Published in final edited form as:

Cancer Immunol Immunother. 2010 November ; 59(11): 1685–1696. doi:10.1007/s00262-010-0895-0.

Generation of a Tumor Vaccine Candidate Based on Conjugation of a MUC1 Peptide to Polyionic Papillomavirus Virus-Like Particles (VLPs)

Sharmila Pejavar-Gaddy², Yogendra Rajawat¹, Zoe Hilioti¹, Jia Xue², Daniel F. Gaddy², Olivera J. Finn², Raphael P. Viscidi³, and Ioannis Bossis¹

¹ Department of Veterinary Medicine, University of Maryland, College Park, MD 20742

² Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

³ Stanley Division of Developmental Neurovirology, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD 21287

Abstract

Virus-like particles (VLPs) are promising vaccine technology due to their safety and ability to elicit strong immune responses. Chimeric VLPs can extend this technology to low immunogenicity foreign antigens. However, insertion of foreign epitopes into the sequence of self-assembling proteins can have unpredictable effects on the assembly process. We aimed to generate chimeric bovine papillomavirus (BPV) VLPs displaying a repetitive array of polyanionic docking sites on their surface. These VLPs can serve as platform for covalent coupling of polycationic fusion proteins. We generated baculoviruses expressing chimeric BPV L1 protein with insertion of a polyglutamic-cysteine residue in the BC, DE, HI loops and the H4 helix. Expression in insect cells yielded assembled VLPs only from insertion in HI loop. Insertion in DE loop and H4 helix resulted in partially formed VLPs and capsomeres, respectively. The polyanionic sites on the surface of VLPs and capsomeres were decorated with a polycationic MUC1 peptide containing a polyarginine-cysteine residue fused to twenty amino acids of the MUC1 tandem repeat through electrostatic interactions and redox-induced disulfide bond formation. MUC1- fully assembled VLPs induced robust activation of bone marrow-derived dendritic cells, which could then present MUC1 antigen to MUC1-specific T cell hybridomas and primary naïve MUC1-specific T cells obtained from a MUC1-specific TCR transgenic mice. Immunization of human MUC1 transgenic mice, where MUC1 is a self-antigen, with the VLP vaccine induced MUC1-specific CTL, delayed the growth of MUC1 transplanted tumors and elicited complete tumor rejection in some animals.

Keywords

Papillomaviruses; Chimeric VLPs; Immunotherapy; MUC1

Introduction

Immunotherapy directed against cancer is a very active area of research and one approach is to induce immune responses to tumor-associated antigens by active vaccination. Human mucin-1 (MUC1) is aberrantly expressed on a wide range of ductal adenocarcinomas and has been intensively studied as a candidate cancer vaccine antigen [1,2]. MUC1 is an

integral membrane protein with an extracellular, transmembrane and cytoplasmic domain. Much of the extracellular domain of MUC1 consists of a tandemly repeating sequence of 20 amino acids. This core peptide (TRD) encodes B and T cell epitopes. Particle-based MUC1 vaccines have received only limited attention. A liposome-based MUC1 vaccine (L-BLP25) that targets the exposed core peptide has shown some promise in phase II trials in lung and prostate cancer patients [3]. A hamster polyomavirus-derived VLP harboring two insertions of a MUC1 HLA-A2 restricted cytotoxic T cell (CTL) core peptide epitope has been shown to activate MUC1-specific CD8⁺ T cells, but the vaccine has not been tested in a tumor model [4].

Virus-like particles (VLPs), which resemble in size and morphology true capsids but do not incorporate viral genetic material are attractive vaccine candidates because they are non-infectious, they have the safety profile of subunit vaccines, but have superior immunological properties [5]. The particulate nature of VLPs, especially those in the size range of 40–50 nm [6], allows efficient uptake by dendritic cells (DCs), central players in initiation of the innate and adaptive immune response. VLPs can stimulate maturation of DCs, induce upregulation of major histocompatibility complexes (MHC) and costimulatory molecules, and lead to production of cytokines. VLPs serve as their own adjuvant, eliciting “danger signals”, often through stimulation via Toll-like receptors. As exogenous antigens, VLPs are processed and presented by MHC class II, but they can also be taken up and processed via the MHC class I pathway by cross presentation [7].

In order to expand the application of VLPs as vaccines, efforts have been made to devise chimeric VLPs that present epitopes of proteins that cannot self assemble [8]. The most common way in which this has been achieved is to construct fusions proteins of a VLP protein and a candidate vaccine peptide. Despite the described successes of this approach, there are limitations to the size and nature of epitopes that can be inserted into VLPs and prior success does not predict future feasibility with a new epitope. Alternatively, foreign vaccine proteins can be chemically conjugated to a pre-formed VLP. Biotinylated peptides and proteins have been linked to biotinylated human papillomavirus-derived VLPs via a streptavidin bridge [9,10]. A strategy initially developed for the purpose of targeting VLPs to specific cells for gene therapy involves coupling of a foreign protein to a mouse polyomavirus VLP via a docking system based on polyionic fusion peptides with complementary charges and an engineered disulphide bond [11]. We adapted this system to bovine papillomavirus (BPV)-based VLPs. We chose bovine over human papillomavirus because there is no pre-existing immunity to bovine viruses in most humans. Herein, we describe the construction of several chimeric VLP vaccine constructs formulated by coupling a 20 amino acid core MUC1 peptide with an N-terminal polyarginine cysteine tag to bovine papillomavirus VLPs with a polyglutamic acid cysteine sequence inserted into a surface exposed region of the L1 major capsid protein. Among all these constructs only the loop substitution that gave fully formed VLP (BPV-HI-E8c-MUC1) was capable of robust DC activation *in vitro* and thus was used as the candidate vaccine for functional and tumor rejection studies *in vivo*. We tested this vaccine in MUC1 transgenic (MUC1-Tg) mice, where human MUC1 is a self-molecule, thus enabling us to evaluate the potential risk for generation of autoimmunity. Our results show that the BPV-MUC1 vaccine elicited a functional anti-MUC1 cellular response and further caused effective tumor rejection in a transplantable tumor model.

