Biochemical and Biophysical Research Communications 430 (2013) 387–393

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Recombinant VP1 protein expressed in *Pichia pastoris* induces protective immune responses against EV71 in mice

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ARTICLE INFO

Article history:
Received 31 October 2012
Available online 15 November 2012

Keywords:
Enterovirus 71
Recombinant VP1
Pichia pastoris
Immunogenicity
Prophylactic efficacy

ABSTRACT

Human enterovirus 71 (EV71) is one of the major causative agents of hand, foot and mouth disease and is also associated with serious neurological diseases in children. Currently, there are no effective antiviral drugs or vaccines against EV71 infection. VP1, one of the major immunogenic capsid proteins of EV71, is widely considered to be the candidate antigen for an EV71 vaccine. In this study, VP1 of EV71 was expressed as a secretory protein with an N-terminal histidine tag in the methylotrophic yeast *Pichia pastoris*, and purified by Ni–NTA affinity chromatography. Immunogenicity and vaccine efficacy of the recombinant VP1 were assessed in mouse models. The results showed that the recombinant VP1 could efficiently induce anti-VP1 antibodies in BALB/c mice, which were able to neutralize EV71 viruses in an *in vitro* neutralization assay. Passive protection of neonatal mice further confirmed the prophylactic efficacy of the antisera from VP1 vaccinated mice. Furthermore, VP1 vaccination induced strong lymphoproliferative and Th1 cytokine responses. Taken together, our study demonstrated that the yeast-expressed VP1 protein retained good immunogenicity and was a potent EV71 vaccine candidate.

1. Introduction

Enterovirus 71 (EV71) is the most frequently detected pathogen in hand, foot and mouth disease (HFMD) patients complicated with the severest forms of neurological disorders [1,2]. Since it was initially identified in 1969 [3], outbreaks and epidemics caused by EV71 have been reported worldwide in the past decades, and hundreds of children have died from severe complications of encephalomyelitis [4–6]. Though there has been a significant increase in EV71 epidemics, effective vaccines or antiviral drugs are not available [7]. There is an urgent need for the development of effective vaccines against EV71.

EV71 belongs to the genus *Enterovirus* in the family *Picornaviridae* [8]. EV71 contains a single-stranded, positive-sense RNA of approximately 7400 nucleotides which is enclosed by capsid proteins VP1, VP2, VP3 and VP4 [9,10]. Because VP1 displays major antigenicity and has been defined as the neutralization determinant [11–13], VP1 is the candidate antigen protein for developing subunit or epitope vaccines [14]. In recent years, several studies have indicated the potential of the VP1 protein to act as an EV71 vaccine candidate. For example, recombinant VP1 protein derived from *E. coli* [15], transgenic tomato [16] or the milk of transgenic mice [17] could induce neutralizing antibodies and protected mice against EV71 infection. However, it was difficult to purify and obtain high-level production of recombinant VP1 proteins in these expression systems.

The methylotrophic yeast *Pichia pastoris* has been widely used in vaccine production with the advantages of easy manipulation, high production levels and low cost [18]. In the present study, the VP1 protein of EV71 was secretory expressed in *P. pastoris* with good antigenicity. The yeast-expressed VP1 induced high levels of neutralizing antibodies, and elicited strong humoral and cellular immune responses in mice, which represents a potential subunit vaccine candidate.

2. Materials and methods

2.1. Cell lines and virus strain

RD cells (rhabdomyosarcoma) were used for the growth of EV71-C4 strain (FY0805, GenBank accession #HQ882182) [19]. EV71 virus stock was collected from the supernatant of infected RD cells 3 days post-infection (dpi) at 37 °C. The virus from the cell culture was purified as described previously [20].

2.2. Codon optimization

The codon-optimized gene was designed based on the protein sequence of VP1 (GenBank Accession #ADC53084) according to the codon bias of *P. pastoris* [21] (http://www.kazusa.or.jp/codon). Codon optimization was performed by using the JCat program [22].

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http://dx.doi.org/10.1016/j.bbrc.2012.11.035

The entire *VP1* gene with *Eco*R I and *Xba* I restriction sites at each end was designed and was in frame with α-factor of pPICZαA vector (Fig. 1C). The designed *VP1* was synthesized by Takara (Dalian, China).

2.3. Construction of plasmids encoding VP1

The synthesized *VP1* gene was inserted in the pUC57 plasmid (pUC57-VP1). Proper construction was confirmed by DNA sequencing. The pUC57-VP1 was double digested with *Eco*R I and *Xba* I, prior to the insertion into plasmid pPICZαA (Invitrogen, USA). The resulting plasmid was designated pPICZαA-VP1.

