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Eglė Mažeikė

GENERATION OF MODEL ANTICANCER VACCINE BASED ON VIRUS-LIKE PARTICLES

Summary of doctoral thesis
Physical sciences, biochemistry (04 P)

Vilnius, 2011

This study has been carried out during 2005-2011 at the Department of Eukaryote Gene Engineering of the Vilnius University Institute of Biotechnology (Vilnius, Lithuania) and at the Friedrich-Löffler Federal Research Institute for Animal Health (Greifswald, Germany).

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The thesis is available at the Library of Institute of Biotechnology and at the Library of Vilnius University.

VILNIAUS UNIVERSITETAS

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MODELINĖS PRIEŠVĖŽINĖS VAKCINOS KONSTRAVIMAS PANAUDOJANT Į VIRUSUS PANAŠIAS DALELES

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Contents

INTRODUCTION

Cancer is second leading cause of death worldwide. Ever year several millions new cases are diagnosed and these numbers are growing up. In our days cancer is treated by all known ways: surgery, chemotherapy, radiation, biotherapy and vaccines. The main task for anticancer vaccine is to stimulate patient's immune system to fight against cancer and to induce cancer cell death.

After identification of tumor associated antigens – short peptides expressed on tumor cells, new anticancer vaccine generation – peptide vaccines were originated. The main goal for these vaccines is to induce CLT immune response against specific tumor associated antigens (Marchand et al., 1995; Rosengerg et al., 1998). However, accumulating evidences suggest that immunization with minimal peptide epitopes often has only limited success or even lead to CTL tolerance (Toes et al., 1996; Zaks ir Rosenberg, 1998). The improvement of peptide vaccines was achieved by using long peptides or polypeptides encompassing a variety of immunogenic CTL and T-helper (Th) epitopes (Perez et al., 2010). Virus-like particles (VLPs) can be an alternative for improvement of unimmunogenic peptide immunogenicity due, repetitive surface epitopes, the virus-like structure and capability to induce humoral and (or) CTL responses (for review see Pumpens et al. 2002).

Recombinant virus-like particles (VLPs) are the icosahedral or rod-like structures comprising of multiple copies of one or more viral structural proteins. VLPs are produced by heterologous synthesis of viral structural proteins resembles native infectious virions in their structural and immunological properties, but are noninfectious because they lack viral nucleic acid. Members of more than 30 viral families have been tested as putative VLP carriers by fusing peptides of interest to the coat protein and exposing them on the surfaces of the assembled VLPs. Some of these modified VLPs were shown to be highly immunogenic antigen carriers, capable to induce strong humoral immune responses against inserted antigens (Buonaguro et al., 2002; Herbst-Kralovetz et al., 2010; Kang et al., 2009; Zvirbliene et al., 2006), and some antigen carrying VLPs were able to induce CTL responses, as well (Ding et al., 2009; Lacasse et al., 2008). However, for induction of a strong CTL response most VLPs required to be administered together with the adjuvant-like molecules such as CpGs or anti-CD40 (Schwarz et al., 2005; Storni et al., 2002). In contrast to exogenous antigens, which usually are processed via MHC class II pathway, non-replicating antigens as VLP are able to enter the MHC class I pathway, the main pathway for CD8+ T cell activation, by cross-presentation (Keller et al., 2010; Storni and Bachmann, 2002; Storni et al., 2004). This is probably due to their macromolecular structure and particulate nature.

Hamster polyomavirus (HaPyV) major capsid protein VP1-based VLPs are powerful vehicles for the antigen transport as they tolerate inserts of different size and origin (from 9 to 120 amino acids (aa) long) at certain VP1 sites (Gedvilaite et al., 2000, 2004). In addition, yeast expressed HaPyV VP1-based chimeric VLPs induce a strong insert-specific humoral response without adjuvant and represent useful tools for generating monoclonal antibodies with desired specificity (Zvirbliene et al., 2006). HaPyV VP1 VLPs are able to trigger more efficiently human

dendritic cell (DC) maturation, IL-12 production and stimulation of CD8+ T cells *in vitro* than VLPs of other polyomaviruses (JCV, BKV, SV40 or MuPyV; Gedvilaite et al., 2006). An evaluation of the capacity of HaPyV VP1 based chimeric VLPs to induce effective epitope-specific CTL immune response *in vivo* is still missing.

The objective of dissertation work was to investigate the potential of recombinant HaPyV VP1 based VLPs for anticancer vaccine generation in model systems including investigation of VP1 applicability for heterologous CTL epitopes insertions, VLPs assembly and ability to induce inserts specific immune response *in vivo.*

The **specific aims** of this study were:

- To produce yeast *Saccharomyces cerevisiae* cells generating chimeric HaPyV VP1-based proteins carrying various CTL epitopes, to purify it and to establish VLPs formation.
- To determine the potential of VP1 as a CLT epitopes carrier and to improve VLPs purification procedure.
- To evaluate the possibility of chimeric VLPs carrying CTL epitope inserts to stimulate humoral immune response in mice.
- To investigate the immunogenicity of chimeric VLPs with GP33 insert *in vitro*: to determine VLPs ability to mature dendritic cells and to stimulate antigen specific CTL cells.
- To evaluate the capacity of HaPyV VP1-based chimeric VLP with GP33 insert to trigger the development of protective GP33 specific long lasting CTL immunity *in vivo*: protect mice against virus challenge and tumor cell growth.

Scientific novelty

In this study potential of HaPyV VP1 based chimeric VLPs harboring CTL epitopes for anticancer vaccine development was investigated. Chimeric VLPs with CTL epitope insertions could be used to strengthen the induction of effective immune response against tumor cells expressing inserted CTL epitope.

For the first time HaPyV VP1 protein ability to tolerate CTL epitope insertions and to assembly into VLPs was studied consistently and extensively. HaPyV VP1 based VLPs carrying CLT epitopes derived from different proteins were generated, most suitable positions for insertion into VP1 protein were selected, the ways to improve assembly and yield of the chimeric VLP were determined and new VLPs purification procedure was created allowing to purify VLPs cheaper, faster and more efficiently.

HaPyV VP1 based VLPs ability to induce humoral and cellular immune response *in vivo* was evaluated for the first time. VLPs formed by majority viral proteins usually induce effective humoral immune response, but only some are able to induce CTL immune response. It was demonstrated that chimeric HaPyV VP1 model VLPs (with GP33 CTL epitope) were able to mature dendritic cells, to stimulate antigen specific CTL cells *in vitro* and *in vivo*, as well as induce GP33-specific long lasting humoral and cellular immune response *in vivo*. It was determined that immunization with chimeric VP1-GP33 model VLPs induced effective insert specific CTL immune response what protected mice from GP33-specific virus infection and killed antigen-specific tumor cells.

Our data proves that HaPyV structural protein VP1 is universal carrier for CTL epitopes, capable not only to tolerate insertions and form VLPs but to induce strong, effective, long lasting immune response against inserted antigens *in vivo*.

Doctoral thesis contents

The doctoral thesis (in Lithuanian) contains the following parts: Introduction, Literature review, Methods and Materials, Results, Discussion, Conclusions, List of references (224 citations), List of publications (4 positions), Participation at conferences (4 positions), Figures (39), Tables (24); 154 pages in total.

MATERIALS AND METHODS

Construction and expression of genes and purification of VLPs

All DNA manipulations were performed according to the standard procedures (Sambrook and Russell, 2001). Cloning and expression of the entire VP1-encoding sequence in yeast *S. cerevisiae* and selection of positions No. 1 and No. 4 in the primary structure of VP1 (corresponding to aa residues between 80–89 and 288–295) for introduction of foreign sequences were described previously (Gedvilaite et al., 2000; Sasnauskas et al., 1999). Oligonucleotide duplexes encoding various CTL peptides were inserted into the VP1-encoding sequence and cloned into the expression cassette of the yeast vector pFX7 (Sasnauskas et al., 1999). Chimeric proteins-encoding sequences were confirmed by DNA sequencing.

The procedure used for VLP generation in the *S. cerevisiae* strain AH22 derivative 214 (a *leu2 his4*) and purification was described earlier (Gedvilaite et al., 2000) and new modified procedure was also used. Briefly, yeast cells were cultured in glucose and then galactose containing induction medium for ~24 h, collected by centrifugation and disrupted by French press in DB buffer (10 mM Tris, 450 mM NaCl, 1 mM $CaCl_2$, 250 mM L-Arg, pH 7,2) containing 2 mM PMSF and ProteoBlock™ protease inhibitor cocktail (Fermentas UAB, Vilnius, Lithuania). The VLPs were purified by ultracentrifugation in 20-60% sucrose gradient (100,000×g, 4 h) followed by 0,5 h incubation with a DNAse-free RNAse (Fermentas UAB, Vilnius, Lithuania) and by ultracentrifugation at 100,000×g for 48 h on CsCl gradient (1,23 to 1,38 g/mL). Fractions containing purified proteins were collected, pooled, dialyzed against PBS, lyophilized and stored at -20°C until further use. All purified VLPs were examined for nucleic acid contaminations according to a protocol described previously (Sasnauskas et al., 1999) and DNA/RNA was not detected.

SDS-PAGE, Western blot analysis and electron microscopy

Preparations of protein samples, SDS-PAGE, and Western blot analysis were performed according to methods described previously (Gedvilaite et al., 2004). As primary antibody anti-HaPyV-VP1 mAb 6D11 (Dr. A. Zvirbliene, Vilnius, Lithuania) or mice serum (1:300) were used.

To confirm VLP assembly purified proteins were placed on 400-mesh carbon coated palladium grids. The samples were stained with 2% aqueous uranyl acetate solution and examined by Morgagni electron microscope (FEI, Oregon, USA).

Peptide and virus

GP33 synthetic peptide (KAVYNFATM) corresponding to an H-2D^b-restricted CTL epitope (amino acid residuals 33–41) from LCMV surface glycoprotein, was purchased from Biosyntan GmbH (Berlin, Germany). The original cysteine at the anchor position 41 in the LCMV GP33 peptide was replaced by methionine (Aichele et al., 1994).

The LCMV-WE isolate used in this study was originally obtained from R. Zinkernagel (Zurich, Switzerland). LCMV-WE was cultivated in MC57 (H-2D^b)

fibrosarcoma cell line using DMEM supplemented with 2 mM L-glutamine, penicillin and streptomycin, 10 % FCS.

Mice were infected i.v. with 200 pfu and viral titers were determined using MC57 cell line as described in Battegay et al. (1991).

CTL epitopes

Muc – CTL epitope from human mucin 1 (MUC1) protein. Amino acid sequence STAPPVHNV.

CEA – carcinoembrionic antigen-derived T cell epitope CAP-1-6D. Amino acid sequence YLSGADLNL.

WT1 – T cell epitope derived from Wilms tumor antigen 1. Amino acid sequence: RMFPNAPYL.

hTERT – T cell epitope derived from human telomerase reverse transcriptase (hTERT $_{540-548}$). Amino acid sequence: ILAKFLHWT.

TRP – T cell epitope derived from tyrosinase related protein 2 (TRP2 $_{185-193}$). Amino acid sequence

MAGE – T cell epitope derived from melanoma antigen 2 (MAGE2$_{116-124}$). Amino acid sequence: LVHPLLLKY.

CMV – T cell epitope derived from cytomegalovirus matrix phosphoprotein (CMV pp65$_{495-503}$). Amino acid sequence: NLVPMVATV.

FLU – T cell epitope derived from Influenza A virus matrix protein (FLU MP$_{58-66}$). Amino acid sequence: GILGFVFTL.

GP33 – CTL epitope derived from LCMV virus surface glycoprotein GP$_{33-41}$. Amino acid sequence: KAVYNFATM.

Mice

C57BL/6 (B6) mice were obtained from breeding stock of the Friedrich-Löffler Institute (FLI, Germany). Transgenic H-2D^b (P14) mice, line 327, carrying the TCR specific for the amino acids 33–41 epitope of LCMV glycoprotein GP33 (Aichele et al., 1994) were purchased from HP. Pircher (Freiburg, Germany).

8 to 16-week-old mice of both sexes were used and were kept under SPF conditions in the animal facility. All animal experiments were carried out in accordance with institutional guidelines and permission of the national authorities.

