

## Exosome Targeting of Tumor Antigens Expressed by Cancer Vaccines Can Improve Antigen Immunogenicity and Therapeutic Efficacy

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### Abstract

MVA-BN-PRO (BN ImmunoTherapeutics) is a candidate immunotherapy product for the treatment of prostate cancer. It encodes 2 tumor-associated antigens, prostate-specific antigen (PSA), and prostatic acid phosphatase (PAP), and is derived from the highly attenuated modified vaccinia Ankara (MVA) virus stock known as MVA-BN. Past work has shown that the immunogenicity of antigens can be improved by targeting their localization to exosomes, which are small, 50- to 100-nm diameter vesicles secreted by most cell types. Exosome targeting is achieved by fusing the antigen to the C1C2 domain of the lactadherin protein. To test whether exosome targeting would improve the immunogenicity of PSA and PAP, 2 additional versions of MVA-BN-PRO were produced, targeting either PSA (MVA-BN-PSA-C1C2) or PAP (MVA-BN-PAP-C1C2) to exosomes, while leaving the second transgene untargeted. Treatment of mice with MVA-BN-PAP-C1C2 led to a striking increase in the immune response against PAP. Anti-PAP antibody titers developed more rapidly and reached levels that were 10- to 100-fold higher than those for mice treated with MVA-BN-PRO. Furthermore, treatment with MVA-BN-PAP-C1C2 increased the frequency of PAP-specific T cells 5-fold compared with mice treated with MVA-BN-PRO. These improvements translated into a greater frequency of tumor rejection in a PAP-expressing solid tumor model. Likewise, treatment with MVA-BN-PSA-C1C2 increased the antigenicity of PSA compared with treatment with MVA-BN-PRO and resulted in a trend of improved antitumor efficacy in a PSA-expressing tumor model. These experiments confirm that targeting antigen localization to exosomes is a viable approach for improving the therapeutic potential of MVA-BN-PRO in humans. *Cancer Res*; 71(15); 5235–44. ©2011 AACR.

### Introduction

MVA-BN-PRO (BN ImmunoTherapeutics) is a candidate immunotherapy product for the treatment of prostate cancer that is currently in a phase I clinical trial (1). This recombinant vector is derived from a clonal isolate of the highly attenuated modified vaccinia Ankara (MVA) virus stock known as MVA-BN and encodes 2 validated tumor-associated antigens (TAA), prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP). PSA is a secreted protein found at elevated levels in the serum of men with prostate cancer (2). Recently, PSA has shown promise as an immunotherapy tumor antigen for prostate cancer patients. In a phase II trial of PROSTVAC (BN Immuno-

Therapeutics), a poxviral-based immunotherapy that targets PSA, treated patients showed an average increase in median survival of 8.5 months compared with those treated with control vectors (3). PAP is a glycosylated phosphatase found in prostate epithelium which occurs either as an intracellular or secreted protein (4). PAP has recently been validated as an antigen for prostate cancer immunotherapy through the approval of the dendritic cell-based therapy sipuleucel-T (5, 6). Therefore, the inclusion of both PSA and PAP as transgenes in MVA-BN-PRO is hoped to provide improved efficacy in prostate cancer patients compared with therapies targeting only a single antigen.

A technology platform called exosome display has recently been described that exploits the exosome pathway for a broad range of applications including vaccine (7, 8) and therapeutic antibody development (9). Exosomes are 50- to 100-nm diameter membrane vesicles secreted into the extracellular space. They bind or fuse with neighboring cells in a process thought to allow cell-to-cell communication at a distance. The effects of exosomes on the immune system have become the subject of increasing interest due to studies showing that they can provide activating signals through several different mechanisms. For example, exosomes produced by antigen-presenting cells (APC) contain MHC class I and class II complexes along with costimulatory proteins, and purified exosomes can activate CD4 or CD8 T cells. Furthermore,

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**doi:** 10.1158/0008-5472.CAN-10-4076

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exosomes purified from other cell types can spread antigens or peptide-loaded MHC complexes to APCs for more efficient presentation (see ref. 10 for a review on the effects of exosomes on immune cells). One modality used for exosome display of antigens is the generation of chimeric expression constructs encoding a protein of interest fused to the C1C2 domain of lactadherin (7). Lactadherin is released via the exosome secretory pathway by binding to the vesicle surface, and chimeric proteins containing the C1C2 domain of lactadherin have been shown to be released into the extracellular milieu bound to exosomes. The potential of this technology for vaccine application was recently illustrated in studies where vaccination of mice with a DNA vector encoding chicken ovalbumin (OVA) fused to the C1C2 domain slowed the growth of OVA-expressing tumors more than vaccination with vectors encoding membrane-bound or secreted OVA (11). Although OVA is a commonly used model antigen in proof-of-principle studies, this technology has not been validated for therapeutically relevant tumor antigens.

Here, we describe studies to test whether the immunogenicity or therapeutic efficacy of MVA-BN-PRO can be improved by targeting PSA or PAP to exosomes. Two additional MVA-BN-PRO vectors were made, one modified to express PSA-C1C2 and wild-type PAP, and the other modified to express PAP-C1C2 and wild-type PSA. Treatment of mice with the MVA-BN-PRO vector encoding exosome-targeted PAP led to a striking increase in the immune response against PAP. More importantly, it resulted in enhanced antitumor activity against a syngeneic tumor expressing PAP when compared with vectors encoding the wild-type transgene. Likewise, treatment with the viral vector encoding exosome-targeted PSA increased the antigenicity of PSA and showed a trend of improved antitumor activity in a PSA-expressing solid tumor model. These experiments show that targeting tumor-associated antigens to exosomes is a viable approach for improving the therapeutic potential of MVA-BN-PRO for the treatment of prostate cancer in humans.

## Materials and Methods

### DNA expression plasmids and cell lines

Four DNA expression vectors were constructed using pcDNA3.1/Zeo or pcDNA3.1/Hygro (Invitrogen) to express either wild-type or C1C2-fused versions of PSA or PAP: pPSA/Zeo, pPSA-C1C2/Zeo, pPAP/Hygro, and pPAP-C1C2/Hygro under the control of the cytomegalovirus promoter. The open reading frame (ORF) of human *PSA* or human *PAP* was amplified by PCR from the pCR2.1 PSA or the pCR2.1 PAP vectors (Bavarian Nordic) using primers that added a 5' Kozak translation initiation sequence and unique restriction sites flanking the ORFs to aid in cloning. To generate constructs encoding C-terminal fusions to the C1C2 domain of lactadherin, this domain was amplified from vector p6mLC1C2 (7) using the forward primer 5'-accgaatacatctgccagtcc-3' and the reverse primer 5'-cactgtaagccttaacagccagcagctccagg-3'. This product was then blunt-end ligated to the PCR-amplified ORF of *PSA* or *PAP* and then cloned into the multiple cloning site of pcDNA3.1/Zeo or pcDNA3.1/Hygro, respectively.

