An Artificial PAP Gene Breaks Self-tolerance and Promotes Tumor Regression in the TRAMP Model for Prostate Carcinoma

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Prostate cancer (PCa) is the most commonly diagnosed type of cancer in men in western industrialized countries. As a public health burden, the need for the invention of new cost-saving PCa immunotherapies is apparent. In this study, we present a DNA vaccine encoding for the prostate-specific antigen prostatic acid phosphatase (PAP) linked to the J-domain and the SV40 enhancer sequence. The PAP DNA vaccine induced a strong PAP-specific cellular immune response after electroporation (EP)-based delivery in C57BL/6 mice. Splenocytes from mice immunized with PAP recognized the naturally processed PAP epitopes, indicating that vaccination with the PAP-J gene broke its self-tolerance against PAP. Remarkably, DNA vaccination with PAP-J inhibited tumor growth in the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mouse model that closely resembled human PCa. Therefore, this study highlights a novel cancer immunotherapy approach with the potential to control PCa in clinical settings.

INTRODUCTION
Prostate cancer (PCa) is the second leading cause of death among men in the United States and the most common form of cancer among men in Europe. PCa, as an age-related disease, will likely become more important among elderly men in the future. As current treatments for metastatic or hormone refractory PCa are very limited in their efficacy, there is an urgent need for the development of new cost-saving therapies (e.g., based on immunotherapy). Unfortunately, with most cancer cases, only self-antigens are available for therapeutic immunizations, and these are generally associated with weak immunogenicity and induction of some level of tolerance. Under these unfavorable conditions, the main challenge for successful immunotherapy is to overcome the self-tolerance against cancer-specific self-antigens and to induce an effective immune response. In the case of PCa, there are several classical tumor-associated antigens, namely prostate-specific membrane antigen (PSMA), prostate stem cell antigen, and prostatic acid phosphatase (PAP), which are mostly restricted to the prostate tissues and are upregulated in PCa. DNA immunization, as one strategy in the field of immunotherapy, has been widely used in the case of PCa in various animal studies. Moreover, PSMA and PAP are undergoing clinical trials (www.clinicaltrials.gov) and, despite promising results in animal models, DNA vaccines against PCa have to date yielded only limited clinical benefits when transferred into humans.

In this study, we developed a DNA vaccine that encodes for murine PAP. We chose PAP as a target antigen because it is highly restricted to the prostate tissues. Moreover, PAP is, in contrast to PSA, present in mice as well as in humans, and therefore, represents a mouse self-antigen that provides a clinically relevant model to study the effects of a prostate-specific DNA vaccine.

We cloned a sequence of PAP that was codon-optimized for use in humans (and is nearly identical to the murine system) into a vector containing a highly modified CpG cassette in the backbone. CpG motifs, which are abundant in bacterial or synthetic DNA, can be recognized by the immune system via Toll-like receptor and therefore, stimulate the innate and adaptive immune systems.

The PAP genes were tested for their ability to induce a PAP-specific cellular immune response and their ability to induce tumor regression in a xenograft tumor model and, more importantly, in the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mouse model (for details see refs. 12,13). This will allow the study of PCa resembling the human disease in several aspects.

To date, the major drawback with active immunotherapy using DNA vaccines against cancer was the failure to transfer successful therapies from rodent models into primates or humans. One possible solution to overcome this hurdle is the use of an alternative DNA delivery system. The electroporation (EP) technique allows the efficient application of low volumes of DNA into muscle cells and can also enhance the uptake of DNA into target cells, leading to an increase in protein expression. Additionally, the procedure by itself causes local tissue damage and inflammation, which promotes humoral and cellular immune responses. Therefore, we took advantage of an EP system that delivers the DNA vaccine via a special TriGrid Array...
(Ichors Medical Systems, San Diego, CA) into the target tissue. This technology combines agent administration and EP into a single device, thereby ensuring that the EP effect is induced consistently at the site of the DNA administration.

In this study, we demonstrated the generation of three different artificial PAP genes and showed that these constructs induced a PAP-specific cellular immune response via needle injection, which was strengthened by using an EP-based delivery system. Furthermore, we showed that one out of the three PAP genes is able to promote tumor regression in C57BL/6 mice and, more importantly, in the TRAMP model for PCa.

RESULTS
We sought to induce a PAP-specific immune response by a therapeutic DNA vaccination, because the self-antigen PAP is mainly restricted to prostate tissues and upregulated during PCa. We attempted to boost the immune response against PCa by using a PAP-expressing plasmid that provided a backbone modified with a highly optimized CpG cassette. We designed three different codon-optimized PAP genes that were modified with the Kozak sequence, the DnaJ-like domain and the SV40 enhancer, as well as via the deletion of the signal peptide. Moreover, we took advantage of an EP system that efficiently delivered the DNA vaccine into the target cells.

