



## Prime-boost with Chikungunya virus E2 envelope protein combined with Poly (I:C) induces specific humoral and cellular immune responses

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### ABSTRACT

Chikungunya virus (CHIKV) is an arbovirus transmitted to humans mainly by the bite of infected *Aedes aegypti* and *Aedes albopictus* mosquitoes. CHIKV illness is characterized by fever and long-lasting arthritic symptoms, and in some cases it is a deadly disease. The CHIKV envelope E2 (E2<sub>CHIKV</sub>) glycoprotein is crucial for virus attachment to the cell. Furthermore, E2<sub>CHIKV</sub> is the immunodominant protein and the main target of neutralizing antibodies. To date, there is no available prophylactic vaccine or specific treatment against CHIKV infection. Here, we designed and produced a DNA vaccine and a recombinant protein containing a consensus sequence of E2<sub>CHIKV</sub>. C57BL/6 mice immunized twice with the E2<sub>CHIKV</sub> recombinant protein in the presence of the adjuvant Poly (I:C) induced the highest E2<sub>CHIKV</sub>-specific humoral and cellular immune responses, while the immunization with the homologous DNA vaccine pVAX-E2<sub>CHIKV</sub> was able to induce specific IFN- $\gamma$  producing cells. The heterologous prime-boost strategy was also able to induce specific cellular and humoral immune responses that were, in general, lower than the responses induced by the homologous E2<sub>CHIKV</sub> recombinant protein immunization. Furthermore, recombinant E2<sub>CHIKV</sub> induced the highest titers of neutralizing antibodies. Collectively, we believe this is the first report to analyze E2<sub>CHIKV</sub>-specific humoral and cellular immune responses after immunization with E2<sub>CHIKV</sub> recombinant protein and DNA pVAX-E2<sub>CHIKV</sub> vaccine platforms.

### 1. Introduction

Chikungunya virus (CHIKV) was first isolated from an infected patient in Tanzania (Africa) in 1952–1953 (Robinson, 1956), although it has been suggested that the infection existed even before, being misdiagnosed as Dengue (Carey, 1971). CHIKV illness was considered a sporadic disease until the 2000s, when several CHIKV outbreaks occurred, and the disease spread to the Americas (Silva and Dermody, 2017). A recent report from the Center for Disease Control and Prevention (CDC) identified CHIKV transmission in more than 100 countries and territories from all continents (Center for Disease Control, 2020). CHIKV transmission occurs mainly by the bite of virus-infected *Ae. aegypti* or *Ae. albopictus* mosquitoes (Lounibos and Kramer, 2016). Usually, CHIKV

infection is not fatal, but it causes very painful and uncomfortable clinical manifestations. About 90% of the infected patients report acute fever and severe joint pain that can last for years. Nevertheless, 3.8–27.8% of the infected patients are asymptomatic (Thiberville et al., 2013).

CHIKV is an alphavirus from the *Togaviridae* family, with a single serotype and 4 lineages: West-African, East-Central-South-African (ECSA), Asian, and Indian Ocean Lineage (IOL) (Volk et al., 2010). The West-African strain is restricted to Africa, while the ECSA strain is observed not only in Africa but also in the Americas. Furthermore, the Asian lineage is observed in America and Asia, while the IOL emerged in Europe and territories surrounded by the Indian Ocean (Weaver and Lecuit, 2015). In Brazil, the Asian and ECSA strains circulate, entering the country independently by Oiapoque and Feira de Santana cities, respectively (Nunes et al., 2015).

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CHIKV has an approximately 12 kb positive-sense single-stranded RNA (ssRNA) genome that encodes non-structural (nsP1, nsP2, nsP3 and nsP4) and structural proteins from the capsid and envelope (capsid (C), E1, E2, E3 and 6 K). E1 and E2 interact with each other forming 80 heterodimers spikes on the virus surface, essentially for the attachment and membrane fusion to the host cell. E3, a small peptide, mediates the folding and association of E1 and E2. The C protein protects the viral genome integrity. The 6 K cleavage product seems to play a role during infection, budding and assembly of the CHIKV (Thiberville et al., 2013).

