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A modified epitope identified for generation and monitoring of PSA-specific T cells in patients on early phases of PSA-based immunotherapeutic protocols

Kajsa Lundberg a,*, Anna-Karin Roos a, 1, Maxim Pavlenko a, Christoph Leder b, Diana Wehrum a, José Guevara-Patiño c, Rikke Sick Andersen d, Pavel Pisa a

a Department of Oncology and Pathology, Cancer Center Karolinska R8:01, Immune and Gene Therapy Laboratory, Karolinska Institute, 171 76 Stockholm, Sweden
b Department of Physiological Chemistry, University of Ulm, D-89081 Ulm, Germany
c Laboratory of Tumor Immunology and Cancer Vaccine Development, Department of Surgery, The University of Chicago, Chicago, IL, USA
d Center for Cancer Immune Therapy (CCIT), Department of Hematology, 54P4, University Hospital Herlev, 2730 Herlev, Denmark

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Efficacy of vaccination in cancer patients on immunotherapeutic protocols can be difficult to evaluate. The aim of this study was therefore to identify a single natural or modified epitope in prostate-specific antigen (PSA) with the ability to generate high levels of PSA-specific T cells to facilitate monitoring in patients after vaccination against prostate cancer. To the best of our knowledge, this study describes for the first time the peptide specificity of T cells stimulated by endogenously processed PSA antigen. The peptide specificity of HLA-A*0201-restricted CD8+ T cells against human and rhesus PSA was investigated both in vivo after DNA vaccination in HLA-A*0201-transgenic mice and in vitro after repetitive stimulation of human T cells with DNA-transfected human dendritic cells (DCs). One of seven native PSA peptides, psa53–61, was able to activate high levels of PSA-specific CD8+ T cells in HLA-A*0201-transgenic mice after PSA DNA vaccination. Psal53–61 was also the only peptide that induced human T cells to produce IFNγ after stimulation with PSA transfected DCs, however not in all donors. Therefore, plasmids encoding modified epitopes in predicted HLA-A*0201 sequences were constructed. One of these modified PSA plasmids consistently induced IFNγ producing CD8+ T cells to the corresponding modified peptide as well as to the corresponding native peptide, in all murine and human T cell cultures. This study demonstrates a novel concept of introducing a modified epitope within a self-tumor antigen, with the purpose of eliciting a reliable T cell response from the non-tolerized immune repertoire, to facilitate monitoring of vaccine efficacy in cancer patients on immunotherapeutic protocols. The purpose of such a modified epitope is thus to not induce therapeutically relevant T cells but rather to, in case of weak or divergent T cell responses to self antigens/peptides, help answer questions about efficacy of vaccine delivery and about the possibility to induce immune responses in the selected and often immunosuppressed cancer patients.

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1. Introduction

Prostate cancer (PC) is one of the most common malignancies in elderly men. As all treatments for patients with hormone-refractory disease are palliative, and the median survival time for these patients is still below 2 years, there is an urgent need for novel treatment approaches. The development of immunotherapeutic vaccination protocols against PC, based on the induction of autoimmunity to prostate tissues, is attractive since the prostate is not a vital organ beyond the reproductive years.

Human prostate-specific antigen (PSA) is one of the prostate differentiation antigens which have been used extensively as a target for T cell induced immunotherapy against PC. The peptides derived from PSA by endogenous processing that are presented on the cell surface in complexes with HLA class I molecules, represent the fine targets recognized by cytotoxic T lymphocytes (CTLs) on tumor cells. Due to the high frequency of the HLA-A*0201 allele in the North American–European population, most of the effort has been focused on characterization of HLA-A*0201-restricted epitopes derived from human PSA. Furthermore, an overrepresentation of the HLA-A2 phenotype has been observed in hormone-refractory PC patients compared to the normal population [1]. A number of
peptides possessing a HLA-A*0201-binding motif have been applied for in vitro generation of PSA-specific CTLs from peripheral blood of healthy donors and PC patients [2–6], as well as for monitoring of T cell responses in patients receiving PSA-based vaccines [7,8].

