Antiviral Research 170 (2019) 104578

Contents lists available at ScienceDirect

Antiviral Research

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

Yeast-produced subunit protein vaccine elicits broadly neutralizing antibodies that protect mice against Zika virus lethal infection

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ARTICLE INFO

Keywords:
Zika virus
EDIII domain
Yeast
Vaccine
Neutralizing antibody

ABSTRACT

Zika virus (ZIKV) infection is a serious public health concern due to its ability to induce neurological defects and its potential for rapid transmission at a global scale. However, no vaccine is currently available to prevent ZIKV infection. Here, we report the development of a yeast-derived subunit protein vaccine for ZIKV. The envelope protein domain III (EDIII) of ZIKV was produced as a secretory protein in the yeast *Pichia pastoris*. The yeast-derived EDIII could inhibit ZIKV infection in vitro in a dose-dependent manner, suggesting that it had acquired an appropriate conformation to bind to cellular receptors of ZIKV. Immunization with recombinant EDIII protein effectively induced antigen-specific binding antibodies and cellular immune responses. The resulting anti-EDIII sera could efficiently neutralize ZIKV representative strains from both Asian and African lineages. Passive transfer with the anti-EDIII neutralizing sera could confer protection against lethal ZIKV challenge in mice. Importantly, we found that purified anti-EDIII antibodies did not cross-react with closely related dengue virus (DENV) and therefore did not enhance DENV infection. Collectively, our results demonstrate that yeast-produced EDIII is a safe and effective ZIKV vaccine candidate.

1. Introduction

Infection with Zika virus (ZIKV), an arthropod-borne virus, not only induces mild symptoms including rash, arthritis, and conjunctivitis (Dick, 1952; Duffy et al., 2009; Lanciotti et al., 2008), but also may lead to neurological and autoimmune complications such as Guillain-Barre syndrome and microcephaly (Cao-Lormeau et al., 2016; Loos et al., 2014; Rasmussen et al., 2016). Large outbreaks of ZIKV infection have occurred since 2007 (Bogoch et al., 2016; Cao-Lormeau et al., 2014; Duffy et al., 2009; Lanciotti et al., 2008; Loos et al., 2014). In particular, the ZIKV outbreak in Brazil has caused estimated 440,000 to 1,300,000 infections, and was declared as a Public Health Emergency of International Concern by the World Health Organization (WHO) in February 2016 (Gulland, 2016). Thus, ZIKV infection has become a serious global public health concern. However, no approved vaccines are currently available for preventing ZIKV infections.

ZIKV belongs to the *Flavivirus* genus of the *Flaviviridae* family and is closely related to yellow fever virus, dengue virus (DENV), West Nile virus (WNV) and Japanese encephalitis virus (Gould and Solomon, 2008). Its genome is a single-stranded, positive-sense RNA of approximately 11 kb, encoding a single polyprotein that can be processed by proteases from host and the virus to produce three structural proteins (C, prM/M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Kuno and Chang, 2007). High-resolution structures of ZIKV mature virions reveal that the viral envelope consists of 180 E protein copies arranged in icosahedral symmetry (Dai et al., 2016; Kostyuchenko et al., 2016; Sirohi et al., 2016; Zhao et al., 2016). The E protein is responsible for virus attachment, entry, and fusion. It contains a transmembrane domain and an ectodomain (also termed E80) which can be divided into domain I (EDI), domain II (EDII), and domain III (EDIII). The distal end of EDII contains the fusion loop that mediates membrane fusion after pH-dependent

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https://doi.org/10.1016/j.antiviral.2019.104578
Received 11 April 2019; Received in revised form 21 July 2019; Accepted 4 August 2019
Available online 05 August 2019

conformational changes. EDIII consists of approximately 100 amino acids and is considered as the receptor binding site (Dai et al., 2016; Kostyuchenko et al., 2016; Zhao et al., 2016). Recent studies show that EDIII displays epitopes recognized by ZIKV–neutralizing monoclonal antibodies (Robbiani et al., 2017; Sapparapu et al., 2016; Stettler et al., 2016; Wang et al., 2016; Zhao et al., 2016).

Many approaches have been tested for the development of safe and effective ZIKV vaccines, yielding some exciting results in pre-clinical studies (Das Neves Almeida et al., 2018; Durbin and Wilder-Smith, 2017; Fernandez and Diamond, 2017; Ghaffar et al., 2018; Lin et al., 2018). For instance, a DNA vaccine encoding ZIKV PrM-E proteins was found to provide protection against viremia in mice and non-human primates (Dowd et al., 2016). Larocca et al. reported that an inactivated virus vaccine also protected mice and rhesus monkeys from ZIKV challenge by inducing E protein-specific neutralizing antibodies (Abbink et al., 2016; Larocca et al., 2016), highlighting the critical role of neutralizing antibodies targeting the envelope protein and preventing ZIKV infection. Shan et al. reported that a live attenuated ZIKV vaccine candidate could induce sterilizing immunity in mouse models (Shan et al., 2017). It was also found that mRNA vaccines encoding ZIKV prM and E genes elicited protective immune responses in mice (Richner et al., 2017) and in non-human primates (Pardi et al., 2017). Based on the encouraging preclinical results, a number of ZIKV vaccine candidates derived from the above mentioned vaccine platforms, including DNA vaccine, mRNA vaccine, inactivated whole virus vaccine, and live attenuated vaccine, have progressed into clinical trials (reviewed in (Das Neves Almeida et al., 2018; Garg et al., 2018; Ghaffar et al., 2018)). Preliminary reports of a phase I trial showed that a consensus DNA vaccine (GLS-5700) was safe and capable of inducing protective antibodies (Tebas et al., 2017). Similarly, a DNA vaccine (VRC5283) was found to be well-tolerated and immunogenic in phase I trials (Gaudinski et al., 2018). Results from phase I trials with a purified formalin-inactivated ZIKV vaccine showed that the vaccine was well-tolerated and could elicit robust neutralizing antibody titers in healthy adults (Modjarrad et al., 2018).