Several studies have shown the importance and the immunodominance of the E2 protein. In infected patients, E2 is the main target of specific antibodies throughout the course of infection (Kam et al., 2012a, 2012b). Human and murine neutralizing monoclonal antibodies targeting the E2 protein are able to block fusion, binding and/or entry of the virus into the target cell (Smith et al., 2015; Jin et al., 2015). In addition, passive transfer of anti-E2 antibodies from convalescent patients is sufficient to reduce or even to eliminate CHIKV infection in adult and neonatal mice (Fox et al., 2015; Selvarajah et al., 2013). Furthermore, most of the subunit vaccines candidates for CHIKV that have entered clinical trials are based in the structural envelope proteins (Reyes-Sandoval, 2019).

Here we tested two different vaccine platforms expressing the Brazilian E2 CHIKV (E2<sub>CHIKV</sub>) consensus sequence in different adjuvant formulations and immunization regimens. C57BL/6 mice immunized twice with the recombinant E2<sub>CHIKV</sub> protein in the presence of the adjuvant Poly (I:C), rather than CpG ODN 1826 or Imiquimod R837, exhibited the highest E2<sub>CHIKV</sub>-specific antibody titer and cellular response. Mice immunized twice with homologous DNA vaccine pVAX-E2<sub>CHIKV</sub> had undetectable anti-E2<sub>CHIKV</sub> IgG titers, but induced specific IFN- $\gamma$  producing cells. However, when mice were vaccinated with the heterologous regimen, first with the DNA vaccine pVAX-E2<sub>CHIKV</sub> and then with the recombinant E2<sub>CHIKV</sub> + Poly (I:C), they were also able to induce specific humoral and cellular immune responses albeit with lower magnitude than homologous E2<sub>CHIKV</sub> protein + Poly (I:C). In addition, recombinant E2<sub>CHIKV</sub> + Poly (I:C) immunized mice presented the highest sera neutralization capacity.

## 2. Materials and methods

### 2.1. Design of optimized E2<sub>CHIKV</sub> sequences

The consensus sequence for the CHIKV Envelope 2 (E2<sub>CHIKV</sub>) protein was generated after the alignment (ClustalW) of 74 CHIKV Brazilian isolate sequences (GenBank accession numbers available at [Supplementary Table 1](#)) and synthesized by GenScript (NJ, USA). For the DNA vaccine, the gene included mammalian codon optimization and a Kozak sequence. The gene was cloned into *HindIII* and *XhoI* sites of pVAX1 vector (Invitrogen). The plasmids were purified using Endofree Plasmid Giga Kit (Qiagen) according to the manufacturer's instructions and analyzed by 1% agarose gel electrophoresis. For E2<sub>CHIKV</sub> recombinant protein production, the gene was codon optimized for bacteria expression and cloned into the pET21a vector using *NheI* and *XhoI* sites.

### 2.2. E2<sub>CHIKV</sub> expression

E2<sub>CHIKV</sub> recombinant protein was produced as inclusion bodies (IB) in BL21(DE3) competent cells transformed with the pET21a-E2<sub>CHIKV</sub> plasmid. Bacteria were inoculated into 1 L of LB medium containing ampicillin (100  $\mu$ g/mL) (Sigma) and grown (37 °C, 200 rpm) to an OD<sub>600nm</sub> between 0.6 and 0.8. Then, protein expression was induced with isopropyl- $\beta$ -D-thiogalactoside (IPTG) 0.5 mM (Sigma) for 4 h at 37 °C and 200 rpm. Bacteria were harvested (15 min, 4 °C and 5900 $\times$ g) and resuspended (Tris-HCl 100 mM, EDTA 5 mM, pH 8.5). Lysozyme (50  $\mu$ g/mL) (Sigma) was then added to the suspension for 15 min at room temperature. Next, sodium deoxycholate (Sigma) was added in order to reach a concentration of 0.1% and then the bacteria pellet was lysed by 3

