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ORIGINAL ARTICLE

Inactivated enterovirus 71 with poly-γ-glutamic acid/Chitosan nano particles (PC NPs) induces high cellular and humoral immune responses in BALB/c mice

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Abstract

Enterovirus 71 (EV71) is the major causative agent of hand-foot-and-mouth disease (HFMD) and many neurological manifestations. Recently, this virus has become a serious concern because of consecutive epidemics in the Asia-Pacific region. However, no effective vaccine for EV71 has been discovered except two EV71 vaccines which are being used in local communities of China. To develop a safe and efficient EV71 vaccine candidate, we generated inactivated EV71 and evaluated its efficacy with γ-PGA/Chitosan nanoparticles (PC NPs), which are safe, biodegradable and effective as an adjuvant. The subcutaneous administration of inactivated EV71 with PC NPs adjuvant induces higher levels of virus-specific humoral (IgG, IgG1, and IgG2a) and cell-mediated immune responses (IFN-γ and IL-4). Additionally, inactivated EV71 with PC NPs adjuvant induces significantly higher virus neutralizing antibody responses compared to the virus only group, and resulted in a long lasting immunity without any noticeable side effects. Together, our findings demonstrate that PC NPs are safe and effective immunogenic adjuvants which may be promising candidates in the development of more efficacious EV71 vaccines.

Introduction

Enterovirus 71 (EV71) is a non-enveloped, single strand positive sense RNA virus which has similar characteristics to poliovirus. It is classified within the species *Enterovirus A* genus *Enterovirus* of the family *Picornaviridae* [1]. Humans are the only known natural host of this virus and EV71 is a highly pathogenic virus causing sporadic outbreaks of HFMD (Hand-Foot-and-Mouth Disease) [2]. HFMD is characterized by usual fever, oral lesions, papulovesicular or maculopapular exanthem on the hands, feet, buttocks, knees and elbows. Children aged from 6 months to 5 years are at the highest risk of infection. Although HFMD is usually a self-limiting mild illness, severe complications can be associated with poliomyelitis-like acute flaccid paralysis, brainstem encephalitis, aseptic meningitis and severe systemic disorders like pulmonary oedema [3]. This virus was first found in California, USA in 1969 and is now a major public health issue across the Asia-Pacific region and beyond [2]. Because of this sudden significant increase in EV71 epidemics, it has attracted immense global health care attention.

There are a number of different approaches that have been taken to develop an effective treatment for EV71 infections. In particular, an inactivated whole virus vaccine approach has demonstrated advancements in recent years [4–9]. In addition to an inactivated whole virus vaccine, several other types of vaccines have also been evaluated for their efficacy. Arita *et al*. (2005) demonstrated that an EV71 mutant live attenuated vaccine (genotype A), which is characterized by attenuated neurovirulence and limited spread of virus,

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could confer effective protection against lethal EV71 infection. However, cynomolgus monkeys who were immunized with the vaccine still displayed mild neurological symptoms [10–12]. Initiatives to produce several types of EV71 subunit vaccine, using VP1 which contains the main neutralizing epitopes of EV71, have been attempted to address shortcomings associated with attenuated vaccines as well as problems such as reverse virulence [5]. VP1-expressing transgenic tomatoes, transgenic mouse milk and VP1 protein epitope-based peptide vaccines have also been evaluated as oral subunit vaccines [13, 14]. Furthermore, virus like particles (VLP) are emerging as vaccine candidates as they contain conserved, fully-conformational epitopes. VLPs pose no risk of virulence and EV71 VLPs have successfully been produced [15–17]. All the above approaches exhibit some positive effects in controlling EV71. In addition, inactivated virus vaccines have the potential to protect neonatal mice by passive transmission via serum from immunized adult mice and maternal antibodies [4, 5, 7].

Vaccine adjuvants are exceedingly important in enhancing vaccine immune responses. An ideal vaccine adjuvant should be biologically safe, highly immunogenic, easy to use, readily available and inexpensive. They should effectively deliver antigen to antigen presenting cells (APC) and improve the antigen processing and presentation by APCs. In addition, they need to induce the production of immunomodulatory cytokines and reduce the amount of antigen needed in the vaccine and the frequency of immunization required to confer protection. Currently, aluminum based mineral salts (alum) are widely used for clinical use. However, in spite of their immunogenicity and enhanced adjuvant properties, aluminum salts can cause several adverse effects, particularly in people with immunological and neurological complications [18–23]. In addition, alum adjuvant is known to be a weak stimulator of cellular immune responses. These issues suggest that it is important to explore alternative options for vaccine adjuvants.

Poly-γ-glutamic acid (γ-PGA) is a promising biodegradable polymer which is naturally produced by *Bacillus subtilis* isolated from Korean traditional fermented soybean paste, "*chungkookjang*". This substance is water soluble, anionic, biodegradable, edible and relatively high in molecular weight (> 2000 kDa). Currently γ-PGA is used in a diverse range of applications which including the food industry, cosmetics and drug delivery [24]. In particular, γ-PGA/Chitosan nano particles (PC NPs) have demonstrated efficient adjuvant capabilities in delivering different antigens [25–29].

In this study, we generated inactivated EV71 and evaluated its vaccine efficacy with γ-PGA/Chitosan nanoparticles (PC NPs). The subcutaneous administration of inactivated EV71 with PC NPs adjuvant induced specific humoral, cell-mediated immune responses and significantly higher virus neutralizing antibody responses in mice.

Material and methods

Cell culture

African green monkey kidney (Vero) cells (ATCC CCL-81) and murine macrophage (Raw264.7) cells (ATCC TIB-71) were maintained in Dulbecco's modified minimum essential medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% antibiotic/antimycotic (A.A) (Gibco, USA) at 37 °C with 5% CO_2 until use.

Murine bone marrow derived dendritic cells (BMDC) were prepared. Briefly, the femurs and tibia were collected from 6-8 week old BALB/c mice under aseptic conditions and washed with 70% alcohol followed by PBS washing. Each epiphysis was cut off and flushed with RPMI (Gibco, USA) medium to collect bone marrow cells. The cell suspension was filtered through a 40 µm cell strainer (BD Falcon, USA) and centrifuged at 1200 rpm for 3 min to collect the cell pellet. The collected cell pellet was then re-suspended with 200 µl of RBC lysis buffer to obtain RBC free bone marrow cells. The purified cells were then seeded in cell culture flasks and stimulated with RPMI medium containing GM-CSF and IL-4 and maintained for 6-7 days at 37 °C with 5% CO_2 until dendritic cells (DC) were formed.

