Published in final edited form as:
J Immunother. 2012 June ; 35(5): 390–399. doi:10.1097/CJI.0b013e3182585d50.

A cytomegalovirus-based vaccine expressing a single tumor-specific CD8+ T cell epitope delays tumor growth in a murine model of prostate cancer

Elena N. Klyushnenkova[1,2], **Diana V. Kouiavskaia**[1], **Christopher J. Parkins**[3], **Patrizia Caposio**[3], **Sara Botto**[3], **Richard B. Alexander**[1,2], and **Michael A. Jarvis**[3,*]

[1]Department of Surgery, Division of Urology, University of Maryland, Baltimore, Maryland [2]VA Maryland Health Care System, Baltimore, Maryland [3]Vaccine and Gene Therapy Institute, Oregon Health & Science University, Beaverton, Oregon

Abstract

Cytomegalovirus (CMV) is a highly immunogenic virus that results in a persistent, life-long infection in the host typically with no ill-effects. Certain unique features of CMV, including its capacity to actively replicate in the presence of strong host CMV-specific immunity, may give CMV an advantage compared to other virus-based vaccine delivery platforms. In the present study, we tested the utility of mouse CMV (mCMV)-based vaccines expressing human prostate-specific antigen (PSA) for prostate cancer immunotherapy in double-transgenic mice expressing PSA and HLA-DRB1*1501 (*DR2b*×*PSA F1*). We assessed the capacity of two mCMV-based vectors to induce PSA-specific CD8 T cell responses and affect the growth of PSA-expressing Transgenic Adenocarcinoma of the Mouse Prostate tumors (TRAMP-PSA). In the absence of tumor challenge, immunization with mCMV vectors expressing either a H2-D^b-restricted epitope PSA_{65-73} (mCMV/PSA_{65-73}) or the full length gene for PSA (mCMV/PSA_{FL}) induced comparable levels of CD8 T cell responses that increased (inflated) with time. Upon challenge with TRAMP-PSA tumor cells, animals immunized with mCMV/PSA_{65-73} had delay of tumor growth and increased PSA-specific CD8 T cell responses, whereas animals immunized with mCMV/PSA_{FL} showed progressive tumor growth and no increase in number of splenic PSA_{65-73}-specific T cells. The data show that a prototype CMV-based prostate cancer vaccine can induce an effective anti-tumor immune response in a “humanized” double-transgenic mouse model. The observation that mCMV/PSA_{FL} is not effective against TRAMP-PSA is consistent with our previous findings that HLA-DRB1*1501-restricted immune responses to PSA are associated with suppression of effective CD8 T cell responses to TRAMP-PSA tumors.

Corresponding authors: Elena N. Klyushnenkova, University of Maryland, School of Medicine, Department of Surgery, 10 S. Pine Street, MSTF 400A, Baltimore, MD 21201. Phone: 410-706-0755; FAX: 410-706-0311; eklyushnenkova@smail.umaryland.edu. Michael A. Jarvis, School of Biomedical & Biological Sciences, Plymouth University, Plymouth, Devon, UK. Phone: +44 (0)1752 588225; FAX: +44 (0)1752 588235; michael.jarvis@plymouth.ac.uk.
*Present address: School of Biomedical & Biological Sciences, Plymouth University, Plymouth, Devon, UK

Financial Disclosure: All authors have declared there are no financial conflicts of interest in regards to this work.

Keywords

cytomegalovirus; prostate-specific antigen; HLA-DR2 transgenic mice; cancer vaccine; prostate cancer

Introduction

Prostate cancer is generally more indolent and slower to progress than most other cancers, but still kills over 30,000 Americans every year. Effective treatments for localized disease exist. However, disease recurs in approximately 30% of patients, and eventually becomes refractory to standard androgen deprivation therapy (becoming castration-resistant prostate cancer; CRPC). Therapeutic interventions for CRPC patients are extremely limited, and these patients have a high likelihood of disease progression and death from prostate cancer (6). Following disease recurrence, many patients have an extended period of time during which they are asymptomatic and immunologically normal. We and others have proposed that these patients may therefore be ideal candidates for therapeutic vaccination to slow or prevent progression of disease. A cell-based personalized therapy using a monocyte-enriched leukopheresis product (sipuleucel-T, PROVENGE®) was approved in April 2010 by the US Food and Drug Administration (FDA) for treatment of asymptomatic or minimally symptomatic CRPC, becoming the first adoptive cellular immunotherapy approved for cancer treatment (see review (3)). A phase III clinical trial demonstrated that sipuleucel-T increased median patient survival by 4.1 months compared to placebo controls (41). However, there was no evidence of an anti-tumor effect, and the mechanisms responsible for increased survival following sipuleucel-T treatment remain unclear (for review, (16;43)).

We hypothesize that immunotherapy using a human cytomegalovirus (hCMV)-based cancer vaccine may enable effective immunological targeting of prostate cancer. CMV is a ubiquitous, but generally benign, species-specific β-herpesvirus that infects the host for life. Although CMV infection does not typically lead to clinical disease in the absence of immunosuppression (such as during transplantation) (30), continual antigenic stimulation provided by chronic CMV replication in the immune-normal host makes CMV one of the most immunogenic viruses known (4;14;15;19;34;38;42;50). In addition to the magnitude of CMV responses, CMV induces T cell memory biased toward distribution to epithelial mucosal sites (11), which may also be particularly relevant to the therapeutic control of cancer given their frequent epithelioid derivation. CMV-based vectors have yet to be assessed in clinical trials. However, a number of laboratories have shown the potential utility of CMV as a vaccine vector in studies using species-specific CMVs (8;10;35;45). In one study, a mouse CMV (mCMV)-based vector was able to break tolerance and induce an immune response against a self-antigen zona pellucida 3 that resulted in immunological sterilization of vaccinated mice (35). In other studies, rhesus CMV-based vectors expressing simian immunodeficiency virus (SIV) antigens protected rhesus macaques from systemic infection following pathogenic SIV challenge (11). Finally, a mCMV vector expressing a single epitope of Ebola virus (*Zaire*) (ZEBOV) nucleoprotein was recently shown to protect against high-dose lethal ZEBOV challenge (45).

Prostate-specific antigen (PSA) is a commonly used target tumor antigen for experimental prostate cancer vaccines due to its restricted prostate tissue distribution and expression by most prostate tumor cells. Many PSA-based vaccination strategies have been explored, including RNA-transfected dendritic cells (9), peptides (5;20), plasmid DNA (32), attenuated strains of *Listeria monocytogenes* (39), and recombinant replication-defective and acute, non-persistent virus vectors (21;23). Currently, two non-persistent viral vector

platforms targeting PSA are being tested in clinical trials: a replication-defective adenovirus (AdV)-based vaccine (21), and a `prime-boost' combination vaccine composed of recombinant vaccinia (rVV) and fowlpox (rFV) viruses (23). Immunity directed against the vector remains a significant limitation of vaccination for most viral vector strategies (37). In contrast, prior immunity to CMV does not prevent CMV re-infection (7). Presumably due to its persistent, low-level replication, CMV immunity is also associated with an `effector memory' T cell ($T_{EM}$)-biased phenotype, whereas the transient antigen expression associated with non-persistent vectors induces a more `central memory' T cell (T_{CM})-biased response ((8;29;40)). Together, we suggest that the ability to re-infect the CMV-seropositive host, combined with the high levels of T_{EM}-biased immune responses, may make CMV-based vectors uniquely suited for development as cancer vaccines.