2.4. VP1 expression and purification

The recombinant expression vector pPICZαA-VP1 was linearized by *Sac* I (TaKaRa) and electroporated into *P. pastoris* GS115 competent cells. The transformants were selected on YPD plates containing Zeocin™ (Invitrogen) at a final concentration of 100 μg/ml. The positive transformants of *P. pastoris* GS115 were grown in buffered complex glycerol media (BMGY) with vigorous shaking (300 rpm) at 30 °C until the culture reached an OD_{600} = 6. The cells were subsequently centrifuged at 3000×g for 5 min and resuspended to an OD_{600} of 1 in buffered complex methanol media (BMMY). The expression was induced by continuous incubation at 30 °C for 72 h and the addition of methanol every 24 h to a final concentration of 0.5% for transcriptional induction. A negative control containing the empty pPICZαA vector was carried out in parallel. The supernatant of the induced culture was collected by centrifugation and subjected to precipitation at 80% ammonium sulfate saturation, and the 80% precipitate was collected by centrifugation at 10,000×g for 15 min. The precipitated protein was dissolved in distilled water and dialyzed extensively against 50 mM TBS (pH 7.2) by changing the dialysis buffer 3 times at 4 °C overnight. The sample was loaded onto a Ni–NTA affinity column pre-equilibrated with TBS (pH 7.2), and eluted with 0.2 M imidazole in 50 mM TBS (pH 7.2) after extensive washing with TBS. The protein samples were analyzed by 12% SDS–PAGE and western blot with anti-His polyclonal antibody. The enriched VP1 fraction was stored at −20 °C.

2.5. Animals and immunization

Three groups, each including twenty 4–6-week old female BALB/c mice, were immunized by intramuscular (i.m.) injection on day 0 with PBS (100 μl), 10 μg VP1 or 10 μg VP1 with Freund's complete adjuvant (50%, v/v) (Sigma, USA). On day 14 and 28, the mice were boosted with PBS (100 μl), 10 μg VP1 or 10 μg VP1 with Freund's incomplete adjuvant (50%, v/v) (Sigma, USA). Blood samples were taken from all the mice on days 0, 14, 28, and 42. The