Peripheral blood mononuclear cells (PBMC) were isolated from different vaccinated animals. Mice were anaesthetized and blood samples were collected by cardiac puncture and PBMC were separated as described previously [30].

Purification and inactivation of EV71

The Enterovirus 71 (TW98-4643) was kindly provided by the microbiology laboratory, Faculty of Medicine, University of Ulsan, South Korea. Virus was amplified in Vero cells containing serum free DMEM for 48-72 hrs. The amplified virus was then layered on a 20% sucrose column and ultracentrifuged at 36000 rpm for 6 hrs using an SW41 rotor (Beckman, USA). The purified virus pellet was then re-suspended in TNE buffer, filtered through a 0.22 µm syringe filter and the viral titer was determined using TCID50. The purified virus was then inactivated by adding 37% formaldehyde (Sigma, USA) at a 1:4000 (v/v) ratio and incubating at 37 °C for 14 days.

Preparation of γ-PGA/Chitosan nano particles

Preparation of the PC NPs was performed by a simple ionic interaction as described previously, with some modifications [24]. The low molecular weight (LMW) chitosan (Sigma–Aldrich) was first dissolved in distilled water and

then chloridized by adding HCl (pH 4). Then, the chloridized chitosan was treated with chitosanase to achieve a lower molecular mass (< 15 kDa). The high molecular weight (HMW) γ-PGA was prepared by collecting the supernatant from *B. subtilis* sub sp. Chungkookjang culture broth and mixing it with 3 volumes of ethanol to precipitate. The precipitated γ-PGA was lyophilized, dissolved in 10 mM Tris–HCl buffer (pH 7.5), treated with proteinase K, and dialyzed against distilled water. Following dialysis, γ-PGA was purified using an ion-exchange chromatography. First, a dialyzed γ-PGA sample was subjected to a Sep-Pak Plus Waters Accell Plus QMA cartridge (Millipore, USA) equilibrated with distilled water. After washing with distilled water, the cartridge column charged with γ-PGA was developed stepwise with NaCl solutions, from 0.1 to 1.0 M. For the preparation of PC NPs, chitosan was prepared at 0.98 mg/ml in 0.9% NaCl solution (pH 5-5.2) and HMW γ-PGA was prepared at 1.4 mg/ml in 0.9% saline (pH 6.2-6.8). After filtering both solutions, the chitosan (0.98 mg/ml) and γ-PGA (1.4 mg/ml) were mixed together into the agitated mixture by dripping. The particle size of NPs was evaluated using a dynamic light scattering (DLS) method with a Zetasizer Nano ZS (Malvern Instruments, UK).

Elisa

PC NPs treated BMDC and Raw264.7 cell culture supernatant were collected at 12 hrs and 24 hrs post treatment and IL-6, IL-12 and IFN-β secretion were measured using commercialized ELISA kits (BD biosciences, USA). The levels of anti-EV71 specific antibodies in the serum were detected by indirect ELISA. Plates were coated with 100 µl/well of 10^5 TCID50 inactivated EV71-4643 virus in carbonate-coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.8) and incubated at 4 °C overnight, which was followed by a 1hr room temperature incubation with 10% skim milk. Diluted sera (1:1000) from immunized mice were added into the plate and incubated for 1.5 hrs at room temperature. After three consecutive washes, horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibody (1:2000) was added into each well. The reaction was developed by 100 µl TMB substrate (3, 3′, 5, 5′-etramethylbenzidine) for 10 min in the dark and the reaction was terminated by adding 50 µl 2M H_2SO_4. The optical densities at 450 nm were determined with a microplate absorbance reader.

qPCR

Murine PBMC cells were collected and stimulated with inactivated EV71 virus. Total mRNA was isolated from cells using an RNeasy Mini Kit (Qiagen) and cDNA was synthesized using a commercially available reverse transcriptase kit (TOYOBO). The gene induction of different targets was quantified by quantitative polymerase chain reaction (qPCR) by using Quanti-tect SYBR Green PCR kit according to the manufacturer's instructions (Qiagen) and gene induction value was obtained using the $2^{-\Delta\Delta CT}$ method as described previously [31]. The primer sequences used for qPCR are displayed in Table 1.

Mice immunization

Female 6 weeks old BALB/c mice were purchased (Samtako, Korea) and grouped according to the experimental plan. Mice were housed in ventilated cages and maintained with pelleted feed and tap water *ad libitum*. Mice immunization was carried out with different treatment combinations as displayed in Table 2. Blood samples were collected from the retro-orbital sinus at different time points as described in the vaccination schedule (Fig. 2A). The collected blood samples were then centrifuged at 12000 rpm for 10 min at 4 °C and the serum was separated and stored at −20 °C until use. At day 42 following the first immunization, five mice from each group were sacrificed to collect splenocytes and PBMC.

Enzyme-linked immunosorbent spot (ELISPOT) assay

ELISPOT assay was performed as described previously [32] with some modifications. Briefly, ELISPOT plates (BD Bioscience, San Diego, USA) were coated either with gamma interferon (IFN-γ) or Interleukin-4 (IL-4) antibodies and incubated at 4 °C for overnight. Subsequently, wells were washed with appropriate buffers and blocked with RPMI media containing 10% FBS. Mice were euthanized and splenocytes were freshly isolated. Subsequent to blocking, plates were washed and splenocytes were added into wells (5×10^5 cells/well) along with either phytohemagglutinin 10 µg/ml (Gibco, USA) or heat inactivated virus (3 µg/ml). After

