Identification of HLA-DRB1*1501–Restricted T-cell Epitopes from Prostate-Specific Antigen

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Abstract The development of immunotherapy for prostate cancer based on the induction of autoimmunity to prostate tissue is very attractive because prostate is not a vital organ beyond the reproductive years. CD4 T cells play an important role in the development of antitumor immune responses, yet the identification of naturally processed MHC Class II–restricted epitopes derived from prostate differentiation antigens has not been described. To facilitate the search for prostate-specific antigen (PSA)–derived MHC class II–restricted peptides, we immunized mice transgenic for *HLA-DRB1*1501* with human PSA and showed a robust dose-dependent immune response to the antigen. Screening a library of overlapping 20-mer peptides that span the entire PSA sequence identified two 20-mer peptides, $PSA_{171-190}$ and $PSA_{221-240}$, which were responsible for this reactivity. Immunization of DR2b transgenic mice with these peptides induced specific responses to the peptide and whole PSA. Identified peptides were used to stimulate CD4 T cells from *HLA-DRB1*1501*+ patients with a rare condition, granulomatous prostatitis, and who seem to have a preexisting immune response directed against the prostate gland. We previously showed a linkage of granulomatous prostatitis to *HLA-DRB1*1501*, suggesting that this disease may have an autoimmune etiology. Peptide-specific CD4 T-cell lines were generated from the peripheral blood of these patients as well as one patient with prostate cancer. These lines also recognized whole, processed PSA in the context of *HLA-DRB1*1501*. This study will be instrumental in understanding the interaction between circulating self-reactive T cells, organ-specific autoimmunity, and antitumor immune response. The use of these peptides for the immunotherapy of prostate cancer is under investigation.

In the last 15 years, two sentinel insights in the immunotherapy of cancer have become clear: (*a*) patients with cancer do, in fact, have an immune response to their tumor and (*b*) the target of the immune response in these patients is usually antigens derived from normal self-proteins to which the cancer-bearing state has somehow released self-tolerance. Hence, cancer reactivity is self-reactivity, and the characteristics of a successful cancer immunotherapy will likely resemble autoimmunity. Specific immunotherapy of cancer has therefore consisted, for the most part, of the identification of and vaccination with such antigens by a variety of strategies in the hopes of augmenting the immune response to cancer antigens and bringing about a therapeutic effect. The development of immunotherapy for prostate cancer based on this model is in its infancy compared with other malignancies. Yet, prostate cancer is ideal for this approach because the disease is common, tends to be slowly progressive, and any destruction of normal prostate tissue is of no apparent consequence.

Effective vaccination against tumors requires generation of both CD8+ and CD4+ T-cell responses. A critical role of CD4+ helper T cells in the development of CD8+ tumor-specific effector function has been shown by numerous studies (reviewed in ref. 1). The role of CD4+ T cells as effectors mediating autoimmune responses, the immunodominant antigens and their epitopes that can trigger the autoimmune responses, as well as susceptible and resistant MHC haplotypes has not been sufficiently studied in application to cancer immunotherapy based on self-antigens. This is particularly true for prostate cancer.

Among prostate differentiation antigens, the immune response to prostate-specific antigen (PSA) has been studied most thoroughly to date. CTLs that recognize the predicted HLA-A2–as well as *HLA-A1*–, *HLA-A3*–, and *HLA-A24*–restricted PSA peptides have been generated from peripheral blood lymphocytes of normal donors and prostate cancer patients in several laboratories (2–6). The diversity of Class II alleles that can elicit a response to PSA has not been studied in detail. Corman et al. (4) identified *HLA-DR4*–binding peptides within the PSA

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Note: A provisional patent application has been filed for $PSA_{171-190}$ and $PSA_{221-240}$.

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sequence; however, their reactivity with the whole protein was not studied.

In search of MHC class II–restricted PSA peptides, we concentrated our effort on *HLA-DRB1*1501* molecule because we have recently shown a strong linkage of this allele with granulomatous prostatitis (7). Granulomatous prostatitis is a chronic inflammatory condition of the prostate of unknown cause where granulomas and a chronic inflammatory infiltrate can destroy prostatic ducts and glands (8), exactly the responses we hope to induce in patients with prostate cancer. PSA-reactive *HLA-DRB1*1501* –restricted CD4 T-cell lines were established from the peripheral blood of a patient with granulomatous prostatitis providing additional evidence for an autoimmune etiology of this disease (9). We reasoned that identification of *HLA-DRB1*1501* –restricted epitopes involved in autoimmune process in patients with granulomatous prostatitis would be logical to test as a vaccination strategy to induce a destructive immune response directed against prostate cancer in *HLA-DRB1*1501* patients.

The association of *HLA-DRB1*1501* allele with multiple sclerosis has led to the development of transgenic mice expressing this molecule for the study of experimental autoimmune encephalitis (10, 11). The mice are termed *DR2b* in reference to the HLA-DR haplotype which contains the *HLA-DRB1*1501* gene. In this report, we show that immunization of *HLA-DR2b* transgenic mice with human PSA resulted in the identification of two peptides that are naturally processed and able to induce immune responses in human CD4 T cells derived from patients with granulomatous prostatitis and prostate cancer.

