1 **Supplementary Information**

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3 Genotype I of Japanese Encephalitis Virus Virus-like Particles Elicit 4 Sterilizing Immunity against Genotype I and III Viral Challenge in 5 Swine

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10 **Protein expression and secretion of GI JEV VLP-expressing plasmids.** VLP is a by- 11 product secreted from JEV infected cells, and is secreted from cells transfected with the 12 plasmid encoding viral premembrane (prM) and envelope (E) genes of GIII JEV[1]. Thus, 13 we constructed a GI JEV VLP-expressing plasmid encoding prM and E genes of GI 14 JEV, namely pVJGI YL2009-4 WT (Supplementary Fig. 1A), and plasmid-transfected 15 COS-1 cells were analyzed by indirect immunoflorescence assay (IFA) using mouse 16 anti-JEV hyperimmune ascitic fluid (HIAF). The signal of VLP antigens were observed 17 in the pVJGI YL2009-4 WT-transfected cells but not in pVAX control-transfected cells 18 (Supplementary Fig. 1B). After establishing a cell clone that continually expressed GI 19 VLP, we constructed another GI VLP-expressing plasmid carrying the neomycin

20 resistance gene named pcDNAJGI YL2009-4 WT, which was derived from pVJGI
21 YL2009-4 WT (Supplementary Fig. 1C). We measured the VLP antigens being
22 expressed and the secretion of pcDNAJGI YL2009-4 WT in heparan sulfate-deficient
23 CHO-pgsA745 cells (CHO-HS(-)), which are low susceptible to JEV infection[2]. The
24 positive signal of VLP antigens was shown in the pcDNAJGI YL2009-4 WT-
25 transfected CHO-HS(-) cells (Supplementary Fig. 1D). Then, we harvested VLP
26 antigens from the day-old culture media of the plasmid-transfected COS-1 and CHO-
27 HS(-) cells, and the end-point VLP titer was determined by the antigen-capture ELISA
28 (Ag-ELISA) with titers in the range of 38 to 75 and 30 to 58, respectively. We compared
29 and examined the viral proteins expressed by COS-1 and CHO-HS(-) cells-derived VLP
30 and JEV-infected culture supernatants by electrophoresis and Western blotting. The
31 viral E, NS1, prM, and M proteins were detectable in JEV sample, and the same size of
32 E and prM proteins were in the VLPs secreted from either COS-1 or CHO-HS(-) cells
33 (Supplementary Fig. 1E). These results indicated VLPs were successfully translated
34 and secreted from pVJGI YL2009-4 WT- and pcDNAJGI YL2009-4 WT-transfected
35 cells.
36
37 **Establishment of the CHO-HS(-) clone to consistently produce GI JEV VLPs**. The
38 use of transiently transfected cells to produce a VLP vaccine requires purified plasmids

39 and a cumbersome process to separate dead and non-antigen producing cells. We
40 selected and established a VLP-expressing CHO-HS(-) cell line, which might reduce
41 the re-attachment of the secreted VLPs on the heparan sulfate-deficient cell surface[2].
42 Thus, VLPs expressed in this cell line may reduce the cell fusion and improve the
43 production of GI JEV VLPs. The pcDNAJGI YL2009-4 WT-transfected and non-
44 transfected CHO-HS(-) cells were cultured with G418, and cell viability was recorded
45 under light microscopy at 0, 5, and 12 days post-treatment (DPT) (Supplementary Fig.
46 S3). The non-transfected CHO-HS(-) cells became rounded and detached from the flask
47 at 5 DPT, with complete cell death at 12 DPT. However, a portion of pcDNAJGI
48 YL2009-4 WT-transformed cells survived at 12 DPT (Supplementary Fig. S3). The
49 G418 resistant GI plasmid-transformed cells were sub-cloned twice using the limited
50 dilution method in 96-well plates, and cell clones secreted VLP antigens were detected
51 by Ag-ELISA. Ten VLP-positive clones secreted VLPs with the Ag-ELISA titers ≥ 128.
52 Five 100% IFA positive cell clones (4-4, 51-2, 51-3, 51-6, and 51-10). The 4-4, 51-6,
53 and 51-10 were selected to determine the stability of VLP secretion (Supplementary
54 Fig. S4A).
55 The dynamic yield of VLP antigens secreted from the selected clones were
56 estimated every day for 6 days (Supplementary Fig. S4B). The VLPs were detectable
57 at day 1, reached a plateau at day 3 and decreased after day 4. The 51-6 and 51-10

