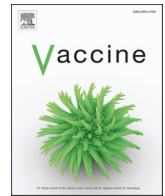


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## Virus-like particles of louping ill virus elicit potent neutralizing antibodies targeting multimers of viral envelope protein

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### ABSTRACT

Louping ill virus (LIV) is a tick-borne flavivirus that predominantly causes disease in livestock, especially sheep in the British Isles. A preventive vaccine, previously approved for veterinary use but now discontinued, was based on an inactivated whole virion that likely provided protection by induction of neutralizing antibodies recognizing the viral envelope (E) protein. A major disadvantage of the inactivated vaccine was the need for high containment facilities for the propagation of infectious virus, as mandated by the hazard group 3 status of the virus. This study aimed to develop high-efficacy non-infectious protein-based vaccine candidates. Specifically, soluble envelope protein (sE), and virus-like particles (VLPs), comprised of the precursor of membrane and envelope proteins, were generated, characterized, and studied for their immunogenicity in mice. Results showed that the VLPs induced more potent virus neutralizing response compared to sE, even though the total anti-envelope IgG content induced by the two antigens was similar. Depletion of anti-monomeric E protein antibodies from mouse immune sera suggested that the neutralizing antibodies elicited by the VLPs targeted epitopes spanning the highly organized structure of multimer of the E protein, whereas the antibody response induced by sE focused on E monomers. Thus, our results indicate that VLPs represent a promising LIV vaccine candidate.

### 1. Introduction

Louping ill virus (LIV) (*Flaviviridae*, *Flavivirus*) is a pathogenic arthropod-borne flavivirus transmitted by *Ixodes ricinus* ticks [1]. The geographical endemic area for LIV is limited to the British Isles, especially in the uplands, and some surrounding areas [2]. Major susceptible species for LIV infection are sheep (*Ovis aries*) and red grouse (*Lagopus lagopus scotica*). In sheep, LIV infection results in morbidity and mortality ranging from 5 % to 60 % depending on herds. It is speculated that infection can occur via bites of infected ticks in weaned lambs whose maternal antibodies have waned [2]. Due to the neurotropic nature of the virus [3], clinical manifestations of symptomatic infected sheep include convulsions, which makes infected sheep leap ('loup' in Scottish) into the air, hence the name 'louping ill' [1]. Red grouse (*Lagopus*

*lagopus scotica*) are reported to be infected after tick bites or ingestion of infected ticks and have mortality rates of up to 80 % [4]. Infection in humans has been reported with limited case numbers, mainly through occupational exposure to infected animals or via accidental laboratory exposure [5]. Serological surveys indicate that infected individuals remain largely asymptomatic. However, clinical cases are characterized by self-limiting influenza-like illness that in half of the affected individuals is followed by a second encephalitic phase [5]. Recent reports described confirmed or suspected cases of LIV infection in dogs [6,7]. Given the close contact of companion dogs and their owners, humans might be at increased risk of LIV infection, particularly if the virus spreads among companion animals.

Like other flaviviruses, LIV has a positive-sense single-stranded RNA genome that encodes three structural proteins i.e., capsid (C), precursor

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of membrane (prM) and envelope (E) protein, and seven non-structural (NS) proteins [8]. The C protein interacts with nascent genomic RNA to form a nucleocapsid core, whilst prM and E proteins form heterodimers that undergo proteolytic cleavage and conformational rearrangement during the particle maturation process eventually leading to the formation of 90 head-to-tail dimers with a herringbone-like architecture arranged on a smooth infectious particle [9,10,11]. Subviral particles (SVPs) or virus-like particles (VLPs) are also produced during infection by assembly of prM-E protein in the same manner as the infectious particles, but they lack the inner nucleocapsid core and hence are non-infectious. These non-infectious particles can be similar in size to the infectious virus (approximately 50 nm) or smaller (approximately 30 nm) [12,13]. Flaviviral VLPs can also be produced recombinantly by expression of viral prM-E gene.

The E protein of flaviviruses is known to bear a receptor binding domain that interacts with a host cell surface protein to initiate endocytosis of the viral particles [14]. The acidic pH of the endosome triggers a conformational rearrangement of the viral E protein from anti-parallel flat dimers to parallel spiky trimers projecting the fusion peptide, located at the tip of the E protein, toward the endosomal lipid membrane and initiating class II fusion process [15]. Several potent neutralizing antibodies interfere with receptor binding or fusion processes either by binding to multiunit of E protein and locking the protein in its dimeric form, or by enhancing dimer stability. Both processes impede protein rearrangement, membrane fusion, and viral infection [16–20].

A preventive vaccine against LIV infection had previously been approved for animal use. However, it has now been discontinued. The vaccine was an inactivated whole virion adjuvanted in paraffin oil and montanide. The potency of this vaccine is believed to be due to its ability to induce neutralizing antibodies targeting the E protein. A major disadvantage of the inactivated vaccine is the need for high containment facilities for infectious virus production, as required by the hazard group 3 status of the virus. A human vaccine against the related tick-borne encephalitis virus (TBEV) may afford cross-protection. However, cost/benefit analysis indicate that the cost/dose of the TBEV vaccine would simply be too high for it to be economically viable for sheep farmers. Therefore, this study aimed to develop alternative protein-based LIV vaccines capable of inducing neutralizing anti-viral E protein antibodies. Recombinant soluble E (sE) protein and VLP antigens were designed, produced, and characterized. sE was the simplest form of E protein whereas the VLPs comprised of mature viral prM and E proteins resembling the authentic viral particles. These VLPs are expected to bear herringbone-like structure of the E protein; thus, resembling the antigenic surface of the inactivated virion vaccine. Following immunization in mice, we found that VLPs elicited better neutralizing antibody response than sE. Further characterization of the elicited antibodies revealed that VLPs had a better propensity to elicit antibodies targeting epitopes spanning the E multimers which likely contributed to their high neutralizing capacity.

## 2. Material and methods

### 2.1. Cells and viruses

A549Npro cells expressing IRF3-degrading BVDV NPro [21] were kindly provided by R.E. Randall, University of St. Andrews, United Kingdom whereas A549 cells (ATCC#CCL-185) were provided by Prof. Ben Hale. A549Npro, A549 and Vero E6 cells (provided by Prof. Michele Bouloy, Institut Pasteur, Paris, France) were cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and penicillin/streptomycin under humidified 5 % CO<sub>2</sub> atmosphere. Expi293F™ cells (Thermo Scientific) were cultured at 37 °C on a shaker in Expi293™ expression medium (Thermo scientific) under humidified 8 % CO<sub>2</sub> atmosphere as per the manufacturer's instructions.

