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Induction of insert-specific immune response in mice by hamster polyomavirus VP1 derived virus-like particles carrying LCMV GP33 CTL epitope

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ABSTRACT

Hamster polyomavirus (HaPyV) major capsid protein VP1 based chimeric virus-like particles (VLPs) carrying model GP33 CTL epitope derived from Lymphocytic choriomeningitis virus (LCMV) were generated in yeast and examined for their capability to induce CTL response in mice. Chimeric VP1-GP33 VLPs were effectively processed in antigen presenting cells *in vitro* and *in vivo* and induced antigen-specific CD8+ T cell proliferation. 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. Moreover, aggressive growth of tumors expressing GP33 was significantly delayed in these mice *in vivo*. Therefore, HaPyV VP1-derived VLP harboring CTL epitopes are attractive vaccine candidates for the induction of insert-specific CTL immune response.

1. Introduction

Recombinant virus-like particles (VLPs) are the icosahedral or rod-like structures comprising multiple copies of one or more viral structural proteins. They are usually similar to the authentic viruses they are derived from, but non-infectious because they are free of viral genetic material (for review see Pumpens and Grens, 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, 2004; Storni et al., 2002). This is probably due to their macromolecular structure and particulate nature.

VLPs may provide an alternative tool for the improvement of non-immunogenic peptide immunogenicity due to their repetitive surface epitopes, the virus-like structure and the capability to induce humoral and/or CTL responses (for review see Pumpens and Grens, 2002). 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 the induction of a strong CTL response most VLPs have to be administered together with adjuvant-like molecules such as CpGs or anti-CD40 (Schwarz et al., 2005; Storni et al., 2002).

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; Gedvilaite et al., 2004). In addition, HaPyV VP1-based chimeric VLPs produced by yeast 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 maturation of human dendritic cells (DC), IL-12 production and stimulation of CD8+ T cells *in vitro* more efficiently than VLPs of other polyomaviruses (JCV, BKV, SV40 or MPyV; Gedvilaite et al., 2006). Interestingly that chimeric mouse polyomavirus capsid protein based VLPs harboring tumor antigens were shown to inhibit tumor growth in a mouse model (Andreasson et al., 2009; Brinkman et al., 2005). In contrast, information about the capacity of HaPyV VP1 based chimeric VLPs to induce effective epitope-specific CTL immune response *in vivo* is still missing.

In this study we have investigated the capacity of HaPyV VP1-based chimeric VLP to trigger the development of effective CTL response by using well established model system dependent on CTL immunity based protection against virus and tumors *in vivo*. 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-2D^b MHC molecules and recognized in this context by specific CD8+ T cells (Pircher et al., 1990);

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(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 the 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).

Chimeric VP1-GP33 VLPs were generated in yeast *Saccharomyces cerevisiae* and were used to study GP33-specific CTL response in mice *in vitro* and *in vivo*.

2. Materials and methods

2.1. 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 #1 and #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). An oligonucleotide duplex encoding the 9 amino acid long LCMV GP33 CTL peptide (Fig. 1A) was inserted into the VP1-encoding sequence into positions #1 and #4 (Fig. 1B) cloned into the expression cassette of the yeast vector pFX7 (Sasnauskas et al., 1999). Chimeric VP1-GP33 protein-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 slightly modified compared to the one described earlier (Gedvilaite et al., 2000). 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

