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Immunization with a prostate cancer xenoantigen elicits a xenoantigen epitope-specific T-cell response

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Abbreviations: CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; HLA, human leukocyte antigen; IFNγ, interferon γ; OVA, ovalbumin; PAP, prostatic acid phosphatase; PMA, phorbol myristate acetate; PSMA, prostate-specific membrane antigen

Vaccines encoding xenoantigens, "non-self" proteins that are highly homologous to their autologous counterparts, have been investigated as a means to increase immunogenicity and overcome tolerance to "self" antigens. We have previously shown that DNA vaccines encoding native prostatic acid phosphatase (PAP) were able to elicit PAP-specific T cells in both rats and humans, but required multiple immunization courses. In this study, we investigated in a preclinical model whether immunizations with a DNA vaccine encoding a xenoantigen could elicit a cross-reactive immune response to the native protein, potentially requiring fewer immunizations. Lewis rats were immunized with a DNA vaccine encoding human PAP and splenocytes from immunized rats were screened with a human peptide library containing overlapping, 15-mer PAP-derived peptides using T-cell proliferation and interferon γ (IFNγ) release as measures of the immune response. One dominant PAP-specific, RT1.A[l]-restricted, epitope was identified. Direct immunization with the immunodominant peptide ($HP_{201–215}$) containing this epitope demonstrated that it included a naturally presented MHC Class I epitope recognized by $CD8^+$ T cells in Lewis rats. However, no cross-reactive immune response was elicited to the corresponding rat peptide despite a difference of only three amino acids. Immunization with DNA vaccines encoding rat PAP (rPAP) in which this foreign dominant epitope was included as well as with DNA vaccines coding for a variant of the xenoantigen from which this epitope was deleted, did not elicit responses to the native antigen. Overall, these results indicate that the immunization with a xenoantigen-coding DNA vaccine can lead to an immune response which potentially favors foreign epitopes and hence limits any cross-reactive response to the native antigen.

Introduction

Prostatic acid phosphatase (PAP) is a prostate cancer-associated tumor antigen. We and others have shown that PAP-specific immune responses can be elicited or augmented in prostate cancer patients using various active PAP-specific vaccination methods. Treatment of prostate cancer patients with sipuleucel-T, a cellular vaccine targeting PAP, elicited PAP-specific T cells in 27.3% of patients and PAP-specific antibody responses in 28.5% of patients.[1] The demonstration that patients treated with sipuleucel-T had a significantly prolonged survival as compared with placebo-treated patients resulted in the first-in-history FDA-approved vaccine for cancer.[1]

Our group has been investigating DNA vaccines encoding PAP. We previously reported that immunization with a DNA vaccine encoding human PAP (xenoantigen) or rat PAP (autologous antigen) in Lewis rats could elicit antigen-specific T_H1-polarized immune responses. However, multiple immunization courses (at least six) with a DNA vaccine encoding the autologous antigen were needed to elicit an immune response to the native antigen.[2,3] Based on these preclinical data, we conducted a Phase I/IIa clinical trial in which prostate cancer patients were immunized six times with a DNA vaccine encoding human PAP. Ten of 22 patients developed PAP-specific T-cell responses of a mainly T_H1/cytotoxic T lymphocyte (CTL) nature. Additionally, some patients developed long-term PAP-specific, interferon γ (IFNγ)–associated immune responses.[4,5] At least for PAP, these results indicate that, in some patients, tolerance to the "self" antigen was overcome with multiple immunizations with a DNA vaccine encoding the autologous antigen. However, while these studies demonstrate that PAP is a targetable antigen, immune responses were not elicited in all patients immunized with either sipuleucel-T or a DNA vaccine, calling for the development of improved immunization strategies.

Vaccines that encode xenoantigens—foreign proteins that are highly homologous to their autologous counterparts—can elicit

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a cross-reactive immune response to the native protein and hence are one method that has been investigated to enhance the immunogenicity and improve the overall efficacy of vaccines. Several preclinical and clinical studies on melanoma have shown that immunization with xenoantigens can allow the host to overcome the immune tolerance to "self" antigens and induce effective antitumor responses. In mouse melanoma models, DNA vaccines encoding several xenogeneic melanoma differentiation antigens, i.e., TRP1/gp75, TRP2/DCT, pMel17/gp100 and tyrosinase, have been shown to elicit antibodies and T cell-mediated antitumor responses.[6–9] Based on these preclinical data, groups have investigated prime/boost strategies using alternating immunization schedules of DNA vaccines encoding xenoantigens and autologous antigens (encoding either gp100 or tyrosinase) in human clinical trials. Researchers have demonstrated the development of peptide ($gp100_{280–288}$)-specific T cells in 5 out of 18 patients, and tyrosinase-specific $CD8^+$ T-cell responses in 7 out of 18 patients, respectively, using these approaches.[10,11] Additionally, a DNA vaccine encoding human tyrosinase has been shown to elicit antitumor responses and to promote long-term survival in dogs affected by malignant melanoma, leading to the first US Department of Agriculture (USDA)-approved anti-cancer vaccine, ONCEPT.[12]

Xenogeneic vaccination approaches have also been used to elicit immune responses to prostate-specific proteins. Mice immunized with a DNA vaccine encoding the human prostate-specific membrane antigen (PSMA), followed by a booster immunization with recombinant human PSMA, developed antibodies against human PSMA as well as against the native mouse PSMA.[13] Based on these preclinical results, prostate cancer patients were immunized with a prime/boost approach alternating between DNA vaccines encoding mouse PSMA and human PSMA. Evidence of immunoreactivity against PSMA was detected in some patients.[14]

PAP has also been targeted with xenogeneic vaccines.[15] Fong, et al. demonstrated that the immunization of Copenhagen rats with vaccinia virus expressing human PAP, but not rat PAP, generated CTL responses and prostatitis, suggesting that T-cell responses were elicited against cross-reactive epitopes.[16] Since a xenogeneic homolog of PAP was reported to be immunogenic in rats, a Phase I clinical trial investigating the safety and efficacy of a xenogeneic tumor vaccine targeting PAP in prostate cancer patients has been conducted. Eleven out of 21 patients who underwent immunization with mouse PAP-loaded dendritic cells (DCs) developed human PAP-specific, T_H1 immune responses.[17] These results suggested that the tolerance to PAP, a "self" antigen, could be overcome by the immunization with a xenoantigen, both in rats and humans. However, the percentage of patients that developed immune responses to PAP after the xenogeneic vaccination was not appreciably higher than what were reported in the sipuleucel-T[1] or pTVG-HP[4] trials. Hence, it remains unclear whether targeting xenoantigens truly conveys an advantage and whether cross-reactive responses are truly elicited by this approach.

In the present study, we investigated whether immunization with a DNA vaccine encoding a xenoantigen would elicit a cross-reactive immune response to the native antigen. For these studies, we focused on PAP, given that the human and rat proteins display 86% homology and 78% identity at the amino acid level.[15] We selected the Lewis rat strain for our studies as this model has been previously used to investigate experimental autoimmune diseases, and because the MHC Class I and Class II molecules of this strain are well characterized, allowing us to investigate PAP-specific T-cell responses at the epitope level.[18,19] Additionally, we have previously demonstrated that Lewis rats develop PAP-specific T-cell responses after immunization with a DNA vaccine encoding PAP.[2] The epitope(s) that were specifically recognized and whether cross-reactive immunity was elicited were determined using peptide libraries. The long-range goal of these studies was to investigate whether PAP might be effectively targeted using DNA vaccines encoding a xenoantigen.

