

## Supplementary Material:

### Materials and Methods

#### Cell lines, media and viruses.

RD cells (human-derived rhabdomyosarcoma cells) were obtained from American Type Culture Collection (ATCC), cultivated in Minimal Essential Medium (GIBCO) supplemented with 10% FBS (PAA) plus 2mM L-glutamine, 100U of penicillin, and 100 µg of streptomycin per ml, and used for preparation of EV71. The EV71 virus were loaded onto a 15-50% continuous sucrose gradient, which resulted in fractions with densities at 20-40% after 3 h ultracentrifugation (32,000 × g, SW41Ti rotor, Beckman). The fractions were collected, pelleted (100,000 × g for 2 h) and re-suspended in PBS. The purified virus was measured for the protein content using the BCA protein assay (Bio-Rad), and was stored in a -80°C freezer. Serially diluted virus samples (from 10<sup>-1</sup> to 10<sup>-10</sup>) were added to RD cells in 96-well plates, and four wells were used at each dilution. The 96-well plates were incubated for 7 days at 37°C, 5% CO<sub>2</sub> and cytopathic effects (CPE) was observed using an inverted microscope. The 50% tissue culture infectious doses (TCID<sub>50</sub>) values were measured by determining CPE and calculated according to the Behrens-Kärber method.

#### Monoclonal antibodies.

Anti-EV71 specific antibodies were produced by our laboratory.<sup>1</sup> A group of 6 Female BALB/c mice (6-8 weeks) were immunized subcutaneously with activated EV71 strain 52-3 (10<sup>4</sup> to 10<sup>5</sup> TCID<sub>50</sub>) emulsified in Freund's complete adjuvant. Two booster doses with the same 50% emulsion with Freund's incomplete adjuvant were delivered to the mice at two-week interval. The same antigen in PBS was directly injected into spleen of mice with the highest serum antibody titers against immunogen 3 days prior to cell fusion. The fusion of spleen cells with mouse myeloma cell was done as described.<sup>2</sup> Fusion of splenocytes with Sp2/0Ag-14 myeloma cells (University of Pavia, Lombardy, Italy) was performed using methods described in detail elsewhere<sup>2, 3</sup> with some modifications. Briefly, hybridomas were selected with GiBCO RPMI1640 plus hypoxanthine-aminopterinthymidine and 20% fetal bovine sera, and supernatants were screened by indirect ELISA or neutralizing test against EV71. Positive clones were further grow in GiBCO RPMI1640 plus 10% FBS for colonial separation. Stable cell lines capable of producing monoclonal antibodies were cultured first using 24-well plates, and then by 100 mL cell culture flask for further amplification. Cells collected from the cell flask were injected into a mouse abdominal cavity. Ascitic fluid was extracted from the mouse after 7-10 days. Purified MAbs were got from ascitic fluid by precipitating with 50% ammonium sulfate, dialyzing with phosphate buffer (PB) at pH 7.4 and purifying with DEAE column by HPLC. EV71-specific monoclonal antibodies were conjugated with horseradish peroxidase (HRP) by the NaIO<sub>4</sub> oxidation method followed by gel filtration chromatography on a Superdex 200HR column. The purified antibodies were saved at -20°C.

### Recombinant EV71 viral proteins.

Viral RNA was extracted from the culture supernatant of the EV71 strain 52-3 infected cells using Trizol reagent (Invitrogen). The cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) with three sets of reverse primers (Table 1). The purified PCR products were restricted by Nde I and Xho I enzymes (TaKaRa, Japan) and cloned into the expression plasmid vector. The recombinant plasmids were verified by sequencing. For cloning and expression experiments, *E.coli* strains of DH5 $\alpha$  and ER2566 (NEB, American) were used. The plasmids to express recombinant VP proteins were transformed into *E. coli* ER2566. After 6 h of induction with 0.2 mM isopropylthio- $\beta$ -D-galactopyranoside (IPTG) at 37°C, cells were centrifuged (9,000  $\times$  g for 5 min) and collected in buffer (20 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0) prior to SDS-PAGE and western blotting analysis. The recombinant EV71 viral proteins were purified by electroelution combined with SDS-PAGE. The concentrations of the recombinant EV71 antigens were determined using a BCA protein assay, and antigens were saved at -20°C.

