

Extracellular Vesicle-Derived gD Protein Vaccine Platform: A Novel Approach to Developing Highly Preventive Vaccines against HSV1/2 Infection and Tumor Metastasis

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Abstract

Although no vaccines are currently available for HSV infection, the development pipeline is rich with candidates in various phases of preclinical and clinical trials. In this study, we introduced a novel preventive vaccine platform based on extracellular vesicles (EVs), representing a significant departure from traditional vaccine strategies. By developing PDGFRN-EV technology, we enabled the selective recruitment of viral membrane antigens onto EV surfaces. This innovative approach delivers HSV glycoprotein gD in its native conformational state within EV lipid membranes, ensuring the preservation of specific epitope structures. The resulting construct, EV^{gD-PDGFRN}, is rapidly absorbed by lymphoid tissues upon administration, where it activates dendritic cells and stimulates both humoral and cellular immune responses. Immunization with EV^{gD-PDGFRN} elicited robust virus-specific neutralizing antibodies, conferring complete protection against lethal HSV-1 and HSV-2 challenges in murine models, with survival rates of 100% and 91%, respectively. Notably, EV^{gD-PDGFRN} immunization also demonstrated efficacy in inhibiting the growth and lung metastasis of melanomas expressing the gD antigen *in vivo*. These results underscore the platform's ability to present and deliver viral membrane proteins in their native membrane-associated conformations. Together, these findings establish EV^{gD-PDGFRN} as a highly versatile and adaptable platform for developing vaccines against viral pathogens and cancers, offering broad potential for future therapeutic applications.

1 Introduction

Herpes simplex virus (HSV) belongs to the Herpesviridae family, a group of spherical viruses measuring 120–200 nm. There are two types of Herpes simplex viruses, Herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2)^{1–3}. These viruses cause lifelong infections that are among the most common viral infections worldwide. HSV is a highly prevalent pathogen, with global seroprevalence estimates ranging from 50–90% for HSV-1 and 10–20% for HSV-2 in adults. HSV infections typically manifest as orofacial or genital lesions, but can also lead to ocular and central nervous system complications as well known. Additionally, individuals with compromised immune systems due to other diseases, such as cancer or COVID-19 infection, are more susceptible to HSV infections^{2, 4–6}. These conditions significantly reduce the quality of life for patients. Current treatment relies primarily on antiviral drugs like acyclovir and valacyclovir, which can alleviate symptoms but do not provide a cure^{7, 8}. Prolonged antiviral use also raises concerns over the development of drug resistance. As such, the development of effective HSV vaccines has become a key research focus⁹. As part of the global effort to control the infections caused by these viruses, many vaccines have been developed; however, to date, none has been licensed for use in humans. It is with regret that GSK (GlaxoSmithKline)'s mRNA HSV vaccine (GSK3943104) did not meet the study's primary efficacy objective in Phase II development and this vaccine will not progress to phase III studies in Sep 11, 2024.

HSV vaccines can be categorized into different types^{10–12}, such as inactivated vaccines, protein subunit vaccines, replication-deficient vaccines, attenuated live vaccines¹³, DNA vaccines¹⁴, peptide vaccines¹⁵ and mRNA vaccine¹⁶. However, these vaccines may still be reactivated and undergo genetic recombination with wild-type strains, posing potential threats to immunocompromised individuals. The development of vaccines utilizing viral surface glycoproteins is currently a predominant strategy in the field, such as gD and gB, as the primary immunogens^{17, 18}. While there are 12 known HSV glycoproteins, gD and gB are the most commonly used in subunit vaccine candidates¹⁹. The gD/gB subunit vaccine developed by Chiron, combined with the MF59 adjuvant, was able to induce high-titer antibodies in 26% of women and provide protection for up to 6 months, but was ineffective in men. In 2007, a GSK subunit vaccine candidate, which included gD, gB and the accessory proteins gH/gL, demonstrated protection in 73% of HSV-1 and HSV-2 seronegative women, but not in men or HSV-1/HSV-2 seropositive women, likely due to anatomical differences between male and female genital tracts. Therefore, gD has emerged as a priority antigen for anti-HSV vaccine development.

Continued basic research and clinical trials are expected to yield safer and more effective HSV vaccine formulations, providing much-needed solutions for the global burden of this ubiquitous viral pathogen. In addition to these conventional vaccine strategies, extracellular vesicle (EV) vaccines are also emerging as a new approach, leveraging the unique properties of EVs^{20, 21}. EVs are nano-sized with a diameter of approximately 50–200 nm, membrane-enclosed structures secreted by various cell types, including immune cells, and they possess the inherent ability to carry diverse biomolecular cargo, including proteins, lipids, and genetic material²². The unique properties of EVs make them an attractive candidate for vaccine development^{23–26}. Firstly, EVs can present antigens in their natural state, which can present antigens to the immune system in a manner close to that of natural pathogen infection, which enables immune cells to recognize and

remember antigens more easily and generate more durable immune protection. Secondly, EVs can serve as natural adjuvants, stimulating robust immune responses without the need for additional adjuvants²⁷. EVs can effectively deliver antigens to antigen-presenting cells (APCs), such as dendritic cells and macrophages, facilitating the induction of both humoral and cellular immune responses. The membrane-bound nature of EVs protects the encapsulated antigens from degradation, ensuring efficient antigen delivery to target cells. Moreover, EVs can be engineered to display specific antigens or to carry immunomodulatory molecules, further enhancing their vaccine potential. This customizability allows for the development of tailored vaccines that can elicit targeted and potent immune responses against a wide range of pathogens, including emerging infectious diseases²⁸ and cancer²⁹. Compared to traditional vaccine approaches, EV-based vaccines offer several advantages³⁰. They are inherently biocompatible and biodegradable, reducing the risk of adverse reactions. Additionally, the small size and natural tropism of EVs for immune cells enable efficient cellular uptake and targeted delivery of vaccine components. Furthermore, EV-based vaccines can be produced in a scalable and cost-effective manner. EV-based vaccines hold the potential to revolutionize the way we prevent and treat a wide range of diseases³¹, paving the way for a new era in vaccine development.

While current HSV vaccine candidates focus on subunit proteins or mRNA platforms, our study pioneers an EV-based approach that preserves native antigen conformation, potentially overcoming limitations in immunogenicity and stability. For concept confirmation, HSV gD, a 395-amino acid envelope glycoprotein that plays a critical role in the virus entry process and is one of the most abundant antigens on the viral surface, was chosen as the viral antigen. It can induce high-titer neutralizing antibodies and effectively suppress the development of viral encephalitis. We engineered the intact gD protein to display on the surface of EVs and then explore its preventive effects against HSV infection. Notably, the EV vaccine displaying gD antigen on its surface demonstrated promising immunogenicity and protective efficacy, even in the absence of adjuvants. This novel vaccine strategy offers new hope for the prevention and control of HSV1/2 infections.

2 Materials and Method

2.1 Materials

The following reagents were obtained from various suppliers: RPMI 1640 medium and DMEM medium were purchased from Thermo Fisher (Massachusetts, USA); FBS was purchased from ExCell Bio (Suzhou, China); Dil was purchased from Beyotime (Shanghai, China); Pierce™ BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Massachusetts, USA); puromycin was purchased from Avantor (Pennsylvania, USA); restriction endonucleases and T4 DNA Ligase were purchased from NEB (Massachusetts, USA). *E. coli* DH5α competent cells were obtained from Takara (Tokyo, Japan), and human herpesvirus type 1 (HSV-1) F strain was a gift from the Guangzhou Institute of Biomedicine and Health.

2.2 Cell Culture

The HEK293T cells (SCSP-502, CAS, China) utilized in this study were sourced from the Typical Cultures Preservation Committee Cell Bank of the Chinese Academy of Sciences (CAS). Vero cells (CCL-81, ATCC, USA), THP-1 cells (TIB-202®TM, ATCC, USA), and the murine melanoma cell line B16F10 (derived from C57BL/6 mice) were obtained from the ATCC Cell Bank. All cell lines were cultured in either high-glucose DMEM (C11995500BT, Gibco, USA) or RPMI-1640 medium (C11875500BT, Gibco, USA), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. The cells were maintained in an incubator set at 37°C with 5% CO₂.

2.3 Plasmid construction

Plasmids pcDNA3.1-gD1-CD63, pcDNA3.1-gD1-PDGFRN-TM, pcDNA3.1-gD1-PTGFRNΔ687-TM, pcDNA3.1-gD1-LAMP2B and pcDNA3.1-gD1-GPI were constructed in our lab. Additionally, pcDNA3.1-gD1-PDGFRN and pcDNA3.1-gD were generated by cloning the gene encoding gD1 into their corresponding vectors. The plasmids pLVX-GFP-P2A-puro (H42827-1-W0618) and pLVX-gD2-P2A-puro (H42827-2-W0624) were constructed by Synbio Technologies company. All constructs generated were confirmed by Sanger sequencing from GENWIZ and Qingke company (Guangzhou, China). All the primers of laboratory-constructed plasmids in this study are described in Table 1.

Table 1
Primers used for construction of laboratory-generated gD plasmids

Plasmid	Primer (5′–3′)	Primer (5′–3′)	Restriction site
pcDNA3.1-gD2	CTTGGTACCATGGGGCGTTTGACCTCCG	CCGGAATTCGTAAAACAATGGCTGGTGCG	KpnI, EcoRI,
pcDNA3.1-gD2-PDGFRN-TM	CTTGGTACCATGGGGCGTTTGACCTCCG	GATTGTACATGTAAAACAATGGCTGGTG	KpnI, BsrGI
pcDNA3.1-gD1	TTGTGTACAGGGGGGGGCTGCCGCCAGGTT	CCGGAATTCGTAAAACAATGGCTGGTGCG	EcoRI, BsrGI
pcDNA3.1-gD1-PDGFRN-TM	CGGGGTACCATGGGGGGGGCTGCCGCCAGGTT	CGCGGATCCGTAAAACAAGGGCTGGTG	KpnI, BamHI

2.4 Construction of stable cell lines

Lentiviral particles were produced by co-transfecting HEK293T cells with three plasmids: transfer vector, packaging plasmid (psPAX2, Addgene #12260), and envelope plasmid (pCAG-VSVG, Addgene #35616) at a 3:2:1 ratio using PolyJet® transfection reagent (SigmaGen Laboratories). Culture supernatants were harvested 48 h post-transfection, filtered through 0.45 µm membranes. For transduction, B16F10 cells were seeded at 1×10^5 cells/well in 24-well plates and incubated with lentivirus in the presence of Polybrene. GFP expression was verified 72 h post-transduction using a fluorescence microscope (Nikon).

2.5 Isolation and purification of EVs from cell culture medium

When the HEK293T cells reached 70–80% confluence, 12 µg of the plasmid described above was transfected into the cells using polyethyleneimine (PEI, 49553-93-7, Polysciences, USA)^{32, 33}. After 6 hours, the transfection medium was discarded and replaced with 2% EVs-free serum medium. Following a 48-hour incubation, the supernatant was collected and subjected to a series of centrifugation steps: first, centrifugation at $300 \times g$ for 10 minutes, followed by $3,000 \times g$ for 10 minutes and $12,000 \times g$ for 30 minutes to remove macromolecular particles such as microbubbles and cellular debris. The resulting supernatants were then further centrifuged at $110,000 \times g$ for 75 minutes, and the EVs were resuspended in sterile PBS. All centrifugation procedures were conducted at 4°C. The EVs were stored in aliquots at -80°C until further examination. The concentration of proteins in the EVs or cells was determined using Pierce™ BCA Protein Assay Kits (23225, Thermo, USA).

2.6 Western blot assay

Purified EVs or cells (used as a positive control) were resuspended in RIPA buffer (KCP102-100, KeyGen BioTECH, China) containing protease inhibitors. Sample buffer was added to the lysate, which was then boiled at 105°C for 10 minutes. 10 µg of protein was loaded onto a 12% acrylamide gel (Tris-glycine/SDS electrophoresis buffer) and subjected to electrophoresis. After electrophoresis, the protein bands were transferred to a PVDF membrane and treated with a blocking solution (5% skim milk powder in wash buffer) for 1 hour at room temperature. The membrane was then washed with TBST and incubated with an HRP-coupled gD monoclonal antibody. EVs from HEK293T cells were used as a negative control. The EV samples were stained with the following HRP-conjugated primary antibodies and visualized by chemiluminescence: CD9 (D801A, Cell Signaling Technology, USA), CD63 (52090S, Cell Signaling Technology, USA), CD81 (52892S, Cell Signaling Technology, USA), Anti-HSV-1 + HSV-2 gD (ab6507, Abcam, UK). After completing the western blotting procedure, ImageJ software was employed to quantify the gray value of the resulting bands.