Results

DNA immunization of Lewis rats with PAP xenoantigen elicits an immune response to a dominant epitope contained within peptide $HP_{201–215}$. We have previously reported that the immunization of Lewis rats with DNA vaccines encoding either human (xenoantigen, hPAP) or rat PAP (native antigen, rPAP) elicited antigen-specific, T_H1-polarized immune responses. When immunization was performed with the native antigen, multiple (at least six) courses of the DNA vaccine were necessary to generate an immune response to rPAP, and T-cell and antibody responses to the xenoantigen were also detected in vitro.[2,3] We consequently sought to investigate whether immunization with the xenoantigen (hPAP) could elicit a cross-reactive response to the native antigen, ideally with fewer immunizations, by identifying the specific epitopes presented and recognized following immunization. Lewis rats were immunized four times with an empty vector (pTVG4) or with a hPAP-encoding construct (pTVG-HP) and immune responses were assessed by T-cell proliferation and IFNγ secretion. As shown in **Figure 1**, rats immunized with pTVG-HP developed T-cell proliferative responses to hPAP, to a library of 38 overlapping 15-mer peptides ($HP_{1–386}$) and to two separate subsets of this peptide library ($HP_{201–255}$ and $HP_{341–386}$). $CD8^+$ T cells proliferated when stimulated with each of the peptide subsets while $CD4^+$ T cells only proliferated in response to the $HP_{201–255}$ subset (**Fig. 1B**). Splenocytes from pTVG-HP-immunized rats were cultured with individual peptides from the subsets and assessed for IFNγ release by ELISA to identify the specific epitopes recognized. Peptide-specific IFNγ release was induced by two 15-mer peptides within these subsets, $HP_{201–215}$ and $HP_{371–386}$ (**Fig. 2** and data not shown). The immune response to peptide $HP_{201–215}$ appeared to be immunodominant since this peptide was recognized by T cells in all hPAP-immunized rats. Conversely, immune responses to peptide $HP_{371–386}$ were only detectable in approximately 50% of hPAP-immunized rats (data not shown) and were not statistically significant (**Fig. 1A**).

A human PAP-specific peptide, but not the corresponding rat PAP peptide, is immunogenic in vivo. To determine whether these hPAP-specific peptides represented true epitopes, Lewis rats were immunized twice directly with the human-specific peptides $HP_{201–215}$ and $HP_{371–386}$, or the corresponding rat-specific peptides, in Freund's adjuvant. Rats immunized with $HP_{201–215}$ developed immune responses to the individual peptide and hPAP,

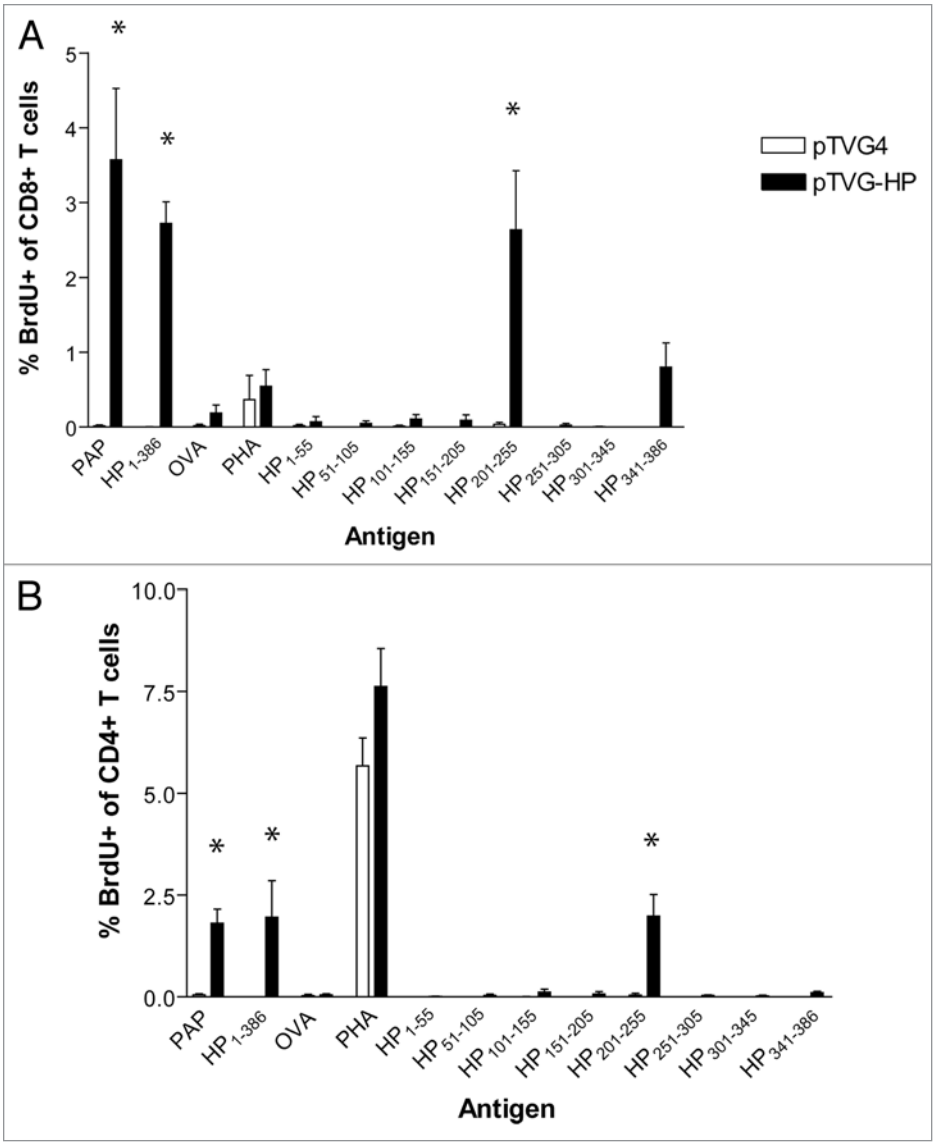

Figure 1. DNA immunization of Lewis rats with a PAP xenoantigen elicits immune responses to PAP-derived peptides. (**A and B**) Ten week-old male Lewis rats received four intradermal immunizations with 100 μg of vector (n = 4) or pTVG-HP (n = 4) and 5 μg GM-CSF at two-week intervals. Splenocytes were cultured with media alone, human PAP protein (PAP), human peptide library ($HP_{1–386}$), human peptide library subsets ($HP_{1–55}$, $HP_{51–105}$, $HP_{101–155}$, $HP_{201–255}$, $HP_{251–305}$, $HP_{301–345}$, or $HP_{341–386}$), ovalbumin (OVA) or phytohemaglutinin (PHA) for 72 h, and then pulsed with BrdU for 8–12 h. Cells were then assessed for antigen-specific $CD8^+$ (**A**) or $CD4^+$ (**B**) proliferation by flow cytometry. Each bar represents the mean frequency of $BrdU^+$ T cells and standard deviation of n = 4 animals per treatment group. Comparisons of the means between the experimental and control groups were made with an unpaired Student's t-test, and p values are shown (*$p < 0.05$). Results are representative of multiple replicate studies.

as indicated by both peptide-specific and PAP-specific $CD8^+$ T-cell proliferation (**Fig. 3A**). Rats immunized with $HP_{371–386}$ developed peptide-specific, but not hPAP-specific, proliferating $CD8^+$ T cells (**Fig. 3B**). Cross-reactive immune responses were not elicited to the corresponding rat-specific peptides $RP_{200–214}$ or $RP_{370–381}$ following immunization with either hPAP peptide (**Fig. 3**). Moreover, the rat-specific peptides $RP_{200–214}$ and $RP_{370–381}$ were not immunogenic, since the immunization of rats with these peptides elicited immune responses neither to the same peptides, neither to the corresponding hPAP-derived peptides, nor to hPAP (**Fig. 3**). The demonstration that hPAP is recognized by T cells following immunization with the $HP_{201–215}$ peptide confirm that this peptide contains an epitope that is naturally processed and presented in Lewis rats. Conversely, the peptide $HP_{371–386}$ appears to not contain a naturally processed and presented PAP epitope, since direct immunization with this peptide did not elicit hPAP-specific T cells. Of note, this peptide is located at the extreme C-terminus of hPAP and contains four additional amino acids compared with the native rPAP amino acid sequence. Thus, this peptide is uniquely foreign in the rat.

