Studies on the mosquito-borne flavivirus serology useful for diagnosis of flavivirus infections

（蚊媒介性フラビウイルス感染症の新規血清診断法の確立に向けた研究）

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Contents

Abbreviations

4G2: D1-4G2-4-15

6E6: BJ-6E6

ADE: Antibody-dependent enhancement

AUC: Area under the curve

BJV: Barkedji virus

BSA: Bovine serum albumin

CFAV: Cell fusing agent virus

CxFV: Culex flavivirus

DENV: Dengue virus

DENV2: Dengue virus type2

DMEM: Dulbecco's Modified Eagle Medium

dsRNA: double stranded RNA

E: Envelope

EDII: Domain II of envelope protein

ELISA: Enzyme-linked immunosorbent assay

FBS: Fetal bovine serum

FFA: Focus forming assays

ffu: focus-forming units

FL: Fusion loop

FRNT: Focus reduction neutralization test

hpi: hours post-infection

HRP: Horseradish peroxidase

IFA: Indirect fluorescent assay

Ig: Immunoglobulin

JEV: Japanese encephalitis virus

LOD: Limit of detection

Mab: Monoclonal antibody

MBFV: Mosquito-borne flavivirus

MEM: Eagle's Minimum essential medium

ML: Maximum-likelihood

MOI: Multiutility of infection

mut: mutant

NS1: Nonstructural protein 1

OD: Optical density

PBS: Phosphate-buffered saline

PBST: 0.01% Phosphate-buffered saline -Tween20

PCR: Polymerase chain reaction

prM: precursor membrane

PSFV: Psorophora flavivirus

RPMI1640: Roswell Park Memorial Institute 1640

RT-qPCR: Quantitative reverse transcription PCR

RT: Room temperature

SC: Subcutaneous

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

sem: Standard error of mean

SS: Signal sequence

SVP: subviral particle

TBFV: Tick-borne flavivirus

Vero cells: Vero9013 cells

WNV: West Nile virus

WT: Wild type

ZIKV: Zika virus

Notes

Chapter I

Tabata K, Itakura Y, Toba S, Uemura K, Kishimoto M, Sasaki M, Harrison JJ, Sato A, Hall WW, Hall RA, Sawa H, Orba Y (2022) Serological characterization of lineage II insect-specific flaviviruses compared with pathogenic mosquito-borne flaviviruses. Biochem Biophys Res Commun 616:115-121

Chapter II

Preparing for submission

General introduction

Members of the *Flaviviridae* comprise enveloped, positive sense, single-stranded RNA viruses, which are mainly divided into mosquito-borne flaviviruses (MBFVs) and tick-borne flaviviruses (TBFVs) [1-4]. Among them, pathogenic MBFVs include dengue virus (DENV), Zika virus (ZIKV), Japanese encephalitis virus (JEV), and West Nile virus (WNV). These viruses have become a major public health concern in the world [5, 6].

In recent years, these flavivirus infectious diseases previously limited to tropical regions are now being seen worldwide because of the effects of climate change, such as La Niña [7-11]. It has also been reported that the endemic areas of flavivirus infections are also spreading along with the expansion of the habitat of mosquitoes [12-14]. In southern Australia, where there had been no record of JEV infection from a geographical or climatic point of view, JEV infection and deaths due to the infection were reported [15, 16]. In France, *Aedes albopictus*, which was not originally found in the country, has been indigenous since 2010, and autochthonous case of DENV transmitted by the mosquito species have been reported [17]. There were no autochthonous cases of DENV in Japan since 1975; however, DENV outbreak was found again in 2014 [18]. Thus, MBFV infection has the potential to cause outbreaks in non-endemic areas and is one of the most important infectious diseases for public health.

Although most infections with JEV and WNV are asymptomatic, their severe cases can cause fatal encephalitis [19-22]. ZIKV infection has been reported to cause microcephaly in newborns at a high rate when pregnant women are infected [23-25]. DENV infection presents classical dengue, including fever, muscle and joint pain symptoms without serious illness at the initial infection [26]. Because of existing serotype 1 to 4 in DENV, a second infection with a different serotype of DENV can cause severe illness and develop dengue hemorrhagic fever due to anti-DENV antibodies acquired during the initial infection [27-29]. Cohort studies have shown that not only antibodies against DENV but also antibodies induced by ZIKV infection and JEV vaccination cause the severity of DENV infection [30-32]. To control these flavivirus infections, there has been concerted efforts to develop specific therapies and vaccines. However, no specific medications have been approved to date, and the number of effective vaccines is limited [33-36].

Approaches for vector control is effective in preventing vector-borne infections [37]. In particular, for MBFVs, genetically engineered mosquitoes have been developed to control mosquito proliferation, and their effectiveness has been experimentally demonstrated in the

field [38-40]. However, the use of the genetically engineered mosquito has been controversial from a variety of perspectives, such as ecological and ethical concerns [41]. Hence, the development of specific therapeutics and vaccines is still an urgent need.

Another effective control of flavivirus infection is epidemiological approach. For flavivirus infections, especially DENV infections, it is important to determine the history of previous flavivirus infections because pre-existing anti-flavivirus antibodies facilitate the risk of severe disease as previously mentioned. There are several serodiagnostic tests, including detection of binding or neutralizing antibodies [42]. While the neutralizing test is the most accurate and specific serological method for the diagnosis of flavivirus infections, this assay acquires high biosafety level facility [42, 43]. Therefore, an easy and accurate serodiagnostic method that can detect viral species-specific antibodies is needed. However, antibodies induced by flavivirus infection cross-react to other flavivirus species, so that it is difficult to determine the accurate history of the flavivirus infection by serodiagnosis [44, 45]. Most of cross-reactive antibodies recognize structural proteins, which are highly conserved among MBFVs [46-48]. The conservation of structural proteins extends not only to MBFVs but also to nonpathogenic insect-specific flaviviruses (ISFVs) [49-52]. A different epidemiological approach is polymerase chain reaction (PCR)-based diagnostic systems that detect viral RNA. While diagnostics for the detection of viral RNA are highly specific and effective, the timing of diagnosis is only limited to acute phase after infection [36]. Serodiagnosis is an effective method to search for a history of the disease, even if a long period of time has passed since infection.

Thus, this study was conducted to develop effective serodiagnostic method, which is able to determine the specific viral species. In Chapter I, the antigenicity of structural proteins of pathogenic and nonpathogenic flaviviruses was investigated. In Chapter II, recombinant viral proteins were engineered to reduce cross-reactive epitopes, and applied to enzyme-linked immunosorbent assay (ELISA)-based serodiagnostic method and vaccine antigen.

Chapter I: Serological characterization of lineage II insect-specific flaviviruses compared with pathogenic mosquito-borne flaviviruses.

Introduction

Flavivirus is an enveloped RNA virus, including TBFVs, MBFVs, and ISFVs. TBFVs and MBFVs infect vertebrates sometimes producing disease. ISFVs are phylogenetically divided into lineage I (also called classical ISFV) and lineage II. In phylogenetic analyses, lineage II ISFVs cluster with the MBFVs [53]. Therefore, lineage II ISFVs are considered to be dual-host affiliated ISFVs, which may be transmitted to mammals other than mosquitoes, as are MBFVs [50, 54]. However, there have been no reports of lineage II ISFVs infecting vertebrate cells under natural conditions [55, 56].

Flaviviruses have positive-sense, single-stranded RNA genomes encoding a polyprotein composed of structural and nonstructural proteins [4]. Reaction analyses using antibodies against the structural protein of MBFVs have demonstrated that those antibodies are mutually cross-reactive among MBFVs, especially DENV, ZIKV, JEV, and WNV [46, 47, 57-59]. These studies suggest that structural proteins are conserved among these MBFVs. Recently, hemagglutination inhibition assays and reaction analyses using a panel of anti-lineage II ISFV monoclonal antibodies showed that lineage II ISFV also possessed antigenically similar structural proteins to MBFVs [50-52].

The lineage II ISFVs are phylogenetically further divided into lineage IIa and IIb, and lineage IIa ISFVs seem to be associated *Aedes* species [60]. While the antigenic relationship between lineage II ISFV and MBFV have been investigated, the characterization of lineage IIa and IIb ISFVs compared with MBFVs are still poorly understood. In this study, antibody-based analyses were performed using anti-lineage IIa and IIb ISFV sera to MBFV antigens to identify antigenic similarity among lineage IIa and IIb ISFVs, and the MBFVs. Therefore, two lineage II ISFVs, Psorophora flavivirus (PSFV, lineage IIa ISFV) isolated from *Psorophora albigenu* in Bolivia, and Barkedji virus (BJV, lineage IIb ISFV) isolated from *Culex* spp. in Zambia, were used [54, 60].

Materials and methods

Cells

Vero9013 cells (Vero cells; JCRB, Ibaraki, Japan) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS). K562 cells (JCRB) were maintained in Roswell Park Memorial Institute 1640 (RPMI1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS. Vero cells and K562 cells were cultured at 37°C and 5% CO_2. *Aedes albopictus*-derived C6/36 mosquito cells (ATCC, Manassas, VA, USA) were cultured at 28°C, 5% CO_2 in Eagle's Minimum essential medium (MEM, Nissui) supplemented with 10% FBS and non-essential amino acids (Thermo Fisher Scientific).

Viruses

DENV type2 (DENV2) hu/INDIA/09-74 strain (GenBank accession no. LC367234), ZIKV MR766-NIID strain (GenBank accession no. LC002520), JEV Beijing strain (GenBank accession no. L48961), and WNV zmq16m11 strain (GenBank accession no. LC318700) were used as vertebrate-infecting MBFVs. PSFV (GenBank accession no. LC567151) and BJV zmq17L31 strain (GenBank accession no. LC497470) were used as lineage IIa and IIb ISFVs, respectively. All viral strains were propagated in C6/36 cells in MEM containing 2% FBS and incubated for three to five days, after which culture supernatants were collected and stored at −80°C until use.

Analysis of homology and molecular phylogenetics

The genome sequences of the flaviviruses used were obtained from GenBank. The sequence similarities of the MBFVs and the lineage II ISFVs used in this study were evaluated using basic local alignment search tool analysis. For phylogenetical analyses, amino acid sequence alignments based on whole amino acid sequences or precursor membrane (prM) and envelope (E) proteins were generated using the MUSCLE algorithm of the CLC Genomics Workbench 20.1 (Qiagen, Hilden, Germany). Maximum-likelihood (ML) phylogenetic trees were constructed using the best-fit models of the Le_Gascuel_2008 model with gamma rate categories with invariant sites, with empirical frequencies (LG+G+I+F) for the whole amino acid sequences and LG+G+I for the prME protein, and bootstrap values of 1,000 replicates in MEGA X software [61]. The phylogenetic trees were visualized using Interactive Tree Of Life version 6.5.2 [62].

Focus forming assay (FFA)

Vero cells (1.5×10^5 cells/well) for MBFVs or C6/36 cells (1.0×10^6 cells/well) for lineage II ISFVs, grown in 24-well plates (Corning, Tewksbury, MA, USA) were inoculated with 10-fold serial dilutions of each virus and incubated for 1 h at 37°C (Vero cells) or 28°C (C6/36 cells). An overlay medium was added (MEM containing both 0.5% methyl cellulose and 2% FBS) and incubated for 32 h (ZIKV, JEV, and WNV) or 72 h (PSFV, BJV, and DENV2). After incubation, the cells were fixed with pre-chilled methanol at −30°C for more than 10 min. The fixed cells were blocked with Phosphate-buffered saline (PBS, pH7.4) containing 1% bovine serum albumin (BSA) at room temperature (RT) for 30 min. Anti-flavivirus nonstructural protein 1 (NS1) monoclonal 4G4 antibody (kindly provided by Prof. Roy, Hall University of Queensland, St Lucia, Queensland, Australia) [63] for MBFVs or anti-flavivirus envelope monoclonal BJ-6E6 (6E6) antibody (kindly provided by Prof. Roy Hall) [52] for lineage II ISFVs in 0.1% BSA-PBS was then added to each well and incubated at RT for 1 h. Alexa Fluor 488-conjugated anti-mouse immunoglobulin (Ig) G antibody (Invitrogen, Waltham, MA, USA) in 0.1% BSA-PBS was applied as a secondary antibody at RT for 1 h, and viral focus images were acquired using a fluorescence microscope (IX73, Olympus, Tokyo, Japan). The virus titers were calculated based on the number of foci and expressed as focus-forming units (ffu) per ml.

Ethical statement

All animal experiments were performed in accordance with the National University Corporation, Hokkaido University, Regulations on Animal Experimentation. The protocol was approved by the Institutional Animal Care and Use Committee of Hokkaido University (approval numbers. 18-0036 and 18-0149).

MBFV-infected serum

AG129 mice deficient in interferon-α/β and -γ receptors were obtained from Marshall BioResources (Hull, East Yorkshire, UK) and bred in-house in an animal facility for use in infection experiments with DENV and ZIKV because these viruses cannot infect immunocompetent mice. Male AG129 mice (10-week-old) were inoculated subcutaneously with 1.0×10^5 ffu of DENV2 or ZIKV. Female BALB/c mice (6-week-old, Japan SLC, Shizuoka, Japan) were inoculated subcutaneously with 1.0×10^6 ffu of JEV or 1.0×10^4 ffu of WNV. The body weight of the virus-inoculated mice was monitored daily. When mice showed a 10% decrease in their body weight, they were euthanized using excess isoflurane

anesthesia, and sera were collected. The mouse sera were inactivated by incubation at 56°C for 30 min and stored at −80°C until use.

Virus purification

At four days post inoculation with either PSFV or BJV in C6/36 cells, the culture supernatants were harvested and filtrated through a 0.45 μm syringe filter and pelleted by ultracentrifugation with a 20% sucrose cushion using an SW32Ti rotor (Beckman Coulter, Brea, CA, USA) at 153,720 ×*g* for 3 h [64]. The pellets were resuspended in PBS. The protein concentrations of the resuspended samples were determined using BCA assays (Thermo Fisher Scientific) conducted according to the manufacture's protocol. Virus suspensions were stored at −80°C until immunization.

Immunization

Female BALB/c mice (6-week-old) were subcutaneously inoculated with 20 μg of PSFV or BJV, or PBS as a negative control. Four weeks post inoculation, the mice were euthanized using excess isoflurane anesthesia, and the sera were collected, inactivated by incubation at 56°C for 30 min and stored at −80°C until use.

ELISA

Vero cells (6.0×10^5 cells/well) or C6/36 cells (4.0×10^6 cells/well) in six-well plates (Corning) were inoculated with MBFV or lineage II ISFV at a multiplicity of infection (MOI) of 0.1. At 72 hours post infection (hpi) (PSFV, BJV, and DENV2) or 48 hpi (ZIKV, JEV, and WNV), the cells were lysed in lysis buffer [1% NP-40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA]. After centrifugation to remove the cell debris, the virus-infected cell lysates were collected from the supernatant. Flat-bottom 384-well plates (Thermo Fisher Scientific) were coated with 50-fold diluted cell lysates at 37°C for 3 h. Each antigen was blocked with 5% skim milk-PBS at RT for 30 min. Immunized mouse sera and pan-flavivirus D1-4G2-4-15 (4G2) monoclonal antibody (ATCC) used as a positive control were serially 10-fold diluted with 1% skim milk-PBS and added to each well. Following incubation for 2 h at 37°C, the plates were washed five times with 0.01% PBS-Tween20 (PBST). Horseradish peroxidase (HRP) conjugated anti-mouse IgG antibody (Thermo Fisher Scientific) was added and incubated at 37°C for 1 h. After washing five times, 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) was added as a HRP substrate. After incubation at RT for 15 min, the reaction was stopped by the addition of 2 M sulfuric acid. The optical density

(OD) value was measured at 450 nm using a GroMax Discover Microplate Reader (Promega, Madison, WI, USA). The binding titer was defined as the endpoint serum dilution, which is the reciprocal of the highest dilution over the cut-off value; the mean plus three times the standard deviation (SD) of non-immunized control sera. The values lower than the cut-off value were expressed as the limit of detection.

Focus reduction neutralization test (FRNT) assay

Vero cells (7.5×10^4 cells/well) or C6/36 (5.0×10^5 cells/well) cells were seeded in 48-well plates (Corning) 24 h before infection. Serum samples were serially 10-fold diluted with DMEM containing 2% FBS and incubated with 50 ffu of each virus at 37°C for 1 h. The cells were inoculated with the serum-virus complexes and incubated at 37°C for 1 h. After incubation, the inoculum was removed, and the cells were treated with overlay media and incubated for 32 h (ZIKV, JEV, and WNV) or 72 h (PSFV, BJV, and DENV2). Fixation and primary antibody treatments were performed, as described in method for FFA. After inoculation with HRP-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific) as a secondary antibody at 37°C 1 h, virus-derived foci were stained with TrueBlue Peroxidase Substrate (Sera Care Life Sciences Inc., Milford, MA, USA) and counted. Neutralizing antibody titers were defined as the reciprocal of the highest serum dilution showing a 50% reduction in the number of foci compared to non-immunized control serum (as $FRNT_{50}$), as previously reported [65].

Antibody-dependent enhancement (ADE) of MBFV infection

Flow cytometry-based assays were performed to measure the ADE of MBFV infection, as previously reported [57]. Serial 10-fold dilutions of serum samples were incubated with MBFV (MOI of 0.1) for 1 h at 37°C, and 5.0×10^4 K562 cells in RPMI containing 10% FBS were added to each well in round-bottom 96-well plates (Corning). At 48 hpi (JEV and WNV) or 72 hpi (DENV2 and ZIKV), cells were fixed with 4% paraformaldehyde, and permeabilized with PBS containing 0.2% BSA and 0.05% saponin. Viral antigens were stained with 4G2 antibody, and Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen). The virus-infected cells were counted using an FACSCanto (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo software version 10.7.1 (Treestar Inc., Ashland, OR, USA). Data were expressed as overall ADE, calculating the area under the curve using GraphPad Prism software version 8.0 (GraphPad Software,

San Diego, CA, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 8.0. The methods of statistical analysis are described in the Figure Legends for each experiment.

