Enhanced Enterovirus 71 Virus-Like Particle Yield From a New Baculovirus Design

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ABSTRACT: Enterovirus 71 (EV71) is responsible for the outbreaks of hand-foot-and-mouth disease in the Asia-Pacific region. To produce the virus-like particle (VLP) vaccine, we previously constructed recombinant baculoviruses to co-express EV71 P1 polypeptide and 3CD protease using the Bac-to-Bac® vector system. The recombinant baculoviruses resulted in P1 cleavage by 3CD and subsequent VLP assembly in infected insect cells, but caused either low VLP yield or excessive VLP degradation. To tackle the problems, here we explored various expression cassette designs and flashBAC GOLD™ vector system which was deficient in v-cath and chid genes. We found that the recombinant baculovirus constructed using the flashBAC GOLD™ system was insufficient to improve the EV71 VLP yield. Nonetheless, BacF-P1-C3CD, a recombinant baculovirus constructed using the flashBAC GOLD™ system to express P1 under the polh promoter and 3CD under the CMV promoter, dramatically improved the VLP yield while alleviating the VLP degradation. Infection of High Five™ cells with BacF-P1-C3CD enhanced the total and extracellular VLP yield to ~268 and ~171 mg/L, respectively, which enabled the release of abundant VLP into the supernatant and simplified the downstream purification. Intramuscular immunization of mice with 5 μg purified VLP induced cross-protective humoral responses and conferred protection against lethal virus challenge. Given the significantly improved extracellular VLP yield (~171 mg/L) and the potent immunogenicity conferred by 5 μg VLP, one liter High Five™ culture produced ~12,000 doses of purified vaccine, thus rendering the EV71 VLP vaccine economically viable and able to compete with inactivated virus vaccines.

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Introduction

Enterovirus 71 (EV71) is a major etiological agent responsible for the outbreaks of hand-foot-and-mouth disease (HFMD) in such Asian countries as China, Taiwan, Malaysia, and Vietnam. EV71 infection of children under 5 years of age may result in severe neurological complications and even death, and EV71 outbreaks in Taiwan led to 78 deaths in 1998 and 14 deaths in 2008 (for review see (Kung et al., 2014)). The epidemics are even more serious in China, causing 7.2 million cases of HFMD and claiming 2457 lives from 2008 to 2012 (Liang and Wang, 2014). The increasing frequency of EV71 epidemics and fatality rates underscore the urgent need to develop vaccines against EV71. Currently, several forms of EV71 vaccines, such as inactivated whole virus (Cheng et al., 2013; Li et al., 2014; Zhu et al., 2013a; Zhu et al., 2013b), recombinant protein (Zhao et al., 2013), synthetic peptides (Liu et al., 2010) and pseudotyped baculovirus that displays the major immunogen VP1 (Kien et al., 2013), have been developed. In particular, inactivated whole virus vaccines are the most exhaustively studied and clinical trials of different phases have been completed in China, Taiwan, and Singapore (for review see
EV71 has a non-enveloped, icosahedral capsid comprised of VP1, VP2, VP3, and VP4 (Plevka et al., 2012). During EV71 replication, the viral structural polyprotein P1 is first cleaved by 3CD protease into VP0, VP1, and VP3. Catalyzed by the encapsidation of viral RNA genome, VP0 is further processed into VP2 and VP4, resulting in the formation of mature virus (Lyu et al., 2015; Wang et al., 2012). In addition, EV71 infection of susceptible mammalian cells results in the production of empty particles consisting of only VP0, VP1, and VP3 while lacking the RNA (Liu et al., 2011; Wang et al., 2012). Inspired by the natural EV71 virus assembly process, we have co-expressed recombinant P1 and 3CD using the baculovirus expression system, which resulted in the cleavage of P1 by 3CD into VP0, VP1, and VP3 and assembly into VLP in the Sf-9 insect cells (Hu et al., 2003). Subsequently, we constructed a recombinant baculovirus, Bac-P1-3CD, that encoded P1 under the polyhedrin (polh) promoter and 3CD under the p10 promoter (Chung et al., 2006). Infection of Sf-9 cells with Bac-P1-3CD successfully led to the P1 and 3CD co-expression and VLP production. After purification, the EV71 VLP was fairly stable (Lin et al., 2014a) and capable of inducing protective humoral immune responses in mice (Chung et al., 2010; Chung et al., 2008; Li et al., 2013) and monkeys (Lin et al., 2012). Importantly, the VLP was able to trigger potent cellular immune responses and confer protection against viral challenge in mice (Chung et al., 2008), thanks to the activation of dendritic cells through toll-like receptor 4 signaling (Lin et al., 2014c).

Materials and Methods

Cell Culture and Preparation of Recombinant Baculoviruses

Sf-9 cells were cultured using TNM-FH medium (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS) for baculovirus production/propagation at 27°C, or using SF-900II serum-free medium (Invitrogen, Carlsbad, CA) for VLP production. High Five™ cells (Invitrogen) were cultured in shaker flasks at 27°C using SF-900II medium.