Materials and methods

Generation of recombinant Baculoviruses and production of BPV VLPs

The entire open reading frame (ORF) of BPV L1 with a Kozak consensus and unique restrictions sites at each end (EcoRI/NotI) was artificially engineered by PCR-based gene

synthesis (GeneScript, Piscataway, NJ) and cloned in a pUC18 vector. The entire ORF was codon-modified for efficient expression in insect cells and contained insertion of a peptide with eight glutamic acid residues and a cysteine residue (E8C). We generated four synthetic L1 constructs, each with deletion of 9 wild type amino acids and insertion of the E8C peptide in the BC_(aa 51–61), DE_(aa 128–138), HI_(aa 346–356) and H4_(aa 412–422) loops, respectively (Supplemental Fig. 1). The modified BPV L1 genes were subcloned between the EcoR1/Not1 sites of the pORB baculovirus transfer vector (Orbigen, San Diego, CA). The transfer vectors were co-transfected with the Diamondback linear baculovirus DNA (Sigma-Aldrich) in *Spodoptera frugiperda* sf9 cells using the Escort reagent (Sigma), as suggested by the manufacturer. Five days posttransfection, the recovered recombinant baculoviruses were further amplified by large scale infections of sf9 cells. Small scale infections to confirm expression of the modified L1 proteins were conducted with 2×10^6 *Trichoplusia ni* (High Five) cells (Invitrogen, Carlsbad, Calif.), growing in 6-well plates and infected with 20 μ l of Baculovirus stocks. 72 hrs post-infection, the cells were lysed in 500 μ l of RIPA buffer and the clarified lysates were subjected to Western blot analysis using a mAb against BPV L1 (Millipore, Temecula, CA). For large-scale production of VLPs, approximately 2×10^9 *Trichoplusia ni* (High Five) cells (Invitrogen, Carlsbad, Calif.) growing in spinner flasks were infected with 40 ml of a high-titer recombinant baculovirus stock in 500 ml of TNM-FH/10%FBS. After 96 h of incubation at 27°C, the cells were harvested, and collected by centrifugation at 2,000 rpm (Sorvall FH18/250 rotor) for 5 min. The cell pellets were resuspended in VLP extraction buffer (50 mM Tris pH=7, 150 mM NaCl, 2 mM MgCl₂, 1 mM CaCl₂), and the VLPs released by 3 freeze-thaw cycles. The lysates were clarified by centrifugation at 8,000 $\times$ g for 30 min and further delipidated by Freon extraction. The lysates were then loaded onto a cushion of 40% sucrose in VLP buffer and centrifuged in a SW-28 rotor at 27,000 rpm for 4 h at 4°C. The resulting pellets were resuspended in VLP buffer with 0.5 M NaCl, loaded on a discontinuous OptiPrep gradient (26%, 32%, 38%), and centrifuged in a SW-40 rotor at 37,000 rpm for 4 h at 16°C. The bands collected at the 26/32 (capsomeres) and 32/38 (capsids) interfaces were diluted 3-fold with VLP buffer, loaded on a discontinuous CsCl gradient (densities of 1.1, 1.2, 1.3, and 1.4 gr/ml), and centrifuged in a SW-40 rotor at 37,000 rpm for 4 h at 4°C. Capsids were collected from the bottom of the 1.3 phase, and capsomeres from the 1.2/1.3 interface and stored frozen at -70 C. A small fraction (< 10% of the overall yield) of VLPs enter the 1.4 phase, and those were collected separately and were not used for the conjugation and immunization studies. These denser VLPs contain considerable more encapsidated nucleic acid. VLPs produced in insect cells may encapsidate some nucleic acid in a non-specific manner, especially after prolonged infections with recombinant Baculoviruses. The density of the light and heavy VLPs was 1.31 and 1.33 gr/ml, respectively. To estimate the amount of encapsidated nucleic acid, 200 μ g of light and heavy WT and chimeric VLPs, purified from 4 different batches, were treated for 2 hrs at 42 C with proteinase K in digestion buffer (20 mM Tris pH=8, 10 mM EDTA, 1% SDS), phenol/chloroform extracted, and the nucleic acid was precipitated by isopropanol. The pellet was resuspended in 10 μ l of water and the concentration of nucleic acid (assumed to be composed by equal amounts of DNA and RNA) was estimated using a NanoDrop ND-1000 spectrophotometer.

Conjugation of BPV polyanionic particles with a poly-arginine MUC1 peptide

To construct a vaccine based on the epithelial antigen mucin-1 (MUC1), we synthesized the 20 amino acids long MUC1 tandem repeat peptide with N-terminal polyarginine, cysteine, and GSG spacer sequences (RRRRRRRCGSGGVTSAPDTRPAPGSTAPPAH), R8C-MUC1. The presence of the polyarginine moiety allows docking of the peptide to the polyanionic site (E8C) inserted in the various loops of the mutant L1 particles. Covalent cross-linking between the two cysteine residues should render this association irreversible under oxidizing conditions. For the conjugation reactions, purified L1 particles were

dialysed in conjugation buffer (20 mM Tris/HCl pH=7.5, 150 mM NaCl, 5% glycerol, 0.5 mM CaCl₂) and then the peptide and the oxidizing reagents were added, allowing the reaction to proceed for 16 hrs at 4 C. Initially we used a 4:1 ratio of oxidized (GSSG):reduced (GSH) glutathione in the conjugation reaction (2 mM GSSG, 0.5 mM GSH) and estimated the highest possible molar ratio of peptide/assembled L1 protein that would not result in aggregation. The ratio of GSSG:GSH was then further optimized by testing ratios from 8:1-2:1. Titration experiments were also conducted to evaluate conjugation efficiency under variable ionic strength (100–400 mM NaCl). At the end of the incubation, the reaction mixtures were applied to a size-exclusion column (Sephadex G-100, Pharmacia, volume 20 ml, flow rate 1 ml/min, 10 mM Tris/HCl (pH=7.4), 150mM NaCl, 0.5 mM CaCl₂) to remove unconjugated peptide and exchange buffer. Conjugated particles that eluted in the void volume were identified by the presence of the L1 protein on SDS-PAGE. The conjugated particles were analyzed by electron microscopy. Conjugation efficiency was estimated using an ELISA assay with an anti-MUC1 mAb (BD Pharmingen, San Diego, CA). Free R8C-MUC1 peptide was used for generating a standard curve.