The aim of the present study was to determine whether vaccination with CMV-based vectors expressing PSA could induce an effective anti-tumor immune response in a mouse model of prostate cancer. Due to the host-specific nature of CMV infection (12;13), mouse CMV (mCMV)-based vectors were used for these studies. The experiments were conducted in a new and stringent tumor challenge model based on Transgenic Adenocarcinoma of the Mouse Prostate tumor cells engineered to express human PSA (TRAMP-PSA) (26), and double transgenic (tg) mice (*DR2b×PSA F1* mice) that co-express human PSA as a self-antigen in the prostate and the human leukocyte antigen (HLA) class II allele, *HLA-DRB1*1501(DR2b*). We have previously shown that *HLA-DRB1*1501* allele supports (is “permissive” for) CD4 T cell mediated responses to PSA (17;18). We tested two different constructs of mCMV expressing PSA to establish a `proof-of-concept' for the use of CMV-based vaccines to target cancer. Our results show that immunization with mCMV-based vectors encoding either a single PSA-specific CD8 T cell epitope (PSA_{65-73}), or full-length PSA induced PSA-specific CD8 T cell responses, which increased in time after immunization (termed `memory inflation'). While the levels of PSA-specific CD8 T cell responses induced by both vectors were comparable in the absence of tumor challenge, the anti-tumor effect was observed only with the PSA_{65-73} epitope-based but not the full-length PSA-based vector. These effective anti-tumor responses in mice immunized with mCMV encoding CD8 epitope PSA_{65-73} were accompanied by increased levels of PSA-specific CD8 T cells. In contrast, mice immunized with a full-length PSA-based vector failed to mount CD8 T cell response to PSA upon tumor challenge. Our data show that CMV-based vectors can induce an effective anti-tumor response in a stringent mouse model of prostate cancer. These results also suggest that tumor antigen design and epitope composition may be critical for cancer vaccine efficacy, particularly for “self” antigens recognized in a context of “permissive” HLA class II alleles, at least in the *DR2b×PSA F1* mouse prostate challenge model.

METHODS

Mice

All animal studies have been approved by the University of Maryland Institutional Animal Care and Use Committee. *DR2b tg* mice bearing chimeric major histocompatibility complex (MHC) class II molecules (DRa1*0101:I-Eα and DRb1*1501:I-Eβ) were provided by Dr. L. Fugger (Aarhus University, Denmark). These mice were engineered on a murine MHC class II knockout C57BL/6J (B6) background (24), and did not express mouse MHC class II molecules. Tumors of B6 origin were rejected by these mice, probably due to the MHC class II mismatch between the host and the tumor cell line (data not shown). To establish a tumor model based on TRAMP-C1 tumor cells, *DR2b tg* mice were crossed to wild type (wt) B6 mice (Jackson Laboratories, Bar Harbor, ME), and tumor experiments were carried out using F1 male offspring. The expression of *HLA-DR* transgene and TRAMP-PSA tumor growth in *DR2b×B6 F1* mice has been previously described (17). Founder *PSA tg* mice

were kindly provided by Dr. M. Kast (UCLA, Los Angeles, CA) (11). *PSA tg* mice on the B6 background were generated by 10 backcrosses to wt B6 mice, and maintained by inbreeding. PSA expression in the prostate of these mice was confirmed at the protein level by immunohistochemical staining with PSA-specific antibodies. Mice expressed PSA in ventral, dorsal and lateral prostate lobes, whereas the anterior prostate did not express detectable PSA (data not shown). *DR2b×PSA F1* double transgenic mice were generated by breeding *DR2b tg* and *PSA tg* (B6) mice.

Vector construction

The mCMV-based vectors expressing PSA antigens were constructed by lambda-based linear recombination (45) using a strategy identical to that which we have used for construction of other CMV recombinants (45). A mCMV (Smith strain) bacterial artificial chromosome (BAC) (27) in which the NK activating m157 MCMV gene has been deleted was used as the genetic background for these vectors. Deletion of m157 (Δm157) was necessary to avoid attenuation of CMV replication by inadvertent high NK control in mouse strains such as B6 that express the corresponding Ly49H NK receptor (1). Inactivation of m157 is common in wild strains of mCMV, and mCMV inactivated for m157 can be regarded as wild-type (48). We designed two distinct recombinant mCMVs expressing either full-length PSA (mCMV/PSA_{FL}) or only a single defined H2-D^b-restricted CTL epitope PSA_{65-73} fused to the carboxy terminus of a non-essential mCMV protein, ie2 (mCMV/PSA_{65-73}) (Figure 1A). For construction of mCMV/PSA_{FL}, the entire human PSA open reading frame (ORF), devoid of the signal peptide was placed under control of the constitutively active EF1α promoter (46). The PSA ORF was also epitope tagged at the carboxy terminus to facilitate analysis of protein expression. For mCMV encoding a CD8 T cell epitope, we used peptide PSA_{65-73} (HCIRNKSVI) that has been previously identified as a single immuno-dominant H2-D^b-restricted epitope in B6 mice (31). PSA_{65-73} was fused `in-frame' to the carboxyl terminus of the mCMV ie2 gene (following a GI linker). The ie2 gene encodes a nonessential mCMV protein that can be deleted or mutationally modified without affecting virus replication *in vivo* (10). For construction of mCMV recombinant BACs, a contiguous frt-flanked kanamycin resistance marker (Kan^R) was inserted into the mCMV BAC genome at the same time as the PSA gene region to enable selection of recombinant BACs on the basis of kanamycin resistance. Following selection of recombinant BACs on the basis of Kan^R, the frt-flanked Kan^R marker was removed by arabinose induction of Flp-recombinase and screening for kanamycin sensitivity. Recombinant viruses were reconstituted from BACs by transfection of BAC DNA into murine embryonic fibroblasts (MEFs). Presence of the BAC cassette within the MCMV genome decreases *in vivo* replication, and serial *in vitro* passage of the BAC-derived virus was performed to remove the BAC cassette (49). Recombinant viruses were assessed for growth *in vitro* on MEFs. Direct DNA sequencing was used to confirm integrity of the PSA genomic region within the mCMV genome. Western analysis using an epitope tag-specific antibody was used to visualize PSA expression for mCMV/PSA_{FL} (Figure 1B).

Immunizations and tumor challenge experiments

Male mice were immunized with either mCMV/PSA_{65-73} or mCMV/PSA_{FL} vectors at 10^4 plaque forming units (PFU)/mouse intraperitoneally (i. p.) and boosted with the same dose of virus four weeks later. The numbers of animals used in each study group were as detailed in Results. A TRAMP tumor cell line engineered to express human PSA (TRAMP-PSA) was provided by Dr. J. Medin (University of Toronto, Canada) (26). Parental TRAMP-C1 line was obtained from American Type Culture Collection (Manassas, VA). Tumor inoculation and monitoring was performed as described (17). *DR2b×PSA F1* male mice between 8 and 12 weeks of age were inoculated subcutaneously (s. c.) in the dorsal neck area with PSA-expressing TRAMP-C1 (TRAMP-PSA) tumor cells ($3×10^6$ cells per mouse

in 100 μl PBS). Tumor growth was monitored by physical measurement weekly for up to 18 weeks. Tumor base area was calculated by measuring two bisecting diameters of the tumor and multiplying these values. Since TRAMP tumors have a tendency to ulcerate and bleed at relatively early time points, survival experiments were not performed due to animal welfare concerns and IACUC regulations. Instead, a tumor base area of 100 mm^2 was used as a surrogate end point for survival.