Results

The prME proteins of lineage IIa ISFVs are phylogenetically closely related to those of MBFVs

ML phylogenetic analysis based on whole amino acid sequences showed that both lineage IIa and IIb ISFVs (PSFV and BJV) were grouped within the cluster of MBFVs (**Fig. 1A**). The cluster of lineage I ISFVs was distinct from those of MBFVs and lineage II ISFVs, as previously reported [66], indicating that lineage II ISFVs have a more similar amino acid sequence to MBFVs compared to lineage I ISFVs.

In general, the amino acid sequences of the prME proteins were highly similar among flaviviruses [2, 3]. Thus, the prME amino acid sequences of the MBFVs and lineage II ISFVs used in this study, were compared. The prME of DENV2, ZIKV, JEV, and WNV had 45-78% amino acid sequence identity and over 60% similarity to one another. The prMEs of PSFV and BJV also had 59–65% amino acid sequence similarity to those of DENV2, ZIKV, JEV, and WNV, which was comparable to the similarity among these MBFVs (**Table 1**). The fusion loop (FL) domain in prME, which is known to be highly conserved in MBFVs, was conserved in both lineage II ISFVs (**Fig. 2**) [67]. ML phylogenic analysis on the prME amino acid sequences of the flaviviruses was also performed. Lineage IIa ISFVs belonged to a clade of MBFVs, while lineage IIb ISFVs were associated with a different clade (**Fig. 1B**). These results suggest that the prME protein of lineage IIa ISFVs is more closely related to MBFVs than that of lineage IIb ISFVs.

Anti-PSFV sera cross-react with MBFV antigens with ADE activity

To evaluate the antigenic similarity between MBFV and lineage II ISFV, serological analysis was conducted using antisera from mice immunized with purified PSFV from lineage IIa ISFV or BJV from lineage IIb ISFV *via* subcutaneous (SC) route.

The production of antibodies against PSFV or BJV in mice sera was evaluated using ELISAs of lineage II ISFV-infected cell lysates. All anti-PSFV or BJV sera (n = 7 each) reacted to cellular lysates infected with each lineage II ISFV, indicating that immunization with each lineage II ISFV *via* SC route induced anti-PSFV or BJV antibody in each serum (**Figs. 3A, 3B, and 4A**). The reactivity of antisera to cellular lysates infected with MBFVs, including DENV2, ZIKV, JEV, and WNV, was evaluated. Pan-flavivirus 4G2 antibody was used as a positive control and reacted against all the lysates infected with MBFVs (**Figs. 3C and 3D**). There were no differences in non-specific binding to cell lysates of non-infected

Vero cells between the anti-PSFV sera, anti-BJV sera, and non-immunized control sera (**Fig. 3E**). ELISA revealed that anti-PSFV sera possessed broad binding activity to cell lysates infected with DENV2 (positivity rate 6/7), ZIKV (positivity rate 6/7), JEV (positivity rate 1/7), and WNV (positivity rate 3/7) (**Figs. 3F and 4B**). On the other hand, anti-BJV sera had binding activity to DENV2 (positivity rate 4/7), ZIKV (positivity rate 3/7), and WNV (positivity rate 1/7) but not to JEV (positivity rate 0/7) (**Figs. 3F and 4B**).

Antibodies which recognize structural proteins such as prME enhance flavivirus infection by incorporating virus-bound antibodies *via* the Fcγ receptor [68, 69]. This enhancement of antibody-dependent viral infection has been called ADE. It was found that anti-PSFV and BJV sera reacted to cell lysates infected with DENV2, ZIKV, JEV, or WNV, suggesting that anti-PSFV and BJV sera may elicit ADE. Therefore, flow cytometry-based ADE assays using Fcγ receptor-expressing K562 cells was performed to evaluate the ADE activity of anti-PSFV or BJV sera. Enhancement of the number of K562 cells infected with DENV2, ZIKV, JEV, or WNV was observed in the presence of anti-PFSV sera (**Fig. 5A**), and the overall ADE activity of anti-PSFV sera was significantly higher than those of control sera (**Fig. 5B**). In contrast, anti-BJV sera had only a slightly enhanced number of infected cells with each MBFV (**Fig. 5A**), and their overall ADE activity was not significantly higher than those of control sera (**Fig. 5B**). These results indicated that anti-PSFV sera potentially cross-reacted to DENV2, ZIKV, JEV, and WNV, accompanied by ADE activity. It has been reported that ADE is elicited by antibodies showing reactivity with structural proteins in flavivirus infection [47]. It was confirmed that structural proteins of MBFVs were recognized by anti-PSFV or BJV sera using western blotting and found that both sera reacted with the prM and E proteins of MBFVs (**Fig. 6**).

Anti-lineage II ISFV sera had no or low neutralizing activity against MBFVs

Then, the neutralizing activity of lineage II ISFV antisera against the lineage II ISFV or MBFV infection, was evaluated. First, inhibition of PSFV or BJV infection in C6/36 cells by anti-PSFV or BJV sera was confirmed using FRNT (**Figs. 7A and 7B**). Some of those antisera neutralized not only their own virus infection but also other lineage II ISFV infections. Next, it was confirmed that the positive controls of MBFV antisera obtained from MBFV-infected mice completely inhibited the foci formation of each virus in Vero cells in a serum dilution-dependent manner (**Fig. 7C**). In contrast, anti-PSFV and BJV sera showed few or no foci reductions against DENV2, ZIKV, JEV, or WNV (**Fig. 7C**). The $FRNT_{50}$ titers

of both anti-ISFV sera were significantly lower than those of positive control sera against all MBFVs (**Fig. 7D**). However, a few anti-PSFV sera suppressed foci formation to below 50% for DENV2 (positivity rate 1/7), ZIKV (positivity rate 1/7), WNV (positivity rate 1/7), and anti-BJV sera also decreased the foci formation of WNV (positivity rate 1/7). These results suggested that the epitopes recognized by anti-MBFV neutralizing antibodies might be present in PSFV and BJV.

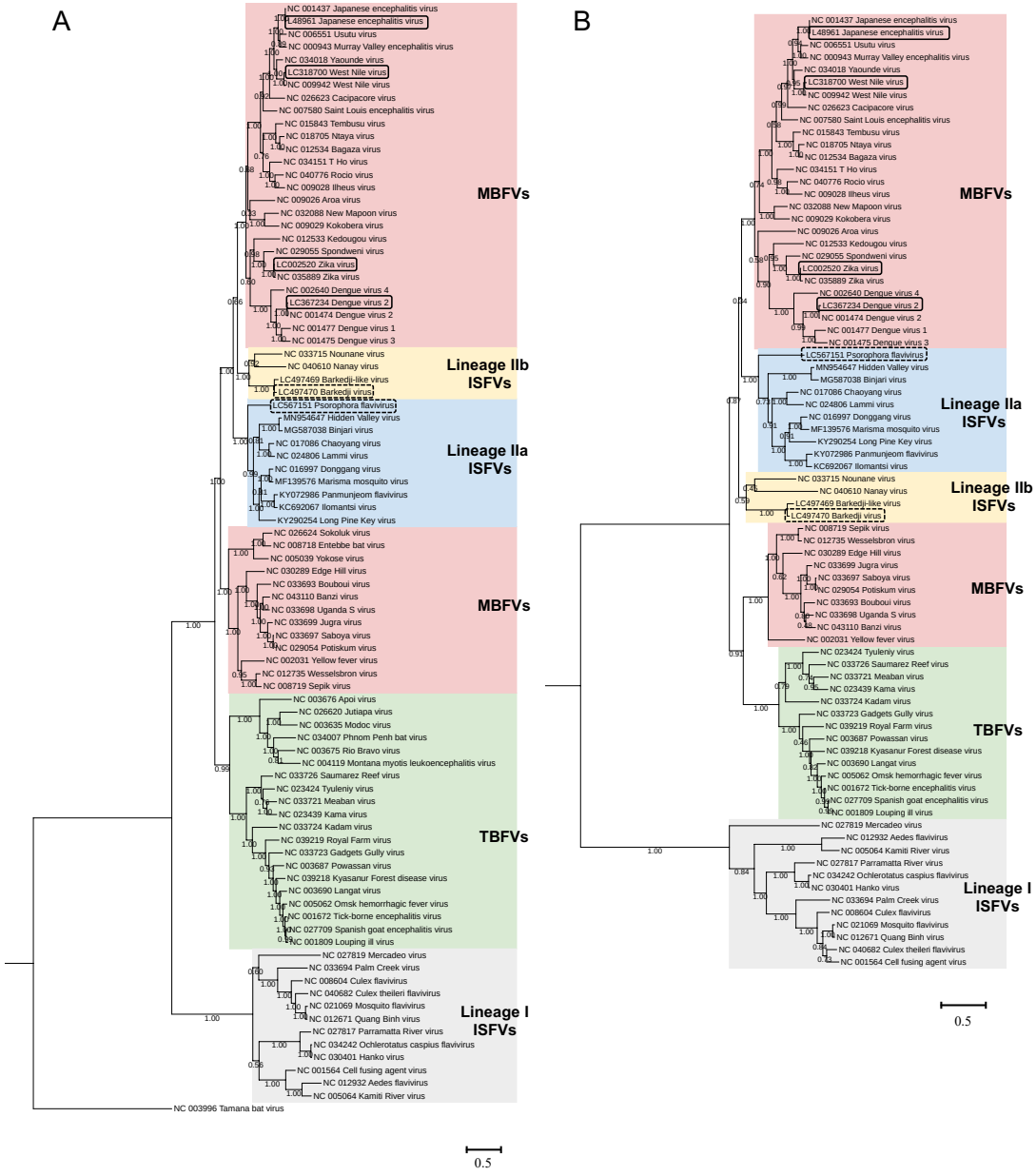


Fig. 1 Phylogenetic analysis of ISFV based on amino acid sequences.

MBFVs, including DENV2, ZIKV, JEV and WNV used in this study are highlighted in the black squares. Lineage II ISFVs, PSFV and BJV, are highlighted in the dot-lined square. Phylogenetic trees were generated based on **(A)** whole amino acid sequence and **(B)** prME by the maximum-likelihood method using best-fit models with bootstrap values of 1,000 replicates.

Table 1. Comparison of amino acid sequences of the prME proteins.

	PSFV		BJV		DENV2		ZIKV		JEV		WNV	
	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
PSFV	-	-	43	60	39	59	42	62	44	64	45	63
BJV			-	-	43	62	43	64	46	65	47	65
DENV2					-	-	51	72	45	64	46	65
ZIKV							-	-	52	74	52	74
JEV									-	-	78	90
WNV											-	-

Fig 2. Comparison of the amino acid sequence of the fusion loop domain. Dots indicate the same amino acid residue with common flaviviruses.

FL domain

common flaviviruses		D	R	G	W	G	N	G	C	G	L	F	G
lineage IIa	**PSFV**	·	·	·	·	·	·	·	·	P	·	·	A
lineage IIb	**BJV**	G	·	·	·	·	·	·	·	A	·	·	·
MBFV	**DENV2**	·	·	·	·	·	·	·	·	·	·	·	·
	ZIKV	·	·	·	·	·	·	·	·	·	·	·	·
	JEV	·	·	·	·	·	·	·	·	·	·	·	·
	WNV	·	·	·	·	·	·	·	·	·	·	·	·

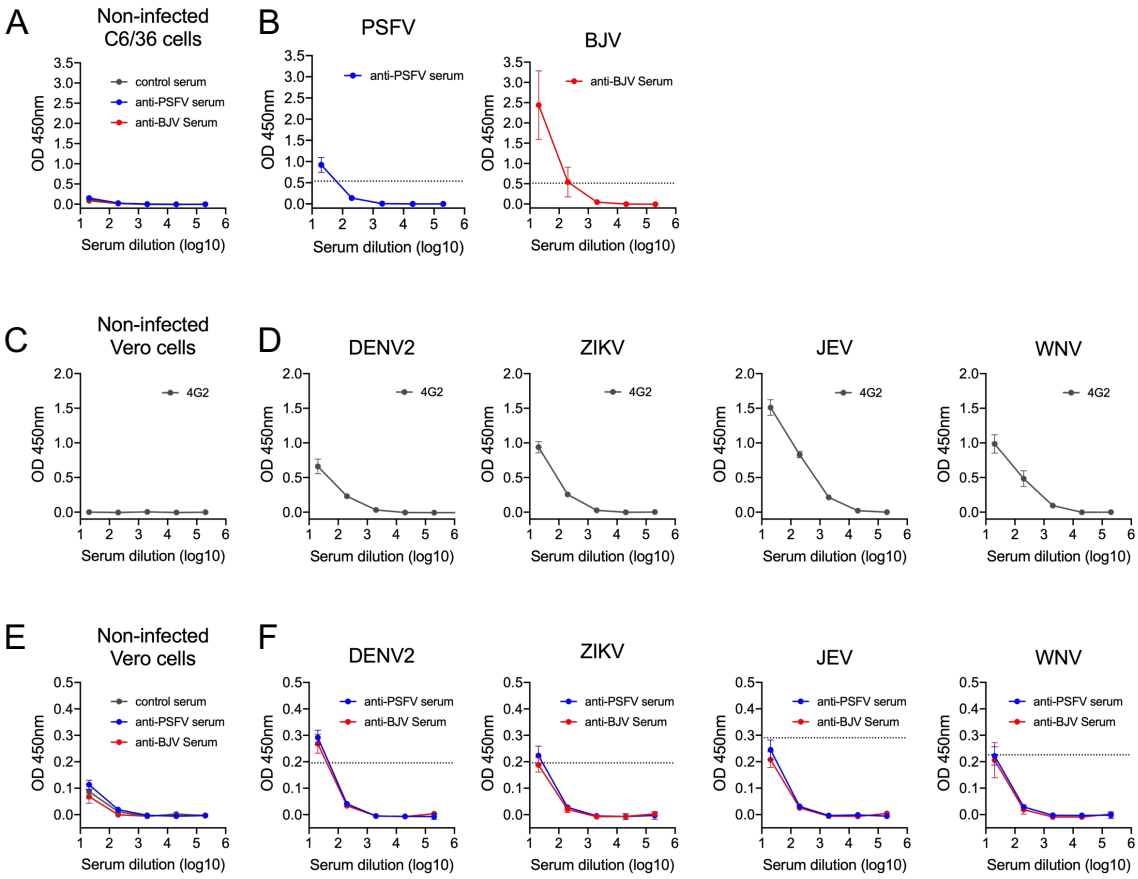


Fig. 3 Anti-PSFV or BJV serum were subjected to ELISA.

(A) Binding activity of non-immunized control sera (control sera), anti-PSFV sera or anti-BJV sera to non-infected C6/36 cell lysates. **(B)** Binding of anti-PSFV or anti-BJV sera to PSFV or BJV-infected C6/36 cell lysates. **(C and D)** Binding of pan-flavivirus 4G2 monoclonal antibody as positive control to non-infected Vero cell lysates (C) and cell lysates infected with DENV2, ZIKV, JEV, and WNV (D). **(E and F)** Binding of control sera, anti-PSFV or anti-BJV sera to non-infected Vero cell lysate (E) and cell lysates infected with DENV2, ZIKV, JEV, and WNV (F). Dotted line indicates cut-off value (mean plus three times the SD of non-immunized control sera). The values in the graphs are expressed as the mean ± SD of seven serum samples (n = 7).

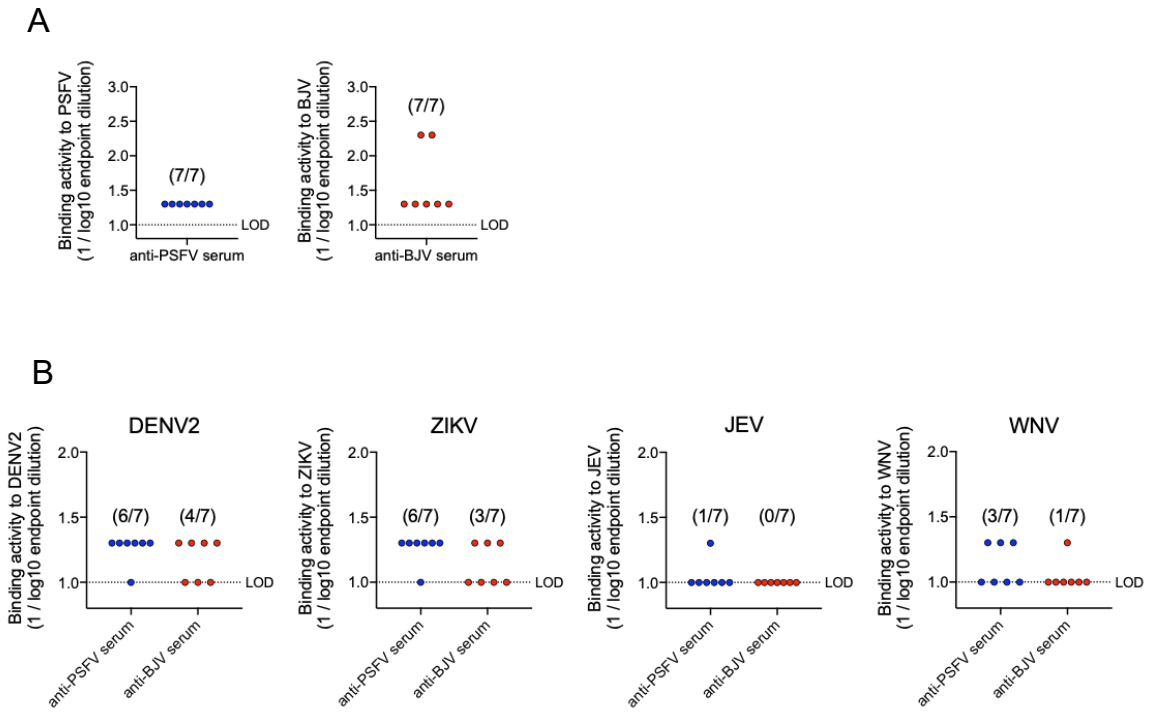


Fig. 4 Binding activity of anti-PSFV or BJV sera to cell lysates infected with each virus. Binding activity of anti-PSFV or BJV sera to cell lysates infected with **(A)** lineage II ISFVs and **(B)** MBFVs was evaluated using ELISA. The dotted line indicates the limit of detection (LOD). The values in the graphs are expressed as the mean ± SD of seven serum samples (*n* = 7).

