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Brief Communication

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Recombinant Japanese Encephalitis Virus Envelope Antigen Presented on CoPoP Liposomes Elicits Cellular Immunity and Protects Mice From Lethal Challenge

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ABSTRACT

Japanese encephalitis virus (JEV) causes severe encephalitis in endemic regions, and vaccination remains the primary preventive measure. To develop subunit vaccines that enhance cellular immunity, we produced a soluble His–tagged recombinant JEV envelope (rJEV-E) ectodomain and particularized it on cobalt porphyrin–phospholipid (CoPoP) immunogenic liposomes containing an monophosphoryl lipid A-related TLR4 agonist, with or without *Quillaja saponaria* saponin fraction 21 (QS-21). CoPoP-mediated particleization enabled stable antigen display and, when combined with QS-21 (rJEV-E/ECLSQ), elicited Th1-biased $CD4^+$ and $CD8^+$ T-cell IFN-γ responses alongside functional neutralizing Abs. In a stringent intracerebral lethal challenge model, rJEV-E/ECLSQ conferred robust protection despite moderate 50% plaque reduction neutralization test titers. JEV-specific IgG responses were sustained for at least one year, with recallable cellular immunity upon late boosting. These findings demonstrate that CoPoP-based particleization enables qualitative modulation of vaccine-induced immunity while maintaining effective protection in a stringent challenge setting.

Keywords: Japanese encephalitis virus; Recombinant envelope (E) antigen; Cobalt porphyrin–phospholipid (CoPoP) liposome; Subunit vaccine; QS-21; Cellular immunity

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Conflict of Interest

The authors declare the following competing interests: Jinil Kim, Cheoljun Choi, Seung Yeon Kwon, Kiho Jeong, and Nari Kim are employees of EuBiologics Co., Ltd., which produced EcML™ and manufactured the rJEV-E antigen used in this study. All other authors declare that they have no competing interests.

Abbreviations

AUC, area under the curve; CoPoP, cobalt porphyrin–phospholipid; dLN, draining lymph node; DLS, dynamic light scattering; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; E, envelope; ECLS, monophosphoryl lipid A-related TLR4 agonist/cobalt porphyrin–phospholipid/1,2-dioleoyl-sn-glycero-3-phosphocholine/cholesterol liposome; ECLSQ, monophosphoryl lipid A-related TLR4 agonist/cobalt porphyrin–phospholipid/1,2-dioleoyl-sn-glycero-3-phosphocholine/cholesterol liposome supplemented with Quillaja saponaria saponin fraction 21; EcML, monophosphoryl lipid A-related TLR4 agonist; Hi5, High Five™; IACUC, Institutional Animal Care and Use Committee; JEV, Japanese encephalitis virus; MPLA, monophosphoryl lipid A; ns, not significant; $PRNT_{50}$, 50% plaque reduction neutralization test; QS-21, Quillaja saponaria saponin fraction 21; rJEV-E, recombinant Japanese encephalitis virus envelope; TCID50, 50% tissue culture infectious dose; Vero-iJEV, Vero cell-derived inactivated Japanese encephalitis virus vaccine.

Author Contributions

Conceptualization: Kang JM, Hwang SS, Lee JY; Data curation: Kim SY, Lee JY; Formal analysis: Kim SY, Jung IR, Park H, Choi JO; Funding acquisition: Kang JM, Hwang SS, Lee JY; Investigation: Kim SY, Jung IR, Park H, Choi JO, Kim SH, Pak J, Kim J, Choi C, Kwon SY, Jeong K, Kim N, Kim J, Shim DH; Resources: Kim J, Choi C, Kwon SY, Jeong K, Kim N, Nam KT, Jeong DG, Lee JM, Kang JM, Hwang SS, Lee JY; Supervision: Kang JM, Hwang SS, Lee JY; Validation: Lee JY; Visualization: Kim SY; Writing - original draft: Kim SY, Jung IR, Park H, Kim N, Lee JY; Writing - review & editing: Kim SY, Kang JM, Hwang SS, Lee JY.

INTRODUCTION

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that remains a major public-health threat in endemic regions across Asia and has recently expanded into parts of Oceania (1-5). An estimated 68,000 clinical cases occur annually, with substantial under-ascertainment, highlighting JEV as an emerging zoonotic disease within a One Health framework (1,6,7). Transmission is maintained primarily by *Culex* mosquitoes in enzootic cycles involving pigs and ardeid birds, whereas humans are incidental, dead-end hosts (1-5). Clinical manifestations range from non-specific febrile illness to severe meningoencephalitis, with reported case-fatality rate of 8%–30% and long-term neuropsychiatric sequelae in 30%–50% of survivors (8-12).

Because no specific antiviral therapy is available (9), vaccination remains the cornerstone of JEV prevention (3,13). Although legacy mouse brain-derived, formalin-inactivated vaccines have been replaced by live-attenuated SA14-14-2 vaccine and Vero cell-derived inactivated vaccines (14,15), immunological limitations persist, including multi-dose schedules, waning neutralizing Ab titers that may necessitate boosters, and reactogenicity considerations (15,16). Collectively, these constraints motivate the development of vaccine candidates that combine favorable safety with durable, high-quality immunity.

Protective immunity to viral infection is multifactorial. While neutralizing Abs are a key component of protection, coordinated cellular responses contribute to clearance of infected cells and the establishment of durable immune memory (17-19). Across diverse viral systems, vaccine-induced $CD8^+$ T cells have been associated with improved quality of protection and accelerated control of infection (20-26). In the context of JEV and flaviviruses, experimental and clinical observations likewise support a contributory role for cellular immunity, particularly $CD8^+$ T-cell responses, in shaping vaccine responses and disease outcomes (22,23,27,28). Nevertheless, currently licensed JEV vaccines may not consistently elicit durable $CD8^+$ T-cell memory, highlighting the need for platforms capable of inducing more balanced and persistent humoral and cellular immunity (27,28).

