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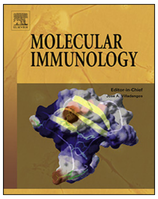

Induction of immune responses by a novel recombinant fusion protein of enterovirus A71 in BALB/c mice

Nipatha Issaro[a], Fenfang Wu[a], Lei Weng[a], Mi Zhou[a], Zhaoxiang Fang[a], Sisi Huang[a], Vinothkumar Rajamanickam[a], Min Liu[a], Haishan Tian[a], Xiaokun Li[b,c], Chao Jiang[a,b,c,*]

[a] School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, 325035, PR China
[b] College of Life and Environmental Sciences, Wenzhou University, Wenzhou, 325035, PR China
[c] Biomedicine Collaborative Innovation Center, Wenzhou University, Wenzhou, 325035, PR China

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ABSTRACT

Fusion protein technology is used in biotechnology and medical developments. In this study, recombinant fusion proteins from enterovirus A71 (EV-A71) subgenotype B5, Thailand were designed based two surface proteins (VP1 and VP2) and an internal protein (VP4), and named "VP0" (consisting of VP4-VP2) and "EV71" (consisting of VP4-VP2-VP1), respectively. The recombinant fusion proteins VP0 and EV71 were expressed in insect cells and successfully produced and secreted into the media. Both recombinant fusion proteins were shown to have immunogenic properties in BALB/c mice when formulated with Freund's complete/incomplete adjuvant (FA). Interestingly, EV71 formulated with FA- induced a level of IgG antibodies level similar to that induced by the recombinant protein VP1 formulated with FA (the positive control). Our results showed that VP1 alone is better at eliciting a strong cell-mediated immune response. Nontheless, EV71 formulated with FA was capable of inducing lymphocyte proliferation and increasing the cytokine-related mRNA expression levels of interferon-γ (IFN-γ), interleukin-2 (IL-2), and IL-10 in mice after immunization. Additionally, the number of $CD4^+$ and $CD8^+$ T lymphocyte cells after stimulation with purified EV71 in splenic cell culture showed highly specific $CD4^+$ and $CD8^+$ T-cell production. We suggest that EV71, which consists of VP4-VP2-VP1, could be used as the foundation for developing a novel recombinant fusion protein-based vaccine for EV-A71.

1. Introduction

Human enterovirus A71 (EV-A71) belongs to the family *Picornaviridae*, the genus *Enterovirus*, and species Enterovirus A, and can be divided into four main genotypes, A–D. EV-A71 is an important etiological agent of hand, foot, and mouth disease (HFMD) in Asia Pacific countries, where cases of HFMD tend to be associated with meningitis, encephalitis, and other severe symptoms, as well as death in children up to 5 years old (Ooi et al., 2010). The virus is transmitted from person to person via direct contact with virus-contaminated oral secretions and by the fecal–oral route (Tang and Holmes, 2017).

EV-A71 is a nonenveloped RNA virus that is 30–35 nm in diameter. It contains a single positive-sense ssRNA molecule and its genome comprises approximately 7500–8500 nucleotides, flanked by a 5′ non-coding region that is joined with a VPg peptide, and a 3′ polyadenine tail (Tuthill et al., 2010). Its single open reading frame codes for a protein that can be cleaved into three polyproteins: one structural protein (P1) and two non-structural proteins (P2 and P3). The P1 protein can be processed into four structural proteins (VP1–4) (Han et al., 2014) (Fig. 1). The VP1–3 proteins are on the outer capsid, whereas the VP4 protein is an inner capsid protein that is myristoylated at its N-terminus and is involved in the viral core, as well as interacting with the viral RNA.

Most research concerning EV-A71 has attempted to develop an EV-A71 vaccine, although an inactivated whole virus vaccine is available in China. Developing countries require a low-cost, high-purity EV-A71 vaccine that is easily stored and simple to prepare. To develop EV-A71 vaccine candidates, it is important to study the virus's structural proteins, which contain immunodominant epitopes. VP1 is used for classifying EV-A71 into subgenotypes (Oberste et al., 1999, 2003; Zhou

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Abbreviations: EV71, recombinant fusion protein consisting of VP4-VP2-VP1; VP0, recombinant fusion protein consisting of VP4-VP2

[*] Corresponding author at: School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, 325035, PR China.

E-mail addresses: nipatha@buu.ac.th (N. Issaro), 1241916342@qq.com (F. Wu), 1530267685@qq.com (L. Weng), 798119842@qq.com (M. Zhou), 952355581@qq.com (Z. Fang), 2267395956@qq.com (S. Huang), biovinoprabath@hotmail.com (V. Rajamanickam), 359923845@qq.com (M. Liu), 1063727451@qq.com (H. Tian), proflxk@163.com (X. Li), chaojiang10@hotmail.com (C. Jiang).

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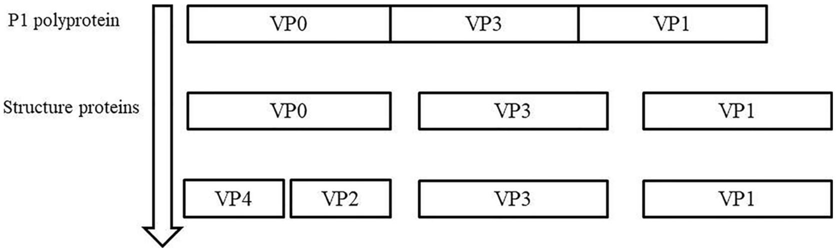

Fig. 1. Schematic representation of the P1 polyprotein processed into four structural proteins (VP1– 4).

et al., 2011) and has been used as an antiviral subunit vaccine (Wang et al., 2013; Zhang et al., 2014). VP2 induces cross-reactivity against coxsackievirus A16 and poliovirus, and mouse immune responses involving mainly single IFN-γ–secreting CD4$^+$ T cells (Tan et al., 2013; Xu et al., 2014). VP3 contains an EV-A71 neutralizing epitope (Kiener et al., 2014), and VP4 induces cross-reactivity against different EV-A71 genotypes and ensures the stability of the capsid (Chow et al., 1987; Zhao et al., 2013). Currently, there is a need for more specific vaccines to treat EV-A71, as well as a multivalent vaccine for different enterovirus 71 genotypes, and this has led to much effort involving the development of a vaccine based on recombinant proteins (Liu et al., 2010; Xu et al., 2016; Xue et al., 2017). The benefits of recombinant protein vaccines (including those involving proteins with T-cell epitopes) include the following: the immune response elicited by the protein structure [the conserved VP1, VP2, and VP4 proteins induce responses that are used for the typing of human enteroviruses (Bengs et al., 2015; Guo et al., 2017; Marttila et al., 2002; Oberste et al., 2003)]; safety (Zhou et al., 2015); cost effectiveness (Yee and Poh, 2016); and a high antibody response when used with Freund's complete/incomplete adjuvant (FA) in animal models (Chou et al., 2012; Zhou et al., 2015).

The use of fusion proteins represents a novel alternative approach to the development of an EV-A71 vaccine. The truncated proteins VP1-VP2-VP3 with linkers have been expressed in *E. coli* and elicited a high level of IgG antibody production, induced cellular immune responses in a BALB/c mouse (Xue et al., 2017), and provided effective protection for newborn mice against EV-A71 infection (Liu et al., 2010). One of the few studies concerning the immunogenicity of the recombinant protein VP4 found that the VP4 core sequence elicits cross-protective antibody responses in neonatal mice, and which could lead to the development of a multivalent vaccine aginst different enterovirus 71 genotypes (Zhao et al., 2013).