Tumor cells

For tumor experiments MCA102 and MCA102-GP33 fibrosarcoma tumor cells were used (Blohm et al., 2002). MCA102-GP33 cells were derived from parental MCA102 tumor cells by gene transfection using the LCMV GP33 minigene (Prevost-Blondel et al., 1998). Both MCA102 cells were cultured in DMEM high glucose supplemented with 10 % FCS, 2 mM L-glutamine, penicillin and streptomycin (PAA Laboratories GmbH, Austria), transfected MCA102-GP33 cells additionally under 600 µg/mL G418 selection (Gibco, Invitrogen, UK).

In tumor animal trials mice were encoded by ear tagging to eliminate subjective bias and injected with 1x10^6 of MCA102-GP33 tumor cells in 100 µL PBS into the flank. Tumor size was measured with caliper independently by two persons

in a blinded, randomized fashion and calculated as the product of bisecting tumor diameters. Mice bearing a tumor with a diameter >10 mm were sacrificed according to animal care regulations. When the experiment was finished mice were decoded and results were analyzed.

Flow cytometry, antibodies

Cell staining for flow cytometry was performed for 20 min at 4°C in FACS buffer (PBS containing 2 % FCS and 0,1 % NaN_3). Cells were analyzed by FACSort flow cytometer (BD Biosciences, USA) using CellQuest-Pro software.

For cytometry analysis the following mAbs were used: biotin conjugated anti-mouse CD11c (Integrin aX) (clone M1/70) restained with FITC or PE conjugated streptavidin; FITC conjugated anti-mouse $I\text{-}A^b$ (clone AF6-120.1), FITC conjugated anti-mouse CD80 (B7-1) (clone 16-10A1); FITC conjugated anti-mouse CD86 (B7-2) (clone GL-1); pure rat anti-mouse CD40 restained with PE conjugated donkey anti-rat IgG (H+L); FITC conjugated anti-mouse CD8a (Ly-2) (clone 53-6.7). All mAbs were purchased from BD PharMingen.

Generation and maturation of dendritic cells *in vitro*

The protocol for generation of mouse bone marrow derived DC was identical to that used by Lutz et al. (1999).

Immature DCs 10^6 cells/well were incubated for 72 h in 6-well plates (3 mL/well) with 10 μg/mL of each VLP construct. Untreated cells served as negative control. For immunophenotypic maturation analysis, pulsed DCs were sampled after 24, 48, 72 h and stained for FACS analysis with specific antibodies against mouse MHCII, CD11c, CD40, CD80, CD86 molecules as described above.

T cell proliferation assay *in vitro*

Single cell suspensions from B6 and P14 mouse spleens were prepared for T cell proliferation assay and stained with CFSE according to the manufacturer's instructions.

GP33 peptide stimulation: CFSE-labeled spleen cells were stimulated with GP33 peptide (final concentration 10^{-7} M) in DMEM media with 10 % FCS, 50 μM β-mercaptoethanol and penicillin/streptomycin in 24-well plates at a concentration of $2x10^6$ cells/mL. Unstimulated spleen cells served as negative control.

Stimulation with GP33 pulsed DCs: DCs were pulsed either with HaPyV VP1 or chimeric VP1-GP33 VLP as described in above. CFSE-labeled spleen cells were cocultivated with variably pulsed DCs, in 24-well plates at a ratio of 10^6 splenocytes to 10^5 DCs/mL at 37°C. Splenocytes incubated with PBS or immature DCs served as negative control, splenocytes stimulated with GP33 peptide were used as positive control.

For B6 or P14 TCR-tg mouse splenocytes restimulation with DCs, DCs pulsed with VLPs (as described earlier) were collected and placed into 24-well plates together with P14 TCR-tg or B6 mouse CFSE-labeled splenocytes (10^6 splenocytes + 10^5 DCs/mL). For negative control, splenocytes were incubated with PBS. As

positive control, splenocytes stimulated with GP33 peptide were used. For additional negative control, splenocytes were stimulated with immature DCs. DCs pulsed with GP33 peptide were used as additional positive control.

The proliferation of CD8+ T cells was determined after 4-5 days of stimulation. CFSE-labeled spleen cells were labeled with PE conjugated anti-mouse CD8 antibodies and analyzed by flow cytometry. Living cells were gated on CD8+ T cells and percentage of proliferating CD8+ T cells (identified by low CFSE level) was determined.

Statistical analysis

T cell proliferation results were compared using Student's *t*-test, values with $p<0.05$ were considered as statistically significant. Statistical significance of virus challenge experiments was calculated using Wilcoxon Mann-Whitney Test.

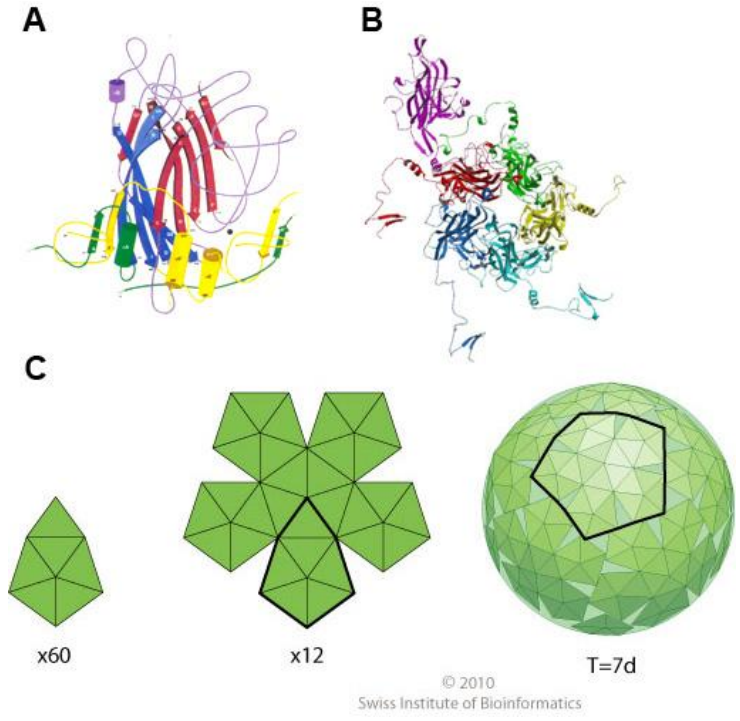
RESULTS

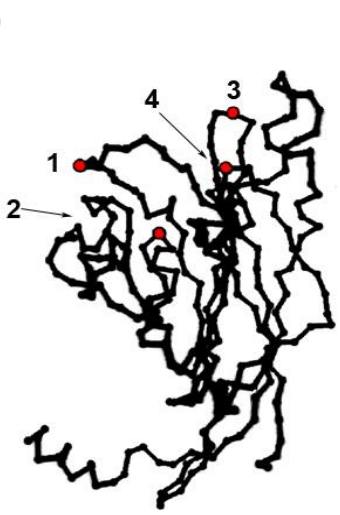
1. Generation of chimeric HaPyV VP1-based VLPs carrying CTL epitopes

In order to evaluate of HaPyV VP1 applicability for anticancer vaccine development, first task was to determine the potential of VP1 as a CLT epitopes carrier.

1.1. Generation of chimeric HaPyV VP1-based VLPs carrying 1, 2, 3, 4 copies of CTL epitope

For the characterization of VP1 proteins positions most suitable for CTL epitope insertion, Muc, CEA, WT1, htert, TRP, MAGE, htert, FLU, CMV CTL epitopes were inserted into four potential insertion sites (No. 1 (81–88 aa), No. 2 (221-224 aa), No. 3 (244–246 aa) and No. 4 (289–294 aa) of VP1 protein (Fig. 1, 2). As multiple copies of CLT epitope inserted into VLPs should ensure stronger immune response induction by using lower doses of such antigen, chimeric HaPyV VP1-based VLPs carrying not only one but also two, three or even four copies of CTL epitope were generated in yeast *S. cerevisiae*. To minimize the influence of inserted CTL epitopes on the assembly of VP1 VLPs, addition of flexible Gly-Ser-Ser-Gly (GSSG) linkers surrounding CLT epitope was tried out for some of the constructs. The list of generated chimeric proteins is shown in Table 1.





Insert position No.	Insert place in VP1 protein
1	80-89 aa
2	221-224 aa
3	244-246 aa
4	280-289 aa

Figure 1. Polyomavirus capsid structure. (A): The tertiary structure of major capsid protein VP1. (B): pentamer structure (5 VP1 proteins form a pentamer) (Liddington et al., 1991). (C): The icosahedral capsid (T=7d) consist of 72 pentamers (http://www.expasy.org/viralzone). Green triangular – one VP1 protein. Capsid is formed by 360 subunits of VP1 (72 pentamers=360). (D): Tertiary structure model of HaPyV VP1 protein. Red spots in model – VP1 protein positions suitable for foreign sequences insertion (Gedvilaitė et al., 2000). The position numbers and they place in VP1 are indicated in lower right corner.

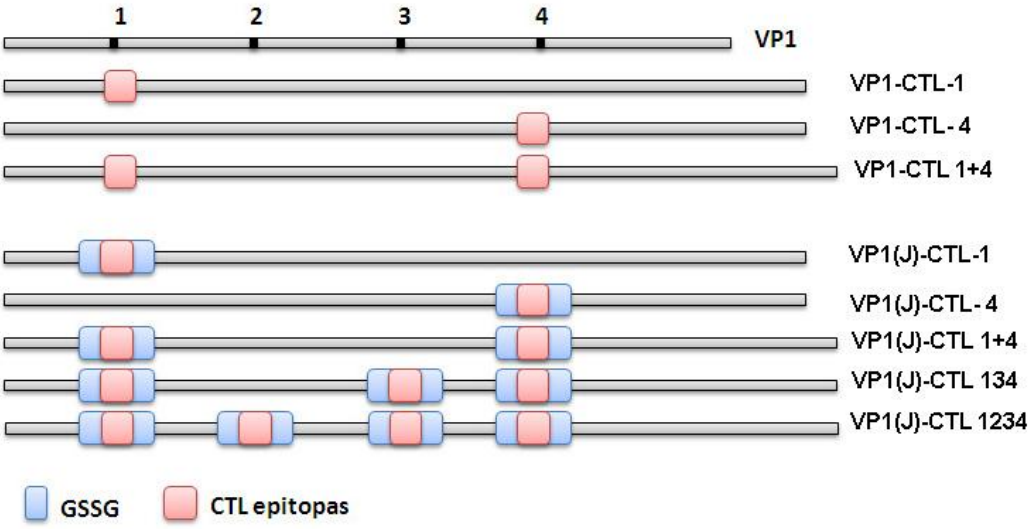


Figure 2. Schematic presentation of chimeric HaPyV VP1 proteins with 1, 2, 3, 4 CTL epitope insertions (with or without GSSG linkers). Gray lines – VP1 protein; black small squares – CTL epitope insertion positions into HaPyV VP1 protein (1: 80-89aa, 2: 221-224aa, 3: 244-246aa, 4: 288-295aa); blue rectangular – GSSG linker; colorful squares – CTL epitopes (e.g. Muc, CEA, WTI, CMV, FLU, TRP, MAGE, hTERT). Protein names are written in right. One CLT epitope was inserted into VP1 positions 1 or 4, two – into positions 1 and for (1+4), three – into positions 1, 3 and 4 (1+3+4), for – into 1, 2, 3, 4 (1+2+3+4).