Generation of the three different versions of the PAP gene
We developed three different versions of the PAP construct, because the combined effects of the insertion of a DnaJ-like domain and the SV40 nuclear targeting sequence for the induction of the cellular immune response were difficult to predict. The designated PAP genes were named PAP-JS, PAP-S, and PAP-J (Figure 1a). The expression of the three different constructs was verified with reverse transcriptase (RT)-PCR and western blotting (Figure 1b,c). The strongest expression at the protein level was detected within PAP-S transfected NIH3T3 cells, which was about eight- to tenfold higher compared to that which was detected in others (PAP-JS, PAP-J). The PAP constructs were cloned in the pPOE-CpG immunization vector, which was successfully used in our group.17

Two mPAP peptides are able to stabilize H2β-restricted MHC-I molecules and show high-binding capacities
A computer-based prediction (http://www-bimas.cit.nih.gov/molbio/hla_bind/BIMAS and http://www.syfpeithi.de/) of potential murine prostatic acid phosphatase (mPAP) epitopes was performed using the murine wild-type PAP sequence. The predicted binding affinities were similar to those of the known Kα- and Dβ-binding peptides (OVA257-264 (ref. 18) and HPV-16 E749-57 (ref. 19), displaying a high affinity for the respective major histocompatibility complex-I (MHC-I) molecules. The two most promising Dβ-binding peptides, mPAP114–122 and mPAP128–136, showed a high-binding affinity comparable to that of HPV-16 E749–57 (Figure 2 and Table 1). All of the predicted Kα epitopes were not able to stabilize the empty MHC-I molecules on the surfaces of the RMA-S cells (Supplementary Figure S1). Thus, we decided to choose

Electroporation of C57BL/6 mice strongly increases the CTL responses against PAP and reveals the superiority of the PAP-J construct
As we were not able to show a significant immune response of our PAP genes in C57BL/6 mice after needle injection or application of adjuvant gene analogues25 into the musculus tibialis anterior,
we decided to take advantage of an EP system that was successfully used in an HPV tumor model by our group.20

All three PAP constructs were able to induce a cytotoxic T lymphocyte (CTL) response as evidenced by the interferon (IFN-γ)-secreting cells/1 × 10⁴ splenocytes (IFN-γ Elispot assay (IFN-γ-secreting cells/1 × 10⁴ splenocytes: PAP-JS: 43 ± 9 versus pPOE: 6 ± 3, P < 0.01, PAP-S: 24 ± 5, P > 0.02 compared with pPOE, PAP-J: 71 ± 11, P < 0.01 compared with pPOE). We received similar results in the granzyme B Elispot assay (granzyme B-secreting cells/1 × 10⁴ splenocytes: PAP-JS: 28 ± 7 versus pPOE: 4 ± 2, P < 0.05, PAP-J: 62 ± 9, P < 0.001 compared to pPOE) (Figure 3b). Additionally, we analyzed the immune response after PAP-J vaccination using a vector lacking the CpG motif (pTHkan: −CpG, pPOE: +CpG). One representative of the two experiments is shown. (a) Ex vivo interferon-γ Elispot responses after DNA immunization. (b) Ex vivo granzyme B and interferon (IFN-γ)-Elispot assays using a immunization vector lacking the CpG motif (pTHkan: −CpG, pPOE: +CpG). One representative of the two experiments is shown.

Figure 3a

| Abbreviations: MHC-I, major histocompatibility complex-I; PAP, prostatic acid phosphatase. A computer-based prediction (SYFPEITHI) of the potential K<sup>a</sup>- and D<sup>b</sup>-binding peptides was made. Comparisons to the known K<sup>a</sup>- and D<sup>b</sup>-binding peptides are shown according to their scores performed in parallel. The score for binding to the K<sup>a</sup> or D<sup>b</sup> molecule was given according to the SYFPEITHI database. |

### Table 1 mPAP peptides fitting the binding motifs for the murine MHC-I molecules K<sup>a</sup> and D<sup>b</sup>

<table>
<thead>
<tr>
<th>MHC-I molecule</th>
<th>Position</th>
<th>Peptide designation</th>
<th>Sequence</th>
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<td>HPV-16 E&lt;sub&gt;7&lt;/sub&gt;&lt;sup&gt;56-63&lt;/sup&gt;</td>
<td>RAHY NIVTF</td>
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**K**<sup>a</sup> 32 mPAP<sup>32-39</sup> KELKVFVTL 22