2.7 Nanoparticle tracking analysis (NTA) and Zeta potential analysis

EVs were diluted 1,000-fold in phosphate-buffered saline (PBS), mixed thoroughly, and aspirated³². The concentration and size distribution of EVs particles were measured using the ZetaView system (Particle Metrix, Germany). Three independent experiments were conducted for each sample, and the resulting data were processed and analyzed using ZetaView Analyze software. The expected particle size range for EVs was 30 to 150 nm. For the measurement of Zeta potential, EVs diluted in sterile PBS were placed in a Malvern zeta potential cuvette. The Zeta potential of the EVs was determined using a Zetasizer Nano (Malvern, UK).

2.8 Transmission electron microscopy (TEM) analysis of EVs

The resuspended EVs were deposited onto a 300-mesh copper mesh carbon support film (BZ110205a, China Mirror Science Instrument, China), and the residual liquid was subsequently removed³². The grids were stained with 3% phosphotungstic acid and incubated in 0.1 M hydrochloric acid (HCl) (Solarbio, G1871, China) for one minute, followed by a wash with purified water. Afterward, the grids were dried at room temperature and examined using a transmission electron microscope (JEOL-1400, Japan) to analyze the EVs. For immunocolloidal gold labeling, EV samples of appropriate concentration were placed on a copper mesh carbon support membrane and fixed with paraformaldehyde for ten minutes. The copper mesh was then blocked by incubation in 5% bovine serum albumin (BSA), filtered through a 0.22 µm microporous filter, for one hour. The grids were incubated overnight at 4°C with the primary antibody diluted to the appropriate concentration. Following this, the copper grids were rinsed with phosphate-buffered saline (PBS) and then incubated with colloidal gold particles (Au Nanoparticles, 15 nm) (R-G015005, Ruixibio, China) conjugated to suitable secondary antibodies at room temperature for 2 hours. The secondary antibodies were removed by washing the grids with PBS, followed by three washes for five minutes each. Finally, the grids were stained and dried with phosphotungstic acid and imaged using a transmission electron microscope.

2.9 Nanoparticle flow cytometry

The flow cytometry antibodies were each diluted tenfold in PBS. Then, the EV samples were exposed to the diluted antibodies (Alexa Fluor 488-CD9, HSV-1 gD Alexa Fluor 488 (sc-21719, Santa Cruz Biotechnology, USA) for 10 hours at 4°C. The antibody-labeled EVs were subsequently diluted 100-fold, and the percentage of labeled antibodies was analyzed using Flow NanoAnalyzer (Nano FCM, China).

2.10 Immunofluorescence experiment

HEK293T cells were seeded onto confocal dishes (1 × 10⁵ cells/dish) and transfected with 500 ng of either pcDNA3.1-gD2-PDGFRN-TM or pcDNA3.1-gD2 plasmid using PolyJet[™] transfection reagent (SignaGen Laboratories) according to the manufacturer's protocol. After 24 hours, cells were fixed with 4% paraformaldehyde (PFA, 15 min, room temperature), permeabilized with 0.1% Triton X-100 (10 min), and blocked with 3% bovine serum albumin in PBS for 1 h. Primary anti-gD antibody (1:200 dilution) was applied overnight at 4°C, followed by three PBS washes. Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:500) for 1 h at room temperature protected from light. Nuclei were counterstained with DAPI for 15 min. Confocal images were acquired using an Olympus FV3000 laser scanning microscope equipped with a 60× oil-immersion objective. Image processing was performed using Olympus FluoView software (FV31S-SW).

2.11 Animal vaccination model

All animal experiments and protocols were approved by the Institutional Animal Care and Use Committee of Southern Medical University. The methods employed in this study were conducted in accordance with approved guidelines. BALB/c mice and C57BL/6 (male, 5–6 weeks old, 20 ± 2g) were obtained from the Experimental Animal Center of Southern Medical University (Guangzhou, China)³⁴. Food and water were administered ad libitum. A therapeutic vaccine inoculation model was established in 4–5-week-old Balb/c mice. The mice received injections of 5 × 10⁹ EV particles into the right abdominal cavity at days 0, 14, and 28. Three days after the final EV injection, the mice were intranasally infected with HSV-1 virus (1 × 10⁵ PFU). Daily monitoring of the mice included recording changes in body weight and infection status. Mice showing significant weight loss or severe symptoms were euthanized, and blood was collected for analysis.

2.12 Real-time quantitative PCR (RT-qPCR)

RNA was extracted from cells using RNA isolation kit (RE-03113, Foregene, China). PrimeScript[™] RT Master Mix (RR036A, Takara, Japan) was used for cDNA synthesis, and Taq Pro Universal SYBR qPCR Master Mix (RR036A, Vazyme, China) was used for real-time PCR on LightCycler 480 system (Roche, Switzerland). Primers were synthesized by GENEWIZ (Suzhou, China), and relative gene expression was analyzed using the 2^{−ΔΔCT} method with GAPDH as a reference control.

Table 2
Primer sequences used for RT-qPCR

Gene	Forward (5'–3' sequence)	Reverse (5'–3' sequence)
GAPDH	TGACCTCAACTACATGGTCTACA	CTTCCCATTCTCGGCCTTG
VP16	AATGTGGTTTAGCTCCCGCA	CCAGTTGGCGTGTCTGTTTC
gD gene	CCAAATACGCCTTAGCAGACC	CACAGTGATCGGGATGCTGG

2.13 Viral plaque assay

Vero cells were cultured overnight in 24-well plates at a density of 1×10^5 cells per well. Mouse serum was diluted 10-fold in DMEM and filtered through a 0.22 μ m filter. Different dilutions of serum were added to Vero cells and incubated at 37°C for 1 hour. After removing the supernatant, cells were cultured in a maintenance medium containing 1.6% methylcellulose for approximately 36–48 hours until cytopathic effects were visible in wells. Cells were fixed with 4% paraformaldehyde and stained with crystal violet (C805211, China, MacKlin) for 30 minutes. Plaques were counted using an enzyme-linked immune spot analyzer as previous reported ³⁵.

2.14 ELISpot

Male Balb/c mice (4–5 weeks old) were immunized with extracellular vesicles (EVs) for three times. After that, their spleens were extracted and prepared into single-cell suspensions using aseptic techniques. To assess IFN- γ levels, the ELISpot Plus Mouse IFN- γ (3321-4APW-2, MabTech, USA) assay protocol was followed. Splenocytes from immunized mice were isolated under aseptic conditions and adjusted to 5×10^6 cells/mL in RPMI-1640 (10% FBS, 1% penicillin-streptomycin). Cells were seeded onto ELISpot plates pre-coated with anti-mouse IFN- γ capture antibody and stimulated with EV for 48 h at 37°C (5% CO₂). Plates were washed, incubated with biotinylated detection antibody for 2 h, and developed using streptavidin-HRP and substrate. After removing cells and washing with PBS, detection antibodies were added, followed by incubation, addition of enzyme-linked secondary antibodies, and color development with substrate solution. Spot enumeration was performed using an enzyme-linked immune spot analyzer.

2.15 Virus neutralization test

In this study, the eyes of mice immunized three times with EVs were removed, and serum was extracted. Vero cells were cultured overnight in 24-well plates at a density of 1×10^6 cells per well. A 10,000-fold dilution of the primary virus in DMEM was then added to the Vero cells, which were incubated at 37°C for one hour. Following this incubation, the supernatant was discarded and replaced with mouse serum diluted in maintenance medium containing 2% FBS. The cells were subsequently incubated for 48 to 72 hours, during which significant lesions were observed in the control cells under a microscope. Cell viability was assessed using the MTT assay.

2.16 T-Cell cytotoxicity assay

The HEK 293T cells were seeded into 24-well plates at a density of 1.5×10^5 cells/ml. Subsequently, the plasmids pcDNA3.1-gD2-PDGFRN-TM and pcDNA3.1-gD2 were transfected into HEK 293T cells using PolyJet transfection reagent, and the cells were incubated for 24 hours. Alternatively, gD2 -B16F10 cells were seeded at the same density and cultured overnight. After euthanizing the mice, splenic T cells were isolated. Different numbers of these T cells were then added to the aforementioned cell cultures. After 48 hours, cell viability was assessed using the MTT assay. Specifically, 20 μ L of 5 mg/ml MTT solution was added to each well and incubated at 37°C for 4 hours. The cells were then carefully lysed, and 150 μ L of DMSO was added to each well. The plates were shaken for 15 minutes, and the absorbance at 570 nm was measured using a microplate reader.

2.17 *In vivo* imaging and tracking of EVs

EVs were labeled with DiI (C1991S, Beyotime, China) by incubating 100 μ g of EVs with an appropriate amount of DiI membrane fluorescent dye at 37°C for 30 minutes. Free dye was removed by ultracentrifugation at 110,000 $\times$ g for 75 minutes, and EVs were resuspended in sterile PBS. DiI-labeled EVs (5×10^9 particles) were injected intraperitoneally into Balb/c mice. One group of mice was subjected to live imaging at different time points, while another group was euthanized at various time points for tissue extraction from the ipsilateral and contralateral inguinal and axillary lymph nodes. The fluorescence of DiI-labeled EVs was visualized using the AniView Phoenix DXA (BLT, China) and analyzed with AniView software.

2.18 Activation of Bone marrow-derived dendritic cells (BMDCs) by EVs *in vitro*

Bone marrow-derived dendritic cells (BMDCs) are typically induced from bone marrow progenitors isolated from mice with GM-CSF. EV^{293T} and EV^{gD2-PDGFRN-TM} were co-incubated with BMDCs (10^7 cells) in 100 μ L of medium for 16 hours. The cells were then washed twice with ice-cold PBS, centrifuged, and resuspended in 100 μ L of cell suspension (equivalent to 10^6 cells) in round-bottomed Eppendorf tubes. The cells were stained with appropriate antibodies, including MO CD86 GL1 PE MAB (2536073, Invitrogen, China) and RAT MHC II HIS19 FITC MAB (2818411, Invitrogen, China). After a 20-minute incubation on ice in the dark, the cells were washed with PBS to remove unbound antibodies and then analyzed using a flow cytometer (Sysmex, China) to assess the staining results.

2.19 Tumor growth suppression study

In this vaccination study, 4- to 5-week-old male C57BL/6 mice were injected intraperitoneally with 5×10^9 EVs (EV) pellets three times on the right side of the body on days 0, 14, and 28. Three days after the completion of immunization, the mice were inoculated subcutaneously on the back with 4×10^5 B16F10 cells. The time taken for the tumor to reach a size of 50 mm³ was recorded. Tumor size was measured daily using calipers, and the volume was calculated using the formula:

$$V = \frac{1}{2} \times \text{length} \times \text{width} \times \text{height}.$$

When tumor growth became more pronounced, the C57BL/6 mice were shaved, and a working solution of D-fluorescein sodium salt (15 mg/mL) (MX4603-100MG, MKBio, China) was prepared in sterile PBS and filtered through a 0.2 μ m filter membrane to eliminate bacteria. The solution was kept ice-cold and protected from light after preparation. An equivalent volume of a 30 mg/mL fluorescein working solution was prepared at a concentration of 10 μ L/g body weight, and this solution was injected intraperitoneally into the mice at two-minute intervals. Imaging analysis was conducted using an In-Vivo FX PRO (Bruker, USA), with analytical processing performed using Bruker MI SE software. Mouse weights were also recorded throughout the study.

2.20 EVs targeting assay

HEK293T cells and THP-1 cells were cultured on confocal dishes at a density of 1×10^5 cells per dish. Differentiation of THP-1 cells was induced by treatment with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (P33390-1, Shanghai Acme Biochemical Technology Co, China) for 24 hours. Following differentiation, both cell types were incubated with DiI-labeled EVs (2×10^9 particles) for durations of 8 hours and 24 hours, respectively. The cells were then fixed with 4% (w/v) paraformaldehyde for 20 minutes. After fixation, the cells were washed once with ice-cold PBS and subsequently stained with DAPI. Imaging was performed using an Olympus FV3000 laser scanning confocal microscope, and the acquired images were analyzed using FluoView software (Olympus FV31S-SW).