We have previously demonstrated that a DNA vaccine encoding full-length human PSA induces PSA-specific CTLs and partial protection against a tumor expressing the PSA protein in C57Bl/6 mice [9,10]. Based on these results, a phase I clinical trial was conducted investigating the T cell responses to a PSA DNA vaccine in patients with hormone-refractory PC [11]. Stimulation of purified CD8+ T cells from the vaccinated PC patients, with three HLA-A*0201-restricted PSA peptides (FLTPKKLQCV165–174, MLLRLSEPAA12–132, and VISNDVCAQV178–187), demonstrated reactivity against all three peptides across the study [12]. However, responses to the different peptides varied between patients, and none of the tested peptides stimulated CD8+ T cells from all patients, which is in line with the variable peptide-specific CD8+ T cell responses observed by others in HLA-A*0201 positive healthy donors and cancer patients [3,4,8,13]. These rather weak and divergent peptide responses suggest that human PSA may not contain any strong immunodominant HLA-A*0201-restricted CTL epitopes or alternatively, that the autologous T cells with reactivity against such epitopes are tolerized during the selection in the thymus or in the periphery. One potential approach to circumvent such obstacles is to design vaccines based on xenogeneic and/or modified PSA sequences containing altered structural motifs that is able to activate PSA-specific T cells in all healthy donors or PC patients investigated, this study aimed at identifying and, if not native, insert such an epitope into the rhesus vaccine. The purpose of such a universal epitope, native or modified, would not primarily be to induce therapeutically relevant T cells but rather to, in case of weak or divergent T cell responses due to PSA peptides with low MHC affinity or to a tolerized immune repertoire, aid immune monitoring after immunotherapy and help answer questions about efficacy of vaccine delivery and about the patient’s immune status.

A recent publication reviewed the importance of applying aimed appropriate tools for monitoring of T-cell responses in cancer vaccine development [16] and especially highlighted the need for surrogate markers for clinical efficacy to bridge immune monitoring assays and clinical endpoints. Furthermore, the interpretation of results obtained from immune monitoring of clinical trials is impedied by the variety of methods and protocols available to detect vaccine-specific T-cell responses. An effort to standardize assays for enumerating antigen specific T cells represents the focus of several recent publications [17,18], where both functional (ELISpot) and structural (tetramer) assays are being compared.

In the present study, a panel of seven native HLA-A*0201-restricted PSA peptides was investigated for HLA-A*0201 binding and for activation of T cells induced after stimulation with endogenously processed PSA. One T cell epitope (psa53–61) was identified after analyzing the peptide-specificity of the CD8+ T cells induced in HLA-A*0201–transgenic mice following vaccination with DNA encoding full-length PSA and after stimulation of human T cells with DNA-transfected autologous dendritic cells (DCs). In vitro stimulations of T cells from PC patients using full-length human or rhesus PSA only induced psa53–61-specific T cells in 1 of 3 patients in cultures stimulated with human PSA, and 2 of 3 patients when stimulated with rhesus PSA. Therefore, with the aim of identifying an epitope able to consistently activate human PSA-specific T cells, we constructed and tested the immunogenicity of DNA plasmids encoding rhesus PSA containing modified HLA-A*0201-restricted epitopes. One of the modified DNA constructs induced PSA-specific CD8+ T cells in all of the HLA-A*0201-transgenic mice and in all in vitro stimulated PBMCs from healthy donors and PC patients. We suggest that the presence of such T cells, activated by a modified PSA epitope, could serve as a marker of successful vaccine delivery and facilitate monitoring of vaccine-induced immune responses in early clinical trials of PC.

2. Material and methods

2.1. Peptides

Non-modified HLA-A*0201-restricted PSA peptides; psa52–60 (GVLVHPQW, psa53–61 (VLVHPQWLV), psa40–49 (VIVASRGRAV), psa122–130 (MLLRLSEPA, psa165–174 (FLTPKKLQCV), psa170–178 (KLQCVDLHV), psa175–183 (DLHVISNDV) and psa178–187 (VISNDVCAQV). Modified HLA-A*0201-restricted PSA peptides; psa7–15 (115V) (FLTL5TSTWV), psa52–60 (V53L) (GLVH- POQW) and psa121–129 (P129V) (LMLLRSEL). Note that peptide starts from the first amino acid in the pro-PSA sequence. Positive/negative control peptides; HLA-A*0201-restricted influenza peptide flu58–66 (GILGFVFTL) and HIV-1pol476–484 (ILKEPVHGV) and the H-2Db-restricted LCMV derived peptide GP33 (KAVYNFATC). All peptides were purchased from GenScript Corporation (NJ, USA) or Proimmune (Oxford, UK) at a purity >95%. The peptides were purified by reverse phase chromatography (HPLC) and peptide composition was verified by MALDI-TOF (Mass-Spec). The peptides were dissolved in DMSO and stored at −20 °C.