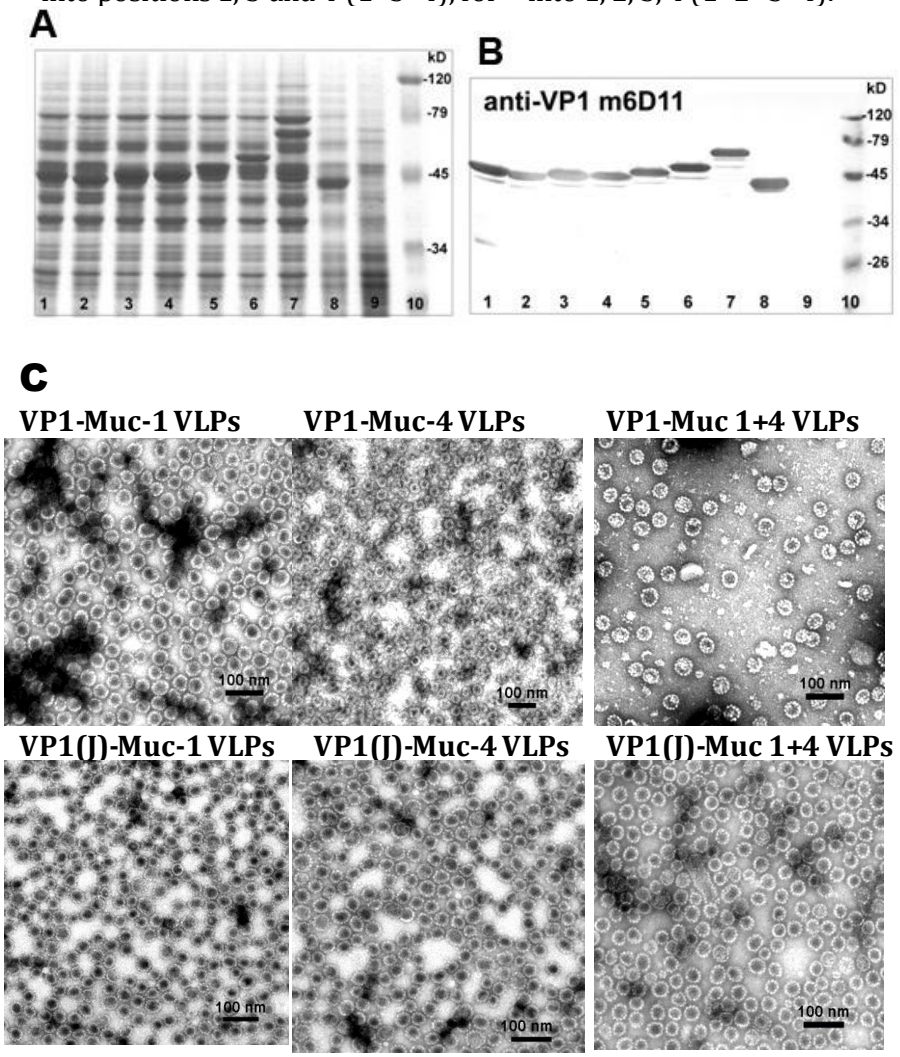


Figure 3.Generation of chimeric VP1–Muc VLPs. (A) SDS-PAGE and Western blot analysis (B) with anti-HaPyV VP1 mAbs 6D11. Yeast cells expressing chimeric proteins lysates, in lanes: 1: VP1-Muc-1, 2: VP1-Muc-4, 3: VP1(J)-Muc-1, 4: VP1(J)-Muc-4, 5: VP1-Muc 1+4, 6: VP1(J)-Muc 1+4, 7: VP1(J)-Muc-1234, 8: VP1 (positive control), 9: no heterologous protein (negatyve control), 10: „Protein Ladder #SM0661“ (Fermentas, Lietuva) (Dorn et al., 2008). (C) Electron microscopy pictures of VLPs stained with 2% aqueous uranyl acetate solution and examined by Morgagni electron microscope.

Table 1. Properties of chimeric proteins carrying different CTL epitopes

Name of CTL epitope	Name of chimeric protein	VP1 positions were CTL epitopes were inserted				The usage of GSSG linker	Chimeric protein expression	VLPs formation	Purified VLPs quantities, mg
		1	2	3	4	(J)			
Muc	VP1-Muc-1	+				-	perfect	perfect	17
	VP1(J)-Muc-1	+				+	perfect	perfect	20
	VP1-Muc-4				+	-	perfect	perfect	15
	VP1(J)-Muc-4				+	+	perfect	perfect	16
	VP1-Muc 1+4	+			+	-	perfect	perfect	15
	VP1(J)-Muc 1+4	+			+	+	perfect	perfect	15
	VP1(J)-Muc 1234	+	+	+	+	+	good	poor	~1
CEA	VP1-CEA-1	+				-	perfect	perfect	16
	VP1(J)-CEA-1	+				+	perfect	perfect	18
	VP1-CEA-4				+	-	perfect	perfect	15
	VP1(J)-CEA-4				+	+	perfect	perfect	17
	VP1-CEA 1+4	+			+	-	perfect	perfect	14
	VP1(J)-CEA 1+4	+			+	+	perfect	perfect	12
	VP1(J)-CEA .1234	+	+	+	+	+	good	poor	~1
WT1	VP1-WT1-1	+				-	good	perfect	18
	VP1(J)-WT1-1	+				+	perfect	perfect	20
	VP1-WT1-4				+	-	good	perfect	17
	VP1(J)-WT1-4				+	+	perfect	perfect	19
	VP1-WT1 1+4	+			+	-	good	good	13
	VP1(J)-WT 1+4	+			+	+	good	good	14
	VP1(J)-WT1 123	+		+	+	+	poor	good	5
	VP1(J)-WT1 1234	+	+	+	+	+	poor	poor	~1
Htert	VP1-htert-1	+				-	poor	good	5
	VP1(J)-htert-1	+				+	poor	good	6
	VP1-htert-4				+	-	poor	good	5
	VP1(J)-htert-4				+	+	poor	good	6
	VP1- htert 1+4	+			+	-	poor	good	4
	VP1(J)-htert 1+4	+			+	+	poor	good	4
	VP1(J)-htert 123	+		+	+	+	poor	poor	3
	VP1(J)-htert 1234	+	+	+	+	+	poor	poor	<1
TRP2	VP1(J)-TRP2 1+4	+			+	+	poor	good	3
MAGE	VP1(J)-MAGE 1+4	+			+	+	poor	good	6
hTERT	VP1(J)-Htert 1+4	+			+	+	poor	good	5
FLU	VP1(J)-FLU 1+4	+			+	+	poor	good	3
CMV	VP1(J)-CMV 1+4	+			+	+	poor	good	7

In column „VP1 positions were CTL epitopes were inserted" symbol (+) shows the positions of VP1 were CTL epitopes inserted. „The usage of GSSG linker" symbol (+) indicates that inserted CTL epitope was surrounded by GSSG sequences. „ Chimeric protein expression": perfect – chimeric protein synthesis level is equal to VP1 synthesis level, good – chimeric protein synthesis is 20% less than VP1, poor – chimeric protein synthesis is >50 % less than VP1. „VLPs formation": „perfect"– indicates that chimeric protein forms the same kind of VLPs as VP1 – 45 nm in diameter, sample contains a lot of VLPs, pentamers are not found; „good"– chimeric protein forms 45 nm VLPs, pentamers are also not found, but the amount of VLPs is less; „poor"– protein sample contains only some 45 nm VLPs, smaller VLPs are also found as well as pentamers. „Purified VLPs quantities" – indicates how many mg of chimeric proteins was purified from 20 g of yeast biomass, for comparison VP1 yield is 20 mg/20 g.

Chimeric proteins synthesis was confirmed by SDS-PAGE and Western blot analysis, proteins were purified by centrifugation in CsCl gradient and VLPs formation was analyzed by electron microscopy (Fig. 3 – example of VLPs formed by one group of chimeric proteins). VP1 protein best tolerated one or two CTL epitope insertions. Some chimeric proteins harboring one or two CTL epitope insertions were synthesized in yeast cell as efficiently as unmodified VP1 (table 1). VP1 with three or four CTL epitope inserts were less soluble, tended to aggregate, VLP formation was poor, and the yield of purified VLPs was low. More copies of inserted CTL epitopes correlated with decreased yield of purified VLPs. Insertion of

additional GSSG linkers surrounding CTL epitopes improved VLP formation in some but not all cases (Table 1). It was observed that CTL epitope sequence hydrophaticity index directly influenced the expression level and VLP assembly efficiency of chimeric protein. Hydrophilic peptide insertions were tolerated better.

1.2. Generation of chimeric HaPyV VP1-based VLPs carrying 3 different CTL epitopes

Although human tumors express multiple CTL epitopes (tumor associated antigens – TAA) that can be recognized by T cells, some of these epitopes can be lost or mutated and immune system is not able to find such tumor cells. Therefore, a vaccine possessing multiple TAA epitopes might be more effective than a vaccine with a single epitope. Polypeptide vaccines may substantially increase the possibility of targeting the tumor cells as one vaccine could activate multiple CD8+ T cells with different specificity. Unfortunately most TAA peptides are hydrophobic and therefore it is difficult to synthesize and purify them.

In this part of the study, we have examined the best way of insertion of multiple different antigens into HaPyV VP1 VLPs. Three different CTL epitopes were inserted into VP1 protein as separate copies or one fused peptide: TRP – tyrosinase related protein-2 epitope (9aa: FVWLHYYSV), MAGE – the MAGE A family protein epitope (9aa: LVHPLLLKY), hTERT – human telomerase reverse transcriptase epitope (9aa: ILAKFLHWT). Three potential insertion sites (No. 1 (81–88 aa), No. 3 (244–246 aa) and No. 4 (289–294 aa)) of the HaPyV major capsid protein VP1 Fig.1) were selected as a target for TRP, MAGE and hTERT TAAs insertions (Fig. 4). Two genes encoding chimeric proteins were constructed. One construct had a TRP epitope inserted into the VP1 site No. 1, MAGE into the site No. 3 and hTERT into the site No. 4 (chimeric protein named VP1(J)-1T3M4H), and another construct had the same three TAAs fused in one polypeptide inserted into the VP1 site No. 4 (chimeric protein named VP1(J)-TMH-4) (Fig. 4) (Aleksaite ir Gedvilaite, 2006). In both cases GSSG linkers were used.

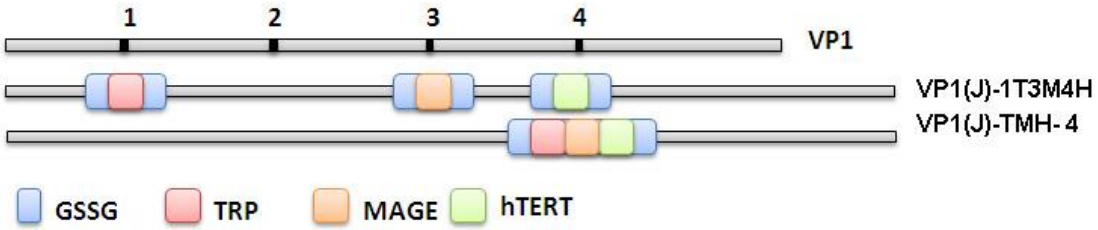


Figure 4. Schematic presentation of chimeric HaPyV VP1 proteins with three different CTL epitopes insertions. Gray lines – VP1 protein; black small squares – CTL epitope insertion positions into HaPyV VP1 protein (1: 80-89aa, 3: 244-246aa, 4: 288-295aa); blue rectangular – GSSG linker; colorful squares – CTL epitopes (TRP, MAGE, hTERT). Protein names are written in right.

Both chimeric proteins were expressed in yeast, and lysates of yeast cells after heterologous protein synthesis induction were examined by SDS-PAGE and Western blot analysis. The expression level of both chimeric proteins was approximately two times lower than unmodified VP1 protein. Purification of yeast-

expressed proteins revealed that both chimeric proteins were less soluble in comparison to authentic VP1, tended to aggregate and sediment. This caused some purification difficulties. The yield of purified VLPs was only 2–4 mg from 20 g of yeast biomass (in a comparison to 20 mg in VP1 case).

After purification, the VLP assembly capacity of VP1(J)-1T3M4H and VP1(J)-TMH-4 chimeric proteins was evaluated by electron microscopy (EM) analysis. Both constructed proteins were capable to form VLPs. VP1(J)-1T3M4H VLPs were similar to the authentic one with the diameter of 40–45 nm. VP1(J)-TMH-4 VLPs were more heterogeneous with the diameter from 20 to 45 nm.

These experiments showed that for generation of chimeric VLPs carrying three different CTL epitopes better solution was the insertion of separate CTL epitopes into different sites of VP1 VLPs. The insertion of a long fused polypeptide unfavorably influenced chimeric protein expression, synthesis as well as VLP assembly and the yield.

