the presence of corresponding PSMA peptides or an irrelevant 15-mer peptide from EBV (EBNA<sub>482</sub>) or non-corresponding PSMA peptides at the same concentration of 20  $\mu$ g/ml. Antibodies against MHC class I and MHC class II molecules (20  $\mu$ g/ml) were added together with specific PSMA peptides to analyze the MHC restriction. Cellular proliferation was measured by [<sup>3</sup>H]thymidine incorporation assays. Mean is shown for duplicate wells. The data shown are representative of three repeated experiments.

peptide-reactive T-cell lines by limiting dilution. T-cell clones specific for PSMA<sub>17</sub> could not be established despite repeated attempts in different donors. The specificity and MHC restriction of PSMA<sub>100</sub>, PSMA<sub>206</sub>, PSMA<sub>459</sub>, PSMA<sub>576</sub>, and PSMA<sub>730</sub> T-cell clones were extensively tested. As shown in

Fig. 3, these T-cell clones responded strongly to stimulation with their corresponding peptides but did not respond to stimulation with irrelevant 15-mer peptides derived from EBNA1 (EBNA<sub>482</sub>) or with non-corresponding PSMA peptides. Furthermore, the responses of these T-cell clones to the correspond-

**Fig. 4** Fluorescence-activated cell sorting analysis of T-cell clones. PSMA-reactive T-cell clones were double-stained with antihuman CD4-FITC and CD8-PE antibodies or isotype controls (mouse IgG-FITC and IgG-PE). Human PBMCs were used as positive control for CD8 staining. The cells were then examined by flow cytometric analysis. More than 95% of the T-cell population was CD4+ and CD8-.



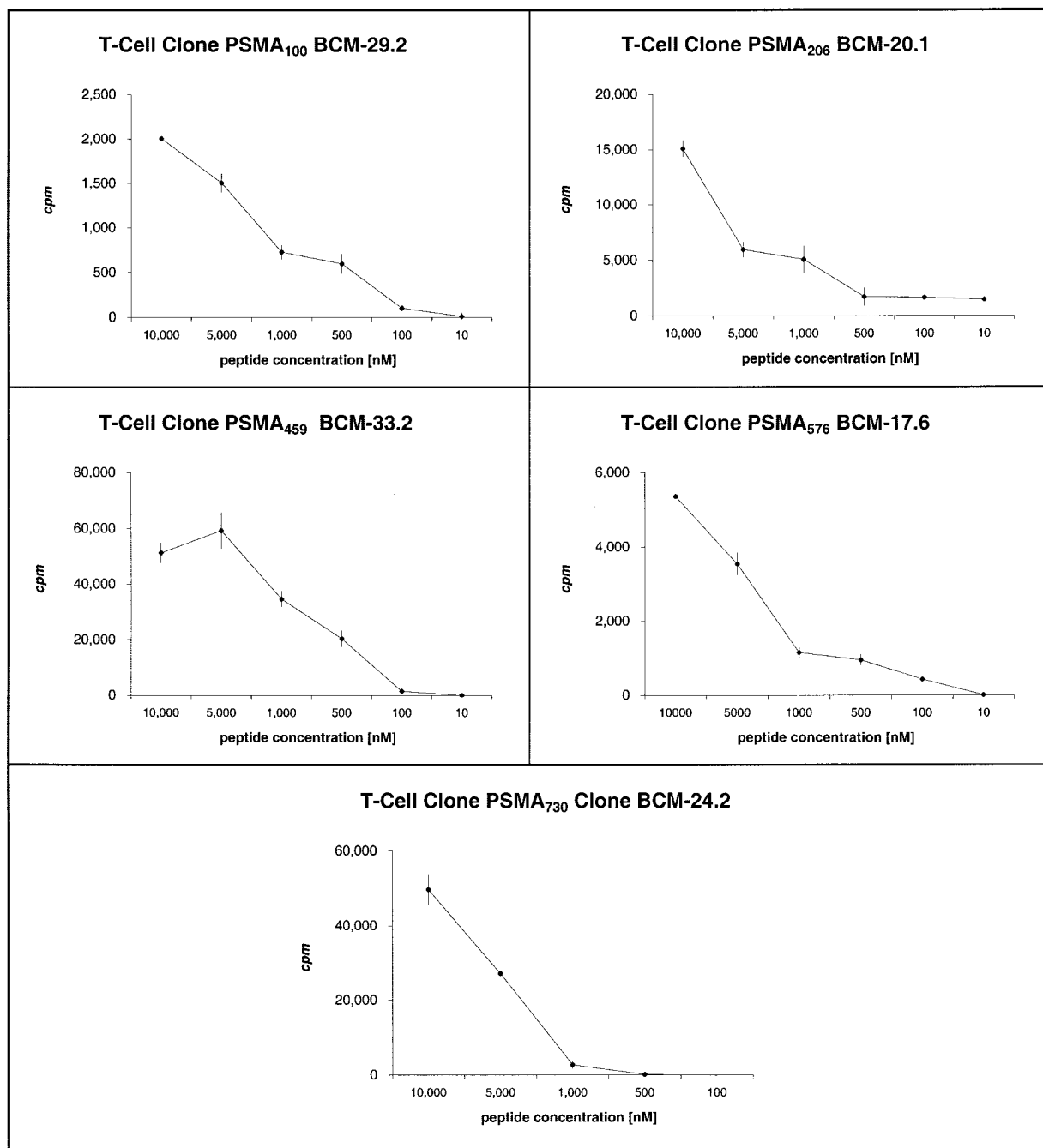
ing peptides were inhibited by an anti-HLA-DR antibody but not by anti-HLA-ABC antibody, indicating that the T-cell responses were HLA-DR restricted, not MHC class I restricted. The response of the PSMA<sub>576</sub>-specific T-cell clone was inhibited by the anti-HLA-DQ antibody, but not by the anti-HLA-DR antibody, indicating that PSMA<sub>576</sub> is an HLA-DQ-restricted epitope. However, the responses of several PSMA<sub>730</sub>-specific T-cell clones from different donors were not blocked by the anti-HLA-DR antibody or by the anti-HLA-DQ antibody but were inhibited by an anti-HLA-DR/DQ antibody and an anti-HLA-DR/DQ/DP antibody, suggesting that the PSMA<sub>730</sub> peptide can be presented by both HLA-DR and HLA-DQ.