58 clones produced a higher amount of VLP, 702.7 and 968.9 ng/ml, respectively. Then,
59 we evaluated the stability of VLP expression by three clones by passaging cells every
60 three days for 10 passages. The 51-6 and 51-10 clones constantly secreted VLP antigens
61 for 10 passages, with an average of 475.1 and 820.4 ng/ml. In contrast, the VLP yield
62 in 4-4 clone was reduced after the 5th passage (Supplementary Fig. S4C).
63 Immunofluorescent assay results showed 100% maintenance of VLP-positive cells of
64 51-6 and 51-10 clones but only half the positive cells of the 4-4 clone at passage 10
65 (Supplementary Fig. S4D). The 51-10 clone was selected, because of its better stability
66 and efficiency in secreting VLPs to produce GI JEV VLP for the following study.
67
68

69 ## Supplementary Methods

70 **Construction of GI JEV VLP-expressing plasmids.** pVJGI YL2009-4 WT and
71 pcDNAJGI YL2009-4 WT were GI JEV VLP-expressing plasmids used in the study
72 and encoded genes in order as human cytomegalovirus early gene promoter (CMV IE),
73 kozak sequence (5'-GCCGCCGCC-3'), the modified JE signal sequence (5'-
74 ATGGGCAAGAGGTCCGCCGGCTCAATCATGTGGCTCGCGAGCTTGGCAGTT
75 GTCATAGCTGGTACAAGCGCT-3'), prM (nucleotide position 478 to 978 in the
76 genome of GI YL2009-4 strain), 80% E genes (nucleotide position 979 to 2175 in the
77 genome of GI YL2009-4 strain) and 20% E genes (nucleotide position 2175 to 2477 in
78 the genome of GIII SA14 strain, KU323483.1) with chimeric human β-globin gene
79 intron (5'-
80 GTAAGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACTGG
81 GCTTGTCGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCACCTATTGGTC
82 TTACTGACATCCACTTTGCCTTTCTCTCCACAG-3'), and bovine growth
83 hormone poly(A) signal. First, the pVJGI YL2009-4 WT was constructed by replacing
84 prM and 80% E genes of the dengue serotype I virus (DENV1) VLP-expressing plasmid
85 (pV8D1-2J-i)[5] with the corresponding region of GI YL2009-4 strain in the reaction of
86 In-Fusion PCR cloning kit (Clontech, USA). Briefly, prM and 80% E genes of GI
87 YL2009-4 strain were amplified from viral cDNA with the forward (5'-

88 ATAGCTGGTACAAGCGCTATGAAGCTATCAAACTTTCAAGG-3') and the

89 reverse (5'-

90 GCCTTGCCCAGCGTGCTTCCAGCTTTGTGCCAGTGATGGTTAATC-3')

91 primers in PCR reaction of Q5® High-Fidelity 2X Master Mix (New England Biolabs,

92 USA). The remaining sequences of pVJGI YL2009-4 WT were amplified from pV8D1-

93 2J-i with the forward (5' GGAAGCACGCTGGGCAAGGC-3') and reverse primers

94 (5'- AGCGCTTGTACCAGCTATGAC-3'). The two PCR products were annealed in

95 the reaction of In-Fusion PCR cloning kit following the manufacturers protocols. The

96 final product of the cloning kit was transformed into competent cells, and it recovered

97 the pVJGI YL2009-4 WT clone. Then, the modified JE signal sequence, prM, and E

98 genes of pVJGI YL2009-4 WT were transferred to pcDNA3.1 with KpnI and NotI

99 restriction enzymes to recover the pcDNAJGI YL2009-4 WT carrying the Neomycin

100 resistant gene.

101

102 **Selection of the stable cell line secreting GI JEV VLP.** GI JEV VLPs were produced

103 from the stable clone transformed with the pcDNAJGI YL2009-4 WT plasmid. The

104 pcDNAJGI YL2009-4 WT plasmid was transfected into CHO-HS(-) cells by using

105 lipofectamine 2000 (Invitrogen) following the manufacturers protocols. The next day,