One of the unique challenges in developing a ZIKV vaccine is a potential immune enhancement of heterologous DENV infection (Fernandez and Diamond, 2017). As ZIKV and DENV are closely related, their infections produce cross-reactive antibodies which may lead to antibody-dependent enhancement (ADE) of heterologous infections, a phenomenon well-documented in different DENV serotypes (Halstead et al., 2010). In fact, it has been reported that anti-ZIKV antibodies could enhance DENV infection in cell cultures (Kawiecki and Christofferson, 2016) and in mouse models (Stettler et al., 2016). Anti-ZIKV antibodies able to enhance DENV infection target mainly the EDI and EDII domains (Stettler et al., 2016). In the case of DENV, antibodies targeting the prM protein have also been shown to facilitate ADE (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010). So far, all the ZIKV vaccine candidates entering clinical trials contain or express full-length prM and E proteins, and therefore bear the risk of enhancing DENV infection. Thus, it is important to continue the search for a safe and effective ZIKV vaccine.

Recently, our group demonstrated that recombinant ZIKV EDIII protein derived from *Drosophila* S2 cells could robustly elicit neutralizing antibodies capable of protecting mice against lethal ZIKV infection (Qu et al., 2018). In the present study, we evaluated whether functional ZIKV EDIII could be produced as a vaccine candidate in yeast, an economic and adaptable recombinant expression system widely used in the vaccine industry. Moreover, we examined whether ZIKV EDIII-immunized mice could produce DENV infection-enhancing antibodies.

2. Materials and methods

2.1. Cells and viruses

Vero E6 cells were grown as described previously (Liu et al., 2011). K562 cells were purchased from the Cell Bank of Chinese Academy of Sciences (www.cellbank.org.cn). ZIKV strain SZ-WIV01 (GenBank accession number: KU963796)has been described in a previous study (Qu et al., 2018). ZIKV strains MR766 and PRVABC59 were obtained from the American Type Culture Collection (ATCC, USA). ZIKV strain NC-2014-5132 has been described previously (Dupont-Rouzeyrol et al., 2017). All ZIKV strains were propagated in Vero cells supplemented with Dulbecco's modified Eagle medium (DMEM) and 2% fetal bovine serum (FBS). DENV serotype 2 strain New Guinea C ((DENV2/NGC) has been described previously (Zou et al., 2011). Virus stocks were titrated by performing plague assays as previously described (Qu et al., 2018).

2.2. Antibodies

Anti-6 × His tag mouse monoclonal antibody was purchased from GenScript (Nanjing, China). Alkaline phosphatase (AP)-conjugated secondary antibodies were purchased from Promega (Madison, WI, USA). Anti-ZIKV-E80 rabbit polyclonal antibody was generated in house by immunizing rabbit with insect cell-produced ZIKV E80 protein (Qu et al., 2018).

2.3. Synthetic peptides

A set of 20 peptides spanning entire amino acid sequence of EDIII (amino acid 297 to 406) of ZIKV/Z1106033 strain (GenBank accession number: KU312312) were synthesized by GL Biochem (Shanghai, China). Each peptide was made up of 15 residues, of which 12 overlapped with the adjacent peptides.

2.4. Vector construction and yeast transformation

The nucleotide coding sequence for EDIII (amino acid 297 to 406) of ZIKV strain Z1106033 was codon optimized, synthesized, and cloned into the backbone vector pPinkα-HC (Invitrogen, USA), yielding the plasmid pPinkα-HC-ZIKV-EDIII.

Yeast transformation was carried out as described previously (Zhang et al., 2015). Briefly, plasmids were linearized by EcoNI digestion and then electroporated into a *P. pastoris*, PichiaPink™ Strain 1 (Invitrogen), by electroporation. The resulting transformed yeast clones were screened for EDIII expression by western blotting as described below.

2.5. SDS-PAGE and Western blot assay

Protein samples were mixed with 5 × SDS sample buffer, boiled, and separated by 15% PAGE. For visualization, SDS-PAGE gels were stained with Coomassie brilliant blue G-250. For western blotting, proteins were transferred to a PVDF membrane. The membrane was probed with either anti-6 × His tag mouse monoclonal antibody (1:5000 dilution) or rabbit anti-ZIKV-E80 polyclonal antibody (1:1000 dilution), followed by incubation with corresponding alkaline phosphatase (AP)-conjugated secondary antibodies.

2.6. Expression and purification of recombinant EDIII protein

To prepare recombinant ZIKV-EDIII, pPinkα-HC-ZIKV-EDIII-transformed yeast strain was grown in 200 ml BMGY medium with shaking at 30 °C for 24 h. The yeast cells were collected by centrifugation and were supplemented with BMMY medium containing 1% methanol for 48 h at 30 °C. The medium supernatant was harvested and then concentrated by ultrafiltration using an Amicon Ultra-3 Centrifugal Filter (Millipore, Germany). The resulting sample was added to 100 ml

binding buffer (0.5 M NaCl, 20 mM Tris, 10 mM imidazole, pH 7.9) and then subjected to affinity purification using Ni-nitrilotriacetic acid (NTA) resins (Millipore). Purified EDIII protein was quantified by Bradford assay with bovine serum albumin (BSA) as the reference standard.

