ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

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

Shih-Yeh Lin,[1] Chia-Tsui Yeh,[2,3] Wan-Hua Li,[1] Cheng-Ping Yu,[2,4] Wen-Chin Lin,[2,5] Jyh-Yuan Yang,[6] Hsueh-Ling Wu,[2] Yu-Chen Hu[1]

[1]Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan; telephone: +886-3-571-8245; fax: +886-3-571-5408; e-mail: ychu@mx.nthu.edu.tw
[2]Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan
[3]Department of Life Science, National Taiwan Normal University, Taipei, Taiwan
[4]Department of Pathology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
[5]Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan
[6]Center for Research, Diagnostics and Vaccine Development, Centers for Disease Control, Taipei, Taiwan

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 *chiA* 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.

Biotechnol. Bioeng. 2015;112: 2005–2015.

KEYWORDS: baculovirus; EV71; VLP; vaccine; hand-foot-and-mouth disease; protease

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 (Kiener 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

Disclosure statement: The authors have no competing financial interests to declare.
Correspondence to: Y.-C. Hu
Contract grant sponsor: Ministry of Science and Technology
Grant numbers: MOST 103-2622-E-007-024-CC1; NSC 102-2622-E-007-022-CC1; NSC 101-2628-E-007-009-MY3; NSC 100-2622-E-007-003-CC2
Contract grant sponsor: Ministry of Health and Welfare
Grant numbers: MOHW103-TDU-PB-211-122021; MOHW102-TDU-PB-111-NSC108; DOH99-TD-I-111-TM022; DOH98-DC-1013
Received 4 March 2015; Revision received 17 April 2015; Accepted 23 April 2015

DOI 10.1002/bit.25625

(Liang and Wang, 2014)). Besides these vaccines, virus-like particle (VLP) is the empty particle composed of viral structural proteins and devoid of viral nucleic acids, and has emerged as a promising vaccine platform (Lin et al., 2014b; Lua et al., 2014; Zhao et al., 2014).

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).

Despite the promise of EV71 VLP vaccine, the yield of extracellular VLP released by Bac-P1-3CD-infected Sf-9 cells was extremely low (≈1.5 mg/L (Chung et al., 2010)). To enhance the VLP yield, we constructed Bac-P1-C3CD which expressed P1 under the *polh* promoter and 3CD under the cytomegalovirus immediate early (*CMV*) promoter. Bac-P1-C3CD infection of Sf-9 cells elevated the VLP yield to ≈40–65 mg/L under optimized culture/infection conditions (Chung et al., 2010). However, excessive VLP degradation was observed during the production. Therefore, here we employed an alternative baculovirus vector system, in conjunction with appropriate baculovirus expression cassette design and cell selection, in attempts to improve the VLP yield and minimize the degradation. The VLP production yield, kinetics, and degradation using different baculovirus vectors were monitored. Whether the resultant VLP was able to trigger cross-reactive immune responses and confer protection against lethal virus challenge was examined.

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^{TM} 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-I3CD (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-I3CD, and P1-C3CD were digested from the pBac-P1-3CD, pBac-P1-I3CD, and pBac-P1-C3CD plasmids by *Sna*BI/*Avr*II, 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 *Eco*RV/*Xba*I to generate pBacPAK8-P1-3CD, pBacPAK8-P1-I3CD, and pBacPAK8-P1-C3CD plasmids. To generate the recombinant baculoviruses, Sf-9 cells were co-transfected with the donor plasmid and flashBAC GOLD^{TM} DNA (Oxford Expression Technologies, UK). The resultant baculoviruses were designated as BacF-P1-3CD, BacF-P1-I3CD, and BacF-P1-C3CD, respectively. The viruses were propagated to passage two by infecting Sf-9 cells, harvested and titered by end-point dilution method (Sung et al., 2014).

VLP Production, Purification, and Characterization

EV71 VLP was produced by infecting Sf-9 or High Five^{TM} cells at a cell density of 2×10^6 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.

For immunization, the extracellular 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^{TM} 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^{TM} 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^{TM} 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 seperated by ultracentrifugation (100,000 × *g*, 4 h) on a 15–35–65% discontinuouse 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/cm^3 cesium chloride solution. The seperated 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 H_2SO_4 and the optical density value at 450 nm (OD_{450}) 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-VP1 polyclonal antibody for VP1 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 (Macherey–Nagel, Germany), and 500 ng of the RNA was reverse transcribed to cDNA using the Omniscript 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: GTTCAGAGCTGACCCTGGAC; Reverse: GCCATAAAGGACCCGGTGAA) and *3CD* (Forward: CTGCCGTGGGTAAAGTGATT; Reverse: TTCTTTGTTGGGCTTCATCC) genes of EV71 (C2 genotype) and for *actin* gene (Forward: CTCCATCGTGCACAGGAAGT; Reverse: ACAAGCGTAATTTGAGCCGC) of High Five™ cells (as internal control). The transcriptional levels of *P1* and *3CD* genes in Bac-P1-C3CD- and BacF-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 *neu* strain (C2 genotype, provided by Dr. Mei-Shang Ho) at MOI 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 ($TCID_{50}$). Briefly, RD cells were inoculated into a 96-well plate (2×10^4 cells/well) and the 10-fold serially diluted virus samples (10^{-1}–10^{-8}) were added, followed by incubation at 37°C for 4 days. The wells of infected RD cells of each dilution were counted and the $TCID_{50}$ 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 (*neu* 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 (*neu* strain, 2×10^6 $TCID_{50}$/well, heat-inactivated at 56°C for 30 min) was added to the wells. The sera were twofold serially diluted (2^4–2^{15} 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 27°C for 90 min and three washes, 100 μL TMB was added for color development for 5 min. Fifty microliter H_2SO_4 (2 N) was added to stop the reaction and OD_{450} 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 2^{15} or less than 2^4 was recorded as 2^{15} or 2^3.