Recombinant baculoviruses Bac-P1-3CD (Chung et al., 2006), Bac-P1-13CD (Chung et al., 2010) and Bac-P1-C3CD (Chung et al., 2010) were constructed previously using the Bac-to-Bac® (Invitrogen) system. The expression cassettes of P1-3CD, P1-13CD, and P1-C3CD were digested from the pBac-P1-3CD, pBac-P1-13CD, and pBac-P1-C3CD plasmids by SnaBI/AvrII, in which the P1 genes were under the control of polh promoter, while 3CD genes were under p10, ie1 and CMV promoters, respectively. The digested fragments were cloned into the pBacPAK8 plasmid (Clontech, Mountain View, CA) by EcoRV/XbaI to generate pBacPAK8-P1-3CD, pBacPAK8-P1-13CD, and pBacPAK8-P1-C3CD plasmids. To generate the recombinant baculoviruses, SF-9 cells were co-transfected with the donor plasmid and flashBAC GOLD™ DNA (Oxford Expression Technologies, UK). The resultant baculoviruses were designated as BacF-P1-3CD, BacF-P1-13CD, and BacF-P1-C3CD, respectively. The viruses were propagated to passage two by infecting SF-9 cells, harvested and titered by endpoint dilution method (Sung et al., 2014).

VLP Production, Purification, and Characterization

EV71 VLP was produced by infecting SF-9 or High Five™ cells at a cell density of 2 × 10⁶ cells/mL at MOI 0.1. The supernatant and cells were harvested at different days post-infection (dpi) by centrifugation (10,000 g for 10 min). The cells were lysed in phosphate-buffered saline (PBS) by three freeze/thaw cycles and centrifuged again as the intracellular VLP sample. To produce the VLP standard for enzyme-linked immunosorbent assay (ELISA), the VLP was purified to a final purity of ≈83% as described (Lin et al., 2014a) with minor modifications. Briefly, the supernatant harvested at 6 dpi was concentrated by tangential flow filtration (TFF) with a 1000 kDa cut-off membrane (Sartorius, Germany) and loaded into a hydroxyapatite chromatography (CHT™ Ceramic Hydroxyapatite, Type I, 40 μm, Bio-Rad, Hercules, CA). The VLP-containing flow-through was collected and concentrated again by TFF with a 300 kDa cut-off membrane (Sartorius). The VLP was purified again by size exclusion chromatography (Sephacryl™ S-400 HR, GE Healthcare, UK). The VLP-containing fractions were collected, concentrated and buffer-exchanged by TFF (300 kDa membrane) with 100 mM sodium phosphate (NaPi) buffer (pH 6.5). The purified VLP samples were aliquoted and stored at −80°C until analysis. The presence of VP0, VP1, and VP3 in the VLP was detected by SDS–PAGE. The VLP morphology was observed by transmission electron microscopy and the particle size was detected by dynamic light scattering as described (Lin et al., 2014a).

To produce the VLP standard for enzyme-linked immunosorbent assay (ELISA), the VLP was purified to high purity (>95%) as described (Chung et al., 2006) with modifications. Briefly, High Five™ cells were infected with Bac-P1-3CD at MOI 0.1. The VLP sample was harvested at 6 dpi, concentrated by ultracentrifugation (100,000 × g, 4 h), and separated by ultracentrifugation (100,000 × g, 4 h) on a 15–35% discontinuous sucrose gradient. The VLP-containing fraction at the interface of 15–35% sucrose was collected and underwent another ultracentrifugation (180,000 × g, 24 h) with a 1.33 g/cm³ cesium chloride solution. The
separated fractions were analyzed by Western blot (see below) and the VLP-containing fractions were pooled, pelleted and resuspended in PBS. The total protein concentration of the purified VLP sample was detected by Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, Waltham, MA) and the VLP purity was calculated by densitometry. The concentration of the purified VLP sample was defined as the total protein concentration × the VLP purity.

ELISA

The VLP concentration was measured by sandwich ELISA as described (Lin et al., 2014a). Briefly, 100 µL of protein A-purified rabbit anti-EV71 VLP polyclonal antibody (3.8 mg/mL, 1:10,000 dilution, provided by Prof. Bor-Luen Chiang) was coated to the 96-well plate at 4°C overnight as the capture antibody. After washes with PBS containing 0.05% Tween 20 (PBST) and blocking with PBST containing 1% bovine serum albumin (BSA) for 1 h, the VLP samples were added. After VLP capture for 2 h at room temperature (RT) and three PBST washes, 100 µL of mouse anti-EV71 IgG (1:20,000 dilution, Cat No. Ab36367, Abcam, Cambridge, MA) was added as the detecting antibody. After incubation at RT for 1.5 h and three PBST washes, the secondary antibody HRP-conjugated anti-mouse IgG (KPL, Gaithersburg, MD) was added, followed by color development with TMB (3, 3′, 5′, 5′-Tetramethylbenzidine Liquid Substrate System for ELISA, Sigma). The reaction was stopped with 2 N H2SO4 and the optical density value at 450 nm (OD450) was measured by Multiskan™ EX ELISA reader (Thermo Scientific).