We also used flow cytometric analysis to test whether the T-cell clones were CD4+ T cells because these T-cell clones were derived from peptide-stimulated PBMCs. As shown in Fig. 4, all of the peptide-specific T-cell clones generated in this study were exclusively CD4+ and CD8-. This result also indicates that the culture and stimulation conditions used in this study selectively stimulated and expanded CD4+ T cells.

**Recognition of Natively Processed Epitopes by CD4+ T-cell Clones.** A critical feature of functional CD4+ T cells is their ability to recognize naturally processed antigen. It is believed that the capacity of peptide-reactive T cells to recognize

naturally processed antigen presented by APCs depends on correct processing of the epitope in the MHC class II pathway and on the avidity of the peptide epitope to its MHC/T-cell receptor complex (38). Thus, we first evaluated the avidity of the specific T-cell clones for their ligands. Peptide titration curves were generated with autologous DCs (Fig. 5). For PSMA<sub>100</sub>, PSMA<sub>206</sub>, PSMA<sub>576</sub>, and PSMA<sub>730</sub>, the peptide concentrations required to obtain half of the maximal proliferation were above 1.0  $\mu\text{M}$ . For peptide PSMA<sub>459</sub>, half-maximal proliferation was observed at a lower concentration between 0.5 and 1.0  $\mu\text{M}$ , suggesting a greater avidity for this peptide.

Furthermore, we tested whether these T-cell clones could recognize and respond to naturally processed and presented epitopes of PSMA. A recombinant PSMA protein that contains all sequences of the predicted peptides tested (Fig. 1B) and an irrelevant Neu-Fc protein were produced and used to pulse DCs. As shown in Fig. 6, the PSMA<sub>459</sub>-specific T-cell clone responded to the PSMA protein-pulsed DCs, as demonstrated by active T-cell proliferation. In contrast, the T-cell clone did not respond to the irrelevant Neu-Fc proteins (31) presented by autologous DCs or control DCs ( $P < 0.01$ , compared with PSMA pulsed DCs). The T-cell response could be inhibited by anti-HLA-DR antibody ( $P < 0.01$ ). T-cell clones specific for the