Table 1 Primer sequences used for qPCR

Primer Name	Primers Sequence
Mouse IFN-γ	5'-TCA AGT GGC ATA GAT GTG GAA-3' (forward) 5'-TGG CTC TGC AGG ATT TTC ATG-3' (reverse)
Mouse IL-2	5'-CCTGAGCAGGATGGAGAATTACA-3' (forward) 5'-GAACATGCCGCAGACAGAG-3' (reverse)
Mouse IL-10	5'-TGGCCCAGAAATCAAGGAGC-3' (forward) 5' -CAGCAGACTCAATACACACT-3' (reverse)
Mouse IL-4	5'-ACAGGAGAAGGGACGCCAT-3' (forward) 5'-GAAGCCCTACAGACGAGCTCA-3' (reverse)
Mouse β actin	5'-AGA GGG AAA TCG TGC GTG AC-3' (forward) 5'-CAA TAG TGA TGA CCT GGC CGT-3' (reverse)

Table 2 Vaccine composition and amount used in the differently vaccinated groups

Vaccinated Groups	Vaccine composition and amount per mouse (µl)			
	Alum adjuvant (10 µg/µl)	PC NPs (20 µg/µl)	Inactivated virus (10^8 TCID50/ml)	PBS
Alum adjuvant only	20	0	0	100
PC NPs only	0	60	0	60
Inactivated virus only	0	0	20	100
Alum adjuvant + Inactivated virus	20	0	20	80
PC NPs + Inactivated virus	0	60	20	40

24 hr incubation at 37 °C with 5% CO_2, detection antibodies (Biotinylated anti-mouse IFN-γ or IL-4) were added. Plates were then conjugated with streptavidin-HRP for one hour followed by washing and finally ELISPOT AEC substrate was added. At the time of optimum spot development, the reaction was terminated by adding distilled water and plates were dried at room temperature. Finally, the spots were enumerated by the ELISPOT plate reader (CTL-Immunospot S5 UV analyzer, Cellular technologies, USA).

Lymphoproliferation assay

Immunized mice were euthanized at 42 days post-immunization and splenocytes were collected. Then RBC-lysed splenocytes were cultured in flat-bottom 96-well plates either with EV71 inactivated virus (3 µg/ml) or phytohemagglutinin/PHA (10 µg/ml) for 72 hrs. The proliferation of EV71 specific lymphocytes were evaluated using MTT assay (Roche Applied Science, USA) as described previously [33].

Virus neutralization assay

Sera from vaccinated mice were heat inactivated at 56 °C for 30 min and serially two fold diluted with DMEM. The diluted sera were then mixed with an equal volume of 100 TCID50 EV71. The mixture was incubated at 4 °C for 18-24 hrs to neutralize the virus. Subsequently, 100 µl from the mixture was added into each well of 96 well plates containing Vero cells. After 4-5 days of incubation at 37 °C with 5% CO_2, the cytopathic effect (CPE) was observed and the TCID50 value was calculated. The 50% neutralization inhibition dose was calculated as the reciprocal of the serum dilution that yielded a 50% reduction in the virus titer using the Reed-Muench method [34].

Cell cytotoxicity assay

Vero cells or Raw264.7 cells were treated with different concentrations of PC NPs and cell cytotoxicity was assessed using the MTT cell cytotoxicity assay (Roche, USA) in Vero cells or by a trypan blue cell viability test in Raw264.7 cells.

Results

PC NPs induce cytokines in murine bone marrow derived dendritic cells (BMDC) and Raw264.7 cells

Previous studies have demonstrated enhanced immune responses with γ-PGA treatment. Specifically, γ-PGA induces TLR2 and TLR4 signaling pathways and upregulates cytokine production [35]. Here we demonstrate the ability of PC NPs to induce different types of cytokines in murine bone marrow derived dendritic cells (BMDC) and murine macrophages (Raw 264.7) in comparison to LPS or recombinant mouse IFN-β (mIFN-β) treatment. We assessed the cytokine induction by PC NPs (1 mg/ml) and compared the efficacy with 100 ng/ml of LPS or 1000 units/ml of mIFN-β treatment. As shown in Fig. 1A and 1B, the secreted IFN-β, IL-6 and IL-12 from PC NPs treated BMDC were comparable to levels induced by LPS treatment. The cytokine secretion by PC NPs treated Raw264.7 cells was also comparable to LPS or mIFN-β treatments. These results suggest that PC NPs possess a potent immune stimulatory effect which is a key feature of an effective vaccine adjuvant. These secreted cytokines would increase the activity of various APCs and enhance antigen presentation.

Evaluation of the stability and safety of PC NPs *in vitro*

Stability and biosafety are top priorities in the development of any vaccine adjuvant. γ-PGA and chitosan are naturally synthesized and biodegradable in nature. However, to confirm their suitability as a treatment in our study, we evaluated the level of cytotoxicity by treating Vero cells or Raw264.7 cells with different concentrations. According to the MTT assay, none of the tested PC NPs solutions (0.156 mg/ml to 10 mg/ml) showed cytotoxicity in Vero cells (epithelial cells) (Fig. 1C). Trypan blue cell viability assay also showed steady cell viability in Raw264.7 cells (immune cells) across the different concentrations that were tested (Fig. 1D). These results collectively demonstrate the high compatibility and safety of PC NPs for biological use.