In parallel, the purified VLP was diluted to 1 µg/mL and then twofold serially diluted. The diluted purified VLP was added to the ELISA plate in lieu of the VLP sample to generate the standard curve.

Western Blot

Western blot analysis of VLP was performed as described (Chung et al., 2010), except that the primary antibodies were rabbit anti-VPI polyclonal antibody for VPI detection (1:2,500 dilution, provided by Prof. Bor-Luen Chiang) and mouse anti-VP2 monoclonal antibody for VP0 detection (1:20,000 dilution, Cat No. MAb979, Millipore, Billerica, MA). The secondary antibodies were HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG, respectively (1:10,000 dilution, KPL, Gaithersburg, MD). The membranes were developed by Western LIGHTING™ Plus-ECL (PerkinElmer, Waltham, MA) and the images were captured by the GeneGnome ECL imager system (J&H Technology, Taiwan).

Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

To quantify the transcription levels of P1 and 3CD genes, High Five™ cells were infected as described above and harvested at 3 dpi by centrifugation (10,000 × g for 10 min). Total RNA of infected cells was extracted by NucleoSpin RNA II Kit (Machery–Nagel, Germany), and 500 ng of the RNA was reverse transcribed to cDNA using the Omniscrypt RT Kit (Qiagen, Germany). The transcribed cDNA was 100-fold diluted and subjected to qPCR reactions using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) with primer sets specific for P1 (Forward: GTTACAGGCTGACCCCTGGAC; Reverse: GCCATAAAGGACCCGGT-GAA) and 3CD (Forward: CTGCCGGTGAAAGTGGT; Reverse: TTCTTTGGTGCTCTCATCC) genes of EV71 (C2 genotype) and for actin gene (Forward: CTCTACGGTGACAGGAAGT; Reverse: ACAAGGTATATTGGAGCGG) of High Five™ cells (as internal control). The transcriptional levels of P1 and 3CD genes in Bac-P1-C3CD- and Bac-F-P1-C3CD-infected cells were normalized against those in Bac-P1-3CD-infected cells.

EV71 Propagation and Purification

EV71 strains belonging to different genotypes were propagated in human rhabdomyosarcoma (RD) cells (kindly provided by Dr. Shin-Ru Shih, Chang Gung University, Taiwan) cultured at 37°C using Dulbecco’s Modified Eagle’s Medium-high glucose (DMEM-HG, Sigma) containing 10% FBS in a biosafety level two (BSL2) laboratory. To produce the positive control in the mouse immunization experiments, RD cells were infected by EV71 neo strain (C2 genotype, provided by Dr. Mei-Shang Ho) at M01 0.001 and the virus in the supernatant was harvested at 3–4 dpi by centrifugation (18,000 × g for 30 min). The harvested viruses were concentrated by TFF with a 1000 kDa cut-off membrane and then purified by ultracentrifugation on a 10%–50% (w/w) continuous sucrose gradient. The fractions containing EV71 viruses were pooled, concentrated by TFF (300 kDa cut-off) and buffer-exchanged to 100 mM NaPi buffer (pH 6.5). The purified viruses were inactivated with formalin (0.2% (v/v)) at 37°C for 3 days and stored at −80°C until use. To evaluate the cross-reactivity of VLP-elicited mouse immune serum, EV71 CL9800002 (C2 genotype), EV71 2010–07146 (C4 genotype) and EV71 2009–02877 (B5 genotype) were propagated by infection of RD cells. The viruses in the supernatants were harvested without purification/inactivation.

The EV71 virus titer was determined by the end-point dilution assay for 50% tissue culture infectious dose (TCID50). Briefly, RD cells were inoculated into a 96-well plate (2 × 104 cells/well) and the 10-fold serially diluted virus samples (10−1–10−6) were added, followed by incubation at 37°C for 4 days. The wells of infected RD cells of each dilution were counted and the TCID50 value of the virus was calculated by Reed-and-Muench method.

Immunization

For immunization, total protein concentrations in the purified VLP and inactivated EV71 samples were quantitated using the Coomassie Plus (Bradford) Assay Kit. The female BALB/c mice (6–8 weeks old, purchased from BioLASCO, Taiwan) were immunized with formulated vaccines containing 5, 1.5 or 0.5 µg of purified VLP (n = 10 for each group), 5 µg of formalin-inactivated EV71 (neo strain, C2 genotype) as positive control (n = 10) and NaPi buffer (pH 6.5) as negative control (n = 5). All of these vaccine antigens (including negative controls) were formulated in a volume of 50 µL with 100 µg aluminum hydroxide (ALHYDROGEL™ 85% 2%, Brenntag Biosector, Denmark). Each mouse was injected intramuscularly (i.m.) with 50 µL formulated
vaccines and received the booster injection in the same manner at week 4.