Electron microscopy and immunogold labeling

To facilitate direct visualization of the constructs, an aliquot of diluted particles was placed on 300-mesh formvar/carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA), negatively stained with 2% phosphotungstic acid (pH=7.0) and examined by transmission electron microscopy (TEM). For immunogold labeling, an aliquot of either conjugated or unconjugated (negative control) diluted particles was adsorbed onto 300-mesh formvar/carbon-coated nickel grids and blocked with 3% BSA in TBS for 2 hrs. The anti-MUC1 monoclonal antibody was diluted 1:25 in 1% BSA/TBS and adsorbed to the grid for 1 hr at room temperature. Bound IgG was detected by incubation for 1 h at room temperature with colloidal-gold-conjugated (6 nm) goat anti mouse IgG (Electron Microscopy Sciences, Hatfield, PA) diluted 1/50 in blocking solution. The grids were negatively stained with 1% sodium silicotungstate (pH=6.5) and examined by TEM.

Cell lines and Mice

Cells were cultured in complete DMEM containing 10% FBS, penicillin and streptomycin, L-glutamine, sodium pyruvate, nonessential amino acids, HEPES buffer and β -mercaptoethanol. The previously described MUC1-specific T cell hybridoma line VF5 was the source of the TCR for generation of VFT mice [12]. VFT mice transgenic for a T cell receptor (TCR) specific for an MHC Class II-restricted epitope, a 12 amino acid peptide GVTSAPDTRPAP derived from the epithelial cell mucin1, were used as source of antigen-specific T cells [13]. MUC1-Tg mice (6–8 wk old) on a C57BL/6 background were purchased from Dr. S. Gendler (Mayo Clinic, Scottsdale, AZ), and conventional C57BL/6 mice (wild type - WT) were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a standard pathogen-free environment at the University of Pittsburgh Cancer Institute and treated in accordance with the guidelines set by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Generation of bone marrow derived dendritic cells (BMDC) and maturation assays

BMDC were generated as described previously [14]. Briefly, bone marrow (BM) cells removed from the tibiae and femurs of C57BL/6 mice were cultured in complete AIM V (cAIM V) medium containing penicillin and streptomycin, L-glutamine, sodium pyruvate, nonessential amino acids and HEPES buffer (without the addition of 2-ME, a reducing agent, in order to prevent destruction of the dicysteine bond between the conjugated R8c-MUC1 peptide and the E8c core of the chimeric VLPs) containing 20 ng/ml each of GM-CSF. Cells were fed on days 2, 4, and 6 by adding 5 ml of cAIM V containing 20 ng/ml GM-CSF. On day 7 of culture, immature DC were harvested and loaded with the various BPV

chimeric particles or soluble antigen for 24h. Following culture, cell supernatants were harvested for the detection of a T cell stimulatory cytokine, IL-12, and the cells were stained for the upregulation of costimulatory and antigen presenting molecules. Briefly, DC were stained with allophycocyanine-conjugated anti-CD11c together with Fluorescein isothiocyanate-conjugated anti-CD40, anti-CD80, anti-CD86 or anti-MHC II antibodies (PharMingen, San Diego, CA). The cells were blocked with Fc block prior to staining to prevent nonspecific binding of antibodies. Flow cytometry was performed using a FACS-LSRII and the data analyzed with FACSDiva software (BD Pharmingen, San Diego, CA). The percent of BMDC expressing high levels of costimulatory molecules was determined by gating on the cells that were positive in untreated cells.

Antigen presentation and T cell stimulation assays

Spleens from VFT mice were harvested and processed to single cell suspension. Following lysis of RBC, 1, 5, or 25 μ g of chimeric MUC-conjugated VLPs were added to total splenocytes in cAIM V media (without the addition of β -ME). Splenocytes were also treated with increasing amounts (10, 50, and 250 ng) of free MUC1 20-mer peptide. Based on the experiments conducted to determine R8c-MUC1 conjugation efficiency on chimeric VLPs, these are the amounts of MUC1 present on 1, 5 and 25 μ g of conjugated chimeric VLPs. Following 3 days of culture in cAIM V, supernatants were harvested to determine IFN γ production as a measure of antigen-specific T cell stimulation, using ELISA (BD Pharmingen, San Diego, CA). The cells were then re-cultured in cAIM V containing 1 μ Ci/100 μ l tritiated thymidine for 24h to measure proliferation. Twenty-four hours following culture, cells were lysed using a semiautomatic cell harvester (Skatron Instruments, Sterling, VA) and the amount of incorporated tritiated thymidine measured using a β -counter.

Vaccination and tumor challenge

MUC1-Tg mice were immunized subcutaneously (s.c.) in the right flank with 5 μ g of BPV-HI-E8c-MUC1 in 100 μ l of PBS (vaccine) or 5 μ g of BPV-HI-E8c (vector control) or PBS (negative control). Two more boosts were administered similarly at 2-wk intervals. Two weeks following the final boost, mice were challenged with 5×10^4 RMA-MUC1 cells s.c. also in the right flank. RMA-MUC1 cells are a T cell lymphoma line on a C57BL6 background that was transfected by electroporation with the pR/CMV-MUC1 plasmid containing full-length MUC1 cDNA with 42 tandem repeats. [15]. Tumor growth and general condition of the mice were monitored every 2–3 days. Tumor size was recorded using vernier calipers and mice were sacrificed according to the University of Pittsburgh IUCAC guidelines, when the tumor reached a size of 2cm.

T cell activation assays

Spleen cells were prepared by mechanical disruption and RBC lysis using red blood cell lysing buffer (Sigma Aldrich, St. Louis, MO). The splenocytes were then stained with Carboxyfluorescein succinimidyl ester (CFSE) as well as antibodies to CD4 and CD8 (BD Pharmingen, San Diego, CA) to evaluate for proliferation and stimulated *in vitro* with BPV-HI-E8c (5 μ g/ml) or BPV-HI-E8c-MUC1 (5 μ g/ml) or concanavalin A (ConA -5 μ g/ml) for 1 or 5 days. On day 1, cells were stained with anti-CD8 and anti-CD107a antibodies (BD Pharmingen, San Diego, CA) and evaluated for lytic capabilities by measurement of CD107a [16]. On day 5, cell culture supernatants were harvested for measurement of IFN γ production by ELISA (BD Pharmingen, San Diego, CA) and cell proliferation was assessed using flow cytometry.

MUC1-specific ELISA

Fourteen days after the last boost, blood samples were collected by tail bleeding, and the serum was tested for the presence of MUC1-specific antibodies with a MUC1-specific ELISA. Briefly, 96-well Immulon 4 plates (Dynatech, Chantilly, VA) were coated at room temperature overnight with 10 µg/ml of 100-aa MUC1 peptide in PBS. The plates were washed three times with PBS and incubated with 1:40 dilution of the immune serum for 2 h at room temperature (RT). After three washes with PBS/0.1% Tween 20, the plates were incubated with polyclonal goat anti-mouse-IgG HRP-conjugated secondary Abs (Sigma, St. Louis, MO) for 1 h at RT. The plates were washed three times with PBS/0.1% Tween 20 and then incubated with the TMB substrate (BD Biosciences, San Diego, CA) for 30 min. The reaction was stopped with 2.5 M sulfuric acid, and the absorbance was measured at 450 nm.