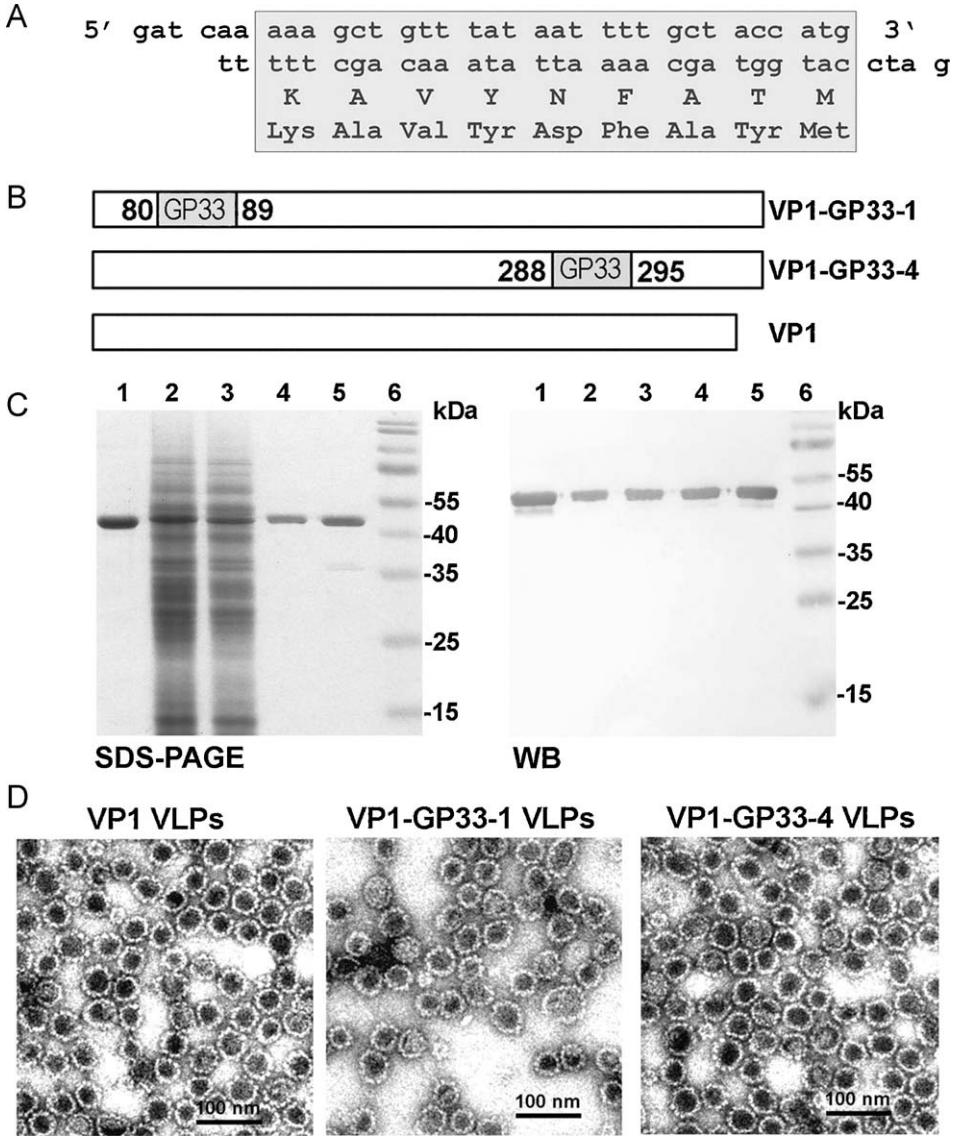

Fig. 1. Generation and purification of HaPyV VP1 based VLPs. (A) Nucleotide and amino acid sequences of LCMV GP33 epitope. (B) Schematic figure of VP1, VP1-GP33-1 and VP1-GP33-4 proteins (aa positions of insertion sites #1 and #4 are indicated) and their identification in SDS-PAGE and Western blot analysis (WB) with anti-HaPyV VP1 mAbs 6D11 (C). 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). (D) Electron microscopy pictures of VLPs, stained with 2% aqueous uranyl acetate solution and examined by Morgagni electron microscope.

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 (1.23–1.38 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.

2.2. 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).

2.3. 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). Statistical significance of virus challenge experiments was calculated using Wilcoxon Mann–Whitney test.

2.4. Mice and tumors

C57BL/6 (B6) mice were obtained from breeding stock of the Friedrich-Loeffler 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 H.P. Pircher (Freiburg, Germany).