2.21 Frozen sectioning of tumor tissues

Tumor tissues were sectioned at 10 μ m using a thermostatic freezing microtome (Leica CM1860, Leica, Germany). Following fixation, the sections were allowed to air dry for 15 minutes at room temperature. Subsequently, the sections were incubated with 10% normal goat serum for 1 hour at room temperature to block non-specific binding. After blocking, a diluted primary antibody or primary working solution was applied to the sections. The slides were then placed in a wet box containing PBS and incubated at 4°C for 1 hour, followed by an incubation at 37°C for an additional hour, all while protected from light. After thorough washing with PBS, the sections were treated with a PBS-diluted secondary antibody for 1 hour at 37°C, again protected from light. Following another round of PBS washing, the sections were stained with a DAPI working solution. Finally, the slides were sealed and visualized using an LSM 880 confocal microscope (Carl Zeiss, Germany).

2.22 Lung metastasis experiments

In the metastasis study³⁶, mice were randomly assigned to receive either PBS as the control or exosomes, administered three times on days 0, 7, and 14. Three days after the final injection, 2×10^5 gD2-B16F10/GFP-B16F10 cells were intravenously injected into C57BL/6 mice via the tail vein. At 20 days post-injection, mice were euthanized for autopsy to assess pulmonary melanoma metastasis.

2.23 Data analyses

All results are expressed as the Mean $\pm$ SD. One-way analysis of variance (ANOVA) was used to analyze data from multiple groups. Student's t-test was performed for comparisons between two groups, and statistical significance was determined accordingly. All statistical analyses were conducted using GraphPad Prism, with the following significance levels: *P < 0.05, **P < 0.01, ***P < 0.001.

3 Results

3.1 Optimization of the gD1-based EV vaccine platform

The purpose of EV based vaccine was to display the intact antigen on the surface of EV to activate effective adaptive immunity. Here, the aim was to develop a flexible system by displaying antigens that can be interchanged depending on the intended need. For proof-of-concept, we first constructed an EV vaccine against HSV1 viruses, to introduce the gD1 antigen (gD of HSV1) to the EV surface (Fig. 1A). The EV vaccine extraction process is shown in Figure 1B as our previous method^{37, 38}. The EV-producing cells were engineered to express a fusion construct of an gD1 and an EV-anchoring protein, to enable enrichment of the gD1 on the surface of EVs. Given that there are various EV membrane-anchoring domains (PTGFRN transmembrane domain-D687, PDGFRN transmembrane domain, CD63, LAMP2B, Glycosylphosphatidylinositol (GPI) Anchors), a systematic comparison of engineering strategies was conducted (Supplementary Fig. 1A). The initial assessment was conducted on the EV-producing cells. A comparison of gD1 abundance on the surface of engineered EVs vaccine was conducted by nanoflow cytometry assay. Fluorescent antibody against gD1 for detecting the gD1 abundance of EV vaccine, while the fluorescent antibody against CD9 for labelling EV, and the ratio of the gD1/CD9 was used to determine the abundance of gD1 of EV vaccine. By comparing the conditioned media of the different EV-producing cells expressing the five different EV-anchoring domains, the PDGFRN outcompeted the other EV-anchoring domains per milliliter, indicating a greater production of EVs vaccine (Supplementary Fig. 1B). Since fusions of the gD1-PDGFRN transmembrane domain (gD1- PDGFRN-TM) produced engineered EVs with the highest expression levels, this combination was chosen as the optimal candidate for EV vaccine production, subsequently referred to as EV^{gD1-PDGFRN}. Fluorescence results showed that compared to the 293T cells producing EV^{gD1}, the EV^{gD1-PDGFRN} generating cells exhibited more aggregation of gD1 within intracellular vesicles (Fig. 1C). In addition, the morphology of the EV vaccine was analyzed using transmission electron microscopy, revealing that all EV vaccine exhibited a classic cup-shaped structure, characteristic of EVs, in contrast to EVs derived from 293T cells (EV^{293T}). These findings indicated that the physical shape of the EV vaccine remained unchanged (Fig. 1D). NTA was employed to analyze the particle size of the EV vaccine, revealing that the EVs loaded with antigens (EV^{gD1}, EV^{gD1-PDGFRN}) did not exhibit significant changes compared to EV^{293T} (Fig. 1E). Additionally, the zeta potential remained around -20 mV among EV^{293T}, EV^{gD1} and EV^{gD1-PDGFRN}. These results indicated that the EV vaccine possesses the typical characteristics of EV (Fig. 1F). The gD1 content in the EV was also assessed using Western blot analysis, confirming the presence of gD1 protein at the expected molecular weight (Fig. 1G). Through ELISA assay, there is about 1% gD1 protein (62.1 pg gD1 in 671 ng EVs) in EV^{gD1} and EV^{gD1-PDGFRN} (Fig. S1C). From the nanoflow cytometry assay, forty percent of the EV^{gD1-PDGFRN} expressed gD1 protein, which was found to be twice as high as that of EV^{gD1} (Fig. 1I). Although the western blot results indicated similar expression levels of gD1 protein between EV^{gD1-PDGFRN} and EV^{gD1}, this result suggested that gD1 is primarily distributed on the surface of EV^{gD1-PDGFRN} vaccine. These classical parameters of EV demonstrated that we successfully constructed the EV^{gD1-PDGFRN} vaccine as expected, indicating that the PDGFRN-TM fusion effectively promotes the surface localization of gD1 on the EV.

3.2 EV^{gD1-PDGFRN} demonstrates potent antiviral efficacy against HSV1 *in vivo*

Next, the protective efficacy of EV^{gD1-PDGFRN} against HSV1 infection was evaluated *in vivo*. The HSV1 mouse infection model, as previously reported by other authors, was used in this study³⁹. The mice were administered three vaccine immunizations at 7-day intervals. One week after the third booster immunization, the mice were infected with the HSV1 virus (Fig. 2A). A rapid and significant decline in body weight was observed in mice administered EV^{293T} by day 5 of the infection period. In contrast, only minor fluctuations in body weight were noted in mice treated with either the EV^{gD1} or EV^{gD1-PDGFRN} vaccine (Fig. 2B). Survival rates of the mice were monitored until all mice in the virus-only infection model group had been sacrificed. It was observed that all mice that had been administered EV^{293T} were sacrificed on the eighth day, whereas all mice that had been given EV^{gD1-PDGFRN} vaccine survived until 12th day (Fig. 2C). The vaccine exhibited complete protection, achieving 100% efficacy. In the group immunized with the EV^{gD1} vaccine, one mouse died on day 10, indicating that its protective efficacy was weaker than that of EV^{gD1-PDGFRN} vaccine.

The morphological assessment of the mice indicated that HSV1 infection led to corneal damage and disheveled fur in those treated with EV^{293T} (Fig. 2D). Conversely, administration of the EV^{gD1-PDGFRN} vaccine markedly mitigated corneal damage and resulted in a well-groomed coat. We also collected serum from HSV1-infected mice and evaluated the viral titer using a plaque assay, with plaques indicating the presence of the virus. It was found that in the group treated with EV^{293T}, there was a significant increase in viral load, while administration of the EV^{gD1} and EV^{gD1-PDGFRN} vaccine led to a substantial decrease in viral titer (Fig. 2E). Additionally, outperformed in terms of efficacy. Under a 1:10 dilution of serum, we observed that the HSV1 viral titer in mice treated with EV^{gD1-PDGFRN} vaccine decreased by approximately 15% compared to those receiving EV^{gD1} vaccine, which aligns with the survival curve of the mice. Furthermore, the analysis revealed that the EV^{gD1-PDGFRN} vaccine markedly decreased the viral gD1 protein levels within the tissues (Fig. 2F). Therefore, we conclude that EV^{gD1-PDGFRN} vaccine effectively prevents viral infection *in vivo*.

3.3 Application of the PDGFRN based EV vaccine platform against HSV2

Although the gD vaccine has shown notable antiviral activity against HSV1, its effectiveness against HSV2 is limited^{1, 40}. Consequently, drawing from the promising results of the EV^{gD1-PDGFRN} vaccine, a gD2 EV vaccine targeting HSV2 was created, and its characteristics were subsequently assessed. As shown in the Fig. 3A, a gD2-PDGFRN-TM fusion plasmid was developed, resulting in the formulation of a EV^{gD2-PDGFRN} vaccine. Morphological and particle size analyses conducted through TEM indicated no notable differences from EV^{293T} to EV^{gD2-PDGFRN} vaccine (Fig. 3B). EV^{gD2-PDGFRN} vaccine did not change the zeta potentials of EV (Fig. 3C). Additionally, nanoparticle flow cytometry assessments demonstrated a fourfold enrichment of gD2 antigen on the surface of EV^{gD2-PDGFRN} vaccine (Fig. 3D). The presence of gD2 antigen in the EV^{gD2-PDGFRN} was further validated by Western blot experiments (Fig. 3E). To mark gD2, colloidal gold particles were used, with dark spots confirming the presence of gD2 antigen on the surface of the EV^{gD2-PDGFRN} (Fig. 3F). These results confirm the validity of the EV^{gD2-PDGFRN} vaccine. Subsequently, the *in vivo* metabolism of the gD2 EV vaccine was evaluated. By Dil stain of EV, fluorescent signals in the blood were analyzed, revealing that both EV^{293T} and EV^{gD2-PDGFRN} achieved peak concentrations in the bloodstream between 4-8 hours, with a marked decrease observed by 24 hours, indicating the similar pharmacokinetic characteristics *in vivo* (Fig. 3G). To further validate the *in vivo* distribution of the EV^{gD2-PDGFRN} vaccine, we conducted *in vivo* imaging and found that the EV^{gD2-PDGFRN} vaccine predominantly localized to the axillary region, reaching peak concentration at 8 hours (Fig. 3H). This phenomenon aligns with their pharmacokinetic characteristics. The axillary tissue is known to be lymph-rich, and we measured the fluorescence intensity in isolated lymphatic tissues. The fluorescence intensity indicated that the EV^{gD2-PDGFRN} vaccine were primarily concentrated in the lymph on the injected side, while the contralateral lymphatic tissue exhibited weaker fluorescence intensity (Fig. 3I). In addition, it was also observed that BMDC significantly uptake both EV^{293T} and EV^{gD2-PDGFRN} vaccines after co-incubation, with no significant difference (Fig. S2B). These findings suggested that there is no significant difference in the *in vivo* distribution between EV^{293T} and EV^{gD2-PDGFRN} vaccine by DC uptake. After administration, it preferentially localized in lymphoid organs, supporting the antigen presentation process of the EV^{gD2-PDGFRN} vaccine. Following Dil labeling, EV^{gD2-PDGFRN} vaccine were co-incubated with THP-1-induced dendritic cells and HEK293 cells. These results demonstrated that, at both 8-hour and 24-hour time points, DCs exhibited a significant enhancement in EVs phagocytosis compared to HEK293 cells, a finding consistent with *in vivo* observations (Fig. 3J).

3.4 EV^{gD2-PDGFRN} vaccine exhibits superior DC-activating effect

Given its accumulation in lymphoid tissues, we further investigated EV vaccine's ability to activate dendritic cells (DCs) *in vitro* and *in vivo*. BMDCs were isolated from healthy mice were incubated with EV^{293T} and EV^{gD2-PDGFRN} for 16 h with the same final concentration of EVs (10⁷ particles/mL), PBS as control group. Activation of DCs were assessed using antibodies against CD86 and MHCII by FACS (Fig. 4B). Compared to the control, EV^{293T} and EV^{gD2-PDGFRN} resulted in a significantly higher percentage of CD86⁺MHCII⁺ DCs (5-6 fold increase, Fig. 4B) and expression level of MHCII⁺ (Fig. 4C) and CD86 (Fig. 4D). It is noteworthy that the both EV^{293T} and EV^{gD2-PDGFRN} could activate DCs in comparison with control (Fig. 4A-C). Consistent with previous reports³¹, the administration of EVs alone can indeed activate DCs. We also studied the dose-dependent DC-activating effect by treating BMDCs with varying concentrations of EV^{gD2-PDGFRN} vaccine (10⁶, 10⁷, 10⁸, 10⁹/mL). At all doses, EV^{gD2-PDGFRN} vaccine resulted in significantly higher expression levels of CD86 and MHCII compared to control (Fig. 4 E-H). As expected, the DCs activation effect increased with higher concentrations of EV^{gD2-PDGFRN} vaccine *ex vivo*. Therefore, we used this concentration for mouse immunization experiments, and we found that following a third immunization, the DCs in the mice were significantly activated. In comparison to the control, both EV^{293T} and EV^{gD2-PDGFRN}

led to a significantly percentage of CD86⁺MHCII⁺ dendritic cells, showing a 2-3 fold increase (Fig. 4 I-J), consistent with the *in vitro* results. These experiments demonstrated the superior DC-activating effect of EV^{gD2-PDGFRN} vaccine.