1.3. Generation of chimeric VLPs carrying CTL epitopes fused to carboxy-terminal region of VP1 protein

The carboxy-terminal region of VP1 protein was predicted to be at least partially surface exposed and demonstrated to represent an immunodominant and highly cross-reactive part of VP1 (Siray at al., 2000). The deletion of C-terminal part of VP1 could reduce unfavorable cross-reactions and represent addition insertion site for CTL epitopes. Due to its putative involvement in the interaction of pentameric subunits of the virion, this VP1 region of HaPyV and other polyomaviruses has never been explored as a potential insertion site for foreign sequences. We showed that certain parts of the carboxy-terminal region of VP1 are not essential for pentamer–pentamer interactions in the capsid (Gedvilaite et al., 2006) and VP1 with deleted 307-384 aa region was able to form VLPs (name of the protein – VP1–4–stop). To explore the possibilities of this fifth insertion site, chimeric proteins carrying CTL epitopes on the end of VP1–4–stop protein were generated (Fig. 5).

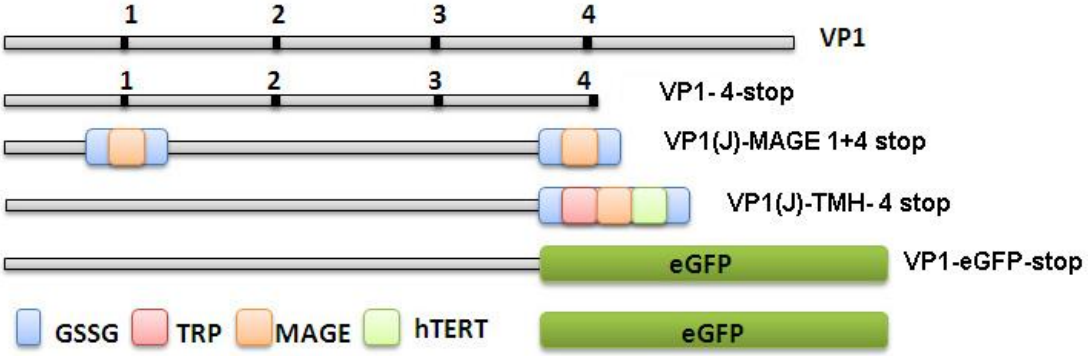


Figure 5. Schematic presentation of chimeric HaPyV VP1-based proteins with CTL epitopes insertions and truncated C-terminal region of VP1. Gray lines – VP1 protein; black small squares – CTL epitope insertion positions into HaPyV VP1 protein (1: 80-89aa, 3: 244-246aa, 4: 288-295aa); blue rectangular – GSSG linker; colorful squares – CTL epitopes (TRP, MAGE, hTERT). Green rectangular – eGFP protein. Protein names are written right side. C-terminal region of VP1 was truncated from position No. 4 (307-384aa).

Unfortunately, the expression and yield of chimeric VP1–4–stop proteins harboring CTL epitopes fused to truncated C terminal region was low and VLPs formation inefficient as fused CTL epitopes interfered with assembly of VLP. Most of constructed the proteins were able to form pentamers but VPLs were rare. Single hydrophobic CTL epitope insertions and eGFP protein (hydrophilic) were tolerated better than highly hydrophobic CTL epitopes (Table 2).

1.4. Generation of mosaic VLPs

Mosaic VLPs are composed from more than one kind of capsid proteins, e.g. chimeric VP1 and unmodified VP1 or VP1 and VP2 proteins. Preliminary studies showed that VLP formation and yield of chimeric VP1-eGFP protein was improved several times when it was coexpressed with unmodified "helper" VP1 protein and mosaic VLPs were formed (data not shown). In order to improve generation of some inefficiently expressed VLPs assembled by chimeric proteins harboring CTL epitopes, they were coexpressed together with "helper" VP1 or VP2 in yeasts, expecting mosaic VLPs formation (Table 2).

The results showed that generation of mosaic VLPs improved expression, quality and yield of majority of problematic chimeric proteins with two or three short CTL epitopes (Table 2). The purification procedure of mosaic VLPs became less complicated. Addition of "helper" VP1 had no influence only on improvement of generation of chimeric VP1–4–stop proteins harboring CTL epitopes fused to truncated C terminal region.

Table 2. Properties of mosaic VLPs

Name of chimeric protein	The usage of "helper" VP1	The usage of "helper" VP2	Chimeric protein expression	VLPs formation	Purified VLPs quantities, mg
VP1(J)-MAGE 1+4			poor	good	6
VP1(J)-MAGE 1+4	+		good	perfect	13
VP1(J)-MAGE 1+4 stop			poor	poor	2
VP1(J)-MAGE 1+4 stop	+		poor	poor	3
VP1(J)-CMV 1+4			poor	good	7
VP1(J)-CMV 1+4	+		good	perfect	15
VP1(J)-1T3M4H			good	poor	4
VP1(J)-1T3M4H	+		good	good	6
VP1(J)-TMH-4			poor	poor	2
VP1(J)-TMH-4	+		poor	poor	2
VP1(J)-TMH-4		+	poor	poor	2
VP1(J)-TMH-4-stop			poor	poor	2
VP1(J)-TMH-4-stop	+		poor	poor	2
VP1(J)-eGFP-4-stop			good	poor	3
VP1(J)-eGFP-4-stop	+		perfect	good	15

In column "The usage of "helper" VP1 (or VP2) symbol (+) indicates that mosaic VLPs were generated from chimeric protein coexpression with unmodified VP1 or VP2 protein. In column „ VP1 positions were CTL epitopes were inserted" symbol (+) shows the positions of VP1 were CTL epitopes inserted. „The usage of GSSG linker" symbol (+) indicates that inserted CTL epitope was surrounded by GSSG sequences. „Chimeric protein expression": perfect – chimeric protein syntheses level is equal to VP1 synthesis level, good – chimeric protein synthesis is 20% less than VP1, poor - chimeric protein synthesis is >50 % less than VP1. „VLPs formation": „perfect" – indicates that chimeric protein forms the same kind of VLPs as VP1 – 45 nm in diameter, sample contains a lot of VLPs, pentamers are not found; „good" – chimeric protein forms 45 nm VLPs, pentamers are also not found, but the amount of VLPs is less; „poor" – protein sample contains only some 45 nm VLPs, smaller VLPs are also found as well as pentamers. „Purified VLPs quantities" – indicates how many mg of chimeric proteins was purified from 20 g of yeast biomass, for comparison VP1 yield is 20 mg/20 g.

2. Generation, purification and characterization of VP1–GP33 chimeric VLPs

For evaluation of the capacity of HaPyV VP1-based chimeric VLP to trigger the development of protective CTL immunity against virus and tumor *in vivo* GP33 CTL epitope inserted into VP1 protein was used. GP33 peptide corresponding to an H-2D^b-restricted CTL epitope (aa residues 33–41) from surface glycoprotein of Lymphocytic choriomeningitis virus (LCMV) was chosen as model antigen (Pircher et al., 1989) based on several already published features: (i) GP33 is a strong CTL epitope presented by H-2Db MHC molecules and recognized in this context by specific CD8+ T cells (Pircher et al., 1990); (ii) GP33 allows tracing of GP33-specific CD8+ T cell response in normal mice; (iii) the GP33 epitope has been widely used as a tumor-associated model antigen. Depending on the tumor cell type and antigen expression levels different tumor specific immune functions were characterized such as: T cell priming (Kundig et al., 1995; Prevost-Blondel et al., 1998), spontaneous tumor regression (Prevost-Blondel et al., 2000), ignorance (Hermans et al., 1998; Ochsenbein et al., 1999; Speiser et al., 1997), or tolerance induction (Ochsenbein et al., 2001).

Figure 6. Schematic presentation of chimeric VP1-GP33 proteins. Gray lines – VP1 protein; black small squares – CTL epitope insertion positions into HaPyV VP1 protein (1: 80-89aa, 4: 288-295aa; pink squares – GP33 epitope (aa: KAVYNFATM). Protein names are written right side.

Chimeric HaPyV VP1–GP33 VLPs with LCMV GP33 epitope inserted into HaPyV VP1 positions No. 1 and No. 4 (Fig. 6) accordingly (Gedvilaite et al., 2000) were produced by heterologous synthesis in the yeast *Saccharomyces cerevisiae.* The generation of both VP1–GP33–1 and VP1–GP33–4 proteins (the expected molecular mass~43 kDa) was confirmed by Coomassie brilliant blue stained SDS-PAGE of yeast lysates and Western blot analysis using HaPyV VP1-specific mAbs (Fig. 7A).

Both chimeric proteins were as soluble as authentic HaPyV VP1. The VLP purification procedure and buffers were modified. The introduced modifications improved the yield of purified VP1–GP33 fusion proteins and it became similar to the yield of unmodified VP1 protein ~1 mg/g of wet yeast. The new modified procedure applied for chimeric VLPs purification allowed to save time, to improve efficiency and to cut chimeric VLP purification price (Table 3).

Both yeast expressed VP1–GP33 fusion proteins assembled into VLPs with the diameter of 40–45 nm typical to polyomavirus or unmodified VP1 VLPs (Fig. 7B).

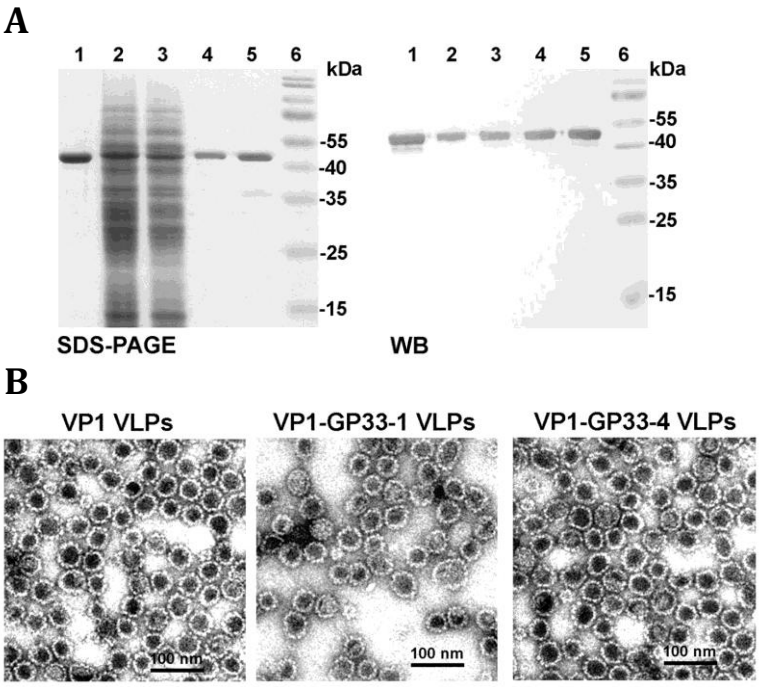


Figure 7. Generation and purification of VP1–GP33 VLPs. (A) VP1, VP1–GP33–1 and VP1–GP33–4 proteins identification in SDS-PAGE and Western blot analysis (WB) with anti-HaPyV VP1 mAbs 6D11. In lanes: 1 – purified VP1 protein; 2 – lysate of yeast cells expressing VP1–GP33–1 VLPs; 3 – lysate of yeast cells expressing VP1–GP33–4 VLPs; 4 – purified VP1–GP33–1 protein; 5 – purified VP1–GP33–4 protein; 6 – PageRuler prestained protein ladder (#SM0671 Fermentas, Lithuania). (B) electron microscopy pictures of VLPs, stained with 2% aqueous uranyl acetate solution and examined by Morgagni electron microscope.

Table 3. Comparison of different purification procedures of HaPyV VP1-based VLPs

Procedure	Classical VLP purification	Modified VLP purification
Yeast cell disruption. *Time – 1h.*	1	1
Cell lysate centrifugation. *Time – 0,5h.*	1	1
Centrifugation through sucrose cushion. *Time - 4 h.*	1	0
Centrifugation through sucrose gradient. *Time - 4 h.*	0	2-3
Centrifugation through CsCl gradient. *Time – 48h.*	2-3	1
VLP concentration by centrifugation. *Time – 12 h.*	1	1
Dialyses. *Time – 24h.*	1	1
Lyophilization. *Time – 24h.*	1	1

Numbers indicates how many times the procedure was performed.