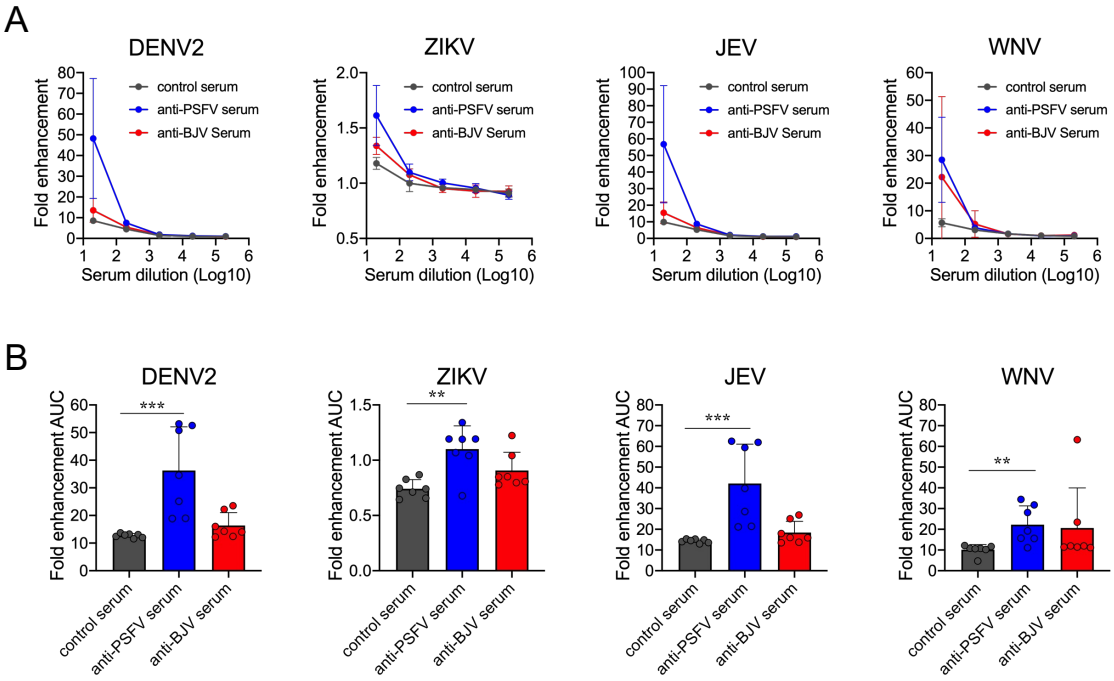


Fig. 5 ADE of MBFV infection by anti-PSFV or BJV sera.

(A) Non-immunized control sera, anti-PSFV sera and anti-BJV sera were evaluated for their enhancement of MBFV infections in K562 cells. **(B)** Overall ADE activity was determined from the area under the curve (AUC) shown in Fig. 3A. The values in the graphs are expressed as the mean ± SD of seven serum samples (n = 7). **: $p < 0.005$, ***: $p < 0.001$ from one-way ANOVA with Dunn's multiple comparison test.

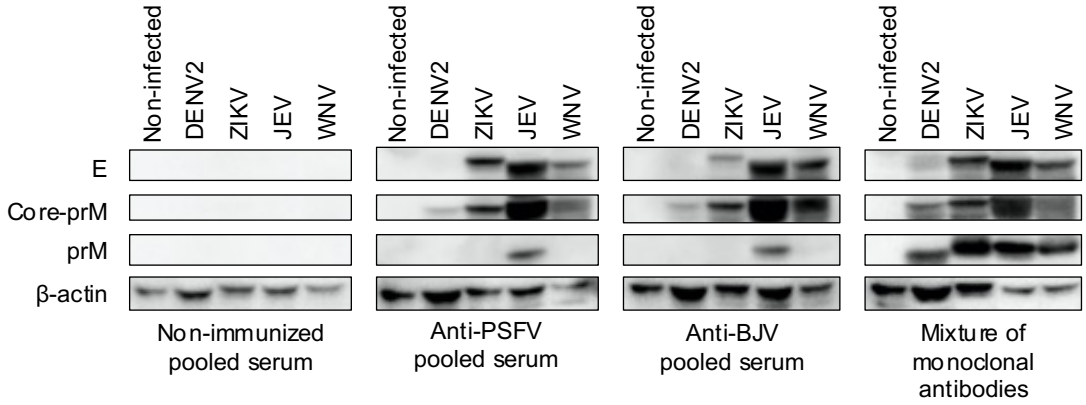


Fig. 6 Viral proteins recognized by anti-PSFV or BJV pooled serum as shown by western blotting of Vero cells infected or uninfected with DENV2, ZIKV, JEV, or WNV.

Mixtures of monoclonal antibodies targeting the prM proteins of DENV2, prM proteins of ZIKV, prM proteins of WNV, and E protein, were used as positive control.

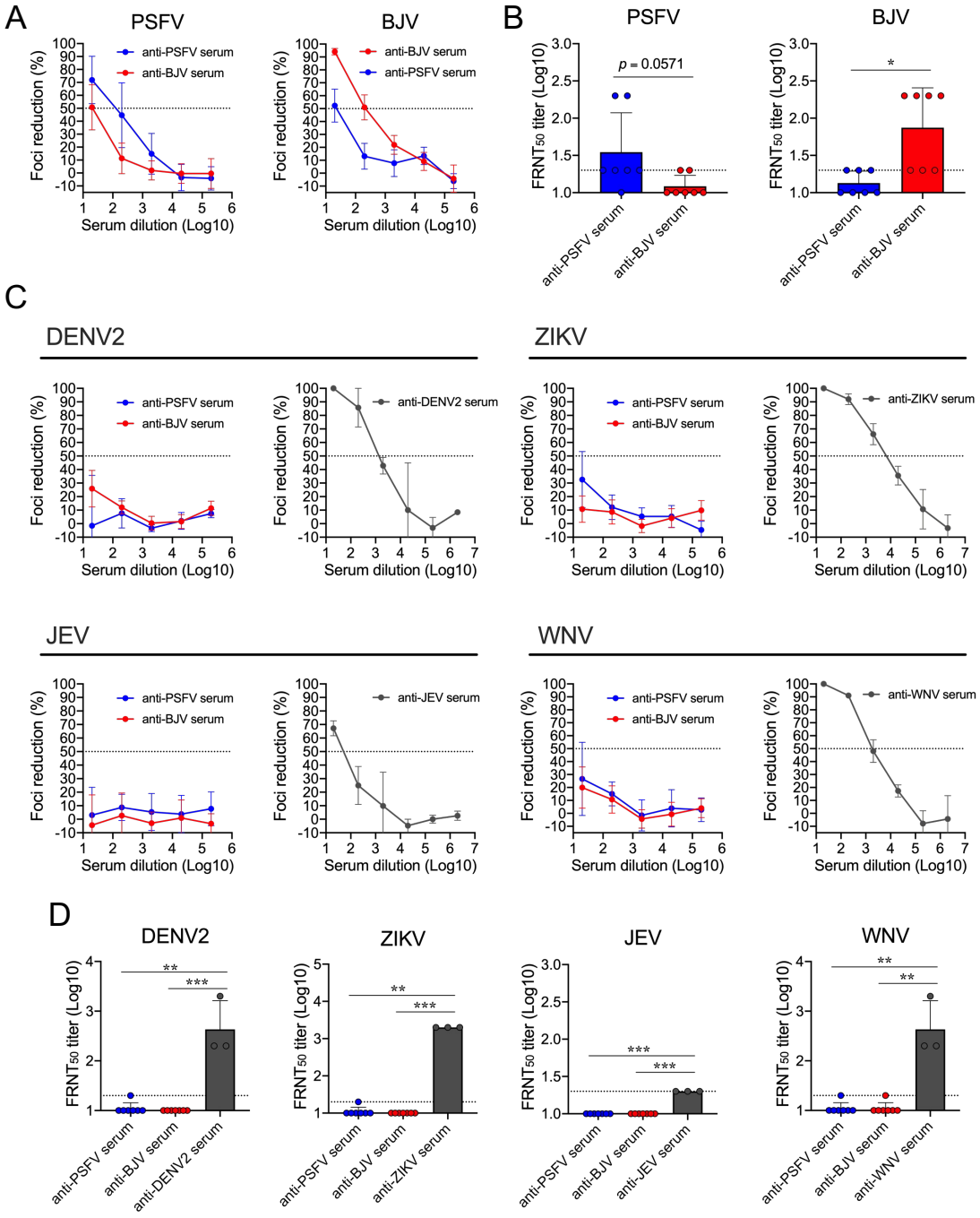


Fig. 7 Neutralizing activities of anti-PSFV or BJV sera against MBFVs. (A) FRNTs against PSFV or BJV were performed using C6/36 cells. **(B)** The $FRNT_{50}$ titer was determined from the data shown in Fig. 4A. *: $p < 0.01$ using t-tests with Mann-Whitney tests. **(C)** FRNT against MBFVs using Vero cells. **(D)** The $FRNT_{50}$ titer was determined from data shown in Fig. 4C. Dotted lines indicate 50% foci reduction (A and C) and detection limits (B and D). The values in the graphs are expressed as the mean ± SD of seven serum samples ($n = 7$). *: $p < 0.01$, **: $p < 0.005$ using one-way ANOVA with Dunn's multiple comparison test.

Discussion

Previous studies using MBFV antisera showed that hemagglutination activities of lineage IIa ISFVs such as Aripo virus, La Tina virus, Marisma mosquito virus and lineage IIb ISFV such as Nanay virus were inhibited by various MBFV antisera [50, 51], suggesting that these lineage II ISFVs antigens were cross-reactive with MBFV antisera. Cross-reactivities of both lineage IIa and IIb ISFVs antisera with MBFVs antigens were evaluated in this study. The results showed that the lineage IIa ISFV PSFV antisera had higher positivity for binding to MBFV antigens and induced more ADE of MBFV infections than lineage IIb ISFV BJV antisera. These results suggest that PSFV is more antigenically similar to MBFVs than BJV. It was further found that both lineage II antisera recognized prM and E proteins of each MBFV (**Fig. 6**). Taken together, these observations suggest that lineage IIa PSFV had prME proteins more antigenically similar to those of DENV2, ZIKV, JEV, and WNV than lineage IIb BJV. This finding was consistent with the result of the ML phylogenetic analysis using prME protein sequences (**Fig. 1B**).

The obtained results and previous studies showed lineage II ISFV possessed antigenically similar viral proteins to MBFVs [50-52]. Furthermore, this study demonstrated the existence of antigenic differences between genetically closely related lineage IIa and IIb ISFVs by antibody-based analyses. It should be noted, however, this study only used a single virus strain as representative of each lineage IIa or IIb ISFV in this study. To clarify the differences in the viral proteins of the lineage II ISFVs, further studies, such as structural analysis or reactive analysis using monoclonal antibodies with other virus strains, will be required.

Summary

The genus Flavivirus includes pathogenic tick- and mosquito-borne flaviviruses as well as non-pathogenic ISFVs. Phylogenetic analysis based on whole amino acid sequences has indicated that lineage II ISFVs have similarities to pathogenic flaviviruses. In this study, we used reactive analysis with immune serum against PSFV as a lineage IIa ISFV, and BJV as a lineage IIb ISFV, to evaluate the antigenic similarity among lineage IIa and IIb ISFVs, and pathogenic MBFVs. Binding and antibody-dependent enhancement assays showed that anti-PSFV sera had broad cross-reactivity with MBFV antigens, while anti-BJV sera had low cross-reactivity. Both of the lineage II ISFV antisera were rarely observed to neutralize MBFVs. These results suggest that lineage IIa ISFV PSFV has more antigenic similarity to MBFVs than lineage IIb ISFV BJV.

Chapter II: Design of flavivirus subviral particle (SVP) to suppress bindings of cross-reactive anti-flavivirus antibodies for serodiagnosis and vaccine development.

Introduction

Flaviviruses are phylogenetically divided into MBFV, TBFVs, lineage I ISFVs, and lineage II ISFVs. It has been demonstrated that lineage II ISFVs are phylogenetically and antigenically close to MBFV; however, lineage I ISFVs are not close to them [49, 52, 56]. Thus far, there is no clear evidence about pathogenicity of lineage I and II ISFVs to vertebrates [55, 56]. Meanwhile, MBFVs such as DENV, ZIKV, JEV, and WNV are able to infect vertebrates and these infections result in lethal encephalitis and hemorrhagic fever [2]. However, no specific therapeutic agents against these infections has yet been developed, and also clinically available vaccines for MBFVs are limited [4].

Structural proteins of flaviviruses contain capsid protein, prM and E proteins. It has been reported that some flavivirus antibodies cross-react with other flavivirus antigens [70, 71], and epitopes for these antibodies are mainly located in prME regions, which are highly conserved among flaviviruses [47]. In particular, FL domain in domain II of E protein (EDII) is known as mostly conserved region in flaviviruses [72]. Therefore, the anti-FL monoclonal antibody (Mab) reacts to the diverse range of flaviviruses, including MBFV and lineage II ISFVs [52].

According to the clinical studies, antisera against DENV, ZIKV, JEV and WNV potentially enhance other flavivirus infections [30, 57, 59]. This ADE of flavivirus infections is caused by cross-reactive antibodies with binding activity to different flavivirus species. If non-neutralizing cross-reactive antibodies are induced by primary flavivirus infection, the antibodies facilitate viral entry through Fcγ receptors upon secondary infection, resulting in the promotion of ADE [73]. The prM and FL domains have been found as predominant epitopes for antibodies eliciting ADE [74-76]. It has also been demonstrated that prM and FL domains in EDII are not effective for neutralization but effective for cross-reaction to the other flaviviruses [77]. Domains I and II in E protein have virus-specific non-neutralizing and neutralizing epitopes, respectively [46, 72, 78-80]. Therefore, an attempt has been made to use E proteins with mutation in FL domain as vaccine antigen without ADE epitopes [67]. Moreover, these mutated E proteins were also used as antigen for serological analysis, which is an important approach for the evaluation of the risk of severe flavivirus disease, including past histories of flavivirus infections which may cause ADE [81]. However, there have been few reports on the design of viral proteins that can be applied as antigens for both vaccines

and serodiagnosis [82, 83].

It has been reported that W101 residue in FL domain is the key epitope of several anti-FL Mab [84-86], and mutation of W101 in FL domain inhibit binding of FL domain-reactive Mab [87]. This mutation not only reduced cross-reactivity but also disrupted neutralizing E dimer epitope or reduced protein expression [77, 86, 88, 89].

Modification of the FL domain is an effective method for the production of flavivirus-specific antibodies, although the mutation must be able to maintain the original neutralizing epitope of the viral particle. In this study, various subviral particles were established, which can detect and induce flavivirus-specific antibody responses, by amino acid mutations in the FL domain.

Materials and Methods

Cell culture

Vero cells (JCRB) were maintained at 37℃, 5% CO_2 in DMEM (Nissui) supplemented with 10% FBS. K562 cells were maintained at 37℃, 5% CO_2 in RPMI1640 (Nissui) supplemented with 10% FBS. Aedes albopictus C6/36 mosquito cells (ATCC, Manassas, VA, USA) were cultured at 28℃, 5% CO_2 in Eagle's MEM supplemented 10% FBS and non-essential amino acids. Expi293F cells (Thermo Fisher Scientific) were at 37℃, 8% CO_2 in Expi293 Expression Medium (Thermo Fisher Scientific).

Viruses

DENV2 hu/INDIA/09-74 strain (GenBank accession no. LC367234), ZIKV MR766-NIID strain (GenBank accession no. LC002520) and PRVABC59 strain (GenBank accession no. KU501215), JEV Beijing strain (GenBank accession no. L48961), WNV NY-99 strain (GenBank accession no. KC407666.1) and Zmq16m11 strain (GenBank accession no. LC318700.1), PSFV (GenBank accession no. LC567151), BJV (GenBank accession no. LC497470), Cell fusing agent virus (CFAV, isolated from *Aedes aegypti* collected in Zambia), Culex flavivirus (CxFV, isolated from *Culex quinquefasciatus* collected in Zambia) were used. All virus strains were propagated in C6/36 cells in MEM containing 2% FBS. After five days, culture supernatant was collected and stored at −80°C until use.

FFA

Vero cells (1.5×10^5 cells/well) in 24-well plates (Corning, Tewksbury, MA, USA) were inoculated with 10-fold serial dilutions of each virus and incubated for 1 h at 37°C (Vero cells) or 28°C (C6/36 cells). An overlay medium was added (MEM containing both 0.5% methyl cellulose and 2% FBS) and incubated for 32 h (ZIKV, JEV, and WNV) or 72 h (DENV2). After incubation, the cells were fixed with pre-chilled methanol at −30°C for more than 30 min. The fixed cells were blocked with PBS containing 1% BSA at RT for 30 min. Anti-flavivirus NS1 monoclonal 4G4 antibody (kindly provided by Prof. Roy Hall) in 0.1% BSA-PBS was then added to each well and incubated at RT for 1 h. The 4G4 antibody Alexa Fluor 488-conjugated anti-mouse IgG antibody (Invitrogen) in 0.1% BSA-PBS was applied as a secondary antibody at RT for 1 h, and viral focus images were acquired using a fluorescence microscope (IX73, Olympus). The virus titers were calculated based on the number of foci and expressed as ffu per ml.