Immunization of Lewis rats with a human PAP-encoding DNA vaccine elicits a xenoantigen dominant immune response that is $RT1.A^l$-restricted. To better characterize the immunodominant epitope, peptide-specific IFNγ secretion was assessed with peptides of different length that flanked the $HP_{201–215}$ peptide. As demonstrated in **Figure 4A**, IFNγ-secreting T cells were elicited by peptides $HP_{201–215}$ and $HP_{204–215}$, while the response was lower with the 10-mer peptide $HP_{204–213,}$ indicating the 12-mer peptide GIWSKVYDPLYC contains the minimal immunogenic epitope within this region. Further, peptide-specific IFNγ immune responses were evaluated using rPAP-specific peptides that corresponded to the hPAP-specific binding regions. As shown in **Figure 4B**, no immune responses to the corresponding rat-specific peptides were elicited in pTVG-HP immunized rats. In the case of $HP_{201–215}$ and $RP_{200–214}$, this occurred despite the fact that these amino acid sequences are highly homologous, differing only by three amino acids (**Fig. 4C**). MHC blocking studies based on antibodies specific for Lewis MHC Class I ($RT1.A^l$) or MHC Class II ($RT1.B^l$) molecules demonstrated that the IFNγ-secreting response to the epitope was MHC Class I-restricted (**Fig. 5A**). Moreover, $CD8^+$ T cells from pTVG-HP-immunized rats produced IFNγ in response to the $HP_{201–215}$ peptide, further demonstrating that this peptide contains an epitope recognized specifically by $CD8^+$ T cells (**Fig. 5B**).

Inclusion or exclusion of the $HP_{201–215}$ immunodominant xenoantigen epitope within a DNA vaccine does not facilitate cross-reactive immune responses to the native PAP antigen. We

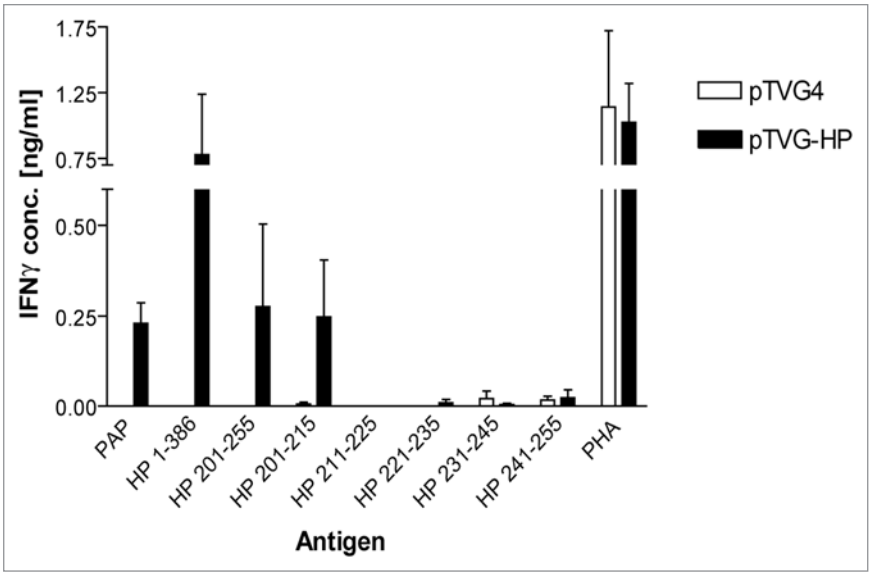

Figure 2. DNA immunization of Lewis rats with a PAP xenoantigen elicits IFNγ-secreting responses specific for defined peptides. Male Lewis rats were immunized as described in the legend to **Figure 1.** Splenocytes were cultured with media alone, human PAP protein, $HP_{1–386}$, individual human peptides within the peptide pool subset, and $HP_{201–255,}$ and assessed for interferon γ (IFNγ) release by quantitative ELISA. Each bar represents the average IFNγ release and standard deviation for n = 3 rats per group, each determined in triplicate. Results are representative of multiple replicate studies.

next sought to investigate whether inclusion of the hPAP-specific immunodominant epitope $HP_{201–215}$, presented in the context of a DNA vaccine encoding native rPAP, or exclusion of this epitope from a DNA vaccine encoding the xenoantigen, could elicit a cross-reactive immune response to native rPAP. Specifically, a modified pTVG-HP-based vaccine (pTVG-HP $RP_{200–214}$) was constructed in which the immunodominant $HP_{201–215}$ region was replaced by PCR-directed mutagenesis with the sequence coding for the corresponding rat amino acids, $RP_{200–214.}$ Similarly, a modified pTVG-RP vaccine (pTVG-RP $HP_{201–215}$) was constructed to encode the immunodominant $HP_{201–215}$ sequence in place of the corresponding region of the pTVG-RP vaccine. Lewis rats were immunized four times with these constructs and control plasmids, a strategy that we have previously found to be suboptimal for eliciting an immune response to rPAP using a DNA vaccine encoding the native antigen.[2] As shown in **Figure 6**, neither human nor rat PAP-specific immune responses were elicited in rats immunized with pTVG-RP. Splenocytes from rats immunized with pTVG-RP $HP_{201–215}$ exhibited detectable immune responses directed to the hPAP protein and dendritic cells (DCs) transfected to express hPAP, but not rPAP (**Fig. 6A**). Moreover, there was no evidence of epitope spread to other rPAP-derived peptides (**Fig. 6B**). When $HP_{201–215}$ was substituted in the hPAP xenoantigen with the $RP_{200–214}$ amino acid sequence, an immune response was still elicited to hPAP, but of a lower intensity and no response to the native rPAP developed. These results indicate that the immune response elicited in Lewis rats by means of a PAP-specific human xenoantigen is human specific and that even the ablation of the dominant human-specific epitope does not result in a cross-reactive response to the native antigen.

Discussion

The use of xenogeneic vaccinations with highly homologous proteins to overcome tolerance to "self" antigens and increase vaccine efficacy has been investigated for the treatment of melanoma and prostate cancer.[12,13,16,17,20,21] We have previously reported that immunization of Lewis rats with a DNA vaccine encoding human PAP (xenoantigen) or rat PAP (autologous antigen) elicits an antigen-specific immune response and that multiple courses of a DNA vaccine encoding rat PAP elicit T_H1-polarized immune response to the native antigen.[2] Similarly, we reported that the repeated immunization of prostate cancer patients with a DNA vaccine encoding human PAP elicited human PAP-specific T-cell proliferative responses and IFNγ secretion.[4,5] Here, in a preclinical model, we explored whether a DNA vaccine encoding a xenoantigen could promote increased immunogenicity, potentially with fewer immunizations. Specifically, we investigated whether the immunization of Lewis rats with a DNA vaccine encoding human PAP would elicit a cross-reactive response to the native antigen, rat PAP. We identified a dominant human PAP-specific epitope, $HP_{201–215}$, that was recognized in all animals following immunization. A second peptide, $HP_{371–386}$, was recognized by some of the animals, but this peptide is located at the C-terminus of the protein, contains an extra four amino acids compared with the rat PAP, and was shown not to be naturally presented. The $HP_{201–215}$ epitope was found to be RT1.A^l-restricted and no cross-reactive immune responses were elicited to the corresponding rat PAP peptide, which differs only by three amino acids. Immunization with modified PAP-encoding DNA vaccines that had the immunodominant human PAP-specific epitope inserted or ablated did not result in cross-reactive immune responses to rat PAP. Overall, our findings demonstrate that, at least in Lewis rats, immunization with a xenoantigen can result in an immune response against a foreign epitope without cross-reactivity to the native antigen.