HEK293-F cells (Invitrogen) were grown as adherent cultures in Dulbecco's modified Eagle's medium (DMEM) media with 4.5 g/L glucose, L-glutamine, sodium pyruvate, and 10% FBS (Mediatech, Inc.) and transfected with plasmid DNA from one of the 4 DNA expression plasmids described above using the Lipofectamine LTX reagent and PLUS Reagent (Invitrogen). Cells were selected on media supplemented with 250 µg/mL of Zeocin or Hygromycin B (Invitrogen) as appropriate for the plasmid. Drug-resistant cells were single-cell cloned to generate cell lines.

E6 cells (12) express human PSA and were grown in DMEM media with 10% FBS (Mediatech). CT26 cells (13) were obtained from American Type Culture Collection and grown in Iscove's DMEM with 2 mmol/L L-glutamine, 25 mmol/L HEPES, and supplemented with 5% FBS or bovine calf serum (BCS; Mediatech, Inc.). To generate CT26-PAP cell lines, CT26 cells were transfected with the pPAP/Hygro plasmid and selected for drug-resistant clones as described for HEK293-F cells. PAP expression was verified in conditioned media with the UBI Magiwell PAP Quantitative Test ELISA (United Biotech). Cell lines have not been independently authenticated.

### Viruses

MVA-BN-PRO was modified using vectors derived from the above DNA expression plasmids to create MVA-BN-PSA-C1C2 and MVA-BN-PAP-C1C2. All viral vectors were produced and assayed for 50% tissue culture infectious dose (TCID<sub>50</sub>) by Bavarian Nordic according to established methods (14). In addition to the TCID<sub>50</sub> assay, the infectious unit (IU) titers of the viral stocks were determined at BN ImmunoTherapeutics using flow cytometry (15).

### Western blotting

CT26 cells grown in 6-well plates were incubated with MVA-BN-PSA-C1C2 or MVA-BN-PAP-C1C2 for 24 hours, scraped from the wells, washed in cold PBS, and resuspended in NP40 Cell Lysis Buffer (Invitrogen) containing the Protease Inhibitor Cocktail Set III (EMD Chemicals). Soluble protein was applied to SDS-PAGE gels, electrophoresed under reducing conditions, and electroblotted onto polyvinylidene difluoride membranes using the iBlot system (Invitrogen). Proteins were detected using the WesternBreeze Chromogenic Immunodetection Kit (Invitrogen) with a mouse anti-human PAP monoclonal antibody (clone 4LJ; Santa Cruz Biotech) or a rabbit anti-human PSA polyclonal antibody (DAKO). Purified PSA or PAP was used as reference protein (Meridian Life Science Inc.).

### Anti-CD81 cross-capture ELISA

To prepare media enriched with exosomes for analysis by the anti-CD81 cross-capture ELISA, HEK293-F cell lines were grown as described above and then seeded at  $2 \times 10^6$  cells/mL and grown as a suspension culture in 100 to 200 mL of the chemically defined, protein-free media CD 293 supplemented with 4 mmol/L L-glutamine (Invitrogen) for 5 to 7 days. Conditioned media was concentrated and filtered as described for a miniscale purification of exosomes (16), except ultracentrifugation over a sucrose cushion was not done.

The anti-CD81 cross-capture ELISA was done as described (9, 16) with modifications. The 96-well plates were coated with an anti-CD81 antibody (clone JS-81, BD Biosciences) at a concentration of 4  $\mu\text{g}/\text{mL}$  in PBS, washed with 0.05% Tween-20 in PBS, blocked with 3% BSA in PBS for 1 hour, and washed again. Two-fold dilutions of exosome-enriched media were added to each well and incubated at room temperature for 12 hours to allow binding of exosomes to the plate via the anti-CD81 antibody. Wells were washed, and PSA was detected with the anti-PSA horseradish peroxidase (HRP)-conjugated antibody and detection protocol from the Human PSA ELISA Kit (Anogen). For the detection of PAP, wells were washed and incubated in 90 mmol/L citrate buffer pH 4.8 with 4 mg/mL *p*-nitrophenyl-phosphate at 37°C for 30 minutes. The reaction was stopped with 0.5 mol/L sodium hydroxide, and the absorbance at 405 nm was read using a Multiskan plate reader (Thermo Electronics). For the detection of other proteins, wells were incubated with biotinylated antibodies in PBS with 3% BSA, washed, incubated with streptavidin HRP (BD Biosciences) in PBS with 3% BSA, washed, and then developed with the TMB substrate solution (EMD Chemicals). Development was stopped with 0.5 mol/L sulfuric acid and the absorbance at 450 nm was measured. The following biotinylated detection antibodies were used: anti-CD81 (clone JS-81 biotinylated with the EZ-Link NHS-PEO Solid Phase Biotinylation Kit, Pierce/Thermo Scientific), anti-MHC class I (clone W6/32; Abnova), and an IgG1 isotype control (BD Biosciences).

#### Animal studies

Male BALB/c or C57BL/6 mice aged 6 to 8 weeks old were obtained from Harlan Sprague Dawley, Inc. Mice were treated with TBS or MVA-BN-vectors injected s.c. For tumor studies,  $5 \times 10^5$  CT26-PAP or  $1 \times 10^5$  E6-PSA cells were implanted intradermally (i.d.), and tumor size was measured twice weekly with calipers. Tumor volume was calculated as  $(\text{length} \times \text{width}^2)/2$ . Statistical significance over the course of the measurements was determined by repeated measure ANOVA (RM-ANOVA) with Bonferroni's multiple comparison test. Differences in tumor size at a particular time point were compared by 1-way ANOVA with Bonferroni's multiple comparison test. All protocols were approved by the BN Immunotherapy Institutional Animal Care and Use Committee.

#### Determination of serum antibody titers by ELISA

The 96-well plates were coated with PSA or PAP protein (Meridian Life Science Inc.) diluted in carbonate buffer or MVA-BN diluted in PBS, then washed and blocked for 1 hour with PBS + 0.05% Tween20. Serial dilutions of mouse sera were added in duplicates, incubated for 1 hour, washed, and incubated with HRP-conjugated secondary antibodies (Southern Biotech). Bound antibodies were detected with the TMB substrate solution (EMD Chemicals), which was stopped with 0.5 mol/L sulfuric acid. Absorbance was measured at 450 nm. Titers were calculated as the reciprocal value of the last dilution with a signal at least 2-fold higher than background.