106 the culture media was replaced with F12 medium supplemented with 10% heat-

107 inactivated FBS and 500 µg Geneticin (G418, ThermoFisher) in 1 ml and incubated in
108 the 37°C incubator until observing death of 90% of cells among the non-transfected
109 CHO-HS(-) cells. After G418 treatment, surviving cells among the pcDNAJGI
110 YL2009-4 WT-transformed CHO-HS(-) cells underwent limiting dilution twice. The
111 single and stable clone was evaluated with the ability and stability of VLP secretion by
112 the yield of VLP and maintenance of JEV-antigen positive cells after ten passages of
113 the clone.
114
115 **Indirect immunoflorescence assay (IFA).** Cells transformed with VLP-expressing
116 plasmids or control vectors were seeded on Millicell EZ SLIDE 8-well glass plates
117 (Merck Millipore). The next day, the transformed cells were fixed with 4%
118 paraformaldehyde (Sigma-Aldrich) in PBS (AppliChem Panreac), permeated with
119 0.05% TritonX 100 (Sigma-Aldrich) in PBS, and then blocked with 5% skimmed milk
120 in PBS. After blocking, the cells expressing JEV antigens were detected with mouse
121 anti-JEV HIAF followed by the addition of anti-mouse IgG (H+L) antibodies
122 conjugated with Alex488 (KPL, Gaithersburg, MD). The cell nucleus was located by
123 staining with DAPI (Invitrogen). Images were viewed and recorded by fluorescence
124 microscopy using OLYMPUS CKX41.
125

126 **Histopathology.** The brain specimens were harvested from JEV challenged pigs and

127 fixed with 10% of formalin (Fisher Scientific). The fixed brain samples were trimmed

128 and embedded in paraffin. Then, the paraffin sample block was cut into 2 μm thickness

129 and stained by hematoxylin and eosin (H&E). The histopathological change was

130 observed and recorded by a light microscopy (BX-51, Olympus Tokyo, Japan).

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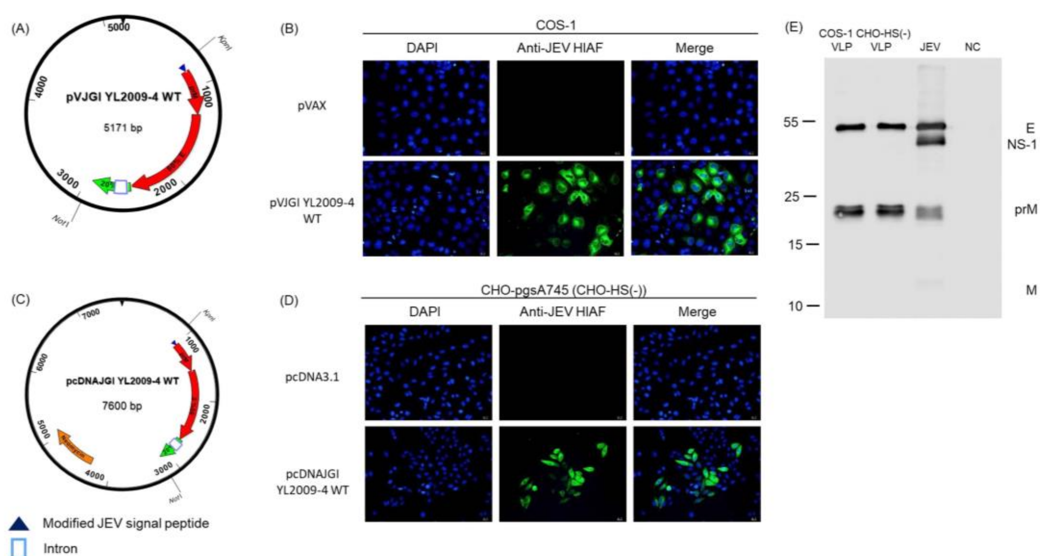

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134 **Supplementary Figure S1. Characterization of GI JEV VLP-expressing plasmids.**

135 The prM and 80% of E genes (ectodomain region) encoded by GI JEV YL2009-4, and

136 20% of the E gene (transmembrane region) of the GIII SA-14 strain[3] were incorporated

137 into pVAX and pcDNA3.1 vectors to establish the plasmids expressing GI JEV VLP,

138 called pVJGI YL2009-4 WT (A) and pcDNAJGI YL2009-4 WT (C). The modified JE

139 signal peptide sequence was located in front of the prM gene, and the intron was

