Vaccine 38 (2020) 3653–3664

Contents lists available at ScienceDirect

Vaccine

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

Homologous prime-boost with Zika virus envelope protein and poly (I:C) induces robust specific humoral and cellular immune responses

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ARTICLE INFO

Article history:
Received 14 December 2019
Received in revised form 18 March 2020
Accepted 19 March 2020
Available online 2 April 2020

Keywords:
Subunit vaccine
DNA vaccine
Prime-boost
Adjuvant
Envelope
Zika virus

ABSTRACT

The recent outbreaks of Zika virus (ZIKV) infection and the potential association with Guillain-Barré syndrome in adults and with congenital abnormalities have highlighted the urgency for an effective vaccine. The ZIKV Envelope glycoprotein (E_{ZIKV}) is the most abundant protein on the virus surface, and has been evaluated together with the pre-membrane protein (prM) of the viral coat as a vaccine candidate in clinical trials. In this study, we performed a head-to-head comparison of the immune response induced by different E_{ZIKV}-based vaccine candidates in mice. We compared different platforms (DNA, recombinant protein), adjuvants (poly (I:C), CpG ODN 1826) and immunization strategies (homologous, heterologous).

The hierarchy of adjuvant potency showed that poly (I:C) was a superior adjuvant than CpG ODN. While poly (I:C) assisted immunization reached a plateau in antibody titers after two doses, the CpG ODN group required an extra immunization dose. Besides, the administration of poly (I:C) induced higher E_{ZIKV}-specific cellular immune responses than CpG ODN. We also show that immunization with homologous prime-boost E_{ZIKV} protein + poly (I:C) regimen induced a more robust humoral response than homologous DNA (pVAX-E_{ZIKV}) or heterologous regimens (DNA/protein or protein/DNA). A detailed analysis of cellular immune responses revealed that homologous (E_{ZIKV} + poly (I:C)) and heterologous (pVAX-E_{ZIKV}/E_{ZIKV} + poly (I:C)) prime-boost regimens induced the highest magnitude of IFN-γ secreting cells and cytokine-producing $CD4^+$ T cells.

Overall, our data demonstrate that homologous E_{ZIKV} + poly (I:C) prime-boost immunization is sufficient to induce more robust specific-E_{ZIKV} humoral and cellular immune responses than the other strategies that contemplate homologous DNA (pVAX-E_{ZIKV}) or heterologous (pVAX-E_{ZIKV}/E_{ZIKV} + poly (I:C), and vice-versa) immunizations.

1. Introduction

Zika virus (ZIKV) is a Flavivirus first identified in 1947 in a febrile sentinel *rhesus* monkey in the Zika forest in Uganda [1]. The first reported case of human infection occurred in 1962 also in Uganda [2,3]. Since 2015, ZIKV spread worldwide and more than 80 countries have reported local transmission [4]. ZIKV infection during pregnancy has been linked to congenital malformations (cerebral calcification, microcephaly and miscarriage) [5,6], while in adults it is linked to Guillain-Barré syndrome (GBS) [7,8]. Transmission mainly occurs by the bite of virus-infected *Aedes* mosquitoes but non-vector-borne transmission (sexual, transfusional and vertical) has also been documented [9].

ZIKV has a 11 kb positive-sense single-stranded RNA (ssRNA) genome that encodes a unique polyprotein that is cleaved into three structural proteins (Capsid (C), pre-membrane/membrane (prM/M) and Envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) involved in virus replication and assembly [10]. The E protein mediates binding and fusion to the target cell receptors and contains three distinct domains (EDI, EDII and EDIII) [11]. Moreover, the E protein is the main target for neutralizing antibodies [12–14] that also confer

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https://doi.org/10.1016/j.vaccine.2020.03.037
0264-410X/

protection in animal infection models [15,16]. To date, several human and mouse monoclonal antibodies (mAbs) targeting the different domains of E_{ZIKV} were generated and inhibited infection both *in vitro* and *in vivo* [14,16–18]. In addition, passive transfer of E_{ZIKV}-specific mAbs reduced maternal-fetal transmission and mortality in mice [13]. Different groups have also recently addressed the fine binding of the neutralizing antibodies. Collins *et al.* have shown that antibodies targeting quaternary epitopes (that bind to E protein dimer on the virus surface) are responsible for high levels of neutralization [19]. Moreover, mAbs directed against other domains, especially EDIII, were also reported as having very high neutralization capacities [14].

Given the effectiveness of some vaccines against flaviviruses such as yellow fever virus (YFV) and Japanese encephalitis virus (JEV), the pursuit of an effective vaccine candidate against ZIKV has been subject of intensive research [20]. Several approaches are now being evaluated including inactivated [21]/ attenuated [22,23] ZIKV, recombinant proteins [24,25], DNA [26,27], RNA [28] and chimeric YFV [29] and JEV [30]. Recently, there has been a tendency to develop recombinant subunit vaccines since they are easily designed, safe and can be scaled up for production with a high degree of purity. The envelope is the most antigenic component in flaviviruses, and a promising candidate as part of a subunit vaccine [31], having already advanced to clinical trials [27,32,33].

Herein, to develop Zika subunit vaccine candidates, we generated a recombinant protein and a DNA vaccine based on the consensus sequence of the envelope protein (E_{ZIKV}) and evaluated its immunogenicity in mice using homologous and heterologous prime-boost regimens.

Our work demonstrated the induction of a robust immune response after different immunization strategies using E_{ZIKV} as a subunit vaccine candidate. This knowledge can contribute for the development of a safe and potent vaccine against ZIKV.

2. Materials and methods

2.1. Design of optimized E_{ZIKV} sequences

The consensus sequence for the ZIKV envelope protein (E_{ZIKV}), lacking the full stem and the transmembrane region (defined as amino acids 291-690 of the ZIKV polyprotein), was generated after alignment (ClustalW) of 69 Brazilian ZIKV isolate sequences (GenBank accession numbers available at Supplementary Table 1) and synthesized (GenScript, NJ). For the DNA vaccine, the gene included mammalian codon optimization, addition of Kozak sequence and immunoglobulin E (IgE) leader peptide sequence (MDWTWILFLVAAATRVHS). The gene was cloned into *HindIII* and *XhoI* sites of pVAX1 vector (Invitrogen). For recombinant protein production, the gene was codon optimized for expression in bacteria and cloned into the pET21a vector using *NheI* and *XhoI* sites. The plasmids were purified using Endofree Plasmid Giga Kit (Qiagen) according to the manufacturer's instructions.

2.2. E_{ZIKV} protein expression and purification

E_{ZIKV} recombinant protein was produced in BL21(DE3)RIL competent cells transformed with the pET21a-E_{ZIKV} plasmid. Bacteria were inoculated into 1 L of LB medium containing ampicillin (100 μg/mL) plus chloramphenicol (25 μg/mL) and grown (37 °C, 200 rpm) to an OD_{600nm} between 0.6 and 0.8. Then, protein expression was induced with isopropy-b-D-thiogalactoside (IPTG) 0.01 mM (Sigma) for 4 h at 37 °C and 200 rpm. Bacteria were then harvested (15 min, 4 °C and 5,900*g*), resuspended (Tris-HCl 100 mM, NaCl 500 mM, glycerol 15%, pH 8) and lysed in a high-pressure system (600 bar, 10 min, 4 °C, APLAB-10, ARTEPEÇAS-Brazil). After centrifugation (40 min, 4 °C, 17,000*g*), the bacterial pellet was resuspended (Tris-HCl 100mM, NaCl 500mM, Urea 8M and pH 8) overnight under constant stirring at 4 °C. Another centrifugation round (40 min, 4 °C, 17,000*g*) was performed to collect the supernatant. For protein refolding, the supernatant was slowly diluted (20x) in buffer (Tris-HCl 100 mM, NaCl 500 mM, glycerol 15%, pH 8 and 2-mercaptoethanol 20 mM). After a final centrifugation (50 min, 4 °C, 17,000*g*), supernatant was collected and the E_{ZIKV} protein was purified using Ni-Sepharose histidine-tagged resin (GE Healthcare) according to the manufacturer's instructions.

2.3. Mice and immunization

Female BALB/c mice (6–8 weeks old) were purchased from Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME) – UNIFESP. All mice were housed in a temperature-controlled, light-cycled facility at Division of Immunology – UNIFESP.

For recombinant protein immunization, mice received two or three doses, at 2-week intervals, with 10 μg of E_{ZIKV} in the presence of poly (I:C) (50 μg; Invivogen) or CpG ODN 1826 (10 μg; Invivogen) in a total volume of 200 μL delivered at the base of the tail. For DNA immunization, mice received two or three doses, at 2-week intervals, with 100 μg of pVAX-E_{ZIKV} in a volume of 100 μL delivered into the *tibialis* anterior muscle (50 μL each). Heterologous prime-boost regimen consisted of a DNA prime (pVAX-E_{ZIKV}) followed by one or two doses of recombinant E_{ZIKV} + poly (I:C), or vice-versa. The control groups received empty pVAX vector or poly (I:C) or CpG ODN 1826. Blood was collected by submandibular vein fourteen days after each dose and mice were euthanized two weeks after the last dose.