The neutralization titer was measured by the microneutralization assay. The heat-inactivated serum was twofold serially diluted (2^3–2^{10} dilutions). Fifty microliter of each dilution was added into wells of a 96-well plate and mixed with 50 μL of EV71 virus (2 $TCID_{50}$/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 2^{10} or less than 2^3 was recorded as 2^{10} or 2^2.

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 (LD_{50}) 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 LD_{50} 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 LD_{50} 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 BacF-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 VP1 production (an indicator of VLP yield), whereas BacF-P1-I3CD and BacF-P1-C3CD appeared to enhance the

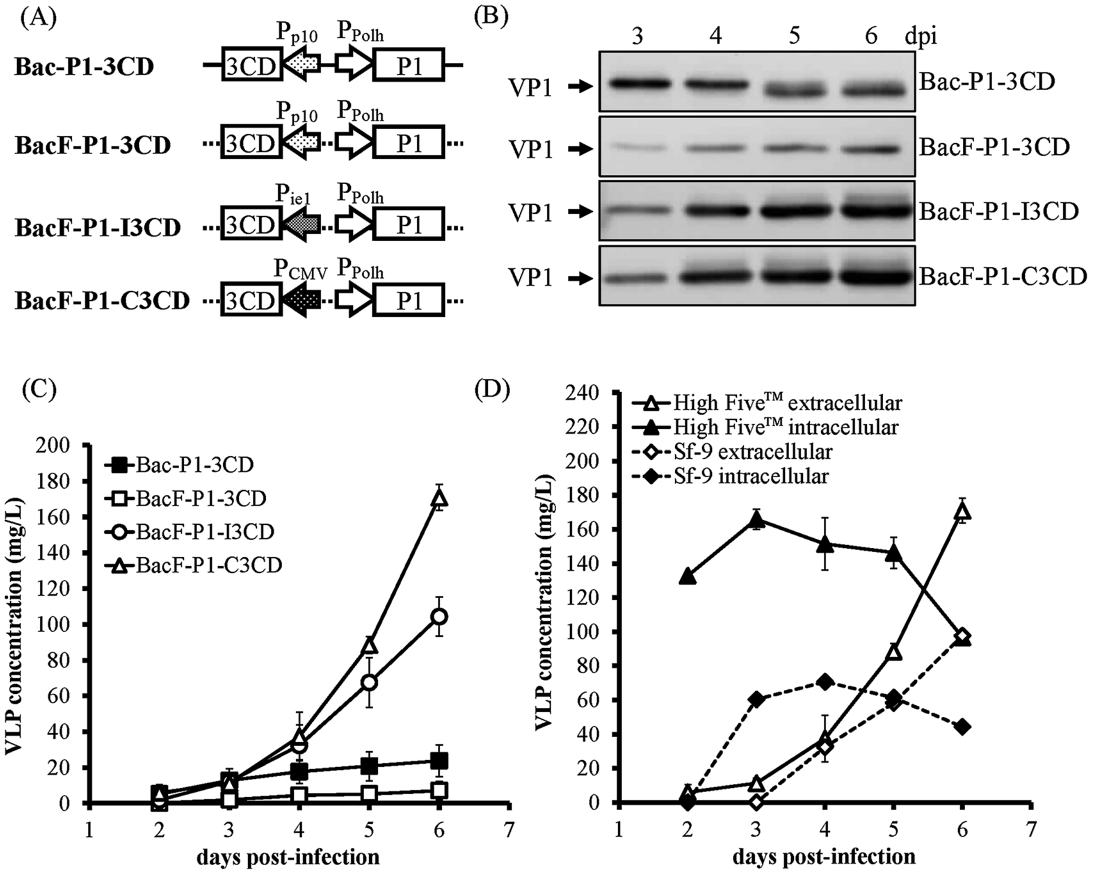

Figure 1. Baculovirus design and EV71 VLP production. (**A**) Schematic illustration of recombinant baculoviruses. (**B**) Western blot analysis of extracellular VP1 protein at 3–6 days after infection of High Five$^{\text{TM}}$ cells. (**C**) Extracellular VLP yield by High Five$^{\text{TM}}$ cells infected with different viruses. (**D**) Extra- and intracellular VLP yield by two cell lines infected by BacF-P1-C3CD. Bac-P1-3CD was constructed using the Bac-to-Bac® system. BacF-P1-3CD, BacF-P1-I3CD, and BacF-P1-C3CD were constructed using the flashBAC GOLD$^{\text{TM}}$ system. The cells were infected by baculovirus under the same condition (MOI 0.1, at 2×10^6 cells/mL). The VLP yield was determined by ELISA.