Immunological assays

IFN-γ ELISPOT and intracellular cytokine staining (ICS) analysis of T cell responses, and ELISA analysis of antibody responses are described in detail in Supplemental Methods (see Supplemental Digital Content 1).

Peptides

The H2-D^b-restricted immunodominant peptide PSA_{65-73} (HCIRNKSVI) (31) and H2-K^b-restricted inflationary epitope M38 peptide $M38_{316-323}$ (SSPPMFRV) (29) have been described previously. An H2-D^b-restricted peptide encoded by a cryptic open reading frame that maps to the non-coding strand of the ampicillin resistance gene (NEO_{49-59}, SSPVNSLRNVV) (47) was used as an irrelevant control peptide. Peptides were synthesized by NeoMPS Inc. (San Diego, CA), and were of >95% purity.

Statistical analysis

For the ELISPOT assay, means and SD of triplicates were calculated for individual mice. The specific responses from these mice were compared to background responses in the absence of antigen using a two-sided Student t-test at $P<0.05$ level. The specific responses for each mouse were calculated by subtracting background levels, and differences between mouse treatment groups were analyzed using a Mann-Whitney U test at $P<0.05$ level. Survival, using a tumor base of 100 mm^2 as a surrogate marker, was calculated by polynomic regression analysis using Excel software (R-squared values > 0.95). Time-to-event analysis was performed by log rank test using MedCalc software (Version 11.6.1).

RESULTS

DR2b×PSA F1 tumor model

We have previously shown that wt B6 mice and *DR2b×B6 F1* mice lacking the *HLA-DR2b* transgene rejected TRAMP-PSA tumors, while *DR2b×B6 F1* littermates expressing *HLA-DR2b* failed to reject the tumors (17). To assess the level of immunological tolerance to PSA in *PSA tg* mice (B6 background), we analyzed the immunogenicity of TRAMP-PSA tumor cells in these mice compared to wt B6 mice. Following s. c. inoculation with TRAMP-PSA tumor cells, both *PSA tg* and wt B6 mice developed MHC class I-restricted PSA-specific CTL responses (based on IFNγ expression by ELISPOT) by two weeks post-inoculation. However, the magnitude of the response was significantly decreased in the *PSA tg* mice compared to wt B6 mice (Figure 2A). This finding suggests that the PSA model tumor antigen in the *PSA tg* mice can be regarded immunologically as a self-antigen with induction of neonatal tolerance. To determine whether this level of immunological tolerance to PSA resulted in increased tumor growth, we challenged the mice with TRAMP-PSA tumor cells. Surprisingly, despite the significantly lower levels of PSA-specific CD8+ T cells in *PSA tg* mice compared to wt B6 mice, TRAMP-PSA tumors were rejected at a comparably high and statistically indistinguishable rate in both strains, reflected as high mouse survival (tumor incidence 11.1% and 12.5% for wt B6 and *PSA tg* mice respectively, Figure 2B). Parental TRAMP-C1 tumors grew equally well in both strains (tumor incidence 89% and 75% for wt B6 and *PSA tg* mice respectively, Figure 2B). We previously reported

that presence of a “permissive” MHC class II allele (*HLA-DRB1*1501* (*DR2b*)) can change the pattern of anti-tumor immune response in a *DR2b×B6* F1 mouse model (17). To test the effect of PSA transgene expression on tumor growth in the `permissive' *DR2b* background, we generated *DR2b×PSA F1* mice and then challenged these mice with TRAMP-PSA tumor cells. As shown in Figure 2C, TRAMP-PSA tumors were established in *DR2b×PSA F1* mice (tumor incidence 95%). Moreover, tumor growth was significantly enhanced in *DR2b×PSA F1* mice compared to *DR2b×B6 F1* mice (median surrogate survival time 7.9 and 10 weeks respectively, p=0.01, log rank test). These data indicate that immunological tolerance to PSA enhances growth of PSA-expressing tumors, but only within a `permissive' MHC class II background.

CD8 T cell responses induced by mCMV/PSA$_{FL}$ and mCMV/PSA$_{65-73}$ constructs in the absence of tumor challenge

PSA-expressing mCMV vectors were constructed as detailed in Figure 1. Immunogenicity of mCMV/PSA$_{FL}$ and mCMV/PSA$_{65-73}$ was first assessed in the absence of TRAMP-PSA tumor challenge in *PSA tg* mice. As shown in Figure 3A, mCMV/PSA$_{FL}$ and mCMV/PSA$_{65-73}$ induced comparable levels of CD8-restricted T cell responses against PSA. PSA-specific responses were consistent with an `inflationary' T cell response (29). Both vectors induced an “acute” phase of the response at week 1 with a contraction phase between weeks 2–16, followed by an `inflationary' memory phase until 32–64 weeks (last time points measured). Next, we compared PSA-specific responses induced by mCMV/PSA$_{65-73}$ in *DR2b×PSA F1* and *PSA tg* mice. In both mouse strains, sustained levels of PSA-specific CD8 T cell responses were induced with a trend toward an increase at 32–64 weeks consistent with an `inflating' response against the PSA epitope. Responses in *DR2b×PSA F1* mice trended toward higher levels than those induced in *PSA tg* mice, but these differences were not statistically significant (Figure 3B). The responses to the irrelevant peptide (Neo$_{49-59}$) were at background (no antigen) levels (Figure 3). The responses in individual mice are shown in Supplemental Figure 1 (see Supplemental Digital Content 2). Immunization with either vector induced IFN-γ responses to the specific peptide (PSA$_{65-73}$) peptide as well as TRAMP-PSA tumor cells, but not to the irrelevant peptide (Neo$_{49-59}$) or parental TRAMP-C1 tumor cells (Supplemental Figure 1). Naïve mice lacked any detectable PSA-specific reactivity (data not shown). Consistent with expression of the full length PSA protein by mCMV/PSA$_{FL}$ immunization with mCMV/PSA$_{FL}$, but not mCMV/PSA$_{65-73}$, induced PSA-specific antibody responses (data not shown). Mice in both groups developed comparable humoral immune responses to mCMV (data not shown).

Effect of mCMV/PSA$_{FL}$ and mCMV/PSA$_{65-73}$ vaccination on TRAMP-PSA tumor growth

Next, we determined the impact of vaccination using the two different mCMV-based vectors on TRAMP-PSA tumor growth. *DR2b×PSA F1* male mice were immunized with mCMV/PSA$_{65-73}$, mCMV/PSA$_{FL}$ or wt mCMV, and boosted with the identical vector four weeks later. Vaccinated mice were then inoculated with TRAMP-PSA tumor cells at either 4 weeks (Figure 4A–D) or 32 weeks (Figure 4E, F) post-boost. As shown in Figure 4, immunization with mCMV vector encoding a single CD8 T cell epitope PSA$_{65-73}$ significantly slowed the progression of TRAMP-PSA tumors. Tumor base area was significantly reduced in mice that received mCMV/PSA$_{65-73}$ vaccine compared to wt mCMV at all time points (two-sided t test, Figure 4A,C), which resulted in the significantly longer time to the 100 mm^2 end-point compared to wt mCMV. In the experiment depicted in Figure 4B, median surrogate survival time was 5.5 and 10.0 weeks for mCMV/PSA$_{65-73}$ and wt mCMV respectively ($p<0.01$, log rank test). These results were reproduced in the experiment depicted in Figure 4D [median surrogate survival time 5.3 and 10.1 weeks for mCMV/PSA$_{65-73}$ and wt mCMV respectively ($p<0.001$, log rank test)]. In contrast, mice vaccinated with mCMV/PSA$_{FL}$ vector showed a delayed tumor growth only at earlier time points while tumor progression at