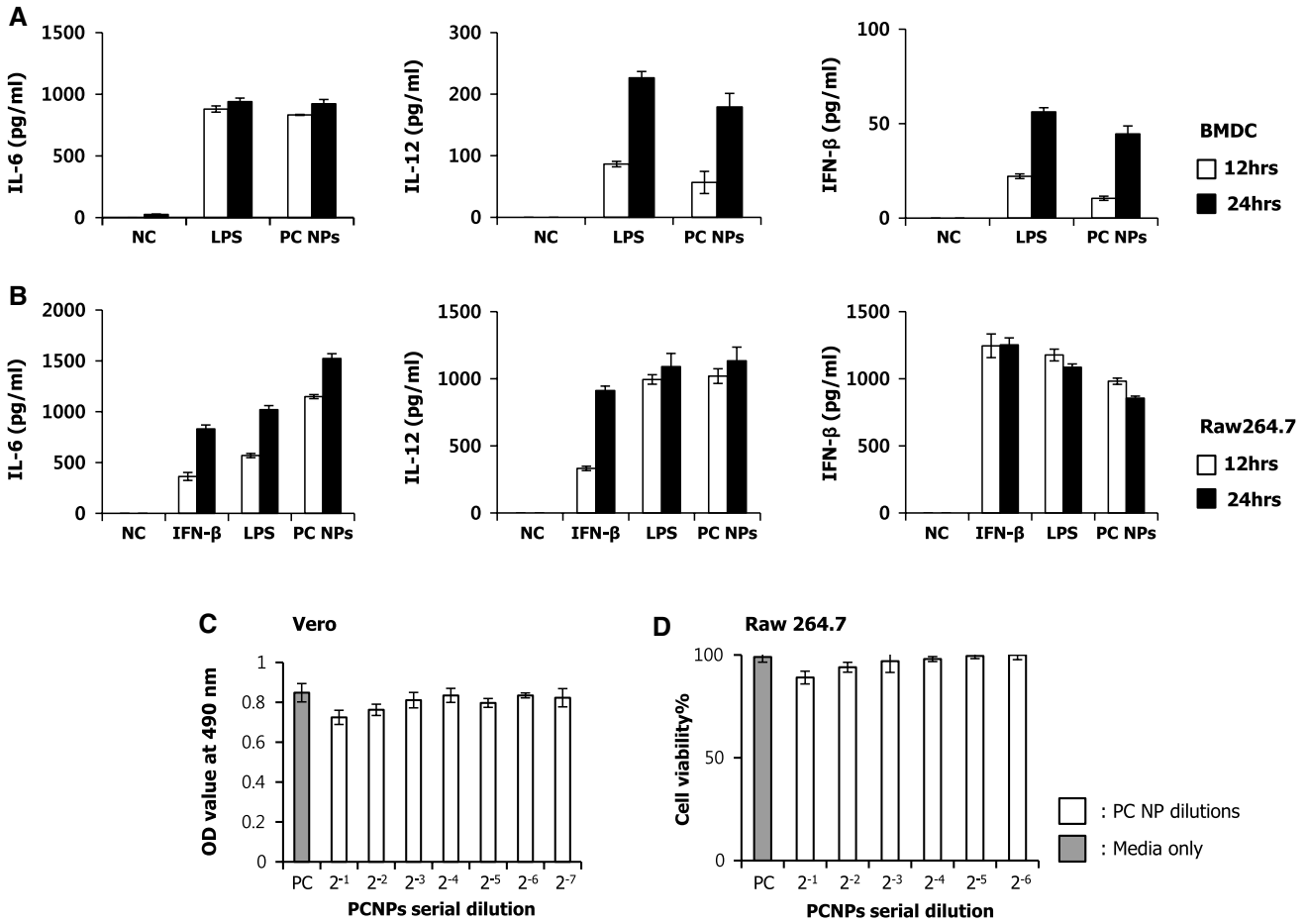

Fig. 1 Characterization of PC NPs *in vitro*. **A** and **B.** Cytokine induction in murine BMDCs and murine macrophages (Raw264.7) upon stimulation with LPS (100 ng/ml) or mIFN-β (1000 units/ml) or PC NPs (1 mg/ ml). At 12 hrs and 24 hrs post treatment, supernatant was collected and IL-6, IFN-β and IL-12 production was measured using ELISA, "x" axis denoted different treatment groups and "y" axis denoted cytokines induction (pg/ml). **C** and **D.** The cytotoxic effects of PC NPs in Vero cells and Raw264.7 cells in response to serial concentrations of PC NPs (10 mg/ml to 0.156 mg/ml) were measured by MTT assay and Trypan blue exclusion test respectively. Cells treated with DMEM containing 10% FBS + 1% A.A were used as the positive control. Bars denote mean ± S.D

Mice vaccinated with inactivated EV71 virus and PC NPs trigger higher humoral immunity

For these experiments we first purified and inactivated EV71 (see material and methods). Inactivation of EV71 was confirmed through the absence of cytopathic effect (CPE) *in vitro* (data not shown). To assess whether the mice vaccinated with inactivated EV71 and PC NPs could induce sufficient level of humoral immune responses, we measured the anti-EV71 serum IgG and its neutralizing activity against EV71. We compared these results with alum adjuvanted EV71 vaccine or with an inactivated virus only group. The immunization and serum collection was conducted as scheduled (Fig. 2A). Sera were diluted 1:1000 and indirect ELISA was performed to measure the anti-EV71 specific serum IgG level in different groups. As shown in Fig. 2B, during the 8th week of the first vaccination, all groups reached their highest serum antibody levels and both alum and PC NPs adjuvanted groups induced similar levels of total serum IgG, significantly more than the virus only group ($P < 0.05$). To determine the virus neutralizing activity in the sera of vaccinated animals, the same sera were serially (two-fold) diluted and treated against 100 TCID50 of virulent EV71 virus (Fig. 2C). Interestingly, alum and PC NPs adjuvant groups elicited similar levels of anti-EV71 neutralizing activity reaching up to $\log2^{11}$-2^{12} while the virus only vaccinated group only achieved $\log2^{8}$. This elevated antibody level suggests that inactivated EV71 virus vaccine with a PC NPs adjuvant can induce sufficient levels of humoral immune induction, and these levels are comparable to the effects of alum adjuvant.