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-I3CD 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$^{\text{TM}}$ 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$^{\text{TM}}$ 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$^{\text{TM}}$ cells conferred higher extracellular/intracellular VLP yield than Sf-9 cells. For High Five$^{\text{TM}}$ 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$^{\text{TM}}$ 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$^{\text{TM}}$-

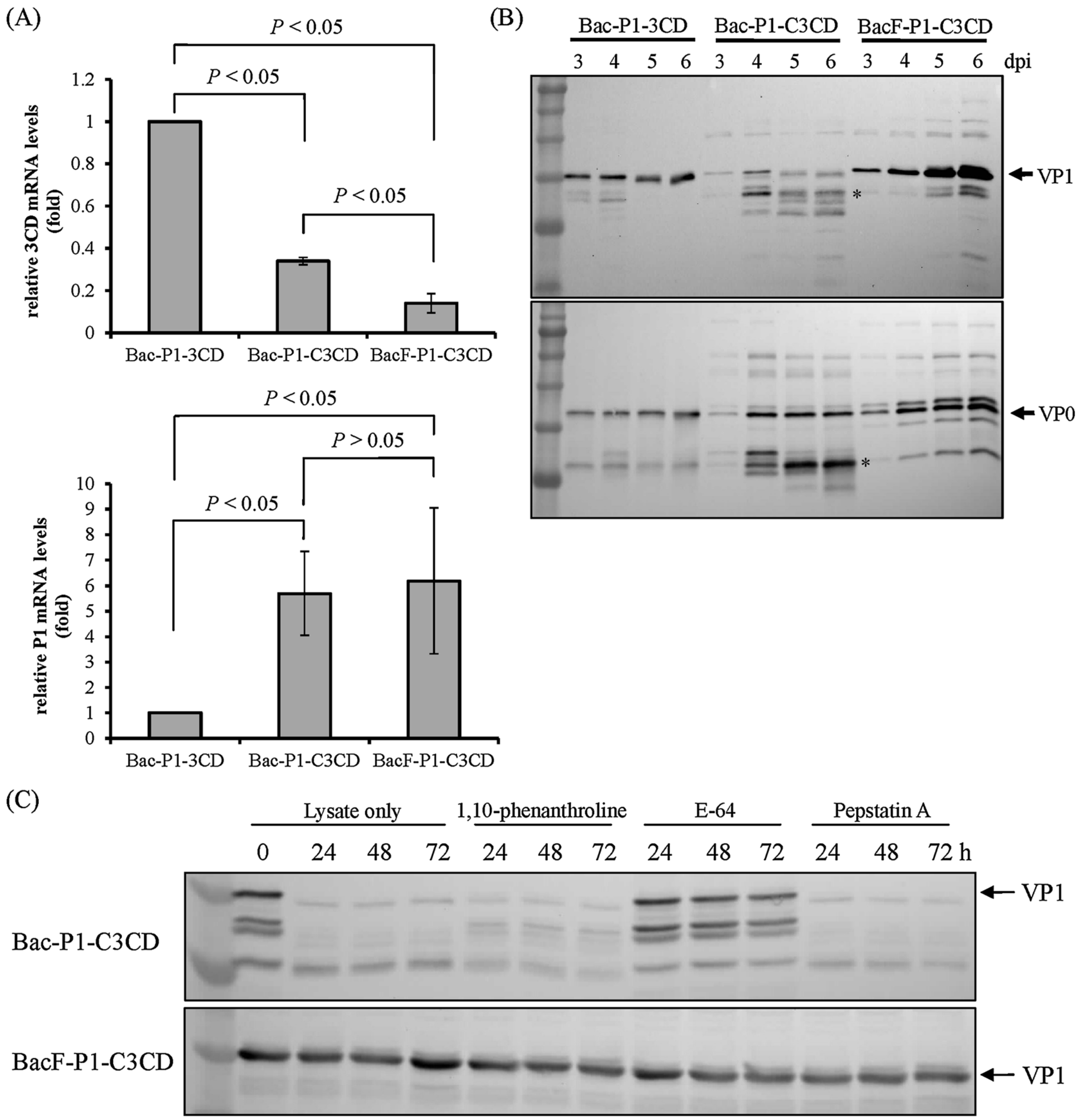

Figure 2. Gene expression and VLP protein degradation. (**A**) Relative *3CD* and *P1* mRNA levels in infected High Five^{TM} cells at 3 dpi. (**B**) The VP1 (upper) and VP0 (lower) protein levels as detected by Western blot. Stars indicate the degradation products. (**C**) Western blot analysis of Bac-P1-C3CD- and BacF-P1-C3CD-infected cell lysates incubated alone or with different protease inhibitors at 27°C for 3 days. Cells were harvested at 3 dpi. 1,10-phenanthroline: metalloprotease inhibitor; E-64: cysteine protease inhibitor; Pepstatin A: carboxyl protease inhibitor.