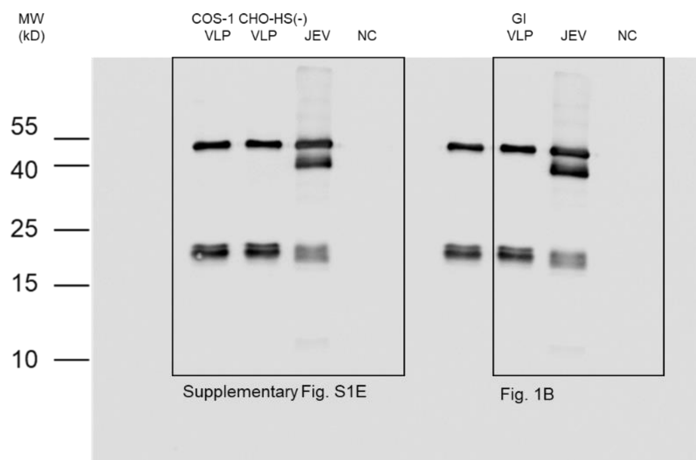
140 inserted within the end of the 20% E gene [4]. The GI VLP antigen was detected in pVJGI

141 YL2009-4 WT-transformed COS-1 cells (B) and pcDNAJGI YL2009-4 WT-

142 transformed CHO-pgsA745 (CHO-HS(-)) cells (D) by IFA with mouse anti-JEV

143 hyperimmune acetic fluid (anti-JEV HIAF) staining. The pVAX- and pcDNA3.1-

144 transfected cells were a negative control (NC). (E) The secreted VLP antigens were
145 harvested and analyzed by Western blot assay stained with anti-JEV HIAF antibody. GI
146 JEV VLP antigens collected from the supernatant of transfected COS-1 or CHO-HS(-)
147 cells are indicated as COS-1 VLP or CHO-HS(-) VLP. The supernatant collected from
148 GI JE YL2009-4 virus-infected or vector-transfected cells are presented as JEV or NC.
149 Full-length Western blots are presented in Supplementary Fig. S2.
150
151 **Supplementary Figure S2. Full-length Western blots for Supplementary Figure**
152 **1E and Figure 1B.**



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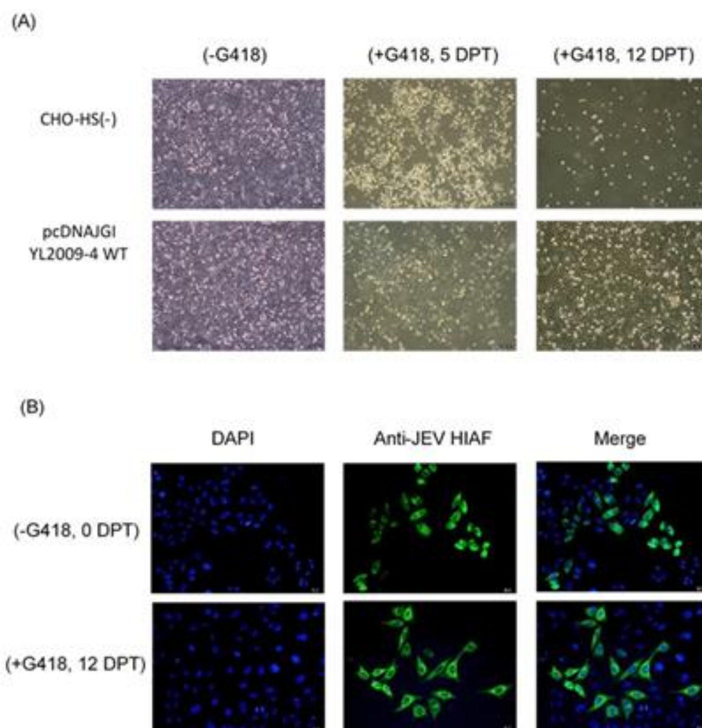

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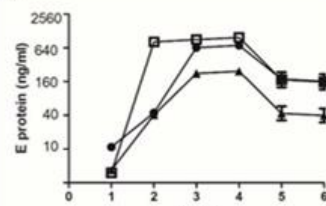
160 **Supplementary Figure S3. G418 selection of the CHO-HS(-) cell-derived stable**
161 **clones expressing GI JEV VLP.** (A) The viability of pcDNAJGI YL2009-4 WT-
162 transformed or non-transformed CHO-HS(-) cells was recorded at day 1, 5, and 12 post-
163 G418 treatment by light microscopy. (B) Before the limiting dilution, JE signals were
164 detected in plasmid-transformed cells that were untreated (-G418, 0 DPT) or treated
165 (+G418, 12 DPT) with G418 by IFA.

