Antiviral Research 104 (2014) 93–101

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Coxsackievirus B3 VLPs purified by ion exchange chromatography elicit strong immune responses in mice

Tiia Koho [a], Minni R.L. Koivunen [b], Sami Oikarinen [c], Laura Kummola [d,f], Selina Mäkinen [a], Anssi J. Mähönen [a], Amirbabak Sioofy-Khojine [c], Varpu Marjomäki [e], Artur Kazmertsuk [e], Ilkka Junttila [d,f], Markku S. Kulomaa [a], Heikki Hyöty [c,d,f], Vesa P. Hytönen [a,f], Olli H. Laitinen [b,g,*]

[a] BioMediTech, University of Tampere and Tampere University Hospital, Biokatu 6, FI-33014 University of Tampere, Finland
[b] Vactech Ltd, Biokatu 8, FI-33520 Tampere, Finland
[c] Department of Virology, School of Medicine, Biokatu 10, FI-33014 University of Tampere, Finland
[d] School of Medicine, FI-33014 University of Tampere, Finland
[e] Department of Biological and Environmental Science/Nanoscience Center, University of Jyväskylä, P.O. Box 35, FI-40014 University of Jyväskylä, Finland
[f] Fimlab Laboratories, Pirkanmaa Hospital District, Biokatu 4, FI-33520 Tampere, Finland
[g] The Center for Infectious Medicine, Department of Medicine HS, Karolinska Institutet, Karolinska University Hospital Huddinge F59, SE-141 86 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 14 October 2013
Revised 20 December 2013
Accepted 20 January 2014
Available online 28 January 2014

Keywords:
VLP
CVB3
Ion exchange chromatography

ABSTRACT

Coxsackievirus B3 (CVB3) is an important cause of acute and chronic viral myocarditis, and dilated cardiomyopathy (DCM). Although vaccination against CVB3 could significantly reduce the incidence of serious or fatal viral myocarditis and various other diseases associated with CVB3 infection, there is currently no vaccine or therapeutic reagent in clinical use. In this study, we contributed towards the development of a CVB3 vaccine by establishing an efficient and scalable ion exchange chromatography-based purification method for CVB3 virus and baculovirus-insect cell-expressed CVB3 virus-like particles (VLPs). This purification system is especially relevant for vaccine development and production on an industrial scale. The produced VLPs were characterized using a number of biophysical methods and exhibited excellent quality and high purity. Immunization of mice with VLPs elicited a strong immune response, demonstrating the excellent vaccine potential of these VLPs.

1. Introduction

Coxsackievirus B3 (CVB3) is an important human pathogen that frequently causes mild infections (Melnick, 1996) but can also lead to serious diseases affecting the heart, pancreas, or central nervous system. CVB3 is among the most common causes of acute and chronic viral myocarditis, which can lead to dilated cardiomyopathy (DCM), often requiring heart transplantation (Selinka et al., 2004; Maier et al., 2004). In addition, CVB3 is associated with meningitis (Wong et al., 2011) and inflammatory diseases of the pancreas (Mena et al., 2000), and it is known to infect stem cells in the neonatal central nervous system (Feuer et al., 2003).

CVB3 is a non-enveloped virus within the genus *Enterovirus* in the *Picornaviridae* family. The 7.4-kb, single-stranded positive-sense RNA genome consists of a single open reading frame (ORF) that is translated into a single long polypeptide containing the P1–P3 regions. The P1 region is further processed by a viral protease to produce the four capsid proteins, VP0 (further cleaved to give VP4 and VP2 during viral maturation), VP3, and VP1. The P2 and P3 regions are processed into seven nonstructural proteins that have roles in polyprotein cleavage and RNA replication (Krausslich et al., 1988; Klump et al., 1990). The single ORF is flanked by a 5′ non-coding region (5′NCR) and a polyadenylated 3′NCR.

Sixty copies of VP1–VP4 form the ~30 nm capsid, which contains canyon-like structures that allow viral attachment through interactions with the host-cell proteins coxsackievirus and adenovirus receptor (CAR) and decay-accelerating factor (DAF) (Park et al., 2009; Bergelson et al., 1994, 1997). Although the VP1 protein contains the main antigenic determinants (Haarmann et al., 1994), changes in the 5′NCR of the genome (Tu et al., 1995; Dunn et al., 2003), as well as in the VP2 and VP3 regions of the capsid, have been found to attenuate the virulence of CVB3 (Knowlton et al., 1996; Stadnick et al., 2004; Park et al., 2009). Potential

Abbreviations: CVB3, Coxsackievirus B3; VLP, virus like particle; DCM, dilated cardiomyopathy; ORF, open reading frame; IEX, ion exchange chromatography.

* Corresponding author at: The Center for Infectious Medicine, Department of Medicine HS, Karolinska Institutet, Karolinska University Hospital Huddinge F59, SE-141 86 Stockholm, Sweden. Tel.: +358 503427273.
E-mail address: olli.laitinen@ki.se (O.H. Laitinen).

http://dx.doi.org/10.1016/j.antiviral.2014.01.013
0166-3542/

CVB-specific T cell epitopes have been mapped to both VP1 and VP2 regions (Huber et al., 1993; Voigt et al., 2010).

Although vaccination against CVB3 could significantly reduce the incidence of serious or fatal viral myocarditis and various other diseases associated with CVB3 infection, there is currently no vaccine or therapeutic reagent in clinical use. In the first attempt to develop a CVB3-specific vaccine, a temperature-sensitive mutant virus was found to elicit serum-neutralizing anti-CVB3 antibodies in mice after vaccination (Godney et al., 1987). Since then, several approaches have been used to demonstrate the potential usefulness of vaccination against CVB3, including a subunit vaccine (Fohlman et al., 1990), several DNA vaccines (Henke et al., 1998; Kim et al., 2005; Xu et al., 2004), and attenuated or inactivated virus vaccines (See and Tilles, 1994; Zhang et al., 1997; Dan and Chantler, 2005; Park et al., 2009; Kim and Nam, 2011). However, these vaccines may have safety issues, or they might not stimulate an ideal immune response (Goldman and Lambert, 2004). Virus-like particles (VLPs) represent a new promising vaccine technology that can overcome these disadvantages, as they offer equally efficient but safer protection than inactivated or live-attenuated viral vaccines.

VLPs are formed by the self-assembly of recombinant viral structural proteins. VLPs are structurally and antigenically similar to the parental infectious virus, but lack viral nucleic acid and are therefore noninfectious. Consequently, they do not carry the risk of reversion to a virulent form. In addition, VLPs lack the safety concerns related to inactivation failures, which may occur when inactivated virus-based vaccines are used. VLPs are highly immunogenic because they present viral epitopes in an authentic conformation and because their receptor binding properties are similar to those of the infective virus (Grgacic and Anderson, 2006; Roy and Noad, 2008). Importantly, the size of VLPs allows uptake by dendritic cells (Fifis et al., 2004). Therefore, VLP vaccines are able to stimulate both humoral and cellular immune responses, overcoming a common major drawback encountered with traditional subunit vaccines (Grgacic and Anderson, 2006). VLPs have emerged as a safe and effective strategy for vaccine development targeting viral diseases (reviewed in Grgacic and Anderson (2006) and Jennings and Bachmann (2008)). Since the first VLP-based vaccines were licensed for clinical use against human papillomavirus (Gardasil, Cervarix) and hepatitis B virus (Recombivax HB, Engerix), VLP vaccine candidates have been developed for many different types of viruses, including enteroviruses (Zhang et al., 2012; Liu et al., 2012; Chung et al., 2008; Rombaut and Jore, 1997). In this study, a scalable and efficient chromatographic production method was developed for CVB3 VLPs. The potential of the resulting high-quality particles to serve as a vaccine was studied by immunizing mice, which resulted in strong immune responses and high titers of neutralizing antibodies.