3.5 EV^{gD2-PDGFRN} vaccine show enhanced cytotoxic T lymphocyte (CTL) response

We next studied the CTL response and antiviral efficacy of EV^{gD2-PDGFRN} vaccine, which is essential for infectious disease⁴¹. BALB/c mice were subcutaneously injected with PBS, EV^{293T} or EV^{gD2-PDGFRN} on days 1, 7, and 14 (Fig. 5A). Due to the lack of gD2-specific CD8⁺ T cell-specific antibodies and corresponding tetramers, Elipot assay was used to analyze the activation of CD8⁺ T cell, which is a highly sensitive assay used to detect and quantify CD8⁺ T cell activation by measuring the cytokines they secrete in response to corresponding antigens. Following the administration of EV vaccine to T cells isolated from the spleen, we assessed the levels of IFN- γ secreted by these T cells. In comparison to the PBS or EV^{293T} treatment groups, a notable presence of blue spots in the EV^{gD2-PDGFRN} vaccine treatment group was observed (Fig. 5A), demonstrating the ability of EV^{gD2-PDGFRN} vaccine to elicit gD2-specific CD8⁺ T cell response. Next, we assessed the dose-response relationship of the EV vaccine and found that CD8⁺ T cell activation increased with higher concentrations of the EV vaccine incubation. Notably, CD8⁺ T cell activated by the EV^{gD2-PDGFRN} vaccine exhibited sixfold greater activity than those from EV^{293T} at the same dosage (10⁹ particles/ml) (Fig. 5B). These findings suggest that the EV^{gD2-PDGFRN} vaccine is more effective at activating CTLs compared to EV^{293T}, as evidenced by the enhanced secretion of IFN- γ (Fig. 5C). Furthermore, T cell cytotoxicity assay was conducted to validate this effect. It was observed that T cells derived from EV^{gD2-PDGFRN} vaccine mice exhibited dose-dependent cytotoxicity against cells expressing the gD2 protein, while showing no cytotoxic effects on cells without this antigen. Compared to the cells lacking gD2, approximately 50% of the target cells underwent cell death with 10⁴ T cells (Fig. 5D). This confirms that the EV^{gD2-PDGFRN} vaccine effectively promotes the expansion of gD2-specific CTLs. In parallel, the levels of gD2 antibody production were evaluated using an ELISA assay. The results indicated that the EV^{gD2-PDGFRN} vaccine significantly enhanced the production of gD2 antibodies (Fig. 5E), which exhibited a dose-dependent capacity to inhibit HSV-2 infection (Fig. 5F). Through the observation of major organs, it was found that compared to the PBS-treated control group, the EVs vaccine-treated group showed no damage to the major organs of the mice (Fig. S2B). These findings robustly confirm that the vaccine efficiently triggers both a cellular immune response and humoral immunity against the gD2 antigen by EV^{gD2-PDGFRN} vaccine.

3.6 Three-dose of the EV^{gD2-PDGFRN} vaccine are more effective than two-dose

To further validate the *in vivo* antiviral efficacy of the EV^{gD2-PDGFRN} vaccine, the differences of vaccination dosage were assessed. BALB/c immunocompetent mice were administered two or three-dose of the EV^{gD2-PDGFRN} vaccine, respectively. One week later, these BALB/c mice were infected with HSV-2 (Fig. 6A). Morphological observations revealed that four days post-infection, the eyes of mice in the virus-infected group were unable to open due to the typical symptoms of HSV keratitis observed in the EV^{293T} treatment group. However, following administration of the EV^{gD2-PDGFRN} vaccine, both the two-dose and three-dose vaccinated groups exhibited no differences in eye appearance compared to the uninfected control group. Furthermore, it was observed that all mice in the EV^{293T} treatment group succumbed to infection seven days post-infection. In contrast, the survival rate for mice receiving two-dose of the EV^{gD2-PDGFRN} vaccine was 48%, while the survival rate significantly increased to 91% for those receiving three-dose of EV^{gD2-PDGFRN} (Fig. 6C). These results demonstrate the substantial antiviral efficacy of the three-dose EV^{gD2-PDGFRN} vaccine regimen. The HE-stained sections of corneal tissue also demonstrate that the inflammation is more pronounced in the corneas of the EV^{293T} and two-dose EV^{gD2-PDGFRN} vaccine treatment group, indicating a significant viral inflammatory response. In contrast, the three-dose EV^{gD2-PDGFRN} treatment resulted in a corneal appearance similar to that of the virus-uninfected group, with no observable inflammation (Fig. 6D). Concurrently, it was observed that the gene level of gD protein in the tissues of virus-infected mice exhibited the lowest concentration following three EV^{gD2-PDGFRN} vaccine immunizations (Fig. 6E). Furthermore, the viral titers in the serum corroborated the aforementioned results (Fig. 6F). Additionally, the anti-gD antibody level in the serum of the mice was tested, and it was found that all of the groups that received the EV^{gD2-PDGFRN} vaccine exhibited significantly elevated levels in comparison to the EV^{293T} group (Fig. 6G). Additionally, we investigated the levels of CTL in mice using ELISPOT, which revealed that the highest levels of IFN- γ secretion were observed in mice that received three-dose of EV^{gD2-PDGFRN} vaccine (Fig. 6H). Furthermore, T cell cytotoxicity assay confirmed the above results (Fig. 6I). The results demonstrate that the administration of three vaccinations with the EV^{gD2-PDGFRN} vaccine is more efficacious than two vaccinations.

3.7 EV^{gD2-PDGFRN} vaccine cross-protection to HSV1 virus infection

HSV-1 and HSV-2 share a multitude of biologically analogous and largely identical immunogenic sequences. Research has shown that, among the immunogenic envelope glycoproteins of HSV, all glycoproteins except glycoprotein G contain immunogenic regions that elicit cross-reactive B and T cell responses⁴². Although previous studies have demonstrated the efficacy of vaccines targeting gD2 in preventing HSV-1 infection, the objective of this study was to determine whether the EV^{gD2}-PDGFRN vaccine possesses a similar inhibitory effect. To this end, mice were immunized with three-dose of EV^{gD2}-PDGFRN vaccine and subsequently infected with HSV1 at day 28, and the resulting outcome was observed. In accordance with the preceding findings, following viral infection, mice that had been administered the EV^{293T} exhibited a rapid loss of weight (Fig. 7A), and all mice succumbed after the ninth day (Fig. 7B). Following the administration of the EV^{gD2}-PDGFRN vaccine, however, there was no significant reduction in body weight, and all mice survived (Fig. 7C). The results demonstrated that the vaccine exhibited 100% protective efficacy, achieving equivalent immune outcomes compared to HSV-1-derived gD in terms of immunogenicity and protective capacity. The corneas of mice that received the EV^{gD2}-PDGFRN vaccine exhibited no lesions, whereas those that received the EV^{293T} displayed notable signs of keratitis (Fig. 7D). Furthermore, the virus titres in the serum of mice were examined by the viral plaque assay, which revealed the presence of virtually no virus particles following the administration of the EV^{gD2}-PDGFRN vaccine (Fig. 7E). Furthermore, the administration of the EV^{gD2}-PDGFRN vaccine was found to significantly reduce the amount of viral glycoprotein gD protein present in the tissues of mice, as determined by the western blotting assay (Fig. 7 G-H). Additionally, the viral genes of HSV1 in the tissues were also significantly reduced, as evidenced by the RT-PCR results (Fig. 7F). This evidence substantiates the hypothesis that the EV^{gD2}-PDGFRN vaccine can effectively induce cross-immunity against HSV-1 infection.

3.8 EV^{gD2}-PDGFRN vaccine inhibits tumor growth with gD2 antigen endogenously expression

To highlight the effectiveness of the EV^{gD2}-PDGFRN vaccine, gD2 was overexpressed on B16F10 melanoma cells stably to evaluate whether the vaccine can suppress the growth of tumors via vaccine-induced CTL response. Initially, we established a B16F10 cell line stably expressing the gD2 antigen or EGFP (as a control) for use in subsequent experiments, confocal microscopy and Western blotting results corroborated the substantial expression of gD2 (Fig. 8A and Fig. S2C). Then we divided the mice into three groups (n=6): the first group received a control vaccine (EV^{293T}) followed by subcutaneously on the dorsal flank inoculation with native B16F10 cells as a positive control for tumor formation; the second group received the EV^{gD2}-PDGFRN vaccine followed by inoculation with B16F10-EGFP cells as a control; and the third group received the EV^{gD2}-PDGFRN vaccine followed by inoculation with gD2-overexpressing B16F10 cells (B16F10-gD2) to evaluate the anti-tumor efficacy of the vaccine (Fig. 8B). Simultaneously, we monitored the body weight in all groups to estimate their potential adverse effects, and the results showed that the body weights of the mice remained stable during the treatment period until after inoculation with tumor cells (Fig. 8C). At the same time, the tumor growth was measured by monitoring the tumor volume (Fig. 8D). It's observed that in the group administered the control vaccine, tumors in mice inoculated with B16F10-gD2 began to grow immediately within 8 days post-inoculation. By day 16, all mice were euthanized once the tumor diameter in any direction exceeded 20 mm, and tumor volume recording was terminated. In contrast, in the group receiving the EV^{gD2}-PDGFRN vaccine, no tumor growth was observed in mice inoculated with B16F10-gD2 cells by day 16 (Fig. 8E). However, significant tumor growth was observed in the other two groups. Meanwhile, there was no significant difference in tumor volume between these two groups, whereas complete tumor regression was observed in the group immunized with the EV^{gD2}-PDGFRN vaccine and challenged with B16F10-gD2 cells (Fig. 8E-F). These results demonstrated that the EV^{gD2}-PDGFRN vaccine significantly inhibits the growth of tumors expressing the gD2 antigen (Fig. 8E-F).

To further validate the efficacy of the vaccine, we examined the activation status of DCs in mice and found that EV^{gD2}-PDGFRN vaccine treatment significantly promoted DCs activation (Fig. 8G). In the Elispot assay, the highest number of IFN- γ spots was observed in T cells from mice vaccinated with EV^{gD2}-PDGFRN vaccine and those bearing B16F10-gD2 tumors. This phenomenon may be attributed to the re-activation effect induced by the presence of gD2 antigen on B16F10-gD2 cells (Fig. 8H). Also, we detected CTLs activity following EV^{gD2}-PDGFRN vaccine treatment. The results showed that CTLs specifically targeted and killed B16F10-gD2 cells but had no cytotoxic effect on B16F10-EGFP cells (Fig. 8G). Furthermore, we measured the levels of gD2-specific antibodies in serum and observed a significant increase in antibody titers in the EVs-based vaccine group compared to the control group (Fig. 8J). These findings demonstrate that the EVs-based vaccine effectively elicits both cellular and humoral immune responses, thereby achieving potent anti-tumor effects. Furthermore, it's observed that administration of the EV^{gD2}-PDGFRN vaccine significantly inhibited lung metastasis of tumor cells (Fig. 8K). Collectively, these results underscore the potent antiviral and antitumor efficacy of the EV^{gD2}-PDGFRN vaccine.

4 Discussion

There are no vaccines currently available for HSV infection, but the pipeline is rich with candidates in various phases of development⁴³. In this study, we constructed a preventive novel EVs-based HSV vaccine platform, which represents a novel vaccine route distinct from those utilizing inactivated vaccines, protein subunit vaccines *et.al*. We have developed an EV-based method that can present and deliver viral surface protein antigens within the environment of lipid membrane. Through fusion to PDGFRN-TM domains, we were able to recruit and package viral membrane proteins onto the surface of EV^{gD-PDGFRN}. Membrane proteins on EV^{gD-PDGFRN} are likely in their natural confirmation as they can be recognized by appropriate antibodies, including neutralizing antibodies. In all cases, we showed that these viral membrane antigens containing EV^{gD-PDGFRN} induce robust production of antibodies that specifically bind to viruses. In the case of the HSV virus, we showed that immunization of viral gD on EV^{gD-PDGFRN} significantly reduced the lethality of the viral infection in mice. These results demonstrated the utility of EV^{gD-PDGFRN} in presenting and delivering a variety of viral membrane proteins in their native membrane associated conformation and established EV^{gD-PDGFRN} as a highly adaptable platform for developing vaccines against viral pathogens.