Baculovirus expression of recombinant proteins in insect cells has been used in studies of eukaryotic systems for over two decades (Aricescu et al., 2006). The baculovirus expression vector system (BEVS) in insect cells uses the strong polyhedrin promoter to drive target protein expression and produces high protein expression levels. Several notable advantages of the BEVS include easy construction of a recombinant baculovirus; potentially high expression levels of recombinant protein; post-translational modifications similar to those in mammalian cells, consequently retaining the biological activity of the original protein; and being an alternative method for many proteins that are difficult to express in an *E. coli* system (Aricescu et al., 2006; Elias et al., 2007). Use of the BEVS has become widespread for small- and large-scale recombinant protein expression, production, and biomedical research applications. Additionally, EV-A71 virus-like particles produced in insect cells have the potential to be used as EV-A71 vaccine candidates because they are highly immunogenic and generate a protective immune response after EV-A71 infection (Chung et al., 2008). To date, human vaccines based on the use of insect cells with the BEVS are commercially available, including human papilloma vaccine and influenza vaccine (Heinimäki et al., 2017).

In this study, we designed a recombinant fusion protein-based vaccine for EV-A71 using VP4-VP2 ("VP0") and VP4-VP2-VP1 ("EV71") proteins that were constructed and transfected into insect cells using the BEVS. Humoral and cell-mediated immune responses to the fusion protein-based vaccines were investigated in BALB/c mice.

2. Materials and methods

2.1. Ethics statement

All procedures were in accordance with the ethical standards established by the Institutional Animal Care and Use Committee at Wenzhou Medical University (WMU), China.

2.2. Reagents

An anti-His mouse monoclonal antibody was acquired from OriGene Technologies, Inc. (Rockville, MD, USA), and an anti-rabbit EV71 polyclonal antibody was purchased from Gentex Inc. (Irvine, CA, USA).

2.3. Construction of recombinant bacmid- VP0 and bacmid- EV71

The *EV71* gene of EV-A71 subgenotype B5, Thailand, listed in GenBank (accession No. MG912011), was synthesized by the Beijing Genomics Institute (BGI). The synthesized DNA fragment of *EV71* had 6× His-tagged tobacco etch virus (TEV) sites at the N-terminal region. It was cloned into the *Eco*RI and *Hin*dIII restriction enzyme sites of a pUC57 vector to create the pUC57-EV71 plasmid (BGI, Beijing, China). The *VP0* gene was then constructed from the pUC57-EV71 plasmid using the polymerase chain reaction (PCR) amplification method with the following specific primers: VP0-Forward (5′-AAAAGAATTCATGAA ACACCACCACCACC-3′) and VP0-Reverse (5′-AAAAAGCT TTTATTGG GTCACAGCTTGACG-3′). Fragments of the VP0 and EV71 constructs were cloned into a pFastBac vector to generate pFastBac-VP0 and pFastBac-EV71, and then transformed into DH10Bac™ competent *E. coli* cells. Recombinant bacmid-VP0 and bacmid-EV71 in DH10Bac™ *E. coli* cells were selected from white colonies, not blue (non-recombinant bacmid DNA), and identified by PCR using the following specific primers: M13 F (5′-GTTTTCCCAGTCACGAC-3′) and M13R (5′-CAGGAAA CAGCTATGAC-3′). The recombinant bacmid-VP0 and bacmid-EV71 samples were stored at 4 °C.

2.4. Recombinant baculovirus production in *Spodoptera frugiperda* (Sf9) cells

The recombinant bacmid DNA expressing either pFastBac-VP0 or pFastBac-EV71 were transfected into Sf9 cells using a transfection method described previously (Chen et al., 2016; Xue et al., 2014). In brief, the transfected cells were placed in six-well plates and incubated at 27.3 °C for 72 h, after which the cellular debris was removed by centrifugation at 1500 rpm for 5 min, followed by filtration (pore size = 0.22 μm). A supernatant P1 viral stock was created, 1–2% fetal bovine serum (FBS) was added, and the mixture solution was covered with foil and stored at 4 °C. The Sf9 cells were transfected with 200 μl of VP0-P1 or 100 μl of EV71-P1 viral stocks to produce the respective high-titer baculoviral stocks. The P3 viral stock determined the high productivity of the recombinant fusion proteins.

2.5. Production optimization and purification of VP0 and EV71 fusion proteins

To set the optimal conditions for VP0 and EV71 expression, multiplicity of infection (MOI), cell density, and harvest time were investigated by shaking 125-ml shaker flasks containing 25 ml of culture medium at 27.3 °C with shaking at 110 rpm. The harvest time was evaluated at a MOI of 10.0 pfu/cell and a cell density of 1.5×10^6 cells/ml, and cells were collected at 24, 48, 72, and 96 h post-infection. Both intracellular and extracellular proteins were detected using Western blot analysis.

The VP0 and EV71 fusion proteins were collected using a ProBond Purification System (Invitrogen, Carlsbad, CA, USA) under native conditions. The Sf9 cells and supernatant were harvested by centrifugation. The supernatant was then filtered and equilibrated in a binding buffer (5 mM imidazole in 50 mM of Tris-buffer [pH 7.5]) using an Amicon ultrafiltration system (Merck Millipore, Burlington, MA, USA). While the pelleted cells were dissolved in ice-cold binding buffer plus 1% phenylmethane-sulfonyl fluoride (Beyotime, Shanghai, China), then homogenized and sonicated on ice to extract the intracellular protein. The clear supernatant was then harvested via centrifugation. Each sample was mixed with Ni-chelating resin, incubated with gentle mixing for 16 h at 4 °C, and was then loaded onto a Ni-NTA column (Invitrogen) for gravity flow chromatography. The bound VP0 and EV71 fusion proteins were eluted with 250 mM imidazole.

The 6× His-tagged fusion protein was treated with 60 μl of resin-coupled TEV protease (Invitrogen) per 1 mg of protein to remove the 6× His-tagged peptides. The native cleavage products accumulated in the flow-through fraction. The native protein was separated by 12% (*v*/*v*) SDS-PAGE and was concentrated by ultrafiltration (molecular weight cutoff = 10–50). The protein concentrations were determined using a BCA kit (Beyotime).

2.6. Mice immunization and serum collection

Each group of four 6–8-week-old female BALB/c mice was immunized via intramuscular injection with one of the following: phosphate-buffered saline (PBS), Freund's complete/incomplete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) (as a negative control), 20 μg of VP1 protein produced from an *E. coli* system (Immune Tech, China) (as a positive control), 20 μg of purified VP0, or 20 μg of purified EV71 protein. Each group of mice was immunized as described previously (Wang et al., 2013). In brief, each protein (VP1, VP0, or EV71) was formulated with/without Freund's complete adjuvant (50% v/v; Sigma-Aldrich, USA) for the primary injection (on day 0) and was formulated with/without Freund's incomplete adjuvant (50% v/v; Sigma-Aldrich, USA) for booster injections (on days 14 and 28). Control mice were injected with PBS or FA following the same procedure. Serum samples were taken using a retro-orbital technique on days 0, 14, 28, and 42, and then stored at −80 °C until enzyme-linked immunosorbent assays (ELISAs) were conducted. Mice from each group were sacrificed on day 42 and their splenocytes were isolated to analyze cell-mediated immune responses.