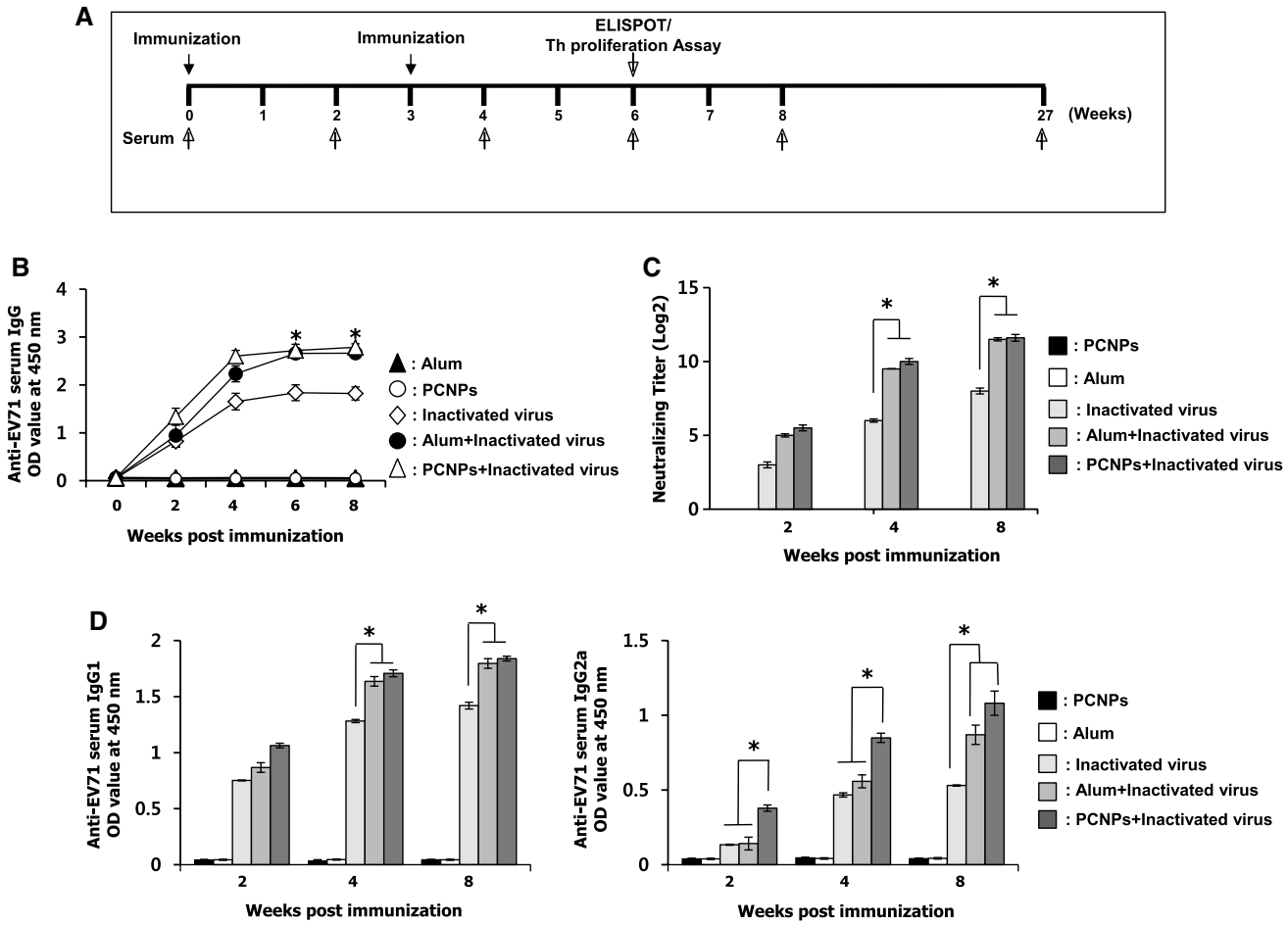

Fig. 2 Humoral immune activation in vaccinated mice. **A.** A schematic representation of the animal experiments. The mice were grouped and received subcutaneous vaccinations as mentioned in Table 2. The close arrows (pointing down) indicate immunization. The open arrows pointing down indicate the time of ELISPOT and Th cell proliferation assays. Similarly, the open arrows pointing up indicate the time of serum collection. Sera were collected on weeks 0, 2, 4, 6, 8 after the 1st vaccination and at week 24 (6-months) following the 2nd vaccination (for long lasting antibody, as shown in fig. 4). Spleens and PBMC were collected on week 6 after 1st vaccination to evaluate cellular immune responses and Th cell proliferation (shown in fig. 3). **B.** Anti-EV71 serum IgG levels for the differently vaccinated animal groups. Sera were collected every 2 weeks for up to 8 weeks and tested for anti-EV71 IgG antibodies using indirect ELISA, "x" axis denotes weeks post immunization while the "y" axis denotes anti-EV71 serum IgG OD value at 450 nm. **C.** The same heat inactivated sera were tested for neutralizing activity against 100 TCID50 of pathogenic EV71. The 50% neutralization inhibition dose was calculated as the reciprocal of the serum dilution that yielded a 50% reduction in the virus titer using the Reed-Muench method, "x" axis denotes weeks post immunization while the "y" axis denotes EV71 neutralizing titer. **D.** The sera of vaccinated animals from the 2nd, 4th and 8th week after 1st vaccination was evaluated for IgG iso-types. IgG1 and IgG2a were tested by indirect ELISA with the "x" axis denoting weeks post immunization and the "y" axis denoting anti-EV71 serum IgG1 and IgG2a levels respectively. Bars denote mean ± S.D. The asterisks indicate significant differences between groups ($p > 0.05$)

Iso-typing of serum IgG of vaccinated mice

Having shown that the PC NPs adjuvanted EV71 vaccinated group demonstrated a high cell mediated immune response, we evaluated the specific IgG iso-types in the sera of vaccinated mice. As shown in Fig. 2D, both PC NPs and alum adjuvanted EV71 vaccinated groups showed higher IgG1 levels compared to IgG2a. However, IgG2a induction in the PC NP group was greater than in the alum group or virus only group. Since IgG2a level in sera is a good indicator of Th1 cell function, these findings suggest that PCNP is able to efficiently activate Th1 subset cells, in comparison to other vaccine groups.

The inactivated EV71 vaccine candidate with PC NPs induced higher cytotoxic T-lymphocyte (CTL) responses

An effective vaccine should ideally induce high cellular and humoral immune responses to defend against

pathogenic infections. Notably, cell mediated immune responses are important to eliminate intracellular pathogens by killing infected cells. Therefore, we investigated the Th1 cell secreting IFN-γ levels in different vaccinated groups by stimulating splenocytes, either with inactivated virus or mitogen (positive control) (Fig. 3A). Our results show that mice immunized with antigen and PC NPs could induce significantly higher levels of IFN-γ when compared to the virus only vaccinated group ($p > 0.05$) (Fig. 3A). These elevated IFN-γ levels reflect elevated $CD8^+$ T cell (CTL) activity and subsequent rapid clearance of infected cells. Additionally, we performed the IL-4 ELISPOT assay to check for Th2 cell activity. The group of mice with inactivated virus mixed with PC NPs showed a higher induction of IL-4 compared to the virus only-vaccinated group but not the alum adjuvanted group. Based on these results, we suggest that our vaccine can induce efficient activation of humoral and cell mediated immune responses in immunized mice.