rounds of sonication (5 min, 30% amp, 30 s pulse on, 30 s pulse off) (Branson Sonifier 450) and centrifuged (20 min, 4 °C and 9500 $\times$ g). The supernatant was discarded and the bacteria pellet was resuspended in 50 mL (Tris-HCl 100 mM, EDTA 5 mM, 0.1% sodium deoxycholate, pH 8.5) and rapidly sonicated to disrupt the aggregates. The suspension was centrifuged (20 min, 4 °C and 9500 $\times$ g) and washed (Tris-HCl 100 mM, EDTA 1 mM, pH 8.5). The IB pellet was resuspended in approximately 20 mL of the same buffer (Tris-HCl 100 mM, EDTA 1 mM, pH 8.5). The IB were diluted with appropriate buffer (CAPS 50 mM pH 11) containing EDTA 1 mM and DTT 1 mM. The IB suspension was maintained at 1 bar for 16 h and placed in the pressure vessel (R4-6-40, High Pressure Equipment) and pressurized at 2.4 kbar with a high-pressure pump (PS-50, High Pressure Equipment) using oil as a transmission fluid for 90 min. Decompression was performed slowly to 0.4 kbar or to 1 bar followed by centrifugation (15 min, 4 °C and 12,000 $\times$ g). The supernatant was collected and dialyzed overnight in Tris-HCl 50 mM pH 8.5. New round of centrifugation (15 min, 4 °C and 12,000 $\times$ g) was performed and the supernatant was collected and stored at -20 °C.

### 2.3. Immunoblot

Approximately 500 ng of recombinant E2<sub>CHIKV</sub> 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 containing 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 3 times with PBS Tween 20 (PBST) 0.05% (v/v) and incubated with mouse (1:500) or human (from CHIKV-infected patients or a healthy individual) (1:500) sera for 2 h at room temperature. 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 goat anti-human IgG (1:5000; KPL) for 1 h at room temperature. After 3 washes with PBST 0.05% (v/v), the reaction was developed with a chemiluminescence detection system ECL (GE Healthcare) according to manufacturer's instructions and analyzed by the Alliance 4.7 software (Uvitec; Cambridge).

### 2.4. DNA vaccine expression

Expression of pVAX-E2<sub>CHIKV</sub> was verified by immunoblot. HEK 293 T cells (5  $\times$  10<sup>5</sup> cells) were seeded in 6-well flat-bottom plates (Costar) containing DMEM (Gibco) supplemented with 1% (v/v) L-glutamine (Gibco), 1% (v/v) penicillin/streptomycin (Gibco) and 10% of fetal bovine serum (FBS, Gibco), and cultured overnight. When the cells reached approximately 70% confluence, the culture medium was replaced by Opti-MEM (Gibco) and cells were transfected with 5  $\mu$ g of DNA plasmid (pVAX-E2<sub>CHIKV</sub> or pVAX1 vector as a control) using Lipofectamine2000 (Invitrogen) transfection method. After 5 days, the supernatants and cell pellets were collected. Supernatants were concentrated approximately 8 times using 30 kDa Centriprep (Millipore). Cell pellets were lysed in cell lysis buffer (150 mM NaCl (Synth), 50 mM Tris (Merck) pH 8 and 1% Triton X-100 (Sigma), fractionated on SDS-PAGE (12%), and transferred to nitrocellulose membranes. Immunoblotting was performed with specific mouse antiserum and horseradish peroxidase-labeled goat anti-mouse IgG (1:5000; KPL) using ECL detection system (GE healthcare).

### 2.5. Mice and immunization

Female C57BL/6 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. All the animal experimental procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) (protocol number #5759150416) and were in compliance with the law and animal care guidelines.

For recombinant protein immunization, mice received two doses, at 2-week intervals, with 10 µg of E2<sub>CHIKV</sub> in the presence of Poly (I:C) (50 µg; Invivogen), CpG ODN 1826 (10 µg; Invivogen) or Imiquimod R837 (20 µg; Invivogen) in a total volume of 200 µL delivered subcutaneously (s.c) at the base of the tail. For DNA immunization, mice received two doses, at 2-week intervals, with 100 µg of pVAX-E2<sub>CHIKV</sub> in a volume of 100 µL delivered into the tibialis anterior muscle (50 µL each leg). Heterologous prime-boost regimen consisted of a DNA prime (pVAX-E2<sub>CHIKV</sub>) followed by one dose of recombinant E2<sub>CHIKV</sub> + Poly (I:C). The control groups received empty pVAX vector, Poly (I:C), CpG ODN 1826 or Imiquimod R837. Blood was collected by submandibular vein, fourteen days after each dose and mice were euthanized two weeks after the last dose.