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

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 10^6 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 >15 mm were killed according to animal care regulations. When the experiment was finished mice were decoded and results were analyzed.

2.5. 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) stained with FITC or PE conjugated streptavidin; FITC conjugated anti-mouse I-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 stained 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.

2.6. 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.

2.7. 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 2×10^6 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 CD8a 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. T cell proliferation results were compared using Student's *t*-test, values with $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Generation, purification and characterization of VP1-GP33 chimeric VLPs

Chimeric HaPyV VP1-GP33 VLPs with LCMV GP33 epitope (Fig. 1A) inserted into HaPyV VP1 positions #1 and #4 (Fig. 1B) accordingly (Gedvilaite et al., 2000) were produced by heterologous synthesis in the yeast *S. 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. 1C).

Both chimeric proteins were as soluble as authentic HaPyV VP1. The yield of VP1-GP33 fusion proteins after purification was ~1 mg/g of yeast cells. Both VP1-GP33 fusion proteins assembled into VLPs similar to unmodified HaPyV-VP1 VLPs with the same polyomavirus-typical diameter of 40–45 nm (Fig. 1D).

3.2. Chimeric VP1-GP33 VLPs were able to stimulate LCMV GP33-specific CD8+ T cells in vitro

The immunogenicity of the model GP33 T cell epitope presented on chimeric VLPs was studied in co-cultivation 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 the GP33 peptide alone (Fig. 2A and B). The stimulatory effect of VP1-GP33-1 and VP1-GP33-4 VLPs was very similar. The GP33 peptide stimulated 70% and VP1-GP33 VLPs – around 50% of P14 CD8+ T cells compared to less than 1% of CD8+ T cell proliferation observed for the negative control. VP1 VLPs without GP33 insert were not able to stimulate P14 CD8+ T cells at all.