later time points was comparable to mice that received wt mCMV. Compared to the wt mCMV group, tumor base area was significantly reduced in mice receiving the mCMV/PSA_{FL} vaccine during the first 6 weeks of observation. However the differences became non-significant at later time points (2-sided t test, Figure 4A). Median surrogate survival time was extended in mice vaccinated with mCMV/PSA_{FL} vector compared to wt mCMV (7.1 and 5.5 weeks respectively), however, the differences were not statistically significant (log rank test, Figure 4B). Median surrogate survival time was significantly longer in mice immunized with mCMV/$PSA_{65\text{-}73}$ vector compared to mCMV/PSA_{FL} vector (10.0 and 7.1 weeks respectively, $p<0.0001$, log rank test, Figure 4B). Protection mediated by mCMV/$PSA_{65\text{-}73}$ was long lasting, with mice challenged with TRAMP-PSA tumor cells at 32 weeks post-boost still demonstrating a significant delay in tumor growth (Figure 4E, F) ($p<0.01$ compared to wt mCMV, log rank test). However, there were no tumor-free survivors in either study group.

CD8 T cell responses induced by mCMV/PSA_{FL} and mCMV/$PSA_{65\text{-}73}$ constructs in the presence of tumor challenge

To determine whether the difference in efficacy against tumor challenge between the two mCMV-based vectors was consistent with a difference in T cell responses following tumor challenge, we measured $PSA_{65\text{-}73}$-specific CD8 T cell responses in mice vaccinated with mCMV/PSA_{FL} or mCMV/$PSA_{65\text{-}73}$ following TRAMP-PSA tumor inoculation. *DR2b×PSA F1* mice were vaccinated with either mCMV/PSA_{FL} or mCMV/$PSA_{65\text{-}73}$, followed by TRAMP-PSA tumor cells 4 weeks after the boost vaccination. Responses to $PSA_{65\text{-}73}$ peptide in the spleen were determined using ICS either two weeks prior to tumor injection, or two weeks after tumor injection. As shown in Figure 5A, TRAMP-PSA tumor challenge induced a significant increase in the frequencies of PSA-specific IFN-γ producing CD8 T cells in animals vaccinated with mCMV/$PSA_{65\text{-}73}$ ($p<0.01$, Mann-Whitney U test). In contrast, tumor inoculation did not result in a significant increase in PSA-specific CD8 T cell responses in animals that had been previously vaccinated with mCMV/PSA_{FL} (Figure 5A). PSA-specific T cell responses were observed only in response to TRAMP-PSA tumor cell challenge. In the absence of tumor challenge, the responses measured by ICS were at the limit of detection in both mCMV/PSA_{FL} or mCMV/$PSA_{65\text{-}73}$ vaccinated groups, and could be quantitated only by the more sensitive IFN-γ ELISPOT assay (Figure 3 and Supplemental Figure 1). Consistent with mCMV infection, all mice had CD8 T cell responses to the mCMV-derived epitope $M38_{316\text{-}323}$ (Figure 5B, frequencies of total IFN-γ + CD8 T cells are shown). CD8 T cell responses to TRAMP-PSA tumor cells in mice immunized with mCMV/$PSA_{65\text{-}73}$ were characterized predominantly by IFN-γ secretion, while TNF-α responses were marginal [median frequency of IFN-γ+ TNF-α+ CD8 T cells 0.4% (0.1% – 1.5%)]. This was in contrast to the underlying immune responses to $M38_{316\text{-}323}$ epitope, which induced strong polyfunctional (IFN-γ+ TNF-α+) CD8 T cell responses [median 2.3% (1.0% – 8.2%) of CD8+ gated events]. Typical responses for individual mice are shown in Supplemental Figure 2 (see Supplemental Digital Content 3).

Discussion

We tested the utility of a mCMV-based vaccine encoding human PSA for prostate cancer immunotherapy in double tg mice expressing *PSA* and *HLA-DRB1*1501(DR2b)*. We have previously shown that HLA-DR2b can present peptides from PSA and support strong PSA-specific CD4 T cell response (17). We further showed that mice expressing the *DR2b* tg demonstrated a significant increase in tumor growth when challenged with TRAMP-PSA, whereas tumors were rejected at high rate in litter-mates not expressing *DR2b* tg or in wt B6 mice (17). These latter two strains of mice express native mouse MHC class II ($I\text{-}A^b$) but this allele does not present PSA peptides, presumably because of lack of appropriate binding. In this study, we showed that the HLA-DR2b-associated differential tumor growth

was also seen in mice that express PSA tg as a self-antigen. *PSA tg* mice rejected TRAMP-PSA tumors at a rate similar to wt B6 mice despite much lower levels of CD8 T cell responses to PSA. Similar to the *DR2b×B6 F1* model, the growth of implanted TRAMP-PSA tumor cells in the *DR2b×PSA F1* mice occurred only in the presence of the *HLA-DR2b* transgene. The data are consistent with a model wherein the effective presentation of the tumor antigen (PSA) by the MHC class II complex (HLA-DR2b) generated a significant humoral immune response and a reciprocal suppression of the CD8 T cell response to PSA. This suppression of the PSA-specific CD8 T cell response to PSA was associated with progressive growth of TRAMP-PSA. Moreover, tumor growth was significantly accelerated in double tg *DR2b×PS*A *F1* mice compared to single tg *DR2b×B6 F1* mice, presumably due to the combined negative regulatory effects of *HLA-DR2b* and the decreased antigenicity of PSA expressed as a `self' antigen. Although this model is highly stringent in terms of permissivity to TRAMP-PSA tumor growth, the precise clinical relevance remains to be more completely defined, as the mechanism responsible for the tumor “permissivity” of the DR2b allele is currently unclear.

We used the *DR2b×PSA F1* model to evaluate the immunogenicity and anti-tumor effect of the CMV vector platform, a novel persistently replicating vaccine vector that has unique immunological characteristics suited to its development as a cancer vaccine. Two PSA-expressing 1 vectors, mCMV/PSA_{FL} and mCMV/PSA_{65-73}, were constructed, and their immunogenicity was initially tested in *PSA tg* and *DR2b×PSA F1* mice in the absence of tumor challenge. Both vectors induced low, but comparable levels of IFN-γ+ CD8 T cell responses against the H2-D^b-restricted epitope PSA_{65-73}, as well as against TRAMP-PSA tumor cells. The levels of CD8 T cell responses were lower at earlier time points (1–16 weeks), but showed a trend towards an increase (inflation) with time (32–64 weeks after boost immunization). Immunological memory inflation is one of the unique features of persistent viruses such as CMV, which may make this virus particularly suited for use as a vaccine delivery platform. Four distinct patterns of memory CD8 T cell responses to chronic mCMV infection have been described based on the pattern of expansion, contraction and memory inflation (29). In our experiments, the responses to PSA_{65-73} showed a pattern characterized by rapid expansion and contraction followed by a phase of memory inflation.