## 2.6. ELISA

ELISA plates (High binding, Costar) were coated overnight at room temperature with 100 ng/well of E2<sub>CHIKV</sub> protein diluted in 50 µL/well of carbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, pH 9.6). The following day, the plates were washed 3 times with PBST 0.02% (v/v) and blocked for 2 h with 150 µL of PBST 0.02%, BSA (1% w/v) and non-fat milk (5% w/v). Plates were washed 3 times with PBST 0.02% and incubated for 2 h with serially diluted serum. Plates were washed 3 times with PBST 0.02% and incubated for 2 h with horseradish peroxidase-labeled goat anti-mouse IgG (1:10000; 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<sub>2</sub>SO<sub>4</sub>. Plates were read at 492 nm (OD<sub>492</sub>) with an ELISA reader (EnSpire Multimode Plate Reader; PerkinElmer). The antibody titer was determined by the highest dilution between an OD<sub>492nm</sub> 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, IgG2b and IgG2c (1:4000; Southern Biotech). To evaluate antibody affinity, we first ran a standard ELISA to determine the serum dilution that gives an OD<sub>492nm</sub> ≈ 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 0.02% and incubated with 100 µL/well of pure PBS or 7 M of urea as a chaotropic agent. Plates were allowed to stand for 5 min at room temperature and then washed to proceed the assay. The percentage of affinity was determined as follows: (OD<sub>492nm</sub> in the presence of urea × 100)/OD<sub>492nm</sub> in the presence of PBS.

## 2.7. Plaque reduction neutralization test (PRNT)

CHIKV ECSA strain Brazilian isolate (Genbank accession number: KP164569) was propagated in Vero E6 cells (ATCC CRL-1586) cultured in MEM medium containing 10% FBS and 1% penicillin/streptomycin (Gibco) for 48 h. The supernatant of infected cells was collected and titrated as previously described (Jang et al., 2016). For the neutralization assay, 24-well flat-bottom plates (Costar) were seeded with approximately  $1 \times 10^5$  Vero E6 cells/well in MEM (Gibco) supplemented with 10% of FBS + 1% penicillin/streptomycin and incubated overnight at 37 °C in 5% CO<sub>2</sub>. Sera samples from pooled immunized mice or a CHIKV-infected patient were heat-inactivated for 30 min at 56 °C and 2-folded diluted in supplemented MEM. Duplicate sera samples from each group were mixed (1:1) with 200 PFU/well of CHIKV and incubated for 1 h at 37 °C in 5% CO<sub>2</sub>. Next, confluent Vero E6 cells were incubated with the antibody-virus mixture for 1 h at 37 °C. Then, the cells were overlaid with complete MEM containing 1.6% carboxymethylcellulose (CMC, Sigma) and incubated further at 37 °C for 3 days. The CMC was removed and the wells were fixed with 4% paraformaldehyde (Sigma), followed by staining with 0.2% crystal violet (Sigma), and washed with distilled water. Stained-fixed cell culture images were acquired and the

number of plaques were counted. The percentage of plaque reduction compared to positive control (Vero E6 infected without sera) was calculated.

## 2.8. Cell suspension

Fifteen days after the last dose, spleen and draining lymph nodes (dLNs) were aseptically removed. After obtaining single cell suspensions, cells from the same group were pooled, and washed in 10 mL of RPMI 1640 (Gibco). Cells were then resuspended in R10 (RPMI supplemented with 10% of FBS, 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,  $5 \times 10^5$  M of 2-mercaptoethanol (all from Gibco) and 20 µg/mL of Cyproflaxacin (Ciprobacter, Isofarma). Cell concentration was estimated with the aid of a cell counter (Countess, Invitrogen) and adjusted in cell culture medium.

## 2.9. T cell ELISpot assay

CHIKV-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<sub>2</sub>, with E2<sub>CHIKV</sub> (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 0.05% and streptavidin-horseradish peroxidase was added and incubated for 45 min, at room temperature in the dark. After extensive washes with PBST 0.05% 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). The number of spots observed in protein-stimulated wells were subtracted from the number of spots detected in the R10 pulsed wells.

## 2.10. 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 E2<sub>CHIKV</sub> protein (100 ng/well) in PBS and incubated overnight at room temperature. Plates were washed 3 times with PBS and blocked for 1 h with R10 at 37 °C and 5% CO<sub>2</sub>. Five hundred thousand lymph nodes cells were added and incubated for 16 h at 37 °C and 5% CO<sub>2</sub>. 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).

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

dLNs single cell suspensions ( $2 \times 10^6$  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). GC B cells were identified as CD4<sup>+</sup>B220<sup>+</sup>GL7<sup>+</sup>CD95<sup>+</sup> and Tfh as CD4<sup>+</sup>B220<sup>-</sup>PD1<sup>+</sup>CXCR5<sup>+</sup> population.