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^{TM} 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 or pepstatin A (upper panel, Fig. 2C), indicating serious protein degradation. Only incubation with E-64 prevented the VP1 degradation with time. 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^{TM} 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 Five$^{\mathrm{TM}}$ 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 μg per dose, $n = 10$ for each group). Formalin-inactivated EV71 virus (inEV71, 5 μg/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 ($2^{12.2}$) was statistically similar ($P > 0.05$) to that by 5 μg inEV71 ($2^{12.3}$) and remarkably ($P < 0.05$) exceeded that by the negative control NaPi ($2^{3.0}$). Lowering the VLP dose to 0.5 and 1.5 μg triggered lower IgG titers ($2^{10.6}$), 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 ($2^{7.2}$) elicited statistically ($P > 0.05$) similar average neutralization titers when compared with 5 μg inEV71 ($2^{4.8}$). 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 ($2^{7.2}$), B5 ($2^{4.6}$), and C4 ($2^{5.8}$) 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 LD_{50}), no mice in the negative control group (NaPi) survived more than 5 days (Fig. 5), but immunization with

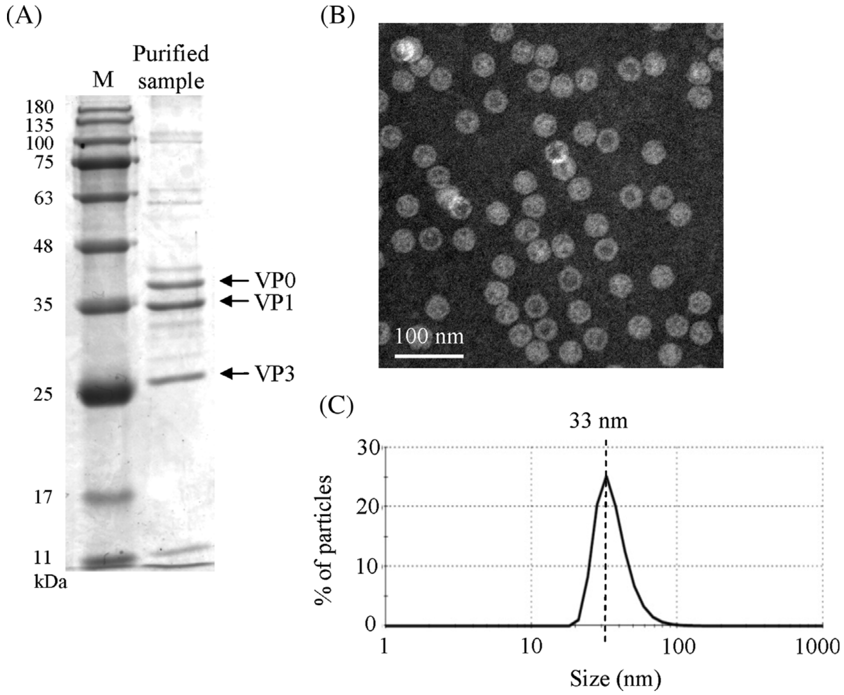

Figure 3. Characterization of purified EV71 VLP. (**A**) SDS–PAGE analysis. Arrows indicate the major capsid proteins VP0, VP1, and VP3. (**B**) Transmission electron micrograph. (**C**) Diameter distribution as determined by dynamic light scattering. The VLP was produced by infecting High Five$^{\mathrm{TM}}$ cells (MOI 0.1, 2×10^6 cells/mL) with BacF-P1-C3CD, harvested at 6 dpi and purified by TFF combined with hydroxyapatite chromatography and size exclusion chromatography.