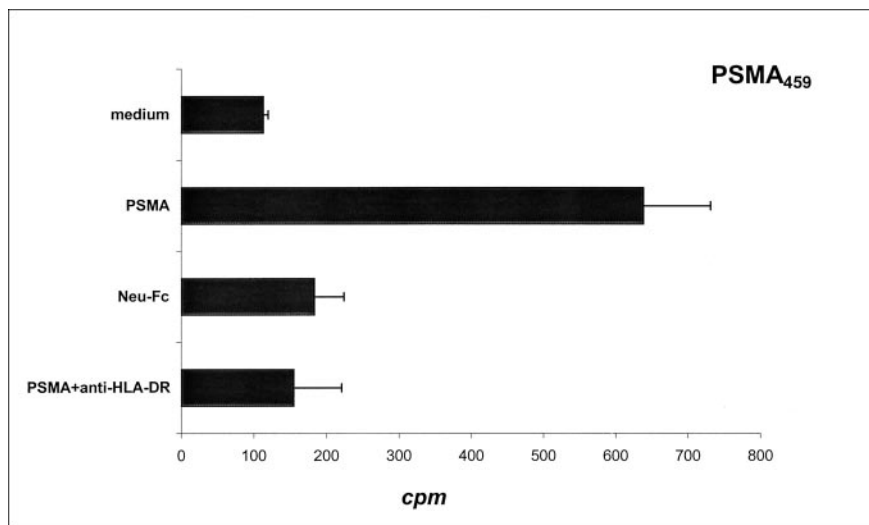
**Fig. 5** Peptide titration assay. Dose-response curves were generated for each peptide-specific T-cell clone to estimate the epitope avidity of the MHC/T-cell receptor complex. T-cell clones ( $3 \times 10^4$  cells/well) were cocultured with autologous PBMC-derived DCs ( $1.5 \times 10^3$ /well) in the presence of PSMA-specific peptides at various concentrations. For PSMA<sub>100</sub>, PSMA<sub>206</sub>, PSMA<sub>576</sub>, and PSMA<sub>730</sub>, the peptide concentrations required to obtain half-maximal proliferation were above  $1.0 \mu\text{M}$ . For PSMA<sub>459</sub>, half-maximal proliferation was observed at lower concentrations between  $0.5$  and  $1.0 \mu\text{M}$ . Each data point represents the mean of triplicate wells. Bars denote SD (if invisible, the SD fell within the size of the symbol). The data are from one experiment that is representative of three experiments performed.

other four peptides (including the clones shown in Figs. 3 and 4) failed to respond to DCs pulsed with various concentrations of PSMA proteins in repeated experiments (data not shown), indicating that these epitopes are cryptic. Taken together, these results indicate that of the five MHC class II peptides tested,

only PSMA<sub>459</sub> represents a naturally processed MHC class II epitope.

**PSMA<sub>459</sub>-induced T-cell Responses in Healthy and Prostate Cancer Individuals.** Using TEPITOPE, the PSMA<sub>459</sub> epitope was predicted to contain a promiscuous bind-

**Fig. 6** Responses of T-cell clone PSMA<sub>459</sub> to natively processed PSMA. PSMA<sub>459</sub>-specific T cells (clone PSMA<sub>459</sub> BCM-33.02;  $2 \times 10^4$ /well) were stimulated with irradiated autologous PBMC-derived DCs ( $1 \times 10^3$ /well) pulsed with recombinant PSMA protein (100 nM) in the presence or absence of the anti-HLA-DR antibody (20  $\mu$ g/ml). The PSMA<sub>459</sub> T-cell clone was also stimulated with DCs pulsed with irrelevant recombinant Neu-Fc proteins (100 nM). T-cell proliferation was determined by [<sup>3</sup>H]thymidine incorporation assay during the last 16 h of a 72-h culture. Values shown are the means of triplicate determinations (bars, SD). The representative result of one of three repeated experiments is shown. The proliferation of T cells cultured with PSMA-pulsed DCs was significantly higher ( $P < 0.01$ , compared with anti-HLA-DR blocking or Neu-Fc-pulsed DCs).