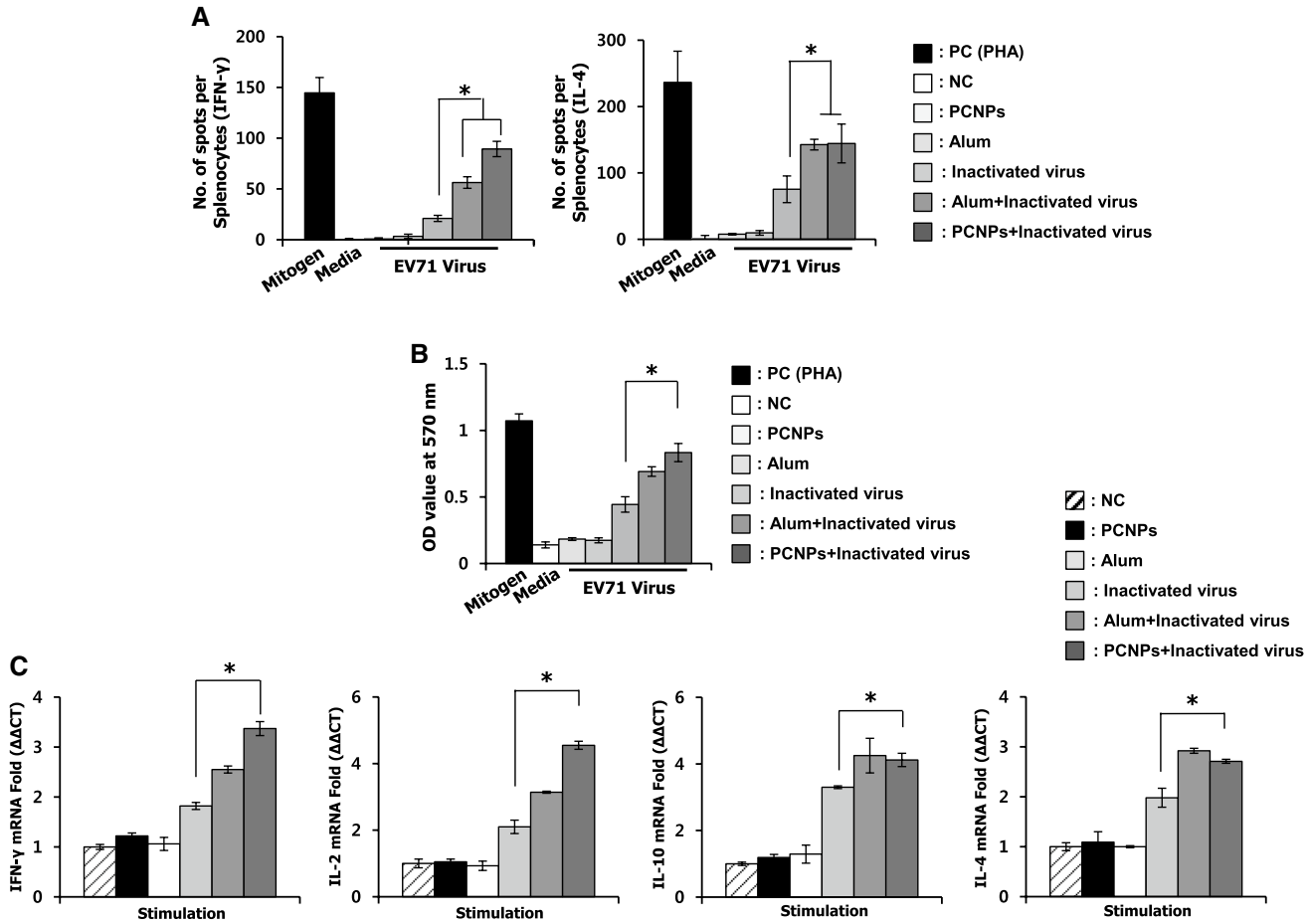

Fig. 3 Cellular immune responses in vaccinated mice. **A.** IFN-γ and IL-4 ELISPOT assay. Splenocytes from vaccinated mice were collected at 6 weeks post vaccination and re-stimulated either with mitogen (Phytohaemagglutinin/ PHA) or with heat inactivated EV71 for 24 hrs and IFN-γ and IL-4 secretion from T-lymphocytes were evaluated by ELISPOT assay. DMEM only media was used as a negative stimulator. The number of spots per well were enumerated using the ELISPOT plate reader, with the "x" axis denoting stimulation of splenocytes from vaccinated mice and the "y" axis denoting the number of spots per 5×10^5 splenocytes. **B.** Lymphoproliferation assay. Splenocytes from mice were collected at 6 weeks post vaccination and stimulated either with PHA or heat inactivated EV71 for 3 days. Cell proliferation was detected using MTT cell proliferation assay kit by measuring the absorbance at 570 nm. The "x" axis denotes stimulation of splenocytes from vaccinated mice while the "y" axis denotes OD value at 570 nm. **C.** Gene induction of Th1 related cytokines (*IFN-γ* and *IL-2*) and Th2 related cytokines (*IL-10* and *IL-4*). PBMCs of vaccinated mice were collected at 6 weeks after vaccination using ficoll-paque density gradient centrifugation. Isolated PBMCs were cultured in 24 well plates and stimulated with inactivated EV71 for 24 hrs. PBMCs from non-vaccinated mice were used as a negative control. Total mRNA was extracted and cDNA was synthesized using reverse transcriptase. *IFN-γ, IL-2, IL-10* and *IL-4* mRNA relative fold induction was assessed using qPCR analysis. The "x" axis denotes re-stimulators of PBMC from vaccinated mice with inactivated EV71 virus while the "y" axis denotes mRNA fold values. Bars denote mean ± S.D. The asterisks indicate significant differences between groups ($p > 0.05$)

Mice vaccinated with inactivated EV71-PC NPs elicit an enhanced T lymphocyte proliferation

To investigate the antigen specific T lymphocyte proliferation in vaccinated animals, splenocytes were collected at 6 weeks after immunization (3 weeks after the 1st boost) and assessed for antigen specific T cell proliferation. PHA stimulation was used as a positive control for general polyclonal responses to nonspecific antigen stimulation. As shown in Fig. 3B, mice vaccinated with inactivated virus mixed with PC NPs showed a high EV71 specific T cell proliferation. As the strength of the T cell response to the *ex vivo* proliferation assay is an indicator of $CD4^+$ T cell activation, this result suggests that the PC NPs adjuvant is able to activate T cells and thus induce cellular immunity.

