Journal of Immunological Methods 512 (2023) 113396

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

A peptide derived from the N-terminal of NS2A for the preparation of ZIKV NS2A recognition polyclonal antibody

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ARTICLE INFO

Keywords:
ZIKV
NS2A
Peptide
Antibody

ABSTRACT

Zika virus non-structural protein NS2A participates in viral replication, organization, and budding, as well as escaping host immunity. NS2A also involved in the induction of microcephaly by ZIKV. However, the above studies were mainly performed through NS2A with a tag due to the lack of available antibodies against NS2A. ZIKV NS2A is a multiplex transmembrane protein, which leads to difficulties in the preparation of its recognition antibodies, thus seriously affecting the study of ZIKV NS2A. In this study, we found that a peptide (GSTDHMDHFSLGVLC) derived from the N-terminal of ZIKV NS2A coupled to KLH induced antibodies recognizing ZIKV NS2A in rabbits. The purified polyclonal antibody recognized ZIKV NS2A in ZIKV-infected cells with high efficiency and specificity, as detected by western blot and immunofluorescence assay. Our study has important implications for the preparation of ZIKV NS2A antibodies and the in-depth study of ZIKV NS2A.

1. Introduction

Zika virus (ZIKV) is a mosquito-borne, enveloped, single-stranded RNA virus, which belongs to the genus *Flavivirus* of the family *Flaviviridae* (Miner and Diamond, 2017). ZIKV genome consists of one open reading frame, approximately 10.8 kb in length, encoding three structural proteins (capsid protein, C; membrane protein and its precursor membrane, M/prM; envelope protein, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Faria et al., 2016; Shi and Gao, 2017). NS1 is the only protein secreted by ZIKV and can be used as a universal vaccine design target for flaviviruses (Modhiran et al., 2021). NS3 has activity of protease and RNA helicase (Xu et al., 2019). NS5 works as ZIKV RNA-dependent RNA polymerase (Tan et al., 2019). NS1, NS3 and NS5 have received a lot of attention with large number of publications. However, ZIKV NS proteins with small molecular weight are poorly studied, especially NS2A.

In fact, NS2A has an important role in ZIKV pathogenesis. NS2A is involved in ZIKV replication, assembly, budding, and helps virus to escape from host immunity (Fanunza et al., 2021; Zhang et al., 2019a). NS2A antagonizes MDA5/RIG-I-mediated interferon-β (Xia et al., 2018) and NF-κB production (Lee et al., 2020). NS2A promotes the degradation of STAT1 and STAT2 to suppress interferon pathway (Fanunza et al., 2021). Even single point mutation of ZIKV NS2A causes change of viral virulence, such as A117V (Ávila-Pérez et al., 2019), A175V (Marquez-Jurado et al., 2018). ZIKV NS2A has also been discovered to induce the degradation of karyopherin subunit alpha 2 *via* chaperone-mediated autophagy, which is the primary nucleocytoplasmic transporter for some transcription factors to activate cellular proliferation and differentiation (He et al., 2020). In our previous study, we found that intracellular expression of ZIKV NS2A inhibited ZIKV infection, the mechanism of which needs to be further investigated (Yu et al., 2022). What is more, ZIKV NS2A disrupts neurogenesis in the cerebral cortex by degrading adherens junction proteins, leading to microcephaly (Yoon et al., 2017). However, these studies are far from sufficient to fully understand the role of NS2A in ZIKV pathogenesis.

ZIKV NS2A consists of 226 amino acids, as shown in Fig. 1A, and contains seven transmembrane segments (pTMS), with the N-terminal region (pTMS1 - pTMS2) near the ER lumen, pTMS3 across the ER membrane, and the C-terminal region (pTMS4 - pTMS7) near the cytoplasm (Zhang et al., 2019b). The multiple transmembrane structures

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https://doi.org/10.1016/j.jim.2022.113396
Received 14 June 2022; Received in revised form 10 November 2022; Accepted 29 November 2022
Available online 1 December 2022

resulted in no available antibodies to the NS2A protein, which may greatly impede the meaningful studies on NS2A. The study of NS2A with tags may not always be a true reflection of ZIKV infection. Therefore, NS2A-specific recognition antibodies are urgently needed. In this study, we found that immunizing New Zealand rabbits with a peptide (GSTDHMDHFSLGVLC) from the N-terminal of ZIKV NS2A, coupled to KLH, resulted in effective and specific ZIKV NS2A recognition antibodies for western blot and immunofluorescence assay. Our study may be important for advancing the in-depth study of ZIKV NS2A.