2.2. HLA-A*0201 reconstitution assay

Human C1R-A2 cells (stably transfected with episcopal vector pHebo-A2 to express HLA-A*0201) were kindly provided by Dr. R. Kiessling (Cancer Center Karolinska, Karolinska Institute, Stockholm, Sweden), and were cultured in RPMI 1640 medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 25 μg/ml gentamycin, 10% FCS and 250 μg/ml selective antibiotic Hygromycin B (Roche Diagnostics GmbH, Mannheim, Germany). The HLA-A*0201 reconstitution assay using C1R-A2 cells was performed as described previously [19] with a few modifications. Briefly, C1R-A2 cells were washed in ice cold PBS and the cell pellet was cooled on ice for 5 min. Subsequently, HLA-A*0201 molecules were disassembled by mild acid treatment (a 3:1 mixture of 0.1 M citric acid and 0.2 M Na2HPO4, pH 3.2) for 90 s. The acid was buffered by adding ice cold serum-free RPMI 1640 to the cells. The cells were washed and resuspended to 3 × 106 cells in 200 μl staining buffer (PBS with 0.5% BSA, 0.05% NaN3) with 100 nM human β2-microglobulin (β2m) (Sigma, St. Louis, MO, USA) and 1, 10 or 100 μM peptide. After 24 h incubation at 4 °C, the cells were stained for HLA-A*0201 expression (mouse anti-HLA-A*0201-FITC, SEROTEC). Control cells were stained with an isotype-matched control (IgG2b-FITC, SEROTEC), and 1% mouse serum was included in all stainings to block unspecific binding. Samples were analysed using a FACS Calibur flow cytometer and CELLQuest software (Becton Dickinson). Relative HLA-A*0201 affinity of each peptide was calculated with the formula: mean HLA-A*0201 fluorescence with PSA peptide/mean HLA-A*0201 fluorescence with H-2Dβ-restricted peptide GP33.

2.3. Assembly assay for peptide binding to class I HLA molecules

HLA-A*0201 binding affinity of the synthetic peptides was carried out as described by Andersen et al. [20,21], only the lysis buffer was modified (Mega-9 was excluded and the protease inhibitors
used were 5 mM iodoacetamide, 2 μg/ml pepstatin and 200 μg/ml pefabloc. In short, the assembly assay is based on stabilization of the HLA class I molecules after loading of different concentrations of peptide to the transporter associated with antigen processing (TAP)-deficient cell line T2. The T2 cells have been metabolically labelled with [35S]-methionine. Stably folded HLA molecules were immune-precipitated and separated by isoelectric focusing (IEF) gel electrophoresis. MHC heavy chain bands were quantified using the FLA-3000 phosphorimager and the Multi Gauge V3.1 software from Fujifilm. The intensity of each band is directly related to the amount of peptide-stabilized HLA class I complex recovered during the assay. The recovery of HLA-A2 was measured in the presence of 40, 4, 0.4, and 0.04 μM of the relevant peptide.

2.4. Plasmids, algorithm-based prediction of modified epitopes and site-directed mutagenesis

Plasmids pVAX/PSA and pVAX/rhPSA were constructed by inserting the genes coding for the full-length human PSA protein [22] (obtained from Dr. T. Ratliff, Washington University, St. Louis, USA) and rhesus PSA protein, respectively, into vector pVAX1 (Invitrogen, Carlsbad, CA). The cDNA encoding rhesus PSA was produced by reverse transcription from prostate mRNA obtained from a rhesus monkey (Macaca mulatta) (kindly provided by Dr. A. King, Cyto Pulse Sciences, MD). Sequence analysis of the rhesus cDNA revealed a nucleotide difference of adenine to guanine at position 578 compared with the published sequence (GenBank accession no. X73560), resulting in a substitution of lysine (K) with glutamic acid (E) at position 194 in the protein sequence (Fig. 1).