**Serological Test**

The serum samples were collected from the submandibular artery at week 8, stored at −80°C and inactivated at 56°C for 30 min before use. The total anti-EV71 IgG titers in sera were measured by sandwich ELISA. Each well in the 96-well plate was coated with 100 μL of protein A-purified rabbit anti-VP1 polyclonal antibody (1:2,500 dilution in PBS, provided by Prof. Bor-Luen Chiang) and incubated at 4°C overnight. After three washes with PBST buffer, the wells were blocked with 300 μL PBST containing 1% BSA at 27°C for 30 min. After washes, 100 μL EV71 virus (new strain, 2 × 10^6 TCID50/well, heat-inactivated at 56°C for 30 min) was added to the wells. The sera were twofold serially diluted (24–215 dilutions) and added into wells. After incubation at 27°C for 90 min, the wells were washed with 300 μL PBST buffer for three times and 100 μL HRP-conjugated goat anti-mouse IgG (H+L) (1:2,500 dilution, KPL) was added into wells. After incubation at 37°C for 90 min and three washes, 100 μL TMB was added to stop the reaction and OD450 was measured. Each plate contained serially diluted non-immunized sera as internal controls. The cut-off OD value was defined as 0.2 plus the average value of each dilution of internal controls in all plates. The total anti-EV71 IgG titer of each sample was determined as the highest dilution at which the OD value of that dilution was higher than the cut-off value. The total anti-EV71 IgG titer larger than 215 or less than 23 was recorded as 215 or 23.

The neutralization titer was measured by the microneutralization assay. The heat-inactivated serum was twofold serially diluted (22–210 dilutions). Fifty microliter of each dilution was added into wells of a 96-well plate and mixed with 50 μL of EV71 virus (2 TCID50/μL). After incubation at 37°C for 1 h for virus neutralization, 100 μL of diluted RD cells (5 × 10^4 cells) were added into each well. The plate was incubated at 37°C for 4 days and the cytopathic effect (CPE) of RD cells was observed and recorded every day. The neutralization titers of the sera were determined as the highest dilution that resulted in 100% inhibition of CPE. The neutralization titer lager than 210 or less than 23 was recorded as 210 or 23.

**Lethal Challenge**

The in vivo protection was evaluated by viral challenge of suckling mice using an EV71 strain MP4 (provided by Prof. Jen–Ren Wang) which was shown to be lethal to neonatal mice (Wang et al., 2004) and the mice were housed in an animal biosafety level two (ABSL2) laboratory at the Institute of Preventive Medicine, National Defense Medical Center. The virus was propagated and titrated as described above and the 50% lethal dose (LD50) was determined by inoculation of neonatal mice. Briefly, BALB/c mice aged 1–3 days were intraperitoneally (i.p.) inoculated with 50 μL of 10-fold serially diluted virus samples (10–2–10–7). The survival of mice for each dilution was recorded for 14 days and the LD50 was calculated according to the Reed-and-Muench method.

For lethal virus challenge, groups of adult female BALB/c mice (purchased from BioLASCO, Taiwan) were i.m. injected with the formulated vaccine containing 5 μg VLP, 5 μg inEV71 or NaPi. These prime-immunized female mice were paired with naïve male mice for mating at week 2, and received a booster injection at week 3. After birth (at week 5), groups of the neonatal mice born to the immunized dams aged 1–3 days were i.p. challenged with 250 LD50 of EV71 MP4 virus (C2 genotype). The survival of challenged neonatal mice was recorded every day for 15 days.

**Ethics Statement**

All animal experiments were performed in compliance with the Institutional Animal Care and Use Committee Guidebook published by the US Office of Laboratory Animal Welfare. The experimental protocols were approved and conducted in accordance with the guidelines and under the supervision of Institutional Committee on Animal Care and Use, Institute of Preventive Medicine, National Defense Medical Center (Approval no. AN-102-01).

**Statistical Analysis**

All in vitro data are expressed as averages of 2–3 independent culture or animal experiments. The data were analyzed by student's t test and P-values less than 0.05 were considered significant.

**Results**

**Improvement of VLP Production Using the FlashBAC GOLD™ Baculovirus System**

Recombinant baculoviruses Bac-P1-3CD and Bac-P1-C3CD were constructed using the Bac-to-Bac® baculovirus system and co-expressed EV71 P1 polypeptide under the polh promoter, and 3CD protease but under p10 or CMV promoter, respectively, after infection of insect cells (Chung et al., 2010; Chung et al., 2006). Co-expression of P1 and 3CD led to the P1 cleavage into VP0, VP1, and VP3 by 3CD, and subsequent VLP assembly. However, Bac-P1-3CD conferred low VLP yield (Chung et al., 2010) while Bac-P1-C3CD resulted in excessive degradation products during the production (data not shown).