Vaccines are currently being developed both to prevent HSV-2 infection (preventive) or to treat HSV-2 infection (therapeutic). While most HSV vaccine research has prioritized HSV-2 rather than HSV-1, cause HSV-2 vaccines may also have benefits in preventing or treating HSV-1 infection given the homology between these viruses^{1, 40}. While our results also confirmed the EV^{gD2-PDGFRN} vaccines possess the cross-protection ability against HSV-1 infection. Prior to the construction of an EV vaccine for HSV-2, an EV vaccine for HSV-1 was developed for proof of concept, because the viral phenomenon of HSV-1 is more readily observable than the keratitis symptoms of HSV-2. gD1 from HSV-1 was first chosen to express on the EV surface as an antigen firstly, because gD1 is widely used as a component of HSV vaccine, such as Herpevac, CJ-gD2, GEN-003. A comparison of several systems revealed that the PDGFRN single transmembrane system could conveniently display the gD1 protein on the surface of EV^{gD1-PDGFRN}. The favorable results gave us the confidence to proceed with the construction of EV^{gD2-PDGFRN}. The physicochemical properties of the EV vaccine were characterized, and it was found that the loading of gD antigen did not alter the typical characteristics of EVs. Furthermore, gD loading on the EVs surface was demonstrated conclusively by nano-FACS and colloidal gold. Furthermore, western blot and ELISA experiments were conducted to confirm the correct conformation of gD. These results demonstrated that the both EV^{gD1-PDGFRN} and EV^{gD2-PDGFRN} vaccine is capable of delivering intact antigens. Also it was observed that EV^{gD2-PDGFRN} vaccine demonstrated notable antiviral efficacy in HSV-1 and HSV-2 infection models. Furthermore, no evidence of toxicity was observed in mice following administration of the EV^{293T} and EV^{gD2-PDGFRN} vaccines (Fig. S2A). These findings collectively demonstrate the efficacy and safety of the EV^{gD-PDGFRN} vaccine.

EV^{gD-PDGFRN} offers several potential advantages compared to existing vaccination platforms. In contrast to recombinant gD-based vaccines, which often require extensive and complex protein engineering to maintain antigens in their native conformation^{11, 44}, EV^{gD-PDGFRN} circumvents this issue by naturally incorporating viral membrane proteins during vesicle budding in producer cells. Since these proteins are expressed on the cell membrane, they are seamlessly loaded onto the surface of EV^{gD-PDGFRN}. Additionally, EV^{gD-PDGFRN} is nonviral in origin, making it less susceptible to interference from pre-existing or induced neutralizing antibodies, a common limitation of adeno-associated virus and other viral-based platforms^{45, 46}. Moreover, EV^{gD-PDGFRN} addresses certain challenges associated with the cutting-edge mRNA platform, which has been successfully utilized in highly effective SARS-CoV-2 vaccines^{47, 48}. Compared to mRNA vaccines, which require ultra-low temperature storage due to RNA instability, EV^{gD-PDGFRN} exhibits remarkable stability at 4°C, significantly reducing logistical challenges in resource-limited settings and overcoming the inherent instability of mRNA. This feature significantly reduces production, transportation, and delivery costs, particularly in resource-limited settings. Another notable benefit of the EV^{gD-PDGFRN} platform is its ability to function effectively without adjuvants. Our studies demonstrated that EV^{gD-PDGFRN} alone, without the addition of Alum, induced robust antibody production and conferred protection against viral infection. This adjuvant-free approach not only lowers costs but also minimizes potential side effects^{49, 50}. The activation of DCs observed after administering natural EV^{293T} suggests that this effect may be driven by the intrinsic immunostimulatory properties of EVs. These vesicles carry a substantial amount of exogenous material, including RNA and heterologous proteins, which may effectively mimic the role of traditional adjuvants, thereby enhancing immune responses.

Accumulating evidence have demonstrated that EV-mediated interactions that result in stimulation of cells of the innate immune system⁵¹. EV^{gD-PDGFRN} vaccine derived from human 293T cell line, which possess the human MHC-I molecules, as

a xenogeneic foreign body for mice, which could not mediate direct activation of mouse T cells⁵². Rather than a direct route for T cell activation, we think high probability that EV^{gD-PDGFRN} vaccine initiate T cell immunity via indirect presentation of viral antigens to the adjacent APCs⁵². Once enter into the mouse, EV^{gD-PDGFRN} vaccine could be captured by DCs at the locality, and process and convert EV^{gD-PDGFRN} vaccine to smaller peptide fragments which are then incorporated into MHC I and II molecules. Upon maturation, the antigen-MHC complexes are located on the external surface of DCs and they start to travel to local lymph nodes where they prime antigen-specific T cell responses. This is similar to our experimental results that a large number of accumulated near the lymphoid tissue nodes was observed at the injection site after EV^{gD-PDGFRN} injection. We also detected a significant increase in the activation level of DC cells, CD4⁺ and CD8⁺ cells with EV^{gD-PDGFRN} injection. Consistent with previous reports⁵³, we found that the EV^{gD-PDGFRN} vaccine did not require immune adjuvant to achieve effective immune effect, which may be due to the strong immunogenicity of the EV vaccine. It can be seen that EV vaccines also have the advantage of being independent of adjuvant.

It is noteworthy that after overexpressing gD2 in melanoma cells B16F10, we found that the vaccine significantly inhibited the growth of gD2-bearing tumors, while it had no effect on the growth of tumors without gD2. This demonstrates that the vaccine indeed exerts an anti-tumor effect. Based on this, we can boldly speculate that since the majority of people have been infected with HSV1/2^{2, 54, 55}, the application of the gD vaccine in the field of anti-tumor therapy might effectively activate the host's anti-tumor immune response. This hypothesis requires further experimental validation.

While this study we provide a new EV^{gD-PDGFRN} vaccine against HSV1/2, which is promising for further development. EV^{gD-PDGFRN} vaccine was focused on membrane antigens of HSV viruses, the EV^{gD-PDGFRN} system can be used to display and deliver membrane antigens of other enveloped viruses such as SARS-CoV-2 and monkeypox. EV^{gD-PDGFRN} engineered with these proteins on surface could lead to the production of neutralizing antibodies and protection against many virus infections. EV^{gD-PDGFRN} may also be used more broadly to present nonviral membrane proteins. We envision an equally effective presentation and delivery of cellular membrane receptors for production of therapeutic antibodies and membrane autoantigens for inducing immune tolerance. Further exploring EV^{gD-PDGFRN} in presenting and delivering an expanding repertoire of viral and nonviral membrane protein antigens will help develop EV^{gD-PDGFRN} into a highly versatile and potentially superior platform for vaccine development and beyond. Several areas of improvement have been identified in our study. Firstly, in addition to PDGFRN-TM domain, more efficient EVs-targeting sequences can be identified to more effectively display the viral proteins on the EVs surface. The second area for improvement is the design of a targeting strategy for DC cells that optimizes their antigen-presenting function. The subsequent objective is to investigate the possibility of delivering viral antigens both externally and internally, beyond merely the EVs surface. These measures may facilitate further enhancement of the potency of EV vaccines.

Declarations

Authorship Contributions

Xingang Yao and Shuwen Liu conceived and designed the research framework. Qiyun Lei, Jing Zhang and Wenyu Wu performed the experimental investigations, including immunological assays and *in vivo* challenge studies. Zhipeng Chen and Renjie Lin established and validated the B16F10 melanoma tumor model. Min Zou and Xiaodong Tang contributed to data interpretation and participated in scientific discussions on experimental design. Shuwen Liu provided critical technical support and supervised the implementation of key methodologies. Xingang Yao and Qiyun Lei drafted the manuscript, which was revised and approved by all authors.

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Conflicts:

All authors declare no conflicts of interest.

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Figures

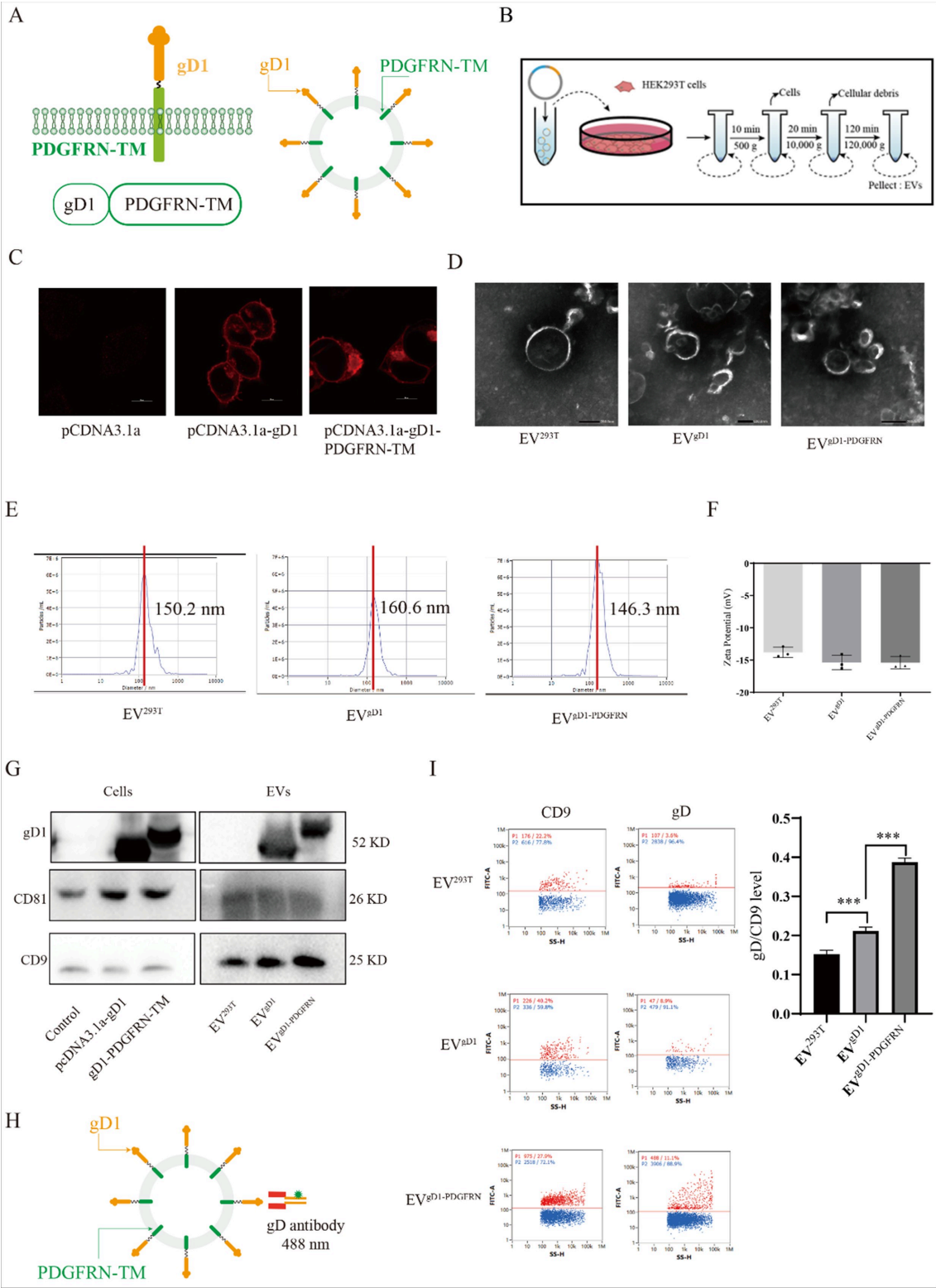


Figure 1

Enhancement of the EV vaccine platform utilizing gD1 antigen

(A) Schematic diagram of the gD1-PDGFRN-TM construct: HSV-1 glycoprotein D (gD1) was genetically fused to the platelet-derived growth factor receptor N-terminal transmembrane domain (PDGFRN-TM) to anchor gD1 on the surface of EVs. **(B)** EV purification workflow from transfected HEK293T cells, including sequential steps of differential centrifugation ($300 \times g$, $3,000 \times g$, $12,000 \times g$) and ultrafiltration ($110,000 \times g$), followed by PBS resuspension. **(C)** Confocal microscopy images of HEK 293T cells transfected with gD1 or gD1-PDGFRN-TM plasmids as indicated. gD1 (Red) was detected by anti-gD antibody (scale bar: 10 μ m). **(D)** TEM images of EV^{293T} (control), EV^{gD1}, and EV^{gD1-PDGFRN}, showing typical cup-shaped morphology (scale bar: 200 nm). **(E)** The size distribution and quantity of EVs were determined using NTA analysis. **(F)** Zeta potential of indicated EVs. **(G)** Western blot analysis of EV markers (CD9, CD63) and gD1 expression in EVs. Lane 1: EV^{293T} (control), Lane 2: EV^{gD1}, Lane 3: EV^{gD1-PDGFRN}. **(H)** Schematic diagram of NanoFCM for detecting surface gD1 expression on EVs using fluorescent anti-gD1 antibodies. **(I)** Quantification of gD1 surface enrichment via NanoFCM, showing a twofold increase in gD1/CD9 ratio for EV^{gD1-PDGFRN} compared to EV^{gD1}. Data are presented as Mean $\pm$ SD from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. *** indicated $P < 0.001$.