**D**<sup>b</sup> 114 mPAP<sup>114-122</sup> OVA<sup>37-44</sup> SIWNPRL 28

6. To determine whether the CTLs of PAP-J immunized animals recognized mPAP<sub>114-122</sub> or mPAP<sub>128-136</sub> epitopes that were externally loaded onto RMA-S cells, we electroporated male mice with the PAP-J-encoding plasmid or the control vector three times on a weekly interval. Splenocytes were in vitro restimulated with RMA-S cells loaded with either mPAP<sub>114-122</sub> or mPAP<sub>128-136</sub> and

Naturally processed PAP epitopes are recognized by CTLs of the PAP-J immunized mice

In order to determine whether the CTLs of PAP-J immunized animals recognized mPAP<sub>114-122</sub> or mPAP<sub>128-136</sub> epitopes that were externally loaded onto RMA-S cells, we electroporated male mice with the PAP-J-encoding plasmid or the control vector three times on a weekly interval. Splenocytes were in vitro restimulated with RMA-S cells loaded with either mPAP<sub>114-122</sub> or mPAP<sub>128-136</sub> and

Next, we analyzed whether the CTLs from the PAP-J immunized mice were able to recognize naturally processed PAP epitopes. Six to seven days after in vitro restimulation of splenocytes with mPAP<sub>128-136</sub> pulsed RMA-S cells a ⁵¹Cr-release assay was performed. CTLs from the PAP-J immunized animals were able to lyse the RMA-S cells loaded with mPAP<sub>128-136</sub> suggesting that this epitope was present after vaccination (specific lysis: PAP-J: 31.5 ± 8% versus pPOE: 3 ± 0.5%, P < 0.05). More importantly, TRAMP-C1 prostate tumor cells<sup>21</sup> were also recognized
demonstrating that PAP-specific priming was induced in vivo (Figure 4a).

To characterize the immune responses in greater detail, a DELFIA EuTDA cytotoxic assay was performed. After immunization of mice, we were able to demonstrate an induction of the CTLs. The PAP-J construct induced CTLs that were able to lyse the target cells pulsed with mPAP 128–136 (PAP-J: 22.5 ± 9.5% versus pPOE: 4.6 ± 4.7%, P < 0.01) (Figure 4c). Additionally, we assessed the number of PAP-specific CTLs using pentamers bearing the PAP epitope mPAP128–136 (Figure 4b). Therefore, spleno-

cytocytes from immunized mice were stained and analyzed via flow cytometry. Approximately 1% of the mPAP128–136-specific CD8+ lymphocytes could be detected (P < 0.05).

Finally, we investigated whether DNA vaccination with PAP-J induced an antibody response. Whole blood samples from immunized C57BL/6 mice were obtained and analyzed. A significant antibody response in the serum of PAP-J-treated mice compared to the serum of control mice (P < 0.001) was detected (Figure 4d).

From these functional assays, we concluded that EP with the PAP-J gene induced the CTLs that were able to recognize the naturally processed PAP epitopes generated by TRAMP-derived C1 PCa cells and to lyse them.

Immunization with the PAP-J construct retards PCA tumor progression in C57BL/6 mice

Next, we investigated whether PAP-specific CTLs were able to recognize TRAMP-C1 prostate tumor cells in vivo. We injected TRAMP-C1 cells subcutaneously into the flanks of male C57BL/6 mice. After 6–8 days the animals received the PAP-J DNA vaccine or the control vector. The tumor growth was assessed two times
Figure 6 Induction of interferon (IFN)-γ-producing splenocytes after immunotherapy with the PAP\textsuperscript{J} gene. Male Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice were immunized with the PAP\textsuperscript{J} plasmid or the control vector (100 μg/mouse, n = 8/group). (a) Seven days after prime immunization, four mice from each group were sacrificed, the spleens were excised and an ex vivo IFN-γ intracellular staining (ICS) assay was performed. (b) A booster immunization with the living TRAMP mice (n = 4/group) was given; 7 days after the boost, the mice were sacrificed, and the spleens were analysed as above. The white bars show the percentage of IFN-γ-producing splenocytes that were not stimulated in the ICS. The black bars show the percentage of IFN-γ-producing splenocytes that were stimulated with mPAP\textsuperscript{128–136} in the ICS. One representative of the two experiments is shown. PAP, prostatic acid phosphatase.

The PAP\textsuperscript{J} gene induces a robust CTL response and effectively suppresses tumor growth in TRAMP mice

We immunized the TRAMP mouse with the PAP\textsuperscript{J} DNA vaccine because this transgene model mirrored the natural development of PCa in the human prostate gland histologically, while also representing a generally accepted tumor model.