Indirect fluorescent assay (IFA)

C6/36 cells were infected with DENV2, JEV, PSFV and BJV at MOI of 1. CFAV and CxFV were 100-fold diluted and inoculated to C6/36 cells. Supernatant (100 µl) of infected cells with CFAV or CxFV were collected at 12 hpi and 72 hpi for Quantitative reverse transcription PCR (RT-qPCR). Following incubation for 4 days, the cells were fixed with 4% paraformaldehyde and blocked with PBS containing 1% BSA for 30 min at RT. Two anti-FL 4G2 (ATCC) and 6E6 (kindly provided by Prof. Roy Hall) Mabs derived from hybridoma, and anti-double stranded RNA (dsRNA) J2 Mab (Nordic MUbio, Susteren, Netherland) were used as primary antibody at RT for 1 h. The hybridoma producing 4G2 and 6E6 Mabs were generated by immunization to mouse with DENV antigens and with lineage II ISFV Binjari virus, respectively [52, 90]. Alexa Fluor 488-conjugated anti-mouse IgG antibody (Invitrogen) in presence of Hoechst 33342 were applied as a secondary antibody at RT for 1 h. Images were acquired using a fluorescence microscope (IX73, Olympus).

RT-qPCR

Aliquots of supernatant from the supernatants of infected C6/36 cells with CFAV and CxFV were extracted using Direct-Zol kit (Zymo research, CA, USA) following the manufacturer's instructions. The amounts of viral RNAs were quantified using PrimeScript One-step RT-PCR kit Ver.2 (Takara, Shiga, Japan) and each virus-specific primer set (for CFAV, primer 1: 5'-AGA AAG CTC ACC AAC CAA CG-3' ,primer 2: 5'-AGG GGT GTC AAC CGA AAA TG-3', for CxFV, primer 3: 5'-AGT TAT ATC AGA TGC CGA CGA C-3', primer4: 5'-AGA GCC CAC AAC ACT TCC GT-3'), under the following thermal condition: 42°C for 5 min, 95°C for 10 sec, and 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Each standard curve was generated using a series of diluted PCR product and RNA copy number of RNA in each sample was calculated.

Protein expression and purification of SVP

The coding sequence for prME from DENV2 hu/INDIA/09-74 strain (1-661), ZIKV MR766-NIID strain (1-672), JEV Beijing strain (1-667), WNV NY-99 strain (1-668) were optimized to homo sapience (GeneArt, Life Technologies) and cloned into pCXSN vector [64] with signal sequence (SS) derived from core protein sequences at N-terminus (23 amino acids) of JEV Beijing strain. The SVPs were expressed in Expi293F cells following the manufacturer's instructions. The SVP mutants were created by inserting substitutions D98N, N103T, G106F/L, L107K/E, and F108W in FL domain. Cultured supernatant fluid was

harvested and clarified by centrifugation and filtration with 0.45 μm. The clarified fluids were pelleted by ultracentrifugation with 20% sucrose cushion at 153,720 ×*g* for 2 h. The pellet was resuspended with PBS/MgCl (phosphate buffered saline supplemented with 1mM $MgCl_2$, and 1mM $CaCl_2$). SVPs were further purified by ultracentrifugation through a 10-50% sucrose gradient in PBS at 153,720 ×*g* for 3 h. Gradient fractions were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie blue staining, and antigen capture ELISA to confirm fractions containing E protein and particle formation. E protein-enriched fractions were pelleted by ultracentrifugation with PBS at 153,720 ×*g* for 2 h. The pellet was resuspended with PBS/MgCl. Amount of E protein in purified SVPs was assessed by SDS-PAGE followed by Coomassie bule staining using bovine serum albumin as a standard.

Western blotting

Protocol of western blotting has already been described [49]. In brief, the purified SVPs were separated using SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA). Following blocking with 5% skim milk-PBS with 0.01% Tween20, primary antibodies were used the following antibodies, FL: 4G2 antibody; E: anti-DENV E antibody, anti-ZIKV E antibody, and anti-JEV E antibody (GeneTex, Irvine, CA, USA), anti-WNV E antibody (Novus Biologicals, CO, USA); prM: anti-DENV prM antibody and anti-ZIKV prM antibody (GeneTex), anti-WNV prM antibody (Abcam, Cambridge, UK). HRP-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific) or rabbit IgG antibody (Thermo Fisher Scientific) were used as secondary antibody and Immobilon Western HRP Substrate (Millipore). The signal was visualized using an ImageQuant 800 (Cytiva, Marlborough, MA, USA).

Antigen capture ELISA

E protein monoclonal antibody clone 402 (kindly provided by Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University, Kagawa, Japan) was coated in 384-well plate overnight at 4°C. The antibodies were blocked with 1% BSA-PBS for 1 h at RT. Gradient fractions were added to each well and incubated for 2 h at 37°C. After washing with PBST at five times, plate was incubated for 1 h at 37°C with HRP-conjugated anti-E monoclonal antibody (clone 402). After washing, plate was added with 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific). After incubation at RT for 15

min, the reaction was stopped by the addition of 2 M sulfuric acid. The OD value was measured at 450 nm using a GloMax Discover Microplate Reader (Promega).

Ethical statement

All animal experiments were performed in accordance with the National University Corporation, Hokkaido University, Regulations on Animal Experimentation. The protocol was approved by the Institutional Animal Care and Use Committee of Hokkaido University (approval numbers 18-0036 and 18-0149).

Serum

For the production of infected serum, interferon-α/β and -γ receptors-deficient AG129 mice were obtained from Marshall BioResources (Hull) and bred in-house in an animal facility. For standard mouse infected serum, 16-18-week-old male AG129 mice (n =3) were inoculated subcutaneously with 1.0×10^5 ffu of DENV2 hu/INDIA/09-74 strain or ZIKV MR766-NIID strain. Six-weeks old female C3H (n =3) or BALB/c mice (n =3) (SLC, Shizuoka, Japan) were inoculated subcutaneously with 1.0×10^4 ffu of JEV Beijing strain or 1.0×10^4 ffu of WNV NY-99 strain, respectively. For challenge mouse infected serum, 10 or 15-week-old AG129 mice (n = 3, two 10-week-old male mice and a 15-week-old female mouse) or 6-week-old female BALB/c mice (n = 6) were inoculated subcutaneously with 1.0×10^3, 1.0×10^4, 1.0×10^5, 1.0×10^6 ffu of ZIKV PRVABC5 strain or 1.0×10^4 ffu of WNV Zmq16m11 strain, respectively. The body weight of the virus-inoculated mice was monitored daily. When mice showed a 10% decrease in their body weight, they were euthanized using excess isoflurane anesthesia, and sera were collected.

For mouse immunization, 6-week-old female BALB/c mice (SLC) were subcutaneously inoculated with SVP (1 μg of E protein content) admixed with equivalent volume of Imject Alum Adjuvant (Thermo Fisher Scientific) twice at a 3-week interval. Three days after second immunization, mice were euthanized using excess isoflurane anesthesia, and sera were collected. All infected and immunized sera were inactivated by incubation at 56°C for 30 min and stored at −80°C until use.

ELISA

Each SVP protein (250 ng/ml of E protein content for antibody titer and standard serum, 500 ng/ml of E protein content for challenge serum) was put onto each well of 384-well plates and incubated overnight at 4°C. SVPs in each well was blocked with 10% horse

serum in PBS for 1 h at RT. Mouse sera were diluted at 100-, 500-, 1,000-, 5,000-, and 10,000-fold with 0.1% horse serum in PBS and incubated for 2 h at 37°C. After washing with PBST at five times, plate was incubated for 1 h at 37°C with HRP-conjugated anti-mouse IgG (Sigma-Aldrich, Merck, St. Louis, MO, USA), IgM (Thermo Fisher Scientific), IgA antibody (Abcam, Cambridge, UK), total Ig (Thermo Fisher Scientific). Following a washing step, signal was detected as described previously in antigen capture ELISA. Endpoint titers were determined as the reciprocal of the highest dilution over eightfold blank values.

FRNT assay

FRNT assay was performed as previous described [49]. Vero cells (7.5×10^4 cells/well) were seeded in 48-well plates (Corning) 24 h before infection. Serial 10-fold diluted serum and 50 ffu of each virus were mixed and incubated for 1 h at 37°C. The cells were then inoculated with the serum-virus complex and incubated for 1 h at 37°C. Following the incubation, the cells were treated with overlay media and incubated for 32 h. Fixation and primary staining were conducted, as described in FFA. HRP-conjugated anti-mouse Ig antibody (Thermo Fisher Scientific). Virus foci were stained with TrueBlue Peroxidase Substrate (Sera Care Life Sciences Inc., Milford, MA, USA) and counted. Neutralizing antibody titers were defined as the reciprocal of the highest serum dilution showing a 50% reduction in the number of foci compared to virus control (as $FRNT_{50}$), as previously reported [65].

ADE assay

Flow cytometry-based ADE assay was slightly modified from previously described [49]. Serial 2-fold dilutions of serum specimens were incubated DENV, ZIKV, JEV and WNV (MOI of 1) for 1 h at 37°C, and 5.0×10^4 K562 cells in RPMI containing 10% FBS were added to each well in round-bottom 96-well plates (Corning). For the evaluation of ADE activity of ZIKV, antibody-virus complex-containing cultured media was changed to fresh RPMI because ZIKV effectively infects to K562 without antibodies. At 48 hpi (JEV and WNV) or 72 hpi (DENV2 and ZIKV), cells were fixed with 4% paraformaldehyde, and permeabilized with PBS containing 0.2% BSA and 0.05% saponin. Virus-infected cells were stained with 4G2 antibody, and Alexa Fluor 488-conjugated goat anti-mouse IgG antibody. The cells were counted using an FACSCanto (BD Biosciences, Franklin Lakes, NJ, USA) or LSRFFortessa (BD Biosciences) and analyzed using FlowJo software version 10.7.1

(Treestar Inc., Ashland, OR, USA). Data were expressed as ADE, calculating the area under the curve using GraphPad Prism software version 8.0 (GraphPad Software, San Diego, CA, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 8.0. The methods of statistical analysis are described in the figure legends for each experiment.

Results

Homology and antigenicity of FL domain

The FL domain is highly conserved among flaviviruses. Therefore, anti-FL domain antibodies exhibit broad cross-reactivity against most flaviviruses. To assess the homology of the FL domain in flaviviruses, alignment analysis using 12 amino acid sequences of FL domains among MBFV, lineage I and II ISFVs was conducted. The analysis revealed that lineage I ISFVs contained more amino acid differences at the FL domain compared to lineage II ISFVs and MBFVs (**Fig. 8A**).

Next, antigenicity of the FL domain was evaluated using two different Mabs for FL domain, clones 4G2 and 6E6, which were obtained from mice immunized with DENV antigens and with lineage II ISFV Binjari virus, respectively [52, 90]. These Mabs were used to detect viral antigen in each virus infected cell by IFA (**Fig. 8B**). Both of Mabs 4G2 and 6E6 bound to DENV2- and JEV-infected cells. Viral antigen was detected in cells infected with lineage II ISFVs, PSFV and BJV, by Mab 6E6 but not 4G2. However, neither of these Mabs bound to cells infected with lineage I ISVFs, CFAV and CxFV. In this experiment, viral infections with all flaviviruses were confirmed by staining for viral dsRNAs using Mab J2. RT-qPCR also showed that viral RNA of these lineage I ISFVs were increased in culture media at 72 hpi compared to 12 hpi (**Fig. 8C**). These results demonstrated that two Mabs which react with different FL epitope did not bind with cells infected by CFAV and CxFV. Thus, The FL domain of lineage I ISFV has different antigenicity compared to those of MBFV and lineage II ISFV.

Mutations of FL domain to avoid binding with anti-FL Mab

Mabs 4G2 and 6E6 were derived from immunization of DENV as MBFVs and Binjari virus as lineage II ISFV, respectively. To determine amino acid residues responsible for the binding of anti-FL antibody, JEV-based SVPs were generated. The JEV SVP wild type (WT) was composed of prME proteins containing the capsid-derived 23 amino acids at the C-terminus as a signal peptide (**Fig. 9A**). SVP mutants were created by replacing amino acid residues of FL domain in JEV with those of lineage I ISFV (**Fig. 9B**).

Western blotting using anti-E antibody exhibited that JEV SVP WT, mutant (mut) 1 (D98N), mut3 (G106F, L107K, and F108W) and mut4 (G106L, L107E, and F108W) were secreted in culture supernatant of the transfected cells, while three SVP mutants (mutant 2, 5, and 6) containing N103T mutation were not detected **(Fig. 9C)**. These results also showed

that the SVP mut4 was the most highly expressed in the supernatant among all of the mutants. Moreover, the SVPs mut3 and 4 were able to impair the binding of anti-FL antibody, but mut1 was still detectable by the Mab. Therefore, SVP mut4, which has the highest expression without functional epitopes for the anti-FL antibody, was used for further experiments.

In addition to JEV, SVP WT and mut4, insertion of G106L, L107E, and F108W, were generated for DENV2, ZIKV and WNV. The purified SVPs by ultracentrifugation were assessed for binding with anti-FL antibody by Western blotting. The result showed that both SVP WT and mut4 were reacted with anti-E and anti-prM antibodies (**Fig. 9D**). Binding of anti-FL antibody was confirmed only for SVP WT, but not for the SVP mut4.

Identification of virus-specific antibody subclasses in infected serum by ELISA using SVP mutants

By replacing a part of the FL domain of MBFV SVP with those of the lineage I ISFV (G106L, L107E, and F108W), SVP mut4 was able to avoid binding of anti-FL antibody. Next, to evaluate the binding activities of flavivirus-infected sera to the SVPs, ELISA was performed using SVP WT or mut4 as antigens. In the ELISA, amounts of SVPs were adjusted with the amount of E protein. Flavivirus-infected sera was produced by infection of each flavivirus, DENV2, ZIKV, JEV, and WNV described in the materials and methods section. At first, the ELISA was performed to measure signals of positive-to-negative ratio (P/N ratio) of IgM, IgG, IgA, and total Ig which bind with each SVP antigen in the flavivirus-infected sera in order to examine the antibody subclass as with high viral species-specific signals.

It was difficult to determine DENV2-specific responses among IgM, IgG, IgA and total Ig, because DENV2-infected sera broadly cross-reacted to heterologous viral antigens, even when the SVP mut4 was used (**Fig. 10A and 10B**). In the ELISA with ZIKV-infected sera, ZIKV SVP WT antigen showed higher signals of IgM, IgG, IgA, and total Ig at each dilution of serum than those of other SVPs (**Fig. 10C**). However, non-specific signals were also detected in all Ig classes. Meanwhile, ZIKV SVP mut4 reacted with ZIKA-infected sera with the marked higher signals of all of Ig subclass compared to those of other mut4 SVPs (**Fig. 10D**). Especially, non-specific antibody responses of IgG and total Ig subclasses to SVP mut4 were markedly suppressed compared with those of SVP WT. JEV-infected sera showed the JEV-specific response with little non-specific responses to both of SVP WT and mut4 (**Fig. 10E and 10F**). In contrast to DENV2 or ZIKV-infected sera, no JEV-specific IgM and IgA responses were detected in JEV-infected sera, while signals of IgG and total Ig to JEV

WT and mut4 SVPs were markedly high. In sera infected with WNV which is the same serotype as JEV, high signals of IgG and total Ig to SVP WT and mut4 of WNV and JEV were observed (**Fig. 10G and 10H**). Similar to the binding properties of JEV-infected sera, there was no specific antibody responses in IgM and IgA subclasses in WNV-infected sera. In summary, virus-specific IgM, IgG, IgA, and total Ig responses were induced in ZIKV-infected sera, while JEV- and WNV-infected sera showed virus-specific antibody responses only in IgG and total Ig. These observations indicate that the IgG and total Ig to each antigen are suitable for the detection of viral species-specific antibodies.

Next, the signals of IgG and total Ig in the flavivirus-infected serum of each individual mouse to each SVP antigen were compared. Heatmap for signals of DENV2-infected sera showed that non-specific IgG and total Ig responses to JEV antigen were the highest signals at 100-fold serum dilution (**Fig. 11A**). When each serum was diluted in 1,000-fold, the signals of IgG and total Ig to DENV2 SVP WT or mut4 were higher than those of other SVP antigens. In ZIKV-infected individual serum, the highest signal of IgG and total Ig to ZIKV SVP antigen were observed (**Fig. 11B**). In particular, non-specific signals to DENV2, JEV, and WNV SVP antigens were markedly decreased in SVP mut4 at all diluted serum. JEV-infected individual serum clearly showed specific binding to JEV SVP antigens without non-specific binding to DENV2, ZIKV and WNV SVPs WT and mut4 (**Fig. 11C**). Although similar signals in WNV SVP WT and JEV SVP WT antigens were observed in WNV-infected individual mouse serum, signals of IgG and total Ig to WNV SVP mut4 antigen were higher than JEV SVP mut4 (**Fig. 11D**). The cross-reactive signals of WNV-infected serum to JEV SVP mut4 antigen were decreased by dilution. These results indicated that the SVP mu4 in ELISA diminished the non-specific binding of cross-reactive antibodies and detected virus species-specific antibodies with high sensitivities.

ELISA with SVP mutants applied to serodiagnosis

The ELISA using SVP mut4 as antigen could detect virus-specific IgG and total Ig responses in flavivirus-infected sera of the same virus species and strain as SVPs. These results suggest that the SVP mut4 are useful for serodiagnosis of flavivirus infections. To assess whether ELISA with SVP mut4 can be utilized as a serodiagnosis, ELISA with serum of mice infected with different viral strains (challenge serum) from SVP antigen was conducted. IgG responses in sera of mice infected with ZIKV (PRVABC59 strain) or WNV (Zmq16m11 strain) were measured by ELISA using SVP mut4 in this experiment. The virus-

specific ELISA signals of challenge sera may be reduced since the viruses used for the infected sera and SVP antigens are the same species but different strains. Therefore, to clearly detect the virus-specific signal, the amount of SVP antigen was increased to 0.5 μg/ml.