2.7. ZIKV infection-inhibition assay

Vero cells cultured in DMEM medium plus 2% FBS were seeded (1×10^5 cells/well) in a 24-well plate and then grown for 24 h. Serially diluted EDIII or BSA was mixed with 100 PFU of ZIKV. The mixtures were added then to the pre-seeded Vero cells. After incubation for 1 h at 37 °C, the protein-virus mixtures were removed and overlay medium (DMEM, 2% FBS, 0.2% low-melting-point agarose) was added to the wells. After 72 h, cultured cells were fixed and stained with 1% crystal violet for plaque visualization.

2.8. Ethics statement

All the animal experiments in this study were approved by the Institutional Animal Care and Use Committee at the Institut Pasteur of Shanghai. Animals were cared for in accordance with institutional guidelines.

2.9. Animal immunization

Prior to immunization, EDIII recombinant protein was formulated with the Alhydrogel adjuvant (Invivogen, USA). Each vaccine dose contained 10 μg of EDIII and 500 μg of aluminum hydroxide. PBS buffer mixed with 500 μg of aluminum hydroxide was prepared as a negative control. Two groups of six female BALB/c mice (6 weeks old, purchased from Shanghai Laboratory Animal Center, Shanghai, China) were injected intraperitoneally (i.p.) with the EDIII vaccine and the adjuvant control, respectively, at weeks 0, 2, and 4. Blood samples were collected at weeks 4 and 6 for antibody measurement.

2.10. Serum antibody measurement

For antibody measurement, 96-well microtiter plates were coated with 50 ng of purified EDIII for 2 h at 37 °C. The wells were then blocked with PBST containing 5% non-fat dry milk for 1 h at 37 °C, incubated with 50 μl serially diluted mouse antisera for 2 h at 37 °C and then with 50 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody for 1 h at 37 °C. After color development, absorbance was measured at 450 nm in a 96-well plate reader.

2.11. Neutralization assay

Neutralization titers of mouse antisera towards ZIKV were determined by performing plaque reduction assay as described previously (Qu et al., 2018). For a given serum sample, the percent reduction of plaques was calculated by comparing the plaque number of this treatment with that of the virus only. Values for 50% plaque reduction neutralization titers (PRNT50) were determined by nonlinear regression analysis using GraphPad Prism version 5.

2.12. ELISPOT assay

For ELISPOT assays, splenocytes were isolated from immunized mice five weeks after the last immunization. ELISPOT assays were performed according to a protocol described previously (Li et al., 2016) with the following modifications: purified EDIII protein or the EDIII peptide pool at a final concentration of 10 μg/ml was used as stimuli in this study. Concanavalin A (ConA) stimulation was set as the positive control and the medium only as the negative control.

2.13. Cross-binding and antibody-dependent enhancement assays

Prior to the assays, mouse sera were pooled for each group and then subjected to IgG purification using HiTrap™ Protein G HP column (GE Healthcare, PA, USA).

For cross-binding assay, pre-seeded Vero cells were inoculated (MOI = 0.01 or 1) with ZIKV strain SZ-WIV01 or DENV2 strain New Guinea C. Two days later, infected cells were fixed, permeabilized, and collected according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). The cells were stained with purified polyclonal antibodies or the 4G2 monoclonal antibody, followed by staining with an anti-mouse Alexa Fluor(R) 488-conjugated secondary antibody (Life Technologies). Then, the samples were analyzed on a BD LSRII flow cytometer.

For ADE assay, serial dilutions of purified IgG or monoclonal antibody 4G2 (positive control) were incubated with DENV2/NGC for 1 h at 37 °C. Then, the mixtures were added to K562 cells at a MOI of 1 and incubated at 37 °C for 2 days. Finally, cells were fixed, permeabilized and stained with Alexa Fluor 488-conjugated anti-DENV rabbit monoclonal antibody (4G2), followed by flow cytometry analysis on a BD LSRII flow cytometer. The results were analyzed with FlowJo software.

2.14. In vivo protection assays

Groups of five-week-old A129 mice (n = 9) deficient in type I interferon receptor were i.p. injected with 400 μl of mouse antisera and after 24 h inoculated with ZIKV/SZ-WIV01 (10^5 PFU per mouse) via the i.p. route. The mice were then monitored daily for survival and weight loss for a period of 14 days. Mice that lost > 20% of their initial body weight were humanely euthanized, counted as moribund sacrifices, and recorded as dead.

2.15. Statistics analysis

All statistical analyses were performed using GraphPad Prism software v5.0. Kaplan–Meier survival curves were compared using log-rank test. Other results were analyzed using Student's two-tailed *t*-test. Statistical significance was indicated as follows: ns, not significant ($P \geq 0.05$); *, $0.01 \leq P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3. Results

3.1. Generation and characterization of yeast-derived EDIII protein

To develop a recombinant ZIKV EDIII protein, an expression vector termed pPinkα-HC-ZIKV-EDIII was constructed. This vector encoded ZIKV EDIII fused with an N-terminal α-mating factor signal peptide and a C-terminal 6 × His tag (Fig. 1A). The pPinkα-HC-ZIKV-EDIII vector was used to transform the PichiaPink™ yeast. From culture supernatant of the pPinkα-HC-ZIKV-EDIII transformed yeast, recombinant ZIKV EDIII protein was purified as described in the Materials and Methods section. SDS-PAGE analysis showed that purified ZIKV EDIII is present as a single protein band of approximately 13 KDa (Fig. 1B). The identity of the recombinant EDIII protein was verified by Western blot analyses with a ZIKV E-specific polyclonal antibody and an anti-6 × His tag monoclonal antibody (Fig. 1C). These results demonstrated that ZIKV EDIII protein was successfully produced by recombinant yeast.