Recombinant protein vaccines offer favorable safety and manufacturability, but often require tailored adjuvants and delivery strategies to elicit potent and durable immunity (25,29,30). The spontaneous nanoliposome antigen particleization platform using cobalt porphyrin–phospholipid (CoPoP) has emerged as a promising approach to enhance the immunogenicity of recombinant antigens (31). CoPoP is embedded within the lipid bilayer and can stably capture polyhistidine-tagged antigens via cobalt coordination, enabling a particulate and repetitive antigen display that can enhance uptake by antigen-presenting cells and promote delivery to draining lymph nodes (dLNs) (32). In addition, immunostimulatory components, including TLR4 agonist monophosphoryl lipid A (MPLA) and *Quillaja saponaria* saponin fraction 21 (QS-21), can be combined to enhance innate activation and promote Th1-skewed and cytotoxic T-cell–biased responses (33-36). Consistent with these mechanisms, CoPoP-based formulations have elicited robust Ab responses and antigen-specific cellular immunity across viral (37-39) and cancer vaccine models (40).

Here, we developed a recombinant JEV envelope (rJEV-E) protein vaccine, the primary target of neutralizing Abs and a key determinant of viral attachment and membrane fusion (41,42). We produced a soluble, His-tagged rJEV-E ectodomain and formulated it on CoPoP liposomes containing an MPLA-related TLR4 agonist (EcML™), with or without QS-21. We evaluated

antigen-specific humoral and cellular immune responses, Ab durability over one year, and protective efficacy in mice using a stringent intracerebral lethal challenge model.

MATERIALS AND METHODS

Cells, virus, and reagents

Spodoptera frugiperda Sf9 insect cells were maintained in Sf-900™ III SFM (Gibco, Waltham, MA, USA), and High Five™ (Hi5; *Trichoplusia ni*) cells were maintained in ESF-921 medium (Expression Systems, Davis, CA, USA). All insect cell cultures were grown in suspension at 27°C with shaking at 125 rpm.

JEV genotype III strain NCCP41304 (GenBank accession No. FJ938224), provided by the Korea National Institute of Health, was used for plaque reduction neutralization tests (PRNTs) and mouse challenge experiments.

CoPoP was purchased from POP Biotechnologies (Buffalo, NY, USA), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). EcML™ (an MPLA-related TLR4 agonist produced from recombinant *Escherichia coli*) was produced by EuBiologics (Chuncheon, Korea). QS-21 was obtained from Desert King (San Diego, CA, USA). Imject® Alum was purchased from Thermo Fisher Scientific (Waltham, MA, USA). A licensed Vero cell-derived inactivated JEV vaccine (Vero-iJEV; Boryung Cell-culture Japanese Encephalitis Vaccine Inj.; Boryung Biopharma, Seoul, Korea) was used as a comparator vaccine and as ELISA coating antigen.

Expression and purification of rJEV-E protein

The JEV envelope (E) ectodomain (residues 1–401) derived from the Nakayama strain was expressed using a baculovirus system. A soluble E construct containing an N-terminal secretion signal and a C-terminal hexahistidine tag was generated and expressed in Hi5 cells. The recombinant E protein was purified from culture supernatants by nickel affinity chromatography followed by size-exclusion chromatography. Purified protein was buffer-exchanged, quantified by absorbance at 280 nm, and stored at −80°C until use. Detailed cloning, expression, and purification procedures are provided in the **Supplementary Data 1** and **Supplementary Fig. 1**.

Preparation of CoPoP-based liposomal adjuvants and antigen formulation

ECLS liposomes were manufactured by combining EcML™, CoPoP, DOPC, and cholesterol at a molar ratio of 1:2:40:10. Lipids dissolved in chloroform were dried by rotary evaporation and nitrogen drying, followed by overnight air-drying, to form a thin lipid film. The film was rehydrated in PBS (pH 7.2) and sonicated in a water bath sonicator (BRANSON CPX8800H-E; Branson Ultrasonics Corp., Danbury, CT, USA) at 62°C±2°C for 2–3 h. The formulation was then microfluidized at 15,000 psi for 3 passes using an LM20 Microfluidizer Processor (Microfluidics, Westwood, MA, USA). Liposomes were diluted to 0.4 mg/ml based on EcML™ concentration, sterile-filtered through a 0.2-μm polyethersulfone filter, and stored at 2°C–8°C.

ECLSQ was prepared by mixing QS-21 with ECLS before immunization. For antigen particleization prior to immunization, rJEV-E antigen, ECLS liposomes, and QS-21 were mixed in PBS (pH 7.2) to final concentrations of 100 μg/ml, 100 μg/ml, and 20 μg/ml,

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respectively, and incubated for 2 h at room temperature, allowing His-tag–mediated coordination of rJEV-E to CoPoP in the liposomal bilayer.

Dynamic light scattering (DLS)

The hydrodynamic diameter and polydispersity index of liposomal formulations were measured using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Samples were diluted 20-fold in 1× PBS (pH 7.2) prior to measurement. For each formulation, 3 independently prepared cuvettes were measured, and each cuvette was read in triplicate; the mean of each cuvette was treated as one technical replicate (n=3). Data are reported as mean ± SD.

Mice, immunization, and sample collection

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University College of Medicine (approval No. 2022-0270) and were performed in accordance with institutional guidelines and applicable national regulations. Six-week-old female BALB/c mice (Orient Bio, Seongnam, Korea) were housed under conventional conditions (12 h light/dark cycle, 23°C±2°C, 50%±5% relative humidity) with ad libitum access to food and water. Mice were randomly allocated to experimental groups. Investigators were blinded to group allocation during data acquisition.

Mice were immunized at 0, 2, and 6 wk with PBS or 5 μg of rJEV-E, either alone or formulated with Imject® Alum, ECLS, or ECLSQ in a 50 μl per dose administered into gastrocnemius muscle. Mice were administered Vero-iJEV intraperitoneally at 0, 2, and 6 wk (200 μl per injection). The 200 μl intraperitoneal dose corresponds to 10 ml/kg for a 20 g mouse and is within commonly used intraperitoneal volume limits.