Naturally occurring, immunological "hotspot" regions in proteins have been reported as regions of interest for vaccine targets. We have previously identified a region in the cancer-testis antigen SSX-2 as one containing nested HLA-A2 and HLA-DR1 binding sites.[22] Such epitope-rich regions, containing both MHC Class I and II epitopes, are of interest as vaccine antigens to generate both antigen-specific $CD4^+$ and $CD8^+$ T cells. Using overlapping peptides, the minimal immunodominant PAP-specific epitope in Lewis rats was mapped to a 12 amino acid peptide, $HP_{204–215}$. The sequence similarity of a 9-mer peptide contained within this region, WSKVYDPLY, to the preferred RT1.A^l binding motif (X-A/S/V-F/Y-X-X-X-X-X-Y/F/L/M), the peptide-elicited production of IFNγ by CD8+ T cells, and the ability of anti-RT1.A^l antibodies to partially block peptide-specific immune

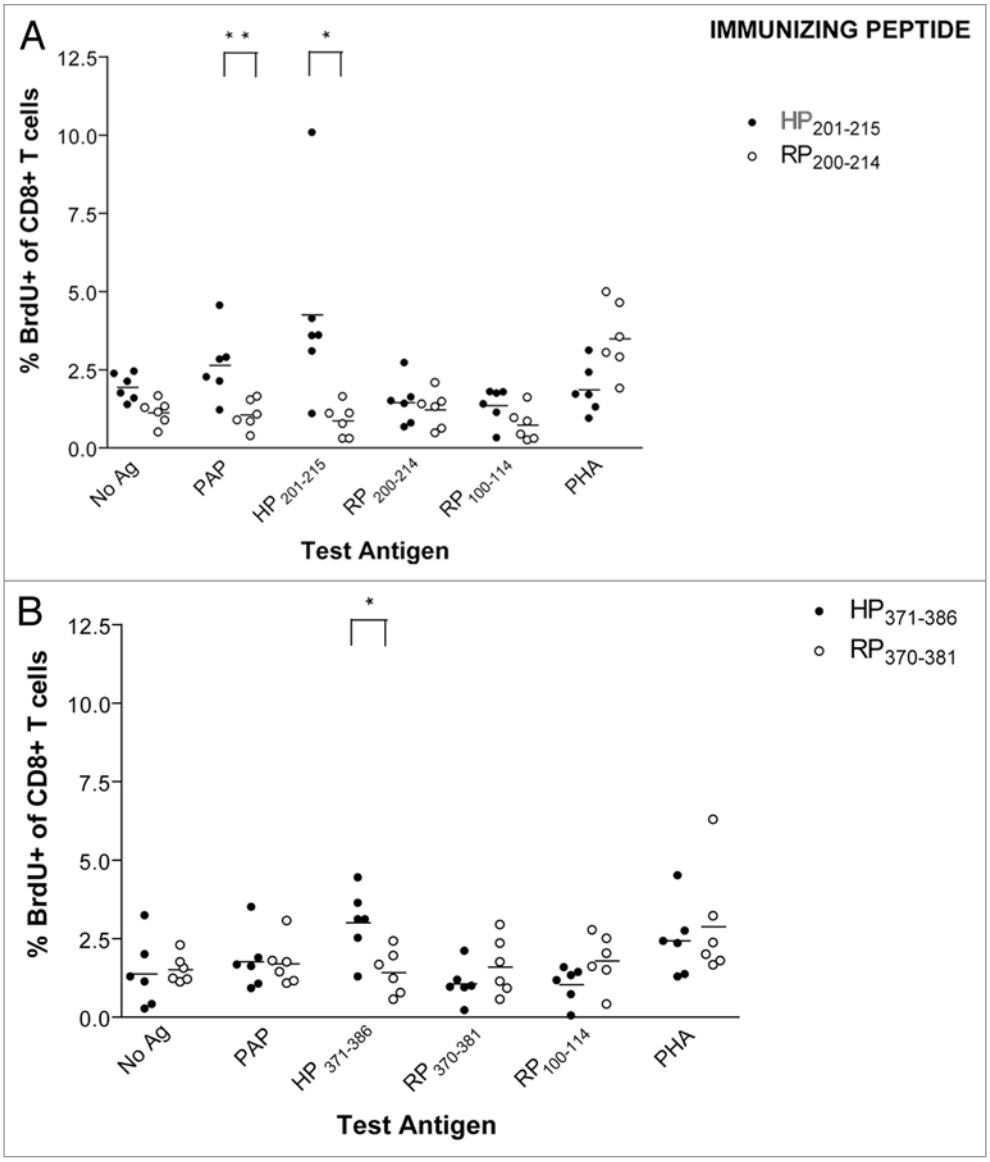

Figure 3. hPAP-specific peptides, but not the corresponding rPAP peptides, are immunogenic in vivo. (**A and B**) Male Lewis rats were immunized twice subcutaneously with 100 μg of $HP_{201–215}$ or $RP_{200–214}$ (**A**), and $HP_{371–386}$ or $RP_{370–381}$ (**B**). Splenocytes were then evaluated for human PAP- and peptide-specific T-cell proliferation as described above. The percentage of proliferating ($BrdU^+$) $CD8^+$ T cells under each antigen-stimulation condition are reported. Each point represents the mean % of proliferating cells for n = 6 rats per group. Comparisons of the means between groups were made with an unpaired Student's t-test, and p values are shown (*$p < 0.05$; **$p < 0.01$).

responses, strongly suggest that this particular epitope is RT1.A^l-restricted.[19] The presence of proliferating $CD4^+$ T cells after stimulation with the $HP_{201–255}$ peptide pool and following direct immunization with the $HP_{201–215}$ peptide suggests that an MHC Class II epitope is likely to be adjacent to or encompassing this epitope. However, we did not characterize this further. RT1.B^l has been the best-characterized MHC Class II molecule in Lewis rats. Still, we did not identify a potential epitope corresponding to the preferred RT1.B^l-binding motif (X-X-T/S/V-F/H/Q-X-A/S/V/T-X-X-E/D), and anti-RT1-B^l antibodies were unable to block peptide-specific immune responses. This does not exclude the possibility that an MHC Class II epitope restricted to other Lewis rat MHC Class II haplotypes exists in this region.

As indicated above, the amino acid sequence of the human immunodominant peptide (GIWSKVYDPLYC) and corresponding rat peptide (EIWSRLYDPLYC) differ only by three amino acids, yet the corresponding rat peptide was not immunogenic in Lewis rats. Based on the predicted RT1.A^l-binding motifs, the anchor residue required for MHC Class I molecule binding was not present in the corresponding rat peptide.[19] It is perhaps not surprising that the sequence of the presented epitope, and not the overall homology of the protein, was most important for the development of a cross-reactive immune response. However, this particular case illustrates that, depending on the MHC type of the host, xenoantigen vaccination may not be advantageous and only elicit immune responses to "foreign" epitopes. Fong and