To evaluate the conformation and function of yeast-produced EDIII protein, we performed ZIKV infection-inhibition assays with the recombinant EDIII and an irrelevant control protein (BSA). As shown in Fig. 2, EDIII inhibited ZIKV infection in a dose-dependent manner with IC50 being 7.60 μg/ml, whereas no inhibitory effect was observed for the BSA control. The results indicated that recombinant EDIII was adequately folded and was able to compete with ZIKV for receptor-binding sites on the cell surface, suggesting that EDIII exhibits a significant potential to elicit neutralizing antibodies against ZIKV.

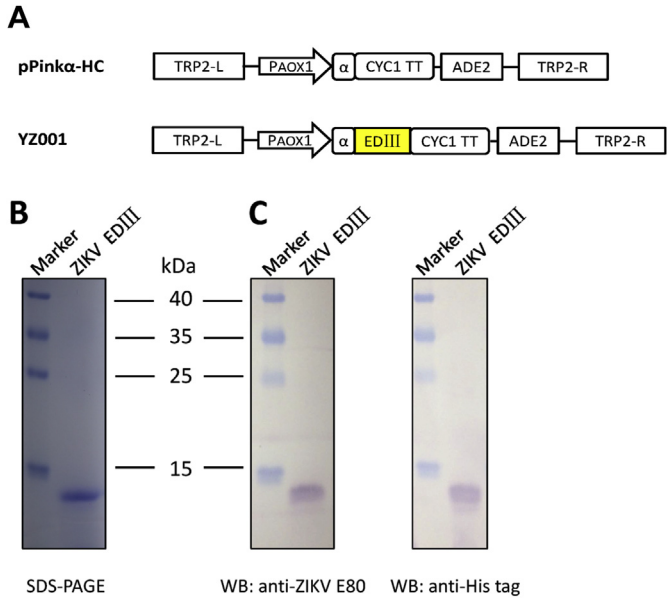

Fig. 1. Expression and purification of ZIKV EDIII proteins in *Pichia pastoris*. (A) Diagrams of the constructs for expressing ZIKV EDIII. TRP2-L and TRP2-R, the up- and down-stream parts of the TRP region; P_{AOX1}, AOX1 promoter; CYC1 TT, CYC1 transcription termination region; ADE2, expression cassette encoding phosphoribosylaminoimidazole carboxylase, used as the selection marker; α, α-factor signal sequence. (B) SDS-PAGE and Coomassie Brilliant Blue staining analysis of the purified ZIKV EDIII antigen used for mouse immunization. (C) Western blot analysis with rabbit-anti-ZIKV-E80 polyclonal antibody (left) and anti-6 × His tag monoclonal antibody (right).

3.2. Yeast-derived EDIII effectively elicited antibody and T-cell responses in mice

To evaluate immunogenicity of yeast-derived EDIII, BALB/c mice were immunized by i.p. injection with ZIKV EDIII protein formulated with Alum adjuvant at weeks 0, 2, and 4. Another group of mice injected with PBS plus Alum adjuvant, served as the control. Serum samples were collected at weeks 4 and 6 and analyzed for EDIII-specific antibody by ELISA using yeast-derived EDIII as the capture antigen. As shown in Fig. 3A, neither sera from week 4 or week 6 from the PBS control group exhibited any binding activity; in contrast, EDIII-binding activity was readily detectable at week 4 in the sera from EDIII-immunized mice and a drastic increase in EDIII-binding was observed for the week-6 anti-EDIII sera. In addition, Fig. 3B showed that geometric mean titers reached 13,626 and 136,258 after the 2nd and 3rd immunization, respectively. These data indicate that EDIII effectively elicited antigen-specific antibody responses in mice.

We then performed ELISPOT assays to assess the vaccine-induced cellular immune responses. As shown in Fig. 4A, splenocytes from the EDIII-immunized mice produced significantly higher amount of IL-4-secreting spot-forming cells (SFCs) upon stimulation with either EDIII protein or EDIII peptide as compared to the mock (medium) treatment; in contrast, only baseline levels of IL-4-secreting SFCs were detected in the PBS control samples regardless of the stimuli. Similarly, EDIII protein or EDIII peptide stimulation drastically increased the number of IFN-γ-secreting SFCs detected in the EDIII-immunized mice but not in the control mice (Fig. 4B). These results demonstrate that EDIII immunization could elicit both ZIKV-specific IFN-γ-secreting and IL-4-secreting T-cell responses in mice.

3.3. Neutralization potency and breadth of the anti-EDIII mouse sera

We performed plaque reduction neutralization assays to determine the antisera's in vitro neutralization efficiency. We found that, when tested against ZIKV strain SZ-WIV01 (Asian lineage), the anti-EDIII sera were able to effectively inhibit plaque formation even at high serum dilutions (e.g. 1: 6,400), whereas only low dilutions (1:25 or 1:100) of the control sera yielded background levels of inhibition (Fig. 5A). The PRNT50 values of individual mice of the two groups are shown in Fig. 5B. Clearly, the anti-EDIII sera had much higher neutralization titers (geometric mean PRNT = 3608) against ZIKV/SZ-WIV01 than the control group.

To assess breadth of neutralization, individual mouse sera collected at two weeks after last immunization were pooled for each group and then subjected to neutralization assays against a panel of representative ZIKV strains, including SZ-WIV01 (Asian lineage), PRVABC59 (Asian lineage), NC-2014-5132 (Asian lineage), and MR766 (African lineage). The result showed that the pooled anti-EDIII sera could effectively neutralize all the strains tested (PRNTs between 905 and 2116) (Fig. 5C), demonstrating a broad neutralization potential of the anti-EDIII sera.