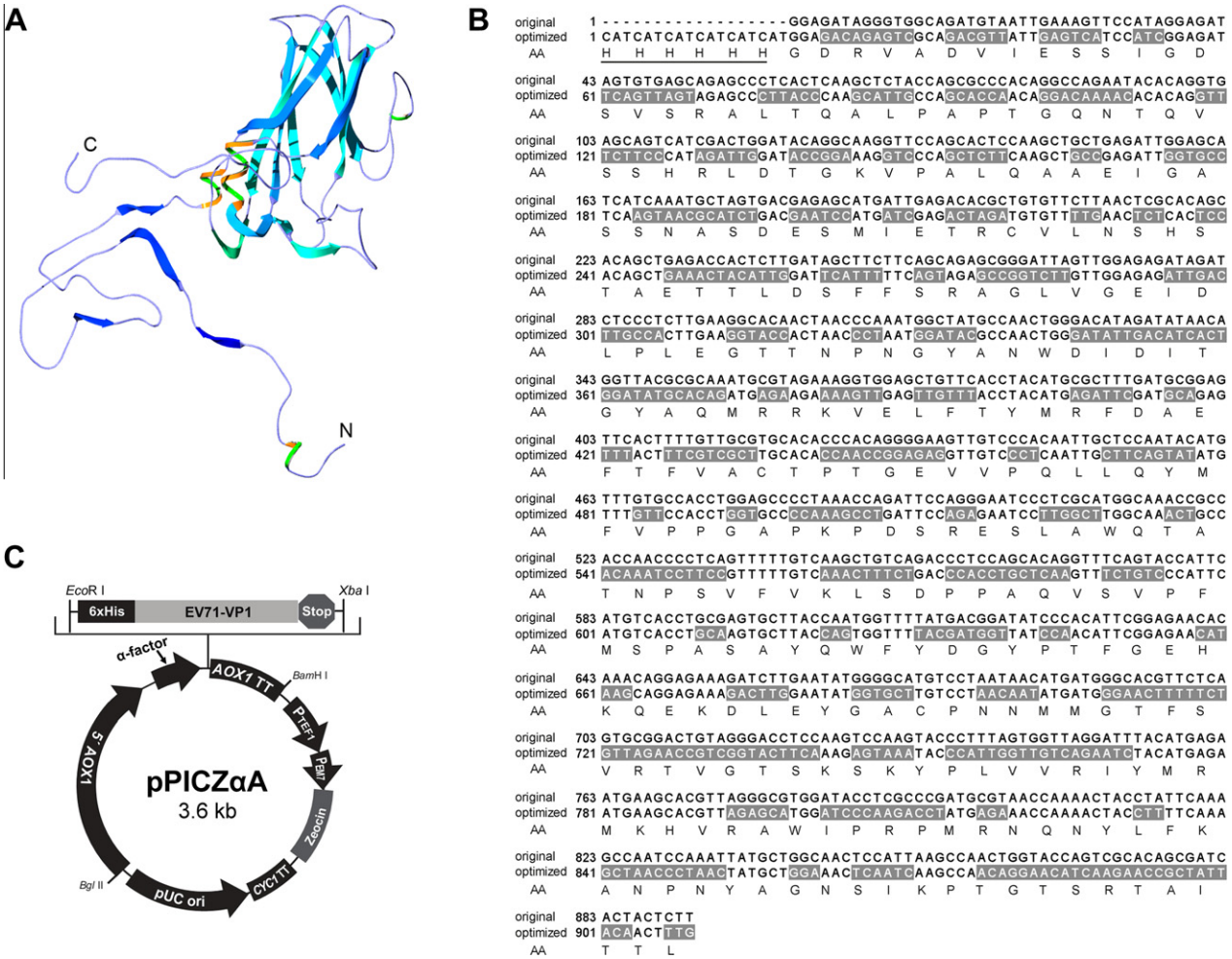

Fig. 1. Construction of the recombinant expression vector pPICZαA-VP1. (A) Crystal structure of VP1. The structure was derived from PDB code 3VBH. (B) Sequence alignment of codon-optimized and original *VP1*. The optimized codons are highlighted in grey; His-tag at the N-terminal is underlined. (C) Schematic diagram of plasmid construct. *VP1* was inserted into pPICZαA for the expression of recombinant protein.

sera were harvested for ELISA and neutralization assay. Procedures were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals.

2.6. Western blot analysis

Recombinant VP1 proteins were separated on 12% SDS–PAGE, transferred to PVDF membrane and reacted with diluted mouse antiserum (1:500) from each group respectively. Mice antibodies were detected with the corresponding HRP-conjugated secondary antibodies (Sigma, USA) and the signals were developed by ECL (Thermo Scientific).

2.7. ELISA

The levels of specific IgG and IgM against VP1 in sera of the immunized mice were determined by ELISA. Briefly, 96-well plates coated overnight with 50 ng purified VP1 in 100 μl of 100 mM carbonate buffer (pH 9.6) per well at 4 °C were washed with PBST and blocked with 2% BSA in PBST. The plates were subsequently incubated with 320 or 160 times diluted sera from immunized mice for 1.5 h at room temperature. After three washes with PBST, HRP-conjugated goat anti-mouse IgG or IgM (1:5000) was added into each well. The plates were washed and developed with tetramethylbenzidine (TMB) substrate (Sigma, USA), stopped with 2 M H_2SO_4 and measured at 450 nm. The mean absorbance value for triplicate wells was used to express serum antibody level.

2.8. Neutralization assay

Following heat-inactivation at 56 °C for 30 min, 50 μl of two-fold serially diluted mice sera were mixed with equal volumes of 100 $TCID_{50}$ EV71 in 96-well plate and incubated at 37 °C for 2 h. Then 1.5×10^4 RD cells in 100 μl of DMEM-10% FBS were added in the mixture and the cytopathic effect (CPE) was read after 3 days. The neutralizing antibody titer was defined as the highest dilution of serum that gave no CPE. The experiment was done in triplicates and the average neutralization titer was recorded.

2.9. In vivo protection against lethal EV71 infection

For the passive protection study, neonatal BALB/c mice were used. The sera from mice were collected on day 42 as described above. Groups of 3 day-of-age BALB/c mice ($n = 10$ each group) were injected intraperitoneally (i.p.) with 100 μl heat-inactivated (56 °C, 30 min) mice immune sera and with EV71 (1000 $TCID_{50}$ per mouse) 24 h later. Control suckling mice ($n = 10$) were injected with sera from PBS immunized mice. Mice were observed daily for mortality until 3 weeks post-infection.

2.10. Spleen lymphocyte proliferation and cytokine production

The spleens were aseptically removed from mice ($n = 3$–5 each group) at 14 days after the third immunization. Lymphocytes were separated by an EZ-Sep™ mouse lymphocyte separation kit (Dakewe, China), seeded at a density of 1×10^6 cells per well in a 96-well plate, and stimulated with recombinant VP1 (5 μg/ml), Con A (5 μg/ml, Sigma, USA) or medium alone (negative control) for 72 h. Then, an MTT assay was carried out as described [23]. The stimulation index (SI) was calculated as the ratio of the average OD_{570} value of wells containing antigen-stimulated cells to the average OD_{570} value of negative control wells.