### Production of recombinant ferritin nanocages.

The vector ferritin plasmid was engineered introduce NcoI and XhoI restriction sites flanking the normal start and stop codons, respectively.<sup>4</sup> A two-step engineering method was used on the pRSF/Ferritin plasmid. Firstly, primers (table 1) were designed to induce BamH I and EcoR I on the N-terminal, C-terminal and Loop zone (163 position) of ferritin, also with a linker next to the digest point. Then, to insert the displayed peptides, primers were designed as below (table 2). The double digested plasmids were inserted with the three nucleotide sequences encoding three different EV71 peptides. The plasmids were used to transform *E. Coli* BL21 (DE3). The expression and purification progress of the vector ferritin and nine engineered ferritins are all the same. A 1-L LB-kanamycin (50 ug/mL) culture of *E. coli* BL21 (DE3), was grown at 37 °C to an OD600 of 0.8, then induced with 1mM IPTG at 37 °C for 4 h. After sonication, the cell lysate was centrifuged at 10,400 rpm (12,930 g) for 30 min to remove cell debris. Supernatant were heated at 60 °C for 10 min. The resultant supernatant was then subjected to size exclusion chromatography with a Superose 6 column to yield the purified proteins.

The purified recombinant proteins were analyzed by 12% SDS-PAGE and were stained by Coomassie blue. Dynamic light scattering assay was performed to determine the size of different ferritin nanoparticles. Ferritin nanoparticles also were analyzed by negative staining electron microscopy. Briefly, Samples were absorbed onto a 200 mesh carbon-coated copper grids for 5 min. Then the grids were washed twice with ddH<sub>2</sub>O and air-dried. Specimens were evaluated by Tecnai Spirit (T12) transmission electron microscope.

Table 1 Primers for peptide display and protein expression.

Primer		Sequence
Amino-terminal	Foward	ATATACCATGGGATCCTTTTTTGAATTCGGTGGTGGTTCT GGTGGTGGTGGCACCACCGCGTCTCCCTC-3'

Carboxyl-terminal	Reverse	5'-CCAGACTCGAGTTAGCTCTCATCACCGTGT-3'
	Forward	5'-ATATACCATGGGCACCACCGCGTC-3'
Loop	Reverse	5'- CTCGAGTTAGAATTCAAAAAAGGATCCACCACCACCAGA ACCACCACCGCTCTCATCACCGTGTCCCAGGGT-3'
	Forward	5'- CTTACGCAAGATGGGAGCCGGTGGTGGTTCTGGTGGTGG TGGATCCTTTTTTGAATTCGGTGGTGGTTCTGGTGGTGGT CCTGAATCTGGCATGGC-3'
	Reverse	5'- GCCATGCCAGATTCAGGACCACCACCAGAACCACCACCG AATTCAAAAAGGATCCACCACCACCAGAACCACCACCG GCTCCATCTTGCCTAAG-3'
	Forward	5'-CATATGGGAGATAGGGTGGCAGATG-3'
EV71-VP1	Reverse	5'-CTCGAGTTAAAGAGTAGTGATCGCTGTGC-3'

Table 2 Primers for protein expression vector engineering.

Primer	Sequence	Coded peptides	
EV71-1	Forward	GATCCAAACAGGAGAAAG	KQEK
	Reverse	AATTCCTTCTCCTGTTG	
EV71-2	Forward	GATCCGAGCACAAACAGGAGAAAGACTTGG	EHKQEKDL
	Reverse	AATCCAAGTCTTCTCCTGTTTGTGCTCG	
EV71-3	Forward	GATCCTATCCTACTTTTGGTGAGCACAAACAG GAGAAAGACTTGGAGTATG	YPTFGEHK QEKDLEY
	Reverse	AATTCATACTCCAAGTCTTCTCCTGTTTGTGC TCACCAAAGTAGGATAG	

#### *In vitro* neutralization assay<sup>5</sup>

RD cell monolayers were prepared at 400,000 cells/ml in MEM (GIBCO) supplemented with 10% FBS (PAA) and seeded at 40,000 cells per well into 96 well plates (NUNC). Serum samples were heat-inactivated at 56°C for 30 min. Two-fold serial dilutions from 1:8 to 1:2,048 were prepared in virus diluents (MEM). Each sample was challenged with 100 TCID<sub>50</sub> per well of the EV71 strain 52-3. After incubated at 37°C, 5% CO<sub>2</sub> for 1 h, the serially diluted samples were incubated with RD cells prepared in 96 well plates. The cultures in the 96-well plates were incubated at 37°C for 7 days, and the TCID<sub>50</sub> values were measured by CPE in infected cells. The neutralization titers were read as the highest dilution in over 50% CPE, taken as the average of the triplicates.