#### Determination of the frequency of IFN- $\gamma$ producing T cells by ELISPOT

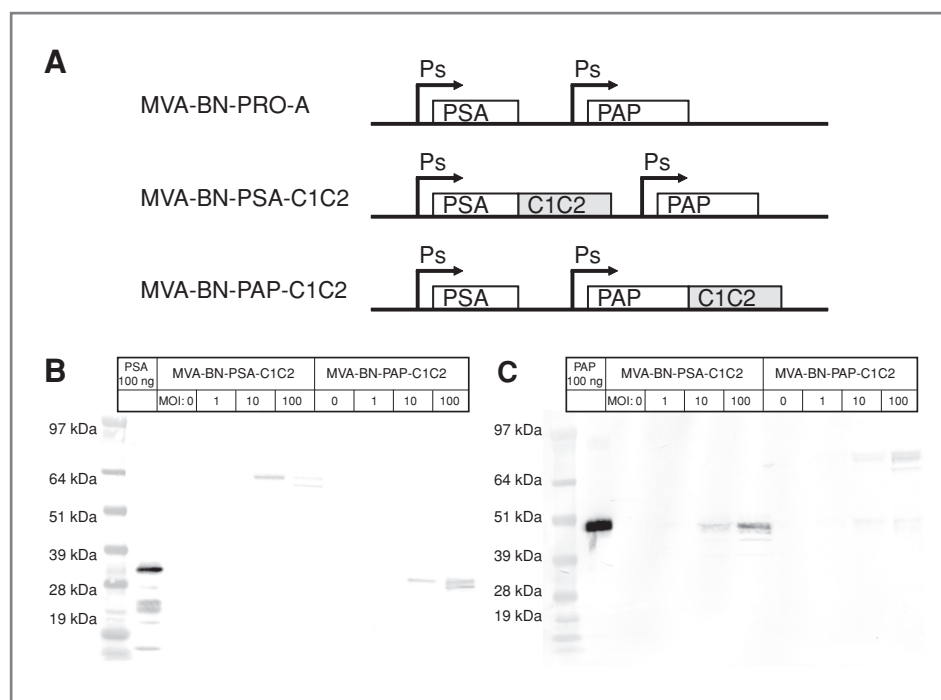
Millipore Multiscreen 96-well filtration plates (Millipore) were coated with rat anti-mouse IFN- $\gamma$  capture antibody (BD Biosciences) overnight at 4°C. Subsequently, plates were washed with PBS, blocked with RPMI-10 media (RPMI + 10% BCS +  $5 \times 10^{-5}$  mol/L  $\beta$ -Mercaptoethanol; Mediatech), washed again, and then splenocytes of treated mice were plated at 5E5 cells per well in RPMI-10. Cells were stimulated with the indicated reagents for 40 hours at 37°C, washed, incubated with a biotin-conjugated anti-IFN- $\gamma$  antibody (AbD Serotec), washed again, and then incubated with Streptavidin-Alkaline Phosphatase (BD Biosciences). Plates were washed and then developed with Vector Blue Substrate (Alkaline Phosphatase Substrate Kit III, Vector Lab Inc.). Spots were counted using an ImmunoSpot plate scanner (Cellular Technology Ltd.).

## Results

#### Construction and characterization MVA-BN-PRO viruses with exosome-targeted transgenes

To target the localization of PSA and PAP to exosomes, 2 new viral vectors were developed that encode fusion proteins linking the C1C2 domain of mouse lactadherin to the C-terminus of PSA or PAP (Fig. 1A). The expression of the transgenes encoded by these vectors was characterized using the mouse colon carcinoma cell line CT26. CT26 cells were incubated with MVA-BN-PSA-C1C2 or MVA-BN-PAP-C1C2 at a multiplicity of infection (MOI) ranging from 1 to 100. Whole cell lysates were prepared 24 hours later for analysis by Western blotting. On blots probed with an anti-PSA antibody, a 64-kDa band was detected in MVA-BN-PSA-C1C2 infected cells consistent with the predicted 66-kDa size of the PSA-C1C2 protein (Fig. 1B). The wild-type PSA protein encoded by MVA-BN-PAP-C1C2 was detected at the expected size of 30 kDa, which was similar to the dominant band present in the lane loaded with purified PSA protein. On blots of the same lysates probed with an anti-PAP antibody, a 50-kDa band was revealed in cells infected with MVA-BN-PSA-C1C2, which matched the size of purified wild-type PAP protein (Fig. 1C). Likewise, an 80-kDa band was detected in lysates from cells infected with MVA-BN-PAP-C1C2, which corresponds to the predicted 79-kDa size of PAP-C1C2. A faint 50-kDa band was also detected in these lysates, which may be a cleavage product of the larger PAP-C1C2 protein. When cells were infected with either virus at the highest MOI, a doublet of bands appeared, which may be a result of cytopathic effects due to the virus being present at a very high MOI.

Conditioned media from similarly infected cultures was analyzed for the presence of secreted transgenic proteins (Supplementary Fig. S1). Low or undetectable amounts of PSA-C1C2 or PAP-C1C2 were measured, compared with high amounts of unmodified PSA or PAP. This is consistent with the expression characteristics of other secreted proteins following linkage to a C1C2 domain (7). Together with the Western blots, these data indicate that infection of cells by MVA-BN-PSA-C1C2 or MVA-BN-PAP-C1C2 results in the



**Figure 1.** A, MVA-BN-PRO encodes wild-type PSA and PAP. MVA-BN-PSA-C1C2 encodes PSA with a C-terminal fusion to the C1C2 domain of lactadherin and wild-type PAP. MVA-BN-PAP-C1C2 encodes PAP with a C-terminal fusion to the C1C2 domain and wild-type PSA. All transgenes are expressed under the control of the synthetic early/late promoter (Ps). B and C, whole cell lysates of CT26 cells infected with either MVA-BN-PSA-C1C2 or MVA-BN-PAP-C1C2 were analyzed by Western blot along with PSA or PAP reference protein. Blots were probed with (B) an anti-PSA antibody or (C) an anti-PAP antibody.

synthesis of protein products of the expected size and expression level for wild-type and exosome-targeted versions of PSA and PAP.

#### Localization of PSA-C1C2 and PAP-C1C2 proteins

To generate large cultures of cells suitable for characterizing protein expression on exosomes, stably transfected HEK293-F cell lines were generated that expressed protein from 1 of 4 DNA expression constructs, encoding either wild-type or exosome-targeted PSA or PAP. Expression of the transgenic proteins in the cell lines was found to be relatively similar to what was observed with the virally infected CT26 cells (data not shown).

Exosome targeting of PSA-C1C2 and PAP-C1C2 was verified as previously described (9, 16) using an anti-CD81-based cross-capture ELISA. In this assay, an antibody against the tetraspan protein CD81 was used to capture exosomes from concentrated conditioned media from each of the HEK cell lines. Subsequently, the presence of PSA or PAP on exosomes was determined using an anti-PSA antibody or an enzymatic assay for PAP in the detection steps. A positive PSA-specific signal was detected in media conditioned by the HEK-PSA-C1C2 cell line but not in the media from the HEK-PSA cell line (Fig. 2A). Likewise, a PAP-specific signal was only detected in media from the HEK-PAP-C1C2 cell line (Fig. 2B). Aliquots of these samples were further tested with anti-CD81 (Fig. 2C) and anti-MHC class I (Fig. 2D) detection antibodies as controls to verify the amount of exosomes captured in the assay. As shown in Fig. 2E, lower signals were obtained from the HEK-PSA-C1C2 sample, which suggests a lower concentration of exosomes in this preparation. Overall, these experiments provide evidence that the C1C2-fusion proteins are localized to exosomes.