The low CD8 T cell responses to PSA in the absence of tumor challenge (detectable only by ELISPOT assay) precluded analysis of phenotype of the PSA-specific T cells induced by mCMV-based vaccines. However, `inflating' CMV-specific immune responses (for example, like those directed against the mCMV M38 protein) have been shown to be qualitatively associated with an `effector memory' T cell (T_{EM})-biased phenotype (8;29;40), characterized by T cells with immediate effector functions (i.e., expression of effector cytokines such as TNFα and type I interferons). These responses are also shifted in their localization toward effector sites such as spleen, liver and mucosal epithelial tissues (2;22;25;36). In contrast, transient antigen expression as occurs with rVV and AdV-expressed proteins, induces a more `central memory' T cell (T_{CM})-biased response. Although T_{CM} responses have greater proliferative potential, these cells are shifted away from epithelial tissue effector sites and are primarily targeted to the central lymphatic tissues. Since T_{EM} cells are biased towards localization to effector sites such as the spleen, liver and mucosal epithelial tissues (2;22;25;36), we believe the distribution of the T_{EM}-biased immune responses induced by CMV may be particularly relevant to the control of epithelioid cancers, such as prostate cancer. T_{EM} responses are also fully functional without a need for *de novo* antigen exposure to induce differentiation into an effector phenotype.

In our experiments, vaccination with a mCMV vector encoding a single H2-D^b-restricted epitope PSA_{65-73} significantly delayed TRAMP-PSA tumor growth. In contrast, animals vaccinated with mCMV encoding the full-length PSA protein had progressive tumor growth.

Animal receiving mCMV/PSA_{65-73} demonstrated a substantial level of PSA-specific CD8 T cells following tumor challenge, whereas animals receiving mCMV/PSA_{FL} did not generate an increased CTL response to PSA. Together, these observations are consistent with a model in which PSA-specific CD4 T cell activation by APC presenting PSA peptides induces a response that includes suppression of an effective CD8 T cell response that would otherwise reject the tumor. One hypothesis for the mechanism of this suppression is the induction of PSA-specific CD4+ CD25+ regulatory T cells (Tregs) in animals expressing *HLA-DR2b*. In this scenario immunogenic epitopes from whole PSA are presented by HLA-DR2b in the mice receiving the mCMV/PSA_{FL} vaccine. This stimulation of PSA-specific CD4 T cells generates Tregs, which inhibit the CD8 T cell response to the tumor antigen. This would explain why the effect is not observed in animals receiving the mCMV/PSA_{65-73} vaccine, as the HLA-DR2b-restricted PSA epitopes are not present in this construct. If this hypothesis is correct, then it implies that the activation or suppressive effect of Tregs in this model is antigen-specific. There is clear evidence that antigen specific Tregs can be induced in murine autoimmunity models including EAE (51) and type I diabetes (44). The potential for involvement of Tregs in this present model is under investigation. Another explanation could be that the PSA_{65-73} epitope is not processed and presented from the full length PSA vaccine, hence animals receiving the mCMV/PSA_{FL} cannot generated an effective CD8 T cell response to the CTL epitope. Evidence against this explanation is that animals without tumors develop a modest CD8 T cell response to the PSA_{65-73} after vaccination with mCMV/PSA_{FL}, which appears to be sufficient for the tumor control at the earlier time points, but is not potent enough to cause a significant delay of tumor growth at later time points.

Although we did not observe a more complete effect on tumor growth, the delay was statistically significant and reproducible. The TRAMP tumor challenge model is accepted as an aggressive challenge model, and a complete effect of vaccination on tumor growth has, to our knowledge, never been demonstrated with any vaccination scheme. The stringency of this model was increased further by placing PSA as a self-antigen into the tumor-permissive HLA-DR2b background. We believe that this model is currently the most stringent mouse prostate cancer model available, most likely with much higher predictive value for the human disease compared to other PSA-based models. In our tumor model, animals were vaccinated prior to TRAMP-PSA tumor challenge. Clearly, this is not the clinical scenario in patients with prostate cancer where the disease is already well-established prior to any attempt at vaccination. The current data are presented as a `proof of concept' that recombinant CMV vaccination is able to provide an effective immunological response to a tumor antigen in a “humanized” mouse tumor model.

The capacity for replication competent CMV to cause disease in immunosuppressed individuals will be a major hurdle to commercialization of CMV-based cancer vaccines. In addition, a replication-competent vector has the ability to spread from the initial vaccine recipient to other members of the population in an uncontrollable fashion. Both of these concerns, although not insurmountable, would be expected to slow translation of a promising CMV-based vector into the clinic. Recent studies indicate that replication-defective versions of CMV maintain their unique immunogenicity in all quantitative and qualitative aspects (28;33). These findings are promising for the use of CMV vectors in potentially immunosuppressed populations such as cancer patients. However, many individuals are already naturally infected with CMV. Hence, we would expect no more disease potential from a therapeutic CMV than would be observed from reactivation of the CMV acquired by natural infection in these patients. We hypothesize that the unique combination of a highly immunogenic and persistent CMV-based vector, combined with selective targeting of an MHC class I-restricted response to PSA to avoid induction of tumor-specific suppression, may be able impact tumor growth in the clinical setting.

However, studies in additional tumor models (both preventative, as in the present study, and therapeutic) will clearly be needed to determine whether this combination of a CMV-based vector together with the use of single MHC class I-restricted cancer antigen epitopes is effective cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: Supported by 1R41CA130493 grant from the National Cancer Institute, National Institutes of Health, a grant from the U.S. Department of Veterans Affairs, and a grant from the Baltimore Research and Education Foundation.

Reference List

1. Arase H, Mocarski ES, Campbell AE, et al. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science. 2002; 296:1323–1326. [PubMed: 11950999]
2. Cheroutre H, Madakamutil L. Mucosal effector memory T cells: the other side of the coin. Cell Mol Life Sci. 2005; 62:2853–2866. [PubMed: 16314932]
3. Drake CG. Prostate cancer as a model for tumour immunotherapy. Nat Rev Immunol. 2010; 10:580–593. [PubMed: 20651745]
4. Gillespie GM, Wills MR, Appay V, et al. Functional heterogeneity and high frequencies of cytomegalovirus- specific CD8(+) T lymphocytes in healthy seropositive donors. J Virol. 2000; 74:8140–8150. [PubMed: 10933725]
5. Hallmeyer S, Perambakam S, Reddy S, et al. A randomized trial of PSA-peptide based, specific active immunotherapy in HLA-A2+ patients with prostate cancer: comparison of two different vaccination strategies. 2005:2519.
6. Han M, Partin AW, Zahurak M, et al. Biochemical (prostate specific antigen) recurrence probability following radical prostatectomy for clinically localized prostate cancer. J Urol. 2003; 169:517–523. [PubMed: 12544300]
7. Hansen SG, Powers CJ, Richards R, et al. Evasion of CD8+ T cells is critical for superinfection by cytomegalovirus. Science. 2010; 328:102–106. [PubMed: 20360110]
8. Hansen SG, Vieville C, Whizin N, et al. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. Nat Med. 2009; 15:293–299. [PubMed: 19219024]
9. Heiser A, Coleman D, Dannull J, et al. Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. J Clin Invest. 2002; 109:409–417. [PubMed: 11828001]
10. Karrer U, Wagner M, Sierro S, et al. Expansion of protective CD8+ T-cell responses driven by recombinant cytomegaloviruses. J Virol. 2004; 78:2255–2264. [PubMed: 14963122]
11. Kast WM. Prostate Cancer Immunotherapy Development in Prostate Specific Antigen Transgenic Mice. Defense Technical Information Center. 1999:1–16.
12. Kern, ER. Animal models of cytomegalovirus infection: Murine CMV. Academic Press; London: 1999.
13. Kern ER. Pivotal role of animal models in the development of new therapies for cytomegalovirus infections. Antiviral Res. 2006; 71:164–171. [PubMed: 16828175]
14. Kern F, Khatamzas E, Surel I, et al. Distribution of human CMV-specific memory T cells among the CD8pos. subsets defined by CD57, CD27, and CD45 isoforms. Eur J Immunol. 1999; 29:2908–2915. [PubMed: 10508265]
15. Khan N, Cobbold M, Keenan R, et al. Comparative Analysis of CD8+ T Cell Responses against Human Cytomegalovirus Proteins pp65 and Immediate Early 1 Shows Similarities in Precursor