3.3. VP1-GP33 VLPs-pulsed DCs were able to stimulate GP33-specific CD8+ T cells in vitro

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

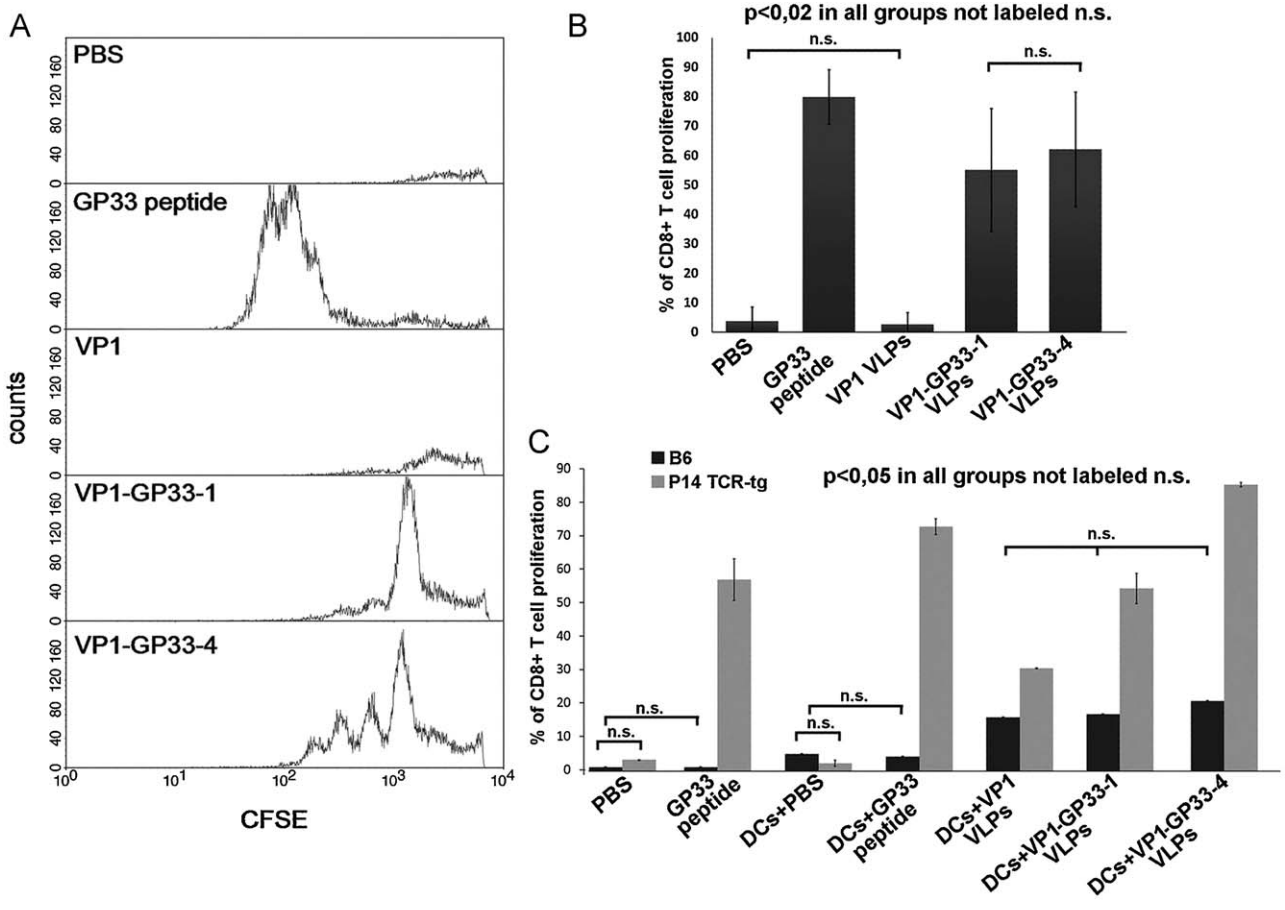

Fig. 2. Proliferation of GP33-specific CD8+ T cells *in vitro*. CFSE-labeled splenocytes were labeled with PE conjugated anti-mouse CD8 antibodies after 4–5 days of stimulation and analyzed by flow cytometry. Living cells were gated on CD8+ T cells and percentage of proliferating CD8+ cells (identified 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 (2 P14 mice per group were used in each experiment) were summarized in diagram (B). Values were shown as mean ± SD. All the differences were statistically significant ($p < 0.02$) if not labeled n.s. – not significant (*t*-test). (C) CFSE-labeled splenocytes from P14 mouse and from B6 mouse (for negative control) were stimulated with DCs treated with 10 μg/mL VP1, VP1-GP33-1, VP1-GP33-4 VLPs (10^6 splenocytes + 10^5 DCs/mL). PBS and untreated DCs were used as negative controls. GP33 peptide and DCs pulsed with GP33 peptide 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 (3 B6 and P14 mice per group were used in both experiments) were shown as mean ± SD. All the differences were statistically significant ($p < 0.05$) if not labeled n.s. (*t*-test).

First, bone marrow derived DCs of naïve C57/BL6 (B6) mice (~80% $CD11c^+/MHCII^+$ cells) were co-cultivated with empty carrier VP1, VP1-GP33-1 and VP1-GP33-4 VLPs, LPS or PBS for 72 h. The expression of co-stimulatory molecules CD80, CD86, CD40 were upregulated following maturation in most of the cells stimulated with either VLPs or LPS. However, it was not observed in cells treated with PBS. The DCs maturation efficiency was comparable for all examined VLPs (data not shown).

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. 2C). A strong specific proliferation of CD8+ T cells from P14 mice was detected compared to a weak non-specific proliferation of CD8+ T cells from B6 mice. DCs treated with chimeric VP1-GP33 VLPs stimulated P14 CD8+ T cells 2–3 times stronger than DCs incubated 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 VLP-treated 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.

3.4. Chimeric VP1-GP33 VLPs were able 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 and intracellular IFNγ measurement 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. 3A).

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 (data not shown).