2. Materials and methods

2.1. Cells and viruses

HMC3, BHK21 or Vero cells were maintained in Minimum Essential Medium (MEM) (Gibco, ThermoFisher Scientific, Wilmington, DE, USA) or Dulbecco's modified Eagle medium (DMEM) (Gibco, ThermoFisher Scientific, Wilmington, DE, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, ThermoFisher Scientific, Wilmington, DE, USA) at 37 °C in 5% CO_2. ZIKV strain SZ01/2016 (GenBank number: KU866423) was isolated from a patient who returned from Samoa (Yu et al., 2022). The origins of ZIKV/FLR, ZIKV/MR766, DENV-2 and YFV-17D were the same as our previously published reference (Yu et al., 2017).

2.2. Antibody preparation

Firstly, the antigenic regions of ZIKV NS2A sequence were analyzed by GenScript Optimum AntigenTM design tool and the candidate peptides were obtained as shown in Table 1. The peptide1 and 4 were synthesized and each was coupled with keyhole limpet hemocyanin (KLH) (GenScript, Nanjing, China). New Zealand rabbits ($n = 2$) were immunized with a mixture of the coupled KLH peptide and GenScript's adjuvant at multiple points in the back, three times at 2-week intervals. One week after the last immunization, antisera were collected from rabbits. The antibody was purified using an antigen affinity purification column by GenScript (Nanjing, China), and the antibody concentration was measured by an NanoDrop at OD280. This study was approved by GenScript IACUC (GS-ANT2101SR0702).

2.3. Enzyme linked immunosorbent assay (ELISA)

The 96 well ELISA plate was coated with 4 μg/ml of peptide 4 in PBS, 100 μl per well, and incubated overnight at 4 °C. On the next day, the plate was washed three times with PBST (PBS including 0.1% Tweeen-20) and 100 μl of 3% BSA was added to each well and incubated at 37 °C for 1 h. After three washes with PBST, 1 mg/ml of purified antibody and rabbit pre-immune serum were diluted from 1:1000 to