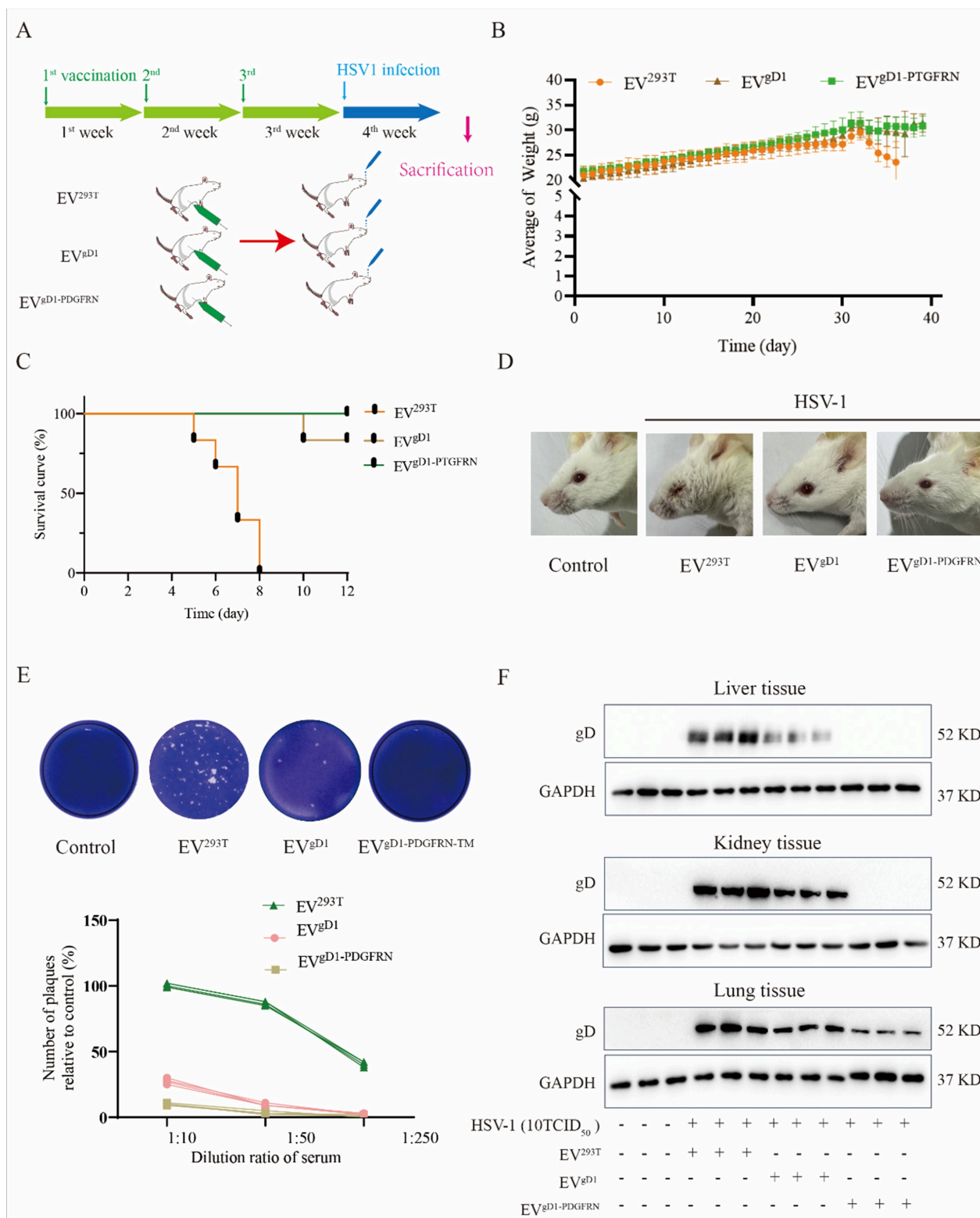


Figure 2

EV^{gD1-PDGFRN} exhibits antiviral activity against HSV1 *in vivo*

(A) EVs immunization schedule: BALB/c mice (n=6/group) received three-dose of EVs (5×10⁸ particles/dose) vaccine by intraperitoneal injection followed by intranasal HSV-1 challenge (1×10⁸ PFU). (B) The body weight of mice from the day of immunization to HSV1 infection (n=6). (C) The survival curves of mice with HSV1 infection. (D) The condition of corneal lesions (keratitis) and fur conditions of HSV1 infected mice. (E) The quantification of serum viral titers by viral plaque assays. (F) The analyses of viral protein gD1 levels in liver, kidney and lung tissues by western blot. Data are presented as Mean ± SD from three independent experiments.

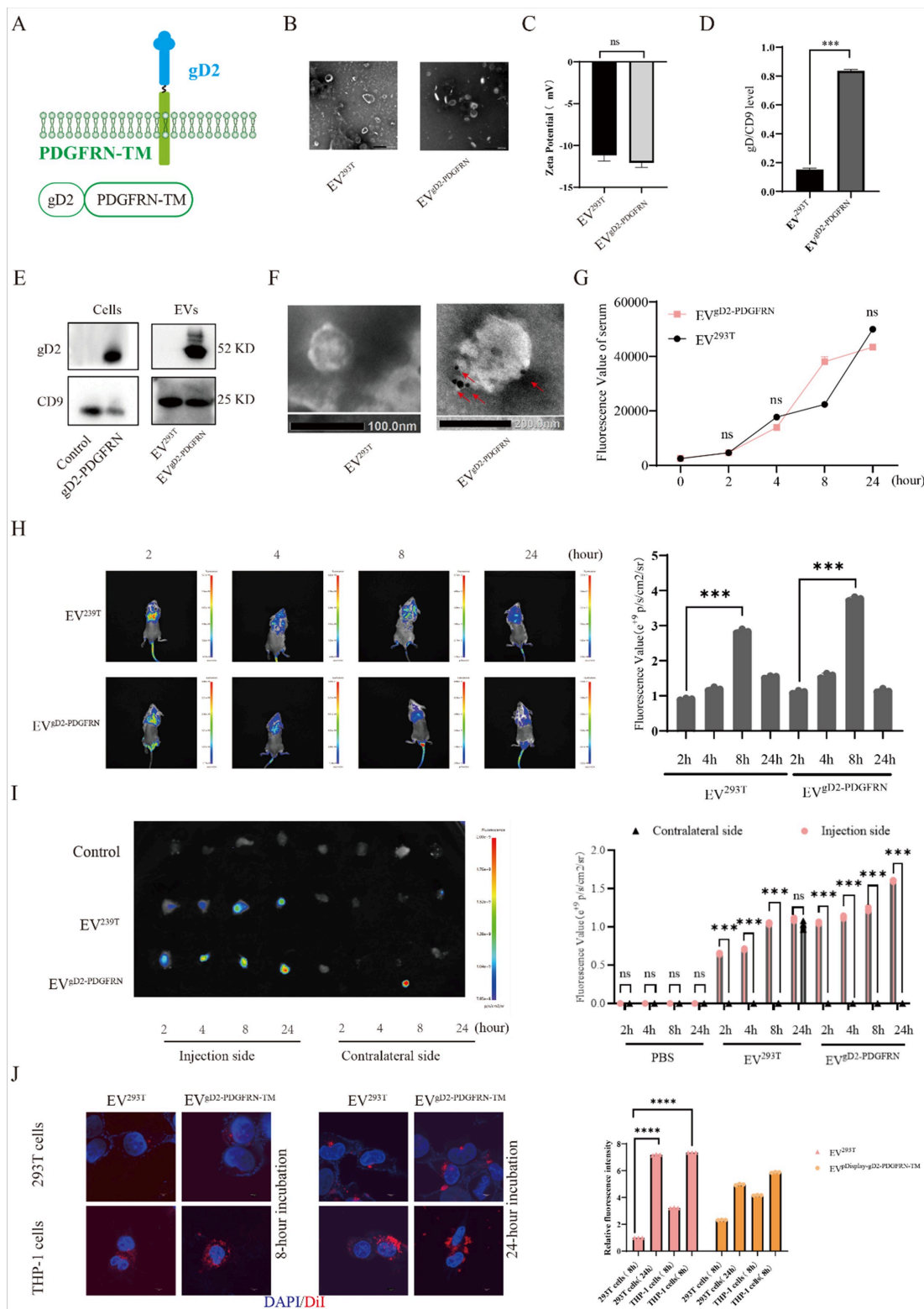


Figure 3

The characters of EV^{gD2-PDGFRN} vaccine

(A) Schematic diagram of the EV^{gD2-PDGFRN} vaccine and gD2-PDGFRN-TM plasmid construct. **(B)** The EV morphology of EV^{293T} and EV^{gD2-PDGFRN} vaccine by TEM (scale bar: 100 nm). **(C)** Zeta potential of EV^{293T} and EV^{gD2-PDGFRN} vaccine. **(D)** The gD2 level of EV^{293T} and EV^{gD2-PDGFRN} vaccine by nanoflow cytometry. **(E)** The gD2 level of EV^{293T} and EV^{gD2-PDGFRN} vaccine by western blot. **(F)** The surface gD2 indicated by red arrows by colloidal gold-labeled anti-gD2 antibodies (arrows, blank dots) through immunogold TEM (scale bar: 50 nm). **(G)** Pharmacokinetics of DiI-labeled EVs. **(H)** Following the administration of the DiI-stained EVs vaccine, the distribution of the vaccine within the mice was

monitored using in vivo imaging technology. The fluorescence quantitative value is on the right-hand side. **(I)** Fluorescence imaging of the injection site and contralateral lymphoid tissues in mice, with fluorescence quantitative data presented on the right-hand side. **(J)** The effect of dendritic cells (THP-1 induced by GM-CSF) versus normal cells (HEK293T) on the extent of EV phagocytosis was observed via confocal microscopy, with the red color representing the exosome vaccine stained with Dil, with fluorescence quantitative data presented on the right-hand side. Data are presented as Mean $\pm$ SD from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. *** indicated $P < 0.001$. **** indicated $P < 0.0001$, ns indicated $p > 0.05$.