The result of ELISA with ZIKV-infected serum showed that little cross-reactive antibodies to DENV2, JEV, or WNV were detected, and the highest signal of IgG to ZIKV SVP mut4 was observed at all the diluted serum (**Fig. 12A**). In WNV-infected serum, cross-reactivity to JEV SVP was observed in all mouse individuals, but the highest signal was detected in the homologous virus combination (**Fig. 12B**). Furthermore, dilution of serum decreased cross-reactivity to JEV SVP mut4, while binding to WNV SVP mut4 was maintained.

Next, the effect of viral infectious dose on the ELISA P/N pattern of challenge serum was examined. ZIKV-infected challenge sera were produced by the inoculation of 10^3, 10^5, or 10^6 ffu of ZIKV to each mouse. As the result, at any amount of ZIKV infection (10^3, 10^5, or 10^6 ffu), ELISA with SVP mut4 demonstrated ZIKV-specific IgG responses without cross-reactive IgG responses (**Fig. 12C, 12D, and 12E**). These results suggest that ELISA with SVP mut4 reduces non-specific detection of cross-reactive IgG antibodies induced by flavivirus infections and clarifies virus-specific IgG responses in a virus species-dependent and virus strain-independent manner.

Induction of virus-specific antibodies by immunization with the SVP mutant

The substitution of amino acids in the FL domains of MBFVs with those of lineage I ISFV was found to reduce non-specific cross-reactive binding to flavivirus SVPs. To further evaluate whether the substitution of FL domain in flavivirus SVPs has potential of eliciting virus-specific antibodies, mice were subcutaneously immunized with the combination of SVP and Alum adjuvant two times at three-weeks interval (**Fig. 13A**). The SVPs of ZIKV and JEV were used as immunogens from different flavivirus serotypes because of their higher protein yield than the SVPs of DENV2 and WNV.

Following the collection of serum from the immunized mice, ELISA was conducted to measure antibody titer to each SVP antigen. There was no clear difference in endpoint titers of ZIKV or JEV SVPs-immunized sera against DENV2 antigen between WT and mut4 (**Fig. 13B**). Likewise, the endpoint titers against ZIKV antigen in those immune sera showed no significant difference between the WT and the mut4 (**Fig. 13B**). The endpoint titers against JEV antigen were significantly lower in sera immunized with ZIKV SVP mut4 compared to

those with the WT, while in the JEV SVP immunization group, there was no difference in the titers between WT and mut4 SVPs (**Fig. 13B**). The titers against WNV antigen tended to decrease, although not significantly, in the sera immunized with ZIKV SVP mut4 compared with those with the WT, and there was a significant decrease in the titers in immunized sera with JEV SVP mut4 compared to those of the WT.

Next, these antibodies induced by immunization with SVPs were assessed for ADE activities. ADE activities against infections of all virus species excepted for ZIKV were significantly inhibited in sera of mice immunized with ZIKV SVP mut4 compared to those with the SVP WT (**Fig. 13C**). ADE activity of JEV SVP mut4-immunized sera to DENV2 infection was also significantly inhibited compared to the WT-immunized sera (**Fig. 13C**). The ADE activity against JEV infection were shown in both JEV SVPs WT and mut4, meanwhile that against WNV, which is same serotype to JEV, was relatively lower in the SVP mut4-immunized sera than the WT SVP-immunized sera (**Fig. 13C**).

In addition, neutralizing activity was also evaluated to ensure that the neutralizing epitope was not destroyed by mutation of the FL domain. PRNT assay showed neutralization activity against ZIKV or JEV in a subset of mouse sera immunized with SVPs of ZIKV and JEV (**Fig. 13D**). Some immune sera from mice immunized with ZIKV SVP mut4 exhibited neutralizing activity against ZIKV infection comparable to those immunized with the WT (**Fig. 13D**). The sera from mice immunized with JEV SVP inhibited ZIKV and JEV infections regardless of whether wild type or mut4 SVP was used as the immunogen (**Fig. 13D**).

A

			FL epitope											
			98	99	100	101	102	103	104	105	106	107	108	109
		common flaviviruses	D	R	G	W	G	N	G	C	G	L	F	G
MBFV	NC_001477	Dengue virus 1	·	·	·	·	·	·	·	·	·	·	·	·
	NC_001474	Dengue virus 2	·	·	·	·	·	·	·	·	·	·	·	·
	NC_001475	Dengue virus 3	·	·	·	·	·	·	·	·	·	·	·	·
	NC_002640	Dengue virus 4	·	·	·	·	·	·	·	·	·	·	·	·
	NC_035889	Zika virus	·	·	·	·	·	·	·	·	·	·	·	·
	NC_001437	Japanese encephalitis virus	·	·	·	·	·	·	·	·	·	·	·	·
	NC_009942	West Nile virus	·	·	·	·	·	·	·	·	·	·	·	·
	NC_006551	Usutu virus	·	·	·	·	·	·	·	·	·	·	·	·
	NC_007580	Saint Louis encephalitis virus	·	·	·	·	·	·	·	·	·	·	·	·
	NC_002031	Yellow fever virus	·	·	·	·	·	·	·	·	·	·	·	·
	NC_009029	Kokobera virus	·	·	·	·	·	·	·	·	·	·	·	·
Lineage II ISFV	LC567151	Psorophora flavivirus	·	·	·	·	·	·	·	·	P	·	·	A
	NC_016997	Donggang virus	·	·	·	·	·	·	·	·	G	·	·	·
	MG587038	Binjari virus	·	·	·	·	·	·	·	·	V	·	·	·
	KC692067	Ilomantsi virus	·	·	·	·	·	·	·	·	G	·	·	·
	MN954647	Hidden Valley virus	·	·	·	·	·	·	·	·	V	·	·	·
	LC497469	Barkedji-like virus	G	·	·	·	·	·	·	·	A	·	·	·
	LC497470	Barkedji virus	G	·	·	·	·	·	·	·	A	·	·	·
	NC_040610	Nanay virus	G	·	·	·	·	·	·	·	G	·	·	·
Lineage I ISFV	NC_001564	Cell fusing agent virus	N	·	·	·	·	T	·	·	F	K	W	·
	NC_008604	Culex flavivirus	N	·	·	·	·	T	·	·	F	K	W	·
	NC_034242	Ochlerotatus caspius flavivirus	N	·	·	·	·	T	·	·	F	K	W	·
	NC_033694	Palm Creek virus	N	·	·	·	·	T	·	·	F	K	W	·
	NC_012932	Aedes flavivirus	N	·	·	·	·	T	·	·	F	K	W	·
	NC_040682	Culex theileri flavivirus	N	·	·	·	·	T	·	·	F	K	W	·
	YP_009259488.1	Nakiwogo virus	N	·	·	·	·	T	·	·	L	E	W	·

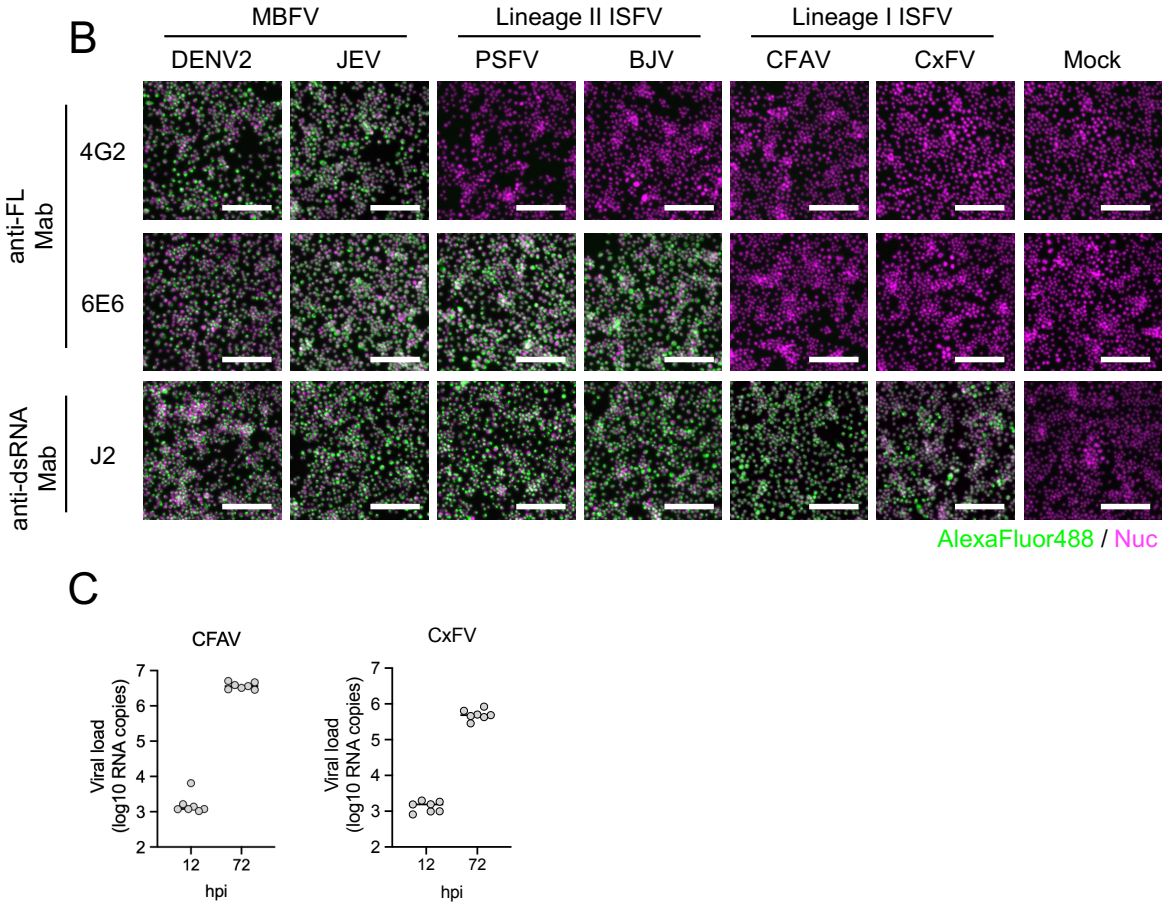


Fig. 8 Comparison of homology and antigenicity for FL domain in flaviviruses.

(A) Sequence alignment of FL domain in MBFVs, lineage I ISFVs, and lineage II ISFVs. **(B)** Antigenicity in FL domain of MBFV, lineage II and lineage I ISFVs were evaluated using anti-FL monoclonal antibodies. Scale bar indicates 100 μm. **(C)** Propagation of CFAV and CxFV were measured by quantitative reverse transcription PCR.

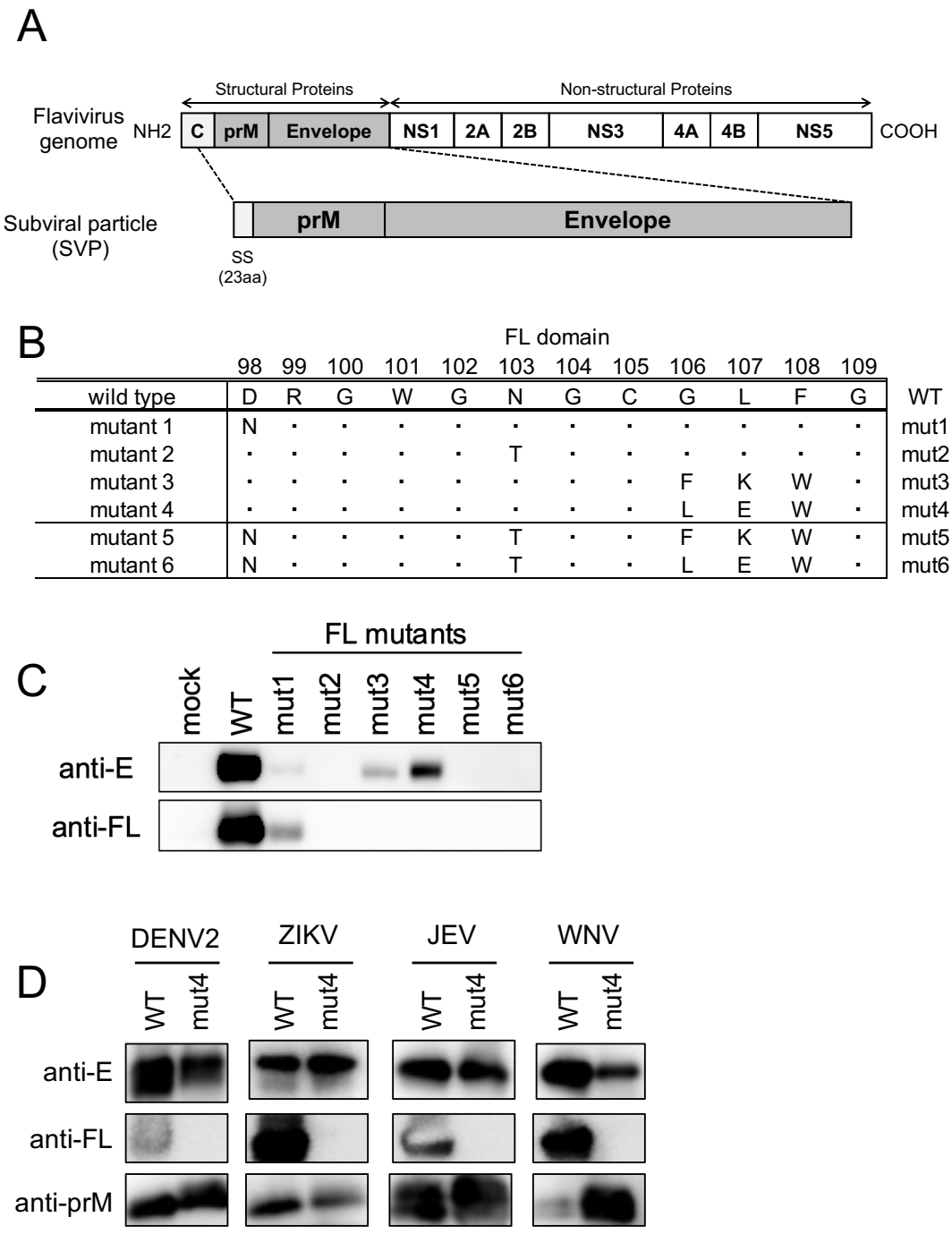


Fig. 9 SVP mutants replaced with FL domain of lineage I ISFV. (A) Scheme of the construction of SVPs. **(B)** FL domains of generated SVP mutants based on JEV SVPs. **(C)** Western blotting of supernatant of transfected with plasmids encoded each SVPs. Anti-JEV E polyclonal antibody and 4G2 Mab was used as anti-E and anti-FL antibodies, respectively. E: envelope. **(D)** Western blotting of purified SVP proteins for DENV2, ZIKV, JEV, and WNV.