2.7. Indirect ELISA for detection of murine IgG antibodies

The antigenicity of VP0 and EV71 was determined via the indirect ELISA method, as previously described (Wang et al., 2013). In brief, the ELISA plate was coated overnight with 100 μl each of purified VP0 (10 μg/ml), EV71 (10 μg/ml), and VP1 proteins (0.2 μg/ml) at 4 °C. For blocking, 200 μl of 5% skimmed milk in PBS containing 0.05% Tween-20 (PBST) was added to each well in the well plate and the plate was then incubated at 37 °C for 2 h. After washing the plate four times with PBST, 100 μl of 3200× diluted serum samples in PBST or PBST alone (control) was added to the wells in quadruplicate. The plate was then incubated for 1 h at 37 °C, and then washed four times. An aliquot of 100 μl of goat anti-mouse horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted in 1:1000 with PBST, was added to each well and the plate was incubated again for 1 h at 37 °C. After washing the plate, a substrate solution of 3,3,5,5-tetramethylbenzidine was added to each well and allowed to react for 30 min in the dark. The reaction was stopped using 2 M H_2SO_4, and the optical density at 450 nm was measured using an ELISA Microplate Reader (Molecular Devices, San Jose, CA, USA). The serum samples from all immunization groups were evaluated in quadruplicate and the results are presented as mean ± standard deviation (SD).

2.8. Splenocyte proliferation assay

All mice from the VP1-, VP0-, and EV71-immunized groups, as well as control groups, were sacrificed at week 6 post-immunization (14 days after the third immunization) and spleens from four mice in each group were harvested under aseptic conditions. Lymphocytes were separated using an EZ-Sep™ mouse lymphocyte separation kit following the manufacture's protocol (Dakewe Biotech, Shenzhen, China). Mouse splenocytes (1×10^6 cells per well) were maintained in a 96-well plate and stimulated with recombinant VP1 (20 μg/ml), VP0 (20 μg/ml), or EV71 (20 μg/ml), Concanavalin A (Con A; positive control) (10 μg/ml) (Sigma-Aldrich) and medium alone (negative control). An MTT assay was performed as previously described (Kumar et al., 2010). The optical density at 570 nm (OD_{570}) was evaluated in an ELISA Microplate Reader (Molecular Devices). The stimulation index (SI) was then calculated using the formula: SI = (OD_{570} value of stimulated sample)/(OD_{570} value of unstimulated sample).

2.9. Analysis of cytokine-related mRNA expression in splenocytes

Mouse splenocytes (5×10^5/ml) from each immunization group were cultured in 24-well plates for 48 h at 37 °C in the presence of 5% CO_2, with and without treatment with 2 μg/ml EV71, VP0, or VP1 protein. Total RNA from each immunization group was extracted using a RNAiso Plus Kit according to the manufacturer's protocol (Takara, Otsu, Japan). qRT-PCR was performed in triplicate under universal cycle conditions (30 s at 95 °C, 40 cycles of 5 s at 95 °C, 5 s at 56–60 °C). In each immunization group, the levels of expression of *IL-2, IL-4, IL-10*, and *IFN*-γ genes were assessed, along with those of the glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*; the endogenous control). The qRT-PCR primers were obtained from a previous study (Overbergh et al., 1999), and the relative mRNA expression level of each target gene was evaluated using the $2^{-\Delta\Delta Ct}$ method (Zhu et al., 2012).

2.10. Flow cytometry analysis of CD4$^+$ and CD8$^+$ T cells in splenocytes

Mouse splenocytes (1×10^6/ml) were seeded in 24-well plates and stimulated with 2 μg/ml VP0, VP1, or EV71 protein for 72 h at 37 °C in the presence of 5% CO_2. The cells were washed with 0.1 M PBS and the pellets were suspended in 200 μl of 0.1 M PBS. Anti-CD3-PerCPCy5.5, anti-CD4-FITC, and anti-CD8-PE antibodies (Biolegend, San Diego, CA, USA) were added and the mixtures were placed on ice in the dark for 30 min, after which the cells were washed and then fixed with staining buffer (5% [FBS] plus 0.1 sodium azide in 0.1 M PBS). The data for the CD3$^+$, CD4$^+$, and CD8$^+$ T cells were evaluated via flow cytometry (BD Bioscience, San Jose, CA, USA). The data presented are from three independent trials.

2.11. Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used to analyze all experimental data and create figures. The Bonferroni method was used to perform individual comparisons between groups.

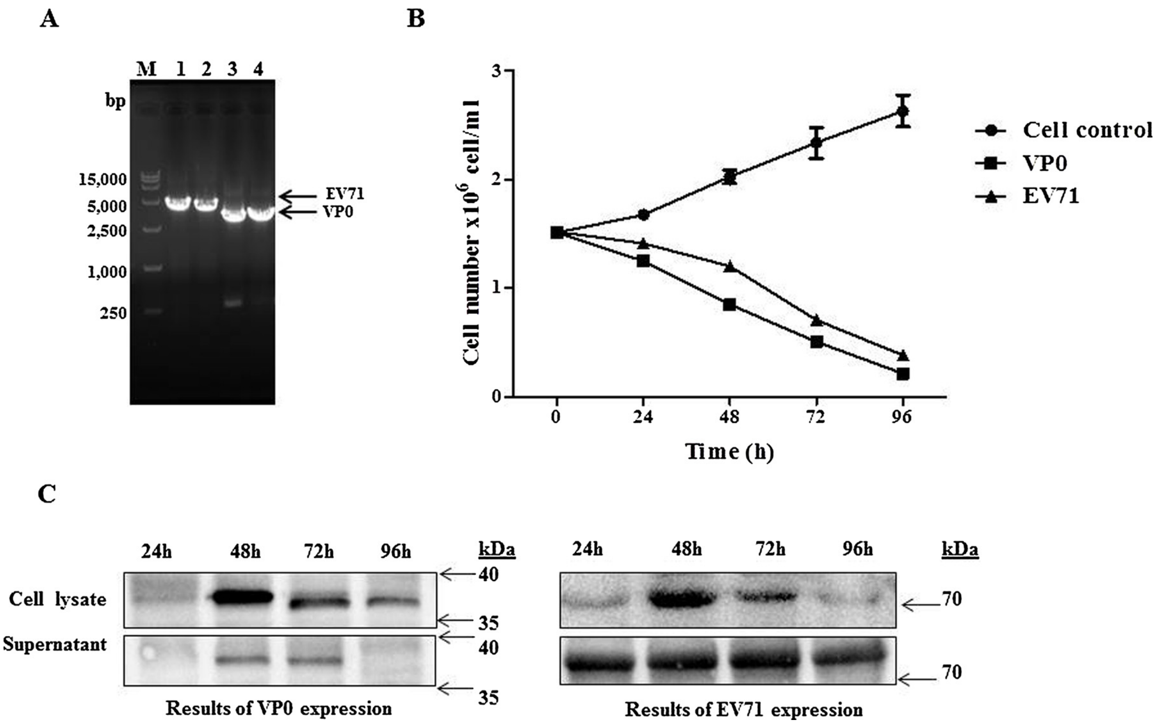

Fig. 2. Agarose gel electrophoresis analysis of pFastBac-EV71 and pFastBac-VP0, a long with optimized protein expression. (A) PCR product of pFastBac-EV71 and pFastBac-VP0 amplification analysis using M13 F and M13R primers. (B) Optimization of VP0 and EV71 expressed in insect cells at a MOI of 10 pfu/cell. (C) Western blot analysis of VP0 and EV71 expression at different post-infection time points (MOI = 10 pfu/cell) in cell lysates and medium supernatant.

