**Supplementary Materials**

**Nanoparticle vaccine based on the envelope protein domain III of JEV elicits robust protective immune responses in mice**

**SUPPLEMENTARY METHODS**

**DCs uptake of nanoparticle proteins in vitro**

Murine bone-marrow-derived DCs (BMDCs) were isolated from 6-week-old C57BL/6 mice as previously described [1]. Briefly, bone marrow cells were isolated from the tibia and femur of sacrificed mice and cultured in RPMI 1640 supplemented with 10% FBS, 10 ng/mL interleukin 4 (IL-4; R&D Systems, USA), 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D Systems, USA), and 50 μM β-mercaptoethanol (Sigma, USA), and 50% of the medium was replaced on 3 and 5 days. After 6 days of culture, loose and nonadherent cells were collected and transferred to 12-well plates at a density of 5 × 105 cells per well and cultured overnight.

Indirect immunofluorescence assay (IFA) was performed to detect whether LS and ED III-LS nanoparticles simulated DCs uptake as previously described [2]. 5×105 immature DCs in a 12-well plate were incubated with identical dose of LS, ED III, or ED III-LS for 4 h. After incubation, the cells were fixed with 4% formaldehyde for 20 minutes before blocking with 5% BSA. 6×His tag mAb (MBL, Japan) and Alexa Fluor 555 conjugated goat anti-mouse IgG antibody (Thermo Fisher, USA) were used to stain intracellular antigens, and 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei for 20 minutes. Images were recorded using a ZEISS Axio Scope microscope. At the same time, the supernatants were collected to further detect DCs cytokine secretion after treatment with PBS, LPS, LS, ED III or ED III-LS for 24 h, TNFα, IL12, and IFN-γ commercial kits (NeoBioscience, Shenzhen, China) were used according to the manufacturer’s protocol.

**Autologous mixed leukocyte reaction (MLR)**

To detect the allogeneic stimulatory activities of different antigen-treated DCs, naïve CD4+ T cells were isolated from the spleens of BALB/c mice with magnetic beads according to the manufacturer’s protocol (Thermo Fisher, USA)， and autologous mixed leukocyte reaction (MLR) was performed as previously described [1]. Briefly, BMDCs were treated with PBS, LPS, LS, ED III, or ED III-LS for 24 h at 37°C with 5% CO2, then co-cultured with naïve CD4+ T cells at a ratio of 1:10 in 96-well plate for 72 h. After that, 10 μL of CCK-8 reagent was added to each well and incubated for another 4 h at 37°C, The OD values of each well were measured at 450 nm to quantify T-cell activation. The stimulation index (SI) was calculated with the following equation: SI = (OD sample well / OD blank well) - (OD CD4+ T cell well/OD blank well).

**Histopathology and** **immunohistochemistry**

The brain tissues collected at 6 days post-challenge (dpc) were fixed in 10% neutral-buffered formalin and embedded in paraffin, then cut into 4-μm thick slices. After deparaffinization and [rehydration](https://www.sciencedirect.com/topics/medicine-and-dentistry/rehydration), the slices were stained with [hematoxylin](https://www.sciencedirect.com/topics/medicine-and-dentistry/haematoxylin) and [eosin](https://www.sciencedirect.com/topics/medicine-and-dentistry/eosin) (HE). For immunohistochemistry detection, sections were incubated with JEV-NS3-specific mAb (1:500, GeneTex, USA) for 18 h at 4°C. The secondary antibody (1: 5000, ABclonal, China) was covered in the section for 30 min at room temperature. Nuclei were counterstained with haematoxylin. Images were captured under a microscope.

1. Du P, Liu R, Sun S, Dong H, Zhao R, Tang R. Biomineralization improves the thermostability of foot-and-mouth disease virus-like particles and the protective immune response induced*. Nanoscale.* 11(47), 22748-22761 (2019).

2. Ren X, Qian P, Hu Z, Chen H, Li X. Genetic characterization of atypical porcine pestivirus from neonatal piglets with congenital tremor in Hubei province, China*.* *Virol J.* 19(1), 51 (2022).