### 2.11. Data analysis

Statistical significance ( $p$ -values) was calculated by One-way ANOVA followed by Tukey honestly significantly different (HSD) post hoc test.  $NT_{50}$  values were calculated by non-linear regression. Statistical analysis and graphical representation were performed using GraphPad Prism version 7.0 software.

## 3. Results

### 3.1. $E2_{CHIKV}$ production in bacteria and expression in eukaryotic cells

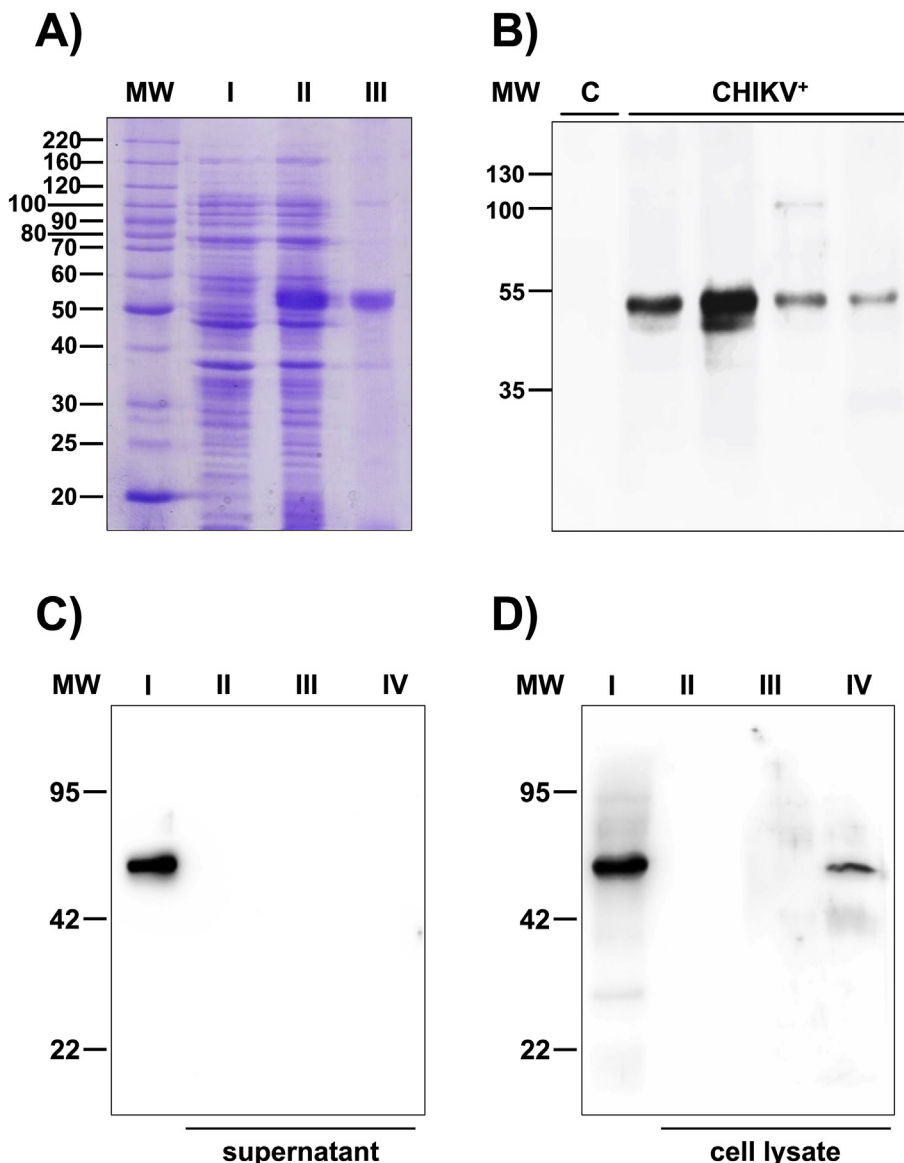
The  $E2_{CHIKV}$  recombinant protein was produced with the expected molecular weight (50 kDa) (Fig. 1A), and retained its antigenicity as confirmed by immunoblotting. Sera from CHIKV-infected patients recognized a specific band corresponding to  $E2_{CHIKV}$  recombinant protein while healthy serum did not (Fig. 1B).