LIV Inverness14 strain (LIV-INV14) (accession number MK007541)

was reverse genetically recovered following circular polymerase extension reaction (CPER) of genomic viral RNA [22]. Briefly, cDNA was generated from LIV-INV14 RNA utilizing SuperScript IV Reverse Transcriptase (Thermo Scientific) as per manufacturer's instructions. The full genome was PCR-amplified in five segments, overlapping by 30 bp, using Phusion® Hot Start Flex DNA Polymerase (New England BioLabs). The primers employed are shown in Table 1 (supplementary data). The fragments were run on a 1 % agarose gel and the desired ~2.5 kb bands were extracted and purified. These purified fragments were combined in equimolar ratios (0.1 pmol of each fragment) with an additional linker fragment which overlaps with the 5' and 3' untranslated regions (UTRs) of LIV and TBEV. This linker fragment encodes the hepatitis delta virus ribozyme (HDVR), the simian virus 40 polyadenylation signal (SV40-PA), a region of filler DNA, and a CMV promoter, as previously described [22] (Fig. S1, supplementary data). The 5 genomic fragments and linker fragment were subjected to CPER to form a circular viral genome using Phusion® Hot Start Flex DNA Polymerase and the following PCR conditions: 95 °C for 45 sec, followed by 20 cycles of 95 °C for 15 sec, 62 °C for 30 min and 72 °C for 7 min, followed by a final extension at 72 °C for 15 min. The final, 50 µl, unpurified CPER product was then transfected into an 80 % confluent 25 cm<sup>2</sup> flask of A549Npro cells using TransIt LT1 (MirusBio) as per manufacturer's instructions. The cells incubated for 4 days until cytopathic effect (CPE) was pronounced, whereupon the supernatant was removed, clarified via centrifugation at 500× g for 10 min, and infectious virus quantified by plaque assay.

### 2.2. Plaque assay

A549Npro, A549 and Vero cells were seeded at a density of  $5 \times 10^5$  cells/well in a 6-well plate for 2–3 h before infection with serially diluted LIV or TBEV at 37 °C. An hour later, cells were overlaid with 4 % carboxymethylcellulose in DMEM supplemented with 1 % FBS and incubated at 37 °C under humidified 5 % CO<sub>2</sub> atmosphere for 7 days. Cell monolayers were fixed with 8 % formaldehyde and stained with 0.1 % crystal violet in 20 % ethanol for 5 min then number of plaques were counted.

### 2.3. Plasmids

Nucleotide sequence expressing LIV E protein without its transmembrane domain [amino acids (aa) 1–399 of E] were amplified from cDNA of LIV-INV14 genome. The sequence was fused in frame at the C-terminus with a sequence expressing the V5 tag (GKPIPNPLGLDGS)-enterokinase cleavage site (DDDDK)-StrepTagII (WSHPQFEK) before being cloned into pVAX vector (described previously [24,25]) backbone resulting in a plasmid expressing sE protein (pVAX-LIV-sE-V5-Strep). In case of biotinylated sE (b-sE), sequence encoding the two molecular tags and enterokinase cleavage site was replaced with that of biotin acceptor peptide (BAP, AviTag) (GLNDIFEAQKIEWHE) and the construct was cloned into a bicistronic pcDNA3 expression vector carrying a sequence encoding the bacterial biotin ligase enzyme, DNA-binding transcriptional repressor/biotin-[acetyl-CoA-carboxylase] ligase BirA [23] (pcDNA3-LIV-sE-BAP-BirA). Nucleotide sequence encoding the last 16 aa of capsid followed by full-length prM and E gene of LIV-INV14 was cloned into pVAX vector resulting in pVAX-LIV-prME which was used to generate VLPs.

### 2.4. Preparation of soluble envelope protein (sE) antigen

Expi293F™ suspension cells were transfected with pVAX-LIV-sE-V5-Strep using Expifectamine™ 293 transfection kit (Thermo Scientific) then incubated at 37 °C. Transfection enhancers were added 18 h post-transfection as recommended by the manufacturer then cells were moved to 28 °C at 24 h post-transfection. After 4–5 days, the cell supernatant was clarified by centrifugation and filtration through 0.45 µm polyethersulfone (PES) membrane and subjected to affinity

chromatography purification using StrepTrap™ HP column (GE Healthcare) equipped with ÄKTA pure (GE Healthcare). The captured protein was eluted by 5 column volumes of 30 % to 80 % linear gradient of elution buffer (2.5 mM desthiobiotin in phosphate buffered saline, PBS). A small aliquot each of the eluted fractions was coated on ELISA plate and assayed for the presence of sE protein using anti-V5 antibody (Abcam, AB15828). Fractions containing the sE protein were pooled, concentrated, and dialyzed against PBS using Amicon® Ultra (10 kDa, Millipore). Total protein concentration of the sE preparation was measured by NanoDropOne (Thermo Scientific).

## 2.5. Preparation of virus-like particle (VLP) antigen

Expi293F™ suspension cells were transfected with pVAX-LIV-prME using ExpiFectamine™ 293 transfection kit (ThermoFisher Scientific) as per the manufacturer's recommendation. Three days after the addition of transfection enhancers, secreted VLPs were purified essentially as described previously [24]. Briefly, VLPs were pelleted from clarified supernatant of the transfected cells through a cushion of 20 % sucrose in TN buffer (20 mM Tris and 120 mM NaCl, pH 8.0) by centrifugation at 115,000× g, 4 °C for 2 h. The pellet was re-suspended in TN buffer then loaded onto discontinuous density gradient composed of sodium potassium tartrate and glycerol. Concentrations of tartrate ranged from 5 % to 30 % with interval of 5 % whilst those of glycerol ranged from 3.75 % to 22.5 % with interval of 3.75 %. The gradient was spun at 174,000× g, 4 °C for 2 h then fractions were collected and analysed by western blot for the presence of E protein using monoclonal antibody (mAb) CR7, a LIV E protein-specific mouse mAb generated in this study (see below). The E-positive fractions were pooled and loaded on to HiPrep™ 16/60 Sephacryl™ S-500 HR column (GE Healthcare) equipped with ÄKTA pure. VLPs were eluted with 1.5 column volume of PBS and 2 ml fractions collected. A small aliquot of each fraction was coated on ELISA plates and assayed for the presence of E protein using mAb CR7. Fractions containing E protein were pooled and concentrated using Amicon® Ultra (100 kDa, Millipore). The concentration of VLPs in the preparation was estimated by comparing E protein band (55 kDa) in silver staining SDS-PAGE against defined concentrations of bovine serum albumin (BSA).

