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Immunogenicity of a truncated enterovirus 71 VP1 protein fused to a Newcastle disease virus nucleocapsid protein fragment in mice

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Summary. – Enterovirus 71 (EV71) is one of the viruses that cause hand, foot and mouth disease. Its viral capsid protein 1 (VP1), which contains many neutralization epitopes, is an ideal target for vaccine development. Recently, we reported the induction of a strong immune response in rabbits to a truncated VP1 fragment (Nt-VP1t) displayed on a recombinant Newcastle disease virus (NDV) capsid protein. Protective efficacy of this vaccine, however, can only be tested in mice, since all EV71 animal models thus far were developed in mouse systems. In this study, we evaluated the type of immune responses against the protein developed by adult BALB/c mice. Nt-VP1t protein induced high levels of VP1 IgG antibody production in mice. Purified VP1 antigen stimulated activation, proliferation and differentiation of splenocytes harvested from these mice. They also produced significant levels of IFN-γ, a Th1-related cytokine. Taken together, Nt-VP1t protein is a potent immunogen in adult mice and our findings provide the data needed for testing of its protective efficacy in mouse models of EV71 infections.

Keywords: enterovirus 71; recombinant VP1 protein; immunogenicity

Introduction

EV71 is a small, non-enveloped virus with an icosahedral shape (Chang *et al.*, 2007), which was first isolated and identified from a fecal specimen in 1969, during an outbreak in California, USA (Schmidt *et al.*, 1974). The virus has been classified into the family of *Picornaviridae*, the genus *Enterovirus* and the species *Human enterovirus A* (King *et al.*, 2000). It is normally associated with uncomplicated diseases such as hand, foot and mouth disease (Melnick, 1996), herpangina and pharyngitis. However, neurological complications such as aseptic meningitis, acute flaccid paralysis and encephalitis may arise (Huang *et al.*, 1999; Chang, 2008).

The risk of symptomatic EV71 infection is inversely proportional to the age of patients (Abzug, 2009). During an outbreak in Taiwan in 1998, most of the victims with fatal cases were children younger than 5 years of age (Ho *et al.*, 1999). Recently, a similar outbreak in China has resulted in more than ten thousand cases, including 50 fatal cases in children (Ding *et al.*, 2009). To prevent further emergence and spread of EV71, good personal hygiene practice, quarantine, and clinical surveillance activities are commonly used (McMinn, 2002; Chang, 2008).

EV71 VP1 protein is one of the structural proteins responsible for EV71 antigenic diversity (Rotbart *et al.*, 1998). It contains many neutralization epitopes (Rueckert, 1990; Oberste *et al.*, 1999), which are crucial for virus identification and evolution studies (Chang *et al.*, 2007). Hence, VP1 has become an ideal target for immunogenicity studies and vaccine development (Wu *et al.*, 2001; Foo *et al.*, 2007b). Recently, we showed that immunization with the first 100 amino acids of VP1 (VP1t) fused to a truncated nucleocapsid protein (Nt) of NDV, which served as a carrier molecule (Kho *et al.*, 2001; Yusoff and Tan, 2001; Rabu

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Abbreviations: EV71 = enterovirus 71; IFN-γ = interferon gamma; IL-2 = interleukin 2; N = full length nucleocapsid protein; NDV = Newcastle disease virus; Nt = truncated nucleocapsid protein; VP1 = full length viral protein 1; VP1t = truncated viral protein 1

et al., 2002), induced strong antibody responses in rabbits (Sivasamugham *et al.*, 2006). This study showed that this recombinant protein, labeled as Nt-VP1t, may represent a useful candidate for EV71 vaccine development. However, its protective effects need to be studied in appropriate animal models of EV71 infection. Thus far mouse models have been found to be the most informative (Wu *et al.*, 2001; Chen *et al.*, 2006; Foo *et al.*, 2007a; Tung *et al.*, 2007). Therefore, prior to an investigation into the protective efficacy of the Nt-VP1t in a mouse model of EV71 infection, it is necessary to investigate the types of mouse immune responses that are mounted against this protein. Hence, in the present study we explored the potential immunogenic characteristics of the purified Nt-VP1t protein in adult female BALB/c mice. Our results indicate that Nt-VP1t is a potent immunogen, which is capable of inducing both humoral and cell-mediated immunity in mice.