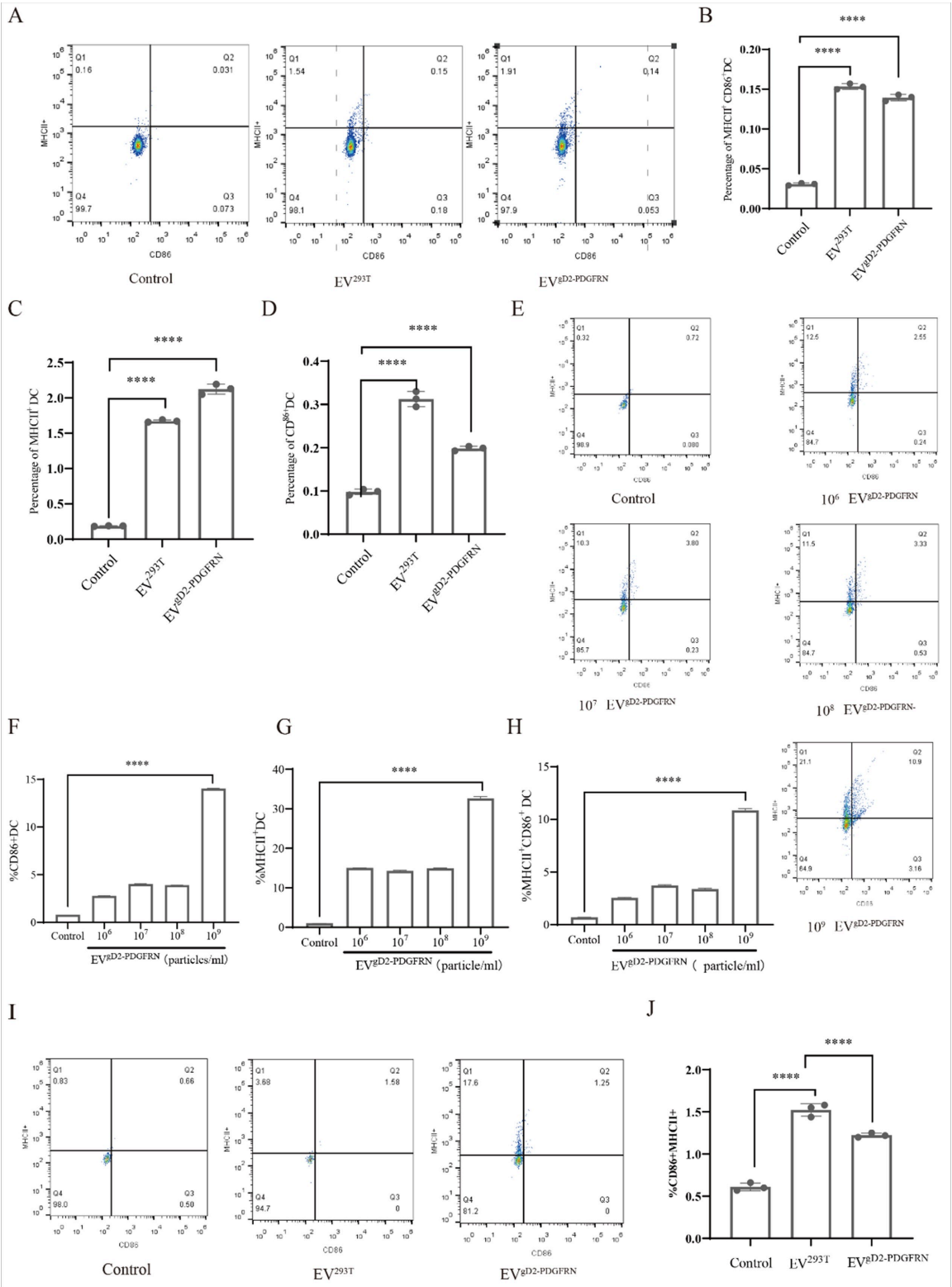


Figure 4

The EV^{gD2-PDGFRN} vaccine induced the dendritic cell activation. (A) DCs were incubated with EVs (1×10^7 /mL) for 16 h, FACS plots of MHCII⁺ DCs and CD86⁺ cells in DCs after treatment with indicated EVs. Shown are (B) percentages of CD86⁺MHCII⁺ DCs, (C) mean MHCII FI of DCs after treatment with indicated EVs and (D) mean CD86 FI of DCs after treatment with indicated EVs (n=3). (E) DCs were incubated with different dose of EV^{gD2-PDGFRN} vaccine (1×10^7 /mL, 1×10^8 /mL and 1×10^9 /mL) for 16 h, FACS plots of MHCII⁺ DCs and CD86⁺ cells in DCs after treatment with indicated EVs. Shown are (F) percentages of CD86⁺MHCII⁺ DCs, (G) mean MHCII FI of DCs after treatment with indicated EVs and (H) mean CD86 FI of DCs after treatment with indicated EVs (n=3). (I) Representative FACS plots of CD86⁺MHCII⁺ DCs in BMDC on day 28 after treatment with indicated EVs. Shown are (J) percentages of CD86⁺MHCII⁺ DCs. Data represent as Mean $\pm$ SD (n=6). Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test.

**** indicated $P < 0.0001$.

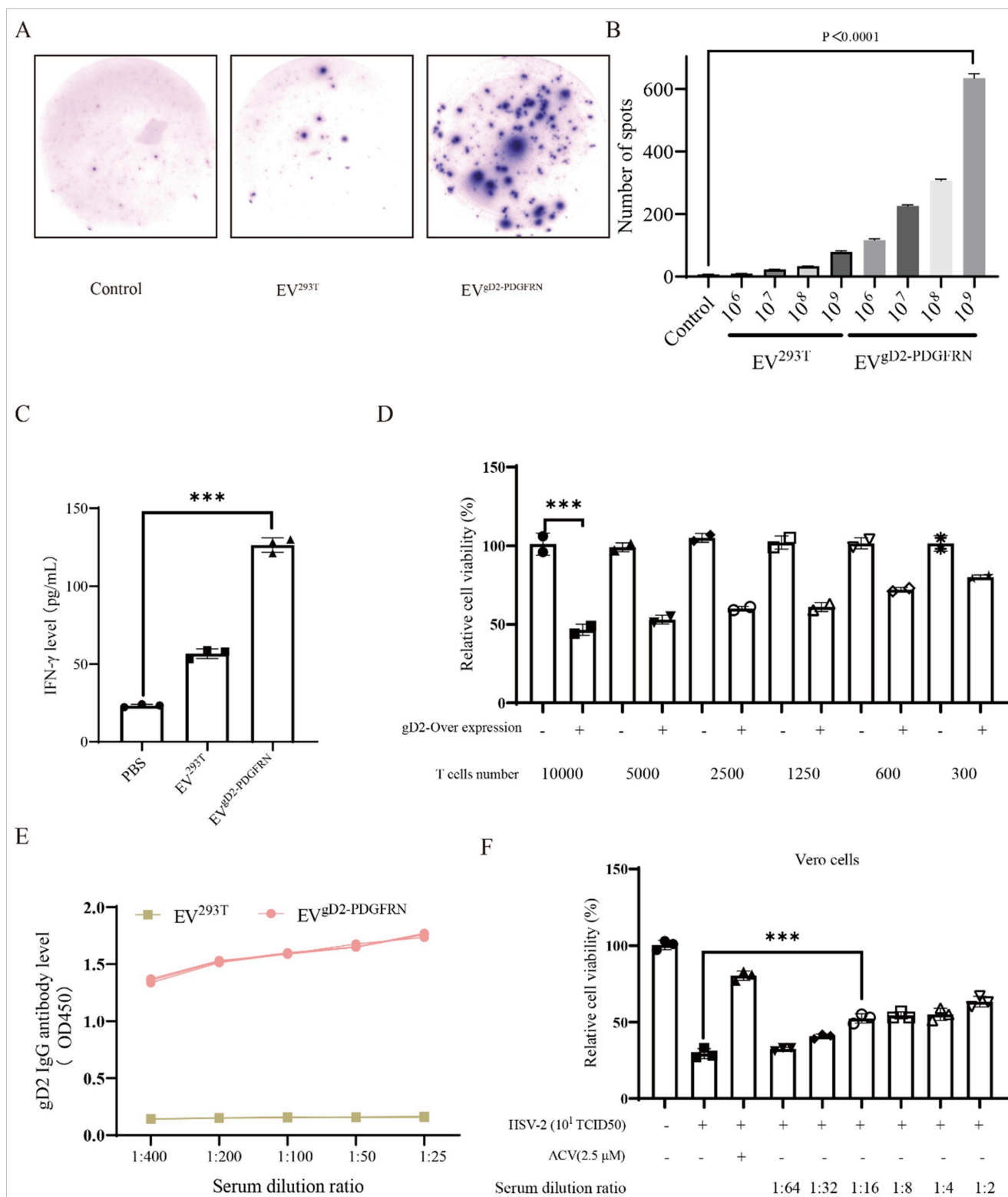


Figure 5

The cytotoxic T lymphocyte (CTL) response of EV^{gD2-PDGFRN} vaccine. (A) After immunizing mice with indicated EVs vaccine, splenic-derived T cells were harvested at day 28 and co-cultured with indicated EV vaccine. The activation of CD8⁺ T cells was subsequently assessed using the ELISPOT assay to detect the IFN- γ level (Blue spots). (B) The activation of CD8⁺ T cells with indicated EV vaccine at different doses through ELISPOT assay. (C) IFN- γ content level was detected by ELISA. (D) After immunizing mice with EV^{gD2-PDGFRN} vaccine, splenic-derived T cells were harvested and incubated with gD2-overexpressed 293T cells. The cellular viability was detected by MTT assay. (E) Serum anti-gD2 IgG titers were detected by ELISA. (F) The anti-HSV1 ability neutralization efficacy of serum with EV^{gD2-PDGFRN} vaccine immunization was evaluated,

with the serum subjected to gradient dilution. Data represent as Mean $\pm$ SD (n=6). Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. *** indicated $P < 0.001$

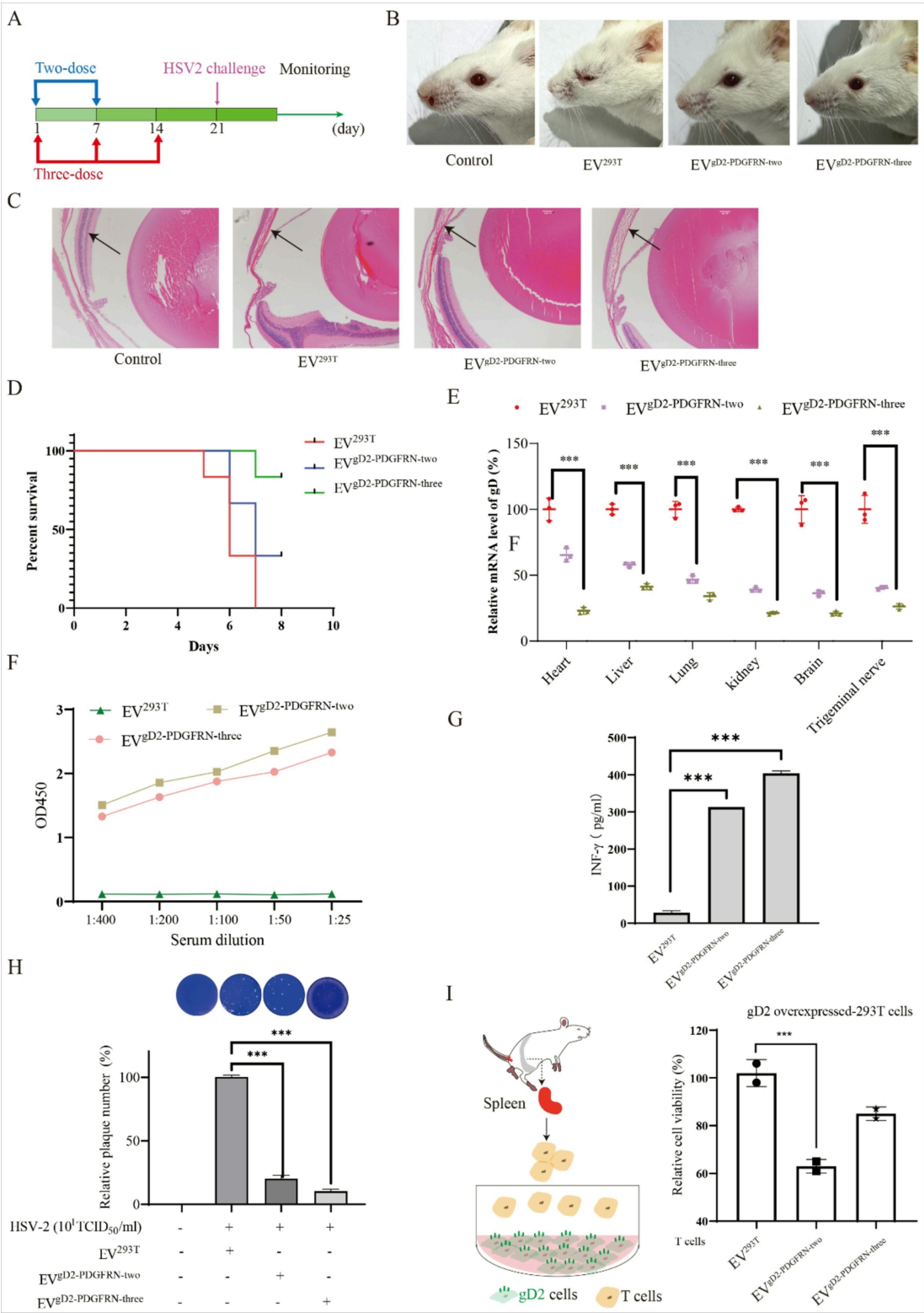


Figure 6

The immunogenic efficacy of three-dose EV^{gD2-PDGFRN} vaccination is superior to that of two-dose vaccination

(A) EVs immunization schedule: BALB/c mice (n=6/group) received two or three-dose of indicated EVs vaccine (5×10^7 particles/dose) by intraperitoneal injection followed by intranasal HSV-2 challenge (1×10^7 PFU). (B) The condition of corneal lesions (keratitis) and fur conditions with HSV1 infected mice. (C) The pathological sections of the eye with HE staining. (D) The survival curves of mice with HSV1 infection. (E) The analyses of viral gene gD2 expression levels in main

tissue of mice by RT-qPCR. (F) Serum anti-gD2 IgG titers measured by ELISA. (G) Serum IFN- γ concentration measured by ELISA. (H) The quantification of serum viral titers by viral plaque assays. (I) The left side illustrates a T cell cytotoxicity experiment. Specifically, T cells are isolated from the spleen and co-cultured with cells overexpressing gD2. The cell survival rate is then measured using the MTT assay. Data represent as Mean $\pm$ SD (n=3). Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. *** indicated P<0.001.