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 were challenged with LCMV 8 weeks after immunization. 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 (Fig. 3B). Reduced virus titers, as compared to naïve and VP1 VLPs immunized mice, 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. 3B), indicating that a single immunization with chimeric VP1-GP33 VLPs without any adjuvant generated a GP33-specific CTL response, which fully protected 70% and partially 30% of mice from LCMV infection.

3.5. 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 GP33-unspecific response against the tumor. The antitumor protective capacity was investigated by time course measurement of tumor progression.

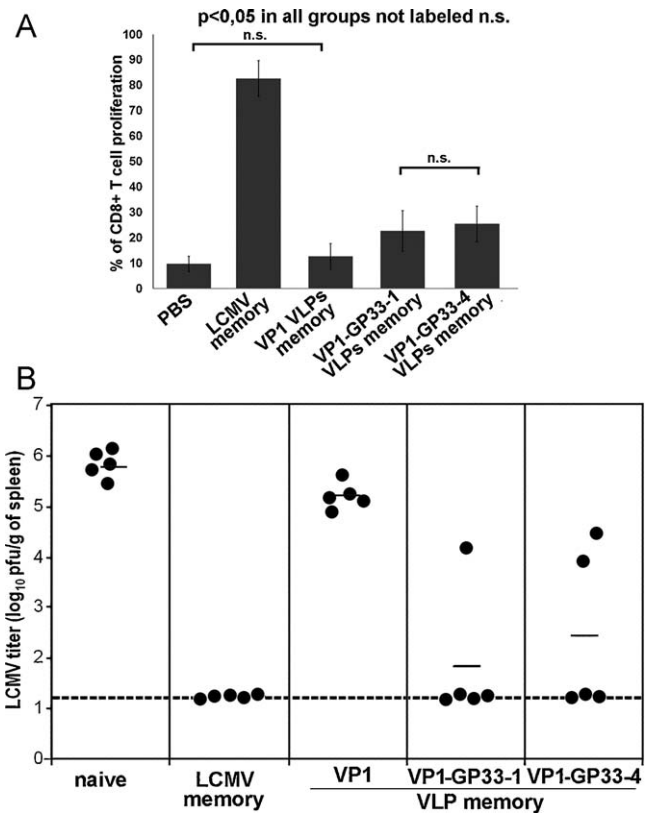

Fig. 3. Detection of chimeric VLPs-induced GP33-specific memory CTL response in mice by CD8+ T cell restimulation *in vitro* (A) and LCMV challenge *in vivo* (B). Adult B6 mice were immunized i.v. with 50 μg of VLPs (VP1, VP1-GP33-1 and VP1-GP33-4), or 200 pfu LCMV, or PBS for positive and negative controls respectively. 8 weeks later CFSE-labeled splenocytes from immunized mice were restimulated with GP33 peptide for 3 days (A) or mice were challenged with LCMV (B). (A) Splenocytes from 3 B6 mice per group were restimulated and 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 (3 B6 mice per group) were shown as mean ± SD. All the differences were statistically significant ($p < 0.05$) if not labeled n.s. (*t*-test). (B): 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. Data from two representative experiments (3 and 2 B6 mice per group) are shown. Differences between groups are statistically significant, $p < 0.05$ according Wilcoxon Mann–Whitney test (except VP1-GP33-1 *vs.* VP1-GP33-4).

The GP33-unspecific response against the MCA102 tumor in all 6 VP1-GP33 VLPs immunized mice (Fig. 4A) 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. 4B) and parental MCA102 tumors in all 6 mice immunized with VP1-GP33 VLPs (Fig. 4A) 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 naïve mice developed tumors of 50 mm^2 size until day 12 (Fig. 3B). 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 immu-