Statistical analysis

The statistical significance for two group comparisons was calculated by a paired, two-tailed t test. Multiple group comparisons were performed using one-way analysis of variance and Fisher's least significant difference. Values of $p < 0.05$ were considered significant.

Results

Production of polyanionic BPV chimeric VLPs

In the present study, we generated recombinant baculoviruses expressing chimeric BPV L1 protein with insertion of a polyglutamic cysteine peptide and replacement of native residues in the BC, DE and HI loops and the H4 helix respectively. Western blot analyses of lysates from Hi5 cells infected with the baculoviruses revealed different patterns of expression for the L1 protein (Fig. 1a). The BPV-BC-E8c baculovirus construct displayed extensive degradation of L1; the H4 had moderate degradation, while degradation of L1 in the BPV-DE-E8c and BPV-HI-E8c was minimal. Large scale infections of insect cells with the four recombinant baculoviruses, and subsequent purification in step gradients revealed different banding patterns for the four constructs. For the BPV-BC-E8c preparation, no L1 reactivity (by Western blot) was observed anywhere in the gradient, suggesting no particle assembly. The majority of the L1 reactivity in the BPV-H4-E8c preparation was detected in fractions with lower density, while, the majority of BPV-HI-E8c was detected in fractions with higher density. The BPV-DE-E8c had L1 reactivity in fractions of both low and heavy density with no obvious peak, indicating the presence of several assembled forms in this preparation. No L1 degradation products were observed in the purified preparations (Fig. 1b). Analysis of the 3 purified preparations by electron microscope confirmed that the BPV-HI-E8c was composed primarily of fully assembled VLPs with approximate size of 45–55 nm (Fig. 1c), BPV-H4-E8c contained capsomeres of approximately 4–5 nm (Fig. 1d), and the BPV-DE-E8c was partially assembled VLPs (Fig. 1e). As was described in the methods section, fully formed VLPs are recovered in two predominant fractions. The light fraction, with density of 1.31gr/ml, is the predominant one, and that was what we used for immunizations. The amount of nucleic acid present in this fraction is 7.8 ± 1.5 ng per µg of VLP. Given that mice are immunized with 5 µg of VLP, it is not expected that this amount of nucleic acid is enough to serve as an adjuvant. Even CpG oligonucleotides, which are designed for increased immunogenicity, are used at doses of several micrograms. The denser fraction, with density of 1.33 gr/ml, contains 56 ± 5 ng per µg of VLP. We will evaluate the potential for increased immunogenicity in these VLPs in future studies.

Conjugation of purified VLPs and capsomeres with the R8C-MUC1 peptide

Based on SDS-PAGE and electron microscopy, particle preparations were more than 90% pure. Therefore, the amount of particles (in μg of protein) was assumed to represent the amount of L1 protein. L1 is composed by 495 aa with a theoretical MW of 55.56 kd, while the 32-mer peptide has a theoretical MW of 3.44 kd. A 16:1 L1/peptide mass ratio was therefore assumed to represent a 1:1 molar ratio. A peptide/L1 molar ratio of greater than 2:1 resulted in substantial aggregation of particles, and subsequent conjugation reactions utilized that ratio which is equivalent to 1 μg of peptide for every 8 μg of purified particles. The effect of ionic strength and the ratio of oxidized/reduced glutathione (GSSG:GSH) in the conjugation reaction was also optimized. The conjugation efficiency, as estimated by the amount of MUC1 reactivity in ELISA assays, was substantially inhibited at NaCl concentrations greater than 150 mM, and a GSSG:GSH ratio of 5:1 was found to be optimal without affecting the morphology of particles and/or inducing aggregation (Supplemental Fig. 2a–b). Based on our quantitative ELISA estimates, each μg of HI VLPs had 8.5 ± 1.0 ng of conjugated peptide (14% conjugation efficiency), while each μg of H4 capsomeres had 12.3 ± 1.1 ng of conjugated peptide (20% conjugation efficiency) (Supplemental Fig. 2c). To further evaluate this estimate, various amounts of conjugated BPV-HI-E8c VLPs (30, 20, 10 μg) and free R8c-MUC1 (100 ng) were subjected to SDS-PAGE and Coomassie brilliant blue staining (Fig. 2a). In comparison with the intensity of staining of 100 ng of free R8c-MUC1, 10 μg of conjugated BPV-HI-E8c VLPs appear to contain ~60–80 ng of peptide, thus confirming our estimations based on the ELISA assays. Furthermore, immunogold labeling an anti-MUC1 mAb verified the integrity of the VLPs and successful attachment of the MUC1 peptide (Fig. 2b). The conjugation efficiency of VLPs in the present study is higher than what has been previously reported for conjugation of antibody fragments in polyomavirus VLPs using similar strategy (11). The reason for increased conjugation efficiency in our studies is probably due to the smaller peptide and reduced steric hindrance (MUC1-32mer vs Fab fragment).

Differential ability of MUC1-conjugated chimeric VLPs and capsomeres to activate DC

Native papillomavirus VLPs are known to activate DC. To determine whether chimeric VLPs and capsomeres retain this property, we exposed bone marrow-derived DC to the chimeric constructs and assessed for increased expression of several DC activation and maturation markers. Immature BMDC were left untreated, or loaded with 250 ng of the 20aa MUC1 free peptide, 5 μg of the various BPV particles without MUC1 or conjugated to MUC1, and wild type BPV. Twenty-four hours later, DC were stained with a monoclonal antibody specific for CD11c, a DC specific marker, and with antibodies specific for activation/maturation markers CD40, CD86, CD80 and MHC Class II and analyzed by flow cytometry. Robust upregulation of costimulatory molecules on DC was seen following treatment with WT BPV (Fig. 3a). The BPV-HI-E8c (unconjugated) and BPV-HI-E8c-MUC1 (conjugated) VLPs retained the ability to significantly (unconjugated vs mock: $p=0.000195$; conjugated vs mock: $p=0.0000035$) increase the expression of activation and maturation molecules on DC (Fig. 3a and Supplemental Fig. 3). The BPV-H4-E8c capsomeres, however, induced an increase in some activation markers but the response was lower than that of fully formed VLPs. We also tested for induction of IL-12 production, which is an important cytokine that promotes generation of T-helper 1 responses [17]. Extending our findings with the cell surface maturation markers, only DC exposed to either WT BPV, conjugated or unconjugated BPV-HI-E8c VLPs produced significant (unconjugated vs. mock: $p=0.0236$; conjugated vs. mock: $p=0.00346684$) levels of IL-12p40 (Fig. 3b). It is important to note that even though there was a significant increase in IL-12 production following treatment with fully formed VLPs, it was lower than the amount produced following treatment with WT BPV. We speculate that this is due to the modifications made in the VLP to accommodate the MUC1 peptide. Therefore, subsequent

in vivo experiments to evaluate the immunogenicity and efficacy were conducted with chimeric MUC-conjugated fully formed VLPs.