#### Enzyme linked immunosorbent assay (ELISA)<sup>6</sup>

The 96-well plates were coated at 4°C overnight with 1 µg/well of proteins. After washed with PBS containing 0.05% Tween 20, the plates were blocked with 0.05% Tween 20 and 1% bovine serum albumin in PBS for 2 h at 37°C. The mAb K8G2 to peptides (1:1,000), the serum samples using 10-fold dilution series, and the first dilution was 100-fold, added to the wells, and were applied for 30 min at 37°C. Horseradish peroxidase-conjugated goat anti-mouse (GAM-HRP) IgG antibody was added into each well in a 1:5,000 dilution, and incubated for 30 min at 37°C. Visualization was done by incubation with o-phenyl-diamine-2HCl (10

µg/mL in 5 mM Tris-HCl, pH7.0) for 15 min, and the reaction was stopped by adding 50 µL of 2 M H<sub>2</sub>SO<sub>4</sub>. The wells were washed 5 times with PBST (0.05% Tween 20 in PBS) between each step. Absorption was measured at A<sub>450/620</sub>.

### **Mice immunization**

For immunization, six female Special pathogen free (SPF) BALB/c mice (6-8 weeks) per group were immunized subcutaneously (s.c.) with a 50% emulsion of Freund's complete adjuvant containing either 100 µg of conjugated recombinant proteins. Three booster doses in 50% emulsions with Freund's incomplete adjuvant were given at two weekly intervals. The immunized animals were bled at 0, 2, 4, 6, 8 weeks for the serological tests, and the serum was collected and stored at -80°C.

### **Passive protection test in mice<sup>1</sup>**

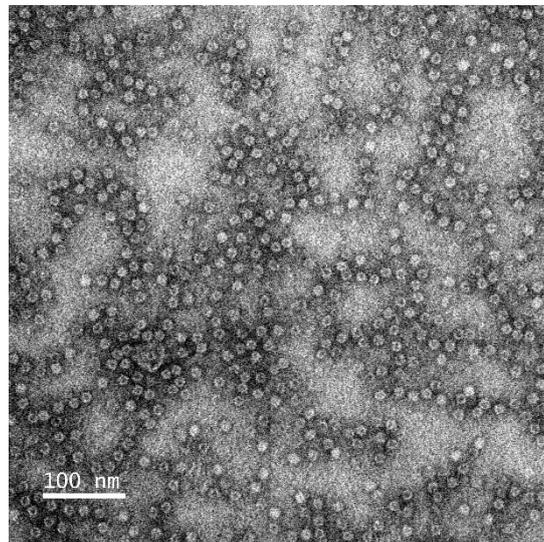
Inbred BALB/c mice were obtained from the Slac Laboratory Animal Co., Ltd., Shanghai, China. All institutional guidelines for animal care and use were strictly followed throughout the experiments. The research project "Ferritin nanocage-based antigen delivery nanoplatfroms: Epitope engineering for peptide vaccine design" has been approved by the Animal Management and Ethics Committee of Xiamen University. The Animal Management and Ethics Committee will monitor and regulate the animal experiments of this project in accordance with relevant state regulations.

Groups of mice (n=6) within day 1 after birth were challenged with 50 µL of EV71 (10<sup>7</sup> TCID<sub>50</sub> per mouse) intraperitoneally (i.p.). For sera protection, 50 µL of mice EV71-Fn immunized sera were administered i.p. 24 h later, suckling mice from control groups were given native ferritin immunized sera. Every group contained two independent experiments. Mice were monitored daily for body weight, clinical illness and death until day 20 post-infection. The grade of clinical disease was scored as follows: 0, healthy; 1, lethargy and inactivity; 2, wasting; 3, limb weakness; 4, hindlimb paralysis; and 5, moribund and death. The protected mice were healthy throughout the experiments.