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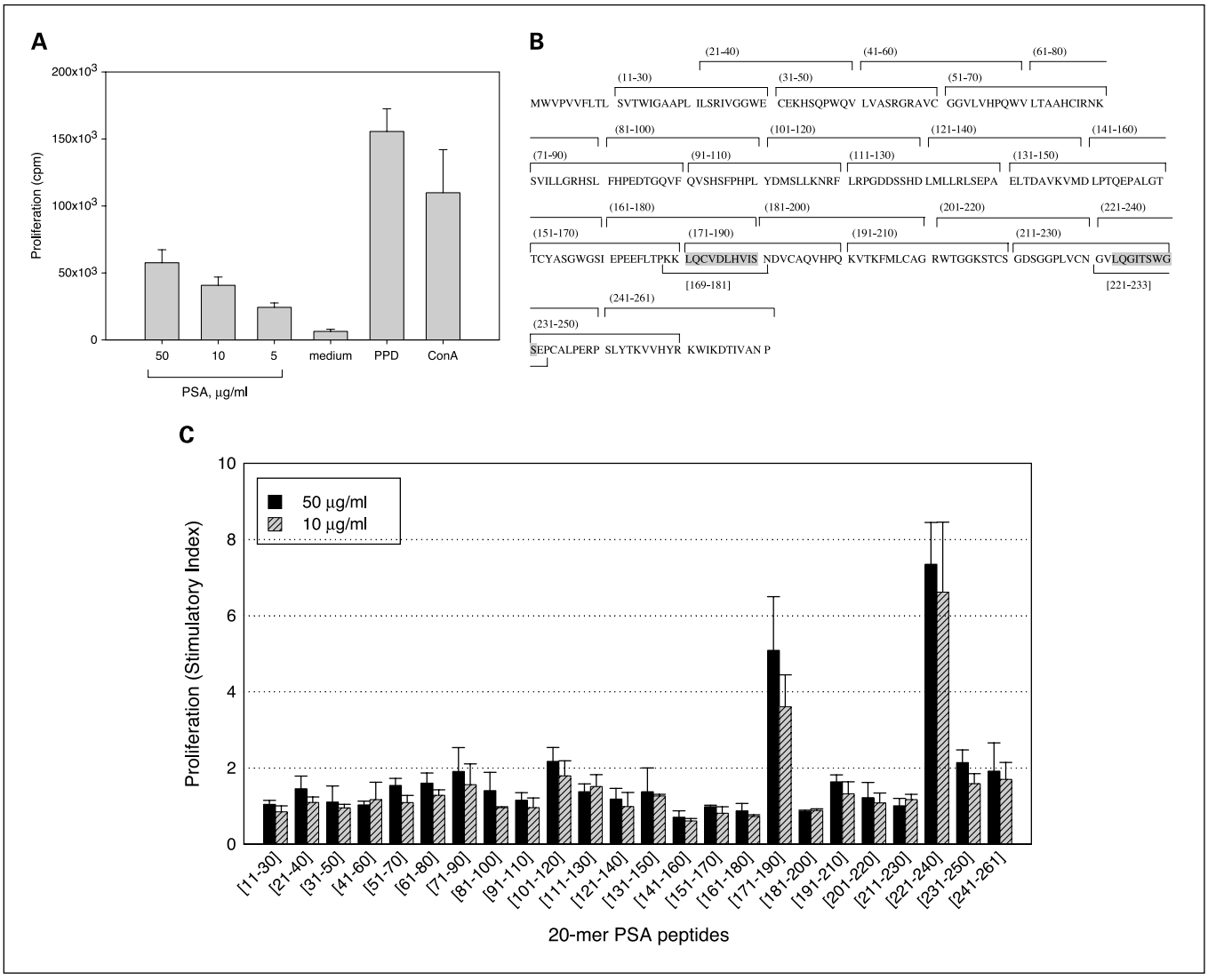

Fig. 1. Recall proliferation assay in draining lymph node cells after PSA immunization. DR2b transgenic mice were vaccinated s.c. with PSA (100 μg/mouse) in complete Freund's adjuvant. Nine days later, draining lymph nodes and spleens were harvested and cultured *in vitro* in medium containing various additives shown for 48 hours. Cultures were pulsed with [^{3}H]thymidine and cpm was determined 18 hours later. Columns, mean of triplicate determinations; bars, SD. *A*, responses to PSA. Draining lymph nodes were cultured *in vitro* in the presence of indicated concentrations of human PSA. Purified protein derivative (*PPD*) was added at 25 μg/mL and concanavalin A (*ConA*) at 0.5 μg/mL. *B*, sequence of human PSA. The peptides are indicated by numbers for the position of the first and the last residues. Nine-mer core sequences for both peptides are highlighted. *C*, responses to PSA-derived overlapping 20-mer peptides. Draining lymph nodes were cultured *in vitro* in medium containing a series of overlapping 20-mer peptides derived from the primary amino acid sequence of PSA. Stimulation index is calculated as $cpm_{peptide}$ / cpm_{medium}.

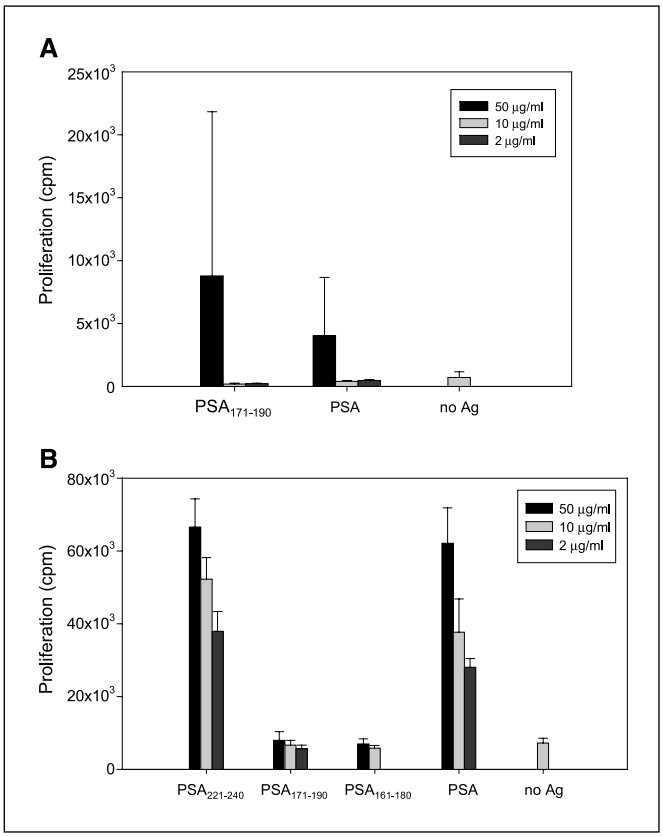

Fig. 2. T-cell responses induced by immunization with PSA peptides. DR2b transgenic mice were immunized s.c. with peptide $PSA_{171-190}$ (*A*) or $PSA_{221-240}$ (*B*; 100 μg/mouse) in complete Freund's adjuvant. Nine days later, draining lymph nodes were harvested and cultured *in vitro* in medium containing various additives for 48 hours. Cultures were pulsed with [^{3}H]thymidine and cpm determined 18 hours later. Columns, mean of triplicate determinations; bars, SD.

Materials and Methods

Peptides. Peptides were synthesized at the Biopolymer Core Facility, University of Maryland (Baltimore, MD) and purified to >90% by reversed-phase high-performance liquid chromatography. The molecular weights were confirmed by mass spectrometry.

Immunizations and in vitro proliferative assay. *HLA-DR2b* transgenic mice bearing chimeric MHC class II molecules consisting of the α1 and β1 sequences of *HLA-DRA1*0101* and *HLA-DRB1*1501* and the α2 and β2 domains of *IEα* and *IEβ*, respectively, were developed as previously described (12, 13). Transgenic offspring were backcrossed twice to the MHC class II knockout mouse, $MHCII^{\Delta/\Delta}$, resulting in deletion of all normal mouse class II molecules (14); hence, T-cell responses in these mice are restricted exclusively by *HLA-DRB1*1501*. The expression of HLA-DR in *DR2b* transgenic mice was analyzed by flow cytometry using anti-pan-DR-PE monoclonal antibody (mAb; Sigma, St. Louis, MO). The mice did not express the endogenous mouse Class II molecules as determined by staining with anti-I-A/E FITC mAb (BD Bioscience PharMingen, San Diego, CA; ref. 10). *HLA-DR2b* transgenic male mice between 8 and 12 weeks of age were immunized s.c. at four sites on the flanks with 0.2 mL of an emulsion comprised of 100 μg PSA purified from human seminal fluid (Fitzgerald Industries International, Concord, MA) or 100 μg peptide in complete Freund's adjuvant containing 400 μg *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI).