To enhance the VLP yield, we reasoned that driving the 3CD expression with weaker promoters may alleviate the competition and enhance the VLP yield. Moreover, to impede the product degradation we employed the flashBAC GOLD™ baculovirus vector system which was shown to improve the recombinant protein integrity (Kaba et al., 2004). We constructed BacF-P1-3CD, BacF-P1-I3CD, and BacF-P1-C3CD (Fig. 1A), which expressed P1 under the polh promoter, but expressed 3CD under promoters with varying strengths: p10 for Bac-P1-3CD, ie1 for BacF-P1-I3CD and CMV for BacF-P1-C3CD. High Five™ cells cultured in the shaker flasks were infected with Bac-P1-3CD, BacF-P1-3CD, BacF-P1-I3CD, or BacF-P1-C3CD under the same condition (MOI 0.1, at 2 × 10^6 cells/mL) and the supernatant was collected at 3–6 days post-infection (dpi) for Western blot using rabbit anti-VP1 polyclonal antibody (Fig. 1B). Compared with Bac-P1-3CD, BacF-P1-3CD conferred lower VPI production (an indicator of VLP yield), whereas BacF-P1-I3CD and BacF-P1-C3CD appeared to enhance the
VLP yield after 4 dpi, as judged from the band intensities in Western blot (Fig. 1B).

ELISA analysis of the supernatants (Fig. 1C) further confirmed that BacF-P1-3CD infection led to lower extracellular VLP yield (≈7 mg/L at 6 dpi) than Bac-P1-3CD (≈24 mg/L at 6 dpi), but BacF-P1-13CD and BacF-P1-C3CD dramatically increased the VLP yield at 6 dpi to ≈104 and ≈171 mg/L, respectively. These data suggested that simply switching the Bac-to-Bac™ system to the flashBAC GOLD™ system did not improve the extracellular VLP yield. Nevertheless, the VLP yield could be remarkably enhanced with appropriate baculovirus design, and BacF-P1-C3CD conferred the highest extracellular yield.

To examine the VLP yield in different cells, High Five™ and Sf-9 cells were infected with BacF-P1-C3CD and the extracellular/intracellular VLP concentrations were measured by ELISA. Fig. 1D reveals that High Five™ cells conferred higher extracellular/intracellular VLP yield than Sf-9 cells. For High Five™ cells, the intracellular VLP yield peaked at 3 dpi (≈166 mg/L) and declined thereafter while the extracellular VLP yield increased with time. The total yield at 6 dpi, including the extracellular (≈171 mg/L) and intracellular (≈97 mg/L) VLP yield, reached ≈268 mg/L at 6 dpi.

Enhanced P1 Expression and Diminished VLP Degradation

BacF-P1-C3CD was constructed based on the assumption that reducing 3CD expression under a weaker CMV promoter was able to enhance the P1 expression. To verify this hypothesis, we infected High Five™ cells with Bac-P1-3CD, Bac-P1-C3CD, or BacF-P1-C3CD at MOI 0.1 and analyzed the mRNA levels at 3 dpi by qRT-PCR (Fig. 2A). Compared with Bac-P1-3CD which expressed 3CD under the p10 promoter, Bac-P1-C3CD and BacF-P1-C3CD, which expressed 3CD under the CMV promoter, indeed conferred significantly (P < 0.05) lower 3CD transcription and higher P1 transcription. However, Western blot analysis of the culture supernatant (Fig. 2B) illustrated that Bac-P1-C3CD, which was constructed using the Bac-to-Bac™ system, led to lower VP1 levels and only slightly higher VP0 levels than Bac-P1-3CD, due to apparent protein degradation. Nonetheless, the flashBAC GOLD™.
based baculovirus BacF-P1-C3CD gave rise to higher VP1 and VP0 levels and less degradation than Bac-P1-C3CD (Fig. 2B).

To examine the roles of proteases in the protein degradation, High Five™ cells were infected by either Bac-P1-C3CD or BacF-P1-C3CD, and the cell lysates at 3 dpi were incubated at 27°C for 72 h alone or with different protease inhibitors (1,10-phenanthroline, E-64, or pepstatin A). Western blot analysis revealed that VP1 protein disappeared at 24 h after incubation when the Bac-P1-C3CD-infected cell lysate was incubated alone or with 1,10-phenanthroline, metalloprotease inhibitor; E-64, cysteine protease inhibitor; Pepstatin A, carboxyl protease inhibitor. Since 1,10-phenanthroline, E-64, and pepstatin A are inhibitors of metalloprotease, cysteine protease and carboxyl protease, respectively, these data indicated that cysteine protease, rather than metalloprotease or carboxyl protease, played pivotal roles in the VLP degradation. In contrast, VP1 degradation was not observed in BacF-P1-C3CD-infected cell lysates that were incubated alone or with any protease inhibitor (lower panel, Fig. 2C), indicating the negligible proteolytic degradation of VLP in the BacF-P1-C3CD-infected cell lysate.