Seven to ten days after each immunization, four mice per group were sacrificed, and the PAP-specific IFN-γ response was assessed ex vivo by intracellular cytokine staining. The spleen cells were then restimulated with splenocytes from naive C57BL/6 mice pulsed with mPAP\textsuperscript{128–136}. After the prime immunization, no PAP-specific immune response was detected. After one boost immunization, however, we observed a robust cellular immune response (Figure 6a,b), revealing ~1% of the IFN-γ-producing splenocytes within the PAP-immunized group (P < 0.01 compared to the control group). However, the second boost immunization did not yield further enhancement of the immune response (Supplementary Figure S2), indicating that two immunizations were sufficient to induce a plateau of the IFN-γ\textsuperscript{+} CTLs.

Subsequently, we investigated whether EP of the PAP-J vaccine can control cancer outgrowth in the natural tumor environment in TRAMP mice. To date, intraepithelial hyperplasia has been shown to prevail in male TRAMP mice.\textsuperscript{11} We immunized 10-week-old TRAMP mice and at week 22, when nearly all of the prostatic glands of the control mice were hyperplastic,\textsuperscript{13} the mice were sacrificed, and the tumor sizes were assessed by magnetic resonance imaging (Figure 7b). The tissues recognized as tumors were verified in part by histology (Figure 7c). A highly significant regression in tumor size could be observed in the PAP-J-treated animals but not in the control group (PAP-J: 21 ± 3 μl versus pPOE: 1,555 ± 232 μl (P < 0.0001)) (Figure 7a). This observation supports the idea that the PAP-J gene delivered by EP was able to control prostatic tumor growth in the TRAMP model.

DISCUSSION

In this study, we describe the generation of a potent therapeutic DNA vaccine against PCa that was able to break the self-tolerance to PAP. More importantly, this artificial gene elicited strong antitumor activity in the C1-transfer and transgenic TRAMP models of PCa.

The treatment of PCa by surgery or radiotherapy is limited to organ-defined tumors. In fact, ~30% of patients with PCa will have progressive or metastatic disease within 10 years after their first assessment.\textsuperscript{22} Additionally, a majority of these relapses become resistant to hormone ablation therapies due to the acquisition of androgen-independent growth properties. These adverse circumstances were responsible for estimated 28,660 deaths in the United States in 2008 and has rendered PCa the second leading cause of death among men in the United States.\textsuperscript{1} Therefore, there is an urgent need for the development of adjuvant treatment options.

Active immunotherapy is a promising possibility for an adjuvant therapeutic treatment against PCa because no conventional treatments are available when the disease becomes recurrent.\textsuperscript{23} One approach for the adjuvant treatment of PCa is the use of DNA-based vaccinations, which were successfully used in preclinical\textsuperscript{24} as well as clinical settings or are already licensed in the fight against other cancers (for an overview, see refs. 24,25). The challenge of inducing a strong cancer-directed immune response arises from the fact that only self-antigens are usually available as targets for immune therapy. In this study, we decided on PAP as our antigen given its restriction to prostate tissues.\textsuperscript{1} In the past, PAP was used as an antigen in therapeutic settings by Johnson et al.\textsuperscript{26} The authors found antigen-specific CTLs in rats after immunization with human recombinant PAP that was crossreactive to rat PAP. Unfortunately, six injections of the rat PAP gene were necessary to break self-tolerance against PAP.

Here, we demonstrated that self-tolerance can be broken with a simple EP-applied therapeutic DNA vaccine that possesses several characteristics enforcing the induction of a strong cellular immune response. All three PAP constructs used in this study were arranged with the Kozak sequence at the 5’-end, for which an enhancement of protein translation was shown.\textsuperscript{27,28} To facilitate
the nuclear entry of the plasmid vector, we took advantage of a nuclear targeting sequence. The SV40 enhancer contains binding sites for different ubiquitously expressed transcription factors (e.g., AP1, AP2, AP3, NF-κB) \(^{29}\) that contain nuclear targeting sequences. It was shown that the DNA-protein complex, consisting of the SV40-DNA and the bound transcription factor, facilitates an increased nuclear import.\(^ {30,31}\) Because the nuclear envelope remains intact in nondividing cells (e.g., muscle cells), it was reported that only 1–10% of the plasmid DNA reaches the nucleus.\(^ {32,33}\) If large numbers of plasmids lacking a nuclear localization signal are delivered to tissues, only some of the plasmids enter the nucleus resulting in low expression.\(^ {34}\) The presence of a nuclear localization signal in the SV40 enhancer greatly increases the gene transfer and expression up to 200-fold.\(^ {35}\)

Third, we fused the large T antigen-derived hsp73-binding DnaJ-like domain in front of the \(\text{PAP}^{a}\) gene in two gene versions in order to achieve a more effective MHC-I crosspresentation and crosspriming of the CTLs. Therefore, the hsp73-bound endogenous antigen was submitted to processing for MHC-I presentation, which facilitated crosspriming.\(^ {36}\) But it is well known that a major hurdle for successful DNA-based immune therapy in human-primates and humans is the delivery method by which the DNA is transported into muscle or peripheral APCs. Plasmid DNA must cross the plasma membrane either by endocytosis and subsequent endosomal escape or via direct penetration. It is believed that passive diffusion of cytoplasmic DNA is extremely inefficient.\(^ {40}\) The delivery of DNA via EP can circumvent this drawback and increase the gene expression up to 100-fold.\(^ {24}\) Indeed, we were able to show that the immune response was strongly enhanced after EP vaccination.