3. Investigation of chimeric VLPs ability to induced antibody response *in vivo*

3.1. VP1-GP33 VLPs induced GP33-specific antibody response in vivo

The humoral immune response induced by immunizations with VP1–GP33–1 or VP1–GP33–4 VLPs was studied in 4 B6 mice (2 mice per group). Serum samples of immunized mice were analyzed for presence of antibodies against chimeric VP1–GP33 VLPs 28 days after the first, second and third immunization. In serum of all four immunized mice VP1–GP33–specific antibodies were detected by enzyme immunoassay starting from the first immunization and were confirmed in Western blotting using serum collected after the third immunization (Fig. 8A). The presence of GP33 specific antibodies in the serum was verified by flow cytometric analysis of serum treated and stained MCA102–GP33 cells expressing GP33 antigen on the cell surface and also parental MCA102 cells without GP33 antigen for negative control. Serum from 3/4 mice showed positive reactions. These mice developed antibodies

against GP33 peptide starting from the second immunization (Fig. 8B). The mouse which failed to develop GP33 specific antibodies was immunized with VP1–GP33–4 VLPs (data not shown).

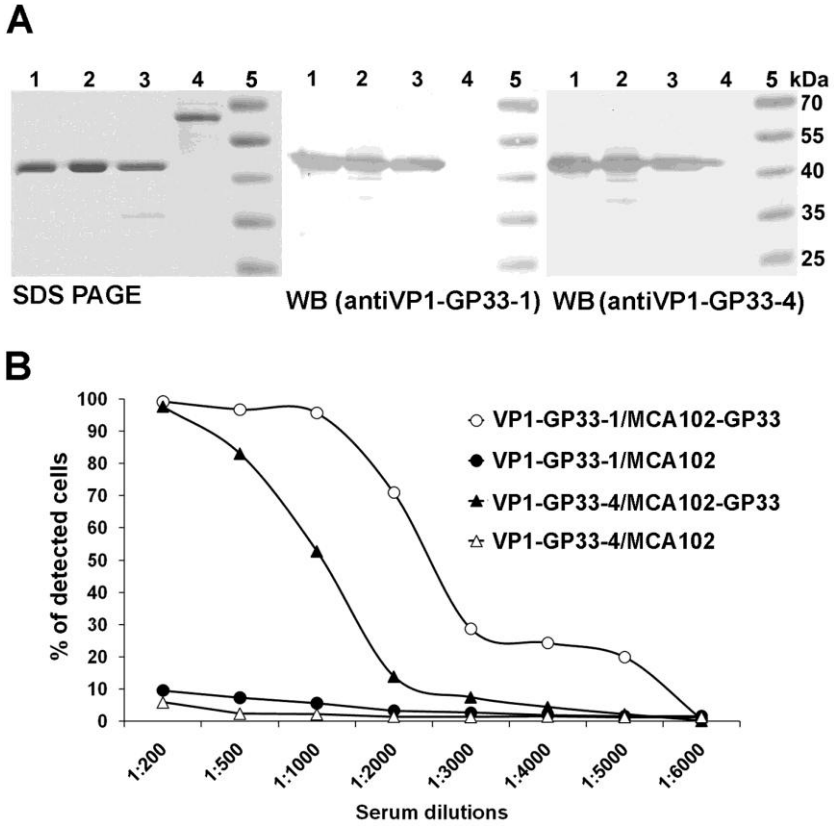


Figure 8. Detection of antiVP1–GP33 antibodies in serum of B6 mice immunized with VP1–GP33 chimeric VLPs. Four B6 mice were immunized with VP1–GP33–1 or VP1–GP33–4 VLPs three times at 28-day intervals. (A): Antibodies against VP1–GP33 VLPs were detected by Western blot analysis with after third immunization from VP1–GP33–1 VLPs (middle panel) and VP1–GP33–4 VLPs (right panel) immunized mice. The SDS-PAGE of same proteins shown in left panel. In lines: 1 – VP1 protein; 2 – VP1–GP33–1; 3 – VP1–GP33–4; 4 – negative control, Bocavirus VP2 protein; 5 – PageRuler prestained protein ladder (#SM0671 Fermentas, Lithuania). (B): GP33 specific antibodies were detected by flow cytometry of MCA102-GP33 cells after incubation with serum samples and staining. Parental MCA102 cells without GP33 antigen were used as negative control.

3.2. VP1-CEA VLPs induced antibody response in vivo

The humoral immune response induced by immunization with different chimeric VP1–CEA proteins was studied in Balb/C mice. All generated chimeric proteins (VP1(J)–CEA–1, VP1–CEA–1, VP1(J)–CEA–4, VP1–CEA–4, VP1(J)–CEA 1+4, VP1–CEA 1+4) formed VLPs with exception of only VP1(J)–CEA–1234 (data not shown). Three mice of each group were immunized intraperitoneally with VP1–CEA fusion proteins or VP1 alone and boosted after 6 weeks with the same amount of protein. There was no adjuvant administered at any time of immunization. Seven days after the boost, blood was taken and samples were screened by indirect ELISA for VP1, BSA, or CEA peptide conjugated to BSA. With the exception of the group immunized with VP1(J)–CEA–1234, in sera from mice of all other groups immunized with chimeric VLPs, CEA-specific antibodies were detected by their

reactivity with BSA-coupled CEA peptide (Table 4). In the groups immunized with VP1(J)–CEA–1, VP1–CEA–1, and VP1(J)–CEA 1+4 all mice developed CEA-reactive antibodies. In contrast, in the groups immunized with VP1(J)–CEA–4, VP1–CEA–4, and VP1–CEA 1+4 only two or one out of three mice showed an CEA-specific antibody response. Furthermore, the average of the titer in the groups immunized with VP1(J)–CEA–1, VP1–CEA–1 was higher than that observed in the other groups. Whereas the linker addition influenced the immunogenicity of the insert for these constructs only slightly, its employment drastically improved the immunogenicity of the construct harboring two CEA insertions. The lack of reactivity of sera from mice immunized with VP1 alone confirmed the specificity of the CEA ELISA (Table 4).

Table 4. ELISA reactivity of sera from mice immunized with chimeric VP1-CEA VLPs 7days and 6 month after final booster immunization (Lawatscheck et al., 2007)

Antigens used for mice immunization	Mouse Nr.	ELISA and-point titers for			
		VP1		**CEA+BSA**	
		7 days	6 months	7 days	6 months
VP1-CEA-1	1	24300	20000	2400	2000
	2	8100	-	2400	-
	3	24300	20000	>9600	3000
VP1(J)-CEA-1	4	24300	10000	4800	600
	5	24300	10000	600	300
	6	72900	20000	4800	600
VP1-CEA-4	7	2700	-	<300	-
	8	8100	-	2400	-
	9	8100	-	1200	-
VP1(J)-CEA-4	10	8100	-	<300	-
	11	24300	-	300	-
	12	72900	-	<300	-
VP1-CEA 1+4	13	2700	-	1200	-
	14	8100	-	1200	-
	15	900	-	2400	-
VP1(J)-CEA 1+4	16	24300	5000	<300	300
	17	24300	5000	<300	300
	18	24300	5000	<300	300
VP1(J)-CEA-1234	19	<100	-	<300	-
	20	<100	-	<300	-
	21	<100	-	<300	-
VP1	22	72900	10000	<300	<300
	23	72900	10000	<300	<300
	24	24300	-	<300	-

"-" – not determined.

All mice immunized with VP1-derived VLPs developed VP1-specific antibodies, except those immunized with VP1(J)–CEA–1234 (Table 4). In comparison with wild-type VP1, all VP1 responses induced by VP1/CEA VLPs were decreased. The immunization of mice with GSSG linker-bearing constructs resulted in higher titers of VP1- specific antibodies. In addition, the anti-VP1 responses of mice immunized with fusion proteins with only one insert were superior to those with two inserts. To prove the induction of long-lasting immunity, sera from mice immunized with the most immunogenic VLPs (VP1(J)–CEA–1, VP1–CEA–1, and VP1(J)–CEA 1+4) were taken 6 months after the final booster immunization and

tested for VP1- and CEA-specific antibodies (Table 4). All mice immunized with chimeric VLPs demonstrated the presence of CEA-specific antibodies with the highest titer being found in animals immunized with VP1–CEA–1 VLPs. In these animals the levels of the titers of anti-CEA and anti-VP1 antibodies after 6 months were only moderately reduced compared with the titers of antibodies analyzed 7 days after the final booster immunization. The reduction of the titers of CEA- and VP1-specific antibodies in the animals immunized with VP1(J)–CEA–1and VP1(J)–CEA 1+4 was larger.

4. Investigation of chimeric VP1–GP33 VLPs ability to stimulate LCMV GP33–specific CD8+ T cells *in vitro*

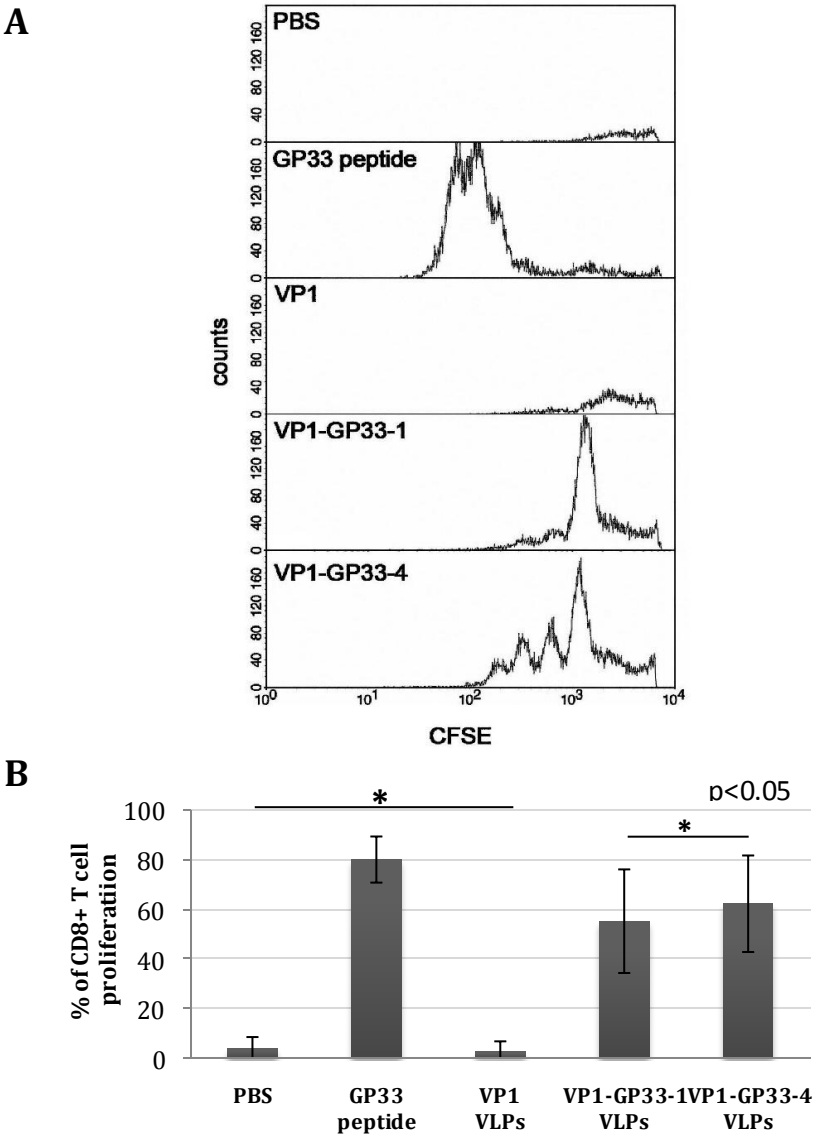


Figure 9. Proliferation of P14 mouse GP33-specific CD8+ T cells *in vitro*. CFSE-labeled splenocytes were labeled with PE conjugated anti-mouse CD8 antibodies after 4–5 days of stimulation and analyzed by flow cytometry. Living cells were gated on CD8+ T cells and percentage of proliferating CD8 cells (identified by low CFSE level) was determined. In (A) flow cytometry data from one representative experiment where CFSE-labeled P14 mouse splenocytes were stimulated with GP33 peptide, PBS or VLPs (VP1, VP1–GP33–1, and VP1–GP33–4 VLPs) were shown. For better visualization and for evaluation of statistical significance data from 4 independent experiments were summarized in diagram (B). Values were shown as mean +/-SD. All the differences statistically were significant (p<0,05) if not labeled (*) – not significant (t-test).