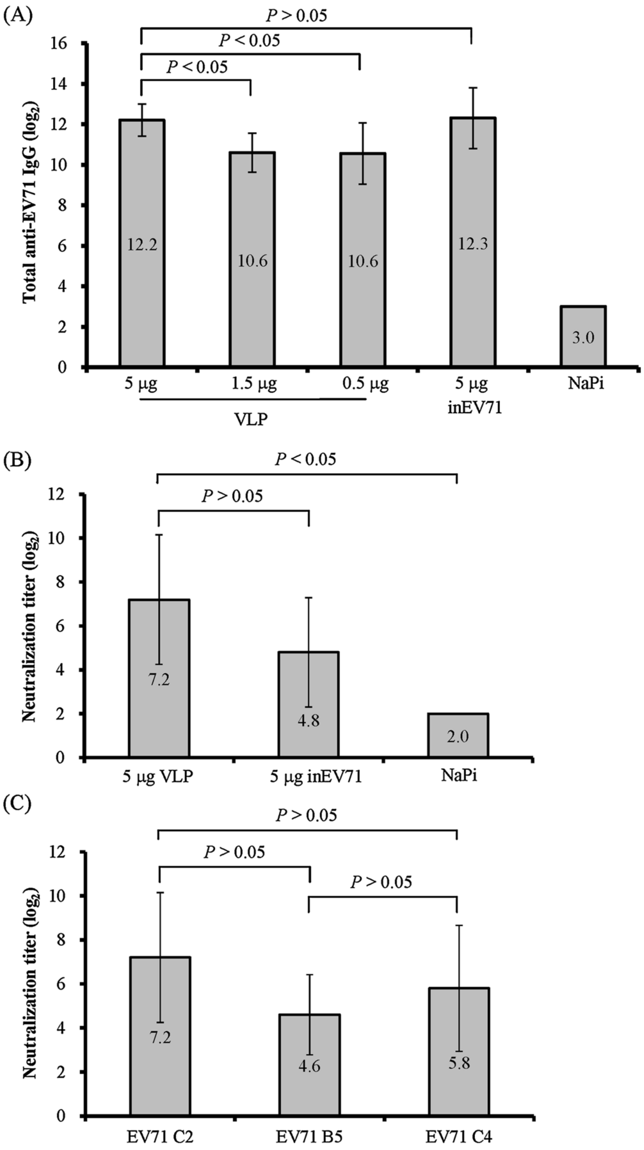

Figure 4. Humoral immune responses elicited by VLP in mouse models. (**A**) Total anti-EV71 IgG titers; (**B**) Neutralization titer against EV71 C2 genotype; (**C**) Cross-neutralizing antibody titer against different EV71 genotypes. The mice were immunized with VLP that was produced and purified as in Fig. 3.

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 coronavirus (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).

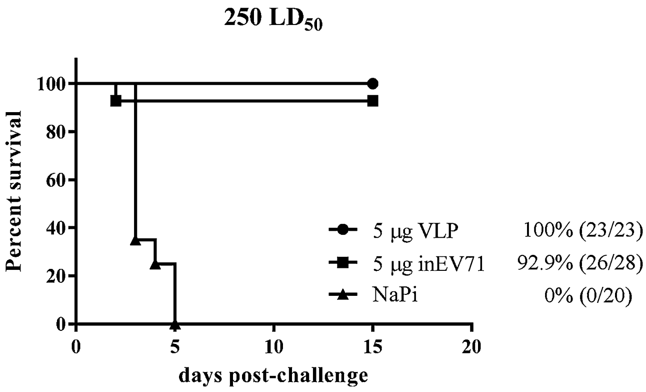

Figure 5. Protection against lethal EV71 virus challenge by VLP. The mice were immunized with VLP produced and purified as in Fig. 3 and mated. The neonatal mice were challenged with 250 LD$_{50}$ of EV71 MP4 virus (C2 genotype).

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 an *hr1* enhancer or an HS4 insulator appended to the 3′ 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). 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 3′ end of *P1* gene or co-expressed P1 and 3CD in tandem under the *polh* promoter, with an internal ribosomal entry site (IRES) element in between (Fig. S3). We also constructed a baculovirus that expressed P1-IRES-3CD under the *polh* promoter and expressed an additional P1 under the *p10* promoter (Fig. S3). However, none of these baculoviruses improved the VLP yield when compared with the original design (co-expressing P1 under the *polh* promoter and 3CD under the *p10* promoter).

The aforementioned recombinant baculoviruses were all constructed using the Bac-to-Bac® system, which lack the *polh* and otherwise have a complete genome. Therefore we adopted a different approach and selected the flashBAC GOLD™ system for the construction of recombinant baculoviruses (Fig. 1A). The flashBAC GOLD™ system was distinct from the Bac-to-Bac® system in that *v-cath* and *chiA* genes were deleted from 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 *ie1* (BacF-P1-I3CD) or *CMV* (BacF-P1-C3CD) promoter could we dramatically enhance the intracellular and extracellular VLP yield. In particular, infection of High Five™ cells with BacF-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 Pr55gag 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 BacF-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 BacF-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 5 μg 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$^{\text{TM}}$ 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$^{\text{TM}}$ cells and the *v-cath*$^-$/*chiA*$^-$ 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 virus 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.