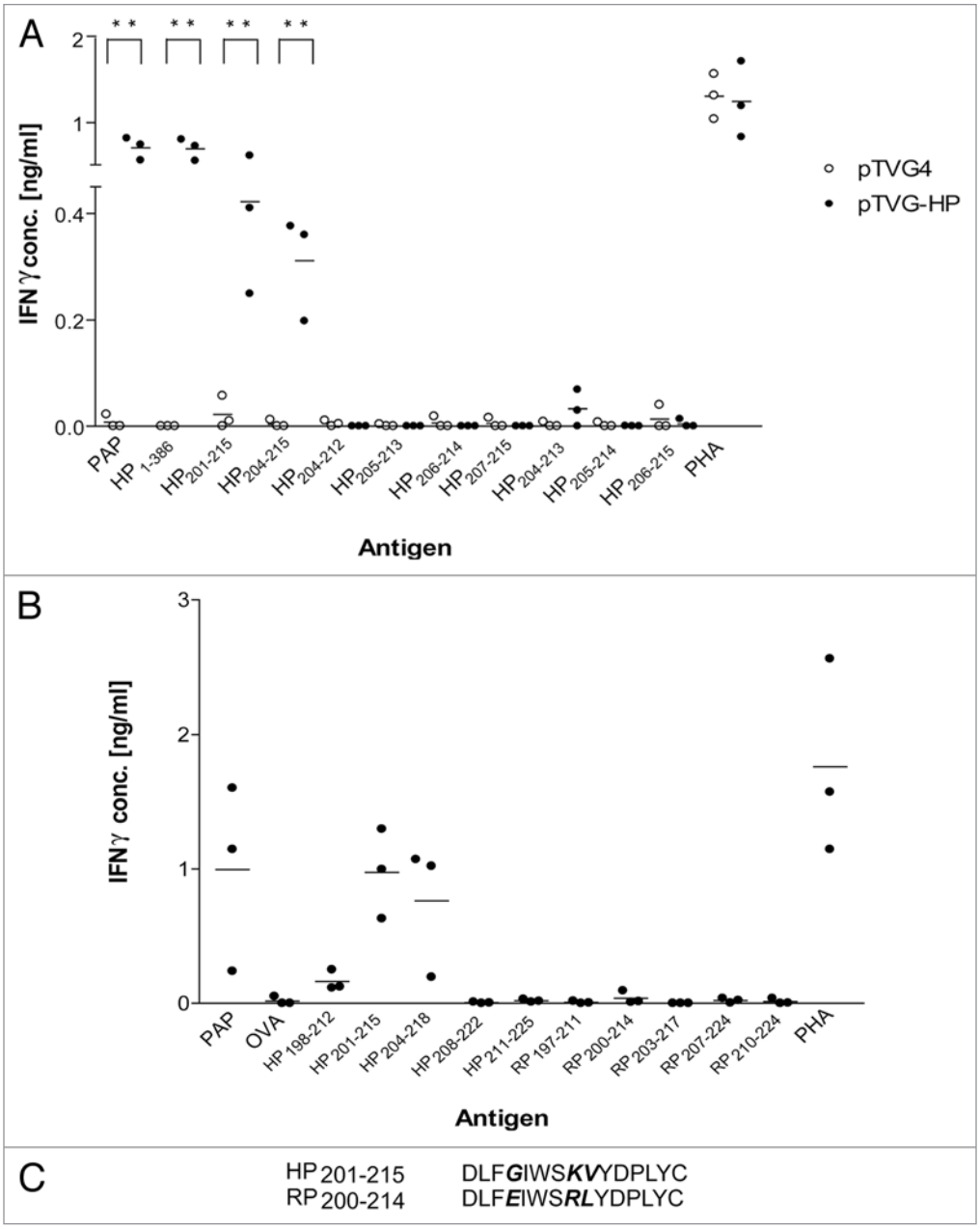

Figure 4. $HP_{204–215}$ contains the immunodominant hPAP epitope recognized by T cells from hPAP-immunized Lewis rats. (**A–C**) Splenocytes obtained from immunized animals were cultured with media alone, human PAP, ovalbumin (OVA, negative control), overlapping human and/or rat PAP peptides, and phytohemaglutinin (PHA) for 72 h. Supernatants were then analyzed for the production of interferon γ (IFNγ) by ELISA. (**A**) Human PAP peptides ranging in size (12-mer, 10-mer, and 9-mer peptides) flanking the immunodominant peptide $HP_{201–215}$ were analyzed. Each dot represents IFNγ production from the splenocytes of each rat (open symbols = pTVG4-immunized rats; closed symbols = pTVG-HP-immunized rats). Comparisons of the means between groups were made with an unpaired Student's t-test, and p values (**$p < 0.01$) are indicated. (**B**) Human ($HP_{198–212}$, $HP_{201–215}$, $HP_{204–218}$, $HP_{208–222}$, $HP_{211–225}$) and the corresponding rat ($RP_{197–211}$, $RP_{200–214}$, $RP_{203–217}$, $RP_{207–224}$, $RP_{210–224}$) PAP peptides overlapping the amino acid region 197–225 were analyzed. Dots represent IFNγ production from the splenocytes of each rat immunized with pTVG-HP. (**C**) The differences in primary sequence between human and rat PAP are highlighted.

colleagues have previously reported that viral vectors encoding hPAP can elicit cross-reactive immune responses in Copenhagen rats, as demonstrated by the generation of CTL responses detectable in vitro and prostate tissue inflammation in vivo.[16] Thus, while the generation of cross-reactive immune responses may certainly occur with xenoantigen vaccines, our results in Lewis rats demonstrate that this is not a generalizable phenomenon. Such a strain-specific cross-reactive immune response following immunization with a xenoantigen is most relevant to human immunization of diverse MHC types, and highlights the disadvantage of generalized conclusions drawn from studies based on inbred rodent strains.

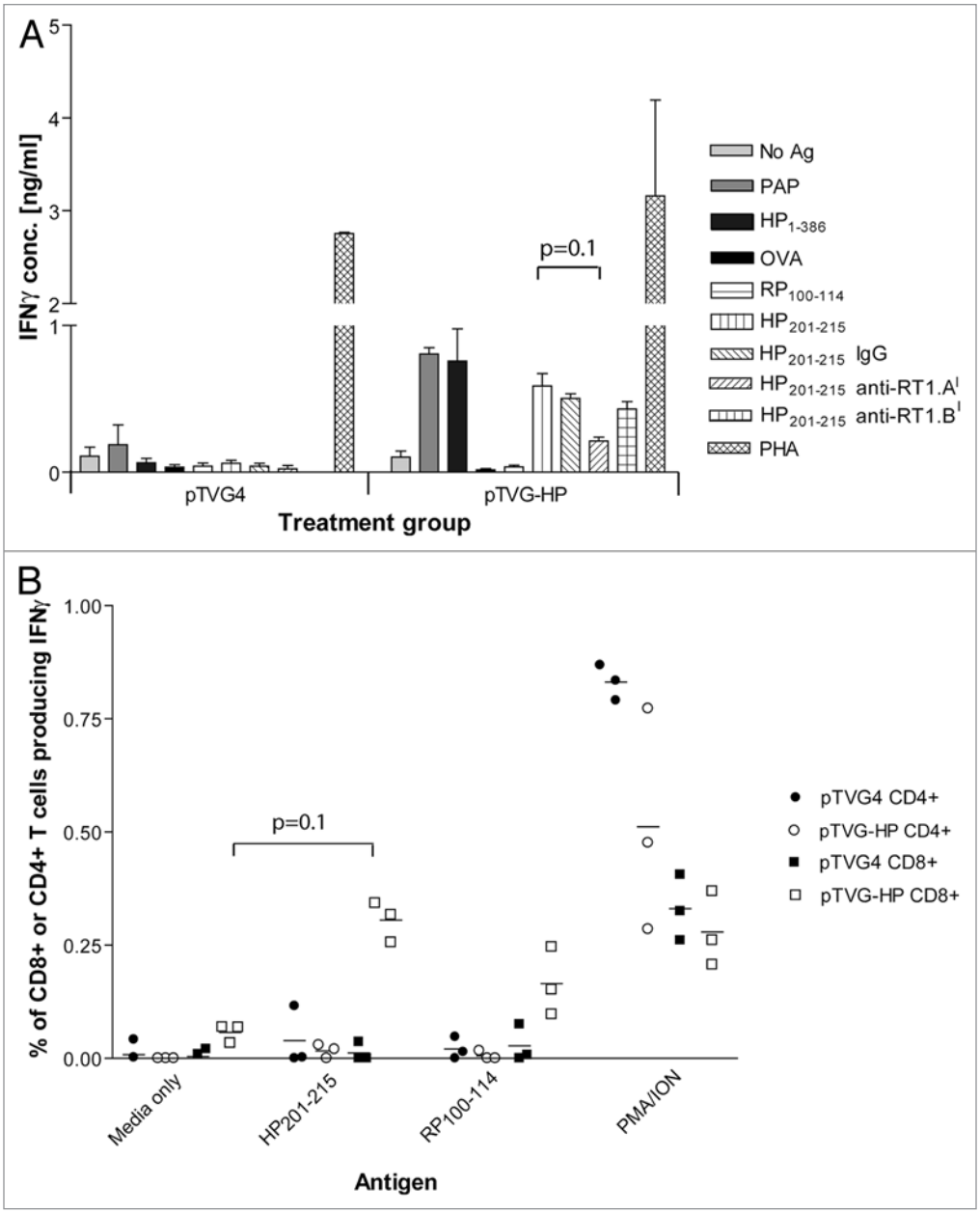

Figure 5. The peptide $HP_{201–215}$ is MHC Class I-restricted. (**A**) Male Lewis rats were immunized four times with pTVG4 or pTVG-HP as described above and splenocytes were cultured with media alone, PAP, ovalbumin (OVA), $HP_{1–386}$, $HP_{201–215}$, non-specific peptide ($RP_{100–114}$), or phytohemaglutinin (PHA) for 96 h, and evaluated for interferon γ (IFNγ) secretion by ELISA. Blocking was performed using 2 μg/mL non-specific IgG, anti-RT1.A^l or anti-RT1.B^l antibodies for 15 min before the addition of the peptide $HP_{201–215}$. Each bar represents mean IFNγ concentration in supernatants and standard error of n = 3 animals per group. (**B**) Splenocytes from rats immunized with plasmid DNA were evaluated for peptide-specific IFNγ expression using flow cytometry. Splenocytes were stimulated with the PAP-derived peptides $HP_{201–215}$ or $RP_{100–114}$, media only, or PMA/Ionomycin as a positive control. Gating was performed on $CD8^+$ or $CD4^+$ T cells, and IFNγ expression was normalized to the signal obtained with an IgG negative control. Each dot represents the percentage of IFNγ producing $CD4^+$ or $CD8^+$ T cells following immunization with plasmid DNA encoding PAP (pTVG-HP) or control vector (pTVG4). Comparisons of the means between the experimental and control groups were made with an unpaired Student's t-test and p values are shown.