Interestingly, the gene version of PAP-J that lacks the signal peptide but contains the DnaJ-like sequence of the SV40 large T antigen induced the strongest immune response. By deleting the signal peptide in the PAP-J construct and therefore blocking the transport to the endoplasmic reticulum, more PAP molecules should be available within the cytoplasm, leading to a TAP-dependent processing of PAP and efficient recognition by the CTLs.\(^ {36}\) Schirmbeck and colleagues showed that the DnaJ-like domain of the SV40 large T antigen enhanced the CTL response of

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**Figure 7** Vaccination with the PAP-J gene inhibited tumor growth in male Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice. Ten-week-old male TRAMP mice were vaccinated with the PAP-J gene (or control vector pPOE) for 3 weeks at weekly intervals (100 μg/mouse, \(n = 50/\text{group}\)) by electroporation (EP). At week 22, all mice were sacrificed, and the tumor growth was assessed by magnetic resonance imaging. (a) The white bar shows the mean ± SEM of the tumor volume (in μl) of the control mice. The black bar shows the mean ± SEM of the tumor volume (in μl) in the mice immunized with the PAP-J gene. (b) The images provide one representative image out of a set of images from the control or PAP-J immunized mice. The white line localizes the tumor in a given section. (c) Tissues assigned as prostate cancer (PCa) were verified in part by histology. The healthy prostate of an untreated C57BL/6 mouse is shown. The small tumors of TRAMP mice treated with the PAP-J gene show clearly defined nodules, in contrast to the tumors of TRAMP mice treated with the empty vector, which developed large tumor cell clusters. All scale bars correspond to 100 μm. PAP, prostatic acid phosphatase.
DNA vaccines only when the binding of the cytosolic heat shock protein hsp73/hsc70 is intact. As such, it is likely that the deletion of the ER leader of PAP in the PAP-J construct enhanced the efficiency of DNA vaccination by promoting the interaction of the PAP-large T fusion protein with hsp73 in the cytoplasm. Hsp73 interacts with carboxy terminus of hsp73 interacting protein and is highly expressed in skeletal muscle cells. Carboxy terminus of hsp73 interacting protein provides E3 ubiquitin ligase activity resulting in substrate ubiquitylation and the consequent degradation of the J-domain-hsp73-carboxy terminus of hsp73 interacting protein complex by the proteasome (for an overview, see ref. 42), which could in part explain the superiority of the PAP-J construct by the optimized MHC loading. This would also be supported by our finding that the PAP-S construct gives a eight- to tenfold stronger signal in the immunoblot compared to the other constructs (PAP-JS and PAP-J), indicating the accumulation of the recombinant protein by a decreased degradation rate.

After the promising results concerning the lysis of TRAMP-C1 cells in the chromium release assays, we challenged the TRAMP-C1 tumor model. In the past, TRAMP-derived tumor cells were used by different groups investigating therapeutic immunization approaches. In our study, PAP-J vaccinated mice displayed a significantly reduced tumor growth compared to control mice. Unfortunately, TRAMP-derived C1/C2 tumor cells do not reflect a realistic PCA situation. In fact, no physiological tumor stroma is provided, which is very likely relevant for the tumor-specific response. Due to these limitations, we decided to use the TRAMP model for subsequent investigations. Garcia-Hernandez and co-workers found a dramatically enhanced survival rate in a therapeutic setting after immunization with the prostate stem cell antigen. Degl’Innocenti and colleagues were able to induce a CTL response against a “self-antigen” in TRAMP animals after immunization with SV40 large T antigen peptide-pulsed DCs, resulting in reduced disease progression. Taking this realistic TRAMP model into account, we vaccinated TRAMP mice with the PAP-J gene to investigate the effects of EP/vaccination on the immune response in this important “physiological” model of PCA. We analyzed the effects of our immunization on the immune response in this important “physiological” model of PCA. We analyzed the effects of our immunization on the immune response in this important “physiological” model of PCA.

Our findings may influence the further development of DNA-based vaccines and suggests that DNA vaccines encoding PAP could be studied in human clinical trials as a potential adjuvant treatment option in PCA patients. Ideally, patients at early stages of PCA or even those displaying rising PSA levels and corresponding histology may be candidates for multiple immunizations. The therapeutic PAP vaccine that provides an important HLA-A2 epitope was submitted for a patent (P. Öhlschlager and M. Groettrup, patent number: 0921088.1), and a transfer to a phase I clinical trial is under preparation.