ing motif for HLA-DRB1\*0301, HLA-DRB1\*0401, HLA-DRB1\*0701, and HLA-DRB1\*1101 (21, 22), suggesting that the PSMA<sub>459</sub> epitope may be able to induce T-cell responses in individuals with diverse DR genotypes. Thus, PBMCs from donors with different HLA-DR alleles were stimulated with the peptide for 1 week using autologous PBMCs as APCs (Fig. 7). The cultures were tested for their capacity to respond to the peptide presented by autologous PBMCs. As shown in Figs. 2 and 7A, T cells from donors with genotypes of DR01/11, DR04/07, DR04/08, DR3/15, and DR03/09 positively responded to the peptide PSMA<sub>459</sub> ( $P < 0.05$ ). We further tested whether T cells from prostate cancer patients can recognize this natively processed epitope. Five prostate cancer patients in disease remission were tested for their T-cell responses to PSMA<sub>459</sub>. T-cell proliferation was assessed as described above. As shown in Fig. 7B, of five prostate cancer patients tested, three elicited positive proliferative T-cell responses to peptide PSMA<sub>459</sub>. These results indicate that the T-cell precursors specific for the PSMA<sub>459</sub> epitope, when stimulated, are readily activated to in healthy individuals and prostate cancer patients with different DR genotypes. Further study is warranted to determine whether this epitope is indeed promiscuously presented by commonly found HLA-DR alleles.

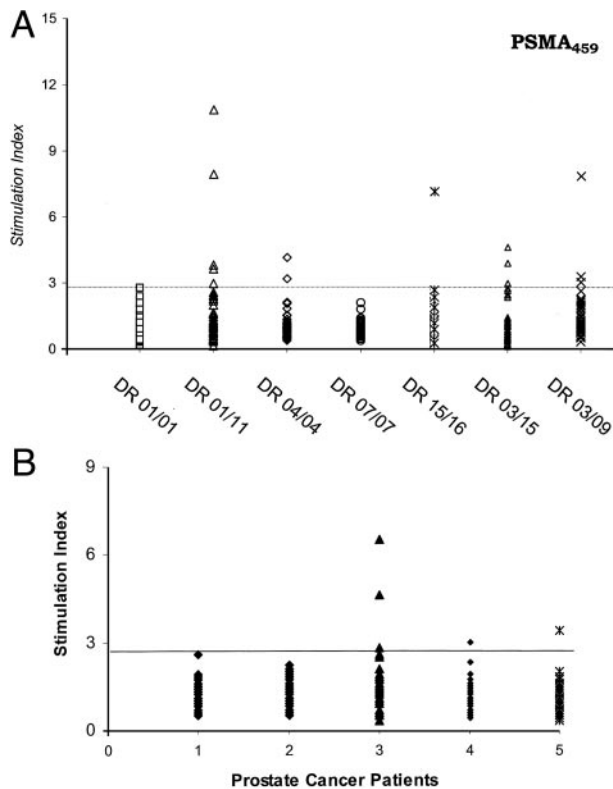
The precursor frequency was primarily estimated as the number of positive wells/total number of T cells in all wells tested because it was demonstrated that an antigen-specific T-cell line derived from a 96-plate well (200,000 cells/well) most likely originates from a single T-cell precursor (28). The estimated precursor frequencies of T cells specific for the PSMA<sub>459</sub> epitope in the positive donors with different DR types range from 0 to  $4.17 \times 10^{-6}$ , in a range of precursor frequencies for other self antigens (28), although a more detailed study is needed to determine the precursor frequency. Interestingly, although T-cell precursors specific for PSMA<sub>206</sub> and PSMA<sub>576</sub> appeared to be more frequent than precursors representing other epitopes, the T-cell clones specific for these two epitopes still did not respond to PSMA protein-pulsed DCs.