In order to measure cytokine secretion, splenocytes were cultured as described above, except that after 48, 72 and 96 h of incubation, the supernatants were collected and assayed for IFN-γ, TNF-α, IL-4 and IL-10 production by commercially available ELISA kits (BD Biosciences).

2.11. Flow cytometry analysis

Mice were then challenged with 100 μl of EV71 fluid containing 5×10^6 $TCID_{50}$/ml via i.p. route at 14 days after the last immunization. Splenocytes and PBMCs were isolated from mice at 3 dpi and 6 dpi. 1×10^6 cells/sample were stained with FITC-conjugated anti-CD3, PE-conjugated anti-CD4 or PE-conjugated anti-CD8 (BD Biosciences). All samples were analyzed by flow cytometry (BD Biosciences).

3. Results

3.1. Expression and purification of VP1

The full length of *VP1* was 891 bp, which encoded 297 amino acids. The crystal structure of VP1 has recently been determined [24]. VP1 has four major surface-exposed loops, which are important neutralizing immunogenic sites [25] (Fig. 1A). The full-length *VP1* was synthesized after codon optimization, and 73 out of 297 codons (almost 24.6%) were substituted by the *P. pastoris* preferred codons. The sequence alignment showed that the optimization did not change the amino acid sequence (Fig. 1B). The optimized *VP1* gene was then inserted into plasmid pPICZαA for protein expression, designated as pPICZαA-VP1 (Fig. 1C).

The *P. pastoris* GS115 cells harboring the pPICZαA-VP1 were induced with methanol to a final concentration of 0.5% for 72 h. The supernatant was collected for SDS–PAGE and western blot analysis. VP1 was secreted in culture supernatant of positive transformant (pPICZαA-VP1/GS115), and the size was consistent with the expected size of 32 kDa (Fig. 2A). Western blot analysis with anti-His antibody further demonstrated the expression of VP1 in *P. pastoris* (Fig. 2B).

The supernatant containing the secreted VP1 was collected and ammonium sulfate was added to precipitate the proteins. After dialysis against TBS, the protein sample was loaded onto the Ni–NTA affinity column and eluted with 0.2 M imidazole after extensive TBS washing (Fig. 2C). Representative purification fractions from the affinity column were run on SDS–PAGE. The eluate fraction yielded a single band (approximately 32 kDa) (Fig. 2D). In addition, anti-His antibody was able to detect the purified VP1 (Fig. 2E).

3.2. Humoral response of vaccinated BALB/c mice

Groups of BALB/c mice were immunized three times at two-week intervals with PBS, purified VP1 or purified VP1 with Freund's adjuvant. The sera were collected from mice at various days after priming and boosting (Fig. 3A). In order to evaluate anti-VP1 antibodies in the sera of immunized mice, purified VP1 protein was subjected to western blot analysis with mice immune sera collected on day 42. The 32 kDa VP1 bands were detected with the antisera from VP1 or VP1 + FA immunized mice, while VP1 could not be detected with antiserum from PBS immunized mice (Fig. 3B). Levels of anti-VP1 IgG and IgM in sera of immunized mice were measured by ELISA. As expected, no detectable IgG antibody was elicited from mice vaccinated with PBS before or after immunization. On the contrary, immunization with VP1 or VP1 + FA induced high levels of IgG at 14 days after the primary immunization, which increased after each booster injection (Fig. 3C). The IgM levels were significantly higher in VP1 or VP1 + FA immunized mice than the control group at 14 days after