The immunogenicity of the model GP33 T cell epitope presented on chimeric VLPs was studied in cocultivation experiments. The complete splenocytes of transgenic H-2D^b (P14) mice, carrying the TCR specific for the amino acids 33–41 epitope of LCMV glycoprotein GP33 (Aichele et al., 1994), were stimulated with VP1, VP1–GP33–1 or VP1–GP33–4 VLPs or GP33 peptide alone. For negative

control, splenocytes were treated with PBS. Both chimeric VP1-GP33 VLPs were able to stimulate P14 mouse derived GP33–specific CD8+ T cells (P14 CD8+ T cells) almost as effective as GP33 peptide alone (Fig. 9). The GP33 peptide stimulated 70% of P14 CD8+ T cells and VP1–GP33 VLPs – around 50 % of these T cells compared to less than 1 % of CD8+ cell proliferation in the negative control. The stimulatory effect of VP1–GP33–4 VLPs was slightly stronger in comparison to VP1-GP33–1 VLPs. VP1 VLPs without GP33 insert were not able to stimulate P14 CD8+ T cells at all.

5. Investigation of VP1-GP33 VLPs pulsed DCs ability to stimulate GP33-specific CD8+ T cells *in vitro*

To evidence and strengthen the determined GP33-specific stimulatory effect of chimeric HaPyV VP1-based VLPs on P14 CD8+ T cells, VP1-GP33 VLPs were used for DCs maturation induction and then for stimulation of specific CD8+ T cells by these VP1-GP33 VLP pulsed DCs *in vitro*.

First, bone marrow derived DCs of naïve C57/BL6 (B6) mice (~80% $CD11c^+/MHCII^+$cells) were cocultivated with empty carrier VP1, VP1–GP33–1 and VP1–GP33–4 VLP, LPS or PBS for 72 h. The expression of costimulatory molecules CD80, CD86, CD40 were upregulated (Fig. 10) following maturation in most of the cells stimulated with VLPs or LPS, but not the cells treated with PBS. The DCs maturation efficiency was alike for all examined VLPs.

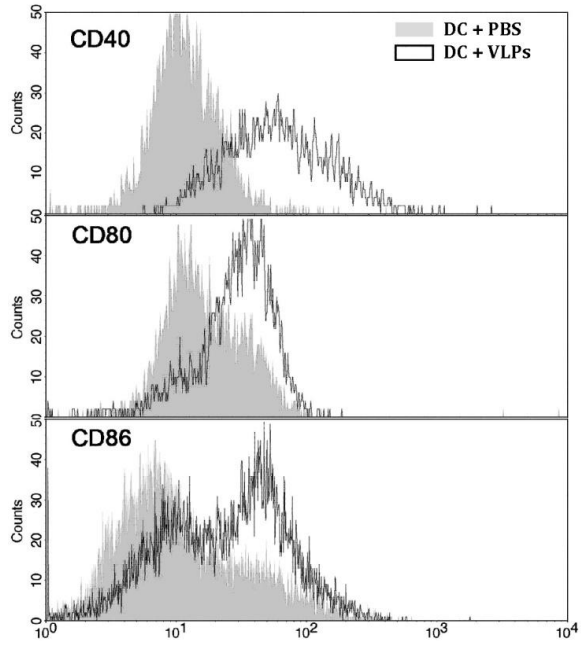


Figure 10. Mouse dendritic cell maturation with VLPs *in vitro*. B6 mouse bone marrow derived dendritic cells (~80 % $CD11c^+MHCII^+$) were maturated for 72h, with 10 µg/mL VLPS (VP1, VP1–GP33–1 or VP1–GP33–4) or with PBS for the negative control. The upregulation of CD80, CD86, CD40 was analyzed by flow cytometry. Cells were stained with FITC labeled mAbs (αCD80, αCD86, αCD40). Gray histograms show expression level of surface markers on immature dendritic cell in comparison with mature cells (black line). The high of the peak indicates the amount of cells; peak shift to the right – upregulation of surface markers. Data of one representative experiment out of three is shown. It his figure data with VP1 VLPs are shown, but all VLPs induced exactly the same upregulation level.

Second, all VLP pulsed DCs were able to stimulate the proliferation of CD8+ T cells from B6 and P14 mice *in vitro*, in contrast to unpulsed DCs (Fig. 11). A strong specific proliferation of CD8+ T cells from P14 mice was detected compared to a weak proliferation of CD8+ T cells from B6 mice. DCs pulsed with chimeric VP1–GP33 VLPs stimulated P14 CD8+ T cells 2–3 times stronger than DCs pulsed with empty carrier VP1 VLPs. A significantly stronger stimulation of GP33-specific CD8+ T cells was detected using VP1–GP33–4 VLP pulsed DCs compared to VP1–GP33–1

pulsed DCs. The effect of VP1–GP33–4 VLP pulsed DCs was as strong as the stimulation using GP33 peptide pulsed DCs or GP33 peptide itself.

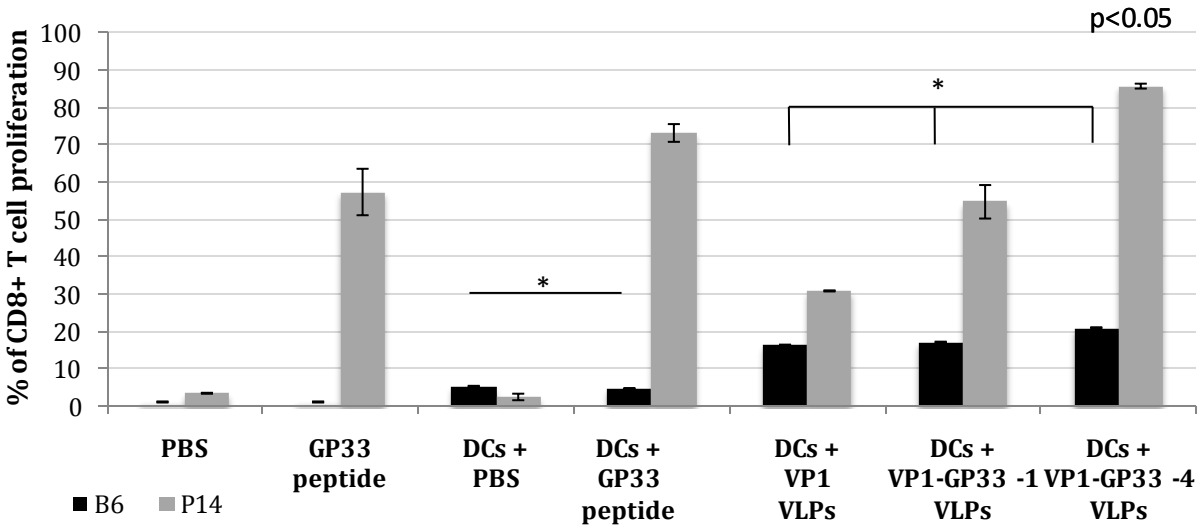


Figure 11. Proliferation of P14 and B6 mouse derived GP33-specific CD8+ T cells stimulated with VLPs pulsed DCs *in vitro*. CFSE-labeled splenocytes from P14 mouse and from B6 mouse (for negative control) were stimulated with DCs pulsed with 10 μg/mL VP1, VP1–GP33–1, VP1–GP33–4 VLPs (10^6 splenocytes + 10^5 DCs/mL). PBS and unpulsed DCs were used as negative controls. GP33 peptides and DCs pulsed with GP33 peptides were used as positive controls. The percentage of proliferating CD8+ T cells for better visualization and for evaluation of statistical significance was shown in diagrams. Values from two independent experiments were shown as mean +/-SD. All the differences statistically were significant ($p<0,05$) if not labeled (*) (t-test).

6. Investigation of chimeric VP1-GP33 VLPs ability to induce GP33-specific memory CTL response *in vivo*

The potential of chimeric VP1–GP33 VLPs to induce GP33–specific T cell response was verified by T cell proliferation assay using splenocytes from mice vaccinated with different VLPs 8 weeks after immunization. LCMV challenge in VLP immune mice was also performed.

T cell proliferation assay confirmed that vaccination of mice with VP1–GP33 VLPs generated detectable GP33–specific T cell response. The proliferation of CD8+ T cells from VP1–GP33 VLPs immunized mice restimulated with GP33 peptide was twice stronger than that of mice immunized with empty VP1 VLPs but ~3 times weaker than the proliferation of T cells from mice infected with LCMV (Fig. 12).

Splenocytes from VP1–GP33 VLP immunized mice restimulated with GP33 peptide for 5 and 24 h were unable to produce notable amounts of intracellular IFNγ compared to high levels of IFNγ produced by splenocytes from LCMV immune control mice.

To find out whether the induced T cell response provides protection from viral infections *in vivo*, mice immunized with VLPs (VP1, VP1–GP33–1 and VP1–GP33–4) without adjuvant 8 weeks after immunization were challenged with LCMV. For control naïve B6 and LCMV immune mice were challenged with LCMV as well. High

virus titers were found in all naïve B6 mice and mice vaccinated with empty carrier VP1 VLPs. Reduced virus titers were measured in 1/5 mice immunized with VP1-GP33–1 VLPs and 2/5 mice immunized with VP1–GP33–4 VLP. No LCMV could be detected in LCMV immune mice, 4/5 mice immunized with VP1–GP33–1 VLPs and 3/5 mice immunized with VP1–GP33–4 VLPs (Fig. 13A), indicating that a single immunization with chimeric VP1–GP33 VLPs without any adjuvant generated a specific CTL response, which fully protected 70% and partially 30% of mice from LCMV infection.

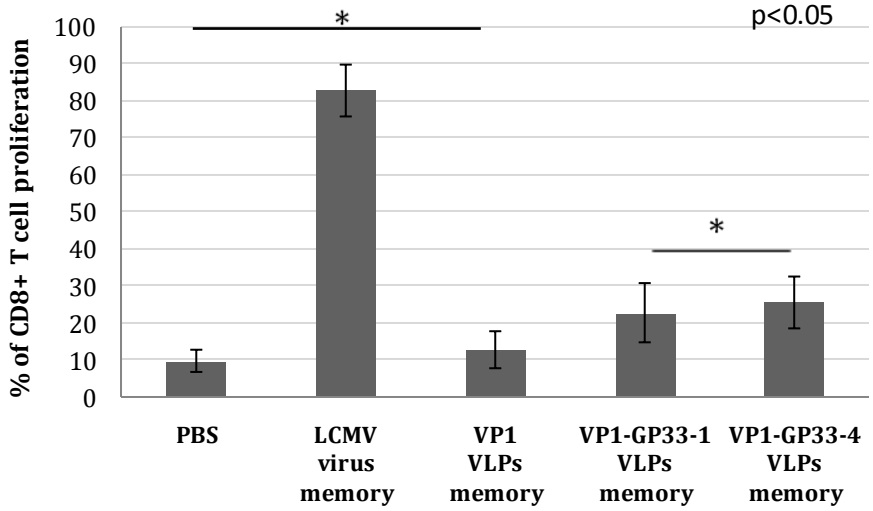


Figure 12. Detection of chimeric VLPs induced GP33-specific memory CTL response in mice by CD8+ T cell restimulation in vitro. 3 B6 mice per group were immunized i.v. with 50 µg VLPs (VP1, VP1–GP33–1, VP1–GP33–4), 200 pfu LCMV virus or PBS and 8 weeks later CFSE-labeled splenocytes were restimulated with GP33 peptide for 3 days. The percentage of proliferating CD8+ T cells for better visualization and for evaluation of statistical significance was shown in diagrams. Values from three independent experiments were shown as mean +/-SD. All the differences statistically were significant ($p<0,05$) if not labeled * (t-test).