**Characterization of the Purified VLP**

To verify the VLP production by BacF-P1-C3CD, High Five™ cells were infected with BacF-P1-C3CD as in Fig. 1 and the VLP in the supernatant was harvested at 6 dpi and purified by chromatography.
as described in Materials and Methods. SDS–PAGE analysis of the purified sample revealed three prominent bands, whose molecular mass corresponded to those of EV71 capsid proteins VP0, VP1, and VP3 (Fig. 3A). The purified VLP resembled the EV71 empty particle (Liu et al., 2011; Wang et al., 2012) in morphology and shape, as evidenced by transmission electron microscopy (Fig. 3B). The dynamic light scattering (Fig. 3C) further confirmed that the average particle size of the purified VLP (33 nm) was similar to that of EV71 empty particle. Taken together, the VLP produced by the BacF-P1-C3CD-infected High FiveTM cells resembled the EV71 empty particle in composition, morphology, and size.

Immune Responses Elicited by the VLP In Mouse Models

To confirm the vaccine efficacy, the purified VLP was injected intramuscularly (i.m.) into female BALB/c mice at different doses (5, 1.5, and 0.5 mg per dose, n = 10 for each group). Formalin-inactivated EV71 virus (inEV71, 5 mg/dose, n = 10) and sodium phosphate (NaPi, n = 5) buffer which was the final buffer for VLP purification were also injected as positive and negative controls, respectively. All of these samples were adjuvanted with 100 μg aluminum hydroxide (Alhydrogel®). Four weeks later, the mice received a booster injection with the same dose and the sera were collected at week 8.

ELISA analysis (Fig. 4A) showed that the total anti-EV71 IgG titer provoked by 5 μg VLP (212.2) was statistically similar (P > 0.05) to that by 5 μg inEV71 (212.3) and remarkably (P < 0.05) exceeded that by the negative control NaPi (23.0). Lowering the VLP dose to 0.5 and 1.5 μg triggered lower IgG titers (210.6), which nonetheless were still remarkably higher than that induced by the negative control.

EV71 has been classified into three genogroups (A, B, and C), which can be further divided into 11 genotypes (A, B1–B5, and C1–C5). The P1 and 3CD genes for VLP production were derived from the C2 genotype. Whether the antibody was capable of neutralizing the homologous virus was examined by the microneutralization assay using live EV71 of the C2 genotype. Fig. 4B delineates that 5 μg VLP (20.2) elicited statistically (P > 0.05) similar average neutralization titers when compared with 5 μg inEV71 (21.8). To explore the cross-reactivity of the antibodies induced by 5 μg VLP, we also performed the microneutralization assay using two other EV71 genotypes (B5 and C4) prevalent in Taiwan and China. Fig. 4C shows that immunization with 5 μg VLP elicited statistically similar (P > 0.05) neutralization titers against EV71 of C2 (20.2), B5 (21.8), and C4 (21.8) genotypes, attesting that the sera from the VLP-immunized mice were able to cross-react with EV71 viruses of homologous (C2) and heterologous (B5 and C4) genotypes.

VLP Conferred Protection Against Lethal Virus Challenge

To evaluate whether the VLP immunization conferred protection to mice, female BALB/c mice were immunized with 5 μg VLP, 5 μg inEV71, or NaPi as described in Materials and methods. These immunized mice were mated and the neonatal mice were challenged with a lethal dose of EV71 (C2 genotype). With a lethal virus dose (250 LD50), no mice in the negative control group (NaPi) survived more than 5 days (Fig. 5), but immunization with

**Figure 3.** Characterization of purified EV71 VLP. (A) SDS–PAGE analysis. Arrows indicate the major capsid proteins VP0, VP1, and VP3. (B) Transmission electron micrograph. (C) Diameter distribution as determined by dynamic light scattering. The VLP was produced by infecting High FiveTM cells (MOI 0.1, 2 x 10⁶ cells/mL) with BacF-P1-C3CD, harvested at 6 dpi and purified by TFF combined with hydroxyapatite chromatography and size exclusion chromatography.
5 μg VLP conferred a survival rate of 100% (23 out of 23 mice survived), which was comparable to that (≈93%, 26 out of 28 mice survived) conferred by the positive control group (inEV71).

Discussion

Baculovirus expression system is a powerful tool for commercial production of VLP vaccines against human papillomavirus (Cervarix®, GlaxoSmithKline) and porcine circovirus type 2 (e.g., Ingelvac CircoFLEX®, Boehringer Ingelheim) and has also been utilized for the production of VLP of numerous viruses, including human immunodeficiency virus (HIV), parvovirus, SARS–CoV and avian influenza virus (for review see (Hu et al., 2008; Lin et al., 2014b; Lua et al., 2014)). However, the VLP yield using conventional baculovirus vectors is usually limited and represents a roadblock to commercial applications. For instance, the yield of chikungunya virus and infectious bursal disease virus VLP falls in the range of 20–30 mg/L (Hu and Bentley, 1999; Wagner et al., 2014) while the VLP yield of Ebola virus is 5–10 mg/L (Ye et al., 2006). Besides, the VLP yield of H1N1 influenza virus, HIV, human papillomavirus type 33, porcine parvovirus and SARS–CoV is generally far lower than 10 mg/L (Baek et al., 2011; Krammer et al., 2010a; Maranga et al., 2002; Mortola and Roy, 2004; Senger et al., 2009).