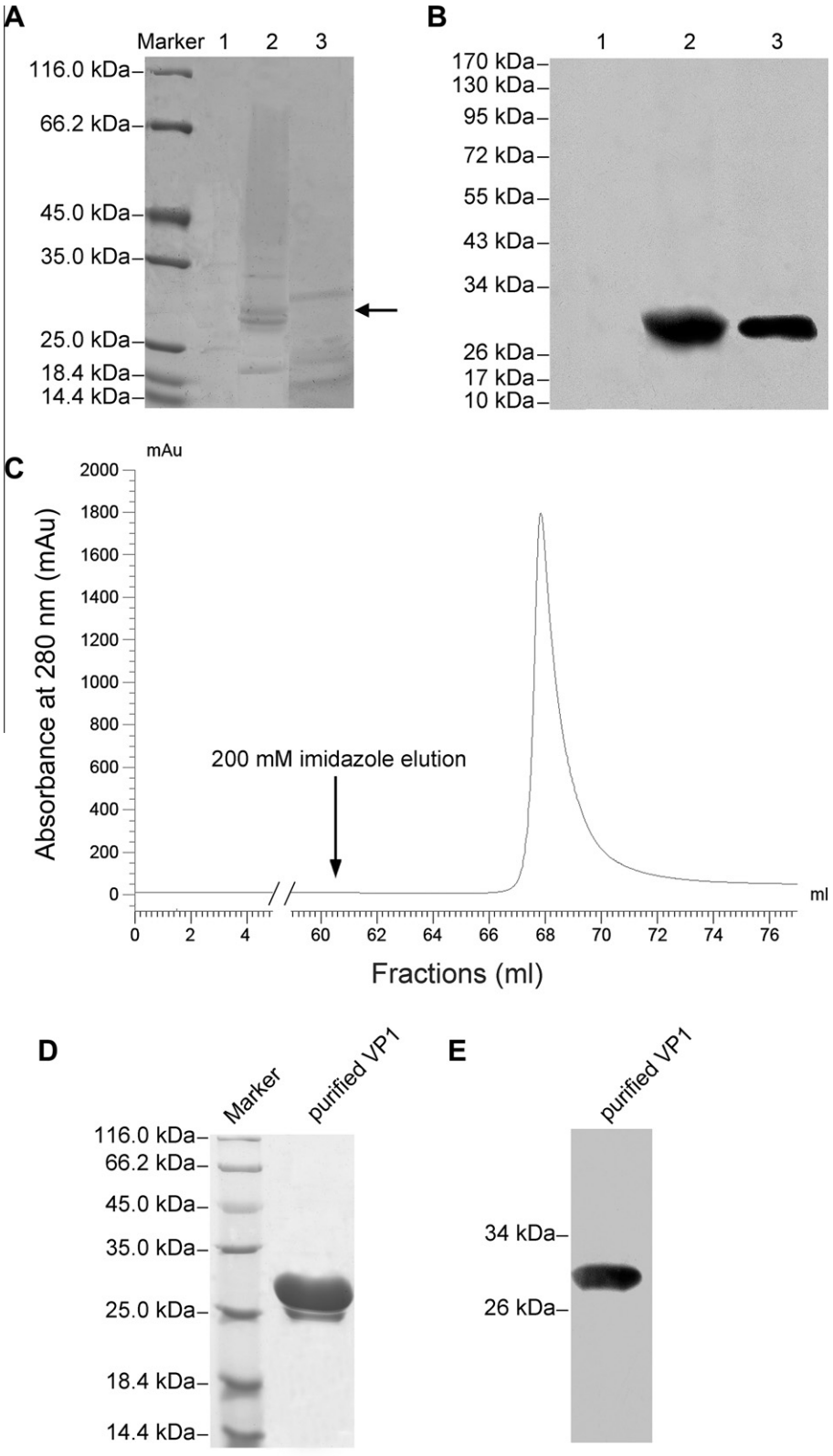

Fig. 2. Expression and purification of VP1. The samples were collected at 72 h post-inducing and then were analyzed by SDS–PAGE (A) and western blot (B). Lane 1: negative control, induced supernatant of control transformant (pPICZαA/GS115); lane 2: induced supernatant of positive transformant (pPICZαA-VP1/GS115); lane 3: cell lysate from positive transformant (pPICZαA-VP1/GS115) after induction. The position of VP1 is indicated. The crude proteins in the supernatant were precipitated and loaded onto Ni–NTA column. After washing with TBS, VP1 was eluted with TBS containing 0.2 M imidazole (indicated by arrow). The image was generated by AKTA prime (Amersham Biosystems) (C). The purified protein was detected by SDS–PAGE (D) and western blot (E).

the primary immunization ($p < 0.05$) and the IgM elicited by VP1 + FA maintained high levels at 28 days ($p < 0.05$) (Fig. 3D).

The neutralization assay showed that VP1 or VP1 + FA immunization induced significantly higher neutralizing antibody titers (up