3.4. Anti-EDIII sera conferred protection against ZIKV challenge in mice

We evaluated the in vivo protective efficacy of the neutralizing antisera in a mouse model of ZIKV infection. This model utilizes A129 mouse which lacks type I IFN receptors and has been shown to be susceptible to ZIKV infection (Liu et al., 2016; Rossi et al., 2016). We injected the anti-EDIII or the control sera into A129 mice and one day later challenged the mice with ZIKV strain SZ-WIV 01 strain. As shown in Fig. 6A, 89% (8 out of 9 mice) of the mice receiving the anti-EDIII sera were protected from ZIKV infection-caused death whereas only 33% of the control sera-treated mice survived the challenge. In addition, the surviving mice in the anti-EDIII treatment group gained more weight than the counterparts in the control group (Fig. 6B). These results demonstrate that the anti-EDIII neutralizing sera can confer in vivo protection against lethal ZIKV infection.

3.5. Anti-ZIKV EDIII antibodies did not cross-react with DENV2 and enhance its infection

To investigate whether ZIKV EDIII immune sera could potentially

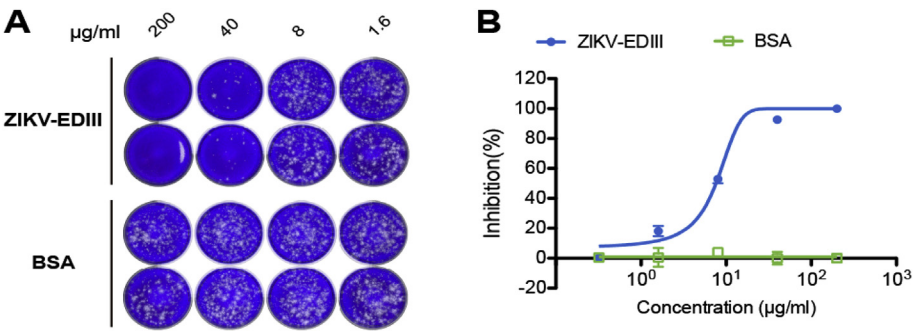

Fig. 2. Blocking of ZIKV infection in Vero cells by ZIKV EDIII. Purified ZIKV EDIII was serially diluted and then mixed with 100 PFU of ZIKV, next the mixture was added to pre-seeded Vero cells immediately. After 1-h incubation, the supernatant containing EDIII and ZIKV was changed into overlay medium, followed by plaque development, fixation, and crystal violet staining. BSA was employed as a negative control. (A) Reduction of plaques yielded in ZIKV EDIII treated cells comparing to that in BSA treated cells. (B) Quantitative analysis of normalized plaque reduction numbers. Means ± SD are shown. The data are representative of two independent experiments.

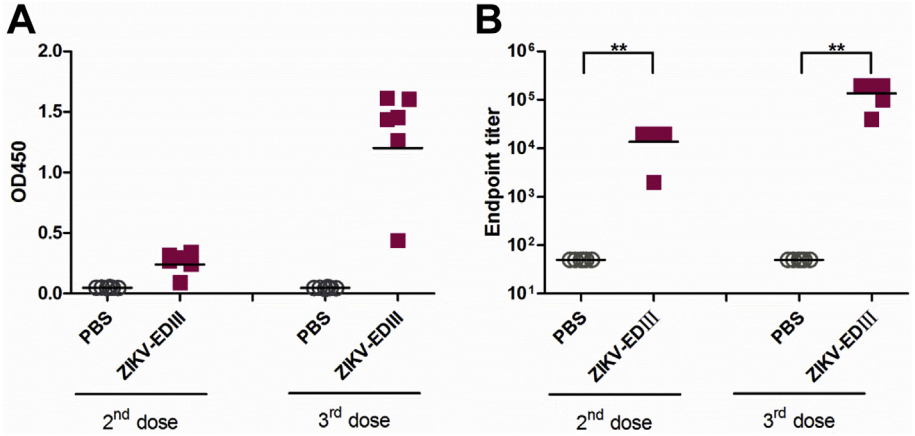

Fig. 3. Antibody responses elicited by ZIKV EDIII. (A) Mice serum samples collected at 2 weeks after the 2nd and 3rd injections were diluted 1:10,000 and subjected to ELISA to detect the ZIKV EDIII-specific antibodies. The horizon bars indicate geometric means of OD 450 of each group. (B) ZIKV EDIII-specific antibodies titers were also determined by performing ELISA. Each symbol represents a mouse and the line indicates geometric mean value of the group. Significant differences were calculated using Student's two-tailed *t*-test and shown as: **, $P < 0.01$.

enhance DENV2 infection, we purified IgG antibodies from the antisera and evaluated their cross-binding against DENV2. As shown in Fig. 7A, the cross-reactive monoclonal antibody 4G2, which targets the conserved fusion loop of flaviviruses, could efficiently bind both ZIKV and DENV2, thus validating the assay. IgG antibodies purified from EDIII-immunized mouse sera positively stained the ZIKV-infected Vero cells but not the DENV-infected cells. As expected, treatment with polyclonal IgG antibodies from the control (PBS) mice only yielded background levels of signal in both ZIKV- and DENV2-infected cells (Fig. 7A). These data indicate that anti-ZIKV-EDIII antibodies did not cross-react with closely related DENV.