We acknowledge the kind gift of antibodies and EV71 MP4 strain from Prof. Bor-Luen Chiang of National Taiwan University Hospital and Prof. Jen-Ren Wang (Center of Infectious Disease and Signaling Research, National Cheng Kung University Hospital, Tainan, Taiwan), respectively. The adjuvant was a gift from Dr. Erik B. Lindblad, Brenntag Biosector, Denmark. The authors gratefully acknowledge the financial support from the Ministry of Science and Technology (MOST 103-2622-E-007-024-CC1, NSC 102-2622-E-007-022-CC1, NSC 101-2628-E-007-009-MY3, NSC 100-2622-E-007-003-CC2), and Ministry of Health and Welfare (MOHW103-TDU-PB-211-122021, MOHW102-TDU-PB-111-NSC108, DOH99-TD-I-111-TM022, DOH98-DC-1013), Taiwan. The authors declare no competing financial interests.

References

Baek JO, Seo JW, Kim IH, Kim CH. 2011. Production and purification of human papillomavirus type 33 L1 virus-like particles from *Spodoptera frugiperda* 9 cells using two-step column chromatography. Protein Expr Purif 75:211–217.

Berger I, Fitzgerald DJ, Richmond TJ. 2004. Baculovirus expression system for heterologous multiprotein complexes. Nat Biotechnol 22:1583–1587.

Cheng A, Fung CP, Liu CC, Lin YT, Tsai HY, Chang SC, Chou AH, Chang JY, Jiang RH, Hsieh YC, Su IJ, Chong PC, Hsieh SM. 2013. A phase I, randomized, open-label study to evaluate the safety and immunogenicity of an enterovirus 71 vaccine. Vaccine 31:2471–2476.

Chung CY, Chen CY, Lin SY, Chung YC, Chiu HY, Chi WK, Lin YL, Chiang BL, Chen WJ, Hu YC. 2010. Enterovirus 71 virus-like particle vaccine: Improved production conditions for enhanced yield. Vaccine 28:6951–6957.

Chung YC, Ho MS, Wu JC, Chen WJ, Huang JH, Chou ST, Hu YC. 2008. Immunization with virus-like particles of enterovirus 71 elicits potent immune responses and protects mice against lethal challenge. Vaccine 26:1855–1862.

Chung YC, Huang JH, Lai CW, Sheng HC, Shih SR, Ho MS, Hu YC. 2006. Expression, purification and characterization of enterovirus 71 virus-like particles. World J Gastroenterol 12:921–927.

Cruz PE, Martins PC, Alves PM, Peixoto CC, Santos H, Moreira JL, Carrondo MJT. 1999. Proteolytic activity in infected and noninfected insect cells: Degradation of HIV-1 Pr55gag particles. Biotechnol Bioeng 65:133–143.

Fernandes F, Teixeira AP, Carinhas N, Carrondo MJ, Alves PM. 2013. Insect cells as a production platform of complex virus-like particles. Expert Rev Vaccines 12:225–236.

Gotoh T, Miyazaki Y, Sato W, Kikuchi K-I, Bentley WE. 2001. Proteolytic activity and recombinant protein production in virus-infected Sf-9 insect cell cultures supplemented with carboxyl and cysteine protease inhibitors. J Biosci Bioeng 92:248–255.

Hawtin RE, Zarkowska T, Arnold K, Thomas CJ, Gooday GW, King LA, Kuzio JA, Possee RD. 1997. Liquefaction of Autographa californica Nucleopolyhedrovirus-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. Virology 238:243–253.

Hitchman RB, Locanto E, Possee RD, King LA. 2011. Optimizing the baculovirus expression vector system. Methods 55:52–57.

Hitchman RB, Possee RD, Crombie AT, Chambers A, Ho K, Siaterli E, Lissina O, Sternard H, Novy R, Loomis K, Bird LE, Owens RJ, King LA. 2010a. Genetic modification of a baculovirus vector for increased expression in insect cells. Cell Biol Toxicol 26:57–68.

Hitchman RB, Possee RD, Siaterli E, Richards KS, Clayton AJ, Bird LE, Owens RJ, Carpentier DCJ, King FL, Danquah JO, Spink KG, King LA. 2010b. Improved expression of secreted and membrane-targeted proteins in insect cells. Biotechnol Appl Biochem 56:85–93.

Hom LG, Volkman LE. 2000. *Autographa californica* M nucleopolyhedrovirus chiA is required for processing of V-CATH. Virology 277:178–183.

Hu YC, Bentley WE. 1999. Enhancing yield of infectious bursal disease virus structural proteins in baculovirus expression systems: Focus on media, protease inhibitors, and dissolved oxygen. Biotechnol Prog 15:1065–1071.

Hu YC, Hsu TA, Huang JH, Ho MS, Ho YC. 2003. Formation of enterovirus-like particle aggregates by recombinant baculoviruses co-expressing P1 and 3CD in insect cells. Biotechnol Lett 25:919–925.

Hu YC, Yao K, Wu TY. 2008. Baculovirus as an expression and/or delivery vehicle for vaccine antigens. Expert Rev Vaccines 7:363–371.