3. Results

3.1. Construction of bacmid-VP0 and bacmid-EV71 fusion genes

The constructed recombinant plasmids of N-terminal 6× His-tagged-TEV *VP0* and *EV71* fusion genes were selected from DH10Bac™ *E. coli* cells. The positive colonies were extracted to generate PCR products 4250 bp in length for the recombinant bacmid-EV71 and 3368 bp in length for the recombinant bacmid-VP0 (Fig. 2A).

3.2. Optimized protein expression and purification

To obtain the optimal conditions for recombinant VP0 and EV71 expression, the expression levels in supernatant and cell lysates were monitored by Western blot analysis at various post-infection time points (using a MOI of 10 pfu/cell). The VP0 and EV71 bands were detected using an anti-His mouse monoclonal antibody. Western blot analysis indicated that the sizes of bands were stable and, as expected, approximately 38 and 72 kDa, respectively. In addition, the number of Sf9 cells had significantly decreased after infection, whereas they increased continuously when used as the control (Fig. 2B). VP0 was detected in the supernatant around 48 h post-infection and reach maximum expression in the cell lysate at 96 h. Interestingly, EV71 in both the supernatant and cell lysate reached maximum expression at 96 h (Fig. 2C). The yields of VP0 and EV71 in the supernatants were 0.72 and 3.12 mg, respectively. Both recombinant fusion proteins from the supernatant and cell lysate were purified using a Ni-NTA column and found to be approximately 38 and 72 kDa, respectively (Fig. 3A and C). Subsequently, Western blot analysis revealed the native proteins after cleavage by the TEV enzyme, and the polyclonal anti-rabbit EV-A71 antibody detected VP0 and EV71 and showed they were 35 and 69 kDa, respectively (Fig. 3B and D).

3.3. Humoral immune response

ELISA was used to evaluate the levels of anti-VP0, anti-VP1, anti-EV71, and IgG in the sera of the immunized mice. The IgG level elicited by each protein with FA increased continuously and reached a maximum at week 6 after the final immunization whereas, as expected, IgG was not elicited in the control mice vaccinated with PBS or FA. In contrast, all three protein regimens induced a significantly higher level of IgG at week 6 compared to that induced in the PBS group (negative control) ($P < 0.001$; Fig. 4). In addition, the data demonstrated that the IgG level induced by EV71 with FA at week 6 was comparable to that induced by VP1 with FA (positive control; $P > 0.05$). VP0, however, produced lower levels of IgG antibody than did VP1 or EV71 ($P < 0.001$). These results demonstrate that the presence of VP1 or EV71, which is an efficient subunit vaccine, induces higher level of antibodies than does VP0.

3.4. Splenic lymphocyte proliferation

The proliferation of splenocytes stimulated by each protein or Con A (positive control) was determined using MTT assays. Compared with the splenocytes from mice in the PBS or FA control groups, the splenocytes from mice in the VP0, VP1, and EV71 with FA groups proliferated significantly after stimulation with the corresponding antigens ($P < 0.01$–0.001; Fig. 5). Furthermore, the splenocyte proliferation level stimulated by VP1 alone, which was the positive control for mice immunization in splenic lymphocytes, was comparatively higher than that stimulated by both EV71 and VP0 ($P < 0.001$). The lymphocyte proliferation in the tread was observed with SI values; VP1 + FA > VP1 > EV71 + FA > EV71 > VP0 + FA > VP0 ($P < 0.05$–0.0001).

3.5. Cytokine-related mRNA expression in splenocytes

To quantitate cytokine-related mRNA expression levels, we used qRT-PCR to detect the mRNA expression levels of IL-2, IL-4, IL-10, and

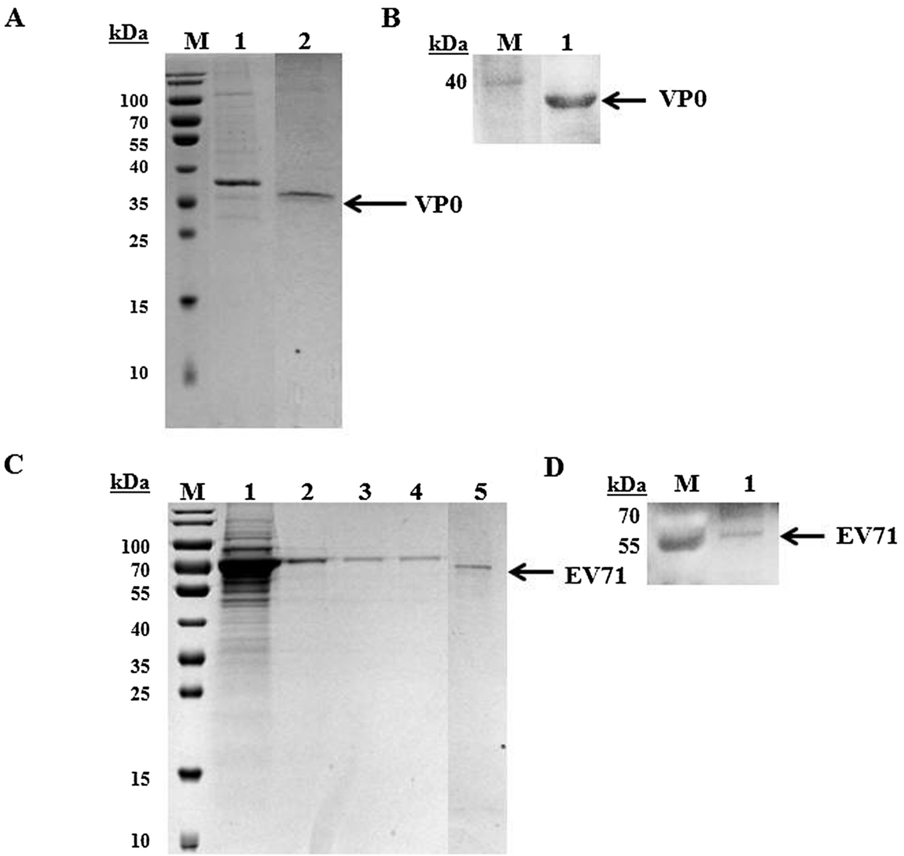

Fig. 3. Purification of VP0 and EV71. (A) SDS-PAGE analysis of VP0 purification before and after TEV digestion: lane M, protein ladder; lane 1, 250 mM imidazole-eluted fraction; lane 2, after TEV digestion. (B) Western blot analysis of VP0: lane M, protein ladder; lane 1, after TEV digestion. (C) SDS-PAGE analysis of EV71 purification before and after TEV digestion: lane M, protein ladder; lane 1, flow-through; lanes 2–4, 250 mM imidazole-eluted fraction; lane 5, after TEV digestion. (D) Western blot analysis of EV71: lane M, protein ladder; lane 1, after TEV digestion.