The modified plasmids rhPSA/II5V, rhPSA/V53L and rhPSA/P129V contain one amino acid substitution at the indicated positions. These plasmids, encoding nanopeptides created for HLA-A*0201, were designed by algorithm-based prediction with a cut-off value for predicted natural processing using the EpitOptimizer program [23]. Three epitopes were selected based on high HLA-A*0201-binding score and the criteria that their native sequences were also present in human PSA. By site-directed mutagenesis in pVAX/rhPSA (QuickChange II kit, Stratagene, AH diagnostics AB, Sweden) plasmids encoding the modified epitopes were created, using the following primers (complementary primers are not shown, mutated bases are underlined); rhPSA/II5V: 5′-GTCCGTGAGTGGGTTGGCGTGACCC-3′; rhPSA/V53L: 5′-GTCCGTGAGTGGGTTGGCGTGACCC-3′; rhPSA/P129V: 5′-CGCCCGCTAGGTTGGCGTGACCC-3′. Mutations were verified by DNA sequence analysis and PSA expression was confirmed through transient transfection of 293 cells, followed by detection of PSA protein in the supernatant by Western blot using rhesus anti-sera (Innovagen, Lund Sweden). All plasmids were amplified in bacteria and purified using the Endotoxin Free Plasmid Purification Kit (Qiagen, Hilden, Germany).

2.5. HLA-A*0201-transgenic mice

HHD homozygous mice [24] were kindly provided by Dr. F. Lemonnier and were bred and housed at the animal facility at Microbiology and Tumor Biology Center at the Karolinska Institute (Stockholm, Sweden). HHD mice express a HLA-A*0201 chimeric monochain (α1 and α2 domains of HLA-A*0201 allele and the α3 and intracellular domains of H-2Dβ allele) covalently linked by its N-terminus to the C-terminus of the human β2m by a 15-amino acid peptide arm [25]. The genes for both the H-2Dβ and mouse β2m have been disrupted and therefore the HHD mice are deprived of cell surface H-2 molecules. All experiments were approved by the Swedish National Board for Laboratory Animals.

2.6. DNA immunization

Mice were anesthetized with 4% isoflurane (Baxter Medical AB, Kista, Sweden) and maintained at 2.5% isoflurane in a mask during immunizations. 20 μg DNA in PBS was injected intradermally on each flank, near the base of the tail. Subsequently, a needle array electrode was placed over the raised skin area of injection and voltage was applied (2 pulses, 1125 V/cm, 50 μs + 8 pulses, 275 V/cm, 10 ms) [26]. The needle array electrode consisted of two parallel rows of four 2-mm pins (Cyto Pulse Sciences, Inc. Glen Burnie, MD). Electroporation was performed using the PA-4000S-Advanced PulseAgile® Rectangular Wave Electroporation System and software (Cyto Pulse Sciences, Inc.).

Fig. 1. Alignment of human PSA (hPSA) and rhesus PSA (rhPSA). Alignment of hPSA and rhPSA protein sequence demonstrates 89.7% identity in amino acid sequence. Brackets show positions of all PSA peptides used in the paper. Boxes indicate positions of the different amino acid substitutions made in the modified PSA peptides and plasmids. Arrow indicates a spontaneous mutation (K → E) detected in the rhesus PSA sequence.
2.7. Murine lymphocyte preparation and in vitro restimulation

Mice were euthanized and spleens harvested 13–15 days after the last immunization. Single-cell suspensions were obtained by homogenizing spleens and passing cells through a 70 µm cell strainer. The erythrocytes were removed by Ammonium Chloride Lysing Reagent (BD Biosciences, PharMingen) and after washings the cells were resuspended to 10 × 10^6 cells/ml in DMEM complete media (DMEM medium supplemented with 10 mM HEPES, 5 µM 2-mercaptoethanol, 25 µg/ml gentamycin, 2 mM l-glutamine, 1% non-essential amino acids, and 5% FCS). The splenocytes were used directly (ex vivo) or restimulated in vitro for 5 days with 100 nM of synthetic peptide in complete DMEM medium supplemented with 20 IU/ml human recombinant IL-2 (PROLEUKIN, Chiron Corporation, Emeryville, CA) at 2 × 10^6 splenocytes/ml.

2.8. Intracellular cytokine staining for murine IFNγ

Freshly prepared or restimulated splenocytes (1 × 10^6/well) were cultured for 5 h in 96-well plates with 10 µM HLA-A*0201-restricted synthetic peptides from PSA or Flu. After 2.5 h of incubation at 37 °C GolgiPlug reagent (BD Biosciences, PharMingen) was added to the cells and incubation continued for another 2.5 h. Intracellular cytokine staining (ICS) was performed as described previously [27].