Materials and Methods

Production and purification of recombinant proteins. Nt-VP1t and the control full length nucleocapsid protein of NDV (N) proteins were produced and purified as described previously (Sivasamugham *et al.*, 2006). The proteins were quantitated by the Bradford assay, electrophoresed in a 12% SDS-PAGE gel and blotted onto a nitrocellulose membrane. Immunodetection using primary antibodies against the VP1 protein (1:2000 dilution) and rabbit NDV antiserum (1:9000 dilution), was used to confirm the presence of each protein.

Immunization of mice. All animal experiments were approved by The Animal Care and Use Committee, Universiti Putra Malaysia (AUP No: 10R84). All animals used in this research were cared for in accordance with The Code to Care and Use of Animals in Research. Eight adult female BALB/c mice, ages six to eight weeks, were innoculated intraperitoneally with 10 µg of purified Nt-VP1t protein. Seven mice served as a control group and were inoculated intraperitoneally with 10 µg of purified N protein. The protein samples (200 µl) were emulsified with 50% Freund's complete adjuvant (Sigma, USA) for primary injection or 50% Freund's incomplete adjuvant (Sigma, USA) for booster injections. Two booster injections were given at 2 and 4 weeks after primary immunization (2-week intervals). The mice were pre-bled prior to each injection. Blood samples were collected at weeks 0, 2, 4, 6, 8, 9, and 10 after immunization. The collected sera were stored at -20°C until used.

ELISA of antibodies. The presence of VP1- and NP-IgG antibodies in sera was determined by ELISA, using purified VP1 and N proteins as the coating antigens. Purified VP1 protein was kindly provided by Prof. Mary Jane Cardosa of the Institute of Health and Community Medicine, Universiti Malaysia Sarawak. Briefly, 1.5 µg/ml of purified VP1 and N proteins were coated onto wells of 96-well ELISA plates. Following blocking and washings, 100 µl of the collected and positive control sera (1:50 dilution) were added in duplicate into individual wells and incubated for 1 hr at room temperature. Reactions were detected using HRP-conjugated goat anti-mouse IgG antibody and the *o*-phenylenediamine dihydrochloride substrate (Sigma Aldrich, USA). Reaction intensities were measured at A490 using an ELISA microplate reader (Model 550, BioRAD, USA). The positive cut off absorbance value was defined as 3 times the absorbance of the pre-immune mice sera.

Western blot analysis. Purified VP1 and N proteins were used to examine the immunoreactivity of sera collected from immunized mice. Briefly, each viral protein was separated by SDS-PAGE and blotted onto nitrocellulose membranes. Individual lanes of the membranes were cut into strips followed by incubation with either sera from the immunized mice or the positive control VP1-antiserum and NDV-antiserum.

Splenocyte proliferation assay. Five mice from both the Nt-VP1t-immunized and control groups were sacrificed at week 9 post-immunization. Spleens from the mice were harvested and dissociated splenocytes were subjected to a Proliferation ELISA Assay (Calbiochem, Germany), according to the manufacturer's protocol. Briefly, the splenocytes were cultured in 96-well tissue culture plates at a concentration of 2×10^5 cells per well. Proliferation was induced with either 3 µg of VP1 protein or phytohemagglutinin (PHA; Sigma Aldrich, USA) at 37°C in a humidified atmosphere with 5% CO_2 for 72 hrs. Spontaneous proliferation of splenocytes in a complete medium served as a background control.

ELISA of cytokines. Culture supernatants of VP1 antigen-induced splenocytes were harvested and assayed for IL-2, IL-4, IL-10, IFN-γ production, using the Mouse Th1/Th2 ReadySETGo! ELISA Set (eBioscience, USA) as suggested by the manufacturer.

Statistical analysis. The Student *t*-test was used to analyze the experimental data in this study. Results were expressed as mean ± standard error (SE). Differences with $p < 0.05$ were considered significant. All of the tests were performed using GraphPad Prism 5 and Windows Microsoft Excel 2007.

Results

Production and purification of recombinant proteins

Nt-VP1t or the control N proteins were purified as previously described (Sivasamugham *et al.*, 2006). Each fractionated protein was analyzed by Western blot analysis and pooled selected fractions were confirmed by SDS-PAGE. N protein fractions showed a band with the expected size of approximately 55 kDa when probed with NDV antibodies (Fig. 1a, arrowhead), while Nt-VP1t fractions showed a band of approximately 60 kDa (Fig. 1b, arrow). A similar 60 kDa band was also observed when the Nt-VP1t fractions were re-probed with VP1 antibodies (Fig. 1c, arrow). Pooled and concentrated N and Nt-VP1t proteins appeared as single bands when separated by SDS-PAGE (Fig. 1d).