2. Materials and methods

2.1. Design and construction of the CVB3 VLP transfer vector and generation of the VLP-producing recombinant baculovirus

Baculoviral transfer vector pFastBac™ Dual (Invitrogen, Carlsbad, CA) containing the desired inserts was ordered from GENEART AG (Regensburg, Germany). The fully sequenced CVB3 strain (GenBank accession number M33854.1) was chosen as a template. The construct contained two separate cassettes (Fig. 1). The first cassette contained the P1 region of the CVB3 strain M33854.1 and the second cassette contained the whole CVB3 genome with the exception of the P1 region, which was replaced with a mCherry coding sequence followed by an artificial recognition site for the enteroviral 3A protease.

The recombinant baculovirus was generated according to the instructions given with the Bac-to-Bac® Baculovirus Expression System (Invitrogen) with the exception that the cassettes were transferred into the F-bacmid baculovirus genome (Karkkainen et al., 2009). An empty pFastBac™ Dual baculoviral transfer vector was used for the generation of empty baculoviruses.

2.2. Production and purification of the CVB3 VLPs

The CVB3 VLPs were expressed in baculovirus-transformed *Spodoptera frugiperda* insect cells (Sf9; Invitrogen) and harvested 5–6 days post-infection. After clarification by centrifugation (10,409×*g* at 4 °C for 20 min), CVB3 VLPs were concentrated from the clarified cell culture supernatant by polyethylene glycol (PEG) precipitation and detergent-treated as previously described (Abraham and Colonno, 1984). The precipitated VLPs were then recovered by centrifugation (10,409×*g* at 4 °C for 5 min) and diluted 1:10 with 20 mM Tris–HCl (pH 7.5) prior to loading onto the chromatography column.

Chromatographic ion exchange purification (IEX) of the pretreated VLPs was performed using monolithic columns (6.7 mM ID × 4.2 mM, V: 1 ml) based on CIM Convective Interaction Media® technology from BIA Separations (Ljubljana, Slovenia) with either quaternary amine (QA) or sulfate (SO3) chemical functionalization. The details of the purification protocol are described in the Supplementary data.

Sf9 insect cells infected with the empty baculovirus were subjected to the same purification process, and the product served as the negative control antigen in the mouse immunization studies.

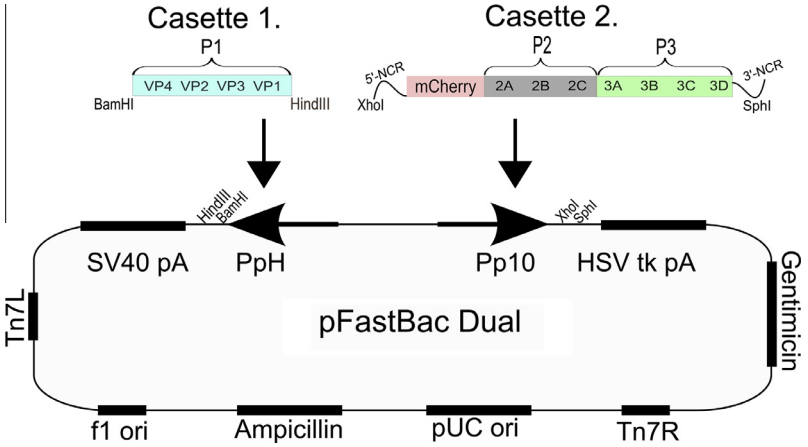

Fig. 1. Cloning of the CVB3 VLP cassettes into the pFastBac™ Dual baculoviral transfer vector.

2.3. Culturing and purification of the CVB3 virus

The CVB3 virus (a Moldova strain variant, GenBank: AY896763.1), was propagated in green monkey kidney cells (GMK) cultured at 37 °C with 5% CO_2 in HyClone SFM4MegaVir protein-free medium (Thermo Fisher Scientific) containing 2 mM L-glutamine and 0.1% penicillin–streptomycin. A MOI of 0.5 plaque-forming units (PFU)/cell was used for the infections. After 2 days of incubation, the viruses were harvested by repeated freeze–thaw cycles and centrifugation (9605×*g* at 4 °C for 20 min). The viruses were precipitated with PEG and detergent-treated as described in Section 2.2. The precipitated viruses were further purified by ultracentrifugation with a 20–5% (w/v) discontinuous sucrose gradient (103,864×*g* at 4 °C for 4 h). The virus-containing fractions were pooled, pelleted, and resuspended in 200–300 μl of Dulbecco's PBS (DPBS) containing Ca/Mg.

The chromatographic purification of the precipitated viruses was performed using a monolithic column (6.7 mM ID × 4.2 mM, V: 1 ml) based on CIM Convective Interaction Media® technology from BIA Separations with the quaternary amine (QA) chemistry. Details of the purification protocol are described in the Supplementary data. The virus titers were determined based on the cytopathic effect (CPE) in infected GMK cells.

2.4. Characterization of the CVB3 VLPs and virus

VLPs and virus samples were run on 12% SDS–PAGE gels and analyzed by Western blotting using either the mouse anti-enterovirus clone 5-D8/1 (DAKO, Glostrup, Denmark) or the mouse anti-baculovirus gp64 clone AcV5 (Santa Cruz Biotechnology Inc., Heidelberg, Germany) at a dilution of 1:3000 and 1:1000, respectively, followed by incubation with an HRP-labeled horse antibody against mouse immunoglobulins (Vector Laboratories Inc., Burlingame, CA) diluted 1:20,000. The samples were visualized using the ECL detection substrate (Thermo Scientific). For assessment of the total protein content, the samples were run on 12% SDS–PAGE gels and subsequently visualized by silver staining (Pierce Silver Stain Kit, Thermo Scientific). The total protein concentrations of the fractions were analyzed using the Pierce BCA Protein Assay kit (Thermo Scientific). The total DNA concentrations of the VLP fractions were analyzed using Quant-iT™ dsDNA Broad-Range Assay Kit (Invitrogen), and the number of baculovirus genomes in the fractions was determined using a qRT-PCR kit (BacPAK™ qPCR titration kit, Clontech Laboratories, Mountain View, CA).

Dynamic light scattering (DLS) analysis was performed with a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). The hydrodynamic diameter was determined using three 10 × 10-second datasets at 25 °C in 20 mM Tris, 20 mM NaCl, and 5 mM $MgCl_2$ (pH 7.5). The samples were further subjected to stepwise heating. Starting at 25 °C, each sample was heated in 5 °C increments and equilibrated for 5 min at each temperature before analysis. The samples were heated to a final temperature of 90 °C, after which they were cooled back to 25 °C.

For the transmission electron microscopy (TEM) analysis, a small aliquot of CVB3 VLPs or infective virus was briefly bound to formvar-coated copper grids that had been glow-discharged just before use. The excess liquid was blotted away, after which a drop of 1% phosphotungstic acid (prepared in water, pH 7) or 2% uranyl acetate was applied to the grid for 1 min and blotted away. The grid was air-dried before visualization with a JEM-1400 (JEOL, Tokyo, Japan) or a Tecnai 10 (FEI, Hillsboro, OR) transmission electron microscope. The scanning electron microscopy (SEM) analysis is presented in the Supplementary data.