PBMCs from mice vaccinated with PC NPs-adjuvanted vaccine showed high induction of Th1 and Th2 cytokine mRNAs

To further evaluate the function of our vaccine with PC NPs on the cellular and humoral immune responses, we evaluated Th1 and Th2 related cytokines at the mRNA level. For this purpose, we collected the PBMCs of vaccinated animals at 6 weeks after vaccination, cultured them in 96-well plates and re-stimulated them with inactivated EV71 virus. After 12 hrs stimulation, total mRNA was extracted, cDNA was synthesized and then analyzed using qPCR. *IL-2* and *IFN-γ* gene induction were measured as a marker of Th1 activation, while *IL-10* and *IL-4* genes were measured as a marker of Th2 activation. Our results demonstrated that PBMCs from mice vaccinated with the PC NPs-adjuvanted vaccine group had high levels of Th1 and Th2 related cytokine gene induction, when compared to virus only vaccinated group (Fig. 3C). These results suggest that our vaccine can induce higher cellular and humoral immune responses.

PC NPs-adjuvanted EV71 vaccine can induce long-lasting immunity

The duration of immunity conferred by a vaccine is a key aspect of its effectiveness and potency. We measured the longevity of our vaccine response by assessing serum IgG levels and neutralizing capability 6 months after final vaccination. As shown in Fig. 4A and 4B, the PC NP-adjuvanted EV71 vaccine induced high levels of serum IgG and high neutralizing activity, comparable to the effects of alum-adjuvanted vaccine. Furthermore, the IgG iso-types within the vaccinated animals demonstrated that the PC NPs-adjuvanted EV71 vaccine could maintain comparatively higher levels of IgG2a for a longer period demonstrating the ability of our vaccine to activate Th1 subset cells for a prolonged duration (Fig. 4C).

Discussion

Since the first discovery of vaccine adjuvants in the 1920s, adjuvants have been widely used in different vaccines to enhance their efficacy [36]. These vaccine adjuvants are important in increasing antibody responses, enhancing cellular immunity, reducing the dose of antigen required, reducing the frequency of vaccination and boosting immune responses in young or elderly people, especially those who respond poorly to vaccines. Although many vaccine adjuvants have been discovered, very few of them are able to use in humans due to safety concerns. There are many controversial ideas concerning commercially available, FDA-approved vaccine adjuvants such as aluminum salts, and many studies are underway to determine the safety risk of those adjuvants [21]. Insoluble aluminum salts (alum) have been widely used in human vaccines for many decades. These alum adjuvants can absorb antigens, stabilize and slowly release to enhance the immune system for longer periods, making them an efficient delivery system. However, alum induces relatively weak cell mediated immune responses. Additionally, aluminum is a known heavy metal and some studies have proven its side effects as a neurotoxin or immunocompromising agent [18, 20, 37–41]. Furthermore, the mechanism of action of alum is not fully understood [42]. Therefore, it is worthwhile to conduct in-depth safety assessments and explore other low risk alternatives for vaccine adjuvants.

Poly-gamma-glutamic acid is an anionic biodegradable substance and an edible capsular polymer secreted by *B.subtilis* which is generally regarded as a safe organism [43]. Currently γ-PGA is used in a diverse range of applications: in the food industry as a health food, thickener and stabilizer; as a moisturizer in cosmetics; a chelating agent in waste water treatment; as a hydrogel (especially super absorbent polymer, SAP) for environmental, agricultural, and biomedical product applications; as a biodegradable packing material; for drug delivery; as an osteoporosis preventing factor; a gene vector; curative biological adhesive; dispersant and enzyme immobilizing material [24]. Besides these applications, it has been used as a potent immunomodulation agent. Chitosan is also a cationic polysaccharide derived from chitin and is a safe and biocompatible polymer. Chitosan has already been tested as a potential nano carrier for delivering antigens in animal models and has been shown to promote effective immune responses [44–47].

In particular, γ-PGA/Chitosan nano particles (PC NPs) have demonstrated efficient adjuvant capabilities. Previously we reported the biological functions of PC NPs as effective mucosal adjuvants and evaluated the protective ability of recombinant proteins with PC NPs as influenza