#### PSMA-specific T-cell Response Induced by PSMA<sub>459</sub> Immunization of HLA-DR Transgenic Mice.

To further assess the therapeutic potential of PSMA<sub>459</sub>, we used HLA-DR4 transgenic mice (31–33) to determine whether immunization with the peptide can induce a CD4<sup>+</sup> Th response specific not only for the peptide but also for the PSMA protein. The HLA-DR molecule expression on transgenic mice was detected by flow cytometric assay. The spleen cells were isolated and stained with FITC-conjugated mouse antihuman HLA-DR, PE-conjugated mouse antihuman CD4, or FITC-conjugated rat antimouse CD4 (BD PharMingen). As shown in Fig. 8A, these transgenic mice are human HLA-DR<sup>+</sup>, human CD4<sup>+</sup>, and mouse CD4<sup>−</sup>. Ten days after the last immunization with PSMA<sub>459</sub> in CFA, the transgenic mice were sacrificed, and the response of their splenocytes to peptides was first examined by using IFN- $\gamma$  ELISPOT assays. The splenocytes of PSMA<sub>459</sub>-immunized mice responded strongly to the PSMA<sub>459</sub> stimulation, producing IFN- $\gamma$  at a high frequency of 220 spots/400,000 splenocytes (Fig. 8B). In contrast, the splenocytes of PBS-immunized control mice did not respond to the PSMA<sub>459</sub> stimulation, producing IFN- $\gamma$  at a background frequency (medium control, without peptide stimulation, 8 spots/400,000 splenocytes). The splenocytes of PSMA<sub>459</sub>-immunized mice did not respond to an irrelevant hTRT<sub>672</sub> peptide stimulation (11 spots/400,000 splenocytes), indicating that T cells induced by peptide immunization specifically respond to the immunized peptide.

Because prostate tumor cells are largely MHC class II negative, CD4<sup>+</sup> T cells induced by peptide immunization could only react with APCs that take up and process the tumor antigen protein. Thus, we tested whether transgenic mouse T cells become activated when cocultured with splenocytes containing T cells and APCs pulsed with the recombinant PSMA proteins. As shown in Fig. 8C, when stimulated with the recombinant PSMA protein, the splenocytes of PSMA<sub>459</sub>-immunized mice produced IFN- $\gamma$  at a frequency of 56 spots/400,000 splenocytes (well), significantly higher than the splenocytes of the PBS-immunized mice ( $P < 0.01$ ). Because the molecular weight of





**Fig. 7** A, PSMA<sub>459</sub> induced T-cell responses in healthy individuals and prostate cancer patients. PBMCs from healthy adult donors (A) with different HLA-DR genotypes and prostate cancer patients (B) were cultured with peptide PSMA<sub>459</sub> for 7 days in 96-well plates ( $2 \times 10^5$  cells/well, 48 wells/donor). [<sup>3</sup>H]Thymidine incorporation by the primed T cells was measured after restimulation with autologous PBMCs ( $1 \times 10^5$ ) as APCs with or without the corresponding peptide PSMA<sub>459</sub>. The results are reported as SI of each tested well of different donors. T cells from healthy donors with genotypes of DR01/11, DR04/07, DR04/08, DR3/15, and DR03/09 positively responded to the peptide PSMA<sub>459</sub> ( $P < 0.05$ ). T cells from prostate cancer patients (donors 3 and 5) positively responded to the peptide PSMA<sub>459</sub> ( $P < 0.05$ ). Positive events were defined if there was a significant difference at a 95% confidence level between the number of positive wells of the peptide-containing wells compared with that of the non-peptide-containing wells.

peptides is much smaller than that of recombinant proteins, the higher proliferation of T cells stimulated by peptides is largely due to the significantly higher concentration of peptides used for stimulation. Furthermore, the splenocytes of PSMA<sub>459</sub>-immunized mice produced IFN- $\gamma$  at a background frequency (10 spots/400,000 splenocytes) when stimulated with irrelevant hTERT proteins (27). These results indicate that PSMA<sub>459</sub> immunization activates T cells that recognize antigenic peptides processed from PSMA proteins.