Frequency, Oligoclonality, and Phenotype. J Infect Dis. 2002; 185:1025–1034. [PubMed: 11930311]

16. Klyushnenkova, EN.; Alexander, RB. Challenges to Prostate Cancer Immunotherapy. In: Arnouk, H., editor. Cancer Immunotherapy. InTech - Open Access Publisher; 2011.
17. Klyushnenkova EN, Kouiavskaia DV, Berard CA, et al. Cutting edge: Permissive MHC class II allele changes the pattern of antitumor immune response resulting in failure of tumor rejection. J Immunol. 2009; 182:1242–1246. [PubMed: 19155468]
18. Klyushnenkova EN, Link J, Oberle WT, et al. Identification of HLA-DRB1*1501-restricted T-cell epitopes from prostate-specific antigen. Clin Cancer Res. 2005; 11:2853–2861. [PubMed: 15837732]
19. Komanduri KV, Viswanathan MN, Wieder ED, et al. Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. Nat Med. 1998; 4:953–956. [PubMed: 9701250]
20. Kouiavskaia DV, Berard CA, Datena E, et al. Vaccination With Agonist Peptide PSA: 154-163 (155L) Derived From Prostate Specific Antigen Induced CD8 T-Cell Response to the Native Peptide PSA: 154-163 But Failed to Induce the Reactivity Against Tumor Targets Expressing PSA: A Phase 2 Study in Patients With Recurrent Prostate Cancer. J Immunother. 2009; 32:655–666. [PubMed: 19483644]
21. Lubaroff DM, Konety BR, Link B, et al. Phase I clinical trial of an adenovirus/prostate-specific antigen vaccine for prostate cancer: safety and immunologic results. Clin Cancer Res. 2009; 15:7375–7380. [PubMed: 19920098]
22. Mackay CR, Marston WL, Dudler L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. J Exp Med. 1990; 171:801–817. [PubMed: 2307933]
23. Madan RA, Arlen PM, Mohebtash M, et al. Prostvac-VF: a vector-based vaccine targeting PSA in prostate cancer. Expert Opin Investig Drugs. 2009; 18:1001–1011.
24. Madsen LS, Andersson EC, Jansson L, et al. A humanized model for multiple sclerosis using HLA-DR2 and a human T-cell receptor. Nat Genet. 1999; 23:343–347. [PubMed: 10610182]
25. Masopust D, Vezys V, Marzo AL, et al. Preferential localization of effector memory cells in nonlymphoid tissue. Science. 2001; 291:2413–2417. [PubMed: 11264538]
26. Medin JA, Liang SB, Hou JW, et al. Efficient transfer of PSA and PSMA cDNAs into DCs generates antibody and T cell antitumor responses in vivo. Cancer Gene Ther. 2005; 12:540–551. [PubMed: 15678150]
27. Messerle M, Crnkovic I, Hammerschmidt W, et al. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. Proceedings of the National Academy of Sciences of the USA. 1997; 94:14759–14763. [PubMed: 9405686]
28. Mohr CA, Arapovic J, Muhlbach H, et al. A spread-deficient cytomegalovirus for assessment of first-target cells in vaccination. J Virol. 2010; 84:7730–7742. [PubMed: 20463067]
29. Munks MW, Cho KS, Pinto AK, et al. Four distinct patterns of memory CD8 T cell responses to chronic murine cytomegalovirus infection. J Immunol. 2006; 177:450–458. [PubMed: 16785542]
30. Pass, RF. Cytomegalovirus. In: David, MK., editor. Fields Virology. Lippincott Williams & Wilkins; Philadelphia: 2001. p. 2675-2705.
31. Pavlenko M, Leder C, Roos AK, et al. Identification of an immunodominant H-2D(b)-restricted CTL epitope of human PSA. Prostate. 2005; 64:50–59. [PubMed: 15651071]
32. Pavlenko M, Roos AK, Lundqvist A, et al. A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer. Br J Cancer. 2004; 91:688–694. [PubMed: 15280930]
33. Popa M, Ruzsics Z, Lotzerich M, et al. Dominant negative mutants of the murine cytomegalovirus M53 gene block nuclear egress and inhibit capsid maturation. J Virol. 2010; 84:9035–9046. [PubMed: 20610730]
34. Rasmussen L, Matkin C, Spaete R, et al. Antibody response to human cytomegalovirus glycoproteins gB and gH after natural infection in humans. J Infect Dis. 1991; 164:835–842. [PubMed: 1658156]

35. Redwood AJ, Messerle M, Harvey NL, et al. Use of a murine cytomegalovirus K181-derived bacterial artificial chromosome as a vaccine vector for immunocontraception. J Virol. 2005; 79:2998–3008. [PubMed: 15709020]
36. Sallusto F, Lenig D, Forster R, et al. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999; 401:708–712. [PubMed: 10537110]
37. Seow Y, Wood MJ. Biological gene delivery vehicles: beyond viral vectors. Mol Ther. 2009; 17:767–777. [PubMed: 19277019]
38. Sester M, Sester U, Gartner B, et al. Sustained high frequencies of specific CD4 T cells restricted to a single persistent virus. J Virol. 2002; 76:3748–3755. [PubMed: 11907214]
39. Shahabi V, Reyes-Reyes M, Wallecha A, et al. Development of a Listeria monocytogenes based vaccine against prostate cancer. Cancer Immunol Immunother. 2008; 57:1301–1313. [PubMed: 18273616]
40. Sierro S, Rothkopf R, Klenerman P. Evolution of diverse antiviral CD8+ T cell populations after murine cytomegalovirus infection. Eur J Immunol. 2005; 35:1113–1123. [PubMed: 15756645]
41. Small EJ, Schellhammer PF, Higano CS, et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. J Clin Oncol. 2006; 24:3089–3094. [PubMed: 16809734]
42. Sylwester AW, Mitchell BL, Edgar JB, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med. 2005; 202:673–685. [PubMed: 16147978]
43. Thara E, Dorff TB, Pinski JK, et al. Vaccine therapy with sipuleucel-T (Provenge) for prostate cancer. Maturitas. 2011; 69:296–303. [PubMed: 21621934]
44. Tonkin DR, He J, Barbour G, et al. Regulatory T cells prevent transfer of type 1 diabetes in NOD mice only when their antigen is present in vivo. J Immunol. 2008; 181:4516–4522. [PubMed: 18802054]
45. Tsuda Y, Caposio P, Parkins CJ, et al. A Replicating Cytomegalovirus-Based Vaccine Encoding a Single Ebola Virus Nucleoprotein CTL Epitope Confers Protection against Ebola Virus. PLoS Negl Trop Dis. 2011; 5:e1275. [PubMed: 21858240]
46. Uetsuki T, Naito A, Nagata S, et al. Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1 alpha. J Biol Chem. 1989; 264:5791–5798. [PubMed: 2564392]
47. van Hall T, van de Rhee NE, Schoenberger SP, et al. Cryptic open reading frames in plasmid vector backbone sequences can provide highly immunogenic cytotoxic T-lymphocyte epitopes. Cancer Res. 1998; 58:3087–3093. [PubMed: 9679975]
48. Voigt V, Forbes CA, Tonkin JN, et al. Murine cytomegalovirus m157 mutation and variation leads to immune evasion of natural killer cells. Proc Natl Acad Sci U S A. 2003; 100:13483–13488. [PubMed: 14597723]
49. Wagner M, Jonjic S, Koszinowski UH, et al. Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution. J Virol. 1999; 73:7056–7060. [PubMed: 10400809]
50. Waldrop SL, Davis KA, Maino VC, et al. Normal human CD4+ memory T cells display broad heterogeneity in their activation threshold for cytokine synthesis. J Immunol. 1998; 161:5284–5295. [PubMed: 9820501]
51. Zhang H, Podojil JR, Chang J, et al. TGF-beta-induced myelin peptide-specific regulatory T cells mediate antigen-specific suppression of induction of experimental autoimmune encephalomyelitis. J Immunol. 2010; 184:6629–6636. [PubMed: 20483764]