## 2.6. Preparation of biotinylated sE (b-sE)

Expi293F™ suspension cells were transfected with pcDNA3-LIV-sE-BAP-BirA using polyethylenimine (PEI, Polysciences, 23966). The transfection reaction was incubated at 37 °C for 24 h then cells were moved to 28 °C. After 4–5 days, cells were pelleted by centrifugation and supernatant clarified by filtration through 0.45 µm PES membrane before being dialyzed against PBS in a dialysis bag (12–14 kDa, Medicell Membranes).

## 2.7. ELISA for antibody titration

ELISA plates (Immulon 2HB flat bottom microtiter plates, Thermo Scientific, Cat. No. 3455) were coated with 5 µg/mL of avidin (Sigma-Aldrich) overnight then blocked with 1 % BSA for 2 h followed by addition of b-sE. Following overnight incubation, serial dilutions of serum samples were incubated with the immobilized antigen for 1 h followed by 1 h incubation with peroxidase-tagged anti-mouse IgG antibody (Sigma-Aldrich, A4416). After addition of TMB (3,3',5,5'-tetramethylbenzidine), the chromogenic reaction was stopped with 0.5 M H<sub>2</sub>SO<sub>4</sub> and measured at OD<sub>450</sub>. Antibody titer was defined as the last dilution of sample giving OD<sub>450</sub> of higher than three times that of the naïve serum.

## 2.8. Neutralization assay

Serum samples were subjected to 3-fold serial dilution with DMEM

supplemented with 2 % FBS in a 96-well plate then incubated at 37 °C with 100 plaque forming units (pfu) of LIV for neutralization. After an hour, A549Npro cells (2 × 10<sup>4</sup> cells/well) were added to the neutralization mixture and incubated at 37 °C, 5 % CO<sub>2</sub> for 2 days. Cells were fixed in 100 % ice-cold methanol for 20 min then cellular biotin was masked by incubation with 1 µg/mL avidin for 30 min followed by 5 µg/mL biotin in 1 % BSA for 45 min. Infected cells were probed with 2 µg/mL b-CR25 (a biotin-labelled anti-LIV E mouse mAb generated in this study, see below) for 45 min followed by 30 min of 1 µg/mL streptavidin Alexa Fluor® 488 (Invitrogen, S32354) and 1:5,000 DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich) in PBST (PBS containing 0.05 % Tween 20). The number of fluorescent (infected) cells in each well were counted by Celigo Imaging Cytometer (Nexcelom Bioscience). Infectivity was calculated based on number of infected cells relative to virus and cell control as the following equation - Infectivity (%) = 100 × (sample-cell control)/(virus control-cell control). The 50 % neutralization titer (NT50) of each serum sample was determined as the last dilution producing infectivity of less than 50 %.

## 2.9. Gel electrophoresis and western blot analysis

Samples were fractionated in 10 % SDS-PAGE then analyzed by silver staining (Pierce™ silver staining kit, Thermo Scientific) or western blotting. For silver staining of gels, protocol provided by kit manufacturer was followed. For western blot, blotted membrane was blocked by Intercept® blocking buffer (LI-COR®) overnight and probed with primary antibody of choice (anti-V5 [AB15828, Abcam] or 1 µg/mL CR16, an in-house-generated anti-LIV E mouse mAb [see below], in PBST) for 1 h followed by incubation with fluorescence-tagged secondary antibody (IRDye®, LI-COR®) for another hour. Images were acquired by ODYSSEY CLx imaging system (LI-COR®).

## 2.10. Electron microscopy

The VLP preparation was adsorbed on Formvar carbon film coated on 400-mesh per inch copper grids (Agar Scientific) and stained with 2 % saturated uranyl acetate (Agar Scientific) followed by washing with distilled water. Micrographs were acquired from transmission electron microscope (LEM-1200 EX II; JEOL) equipped with a charge-coupled-device (CCD) camera (Orius, Gatan) at 80 kV acceleration voltage.

## 2.11. Animal immunization

Groups of 6–8-weeks-old female BALB/c mice (n = 6) were subcutaneously immunized with either sE (10 µg), VLPs (2 µg or 0.4 µg) or sham PBS adjuvanted (1:1) with AddaVax (Invivogen). Animals were immunized three times at three-weeks interval. Blood was collected for antibody titration and neutralization assay at 3 weeks following administration of the final boost. Animal procedures were approved by the University of Glasgow Animal Welfare and Ethical Review Board and carried out under the Animals (Scientific Procedures) Act 1986 of the United Kingdom with project licence number P9722FD8E.

## 2.12. Adsorption assay

Immunotubes (Maxisorp, Thermo Scientific) were coated with 5 µg/mL avidin overnight then blocked with 1 % BSA for 2 h followed by further overnight incubation with b-sE. Sera were 5-fold diluted in PBST and divided into two halves. First half was left un-frozen as a control. The second half was serially incubated with the immobilized antigen in immunotubes for 10 tubes (at least 1 h/tube). Antibodies binding to immobilized antigen were collectively eluted from the 10 immunotubes by incubating with 0.1 M glycine-HCl pH 2.7 (5 min/tube) followed by neutralization with 1 M Tris pH 8.2.