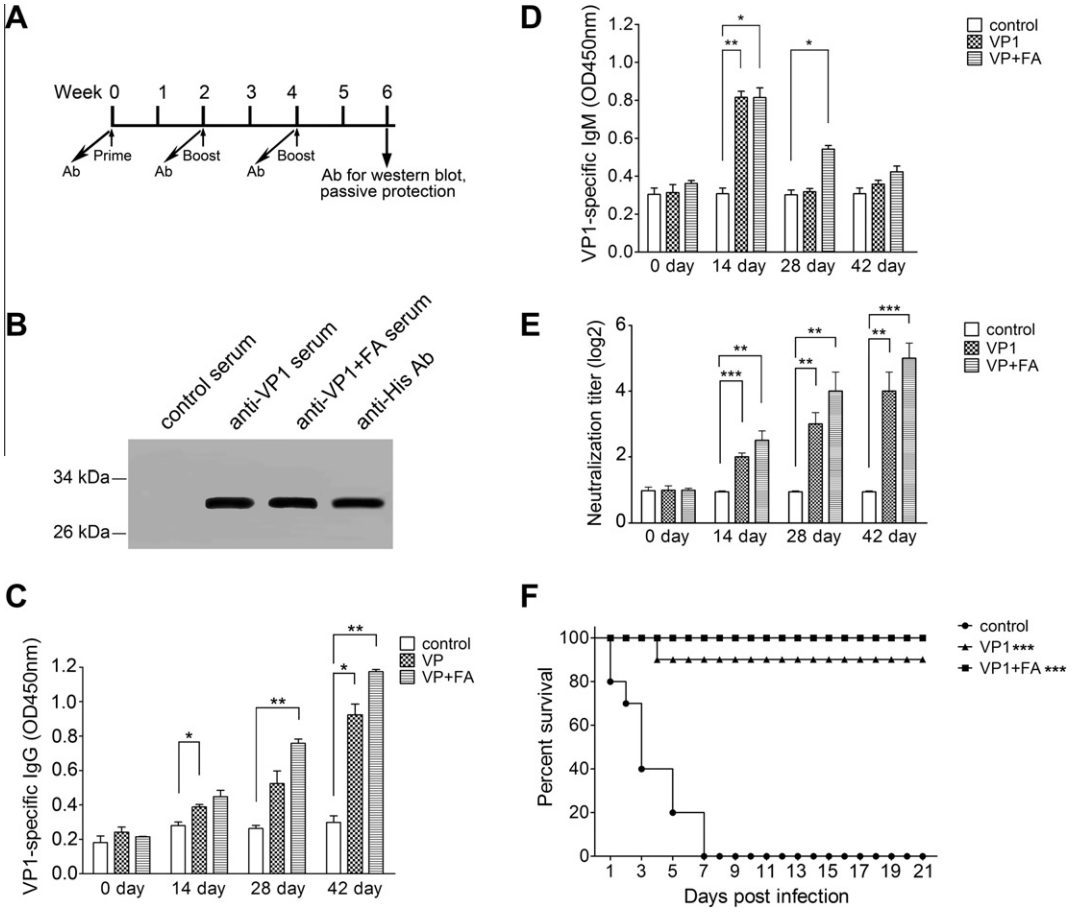

Fig. 3. Humoral immune responses in vaccinated mice. (A) Immunization regimen showing when immunizations were performed and when sera were collected for antibodies assay and passive protection experiment. (B) Western blot analysis. The recombinant VP1 proteins were resolved in 12% SDS–PAGE, transferred onto PVDF and reacted with mice immune sera collected on day 42. IgG (C) and IgM (D) antibodies induced by VP1 immunization were detected by ELISA. The sera of (C) and (D) were diluted with 320 and 160 fold, respectively. (E) *In vitro* neutralization assay. The sera were collected from the immunized mice at different time points were serially diluted, mixed with EV71 virus and used to infect RD cells. After 3 days, CPE was monitored and the maximum dilution that gave no CPE was determined as the neutralization titer. (F) Passive protection of EV71-infected suckling mice. Mice were observed daily for mortality until 3 weeks post-infection. VP1 + FA: recombinant VP1 protein with Freund's adjuvant. All the data presented were the mean ± SD of three replicate wells ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

to 2^5) whereas antisera from control mice had no neutralizing activity ($p < 0.01$) (Fig. 3E). The results suggested that antibodies raised against the recombinant VP1 were efficient in neutralizing EV71 viruses.

3.3. Protection against lethal EV71 challenge in suckling mice

The protective efficacy of the VP1 vaccine was then evaluated in a passive immunization study. 90% and 100% survival rates ($n = 10$ each group) were observed by day 21 post-infection for groups of EV71-infected suckling mice that received the anti-VP1 serum and anti-VP1 + FA serum respectively, whereas EV71-infected suckling mice which received antiserum from PBS immunized mice died by day 7 post infection (Fig. 3F). Moreover, injection with anti-VP1 serum or anti-VP1 + FA serum significantly increased the survival rate of EV71-infected suckling mice compared with antiserum from PBS immunized mice ($p < 0.001$).

3.4. VP1-induced splenocyte proliferation and Th1 cytokine production

Since lymphocyte proliferative responses are generally related to the cell-mediated immunity [26], VP1-specific lymphocyte proliferation assay was evaluated using MTT methods. The spleen lymphocytes were isolated from immunized mice at 14 days after the last immunization and stimulated with VP1 or Con A for 72 h. Splenocytes of VP1 or VP1 + FA immunized mice showed a greater increase in cell proliferation than the control group ($p < 0.01$, Fig. 4A).