Spleens and inguinal lymph nodes from *HLA-DR2b* transgenic mice were recovered 9 to 11 days after immunization and processed into single-cell suspensions. T-cell proliferation responses were assessed by plating 4×10^5 cells/well in a 96-well flat-bottomed tissue culture plate in triplicate in media alone (control) or in the presence of antigens in 2% fetal bovine serum–containing RPMI 1640 supplemented with 0.05 mmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 g/mL penicillin G, and 100 g/mL streptomycin (Invitrogen GIBCO, Carlsbad, CA). Cultures were incubated for 72 hours at 37°C in 7% CO_2. Wells were pulsed for the final 18 hours with 0.5 Ci of [^{3}H]thymidine per well (Amersham, Arlington Heights, IL). The cells were harvested onto glass fiber filters and [^{3}H]thymidine uptake was measured using a liquid scintillation counter (1205 Betaplate, Wallac, Turku, Finland). Mean counts per minute (cpm) ± SD was calculated for triplicate wells.

Patients and HLA typing. Five *HLA-DRB1*1501+* patients with granulomatous prostatitis were identified through clinical practice at the VA Maryland Health Care System and University of Maryland. Patients underwent automated leukopheresis to obtain peripheral blood mononuclear cells (PBMC) in the University of Maryland Cancer Center apheresis unit under Institutional Review Board–approved protocol. PBMCs were separated by centrifugation on

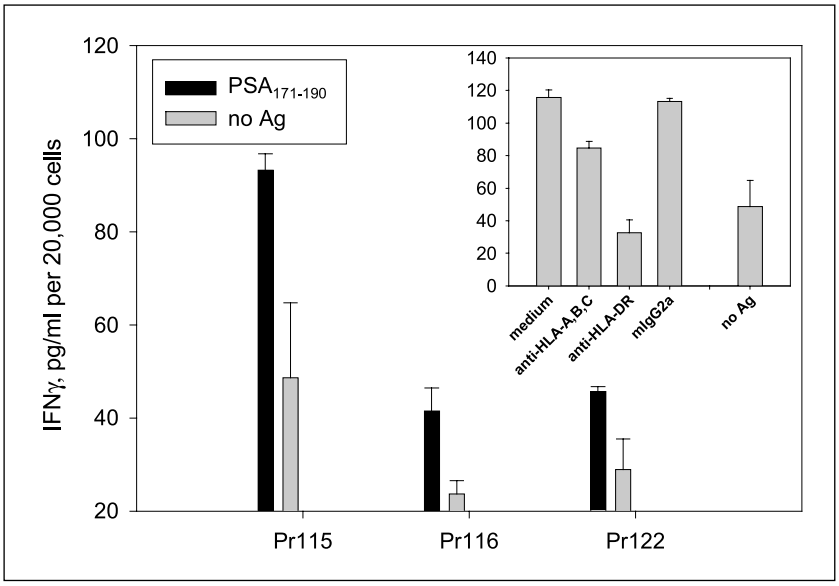

Fig. 3. The responses of human CD4 T-cell lines to peptide $PSA_{171-190}$ presented by autologous DC. CD4 T-cell lines were generated from PBMC of granulomatous prostatitis patients by multiple stimulations with peptide $PSA_{171-190}$ in the presence of irradiated autologous PBMC. T cells were harvested and incubated at 20,000 cells/well with mature autologous DCs (5,000 cells/well) in the presence or absence of the specific peptide (20 μg/mL). IFNγ secretion was measured in culture supernatants after 48 hours of culture. Columns, mean of triplicates; bars, SD. Responses at 3rd *in vitro* stimulation (28 days after initiation of culture). Inset, T cells were stimulated in the presence of anti-HLA-A,B,C (clone W6/32), anti-HLA-DR (clone L243) antibodies or control IgG2a (clone IA14; all mAbs were added at 5 μg/mL).

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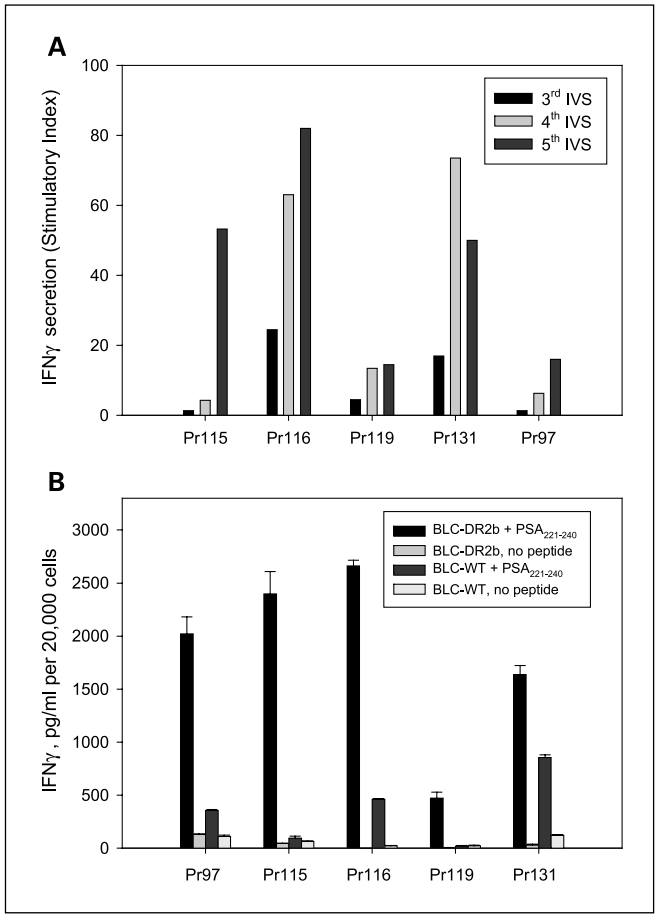

Fig. 4. The responses of human CD4 T-cell lines to peptide $PSA_{221-240}$ presented by BCL-DR2b cell line. CD4 T-cell lines were generated from five different patients by multiple stimulations with peptide $PSA_{221-240}$ in the presence of irradiated autologous PBMC. T cells were harvested and incubated at 20,000 cells/well with BLC-DR2b or parental BLC cell lines (10,000 cells/well, irradiated at 10,000 rad) in the presence or absence of peptide $PSA_{221-240}$ (20 μg/mL). IFNγ secretion was measured in culture supernatants after 48 hours of incubation. *A,* T cells were tested at 3rd, 4th, and 5th *in vitro* stimulation (*IVS*; 28, 42, and 56 days after initiation of culture); stimulatory index is calculated as $Response_{peptide}$ / $Response_{medium}$. *B,* responses at 4th *in vitro* stimulation.

lymphocyte separation medium (ICN Biomedicals, Inc., Aurora, OH) and cryopreserved. HLA typing at intermediate resolution and high-resolution typing of *DR15* alleles were determined at the American Red Cross National Histocompatibility Laboratory at the University of Maryland.