Prostate cancer patients immunized with vaccines targeting PAP have developed PAP-specific immune responses. In a Phase I clinical trial, Fong, et al. demonstrated that prostate cancer patients treated with mouse PAP (xenoantigen)-loaded DCs developed T-cell responses to mouse PAP, and 11 out of 21 of these patients also developed cross-reactive human PAP-specific T_H1 responses.[17] We showed that 10 out of 22 prostate cancer patients immunized with a DNA vaccine encoding human PAP developed a T_H1-polarized/CTL response to the native PAP with long-term memory to PAP in some patients.[4,5] In addition, the development of human PAP-specific T cells and antibodies has been documented in 28% patients treated with sipuleucel-T.[1] Although PAP was targeted using different immunization methods, patients generated PAP-specific immune responses regardless of whether they were

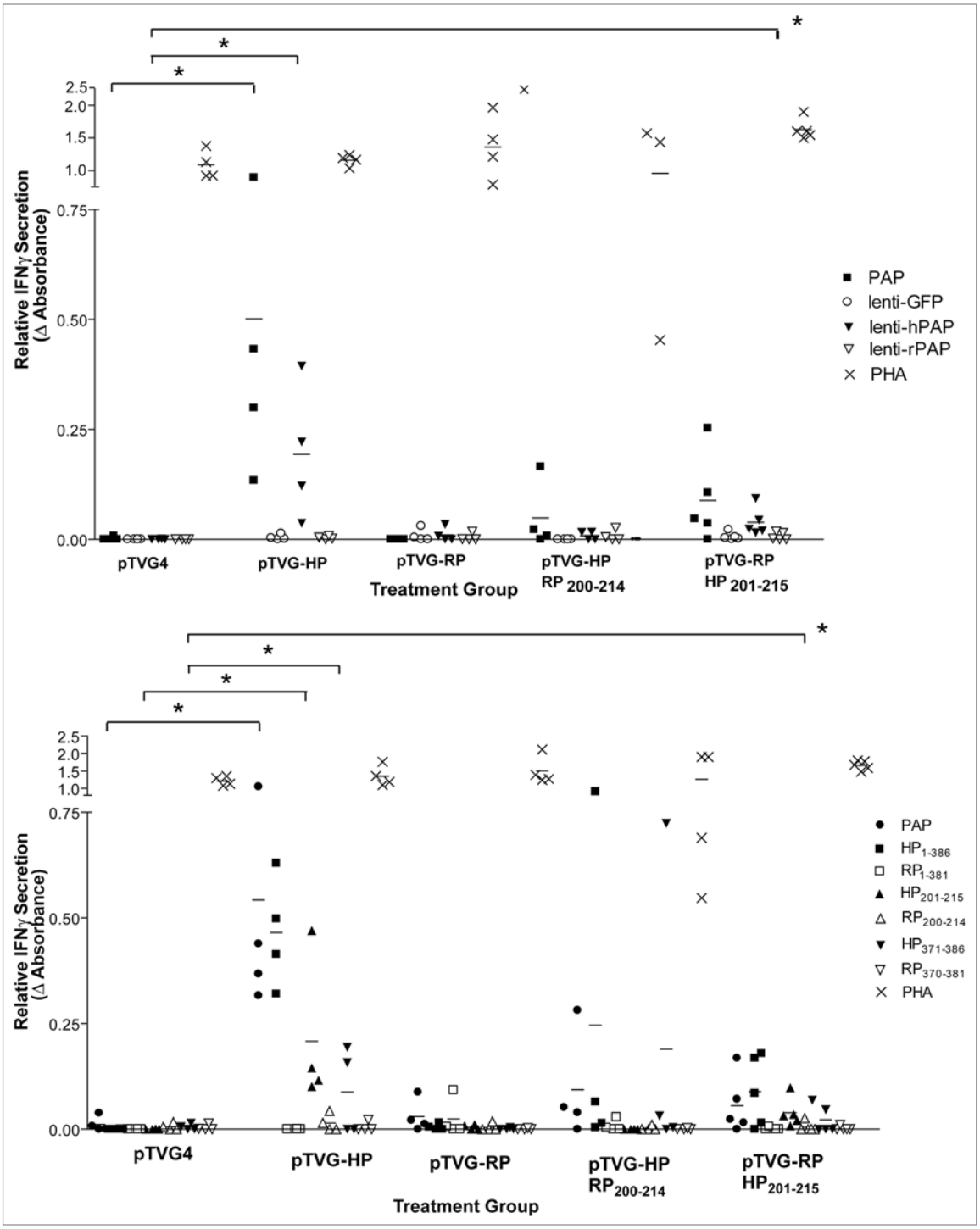

Figure 6. Inclusion or exclusion of the $HP_{201-215}$ immunodominant xenoantigen epitope in a DNA vaccine does not lead to cross-reactive immune responses to the native PAP antigen. (**A and B**) Lewis rats were immunized four times with 100 μg vector (pTVG4, n = 4), pTVG-HP (n = 4), pTVG-RP (n = 4), or the modified DNA vaccines pTVG-HP $RP_{200-214}$ (n = 4) or pTVG-RP $HP_{201-215}$ (n = 5) at two-week intervals. Splenocytes were cultured with media alone, phytohemaglutinin (PHA), dendritic cells (DCs) transduced to express GFP (control), hPAP, or rPAP (**A**) or the peptides HP_{1-386}, $HP_{201-215}$, $RP_{200-214}$, $HP_{371-386}$, $RP_{370-381}$ (**B**) for 72 h. Culture supernatants were analyzed for the production of interferon γ (IFNγ) by ELISA. Each dot represents the average antigen-specific relative IFNγ secretion (Δ absorbance = antigen-specific absorbance - absorbance obtained with medium) from triplicate assessments. Comparisons of the means between groups were made with an unpaired Student's t-test, and p values < 0.05 (*) are shown.

immunized with a xenoantigen or with the native antigen. Thus, even though xenogeneic immunization has been shown to elicit cross-reactive immune responses, there is not an obvious advantage of using a xenoantigen approach targeting this antigen in humans. To determine whether xenoantigen immunization can improve the immunogenicity of a particular vaccine approach in humans, it

will be important to determine whether antigen-specific epitopes recognized in patients following xenoantigen immunization are identical or not to those of the native antigen.

We have previously reported that, in both a preclinical model and a clinical trial, multiple immunizations (at least six) with a DNA vaccine encoding an autologous antigen were necessary to overcome tolerance and elicit a T_H1-polarized/CTL immune response to native PAP.[2,4] While cumbersome, multiple immunizations with a DNA vaccine encoding an autologous antigen may be preferable as compared with a xenoantigen-coding DNA vaccine. Other potential means to increase the immunogenicity of this approach perhaps include different prime/boost strategies based on alternating immunization schedules of DNA vaccines encoding native and xenogeneic PAP. Such prime/boost strategies using DNA vaccines encoding xenoantigens and autologous antigens have been explored in melanoma and prostate cancer models.[10,11,14] A prime/boost approach using xenogeneic DNA vaccines encoding mouse or human PSMA has specifically been explored in patients with prostate cancer.[14] A priori, however, our data would suggest that priming or boosting the immune system with a DNA vaccine coding for a xenoantigen may promote an immune response against a dominant foreign epitope regardless of priming or boosting with a DNA vaccine encoding the autologous antigen. Alternatively, approaches to specifically modify known epitopes might be advantageous. This is notably feasible in the case of PAP, for which multiple HLA-A2, HLA-A24, HLA-A3 and HLA-DR1 epitopes have already been identified.[23–28]

In conclusion, we identified a RT1.A^l-restricted immunodominant human PAP-specific peptide that is naturally processed, presented and recognized in Lewis rats following a xenogeneic immunization with a DNA vaccine encoding human PAP. However, no cross-reactive immune response was elicited to the corresponding peptide from the native antigen. Overall, these results indicate that immunization with a DNA vaccine encoding a xenoantigen may result in immune responses toward specific foreign epitopes, with no cross-reactive responses to the autologous antigen. For the specific targeting of PAP, our results suggest that a preferred vaccination strategy may be to immunize with vaccines encoding the autologous antigen. Future directions could be to modify DNA vaccines at specific epitopes, in order to increase MHC Class I and II molecule binding and/or T-cell receptor recognition, in order to directly target known MHC Class I and II epitopes rather than using foreign homologous proteins, a setting in which random epitope differences may not be advantageous.