### 2.13. Isolation and characterization of mAb specific to LIV E protein

A group of six female BALB/c mice were subcutaneously immunized four times with LIV-sE adjuvanted with 1 % aluminium hydroxide and 5 µg monophosphoryl lipid A (MPLA), kindly provided by Dr Hannah Scales, University of Glasgow. Two animals were further boosted intraperitoneally with antigen and their spleens were excised five days later. Collected splenocytes were fused with mouse myeloma Sp2/0 cells (Sigma-Aldrich) in the presence of polyethylene glycol (Sigma-Aldrich, P-7181) and the resulting hybridoma cells were selected in medium supplemented with hypoxanthine-aminopterin-thymidine (HAT) for 14 days. Hybridoma cells secreting LIV E-specific antibodies, screened by ELISA against b-sE as described above, were single-cell cloned by limiting dilution and further cultured for 12 days. Monoclonal cells were screened for secretion of anti-LIV E mAb using the ELISA against b-sE. Supernatant of cells secreting mAb CR7, CR16, and CR25 were collected and purified using protein G column (GE Healthcare) according to manufacturer's protocol and dialysed against PBS using Vivaspin® (30 kDa, Sartorius). Protein concentrations were determined by NanoDropOne (Thermo Scientific). The reactivity of these mAbs to LIV E was subsequently confirmed by western blot, immunofluorescence assay against infected cells as well as E-specific ELISA.

### 2.14. Statistical analysis

Antibody titer and neutralization titer were transformed into logarithmic form before being tested for normality using the Ryan Joiner model. The normally distributed data were compared by 2-sided analysis of variance (ANOVA) with Tukey Pairwise comparison at 95 %

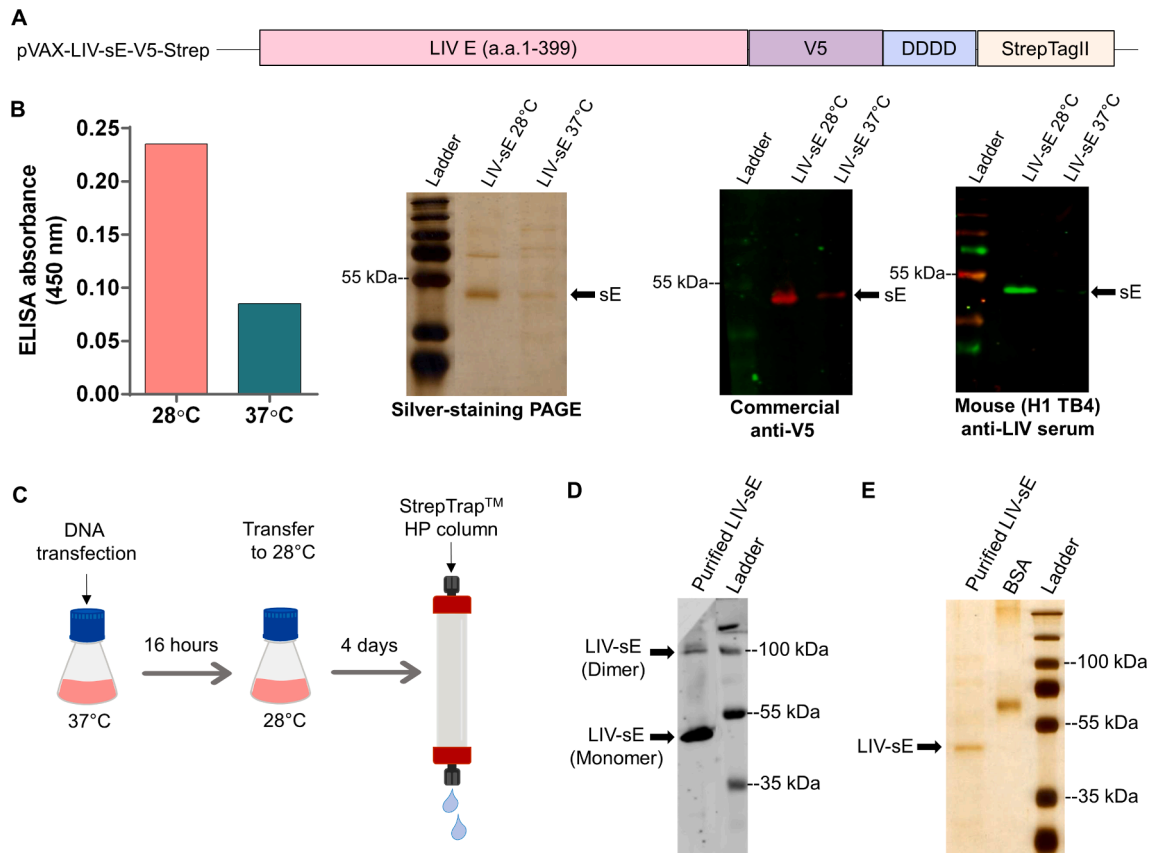
confidence level. The statistical analyses were carried out using Minitab Statistical Software. Levels of significance were displayed by asterisks (\* $p < 0.05$  and \*\*\*  $p < 0.001$ ).

## 3. Results

### 3.1. Preparation of sE antigen

sE was expressed in Expi293F™ suspension cells following transfection of a plasmid encoding E protein of LIV strain Inverness14 without its transmembrane domain (TMD) (aa 1–399 of E protein). Two molecular tags, V5 and StrepTagII, were included in-frame to facilitate detection and purification, respectively, of the translated protein (Fig. 1A). Protein expression was optimized by varying incubation temperature of the transfected cells then the culture medium was analyzed for relative abundance of the protein by ELISA using anti-V5 tag antibody, silver staining SDS-PAGE and western blot analysis (Fig. 1B). Results from 2 independent transfections showed that, at 4 days post-transfection, the sE was better expressed/secreted after incubation at 28 °C than 37 °C. Similar benefits of lowering incubation temperature facilitating expression and secretion of flaviviral sE through proper folding and protein dimerization have been reported previously [24,25]. The expressed protein was purified by affinity chromatography using the specific interaction between StrepTagII and StrepTrap™ HP column. After elution, the chromatographic fractions containing sE, as detected using the anti-V5 ELISA, were pooled and dialyzed against PBS before determination of protein concentration by NanoDrop spectrophotometer (Fig. 1C).

Western blot analysis of purified sE using anti-V5 antibody showed a



**Fig. 1.** (A) Schematic of the expression cassette for sE antigen showing gene encoding sE followed by V5 tag, enterokinase cleavage site (DDDDK) and StrepTagII. (B) Expression level of sE antigen at 4 days post-transfection following incubation at 28 °C and 37 °C estimated by anti-V5 ELISA, silver-staining PAGE and western blot using anti-V5 or mouse anti-LIV E polyclonal antiserum. (C) Schematic depicting the preparation and purification of sE antigen. (D) Anti-V5 western blot and (E) Silver-stained gel electrophoresis of purified sE preparation.



major band of ~50 kDa, an expected molecular size of flaviviral sE [25,26] (Fig. 1D). Another less intense band, possibly representing a dimeric form of sE, was also observed at 100 kDa. Silver-stained SDS-PAGE revealed sE as the major component confirming the relative purity of the sE preparation (Fig. 1E). However, other less well-represented bands including the one likely to be dimeric sE were also observed.