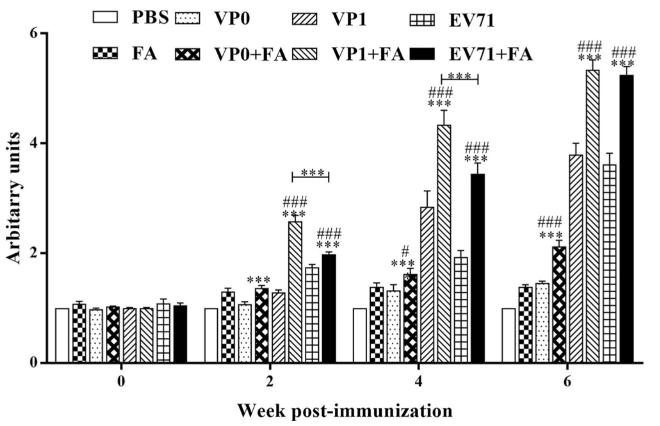

Fig. 4. Humoral immune responses to each protein in immunized mice and control group as found by ELISA. IgG antibodies induced by the negative controls (PBS and Freund's complete/incomplete adjuvant [FA]), the tested protein (VP0, VP1 [positive control], or EV71), and the tested protein with FA in BALB/c mice. Sera were collected at weeks 0, 2, 4, and 6 after immunization. The IgG antibody levels are represented in arbitrary units as mean ± SD ($n = 4$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Asterisks (*) and hashes ($^{\#}$) above the bars indicate statistically significant differences among the PBS and FA control groups, respectively.

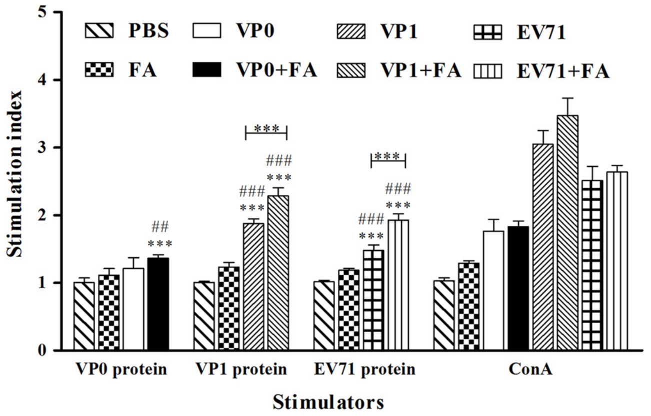

Fig. 5. Splenocyte proliferation responses in immunized mice from each immunization group were assessed 14 days after the final immunization. Lymphocyte proliferation was detected after stimulation with VP0, VP1, EV71, or Con A for 72 h. Data are presented as mean ± SD ($n = 4$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Asterisks (*) and hashes ($^{\#}$) above the bars indicate statistically significant differences among the PBS and Freund's complete/incomplete adjuvants control groups, respectively.

IFN-γ in mouse splenocytes after stimulation for 48 h with and without VP0, VP1, and EV71. Prior immunization with VP1 + FA (positive control) and EV71 + FA significantly ($P < 0.001$) up-regulated the mRNA expression of IL-2, IFN-γ, and IL-10 in comparison to the effects of PBS or FA immunization (Fig. 6A and B). The mRNA expression level of IL-10 in mice immunized with VP1 + FA (positive control) was significantly higher than that in mice immunized with EV71 + FA and VP0 + FA ($P < 0.05$–0.001), whereas VP0 + FA immunization significantly ($P < 0.001$) up-regulated the mRNA expression of IL-10, in comparison to PBS immunization. Meanwhile, the levels of IL-4 in all protein-immunized groups were similar to those in the PBS and FA control groups ($P > 0.05$; Fig. 6A). The recombinant VP0 fusion protein, however, did not significantly induce mRNA expression of IL-2, IL-4, or IFN-γ ($P > 0.05$).

3.6. Results of $CD4^{+}$ and $CD8^{+}$ t cell counts

To analyze the T lymphocyte subsets in mice after immunization, we

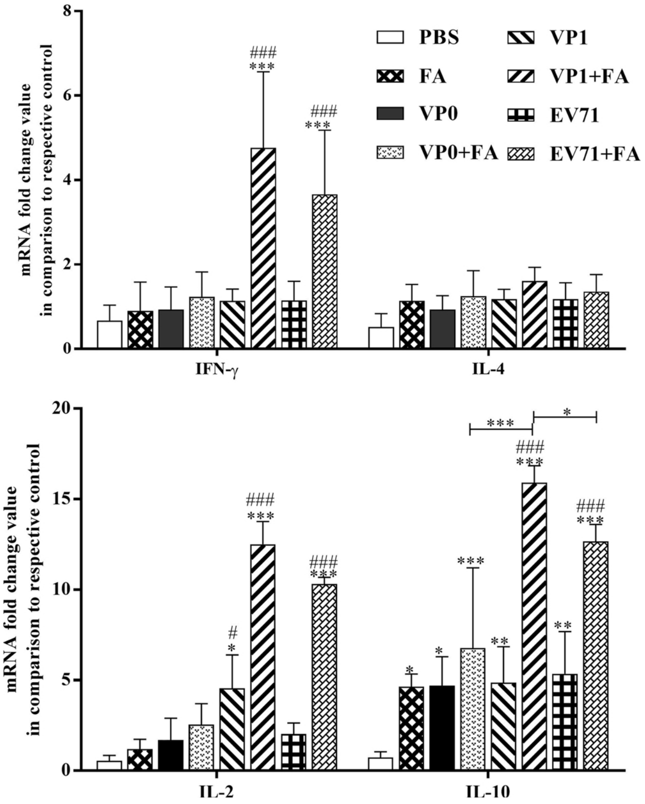

Fig. 6. Cytokine mRNA expression levels of mice splenocytes. (A, B) mRNA transcription levels of IL-2, IL-4, IL-10, and IFN-γ were evaluated using qRT-PCR with murine splenocytes stimulated with VP0, VP1, and EV71. The values of the relative fold change of each group of mice are expressed as mean ± SD ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Asterisks (*) and hashes ($^{\#}$) above the bars indicate statistically significant differences among the PBS and Freund's complete/incomplete adjuvant control groups, respectively.

used flow cytometry to study the induction of immune response by determining the percentages of CD4^{+} and CD8^{+} T cells in the immunized mice after the splenic T cells were stimulated with each protein for 72 h. Percentage of CD3^{+}CD8^{+}CD4^{-} T cells and CD3^{+}CD4^{+}CD8^{-} T cells were significantly increased in the VP0 + FA, VP1, VP1 + FA, EV71, and EV71 + FA groups compared to those in the PBS and FA groups ($P < 0.01$–0.001; Fig. 7A–C) with the highest increase in percentage observed in EV71 + FA. There were no significant differences in the percentages of CD4^{+} and CD8^{+} T cells between the PBS and FA control groups ($P > 0.05$).

4. Discussion

The VP1 protein has been considered an antiviral subunit vaccine (Wang et al., 2013; Zhang et al., 2014; Zhou et al., 2015) and has been the target of several initial studies concerning EV-A71 vaccine development (Xu et al., 2016; Xue et al., 2017). In the present study, we attempted to develop an EV-A71 vaccine using two fusion proteins, VP0 (VP4-VP2) and EV71 (VP4-VP2-VP1), which include important immunodominant epitopes of the VP1, VP2, and VP4 proteins. Protein expression was performed using the baculovirus expression system via transfection of Sf9 cells. VP0 and EV71 were secreted effectively and efficiently into the media, resulting in easy purification under native conditions.