Induction of specific antibodies in mice

To determine the immunogenicity of the Nt-VP1t protein, immunization of adult BALB/c mice was performed. Results of an indirect ELISA on the collected sera, using VP1 as a coating antigen, showed that Nt-VP1t protein elicited high titers of VP1 IgG antibody (Fig. 2a, shaded bars). The antibody titers significantly increased ($p < 0.05$) after the primary immunization and were further enhanced following subsequent booster injections (Fig. 2a, arrows and shaded bars). The highest titers were reached after the last booster injection and the level was maintained until week 9 post-immunization. No significant VP1 IgG antibody level was detected in sera of mice immunized with the control N protein (Fig. 2a, white bars). When purified N protein was used as the capturing antigen, titers of N IgG antibodies were also found to be high in sera of both the Nt-VP1t-immunized (Fig. 2b, shaded bar) as well as in the control N-immunized sera (Fig. 2b, white bar).

In addition to the analysis by ELISA, the collected sera were also assayed against purified VP1 (Fig. 2c, upper panel) and N (Fig. 2c, lower panel) proteins by Western blot to confirm the presence of VP1- and N-antibodies, respectively. Pooled sera from Nt-VP1t-immunized mice gave a band of ~40 kDa when probed against separated VP1 protein (Fig. 2c, arrow) and a band of ~55 kDa when probed against separated N protein (Fig. 2c, arrowhead). As expected, pre-immune sera showed no reactivity with both proteins.

Stimulation of splenocytes in immunized mice

Cell-mediated immunity is important for viral clearance. In the present study, a splenocyte proliferation assay was used to monitor and investigate T-cell responses after the Nt-VP1t immunization. PHA was used as a positive stimulator to give a non-specific T-cell stimulation. When the splenocytes were activated either by PHA or VP1 proteins, the cells formed clusters (Fig. 3a, arrows). Upon PHA stimulation (Fig. 3b, white bar), there was no difference in splenocyte proliferation ($p > 0.05$) between the control and the Nt-VP1t-immunized groups. The Nt-VP1t-immunized group, on the other hand, showed significantly increased level of T-cell proliferative responses ($p < 0.05$) to VP1 protein when compared to the control group (Fig. 3b, shaded bar). The stimulation index (S.I.) for this group (S.I. = 1.267 ± 0.104) was approximately 1.7 fold greater than the S.I. value for the control group.

Production of cytokines in stimulated splenocytes

To determine the secreted cytokine levels in culture supernatants of the splenocytes, a Mouse Th1/Th2 ReadySETGo! ELISA Set (eBioscience, USA) was employed. The VP1 antigen-induced splenocytes secreted a number of Th1/Th2 cytokines such as IL-2, IL-4, IL-10, and IFN-γ (Fig. 4) into the culture supernatant. Both of the groups produced high levels of cytokines but IL-2, IL-4, and IL-10 showed no significant differences ($p > 0.05$) between the control (Fig. 4, white bar) and the Nt-VP1t-immunized groups (Fig. 4, shaded bar). In contrast, IFN-γ, a Th1-related cytokine, was produced at significantly higher levels ($p < 0.05$) by splenocytes from Nt-VP1t-immunized mice compared to those from the control group. This result strongly suggests that the Nt-VP1t protein induced a Th1 cellular response.