Two weeks after the final vaccination (8 wk), blood, spleens, and popliteal dLNs were collected for immunological assays. For long-term immunogenicity evaluation, sera were collected at 8, 17, 26, 35, 44, and 52 wk for ELISA. At 52 wk, mice received a booster immunization with the same formulation, dose, and route as the primary immunizations, and blood, spleens, and dLNs were collected 2 wk later (54 wk). Serum samples were stored at −80°C until analysis.

Intracerebral lethal challenge and post-infection analyses

Two weeks after the final immunization (8 wk), mice were challenged by intracerebral inoculation with JEV NCCP41304 (1×10^4 50% tissue culture infectious dose [$TCID_{50}$] in 50 μl) under ketamine/xylazine anesthesia and monitored daily for survival and clinical signs for 21 days post-infection.

Clinical scores were assessed using a predefined rubric: 0=normal; 1=mild lethargy/ruffled fur; 2=hunched posture/reduced mobility; 3=neurologic signs such as tremor or paresis; 4=severe neurologic impairment/moribund; 5=death. Humane endpoints were applied according to the IACUC protocol (e.g., severe neurologic signs or ≥20% weight loss), and animals meeting endpoints were euthanized using ketamine/xylazine anesthesia.

For viral load analysis, brains were collected after challenge and viral titers were quantified by plaque assay using BHK-21 cells. Detailed procedures are described in the **Supplementary Data 1**.

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Immunological assays

JEV-specific Ab responses were measured by ELISA using Vero-iJEV as the coating antigen. Neutralizing Ab titers were determined by 50% plaque reduction neutralization test ($PRNT_{50}$) using JEV NCCP41304. Cellular immune responses were evaluated by IFN-γ ELISpot following stimulation with JEV-derived MHC class II or class I peptides. Detailed assay procedures including ELISA, PRNT, ELISpot are described in the **Supplementary Data 1**.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 10.6.1; GraphPad Software, San Diego, CA, USA). Significance was set at $p<0.05$. Appropriate parametric or nonparametric tests were applied as indicated, including 1- or 2-way ANOVA with Tukey's multiple comparisons test and the Mantel–Cox log-rank test for survival analysis. Specific statistical methods are described in the corresponding figure legends.

For more details on the materials and methods used, please refer to the **Supplementary Data 1**.

RESULTS

Production of soluble rJEV-E and formulation on CoPoP-based liposomal adjuvants

To generate a soluble rJEV-E protein antigen, we expressed a C-terminally truncated E protein lacking the transmembrane region (residues 1–401 of the Nakayama strain). The coding sequence was cloned into pAcGP67A vector including a C-terminal TEV protease site followed by a hexahistidine tag (6×His) for purification and detection, and an IE-1 sequence was inserted via EcoRV digestion to generate the pIP-JEV-E plasmid (**Fig. 1A**). Recombinant rJEV-E was produced in a baculovirus/insect cell expression system. Purified rJEV-E recovered from culture supernatants migrated at ~45 kDa by SDS-PAGE and was detected as a corresponding band by western blotting, consistent with its predicted molecular mass (**Fig. 1B and C**).

We next formulated His-tagged rJEV-E on CoPoP-based immunogenic liposomes. ECLS liposomes were composed of DOPC, cholesterol, EcML™, and CoPoP, and the QS-21-containing formulation (ECLSQ) was generated by supplementing ECLS with QS-21. The His-tag enables directional coordination of rJEV-E to cobalt within the bilayer, facilitating particulate surface display (**Fig. 1D**). DLS showed that empty liposomes and antigen-formulated particles had comparable hydrodynamic diameters (112.9–121.6 nm) and similar polydispersity indices (~0.22), indicating that antigen loading and QS-21 supplementation did not materially alter particle size distribution under the tested conditions (**Fig. 1E and F**).

QS-21–containing CoPoP liposomes enhance humoral and cellular immunogenicity of rJEV-E

We evaluated the immunogenicity of rJEV-E formulated with ECLS or ECLSQ in BALB/c mice immunized intramuscularly at 0, 2, and 6 wk with PBS, rJEV-E alone, rJEV-E/Alum, rJEV-E/ECLS, or rJEV-E/ECLSQ. Serum and spleens were collected 2 wk after the final dose (8 wk) (**Fig. 2A**).

Serum Ab titers measured by ELISA showed that adjuvantation increased JEV-specific IgG relative to antigen alone, with rJEV-E/ECLSQ eliciting the highest responses among the