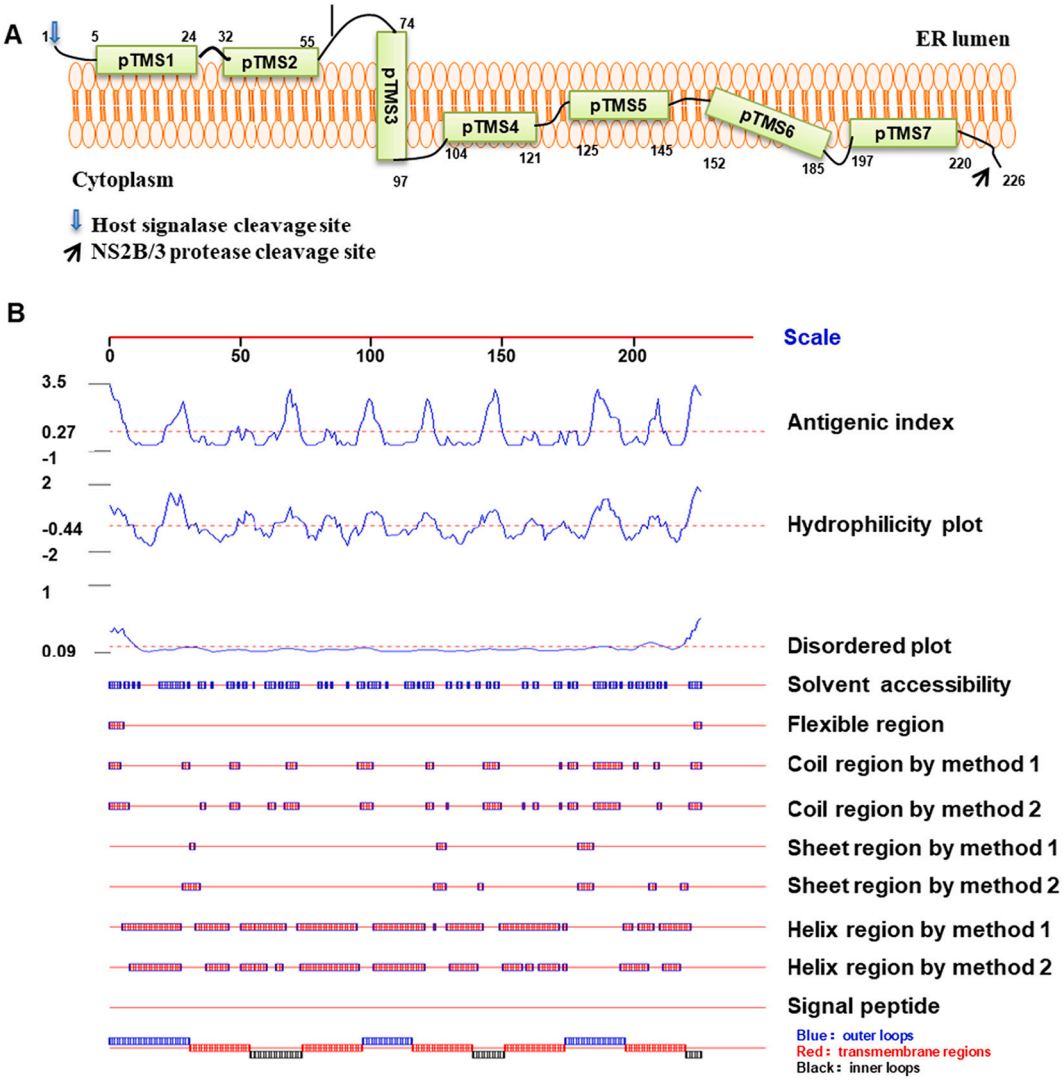

Fig. 1. Structural features of ZIKV NS2A. A, Topology structure of ZIKV NS2A (Zhang et al., 2019b). B, Sequence of ZIKV NS2A was analyzed by GenScript Optimum AntigenTM design tool.

Table 1
Antigen design result of ZIKV NS2A.

No.	Start	Antigenic Determinant	Length	Antigenicity/Surface/ Hydrophilicity	Disordered Score	Synthesis	Mus_musculus | Rattus_norvegicus | Oryctolagus_cuniculus blast
1	183	CLSLKGKGSVKKNLP	14	1.44/0.64/0.30	NONE	Easy	42% | 56% | 56%
2	62	GATFAEMNTGGDVAC	14	0.88/0.71/−0.22	NONE	Easy	42% | 56% | 42%
3	139	CIRAMVVPRTDNITL	14	0.84/0.43/−0.21	NONE	Normal	42% | 49% | 42%
4	1	GSTDHMDHFSLGVLC	14	0.83/0.64/−0.25	0.2043	Easy	42% | 42% | 49%
5	213	CNVVGLLLLTRSGKR	14	0.79/0.43/−0.11	0.1850	Easy	64% | 64% | 57%
6	22	EGLKKRMTTKIIISC	14	0.75/0.71/0.34	NONE	Easy	50% | 49% | 42%
7	92	CSFIFRANWTPRESM	14	0.71/0.64/−0.32	NONE	Easy	50% | 70% | 42%
8	115	CQTAISALEGDLMVL	14	0.38/0.57/−0.40	NONE	Hard	56% | 56% | 64%

Note:
1. An extra "C" (high-lighted as green) is added to the C-terminus (or N-terminus) to facilitate conjugation.
2. Positive charged residues (K, R, H) are in blue. Negative charged residues (D, E) are in red.
3. The underline regions are predicted as disordered regions.

1:512,000 in a two-fold ratio. Then they were added to the plate and incubated at 37 °C for 1 h. After five washes with PBST, HRP-labeled anti-rabbit secondary antibody was added and incubated at 37 °C for 1 h. After 5 washes with PBST, the TMB agent was used for color development for 5–20 min. Finally, the reaction was terminated by 2 M sulfuric acid. The data were collected by a microplate reader at OD450.

2.4. Western blot assay (WB)

To determine whether antibody prepared from the N-terminal peptide 4 (peptide 4 antibody) recognized ZIKV NS2A, HMC3 cells were spread in a 6-well plate and incubated at 37 °C, 5% CO_2 for 24 h. Then the cells were infected by 0.1 MOI of ZIKV/SZ01 at 37 °C, 5% CO_2. 0, 10, 24, 36, 48, and 60 h post infection (p.i.), the cells were collected for western blot assay. Peptide 4 antibody (1 μg/ml) was used as the primary antibody. The ZIKV E (B1845, Biodragon-immunotech, Beijing, China) and NS1 rabbit (GTX634158, GeneTex, CA, USA) antibody at concertration of 1 μg/ml were set as the positive controls. The HRP-labeled anti-rabbit secondary antibody (Proteintech, Wuhan, China) was used as the secondary antibody.