Kaba SA, Salcedo AM, Wafula PO, Vlak JM, van Oers MM. 2004. Development of a chitinase and v-cathepsin negative bacmid for improved integrity of secreted recombinant proteins. J Virol Methods 122:113–118.

Kiener TK, Premanand B, Kwang J. 2013. Immune responses to baculovirus-displayed enterovirus 71 VP1 antigen. Expert Rev Vaccines 12:357–364.

Krammer F, Nakowitsch S, Messner P, Palmberger D, Ferko B, Grabherr R. 2010a. Swine-origin pandemic H1N1 influenza virus-like particles produced in insect cells induce hemagglutination inhibiting antibodies in BALB/c mice. Biotechnol J 5:17–23.

Krammer F, Schinko T, Palmberger D, Tauer C, Messner P, Grabherr R. 2010b. Trichoplusia ni cells (High Five$^{\text{(TM)}}$) are highly efficient for the production of influenza A virus-like particles: A comparison of two insect cell lines as production platforms for influenza vaccines. Mol Biotechnol 45:226–234.

Kung YA, Hung CT, Liu YC, Shih SR. 2014. Update on the development of enterovirus 71 vaccines. Expert Opin Biol Ther 14:1455–1464.

Li HY, Han JF, Qin CF, Chen R. 2013. Virus-like particles for enterovirus 71 produced from *Saccharomyces cerevisiae* potently elicits protective immune responses in mice. Vaccine 31:3281–3287.

Li YP, Liang ZL, Xia JL, Wu JY, Wang L, Song LF, Mao QY, Wen SQ, Huang RG, Hu YS, Yao X, Miao X, Wu X, Li RC, Wang JZ, Yin WD. 2014. Immunogenicity, safety, and immune persistence of a novel inactivated human enterovirus 71 vaccine: A phase II, randomized, double-blind, placebo-controlled trial. J Infect Dis 209:46–55.

Liang Z, Wang J. 2014. EV71 vaccine, an invaluable gift for children. Clin Transl Immunology 3:e28.

Lin SY, Chiu HY, Chiang BL, Hu YC. 2015. Development of EV71 virus-like particle production and purification processes. Vaccine. Epub ahead of print doi: 10.1016/j.vaccine.2015.04.077

Lin SY, Chung YC, Chiu HY, Chi WK, Chiang BL, Hu YC. 2014a. Evaluation of the stability of enterovirus 71 virus-like particle. J Biosci Bioeng 117:366–371.

Lin SY, Chung YC, Hu YC. 2014b. Update on baculovirus as an expression and/or delivery vehicle for vaccine antigens. Expert Rev Vaccines 13:1501–1521.

Lin YL, Hu YC, Liang CC, Lin SY, Liang YC, Yuan HP, Chiang BL. 2014c. Enterovirus-71 virus-like particles induce the activation and maturation of human monocyte-derived dendritic cells through TLR4 signaling. PLoS ONE 9: e111496.

Lin YL, Yu CI, Hu YC, Tsai TJ, Kuo YC, Chi WK, Lin AN, Chiang BL. 2012. Enterovirus type 71 neutralizing antibodies in the serum of macaque monkeys immunized with EV71 virus-like particles. Vaccine 30:1305–1312.

Liu CC, Guo MS, Lin FHY, Hsiao KN, Chang KHW, Chou AH, Wang YC, Chen YC, Yang CS, Chong PCS. 2011. Purification and characterization of enterovirus 71 viral particles produced from vero cells grown in a serum-free microcarrier bioreactor system. PLoS ONE 6:e20005.

Liu J, Wang W, Duo J, Hao Y, Ma C, Li W, Lin S, Gao X, Liu X, Xu Y. 2010. Combined peptides of human enterovirus 71 protect against virus infection in mice. Vaccine 28:7444–7451.

Lua LHL, Connors NK, Sainsbury F, Chuan YP, Wibowo N, Middelberg APJ. 2014. Bioengineering virus-like particles as vaccines. Biotechnol Bioeng 111:425–440.

Lyu K, Wang GC, He YL, Han JF, Ye Q, Qin CF, Chen R. 2015. Crystal structures of EV71 recombinant virus particles provide insights into vaccine design. J Biol Chem Epub ahead of print doi: 10.1074/jbc. M1114. 624536.

Mahonen AJ, Airenne KJ, Purola S, Peltomaa E, Kaikkonen MU, Riekkinen MS, Heikura T, Kinnunen K, Roschier MM, Wirth T, Yla-Herttuala S. 2007. Post-transcriptional regulatory element boosts baculovirus-mediated gene expression in vertebrate cells. J Biotechnol 131:1–8.

Maranga L, Rueda P, Antonis AF, Vela C, Langeveld JP, Casal JI, Carrondo MJ. 2002. Large scale production and downstream processing of a recombinant porcine parvovirus vaccine. Appl Microbiol Biotechnol 59:45–50.

Mortola E, Roy P. 2004. Efficient assembly and release of SARS coronavirus-like particles by a heterologous expression system. FEBS Lett 576:174–178.