We further determined whether anti-ZIKV-EDIII antibodies could enhance DENV2 infection in vitro by performing ADE assays with the monoclonal antibody 4G2 as the positive control. As shown in Fig. 7B, 4G2 treatment produced a typical antibody dose-dependent enhancement profile of DENV2 infection in K562 cells. In contrast, polyclonal IgG antibodies isolated from the ZIKV EDIII immune sera or from the control sera did not exhibit significant enhancing effect on DENV2 infection regardless of the antibody doses used. Collectively, the above results demonstrated the anti-ZIKV-EDIII antibodies did not bind DENV2 and therefore could not promote ADE towards DENV2 infection.

4. Discussion

The present study was aimed at investigating the possibility of developing a ZIKV subunit protein vaccine based on yeast-produced EDIII. We found that EDIII could be readily produced in *P. pastoris* yeast and vaccination with yeast-derived EDIII could induce protective immunity in mice. Yeast-derived EDIII appeared to fold adequately to display native conformation as it could inhibit ZIKV infection in vitro (Fig. 2) probably by competing with the virus for receptor binding sites on the cell surface. The experimental results are consistent with the notion that EDIII of flaviviruses encodes a primary viral receptor-binding motif (Rey et al., 2017). It has been reported that ZIKV EDIII displays epitopes recognized by strongly neutralizing monoclonal antibodies (Gromowski and Barrett, 2007; Pierson et al., 2008; Sukupolvi-Petty et al., 2007; Zhao et al., 2016), suggesting that ZIKV EDIII is a major inducer of neutralizing antibodies. In agreement with these findings, our results showed that yeast-derived EDIII indeed potently elicited ZIKV-neutralizing antibodies in mice (Fig. 5). Notably, anti-EDIII sera could efficiently neutralize ZIKV representative strains of both Asian and African lineages (Fig. 5), suggesting the potential utility of yeast-derived EDIII as a universal ZIKV vaccine.

In the present study, we demonstrated that passive transfer with neutralizing antisera protected recipient mice against ZIKV lethal challenge (Fig. 6). Similar observations have been made by other groups (Abbink et al., 2016; Larocca et al., 2016; Qu et al., 2018). Together, these findings indicate that neutralizing antibodies play a key role in protection against ZIKV infection. We also found that our EDIII vaccine could elicit robust IFN-γ- and IL-4-secreting T-cell responses (Fig. 4). These T-cell responses are likely important for B-cell maturation (Blom et al., 2013) and production of high-titer neutralizing antibodies (Kohler et al., 2012) and may provide additional protective effects (Larocca et al., 2016). Therefore, the ability to evoke both humoral and cellular immune responses is a desirable trait for the yeast-derived EDIII vaccine.

Perhaps the most significant finding from this study was that yeast can be utilized to robustly produce protective EDIII vaccine. Recombinant EDIII protein was secreted into supernatant of yeast cultures and readily purified at levels around 4.5 mg/L under our laboratory conditions. Such a yield is much higher than that of inactivated whole-virion ZIKV vaccine. It is likely that EDIII production in yeast can be further increased by optimization of the production process,such as screening and identification of high-expressing clones, characterization of biomass growth and product formation, and development of fed-batch processes (Looser et al., 2015). As recombinant expression systems, yeasts possess properties desirable for vaccine production

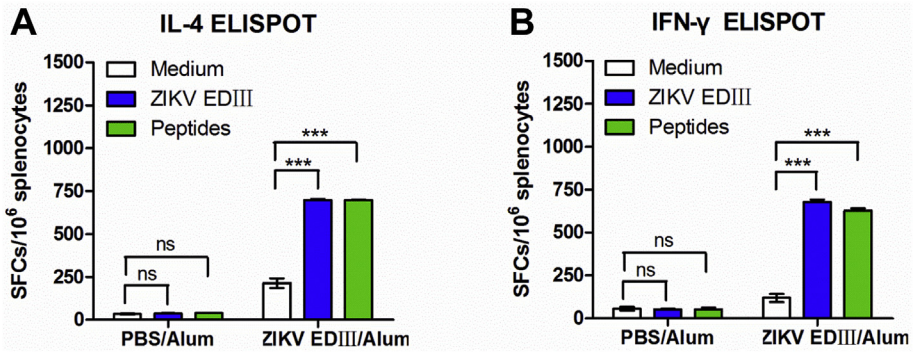

Fig. 4. Cellular immune responses in EDIII-immunized mice. (A) IL-4 ELISPOT assay. (B) IFN-γ ELISPOT assay. Splenocytes from three mice at week 5 after last immunization were isolated and pooled. Approximately 10^6 Splenocytes were plated out per well and stimulated with yeast-derived ZIKV EDIII or peptide pool spanning the entire ZIKV EDIII for 48 h. The cytokine production is expressed as spot-forming cells (SFCs) per 10^6 splenocytes. Means and SEM of triplicate wells are shown. The asterisks represent significant differences between medium and ZIKV EDIII stimulation or between medium and peptides in each group. ns, no significance ($P \geq 0.05$); *, $0.01 \leq P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