To determine if the VLPs contained the modified genome, a 20-μl aliquot was digested with 25 U of RNase If enzyme (New England Biolabs) in 1× NE buffer at 37 °C for 2 h. Then the enzyme was inactivated at 70 °C for 20 min. Another 40-μl aliquot of VLPs was digested at RT for 20 min with 2.7 Kunitz units of DNase I (Qiagen) in 1× RDD buffer. Both digestions were followed by nucleic acid extraction (Qiagen Viral RNA Kit), and samples were analyzed by enterovirus- and mCherry-specific PCR with and without a preceding reverse transcriptase reaction. The PCR run was performed using the primers and probes shown in Table 1 according to the instructions provided with the Quantitect Probe kit (Qiagen) using Taqman chemistry.

2.5. Vaccination and sampling of the mice

The vaccination trial was performed according to the guidelines of the Tampere University Animal Welfare program under the approval number ESAVI/4588/04.10.03/2012 from the Regional State Administrative Agency. Twelve female Balb/c mice, 6–8 weeks old were randomly divided into two groups of six. The first group was administered 5 μg of CVB3 VLPs, and the second group was given an identical volume of negative control vaccine (see Section 2.2). In addition, positive control experiment was performed by administering formalin-inactivated CVB3 virus to a group of six mice. All vaccine preparations were mixed with an equal volume of either complete (primary vaccination) or incomplete (booster vaccinations) Freund's adjuvant (Sigma–Aldrich) immediately before administration.

The primary vaccination was given in a volume of 100 μl s.c. and the two booster vaccinations (at day 21 and day 42) were given in volumes of 200 μl i.p. Blood samples were collected before vaccination, at days 21 and 42, and by heart puncture at day 63 when the mice were sacrificed. Spleens were collected, disrupted mechanically, and used to prepare into single-cell suspensions as follows: cells were washed with PBS, and red blood cells were lysed by incubating them for 1 min in ACK lysing buffer (Lonza). Splenocytes were suspended in 50 ml of 2% FBS, 0.5% penicillin–streptomycin, and 1% L-glutamine supplemented RPMI-1640 medium (Lonza).

2.6. ELISA and neutralization assays

For the ELISA assay, 96-well plates were coated with 150 ng/well of either purified CVB3 VLPs or virus. The wells were blocked at room temperature for 30 min using PBST supplemented with 0.1% (w/v) BSA, followed by incubation at 37 °C for 1 h with day 63 mouse antisera serially diluted in PBST containing 1% BSA (50 μl/well). HRP-conjugated horse anti-mouse IgG (Vector) diluted 1:2300 in 1% BSA in PBST (50 μl/well) was used as a secondary antibody, and the antibodies were detected using *O*-phenylenediamine dihydrochloride (Sigma–Aldrich) as a substrate. The reaction was stopped with 0.5 M sulfuric acid after a 30-min incubation at 37 °C, and the OD values were measured at 490 nm using an ELISA plate reader (Victor^2 1420 Multilabel counter, Perkin Elmer, Waltham, MA). The values were reported as the mean OD values for each dilution of the antisera. Statistical significance was determined by the unpaired two-tailed *t*-test using GraphPad Prism version 6.

Table 1
Primers and probes used in the study.

Primer/probe	Sequence (5′–3′)
5′NCR-for	CGG CCC CTG AAT GCG GCT AA
5′NCR-rev	GAA ACA CGG ACA CCC AAA GTA
5′NCR-probe	FAM-TCT GCA GCG GAA CCG ACT A-TAMRA
mCherry-for	CAC TAC GAC GCT GAG GTC AA
mCherry-rev	TAG TCC TCG TTG TGG GAG GT
mCherry-probe	VIC®-TGT GGG AGG TGA TGT C-MGB

The presence of neutralizing antibodies in mice against the ATCC reference strain Nancy (ATCC number VR-30) was analyzed using a plaque seroneutralization assay (Roivainen et al., 1998). The details of the neutralizing assay are described in the Supplementary data.

2.7. Immunological assays

To analyze immune cells using flow cytometry, mouse spleens were disrupted mechanically. Single-cell splenocyte suspensions (2 ml of per mouse) in RPMI medium were analyzed. The cells were washed with 0.1% BSA in PBS and stained for 20 min at +4 °C with the following antibodies (dilution, 1:400): CD3-APC (a pan-T cell marker), CD4-PerCP/Cy5.5 (a $CD4^+$ T cell marker), CD8-FITC (a $CD8^+$ T cell marker), CD44-APC/eFluor780 (a differentiated T cell marker), CD62L-PE (a naïve T cell marker), and B220-PE/Cy7 (a B cell marker). All antibodies were from eBioscience (San Diego, CA, USA). The cells were then filtered with cell strainer cap FACS tubes (BD, Franklin Lakes, NJ, USA) and washed with 0.1% BSA in PBS. The FACS analysis was performed using a FACSCanto™II flow cytometer (BD). Flow cytometric data was analyzed using FlowJo (Tree Star, Ashland, OR, USA). A total of 50,000 cells was analyzed for each sample. Live cells were gated, and the number of live cells was confirmed to be similar in each of the sample groups. Among the live cells, the $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cell populations were gated by plotting CD3-positive cells vs. CD4- or CD8-positive cells. The effector-memory (EM) T cell populations in these two populations were examined by plotting CD62L vs. CD44.

3. Results

3.1. Ion exchange purification resulted in highly purified CVB3 VLPs and CVB3 virus that were recognized by enterovirus-specific antibodies

Recombinant CVB3 VLPs were recovered from clarified insect cell culture supernatants by PEG precipitation and then subjected to further purification with either anion or cation exchange chromatography. The VLPs eluted from the QA anion exchange column at a NaCl concentration of 100 mM and from the SO3 cation exchange column between 410 and 550 mM NaCl (Fig. 2A). The yield was approximately 0.5 mg of VLPs per liter of insect cell culture.

Isolation of CVB3 virus by PEG precipitation and purification by sucrose gradient ultracentrifugation resulted in approximately 1.4 mg CVB3 virus per liter of cell culture with an infectivity of 2.8×10^{10} PFU. The PEG-precipitated virus that was further purified by anion exchange chromatography and eluted from the QA column at 60 mM NaCl (Fig. 3A) resulted in a yield of approximately 2 mg of viruses per liter of cell culture with an infectivity of 5.6×10^{10} PFU.

SDS–PAGE analysis and subsequent silver staining of CVB3 virus samples showed the presence of four proteins of approximately 34 kDa, 30 kDa, 26 kDa, and 8 kDa in size (Fig. 3B, lanes 4 and 5). These molecular weights correspond well with the estimated molecular weights of the four CVB3 capsid proteins, VP1, VP2, VP3, and VP4, respectively (Cunningham et al., 1992). The purified VLP sample contained three protein bands in the silver-stained SDS–PAGE gel (Fig. 2B, lane 4) that correlated with the molecular weights of the capsid proteins VP0, VP1, and VP3. VP0 appears not to undergo cleavage to yield the VP4 and VP2 proteins in insect cells. The purity confirmed by silver staining was very high for all the purified material (Fig. 2B, lane 4; Fig. 3B, lanes 4 and 5) and was estimated to be >95% for both CVB3 VLPs and CVB3 virus. No residual protein impurities were detected in any of the purified materials. The total DNA content of the purified VLPs was very low (0.82 ± 0.38 ng/µl), measured from three independent purifications. The baculovirus genome content of the samples was 0.02 ± 0.02 ng/µl, measured from three independent purifications. Both the virus and the VLPs were recognized by an antibody against enterovirus VP1 (Figs. 2C and 3C). None of the proteins were recognized by an antibody against baculovirus (Supplementary Fig. S5).

3.2. The assembly and homogeneity of the IEX-purified CVB3 VLPs and viruses were confirmed by dynamic light scattering and electron microscopy

DLS analysis showed that a majority (98.2%) of the particles in the VLP sample purified using SO3-based ion exchange chromatography had an average diameter of 30.7 nm, and the sample was relatively monodisperse (polydispersity index, PdI = 0.316) (Fig. 4A).