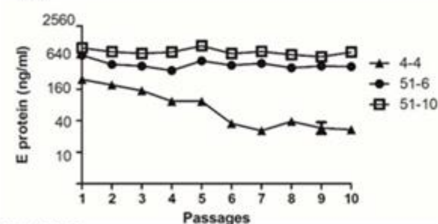
166

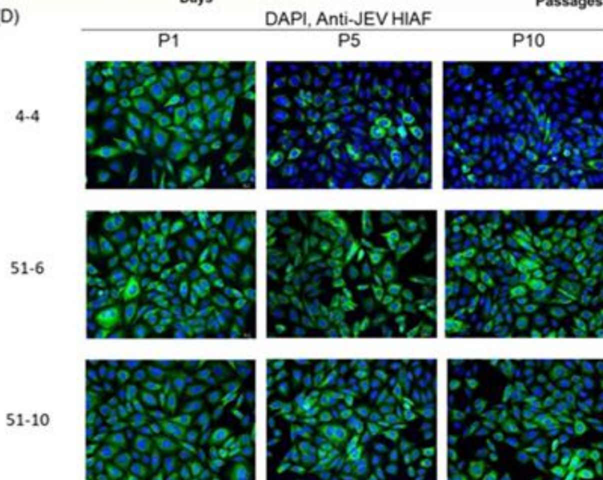
(A)

Clones[a]	VLP secretion[b]	Positive cells[c] (%)
4-1	16	93
4-4	64	100
4-6	16	96
51-2	128	100
51-3	128	100
51-6	128	100
51-10	256	100
53-1	128	69
53-2	128	64
53-5	64	58
53-6	128	75
53-12	128	91
62-7	128	57
62-9	128	54
62-12	16	20

[a] Clones were selected after G418 treatment followed by the limiting dilution
[b] VLP secretion was indicated as the endpoint titer of Ag-ELISA
[c] Positive cells expressing JEV antigens were calculated in IFA







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168 **Supplementary Figure S4. Establishment of the GI VLP-expressing CHO-HS(-)**

169 **stable clones.** (A) After incubation with G418 for 12 days, surviving pcDNAJGI

170 YL2009-4 WT-transformed CHO-HS(-) cells were limiting diluted and cultured twice.

171 The single clone was selected and VLP secretion was measured by Ag-ELISA and JEV-

172 positive rate by IFA. (B) The dynamic yield of VLP antigens secreted from the selected

173 4-4, 51-6, and 51-10 clones was estimated every day for 6 days. (C) The stability of 4-

174 4, 51-6, and 51-10 clones on VLP expression was evaluated by passaging every 3 days

175 for 10 passages. The yield was estimated by Ag-ELISA by using the standard of purified

176 GI VLP and presented as the concentration of E protein (nanograms per ml). (D) The

177 positive rate of JEV signal was determined among the 4-4, 51-6, and 51-10 clones at

178 passage 1 (P1), 5 (P5), and 10 (P10) by IFA.

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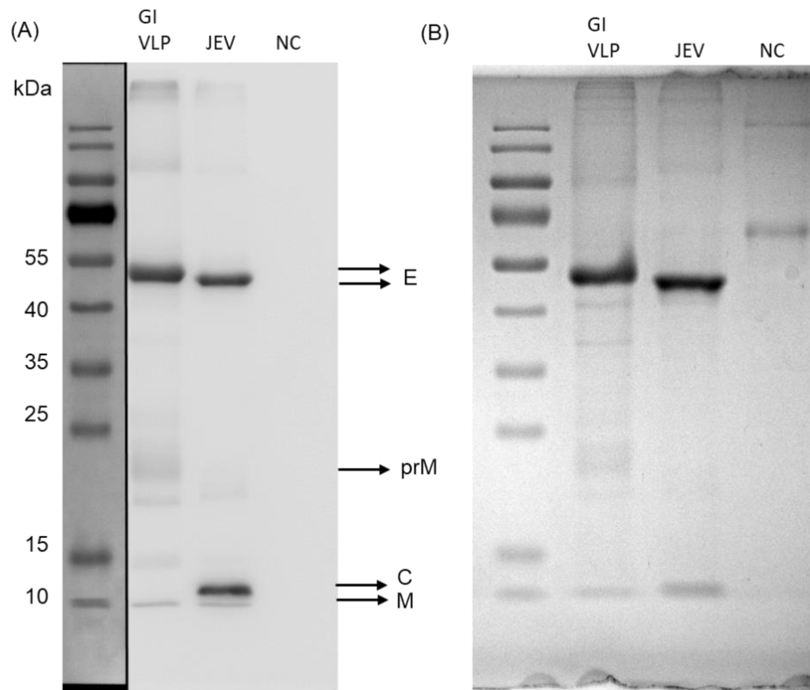