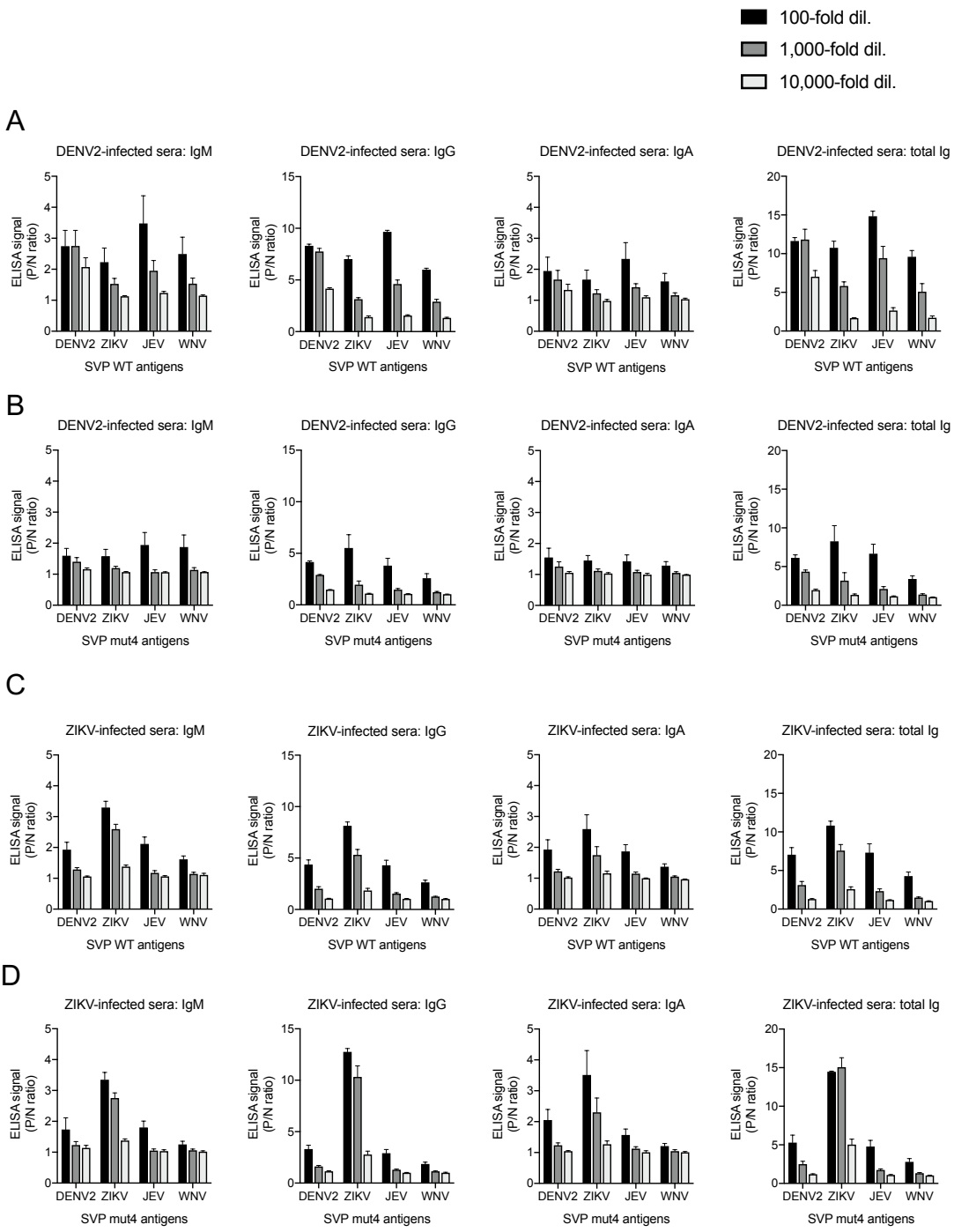
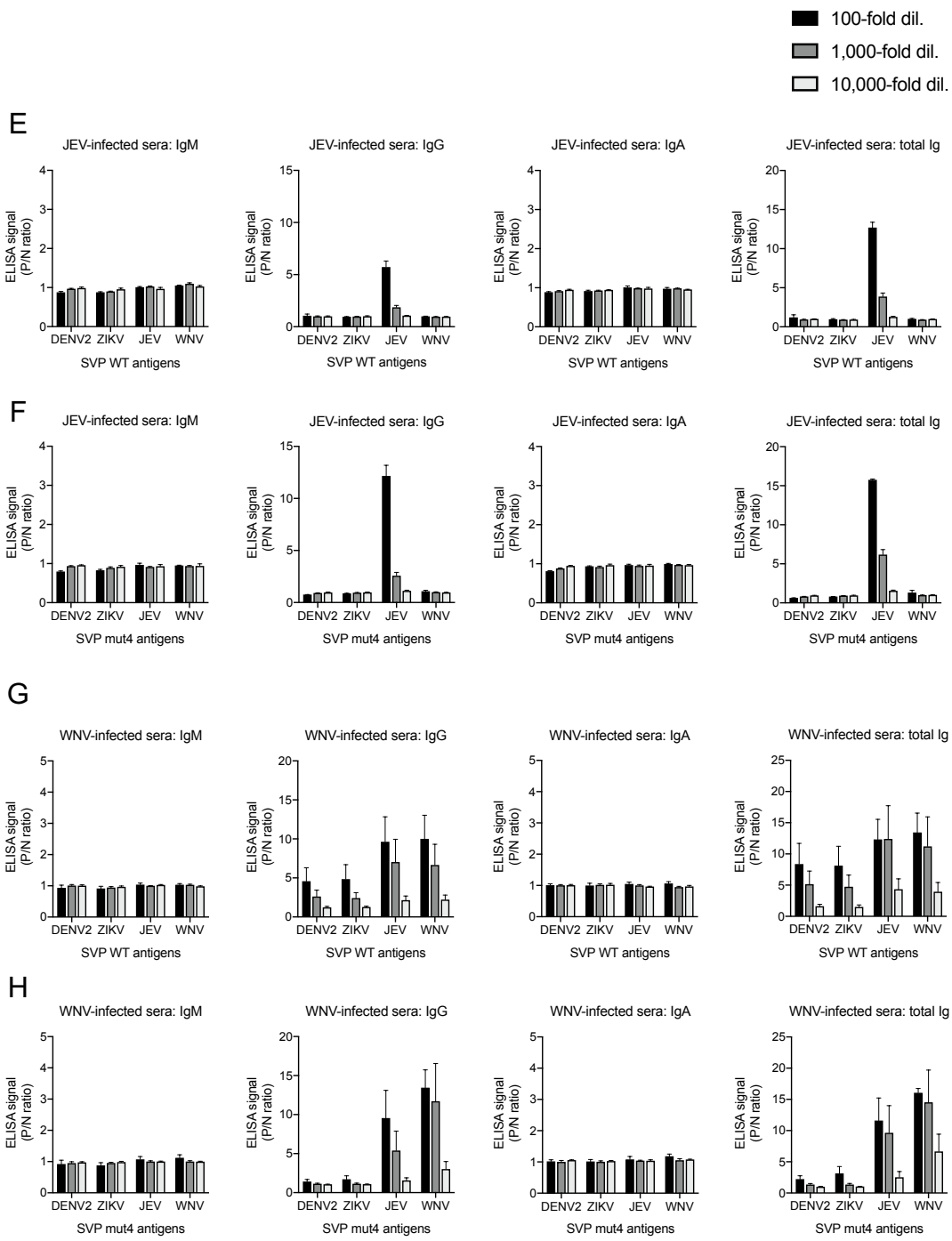


Fig. 10 ELISA with SVPs WT and mut4 using flavivirus-infected sera.

(A to H) IgM, IgG, IgA and total IgG to each SVP antigen in mouse sera infected with DENV2 (A and B), ZIKV (C and D), JEV (E and F) or WNV (G and H) were measured by ELISA. SVP WT (A, C, E, and G) and SVP mut4 (B, D, F, and H) were used as ELISA antigens. The values in the graphs are expressed as the mean ± sem of serum samples (n = 3).

(Figure 10 continued)



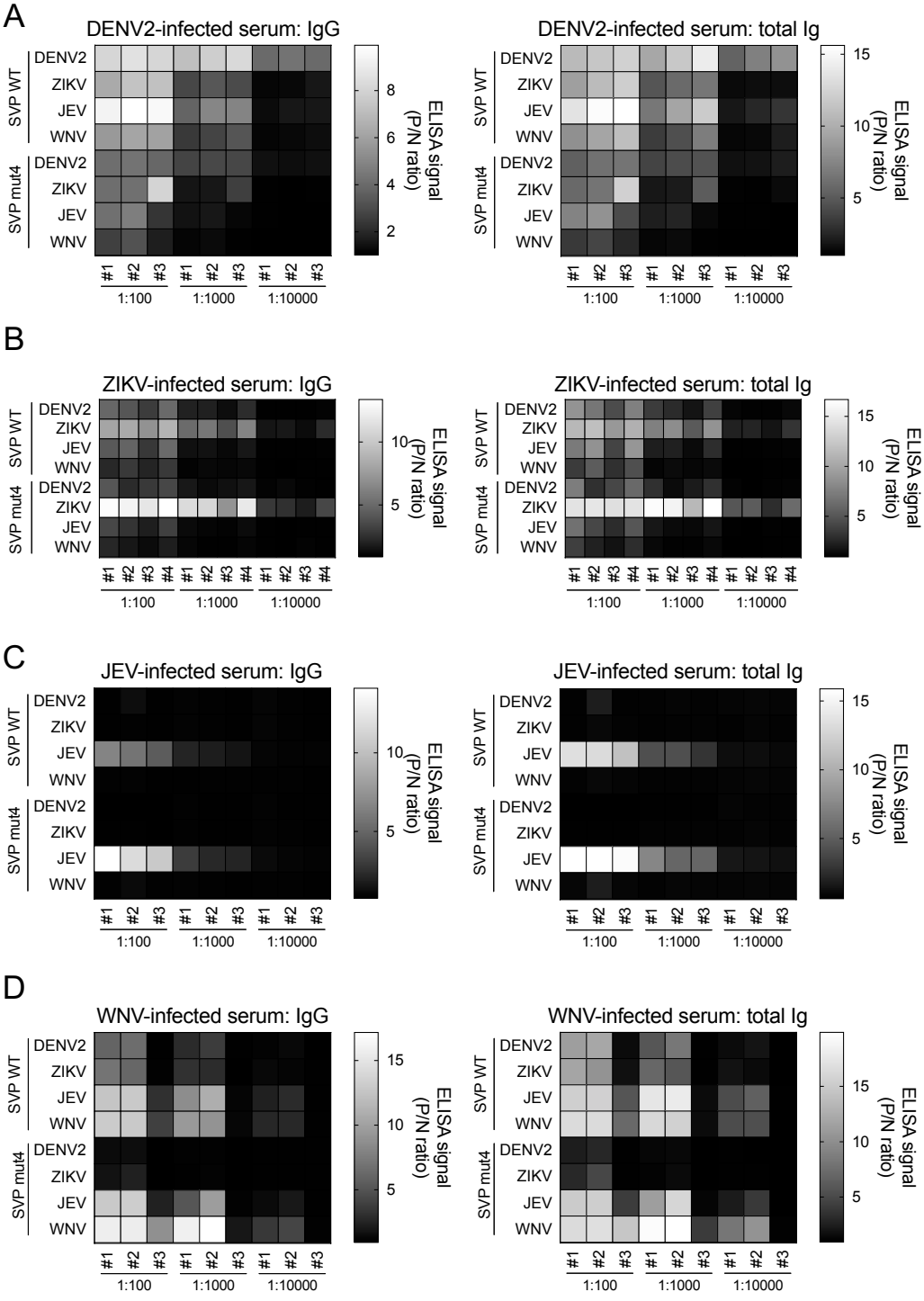


Fig. 11 Heatmap for cross-reactivities of IgG and total Ig in individual flavivirus-infected serum to each SVP WT and mut4 antigen.

(A to D) Heatmaps of IgG and total Ig from individual mouse serum infected with DENV2 (A), ZIKV (B), JEV (C), and WNV (D). #, each individual mouse.

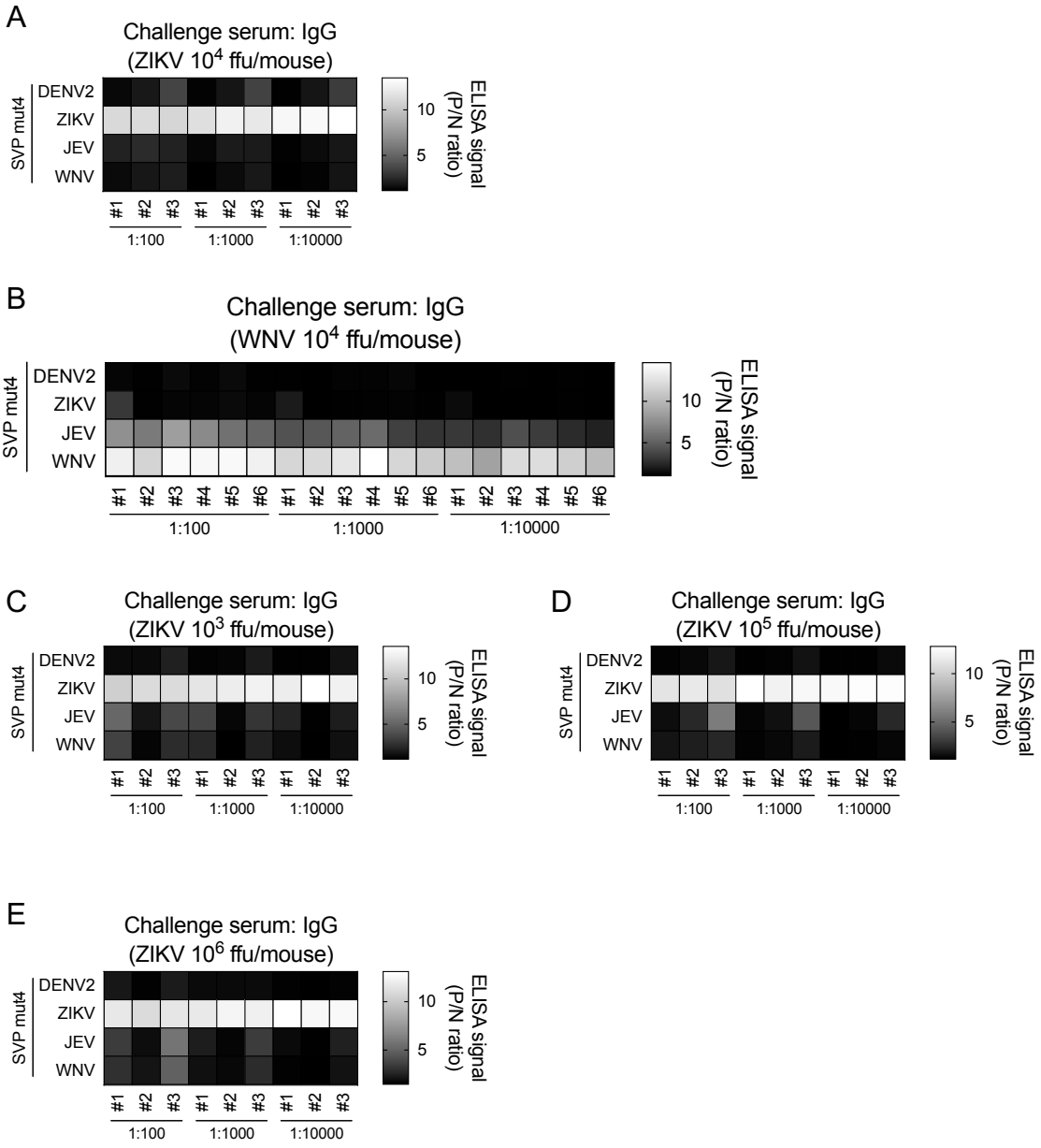


Fig. 12 Heatmap for cross-reactivity of IgG in serum from infected mouse with ZIKV (PRVABC59 strain) and WNV (Zmq16m11 strain) to each SVP mut4 antigen.

(A and B) Heatmaps of IgG from individual mouse serum infected with ZIKV (A) and WNV (B) at 10^4 ffu/mouse. (C to E) ZIKV-infected mouse serum was produced by inoculation with 10^3, 10^5, and 10^6 ffu/mouse, respectively. Heatmap for IgG from individual mouse serum infected with ZIKV at 10^3 (C), 10^5 (D), and 10^6 (E) ffu/mouse. #, each individual mouse.

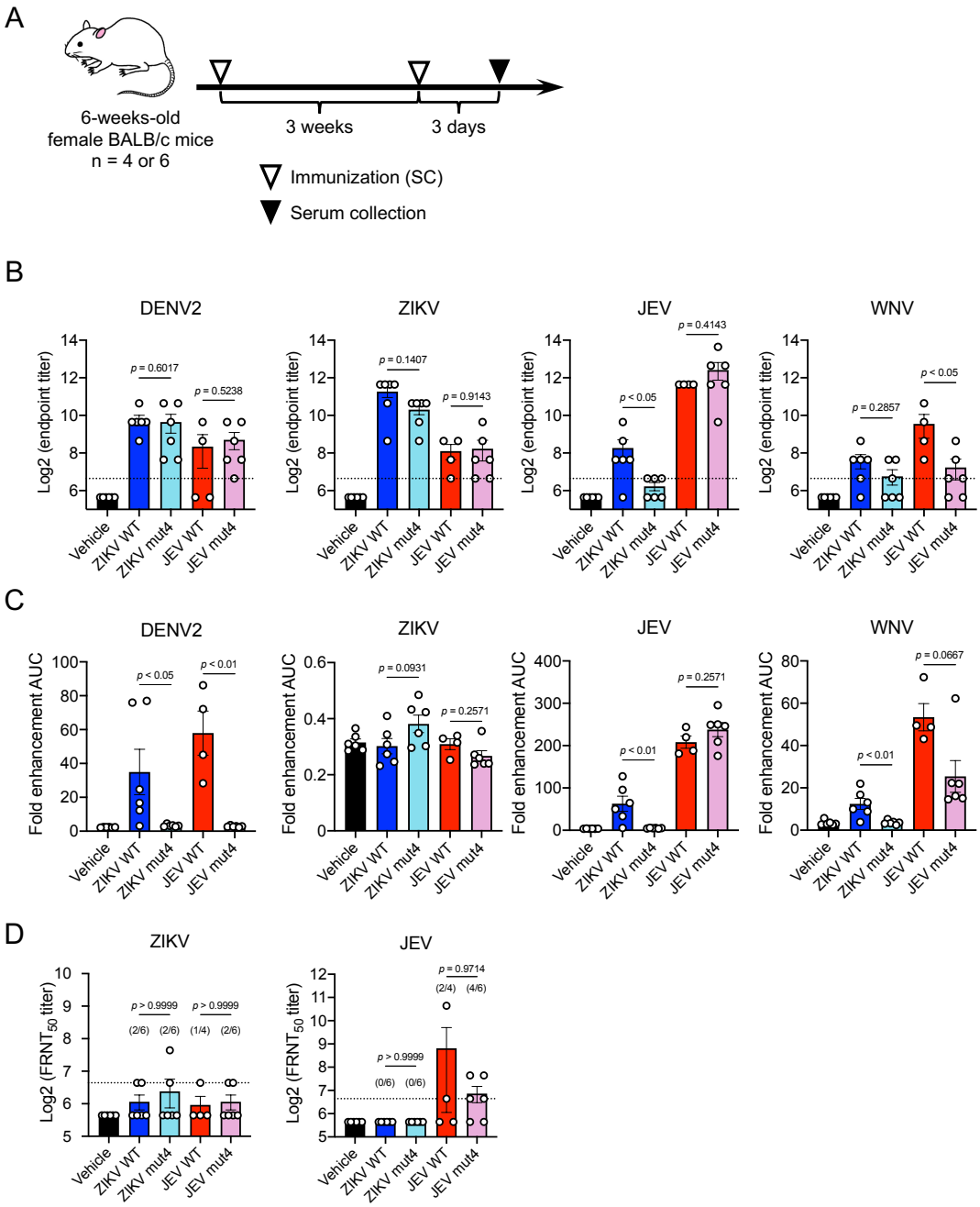


Fig. 13 Binding, ADE and neutralization activities of antibodies induced by immunization with SVP mut4.

(A) Schedule of immunization to mice. **(B)** Binding activities of each immunized sera were measured by ELISA using SVP WT antigen. **(C)** Each serum wase evaluated for their enhancement of MBFV infections in K562 cells by flow cytometry. **(D)** Neutralization activity of each serum was measured by $FRNT_{50}$. The values in the graphs are expressed as the mean ± sem of serum samples (n = 6; vehicle, ZIKV WT, ZIKV mut4, and JEV mut4, n = 4; JEV mut4). Dotted lines indicate detection limits. P values were analyzed with Mann-Whitney U test.