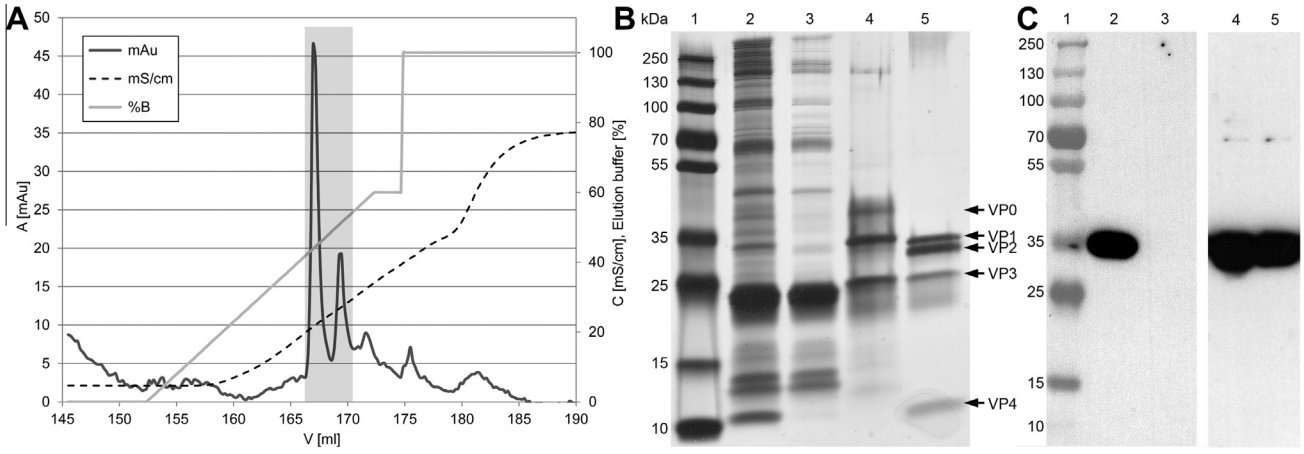

Fig. 2. Characterization of the chromatography-purified CVB3 VLPs. (A) PEG-precipitated VLPs were loaded onto a cation exchange column and were eluted from the column with a linear gradient using 1 M NaCl, 20 mM Tris–HCl, and 5 mM $MgCl_2$ (pH 7.4) as the elution buffer. A flow rate of 1 ml/min was used. The peak fractions (indicated by shading) eluted from the column between 410 and 550 mM NaCl. A: absorbance at 280 nm, V: volume, C: conductivity. (B) Analysis of the silver-stained SDS–PAGE gel showed that the VLPs were efficiently purified and concentrated during the chromatography purification process. Lane 1: molecular weight marker; lane 2: chromatography input sample; lane 3: flow-through sample; lane 4: VLP-containing elution sample; lane 5: ultracentrifugation-purified CVB3 virus control sample. (C) Western blot analysis of chromatography-purified CVB3 VLPs revealed that the VLPs were recognized by an anti-enterovirus VP1 antibody. Lane 1: molecular weight marker; lane 2: chromatography input sample; lane 3: flow-through sample; lanes 4 and 5: VLP-containing elution samples.

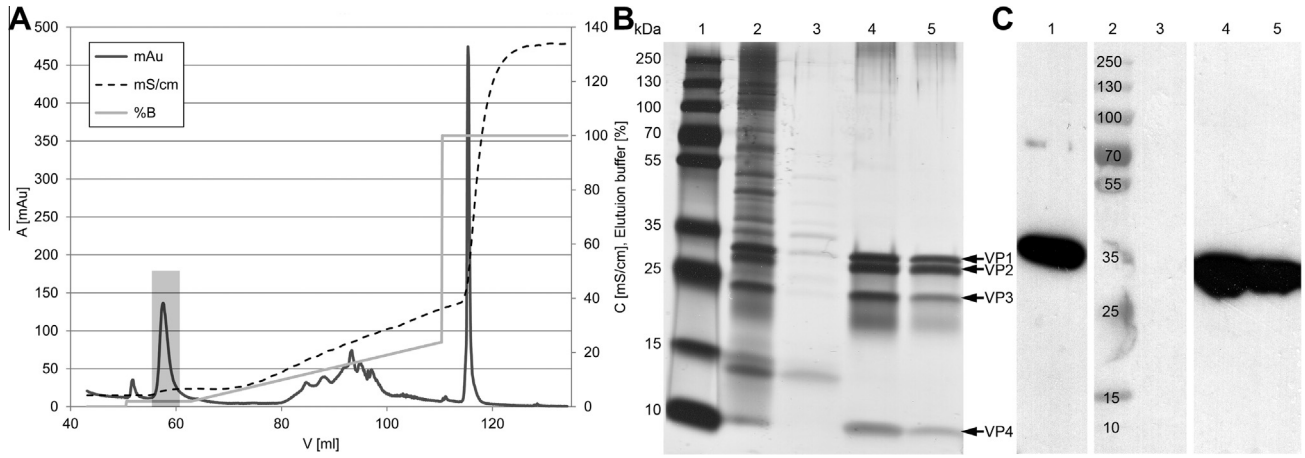

Fig. 3. Chromatographic purification of the CVB3 virus and comparison of the purified virus with the ultracentrifugation-purified virus. (A) PEG-precipitated viruses were loaded onto an anion exchange column and were eluted from the column using a combination of stepwise and linear gradients as shown in the figure. A flow rate of 1 ml/min was used, and the elution buffer contained 2 M NaCl, 20 mM Tris–HCl, and 5 mM $MgCl_2$ (pH 7.4). The peak fractions (indicated by shading) eluted from the column at a NaCl concentration of 60 mM. A: absorbance at 280 nm, V: volume, C: conductivity. (B) Analysis of silver-stained SDS–PAGE gels showed that the viruses were efficiently purified by both chromatography and ultracentrifugation. Lane 1: molecular weight marker; lane 2: chromatography input sample; lane 3: flow-through sample; lane 4: virus-containing elution sample; lane 5: ultracentrifugation-purified CVB3 virus sample. (C) Western blot analysis of chromatography-purified CVB3 viruses revealed that the viruses were recognized by an anti-enterovirus VP1 antibody. Lane 1: chromatography input sample; lane 2: molecular weight marker; lane 3: flow-through sample; lanes 4 and 5: virus-containing elution samples.