Ohkawa T, Majima K, Maeda S. 1994. A cysteine protease encoded by the baculovirus Bombyx mori nuclear polyhedrosis virus. J Virol 68:6619–6625.

Plevka P, Perera R, Cardosa J, Kuhn RJ, Rossmann MG. 2012. Crystal structure of human enterovirus 71. Science 336:1274.

Possee RD, Thomas CJ, King LA. 1999. The use of baculovirus vectors for the production of membrane proteins in insect cells. Biochem Soc Trans 27:928–932.

Senger T, Schadlich L, Gissmann L, Muller M. 2009. Enhanced papillomavirus-like particle production in insect cells. Virology 388:344–353.

Sung LY, Chen CL, Lin SY, Li KC, Yeh CL, Chen GY, Lin CY, Hu YC. 2014. Efficient gene delivery into cell lines and stem cells using baculovirus. Nat Protocols 9:1882–1899.

Viswanathan P, Venkaiah B, Kumar MS, Rasheedi S, Vrati S, Bashyam MD, Hasnain SE. 2003. The homologous region sequence (hr1) of *Autographa californica* multinucleocapsid polyhedrosis virus can enhance transcription from non-baculoviral promoters in mammalian cells. J Biol Chem 278:52564–52571.

Vlak JM, Klinkenberg FA, Zaal KJ, Usmany M, Klinge-Roode EC, Geervliet JB, Roosien J, van Lent JW. 1988. Functional studies on the p10 gene of Autographa californica nuclear polyhedrosis virus using a recombinant expressing a p10-beta-galactosidase fusion gene. J Gen Virol 69:765–776.

Wagner JM, Pajerowski JD, Daniels CL, McHugh PM, Flynn JA, Balliet JW, Casimiro DR, Subramanian S. 2014. Enhanced production of chikungunya virus-like particles using a high-pH adapted Spodoptera frugiperda insect cell line. PLoS ONE 9:e94401.

Wang X, Li L, Ding S, Huang X, Zhang J, Yin J, Zhong J. 2009. Chicken HS4 insulator significantly improves baculovirus-mediated foreign gene expression in insect cells by modifying the structure of neighbouring chromatin in virus minichromosome. J Biotechnol 142:193–199.

Wang X, Peng W, Ren J, Hu Z, Xu J, Lou Z, Li X, Yin W, Shen X, Porta C, Walter TS, Evans G, Axford D, Owen R, Rowlands DJ, Wang J, Stuart DI, Fry EE, Rao Z. 2012. A sensor-adaptor mechanism for enterovirus uncoating from structures of EV71. Nat Struct Mol Biol 19:424–429.

Wang YF, Chou CT, Lei HY, Liu CC, Wang SM, Yan JJ, Su IJ, Wang JR, Yeh TM, Chen SH, Yu CK. 2004. A mouse-adapted enterovirus 71 strain causes neurological disease in mice after oral infection. J Virol 78:7916–7924.

Ye L, Lin JG, Sun YL, Bennouna S, Lo M, Wu QY, Bu ZG, Pulendran B, Compans RW, Yang CL. 2006. Ebola virus-like particles produced in insect cells exhibit dendritic cell stimulating activity and induce neutralizing antibodies. Virology 351:260–270.

Zhao L, Seth A, Wibowo N, Zhao CX, Mitter N, Yu C, Middelberg APJ. 2014. Nanoparticle vaccines. Vaccine 32:327–337.

Zhao M, Bai Y, Liu W, Xiao X, Huang Y, Cen S, Chan P, Sun X, Sheng W, Zeng Y. 2013. Immunization of N terminus of enterovirus 71 VP4 elicits cross-protective antibody responses. BMC Microbiol 13:287.

Zhu FC, Liang ZL, Li XL, Ge HM, Meng FY, Mao QY, Zhang YT, Hu YM, Zhang ZY, Li JX, Gao F, Chen QH, Zhu QY, Chu K, Wu X, Yao X, Guo HJ, Chen XQ, Liu P, Dong YY, Li FX, Shen XL, Wang JZ. 2013a. Immunogenicity and safety of an enterovirus 71 vaccine in healthy Chinese children and infants: A randomised, double-blind, placebo-controlled phase 2 clinical trial. The Lancet 381:1037–1045.

Zhu FC, Meng FY, Li JX, Li XL, Mao QY, Tao H, Zhang YT, Yao X, Chu K, Chen QH, Hu YM, Wu X, Liu P, Zhu LY, Gao F, Jin H, Chen YJ, Dong YY, Liang YC, Shi NM, Ge HM, Liu L, Chen SG, Ai X, Zhang ZY, Ji YG, Luo FJ, Chen XQ, Zhang Y, Zhu LW, Liang ZL, Shen XL. 2013b Efficacy, safety, and immunology of an inactivated alum-adjuvant enterovirus 71 vaccine in children in China: A multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. Lancet 381:2024–2032.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.