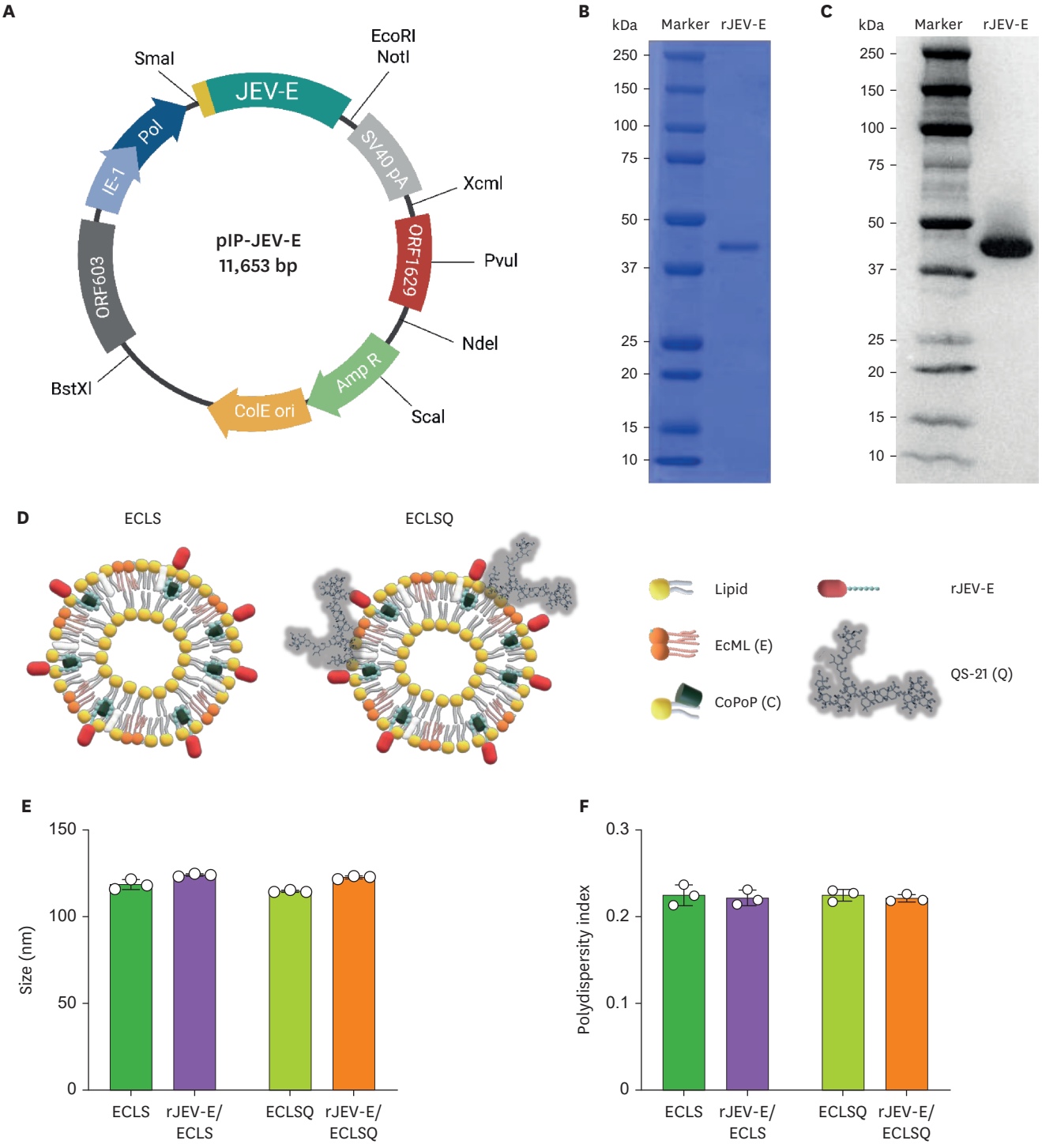

Figure 1. Generation and characterization of rJEV-E antigen formulated with CoPoP-based liposomal adjuvants. (A) Schematic map of the pIP-JEV-E expression vector encoding soluble rJEV-E. (B) Coomassie-stained SDS-PAGE of purified rJEV-E (~45 kDa). (C) Western blot analysis confirming rJEV-E at the expected molecular mass. Molecular weight markers (kDa) are indicated. (D) Schematic representation of CoPoP-based liposomal adjuvants. ECLS consists of DOPC, cholesterol, EcML™ (MPLA-related TLR4 agonist), and CoPoP. ECLSQ denotes ECLS supplemented with QS-21. His-tag–mediated coordination enables surface display of rJEV-E on CoPoP liposomes. (E, F) DLS analysis of hydrodynamic diameter (E) and polydispersity index (F) of empty liposomes (ECLS, ECLSQ) and antigen-formulated particles (rJEV-E/ECLS, rJEV-E/ECLSQ). Samples were diluted 20-fold in PBS prior to measurement. Data are shown as mean ± SD of 3 independent cuvettes (n=3); each cuvette was read in triplicate and averaged.

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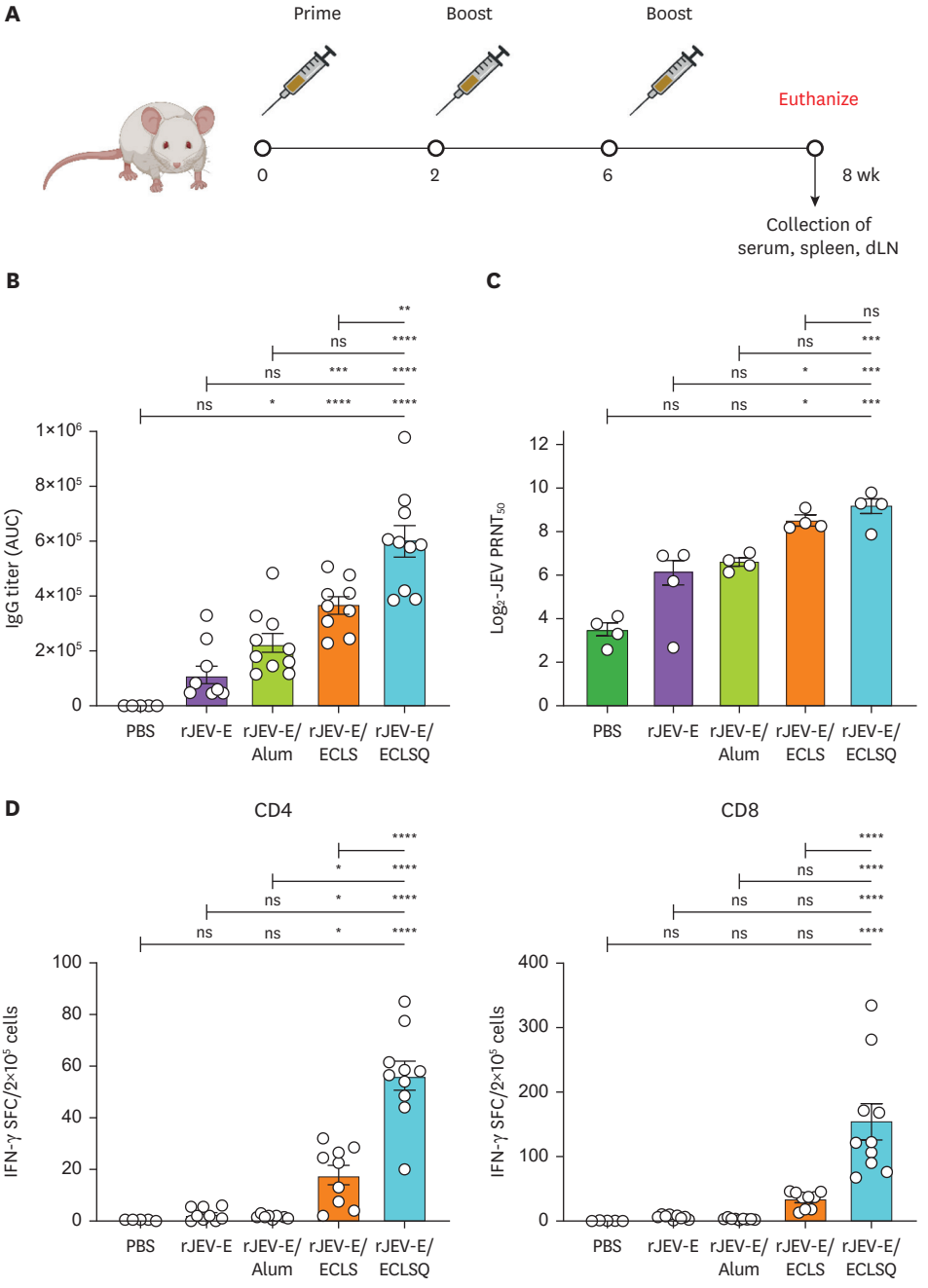