#### Humoral responses in mice treated with MVA-BN-PRO vectors encoding wild-type or exosome-targeted PSA and PAP

To assess whether exosome targeting improved the humoral response against PSA or PAP, male BALB/c or C57BL/6 mice were treated with 5E7 TCID<sub>50</sub> of the MVA-BN-PRO viral vectors once every 2 weeks for a total of 3 treatments. Serum was collected 2 weeks after each treatment, and the titer of anti-PSA IgG, anti-PAP IgG, or anti-MVA IgG was evaluated in pooled sera from each group by ELISA. As shown in Fig. 3A, BALB/c mice treated with any of the 3 viruses developed anti-PSA IgG titers with similar kinetics that reached a titer of 64,000 by day 42. Notably, anti-PSA IgG titers were undetectable in C57BL/6 mice treated with the viruses encoding wild-type PSA, whereas low titers did develop in mice treated with MVA-BN-PSA-C1C2 (Fig. 3B).

The anti-PAP IgG titers induced by treatment with the viruses encoding wild-type PAP tended to be low in BALB/c and C57BL/6 mice and required 2 or 3 treatments for detection (Fig. 3C and D). In contrast, anti-PAP IgG titers were 16- to 128-fold higher in BALB/c mice treated with MVA-BN-PAP-C1C2. Furthermore, anti-PAP IgG titers were detectable after only a single dose (day 14). Similar titers were reached even when mice were treated with 10-fold lower amounts of this virus (data not shown). An analysis of titers from individual mice revealed that all BALB/c mice treated with MVA-BN-PAP-C1C2 developed anti-PAP IgG titers detectable at the lowest dilution tested (1:125), compared with only 7 or 10 mice treated with MVA-BN-PRO or MVA-BN-PSA-C1C2 (Supplementary Fig. S2). In C57BL/6 mice, MVA-BN-PAP-C1C2 treatment also led to higher anti-PAP IgG titers than the other viruses (Fig. 3D). The anti-MVA IgG titers raised by the 3 viral vectors were similar between all 3 viruses (Fig. 3E and F),

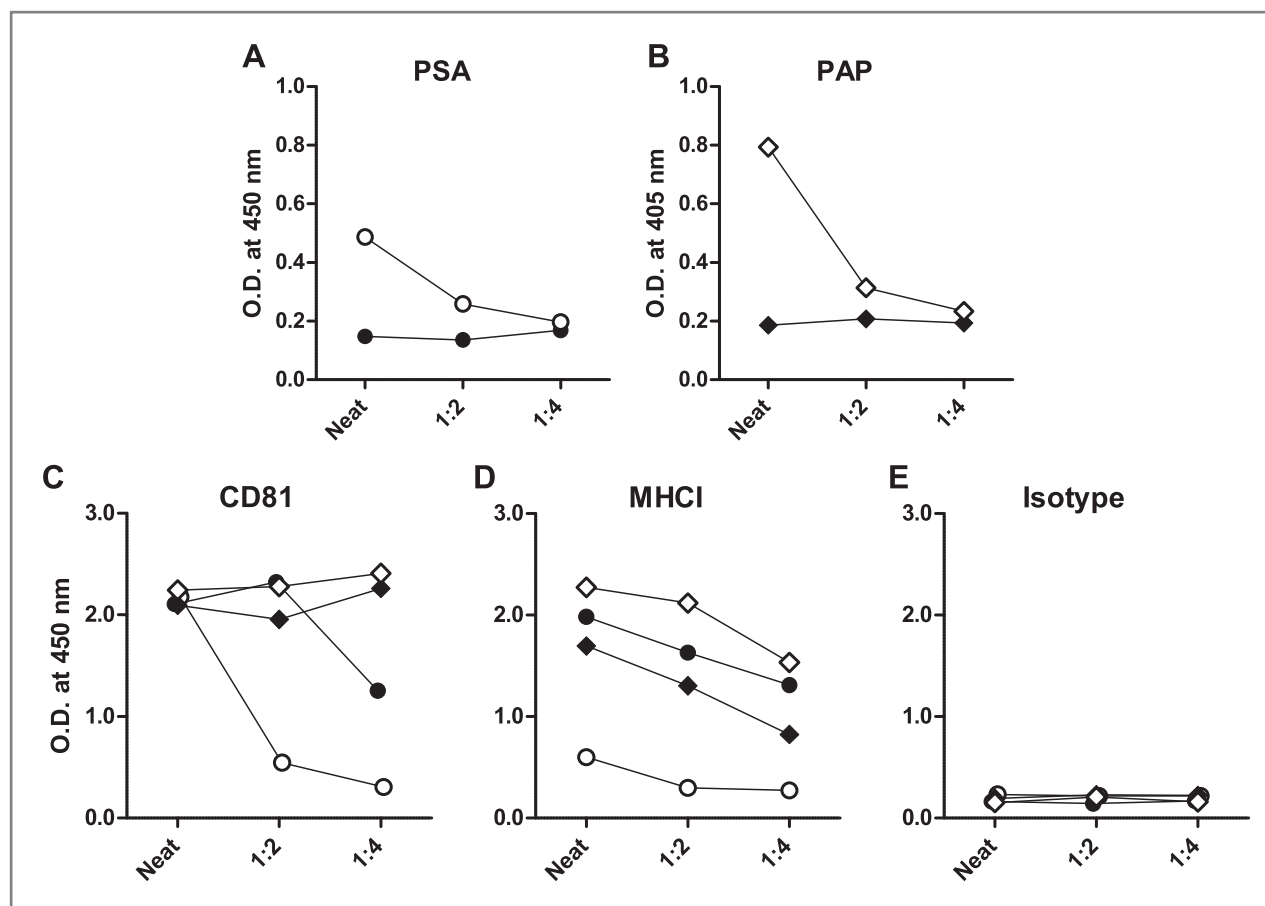


Figure 2. Protein expression on exosomes was analyzed with an anti-CD81 cross-capture ELISA. The HEK-PSA (●), HEK-PSA-C1C2 (○), HEK-PAP (◆), and HEK-PAP-C1C2 (◇) cell lines were grown for 5 to 7 days in protein-free media, and concentrated media from each cell line was incubated in wells coated with an anti-CD81 antibody to capture exosomes. Wells were probed with a detection antibody against (A) PSA or (B) PAP-derived enzymatic activity was detected with a phosphatase assay. Detection antibodies were also used against (C) CD81 or (D) MHC class I. E, detection with an isotype control antibody.

supporting that the differences detected between groups were not due to variable virus dosing.