MATERIALS AND METHODS

Generation of the PAP DNA vaccine. Three different versions of the PAP DNA vaccine were generated and cloned via S-HindIII and S-Xhol into the pPOE-CpG immunization vector that provided a CpG cassette highly optimized for both murine and human systems. Additionally, a CpG-lacking vector, pTHkan, was used. The PAP genes were assembled from synthetic oligonucleotides based on the murine wild-type PAP sequence (NT_039477.7) and codon-optimized for the human system by GENEART (Regensburg, Germany). The Kozak sequence and the J-domain were attached on the 5′-end. The SV40 enhancer sequence was attached on the 3′-end of the PAP gene. For the expression analysis, an HA-tag was attached to the 3′-end. Finally, the expression was verified by RT-PCR and western blot analysis after transfection of the NIH3T3 cells with three PAP genes (Figure 1b, c). The sequences of the three PAP genes are as follows:

- PAP-JS: (5′-Kozak_7, J-domain_1000, signal peptide_1000–1351, HA-tag_1352–1560) PAP gene SV40 enhancer_1561–1587, HA-tag_1588–1653
- PAP-J: (5′-Kozak_7, J-domain_1000, PAP gene_1384–1489, SV40 enhancer_1490–1560, HA-tag_1561–1587)

RT-PCR analysis. RNA from the transfected NIH3T3 cells (Effector Transfection Reagent; Qiagen, Hilden, Germany) was isolated by Nucleospin total RNA isolation (Macherey-Nagel, Duren, Germany) according to the manufacturer’s protocol. For RT-PCR, a One-Step RT-PCR kit (Qiagen) was used according to the user manual. Primers for RT-PCR were as follows: 5′-GAA CTG AGG TTC GTG ACC CTG -3′ (mPAP RT forward) and 5′-GAC CCG ATG TAG ATC TGG TCG -3′ (mPAP RT reverse). DNA was amplified for 30 minutes at 50°C (1 cycle), 15 minutes at 95°C (1 cycle), [30 seconds at 94°C, 30 minutes at 60°C, 30 seconds at 72°C (30 cycles)] and 5 minutes at 72°C (1 cycle).

Western blot analysis. Protein expression of the cloned PAP genes were detected by western blot. NIH3T3 cells (2.5 × 105) were lysed 24 hours after transfection (Effecntene Transfection Reagent; Qiagen) by boiling for 10 minutes in a SDS sample buffer and direct separation by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Millipore, Eschborn, Germany) by a semidry Fastblot system (Biometra, Goettingen, Germany) and detected via the aforementioned HA-tag. To detect the PAP proteins, the monoclonal mouse anti-HA antibody (1:10,000, 1 mg/ml, H3663, clone HA-7) (Sigma-Aldrich, St Louis, MO) was used. For α-tubulin detection, we used a monoclonal anti-tubulin antibody (1:1,000, dilution 1:5–1:1) (Sigma-Aldrich). The corresponding secondary antibody, polyclonal goat anti-mouse immunoglobulin/horseradish peroxidase (1:1,000, P0447) (DakoCytonation, Glostrup, Denmark), was also used.

Peptide-binding assay. Murine PAP epitopes were chosen by computer-based predictions [BIMAS (www.bimas.cit.nih.gov/molbio/hla_bind) and SYFPEITHI (www.syfpeithi.de)]. After the screening for eight potential K- and D-binding peptides, the two most promising peptides that bound to the murine MHC-I molecule H-2Dβ were used for in vitro restimulation. The binding affinity of the peptides was verified using a RMA-S binding assay. Briefly, RMA-S cells were cultured for 24 hours at room temperature (RT) and were added to serial dilutions of the peptide. After 4 hours of incubation at 37°C, the cells were washed two times with phosphate-buffered saline (PBS) and stained with the fluorescein isothiocyanate anti-mouse H-2Dβ antibody (clone AF6-88.5; BD Pharamingen, San Diego, CA) or with the fluorescein isothiocyanate anti-mouse H-2Kb.
antibody (clone K9H5; BD Pharmingen). Fluorescence was determined using a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA). The peptides were synthesized by Eurogentec S.A. (Seraing, Belgium).

Cell lines and culture conditions. All of the cell lines used were of C57BL/6 origin (H2^b^ context). RMA-S cells and DC2.4 (kindly provided by K. Rock, University of Massachusetts Medical School Worcester, Worcester, MA) were cultured in RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; Gibco, Eggenstein, Germany), penicillin (100 U/ml) and streptomycin (100 μg/ml). The TRAMP-C1 cell line was cultured as mentioned elsewhere.21 The splenocytes were cultured as previously mentioned.21

The NIH3T3 cells were cultured in Dulbecco’s modified eagle medium with 10% CS, 2 mmol/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cultures were grown at 37 °C and 7.5% CO$_2$ in a humidified incubator.