Discussion

Mutations of G106, L107E, and F108W in the FL domain of SVP inhibited binding of non-specific cross-reactive antibodies in sera infected with DENV2, ZIKV, JEV, and WNV. However, DENV2-infected sera were strongly cross-reactive to heterologous viral antigens at their high concentration (**Fig. 11A**). Previous study has shown that more than 80% of anti-DENV E Mabs isolated from DENV-infected patients cross-react with antigens of DENV group and JEV [47]. These results suggest that the prME protein of DENV2 may have more cross-reactive epitopes than those of other viruses. To inhibit bindings of non-specific cross-reactive antibodies to DENV2 SVP, further study is needed to find the other cross-reactive epitopes.

The use of SVP mut4 as the ELISA antigen diminished the signals of non-specific cross-reactive antibodies in ZIKV- and WNV-infected serum (**Fig. 12**). These results suggest that antibodies to the FL domain may be induced in murine serum at a high frequency by these viral infections. These antibodies induced by ZIKV or WNV infections could cause ADE. Previous studies have demonstrated that ZIKV- and WNV-infected sera have the potential to induce ADE of the other flavivirus infections in mouse, non-human primate and human [31, 32, 57, 91].

Furthermore, it was found that immunization with SVP mut4 induced neutralizing antibodies without ADE activity (**Fig. 13C and 13D**). Vaccination of adenovirus vectors carrying ZIKV prME with FL domain of lineage I ISFV has also been indicated to elicit neutralizing antibodies that do not cause ADE [86]. Hence, it is suggested that flavivirus structural proteins carrying the FL domain of the lineage I ISFV may be useful as vaccine antigens against several flavivirus infections. However, further studies are needed to understand the detailed mechanism of ADE, since there is a discrepancy between binding and ADE activities in the SVP mut4 immune sera (**Fig. 13B and 13C**). Previous study has shown that Mab reacting to flavivirus antigens regardless with or without neutralizing activity, exhibits ADE activity in an antibody concentration-dependent manner [92]. Immune sera contain polyclonal antibodies with or without neutralizing and ADE activities. Therefore, the combined action of these polyclonal antibodies may have resulted in a discrepancy between binding activity and ADE activity [93, 94]. Accordingly, it is speculated that further ADE verification under polyclonal conditions using multiple Mabs is needed.

Taken together, this study shows that MBFV SVPs with amino acid substitutions to

lineage I ISFV may have potential applications as serodiagnosis and vaccine antigens. However, this SVP mutant still contains cross-reactive epitopes. Therefore, the generation of non-cross-reactive antigens by further substitution of other possible cross-reactive epitopes using the lineage I ISFV is expected.

Summary

The FL domain in envelope protein is highly conserved among the diverse species of flavivirus. It has been difficult to establish accurate serodiagnostic method and safe vaccine because anti-FL antibodies, which have properties of highly cross-reactivity, poor neutralizing, and ADE activities to a wide range of flaviviruses, are induced by natural infection and vaccination. In this study, it was found that the FL domain of lineage I ISFV has different antigenicity from those of pathogenic MBFV and lineage II ISFV, and JEV-based SVPs with amino acid mutations (G106L, L107E, and F108W), which are originally contained in the FL domain of lineage I ISFV, avoid binding of anti-FL antibody. In addition to JEV, these mutations were also applied to SVPs of DENV2, ZIKV, and WNV. ELISA using SVP mutants allowed inhibition of non-specific cross-reactive antibodies and clearly detection of viral species-specific IgG and total Ig signals in ZIKV-, JEV-, and WNV-infected sera. When sera of mice infected with the different strain of ZIKV or WNV was applied to the ELISA, the virus-specific IgG signal was also observed. Furthermore, it was verified whether these SVP mutants could induce virus-specific antibodies without ADE activities. Immunization of ZIKV or JEV SVP mutant elicited antibody responses. These antibodies significantly suppressed ADE of heterologous viral infection compared the those of wild type SVP immunized mice and possessed neutralization activities against ZIKV or JEV. This study suggests that SVP mutants, substitution of G106L, L107E, and F108W, have potential to apply to ELISA-based serodiagnostic method and vaccine against flavivirus infections.

Conclusion

MBFVs have highly conserved amino acid sequences of structural proteins among different virus species. Therefore, antibodies to these structural proteins are cross-reactive. Because the cross-reactive antibodies bind to a variety of flavivirus structural proteins, serodiagnostic methods capable of detecting viral species-specific antibodies have not yet been developed. In this study, the antigenicity of MBFV and ISFV was evaluated, and viral antigens were designed to reduce nonspecific binding of cross-reactive antibodies using amino acid sequences of viruses with different antigenic properties.

In chapter I, reaction analysis with immune serum against lineage IIa ISFV, PSFV, and lineage IIb ISFV, BJV, was conducted to evaluate the antigenic similarity among lineage IIa and IIb ISFVs, and MBFV. Binding and ADE assays demonstrated that anti-PSFV sera had higher cross-reactivity with MBFV antigens than anti-BJV sera, while their neutralization activities against MBFV were not observed. These results show that lineage IIa ISFV, PSFV, has more similar structural proteins to MBFVs than lineage IIb ISFV, BJV. This suggests that when the ISFV migrates to humans and other vertebrates *via* of the virus-infected mosquito bites, memory B cells induced by past MBFV infections may be reactivated. However, it is undetermined whether these ISFVs infect mosquitoes and are secreted into their saliva. Therefore, further experiments using alive mosquitoes are needed.

In chapter II, MBFV SVPs (SVP mut4) with mutations in FL domain of lineage I ISFV were generated and applied to ELISA antigens. The SVP mut4 used as ELISA antigen suppressed non-specific cross-reactive binding of infected sera with MBFVs, especially ZIKV and WNV compared to SVP WT. Moreover, the SVP mut4 also decreased the non-specific cross-reactive binding of and clearly detected the signal of infected sera with different strain of ZIKV and WNV. These results suggest that the ELISA using SVP mut4 antigen becomes one of the candidates for serological test to diagnose flavivirus infection. SVP mut4-immunized sera also showed low cross-reactivity and comparatively specific binding to the antigen same as immunogen than SVP WT-immunized sera. These sera induced by immunization of the SVP mut4 suppressed significantly or more strongly ADE activities against heterologous viral infections than those of the SVP WT. A part of SVP mut4- or SVP WT-immunized sera neutralized infections of ZIKV or both of ZIKV and JEV

infections. These findings suggest that SVP mut4 could be a vaccine antigen which does not induce ADE activity.

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References

1. Guarner J, Hale GL (2019) Four human diseases with significant public health impact caused by mosquito-borne flaviviruses: West Nile, Zika, dengue and yellow fever. Semin Diagn Pathol 36:170-176
2. Pierson TC, Diamond MS (2020) The continued threat of emerging flaviviruses. Nat Microbiol 5:796-812
3. Krol E, Brzuska G, Szewczyk B (2019) Production and Biomedical Application of Flavivirus-like Particles. Trends Biotechnol 37:1202-1216
4. Neufeldt CJ, Cortese M, Acosta EG, Bartenschlager R (2018) Rewiring cellular networks by members of the Flaviviridae family. Nat Rev Microbiol 16:125-142
5. Daep CA, Munoz-Jordan JL, Eugenin EA (2014) Flaviviruses, an expanding threat in public health: focus on dengue, West Nile, and Japanese encephalitis virus. J Neurovirol 20:539-560
6. Hombach J, Barrett AD, Cardosa MJ, Deubel V, Guzman M, Kurane I, Roehrig JT, Sabchareon A, Kieny MP (2005) Review on flavivirus vaccine development. Proceedings of a meeting jointly organised by the World Health Organization and the Thai Ministry of Public Health, 26-27 April 2004, Bangkok, Thailand. Vaccine 23:2689-2695
7. Andersen LK, Davis MD (2017) Climate change and the epidemiology of selected tick-borne and mosquito-borne diseases: update from the International Society of Dermatology Climate Change Task Force. Int J Dermatol 56:252-259
8. Gould EA, Higgs S (2009) Impact of climate change and other factors on emerging arbovirus diseases. Trans R Soc Trop Med Hyg 103:109-121
9. Harrigan RJ, Thomassen HA, Buermann W, Smith TB (2014) A continental risk assessment of West Nile virus under climate change. Glob Chang Biol 20:2417-2425
10. Munoz AG, Thomson MC, Goddard L, Aldighieri S (2016) Analyzing climate variations at multiple timescales can guide Zika virus response measures. Gigascience 5:1-6
11. Zell R, Krumbholz A, Wutzler P (2008) Impact of global warming on viral diseases: what is the evidence? Curr Opin Biotechnol 19:652-660
12. Paz S (2015) Climate change impacts on West Nile virus transmission in a global context. Philos Trans R Soc Lond B Biol Sci 370
13. Semenza JC, Rocklov J, Ebi KL (2022) Climate Change and Cascading Risks from Infectious Disease. Infect Dis Ther 11:1371-1390
14. Walther GR, Post E, Convey P, Menzel A, Parmesan C, Beebee TJ, Fromentin JM, Hoegh-Guldberg O, Bairlein F (2002) Ecological responses to recent climate change. Nature 416:389-395
15. Waller C, Tiemensma M, Currie BJ, Williams DT, Baird RW, Krause VL (2022) Japanese Encephalitis in Australia - A Sentinel Case. N Engl J Med 387:661-662
16. Yakob L, Hu W, Frentiu FD, Gyawali N, Hugo LE, Johnson B, Lau C, Furuya-Kanamori L, Magalhaes RS, Devine G (2022) Japanese encephalitis emergence in Australia: the potential

population at risk. Clin Infect Dis
17. Marchand E, Prat C, Jeannin C, Lafont E, Bergmann T, Flusin O, Rizzi J, Roux N, Busso V, Deniau J, Noel H, Vaillant V, Leparc-Goffart I, Six C, Paty MC (2013) Autochthonous case of dengue in France, October 2013. Euro Surveill 18:20661
18. Kobayashi D, Murota K, Fujita R, Itokawa K, Kotaki A, Moi ML, Ejiri H, Maekawa Y, Ogawa K, Tsuda Y, Sasaki T, Kobayashi M, Takasaki T, Isawa H, Sawabe K (2018) Dengue Virus Infection in Aedes albopictus during the 2014 Autochthonous Dengue Outbreak in Tokyo Metropolis, Japan. Am J Trop Med Hyg 98:1460-1468
19. Misra UK, Kalita J (2010) Overview: Japanese encephalitis. Prog Neurobiol 91:108-120
20. Sharma S, Mathur A, Prakash V, Kulshreshtha R, Kumar R, Chaturvedi UC (1991) Japanese encephalitis virus latency in peripheral blood lymphocytes and recurrence of infection in children. Clin Exp Immunol 85:85-89
21. Chancey C, Grinev A, Volkova E, Rios M (2015) The global ecology and epidemiology of West Nile virus. Biomed Res Int 2015:376230
22. Hayes EB, O'Leary DR (2004) West Nile virus infection: a pediatric perspective. Pediatrics 113:1375-1381
23. Slavov SN, Otaguiri KK, Kashima S, Covas DT (2016) Overview of Zika virus (ZIKV) infection in regards to the Brazilian epidemic. Braz J Med Biol Res 49:e5420
24. Brasil P, Pereira JP, Jr., Moreira ME, Ribeiro Nogueira RM, Damasceno L, Wakimoto M, Rabello RS, Valderramos SG, Halai UA, Salles TS, Zin AA, Horovitz D, Daltro P, Boechat M, Raja Gabaglia C, Carvalho de Sequeira P, Pilotto JH, Medialdea-Carrera R, Cotrim da Cunha D, Abreu de Carvalho LM, Pone M, Machado Siqueira A, Calvet GA, Rodrigues Baiao AE, Neves ES, Nassar de Carvalho PR, Hasue RH, Marschik PB, Einspieler C, Janzen C, Cherry JD, Bispo de Filippis AM, Nielsen-Saines K (2016) Zika Virus Infection in Pregnant Women in Rio de Janeiro. N Engl J Med 375:2321-2334
25. Salvador FS, Fujita DM (2016) Entry routes for Zika virus in Brazil after 2014 world cup: New possibilities. Travel Med Infect Dis 14:49-51
26. Vasilakis N, Cardosa J, Hanley KA, Holmes EC, Weaver SC (2011) Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health. Nat Rev Microbiol 9:532-541
27. Whitehead SS, Blaney JE, Durbin AP, Murphy BR (2007) Prospects for a dengue virus vaccine. Nat Rev Microbiol 5:518-528
28. Halstead SB (1988) Pathogenesis of dengue: challenges to molecular biology. Science 239:476-481
29. Halstead SB (2003) Neutralization and antibody-dependent enhancement of dengue viruses. Adv Virus Res 60:421-467
30. Anderson KB, Gibbons RV, Thomas SJ, Rothman AL, Nisalak A, Berkelman RL, Libraty DH, Endy TP (2011) Preexisting Japanese encephalitis virus neutralizing antibodies and increased

symptomatic dengue illness in a school-based cohort in Thailand. PLoS Negl Trop Dis 5:e1311

31. Katzelnick LC, Gresh L, Halloran ME, Mercado JC, Kuan G, Gordon A, Balmaseda A, Harris E (2017) Antibody-dependent enhancement of severe dengue disease in humans. Science 358:929-932
32. Katzelnick LC, Narvaez C, Arguello S, Lopez Mercado B, Collado D, Ampie O, Elizondo D, Miranda T, Bustos Carillo F, Mercado JC, Latta K, Schiller A, Segovia-Chumbez B, Ojeda S, Sanchez N, Plazaola M, Coloma J, Halloran ME, Premkumar L, Gordon A, Narvaez F, de Silva AM, Kuan G, Balmaseda A, Harris E (2020) Zika virus infection enhances future risk of severe dengue disease. Science 369:1123-1128
33. Barrows NJ, Campos RK, Powell ST, Prasanth KR, Schott-Lerner G, Soto-Acosta R, Galarza-Munoz G, McGrath EL, Urrabaz-Garza R, Gao J, Wu P, Menon R, Saade G, Fernandez-Salas I, Rossi SL, Vasilakis N, Routh A, Bradrick SS, Garcia-Blanco MA (2016) A Screen of FDA-Approved Drugs for Inhibitors of Zika Virus Infection. Cell Host Microbe 20:259-270
34. Boldescu V, Behnam MAM, Vasilakis N, Klein CD (2017) Broad-spectrum agents for flaviviral infections: dengue, Zika and beyond. Nat Rev Drug Discov 16:565-586
35. Kok WM (2016) New developments in flavivirus drug discovery. Expert Opin Drug Discov 11:433-445
36. Stiasny K, Aberle JH, Chmelik V, Karrer U, Holzmann H, Heinz FX (2012) Quantitative determination of IgM antibodies reduces the pitfalls in the serodiagnosis of tick-borne encephalitis. J Clin Virol 54:115-120
37. Kauffman EB, Kramer LD (2017) Zika Virus Mosquito Vectors: Competence, Biology, and Vector Control. J Infect Dis 216:S976-S990
38. Harris AF, McKemey AR, Nimmo D, Curtis Z, Black I, Morgan SA, Oviedo MN, Lacroix R, Naish N, Morrison NI, Collado A, Stevenson J, Scaife S, Dafa'alla T, Fu G, Phillips C, Miles A, Raduan N, Kelly N, Beech C, Donnelly CA, Petrie WD, Alphey L (2012) Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nat Biotechnol 30:828-830
39. Gabrieli P, Smidler A, Catteruccia F (2014) Engineering the control of mosquito-borne infectious diseases. Genome Biol 15:535
40. Harris AF, Nimmo D, McKemey AR, Kelly N, Scaife S, Donnelly CA, Beech C, Petrie WD, Alphey L (2011) Field performance of engineered male mosquitoes. Nat Biotechnol 29:1034-1037
41. Sabet A, Goddard J (2022) Promise or Peril: Using Genetically Modified Mosquitoes in the Fight Against Vector-Borne Disease. Am J Med 135:281-283
42. Maezono K, Kobayashi S, Tabata K, Yoshii K, Kariwa H (2021) Development of a highly specific serodiagnostic ELISA for West Nile virus infection using subviral particles. Sci Rep 11:9213