Figure 2. Humoral and cellular immune responses induced by rJEV-E formulated with liposomal adjuvants.
(A) Immunization schedule (0, 2, and 6 wk) and sampling at 8 wk (serum, spleen, and dLNs). (B) JEV-specific binding IgG responses measured by ELISA and summarized as AUC. (C) Neutralizing Ab titers measured by $PRNT_{50}$ (shown as log2-transformed titers). (D) IFN-γ ELISpot responses of splenocytes and dLN cells following stimulation with MHC class II (CD4) or class I (CD8) peptides, shown as SFCs per 2×10^5 cells. ELISA and ELISpot assays were performed on individual mice, with each symbol representing one mouse (n=9–10 mice per group). $PRNT_{50}$ was conducted using pooled sera from 2–3 mice per group, with each symbol representing one pool of sera (n=4 pools per group). Data are pooled from 2 independent experiments and are presented as the means ± SEM.
SFC, spot-forming cell.
Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparisons test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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tested formulations (**Fig. 2B**). Neutralizing activity assessed by $PRNT_{50}$ followed a similar trend, with rJEV-E/ECLSQ yielding the highest neutralizing activity (**Fig. 2C**). Cellular immunity assessed by IFN-γ ELISpot after stimulation with MHC class II (CD4) and MHC class I (CD8) peptides showed that rJEV-E/ECLSQ induced the strongest IFN-γ responses to both peptide stimulations, exceeding PBS, alum, ECLS, and unadjuvanted groups (**Fig. 2D**). Collectively, these data identify rJEV-E/ECLSQ as the most immunogenic formulation among those tested, inducing robust humoral responses together with a pronounced IFN-γ T-cell response profile.

rJEV-E/ECLSQ confers complete protection against lethal intracerebral JEV challenge

Based on the immunogenicity data, we assessed protective efficacy of rJEV-E/ECLSQ against JEV using a stringent intracerebral lethal challenge model. Two weeks after the final immunization (8 wk), mice were challenged intracerebrally with JEV genotype III NCCP41304 at a target dose of 1×10^4 $TCID_{50}$ per mouse. Animals were monitored daily for 21 days post-infection for survival and clinical disease scores (**Fig. 3A**). PBS-treated mice developed progressive disease with substantial mortality, whereas rJEV-E/ECLSQ conferred complete protection with minimal clinical signs, whereas both vaccinated groups achieved complete survival (**Fig. 3B and C**). To quantify central nervous system viral replication, brain viral titers were measured by plaque assay at 8 days post-infection. Both rJEV-E/ECLSQ and Vero-iJEV markedly reduced brain viral loads relative to PBS controls, with no significant difference between the 2 vaccinated groups under these conditions (**Fig. 3D**).

To further compare immune profiles associated with protection, we assessed humoral and cellular responses induced by rJEV-E/ECLSQ versus Vero-iJEV in a parallel immunized cohort at 8 wk. While both vaccines induced robust JEV-specific Ab responses (**Fig. 3E**), Vero-iJEV generated higher neutralizing Ab titers than rJEV-E/ECLSQ (**Fig. 3F**). In contrast, rJEV-E/ECLSQ induced substantially higher IFN-γ ELISpot responses to both CD4 and CD8 peptide stimulation compared with Vero-iJEV (**Fig. 3G**). These findings indicate distinct immune signatures between the subunit liposomal platform and the licensed inactivated vaccine and are consistent with the possibility that cellular immune responses contribute to protection in this stringent intracerebral challenge setting.

rJEV-E/ECLSQ induces durable Ab responses and recall cellular immunity after late boosting

To evaluate durability, JEV-specific IgG was measured longitudinally by ELISA through one year after primary immunization. IgG responses peaked after completion of the primary series and gradually declined over time but remained above background through 52 wk. Across follow-up, rJEV-E/ECLSQ elicited higher JEV-specific IgG levels than Vero-iJEV at early time points, and although the differences became less pronounced after 26 wk, JEV-specific IgG remained detectable throughout the study period (**Fig. 4B**). At 52 wk, mice received a booster immunization, and humoral and cellular responses were assessed 2 wk later (54 wk) (**Fig. 4A**). Post-boost JEV-specific IgG titers were comparable among groups (**Fig. 4C**), whereas neutralizing titers in the rJEV-E/ECLSQ group remained lower than those in the Vero-iJEV group (**Fig. 4D**). Nevertheless, rJEV-E/ECLSQ elicited the highest IFN-γ ELISpot responses, particularly following MHC class I (CD8) peptide stimulation (**Fig. 4E**). Taken together, these data indicate that rJEV-E/ECLSQ induces durable humoral immunity and robust cellular immune recall responses over an extended follow-up period.