To test the specificity of peptide 4 antibody for ZIKV NS2A, Vero cells were seeded in 6-well culture plates and grown at 37 °C with 5% CO_2 overnight. Then the cells were infected by ZIKV/SZ01, ZIKV/FLR, ZIKV/MR766, DENV-2 or YFV-17D at MOI of 0.1 respectively. At 36 h p.i., the cells were collected for western blot assay as described above.

2.5. Immunofluorescence assay (IF)

To determine whether antibody prepared from the N-terminal peptide 4 recognized ZIKV NS2A,Vero cells were spread in a 24-well plate with sterile coverslips and incubated at 37 °C, 5% CO_2 for 24 h. Then the cells were infected by 0.1 MOI of ZIKV/SZ01 at 37 °C with 5% CO_2 for 48 h. The supernatants were discarded and the cells were fixed by 4% paraformaldehyde for 10 min. After treating with 0.2% TritonX-100 for 10 min, the cells were blocked by 3% BSA for 30 min. Then peptide 4 antibody (5 μg/ml) in 1% BSA was added to cells and incubated at room temperature for 1 h. The ZIKV E mouse antibody prepared from 4G2 hybridoma cells and NS1 rabbit (GTX634158, GeneTex, CA, USA) antibody were set as the positive controls. After 3 washes with PBS, secondary antibody Alexa Fluor 488 donkey anti-mouse or rabbit IgG (1:1000, ThermoFisher Scientific, Wilmington, DE, USA) was added to cells and incubate at room temperature for 1 h. After 5 washes with PBS, the coverslips were sealed with antifade reagent with DAPI (Beyotime Biotechnology, Shanghai, China) and observed under the Olympus FLUOVIEW FV3000 confocal microscope.

To detected whether the peptide 4 antibody recognizes ZIKV NS2A specifically, BHK-21 cells were seeded in 24-well plates with sterile coverslips and grown at 37 °C with 5% CO_2 overnight. Then the cells were infected by ZIKV/SZ01, ZIKV/FLR, ZIKV/MR766, DENV-2 or YFV-17D at MOI of 0.1 respectively. At 30 h p.i., immunofluorescence assay was performed as described above.

3. Results

3.1. Peptide sequence screening

To screen the antigenic regions, the NS2A sequence was firstly analyzed on multiple aspects by GenScript Optimum AntigenTM design tool, including antigenicity, hydrophilicity, surface probability, transmembrane, homology, flexible region, helix region, sheet region, signal peptide. As shown in Fig. 1 and Table 1, we obtained 8 candidate peptides. Peptide 1 (CLSLKGKGSVKKNLP) has the highest antigenicity, noticeably higher than other peptides. Therefore, we chose peptide 1 for GenScript polyexpress™ polyclonal antibody production service. Generally, the peptide close to C terminus or N terminus may generate antibodies with better performance in western blot and immunoprecipitation. C terminus of NS2A, peptide 5 (NVVGLLLLTRSGKR), consists of repetitive sequences, which may be unfavorable for antibody preparation. Therefore, we chose N terminus of NS2A, peptide 4 (GSTDHMDHFSLGVLC) for GenScript custom polyclonal antibody production service.

3.2. Antibody prepared from the N-terminal peptide 4 recognized ZIKV NS2A

To prepare antibodies against ZIKV NS2A, rabbits were immunized with peptide 1 or 4 conjugated with KLH. However, antibody prepared by peptide 1 did not recognize ZIKV NS2A in WB and IF (results not shown). To our surprise, antibodies prepared by peptide 4 showed effective recognition of ZIKV NS2A. Firstly, the binding ability of the purified polyclonal antibody to peptide 4 was detected by ELISA. As shown in Fig. 2A, the ELISA titer of peptide 4 antibody was 1:521,000, indicating that peptide 4 antibody can effectively bind peptide 4. To determine antibody recognition of the linear epitopes of ZIKV NS2A, HMC3 cells were infected with ZIKV/SZ01 and cell lysates were collected for WB. The results showed that peptide 4 antibody was able to recognize the linear epitope of ZIKV NS2A (Fig. 2B). Similar to the results of the commercial ZIKV NS1, E antibodies, the expression of ZIKV NS2A protein gradually increased with the duration of viral infection. To test whether peptide 4 antibody recognizes the structural epitope of ZIKV NS2A, we performed IF using Vero cells infected by ZIKV/SZ01 for 48 h. As shown in Fig. 2C, peptide 4 antibody effectively recognized the structural epitope of ZIKV NS2A. In summary, peptide 4 derived from the N-terminal of ZIKV NS2A induces antibodies that effectively recognize ZIKV NS2A and can be used in WB and IF.