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181 **Supplementary Figure S5.** The CHO-HS(-) derived 51-10 clone-produced and

182 purified GI JEV VLP sample was analyzed by Western blot assay (A) with anti-JEV

183 HIAF and is indicated as GI VLPs. Total protein of the GI JEV VLP-purified sample

184 was examined in Coomassie blue-stained SDS page (B). The supernatant collected from

185 GI JE YL2009-4 virus-infected or the non-infected C6/36 cells (*Aedes albopictus* clone)

186 is presented as JEV or NC (negative control).

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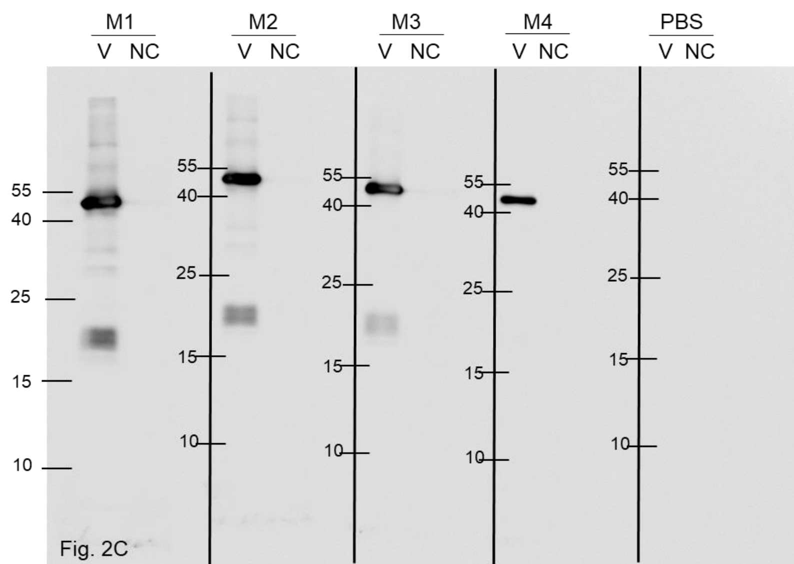
190 **Supplementary Figure S6. Full-length Western blots for Figure 2C**



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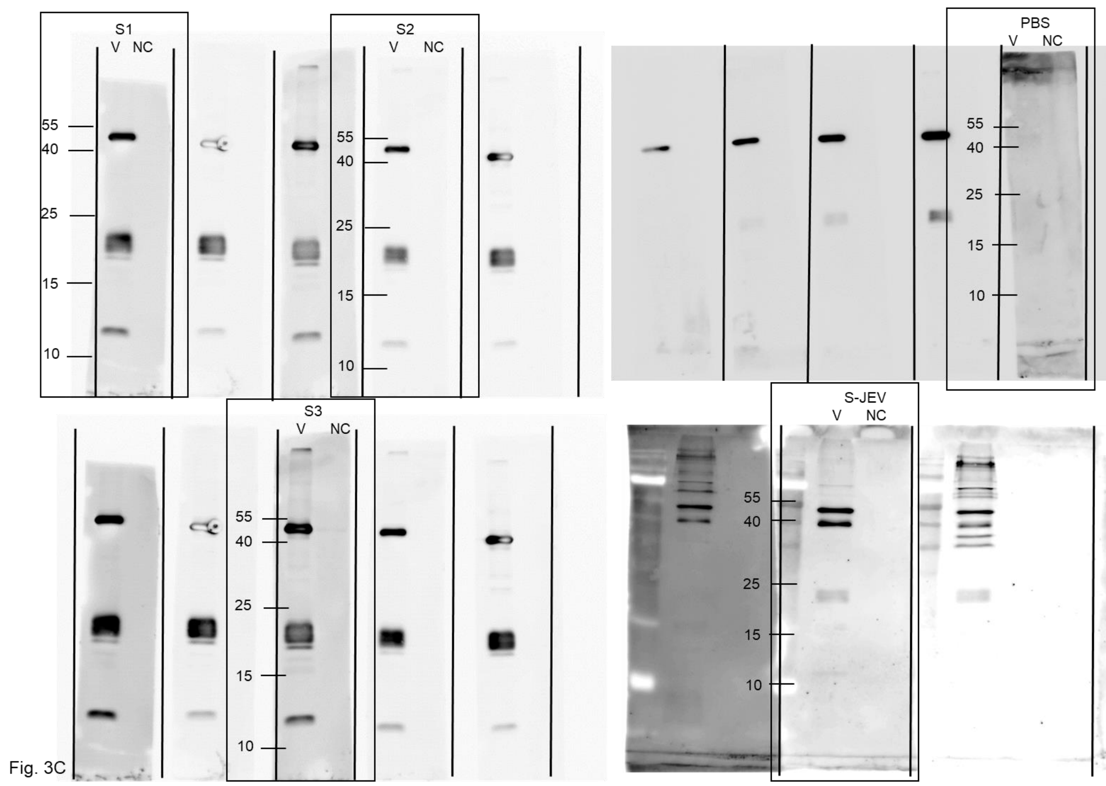
193 **Supplementary Figure S7. Full-length Western blots for Figure 3C**