7. MCA102–GP33 tumor challenge *in vivo*

To investigate the capability of memory T cells from VLP immunized mice to lyse tumor cells, VP1–GP33 (or PBS for negative control) immunized mice were injected with MCA102–GP33 fibrosarcoma cell suspension. In parallel 6 VP1–GP33 VLPs immunized mice were injected with cell suspension of parental cell line MCA102 not expressing GP33, as a control for non GP33-specific response against the tumor. The antitumor protective capacity was evaluated by time course measurement of tumor progression. GP33-unspecific response against the tumor in all 6 VP1–GP33 VLPs immunized mice (Fig. 13C) was very similar to the response in PBS treated control mice: the tumor growth was progressive. MCA102–GP33 tumors in all 7 PBS treated control mice (Fig. 13C) and all parental MCA102 tumors in mice immunized with VP1–GP33 VLPs (Fig. 13B) reached the size of 50 mm^2 until day 12 (in VP1–GP33–1 VLPs treated mice) or day 14 (in VP1–GP33–4 VLPs treated mice) post tumor injection. In contrast prophylactic immunization with VLPs partly protected mice from MCA102–GP33 tumor growth: only 1/7 VP1–GP33–4 VLP immunized mice, 0/7 VP1–GP33–1 VLP immunized mice and 3/7 mice

immunized with empty carrier VP1 VLP compared to 7/7 control mice developed tumors of 50 mm^2 size until day 12 (Fig. 13C). The vaccination of mice with chimeric VP1–GP33 VLPs had slightly stronger effect on tumors growth than the immunization with empty VP1 carrier. 10 days later (day 22) tumors in all 7/7 (100%) mice immunized with carrier VP1 VLP were bigger than 50 mm^2 and had reached 100 mm^2 size in 5/7 (71%) mice. By that time 3/7 (42%) and 4/7 (57%) mice immunized with VP1–GP33–1 and VP1–GP33–4 VLPs accordingly had tumors smaller than 50 mm^2 and only 1/7 and 1/7 (14%) mice developed tumors bigger than 100 mm^2. In all mice immunized with empty carrier VP1 VLPs tumors had reached the size of 100 mm^2 until day 27. In both groups of mice immunized with VP1–GP33 VLPs 8 mice (4/7 and 4/7 or 57%) had tumor size smaller than 100 mm^2 on day 27. Moreover, 2/7 (28%) mice immunized with VP1–GP33–4 VLPs stayed free of tumors for the whole experiment.

8. The effect of therapeutic injections of HaPyV VP1 and VP1–GP33 VLPs into MCA102-GP33 tumor bearing mice

HaPyV VP1-based VLPs were used for therapeutic purpose in mice bearing solid MCA102–GP33 tumors. At day 9 post tumor inoculation, when the MCA102-GP33 tumors had reached a size around 25 mm^2, mice were injected i.v. with 10 μg of VLPs (empty carrier VP1, VP1–GP33–1, VP1–GP33–4) or with PBS for negative control. VLPs injections were repeated three times (on days 9, 11, 13). Whereas in control mice tumors grew progressively and on day 12 had reached a size of more than 50 mm^2 in all six mice, only 2/6 VP1 VLPs treated mice were not responding to the therapy (Fig. 13D). Tumor progression arrest and tumor growth delay was observed in all mice groups receiving VLP therapy immediately after the first injection of VLPs, but the effect was slightly different in mice treated with empty carrier VP1 and VP1–GP33 VLPs. In mice injected with VP1 VLPs tumors were growing slower than in control mice but progressively, except one mice where tumor size regression was seen starting from day 15 until 19. In contrast VP1-GP33 VLPs injections induced tumor growth arrest or regression for some time what was similar to tumor regression pattern characteristic for LCMV infected mice (Prevost-Blondel et al., 1998). On day 21 tumors reached size of 50 mm^2 in all 6/6 mice treated with VP1 VLPs, but only in half (3/6 and 3/6 or 50%) mice injected with VP1–GP33 VLPs. Despite strong initial VLP–induced therapeutic effect at the end of experiment all tumors grew progressively.

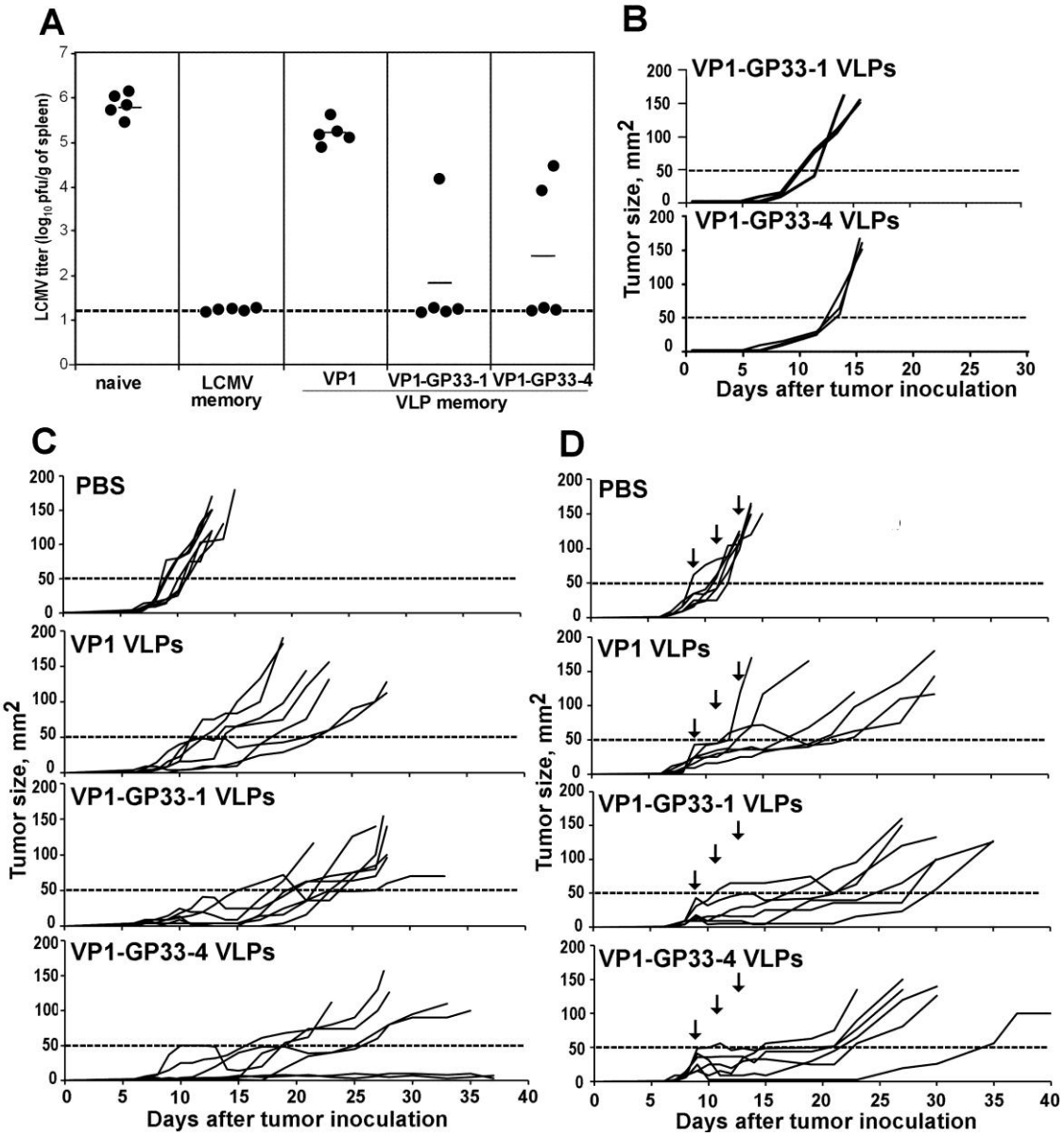


Figure 13. Protective efficacy of HaPyV VP1 based chimeric VLPs *in vivo*. Adult B6 mice were immunized i.v. with 50 μg of VLPs (VP1, VP1-GP33–1 and VP1-GP33–4), or 200 pfu LCMV virus, or PBS for positive and negative controls respectively. 8 weeks later mice were challenged with LCMV virus (A) or tumor not expressing (B) and expressing GP33 (C). (A): Mice were challenged i.v. with 200 pfu of LCMV; viral titer in the spleen was determined 4 days after the challenge and expressed as pfu/g of spleen tissue. Each circle represents an individual mouse, horizontal dash – mean titer value in the group. The dotted line represents the assay cutoff. Two representative experiments are shown. Differences between groups are statistically significant, $p<0,05$, Wilcoxon Mann-Whitney test (except VP1-GP33–1 *vs* VP1-GP33–4). (B and C): Mice were challenged by injecting 10^6 of MCA102 tumor cells (B) or MCA102-GP33 tumor cells (C) s.c. into the flank. Progression in time of tumor size of individual mice calculated as the product of bisecting tumor diameters is displayed. The horizontal dotted line marks tumor size 50 mm^2. (D): Therapeutical effect of HaPyV VP1 and VP1-GP33 VLPs injections on MCA102-GP33 tumor growth. B6 mice were injected with 10^6 of MCA102-GP33 tumor cells s.c. into the flank. When tumors reached ~25 mm^2 mice were treated i.v. with 10 μg VLPs or with PBS. Progression in time of tumor size of individual mice calculated as the product of bisecting tumor diameters is displayed. The horizontal dotted line marks tumor size 50 mm^2. Schedule of immunization on days 9, 11 and 13 when the treatments were administrated is shown by arrows.

DISCUSSION

In this study we wanted to evaluate the potential of recombinant HaPyV VP1 based VLPs for anticancer vaccine generation in model systems including investigation of VP1 applicability for heterologous CTL epitopes insertions, VLPs assembly and ability to induce inserts specific immune response *in vivo.*

It was shown that HaPyV VP1 tolerated various CTL epitope insertions and chimeric VLP were successfully generated in yeasts. It was determined that VP1 best tolerated one or two CTL epitopes insertions. The usage of GSSG linkers for CTL epitope insertions into VP1 improved VLPs formation only in some cases. For the generation of chimeric VLPs carrying three different CTL epitopes better solution was the insertion of separate CTL epitopes into different sites of VP1 VLPs as the insertion of a long fused polypeptide of several CTL epitopes unfavorably influenced chimeric protein expression, synthesis as well as VLP assembly and the yield. The fusing of CTL epitopes to truncated C-terminal end (aa 307-384) of the VP1 protein was not well tolerated and interfered with assembly of VLP. The improvement of chimeric VLP expression, assembly and yield sometimes was achieved by generating mosaic VLPs composed of chimeric VP1 carrying CTL epitope insertion and unmodified VP1 or VP2 proteins coexpressed together. The new modified procedure applied for chimeric VLPs purification allowed to save time, to improve efficiency and to cut chimeric VLP purification price.

HaPyV VP1-based VLPs demonstrated great capacity as excellent stimulators for antibody production (Zvirbliene et al., 2006) and human DC maturation *in vitro* (Dorn et al., 2008). Whereas evaluation of their capability to induce CTL immune response *in vivo* was still missing, in this study we evaluated the potential of HaPyV VP1 VLPs as carrier for CTL epitopes and capacity of HaPyV VP1-based chimeric VLP to trigger the development of protective CTL immunity in mice by employing the model epitope GP33 inserted into VP1 VLPs. Unlike GP33 peptide used as positive control in this study, chimeric VP1-GP33 VLPs were not able to bind directly to MHC class I molecules but have to undergo processing for correct antigen presentation by DCs and for stimulation of CD8+ T cells. Despite this, they induced DC maturation and stimulated GP33-specific P14 mouse splenocytes *in vitro* as effectively as GP33 peptide. Moreover, VP1-GP33-4 VLP pulsed DCs were able to stimulate GP33 CD8+ T cells *in vitro* more strongly than GP33 peptide pulsed DCs or GP33 peptide without DCs. These results clearly demonstrated that the GP33 CTL epitope inserted into HaPyV VP1 VLPs was efficiently processed and cross-presented by DCs as had been shown for other non replicating VLPs (Keller et al 2010; Ruedl et al., 2002; Storni and Bachmann 2004; Storni et al., 2002).