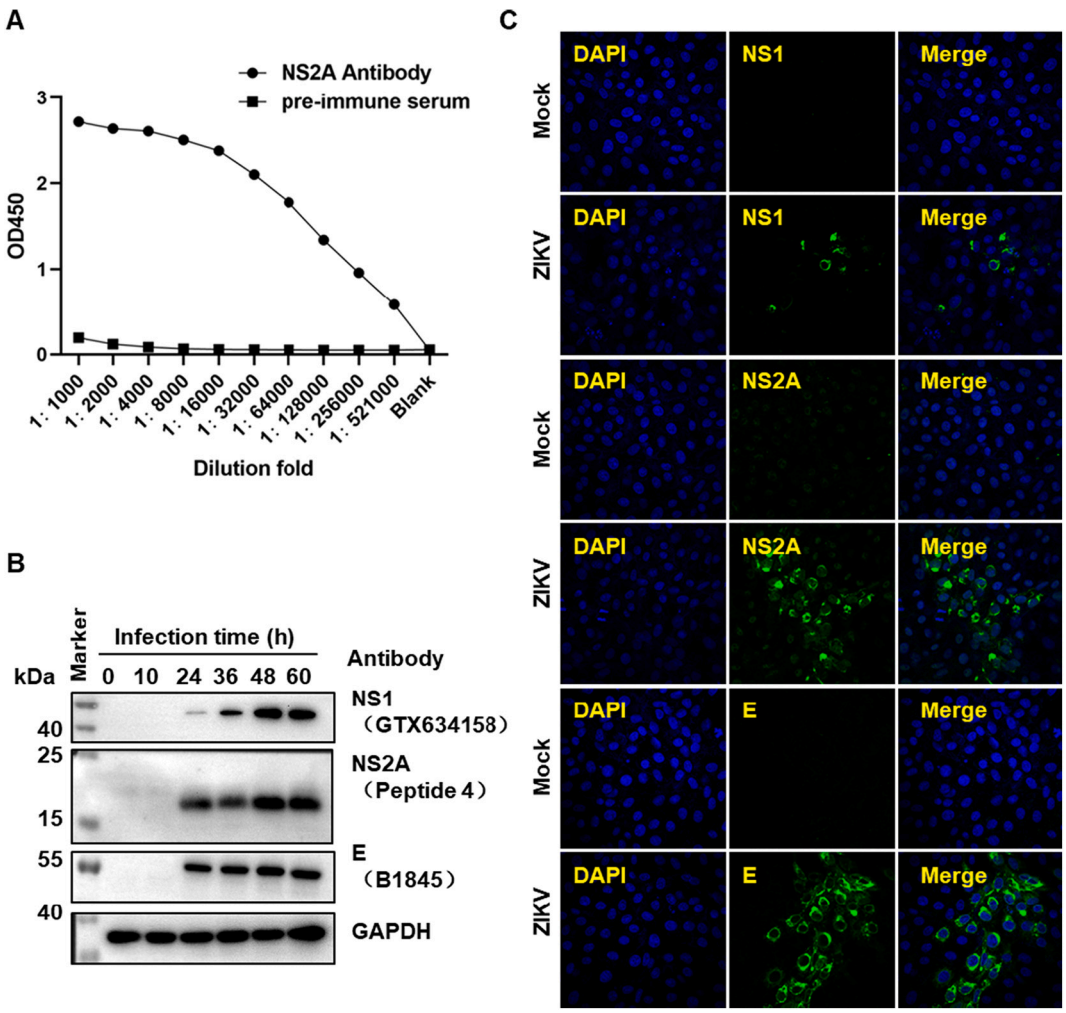

Fig. 2. Antibody prepared from the N-terminal peptide 4 recognized NS2A of ZIKV/SZ01. A, Antibody recognition of peptide 4 assayed by ELISA. B, Antibody recognition of ZIKV/SZ01 NS2A assayed by WB. C, Antibody recognition of ZIKV/SZ01 NS2A assayed by IF. ZIKV NS1, E, NS2A were detected by anti-NS1 (GTX634158), anti-E (B1845, WB; 4G2, IF), and anti-NS2A (prepared from the N-terminal peptide 4) antibodies, respectively.