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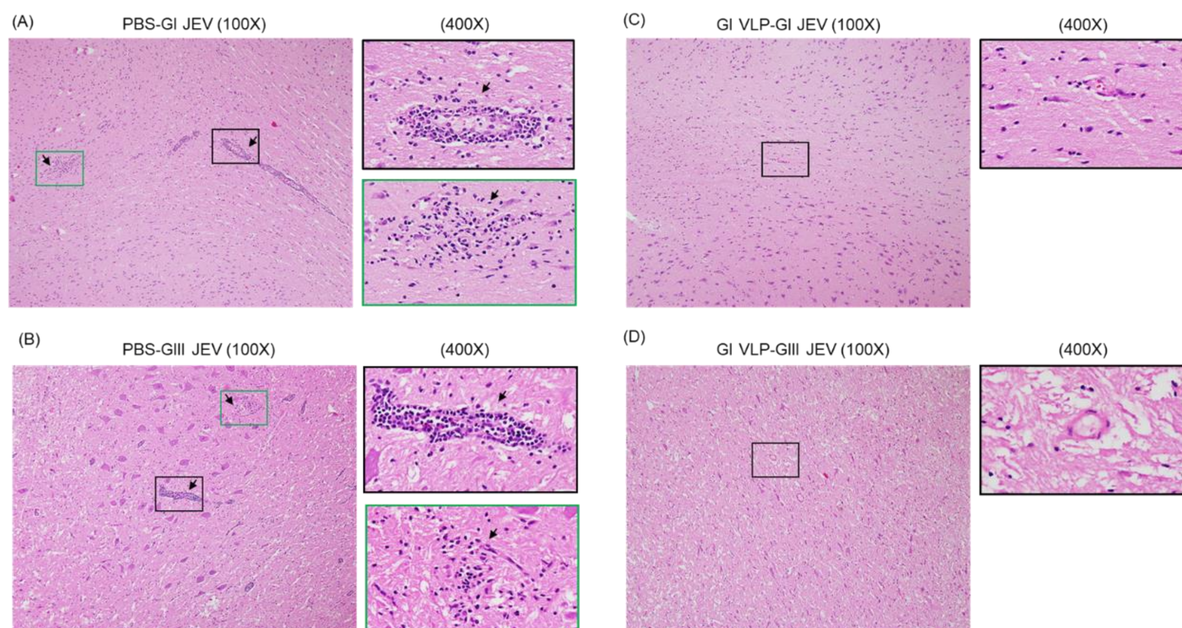

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199 **Supplementary Figure S8. Histopathological changes of immunized SPF pig brain**

200 **after JEV challenge.** Immunized pigs at age 10 weeks were subcutaneously injected

201 with 10^7 ffu JEVs. Brains were harvested from PBS-immunized pigs (A and B) and GI

202 VLP-immunized pigs (C and D) at 8 days after challenge with GI YL2009-4 virus (A

203 and C) or GIII CH1392 virus (B and D). Histology was examined by hematoxylin and

204 eosin staining under low magnification (100X) and high magnification (400X).

205 Multifocal lymphocytic perivascular cuffing and gliosis are indicated with arrows and

206 highlighted with black and green rectangles, respectively.

207

208 **Supplementary References**

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