Mice. Male C57BL/6 mice (own breed) and the SV40 transgene positive male TRAMP mice (own breed) were kept under SPF isolation conditions and fed a standard diet at the animal facilities of the University of Constance, Constance, Germany. All animal experiments were performed with approval by and in accordance with regulatory guidelines and standards set by the institutional review board at Regierungspraesidium, Freiburg, Germany.

DNA vaccination. Agarose-gel verified plasmids (>95% supercoiled) (Qiagen EndoFree Plasmid Kit; preparations containing fewer than 0.1 endotoxin units/μg plasmid DNA, as tested earlier by Limulus endotoxin assay) were administered to 6–8-week-old male C57BL/6 mice into each muscle tibialis anterior [50 μg of plasmid DNA per muscle (100 μg in total), 1 μg/μl in PBS].

DNA vaccination/EP. In the DNA vaccination/EP experiments, the electrode array of the EP unit (Ichor Medical Systems, San Diego, CA) was directed into the muscle tibialis anterior of 6–8-week-old C57BL/6 or 10-week-old TRAMP mice. The DNA vaccination/EP procedure was conducted as described earlier.27

In vitro restimulation of murine CTL lines. The in vitro restimulation was performed as mentioned in ref. 20, but instead of the RMA cells, RMA-S cells and DC2.4 cells were used. For the EP experiments, irradiated splenocytes (100 Gy) of naive C57BL/6 mice, instead of RMA-S and DC2.4 cells, were pulsed with the designated peptides. Finally, 2 × 10⁶ cells were added in each 25 cm² cell culture flask for restimulation.

IFN-γ/granzyme B Elispot assays. Murine IFN-γ Elispot assays were performed ex vivo and 6–7 days after each in vitro restimulation. Spots were quantified with an Elispot reader (Cellular Technology, Shaker Heights, OH). The spots of the negative-control wells were subtracted from the spots of the test samples (mPAP114–122 or mPAP128–136 peptide stimulated). An animal was scored as positive when the number of IFN-γ-secreting cells was at least 100% above the highest number of IFN-γ secreting cells of the control animal. A 96-well plate (MultiScreen; Millipore) was coated overnight with purified monoclonal anti-mouse IFN-γ antibody (100 ng/well, clone R4-6A2) (BD Pharmingen) in PBS and blocked with RPMI containing 10% FCS. Splenocytes (2 × 10⁴) were seeded onto MultiScreen plates. After 20 hours, the cells were washed off with PBS/Tween 20 (0.05%). The staining reaction was developed with fluorescein-conjugated mouse anti-CD8 (clone 53-6.7; BD Pharmingen). Following fixation with 4% paraformaldehyde at 4 °C for 5 minutes, the cells were washed overnight with fluorescein-conjugated mouse anti-IFN-γ (clone XMG1.2; BD Pharmingen) in PBS containing 2% FCS and 0.1% (wt/vol) saponin (Sigma-Aldrich). The samples were washed twice and analyzed with a FACSscan flow cytometer (BD Biosciences, Heidelberg, Germany).

Pentamer staining of mPAP128–136-specific splenocytes. To detect CD8⁺ lymphocytes that were able to recognize the mPAP128–136 epitope, H2-D^b-specific mPAP128–136 pentamers (ProImmune, Oxford, UK) were ordered, and 6–8-week-old male C57BL/6 mice were immunized with either PAP-J or the empty vector as described above. Seven days after the final immunization, the mice were sacrificed, and the spleens were excised. After the homogenization and depletion of the erythrocytes, the splenocytes were washed twice with PBS (supplemented with 2% FCS). Pentamer staining was performed according to the manufacturer’s protocol (www.proimmune.com). Briefly, 10 μl of pentamer solution was added to 50 μl of splenocytes/PBS solution and incubated for 10 minutes at RT. The cells were washed twice with PBS and stained with an APC-labeled rat anti-mouse CD8α antibody (eBioscience, San Diego, CA, clone 53-6.7, 1:150 dilution) for 30 minutes at 4 °C. After washing twice with PBS, mPAP128–136-specific splenocytes were detected using FACSAria IIu.