The relative ratio of antibody isotypes produced against an antigen (IgG2a/IgG1 for BALB/c or IgG2c/IgG1 for C57BL/6 mice) can be used to indicate whether the response is more biased toward a Th1 response (higher ratio) or a Th2 response (lower ratio; refs. 17, 18). In BALB/c mice, the ratios for anti-PSA and anti-PAP antibody titers were similar among all groups and were consistent with a Th1 response (data not shown). In C57BL/6 mice, the antibody titers were also consistent with a Th1 response, but responses generated against the nonexosome-targeted antigens were so low that ratios could not be determined (data not shown).

#### T-cell responses in mice treated with MVA-BN-PRO vectors encoding wild-type or exosome-targeted PSA and PAP

To investigate the effects of immunization with exosome-targeted antigens on cellular immunity, BALB/c mice were immunized every 2 weeks for a total of 4 treatments with MVA-BN-PRO, MVA-BN-PSA-C1C2, or MVA-BN-PAP-C1C2.

Five days after the last immunization, the frequency of PSA, PAP, or MVA-specific T-cell responses in restimulated splenocytes was enumerated by ELISPOT. As shown in Fig. 4A, treatment with MVA-BN-PSA-C1C2 induced a 2 to 4-fold higher frequency of PSA-specific T cells than the other groups. Furthermore, the frequency of PAP-specific T cells was 4- to 6-fold higher in mice treated with MVA-BN-PAP-C1C2 than the other groups (Fig. 4B). The frequencies of anti-MVA T cells between each group were at most 40% different from each other (Fig. 4C), suggesting that the differences seen for PSA and PAP were specific to exosome targeting of these antigens. Depletion of CD4 or CD8 T cells prior to restimulation with PSA or PAP overlapping peptide library (OPL) showed that the ratio of CD4:CD8 T cells responding to the stimuli was similar in mice treated with the viruses encoding either exosome-targeted or wild-type antigens (data not shown).

#### Therapeutic activity of MVA-BN-PRO viruses in mouse models of prostate cancer

The therapeutic efficacy of MVA-BN-PRO viruses encoding exosome-targeted or wild-type transgenes was compared in