2.4. Western blot

Approximately 500 ng of recombinant E_{ZIKV} protein were submitted to SDS-PAGE gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes (Hybond-C extra nitrocellulose - GE Healthcare). Next, nitrocellulose membranes were blocked with PBS Tween 20 (PBST) (0.02% v/v), non-fat milk (5% w/v) and BSA (2.5% w/v) or PBS BSA (5% w/v), overnight at 4 °C. The membranes were washed 3x with PBST (0,05% v/v) and incubated with mouse (1:500) or human sera (ZIKV-infected patient or healthy individual) (1:500) for 2 h. After 3 washes with PBST (0.05% v/v) the membranes were incubated with horseradish peroxidase-labeled goat anti-mouse IgG (1:5000; KPL) or alkaline phosphatase AffinePure goat anti-human IgG (1:2000; Jackson ImmunoResearch) for 1 h. After 3 washes with PBST, the reaction was developed with a chemiluminescence detection system ECL (GE Healthcare) or NBT/BCIP (Thermo Fisher Scientific) according to manufacturer's instructions and analyzed by Alliance 4.7 software (Uvitec; Cambridge).

2.5. ELISA

ELISA plates (High binding, Costar) were coated overnight at room temperature with 100 ng/well of E_{ZIKV} protein diluted in 50 μL/well of carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). The following day, the plates were washed 3x with PBST (0.02% v/v) and blocked for 2 h with 150 μL of PBST, BSA (1% w/v) and non-fat milk (5% w/v). Plates were washed 3x with PBST and incubated for 2 h with serially diluted serum. Plates were washed 3x with PBST and incubated for 2 h with horseradish peroxidase-labeled goat anti-mouse IgG (1:5000; KPL). The plates were vigorously washed and the enzymatic reaction was developed by the addition of 1 mg/mL of o-phenylenediamine (Sigma) diluted in phosphate–citrate buffer, pH 5, containing 0.03% (v/v) hydrogen

peroxide. The enzymatic reaction was stopped by the addition of 50 μL of a solution containing 4 N H_2SO_4. Plates were read at 492 nm (OD_{492}) with an ELISA reader (EnSpire Multimode Plate Reader; PerkinElmer). The antibody titer was determined by the highest dilution between an OD_{492nm} of 0.1–0.2. ELISA to detect mouse IgG subclasses was performed as described above, except that the secondary antibodies were specific for mouse IgG1, IgG2a and IgG2b (1:4000; Southern Biotech). To evaluate antibody affinity, we first ran a standard ELISA to determine the serum dilution that gives an $OD_{492nm} \approx 1.0$. Then, a second ELISA was performed using the established serum dilution (in triplicate) to ensure the use of the same amount of antibodies from different groups. Following incubation with pooled sera, the wells were washed with PBST and incubated with 100 μL/well of PBS or increasing concentrations of ammonium thiocyanate (0–8 M). Plates were allowed to stand for 15 min at room temperature and then washed to proceed the assay. The percentage of affinity was determined as follows: (OD_{492nm} in the presence of ammonium thiocyanate treatment × 100)/OD_{492nm} in the presence of PBS.

2.6. Immunofluorescence assay (IFA)

For IFA analysis, ZIKV-infected Vero cells (MOI = 0.1) were added to multi-well glass slides (1×10^4/well) for 1 h at room temperature, fixed with acetone 80% (v/v) and incubated at −20 °C for 30 min. The cells were washed 3x with PBS and incubated for 30 min with primary antibody (mouse immune sera, 1:500). After a wash with PBS, goat anti-mouse IgG conjugated with FITC (1:750; Sigma) was added for 30 min. Slides were imaged using fluorescence microscopy (Olympus BX21) and the images were captured by cellSens software (Olympus Life Science).

2.7. Cell suspension

Fifteen days after the last dose, spleen and draining lymph nodes (DLNs) were aseptically removed. After obtaining single cell suspensions, cells were washed in 10 mL of RPMI 1640 (Gibco). Cells were then resuspended in R10 (RPMI supplemented with 10% of fetal bovine serum, 2 mM L-glutamine, 1% v/v vitamin solution, 1 mM sodium pyruvate, 1% v/v non-essential amino acids solution, 40 μg/mL of gentamicin, 5×10^{-5} M of 2-mercaptoethanol (all from Gibco) and 20 μg/mL of Cyprofloxacin (Ciprobacter, Isofarma). Cell concentration was estimated with the aid of a cell counter (Countess, Invitrogen) and adjusted in cell culture medium.

2.8. T cell ELISpot assay

ZIKV-specific T cell responses were assessed using IFN-γ ELISpot Ready-SET-Go! kit (eBiosciences). Briefly, 96-well plates (MAIPS 4510, Millipore) were coated with purified IFN-γ capture antibody and incubated overnight at 4 °C. The following day, the plates were washed with Coating buffer (eBiosciences) and blocked for 2 h with R10 at room temperature. Three hundred thousand splenocytes were added and stimulated, for 18 h at 37 °C in 5% CO_2, with E_{ZIKV} (5 μg/mL), Concanavalin A (ConA-2.5 μg/mL; positive control), or R10 (negative control). The plates were washed with PBST (0.05% v/v) and incubated with biotinylated anti-mouse IFN-γ for 2 h at room temperature. The plates were then washed 3 times with PBST and streptavidin-horseradish peroxidase was added and incubated for 45 min, at room temperature in the dark. After extensive washes with PBST and PBS, the reaction was developed by 3-amino-9-ethylcarbazole (AEC) (BD Biosciences) and the spots were counted using AID ELISpot Reader System (Autoimmun Diagnostika GmbH, Germany).

2.9. B cell ELISpot assay

The frequency of antigen-specific antibody secreting cells (ASC) was determined by ELISpot assay. Briefly, 96-well plates (MAIPS 4510, Millipore) were coated with E_{ZIKV} protein (100 ng/well) in PBS and incubated overnight at room temperature. Plates were washed 3x with PBS and blocked for 1 h with R10 at 37 °C and 5% CO_2. Five hundred thousand lymph nodes cells were added and incubated for 16 h at 37 °C and 5% CO_2. The plates were washed with PBS and incubated with horseradish peroxidase labeled goat anti-mouse IgG (1:1000; KPL) for 2 h at room temperature. After extensive washes with PBS, the reaction was developed by 3-amino-9-ethylcarbazole (AEC; BD Biosciences) and the spots were counted using AID ELISpot Reader System (Autoimmun Diagnostika).

2.10. Identification of T follicular helper (Tfh) cells and germinal center (GC) B cells by flow cytometry

DLNs single cell suspensions (2×10^6 cells) were stained with CXCR5-APC (clone 2G8) in FACS buffer (BSA 0.5% w/v and 2 mM EDTA in PBS) for 1 h in the dark at 37 °C. Next, cells were washed with FACS buffer and stained with CD4-Pacific Blue (clone RM4-5), B220-PerCP (clone RA3-6B2), CD279-PE (PD1, clone J43), GL7-FITC (clone GL7) and CD95-PE (clone Jo2), and incubated at 4 °C for 30 min in the dark. All monoclonal antibodies were purchased from BD Pharmingen. Cells were washed twice and resuspended in FACS buffer. Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences) and then analyzed using FlowJo software (version 9.9.4, Tree Star). Germinal center B cells were identified as $CD4^-B220^+GL7^+CD95^+$ and Tfh as $CD4^+B220^-PD1^+CXCR5^+$ population.