2.9. Patients and healthy donors

HLA-A*0201 positive patients with prostate adenocarcinoma, stage II or III, were HLA-typed by PCR (Clinical Immunology Laboratory, Karolinska University Hospital, Huddinge, Sweden). Male healthy donors were confirmed to be HLA-A2 positive by double positive staining with supernatants from hybridoma HB-54 and HB-82 (kindly provided by Dr. D. Schendel, National Research Center, Munich, Germany). The study was approved by the local ethics committee, and written informed consent was obtained from all individuals in accordance with the declaration of Helsinki.

2.10. Generation of mature PSA-transfected human dendritic cells

PC patients were leukapheresed and lymphocytes and monocytes were separated by elutriation (ELUTRA, Gambro, Sweden). Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained by centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Sweden). All cells were aliquoted and cryopreserved in liquid nitrogen. Monocytes from healthy donors were obtained by CD14 antibody-coated magnetic bead selection (Miltenyi, Germany) from PBMCs. Monocytes were cultured in Cellgro media (Cellgenix, Germany) supplemented with 40 ng/ml IL-4 (Cellgenix) and 100 ng/ml GM-CSF (Leucomax). Fresh media and cytokines were added on day 2 of culture. On day 5 immature DCs were transfected with PSA plasmids by electroporation, applying the Amaxa device according to the manufacturer’s instructions for human DCs (Human Dendritic Cell Nucleofector™ Kit, Amaxa biosystems, Germany). After electroporation DC maturation was induced by adding 50 µg/ml poly I:C (Amersham Pharmacia Biotech, Sweden), and 15 h later mature DCs were harvested.

2.11. In vitro human T cell stimulations and IFNγ ELISPOT assay

T cell stimulations with autologous PSA-transfected DCs were initiated at a responder to stimulator ratio of 20:1 in X-VIVO 15 media (Bio Whittaker, In vitro, Sweden) supplemented with 2% autologous plasma, 25 ng/ml IL-7 (Peprotech, UK) and 20 ng/ml IL-12 (Peprotech). CD8+ T cells from the DC stimulation cultures were isolated for further stimulation on day 7, by anti-CD8 antibody-coated magnetic beads (EasySep, StemCell Technologies, UK). On day 14 another identical round of restimulation was performed. 48 h after every restimulation IL-2 (PROLEUKINE, Chiron Corporation) was added to a final concentration of 10 or 20 U/ml after the second and third stimulation, respectively. Seven days after the last restimulation, 5 × 10^4 CD8+ T cells (effectors) and 5 × 10^5 CIR-A2 cells pulsed with 25 µM of HLA-A*0201-restricted peptide (targets) were added to a 96-well nitrocellulose plate in triplicates (Multi-screen IP white, Millipore, Sweden) at an effector to target ratio of 10:1, and incubated at 37 °C for 4 h. IFNγ detection by ELISPOT was carried out according to the manufacturer’s instructions (IFNγ ELISPOT kit, Mabtech, Sweden). Number of positive spots was calculated by removing background spots against control peptides HIV and Flu, responses are multiplied by two and shown as spots per 1 × 10^6 cells. Quantification of spots was performed by an automated reader (Zeiss Axioplan 2, Germany). To statistically compare the number of spots in psa peptide wells with that of their paired control wells we applied a Student’s t-test.

3. Results

3.1. Binding of PSA peptides to HLA-A*0201 molecules

A panel of seven native peptides present in human PSA, of which three (psa40–49, psa53–61 and psa122–130) are also present in rhesus PSA, were synthesized (Fig. 1). These peptides have been investigated separately in previous studies as potential HLA-A*0201-restricted CTL epitopes [3,4,6,13], but to our knowledge these commonly used peptides have not been tested side by side. To compare and determine their relative affinity for HLA-A*0201 molecules, the peptides were tested in a HLA-A*0201 reconstitution assay with the CIR-A2 cell line. All peptides were tested at concentrations of 1, 10 or 100 µM and the binding affinity to HLA-A*0201 molecules differed up to twofold between the different PSA peptides (Fig. 2). Of the seven PSA peptides, psa165–174 and psa178–187 bound HLA-A*0201 molecules with relatively high affinity; psa53–61, psa40–49 and psa122–130 with intermediate affinity and psa170–178 and psa175–183 with very low affinity (Fig. 2). Despite the low HLA-A*0201-binding of psa170–178 and psa175–183, these peptides were not excluded from further investigation since in vitro HLA-A*0201 reconstitution measures binding of peptides at rather non-physiological concentrations, and might therefore not be sensitive enough to detect binding of all natural peptides.