Development of human CD4 T-cell lines specific for prostate-specific antigen peptides. CD4 T cells were prepared from PBMC by negative selection using human CD4 T-cell Negative Isolation Kit (Dynal Biotech, Inc., Lake Success, NY). Irradiated (3,300 rad) autologous PBMCs were used as antigen-presenting cells (APC). CD4 T cells were added at 1×10^6 cells/well, irradiated PBMC at 2×10^6 cells/well, and peptides at 20 μg/mL. Cells were incubated at 2 mL/well in 24-well plates in Iscove's modified Dulbecco's media with the following supplements: 2 mmol/L L-glutamine, 0.1 mmol/L MEM nonessential amino acids, 1 mmol/L MEM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.05 mmol/L 2-mercaptoethanol (all reagents from Invitrogen), 5% human AB serum (Gemini Bio-Products, Woodland, CA), and 10 ng/mL recombinant human interleukin-7 (PeproTech, Rocky Hill, NJ) at 37°C in a humidified 5% CO_2 atmosphere. Human recombinant interleukin-2 (Chiron Corp., Emeryville, CA) was added at 30 IU/mL on day 2. Media containing interleukin-2 and interleukin-7 was replaced twice a week. Cells were restimulated every 14 days. The phenotype of surface markers expressed by T-cell lines was determined by flow cytometry using anti-CD4-FITC/anti-CD8-PE, anti-TcRα/β-FITC, and IgG2a-FITC/IgG2a-PE mAbs (Caltag Laboratories, Burlingame, CA).

Functional characterization of human peptide-specific T-cell lines. CD4 T cells were taken to the assay at resting stage, 2 to 3 weeks after the exposure to antigen in a previous stimulation cycle, washed thrice with culture media to remove cytokines, and plated into round-bottomed 96-well tissue culture plates at 20,000 cells/well. Autologous PBMCs were irradiated at 3,300 rad and added at 50,000 cells/well. Peptides or whole PSA was added at different concentrations (5-40 μg/mL). HEK 293 cell line expressing PSA was prepared by transfection of parental cell line with pSecTag2/Hygro/PSA plasmid (Invitrogen). Cells transfected with pSecTag2/Hygro/A plasmid served as a negative control. PSA concentration was determined in culture supernatants by ELISA as previously described (9). To generate dendritic cells (DC), CD14+ cells (>95% pure) were prepared from PBMC by negative selection using Monocyte Negative Isolation kit (Dynal Biotech). Immature DCs were cultured in the presence of recombinant human interleukin-4 and recombinant human granulocyte macrophage colony–stimulating factor (PeproTech) at 100 ng/mL each for 7 days; half of the media with fresh cytokines was replaced on day 4. To ensure optimal antigen uptake and processing, PSA or cell lysates were added at different concentrations to immature DCs on day 4 after initiation of DC culture for 6 to 8 hours followed by incubation with recombinant human tumor necrosis factor α (30 ng/mL) and recombinant human IFNα (50 units/mL; R&D Systems, Inc., Minneapolis, MN) for the remaining 3 days (15). The phenotype of mature DCs was assessed by staining with anti-CD14-FITC (<1%), anti-CD83-PE (18-22%), anti-CD80-PE (13-25%), anti-HLA-DR-PE (70-80%), and anti-CD86-FITC (98%; all mAbs from Pharmingen). DCs were harvested by gentle pipetting, washed four times to remove cytokines, and added at 5,000 cells/well without irradiation. Monoclonal antibodies W6/32 (anti-HLA-A,B,C), L243 (anti-HLA-DR), and IA14 (IgG2a; American Type Culture Collection, Manassas, VA) for blocking studies were produced by culturing hybridoma cells, purified from conditioned culture supernatant using protein A/G columns (Pierce Biotechnology, Inc., Rockford, IL) and were added at 5 to 10 μg/mL. BLC cell line expressing *DR2b* (*DRB1*1501*) and parental BLC line were kindly provided by Dr. W. Kwok (University of Washington, Seattle, WA; ref. 16). The lines were maintained in complete Iscove's modified Dulbecco's media (supplemented with 2 mmol/L L-glutamine, 0.1 mmol/L MEM nonessential amino acids, 1 mmol/L MEM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum). BLC-DR2b line was cultured in the presence of G418 (Invitrogen) at 1 mg/mL. HLA type of BLC lines was determined by PCR-based typing using Micro SSP Generic HLA Class II DNA typing trays; the expression of *HLA-DR1501* in BLC-DR2b line was confirmed using Micro SSP Allele-specific HLA Class II DNA typing trays—DRB1*15 (One Lambda, Inc., Canoga Park, CA). Cells were washed twice before mixing with T cells, irradiated at 10,000 rad, and added at 10,000 cells/ well. Cultures were incubated for 48 hours. IFNγ secretion was measured in culture supernatants by ELISA using a pair of capture and biotinylated detecting mAbs (Pierce/Endogen) as described earlier (9). To determine the level of DNA synthesis, [^{3}H]thymidine (Amersham, specific activity 5 Ci/mmol) was added at 1 μCi / well for additional 18 hours. Cultures were harvested using a Mach III M 96-well cell harvester. Isotope incorporation was determined using a liquid scintillation counter.

Results

Immunization of DR2b transgenic mice with whole prostate-specific antigen. To search for *HLA-DR1501*–restricted immunogenic PSA peptides, we immunized *DR2b* transgenic mice with PSA in complete Freund's adjuvant. After 9 days, draining

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lymph nodes and spleen were harvested and tested for recall responses to the immunizing antigen. The responses to purified protein derivative and concanavalin A served as positive controls. DNA synthesis was determined by [^{3}H]thymidine uptake. The data show a robust dose-dependent immune response to PSA in draining lymph nodes (Fig. 1A). A similar response in spleen was also present although it was lower in magnitude (data not shown). To determine PSA epitopes that are involved in the observed reactivity, DR2b transgenic mice were immunized s.c. with whole PSA in complete Freund's adjuvant. Lymphocytes recovered from immunized animals were stimulated with a PSA peptide library consisting of 20-mers that overlap by 10 amino acids and span the entire PSA amino acid sequence. Figure 1B shows the sequence of PSA and the positions of the first and the last residues for each 20-mer peptide. We found that of the 24 peptides tested in this experiment, two 20-mers derived from the PSA sequence, $PSA_{171\text{-}190}$ and $PSA_{221\text{-}240}$, stimulated proliferative responses both in draining lymph nodes (Fig. 1C) and spleen (data not shown).