### 3.2. Generation of LIV-derived VLPs

VLPs were expressed, either at 28 °C or 37 °C, in Expi293F™ suspension cells after transfection of a plasmid encoding full-length prM-E protein of LIV strain Inverness14. Nucleotide sequence of the anchoring domain of capsid protein (Ca) (last 16 aa of the C protein) was included 5' to the prM-E gene and served as a signal peptide aiding secretion of the VLPs (Fig. 2A). Unlike sE, the VLPs were found to be better expressed at 37 °C (Fig. 2B). Secreted VLPs were purified using a combination of density gradient centrifugation and size-exclusion chromatography (SEC) as described in Methods (Fig. 2C).

Purified preparations of VLPs were characterized by western blot analysis using CR16, a mouse mAb recognizing the LIV E protein. The blot showed an E protein band of ~55 kDa in the purified preparation (Fig. 2D). The molecular size of E protein in VLPs was a little higher than that of the sE. This was expected as the VLPs contained full-length E whereas sE lacked the TMD. The E protein band was visible along with other protein bands in silver-stained SDS-PAGE indicating incomplete purification of the VLPs (Fig. 2E). Electron microscopy (EM) examination of the purified VLPs preparation (Fig. 2F) illustrated particles of about 30 and 50 nm diameter corresponding to the two different sizes of VLPs and authentic flaviviral particles [12,13]. Small size, non-particle impurities were also observed in the EM image which was in concordance with the result of silver-stained SDS-PAGE.

### 3.3. LIV sE and VLPs antigens are immunogenic in mice

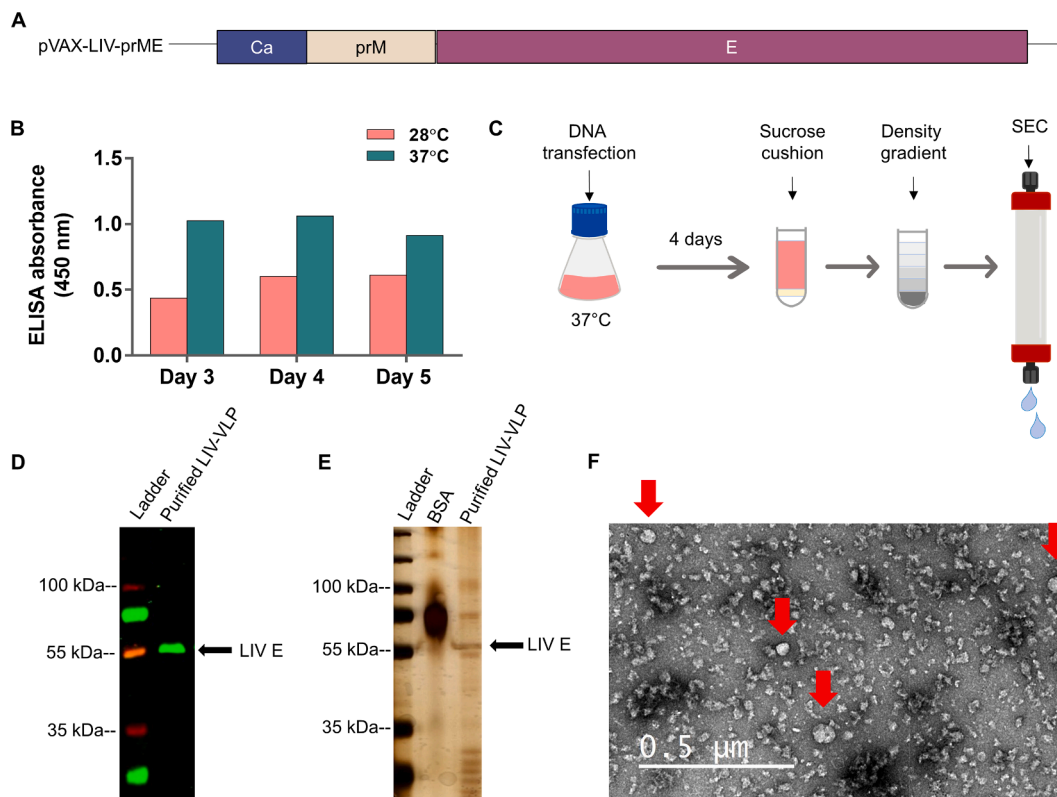
A cohort of 6–8 weeks old female BALB/C mice (n = 6/group) were subcutaneously immunized three times at three-week intervals with AddaVax-adjuvanted sE (10 µg), VLP<sub>high dose</sub> (2 µg), VLP<sub>low dose</sub> (0.4 µg) or PBS. Blood samples were collected three weeks after final immunization for determination of antibody titer and virus neutralizing activity (Fig. 3A).

Antibody responses were assessed by serum titration against immobilized monomeric sE which was tagged at C-terminus with biotin acceptor peptide (BAP, AviTag), enabling site of specific biotinylation in the presence of biotin ligase enzyme supplied *in trans* [23]. The biotinylated sE (b-sE) secreted from transfected Expi293F™ cells was incubated in ELISA plates pre-coated with avidin, to allow b-sE binding (Fig. 3B, C). These plates were used to determine the levels of anti-E antibodies in serially diluted serum samples as described in Methods.