3.2. Peptide specificity of HLA-A*0201-restricted murine and human CD8+ T cells after human and rhesus PSA DNA vaccination/stimulation

To investigate if the PSA peptides are endogenously processed and presented on HLA-A*0201 molecules for priming of CD8+ T cells in vivo, HLA-A*0201-transgenic mice were vaccinated with plasmids encoding full-length human (pVAX/hPSA) or rhesus (pVAX/rhPSA) PSA. Splenocytes from individual mice were then stimulated in separate in vitro cultures with each of the native peptides.

Splenocytes from mice vaccinated once with plasmid pVAX/hPSA were tested against each of the seven PSA peptides, and splenocytes from mice vaccinated with plasmid pVAX/rhPSA were tested only against the three peptides present in the rhesus PSA sequence. One peptide, psa53–61, out of the seven investigated, was able to induce proliferation and production of IFNγ by CD8+ T cells after 5 days of in vitro restimulation (Fig. 3). These psa53–61 specific T cells could be detected at an average level of
To ensure that the absence of HLA-A*0201-restricted CD8+ T cells specific for any of the other six PSA peptides were not due to insufficient stimulation of T cells after a single vaccination, mice were vaccinated with a prolonged schedule consisting of three vaccinations, 3 weeks apart. When splenocytes were tested against the seven peptides after 5 days of in vitro restimulation, CD8+ T cells from 4/4 mice produced IFNγ both in response to peptide psa53–61 and to peptide psa178–187 (Fig. 4). However, the levels of IFNγ producing CD8+ T cells specific for psa178–187 (average 6% ± 5.4) were considerably lower compared to stimulation with peptide psa53–61 (average 26% ± 22.3).

As mice do not express a homologue to human PSA questions of tolerance could not be addressed in this mouse model. Therefore, to investigate the peptide specificity of T cells induced with PSA DNA in a PSA-tolerant setting, PBMCs from HLA-A*0201+ PC patients were utilized. Human DCs were transfected by electroporation with DNA encoding full-length human or rhesus PSA and used to stimulate autologous T cells. After three rounds of weekly stimulations CD8+ T cells were tested against C1R-A2 cells separately pulsed with each of the seven native PSA peptides in an IFNγ ELISpot assay. Three separate experiments, each with two parallel cultures using human or rhesus PSA-loaded DCs, were performed to test the PSA-peptide specificity of T cells. This demonstrated that only peptide psa53–61 induced IFNγ production by human T cells (data not shown). Although, 1 of 3 patients responded in cultures stimulated with human PSA, and 2 of 3 patients responded when stimulated with rhesus PSA, responses were very low (data not shown).

3.3. Modification of PSA epitopes

As responses to all of the seven native PSA peptides were either undetectable or modest (for psa53–61) in PC patients, an algorithm-based approach (EpitOptimizer) was used to design modified epitopes within PSA to create epitopes with higher potential of activating CD8+ T cells. Three modified PSA peptides were synthesized, all with one amino acid substitution at anchor position two or nine, predicted to increase their binding to HLA-A*0201 (Table 1). The native sequences of these modified peptides are present in both the human and rhesus PSA protein (Fig. 1), thus no xenogeneic sequences in rhesus PSA were mod-
Table 1

<table>
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3.4. Recognition of modified PSA peptides by murine and human CD8+ T cells after vaccination/stimulation with modified full-length PSA plasmids

To further examine if our modified peptides were naturally processed and presented in vivo, specific point mutations creating these altered epitopes were introduced into the gene encoding the full-length rhesus PSA, resulting in plasmids rhPSA/I15V, rhPSA/V53L, and rhPSA/P129V. HLA-A*0201-transgenic mice were vaccinated with these modified plasmids, and splenocytes were then stimulated with the respective modified peptide. Only T cells from mice vaccinated with the modified plasmid rhPSA/V53L responded specifically to in vitro stimulation with its corresponding modified epitope (psa52–60(V53L)), as demonstrated by ICS for IFNγ after 5 days of restimulation (Fig. 5). As a positive control for vaccinations, splenocytes were stimulated with peptide psa53–61 in parallel in vitro cultures, and CD8+ IFNγ producing T cells were detected after 5 h of restimulation among splenocytes from both rhPSA/I15V and rhPSA/P129V vaccinated mice (data not shown). To address if T cells induced by rhPSA/V53L DNA also recognize the native peptide psa52–60, HLA-A*0201-transgenic mice were vaccinated with rhPSA/V53L DNA and splenocytes from individual mice were then stimulated with peptide psa52–60 and psa52–60(V53L). To compare the recognition of peptide psa52–60 by T cells induced after rhPSA/V53L vaccination with that of T cells induced after wt PSA vaccination, parallel groups of mice were vaccinated with plasmids expressing human and rhesus PSA. As shown in Fig. 5 non-modified peptide psa52–60 was only recognized by CD8+ T cells from mice vaccinated with rhPSA/V53L DNA, but not by CD8+ T cells from mice vaccinated with wt human or rhesus PSA DNA.