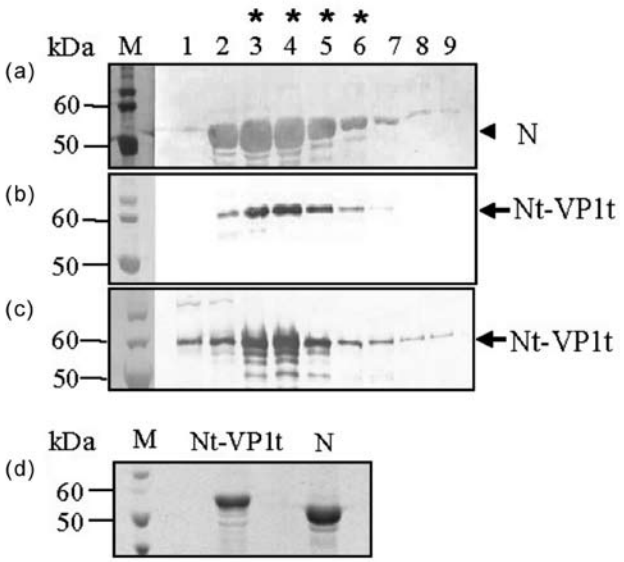

Fig. 1

Purification and concentration of N and Nt-VP1t recombinant proteins

(a-c) Western blot analysis of fractions collected after sucrose gradient centrifugation. The blots were probed using either a NDV-antiserum (a, b), or a VP1-antiserum (c). Asterisks indicate the fractions that were pooled and used for the SDS-PAGE in d. (d): SDS-PAGE of the pooled and concentrated Nt-VP1t and N proteins. M, Marker.

Discussion

Further purification and concentration of the N and Nt-VP1t proteins yielded highly purified recombinant proteins. However, minor impurities were noted when the proteins were separated on a SDS-PAGE. Similar observations were also reported by others (Kho *et al.*, 2001; Sivasamugham *et al.*, 2006), suggesting that there was a slight degradation of the proteins following concentration and purification. Other studies also suggested that the N recombinant proteins may generate small molecular mass proteins as a result of proteolytic degradation (Mountcastle *et al.*, 1970; Makkay *et al.*, 1999).