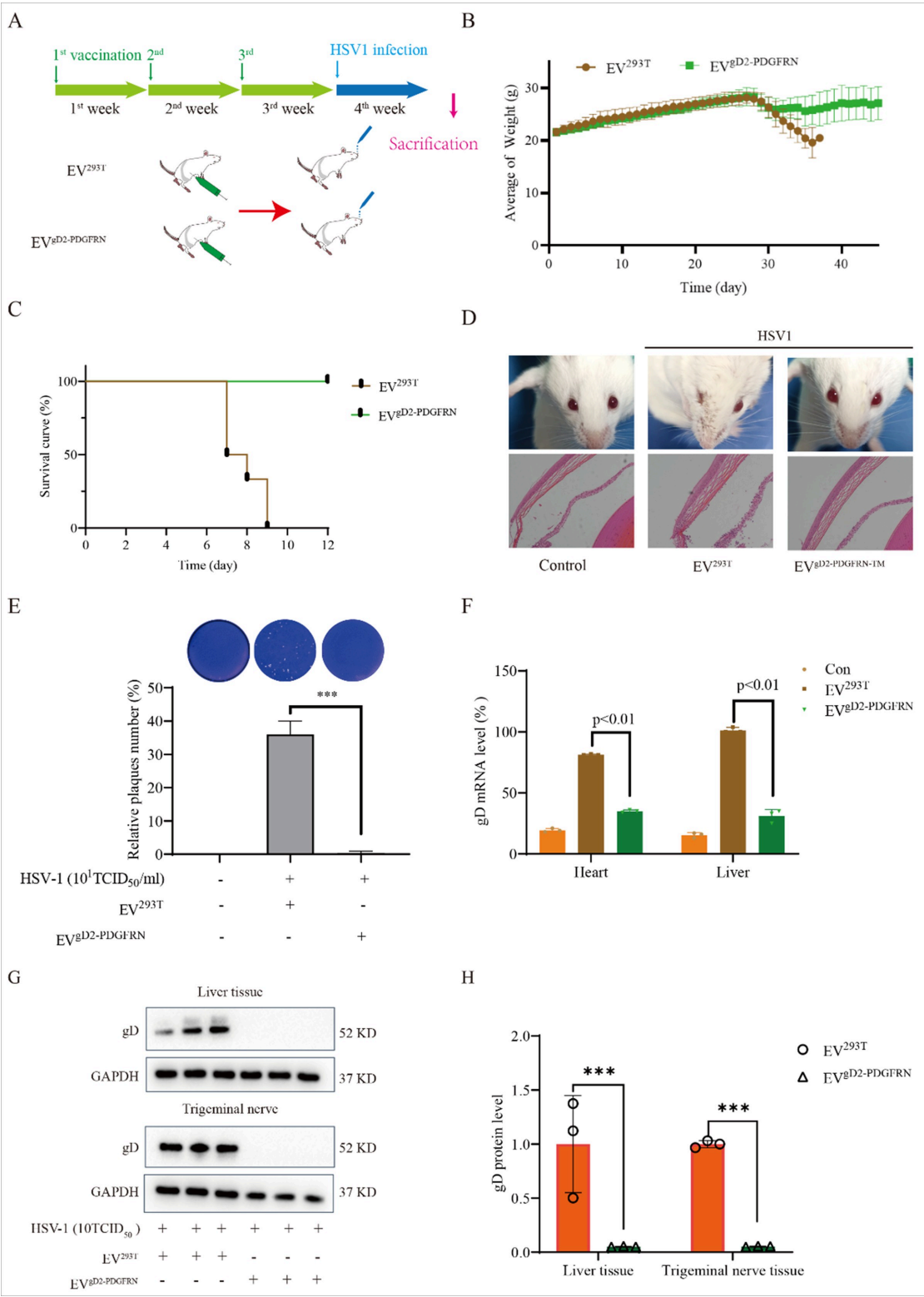


Figure 7

The EV^{gD2-PDGFRN} vaccine provides cross-protective immunity against HSV1 infection

(A) EVs immunization schedule: BALB/c mice (n=6/group) received three-dose of EV^{293T} or EVgD2-PDGFRN (5×10¹⁰ particles/dose) vaccine by intraperitoneal injection followed by intranasal HSV-1 challenge (1×10⁶ PFU). **(B)** The body weight of mice from the day of EVs immunization to HSV1 infection (n=6). **(C)** The survival curves of mice with HSV1 infection. **(D)** The up figures are the condition of corneal lesions (keratitis) and fur conditions with HSV1 infected mice. The down figures are the pathological sections of the eye with HE staining. **(E)** The quantification of serum viral titers by viral plaque assays. **(F)** The analyses of viral gene gD1 expression levels in heart and liver by RT-qPCR. **(G)** The analyses of viral protein gD1 levels in liver and trigeminal nerve by western blot. **(H)** Densitometric quantification of gD protein levels from **(G)**, values normalized to GAPDH and expressed as fold-change relative to EV^{293T} group. All histogram data are presented as Mean ± SD (n=6). Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. *** indicated P<0.001.

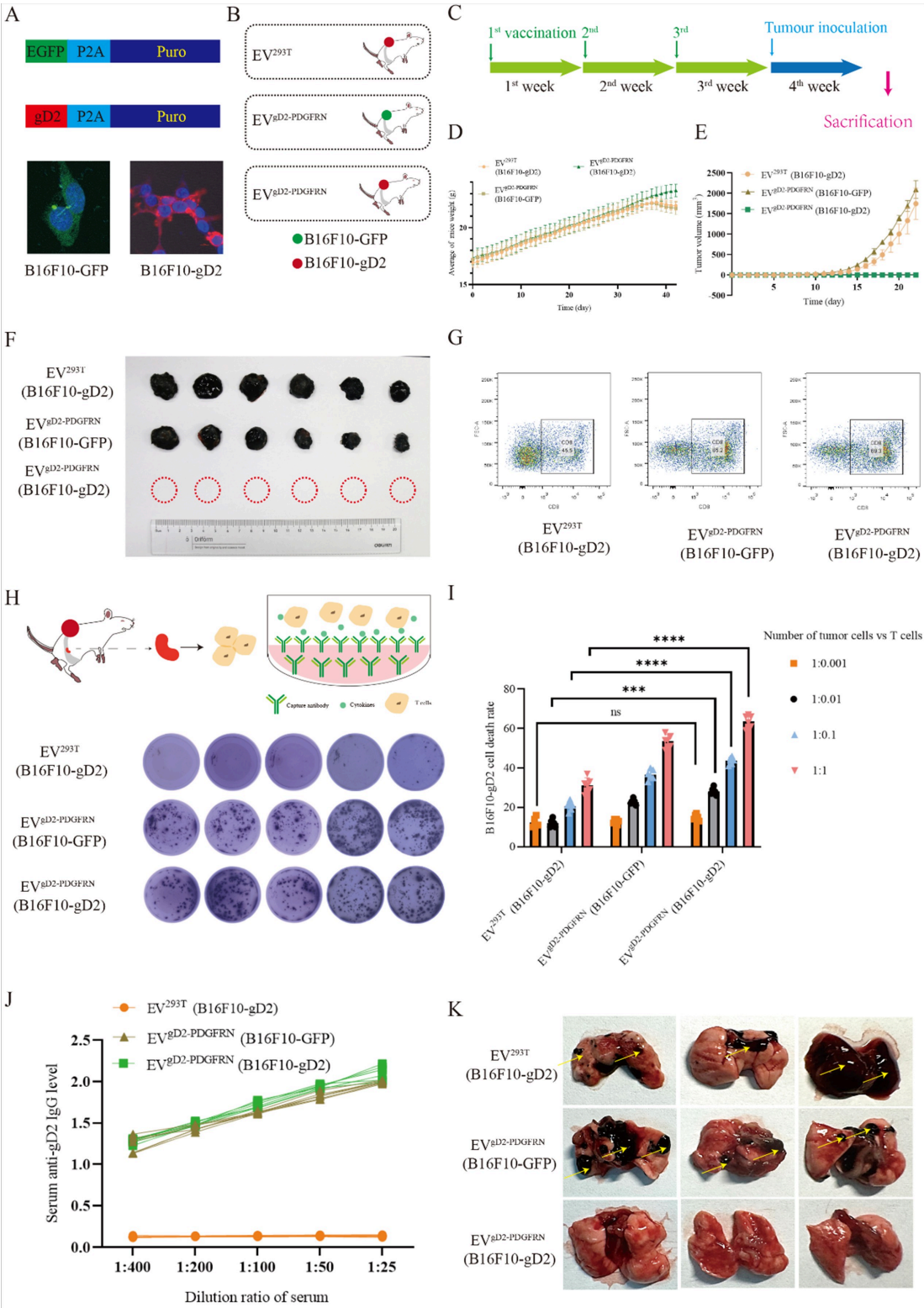


Figure 8

The EV^{gD2-PDGFRN} vaccine immunization inhibited the growth of melanoma cells expressing gD2 antigen

(A) The schematic diagram illustrates the construction of the EGFP and GD2 overexpression plasmids. The following figure presents confocal microscopy images of B16F10 cells stably expressing EGFP or gD2, designated as B16F10-GFP cells, and B16F10-gD2 cells. Scale bar: 20 μ m. **(B)** The schematic diagram of mouse immunization involves three groups of mice: 1. Mice immunized with the EV^{293T} vaccine, followed by inoculation with B16F10-gD2 cells. 2. Mice immunized with the EV^{gD2-PDGFRN} vaccine, followed by inoculation with B16F10-GFP cells (green). 3. Mice immunized with the EV^{gD2-PDGFRN} vaccine, followed by inoculation with B16F10-gD2 cells (red). **(C)** EVs immunization schedule: C57/BL6 mice (n=6/group) received three-dose of EV^{293T} or EV^{gD2-PDGFRN} (5×10^8 particles/dose) vaccine by intraperitoneal injection followed by indicated B16F10 inoculation. **(D)** Mouse body weight data from vaccine immunization. **(E)** Recording of tumor growth volume. **(F)** The image above shows a tumor (black) excised from the B16F10 cells inoculated mice, with a standard ruler of total length 20 cm placed below it. **(G)** Flow cytometry analysis of dendritic cell (DC) activation in tumor-draining lymph nodes. **(H)** ELISPOT assay demonstrating the IFN- γ spot formation by splenocytes from immunized mice. **(I)** The cytotoxic activity of T cells from mice immunized with EV vaccines against B16F10-gD2 cells. **(J)** Serum anti-gD2 IgG titers measured by ELISA. **(K)** The experiment on lung metastasis of B16F10 cells after indicated EVs vaccine immunization. Data are presented as Mean $\pm$ SD (n=6). Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ns indicated $P > 0.05$, *** indicated $P < 0.001$, **** indicated $P < 0.0001$.

Supplementary Files

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