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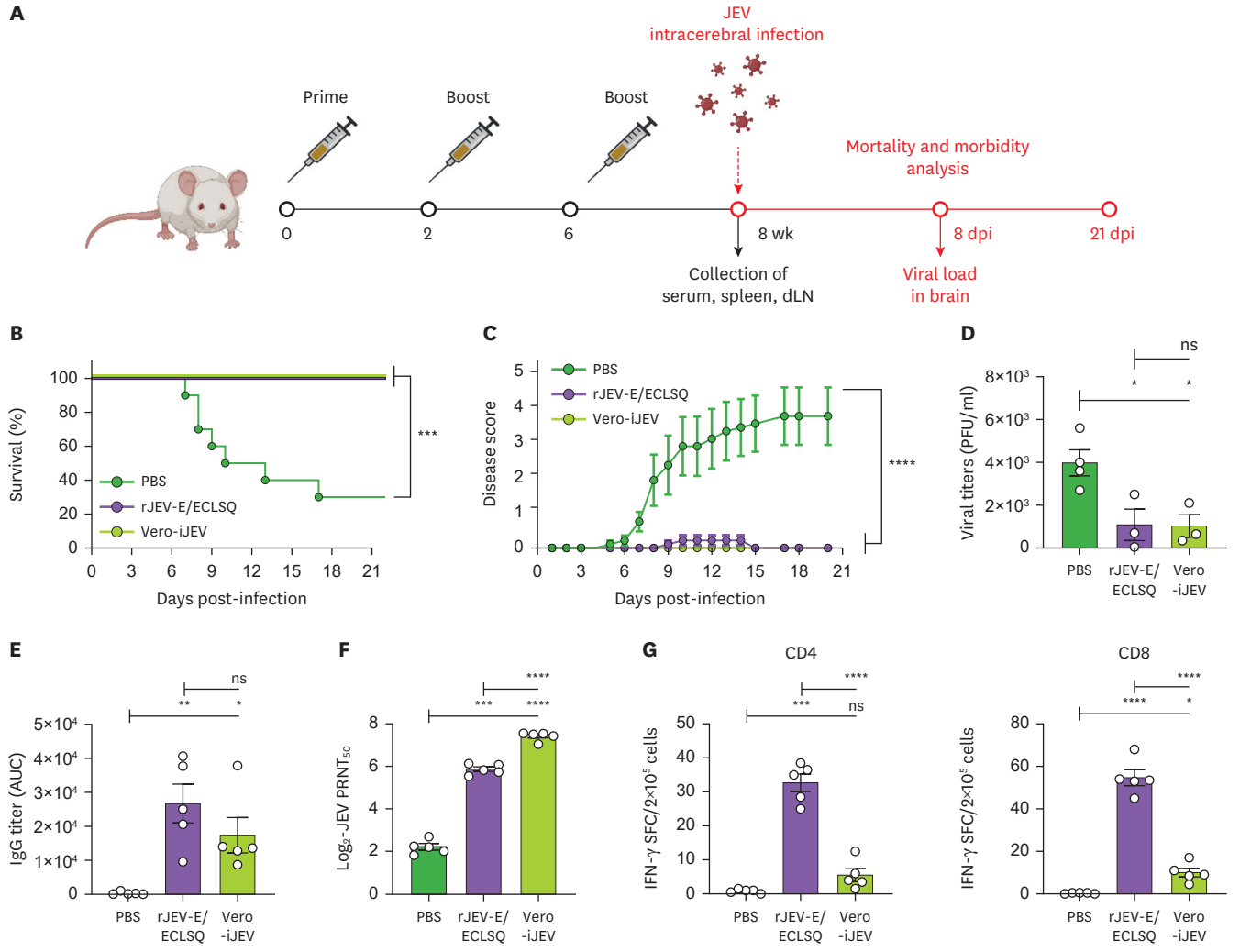