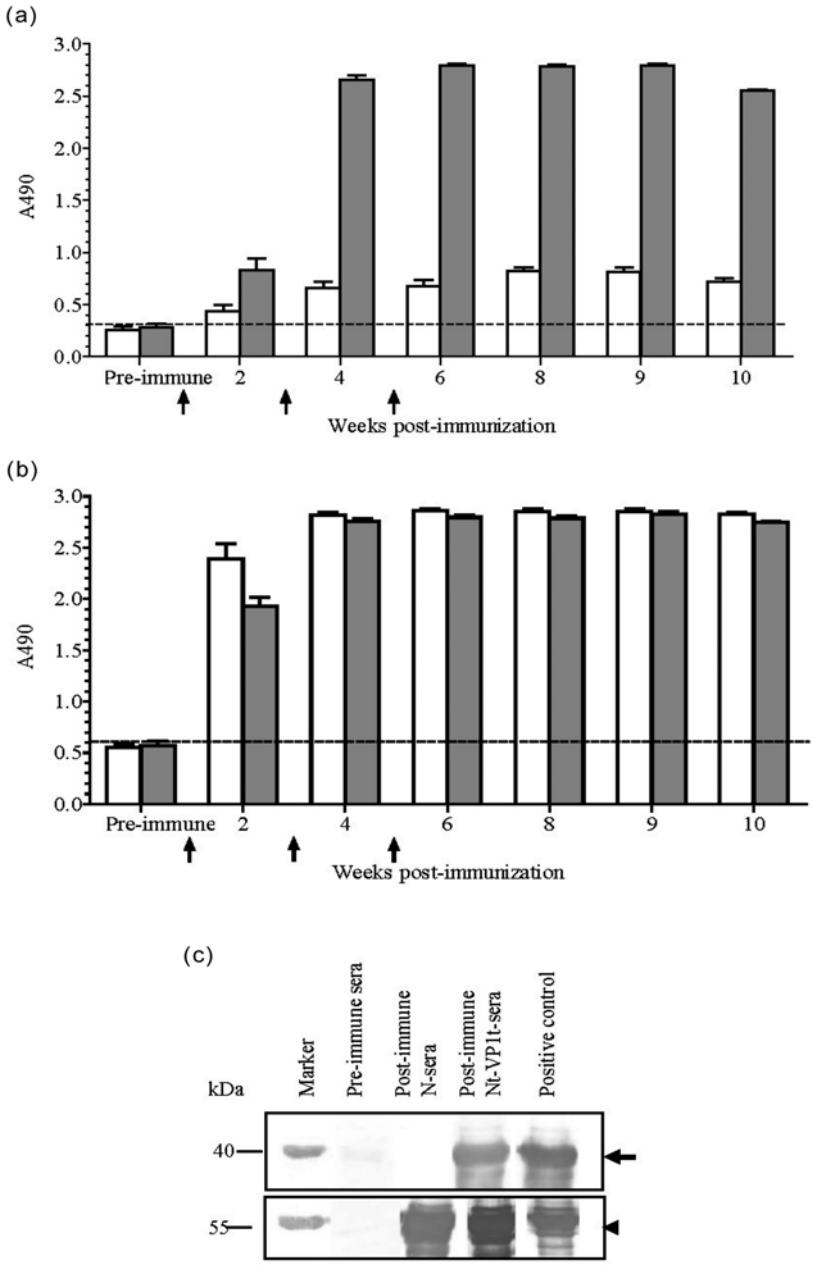

Fig. 2

IgG antibodies to N and Nt-VP1t recombinant proteins

(a, b) ELISA of VP1- (a) and N- (b) antibodies at different times post immunization with N (white bars) and Nt-VP1t (shaded bars) proteins. Broken lines indicate the cut-off values. Arrows indicate immunization time points. (c) Western blot analysis of purified VP1 (upper panel) and N (lower panel) probed with pooled sera from the immunization experiment shown in Fig. 2 a and b. VP1, EV71 viral capsid protein 1; N, full length nucleocapsid protein of NDV.

IgG is the most abundant form of immunoglobulin in serum, playing important roles in complement activation and opsonization (Wood, 2006). In most cases, high levels of IgG are formed after a secondary immunization (Wood, 2006). In the present study, purified N protein of NDV, and VP1 protein of EV71 were used to determine murine IgG responses, as measured by ELISA and Western blot analyses. The potential of the individual N (Makkay *et al.*, 1999) and VP1 proteins (Shih *et al.*, 2000) as diagnostic agents has been evaluated. In our study, a recombinant N-VP1 fusion protein,

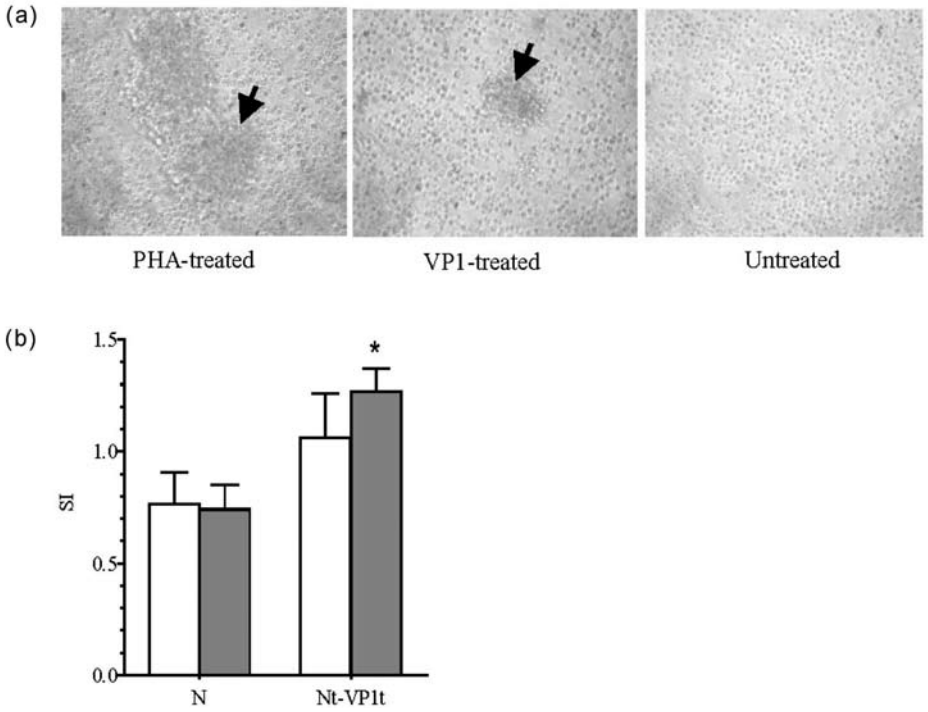

Fig. 3

Activation of proliferation of splenocytes from the mice immunized with recombinant proteins

(a) Clumping of splenocytes stimulated with VP1 or PHA (arrows). Magnification 200x. (b) Splenocyte proliferation assay, using splenocytes harvested from mice immunized with either N and Nt-VP1t proteins, and stimulated with VP1 (shaded column) or PHA (white column). *p < 0.05 for the Nt-VP1t- versus the N-vaccinated mice.

namely Nt-VP1t was used. Since the N protein was used as a carrier for the VP1 protein, it was also used as an antigen for the control group. It was also chosen because it has a molecular mass similar to that of the Nt-VP1t protein.