MUC1 conjugated on chimeric BPV particles can be processed and presented to primary MUC1-specific T cells

In order to induce adaptive, antigen-specific immunity, APC need to be able to uptake and also process the correct peptides, when antigen is delivered by various vehicles, such as chimeric VLPs. To test the ability of the MUC1-conjugated chimeric BPV VLPs to activate T cells in the context of many different APC, we used splenocytes from MUC1-specific TCR transgenic VFT mice that provide the APC and also a high frequency of naive MUC1-specific T cells. We cultured splenocytes for three days with various amounts of soluble MUC1 peptide or with chimeric MUC1-conjugated or unconjugated VLPs. Supernatant was harvested for evaluation of IFN γ production, and the cells were cultured for an additional day in media containing [3 H]-thymidine to evaluate T cell proliferation. Following culture with the MUC1 decorated chimeric BPV particles, but not with the unconjugated BPV particles, MUC1-specific TCR transgenic splenocytes underwent proliferation (data not shown) and secreted significant ($p < 0.01$ for both conjugated VLP and capsomeres against peptide alone for each concentration shown) amounts of IFN γ (Fig. 4). The response was significantly higher when MUC1 was delivered conjugated to VLPs than as a free peptide.

BPV-HI-E8c-MUC1 vaccine activates primarily CD8 $^+$ T cells

MUC1-Tg mice contain human MUC1 that is both spatially and temporally expressed similarly to that in humans and serves as a model to assess the ability of the vaccine to overcome potential tolerance in these mice to this endogenous tumor-associated antigen. Thus, MUC1-Tg mice were immunized three times, 2 weeks apart with 5 μ g per dose, each of vector alone (BPV-HI-E8c) or the vaccine (BPV-HI-E8c-MUC1), or PBS (controls). Two weeks following the last booster, mice were injected with RMA-MUC1 tumor cells to mimic tumor development in humans in the presence of pre-existing immunity. Approximately 11 days following vaccination, some mice from each group were sacrificed and spleens were harvested to evaluate anti-MUC1 immunity. Splenocytes were CFSE-labeled and cultured in the presence of BPV-HI-E8c-MUC1 or BPV-HI-E8c to further expand the number of MUC1-specific T cells. Five days post culture, supernatant was harvested to evaluate IFN γ , and cell proliferation was measured using CFSE dilution detected by flow cytometry. A significant increase in proliferation of MUC1-specific CD8 $^+$ T cells was seen in MUC1-Tg mice vaccinated with BPV-HI-E8c-MUC1 as compared to PBS treated controls and vector control BPV-HI-E8c (Fig. 5a). However, we detected only a slight increase in specific CD4 $^+$ T cell proliferation (Fig 5b). Further evaluation of the functional capacity of the splenocytes showed a trend ($p=0.058$) towards the production of increased levels of an important immunomodulatory cytokine, IFN γ , by bulk splenocytes from mice that were vaccinated compared to controls treated with vector or PBS (Fig. 5C). Based on the proliferation data, we hypothesize that the activated CD8 $^+$ T cells were the major producers of IFN γ . Increased activation of CD8 $^+$ T cells in MUC1-Tg mice immunized with the MUC1 vaccine was further seen in the expression of lysosome-associated markers, CD107a (LAMP-1) (data not shown). Appearance of this marker has been shown to be indicative of cytolytic activity of CTL [18].

Further evaluation of the humoral arm of the immune response showed no detection of anti-MUC1 antibodies (Supplemental Fig. 4) in serum samples from infected mice. This was not surprising considering that the short MUC1 peptide contained in the VLP is processed into shorter epitopes by DC for presentation primarily to T cells. These mice were however able to generate a robust humoral immunity against BPV VLP, showing that they still had a functional B cell compartment (data not shown).

Slower growth kinetics and decreased tumor mass in vaccinated mice

To determine if the vaccine-elicited immune response could affect tumor growth, we monitored the tumor size every 2–3 days up to 60 days. By day 30, 100% of PBS-treated control MUC1-Tg mice were sacrificed because their tumors reached a size of 2 cm (Fig. 6a). In contrast, mice immunized with the BPV-HI-E8c vector control (5 μ g per dose) or the BPV-HI-E8c-MUC1 vaccine (5 μ g per dose), both showed a lag in tumor appearance, as well as slower growth kinetics, with a strikingly longer time to appearance documented in BPV-HI-E8c-MUC1 vaccinated animals (Fig. 6b–c). Measuring the tumors in the two surviving groups on the day that the PBS-treated group had to be sacrificed due to the tumors reaching the size of 2cm, showed a significantly smaller tumor mass in mice that received the BPV-HI-E8c-MUC1 vaccine (Fig. 6d) compared to vector or PBS-treated animals.

Discussion

Vaccines based on the tumor antigen MUC1 could have wide applications against several adenocarcinomas including breast, lung, pancreatic and colon [19,20,21]. Human MUC1 is one of the few well-characterized tumor neoantigens [2]. In several epithelial tumors, polarized expression of MUC1 is lost, and the normally heavily glycosylated protein is over-expressed in hypo- and unglycosylated forms [22]. This abnormal glycosylation exposes novel B and T cell epitopes within the TRD making this an immunodominant region and attractive as a candidate cancer vaccine antigen [2]. Low-frequency CTL and low-titer IgM responses against MUC1 are present in cancer patients but do not prevent cancer growth [23,24,25]. Therefore, boosting MUC1-specific immunity with VLP vaccines could lead to the development of successful cancer immunotherapy.