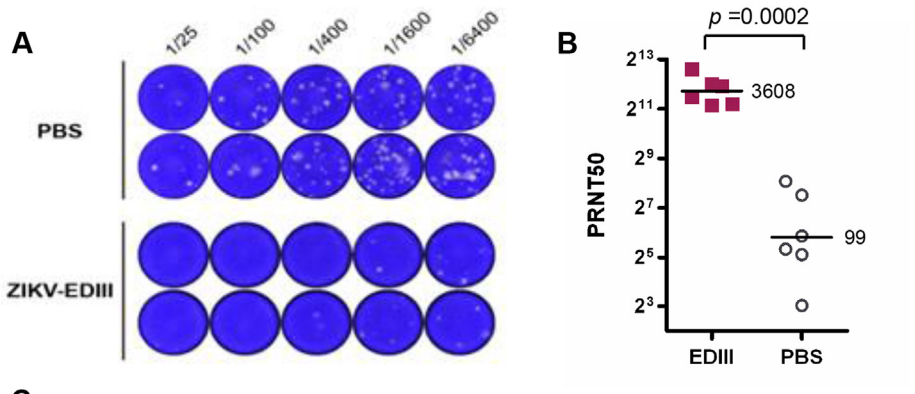

Fig. 5. Anti-EDIII sera were broadly neutralizing. (A) Anti-EDIII sera neutralized ZIKV infection in vitro. Antisera collected two weeks after the 3rd immunization were subjected to plaque reduction neutralization assay. Representative results are shown. (B) PRNT50 values of individual mice. The geometric means of PRNT50s for each group and p-values are indicated. (C) Neutralization titers of the pooled antisera against a panel of ZIKV strains.

C

Neutralization potency of the antisera against a panel of ZIKV strains

Pooled sera	PRNT50 against ZIKV strains			
	SZ-WIV01	PRVABC59	NC-2014-5132	MR766
Control sera	169	92	202	193
Anti-EDIII sera	2116	1254	905	1020

including high scalability, easy manipulation, and relatively high yield. Vaccine manufacturers in some developing countries where ZIKV vaccines are needed have already developed facilities and gained experience in producing yeast-derived vaccines, such as HBV and HPV vaccines. Therefore, the identification of *P. pastoris* yeast as an EDIII production platform will undoubtedly facilitate future development and production of EDIII-based ZIKV vaccine.

One of the major concerns in the development of flavivirus vaccines is the risk of vaccine-induced ADE towards heterologous flaviviruses (Halstead, 2016; Halstead and Russell, 2016; Morens, 1994; Ng et al., 2014). The phenomenon of ADE is largely attributed to the presence of cross-reactive and non-neutralizing heterotypic antibodies prior to infection (Halstead et al., 2010). ZIKV shares high-degree of similarity in amino acid sequence and structure of the prM and E proteins with other flaviviruses, with the majority being located in EDII and its neighboring region (Dai et al., 2016; Dejnirattisai et al., 2016; Sirohi et al., 2016). Therefore, it is likely that antibodies against the prM or E proteins of ZIKV could cross-react with the counterparts on closely related flaviviruses such as DENV or WNV and therefore promote ADE. Indeed, it has been shown that antibodies targeting the ZIKV EDI/II were cross-reactive and enhanced DENV infection both in vitro and in vivo (Stettler et al., 2016). Recently, Richner et al. reported that mRNA vaccines containing the unmodified prM-E sequence induced cross-reactive antibodies promoting ADE for DENV in cells and in mice, and the fusion loop located on EDII was implicated as the inducer of ADE antibodies (Richner et al., 2017). In the present study, we used EDIII, which does not contain potential enhancing antibody-inducing EDI/II regions, as the vaccine antigen. We demonstrated that anti-EDIII antibodies did not bind DENV and enhance DENV infection in vitro (Fig. 7). Our results are in agreement with previous reports showing that monoclonal antibodies targeting ZIKV EDIII are type-specific, robust neutralizing antibodies (Stettler et al., 2016; Wang et al., 2016; Zhao et al., 2016). These findings indicate that EDIII as an antigen is superior to full-length E protein in terms of minimal risk to induce cross-reactive enhancing antibodies and therefore should be considered in the design of new-generation ZIKV vaccines.

Collectively, our results show that ZIKV EDIII protein can be robustly produced in yeast and it potently induces protective immunity against ZIKV infection without elicitation of cross-reactive and infection-enhancing antibodies for DENV, thus demonstrating that yeast-produced EDIII is a safe and effective ZIKV vaccine candidate worthy of further development.

Acknowledgements

We thank the Microorganisms & Viruses Culture Collection Center, Wuhan Institute of Virology, Chinese Academy of Sciences, for providing the ZIKV strain SsZ-WIV01. This study was supported by a grant (2016YFE0133500) from the Chinese Ministry of Science and Technology for EU-China Inter-governmental S&T Cooperation

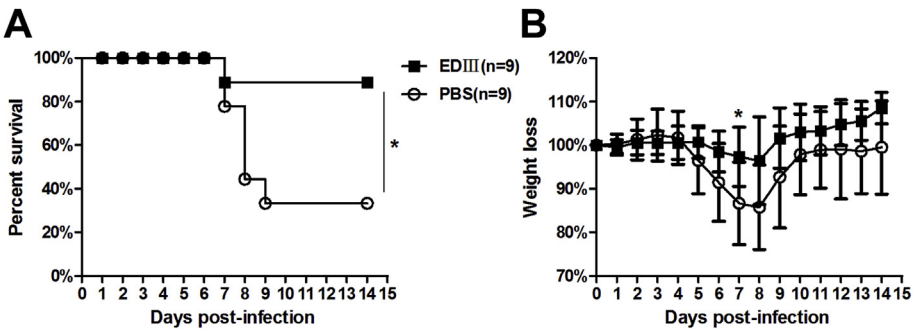

Fig. 6. Passive transfer of anti-EDIII sera protected A129 mice against ZIKV lethal infection. Five-week-old mice lacking type I interferon receptor (A129 mice) were intraperitoneally injected with either ZIKV EDIII antisera (n = 9) or PBS antisera (n = 9), followed by inoculation with ZIKV (10^5 PFU/mouse) via the intraperitoneal route after 24 h. Survival and weight loss were monitored daily. Mice that lost > 20% of their initial body weight were euthanized and considered as dead. Significant difference was determined by performing Log-rank test and two-tailed Student's *t*-test. *, $0.01 \leq P < 0.05$.