Profiles of cytokine released from VP1-stimulated splenocytes were examined to determine the primed pathway of immune responses. The Th1 (IFN-γ and TNF-α) and Th2 (IL-4 and IL-10) cytokines were detected in the cell-free supernatants at 48, 72 and 96 h after incubation with VP1 (Fig. 4B–E). Splenocytes of mice immunized with VP1 or VP1 + FA produced higher levels of IFN-γ and TNF-α than the control mice ($p < 0.05$). However, the levels of IL-4 and IL-10 were slightly higher in the two vaccine groups. The results suggested that VP1 immunization greatly stimulated Th1-type cytokine production, and the change in cytokines suggested the activation of Th1 immune response.

3.5. Proliferation of $CD4^+$ and $CD8^+$ T cells in the spleen and peripheral blood of immunized mice

To gain a better understanding of the mechanisms of immune activation by the VP1 vaccine, we studied the systemic response of the $CD4^+$ and $CD8^+$ T cells in the immunized mice. At 3 dpi or 6 dpi, $CD4^+$ and $CD8^+$ T cells in spleens and peripheral blood were stained and detected by flow cytometry (Fig. 4F and G). The proportions of $CD4^+$ and $CD8^+$ T cell populations in spleens of VP1 or VP1 + FA immunized mice were comparable to those measured