51Cr-release assay. The 51Cr-release assays were performed 6–7 days after the in vitro restimulation of the murine spleen cells in parallel with the Elispot/intracellular staining assays, as described elsewhere.28

DELFIA EuTDA cytotoxicity assay. The cytotoxicity assay was performed according to the manufacturer’s protocol (CatNo. AD0116; PerkinElmer, Boston, MA). Briefly, 6–8-week-old male C57BL/6 mice were electroporated as described above. The mice were sacrificed, and the spleens were homogenized. After the depletion of the erythrocytes, 1 × 10⁶ splenocytes were incubated with 1 × 10⁶ peptide-pulsed RMA-S target cells for 2 hours at 37 °C (in triplicates). The RMA-S cells were pulsed with mPAP128–136 as described above and stained with the BATDA labeling reagent according to the manufacturer’s protocol. After adding the Europium solution, signals were determined with a Tecan Infinite 200 Pro using the following conditions: an excitation wavelength of 340 nm and bandwidth of 9 nm, an emission wavelength of 650 nm and a band length of 20 nm, gain 255, 100 flashes, an integration time 400 ms, a lag time 400 ms and a settle time 200 ms.

Serum ELISA. Ninety-six well ELISA plates were coated with 200 μl of purified human PAP protein (10 μg/ml) and incubated overnight at 4 °C (provided by A. Aichem, BITg, Switzerland). The plates were washed three times with PBS-Tween-20 (0.05%) and blocked with PBS-Tween-20 (containing 3% bovine serum albumin) for 1 hour at RT. After washing the plates twice with PBS-Tween-20, serial dilutions of 1:100–1:102,400 of sera from the immunized mice were prepared and 200 μl of sera was pipetted into each well. The sera were incubated for 3 hours at RT. The plates were washed six times with PBS-Tween-20, and a polyclonal goat anti-mouse antibody [DakoCytomation, 1:2,000 in PBS-Tween-20 (0.05%)-bovine serum albumin (1%)] was added to the cells and incubated at RT for 2 hours. After washing the plates three times, the signal was detected by adding 100 μl of TMB substrate (BD OptEIA; BD Biosciences). The reaction was stopped with 50 μl of 1 mol/l H₂SO₄, and the absorbance was read at 450 nm with a TECAN SPECTRAFluor Plus plate reader.
Tumor regression studies. In the C57BL/6 mouse tumor regression experiment, TRAMP-C1 cells were injected subcutaneously into the flanks of 6–8-week-old mice (1 × 10^6 cells in 100 μl PBS) with a 20G 1½” needle (BD Microlance 3; Becton Dickinson, Mountain View, CA). Eight days after the application of the tumor cells, mice were electroporated four times on every 7th day with 50 μg of plasmid DNA (PAP-J or empty vector) per musculus tibialis anterior (100μg/mouse). As soon as the small tumors were palpable (tumor size of 1–2 mm in diameter), their growth was assessed two times a week. Mice were sacrificed when the tumors in the control group reached a size of 15 mm in diameter (measured by a caliper). The tumor sizes of the mice within a group were calculated as the arithmetic mean of the tumor area with standard error of the mean values (SEM). The application of tumor cells was performed under isoflurane anesthesia (CuraMed, Karlruhe, Germany).

In the TRAMP mouse tumor regression experiment, the animals were sacrificed at 22 weeks when nearly all of the prostatic glands of the control mice were hyperplastic. Tumor regression studies.

Magnetic resonance imaging of TRAMP mice. Fifty animals of each group were imaged ex vivo using a 9.4 T small bore animal scanner (BioSpec 94/20; Bruker Biospin, Ettlingen, Germany) equipped with a cylindrical quadrature birdcage resonator with an inner diameter of 38 mm, specifically designed for whole body mouse imaging (Figure 7b). The magnetic resonance imaging protocol consisted of a localizer and a T2-weighted echo-planar imaging (EPI) sequence with a FOV of 15 mm x 15 mm x 15 mm and a TR/TE/eff/FA: 3,000 ms/36 ms/180°, echo train length 8) featured a FOV of 15 cm x 15 cm x 1 mm.

Formed to delineate the prostatic tumors from the surrounding abdominal fat. Resonance imaging at the Department of Radiology/Medical Physics at the University of Constance and Biotechnology Institute Thurgau, Switzerland) for their cooperation in preparing the histological analyses and for helpful discussions. We thank Gerald Mende (Animal Facilities of the University of Constance) for helpful discussions.

Histological analysis. Prostate tissues from all of the study groups were fixed and H&E stained (using standard procedures). The tissues assigned to tumors were verified in part by histology at the Institute of Veterinary Pathology, Ludwig-Maximilians-University, Munich, Germany.

Statistical analysis. Differences of the mean values between the experimental and control group were considered statistically significant when P was < 0.05 by an unpaired Student’s t-test.

SUPPLEMENTARY MATERIAL

Figure S1. Peptide-binding assay.

Figure S2. A second boost immunization with the PAP-J gene does not lead to a further immune response as measured by ICS.

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