Immunization with peptides $PSA_{171\text{-}190}$ and $PSA_{221\text{-}240}$ induces recognition of whole prostate-specific antigen. To ensure that identified 20-mer peptides are immunogenic, *DR2b* transgenic mice were immunized s.c. with peptides $PSA_{171\text{-}190}$ or $PSA_{221\text{-}240}$ in complete Freund's adjuvant; draining lymph node cells and splenocytes were harvested 9 days later. Draining lymph node T cells from the peptide $PSA_{171\text{-}190}$-vaccinated *DR2b* transgenic mice recognized $PSA_{171\text{-}190}$ as well as whole PSA (Fig. 2A) although the responses were moderate and seen only at high antigen concentration (50 μg/mL). Similar data were obtained with splenocytes (data not shown). Immunization with peptide $PSA_{221\text{-}240}$ induced strong responses to the peptide as well as whole PSA in draining lymph nodes (Fig. 2B) and spleen (data not shown); no cross-reaction with peptides $PSA_{161\text{-}180}$ or $PSA_{171\text{-}190}$ was observed. The responses to the specific peptide were much higher in $PSA_{221\text{-}240}$-immunized mice compared with peptide $PSA_{171\text{-}190}$-immunized mice, and were seen in a broad range of peptide concentrations. These data show that the peptides $PSA_{171\text{-}190}$ and $PSA_{221\text{-}240}$ are naturally processed and presented by mouse APC expressing *HLA-DRB1*1501*.

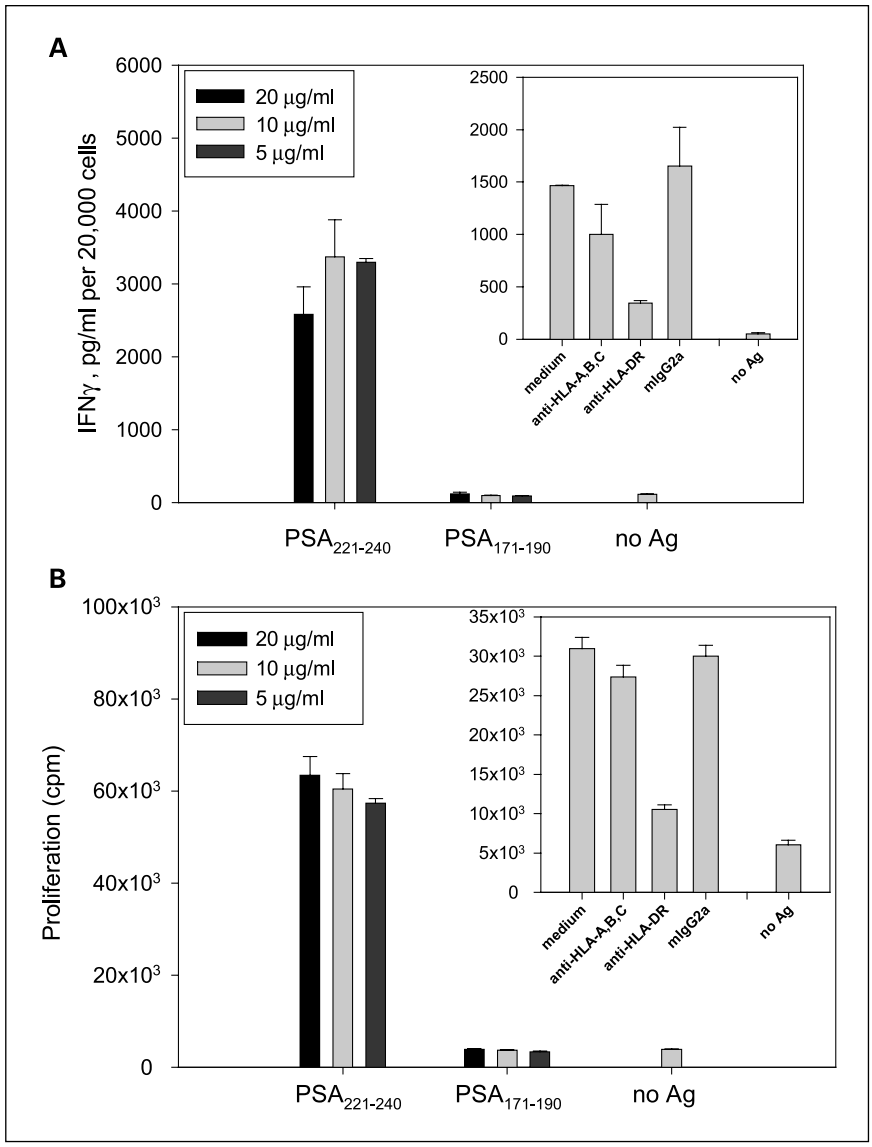

Fig. 5. The responses of human CD4 T-cell lines to peptide $PSA_{221\text{-}240}$ presented by autologous DCs. CD4 T cells specific to peptide $PSA_{221\text{-}240}$ were incubated with mature autologous DCs (5,000 cells/well) and peptides at different concentrations. IFNγ secretion was measured in culture supernatants after 48 hours of culture (*A*). Cultures were then pulsed with [^{3}H]thymidine and cpm determined 18 hours later (*B*). Columns, mean of triplicate determinations; bars, SD. Response for patient Pr131 at 4th *in vitro* stimulation. Inset, cells were cultured in the presence of anti-HLA-A,B,C, anti-HLA-DR antibodies, or control IgG2a. Response for patient Pr131 at 5th *in vitro* stimulation.

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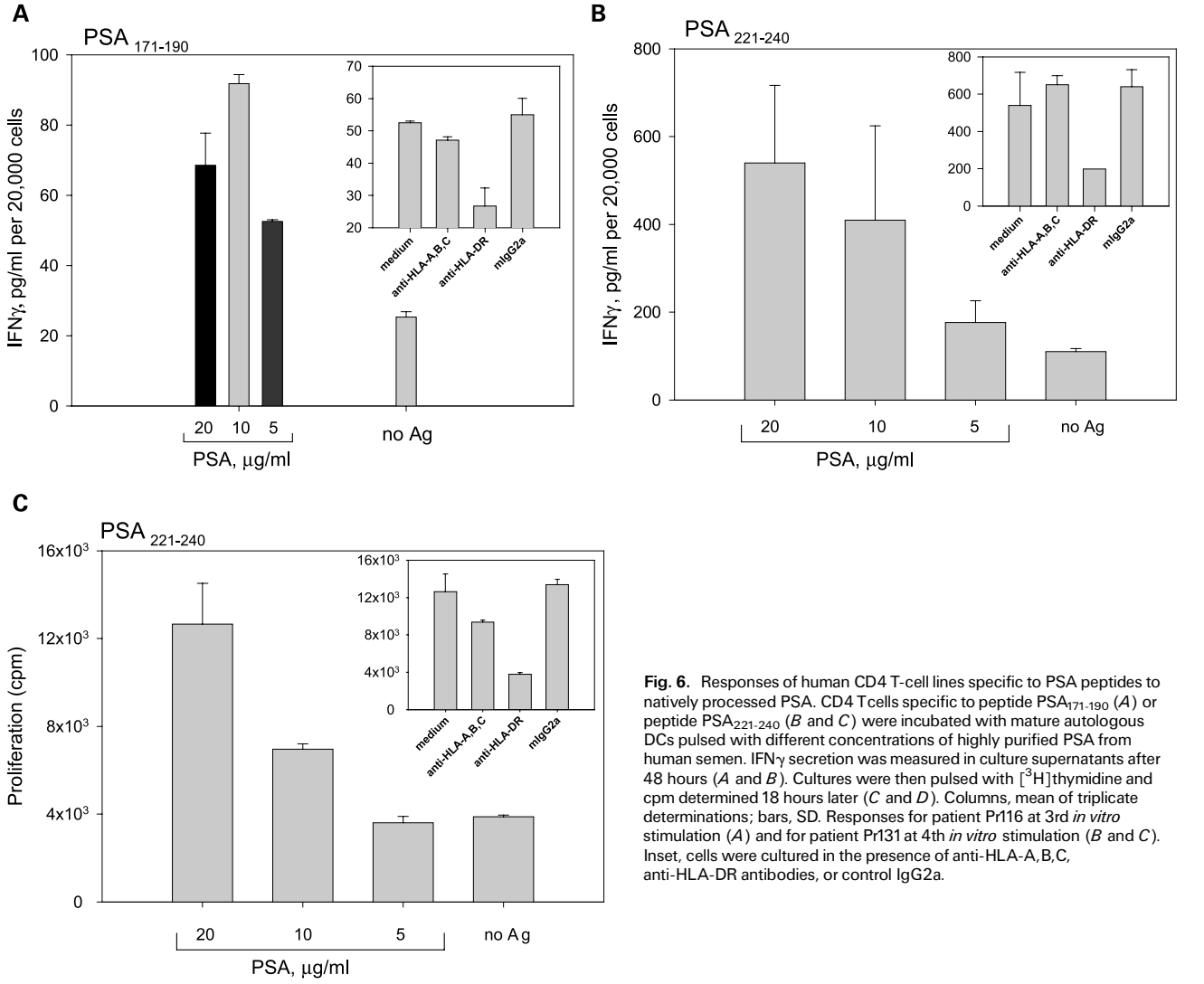