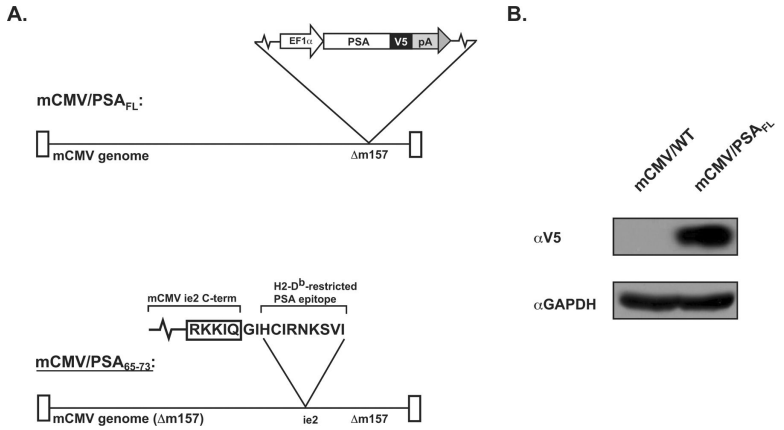

Figure 1. Schematic of mCMV/PSA$_{FL}$ and mCMV/PSA$_{65-73}$vectors

Recombinant vectors were constructed by lambda-based linear recombination using the pSMfr3 (Smith strain) BAC. ***A.*** *Vector maps.* mCMV/PSA$_{FL}$: A V5 epitope-tagged full-length PSA open reading frame (ORF) was placed under control of the EF1α promoter and inserted into the mCMV genome to replace the m157 gene within the mCMV BAC, pSMfr3. The PSA ORF consists of the full-length PSA coding region except for the predicted signal peptide (amino acids 2–24). mCMV/PSA$_{65-73}$: An H2^{b} -restricted T cell epitope from PSA (HCIRNKSVI) was fused `in-frame' to the carboxyl terminus of mCMV ie2 using pSMfr3 deleted for m157 as the genetic background. ***B.*** *Western blot analysis*. Analysis of PSA expression in cell lysates of MEFs infected with mCMV/PSA$_{FL}$ showing *in vitro* expression of the V5-tagged PSA$_{FL}$ at day 6 post-infection compared to wtmCMV. PSA (top panel) and GAPDH (bottom panel) were visualized using polyclonal rabbit antibodies against V5 (1:1000) and GAPDH (1:1000).

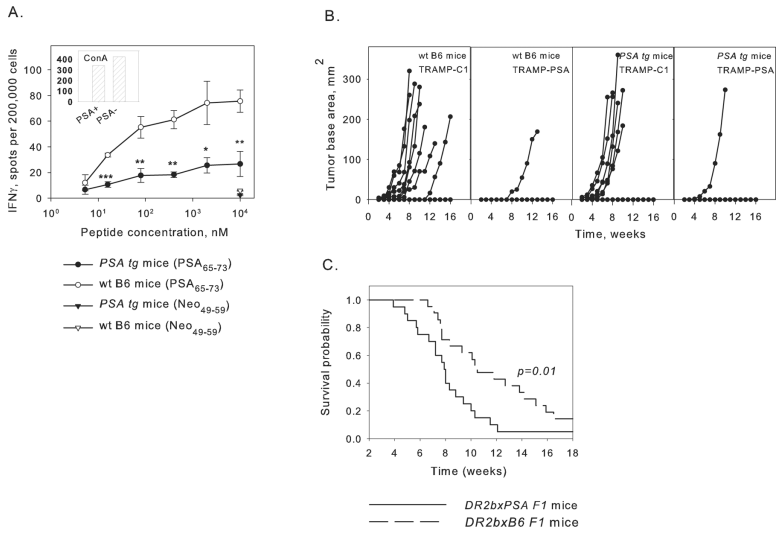

Figure 2. The response to TRAMP-PSA tumors in PSA tg mice

A. *CD8 T cell responses. PSA tg* or wt B6 male mice were inoculated s. c. with TRAMP-PSA tumors. The responses to the PSA_{65-73} peptide or control peptide Neo_{49-59} were measured two weeks later by IFN-γ ELISPOT assay. Data are presented as mean ± SD of triplicates (pool of 3 mice per group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (unpaired t-test). *Inset*: response to concanavalin A (ConA) of *PSA tg* (PSA+) and wt B6 (PSA-) mice. ***B.*** *TRAMP tumorgrowth in PSA tg mice. PSA tg* or wt B6 male mice were inoculated s. c. with either parental TRAMP-C1 or TRAMP-PSA tumor cells. Tumor growth was monitored for up to 16 weeks. Tumor base area was calculated by measuring two bisecting diameters of the tumor and multiplying these values. Individual growth curves from a representative experiment are shown (wt B6 mice, TRAMP-C1 tumors: n=9; wt B6 mice, TRAMP-PSA tumors: n=9; *PSA tg* mice, TRAMP-C1 tumor: n=8; *PSA tg* mice, TRAMP-PSA tumors, n=8). Absence of TRAMP-PSA tumor growth in *PSA tg* mice was further confirmed in two additional independent experiments [13.5% tumor incidence (n=37), data not shown]. ***C.*** TRAMP-PSA tumorgrowth in DR2b+ F1 mice: time-to-event analysis. TRAMP-PSA tumor cells were inoculated into either *DR2b×B6 F1* (n=21) or *DR2b×PSA F1* (n=20) male mice. Time when tumor base area reached 100 mm^2 was used as a surrogate end point for the survival analysis, and calculated by polynomic regression analysis (R-squared values > 0.95). Time-to-event analysis was performed by log rank test using MedCalc software. *P* value is shown on the graph. Combined results from two independent experiments are shown.