In the present study, we constructed chimeric papillomavirus VLP that displayed a repetitive array of polyanionic docking sites on its surface. This type of VLP can serve as a generic vaccine platform for the covalent coupling of polycationic fusion proteins and/or oligopeptides. The major advantage of this approach is that a new VLP will not have to be designed and produced for every new immunogen. In addition, the presence of several docking sites on the VLP would allow for coupling of several epitopes. The feasibility of this approach has been previously demonstrated with polyionic mouse polyomavirus VLPs decorated with by-specific antibodies for the purpose of gene targeting on specific cells [11].

Several papillomavirus VLPs are potent activators of bone marrow derived DCs and can induce their phenotypic and functional maturation [26,27,28]. In contrast, most studies have shown that polyomavirus-based VLPs do not induce maturation of DCs in vitro [29–31]. It is worth mentioning that polyomaviruses and papillomaviruses exhibit differences in receptor specificity and internalization pathways [32]. Those properties may have arisen from differences in selective pressure during evolution of papillomaviruses and polyomaviruses, and may explain their ability in activating professional antigen presenting cells. Prime examples of successful papillomavirus VLP vaccines are Gardasil, which is composed of HPV-6, -11, -16, 18 VLPs, and Cervarix, which contains HPV-16, -18 VLPs. These vaccines reduce HPV disease by greater than 90%, and are currently used as cervical cancer vaccines worldwide [33]. The excellent immunological properties, as well as their safety profiles, makes papillomavirus VLPs ideal candidates for designing a generic vaccine platform.

The papillomavirus virion contains 72 pentamers (capsomeres) of L1 protein [34]. The L1 protein is capable of self-assembly into capsid-like structures that are morphologically indistinguishable from native virions when expressed in eukaryotic cells [35,36]. Deletions of native amino acids and/or insertion of foreign amino acid sequences into self-assembling proteins can have unpredictable and often deleterious effects on the assembly process, and

only relatively short amino acid sequences can be inserted [37–40]. The L1 monomer contains 12 β -strands, 6 loops (BC, CD, DE, EF, FG, HI), and 5 helices (H1–H5). Most of the loops are highly exposed towards the outer surface of the capsid, and insertion of the polycationic docking site in these areas is predicted to be displayed on the outer surface of VLPs [41]. In the present study, we explore the possibility of inserting a polyglutamic residue in the BC, DE and HI loops and the H4 helix of BPV L1. Even though the replacement of amino acids in the 3 loops were made in regions of the capsid that theoretically are not part of the structural core, only replacement in the HI loops yielded fully formed VLPs. These observations reaffirm that even small alteration on the capsid of papillomavirus VLPs cannot be tolerated.

Conjugation of the fusion peptide was successful in both chimeric VLPs and capsomeres, albeit with different efficiency. However, only the construct that consisted of fully formed VLP made by substitutions in the HI loop (BPV-HI-E8c-MUC1) caused robust maturation of DC, both in terms of up-regulation of costimulatory molecules, as well as, cytokine production. Importantly, the loop substitution that gave only capsomeres did not cause significant DC maturation.

Vaccination using this MUC1 vaccine (BPV-HI-E8c-MUC1) in MUC1-Tg mice, where human MUC1 is a self molecule (model for tolerance), showed us that this vaccine was capable of activating MUC1-specific CD8⁺ cytotoxic T cell (CTL), both in terms of proliferation and function. Only a slight increase in MUC1-specific CD4⁺ T cell proliferation was observed in MUC1-Tg mice following vaccination. This may be due to the extremely low antigen dose contained within the vaccine. Significant T-helper (CD4⁺ T cells) activation may require much higher amounts of antigen that can be achieved by conjugating a longer peptide with a higher number of tandem repeats, an approach which we are currently exploring.

In line with the generation of an anti-MUC1 immunity following vaccination, a significant delay in the MUC1+ tumor appearance and growth and a decreased tumor size was observed in MUC1-Tg mice. The BPV-HI-8Ec-MUC vaccine was administered before tumor challenge, demonstrating its potential efficacy for prevention of recurrent disease. In future studies, we plan to explore the efficacy of the vaccine to treat established tumors. A certain degree of protection was also seen in the vector control treated animals. This non-specific protection is probably due to the significant anti-viral innate immunity that might act as by-stander immunity against the tumor cells. Apparently, in this tumor model, MUC1-specific CTL was the only measurable effector mechanism, and was sufficient to see significant efficacy. However, activation of CD4⁺ T cells may be needed in order to achieve better results than those observed. It is accepted that for a better CD8⁺ T cell activation CD4⁺-help is required. Although the known immunogenic properties of papillomavirus VLPs are likely important for the efficacy of the BPV-HI-8Ec-MUC vaccine, future studies are needed to further characterize the mechanism of immune protection afforded by the vaccine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the Virginia-Maryland Regional College of Veterinary Medicine (to I.B.) and NIH grant P01 CA073743 (to O.J.F.). SPG was supported by The Sass Foundation for Medical Research Postdoctoral Fellowship We would like to thank Hamp Edwards and Kristen Gambles from the University of Maryland for help with electron microscopy and insect cell culture work.