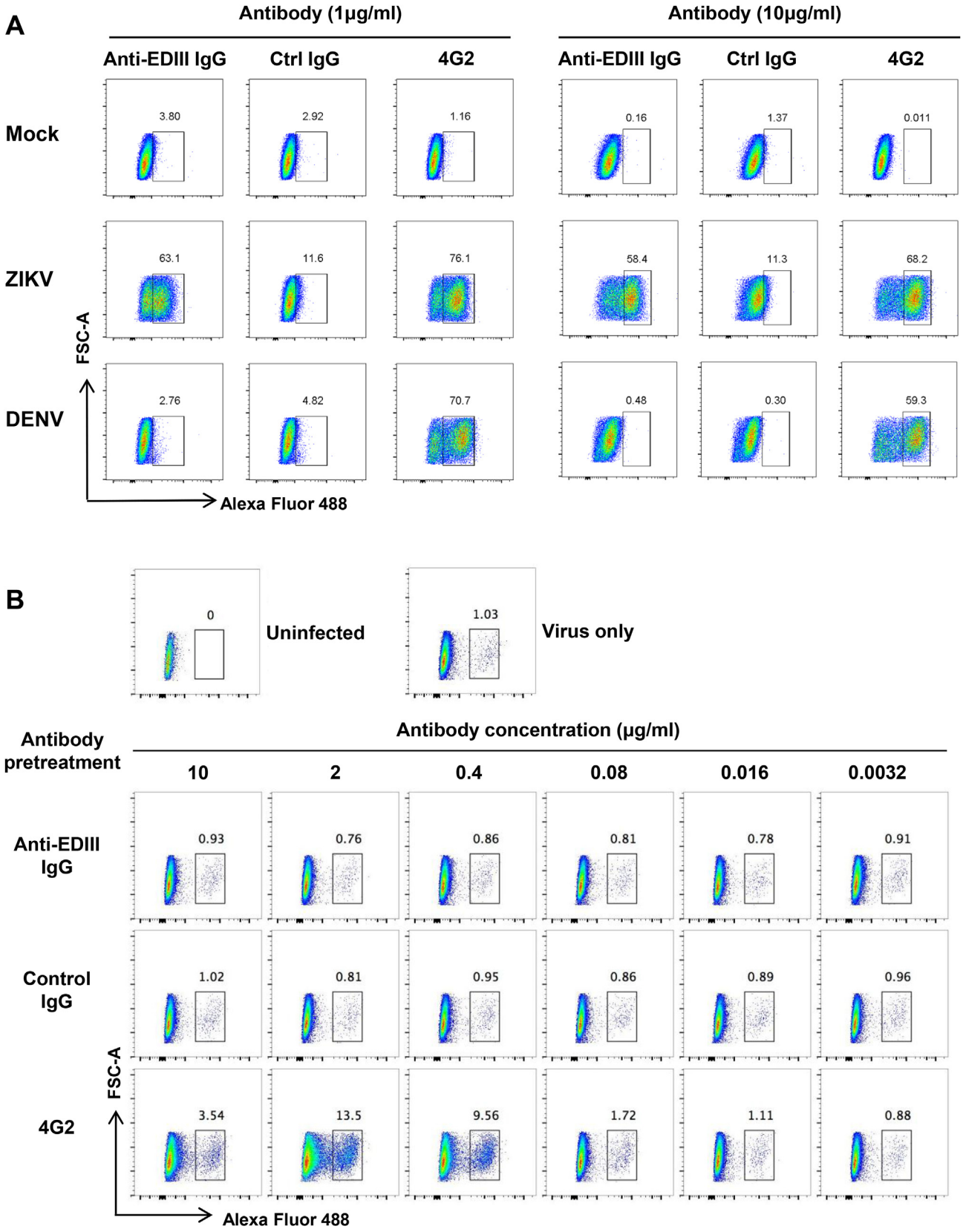

Fig. 7. Assessment of the anti-EDIII antibodies for cross-reactivity and ADE for DENV. (A) Cross-binding assay. Pre-seeded Vero cells were infected with ZIKV or DENV2 for 2 days. Infected cells were consecutively stained with a primary antibody (IgG antibodies purified from pooled mouse sera or the monoclonal antibody 4G2 at the concentration of 1 μg/ml or 10 μg/ml) and a secondary antibody, and then analyzed by flow cytometry. (B) ADE assay. Serial dilutions of purified IgG or monoclonal antibody 4G2 (positive control) were incubated with DENV2/NGC for 1 h at 37 °C. The mixtures were added to pre-seeded K562 cells, followed by incubation at 37 °C for 2 days. Infected cells were stained with the Alexa Fluor 488-conjugated 4G2 antibody and then analyzed by flow cytometry. The data shown are representative results of two independent experiments.

program, a grant (2016YFC1201000) from the National Major R&D Program of China, grants (XDB29040300 and 153831KYSB20170043) from the Chinese Academy of Sciences, a grant (ZIKAlliance Grant Agreement 734548) from the European Union Horizon 2020 Research and Innovation program, a grant (81860367) from the National Natural Science Foundation of China, a grant (no. ZDYF2017091) from the Key Research and Development Plan of Hainan Province, Ministry of Science and Technology, and a grant (no. Hnky2017ZD-16) from the Higher Educational Scientific Research Project of Hainan Province, the Ministry of Education.

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