Likewise, the EV71 VLP yield conferred by our first version of recombinant baculovirus (Bac-P1-3CD) was limited (Chung et al., 2006). Although the second generation recombinant baculovirus Bac-P1-C3CD was able to enhance the VLP yield (Chung et al., 2010), the VLP proteins appeared to be unstable during the production phase. To enhance the VLP yield by boosting the P1 expression, we designed a panel of baculoviral constructs which co-expressed P1 and 3CD, yet with a hr1 enhancer or an HS4 insulator appended to the 30 end of the P1 gene (Fig. S1), which were reported to enhance the recombinant protein yield (Viswanathan et al., 2003; Wang et al., 2009). However, infection of High Five™ cells with these constructs failed to increase the VLP yield when compared with Bac-P1-3CD (Fig. S1). To reduce the 3CD expression by attenuating the p10 promoter strength, we employed truncated p10 promoters to drive the 3CD expression and used polh promoter to drive the P1 expression. However, the resultant baculovirus vectors still gave inferior VLP yield (Fig. S2). Using the B5 genotype as an example, we also attempted to append a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE, which reportedly enhances the RNA stability and protein production (Mahonen et al., 2007)) to the 30 end of P1 gene (Fig. S1), which were reported to enhance the recombinant protein yield (Viswanathan et al., 2003; Wang et al., 2009). However, infection of High Five™ cells with these constructs failed to increase the VLP yield when compared with Bac-P1-3CD (Fig. S1). To reduce the 3CD expression by attenuating the p10 promoter strength, we employed truncated p10 promoters to drive the 3CD expression and used polh promoter to drive the P1 expression. However, the resultant baculovirus vectors still gave inferior VLP yield (Fig. S2). 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the baculoviral genome hampers the liquefaction of hosts to some extent (Kaba et al., 2004) as well as elevates the expression yield of secreted VLP yield. In particular, infection of High Five™ cells with BacF-P1/C25 infected the baculoviral genome. Baculovirus cathepsin encoded by v-cath is a papain-like cysteine protease (Ohkawa et al., 1994) while chitinase encoded by chiA functions in concert with cathepsin and promotes liquefaction of the host in the latter stages of infection, resulting in the release of viruses to infect more cells (Hawtin et al., 1997). Both v-cath and chiA are dispensable for viral replication/polyhedron production in insect cells, thus deletion of v-cath and/or chiA from the baculoviral genome hampers the liquefaction of hosts to some extent. Consequently, infection of insect cells with the recombinant baculovirus deficient in v-cath and chiA genes ameliorates the integrity of both intracellular and secreted recombinant proteins (Kaba et al., 2004) as well as elevates the expression yield of secreted and membrane-targeted proteins (Hitchman et al., 2010b).

However, the flashBAC GOLD™-based baculovirus (BacF-P1-3CD) with an expression cassette design similar to our first generation baculovirus (Bac-P1-3CD) also failed to increase the yield (Fig. 1B and 1C), indicating that deletion of v-cath and chiA was insufficient to promote the EV71 VLP yield. Only when the flashBAC GOLD™ system was employed and 3CD was expressed under the icel (BacF-P1-3CD) or CMV (BacF-P1-C3CD) promoter could we dramatically enhance the intracellular and extracellular VLP yield. In particular, infection of High Five™ cells with Bac-F-P1-C3CD enhanced the total and extracellular VLP yield to ≈268 and ≈171 mg/L, respectively. The extracellular yield was ≈114- and ≈7-fold the yield conferred by Bac-P1-3CD-infected Sf-9 (≈1.5 mg/L (Chung et al., 2010) and High Five™ cells (≈24 mg/L, Fig. 1C). Such high extracellular VLP yield also significantly exceeded the yield of many other VLP as mentioned above.

The remarkable enhancement of EV71 VLP yield was ascribed to the combination of three determinants. First, here we employed High Five™ cells for VLP production, which gave significantly higher intracellular and extracellular VLP yield than Sf-9 cells (Fig. 1C). This result echoed the findings that High Five™ cells conferred higher yield of H1N1 influenza VLP (Krammer et al., 2010a) and extracellular proteins (for review see (Fernandes et al., 2013)) than Sf-9 cells. Furthermore, High Five™ cells result in a much lower virus background of the final VLP preparation than Sf-9 cells (Krammer et al., 2010b) and are exploited for the commercial production of the HPV VLP vaccine (Cervarix™), which further support the use of High Five™ cells for EV71 VLP production.

