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 \(\gamma\) (IFN\(\gamma\))–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...
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\(_h\)\(^1\) 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\(^\circ\) or pTVG–HP\(^\circ\) 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\(_h\)\(^1\)-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 IFN\(_\gamma\) 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\(_{341–360}\)) and to two separate subsets of this peptide library (HP\(_{201–215}\) and HP\(_{341–360}\)).

CD8+ T cells proliferated when stimulated with each of the peptide subsets while CD4+ T cells only proliferated in response to the HP\(_{201–215}\) subset (Fig. 1B). Splenocytes from pTVG–HP-immunized rats were cultured with individual peptides from the subsets and assessed for IFN\(_\gamma\) release by ELISA to identify the specific epitopes recognized. Peptide-specific IFN\(_\gamma\) 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,
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 271–386 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.A1-restricted. To better characterize the immunodominant epitope, peptide-specific IFNγ secretion was assessed with peptides of different length that flanked the HP201–215 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 HP201–215 and RP200–214 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.A1) or MHC Class II (RT1.B1) 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 HP201–215 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
next sought to investigate whether inclusion of the hPAP-specific immunodominant epitope HP_{201–215}^{hp1–386} was 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}^{hp1–386}) was constructed in which the immunodominant HP_{201–215}^{hp1–386} region was replaced by PCR-directed mutagenesis with the sequence coding for the corresponding rat amino acids, RP_{200–214}^{hp1–386}. Similarly, a modified pTVG-RP vaccine (pTVG-RP HP_{201–215}^{hp1–386}) was constructed to encode the immunodominant HP_{201–215}^{hp1–386} 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. As shown in Figure 6, neither human nor rPAP-specific immune responses were elicited in rats immunized with pTVG-RP. Splenocytes from rats immunized with pTVG-RP HP_{201–215}^{hp1–386} 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}^{hp1–386} was substituted in the hPAP xenoantigen with the RP_{200–214}^{hp1–386} 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 rPAP-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.^{1} 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. 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}^{hp1–386}, that was recognized in all animals following immunization. A second peptide, HP_{371–386}^{hp1–386}, 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}^{hp1–386} epitope was found to be RT1.A-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 rPAP. 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. 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}^{hp1–386}. The sequence similarity of a 9-mer peptide contained within this region, WSKVYDPLY, to the preferred RT1.A binding motif (X-A/S/V-F/Y-X-X-X-Y/F/L/M), the peptide-elicited production of IFNγ by CD8+ T cells, and the ability of anti-RT1.A^{A} antibodies to partially block peptide-specific immune...
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.Al-binding motifs, the anchor residue required for MHC Class I molecule binding was not present in the corresponding rat peptide. 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 3](image-url)

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 HP201–215 or RP200–214 (A), and HP371–386 or RP370–381 (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).
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.

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. 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 4. HP204–215 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 phytohemagglutinin (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 HP201–215 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 (HP208–222, HP201–215, HP204–218, HP211–225) and the corresponding rat (RP200–214, RP203–217, RP207–224, RP210–224) 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.
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 Th1 responses. We showed that 10 out of 22 prostate cancer patients immunized with a DNA vaccine encoding human PAP developed a Th1-polarized/CTL response to the native PAP with long-term memory to PAP in some patients. In addition, the development of human PAP-specific T cells and antibodies has been documented in 28% patients treated with sipuleucel-T. 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 HP201–215 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-hpRp200–214 (n = 4) or pTVG-RpHp201–215 (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 hP1–386, hP201–215, Rp200–214, hP371–386, Rp370–381 (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 xenogeneic 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<sub>h</sub><sup>1</sup>-polarized/CTL immune response to native PAP. While cumbersome, multiple immunizations with a DNA vaccine encoding an autologous antigen may be preferable as compared with a xenogeneic-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 xenogeneic antigens and autologous antigens have been explored in melanoma and prostate cancer models. A prime/boost approach using xenogeneic DNA vaccines encoding mouse or human PSMA has specifically been explored in patients with prostate cancer. A priori, however, our data would suggest that priming or boosting the immune system with a DNA vaccine coding for a xenogeneic 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.

In conclusion, we identified a RT1.A<sup>+</sup>-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 xenogeneic antigen may result in immune responses towards 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.

**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). Individual rat PAP peptides corresponding to the human PAP regions were synthesized (ProImmune). The peptides used for direct immunization were the following: HP<sub>201–215</sub> (DLFGIWSVKYDPY), RP<sub>200–214</sub> (DLFEIWSRYDPY), HP<sub>219–223</sub> (ECMTTNSHOGTEDST) and RP<sub>370–381</sub> (ECMTTNSHQA). Overlapping human peptides (HP<sub>198–212</sub> HGQDLFEIWSRKVDP, HP<sub>224–238</sub> GIWSVKYDPYCESV, HP<sub>226–232</sub> VYDPLICESYHNFT and HP<sub>21–225</sub> DPLYCESVHNFTLP) and the corresponding rat peptides (RP<sub>197–211</sub> EDQDLFEIWSRLYDP, RP<sub>201–217</sub> EIWRSRLYDPYC, RP<sub>207–224</sub> RLYDPRICESVHNFT and RP<sub>210–224</sub> DPLYCESVHNFTLP) around the HP<sub>201–215</sub> 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. Oligonucleotide-directed mutagenesis (Phusion site-directed mutagenesis kit, New England Biolabs) was used to modify human PAP DNA vaccine (pTVG-HP RP<sub>200–214</sub>) so that the RP<sub>200–214</sub> amino acid region was inserted into the HP<sub>201–215</sub> amino acid coding sequence of the human PAP-coding DNA vaccine. Similarly, a modified rat PAP vaccine (pTVG-RP HP<sub>201–215</sub>) was constructed in which the HP<sub>201–215</sub> amino acid region was inserted into the RP<sub>200–214</sub> 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.

**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 pTVG-4 (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. Specifically, 2 × 10<sup>6</sup> 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<sub>2</sub> atmosphere. Then, cultures were pulsed with 1 μM BrdU (BD Biosciences) for eight to 12 h. Antigen-specific, proliferating (BrdU<sup>+</sup>) CD4<sup>+</sup> or CD8<sup>+</sup> 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.

**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.3,2 The presence of IFNγ in the culture media was determined using a quantitative capture ELISA specific for rat IFNγ as described previously.3,2 Where indicated, splenocytes were pre-incubated for 15 min with 2 μg/mL antibodies specific for Lewis rat anti-RT1.A (BD Biosciences), anti-RT1. B (Cedarlane Laboratories), or mouse IgG as a negative control, prior to adding stimulator peptides. Results are reported as concentration (μg/mL) or specific for Lewis rat anti-RT1.A (BD Biosciences), anti-RT1. B (Cedarlane Laboratories), or mouse IgG as a negative control, prior to adding stimulator peptides. Results are reported as concentration (μg/mL) or relative 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 (HP20–219) or non-specific (RP100–111) 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, Biologend) using an LSR II flow cytometer (BD Biosciences). 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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