To investigate in vivo processing and presentation of the modified epitopes in human antigen presenting cells (APCs) and the epitopes potential to activate CD8+ T cells, in vitro cultures were performed. T cells from both PC patients and healthy donors were stimulated three times with DCs transfected with the modified rhesus plasmids and assessed for IFNγ secretion in response to C1R-A2 cells pulsed with the modified peptides. After T cell stimulations with the modified plasmids, rhPSA/I15V or rhPSA/P129V, identified. Notably, one of the modified epitopes (psa52–60(V53L)), identified by the algorithm-based prediction tool was shifted by one amino acid from the native epitope psa53–61. Nevertheless, this modified epitope was included for further investigation even though this modification could disrupt a possible therapeutic epitope.
peptide-specific T cells for each of the respective modified peptides were detected at low frequencies in one of seven stimulations (Table 2). In contrast, T cells from all patients and healthy donors tested were stimulated with rhPSA/V53L-transfected DCs responded specifically to peptide psa52–60(V53L) (Table 2). This clearly demonstrates that peptide psa52–60(V53L) is naturally processed and presented by human APCs and that this peptide activates CD8+ human T cells in all cultures tested (p < 0.05). Furthermore, the same healthy donors were in parallel assessed for their IFNγ response to the native peptide psa52–60. After in vitro stimulation with rhPSA/V53L-transfected DCs 5/5 T cell cultures recognized the native peptide psa52–60 while only 2/5 and 1/5 cultures stimulated with rhPSA/I15V or rhPSA/P129V-transfected DCs recognized this peptide (Table 2).

To determine if this improvement in T cell induction could be explained by increased binding of the modified peptide, we performed a sensitive HLA-A*0201 assembly assay. This assay demonstrated that the native peptide psa52–60 is a poor HLA-A*0201 binder at low concentrations as compared to the modified peptide psa52–60(V53L), which stabilized HLA-A*0201 already at 0.4 μM (Fig. 6).

### Table 2
IFNγ responses after in vitro stimulation with the modified DNA constructs.

<table>
<thead>
<tr>
<th>Plasmid stimulation</th>
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<th>rhPSA/P129V</th>
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<td>psa52–60(V53L)b **</td>
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<td>Patient 1</td>
<td>0</td>
<td>n.d.</td>
<td>32</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0</td>
<td>n.d.</td>
<td>62</td>
</tr>
<tr>
<td>Healthy donor 1</td>
<td>26</td>
<td>33</td>
<td>54</td>
</tr>
<tr>
<td>Healthy donor 2</td>
<td>0</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>Healthy donor 3</td>
<td>0</td>
<td>32</td>
<td>86</td>
</tr>
<tr>
<td>Healthy donor 4</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Healthy donor 5</td>
<td>0</td>
<td>0</td>
<td>37</td>
</tr>
</tbody>
</table>

* Number of IFNγ secreting cells per 10⁶ CD8⁺ T cells.

** Peptide readout.

n.d. = not determined.

** p < 0.005 significantly higher IFNγ production compared to stimulation with negative control peptide (flu).

### 4. Discussion

This study aimed at the identification of a single natural or modified epitope in PSA with the ability to consistently generate PSA-specific T cells to facilitate monitoring after vaccination. We first tested a panel of the most commonly used HLA-A*0201-restricted human PSA peptides by PSA DNA vaccination of HLA-A*0201-transgenic mice and after repetitive stimulation of human T cells with PSA DNA-transfected DCs, to determine which peptides are naturally processed and most efficiently presented from full-length human PSA. Several other studies have investigated the potential of HLA-A*0201-restricted PSA peptides to activate human T cells in vitro [3–6,13]. However, to our knowledge, no previous studies have investigated the peptide-specificity of human or murine T cells induced by stimulation with only endogenously processed PSA. These experiments are necessary, since only induction of T cells by endogenously processed antigen are relevant for immunotherapeutical protocols with whole gene vaccines.