2.11. Flow cytometry analysis of E_{ZIKV}-specific $CD4^+$ T cell responses

To assess E_{ZIKV}-specific $CD4^+$ T cell proliferation and cytokine production, splenocytes were collected and labeled with carboxyfluorescein succinimidyl ester (CFSE), as previously described [34]. In summary, fresh splenocytes (50×10^6 cells/mL) were isolated, resuspended in PBS at 37 °C and labeled with 1.25 μM of CFSE (Molecular Probes) for 10 min at 37 °C. The reaction was quenched with R10 and cells were cultured in 96-well round-bottomed plates ($5x10^5$ cells/well, in triplicates) for 5 days at 37 °C and 5% CO_2 with medium only or E_{ZIKV} recombinant protein (5 μg/mL) or ConA (2.5 μg/mL). After 4 days, cells were re-stimulated with the same antigen in the presence of anti-CD28 (2 μg/mL) (BD Pharmigen) and Brefeldin A GolgiPlug™ (BD Pharmigen) for further 12 h. Then, cells were washed with FACS buffer and surface stained for 30 min on ice with the antibodies CD3-APCCy7 (clone 145-2C11) and CD4-PerCP (clone RM4-5) for 30 min. Cells were then fixed and permeabilized using Cytofix/Cytoperm™ kit (BD Pharmigen) according to the manufacturer's instructions. Next, cells were washed with Perm/Wash buffer (BD Pharmigen) and stained on ice with the antibodies TNFα-PECy7 (clone MP6-XT22) and IFNγ-APC (clone XMG1.2) for 30 min at 4 °C. Cells were washed twice and resuspended in FACS buffer. All antibodies were from BD Pharmigen. Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences) and then analyzed using FlowJo software (version 9.9.4, Tree Star). To analyze polyfunctional $CD4^+$ T cells, we used the Boolean gating platform (FlowJo software) to create combinations of the two cytokines (TNF-α and IFN-γ). To allow proper compensation, unstained and all single-color controls were performed.

2.12. Data analysis

Statistical significance (*p*-values) was calculated by One-way ANOVA followed by Tukey honestly significantly different (HSD) post hoc test or Two-way ANOVA followed by Bonferroni's post hoc test. Statistical analysis and graphical representation were performed using GraphPad Prism version 7.0 software.

3. Results

3.1. Synthetic development of Zika subunit vaccines

The consensus ZIKV envelope sequence for the DNA vaccine (pVAX-E_{ZIKV}) and the recombinant protein (E_{ZIKV}) were generated based on contemporary ZIKV sequences from 69 Brazilian isolates (Supplementary Table 1). In order to improve transgene product expression after DNA vaccine administration, the codon optimized gene also included a Kosak sequence and an immunoglobulin E (IgE) leader peptide sequence that has been demonstrated to enhance the stability of mRNA and promote efficient protein translation and secretion [35]. The recombinant E_{ZIKV} protein was purified by affinity chromatography (Supplementary Fig. 1a, with a yield of approximately 7 mg/L) and its antigenicity was evaluated by immunoblot. As expected, purified E_{ZIKV} protein was recognized by antibodies present in the serum of a Zika convalescent patient Supplementary Fig. 1b) showing that the recombinant protein retained its antigenic properties.

3.2. Poly (I:C) is superior to CpG ODN 1826 to induce ZIKV-specific cellular immune responses

The choice of a suitable adjuvant formulation is considered a critical issue for subunit vaccines since it can directly impact the immunogenicity of the formulation. Since poly (I:C) and CpG ODN are suitable for human use, we first tested if these two clinically relevant adjuvants could induce E_{ZIKV} specific immune responses. For this purpose, BALB/c mice were immunized three times with E_{ZIKV} recombinant protein in the presence of poly (I:C) or CpG ODN. After the first dose, no significant specific antibody response was detected in sera from any tested group (Fig. 1a). After the second dose, sera from the E_{ZIKV} + poly (I:C) group exhibited significantly higher IgG anti-E_{ZIKV} titers than E_{ZIKV} + CpG ODN 1826 group. On the other hand, after the third immunization, the group that received E_{ZIKV} + CpG ODN was able to produce an antibody response similar to the E_{ZIKV} + poly (I:C) group. In contrast, control groups did not produce specific antibodies. Both adjuvants induced IgG1, IgG2a and IgG2b, but the IgG1/IgG2a ratio was lower in the E_{ZIKV} + poly (I:C) group (Fig. 1b). These results indicate that

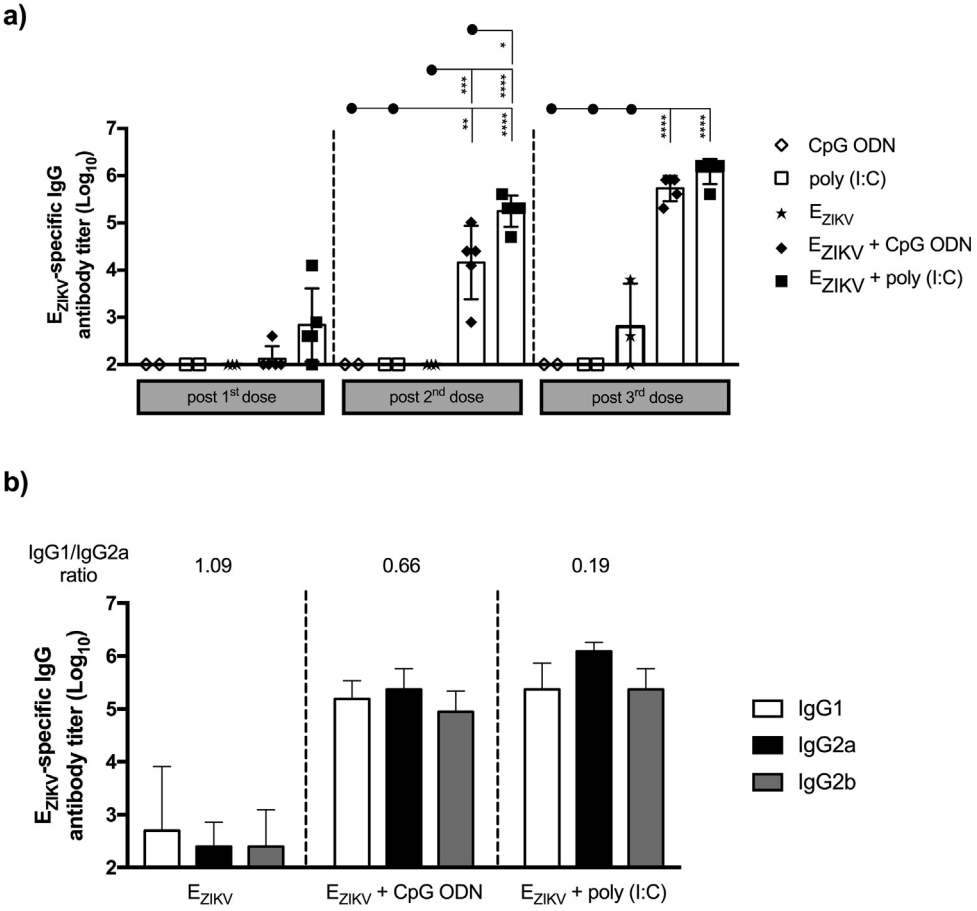

Fig. 1. Three doses of E_{ZIKV} recombinant protein combined with CpG ODN 1826 induced similar humoral response than poly (I:C) assisted immunization. BALB/c mice (n = 2 or 3 for control groups and n = 5 for experimental groups) were immunized (SC) three times with E_{ZIKV} combined with poly (I:C) or CpG ODN. Control groups received poly (I:C), CpG ODN 1826 or E_{ZIKV} in PBS. Mice were bled 14 days after each dose to evaluate specific humoral response by ELISA. (a) Total anti-E_{ZIKV} IgG antibody titers on a logarithmic scale (Log_{10}). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (b) Anti-E_{ZIKV} IgG subclasses antibody titers (Log_{10}) were evaluated 15 days after the last dose. Data represent mean ± SD and are representative of 2 independent experiments. SC: subcutaneous.

immunization with E_{ZIKV} in the presence of poly (I:C) or CpG ODN could induce strong and similar specific antibody responses. To extend the analysis, cellular immune responses were evaluated in secondary lymphoid organs after the last dose. Fig. 2 shows that poly (I:C) assisted vaccination produced higher numbers of specific IFN-γ (Fig. 2a) and antibody secreting cells (Fig. 2b). E_{ZIKV} + poly (I:C) combination also induced higher frequency of germinal center (GC) B cells and T follicular helper (Tfh) cells when compared to vaccination using CpG ODN 1826 as adjuvant (Fig. 2c and d, respectively).

These data supported the use of the poly (I:C) as a suitable adjuvant for strong humoral and cellular immune responses, allowing us to select the proper adjuvant and move on to the different immunization strategies.

3.3. Homologous and heterologous prime-boost immunizations with subunit vaccines induces potent specific humoral responses

The antigenicity and immunogenicity of the subunit vaccines were investigated in BALB/c mice as shown in Fig. 3a. BALB/c mice were immunized twice in a homologous prime-boost regimen with the DNA vaccine pVAX-E_{ZIKV} or the E_{ZIKV} recombinant protein in the presence of poly (I:C) (E_{ZIKV+} poly (I:C)). We also included two heterologous prime-boost groups, in which mice received a prime with pVAX-E_{ZIKV} and then a boost with E_{ZIKV} + poly (I:C) or vice-versa. Fig. 3b (lanes III-VI) shows that antibodies elicited after immunization with different strategies recognized specifically the recombinant E_{ZIKV} protein by western blot. Furthermore, immunofluorescence assay (IFA) showed that sera from immunized mice recognized ZIKV-infected cells (Fig. 3c; III-VI). In contrast, control mice immunized with empty pVAX vector or the adjuvant poly (I:C) alone did not produce specific antibodies capable to recognize E_{ZIKV} protein (Fig. 3b; I and II) or ZIKV-infected cells (Fig. 3c; I and II).