The proliferation of GP33-specific CD8+ T cells from spleens of VP1-GP33 VLP immunized mice indicated that T cells were able to recognize GP33 antigen, to divide and to differentiate but did not reveal effectors' function which they mediated. LCMV challenge and MCA102-GP33 tumor experiments allowed us to assess the ability of vaccinated mice to eliminate GP33 expressing cells and to address directly CD8+ T cell function *in vivo*. As it is known that LCMV infection *in vivo* is exclusively controlled by CD8+ T cells, an absence of virus elimination

reflects an absence of cytotoxic T cell activity (Bachmann, 1997). Three times weaker proliferation of T cells from mice immunized with VP1-GP33 VLPs compare to proliferation of T cells infected with LCMV (Fig. 13A) showed that after single immunization lower insert specific T cell quantities were induced in these mice. Full protection from virus infection requires some threshold amount of T cells and there is a possibility that the amount of T cells induced by VLPs after one immunization was not sufficient to ensure full protection in all mice. However, immunization with chimeric VP1-GP33 VLPs without adjuvant induced protective immune response in mice: 7/10 (70%) mice were fully and 3/10 (30%) mice partially protected against LCMV challenge, suggesting effective insert-specific CD8+ T cell induction *in vivo*. Not all VLPs are able to stimulate effective and long lasting CTL response *in vivo*, especially without additional adjuvant (Ding et al., 2009; Lacasse et al., 2008; Martinez et al., 2003). Interestingly, immunization with chimeric hepatitis B core Ag VLPs harboring LCMV GP33 peptide fully protected mice from LCMV challenge infection only when they were applied with CpG-rich DNA (Storni et al., 2002).

To strengthen LCMV challenge result which showed effective CTL immune response generated by VP1-GP33 VLPs, the very aggressive MCA102-GP33 tumor model was also used. Only in LCMV immune mice MCA102-GP33 tumor cells are fully rejected. LCMV therapy only temporarily eliminates this tumor but at the end tumor escape variants appear (Blohm et al., 2002). In mice vaccinated with chimeric VP1-GP33 VLPs and challenged with MCA102-GP33 tumor, tumor growth was delayed compared to mice injected with PBS which means that memory CD8+ CTLs were activated. This capability of HaPyV VP1-based VLPs to induce effective CTL response in mice was supported also by a therapeutic effect against MCA102-GP33 tumor. CD8+ T cells activated by VLPs were able to find and to eliminate not only inoculated tumor cell suspension but also solid tumor cells in real situation *in vivo.* However, weaker antitumor effect induced by VLPs comparing to LCMV induced antitumor effect (Blohm et al., 2002) could be the result of IFNγ production deficiency in splenocytes after stimulation with VLPs because it is known that IFNγ is the only effector molecule in MCA102 tumor model (Blohm et al., 2006).

Surprisingly, immunization with empty VP1 VLPs showed quite strong protective antitumor effect and slight protection against LCMV challenge. CD8+ T cell activation *in vivo* induced by empty VP1 VLPs was not as efficient as that induced by chimeric VP1-GP33 VLPs but clearly detectable. The tumors in all 7 VP1 VLPs immunized mice reached the 50 mm^2 size on 22 day but only half of VP1-GP33 VLPs immunized mice had the tumors of this size on this day. Because of severity of the tumor model even in mice injected with LCMV, tumors reaches the 50 mm^2 size on days 25-30 (Blohm et al., 2002). The possibility that injection of the VP1 VLP induced a non specific activation of the immune system in mice which facilitated partial virus clearance by the mice or had an antitumor effect could be argued. VP1-G33 VLPs had GP33-specific stimulatory effect on GP33-specific CD8+ T cells *in vitro* (Fig. 13B) and prophylactic immunization with VLPs clearly delayed MCA102-GP33 but not MCA102 tumor growth in mice (Fig. 13C). More likely that VP1 VLPs induced activation of cross-reactive memory CD8+ T cells. It is known that only a

few TCR contact residues on the peptide presented by MHC molecules are required for activation, although a TCR of CD8+ T cell can tolerate certain amino acid substitutions in the peptide sequence and still become activated. Therefore antigen specific CD8+ T memory cells can cross-react with different epitopes derived either from the same or a related antigen, or a totally unrelated one. Examples of cross-reactive T-cell responses involving completely unrelated viruses such as LCMV and vaccinia virus (Kim et al., 2005), influenza virus and HIV (Acierno et al., 2003), human papilloma virus and coronavirus (Nilges et al., 2003), have been reported. The analysis of Immune Epitope Database results and multiple sequences alignments revealed the presence of six potential H-2 D^b CTL epitopes within the HaPyV VP1 protein which exhibited sufficient homology to GP33 peptide for cross-reacting T cell epitopes (Selin et al., 2006). To confirm this hypothesis, it would be interesting to mutate these TCR contact residues in the VP1 molecule to eliminate cross-reactivity and to study the protection induced by mutant variants.

Our results demonstrated that efficient antibody response against VP1-GP33 VLPs in mice had not inhibited CTL response. Although there were some reports that influence of VLP-specific antibodies on antigen presentation impaired the induction of protective immune responses (Da Silva et al., 2001), more often specific T-cell responses for non-replicating vaccines were rather improved than reduced by the presence of specific antibodies (Den Hann and Bevan, 2002; Kalergis and Ravetch, 2002; Ruedl et al., 2005). The specific antibodies may facilitate CTL activation by enhancing opsonization of VLPs leading to a stronger antigen presentation and CTL activation. So, the presence of specific antibodies could be an advantage for enhancing GP33 specific CTL response induced by chimeric VP1-GP33 VLPs.

In conclusion, VP1-GP33 VLPs were well processed, cross-presented by antigen presenting cells to immune system and induced insert-specific CD8+ T cell response. VLPs induced CTL response had a protective and therapeutic capacity and was not inhibited by B cell immune response. Our results demonstrated the potential of chimeric HaPyV VP1-derived VLPs as carriers of CTL epitopes suitable for the induction of CTL immune response and for the development of vaccines against desired antigens.

CONCLUSIONS

1. HaPyV major capsid protein VP1 tolerated CTL epitope insertions and chimeric VLPs were successfully generated in yeast *Saccharomyces cerevisiae.*

2. Sequences of inserted CTL epitopes influenced chimeric VLPs properties. Improvement of chimeric VLP formation and yield of purified VLPs harboring particular epitope can be achieved by selecting favorable insertion position and the number of insertions into VP1 protein, applying additional linker sequences or adding unmodified VP1 or VP2 proteins for mosaic VLPs formation.

3. Chimeric VLPs based on HaPyV VP1 induced insert-specific and carrier-specific humoral immune response in mice.

4. Chimeric VLPs based on HaPyV VP1 induced dendritic cells maturation *in vitro* whereas mature dendritic cells effectively stimulated antigen specific CTL cells.

5. Chimeric model VLPs harboring GP33 CTL insert induced GP33-specific long lasting immune response *in vivo*:

    5.1 Mice immunized only once with VP1-GP33 VLPs without adjuvant developed an effective GP33-specific memory T cell response: 70% were fully and 30% partially protected from LCMV infection;

    5.2 Prophylactic immunization with model chimeric VP1-GP33 VLPs without any adjuvant suppressed GP33 specific tumor cell growth and repetitive therapeutic VLPs injections induced tumor growth delay or progression arrest of solid antigen specific tumor in mice.

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REZIUMĖ

Į virusus panašios dalelės (VPD) galėtų būti perspektyvi alternatyva peptidinėms vakcinoms, skirtoms imuniniam atsakui prieš CTL epitopus sukelti. VPD pasižymi virusams būdingomis savybėmis – pasikartojančiu paviršiumi, virusine kilme, dydžiu, gebėjimu sukelti humoralinį ir (ar) ląstelinį imuninį atsaką. Šiame darbe buvo parodytos rekombinantinių žiurkėno poliomos viruso (HaPyV) struktūrinių VP1 baltymų formuojamų VPD panaudojimo priešvėžinių vakcinų kūrimui galimybės, įvertintas svetimų CTL epitopų įterpimo į VP1 baltymą toleravimas, VPD formavimosi efektyvumas bei įterptam antigenui sukeltas specifinis imuninis atsakas.

Pirmiausia buvo sukurti mielių *Saccharomyces cerevisiae* producentai, gaminantys HaPyV VP1 pagrindu sukonstruotus chimerinius (su įvairiais CTL epitopų intarpais) baltymus, su kuriais buvo ištirtos HaPyV viruso pagrindinio kapsidės baltymo VP1 – svetimų CTL epitopų nešiklio, savybės. Buvo nustatyta, kad HaPyV VP1 toleruoja įvairius CTL epitopų intarpus, todėl šis baltymas tinka chimerinių VPD gamybai mielėse *S. cerevisiae*. Buvo parodyta, kad HaPyV VP1 baltymas geriausiai toleravo vieno ir dviejų CTL epitopų kopijų įterpimą. GSSG jungtukų panaudojimas svetimų sekų įterpimui palengvino chimerinių VPD formavimąsi tik atskirais atvejais. Eksperimentai patvirtino, kad chimerinių VPD su trimis skirtingais CTL epitopais gavimui geriau buvo po vieną CTL epitopus įterpti į skirtingas VP1 baltymo pozicijas, negu ilgesnio polipeptido, gauto suliejus tris CTL epitopus, įterpimas į vieną VP1 baltymo vietą. Pasirodė, kad VP1 baltymo su sutrumpintu C-galu panaudojimas CTL epitopų prijungimui netinka, tačiau pasiteisino mozaikinių VPD, sudarytų iš „pagalbinio“ nemodifikuoto VP1 baltymo ir chimerinių VP1 baltymų gamyba, nes pagerėjo chimerinių baltymų raiška ir išgrynintų VPD išeigos. Darbo eigoje buvo sukurta efektyvesnė chimerinių VPD gryninimo schema, kuri sutrumpino ir atpigino chimerinių VPD gryninimą, padidino išgrynintų VPD išeigas.

Chimerinių VPD su įterptais CTL epitopais gebėjimas sukelti antikūnų prieš įterptus peptidus sintezę buvo tiriamas pelėse. Buvo nustatyta, kad chimerinės VPD su CTL epitopų intarpais (MUC, CEA, GP33) pelėse sukėlė VP1 baltymui bei įterptiems 9 aminorūgščių ilgio CTL epitopams specifinį humoralinį imuninį atsaką. Stipresnis atsakas sukėlė VPD, kurių CTL epitopas buvo įterptas 1-ojoje VP1 baltymo pozicijoje (80-89 ar.).

Norint ištirti HaPyV VP1 pagrindu sukurtų chimerinių VPD gebėjimą sukelti įterptai sekai specifinį CTL imuninį atsaką, buvo sukurtos chimerinės VP1-GP33 VPD su įterptu modeliniu GP33 CTL epitopu.

Dendritinių ląstelių stimuliavimas modelinėmis chimerinėmis VPD subrandino dendritines ląsteles, kurios stimuliavo GP33 antigenui specifines CTL ląsteles *in vitro*. Taip buvo patvirtinta, kad chimerinės VPD su CTL epitopų intarpais žmogaus bei pelės dendritinių ląstelių buvo teisingai procesuojamos.

Tiriant modelinių chimerinių VP1-GP33 VPD gebėjimą sukelti GP33 specifinį CTL imuninį atsaką *in vivo*, buvo parodyta, kad vienkartinė imunizacija VPD be jokių adjuvantų pelėse sužadino ilgalaikio GP33 antigenui specifinio imuninio

atsako susidarymą, o susidaręs imuninis atsakas buvo efektyvus – jis iš dalies arba visiškai apsaugojo peles nuo LCMV infekcijos. Efektyvaus CTL atsako susidarymą pelėse patvirtino ir eksperimentai su vėžinėmis ląstelėmis. Profilaktinė pelių imunizacija modelinėmis VPD be adjuvantų pristabdė modeliniam antigenui specifinių vėžinių ląstelių augimą, o pakartotiniai terapiniai VPD sušvirkštimai slopino jau esamų antigenui specifinių auglių augimą.

HaPyV VP1 baltymas gali būti naudojamas CTL epitopų nešikliu, o jo pagrindu sukurtos chimerinės VPD tinka vakcinų, skirtų imuniniam atsakui prieš norimą CTL epitopą sukelti, kūrimui.

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