Materials and Methods

Animals. Male Lewis rats of approximately 70–77 d of age (251–275 g) were obtained from Charles River Laboratories. They were housed in an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. Rats were fed a chow diet (8604 Teclab), consumed distilled water ad libitum, and maintained on a 12-h dark/12-h light schedule. All experimental protocols were received and approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC).

Peptides. Peptide libraries containing peptides of 15 amino acids length and overlapping by ten amino acids, were synthesized spanning the human PAP protein (SynPep Dublin and ProImmune).[28] Individual rat PAP peptides corresponding to the human PAP regions were synthesized (Proimmune). The peptides used for direct immunization were the following: $HP_{201–215}$ (DLFGIWSKVYDPLYC), $RP_{200–214}$ (DLFEIWSRLYDPLYC), $HP_{371–386}$ (ECMTTNSHQGTEDSTD) and $RP_{370–381}$ (ECMGTSNHQASL). Overlapping human peptides ($HP_{198–212}$ HGQDLFGIWSKVYDP, $HP_{204–218}$ GIWSKVYDPLYCESV, $HP_{208–222}$ KVYDPLYCESVHNFT and $HP_{211–225}$ DPLYCESVHNFTLPS) and the corresponding rat peptides ($RP_{197–211}$ EDQDLFEIWSRLYDP, $RP_{203–217}$ EIWSRLYDPLYCESV, $RP_{207–224}$ RLYDPLYCESVHNFT and $RP_{210–224}$ DPLYCESVHNFTLPT) around the $HP_{201–215}$ immunodominant region were analyzed.

DNA vaccines. DNA vaccines encoding human PAP (pTVG-HP) or rat PAP (pTVG-RP) used for immunizations have been previously described.[2,3] Oligonucleotide-directed mutagenesis (Phusion site-directed mutagenesis kit, New England Biolabs) was used to modify human PAP DNA vaccine (pTVG-HP $RP_{200–214}$) so that the $RP_{200–214}$ amino acid region was inserted into the $HP_{201–215}$ amino acid coding sequence of the human PAP-coding DNA vaccine. Similarly, a modified rat PAP vaccine (pTVG-RP $HP_{201–215}$) was constructed in which the $HP_{201–215}$ amino acid region was inserted into the $RP_{200–214}$ amino acid coding sequence of the rat PAP-coding DNA vaccine. Protein expression was confirmed by transfecting CHO cells with the DNA vaccines and measuring PAP expression using an assay of phosphatase activity in the culture supernatant and cell lysates, as previously described.[29]

Immunization. For DNA immunization, Ten-11 week-old male Lewis rats were injected four times intradermally at 14-d intervals with 100 μg plasmid DNA, including pTVG4 (vector control), pTVG-HP (encoding hPAP), pTVG-RP (encoding rPAP) and modified variants. For each immunization, 5 μg rat granulocyte-macrophage colony-stimulating factor (GM-CSF) was co-administered as an adjuvant. For peptide immunizations, rats were injected subcutaneously with 100 μg peptide in complete Freund's adjuvant (CFA), and boosted 14 d later with peptide in incomplete Freund's adjuvant. Rats were euthanized and spleens were harvested two weeks after the last immunization for immunological analyses.

Antigen-specific T-cell proliferation. Splenocytes harvested from immunized animals were evaluated using methods previously described.[3] Specifically, 2×10^5 cells were cultured with 2 μg/mL hPAP (Chemicon Int., Temecula, CA), 2 μg/mL peptide library or individual peptides, or 10 μg/mL phytohemaglutinin (PHA) (Sigma) for 72 h at 37°C in an 5% CO_2 atmosphere. Then, cultures were pulsed with 1 μM BrdU (BD Biosciences) for eight to 12 h. Antigen-specific, proliferating ($BrdU^+$) $CD4^+$ or $CD8^+$ T cells were detected using an intracellular flow cytometric staining method (BD Flow kit, BD Biosciences) according to the manufacturer's standard protocol, and BrdU incorporation was measured and analyzed as previously described.[3]

Interferon γ (IFNγ)-specific ELISA. Splenocytes from immunized rats were cultured with hPAP protein, peptide library,

peptide subsets, or individual peptides for 72 or 96 h as described above. Where indicated, rat dendritic cells (DCs) were used as antigen-presenting cells following transduction with lentivirus-expressing rPAP, hPAP or green fluorescent protein (GFP, negative control). For these studies, DCs were collected from spleens of naïve Lewis rats using anti-OX-62-PE (AbD Serotec) and isolated by PE positive selection (StemCell Technologies, Inc.) as described previously.[2] The presence of IFNγ in the culture media was determined using a quantitative capture ELISA specific for rat IFNγ as described previously.[2,3] Where indicated, splenocytes were pre-incubated for 15 min with 2 μg/mL antibodies specific for Lewis rat anti-RT1.A^{l} (BD Biosciences), anti-RT1.B^{l} (Cedarlane Laboratories), or mouse IgG as a negative control, prior to adding stimulator peptides. Results are reported as the mean IFNγ concentration and standard deviation, or relative concentration (Δ absorbance) in the absence of a standard curve for reference, from multiple replicates.