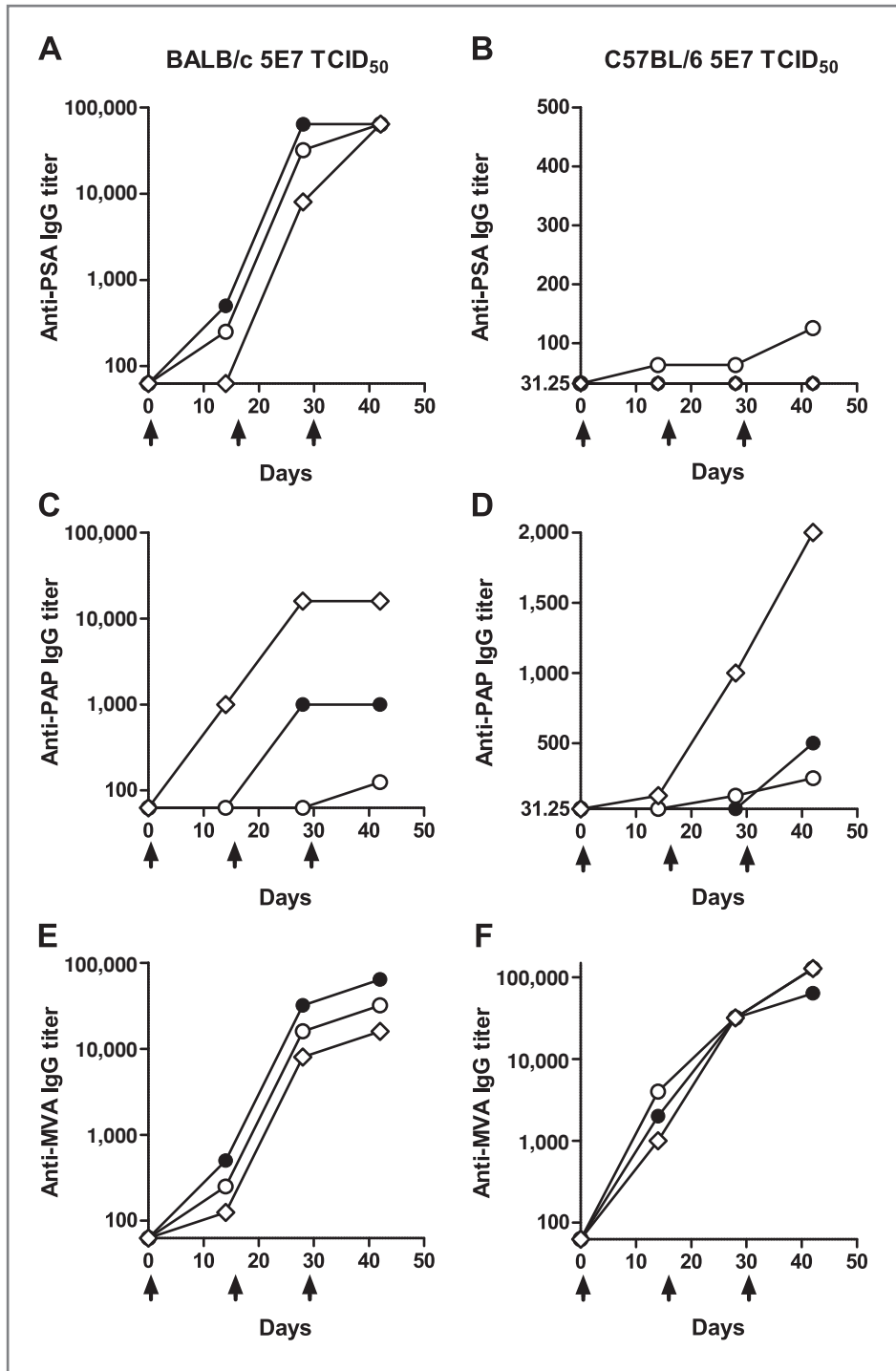


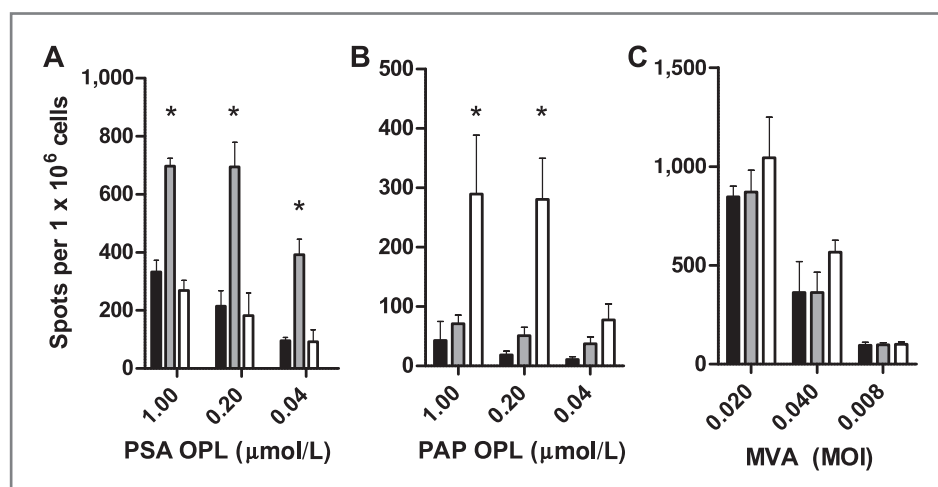
Figure 3. A and B, anti-PSA IgG, (C), (D) anti-PAP IgG, and (E) and (F) anti-MVA IgG titers were determined by ELISA from BALB/c (left column) or C57BL/6 mice (right column) treated with MVA-BN-PRO (●), MVA-BN-PSA-C1C2 (○), or MVA-BN-PAP-C1C2 (◇). Number of mice per group is 5; treatments indicated by black arrows. Data are representative of at least 2 independent experiments. The difference between the anti-PAP IgG titers of the MVA-BN-PRO and MVA-BN-PSA-C1C2-treated mice shown in (B) is not reproducible. It is due to random variation of titers between individual mice (see Supplementary Fig. S2).

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tumor models of prostate cancer. To evaluate the antitumor activity of these viruses against PAP-expressing tumors, new CT26-PAP cell lines were established and used in prevention therapy experiments. Whereas treatment with all 3 MVA-BN-PRO viruses showed significant protection as compared with TBS or MVA-BN-treated groups ( $P < 0.001$ ; RM-ANOVA), the

greatest suppression of tumor growth occurred in mice treated with MVA-BN-PAP-C1C2 (Fig. 5A–C). This difference was not only statistically significant against the TBS- or MVA-BN-treated groups ( $P < 0.001$ ), but also against the other PRO virus treatment groups ( $P < 0.01$ ). Likewise, in an evaluation of tumor size on day 20 alone, only the MVA-BN-PAP-C1C2

**Figure 4.** ELISPOT determination of the frequency of IFN- $\gamma$  producing T cells in restimulated splenocytes from BALB/c mice ( $n = 5/\text{group}$ ) treated with 5E7 TCID<sub>50</sub> of MVA-BN-PRO (black bars), MVA-BN-PSA-C1C2 (gray bars), or MVA-BN-PAP-C1C2 (white bars). Splenocytes were restimulated for 36 hours with (A) PSA OPL, (B) PAP OPL, or (C) MVA-BN (asterisks denote  $P < 0.05$  vs. the other 2 groups). Fewer than 10 spots per well were detected in restimulated splenocytes from TBS-treated mice or in media control wells for all groups (data not shown). Data are representative of 2 independent experiments.



treatment group had significantly smaller tumors compared with both TBS and MVA-BN (Fig. 5B;  $P < 0.001$ ; ANOVA). Similar results were obtained in tumor efficacy experiments using an independently isolated CT26-PAP clone (data not shown). Treatment with MVA-BN-PRO or MVA-BN-PAP-C1C2 did not reduce tumor growth in CT26-PAP tumor models when used in a therapeutic setting (data not shown).

In an effort to correlate antitumor efficacy with immune responses against PAP, the anti-PAP IgG titers and IgG2a/IgG1 isotype ratios were determined from individual mice at the end of the study (Fig. 5D–G). Mice treated with TBS had tumor-induced anti-PAP IgG antibody titers with a median value of 5,000 (Fig. 5D), and a low IgG2a/IgG1 ratio (Fig. 5G, median = 0.05). Treatment with MVA-BN-PRO or MVA-BN-PSA-C1C2 led to a similar anti-PAP IgG antibody titer and a slightly higher IgG2a/IgG1 ratio (Fig. 5G, median = 0.19 and 0.13, respectively). In contrast, mice treated with MVA-BN-PAP-C1C2 had overall higher anti-PAP IgG antibody titers than those induced by the growing tumor (Fig. 5D, median = 64,000). This was mainly due to a considerable increase in anti-PAP-specific antibodies of the IgG2a isotype (Fig. 5E, median = 64,000;  $P < 0.01$ ) resulting in a much higher IgG2a/IgG1 ratio as compared with the other groups (Fig. 5G, median = 2.00;  $P < 0.01$ ). These results indicate that growth of the CT26-PAP tumors promoted a Th2-biased humoral response against PAP as indicated by the relatively low IgG2a/IgG1 ratio in the mice treated with TBS. However, previous treatment with MVA-BN-PAP-C1C2 but not MVA-BN-PRO or MVA-BN-PSA-C1C2, significantly increased the anti-PAP IgG2a/IgG1 ratio, suggesting that this therapy induced a stronger and more Th1-biased immune response against the tumor antigen.

Therapeutic efficacy was also tested in an immunotherapy setting using the PSA-expressing E6 cell line, and the data from 2 replicate studies are shown combined in Fig. 6. The tumors in mice treated with any of the 3 MVA-BN-PRO viruses grew significantly slower than in mice treated with TBS or MVA-BN ( $P < 0.01$  or 0.001). Furthermore, a trend of improved efficacy was seen in the mice treated with MVA-BN-PSA-C1C2.

These mice had the smallest tumor volume at multiple time points (Fig. 6A and C) and no tumors larger than 350 mm<sup>3</sup> on days 17 or 18 (Fig. 6B). As described before in the PAP tumor model, anti-PSA IgG titers and IgG2a/IgG1 isotype ratios were determined in individual mice from each group at the end of the study (Fig. 6D–G). The growing tumor by itself induced a Th2-biased anti-PSA immune response with a low IgG2a/IgG1 ratio (Fig. 6G TBS group, median ratio = 0.125). Notably, treatment with MVA-BN-PSA-C1C2 significantly increased the anti-PSA IgG2a/IgG1 ratio above that of all other groups (Fig. 6G, median = 0.5;  $P < 0.01$ ). Therefore, in the immunotherapeutic setting only treatment with the vector encoding exosome-targeted PSA was capable of shifting the tumor-induced PSA response toward a Th1 bias. In prevention experiments, treatment with any of the 3 MVA-BN-PRO viruses provided similar, strong protection (data not shown).

Overall, these experiments showed that treatment with MVA-BN-PAP-C1C2 led to the strongest antitumor activity in a PAP-expressing tumor model. This enhancement correlated with an increased anti-PAP humoral response characterized by a high IgG2a/IgG1 ratio. Moreover, MVA-BN-PSA-C1C2 treatment resulted in a trend of improved antitumor activity against a PSA-expressing tumor model and also promoted an anti-PSA humoral response with a high IgG2a/IgG1 ratio.