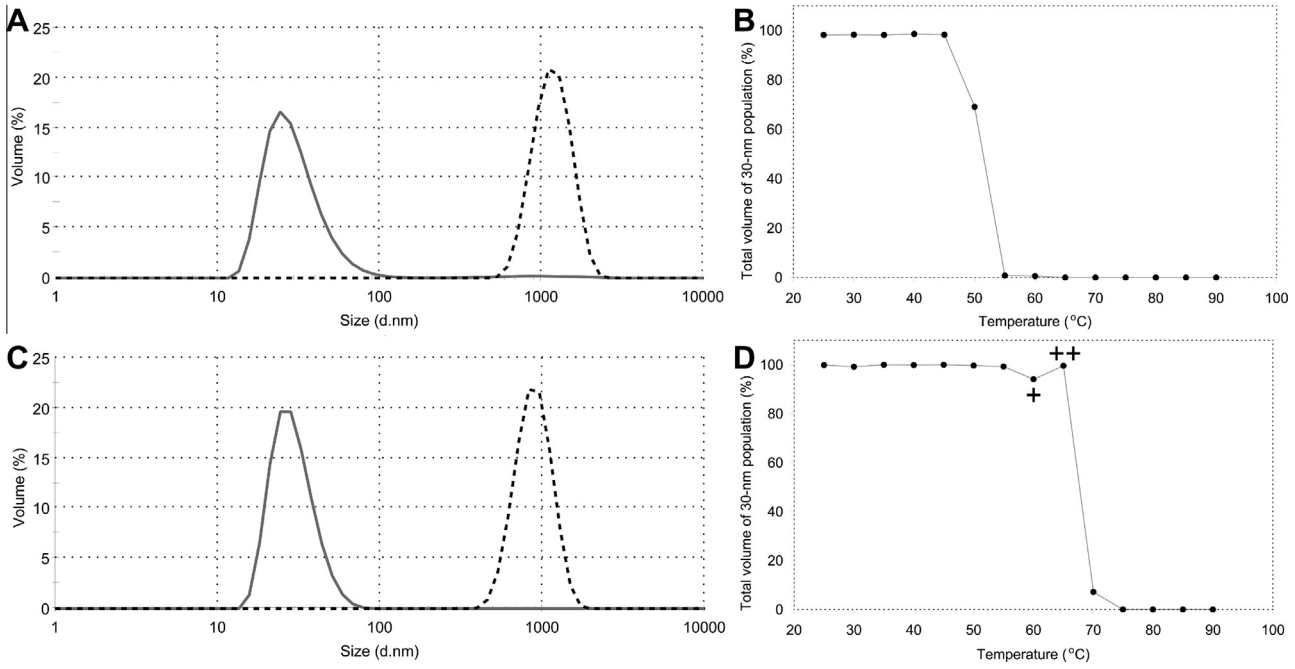

Fig. 4. Dynamic light scattering (DLS) analysis of the CVB3 VLPs and viruses. (A) Chromatography-purified VLP sample contained 98.2% particles (determined by particle volume) with a hydrodynamic diameter of 30.7 nm (solid gray line). After gradual heating of the sample to 90 °C, followed by cooling of the sample to 25 °C, the particles were aggregated with an average particle size of 1191 nm (dotted line). (B) Volume-% of the 30-nm particle population in the VLP sample over the temperature range analyzed (25–90 °C). (C) DLS analysis of the CVB3 virus as described in (A). The virus sample contained 100% particles with an average hydrodynamic diameter of 29.8 nm. (D) Heating of the virus sample indicated the onset of thermal denaturation at 60 °C, and an increase in the average particle diameter (40.8 nm) was observed (+). Further heating to 65 °C led to an increase in the average size to 82.0 nm (++), after which larger aggregates were formed (200–500 nm). When the sample was cooled to 25 °C, 898.7 nm particles were observed (C, dotted line).

A sample of chromatography-purified CVB3 virus contained mainly (99.9%) particles with an average diameter of 28.2 nm (Fig. 4C). The sample was homogenous, with a PdI of 0.173.

The heating of both the VLPs and the virus from 25 °C to 95 °C led to aggregation of the sample, resulting in particles of 600–1000 nm in diameter (Supplementary Figs. S6 and S7). After cooling to 25 °C, a further increase in the particle size was observed, indicating irreversible thermal unfolding. A difference between the VLPs and the virus was observed in the temperature at which they aggregated. The VLPs showed signs of thermally induced aggregation at 50 °C and were completely aggregated at 55 °C (Fig. 4B), whereas the virus sample showed the first signs of aggregation at 60 °C and was completely aggregated at 75 °C (Fig. 4D). Therefore, the thermally induced events occurred over much

broader temperature range in case of CVB3 virus and the midpoint of the thermal aggregation was 10–15 °C higher than in the case of the VLPs.

Examination of the CVB3 VLP and virus samples by both transmission and scanning electron microscopy showed the presence of intact and uniform particles with the correct size (approximately 30 nm in diameter) and morphology (Figs. 5 and 6), although in the SEM images, the VLPs appeared somewhat smaller in size than the virus particles.

To determine whether modified viral RNA or baculoviral DNA was packaged into the VLPs, RNase If or DNase I digestions for the supernatant aliquots were performed. Nucleases will digest RNA or DNA in the supernatant, but not inside the VLP. Digestion was followed by nucleic acid extraction and PCR amplification with and without reverse transcriptase (RT-) step to control whether PCR signal originates from RNA or DNA. The supernatant DNase I-digested samples were negative indicating that the supernatant was free of viral RNA, and that the VLP particles did not contain RNA or DNA, both of which would have given signals in the RT-PCR and/or in the PCR if positive. Therefore, the DNA in the supernatant was digested. The VLP supernatants digested with RNase If were positive with and without the RT-step, which demonstrated that the samples contained traces of baculovirus-derived DNA outside the particle.

3.3. CVB3 VLP vaccination generated a strong antibody-mediated immune response in mice

The antibodies generated in immunized mice were quantified using an ELISA (Fig. 7). The negative control vaccine did not elicit any reactivity against either inactivated CVB3 (ATTC, Nancy strain) or the VLP proteins. In contrast, the sera of the VLP-vaccinated mice reacted strongly to both antigens, and high IgG titers were generated in all but one mouse (mouse no. 11, Supplementary Fig. S8). The mean titers were slightly higher when the VLPs were used as an antigen rather than when intact CVB3 particles were used.

Sera from mice immunized with CVB3 VLPs and the negative control vaccine were further evaluated for their ability to neutralize infective CVB3 *in vitro*. The end-point neutralizing titers (up to 1:4096) were determined for sera collected on day 0 and day 63. The negative control vaccine failed to elicit any neutralization at 1:4, whereas high neutralizing antibody titers were induced in mice immunized with CVB3 VLPs (Table 2). The mean neutralizing titer in the VLP-vaccinated group at day 63 was over 1:1100, but considerable variations were observed between individual mice. One mouse (mouse no. 7) generated neutralizing antibodies only after the second booster dose; however the final end-point antibody titer was higher than those seen in other mice. Additionally, one mouse (mouse no. 11) failed to generate neutralizing antibodies after vaccination. The mean neutralizing titer of the mice infected with purified formaldehyde-inactivated CVB3 virus, which was used as a positive control was 1:2200 at day 63 ($n = 6$).

3.4. CVB3 VLPs induced cell-mediated immunity in mice

The spleen cells of CVB3 VLP- and negative control-vaccinated mice were analyzed for markers of immune activation (Supplementary Table 1). Initially, when the numbers of B cells, $CD4^+$ T cells, and $CD8^+$ T cells were determined, there was no statistically significant expansion of effector B or T cells, nor differences between the study groups. When activated by virus infection, EM-T cells expanded in number and modulated their expression of cell surface molecules such as CD62L and CD44. Upon EM-generation, T cells down-regulate CD62L and up-regulate CD44 (Sprent, 1997). Thus, the numbers and proportions of the memory cell populations of CD3 and CD4/CD8 double-positive cells were measured based on the expression of CD44 and CD62L. We found that 9 weeks after the initial VLP immunization, the numbers of $CD62L^{low}$ $CD44^{high}$ cells in both the CD4 and CD8 cell populations

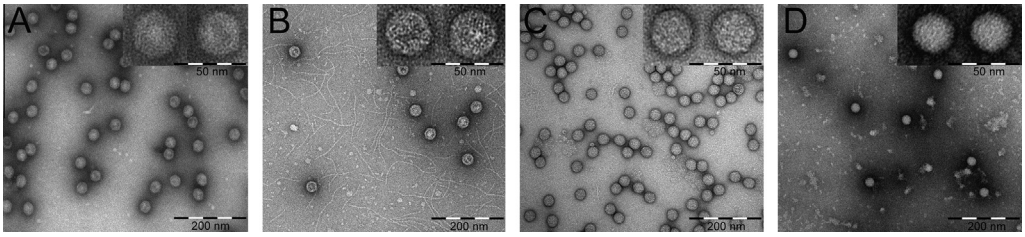

Fig. 5. Transmission electron micrographs of (A) S03 chromatography-purified VLPs, (B) QA chromatography-purified VLPs, (C) ultracentrifugation-purified viruses, and (D) QA chromatography-purified viruses. Scale bars, 200 nm and 50 nm (close-up).