Second, here we employed the weaker CMV promoter in lieu of the stronger p10 promoter for transcriptional control of 3CD, which could theoretically enhance the polh-driven P1 expression by reducing the transcriptional/translational competition and indeed elevated P1 expression (Fig. 2A). This approach was justified by the finding that inhibition/deletion of p10 promoter enhances the polh-controlled protein production (Vlak et al., 1988). The importance of replacing p10 promoter was further evidenced by our supplementary data which unveiled that 3CD expression driven by the full-length or truncated p10 promoter gave rise to inferior VLP yield, either using the Bac-to-Bac™ (Fig. S1) or flashBAC GOLD™ (Fig. S4) baculovirus system. As such, the replacement of p10 promoter was critical for the enhanced EV71 VLP yield.

Third, it has been shown that proteolytic degradation is responsible for low VLP yield (as in the case of HIV Pr55ag particle (Cruz et al., 1999)) in the supernatant of baculovirus-infected cell culture. Cathepsin encoded by v-cath is a cysteine protease synthesized late in infection as an inactive pro-enzyme located in the endoplasmic reticulum (ER) and is activated by proteolytic cleavage upon cell death (Hitchman et al., 2011). Cysteine protease activity is the most abundant protease activity in the medium of virus-infected Sf9 cells (Gotoh et al., 2001), which led to significant VLP degradation in the Bac-P1-C3CD-infected cell culture (Figs. 2B–2C). Since the flashBAC GOLD™-based BacF-P1-C3CD was deficient in v-cath and chiA, the VLP degradation was minimal in the Bac-P1-C3CD-infected cell lysate (Figs. 2B–2C). Such reduction of proteolysis was also observed in Sf9 cells infected with a v-cath/chiA baculovirus (for review see (Hitchman et al., 2011)). Furthermore, chitinase is targeted to the ER and may act as a chaperone for the correct folding of pro-v-cath in the ER (Hom and Volkman, 2000). Expression of chiA could not only activate the cathepsin function but also obstruct the ER, severely compromising the function and efficacy of the secretory pathway (Possee et al., 1999). Altogether, deletion of v-cath and chiA could attenuate the VLP degradation and deterioration of cellular functions after baculovirus infection, thus facilitating the VLP protein production and assembly.

In addition to flashBAC GOLD™, currently several other baculovirus vector systems with v-cath and chiA deletion have been publicly available. These include the BestBac System (Expression Systems), BacVector-3000 (Novagen) and MultiBac (European Molecular Biology Laboratory). The MultiBac system remarkably improves the recombinant protein quality, reduces proteolytic breakdown (Berger et al., 2004) and has been exploited for enhancing the yield of HPV VLP (Senger et al., 2009). Furthermore, a baculovirus vector (flashBAC ULTRA™, Oxford Expression Technologies) with 5 non-essential genes (v-cath, chiA, p10, p26, and p74) removed from the virus genome was developed, which was shown to further enhance the yield of recombinant proteins when compared with flashBAC GOLD™ deficient in only v-vath/chiA (Hitchman et al., 2010a). More recently, a new flashBAC PRIME™ system (Oxford Expression Technologies) was developed, which claimed to increase the yield of VLP by inducing cell lysis at a very late stage of infection in Trichoplusia ni-derived cell lines (http://oetltd.com/products/product/flashBAC_PRIME/). These systems, together with the intricate expression cassette design, may also improve the EV71 VLP yield.

It is worth of emphasizing that using this Bac-F-P1-C3CD/High Five™ combination a large portion of VLP was released into the supernatant, thereby simplifying the downstream purification. The chromatography process we used allowed for the recovery of ≈36% purified VLP (Lin et al., 2015). The purified VLP was similar to the EV71 empty particle in size, shape and composition (Fig. 3). As a result, immunization of mice with 5μg VLP was sufficient to induce humoral immune
responses that were as potent as those induced by same amount of inactivated EV71 virus (Figs. 4A–4B). Importantly, 5 μg purified EV71 VLP per dose induced immune responses capable of neutralizing homologous and heterologous EV71 strains (Fig. 4C) and conferred protection against lethal virus challenge (Fig. 5). Given the significantly improved extracellular VLP yield (∼171 mg/L), the recovery efficiency (∼36%) of extracellular VLP (Lin et al., 2015), and the potent immunogenicity/ protection conferred by 5 μg VLP, one liter High Five™ culture was able to provide ∼60 mg purified VLP, which was equivalent to ∼12,000 doses of vaccine. Such high VLP yield renders the EV71 VLP vaccine economically viable, which should enable the VLP vaccine to compete with the inactivated virus vaccines.

Conclusions

In summary, the combined use of High Five™ cells and the v-cath ichia baculovirus vector that expressed P1 under the polh promoter and 3CD under the CMV promoter allowed for the production of EV71 VLP capable of inducing cross-protective humoral immune responses and conferring protection against lethal viral challenge. This approach improved the extracellular VLP yield to ∼171 mg/L, which after purification was equivalent to ∼12,000 doses of vaccines, thus rendering the EV71 VLP vaccine economically viable and allowing for its competition with inactivated virus vaccines.

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Supporting Information

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