HLA-A*0201-transgenic mice have been widely used to examine the in vivo immunogenicity of potential CTL epitopes identified as 8–10-mer peptides capable of binding to HLA-A*0201 molecules [24,28,29]. A general consensus exists between the identification of peptides after DNA vaccination of HLA-transgenic mice and after in vitro stimulation of human PBMCs [30–32]. Both approaches are therefore regarded as valid for the identification of potential CTL epitopes from human antigens.

We demonstrate the induction of CD8⁺ T cells specific for two native peptides, psa53–61 (VLVHPQWVL) and to a lower extent also psa178–187 (VISNDVCAQV), after pVAX/hPSA vaccination of HLA-A*0201-transgenic mice (Figs. 3 and 4). Psal187–187, often referred to as PSA-3, is one of the most commonly used and investigated PSA peptides [6,15,33,34]; however, our data suggest that peptide psa53–61 after natural processing more efficiently induce IFNγ-producing CD8⁺ T cells in HLA-A*0201-transgenic mice (Figs. 3 and 4) and humans (data not shown). Psal53–61 was originally identified by Alexander et al., by the application of a HLA-A2 prediction tool and by an in vitro HLA-A2 stabilization assay [4]. A recent publication by Forsberg et al., demonstrate that 3/13 PC patients have psa53–61 reactive CD8⁺ T cells [35]. Furthermore, our previously performed clinical trial with PSA DNA vaccination demonstrated that only 1/3 patients in the highest dose group responded specifically to peptide PSA-3, even though they all generated T cell responses to full-length PSA protein [10,12].

Our results show that T cells from PC patients stimulated, in vitro with wt human or rhesus PSA DNA, exhibit lower responder frequencies to peptide psa53–61 than T cells from DNA vaccinated mice. Low levels of peptide-specific T cells and variations between individuals (both within and between studies) are common attributes of human T cell stimulations with PSA peptides.
but only in 2/5 and 1/5 human T cell cultures stimulated with rhPSA/V53L-transfected DCs in all healthy donors, low peptide concentrations as compared to the modified peptide. This difference in T cell induction might be due to the lower potency of the modified plasmids, rhPSA/V53L, was shown to consistently induce T cells specific for peptide psa53–60 after processing and presentation by both murine and to identify at least one epitope with a high potential of inducing vaccine–specific T cells across individuals, additional rationally modified epitopes were introduced into rhesus PSA. One of the modified plasmids, rhPSA/V53L, was shown to consistently induce T cells specific for the corresponding modified peptide psa52–60 (V53L) after processing and presentation by both murine (Fig. 5) and human (Table 2) APCs. Furthermore, native peptide psa52–60 was only recognized by CD8+ T cells from mice vaccinated with rhPSA/V53L DNA, but not by CD8+ T cells from mice vaccinated with wt human or rhesus PSA DNA (Fig. 5). Likewise, native peptide psa52–60 was recognized by human CD8+ T cells after in vitro stimulation with rhPSA/V53L-transfected DCs in all healthy donors, but only in 2/5 and 1/5 human T cell cultures stimulated with rhPSA/115V or rhPSA/P129V-transfected DCs, respectively (Table 2). This difference in T cell induction might be due to the lower potential of the native psa52–60 peptide to assemble HLA-A2 at low peptide concentrations as compared to the modified peptide psa52–60 (V53L) (Fig. 6).

Furthermore, this indicates that our epitope created for monitoring purposes additionally could have a therapeutic effect. Although T cells from some healthy donors responded to peptide psa52–60 after stimulation with unmodified PSA (Table 2), indicating that it might be naturally processed, we currently do not know whether the native peptide, psa52–60, is naturally processed and presented by PC cells. Inclusion of the psa52–60 (V53L) epitope in PSA-based immunotherapy may thus indicate processing and presentation of the vaccine to the naive immune repertoire, but does not necessarily represent recognition of any other epitope in PSA which might have clinical benefit.

In conclusion, we have identified a modified epitope, psa52–60 (V53L), with two important properties; a higher potential than known native epitopes to induce CD8+ human T cell responses and to induce T cells that are cross-reactive to the native, but maybe not naturally processed, epitope psa52–60. Importantly, this modified epitope was identified by endogenously processed PSA antigen. Therefore, inclusion of this epitope in PSA-based vaccines might facilitate in vitro monitoring of vaccine efficacy, as a complement to the clinically relevant response against the whole antigen, in patients on early phases of immunotherapeutic protocols.

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References