References

1. Li Y, Cozzi PJ. MUC1 is a promising therapeutic target for prostate cancer therapy. *Curr Cancer Drug Targets*. 2007; 7:259–271. [PubMed: 17504123]
2. Vlad AM, Kettel JC, Alajez NM, et al. MUC1 immunobiology: from discovery to clinical applications. *Adv Immunol*. 2004; 82:249–293. [PubMed: 14975259]
3. North S, Butts C. Vaccination with BLP25 liposome vaccine to treat non-small cell lung and prostate cancers. *Expert Rev Vaccines*. 2005; 4:249–257. [PubMed: 16026241]
4. Dorn DC, Lawatscheck R, Zvirbliene A, et al. Cellular and humoral immunogenicity of hamster polyomavirus-derived virus-like particles harboring a mucin 1 cytotoxic T-cell epitope. *Viral Immunol*. 2008; 21:12–27. [PubMed: 18355119]
5. Grgacic EV, Anderson DA. Virus-like particles: passport to immune recognition. *Methods*. 2006; 40:60–65. [PubMed: 16997714]
6. Fifis T, Gamvrellis A, Crimeen-Irwin B, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. *J Immunol*. 2004; 173:3148–3154. [PubMed: 15322175]
7. Dickgreber N, Stoitzner P, Bai Y, et al. Targeting antigen to MHC class II molecules promotes efficient cross-presentation and enhances immunotherapy. *J Immunol*. 2009; 182:1260–1269. [PubMed: 19155471]
8. Boisgérault F, Morón G, Leclerc C. Virus-like particles: a new family of delivery systems. *Expert Rev Vaccines*. 2002; 1:101–109. [PubMed: 12908517]
9. Chackerian B, Lowy DR, Schiller JT. Conjugation of a self-antigen to papillomavirus-like particles allows for efficient induction of protective autoantibodies. *J Clin Invest*. 2001; 108:415–423. [PubMed: 11489935]
10. Peacey M, Wilson S, Baird MA, et al. Versatile RHDV virus-like particles: incorporation of antigens by genetic modification and chemical conjugation. *Biotechnol Bioeng*. 2007; 98:968–977. [PubMed: 17546687]
11. Stubenrauch K, Gleiter S, Brinkmann U. Conjugation of an antibody Fv fragment to a virus coat protein: cell-specific targeting of recombinant polyoma-virus-like particles. *Biochem J*. 2001; 356:867–873. [PubMed: 11389696]
12. Vlad AM, Muller S, Cudic M, et al. Complex carbohydrates are not removed during processing of glycoproteins by dendritic cells: processing of tumor antigen MUC1 glycopeptides for presentation to major histocompatibility complex class II-restricted T cells. *J Exp Med*. 2002; 196:1435–1446. [PubMed: 12461079]
13. Alajez NM, Schmielau J, Alter MD, et al. Therapeutic potential of a tumor-specific, MHC-unrestricted T-cell receptor expressed on effector cells of the innate and the adaptive immune system through bone marrow transduction and immune reconstitution. *Blood*. 2005; 105:4583–4589. [PubMed: 15746083]
14. Turner MS, Cohen PA, Finn OJ. Lack of effective MUC1 tumor antigen-specific immunity in MUC1-transgenic mice results from a Th/T regulatory cell imbalance that can be corrected by adoptive transfer of wild-type Th cells. *J Immunol*. 2007; 178:2787–2793. [PubMed: 17312122]
15. Soares MM, Mehta V, Finn OJ. Three different vaccines based on the 140-amino acid MUC1 peptide with seven tandemly repeated tumor-specific epitopes elicit distinct immune effector mechanisms in wild-type versus MUC1-transgenic mice with different potential for tumor rejection. *J Immunol*. 2001; 166:6555–6563. [PubMed: 11359807]
16. Devèvre E, Romero P, Mahnke YD. LiveCount Assay: concomitant measurement of cytolytic activity and phenotypic characterisation of CD8(+) T-cells by flow cytometry. *J Immunol Methods*. 2006; 311:31–46. [PubMed: 16527300]
17. Goriely S, Goldman M. Interleukin-12 family members and the balance between rejection and tolerance. *Curr Opin Organ Transplant*. 2008; 13:4–9. [PubMed: 18660699]
18. Aktas E, Kucuksezer UC, Bilgic S, et al. Relationship between CD107a expression and cytotoxic activity. *Cell Immunol*. 2009; 254:149–154. [PubMed: 18835598]
19. Acres B, Limacher JM. MUC1 as a target antigen for cancer immunotherapy. *Expert Rev Vaccines*. 2005; 4:493–502. [PubMed: 16117706]

20. Byrd JC, Bresalier RS. Mucins and mucin binding proteins in colorectal cancer. *Cancer Metastasis Rev.* 2004; 23:77–99. [PubMed: 15000151]
21. Karsten U, von Mensdorff-Pouilly S, Goletz S. What makes MUC1 a tumor antigen? *Tumour Biol.* 2005; 26:217–220. [PubMed: 16006776]
22. Brockhausen I, Yang JM, Burchell J, et al. Mechanisms underlying aberrant glycosylation of MUC1 mucin in breast cancer cells. *Eur J Biochem.* 1995; 233:607–617. [PubMed: 7588808]
23. Kotera Y, Fontenot JD, Pecher G, et al. Humoral immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic, and colon cancer patients. *Cancer Res.* 1994; 54:2856–2860. [PubMed: 7514493]
24. McKolanis JR, Finn OJ. Analysis of the frequency of MHC-unrestricted MUC1-specific cytotoxic T-cells in peripheral blood by limiting dilution assay. *Methods Mol Biol.* 2000; 125:463–470. [PubMed: 10820782]
25. von Mensdorff-Pouilly S, Vennegoor C, Hilgers J. Detection of humoral immune responses to mucins. *Methods Mol Biol.* 2002; 125:495–500. [PubMed: 10820785]
26. Bontkes HJ, Ruizendaal JJ, Kramer D, et al. Plasmacytoid dendritic cells are present in cervical carcinoma and become activated by human papillomavirus type 16 virus-like particles. *Gynecol Oncol.* 2005; 96:897–901. [PubMed: 15721448]
27. Rudolf MP, Fausch SC, Da Silva DM, et al. Human dendritic cells are activated by chimeric human papillomavirus type-16 virus-like particles and induce epitope-specific human T cell responses in vitro. *J Immunol.* 2001; 166:5917–5924. [PubMed: 11342606]
28. Yang R, Murillo FM, Cui H, et al. Papillomavirus-like particles stimulate murine bone marrow-derived dendritic cells to produce alpha interferon and Th1 immune responses via MyD88. *J Virol.* 2004; 78:11152–11160. [PubMed: 15452235]
29. Andreasson K, Tegerstedt K, Eriksson M, et al. Murine pneumotropic virus chimeric Her2/neu virus-like particles as prophylactic and therapeutic vaccines against Her2/neu expressing tumors. *Int J Cancer.* 2009; 124:150–156. [PubMed: 18839427]
30. Tegerstedt K, Franzén A, Ramqvist T, Dalianis T. Dendritic cells loaded with polyomavirus VP1/VP2Her2 virus-like particles efficiently prevent outgrowth of a Her2/neu expressing tumor. *Cancer Immunol Immunother.* 2007; 56:1335–1344. [PubMed: 17268793]
31. Gedvilaite A, Dorn DC, Sasnauskas K, et al. Virus-like particles derived from major capsid protein VP1 of different polyomaviruses differ in their ability to induce maturation in human dendritic cells. *Virology.* 2006; 354:252–260. [PubMed: 16904154]
32. Yan M, Peng J, Jabbar IA, et al. Despite differences between dendritic cells and Langerhans cells in the mechanism of papillomavirus-like particle antigen uptake, both cells cross-prime T cells. *Virology.* 2004; 324:297–310. [PubMed: 15207617]
33. Schiller JT, Castellsagué X, Villa LL, et al. An update of prophylactic human papillomavirus L1 virus-like particle vaccine clinical trial results. *Vaccine (Suppl).* 2008; 10:K53–K61.
34. Trus BL, Roden RB, Greenstone HL, et al. Novel structural features of bovine papillomavirus capsid revealed by a three-dimensional reconstruction to 9 Å resolution. *Nat Struct Biol.* 1997; 4:413–420. [PubMed: 9145113]
35. Buck CB, Cheng N, Thompson CD, et al. Arrangement of L2 within the papillomavirus capsid. *J Virol.* 2008; 82:5190–5197. [PubMed: 18367526]
36. Roy P, Noad R. Virus-like particles as a vaccine delivery system: myths and facts. *Hum Vaccin.* 2008; 4:5–12. [PubMed: 18438104]
37. Miyamura K, Kajigaya S, Momoeda M, et al. Parvovirus particles as platforms for protein presentation. *Proc Natl Acad Sci USA.* 1994; 91:8507–8511. [PubMed: 8078912]
38. Müller M, Zhou J, Reed TD, et al. Chimeric papillomavirus-like particles. *Virology.* 1997; 234:93–111. [PubMed: 9234950]
39. Tindle RW, Herd K, Londoño P, et al. Chimeric hepatitis B core antigen particles containing B- and Th-epitopes of human papillomavirus type 16 E7 protein induce specific antibody and T-helper responses in immunised mice. *Virology.* 1994; 200:547–557. [PubMed: 7513919]
40. Wagner R, Deml L, Schirmbeck R, et al. Construction, expression, and immunogenicity of chimeric HIV-1 virus-like particles. *Virology.* 1996; 220:128–140. [PubMed: 8659105]