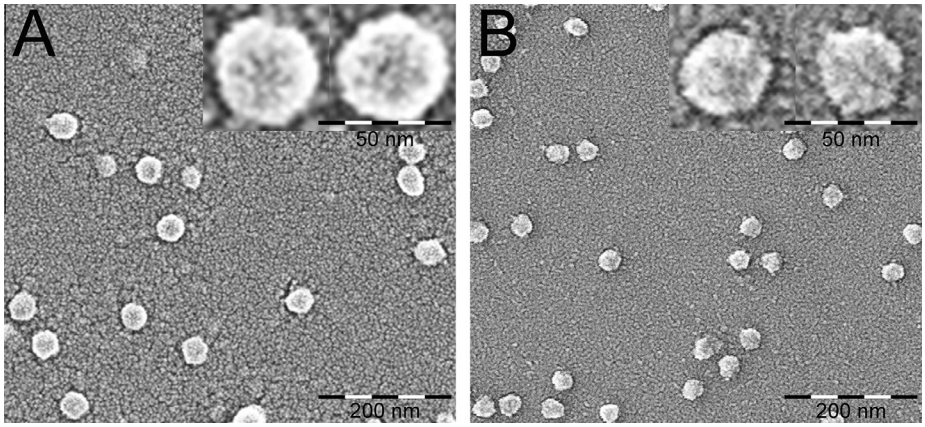

Fig. 6. Scanning electron micrographs of (A) the S03 chromatography-purified viruses and (B) VLPs. Scale bars, 200 nm and 50 nm (close-up).

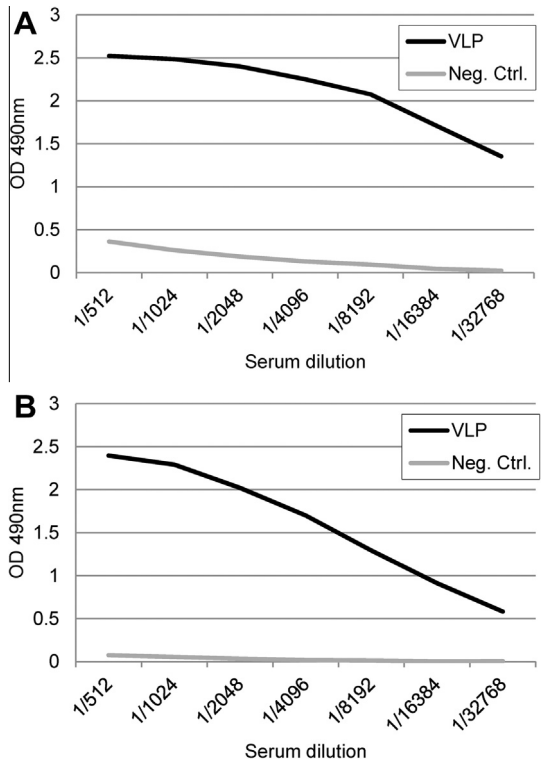

Fig. 7. Mean antibody responses elicited in mice by CVB3 VLP immunization. Two groups of mice were injected on days 0, 21, and 42 with 5 μg of CVB3 VLPs or an equivalent amount of negative control vaccine. The immunized mice were sacrificed on day 63, and serum samples were collected. The results represent the mean of three replicate experiments. (A) The binding activity of the antisera to a CVB3 VLP antigen. (B) Binding activity of the antisera to a CVB3 virus.

Table 2
Neutralizing titers analyzed by seroneutralization assay.

Vaccination group	Neutralizing titer[a]			
	Day 0	Day 21[b]	Day 42[b]	Day 63
Negative control				
Mouse 1	–	–	–	–
Mouse 2	–	–	–	–
Mouse 3	–	–	–	–
Mouse 4	–	–	–	–
Mouse 5	–	–	–	–
Mouse 6	–	–	–	–
CVB3 VLP				
Mouse 7	–	–	1:16	1:4096
Mouse 8	–	1:16	1:16	1:256
Mouse 9	–	1:16	1:16	1:1024
Mouse 10	–	1:16	1:16	1:1024
Mouse 11	–	–	–	–
Mouse 12	–	1:16	1:16	1:256

[a] Titers were determined against ATCC reference strain Nancy (ATCC number VR-30). –, no detectable neutralizing antibodies (titer <1:4).
[b] Neutralizing titers were analyzed only at dilutions of 1:4 and 1:16.

were consistently higher than in control mice. For CD4 cells, the induction was from ~500 to 700 cells, and for CD8 cells, the induction was from ~30 to 45 cells. No significant differences in the total number of $CD4^+$ or $CD8^+$ T cells were observed between the study groups.

4. Discussion

In this article, we describe a technology that allows the production of high-quality enterovirus VLPs for different applications. We have shown herein that the produced VLPs were stable and highly purified. The preparations were determined to be free of contaminating proteins by silver stain, which has a detection limit of 0.25 ng for proteins, thus verifying purity >95%. In addition, the VLP samples were virtually free of contaminating DNA (0.82 ± 0.38 ng/μl). The VLPs were indistinguishable from the native virus in size, composition, and appearance, as determined by SDS–PAGE, Western blotting, and transmission electron microscopy. Moreover, the VLPs were also almost as stable as native viruses when exposed to elevated temperatures, and they also withstood a year-long storage at −20 °C without declining (data not shown). Most importantly, VLPs were highly immunogenic, as shown by the vaccination experiments in mice.

Since the launch of polio vaccines in 1950s, no other enterovirus vaccines have been developed and registered for human use. One reason for the lack of enterovirus vaccines is due to the large number of different enterovirus serotypes (over 100), which circulate around the world and are rapidly and continuously recombining and evolving. It has not, therefore, been easy to identify causal serotype-disease relationships. There are, however, certain virus-disease associations that are currently well established which make the development of novel vaccines worthy of consideration. Among such enteroviruses are EV71 and CVB3, both of which commonly cause severe infections that can be fatal (McMinn, 2012; Maier et al., 2004). The recent progress in the development of an EV71 vaccine has been encouraging. Several vaccine formulae have been studied in animal models, and human clinical trials have yielded promising results (Liang et al., 2013). However, to date, such breakthroughs in the development of a CVB3 vaccine have not been made.

In research settings, enteroviruses are routinely purified by sucrose gradient ultracentrifugation. Although meticulously performed ultracentrifugation generates high-quality virus material, the procedure is cumbersome, and the yields are modest considering the laborious nature of the method. On the industrial scale, other methods such as tangential flow filtration, ultrafiltration, and chromatography-based purification are more feasible (Morenweiser, 2005). In response to this demand, we developed an easily scalable ion exchange chromatography-based purification system for the CVB3 VLP vaccine. We also developed an analogous ion exchange chromatography-based purification method for the CVB3 virus. The single-step chromatography procedure yielded highly pure and stable CVB3 VLP and virus preparations, and the developed methodology is therefore an easily scalable purification system for industrial-scale vaccine production.