The antigen-specific humoral response was also analyzed by ELISA. After prime, only groups that received the recombinant E_{ZIKV} + poly (I:C) produced specific antibodies (Fig. 4a). After boost, sera from all groups immunized with the E_{ZIKV} subunit vaccines presented antigen-specific antibodies. However, a systematic comparison revealed that homologous E_{ZIKV} + poly (I:C) immunized mice presented the highest antibody titers. No significant difference was observed between the antibody titers of heterologous groups (pVAX-E_{ZIKV} prime/E_{ZIKV} + poly (I:C) boost, and vice-versa). As indicated in Fig. 4b, sera from heterologous E_{ZIKV} + poly (I:C)/pVAX-E_{ZIKV} group presented slightly higher affinity than the others. IgG subclasses profile analysis demonstrated the production of IgG1, IgG2a and IgG2b, although sera from mice that received the homologous E_{ZIKV} + poly (I:C) and heterologous pVAX-E_{ZIKV} prime/E_{ZIKV} + poly(I:C) boost presented lower IgG1/IgG2a ratio suggesting a Th1 biased immune response (Fig. 4c).

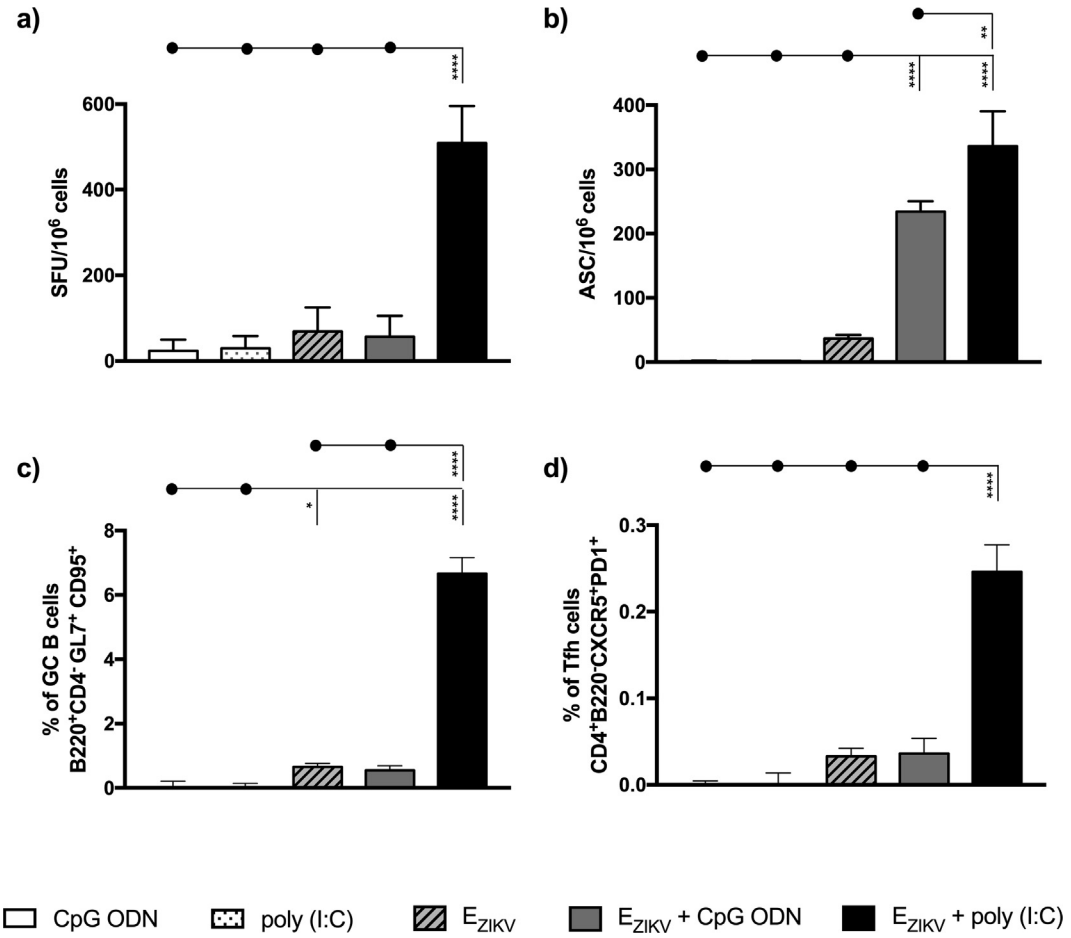

Fig. 2. Poly (I:C) is superior to CpG ODN 1826 to induce E_{ZIKV}-specific cellular immune responses. BALB/c mice were immunized as described in Fig. 1. (a) Fifteen days after the last dose, splenocytes were cultured in the presence of recombinant E_{ZIKV} for 18 h to evaluate the number of IFN-γ producing cells by ELISpot assay. SFU: spot forming units. (b) Cells from draining lymph nodes were placed in culture for 16 h to evaluate the number of E_{ZIKV}-specific antibody secreting cells (ASC) by ELISpot. Cells were also labeled with fluorescent antibodies to determine the frequency of (c) germinal center (GC) B cells ($CD4^-B220^+CD95^+GL7^+$) or (d) T follicular helper (Tfh) cells ($CD4^+B220^-PD1^+$-$CXCR5^+$). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Data represent mean ± SD and are representative of 2 independent experiments.