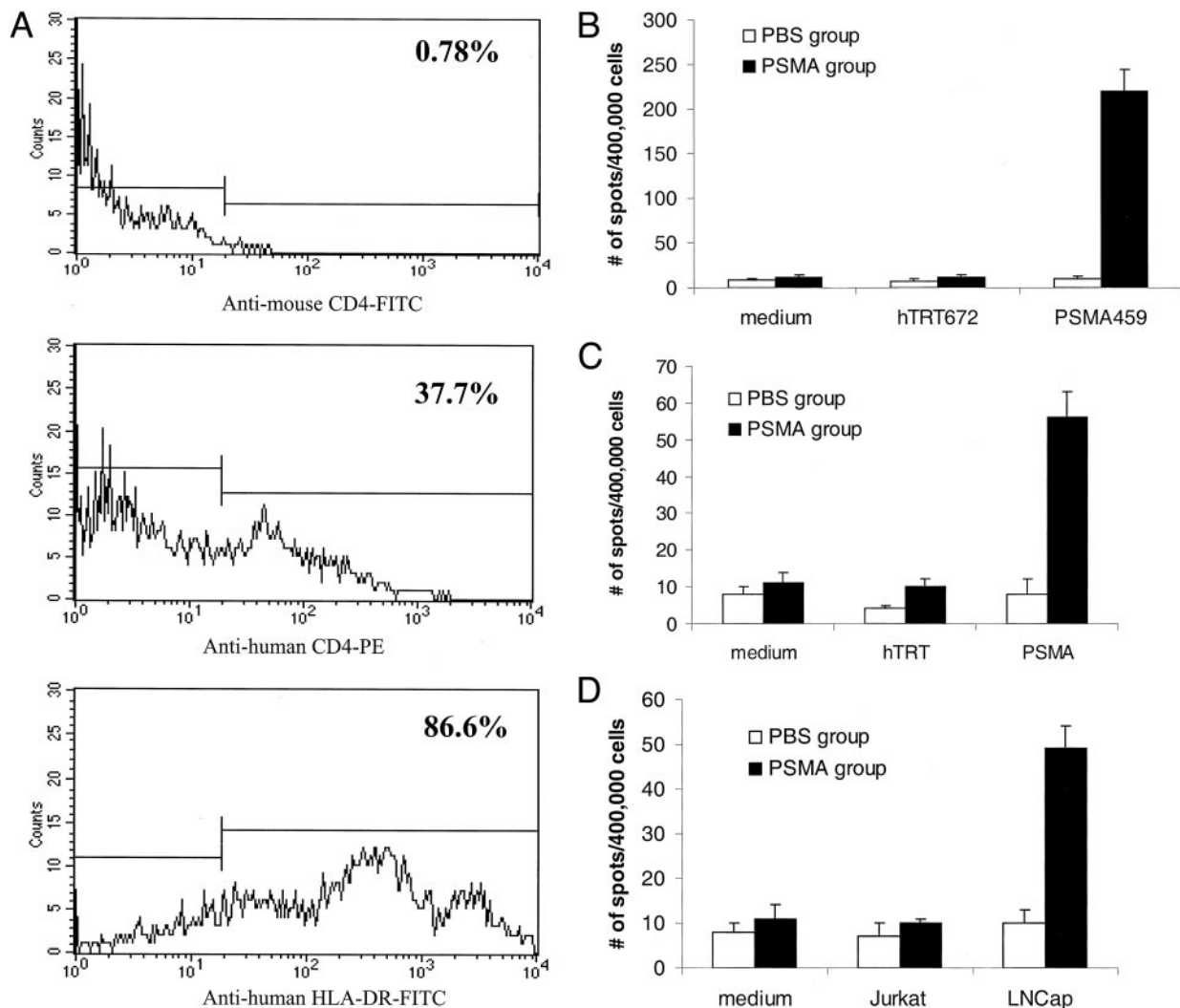
Finally, we tested whether activated CD4<sup>+</sup> T cells can react with APCs that directly take up and process the tumor antigen from tumor cells. Prostate tumor cells (LNCaP) that express high levels of PSMA (36, 37) were used for this assay. As shown in Fig. 8D, when stimulated with LNCaP cell lysates, the splenocytes of PSMA<sub>459</sub>-immunized mice produced IFN- $\gamma$  at a frequency of 49 spots/400,000 splenocytes, significantly

higher than the splenocytes of the PBS-immunized mice at a frequency of 8 spots/400,000 cells ( $P < 0.01$ ). The splenocytes of PSMA<sub>459</sub>-immunized mice produced IFN- $\gamma$  at a background frequency (medium control, 10 spots/400,000 cells) when stimulated with PSMA-negative Jurkat tumor lysates (11 spots/400,000 cells), indicating that T cells activated by PSMA<sub>459</sub> immunization specifically respond to antigenic peptides derived from PSMA-positive tumor.