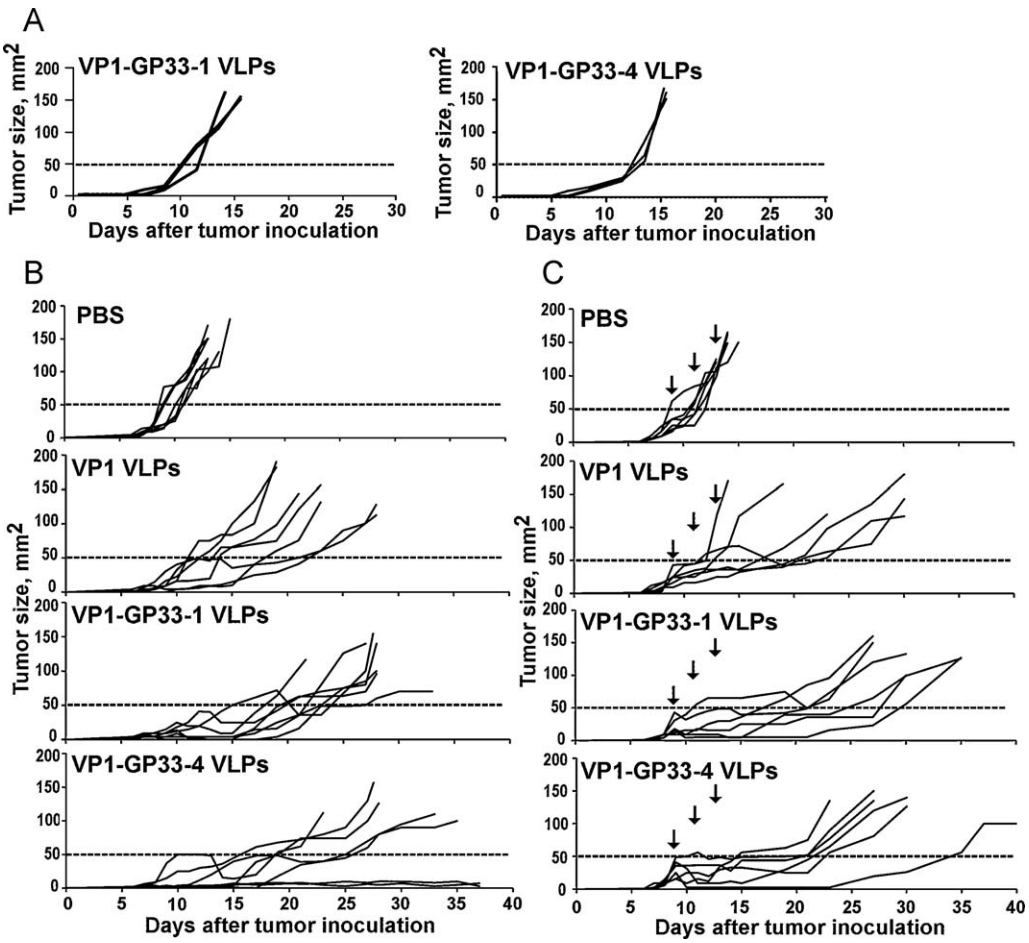

Fig. 4. Protective and therapeutical efficacy of HaPyV VP1 based chimeric VLPs *in vivo* using tumor model system. (A and B) Adult B6 mice were immunized i.v. with 50 μg of VLPs (VP1, VP1-GP33-1 and VP1-GP33-4), or 200 pfu LCMV, or PBS for positive and negative controls respectively. 8 weeks later mice were challenged by injecting s.c. into the flank (A) 10^6 of MCA102 tumor cells not expressing GP33 (3 B6 mice per group) or (B) MCA102-GP33 tumor cells expressing GP33 (7 B6 mice per group). 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) B6 mice were injected with 10^6 of MCA102-GP33 tumor cells s.c. into the flank. Therapeutical effect of HaPyV VP1 and VP1-GP33 VLPs injections on MCA102-GP33 tumor growth was examined when tumors reached ~25 mm^2 and mice were treated i.v. with 10 μg VLPs or with PBS (6 B6 mice per group). 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.

nized 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.