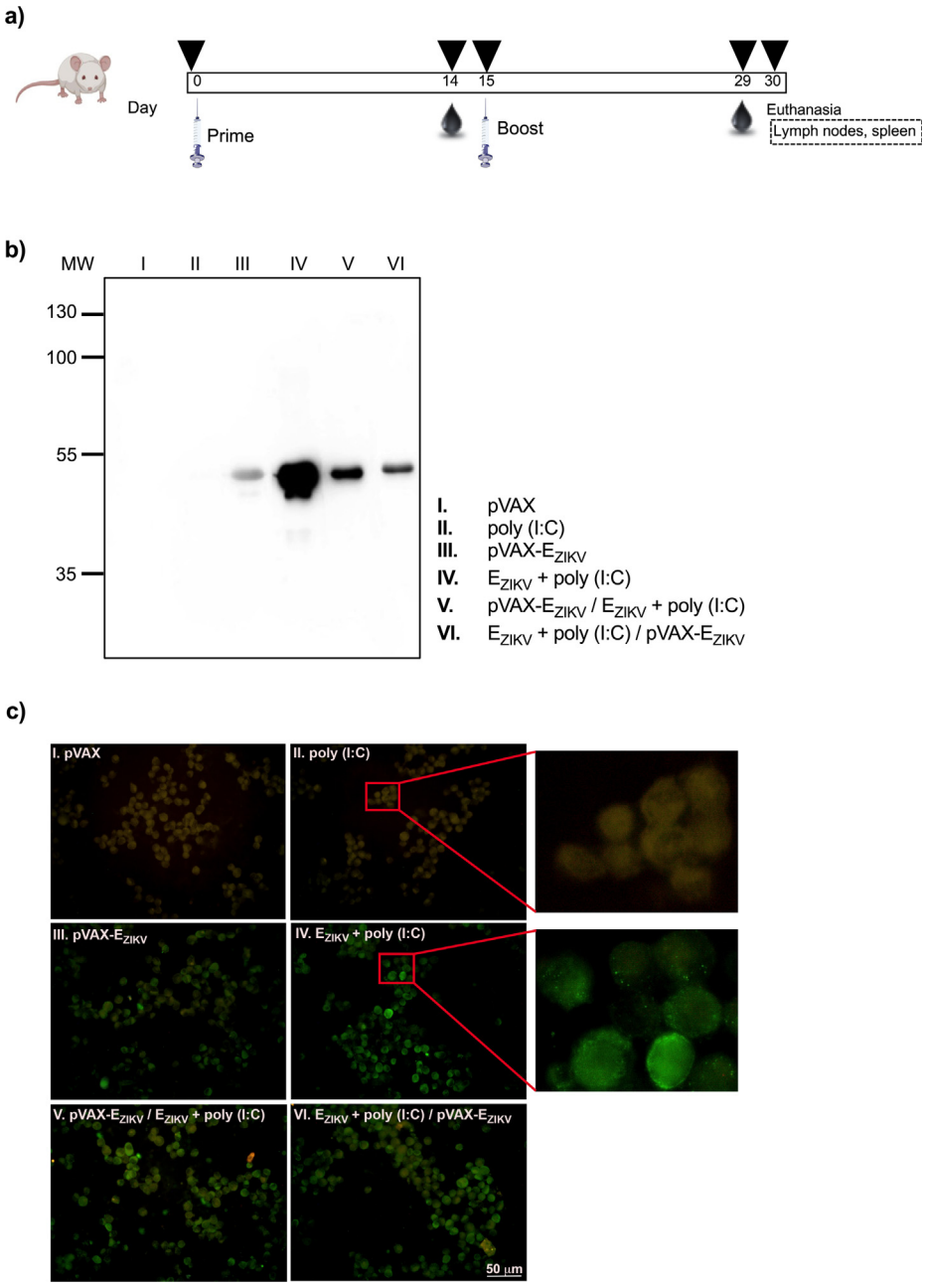

Fig. 3. Recognition of the recombinant E_{ZIKV} protein and ZIKV-infected cells by sera from immunized mice. (a) Immunization strategy. BALB/c mice (n = 2 for control groups and n = 5 for experimental groups) were immunized twice with 100 µg of the DNA vaccine pVAX-E_{ZIKV} (IM) or 10 µg of the E_{ZIKV} combined with 50 µg poly (I:C) (SC). For the heterologous protocol, mice first received 100 µg of the DNA vaccine pVAX-E_{ZIKV} (IM) followed by 10 µg of the E_{ZIKV} combined with 50 µg poly (I:C) (SC), or vice-versa. Control groups received empty pVAX or poly (I:C) alone. Fourteen days after the last dose, mice sera were used to evaluate specific recognition of **(b)** recombinant E_{ZIKV} through western blot or **(c)** ZIKV-infected Vero cells (MOI = 0.1). For the IFA assay, mice sera and goat anti-mouse IgG-FITC were used as primary and secondary antibodies, respectively. I: pVAX; II: poly (I:C); III: pVAX-E_{ZIKV}; IV: E_{ZIKV} + poly (I:C); V: pVAX-E_{ZIKV}/E_{ZIKV} + poly (I:C); VI: E_{ZIKV} + poly (I:C)/ pVAX-E_{ZIKV}. MW: Molecular weight in kDa; IM: intramuscular; SC: subcutaneous.

3.4. Subunit recombinant E_{ZIKV} in the presence of poly (I:C) induces robust cellular immune responses

Cell-mediated immunity was assessed in secondary lymphoid organs, 15 days after the boost using flow cytometry and enzyme-linked immunospot (ELISpot). Fig. 5a shows that splenocytes from mice that received the homologous E_{ZIKV} + poly (I:C) presented higher number of specific IFN-γ producing T cells than both heterologous prime-boost regimens. On the other hand, homologous pVAX-E_{ZIKV} immunization induced weaker T cell ELISpot responses. We next assessed B cell recall responses by ELIspot and the homologous E_{ZIKV} + poly (I:C) group displayed higher number of specific antibody secreting cells than all the other groups (Fig. 5b). The magnitude of induced B cell responses after vaccination with homologous E_{ZIKV} + poly (I:C) was higher than all other groups. In fact, immunophenotyping of lymph nodes cells revealed higher frequencies of GC B cells (Fig. 5c) and Tfh (Fig. 5d) in mice that received homologous E_{ZIKV} + poly (I:C) immunization when compared to the other groups.

Given the fundamental role of CD4 T cell help to promote B cell memory and antibody affinity maturation [36], we further analyzed the functional profile of E_{ZIKV} specific $CD4^+$ T cells. We

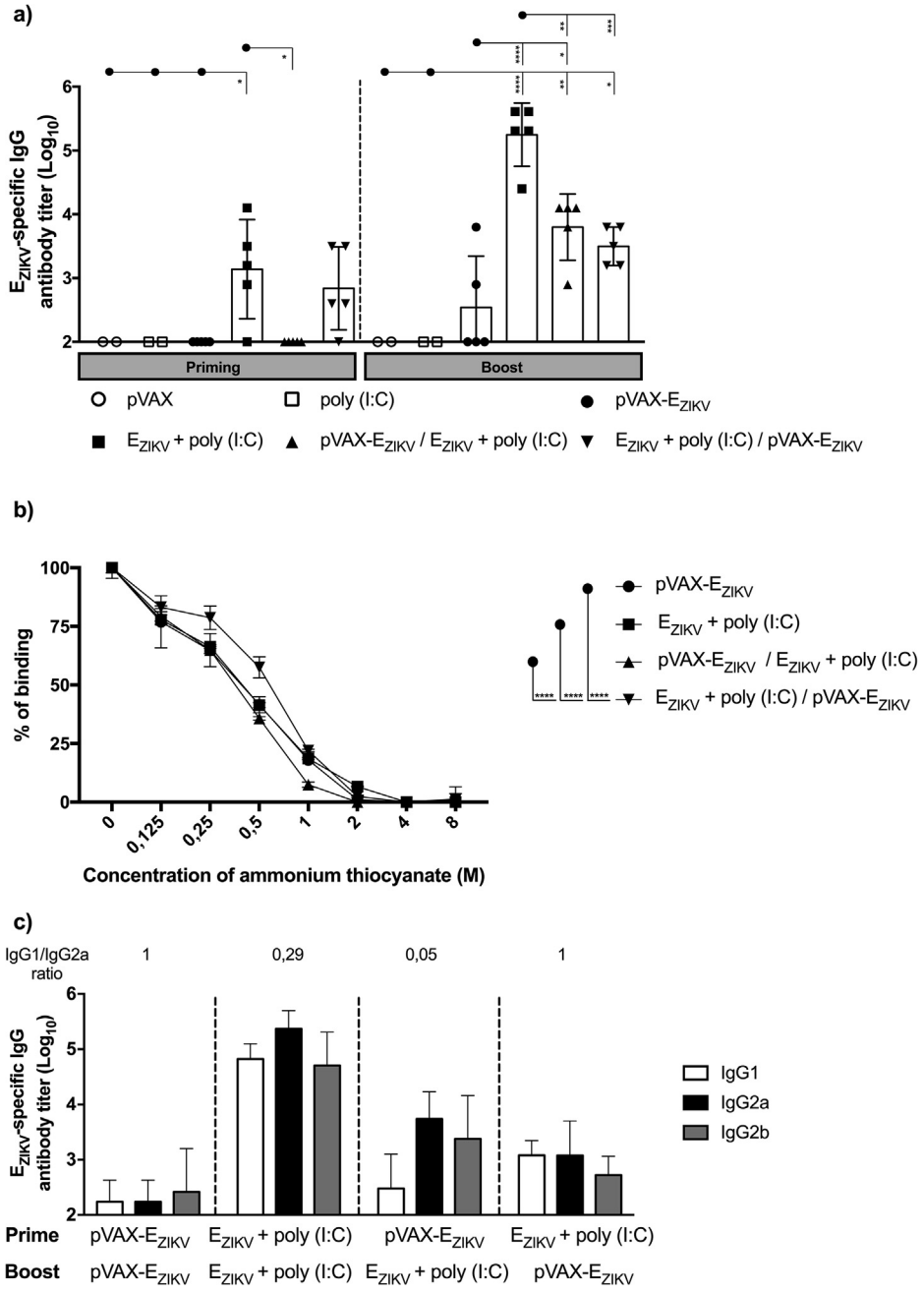

Fig. 4. Specific humoral immune response elicited after homologous and heterologous immunization with pVAX-E_{ZIKV} and/or E_{ZIKV} recombinant protein. BALB/c mice (n = 2 for control groups and n = 5 for experimental groups) were immunized as described in Fig. 3. Mice were bled 14 days after each dose to evaluate specific humoral response by ELISA. **(a)** Total anti-E_{ZIKV} IgG antibody titers on a logarithmic scale (Log_{10}). Statistical significance was measured by One-way ANOVA followed by Tukey's post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; **(b)** Antibody affinity was determined by ELISA treatment using increasing concentrations of ammonium thiocyanate. Statistics were performed with 0.5 M ammonium thiocyanate treatment. ****p < 0.0001 **(c)** Anti-E_{ZIKV} IgG subclasses antibody titers (Log_{10}) were evaluated 14 days after the last dose. Data represent mean ± SD and are representative of 3 independent experiments. IM: intramuscular; SC: subcutaneous.

assessed T cell proliferation and cytokine production after *in vitro* recall with recombinant E_{ZIKV}. The flow cytometry profile demonstrated that immunization induced T cell responses in an antigen-specific way in the $CD4^+$ compartment (Fig. 6). The magnitude of $CD4^+$ T cell proliferation was similar among groups that received homologous or heterologous vaccination but higher than control groups (Fig. 6a). Boolean analysis was used to determine the ability of single $CD4^+$ T cells to produce IFN-γ and TNF-α. Homologous E_{ZIKV} + poly (I:C) group displayed higher frequency of $CD4^+$ T cells that produced IFN-γ and TNF-α simultaneously (IFN-γ^+/TNF-α^+) when compared to other groups (Fig. 6b). In addition, pVAX-E_{ZIKV} prime/E_{ZIKV} + poly (I:C) boost vaccination induced higher frequency of IFN-γ^+/TNF-α^+ $CD4^+$ T cells than E_{ZIKV} + poly (I:C) prime/pVAX-E_{ZIKV} boost or homologous pVAX-E_{ZIKV}. Analysis of single cytokine producing cells revealed that homologous E_{ZIKV} + poly (I:C) and heterologous groups present similar frequencies of single IFN-γ^+ or TNF-α^+ producing $CD4^+$ T cells. The homologous pVAX-E_{ZIKV} presented lower frequency of double positive and single positive cytokine producing $CD4^+$ T cells. Control groups responses were negligible.