Fig. 6. Responses of human CD4 T-cell lines specific to PSA peptides to natively processed PSA. CD4 T cells specific to peptide $PSA_{171-190}$ (*A*) or peptide $PSA_{221-240}$ (*B* and *C*) were incubated with mature autologous DCs pulsed with different concentrations of highly purified PSA from human semen. IFNγ secretion was measured in culture supernatants after 48 hours (*A* and *B*). Cultures were then pulsed with [^{3}H]thymidine and cpm determined 18 hours later (*C* and *D*). Columns, mean of triplicate determinations; bars, SD. Responses for patient Pr116 at 3rd *in vitro* stimulation (*A*) and for patient Pr131 at 4th *in vitro* stimulation (*B* and *C*). Inset, cells were cultured in the presence of anti-HLA-A,B,C, anti-HLA-DR antibodies, or control IgG2a.

Development of human CD4 T-cell lines specific to prostate-specific antigen–derived 20-mer peptides $PSA_{171-190}$ and $PSA_{221-240}$. To determine whether the PSA epitopes identified in the experiments with transgenic mice can stimulate immune responses in *HLA-DR1501+* individuals, short-term CD4 T-cell lines were established from PBMC of several *HLA-DRB1*1501+* patients with granulomatous prostatitis and one patient with prostate cancer (Pr97) by repeated stimulation with either $PSA_{171-190}$ or $PSA_{221-240}$ presented by irradiated autologous PBMC. Cultures were tested for the reactivity with specific peptides using irradiated autologous PBMC, mature DC, or BLC-DR2b cells as APC. Relatively weak reactivity with peptide $PSA_{171-190}$ measured by IFNγ secretion was observed in three of five patients with granulomatous prostatitis after 2nd *in vitro* stimulation (Figs. 3 and 8A); further expansion of the cultures was unsuccessful. The responses to the peptide were HLA-DR restricted because IFN-γ secretion was blocked by the addition of anti-HLA-DR but not with anti-HLA-A,B,C mAb (Fig. 3, inset).

When peptide $PSA_{221-240}$ was used for stimulation, long-term CD4 T-cell lines from all five patients were established that showed peptide-specific reactivity at 3rd, 4th, and 5th *in vitro* stimulation (Fig. 4A). To prove that the responses are *HLA-DRB1*1501* restricted, BCL cell line engineered to express *HLA-DRB1*1501* molecule (*DR2b*) was used as APC. CD4 T-cell lines from all five patients showed strong specific responses to the peptide $PSA_{221-240}$ presented by BCL-DR2b cells as measured by IFNγ secretion (Fig. 4A and B). No responses to the peptide presented by parental BCL cells (*HLA-DRB1*1501* negative) were seen in patients Pr115 and Pr119 whereas T-cell lines from three other patients (Pr97, Pr116, and Pr131) showed low levels of reactivity with the peptide in an *HLA-DR2b*–independent fashion (Fig. 4A). The parental BCL line expressed *HLA-DRB1*03*, *HLA-DRB1*11*, *HLA-DRB3*01*, and *HLA-DQB1*05* alleles as shown by intermediate resolution PCR-based typing. The proliferation and IFNγ secretion were seen in a broad range of peptide concentrations (from 40 to 5 μg/mL) as shown in representative

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experiments for patient Pr131 (Fig. 5A and B) and patient Pr97 (Fig. 8B and C); no cross-reactivity with peptide $PSA_{171-190}$ was observed. The HLA Class II restriction of $PSA_{221-240}$-specific T-cell lines was confirmed using blocking mAbs to *HLA-DR* (Fig. 5A and B, insets). The phenotype of all T-cell lines was exclusively CD4 positive and CD8 negative as determined by immunofluorescence staining at 3rd *in vitro* stimulation (data not shown). The analyses of sequence similarity of both epitopes to the human proteome were conducted using the PIR nonredundant reference protein database and peptide match program (17). For any 5-mer motifs within 20-mer amino acid sequences, $PSA_{171-190}$ showed a maximum of four matches, and $PSA_{221-240}$ a maximum of seven matches (data not shown).

Peptide-specific human T-cell lines recognize whole prostate-specific antigen. Next we tested whether peptide-specific T-cell line could recognize and respond to naturally processed whole PSA. Because immature rather than mature DCs are most effective in the endocytosis and processing of whole proteins, purified PSA was added to immature DC cultures on day 4 after initiation of DC culture. After 6 to 8 hours of incubation, recombinant human tumor necrosis factor α and recombinant human IFNα were added for an additional 3 days to induce maturation. As shown in Fig. 6A, cultured CD4 T cells from patient Pr116 stimulated *in vitro* with peptide $PSA_{171-190}$ produced IFNγ in response to DCs pulsed with whole PSA. The responses were blocked by anti-*HLA-DR* but not by *HLA-A,B,C* – specific mAbs (Fig. 6A, inset). Similar data were obtained with CD4 T cells from granulomatous prostatitis patients Pr115 and Pr122 (data not shown). Similarly, cultured CD4 T-cell lines stimulated *in vitro* with peptide $PSA_{221-240}$ secreted IFNγ (Fig. 6B) and proliferated (Fig. 6C) in response to DCs pulsed with whole protein. To ensure that peptide $PSA_{221-240}$-specific T cells can recognize PSA endogenously expressed in tumor cells, we pulsed DCs with cell lysates prepared from 293 cell line engineered to express PSA. Lysates prepared from 293 line transfected with the empty vector served as a negative control. As shown in Fig. 7, $PSA_{221-240}$-specific T cells from patient Pr131 responded to DCs pulsed with PSA-containing cell lysate, but not with control lysate. The responses to the 293/PSA lysate were *HLA-DR* restricted (Fig. 7, inset). Similar results were obtained for other granulomatous prostatitis patients (Pr115, Pr116, and Pr119) and prostate cancer patient Pr97 (data not shown). These experiments show that the peptides identified in the *DR2b* transgenic mice stimulated human T cells and these T cells are capable of recognizing naturally processed and presented PSA.