3.6. Therapeutic injections of HaPyV VP1 and VP1-GP33 VLPs temporally delayed MCA102-GP33 tumor growth in 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 6 mice, only 2/6 VP1 VLPs-treated mice were not responding to the therapy (Fig. 4C). Tumor progression arrest and tumor growth delay were 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 transient tumor growth arrest or regression similar to the tumor regression pattern observed 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 mice (3/6 and 3/6 or 50%) injected with VP1-GP33 VLPs. Despite strong initial VLP-induced therapeutic effects all tumors grew progressively at the end of experiment.

3.7. 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 (data not shown) and were confirmed by Western blotting using serum collected after the third immunization (Fig. 5A). The presence of GP33-specific antibodies in the serum was verified by flow

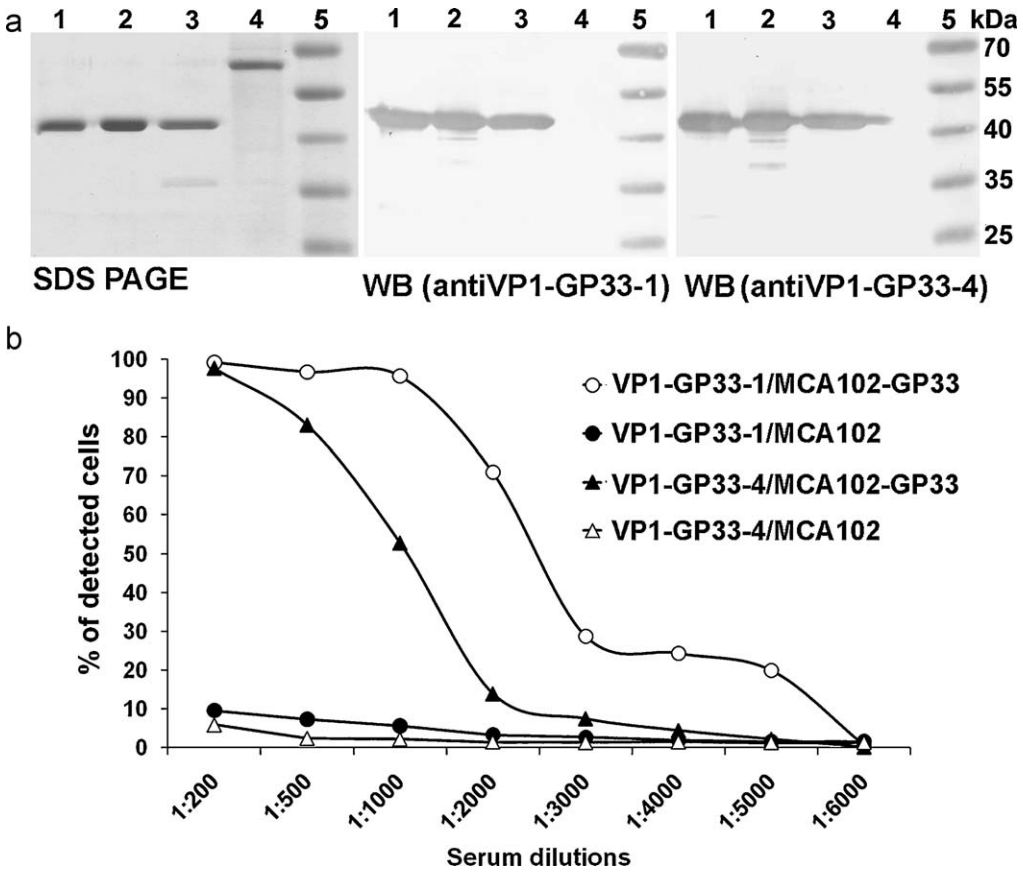

Fig. 5. Detection of antiVP1-GP33 antibodies in serum of B6 mice immunized with VP1-GP33 chimeric VLPs. Four B6 mice were immunized with VP1-GP33-1 or VP1-GP33-4 VLPs three times at 28-day intervals. (A) Antibodies against VP1-GP33 VLPs were detected by Western blot analysis with after third immunization 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.

cytometric analysis of sera-treated MCA102-GP33 cells expressing GP33 on the cell surface and parental MCA102 cells without GP33 for negative control. Serum from 3/4 mice showed positive reactions. These mice developed antibodies against GP33 peptide starting from the second immunization (Fig. 5B). The mouse which failed to develop GP33 specific antibodies was immunized with VP1-GP33-4 VLPs (data not shown).