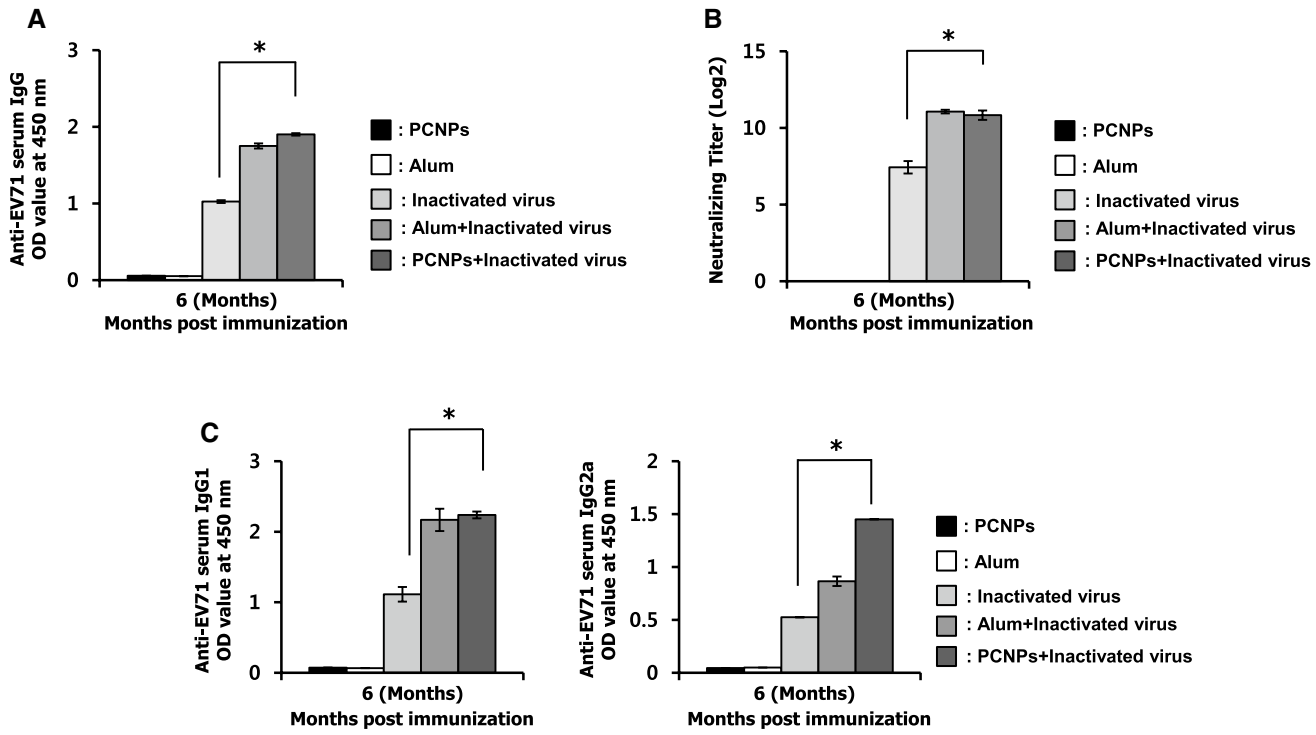

Fig. 4 Long-lasting immune induction following vaccination. **A.** Long-lasting anti-EV71 serum IgG antibody levels in vaccinated mice. Six months after the 2nd vaccination, sera were collected and tested for anti-EV71 IgG antibodies using indirect ELISA. The "x" axis denotes vaccinated groups 6 months after the 2nd vaccination while the "y" axis denotes anti-EV71 serum IgG OD values at 450 nm. **B.** The same sera were tested for neutralizing activity against 100 TCID50 of pathogenic EV71; "x" axis denotes vaccinated groups 6 months after the 2nd vaccination while the "y" axis denotes EV71 neutralizing titers. **C.** Serum IgG isotyping of vaccinated mice. Sera from mice 6 months after the 2nd immunization were collected and IgG1 and IgG2a isotypes were tested; "x" axis denotes vaccinated groups 6 months after the 2nd vaccination and "y" axis denotes anti-EV71 serum IgG1 and IgG2a levels respectively. Bars denote mean ± S.D. The asterisks indicate the significantly different groups ($p > 0.05$)

vaccine candidates [27, 28]. In the current study, we used PC NPs as a safe and effective adjuvant for an inactivated, whole EV71 virus, vaccine candidate administered subcutaneously - determining its effectiveness by assessing different immune responses.

First, we confirmed that PC NPs induce various inflammatory cytokines and type I interferon in APCs (Fig. 1A). High secretion of IL-12 can induce IFN-γ secretion by T cells and activate cytotoxic T lymphocytes, macrophages, NK cells and Th1 cell differentiation [48]. Moreover, the activated CTLs and NK cells continue to secrete IFN-γ, leading to macrophage activation which kill pathogens through phagocytosis [49]. In addition, increased levels of type I interferons result in the dampening of viral replication by activating several interferon stimulatory genes (ISGs) and enhancing antiviral protein production. Previously, Liu *et al.* (2005) showed type I interferons can protect mice against lethal EV71 infections [50]. In addition, type I interferons can enhance MHC class I molecules and activate $CD8^+$ T cells. The activation of these various signaling pathways and cell mediated immune responses results in the rapid clearance of intracellular pathogens [51]. Based on our results, the PC NPs-adjuvanted group clearly displayed enhanced cell mediated immune responses when compared to the alum adjuvant group (Fig. 3A). These results are consistent with the results of previous studies published by Okamoto et al which showed significantly higher cellular immune responses with PCNPs when they used it together with an influenza hemagglutinin vaccine [52]. Intriguingly, Chang et al. (2006) demonstrated that induction of the Th1 cellular response is critical for EV71 vaccine development against severe infections. This is because pathogenic EV71 infections can drastically reduce host cellular immune responses, especially in younger children who are genetically determined with low levels of cellular immunity [53]. Therefore, our EV71 vaccine candidate, with PC NPs, could address these issues, particularly during active outbreaks, as it can effectively induce cell-mediated immunity.

Induction of humoral immunity is also a main priority for many vaccines. This controls extracellular pathogens through antibody binding and neutralization. Our virus neutralization assay showed the PC NPs-adjuvanted EV71 vaccinated group could neutralize pathogenic EV71 activity as effectively as the alum-adjuvanted group (Fig. 2C).

According to Cao *et al*. (2013), the IgG1 subclass is the main player in EV71 virus neutralizing activity [54]. This explains the similar neutralizing capacity of the alum and PC NPs vaccines as they induced similar levels of IgG1 (Fig. 2D). The elevated Th2 activity reflects the enhanced IgG1 levels in both groups. IL-4 and IL-10 are the main inducers of Th2 cell development from naive $CD4^+$ T helper cells. Interestingly, we observed comparable IL-4 and IL-10 induction in re-stimulated PBMCs in the vaccinated groups (Fig. 3C).

The development of a commercial EV71 vaccine is a significant milestone. For the EV71 vaccine, enhanced delivery options such as nano-particles and superior immunogenic adjuvants with less or no adverse effects are required. Consequently, in this study we evaluated the efficacy and the immunogenicity of PC NPs as a potent adjuvant for the EV71 vaccine. PC NPs may be promising candidates in the development of more efficacious EV71 vaccines.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants by any of the authors. All animal experiments were conducted strictly in accordance with Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) with the approval of Institutional Animal Care and Use committee of Bioleaders Corporation, Daejeon, South Korea. (reference number: BSL-ABSL-14-000).

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