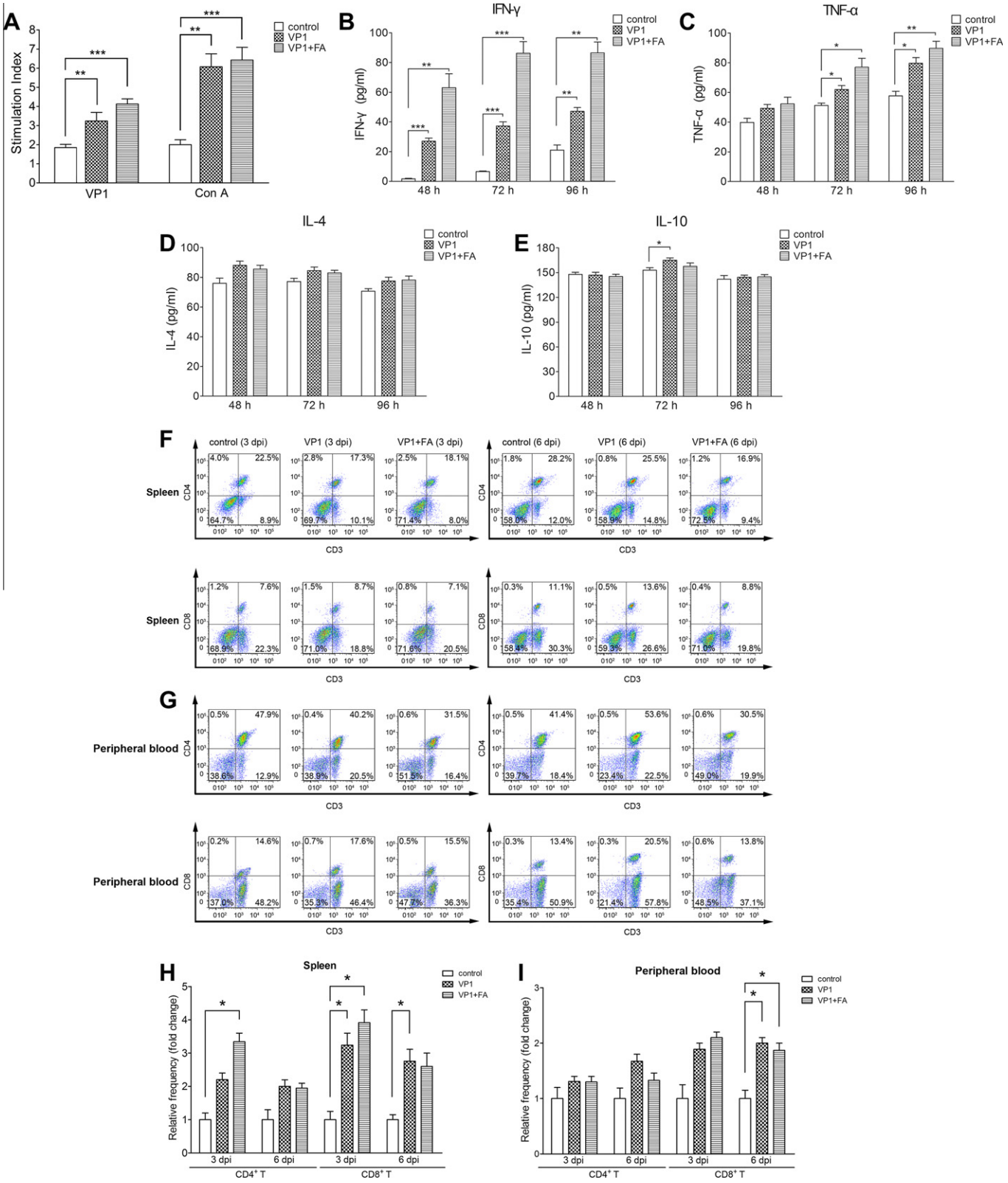

Fig. 4. Cellular immune responses in vaccinated mice. (A) Spleen lymphocytes from each group were isolated at 14 days after the last immunization. The proliferation of lymphocytes was detected after stimulation with VP1 or Con A for 72 h. Concentrations of IFN-γ (B), TNF-α (C), IL-4 (D) and IL-10 (E) in the culture supernatants of splenocytes were determined by ELISA at 48, 72 and 96 h. All the results were expressed as mean ± SD of at least three mice. Representative FACS plots for single-cell suspensions in spleen (F) and peripheral blood (G) were shown. At 3 dpi and 6 dpi, the lymphocytes from spleen and peripheral blood were harvested, stained with fluorescence labeled anti-CD3, anti-CD4 or anti-CD8 and analyzed by flow cytometry. Relative frequencies of $CD4^+$ and $CD8^+$ T cells in spleen (H) and peripheral blood (I) were shown. Relative frequency was calculated as the ratio of the average number of $CD4^+$ or $CD8^+$ T cells in VP1 or VP1 + FA immunized mice to the average number of that in the controls. Statistically significant values ($p < 0.05$) were indicated by an asterisk.

in the control group. In contrast, a slight decrease in the proportion of $CD4^+$ T cells, in parallel with an increase in the proportion of $CD8^+$ T cells, was observed in blood of the two vaccine groups compared with the control group. The absolute numbers of splenocytes and PBMCs increased markedly in VP1 and VP1 + FA immunized mice (data not shown). So the numbers of $CD4^+$ and $CD8^+$ T cells were dramatically higher in spleen and blood of the two vaccine groups (Fig. 4H and I). These results suggested that $CD4^+$ and $CD8^+$ T cells were involved in VP1 vaccine mediated antiviral defense.

4. Discussion

As a major capsid protein on the surface of EV71, VP1 has long been a focal point of EV71 research. In this study, high-level secretory production of VP1 was obtained in *P. pastoris* by methanol induction (approximately 500 mg/l, data not shown) and its potential use as a subunit vaccine was evaluated.

Groups of female BALB/c mice were inoculated with VP1 and the serological responses were detected to evaluate the efficacy of VP1 as a vaccine. Compared with the control of PBS injection, serum VP1 specific IgG and IgM could be detected at 2-week intervals post the initial vaccination in the two vaccine groups, and the vaccination with VP1 + FA could induced even higher IgG level than VP1 alone. It indicated that the presence of adjuvant could enhance VP1-specific immune responses in mice. The anti-VP1 antibodies were efficient in neutralizing EV71 *in vitro*, and the neutralizing titers increased following the booster injections. A passive immunization study showed the suckling mice that received the antiserum from VP1 + FA immunized mice were fully protected from EV71 infection. The animal experiments indicated that the recombinant VP1 was able to induce strong protective immune responses against EV71 infection.

We found that splenocytes of mice immunized with VP1 or VP1 + FA produced significantly higher levels of IFN-γ and TNF-α compared with the control group. The observation suggested that the immune responses of VP1 vaccination were provoked through Th1-type route. Furthermore, the $CD4^+$ and $CD8^+$ T cells were detected in spleen and peripheral blood by using flow cytometry. Our data showed that the numbers of $CD4^+$ and $CD8^+$ T cells increased in VP1 and VP1 + FA groups. Both $CD4^+$ and $CD8^+$ T cells were reported to protect mice from EV71 infection by reducing viral loads in tissues [27]. Our results indicated that VP1 immunization induced effective T-cell immune responses, which contributed to the clearance of EV71 virus and thus protected mice from EV71 infection.

In conclusion, we have successfully expressed the VP1 protein of EV71 in *P. pastoris* with high production levels and strong immunogenicity. The recombinant VP1 elicited high levels of neutralizing antibodies and induced strong T-cell immune responses. Moreover, *P. pastoris* yeast is a convenient and efficient expression system. We believe that the *P. pastoris*-expressed VP1 is a promising EV71 vaccine candidate for industrial purpose.

Acknowledgments

This work was supported by the National Basic Research Program of China (973 Program, no. 2010CB530101).

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