Altogether, these results demonstrate that two doses of E_{ZIKV} recombinant protein along with poly (I:C) induces superior

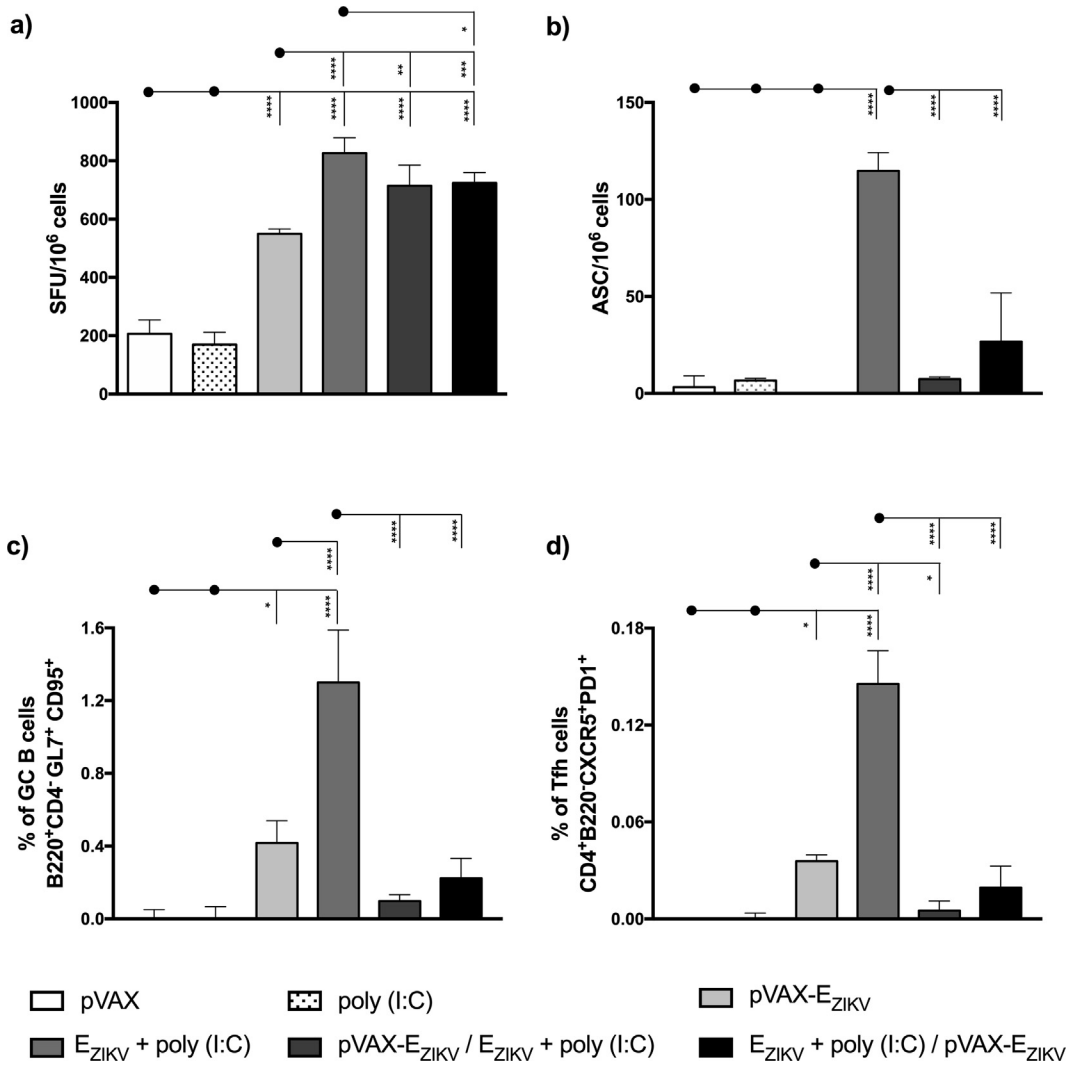

Fig. 5. Properties of E_{ZIKV}-specific cellular immune response after homologous or heterologous regimens. BALB/c mice were immunized as in Fig. 3. (a) Fifteen days after the boost, the splenocytes were cultured in the presence of recombinant E_{ZIKV} for 18 h to evaluate the number of IFN-γ producing cells by ELISpot assay. SFU: spot forming units. (b) Cells from draining lymph nodes were placed in culture for 16 h to evaluate the number of E_{ZIKV}-specific antibody secreting cells (ASC) by ELISpot; labeled with fluorescent antibodies to determine the frequency of (c) germinal center (GC) B cells ($CD4^-B220^+CD95^+GL7^+$) or (d) T follicular helper (Tfh) cells ($CD4^+B220^-PD1^+CXCR5^+$). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data represent mean ± SD and are representative of 3 independent experiments.

E_{ZIKV}-specific humoral and cellular immune responses than other immunization strategies.

3.5. An additional dose in the heterologous regimen enhances specific humoral responses

We next sought to evaluate if an additional vaccine dose would increase the specific immune responses in heterologous prime-boost groups. For this purpose, BALB/c mice were immunized three times in the homologous regimen with E_{ZIKV} + poly (I:C) or pVAX-E_{ZIKV}, while the heterologous groups received a prime with pVAX-E_{ZIKV} followed by two boosts with E_{ZIKV} + poly (I:C), or vice-versa (Fig. 7a). After a third dose, vaccination with homologous E_{ZIKV} + poly (I:C) induced specific IgG anti-E_{ZIKV} titers higher than both heterologous groups (Fig. 7b). However, the antibody titers of the heterologous pVAX-E_{ZIKV} prime/E_{ZIKV} + poly (I:C) boost group reached similar levels than homologous E_{ZIKV} + poly (I:C). This was probably due to the second E_{ZIKV} + poly (I:C) boost, indicating that two doses of E_{ZIKV} + poly (I:C) are sufficient to induce a robust E_{ZIKV}-specific immune response. Although mice immunized with the heterologous E_{ZIKV} + poly (I:C)/pVAX-E_{ZIKV} displayed an increase in anti-E_{ZIKV} IgG titers after the additional dose, it was still lower than the previous two groups. Homologous pVAX-E_{ZIKV} group was not significantly different from its pVAX control group. Antibody affinity remained slightly higher in the E_{ZIKV} + poly (I:C)/pVAX-E_{ZIKV} immunized group, followed by the homologous E_{ZIKV} + poly (I:C) and heterologous pVAX-E_{ZIKV}/E_{ZIKV} + poly (I:C) group. Interestingly, the homologous pVAX-E_{ZIKV} group presented significant lower affinity than the other platforms (Fig. 7c). The IgG1/IgG2a ratio was below 1 in all immunized groups, suggesting Th1 biased responses (Fig. 7d).