## Discussion

Recently, prostate cancer immunotherapy has made great progress in the clinic and shows that the immune system can be utilized to extend patient survival (3, 5, 6). MVA-BN-PRO is being developed as a next-generation immunotherapeutic that may provide a higher frequency of protection or more prolonged survival by encoding 2 TAA, PSA, and PAP. To further enhance the activity of this vector, we have targeted PSA or PAP localization to exosomes. Exosome targeting has been shown to improve the antigenicity of poorly immunogenic proteins (7, 9) and has been explored with DNA vectors for improving antitumor efficacy with the model antigen OVA (11). We show here that the immunogenicity of PSA and PAP

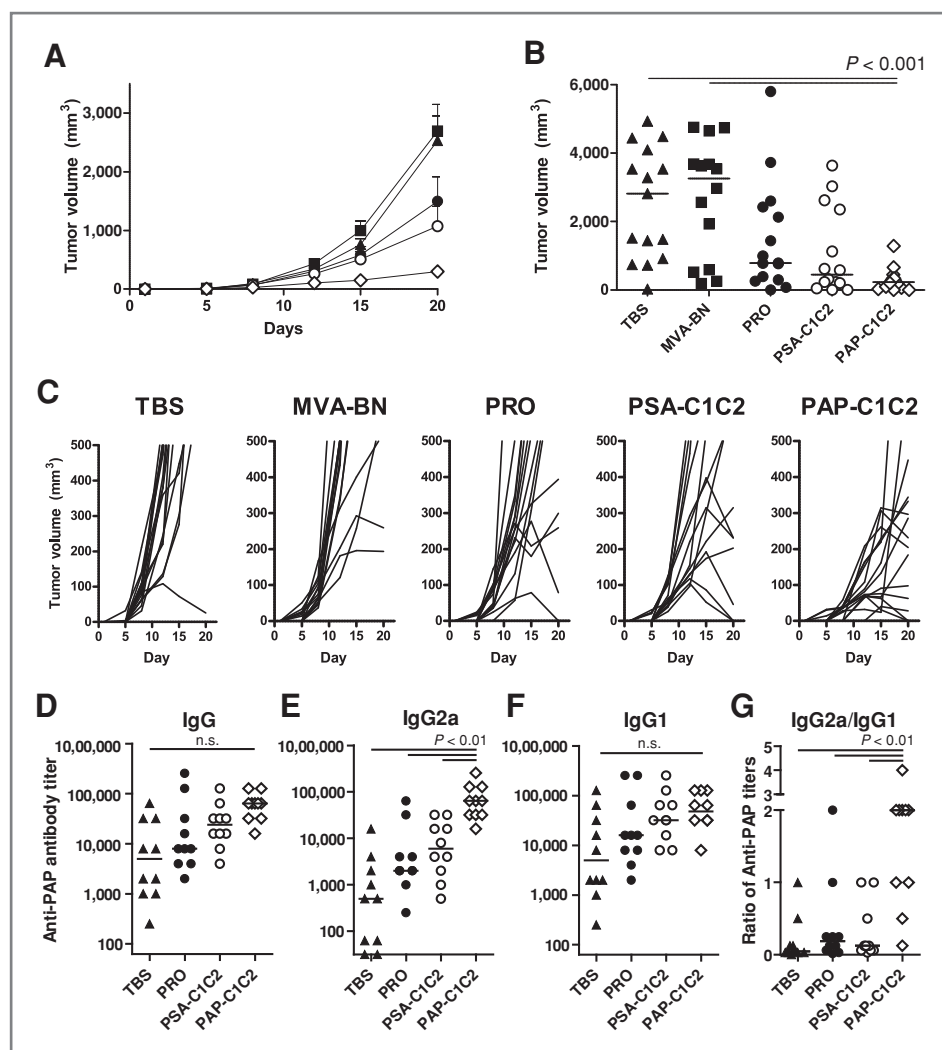


Figure 5. A–C, antitumor efficacy of MVA-BN-PRO viruses in the CT26-PAP tumor model. D–G, humoral responses of mice at the end of the study. BALB/c mice were treated q2wk  $\times$  3 with TBS ( $\blacktriangle$ ) or  $1 \times 10^8$  IU of MVA-BN ( $\blacksquare$ ), MVA-BN-PRO ( $\bullet$ ), MVA-BN-PSA-C1C2 ( $\circ$ ), or MVA-BN-PAP-C1C2 ( $\diamond$ ).  $1 \times 10^8$  IU was approximately equivalent to  $5 \times 10^7$  TCID<sub>50</sub> (data not shown). Fourteen days after the last treatment, mice were challenged by i.d. injection of  $1 \times 10^6$  CT26-PAP cells ( $n = 15$  mice/group; the day of tumor implantation was defined as day 1). A, average tumor volume of each group  $\pm$  SEM. B, tumor sizes of individual mice on day 20. A black line depicts the median value of each group. C, tumor size of each mouse over time with consecutive measurements from an individual mouse depicted as a continuous line. Serum was collected 35 days after tumor implantation (or earlier if mice were euthanized due to excessive tumor size) and anti-PAP IgG titers of the indicated isotype were determined by ELISA: (D) whole IgG, (E) IgG2a, and (F) IgG1 anti-PAP IgG titers, and (G) the calculated ratio of IgG2a/IgG1 anti-PAP IgG titers. A black line depicts the median value of each group.

can be improved by fusing either protein to the C1C2 domain of lactadherin. Compared with MVA-BN-PRO, treatment of mice with MVA-BN-PAP-C1C2 caused a striking improvement in the immune response against PAP and significantly enhanced antitumor activity against a PAP-expressing tumor. Similarly, the immune response against PSA was improved in mice treated with MVA-BN-PSA-C1C2, and a trend of enhanced efficacy was also found against a PSA-expressing tumor. The enhancement of antigenicity was specific to the exosome-targeted antigens, whereas the immunogenicity of the other wild-type transgene in the constructs was unchanged.