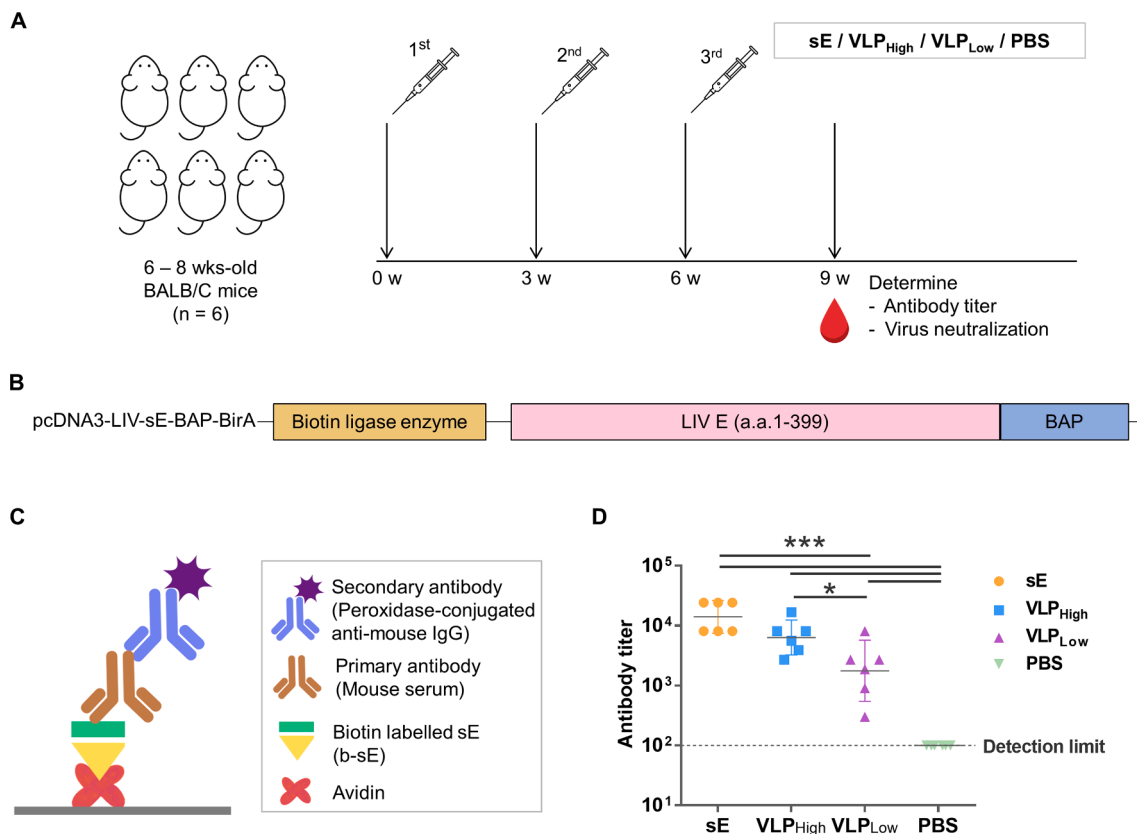
sE and VLPs, administered with both low and high doses, induced high anti-E antibody titers (Fig. 3D). Particularly, sE and VLP<sub>high dose</sub> induced comparable levels of anti-E antibodies whilst VLP<sub>low dose</sub> produced significantly lower antibody titer than sE.

### 3.4. LIV VLPs induce robust neutralizing antibody response

Virus neutralizing activity of the elicited serum antibodies was assessed in a neutralization assay developed in this study (Fig. 4B). First, three cell lines i.e., Vero, A549 and A549Npro were assessed for susceptibility to virus infection by plaque assay. LIV strain Inverness14 produced higher number of plaques in the A549 and A549Npro cells indicating higher susceptibility to the infection of these cells (Fig. 4A). We next used A549NPro cells to determine serum neutralizing activity against LIV.



**Fig. 2.** (A) Schematic of expression cassette for VLP antigen as detailed in the text. (B) Expression level of VLP at 3–5 days post-transfection following incubation at 28 °C and 37 °C measured by sandwich ELISA using mAb CR25 as coating antibody for VLP capture and biotinylated mAb b-CR7. The bound b-CR7 was detected using streptavidin-tagged peroxidase. (C) Schematic depicting preparation and purification of VLP antigen. (D) E protein western blot using mAb CR16, (E) Silver-stained gel electrophoresis and (F) scanning electron micrograph of purified VLP preparation.



**Fig. 3.** (A) Schedule of immunization experiment (B) Expression cassettes of biotin ligase enzyme and sE-BAP in a bi-cistronic plasmid. (C) Schematic diagram of anti-E antibody titration using b-sE ELISA. (D) Measurement of anti-E antibody titers at three weeks after the third dose of immunization. Each data point represents geometric mean of three technical replicates.

As shown in Fig. 4C, sE elicited neutralizing antibody response in 33.3 % of mice (2/6) while VLPs, both at low and high dose, induced the response in 66.7 % of mice (4/6). As expected, PBS did not induce neutralizing activity in any mice (0/6); however, a titer of 8.3 was assigned to each mouse of this group to set a lower limit of neutralization. Statistical analysis revealed that only immunization with VLP<sub>high</sub> dose induced significantly higher neutralizing activity than the PBS control. This was despite the sera of sE- and VLP<sub>high</sub> dose-immunized animals having comparably high anti-E antibody titers (Fig. 3D). Furthermore, neutralizing activities of VLP<sub>low</sub> dose-immune sera were comparable to those of the sE sera, despite the former producing significantly lower antibody titers. These differences led us to hypothesize that VLPs could induce another class of neutralizing antibodies targeting complex epitopes spanning on multimeric E protein. This would be expected from VLP immunization as the antigen presents multiple copies of E protein arranged in a highly organized way, likely mimicking structural arrangements found on authentic virus particles.