To confirm the expression of the DNA vaccine pVAX- $E2_{CHIKV}$ , HEK293T cells were transiently transfected. The immunoblotting analysis demonstrated that  $E2_{CHIKV}$  was not present in the cells supernatant (Fig. 1C), but rather in the cell lysate (Fig. 1D).

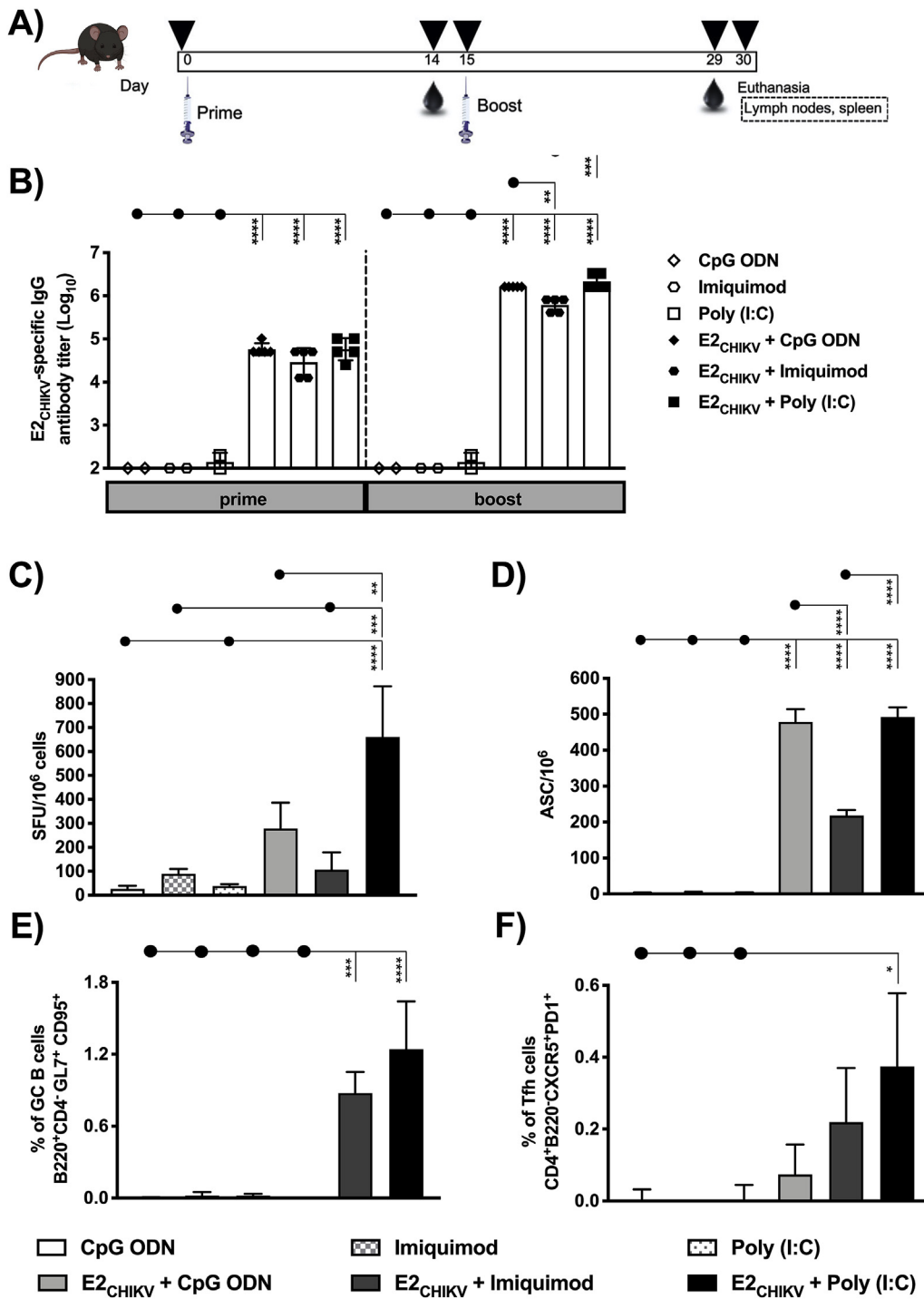
### 3.2. Poly (I:C) is a superior adjuvant for $E2_{CHIKV}$ recombinant protein vaccine