41. Chen XS, Garcea RL, Goldberg I, et al. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Mol Cell*. 2000; 5:557–567. [PubMed: 10882140]

Figure 1.

Expression profile of chimeric L1 constructs and electron micrographs of purified chimeric particles. a) Western blot analysis using a monoclonal anti-BPV L1 in lysates of Hi5 cells infected with the four recombinant baculoviruses BPV-BC-E8c, BPV-DE-E8c, BPV-HI-E8c, BPV-H4-E8c, and BPV-WT expressing the chimeric and native L1 proteins. b) Western blot analysis of purified BPV-DE-E8c, BPV-HI-E8c, and BPV-H4-E8c. The BPV-BC-E8c did not result in any particle formation. c) BPV-HI-E8c VLPs, magnification 30 K, the scale bar is 100nm; d) BPV-H4-E8c capsomeres, magnification at 70 K, scale bar is 50 nm; e) BPV-DE-E8c partially assembled VLPs, magnification at 30 K, the scale bar is 100 nm. For electron microscopy, the purified particles were loaded on carbon-coated copper grids, negatively stained with 2% potassium phosphotungstate (pH=7), and visualized under a JEOL 1200 TEM.

Figure 2.

Quantitative and qualitative assessment of MUC1 peptide conjugation on chimeric VLPs. a) SDS-PAGE and Coomassie brilliant blue staining of conjugated BPV-HI-E8c VLPs. 10, 20, and 30 µg of conjugated VLPs and 100 ng of R8c-MUC1 (mass standard) were loaded on separate lanes. Due to overloading of the gel (at 30 µg), delayed band migration is observed. The L1 runs according to the theoretical MW of 56 kd. The R8c-MUC1 runs as ~6 kd. b) Immunogold labeling of BPV-HI-E8c VLPs conjugated with the R8c-MUC1 peptide. The conjugated VLPs were adsorbed on formvar/carbon-coated nickel grids. The primary antibody was monoclonal anti-MUC1 IgG, and the secondary was colloidal-gold-conjugated (6 nm) goat anti mouse IgG. Negative staining was performed with 1% sodium silicotungstate (pH=6.5). Magnification is at 40K and the scale bar is 100 nm.

Figure 3.

BMDC activation following uptake of BPV and BPV-MUC1. a) Bone marrow DC were loaded with various BPV constructs (WT BPV, BPV-HI-E8c-MUC1, BPV-HI-E8c; BPV-H4-E8c-MUC1, BPV-H4-E8c) for 24h, and subsequently were stained for standard DC maturation markers CD40, CD80, CD86 and MHC class II and analyzed by flow cytometry (unconjugated vs mock: $p=0.000195$; conjugated vs mock: $p=0.0000035$). b) Supernatants harvested from DC cultures, 24h post-treatment with various constructs, were used to assess IL-12 secretion using IL-12 ELISA (unconjugated vs mock: $p=0.0236$; conjugated vs mock: $p=0.00346684$). DC alone (untreated - UT), MUC1 peptide (250ng-GVTSAPDTRPAPGSTAPPAH)(pep). * $p < 0.05$; ** $p < 0.01$

Figure 4.

MUC1 conjugated to chimeric BPV VLPs can be cross-presented to primary, naïve MUC1-specific T cells. IFN γ production following mock-treatment (untreated), addition of MUC1 peptide (10, 50, 250ng), or treatment with unconjugated (BPV-HI-E8c) and MUC1-conjugated (BPV-HI-E8c-MUC1) chimeric VLPs (1, 5, 25 μ g). p values were calculated against peptide alone for each concentration shown. **p < 0.01;

Figure 5.

T cell activation in MUC1-Tg mice. Proliferation of MUC1-specific CD8⁺ T cells (a) and CD4⁺ T cells (b) following *in vitro* culture of CFSE-labeled splenocytes for 5 days with the immunizing antigens as indicated. c) On day 5, supernatants were harvested and measured for the presence of IFN γ using ELISA. The results shown are for individual mice. * Values of $p < 0.05$ were considered significant. $n = 6-7$ mice per group.

Figure 6.

Tumor progression following vaccinations. Mice were injected with 5×10^4 RMA-MUC1 tumor cells 2 weeks following the final vaccine boost ($5 \mu\text{g}$ per dose). Tumor progression in individual MUC1-Tg treated with PBS (a), vector alone BPV-HI-E8c, (b) and vaccine BPV-HI-E8c-MUC1 (c) was followed for 60 days. On day 21 (day before the first mouse in the PBS negative control groups was sacrificed), tumors in all mice in all groups were measured (d). * Values of $p < 0.05$ were considered significant; ** $p < 0.01$. $n = 21$ per group for vaccine and vector group and $n = 9$ mice per group for PBS group.