Immunization of BALB/c mice with VP0 + FA and EV71 + FA induced a strong humoral immune response. Interestingly, EV71 + FA elicited a high level of antibody production, comparable to that induced by VP1 + FA (which was used as a positive control). In addition, all immunizations using protein + FA induced an IgG level higher than that caused by immunization using protein alone, showing that the presence of an adjuvant is important for eliciting an effective immune response in mice. The immune system requires either a stimulus to increase the ability of the protein vaccine to stimulate an immune response or an enhancer to induce an immune response in animal models (Babu et al., 2008).

With respect to estimating the overall cell-mediated immunity after stimulation with each fusion protein via splenocyte proliferation assay, we found that the recombinant fusion proteins efficiently stimulated cellular proliferation responses in the VP0 + FA, EV71, and EV71 + FA immunized groups. In addition, we used qRT-PCR assays to study the basic immune function of cytokine-related mRNA expression levels (IL2, IL4, IL10, and IFN-γ) directly affecting the development of effector T- helper (Th) cells in the splenic lymphocyte. Cytokine-related mRNA for IL-2, IFN-γ, and IL-10 was expressed at significantly higher levels in mice immunized with EV71 + FA and VP1 + FA than in mice in the PBS control group, FA control group, or protein alone group. In past studies, qRT-PCR has allow the quantification of cytokines-related mRNA expression levels from mouse spleens after immunization (Gupta et al., 2016; Overbergh et al., 1999; Liu et al., 2017; Won and John, 2017) and has also been used to measure relative levels of IFN-γ, IL-2, IL-4, IL-10, and TNF-α mRNA in rabbits (Espino and Rivera, 2010; Godornes et al., 2007). This method has been useful in the study of basic immune functions in animal models, and eventually in humans, because it is a rapid, precise, and relatively inexpensive way to quantify cytokine responses (Abdalla et al., 2003; Overbergh et al., 1999). IL-2, IL-4, IL-10, and IFN-γ are major cytokines that have been shown to play crucial roles in immune responses to EV-A71 (Chung et al., 2008; Zhou et al., 2015). Interestingly, although IL-4 in particular is produced by many cell types and is involved in promoting Th2 cell differentiation (Zhu, 2015), IL-4-related mRNA expression in mouse splenocytes was observed to be low in current study.

The activation of antigen-specific CD4^{+} and CD8^{+} T cells is an early strategy by which to activate cell-mediated immunity (Liu et al., 2017). CD4 and CD8 molecules are major surface coreceptors. CD4^{+} cells recognize an antigen on the antigen-presenting cell (APC) surface of major histocompatibility complex (MHC) class II molecules and secrete cytokines for effector T helper (Th) cells (Th1/Th2). Conversely, CD8^{+} cells recognize an antigen on the APC surface of MHC class I molecules and form effector cytotoxic T lymphocytes (Broere et al., 2011). The foreign antigen-MHC class I molecule complex of antigen-specific CD8^{+} cells can exert a direct cytotoxic effect and eliminate the infected/malignant cells (Foster et al., 2010). Furthermore, the detection of both CD4^{+} and CD8^{+} T cells that has been recognized as the most meaningful parameter for evaluating the immune response in the design of protein-based vaccines for enterovirus EV-A71 (Yee and Poh, 2018). The proliferation of CD4^{+} and CD8^{+} T lymphocyte cells was detected using flow cytometry on splenocyte cultures after stimulation with each fusion protein. The number of CD4^{+} and CD8^{+} T lymphocyte cells increased in all groups immunized with protein formulated with FA, in comparison to groups immunized with protein alone, PBS, or only FA, indicating that the fusion proteins may have enhanced the antigen-specific immune response of mice after immunization and activated the immune system via proteins formulated with FA, as well as upregulating IL-2, IL-10, and IFN-γ mRNA, which mediate the differentiation of Th1/Th2 cells.

This study demonstrated that the recombinant fusion protein EV71, comprising portions of the VP1, VP2, and VP4 proteins, induced highly specific responses by CD4^{+} and CD8^{+} T cell in splenic cell culture and thus has the potential to induce immune responses in a murine model. Conversely, the recombinant fusion protein VP0, which does not contain the VP1 protein, produced lower levels of IgG antibodies and a lower cell-mediated immune response in a mouse model. The presence of fewer CD4^{+} and CD8^{+} T lymphocyte cells in relation to immunization with VP0 than with EV71 may be due to the lower immunogenicity of the hollow core in VP0 (Liu et al., 2011; Wang et al.,