Most importantly, the anti-VLP sera raised by CVB3 VLP vaccination exhibited a strong neutralizing capacity against the homologous CVB3 strain. In addition, we showed by ELISA that mice immunized with CVB3 VLPs had a strong serum IgG antibody response and that the generated antibodies reacted with both VLP and virus antigens within the same genogroup. There are several previous studies showing the efficacy of enteroviral VLPs as immunogens (Zhang et al., 2012; Liu et al., 2012; Lin et al., 2012). All of these studies measured immune responses by ELISA and/or seroneutralization as part of their main analyses, and our results are very similar. Closely analogous to our study is the work performed by Zhang et al. (2012), who obtained neutralizing titers against their CVB3 VLPs of up to 1:320. In comparison, our titers had an average over 1:1100. Zhang et al. used a similar vaccination regime and similar adjuvants as to those used in the present study, but in contrast to the 5 μg dose used in the present study, they used one of 20 μg. We therefore were able to generate comparable or even higher neutralizing titers with smaller doses, thus highlighting the purity and high immunogenicity of the chromatography-purified VLPs. The efficacy of the VLP vaccine was also found comparable to the positive control, formaldehyde-inactivated virus. The

small difference in the efficacy of these two formulations may be due to the formaldehyde used to inactivate the virus or the lack of VP0 maturation cleavage in VLP. One mouse vaccinated with VLP did not generate measurable amounts of neutralizing antibodies, and may represent somehow immunocompromised animal.

Our analysis of the B cell, $CD8^+$, and $CD4^+$ T cell responses induced by CVB3 VLPs revealed a limited degree of activation. However, the effector-memory T cell populations were consistently increased by CVB3 VLPs indicating that an immunological response to the VLP was obtained. These findings are consistent with similar studies carried out with CVB3 virus (Slifka et al., 2001; Kemball et al., 2008). When comparing the increase in the size of the EM-T cell pool with the efficacy of neutralizing antibody production, the degrees of the responses appear to be consistent. A future challenge will be to construct a tetramer that could indicate that $CD8^+$ and $CD4^+$ memory T cells have been induced specifically by CVB3 VLPs.

In conclusion, we present the construction, production, and purification of an immunologically efficient and safe vaccine candidate for CVB3-related diseases in the form of VLPs. The introduced purification system in particular is highly relevant for industrial-scale vaccine production, which enables a smooth shift from pre-clinical studies to human clinical trials. The produced VLP vaccine was able to induce a strong immune response in mice, as determined by seroneutralization and ELISA assays and also by immune cell assay. In addition, we developed an efficient and scalable purification method for the CVB3 virus, which will facilitate its further characterization and provides the possibility of using the virus as the basis of a conventional attenuated or killed vaccine.

Acknowledgments

The authors thank Tanja Rämö, Laura Kananen, Ulla Kiiskinen, Outi Väätäinen, Tuula Koivuharju, Jussi Lehtonen, Niila Saarinen, Juha Määttä, and Outi Tolonen for their valuable technical assistance. Anni Laitinen and Pär Larsson are acknowledged for their help with the mouse experiments and Sanna Hämäläinen for the assistance with the flow cytometry experiments. Mari Leinonen, Tommi Isoniemi, Lars Haag, and Josefina Nilsson are acknowledged for their support with the electron microscopy. The authors thank Raimo Harju for many helpful suggestions during the study. The work was supported by grants from the Academy of Finland (136288, 140978, 273192, 257125), TEKES (FiDiPro project "NOVAC", Novel methods for vaccination and virus detection), Competitive Research Funding of the Tampere University Hospital District, the National Doctoral Programme in Informational and Structural Biology (ISB), the European Commission, PEVNET (FP-7 Programme, contract No. 261441), and Vactech Ltd.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.01.013.

References

Abraham, G., Colonno, R.J., 1984. Many rhinovirus serotypes share the same cellular receptor. J. Virol. 51, 340–345.
Bergelson, J.M., Chan, M., Solomon, K.R., St John, N.F., Lin, H., Finberg, R.W., 1994. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc. Natl. Acad. Sci. USA 91, 6245–6248.
Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L., Finberg, R.W., 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275, 1320–1323.
Chung, Y., Ho, M., Wu, J., Chen, W., Huang, J., Chou, S., Hu, Y., 2008. Immunization with virus-like particles of enterovirus 71 elicits potent immune responses and protects mice against lethal challenge. Vaccine 26, 1855–1862.
Cunningham, M.W., Antone, S.M., Gulizia, J.M., McManus, B.M., Fischetti, V.A., Gauntt, C.J., 1992. Cytotoxic and viral neutralizing antibodies crossreact with streptococcal M protein, enteroviruses, and human cardiac myosin. Proc. Natl. Acad. Sci. USA 89, 1320–1324.
Dan, M., Chantler, J.K., 2005. A genetically engineered attenuated coxsackievirus B3 strain protects mice against lethal infection. J. Virol. 79, 9285–9295.
Dunn, J.J., Bradrick, S.S., Chapman, N.M., Tracy, S.M., Romero, J.R., 2003. The stem loop II within the 5′ nontranslated region of clinical coxsackievirus B3 genomes determines cardiovirulence phenotype in a murine model. J. Infect. Dis. 187, 1552–1561.
Feuer, R., Mena, I., Pagarigan, R.R., Harkins, S., Hassett, D.E., Whitton, J.L., 2003. Coxsackievirus B3 and the neonatal CNS: the roles of stem cells, developing neurons, and apoptosis in infection, viral dissemination, and disease. Am. J. Pathol. 163, 1379–1393.
Fifis, T., Gamvrellis, A., Crimeen-Irwin, B., Pietersz, G.A., Li, J., Mottram, P.L., McKenzie, I.F., Plebanski, M., 2004. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J. Immunol. 173, 3148–3154.
Fohlman, J., Ilback, N.G., Friman, G., Morein, B., 1990. Vaccination of Balb/c mice against enteroviral mediated myocarditis. Vaccine 8, 381–384.
Godney, E.K., Arizpe, H.M., Gaunti, C.J., 1987. Characterization of the antibody response in vaccinated mice protected against Coxsackievirus B3-induced myocarditis. Viral Immunol. 1, 305–314.
Goldman, M., Lambert, P.H., 2004. Immunological safety of vaccines: facts hypothesis and allegations. In: Kaufmann, S.H.E. (Ed.), Novel Vaccination Strategies. Wiley-VCH, Weinheim, Germany, pp. 595–611.
Grgacic, E.V., Anderson, D.A., 2006. Virus-like particles: passport to immune recognition. Methods 40, 60–65.
Haarmann, C.M., Schwimmbeck, P.L., Mertens, T., Schultheiss, H.P., Strauer, B.E., 1994. Identification of serotype-specific and nonserotype-specific B-cell epitopes of coxsackie B virus using synthetic peptides. Virology 200, 381–389.
Henke, A., Wagner, E., Whitton, J.L., Zell, R., Stelzner, A., 1998. Protection of mice against lethal Coxsackievirus B3 infection by using DNA immunization. J. Virol. 72, 8327–8331.
Huber, S., Polgar, J., Moraska, A., Cunningham, M., Schwimmbeck, P., Schultheiss, P., 1993. T lymphocyte responses in CVB3-induced murine myocarditis. Scand. J. Infect. Dis. Suppl. 88, 67–78.
Jennings, G.T., Bachmann, M.F., 2008. The coming of age of virus-like particle vaccines. Biol. Chem. 389, 521–536.
Karkkainen, H.R., Lesch, H.P., Maatta, A.I., Toivanen, P.I., Mahonen, A.J., Roschier, M.M., Airenne, K.J., Laitinen, O.H., Yla-Herttuala, S., 2009. A 96-well format for a high-throughput baculovirus generation, fast titering and recombinant protein production in insect and mammalian cells. BMC Res. Notes 2, 63. http://dx.doi.org/10.1186/1756-0500-2-63.
Kemball, C.C., Harkins, S., Whitton, J.L., 2008. Enumeration and functional evaluation of virus-specific CD4+ and CD8+ T cells in lymphoid and peripheral sites of Coxsackievirus B3 infection. J. Virol. 82, 4331–4342.
Kim, D.S., Nam, J.H., 2011. Application of attenuated Coxsackievirus B3 as a viral vector system for vaccines and gene therapy. Hum. Vaccine 7, 410–416.
Kim, J.Y., Jeon, E.S., Lim, B.K., Kim, S.M., Chung, S.K., Kim, J.M., Park, S.I., Jo, I., Nam, J.H., 2005. Immunogenicity of a DNA vaccine for Coxsackievirus B3 in mice: protective effects of capsid proteins against viral challenge. Vaccine 23, 1672–1679.
Klump, W.M., Bergmann, I., Muller, B.C., Ameis, D., Kandolf, R., 1990. Complete nucleotide sequence of infectious Coxsackievirus B3 cDNA: two initial 5′ uridine residues are regained during plus-strand RNA synthesis. J. Virol. 64, 1573–1583.
Knowlton, K.U., Jeon, E.S., Berkley, N., Wessely, R., Huber, S., 1996. A mutation in the puff region of VP2 attenuates the myocarditic phenotype of an infectious cDNA of the Woodruff variant of Coxsackievirus B3. J. Virol. 70, 7811–7818.
Krausslich, H.G., Nicklin, M.J., Lee, C.K., Wimmer, E., 1988. Polyprotein processing in picornavirus replication. Biochimie 70, 119–130.
Liang, Z.L., Mao, Q.Y., Wang, Y.P., Zhu, F.C., Li, J.X., Yao, X., Gao, F., Wu, X., Xu, M., Wang, J.Z., 2013. Progress on the research and development of inactivated EV71 whole-virus vaccines. Hum. Vaccine Immunother. 9, 6.
Lin, Y.L., Yu, C.I., Hu, Y.C., Tsai, T.J., Kuo, Y.C., Chi, W.K., Lin, A.N., Chiang, B.L., 2012. Enterovirus type 71 neutralizing antibodies in the serum of macaque monkeys immunized with EV71 virus-like particles. Vaccine 30, 1305–1312.
Liu, Q., Yan, K., Feng, Y., Huang, X., Ku, Z., Cai, Y., Liu, F., Shi, J., Huang, Z., 2012. A virus-like particle vaccine for Coxsackievirus A16 potently elicits neutralizing antibodies that protect mice against lethal challenge. Vaccine 30, 6642–6648.
Maier, R., Krebs, P., Ludewig, B., 2004. Immunopathological basis of virus-induced myocarditis. Clin. Dev. Immunol. 11, 1–5.
McMinn, P.C., 2012. Recent advances in the molecular epidemiology and control of human enterovirus 71 infection. Curr. Opin. Virol. 2, 199–205.
Melnick, J.L., 1996. Enteroviruses: polioviruses, Coxsackieviruses, echoviruses and newer enteroviruses. In: Fields, B.N., Knipe, D.M., Howley, P.M., Channock, R.M., Melnick, J.L., Monath, T.P., Roizman, B., Straus, S.E. (Eds.), Fields Virology, third ed. Lippincott-Raven Publishers, Philadelphia, PA, pp. 655–712.
Mena, I., Fischer, C., Gebhard, J.R., Perry, C.M., Harkins, S., Whitton, J.L., 2000. Coxsackievirus infection of the pancreas: evaluation of receptor expression, pathogenesis, and immunopathology. Virology 271, 276–288.
Morenweiser, R., 2005. Downstream processing of viral vectors and vaccines. Gene Ther. 12 (Suppl. 1), S103–S110.