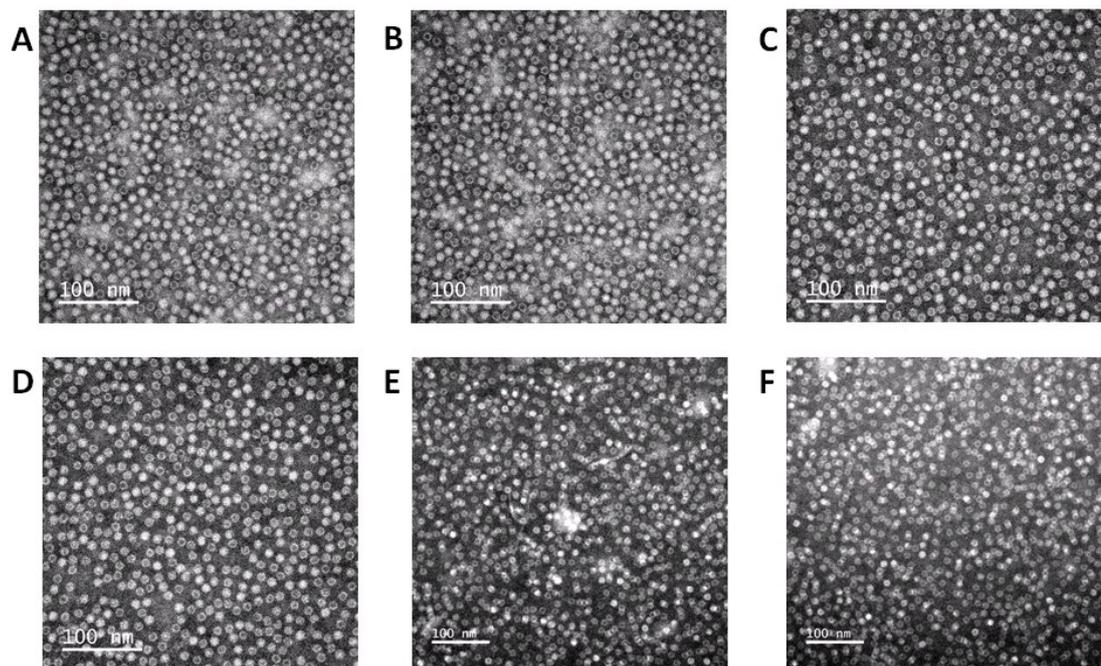
### **Histopathologic and immunohistochemical staining**

At day 5 to day 8 post-infection, challenged mice were subjected to histopathologic and immunohistochemical examination. Brain, spine, heart, liver, lung, intestines and limb skeletal muscles were separately harvested after euthanization, and then fixed by immersion in 4% PBS-buffered formalin for at least 72 h at room temperature. Fixed tissues were bisected, embedded in paraffin and sectioned on 4 mm thick. For histopathologic test, tissue sections were stained with hematoxylin and eosin. Immunohistochemical examination was performed by using Ultrasensitive TMS-P kit (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) and DAB Detection Kit (Streptavidin-Biotin; Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) according to manufacturer's recommendation. The primary antibody, I2D7, was a mouse anti-EV71 VP1 monoclonal antibody (1 mg/mL, 1:1,000 dilution).