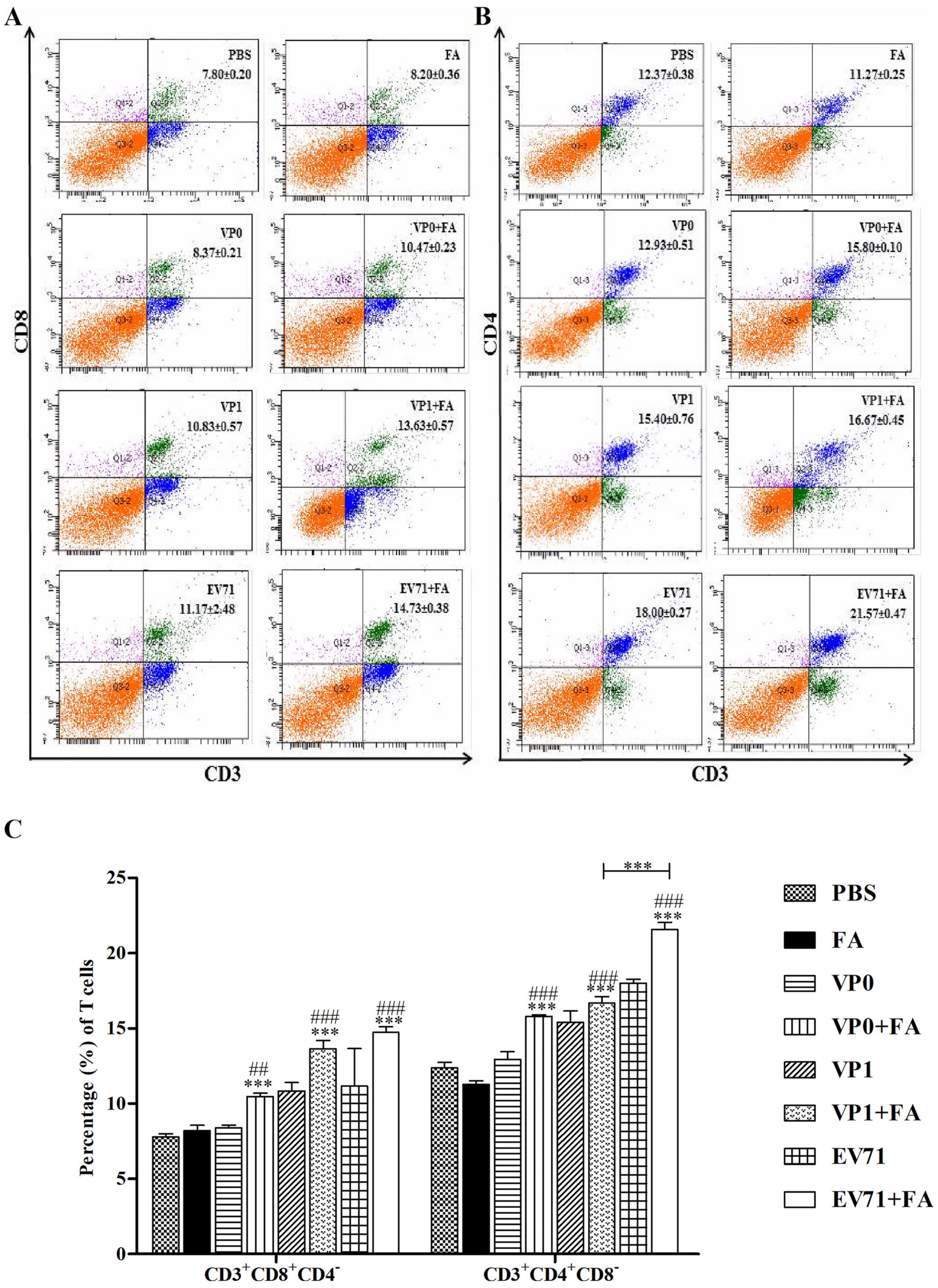

Fig. 7. Splenic T-cell populations in immunized mice from the PBS control group, Freund's complete/incomplete adjuvant (FA) control group, and each protein group formulated with and without FA that was stimulated with VP0, VP1, and EV71, as determined by flow cytometry. (A) Representative flow cytometry images of CD3$^+$CD8$^+$CD4- T lymphocytes in murine spleen cells. (B) Representative flow cytometry images of CD3$^+$CD4$^+$CD8- T lymphocytes in murine spleen cells. (C) Percentages of CD3$^+$CD8$^+$CD4- and CD3$^+$CD4$^+$CD8- T lymphocytes in murine spleen cells. The results are presented as mean ± SD ($n = 3$); $^{**}P < 0.01$, $^{***}P < 0.001$. Asterisks (*) and hashes ($^\#$) above the bars indicate statistically significant difference differences among the PBS and FA control groups, respectively.

2012). The effects of murine immunogenicity were due to the VP1 protein in EV71. VP1 is therefore a useful tool in the design of a fusion protein-based vaccine for EV-A71.

We have demonstrated that the recombinant fusion protein EV71 formulated with FA induced a greater immune response than did the fusion protein alone. To our knowledge, this is the first report of this phenomenon. Our study shows that EV71 could be the foundation for an immunogenicity study in a BALB/c mouse model of a novel recombinant fusion protein developed from VP1, VP2, and VP4 proteins. Finally, the protective immune response against EV-A71 infection that is induced by a fusion protein vaccination should be evaluated, and both neutralization research and research concerning different genotypes of enterovirus 71 should be performed.

Competing interest statement

The authors have no commercial conflicts of interest to declare.

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References

Abdalla, A.O., Kiaii, S., Hansson, L., Rossmann, E.D., Jeddi-Tehrani, M., Shokri, F., Osterborg, A., Mellstedt, H., Rabbani, H., 2003. Kinetics of cytokine gene expression in human CD4$^+$ and CD8$^+$ T-lymphocyte subsets using quantitative real-time PCR. Scand. J. Immunol. 58, 601–606.

Aricescu, A.R., Assenberg, R., Bill, R.M., Busso, D., Chang, V., Davis, S., Dubrovsky, A., Gustafsson, L., Hedfalk, K., Heinemann, U., 2006. Eukaryotic expression: developments for structural proteomics. Acta Crystallogr. D Biol. Crystallogr. 62, 1114–1124.

Babu, J.P., Pattnaik, P., Gupta, N., Shrivastava, A., Khan, M., Rao, P.V., 2008. Immunogenicity of a recombinant envelope domain III protein of dengue virus type-4 with various adjuvants in mice. Vaccine 26, 4655–4663.

Bengs, S., Marttila, J., Susi, P., Ilonen, J., 2015. Elicitation of T-cell responses by structural and non-structural proteins of coxsackievirus B4. J. Gen. Virol. 96, 322–330.

Broere, F., Apasov, S.G., Sitkovsky, M.V., van Eden, W., 2011. A2 T cell subsets and T cell mediated immunity. In: Nijkamp, F.P., Parnham, M.J. (Eds.), Principles of Immunopharmacology. Birkhäuser Basel, pp. 15–27.

Chen, N., Ma, J., Zhao, Y., Wu, M., Yang, H., Gong, W., Chao, J., Li, X., 2016. Expression of functional recombinant human fibroblast growth factor 8b and its protective effects on MPP(+)-lesioned PC12 cells. Appl. Microbiol. Biotechnol. 100, 625–635.

Chou, A.H., Liu, C.C., Chang, J.Y., Lien, S.P., Guo, M.S., Tasi, H.P., Hsiao, K.N., Liu, S.J., Sia, C., Wu, S.C., 2012. Immunological evaluation and comparison of different EV71 vaccine candidates. Clin. Dev. Immunol. 2012.

Chow, M., Newman, J.F., Filman, D., Hogle, J.M., Rowlands, D.J., Brown, F., 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature 327, 482–486.

Chung, Y.C., Ho, M.S., Wu, J.C., Chen, W.J., Huang, J.H., Chou, S.T., Hu, Y.C., 2008. Immunization with virus-like particles of enterovirus 71 elicits potent immune responses and protects mice against lethal challenge. Vaccine 26, 1855–1862.

Elias, C.B., Jardin, B., Kamen, A., 2007. Recombinant protein production in large-scale agitated bioreactors using the baculovirus expression vector system. Methods Mol. Biol. 388, 225–246.

Espino, A.M., Rivera, F., 2010. Quantitation of cytokine mRNA by real-time RT-PCR during a vaccination trial in a rabbit model of fascioliasis. Vet. Parasitol. 169, 82–92.

Foster, S., Duvall, C.L., Crownover, E.F., Hoffman, A.S., Stayton, P.S., 2010. Intracellular delivery of a protein antigen with an endosomal-releasing polymer enhances CD8 T-cell production and prophylactic vaccine efficacy. Bioconjug. Chem. 21, 2205–2212.

Godornes, C., Leader, B.T., Molini, B.J., Centurion-Lara, A., Lukehart, S.A., 2007. Quantitation of rabbit cytokine mRNA by real-time RT-PCR. Cytokine 38, 1–7.

Guo, H., Gao, L., Guo, J., Hou, X., Yan, W., 2017. Molecular assay and genotyping of childhood enterovirus infection in central nervous system. Int. J. Clin. Exp. Pathol. 10, 523–529.

Gupta, J., Misra, S., Misra-Bhattacharya, S., 2016. Immunization with Brugia malayi myosin as heterologous DNA prime protein boost induces protective immunity against B. malayi infection in Mastomys coucha. PLoS One 11, e0164991.

Han, X., Ying, X.L., Zhou, S.L., Han, T., Huang, H., Jin, Q., Yang, F., Sun, Q.Y., Sun, X.X., 2014. Characterization of the enterovirus 71 P1 polyprotein expressed in *Pichia pastor* as a candidate vaccine. Hum. Vaccin. Immunother. 10, 2220–2226.