Intracellular IFNγ analysis. Splenocytes from immunized rats were cultured for 18 h with 2 μg/mL specific ($HP_{201–215}$) or non-specific ($RP_{100–114}$) PAP peptides, media only, or 40 ng/mL PMA plus 1 μg/mL ionomycin, for 6 h. Monensin (2 μg/mL) was added to all cultures for the final 6 h. The production of IFNγ by $CD4^+$ and/or $CD8^+$ T cells was detected by standard methodologies (BD Cytofix/Cytoperm kit, $CD8^+$: clone OX-8, $CD4^+$: clone OX-35, BD Biosciences; IFNγ: clone DB-1, Biolegend) using an LSR II flow cytometer (BD Biosciences).[30] Gating was performed on $CD8^+$ and $CD4^+$ T cells and the percentage of IFNγ–expressing cells was determined by comparison with a fluorochrome-labeled IgG control under identical antigen stimulation conditions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al.; IMPACT Study Investigators. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 2010; 363:411-22; PMID:20818862; http://dx.doi.org/10.1056/NEJMoa1001294.
2. Johnson LE, Frye TP, Chinnasamy N, Chinnasamy D, McNeel DG. Plasmid DNA vaccine encoding prostatic acid phosphatase is effective in eliciting autologous antigen-specific CD8+ T cells. Cancer Immunol Immunother 2007; 56:885-95; PMID:17102977; http://dx.doi.org/10.1007/s00262-006-0241-8.
3. Johnson LE, Frye TP, Arnot AR, Marquette C, Couture LA, Gendron-Fitzpatrick A, et al. Safety and immunological efficacy of a prostate cancer plasmid DNA vaccine encoding prostatic acid phosphatase (PAP). Vaccine 2006; 24:293-303; PMID:16115700; http://dx.doi.org/10.1016/j.vaccine.2005.07.074.
4. McNeel DG, Dunphy EJ, Davies JG, Frye TP, Johnson LE, Staab MJ, et al. Safety and immunological efficacy of a DNA vaccine encoding prostatic acid phosphatase in patients with stage D0 prostate cancer. J Clin Oncol 2009; 27:4047-54; PMID:19636017; http://dx.doi.org/10.1200/JCO.2008.19.9968.
5. Becker JT, Olson BM, Johnson LE, Davies JG, Dunphy EJ, McNeel DG. DNA vaccine encoding prostatic acid phosphatase (PAP) elicits long-term T-cell responses in patients with recurrent prostate cancer. J Immunother 2010; 33:639-47; PMID:20551832; http://dx.doi.org/10.1097/CJI.0b013e3181dda23e.
6. Gold JS, Ferrone CR, Guevara-Patiño JA, Hawkins WG, Dyall R, Engelhorn ME, et al. A single heteroclitic epitope determines cancer immunity after xenogeneic DNA immunization against a tumor differentiation antigen. J Immunol 2003; 170:5188-94; PMID:12734366.
7. Bowne WB, Srinivasan R, Wolchok JD, Hawkins WG, Blachere NE, Dyall R, et al. Coupling and uncoupling of tumor immunity and autoimmunity. J Exp Med 1999; 190:1717-22; PMID:10587362; http://dx.doi.org/10.1084/jem.190.11.1717.
8. Naftzger C, Takechi Y, Kohda H, Hara I, Vijayasaradhi S, Houghton AN. Immune response to a differentiation antigen induced by altered antigen: a study of tumor rejection and autoimmunity. Proc Natl Acad Sci U S A 1996; 93:14809-14; PMID:8962137; http://dx.doi.org/10.1073/pnas.93.25.14809.
9. Hawkins WG, Gold JS, Dyall R, Wolchok JD, Hoos A, Bowne WB, et al. Immunization with DNA coding for gp100 results in CD4 T-cell independent antitumor immunity. Surgery 2000; 128:273-80; PMID:10923004; http://dx.doi.org/10.1067/msy.2000.107421.
10. Yuan J, Ku GY, Gallardo HF, Orlandi F, Manukian G, Rasalan TS, et al. Safety and immunogenicity of a human and mouse gp100 DNA vaccine in a phase I trial of patients with melanoma. Cancer Immun 2009; 9:5; PMID:19496531.
11. Wolchok JD, Yuan J, Houghton AN, Gallardo HF, Rasalan TS, Wang J, et al. Safety and immunogenicity of tyrosinase DNA vaccines in patients with melanoma. Mol Ther 2007; 15:2044-50; PMID:17726460; http://dx.doi.org/10.1038/sj.mt.6300290.
12. Bergman PJ, McKnight J, Novosad A, Charney S, Farrelly J, Craft D, et al. Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. Clin Cancer Res 2003; 9:1284-90; PMID:12684396.
13. Gregor PD, Wolchok JD, Turaga V, Latouche JB, Sadelain M, Bacich D, et al. Induction of autoantibodies to syngeneic prostate-specific membrane antigen by xenogeneic vaccination. Int J Cancer 2005; 116:415-21; PMID:15800947; http://dx.doi.org/10.1002/ijc.21014.
14. Slovin S, Gregor P, Wolchok J, Pedraza A, Orlandi F, Jefferson M, et al. A xenogeneic PSMA DNA vaccine for patients with non-castrate metastatic (NCMPC) and castrate metastatic prostate cancer (CMPC)-A phase I trial of proof of principle. J Clin Oncol 2007; 25:3073.
15. Hassan MI, Aijaz A, Ahmad F. Structural and functional analysis of human prostatic acid phosphatase. Expert Rev Anticancer Ther 2010; 10:1055-68; PMID:20645695; http://dx.doi.org/10.1586/era.10.46.
16. Fong L, Ruegg CL, Brockstedt D, Engleman EG, Laus R. Induction of tissue-specific autoimmune prostatitis with prostatic acid phosphatase immunization: implications for immunotherapy of prostate cancer. J Immunol 1997; 159:3113-7; PMID:9317107.
17. Fong L, Brockstedt D, Benike C, Breen JK, Strang G, Ruegg CL, et al. Dendritic cell-based xenoantigen vaccination for prostate cancer immunotherapy. J Immunol 2001; 167:7150-6; PMID:11739538.
18. Reizis B, Mor F, Eisenstein M, Schild H, Stefanoviç S, Rammensee HG, et al. The peptide binding specificity of the MHC class II I-A molecule of the Lewis rat, RT1.BI. Int Immunol 1996; 8:1825-32; PMID:8982766; http://dx.doi.org/10.1093/intimm/8.12.1825.
19. Reizis B, Schild H, Stefanovic S, Mor F, Rammensee H, Cohen IR. Peptide binding motifs of the MHC class I molecules (RT1.Al) of the Lewis rat. Immunogenetics 1997; 45:278-9; PMID:9002449; http://dx.doi.org/10.1007/s002510050204.
20. Mincheff M, Zoubak S, Makogonenko Y. Immune responses against PSMA after gene-based vaccination for immunotherapy-A: results from immunizations in animals. Cancer Gene Ther 2006; 13:436-44; PMID:16276349; http://dx.doi.org/10.1038/sj.cgt.7700914.
21. Gregor PD, Wolchok JD, Ferrone CR, Buchinshky H, Guevara-Patiño JA, Perales MA, et al. CTLA-4 blockade in combination with xenogeneic DNA vaccines enhances T-cell responses, tumor immunity and autoimmunity to self antigens in animal and cellular model systems. Vaccine 2004; 22:1700-8; PMID:15068853; http://dx.doi.org/10.1016/j.vaccine.2003.10.048.
22. Smith HA, McNeel DG. Vaccines targeting the cancer-testis antigen SSX-2 elicit HLA-A2 epitope-specific cytolytic T cells. J Immunother 2011; 34:569-80; PMID:21904219; http://dx.doi.org/10.1097/CJI.0b013e31822b5b1d.
23. Olson BM, Frye TP, Johnson LE, Fong L, Knutson KL, Disis ML, et al. HLA-A2-restricted T-cell epitopes specific for prostatic acid phosphatase. Cancer Immunol Immunother 2010; 59:943-53; PMID:20140431; http://dx.doi.org/10.1007/s00262-010-0820-6.
24. Peshwa MV, Shi JD, Ruegg C, Laus R, van Schooten WC. Induction of prostate tumor-specific CD8+ cytotoxic T-lymphocytes in vitro using antigen-presenting cells pulsed with prostatic acid phosphatase peptide. Prostate 1998; 36:129-38; PMID:9655265; http://dx.doi.org/10.1002/(SICI)1097-0045(19980701)36:2<129::AID-PROS8>3.0.CO;2-D.
25. Harada M, Matsueda S, Yao A, Ogata R, Noguchi M, Itoh K. Prostate-related antigen-derived new peptides having the capacity of inducing prostate cancer-reactive CTLs in HLA-A2+ prostate cancer patients. Oncol Rep 2004; 12:601-7; PMID:15289844.

26. Machlenkin A, Paz A, Bar Haim E, Goldberger O, Finkel E, Tirosh B, et al. Human CTL epitopes prostatic acid phosphatase-3 and six-transmembrane epithelial antigen of prostate-3 as candidates for prostate cancer immunotherapy. Cancer Res 2005; 65:6435-42; PMID:16024648; http://dx.doi.org/10.1158/0008-5472.CAN-05-0133.
27. Matsueda S, Takedatsu H, Yao A, Tanaka M, Noguchi M, Itoh K, et al. Identification of peptide vaccine candidates for prostate cancer patients with HLA-A3 supertype alleles. Clin Cancer Res 2005; 11:6933-43; PMID:16203785; http://dx.doi.org/10.1158/1078-0432.CCR-05-0682.
28. Johnson LE, McNeel DG. Identification of prostatic acid phosphatase (PAP) specific HLA-DR1-restricted T-cell epitopes. Prostate 2012; 72:730-40; PMID:22529020; http://dx.doi.org/10.1002/pros.21477.
29. Choe BK, Pontes EJ, Bloink S, Rose NR. Human prostatic acid phosphatases: I. Isolation. Arch Androl 1978; 1:221-6; PMID:83814; http://dx.doi.org/10.3109/01485017808988340.
30. Caraher EM, Parenteau M, Gruber H, Scott FW. Flow cytometric analysis of intracellular IFN-gamma, IL-4 and IL-10 in CD3(+)4(+) T-cells from rat spleen. J Immunol Methods 2000; 244:29-40; PMID:11033016; http://dx.doi.org/10.1016/S0022-1759(00)00249-0.