ELISA analyses showed that mice that received Nt-VP1t protein generated statistically significant high levels of VP1 IgG antibodies when compared to the control group. This suggests that the Nt-VP1t protein acted as a strong immunogen for the humoral response. It is well known that when naïve B-cells encounter specific antigen(s), they will divide rapidly and differentiate into immunoglobulin- producing plasma cells (Goldsby *et al.*, 2003). In the present study, two booster injections of the Nt-VP1t probably led to memory B-cell activation at weeks 2 and 4 post-immunization. Consequently, greater magnitudes of antibody response were generated.

To study the pattern of cell-mediated immune responses in these mice, their spleens were harvested and further processed. In the present study, increased levels of splenocyte proliferation in the vaccinated group were observed. This suggested that this group was able to generate better immune responses, especially cell-mediated immunity, when compared to the control group. Feng *et al.* (2009) suggested that splenocyte proliferation reaction is directly proportional

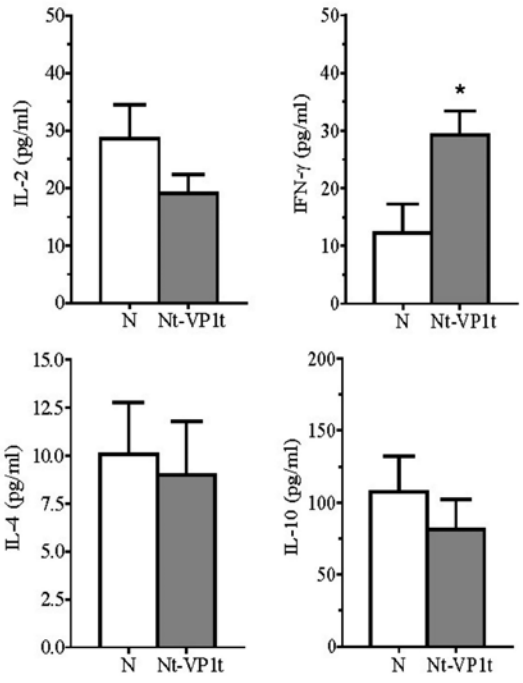

Fig. 4

Cytokines produced by VP1-stimulated splenocytes harvested from mice immunized with N and Nt-VP1t recombinant proteins

Levels of IL-2, IL-4, IL-10 and IFN-γ were detected with ELISA. *p < 0.05. White bars – control, shaded bars – Nt-VP1t.

to the cellular immune response that is important for viral killing. In another study, mice which received inactivated EV71 virus showed greater splenocyte proliferation than mice that received subunit vaccines (Wu *et al.*, 2001). At a high EV71 virus challenge dose, these mice also showed 80% survival after viral challenge, while the subunit vaccine failed to give protection (Wu *et al.*, 2001).

According to Wu *et al.* (2001), immunization of mice using the full-length VP1 recombinant protein resulted in a mixed Th1 and Th2 response. Th1 and Th2 subsets of helper T-cells express distinct cytokine patterns, reflecting the different immune response pathways (Del Prete and Romagnani, 1994). Th1-cells are known to be involved in cell-mediated immunity, while Th2-cells function as helper T-cells in humoral immunity (Goldsby *et al.*, 2003). In the present study, significantly higher levels of IFN-γ in culture supernatants of splenocytes from the Nt-VP1t-vaccinated group suggest that this protein promoted the Th1 immune response. This type of immune response is observed in responses to viruses and other intracellular pathogens (Bergmann *et al.*, 2001). Human patients infected with EV71 and suffering from pulmonary edema showed weaker cellular IFN-γ responses (Chang *et al.*, 2006). Since the Nt-VP1t immunization produced high levels of IFN-γ response, this subunit vaccine may hold promise as an effective EV71 vaccine and improve the outcome of EV71 infections. This notion is further supported by Cafruny and colleagues (1997), who demonstrated that mice that received IFN-γ treatment were protected from paralytic disease in cases of poliomyelitis (Cafruny *et al.*, 1997).

Altogether, our findings suggested that the Nt-VP1t protein is an ideal candidate vaccine, since it is capable of inducing humoral immunity as well as cell-mediated immunity in adult mice. However, strong antibody responses generated after immunization can only indicate good immunogenicity of the protein. Additional studies such as viral challenge should be performed in suitable animal models to evaluate the protective efficacy of Nt-VP1t against EV71 infection.

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