### 3.5. LIV VLPs induce neutralizing antibodies targeting complex epitopes of multimeric E protein

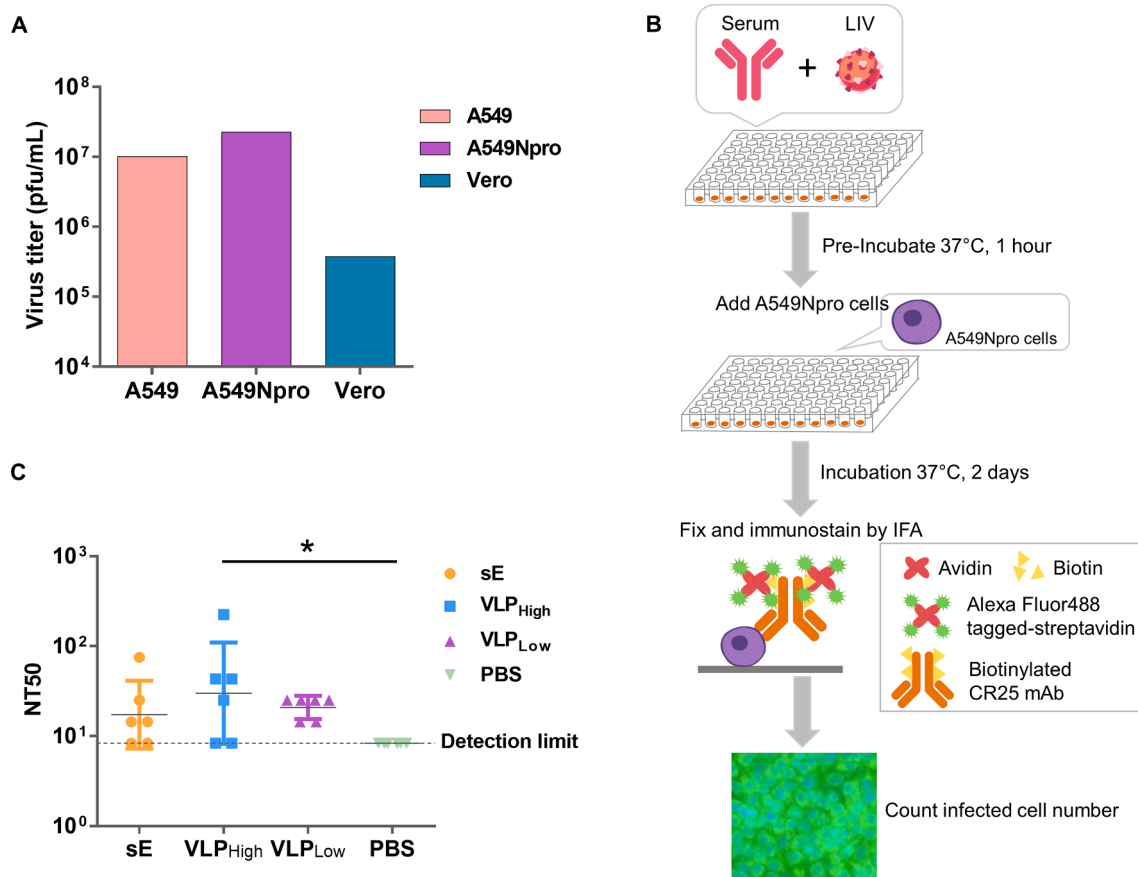
To examine whether the VLPs elicit neutralizing antibodies targeting epitopes on the multimeric E protein, two representative immune sera from each vaccinated group were repeatedly incubated with immobilized monomeric b-sE in order to deplete them of antibodies binding to the E monomer and thus enrich with antibodies specific to epitopes on the multimer, possibly the raft structure, of the E protein (Fig. 5A). The E-monomer-adsorbed antibodies were subsequently eluted. The E-monomer-depleted sera, together with the control un-adsorbed sera and the eluted monomeric E-specific antibodies, were tested for virus-neutralizing activity as described above.

As shown in Fig. 5B, the levels of anti-monomeric E antibody titers in the depleted VLP<sub>high</sub>-or VLP<sub>low</sub>-immunized serum samples, respectively, decreased to 1:100 or 1:33 (representing lower limit of detection). In contrast, titers of the antibodies in depleted sE sera decreased only slightly compared to the control un-adsorbed sera indicating that the adsorption procedure had not completely depleted monomeric E-specific antibodies, likely due to the abundance of such antibodies elicited by sE immunization. An obvious decrease in level of anti-monomeric E antibodies after adsorption of the VLP sera indicated that the VLP antigen induced fewer E monomer-specific antibodies than the sE upon immunization.

In keeping with our hypothesis, the depleted VLP<sub>high</sub> dose sera were able to neutralize the virus although the NT50 titers were lower than control input sera (Fig. 5C). In the case of sE and VLP<sub>low</sub> dose sera, the two input control immune sera of each immunization group had similar monomeric E-specific antibody titers but only one from each group exhibited virus neutralizing activity which was lost after adsorption. This indicated that the neutralizing activity of sera elicited by either sE or low dose of the VLPs was majorly, if not solely, contributed by the E monomer-specific antibodies.

## 4. Discussion

This study reports likelihood of VLP antigen of LIV in inducing neutralizing antibodies recognizing multimeric E protein possibly via its quaternary epitopes. Potent neutralizing activity of such a class of antibodies have been demonstrated for other vector-borne flaviviruses including Zika virus, dengue virus, West Nile virus (WNV) and TBEV [16–18,20]. Induction of such class of antibodies was expected following immunization of VLP antigen of LIV because the VLPs comprise of viral prE protein potentially imitating assembly pathway



**Fig. 4.** (A) Determination of virus susceptibility of cell lines by plaque assay using fixed virus concentration. The bars indicate average of two technical replicates. (B) Schematic diagram of virus neutralization assay. (C) Measurement of serum neutralization titres (NT50s) at three weeks after the third dose of immunization. Each data point represents geometric mean of two technical replicates.

and antigenic structure of the authentic LIV particles. Therefore, presentation of such antigenic structure may focus the host humoral responses to target epitopes spanning multimeric E protein of the infectious particles. This might lead to neutralization of the virus infectivity through blockage of virus-receptor interaction or prevention of rearrangement of the E protein requisite for initiation of infection as shown in several mechanistic studies of other flaviviruses [16–20]. On the other hand, sE antigen was expressed as a monomer hence induction of neutralizing antibodies binding to multimeric E protein was not likely to be achieved. However, induction of neutralizing antibodies binding a single molecule of E protein remained possible.