4. Discussion

The recent spread of ZIKV infection worldwide together with its associated neurological morbidity in newborn and adults highlighted the urgency of an effective vaccine. Synthetic subunit vaccines offer several advantages over traditional vaccine approaches, with safety being the most striking. In this study, we describe the development of synthetic subunit vaccine candidates based on E_{ZIKV} consensus sequence and analyze the specific humoral and cellular immune responses upon immunization. The vaccine

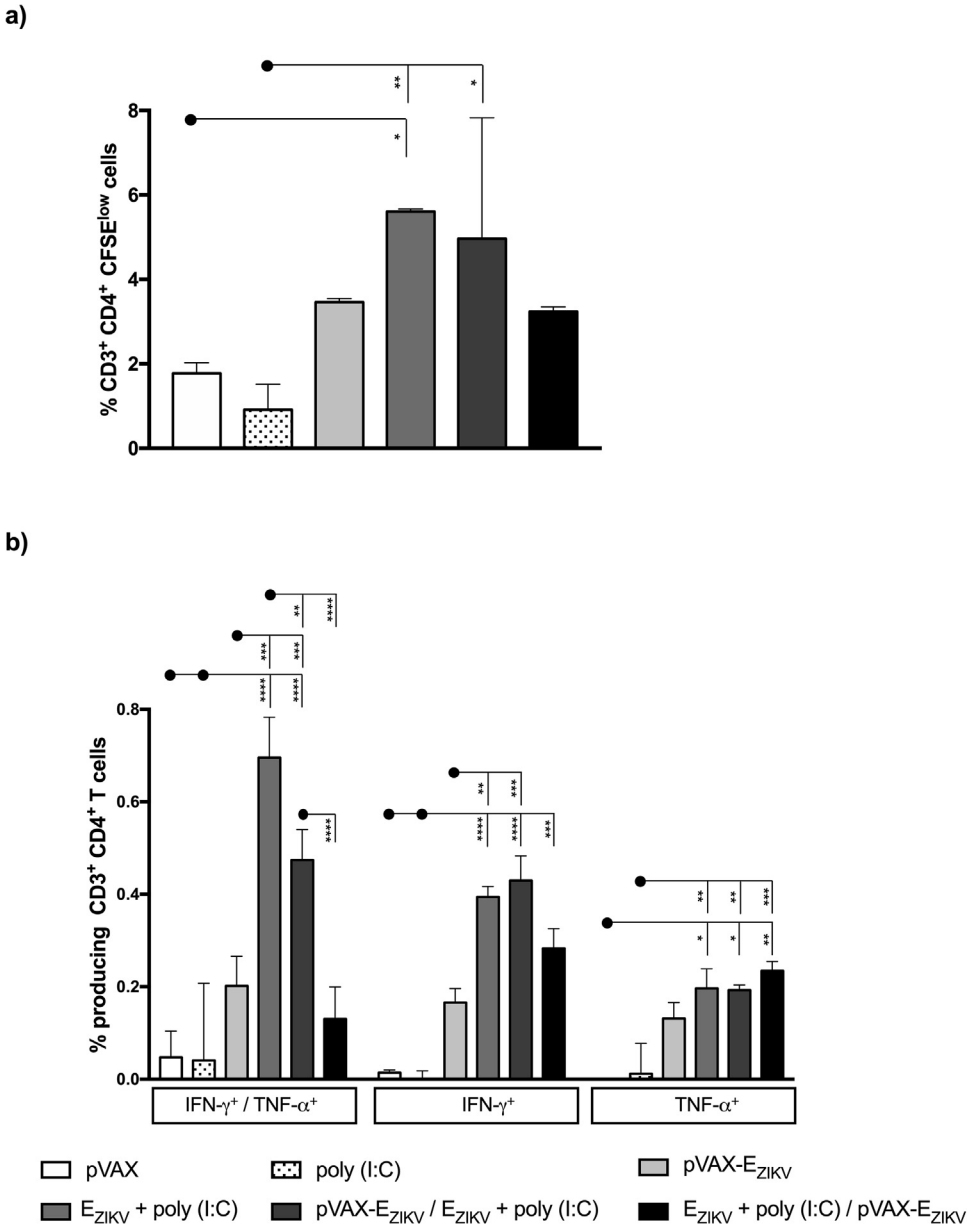

Fig. 6. Homologous and heterologous prime-boost immunization induces ZIKV CD4$^+$ T cell responses. BALB/c mice were immunized as in Fig. 3. Fifteen days after the last dose, the splenocytes were labeled with CFSE (1.25 μM) and cultured in the presence of E_{ZIKV} to evaluate specific proliferation and cytokine production by multiparameter flow cytometry. (a) CFSE dilution on gated CD3$^+$ CD4$^+$ cells was used as readout for antigen-specific proliferation. The frequency of proliferating CD4$^+$ CFSE^{low} cells was determined in the CD3$^+$ T cell population. The frequency of proliferating T cells was calculated by subtracting the values of stimulated from non-stimulated cultures. *p < 0.05, **p < 0.01. (b) For intracellular cytokine detection, cells were pulsed for the last 12 h with E_{ZIKV}, anti-CD28 and brefeldin A. Boolean combinations were created using FlowJo software to determine the frequency of each response based on all possible combinations of cytokine-producing CD3$^+$ CD4$^+$ T cells. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data represent mean ± SD and are representative of 3 independent experiments.

platforms include a bacteria expressed recombinant protein and a plasmid DNA that was optimized for increased transgene expression [35,37]. Bacterial systems are often used for the production of subunit vaccines because of the high yields obtained. Nonetheless, expression in some eukaryotic systems, like insect cells, may yield comparable or even higher protein levels, albeit with higher costs. In fact, immunization with eukaryotic or prokaryotic E_{ZIKV} expressed proteins did not show marked differences in protection [38]. A central feature for subunit vaccine development is the choice of a proper adjuvant as it can impact both specific humoral and cellular immune responses [39]. In the past years, different microbial compounds have been characterized and used as adjuvants to enhance the magnitude and modulate immune responses [40]. Polyinosinic:polycytidylic acid (poly (I:C)) is a synthetic analog of viral double-stranded RNAs (dsRNAs) that activates TLR3 and RIG-I-like receptors (retinoic acid-inducible gene -I- like receptors, or RLRs) [41]. On the other hand, CpG oligodeoxynucleotides (ODN) are unmethylated CpG motifs that are sensed by TLR9 and induce pro-inflammatory cytokine production by dendritic cells [42]. Multiple clinical trials were conducted and CpG ODN emerged as a potent adjuvant to induce high antibody titers [43]. Likewise, poly (I:C), and its derivative poly ICLC, have been successfully used in clinical trials as cancer vaccine adjuvants [44]. The promising results obtained so far suggest that poly ICLC may be a feasible adjuvant for use in cancer immunotherapy. Our results demonstrated a robust humoral response after the boost dose with