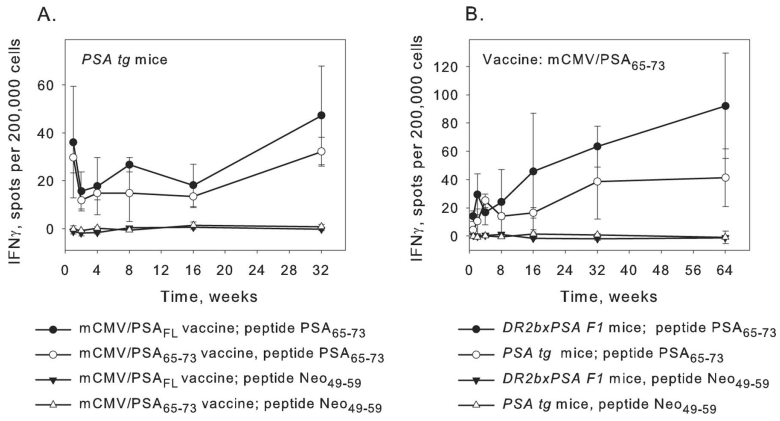

Figure 3. Kinetics of PSA-specific CD8 T cell response to mCMV/PSA $_{65-73}$or mCMV/PSA$_{FL}$ vectors in the absence of tumor challenge

A. *PSA tg* male mice were immunized with either mCMV/PSA$_{65-73}$ or mCMV/PSA$_{FL}$ vectors at 10^4 PFU/mouse (i. p.) and boosted with the same dose of virus four weeks later. ***B***. *PSA tg* or *DR2b×PSA F1* male mice were immunized with mCMV/PSA$_{65-73}$ vector as described above. Frequencies of PSA$_{65-73}$ peptide-specific T cells were measured by IFN-γ ELISPOT at indicated time points after boost immunization, the responses to peptide Neo$_{49-59}$ served as negative controls. Data are means ± SE (following subtraction of background in the absence of antigen, 3–6 mice per group). Responses from individual mice are shown in Supplemental Figure 1 (see Supplemental Digital Content 2). Comparison between groups was performed using Mann-Whitney U-test and showed no statistical significance at $P<0.05$.

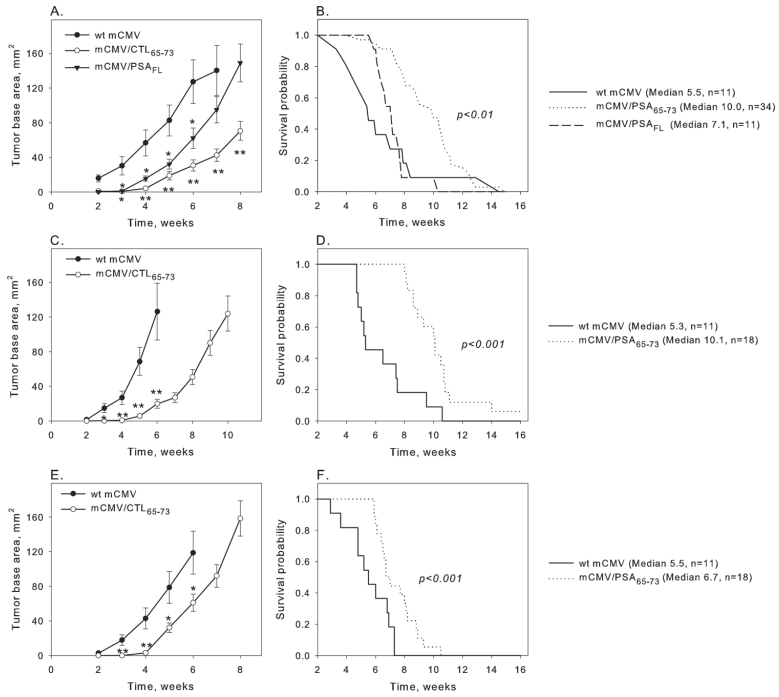

Figure 4. Effect of immunization with mCMV vectors on TRAMP-PSA tumor growth

DR2b×PSA F1 male mice were immunized with mCMV/PSA_{65-73}, mCMV/PSA_{FL}, or Δm157 (wt) mCMV as described in Figure 3.***A-D***: TRAMP-PSA tumor cells were inoculated s. c. four weeks after boost immunization; ***E-F***: TRAMP-PSA tumor cells were inoculated s. c. thirty-two weeks after boost immunization. *Left panels* (***A, C, E***) depict changes in the tumor base area. Tumor base area was calculated by measuring two bisecting diameters of the tumor and multiplying these values. Data are means ± SE. Differences between PSA expressing mCMV vector(s) and control wt mCMV vector were analyzed for each time point using a two-sided Student t-test (* $p<0.05$; ** $p<0.01$). *Right panels* (***B, D, F***) represent time-to-event (surrogate survival) analyses for the same experiments. Tumor growth (time to tumor base area of 100 mm^2) was analyzed by log rank test using MedCalc software, p values are shown on the graphs (in ***B,*** *p* value is shown for "mCMV/PSA_{65-73}" group compared to "wt mCMV" group). Median time-to-event and group size are shown under corresponding panels.

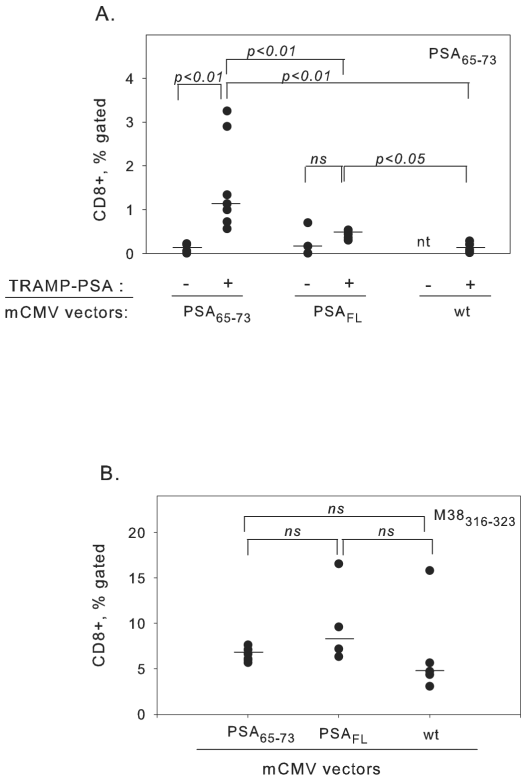

Figure 5. TRAMP-PSA tumor challenge results in significant differences in PSA-specific CD8 T cellresponses between mCMV/PSA$_{65-73}$ and mCMV/PSA$_{FL}$ immunized mice

DR2b×PSA F1 male mice were immunized with mCMV/PSA$_{65-73}$, mCMV/PSA$_{FL}$ or Δm157 (wt) mCMV constructs as described in Figure 3. Mice were inoculated with TRAMP-PSA tumor cells 4 weeks after the boost immunization. Splenocytes were harvested either two weeks before or two weeks after tumor challenge. ICS was performed after 16–18 hr of incubation with either the peptide PSA$_{65-73}$ or mCMV epitope M38$_{316-323}$, both in the presence of Brefeldin A. Frequencies of total IFN-γ -producing CD8 T cells in response to peptides PSA$_{65-73}$ ***(A)*** or M38$_{316-323}$ ***(B)*** are shown for CD8+ gated events for individual mice with background values in the absence of antigen subtracted. Comparison between groups was performed using Mann-Whitney U-test; horizontal lines indicate medians, and p values are shown (*ns* - not significant). Combined results from two independent experiments are shown (nt - not tested). In ***(B)***, only the responses following TRAMP-PSA tumor challenge are shown. No statistically significant differences were observed between the groups in response to M38$_{316-323}$ before tumor inoculation (data not shown). Responses from typical individual mice are shown in Supplemental Figure 2 (see Supplemental Digital Content 3).