Park, J.H., Kim, D.S., Cho, Y.J., Kim, Y.J., Jeong, S.Y., Lee, S.M., Cho, S.J., Yun, C.W., Jo, I., Nam, J.H., 2009. Attenuation of Coxsackievirus B3 by VP2 mutation and its application as a vaccine against virus-induced myocarditis and pancreatitis. Vaccine 27, 1974–1983.

Roivainen, M., Knip, M., Hyoty, H., Kulmala, P., Hiltunen, M., Vahasalo, P., Hovi, T., Akerblom, H.K., 1998. Several different enterovirus serotypes can be associated with prediabetic autoimmune episodes and onset of overt IDDM. Childhood diabetes in Finland (DiMe) study group. J. Med. Virol. 56, 74–78.

Rombaut, B., Jore, J.P., 1997. Immunogenic, non-infectious polio subviral particles synthesized in *Saccharomyces cerevisiae*. J. Gen. Virol. 78 (Pt 8), 1829–1832.

Roy, P., Noad, R., 2008. Virus-like particles as a vaccine delivery system: myths and facts. Hum. Vaccine 4, 5–12.

See, D.M., Tilles, J.G., 1994. Efficacy of a polyvalent inactivated-virus vaccine in protecting mice from infection with clinical strains of group B Coxsackieviruses. Scand. J. Infect. Dis. 26, 739–747.

Selinka, H.C., Wolde, A., Sauter, M., Kandolf, R., Klingel, K., 2004. Virus-receptor interactions of Coxsackie B viruses and their putative influence on cardiotropism. Med. Microbiol. Immunol. 193, 127–131.

Slifka, M.K., Pagarigan, R., Mena, I., Feuer, R., Whitton, J.L., 2001. Using recombinant Coxsackievirus B3 to evaluate the induction and protective efficacy of CD8+ T cells during picornavirus infection. J. Virol. 75, 2377–2387.

Sprent, J., 1997. Immunological memory. Curr. Opin. Immunol. 9, 371–379.

Stadnick, E., Dan, M., Sadeghi, A., Chantler, J.K., 2004. Attenuating mutations in Coxsackievirus B3 map to a conformational epitope that comprises the puff region of VP2 and the knob of VP3. J. Virol. 78, 13987–14002.

Tu, Z., Chapman, N.M., Hufnagel, G., Tracy, S., Romero, J.R., Barry, W.H., Zhao, L., Currey, K., Shapiro, B., 1995. The cardiovirulent phenotype of coxsackievirus B3 is determined at a single site in the genomic 5′ nontranslated region. J. Virol. 69, 4607–4618.

Voigt, A., Jakel, S., Textoris-Taube, K., Keller, C., Drung, I., Szalay, G., Klingel, K., Henklein, P., Stangl, K., Kloetzel, P.M., Kuckelkorn, U., 2010. Generation of in silico predicted Coxsackievirus B3-derived MHC class I epitopes by proteasomes. Amino Acids 39, 243–255.

Wong, A.H., Lau, C.S., Cheng, P.K., Ng, A.Y., Lim, W.W., 2011. Coxsackievirus B3-associated aseptic meningitis: an emerging infection in Hong Kong. J. Med. Virol. 83, 483–489.

Xu, W., Shen, Y., Jiang, Z., Wang, Y., Chu, Y., Xiong, S., 2004. Intranasal delivery of chitosan-DNA vaccine generates mucosal SIgA and anti-CVB3 protection. Vaccine 22, 3603–3612.

Zhang, H., Morgan-Capner, P., Latif, N., Pandolfino, Y.A., Fan, W., Dunn, M.J., Archard, L.C., 1997. Coxsackievirus B3-induced myocarditis. Characterization of stable attenuated variants that protect against infection with the cardiovirulent wild-type strain. Am. J. Pathol. 150, 2197–2207.

Zhang, L., Parham, N.J., Zhang, F., Aasa-Chapman, M., Gould, E.A., Zhang, H., 2012. Vaccination with Coxsackievirus B3 virus-like particles elicits humoral immune response and protects mice against myocarditis. Vaccine 30, 2301–2308.