4. Discussion

HaPyV VP1-based VLPs demonstrate great capacity as epitope display system (Gedvilaite et al., 2000; Gedvilaite et al., 2004), they are excellent stimulators for antibody production (Zvirbliene et al., 2006) and human DC maturation *in vitro* (Dorn et al., 2008). However, until now not much is known about their capability to induce CTL immune response *in vivo*. Thus, in our present study we have analyzed the potential of HaPyV VLPs as carrier for CTL epitopes to induce effective CTL immune response in mice.

Unlike the GP33 peptide, chimeric VP1-GP33 VLPs have to be processed by DCs for correct antigen presentation, binding to MHC class I molecules and stimulation of CD8+ T cells. Nevertheless, they induced DC maturation and stimulated GP33-specific P14 mouse splenocytes *in vitro* as effectively as the GP33 peptide (Fig. 2A and B). Moreover, VP1-GP33-4 VLP pulsed DCs were able to stimulate GP33 CD8+ T cells *in vitro* much more strongly than GP33 peptide-pulsed DCs or GP33 peptide without DCs (Fig. 2C). These results clearly demonstrated that the GP33 CTL epitope inserted into HaPyV VP1 VLPs was efficiently processed and cross-presented by DCs like 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 clearly indicated that T cells were able to recognize the GP33 antigen. Furthermore, 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). 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 (Fig. 3B) suggesting effective insert-specific CD8+ T cell induction *in vivo*. Not all VLPs were 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 show effective CTL immune response generated by VP1-GP33 VLPs, we also used the very aggressive MCA102-GP33 tumor model. In LCMV immune mice MCA102-GP33 tumor cells are fully rejected. However, LCMV therapy only temporarily eliminates this tumor and finally 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 (Fig. 4B). 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 (Fig. 4C). CD8+ T cells activated by VLPs were able to identify and eliminate not only inoculated tumor cell suspensions but also solid tumor cells under *in vivo* situations. However, the weaker antitumor effect induced by VLPs when compared with the 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 seems to be the only effector molecule in MCA102 tumor model (Blohm et al., 2006).

Surprisingly, immunization with empty VP1 VLPs showed to some extent a protective antitumor effect. CD8+ T cell activation *in vivo* induced by empty VP1 VLPs was not as efficient as the activation observed for chimeric VP1-GP33 VLPs but clearly detectable. It might be possible that injection of the VP1 VLP induced in mice a non-specific activation of the immune system which in turn was responsible for an antitumor effect. However, it should be noted that VP1-G33 VLPs had a GP33-specific stimulatory effect on GP33-specific CD8+ T cells *in vitro* (Fig. 2A and B). Furthermore, prophylactic immunization with VLPs clearly delayed MCA102-GP33 tumor growth but had no effect on parental MCA102 tumor cell line growth in mice (Fig. 4A and B). Thus, it is 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 and a TCR of CD8+ T cell can tolerate certain amino acid substitutions in the peptide sequence. 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.

Our results demonstrated that efficient antibody response against VP1-GP33 VLPs in mice had not inhibited CTL response. Although one report that VLP-specific antibodies have a negative influence on antigen presentation impaired the induction of protective immune responses (Da Silva et al., 2001), several other studies described that specific T-cell responses to 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 and cross-presented by antigen presenting cells to insert-specific CD8+ T cells. VLPs-induced CTL responses had a protective and therapeutic capacity and were not inhibited by B cell-mediated immune reactions. Our results demonstrated the potential of chimeric HaPyV VP1-derived VLPs as carriers of CTL epitopes suitable for the induction of CTL immune response against desired antigens.

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