43. Inagaki E, Sakai M, Hirano M, Muto M, Kobayashi S, Kariwa H, Yoshii K (2016) Development of a serodiagnostic multi-species ELISA against tick-borne encephalitis virus using subviral particles. Ticks Tick Borne Dis 7:723-729
44. Martin DA, Muth DA, Brown T, Johnson AJ, Karabatsos N, Roehrig JT (2000) Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays for routine diagnosis of arboviral infections. J Clin Microbiol 38:1823-1826
45. Kuno G (2003) Serodiagnosis of flaviviral infections and vaccinations in humans. Adv Virus Res 61:3-65
46. Crill WD, Chang GJ (2004) Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. J Virol 78:13975-13986
47. Dejnirattisai W, Jumnainsong A, Onsirisakul N, Fitton P, Vasanawathana S, Limpitikul W, Puttikhunt C, Edwards C, Duangchinda T, Supasa S, Chawansuntati K, Malasit P, Mongkolsapaya J, Screaton G (2010) Cross-reacting antibodies enhance dengue virus infection in humans. Science 328:745-748
48. Rathore APS, St John AL (2020) Cross-Reactive Immunity Among Flaviviruses. Front Immunol 11:334
49. Tabata K, Itakura Y, Toba S, Uemura K, Kishimoto M, Sasaki M, Harrison JJ, Sato A, Hall WW, Hall RA, Sawa H, Orba Y (2022) Serological characterization of lineage II insect-specific flaviviruses compared with pathogenic mosquito-borne flaviviruses. Biochem Biophys Res Commun 616:115-121
50. Auguste AJ, Langsjoen RM, Porier DL, Erasmus JH, Bergren NA, Bolling BG, Luo H, Singh A, Guzman H, Popov VL, Travassos da Rosa APA, Wang T, Kang L, Allen IC, Carrington CVF, Tesh RB, Weaver SC (2021) Isolation of a novel insect-specific flavivirus with immunomodulatory effects in vertebrate systems. Virology 562:50-62
51. Guzman H, Contreras-Gutierrez MA, Travassos da Rosa APA, Nunes MRT, Cardoso JF, Popov VL, Young KI, Savit C, Wood TG, Widen SG, Watts DM, Hanley KA, Perera D, Fish D, Vasilakis N, Tesh RB (2018) Characterization of Three New Insect-Specific Flaviviruses: Their Relationship to the Mosquito-Borne Flavivirus Pathogens. Am J Trop Med Hyg 98:410-419
52. Harrison JJ, Hobson-Peters J, Colmant AMG, Koh J, Newton ND, Warrilow D, Bielefeldt-Ohmann H, Piyasena TBH, O'Brien CA, Vet LJ, Paramitha D, Potter JR, Davis SS, Johansen CA, Setoh YX, Khromykh AA, Hall RA (2020) Antigenic Characterization of New Lineage II Insect-Specific Flaviviruses in Australian Mosquitoes and Identification of Host Restriction Factors. mSphere 5
53. Hobson-Peters J, Harrison JJ, Watterson D, Hazlewood JE, Vet LJ, Newton ND, Warrilow D, Colmant AMG, Taylor C, Huang B, Piyasena TBH, Chow WK, Setoh YX, Tang B, Nakayama E, Yan K, Amarilla AA, Wheatley S, Moore PR, Finger M, Kurucz N, Modhiran N, Young PR, Khromykh AA, Bielefeldt-Ohmann H, Suhrbier A, Hall RA (2019) A recombinant platform

for flavivirus vaccines and diagnostics using chimeras of a new insect-specific virus. Sci Transl Med 11

54. Wastika CE, Harima H, Sasaki M, Hang'ombe BM, Eshita Y, Qiu Y, Hall WW, Wolfinger MT, Sawa H, Orba Y (2020) Discoveries of Exoribonuclease-Resistant Structures of Insect-Specific Flaviviruses Isolated in Zambia. Viruses 12
55. Piyasena TBH, Newton ND, Hobson-Peters J, Vet LJ, Setoh YX, Bielefeldt-Ohmann H, Khromykh AA, Hall RA (2019) Chimeric viruses of the insect-specific flavivirus Palm Creek with structural proteins of vertebrate-infecting flaviviruses identify barriers to replication of insect-specific flaviviruses in vertebrate cells. J Gen Virol 100:1580-1586
56. Colmant AMG, Hobson-Peters J, Slijkerman TAP, Harrison JJ, Pijlman GP, van Oers MM, Simmonds P, Hall RA, Fros JJ (2021) Insect-Specific Flavivirus Replication in Mammalian Cells Is Inhibited by Physiological Temperature and the Zinc-Finger Antiviral Protein. Viruses 13
57. Bardina SV, Bunduc P, Tripathi S, Duehr J, Frere JJ, Brown JA, Nachbagauer R, Foster GA, Krysztof D, Tortorella D, Stramer SL, Garcia-Sastre A, Krammer F, Lim JK (2017) Enhancement of Zika virus pathogenesis by preexisting antiflavivirus immunity. Science 356:175-180
58. Garg H, Yeh R, Watts DM, Mehmetoglu-Gurbuz T, Resendes R, Parsons B, Gonzales F, Joshi A (2021) Enhancement of Zika virus infection by antibodies from West Nile virus seropositive individuals with no history of clinical infection. BMC Immunol 22:5
59. Saito Y, Moi ML, Takeshita N, Lim CK, Shiba H, Hosono K, Saijo M, Kurane I, Takasaki T (2016) Japanese encephalitis vaccine-facilitated dengue virus infection-enhancement antibody in adults. BMC Infect Dis 16:578
60. Orba Y, Matsuno K, Nakao R, Kryukov K, Saito Y, Kawamori F, Loza Vega A, Watanabe T, Maemura T, Sasaki M, Hall WW, Hall RA, Pereira JA, Nakagawa S, Sawa H (2021) Diverse mosquito-specific flaviviruses in the Bolivian Amazon basin. J Gen Virol 102
61. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 35:1547-1549
62. Letunic I, Bork P (2021) Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res 49:W293-W296
63. Clark DC, Lobigs M, Lee E, Howard MJ, Clark K, Blitvich BJ, Hall RA (2007) In situ reactions of monoclonal antibodies with a viable mutant of Murray Valley encephalitis virus reveal an absence of dimeric NS1 protein. J Gen Virol 88:1175-1183
64. Sasaki M, Anindita PD, Phongphaew W, Carr M, Kobayashi S, Orba Y, Sawa H (2018) Development of a rapid and quantitative method for the analysis of viral entry and release using a NanoLuc luciferase complementation assay. Virus Res 243:69-74
65. Slon Campos JL, Poggianella M, Marchese S, Mossenta M, Rana J, Arnoldi F, Bestagno M, Burrone OR (2017) DNA-immunisation with dengue virus E protein domains I/II, but not

domain III, enhances Zika, West Nile and Yellow Fever virus infection. PLoS One 12:e0181734

66. Blitvich BJ, Firth AE (2015) Insect-specific flaviviruses: a systematic review of their discovery, host range, mode of transmission, superinfection exclusion potential and genomic organization. Viruses 7:1927-1959
67. Berneck BS, Rockstroh A, Fertey J, Grunwald T, Ulbert S (2020) A Recombinant Zika Virus Envelope Protein with Mutations in the Conserved Fusion Loop Leads to Reduced Antibody Cross-Reactivity upon Vaccination. Vaccines (Basel) 8
68. Priyamvada L, Quicke KM, Hudson WH, Onlamoon N, Sewatanon J, Edupuganti S, Pattanapanyasat K, Chokephaibulkit K, Mulligan MJ, Wilson PC, Ahmed R, Suthar MS, Wrammert J (2016) Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus. Proc Natl Acad Sci U S A 113:7852-7857
69. Goncalvez AP, Engle RE, St Claire M, Purcell RH, Lai CJ (2007) Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. Proc Natl Acad Sci U S A 104:9422-9427
70. Mansfield KL, Horton DL, Johnson N, Li L, Barrett ADT, Smith DJ, Galbraith SE, Solomon T, Fooks AR (2011) Flavivirus-induced antibody cross-reactivity. J Gen Virol 92:2821-2829
71. Allwinn R, Doerr HW, Emmerich P, Schmitz H, Preiser W (2002) Cross-reactivity in flavivirus serology: new implications of an old finding? Med Microbiol Immunol 190:199-202
72. Chiou SS, Crill WD, Chen LK, Chang GJ (2008) Enzyme-linked immunosorbent assays using novel Japanese encephalitis virus antigen improve the accuracy of clinical diagnosis of flavivirus infections. Clin Vaccine Immunol 15:825-835
73. Rey FA, Stiasny K, Vaney MC, Dellarole M, Heinz FX (2018) The bright and the dark side of human antibody responses to flaviviruses: lessons for vaccine design. EMBO Rep 19:206-224
74. Huang KJ, Yang YC, Lin YS, Huang JH, Liu HS, Yeh TM, Chen SH, Liu CC, Lei HY (2006) The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. J Immunol 176:2825-2832
75. de Alwis R, Williams KL, Schmid MA, Lai CY, Patel B, Smith SA, Crowe JE, Wang WK, Harris E, de Silva AM (2014) Dengue viruses are enhanced by distinct populations of serotype cross-reactive antibodies in human immune sera. PLoS Pathog 10:e1004386
76. Gunawardana SA, Shaw RH (2018) Cross-reactive dengue virus-derived monoclonal antibodies to Zika virus envelope protein: Panacea or Pandora's box? BMC Infect Dis 18:641
77. Vogt MR, Dowd KA, Engle M, Tesh RB, Johnson S, Pierson TC, Diamond MS (2011) Poorly neutralizing cross-reactive antibodies against the fusion loop of West Nile virus envelope protein protect in vivo via Fcgamma receptor and complement-dependent effector mechanisms. J Virol 85:11567-11580
78. Roehrig JT, Bolin RA, Kelly RG (1998) Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. Virology 246:317-328

79. Stiasny K, Kiermayr S, Holzmann H, Heinz FX (2006) Cryptic properties of a cluster of dominant flavivirus cross-reactive antigenic sites. J Virol 80:9557-9568
80. Sukupolvi-Petty S, Austin SK, Purtha WE, Oliphant T, Nybakken GE, Schlesinger JJ, Roehrig JT, Gromowski GD, Barrett AD, Fremont DH, Diamond MS (2007) Type- and subcomplex-specific neutralizing antibodies against domain III of dengue virus type 2 envelope protein recognize adjacent epitopes. J Virol 81:12816-12826
81. Rockstroh A, Moges B, Barzon L, Sinigaglia A, Palu G, Kumbukgolla W, Schmidt-Chanasit J, Sarno M, Brites C, Moreira-Soto A, Drexler JF, Ferreira OC, Ulbert S (2017) Specific detection of dengue and Zika virus antibodies using envelope proteins with mutations in the conserved fusion loop. Emerg Microbes Infect 6:e99
82. Kam YW, Leite JA, Amrun SN, Lum FM, Yee WX, Bakar FA, Eng KE, Lye DC, Leo YS, Chong CY, Freitas ARR, Milanez GP, Proenca-Modena JL, Renia L, Costa FTM, Ng LFP, Zika-Unicamp N (2019) ZIKV-Specific NS1 Epitopes as Serological Markers of Acute Zika Virus Infection. J Infect Dis 220:203-212
83. Wan J, Wang T, Xu J, Ouyang T, Wang Q, Zhang Y, Weng S, Li Y, Wang Y, Xin X, Wang X, Li S, Kong L (2021) Novel Japanese encephalitis virus NS1-based vaccine: Truncated NS1 fused with E. coli heat labile enterotoxin B subunit. EBioMedicine 67:103353
84. Dejnirattisai W, Wongwiwat W, Supasa S, Zhang X, Dai X, Rouvinski A, Jumnainsong A, Edwards C, Quyen NTH, Duangchinda T, Grimes JM, Tsai WY, Lai CY, Wang WK, Malasit P, Farrar J, Simmons CP, Zhou ZH, Rey FA, Mongkolsapaya J, Screaton GR (2015) A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. Nat Immunol 16:170-177
85. Tsai WY, Lai CY, Wu YC, Lin HE, Edwards C, Jumnainsong A, Kliks S, Halstead S, Mongkolsapaya J, Screaton GR, Wang WK (2013) High-avidity and potently neutralizing cross-reactive human monoclonal antibodies derived from secondary dengue virus infection. J Virol 87:12562-12575
86. Dai L, Xu K, Li J, Huang Q, Song J, Han Y, Zheng T, Gao P, Lu X, Yang H, Liu K, Xia Q, Wang Q, Chai Y, Qi J, Yan J, Gao GF (2021) Protective Zika vaccines engineered to eliminate enhancement of dengue infection via immunodominance switch. Nat Immunol 22:958-968
87. Chabierski S, Barzon L, Papa A, Niedrig M, Bramson JL, Richner JM, Palu G, Diamond MS, Ulbert S (2014) Distinguishing West Nile virus infection using a recombinant envelope protein with mutations in the conserved fusion-loop. BMC Infect Dis 14:246
88. Urakami A, Ngwe Tun MM, Moi ML, Sakurai A, Ishikawa M, Kuno S, Ueno R, Morita K, Akahata W (2017) An Envelope-Modified Tetravalent Dengue Virus-Like-Particle Vaccine Has Implications for Flavivirus Vaccine Design. J Virol 91
89. Slon-Campos JL, Dejnirattisai W, Jagger BW, Lopez-Camacho C, Wongwiwat W, Durnell LA, Winkler ES, Chen RE, Reyes-Sandoval A, Rey FA, Diamond MS, Mongkolsapaya J, Screaton GR (2019) A protective Zika virus E-dimer-based subunit vaccine engineered to abrogate

antibody-dependent enhancement of dengue infection. Nat Immunol 20:1291-1298

90. Henchal EA, Gentry MK, McCown JM, Brandt WE (1982) Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am J Trop Med Hyg 31:830-836

91. Pantoja P, Perez-Guzman EX, Rodriguez IV, White LJ, Gonzalez O, Serrano C, Giavedoni L, Hodara V, Cruz L, Arana T, Martinez MI, Hassert MA, Brien JD, Pinto AK, de Silva A, Sariol CA (2017) Zika virus pathogenesis in rhesus macaques is unaffected by pre-existing immunity to dengue virus. Nat Commun 8:15674

92. Willis E, Hensley SE (2017) Characterization of Zika virus binding and enhancement potential of a large panel of flavivirus murine monoclonal antibodies. Virology 508:1-6

93. Okuya K, Hattori T, Saito T, Takadate Y, Sasaki M, Furuyama W, Marzi A, Ohiro Y, Konno S, Hattori T, Takada A (2022) Multiple Routes of Antibody-Dependent Enhancement of SARS-CoV-2 Infection. Microbiol Spectr 10:e0155321

94. Takada A, Ebihara H, Feldmann H, Geisbert TW, Kawaoka Y (2007) Epitopes required for antibody-dependent enhancement of Ebola virus infection. J Infect Dis 196 Suppl 2:S347-356

Summary in Japanese

病原性蚊媒介性フラビウイルス（MBFV）は、異なるウイルス種間で構造タンパク質のアミノ酸配列が高度に保存されている。そのため、これらの構造タンパク質を認識する抗体は、交差反応性を示す。その交差反応性抗体は、幅広いフラビウイルスの構造タンパク質に結合するため、ウイルス種特異的な抗体を検出可能な血清診断法は未だ開発されていない。本研究では、これまで報告されてきた MBFV 間ではなく、MBFV と昆虫特異的フラビウイルス（ISFV）間の抗原性を比較し、異なる抗原性を有するウイルスアミノ酸配列を用いて、交差反応性抗体の非特異的な結合を低減させるウイルス抗原作出を試みた。

第一章　MBFV と比較した lineage II ISFV の血清学的性状解析

第一章では、分子系統学的に MBFV に近縁な lineage II ISFV の構造タンパク質の抗原性を MBFV の構造タンパク質と比較した。まず、系統樹解析を実施した結果、lineage II ISFV は lineage IIa ISFV と lineage IIb ISFV にクラスターが別れることが明らかになった。そこで、これら二種類の lineage II ISFV の抗原性をそれぞれのウイルスに対する抗血清を用いて評価した。これまでに所属研究室で分離してきた lineage IIa ISFV である PSFV と lineage IIb ISFV である BJV をマウスに免疫することにより、抗血清を作出した。それらの抗血清は一部の MBFV（DENV:デングウイルス、ZIKV:ジカウイルス、JEV:日本脳炎ウイルスおよび WNV:ウエストナイルウイルス）の感染細胞抗原に対し結合活性を示し、更に、それらの抗体は、フラビウイルス感染症で問題となっている抗体依存性感染増強（ADE）活性をそれぞれの MBFV に対して有していることを示した。また、PSFV 及び BJV に対する抗血清間で、MBFV の ADE 活性が異なることも明らかにした。本結果から、lineage IIa ISFV と lineage IIb ISFV では、抗原性が異なることが示唆され、lineage IIa ISFV である PSFV と lineage IIb ISFV である BJV が MBFV（DENV、ZIKV、JEV 及び WNV）と類似した抗原性の構造タンパク質を有していることを示した。本結果により、MBFV 感染歴のある患者が、ISFV 感染蚊に吸血されることにより、MBFV に対する抗体が再活性化され、更にその抗体が ADE を誘導する可能性が示唆された。しかしながら、ISFV が感染した蚊の吸血により吸血対象へウイルスが導入されるかについては不明であるため今後、更なる研究が必要である。

第二章　フラビウイルス種特異的な抗体の検出及び誘導を可能にするウイルスタンパク質デザイン

第二章では、MBFV、lineage II ISFV 及び lineage I ISFV の fusion loop（FL）ドメインの抗原性を利用した交差反応を低減させるウイルスタンパク質の創出を試みた。FL ドメインは、異なるフラビウイルス種間で高度に保存されており、抗 FL 抗体は幅広いフラビウイルスで交差反応することが報告されている。本研究では、lineage I ISFV の FL ドメインは MBFV や lineage II ISFV と異なる抗原性を有することを明らかにした。更に、lineage I ISFV の FL ドメインの一部を搭載した MBFV（DENV、ZIKV、JEV 及び WNV）の変異型ウイルス様粒子（SVP）を作出し、その変異型 SVP へのフラビウイルス感染血清の交差反応性と、その免疫により誘導される抗体の交差反応性を評価した。その結果、野生型 SVP と比較して、変異型 SVP ではフラビウイルス感染血清（同ウイルス種・株）の交差反応性を低減させ、ウイルス種特異的な結合シグナルを検出できた。また、チャレンジフラビウイルス（同ウイルス種・異なるウイルス株）の感染血清においても、強いウイルス種特異的な結合シグナルを検出することができた。さらに、変異型 SVP を免疫した血清において、交差反応性が低下し、ADE 活性が抑制された抗体が誘導され、それらの抗体は中和活性を有することを示した。本成果は、変異型 SVP を ELISA 抗原として用いることにより、交差反応性抗体の非特異的な結合を減少させ、ウイルス特異的な抗体が検出できる血清診断法への応用可能性を示した。更に、変異型 SVP 免疫血清では、野生型 SVP と比べ劇的に ADE 活性が抑制され、一部に中和活性が認められた。以上の結果から、変異型 SVP は新規フラビウイルスワクチン抗原の候補になり得ることが期待される。しかしながら、交差反応エピトープは FL ドメイン以外にも存在する。そのため、今後、更なる交差エピトープを抗原性が異なる lineage I ISFV に置換することにより、血清診断法及びワクチン開発に応用可能なウイルス抗原の作出を目指す。