3.3. Peptide 4 antibody specifically recognized the NS2A of ZIKV

Flaviviruses include DENV, YFV, ZIKV and the others. To test whether peptide 4 antibody specifically recognizes ZIKV NS2A, BHK21 cells were infected by different strains of ZIKV (ZIKV/SZ01, ZIKV/FLR, ZIKV/MR766) and DENV-2, YFV-17D, and then analyzed by IF using peptide 4 antibody. Meanwhile, Vero cells were infected by the above viruses, followed by WB using peptide 4 antibody as primary antibody. As shown in Fig. 3A and B, peptide 4 antibody effectively recognized NS2A in cells infected by ZIKV/SZ01, ZIKV/FLR, and ZIKV/MR766, but not NS2A of DENV-2- and YFV-17D- infected cells in IF and WB. These results indicate that peptide 4 antibody can specifically recognize ZIKV NS2A, but not NS2A of other flaviviruses, such as DENV-2 and YFV-17D. This may be due to the fact that the sequence of ZIKV NS2A (1-14aa) is 100% homologous with peptide 4; while the homologies of DENV-2 and YFV-17D NS2A (1-14aa) with peptide 4 are 57.1%, 14.3%, respectively (Fig. 3C).

4. Discussion

NS2A proteins of flaviviruses, as multiplex transmembrane proteins, are difficult to obtain *in vitro* for the preparation of NS2A specific antibodies. In this study, we first found that a peptide derived from the N-terminal of ZIKV NS2A induced the production of NS2A antibody, which specifically recognized the NS2A of ZIKV in IF and WB. NS2A antibody can be used to elucidate the mechanism of NS2A in viral replication, assembly and budding by detecting the intracellular localization changes of NS2A during viral infection. NS2A antibody can be applied to detect the expression of NS2A protein during viral infection to analyze how NS2A regulates immune signaling pathways. NS2A antibody makes it available to detect the interaction of NS2A with host proteins during viral infection to further clarify how NS2A involves in the pathogenesis of microcephaly in infants. Altogether, NS2A antibody can enable the study of NS2A proteins back to the process of viral infection, which is more favorable for the understanding of ZIKV NS2A functions. ZIKV belongs to flaviviruses. We believe our study provides a direction for the preparation of NS2A antibodies for flaviviruses.

Peptides with higher antigenicity predicted by antigen analysis software may not induce specific antibodies when immunized animals. Although software takes into account the possible secondary structures, it mainly analyzes the amino acid sequence of protein and may lose some information. The analysis software is not to be substituted for considering the overall conformation of the protein when there is availability of a crystal structure. Therefore, antigen prediction, animal immunity and antibody specificity assay are indispensable for antibody preparation by peptide.

Author contributions

Conceptualization, Y.Y.; methodology, J.W., Y.Y., P.Z., Y.C., X.F.;