## DISCUSSION

The main focus of current tumor vaccination efforts has been to induce CTL responses (8, 9). Numerous MHC class I epitopes from different prostate tumor antigens, including prostate-specific antigen, PSMA, and prostatic acid phosphatase (39), have been identified. Because CD4<sup>+</sup> Th cells play critical roles in initiating, regulating, and maintaining CTL responses (8, 9), in addition to other antitumor activities (8, 11–15), the potency of current tumor vaccines could be enhanced by combined application of CTL epitopes with Th epitopes. Thus, increasing effort has been directed to the identification of Th epitopes in TAAs. Thus far, only a limited number of human TAAs recognized by Th cells have been identified, and even less is known about the Th epitopes in prostate tumor-associated antigens (26). Here, we used *in vitro* T-cell biological analysis, together with the MHC class II prediction software, to initially identify PSMA epitope candidates. We then established CD4<sup>+</sup> T-cell clones specific for these potential epitopes and found that one of the T-cell clones can recognize the naturally processed and expressed PSMA<sub>459</sub> epitope (NYTLRVDCTPLMYSL). We found that the T-cell precursors specific for the PSMA<sub>459</sub> epitope are readily activated to in healthy individuals and prostate cancer patients with different DR genotypes. Furthermore, PSMA<sub>459</sub> peptide immunization of HLA-DR4 transgenic mice activates T cells that specifically responded to antigenic peptides derived from PSMA proteins and PSMA-positive tumor. In summary, the identification of MHC class II-restricted epitope(s) in PSMA, which is highly expressed in prostate cancer, should facilitate the development of improved vaccines for treating prostate cancer through the simultaneous stimulation of PSMA-specific CTL and Th responses.

In contrast to successful identification of MHC class I epitopes in tumor antigens, the identification of Th epitopes has been difficult, as demonstrated by the fact that only a few class II-restricted epitopes on human TAAs have been identified (22, 38, 40–46). Two approaches to identify Th epitopes are in current use: (a) sequencing of eluted peptides bound to specific MHC class II molecules on tumor cells; and (b) screening panels of overlapping peptides derived from TAAs. The first method has been used to identify MHC class II epitopes from the melanoma tumor antigens tyrosinase (43, 47) and gp100 (48). However, this method is cumbersome and difficult because most tumor cells are MHC class II negative, and even if the tumor cells are class II positive, they contain mixed epitopes from multiple antigens in minuscule amounts, in addition to the observation that the length of class II epitopes is more variable than that of class I epitopes (49). By screening panels of overlapping peptides derived from TAAs, several Th epitopes from the MAGE-3 and MAGE-1 melanoma tumor antigens have been



**Fig. 8** PSMA-specific Th responses induced by PSMA<sub>459</sub> immunization of HLA-DR transgenic mice. **A**, HLA-DR expression in transgenic mice by flow cytometric assay. Splenocytes from the transgenic mice with HLA-DR4 and human CD4 molecules were stained with FITC antihuman HLA-DR, PE antihuman CD4, FITC antimouse CD4, and the corresponding isotype controls, respectively. The cells were then examined by flow cytometric analysis. All of the transgenic mice are human HLA-DR+, human CD4+, and mouse CD4-. **B**, peptide-specific Th response induced by immunization with PSMA<sub>459</sub>. HLA-DR4 transgenic mice (4 mice/group) were immunized twice with 100 μg of PSMA<sub>459</sub> or PBS (100 μl) emulsified with CFA. Ten days later, after the second immunization, the splenocytes of each mouse group were pooled and tested for peptide PSMA<sub>459</sub>-induced production of IFN-γ by ELISPOT. Data show the mean and SD of spot number from triplicate wells from one experiment that is representative of two experiments performed. The frequency of peptide-specific T cells of PSMA<sub>459</sub>-immunized mice was significantly higher than that of PBS control group ( $P < 0.001$ ). The background IFN-γ spot (without peptide stimulation) was <11 spots/400,000 cells in both PSMA<sub>459</sub>-immunized and PBS control mouse groups. **C** and **D**, response to antigenic peptides derived from PSMA proteins and tumor lysates. Splenocytes from HLA-DR4 transgenic mice (4 mice/group) immunized with 100 μg of PSMA<sub>459</sub> or PBS (100 μl) emulsified with CFA were assessed for the production of IFN-γ by ELISPOT. Recombinant PSMA proteins or hTTR proteins (20 μg/ml; **C**) and PSMA-positive LNCaP tumor lysate or PSMA-negative Jurkat cell lysate (50 μl; **D**) were used to pulse the splenocytes. Data show the mean and SD of spot number from triplicate wells from one experiment that is representative of two experiments performed. The frequency of IFN-γ+ cells in the PSMA<sub>459</sub>-immunized mouse group when stimulated with PSMA proteins or tumor lysates was significantly higher than that of PBS control mouse group [ $P < 0.01$  (**C**) and  $P < 0.01$  (**D**), respectively]. The background IFN-γ spots were <11 spots/400,000 cells in PSMA<sub>459</sub>-immunized mice stimulated with recombinant hTTR proteins or PSMA-negative Jurkat cell lysate.