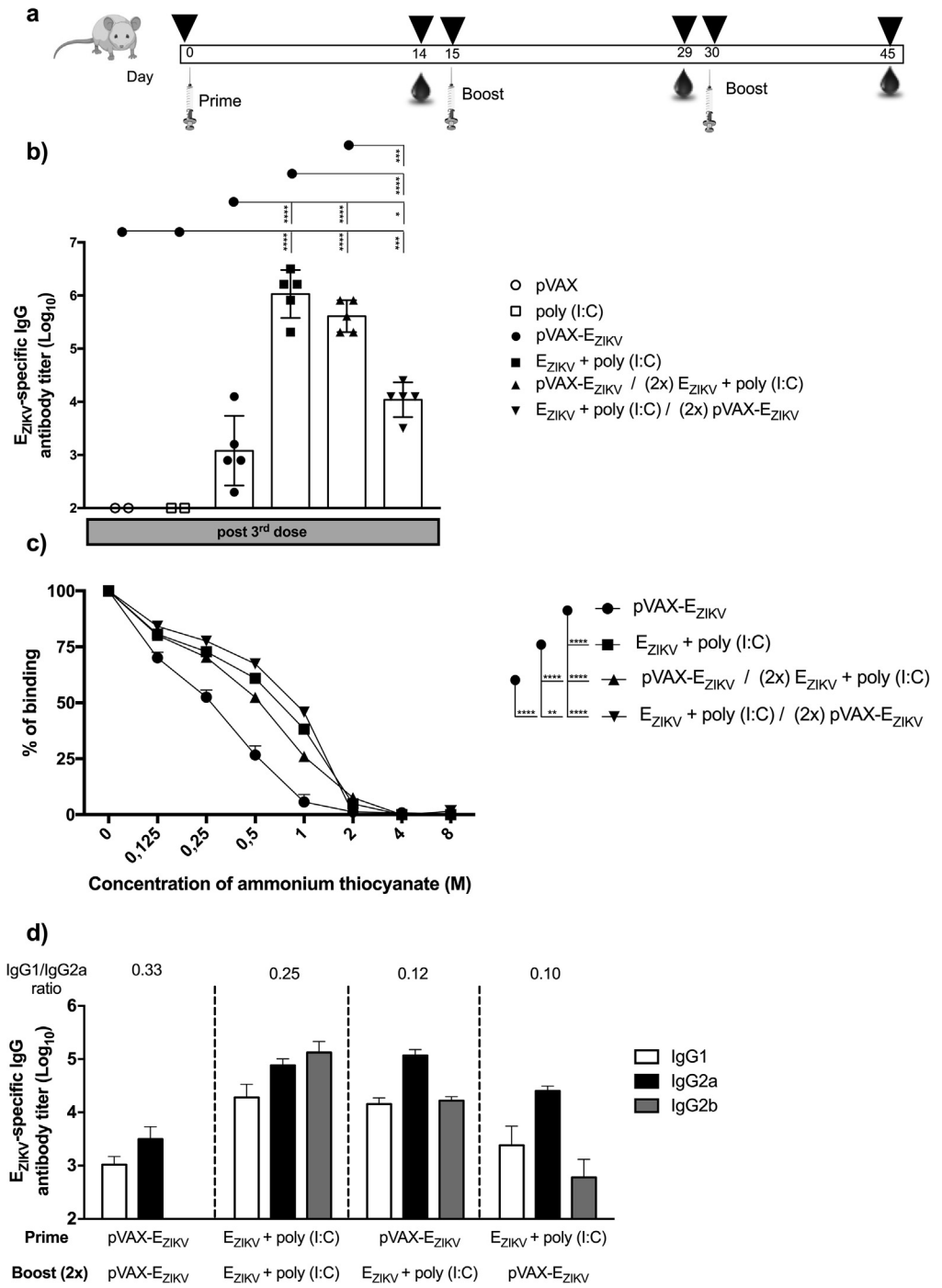

Fig. 7. An additional dose in the heterologous pVAX-E_{ZIKV}/E_{ZIKV} + poly (I:C) regimen enhances specific humoral response to similar potency than homologous E_{ZIKV} + poly (I:C). (a) BALB/c mice (n = 2 for control groups and n = 5 for experimental groups) were immunized three times with 100 μg of the DNA vaccine pVAX-E_{ZIKV} (IM) or 10 μg of the E_{ZIKV} combined with 50 μg poly (I:C) (SC). For the heterologous protocol, mice first received 100 μg of the DNA vaccine pVAX-E_{ZIKV} (IM) followed by two doses of 10 μg of the E_{ZIKV} combined with 50 μg poly (I:C) (SC), or vice-versa. Mice were bled 14 days after each dose to evaluate specific humoral response by ELISA. (b) Total anti-E_{ZIKV} IgG antibody titers on a logarithmic scale (Log_{10}). (c) Antibody affinity was determined by ELISA treatment using increasing concentrations of ammonium thiocyanate. Statistics were performed with 0.5 M ammonium thiocyanate treatment. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (d) Anti-E_{ZIKV} IgG subclasses antibody titers (Log_{10}) were evaluated 15 days after the last dose. Data represent mean ± SD and are representative of 2 independent experiments. IM: intramuscular; SC: subcutaneous.

E_{ZIKV} + poly (I:C) that did not increase significantly after an additional dose. On the other hand, CpG ODN 1826 assisted immunization only reached similar antibody levels after the third dose. Moreover, immunization with E_{ZIKV} + poly (I:C) increased the magnitude of IFN-γ and antibody secreting cells, and the frequency of GC B cells and Tfh when compared to the administration of the protein in the presence of CpG ODN 1826. This result clearly highlights that the selection of a suitable adjuvant may be beneficial for completion of a vaccine schedule, as the number of doses could be decreased to achieve the desired immune response. High and protective anti-ZIKV antibody titers were observed after immunization with bacteria-expressed truncated E_{ZIKV} protein in the presence of aluminum salt (alum) [24]. Likewise, C57BL/6 mice immunized with prokaryotic expressed $EDIII_{ZIKV}$ plus the adjuvants alum or TiterMax Gold presented protective humoral immunity [45]. Recently, no significant differences in immunogenicity nor

in protection were observed after vaccination with recombinant E_{ZIKV} protein expressed in eukaryotic when compared to prokaryotic system [38].

In the first set of prime-boost regimens using DNA and/or recombinant protein, we compared the immunogenic properties of both subunit ZIKV vaccine candidates. BALB/c mice were immunized with homologous (two doses of the DNA vaccine pVAX-E_{ZIKV} or two doses of the recombinant protein E_{ZIKV} + poly (I:C)) or heterologous (pVAX-E_{ZIKV}/E_{ZIKV} + poly (I:C), and vice-versa) prime-boost regimens. The homologous E_{ZIKV} + poly (I:C) immunization induced the most potent humoral response when compared to other strategies. A previous report demonstrated that immunization with virus-like-particles (VLP) containing EDIII from ZIKV in the presence of poly (I:C) induced a strong humoral response in mice [25]. Despite the fact that our DNA vaccine pVAX-E_{ZIKV} was not able to induce robust specific humoral response compared to the other strategies, successful pre-clinical studies with E_{ZIKV}-expressing DNA vaccines were reported in mice and nonhuman primates [15,21,26,28,46]. Notably, these DNA vaccines not only express the E_{ZIKV} gene but also the pre-membrane (prM) sequence from ZIKV [15,26,47,48]. Since the prM_{ZIKV} plays an important role in the folding of the E_{ZIKV} protein and also in allowing virion release, this extra prM sequence in the DNA vaccine may enhance its gene expression and proper secretion [49]. Furthermore, it is well known that an effective DNA vaccine requires optimal plasmid uptake that can be achieved, for example, by electroporation [50]. If electroporation will be able to potentiate the immune response in our DNA vaccine pVAX-E_{ZIKV}, remains to be addresed.

While antibodies are the primary correlates of protection against ZIKV infection, T cell immunity also plays a role in controlling virus replication [51]. A previous report demonstrated that depletion or genetic absence of $CD8^+$ T cells during ZIKV infection enhanced mortality in mice [52]. Furthermore, DENV specific $CD8^+$ T cells can induce cross-protection against ZIKV infection [53], including during pregnancy [54]. Recently, it was elegantly shown a pivotal role for $CD4^+$ T cells and IFN-γ signaling in protection during Zika virus infection [55]. Transfer experiments revealed that $CD4^+$ T cells are necessary to protect against lethal ZIKV challenge [56]. $CD4^+$ T cells participate in the generation of protective immunity since their depletion reduced the generation of anti-ZIKV antibodies [57,58] and cross-reactive $CD8^+$ T cell responses [54]. Moreover, in a mouse model of neuroinvasive ZIKV infection, the absence of $CD4^+$ T cells generates more neurological sequela and increased viral titers in the central nervous system [56]. The presence of polyfunctional $CD4^+$ T cell responses is also implicated in protection against flavivirus infections (JEV) [59], and is a hallmark after effective YFV vaccination [60,61]. Indeed, a detailed evaluation of T cell responses demonstrated that two doses of the homologous (E_{ZIKV} + poly (I:C)) and heterologous (pVAX-E_{ZIKV}/E_{ZIKV} + poly (I:C)) prime-boost regimens induced the highest magnitude of IFN-γ secreting cells and frequency of cytokine-producing $CD4^+$ T cells.

Collectively, our observations demonstrated that homologous protein- or heterologous DNA/protein-based regimens induced ZIKV-specific humoral and cellular immune responses. However, it will be important to determine the efficacy of the subunit vaccine candidates in future challenge experiments.

5. Conclusions

In summary, our study performed a head-to-head comparison of the quality of the immune response induced by different vaccine platforms (DNA, recombinant protein), adjuvants (poly (I:C) vs CpG ODN 1826) and regimens (homologous vs heterologous). Overall, our findings demonstrate that homologous E_{ZIKV} + poly (I:C) prime-boost immunization is sufficient to induce robust and E_{ZIKV}-specific humoral and cellular immune responses than the other strategies that contemplate homologous DNA (pVAX-E_{ZIKV}) or heterologous (pVAX-E_{ZIKV}/E_{ZIKV} + poly (I:C), and vice-versa) candidates.

6. Ethics statement

All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) (#5759150416) and carried out in accordance with the recommendations of the Federal Law 11.794 (2008), the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (CONCEA) and the ARRIVE guidelines.

Funding

This research was supported by São Paulo Research Foundation [FAPESP, grant number 2017/17471-7], the Brazilian National Research Council (CNPq)/Institute for Investigation in Immunology [CNPq, grant number 465434/2014-2] and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior [CAPES, Finance Code 001]. JSA, VASL, FCC, ERF, NT, SBB and DSR received fellowships from CNPq/FAPESP. MPA and HFSS received fellowships from CAPES.

CRediT authorship contribution statement

Marcelo Pires Amaral: Investigation, Methodology, Formal analysis, Validation, Writing - original draft, Visualization. **Juliana de Souza Apostolico:** Investigation, Methodology, Validation, Visualization, Formal analysis. **Nádia Tomita:** Investigation, Validation. **Fernanda Caroline Coirada:** Investigation. **Victória Alves Santos Lunardelli:** Investigation. **Edgar Ruz Fernandes:** Investigation. **Higo Fernando Santos Souza:** Investigation. **Renato Mancini Astray:** Resources. **Silvia Beatriz Boscardin:** Conceptualization, Methodology, Resources, Writing - review & editing. **Daniela Santoro Rosa:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Acknowledgments

We thank Mr. Geová Santos for assistance in the animal facility.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2020.03.037.

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