Heinimäki, S., Tamminen, K., Malm, M., Vesikari, T., Blazevic, V., 2017. Live baculovirus acts as a strong B and T cell adjuvant for monomeric and oligomeric protein antigens. Virology. 511, 114–122.

Kiener, T.K., Jia, Q., Meng, T., Chow, V.T., Kwang, J., 2014. A novel universal neutralizing monoclonal antibody against enterovirus 71 that targets the highly conserved "knob" region of VP3 protein. PLoS Negl. Trop. Dis. 8, e2895.

Kumar, H.M., Singh, P.P., Qazi, N.A., Srinivas, J., Malik, F., Sidiq, T., Gupta, A., Khajuria, A., Suri, K.A., Satti, N.K., Qazi, G.N., 2010. Development of novel lipidated analogs of picroside as vaccine adjuvants: acylated analogs of picroside-II elicit strong Th1 and Th2 response to ovalbumin in mice. Vaccine 28, 8327–8337.

Liu, J.N., Wang, W., Duo, J.Y., Hao, Y., Ma, C.M., Li, W.B., Lin, S.Z., Gao, X.Z., Liu, X.L., Xu, Y.F., Xu, W.B., Qin, C., Zhang, L.F., 2010. Combined peptides of human enterovirus 71 protect against virus infection in mice. Vaccine 28, 7444–7451.

Liu, C.C., Guo, M.S., Lin, F.H., Hsiao, K.N., Chang, K.H., Chou, A.H., Wang, Y.C., Chen, Y.C., Yang, C.S., Chong, P.C., 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, C., Shen, Q., Zheng, W., Lv, Y., Chen, X., Li, X., Zhu, Q., Guo, X., Ge, R., Li, C., 2017. Poly(anhydride) nanoparticles act as effective adjuvants to elicit a persistent immune response. RSC Adv. 7 55495–55470.

Marttila, J., Hyoty, H., Vilja, P., Harkonen, T., Alho, A., Roivainen, M., Hyypia, T., Ilonen, J., 2002. T cell epitopes in coxsackievirus B4 structural proteins concentrate in regions conserved between enteroviruses. Virology 293, 217–224.

Oberste, M.S., Maher, K., Kilpatrick, D.R., Flemister, M.R., Brown, B.A., Pallansch, M.A., 1999. Typing of human enteroviruses by partial sequencing of VP1. J. Clin. Microbiol. 37, 1288–1293.

Oberste, M.S., Nix, W.A., Maher, K., Pallansch, M.A., 2003. Improved molecular identification of enteroviruses by RT-PCR and amplicon sequencing. J. Clin. Virol. 26, 375–377.

Ooi, M.H., Wong, S.C., Lewthwaite, P., Cardosa, M.J., Solomon, T., 2010. Clinical features, diagnosis, and management of enterovirus 71. Lancet Neurol. 9, 1097–1105.

Overbergh, L., Valckx, D., Waer, M., Mathieu, C., 1999. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. Cytokine 11, 305–312.

Tan, S., Tan, X., Sun, X., Lu, G., Chen, C.C., Yan, J., Liu, J., Xu, W., Gao, G.F., 2013. VP2 dominated CD4$^+$ T cell responses against enterovirus 71 and cross-reactivity against coxsackievirus A16 and polioviruses in a healthy population. J. Immunol. 191, 1637–1647.

Tang, J.W., Holmes, C.W., 2017. Acute and chronic disease caused by enteroviruses. Virulence 8, 1062–1065.

Tuthill, T.J., Groppelli, E., Hogle, J.M., Rowlands, D.J., 2010. Picornaviruses. Curr. Top. Microbiol. Immunol. 343, 43–89.

Wang, X., Peng, W., Ren, J., Hu, Z., Xu, J., Lou, Z., Li, X., Yin, W., Shen, X., Porta, C., 2012. A sensor-adaptor mechanism for enterovirus uncoating from structures of EV71. Nat. Struct. Mol. Biol. 19, 424–429.

Wang, M., Jiang, S., Wang, Y., 2013. Recombinant VP1 protein expressed in *Pichia pastoris* induces protective immune responses against EV71 in mice. Biochem. Biophys. Res. Commun. 430, 387–393.

Won, G., John, Hwa.L., 2017. Potent immune responses induced by a *Salmonella* ghost delivery system that expresses the recombinant Stx2eB, FedF, and FedA proteins of the *Escherichia coli*-producing F18 and Shiga toxin in a murine model and evaluation of its protective effect as a porcine vaccine candidate. Vet. Q. 37, 81–90.

Xu, L., He, D., Li, Z., Zheng, J., Yang, L., Yu, M., Yu, H., Chen, Y., Que, Y., Shih, J.W.K., Liu, G., Zhang, J., Zhao, Q., Cheng, T., Xia, N., 2014. Protection against lethal enterovirus 71 challenge in mice by a recombinant vaccine candidate containing a broadly cross-neutralizing epitope within the VP2 EF loop. Theranostics 4, 498–513.

Xu, B., Wk, R., Lee, S., Liu, W., Im, D., Bj, P., Dl, K., Tzipori, S., 2016. Silk-based microneedle transdermal immunization with EV71 vaccines elicits potent immune responses in mice. Int. J. Vaccines Immune Syst. 2, 1–8.

Xue, P., Zhu, X., Shi, J., Fu, H., Zhang, J., Liu, M., Jiang, C., Li, X., 2014. Highly efficient expression of functional recombinant human keratinocyte growth factor 1 and its protective effects on hepatocytes. Appl. Microbiol. Biotechnol. 98, 3933–3945.

Xue, L., Liu, J., Wang, Q., Zhang, C., Xu, L., Luo, J., Wang, J., Qin, C., Liu, Y., Su, Z., 2017. Purification and assembling a fused capsid protein as an enterovirus 71 vaccine candidate from inclusion bodies to pentamer-based nanoparticles. Biochem. Eng. J. 117, 139–146.

Yee, P.T.I., Poh, C.L., 2016. Development of novel vaccines against enterovirus-71. Viruses. 8, 1.

Yee, P.T.I., Poh, C.L., 2018. T cell immunity to enterovirus 71 infection in humans and implications for vaccine development. Int. J. Med. Sci. 15, 1143–1152.

Zhang, F., Hao, C., Zhang, S., Li, A., Zhang, Q., Wu, W., Liu, L., Li, C., Liang, M., Li, X., Li, D., 2014. Oral immunization with recombinant enterovirus 71 VP1 formulated with chitosan protects mice against lethal challenge. Virol. J. 11, 80.

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

Zhou, F., Kong, F., Wang, B., McPhie, K., Gilbert, G.L., Dwyer, D.E., 2011. Molecular characterization of enterovirus 71 and coxsackievirus A16 using the 5' untranslated region and VP1 region. J. Med. Microbiol. 60, 349–358.

Zhou, S.L., Ying, X.L., Han, X., Sun, X.X., Jin, Q., Yang, F., 2015. Characterization of the enterovirus 71 VP1 protein as a vaccine candidate. J. Med. Virol. 87, 256–262.

Zhu, J., 2015. T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. Cytokine. 75, 14–24.

Zhu, B., Ye, J., Lu, P., Jiang, R., Yang, X., Fu, Z.F., Chen, H., Cao, S., 2012. Induction of antigen-specific immune responses in mice by recombinant baculovirus expressing premembrane and envelope proteins of West Nile virus. Virol. J. 9, 132.