Determination of the minimal epitope. The amino acid sequences for the peptides $PSA_{171-190}$ and $PSA_{221-240}$ were analyzed using the ProPred computer algorithm (18). The analysis indicated that both 20-mers contain 9-amino-acid *HLA-DR1501* – binding predicted core motifs, $PSA_{171-179}$ and $PSA_{223-231}$, with a score of 2.9 (30% of maximal achievable score) for both sequences. Based on the core sequences and the fact that no responses were observed with adjacent overlapping 20-mer peptides, 13-mer peptides, $PSA_{169-181}$ and $PSA_{221-233}$, were designed by adding two flanking amino acids to both sides of the core peptide (Fig. 1B). CD4 T-cell lines specific to either 20-mer peptide $PSA_{171-190}$ (Fig. 8A, patient Pr115) or peptide $PSA_{221-240}$ (Fig. 8B and C, patient Pr97) responded to the corresponding 13-mer peptides, suggesting that the minimal T-cell determinants presented by *HLA-DR1501* molecule are present within these sequences.

Discussion

In this study, we immunized transgenic mice that express an *HLA-DRB1*1501* chimeric construct on a mouse class II knockout background with human PSA and observed a robust recall proliferative response to the antigen. By screening a 20-mer peptide library that overlapped by 10 amino acids and spanned the entire mature, secreted PSA sequence, we identified two 20-mer peptides, $PSA_{171-190}$ and $PSA_{221-240}$, that were immunogenic *in vivo* in *DR2b* transgenic mice. We found that these epitopes determined in mice were capable of stimulating human CD4 T

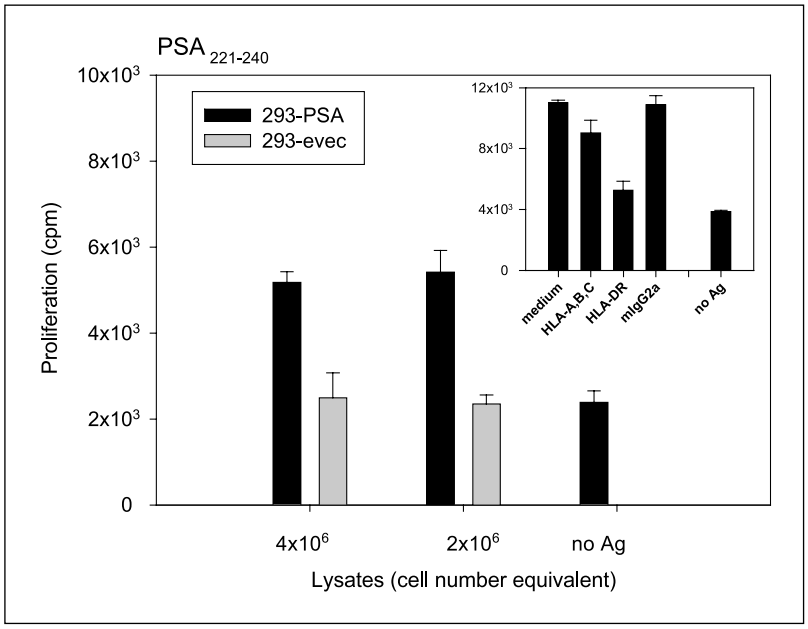

Fig. 7. Responses of human CD4 T-cell lines specific to peptide $PSA_{221-240}$ to antigen endogenously expressed in tumor cells. Peptide-specific T cells were incubated with mature autologous DCs pulsed with different concentrations of lysate prepared from HEK 293 cell line transfected with PSA (*293-PSA*) or control "empty" vector (*293-evec*). After 48 hours of incubation, cultures were pulsed with [^{3}H]thymidine and cpm determined 18 hours later. Columns, mean of triplicate determinations; bars, SD. Response for patient Pr131 at 4th *in vitro* stimulation. Inset, cells were cultured in the presence of anti-HLA-A,B,C, anti-HLA-DR antibodies, or control IgG2a. Response for patient Pr131 at 5th *in vitro* stimulation.

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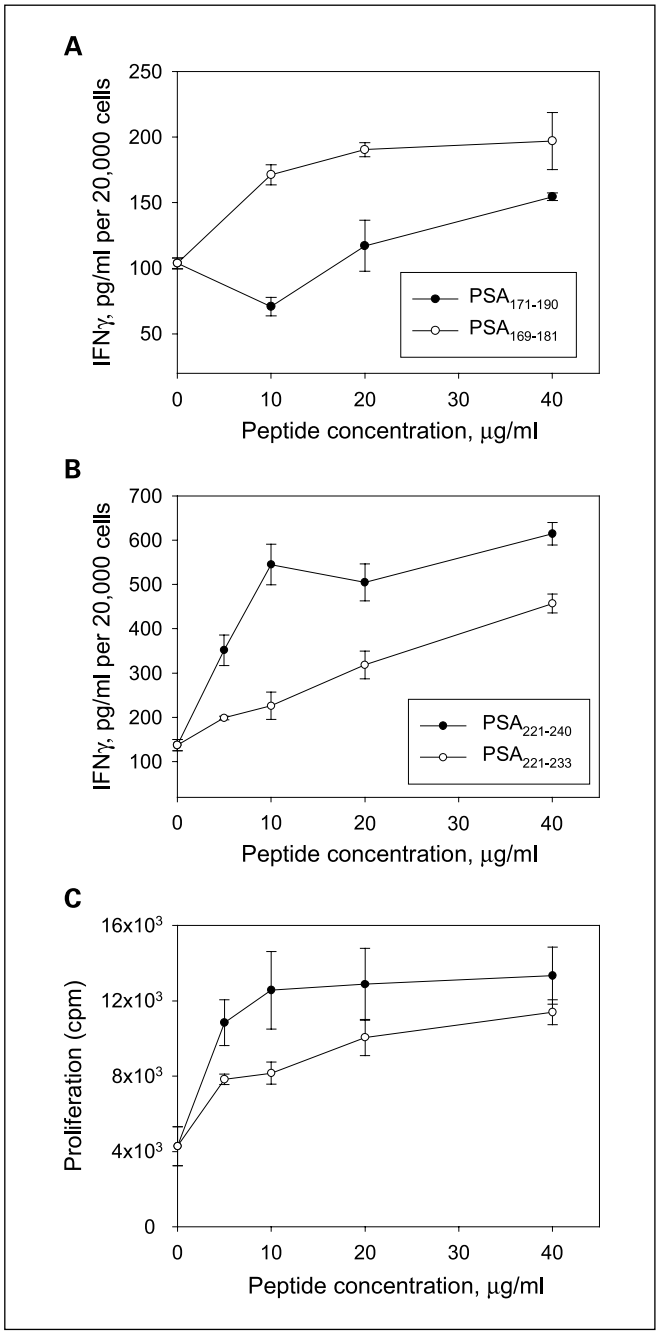