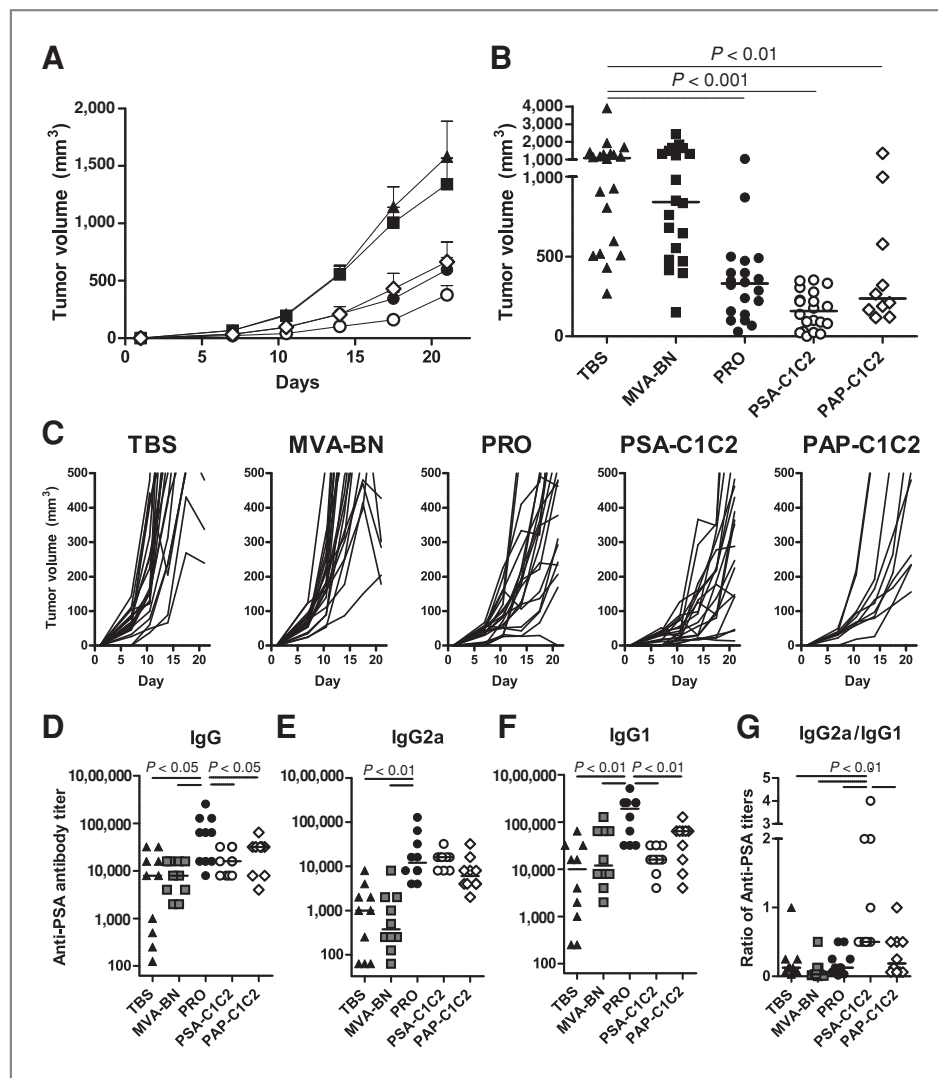
Treatment of mice on 2 different genetic backgrounds with MVA-BN-PAP-C1C2 resulted in dramatically higher, more rapidly induced antibody titers against PAP than mice treated with viruses encoding wild-type PAP. Wild-type PAP seems to be a particularly weak B-cell antigen in mice as compared with other transgenic antigens expressed by MVA or even with the use of PAP mixed in Freund's adjuvant (data not shown). Therefore, it is of particular significance that immunization with MVA-BN expressing exosome-targeted PAP led to an

improved B-cell response. Exosome targeting also improved humoral responses against PSA in C57BL/6 mice, which failed to raise antibodies against PSA when treated with viruses encoding wild-type PSA. This example shows that targeting a tumor-associated antigen like PSA for exosome localization can improve responses in situations where raising a humoral response is very difficult. This may be relevant for treating self-tolerant human patients in whom raising anti-PSA antibodies is challenging (19).

In these experiments, exosome targeting modulated the immune responses against PSA and PAP differently. Treatment of mice with constructs encoding exosome-targeted PSA led to an enhanced cellular response, but the antibody titers against PSA were only improved in C57BL/6 mice, and the induced titers were low. Mice treated with constructs encoding exosome-targeted PAP also had an increased cellular response, but in contrast to PSA, the humoral response against PAP was dramatically enhanced in BALB/c and C57BL/6 mice. It is unknown why exosome targeting affected the immune responses against these 2 antigens differently, but distinct



**Figure 6.** Antitumor efficacy in the E6 PSA-expressing tumor model. BALB/c mice were injected i.d. with  $1 \times 10^5$  E6 cells on day 1 and treated on day 1 and 15 with TBS ( $\blacktriangle$ ) or 5E7 TCID<sub>50</sub> of MVA-BN ( $\blacksquare$ ), MVA-BN-PRO ( $\bullet$ ), MVA-BN-PSA-C1C2 ( $\circ$ ), or MVA-BN-PAP-C1C2 ( $\diamond$ ). The data from 2 studies were combined ( $n = 20$  mice/group except  $n = 10$  for MVA-BN-PAP-C1C2 because it was only in 1 of the studies). A, average tumor volume of each of each group  $\pm$  SEM. At 2 time points the day of measurement differed by 1 day between the studies and was therefore graphed at an average time point (10.5 or 17.5). B, tumor volume of individual mice measured on either day 17 or 18, depending on the study. A black line depicts the median value of each group over time. C, tumor size of each mouse over time with consecutive measurements from an individual mouse depicted as a continuous line. Serum was collected 35 days after tumor implantation (or earlier if mice were euthanized due to excessive tumor size) and anti-PSA IgG titers of the indicated isotype were determined by ELISA: (D) whole IgG, (E) IgG2a, and (F) IgG1 anti-PSA IgG titers, and (G) the calculated ratio of IgG2a/IgG1 anti-PSA IgG titers. A black line depicts the median value of each group.



antigens induce qualitative and quantitative differences in immune responses. Furthermore, fusion of the 30-kDa C1C2 domain to different proteins could differentially affect a variety of characteristics predicted to affect immunogenicity. These include the efficiency of exosome localization, protein half-life, alterations to protein conformation caused by the C1C2 fusion, and levels of proteolytic processing.

Exosome targeting improved antitumor activity in 2 different tumor models. Although the immunologic mechanisms behind the enhanced antitumor efficacy are currently unknown, exosome targeting improved both the magnitude and quality of the immune responses. Mice treated with viruses encoding exosome-targeted PSA or PAP had an increased frequency of PSA- or PAP-specific T cells compared with mice treated with viruses encoding wild-type transgenes. Furthermore, in the context of tumors that promoted Th2-biased immunity against PSA or PAP, only treatment with the virus encoding the exosome-targeted form of the tumor antigen shifted immunity toward a Th1-biased response.

Although the most effective treatments induced a Th1-biased humoral response, no direct correlation was observed between antibody titers and tumor size when individual mice were analyzed (data not shown). Further work will be needed to determine the contribution of different aspects of immunity toward the enhanced therapeutic activity. However, the characteristics of immune responses provided by vectors encoding exosome-targeted antigens are desirable for treating prostate cancer patients.

In summary, this study shows that exosome targeting is a viable method for improving the immunogenicity and therapeutic efficacy of viral-based immunotherapies and warrants further investigation of modified PSA and PAP in poxvirus vectors for the treatment of prostate cancer.

#### Disclosure of Potential Conflicts of Interest

The authors declare that they are current or former employees and stockholders of Bavarian Nordic or BN ImmunoTherapeutics.

## Acknowledgments

The authors thank Tobias Njoku and Fareed Yahya for expert animal handling and husbandry.

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Received November 15, 2010; revised May 24, 2011; accepted June 9, 2011; published OnlineFirst June 13, 2011.

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