Figure 3. Protective efficacy of rJEV-E/ECLSQ against lethal intracerebral JEV challenge and immune profiling versus a licensed inactivated vaccine.
(A) Immunization and challenge scheme. Mice immunized with PBS (i.m.; n=10), rJEV-E/ECLSQ (i.m.; n=10), or Vero-iJEV (i.p.; n=5) were challenged intracerebrally with JEV genotype III NCCP41304 at 8 wk (1×10^4 $TCID_{50}$ per mouse) and monitored for 21 dpi. Brain viral load was assessed at 8 dpi. (B) Kaplan–Meier survival curves. Significant differences in survival rate were determined using the log-rank (Mantel–Cox) test. (C) Clinical disease scores over time after challenge. Data are pooled from 2 independent experiments and presented as mean ± SEM. Statistical comparisons were performed using 2-way ANOVA followed by Tukey's multiple comparisons test. (D) Viral titers in brains determined by plaque assay at 8 dpi (PBS, n=4; rJEV-E/ECLSQ, n=3; Vero-iJEV, n=3). Statistical comparisons were performed using one-way ANOVA with Tukey's multiple comparisons test. (E, F) JEV-specific IgG titers (AUC) (E) and neutralizing Ab titers ($PRNT_{50}$; log2-transformed) (F) measured 2 wk after the final immunization (8 wk) in a parallel immunized cohort (each symbol represents one mouse; n=5 per group). (G) IFN-γ ELISpot responses following MHC class II (CD4) or class I (CD8) peptide stimulation in the same parallel cohort (n=5 per group). For panels (D-G), data are presented as the means ± SEM.
i.m., intramuscular injection; i.p., intraperitoneal injection; dpi, days post-infection.
Multiple-group comparisons were performed using one-way ANOVA followed by Tukey's multiple comparisons test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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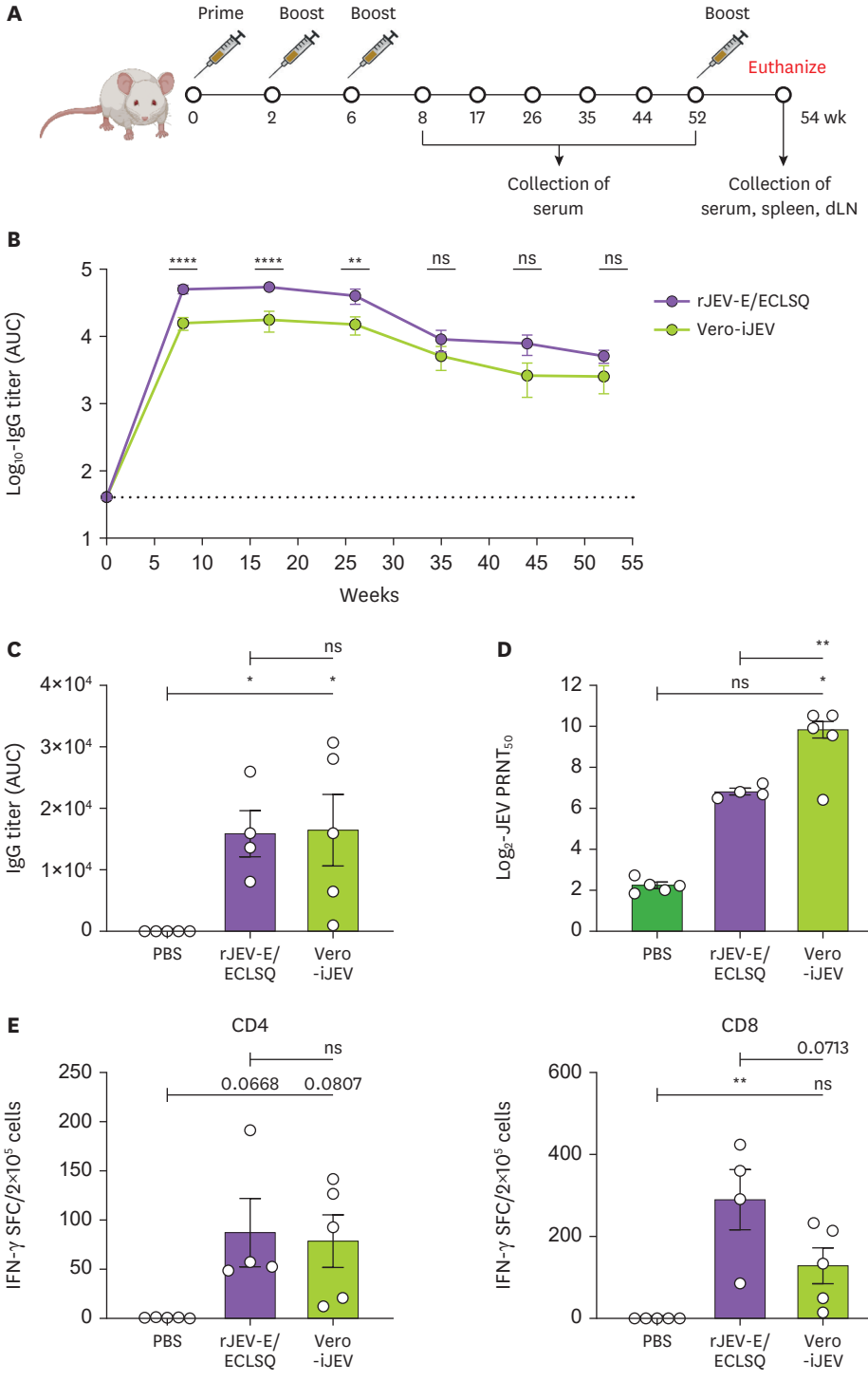

Figure 4. Longitudinal Ab persistence and recall cellular immunity after rJEV-E/ECLSQ immunization. (A) Study design for long-term follow-up. Serum was collected longitudinally through 52 wk; a booster was administered at 52 wk; terminal sampling was performed at 54 wk (serum, spleen, and dLN collection). (B) Longitudinal JEV-specific IgG titers (AUC) measured by ELISA and summarized as log10-transformed AUC at the indicated weeks. The dotted line indicates background signal (PBS). Statistical comparisons between vaccine groups at each time point were performed using 2-way (repeated-measures) ANOVA with Tukey's multiple comparisons test. (C, D) JEV-specific IgG titers (AUC) (C) and $PRNT_{50}$ neutralizing titers (log2-transformed) (D) 2 wk after the 52 wk booster (54 wk). (E) IFN-γ ELISpot responses 2 wk after boosting (54 wk), following MHC class II (CD4) or class I (CD8) peptide stimulation. For panels (C-E), each symbol represents one mouse (n=4–5 per group). Data are presented as mean ± SEM.
Statistical comparisons were performed using one-way ANOVA with Tukey's multiple comparisons test unless otherwise indicated; *p<0.05, **p<0.01, ****p<0.0001; Exact p-values are shown where indicated.

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DISCUSSION

JEV remains a significant public-health concern, and continued development of vaccine platforms that can elicit durable and high-quality immunity is warranted (1-5,15,16). In this study, we evaluated a soluble rJEV-E ectodomain formulated on CoPoP-based immunogenic liposomes containing an EcML™, with or without QS-21 supplementation. Among the tested formulations, QS-21 supplemented CoPoP liposomes (ECLSQ) elicited the strongest overall immunogenicity, including Ab responses, the highest neutralizing activity, and the most pronounced IFN-γ producing type 1 (Th1) immune responses to both MHC class II and class I peptides. Importantly, in a stringent intracerebral challenge model, both rJEV-E/ECLSQ and Vero-iJEV achieved complete survival and marked reduction of brain viral loads.