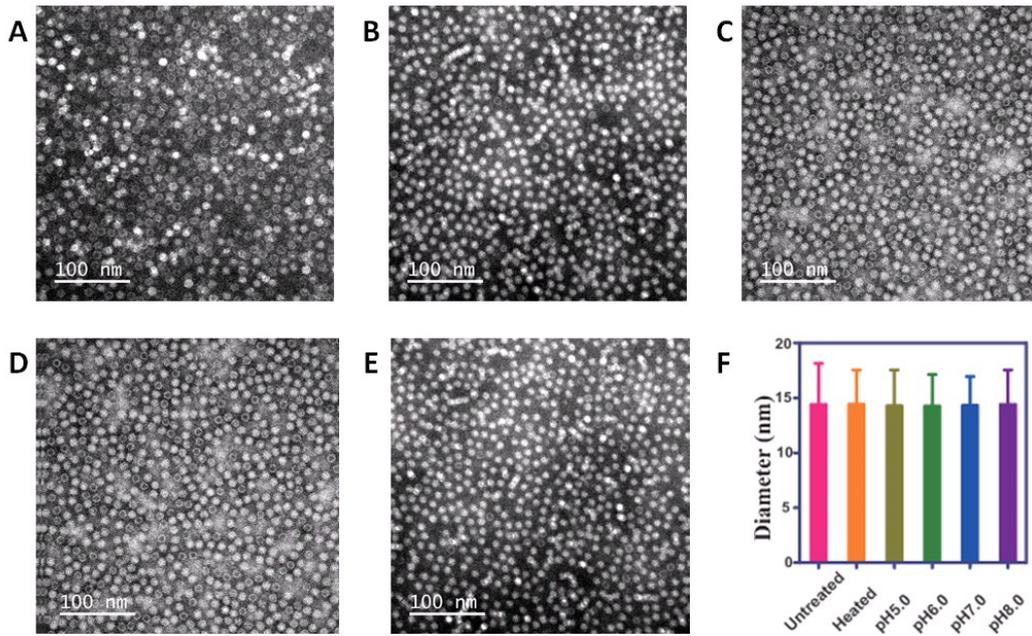
**Supplementary Figures:**



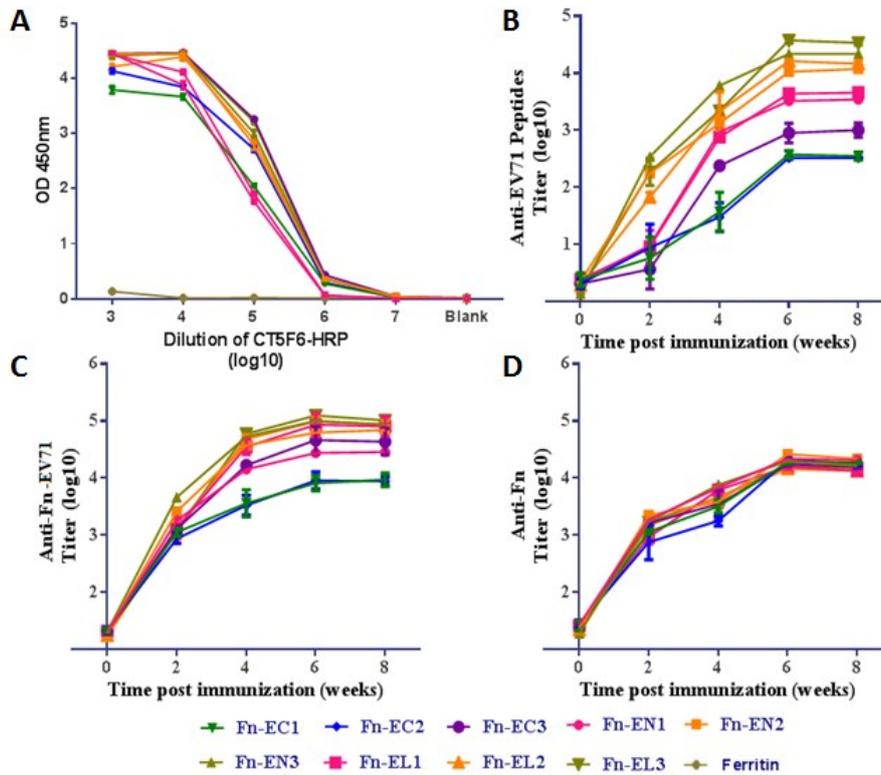
**Figure S1.** TEM image of native ferritin.



**Figure S2.** TEM of 6 ferritins. A. Fn-EC1. B. Fn-EC2. C. Fn-EN1 D. Fn-EN2. E. Fn-EL1. F. Fn-EL2.

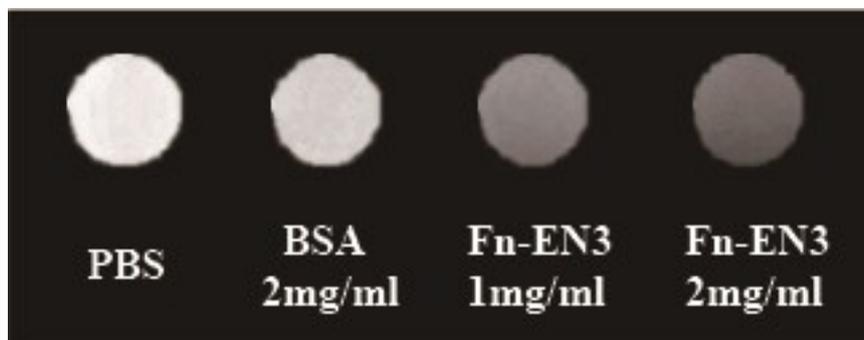


**Figure S3. Stability study of Fn-EN3.** A. TEM images of Fn-EN3 heated in 70 °C for 5 min. B. Dialyzed into different solutions MES pH 5.0. C. Phosphate buffer pH 6.0. D. Phosphate buffer pH 7.0. E. Tris-HCl buffer pH 8.0 F. DLS study of the treated five ferritins.



**Figure S4.** Dynamic changes of antibody titer in immunized mice. Ten groups of mice, each including 6 female BALB/c mice, were immunized subcutaneously with four injections of 100 µg of different recombinant proteins on weeks 0, 2, 4, 6 and were bled at 0, 2, 4, 6, 8 for the serological tests. (A) The reactivity between different ferritins and anti-VP1 (208-222aa) antibody. (B) rVP1 as coated protein to detect VP1-epitope specific antibodies. (C) Nine

ferritins as coated proteins to detect anti Fn-EV71 VP1 antibodies. (D) Native ferritin as coated protein to detect anti ferritin antibodies.



**Figure S5.** MR images of different proteins. MRI studies were performed using a 9.4T MR scanner, a commercially available volume coil (RF RES 1H 75/40 Q TR, Germany) of 40mm in diameter was used. T2-weighted TueboRARE images (TR 2,500ms; TE 33ms; FOV 4 × 4cm; slice thickness 1mm) were acquired from sagittal slices.

### Supplementary references

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