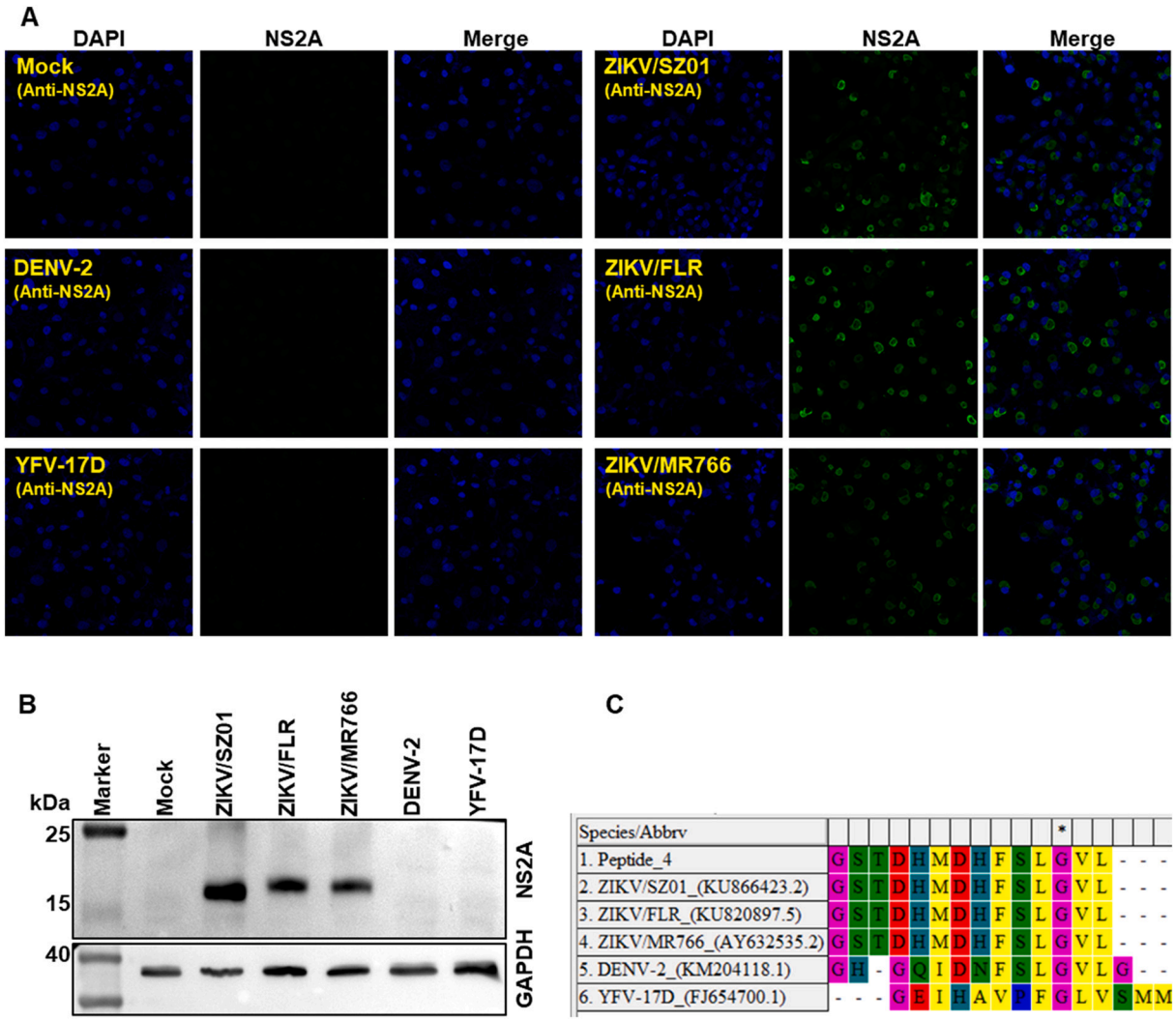

Fig. 3. Peptide 4 antibody specifically recognized the NS2A of several ZIKV strains. A, Peptide 4 antibody specifically detected NS2A of several ZIKV strains by IF. B, Peptide 4 antibody specifically detected NS2A of several ZIKV strains by WB. C, Homology analysis of NS2A (1-14aa) sequences of ZIKV, DENV-2, and YFV-17D with peptide 4.

validation, S.Q., Y.Y.; investigation, Y.Y., Z.H., P.Z.; resources, J.W., Y.Y, P.Z.; data curation, Y.Y., S.H., Y.C., X.F.; writing—original draft preparation, Y.Y.; writing—review and editing, Y.Y., P.Z., Y.C.; visualization, Y.Y., Y.C.; supervision, Z.H.; funding acquisition, Y.Y., P.Z. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by National Natural Science Foundation of China (82102388 to Y.Y.) and was also supported by funding from Shanghai Public Health Clinical Center (KY-GW-2017-17 to P.Z.).

Institutional review board statement

The animal study protocol was approved by GenScript IACUC (protocol code GS-ANT2101SR0702).

Informed consent statement

Not applicable.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request. The data presented in this study are available on request from the corresponding author.

Acknowledgments

We acknowledge GenScript's help in the antibody preparation. We thank Prof. Lifang Jiang at Sun Yat-sen University for providing the 4G2 hybridoma cells; We are grateful to Prof. Zheng Xing of Nanjing University for his support for this study.

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