The JEV-E protein mediates virion attachment and membrane fusion and constitutes a dominant target of virus-neutralizing Abs, which represent an important component of protection against JEV infection (41,42). For this study, we selected the Nakayama strain as the recombinant E source because it is a well-characterized genotype III reference with established recombinant expression performance and demonstrated immunogenicity (43,44). Nevertheless, as a recombinant subunit antigen, optimal immunogenicity depends on appropriate formulation strategies to ensure effective antigen presentation and immune activation. The improved performance of ECLSQ relative to ECLS is compatible with established adjuvant properties of QS-21 in promoting type 1-skewed immunity and supporting cytotoxic T-cell priming and differentiation (45,46). While mechanistic dissection was beyond the scope of this study, the enhanced IFN-γ ELISpot responses observed with QS-21 supplementation are consistent with improved antigen presentation and/or T-cell priming *in vivo*.

Neutralizing Ab titers, including $PRNT_{50}$, are well-established correlates of protection in peripheral JEV infection and remain an important benchmark for vaccine evaluation (47). In the present study, Vero-iJEV induced higher $PRNT_{50}$ titers than rJEV-E/ECLSQ. However, in the stringent intracerebral challenge model, both vaccines conferred complete survival with minimal clinical signs and comparably reduced brain viral loads. These findings indicate that serum $PRNT_{50}$ magnitude alone did not segregate protection under these experimental conditions. Because intracerebral inoculation represents a deliberately stringent model that does not recapitulate the natural peripheral route of infection, protection in this setting may not be fully predicted by circulating neutralizing Ab titers alone. Accordingly, immune components not captured by standard $PRNT_{50}$ assays, potentially including cellular immunity and/or qualitative Ab effector functions (e.g., Fc-mediated activity), may contribute to protection in this context. We do not dispute the established role of $PRNT_{50}$ as a validated surrogate marker for peripheral protection. We therefore interpret the intracerebral model as a complementary, hypothesis-generating system to probe additional protective features of vaccine platforms designed to enhance cellular immunity, rather than as a replacement for established peripheral correlates of protection.

Durability analyses further supported the immunologic potential of rJEV-E/ECLSQ. Over a one-year follow-up, rJEV-E/ECLSQ elicited sustained JEV-specific IgG titers, and a late booster elicited robust recall cellular responses, particularly following MHC class I peptide stimulation. These findings are consistent with the maintenance of antigen-experienced memory T cells and durable humoral immunity, potentially supported by sustained germinal center activity, long-lived plasma cells, and T follicular helper cell responses.

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However, as these subsets were not directly assessed in the present study, mechanistic immunophenotyping will be required in future work to define the cellular pathways underlying Ab persistence and recall responses.

Several limitations and translational priorities emerge from this work. First, peripheral challenge models together with measurements of viral burden and neuropathology will be needed to strengthen biological relevance and link immune profiles to *in vivo* control. Second, mechanistic experiments, such as $CD8^+$ T-cell depletion and antigen specific memory T cell formation, will be required to define the relative contributions of cellular immunity and effector functions. Third, the rJEV-E (Nakayama strain), comparator vaccine (Beijing-1 strain, as reported), and challenge virus (NCCP41304) were all genotype III and demonstrated >99% amino-acid identity in the E-protein (**Supplementary Fig. 2**), minimizing the likelihood of major antigenic mismatch. Importantly, protective efficacy was evaluated using the same challenge virus across all groups, thereby avoiding group-specific challenge bias. Nevertheless, even minor sequence variation may influence quantitative immunogenicity readouts. This underscores the need for future studies using fully strain-matched antigens, comparator vaccines, and challenge viruses to refine immunogenicity comparisons and clarify immune correlates. Finally, interpretation of cross-platform comparisons should also consider differences in immunization route and dose regimen used in this study. Vero-iJEV was administered intraperitoneally, whereas rJEV-E formulations were delivered intramuscularly. This comparator regimen was selected to provide a reproducible positive-control benchmark for protection in mice under intracerebral challenge conditions rather than to model clinical vaccination in humans. In pilot experiments under the same intracerebral challenge conditions, intramuscular or subcutaneous administration of Vero-iJEV did not consistently confer survival, whereas intraperitoneal administration provided reproducible protection (**Supplementary Fig. 3**). Although this approach enabled benchmarking against a licensed product under practical mouse-study constraints, route mismatch and non-harmonized dose regimens limit direct immunogenicity comparisons. Future head-to-head studies using matched routes, harmonized dosing, individual-level neutralization assays, and defined mechanistic perturbations will further clarify correlates and mechanisms of protection.

In summary, particulate display of rJEV-E antigen on CoPoP liposomes adjuvanted with EcML™ and supplemented with QS-21 was associated with strong IFN-γ cellular responses, complete protection in a stringent lethal challenge model, and durable Ab responses with robust recall cellular immunity. These findings support further evaluation of this CoPoP-based liposomal subunit vaccine approach in models and assays designed to define breadth, mechanism, and translational relevance.

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SUPPLEMENTARY MATERIALS

Supplementary Data 1

Materials and methods

Supplementary Figure 1

Sequence alignment of $CD4^+$ (DLALPWTPPSSTAW) and $CD8^+$ (CYHASVTDI) T cell epitopes between the Nakayama and Beijing-1 strains. Sequences were obtained from the National Center for Biotechnology Information (NCBI) database, and the corresponding $CD4^+$ and $CD8^+$ T cell peptide region was aligned to assess sequence conservation between the Nakayama and Beijing-1 strains. Conserved sequences shared by both strains are highlighted with red boxes.

Supplementary Figure 2

Alignment of E protein amino acid sequences across strains. Sequences were retrieved from the National Center for Biotechnology Information (NCBI) database and aligned to compare conserved and variable regions among the Nakayama, Beijing-1, and NCCP41304 strains. Red arrows indicate differences in amino acid sequences among the strains.

Supplementary Figure 3

Impact of vaccination route on survival after viral challenge. Survival rates of mice (n=5 per group) vaccinated with PBS (i.m.) or Vero-iJEV via different routes (s.c., i.p., or i.m.) and challenged with JEV genotype III NCCP41304 at 8 wk (1×10^4 $TCID_{50}$ per mouse). Mice were monitored for 14 days after viral challenge.

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