Fig. 8. The responses of human CD4 T-cell lines specific to 20-mer peptide $PSA_{171-190}$ and $PSA_{221-240}$ to the minimal T-cell determinant. CD4 T-cell lines specific to $PSA_{171-190}$ (*A*) or $PSA_{221-240}$ (*B* and *C*) were incubated with irradiated autologous PBMC in the presence of different concentrations of original 20-mer peptide (●) or 13-mer peptide containing core *HLA-DRB1*1501* – binding epitope (○). IFNγ secretion was measured in culture supernatants after 48 hours of culture (*A* and *B*). Cultures were then pulsed with [^{3}H]thymidine and cpm determined 18 hours later (*C*). Columns, mean of triplicate determinations; bars, SD. Responses for patient Pr115 at 3rd *in vitro* stimulation (*A*) and patient Pr97 at 5th *in vitro* stimulation (*B* and *C*).

cells derived from patients with granulomatous prostatitis and prostate cancer that are *HLA-DRB1*1501* positive and were naturally processed by human APC. Of the two epitopes detected, $PSA_{221-240}$ proved to be immunodominant. This is a first demonstration of *HLA-DRB1*1501* – restricted peptides derived from PSA that are naturally processed.

The advantage of the *DR2b* transgenic mouse system is especially apparent for identification of naturally processed peptides. Because antigen processing in various mammalian cells is quite similar, it was likely that peptides identified in the *DR2b* transgenic mice would be immunogenic in human *HLA-DRB1*1501*+ PBMC cultures. The correlation of such responses is best shown by the work of Madsen et al. (11) who showed that when the *DR2b* transgenic mice were also made transgenic for a human T-cell receptor that recognized a peptide from human myelin basic protein (84-102), the animals developed a multiple sclerosis – like disease showing that the same peptide from patients was presented in the mice. This provides strong evidence for the notion that peptides identified in *DR2b* mice vaccinated with human PSA will also be naturally processed from whole PSA in humans. Indeed, $PSA_{171-190}$- and $PSA_{221-240}$-specific T cells were able to recognize whole PSA in an MHC-restricted manner showing that these peptides are naturally processed and presented by mouse and human APC.

Our previous attempts to identify Class-II – restricted PSA epitopes using TEPITOPE (19) or ProPred (18) programs predicting peptide binding has not been successful probably because this approach concentrates on high-score (high affinity) epitopes. Yet numerous data indicate that the majority of autoreactive peripheral blood T cells are of low affinity. This is particularly important for naturally processed peptides because T cells recognizing these peptides with high affinity undergo negative selection in thymus. Both $PSA_{171-190}$ and $PSA_{221-240}$ peptides contained 9-mer core amino acid sequences with relatively low potential binding to *HLA-DRB1*1501* (30% of the highest achievable score, ProPred program). However, both fragments showed a low level of similarity to other human proteins when analyzed using PIR nonredundant reference protein database and peptide match program (17). It has been shown recently for melanoma-derived antigens that epitopes with low similarity level to the human proteome are preferred immunogenic epitopes (20). Low level of similarity is also important when a peptide is considered as a candidate for immunotherapy because it indicates that the probability of cross-reaction with other tissues after immunization with these peptides is low.

Significant difficulties in generation of human antigen-specific CD4 T-cell lines from peripheral blood emphasize the value of selecting donors that were exposed to the antigen in the past and have memory T cells in peripheral circulation. We overcame this problem by utilizing a unique population of patients with granulomatous prostatitis that may manifest naturally the immune responses to prostatic antigens. Even within this group, it required more than two *in vitro* stimulation to show reliable and reproducible antigen-specific responses. Although the antigen responsible for the autoimmune process in granulomatous prostatitis patients has not been identified yet, PSA is a good candidate to be such an antigen. We showed earlier that the reactivity to PSA but not to other secretory proteins of prostate, such as prostatic acid phosphatase or β-seminoprotein, has been detected in peripheral blood of patients with chronic prostatitis/chronic pelvic pain syndrome, and such a response has not been seen in healthy men (21). PSA-reactive CD4 and CD8 T-cell lines were established from peripheral blood of a patient with granulomatous prostatitis (9). Whereas PSA was the most immunogenic of the three major

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secreted soluble proteins made by the prostate epithelium, there may be other antigens in the gland that manifest a stronger immune response or an immunodominant response in the context of *HLA-DRB1*1501*. The search for other antigens in the prostate will also be facilitated by the use of *DR2b* transgenic mice. Given the identification of PSA peptides defined here, studies of prostate antigen reactivity using peptide class II tetramers (22) in *HLA-DRB1*1501* men with granulomatous prostatitis and normal donors will likely clarify the role of PSA in the development of granulomatous prostatitis. Patients with granulomatous prostatitis should have higher reactivity with prostate antigens if autoimmunity is important in the etiology of the disease.

The choice of the restriction element for our study was also dictated by our previous finding that granulomatous prostatitis is linked to *HLA-DRB1*1501* allele in Caucasians (7). It has been shown in melanoma that positive clinical outcome of immunotherapy correlated with the development of autoimmune responses to normal melanocytes (vitiligo; ref. 23). Similarly, the induction of autoimmune responses to prostate antigens in prostate cancer patients with appropriate susceptible HLA type seems to be very attractive because prostate is not a vital organ beyond reproductive years. We were able to establish CD4 T-cell line specific to $PSA_{221-240}$ from peripheral blood of a patient with prostate cancer. These data suggest that the response to this epitope can occur naturally during antitumor immune recognition. This observation needs to be confirmed further using a larger group of *HLA-DR15*+ patients with prostate cancer. It also needs to be determined whether the disease progression and survival correlates with *HLA-DR* phenotype and the presence and phenotype of circulating CD4+ T cells reactive to prostate antigens.

In summary, we have identified two *HLA-DRB1*1501*–restricted peptides derived from human PSA that are naturally processed by mouse and human APCs and are recognized by *HLA-DRB1*1501*–restricted CD4 T cells from patients with granulomatous prostatitis and prostate cancer. The $PSA_{221-240}$ epitope induced stronger responses compared with $PSA_{171-190}$ in both the mice and patients. The use of these peptides in vaccination strategies for the treatment of prostate cancer is under investigation. This study will be instrumental in attempting to understand the interaction between circulating self-reactive T cells, organ-specific autoimmunity, and the immune response to prostate tumors.

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