recently identified (50). Because Th precursors to TAAs were found to be rare among patients, Th precursors require extensive antigen-specific stimulation and expansion *in vitro* by antigen-pulsed APCs, which poses a difficult challenge. Furthermore, T cells stimulated and expanded *in vitro* may be specific for contaminants in purified recombinant proteins used for *in vitro* T-cell stimulation because they are usually highly immuno-

genic, in contrast to self tumor-associated antigens (46). In addition, it is difficult to ensure that T cells specific for all epitopes are stimulated and expanded *in vitro*. These considerations led us to use an algorithm prediction, combined with *in vitro* T-cell biological analysis, to identify MHC class II epitopes, as used in our recent study (27) and by others (38, 40–42, 44, 45).

In this study, we paid particular attention to the generation of peptide-specific T-cell clones because in-depth biological analysis would be not feasible without a peptide-specific T-cell clone. There is only limited information on the correlation of T-cell precursor frequency and the robustness of the T-cell response to a given peptide with the biological responsiveness of the peptide-specific T cells to tumor antigens. We established a simple, consistent method that successfully generated peptide-reactive T-cell clones for five of six peptides tested. By studying these peptide-specific T-cell clones, we found that higher T-cell precursor frequency and robust T-cell responses to a synthetic peptide do not correlate with the ability of T cells to recognize the naturally processed PSMA antigen. Cryptic epitopes appear to induce a higher frequency of stronger peptide-specific T-cell responses. This observation raises questions concerning the current preclinical evaluation and clinical trials of peptide-based tumor vaccines. Selection of peptides based on their *in vitro* MHC binding affinity and robustness of T-cell responses to synthetic peptides may be insufficient and misleading. Given the lack of correlation of the T-cell precursor frequency and robustness of T-cell responses to a given peptide with T-cell ability to recognize naturally processed epitopes, it seems essential to establish and test peptide-specific T-cell clones to identify naturally processed epitopes for potential tumor immunotherapy.

Peptide vaccination to elicit antitumor immunity is a promising means of treating cancer patients. However, current peptide vaccination mainly utilizes CTL epitopes. A naturally processed epitope, PSMA<sub>459</sub>, identified in this study indicates that the clonal deletion for T cells specific for self-antigens is incomplete. Furthermore, these T cells are a normal part of the human T-cell repertoire and can be readily activated with proper stimulation. A concern for peptide-based therapy is the MHC genotype restriction because class I-restricted epitopes are largely restricted by a particular class I genotype (38, 51–53). We found that T-cell precursors specific for the PSMA<sub>459</sub> epitope, when stimulated, are readily activated to in healthy individuals and prostate cancer patients with different DR genotypes, suggesting that the PSMA<sub>459</sub> epitope may be promiscuously presented by HLA-DR alleles. In summary, the identification of a natively processed class II-restricted Th epitope in PSMA raises the possibility of developing a multivalent peptide vaccine that contains both class I- and II-restricted PSMA epitopes for prostate cancer immunotherapy. Experiments are under way to systemically evaluate the CD4<sup>+</sup> T-cell responses in prostate cancer patients and the possible enhancement of PSMA-specific CTL responses induced by PSMA Th peptides in transgenic mouse models.

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