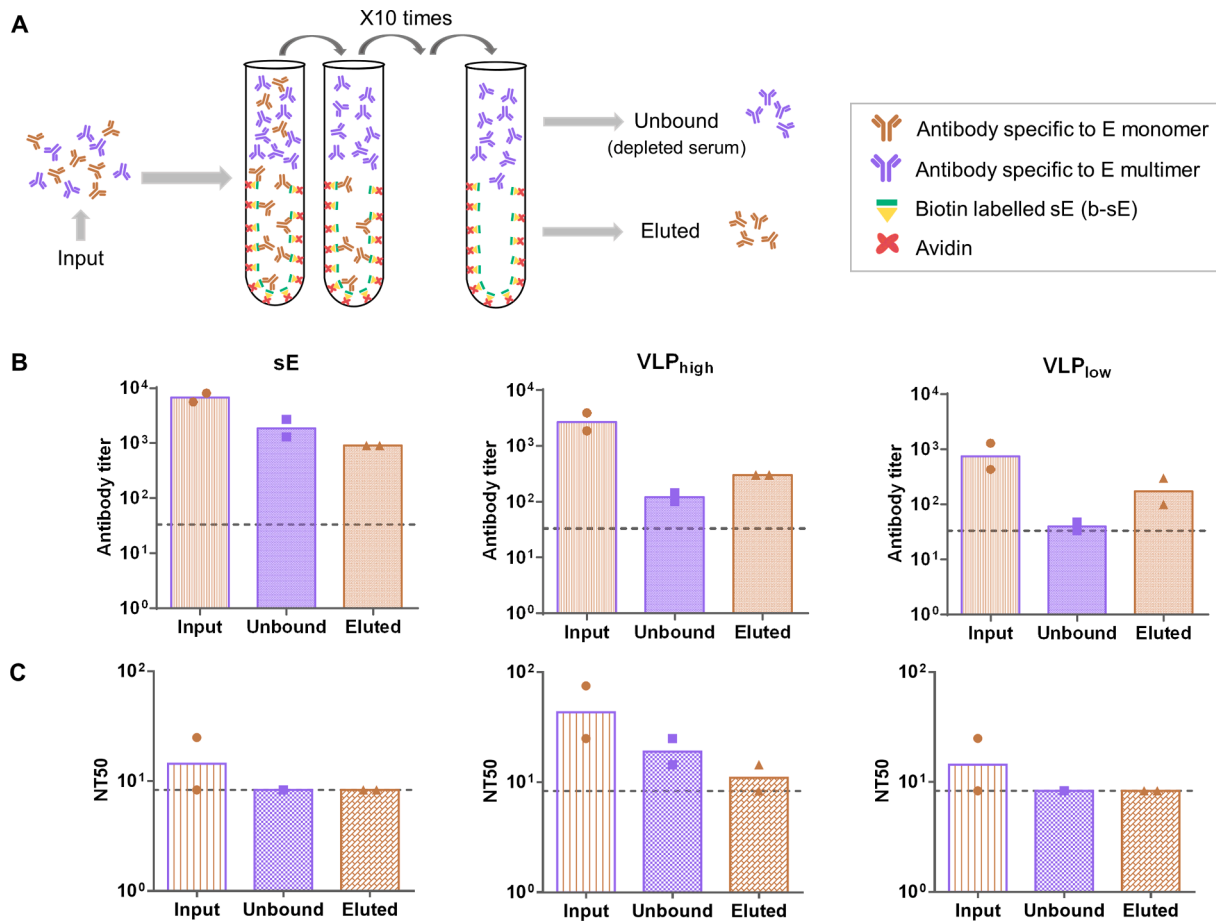
The failure of LIV E protein to protect animals against challenge with LIV or TBEV following vaccination with vaccinia virus-expressed LIV E has been demonstrated previously [27]. Similar failure was observed following immunization of LIV E protein expressed in *Spodoptera frugiperda* (Sf) cells by infection of recombinant baculovirus expressing full-length LIV E protein [28]. In both cases, the TMD of the E protein was included. The TMD was previously shown to hamper secretion of soluble E protein [26]. Immunization with vaccinia virus expressing full-length prM and E without TMD (prM-EΔTMD) which results in expression and secretion of LIV-sE antigen was demonstrated to deliver partial protection [27]. In our study, although a purified preparation of secreted sE was used for immunization, the obtained serum neutralizing activity was still not satisfactory.

Interestingly, Fleeton et al. [29] used recombinant Semliki Forest virus (SFV) particles to deliver recombinant RNA expressing LIV prM-E in mice and showed neutralization activity of sera obtained and protection following LIV challenge. However, the authors did not demonstrate whether VLPs were formed following expression of prM-E by such

constructs. The protection afforded by this SFV-based immunogen was at best partial in strains carrying antibody-escape mutations.

In this study, we developed a protocol to produce LIV VLPs. Electron micrograph revealed a heterogeneous-size population of VLPs, some of approximately 50 nm likely resembling authentic virions while others were smaller, possibly comprising of 180 or 60 copies of a viral E protein, respectively [30]. Such size differences have previously been reported for WNV VLPs [13]. Indeed, that study showed that 50 nm-WNV VLPs exhibited higher antiviral efficacy probably due to their close resemblance, both in size and antigenic presentation, to the authentic infectious virus.

Here, we demonstrate that LIV VLPs comprising prM-E elicit a more potent neutralizing antibody response than sE, even though the total anti-envelope IgG content induced by the two antigens was similar. Furthermore, these VLPs induce a more focused and potent antibody response mainly targeting neutralizing epitopes that span the multimer of E protein rather than poorly neutralizing epitopes presented on monomeric E. This is important in the context of flavivirus as poorly neutralising antibodies recognising immunodominant epitopes, besides being weakly protective, are likely to contribute to ADE of infection by related viruses. On the other hand, most potent neutralizing antibodies often recognize complex epitopes that span multiple adjacent E proteins presented on the virion or structures mimicking it, such as VLPs. Furthermore, such antibodies represent excellent tools to explore the antigenic structure of LIV and as such broaden our understanding of flavivirus immunology. Overall, our data show that the LIV VLP represents an excellent candidate vaccine that merits further *in vitro* and *in vivo* efficacy trials, and studies assessing different expression and purification systems to enhance quality, scalability and safety, to progress it



**Fig. 5.** (A) Schematic depicting fractionation of serum antibodies (input) into E monomer-specific (eluted) and E multimer-specific antibodies (unbound) using b-sE. (B) Measurement of anti-E antibody titres and (C) neutralization titres of fractionated and non-fractionated sera presented as geometric mean values of three and two technical replicates, respectively.

for its eventual translational end-use.

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## CRediT authorship contribution statement

**Rapeepat Tandavanitj:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Chayanee Setthapramote:** Formal analysis, Investigation, Methodology, Resources, Supervision, Visualization, Writing – original draft. **Giuditta De Lorenzo:** Formal analysis, Investigation, Methodology, Supervision, Validation. **Ricardo Sanchez-Velazquez:** Methodology, Validation. **Jordan J. Clark:** Formal analysis, Investigation, Methodology, Resources. **Mara Rocchi:** Resources, Writing – review & editing. **Colin McInnes:** Resources, Writing – review & editing. **Alain Kohl:** Conceptualization, Funding acquisition, Resources, Writing – original draft. **Arvind H. Patel:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data shared at the Attached File step

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2024.03.008>.

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