Since adjuvants play a pivotal role in modulating immunogenicity, we first assessed three promising adjuvants to use with the  $E2_{CHIKV}$  protein. C57BL/6 mice were immunized with the  $E2_{CHIKV}$  plus the adjuvants CpG ODN, Imiquimod (R837) or Poly (I:C) in the homologous prime-boost protocol while the control groups received only the adjuvants (Fig. 2A). After prime, all experimental groups developed similar  $E2_{CHIKV}$ -specific IgG antibody titers. After boost, mice immunized with  $E2_{CHIKV}$  in the presence of Poly (I:C) or CpG ODN displayed the highest specific IgG titers. Mice immunized with  $E2_{CHIKV}$  + Imiquimod presented significantly lower specific IgG titers when compared to other experimental groups. In contrast, control groups displayed negligible  $E2_{CHIKV}$ -specific IgG titers (Fig. 2B).

To assess cellular immune responses 15 days after boost, we collected the spleens to evaluate the number of specific IFN- $\gamma$  producing cells, and the draining lymph nodes (dLNs) to estimate the number of antibody secreting cells (ASC) and the frequency of germinal center (GC) B cells and T follicular helper (Tfh) cells. Mice immunized with  $E2_{CHIKV}$  + Poly (I:C) presented the highest number of  $E2_{CHIKV}$ -specific IFN- $\gamma$  producing



**Fig. 1.** Expression and characterization of  $E2_{CHIKV}$  recombinant protein and pVAX- $E2_{CHIKV}$  DNA vaccine. **A)** SDS-PAGE analysis of the  $E2_{CHIKV}$  recombinant protein expressed in bacteria. Lane I: BL21(DE3) lysate; Lane II: BL21(DE3) lysate after IPTG induction; Lane III: purified  $E2_{CHIKV}$  recombinant protein. **B)**  $E2_{CHIKV}$  recombinant protein (500 ng/lane) was resolved in a SDS-PAGE 12% polyacrylamide gel, under reducing conditions, and transferred to a nitrocellulose membrane. The membrane was incubated with human sera and horseradish peroxidase-labeled goat anti-mouse IgG. **C)** Immunoblot using (I)  $E2_{CHIKV}$  recombinant protein (500 ng/lane) as positive control and (II) Bovine serum albumin (BSA) as negative control, 50  $\mu$ L/lane of supernatant or 10  $\mu$ L/lane of cell lysate after HEK293T transfection with (III) pVAX or (IV) pVAX- $E2_{CHIKV}$  plasmid. The immunoblot was developed with specific mouse antiserum and horseradish peroxidase-labeled goat anti-mouse IgG. MW: Molecular weight in kDa; C: healthy control.



**Fig. 2.** Poly (I:C) adjuvant induces higher E2<sub>CHIKV</sub>-specific humoral and cellular immune responses than CpG ODN or Imiquimod. **A)** Immunization strategy. C57BL/6 mice (n = 2 for control groups and n = 5 for experimental groups) were immunized s.c. twice with E2<sub>CHIKV</sub> in the presence of CpG ODN, Imiquimod (R837) or Poly (I:C). Control groups received only CpG ODN, Imiquimod or Poly (I:C) in PBS. Mice were bled 14 days after each dose to evaluate the specific humoral response by ELISA. **B)** Total anti-E2<sub>CHIKV</sub> IgG antibody titers on a logarithmic scale (Log<sub>10</sub>). **C)** Fifteen days after the boost, pooled splenocytes were cultured in the presence of recombinant E2<sub>CHIKV</sub> for 18 h to evaluate the number of IFN- $\gamma$  producing cells by ELISpot assay. SFU: spot forming units. Alternatively, cells from draining lymph nodes (dLNs) **(D)** were placed in culture for 16 h to evaluate the number of E2<sub>CHIKV</sub>-specific antibody secreting cells (ASC) by ELISpot; or immediately labeled with fluorescent antibodies to determine the frequency of **(E)** germinal center (GC) B cells (B220<sup>+</sup>CD4<sup>-</sup>GL7<sup>+</sup>CD95<sup>+</sup>) or **(F)** T follicular helper (Tfh) cells (CD4<sup>+</sup>B220<sup>-</sup>CXCR5<sup>+</sup>PD1<sup>+</sup>). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001. Data represent mean  $\pm$  SD. s.c.: subcutaneous.

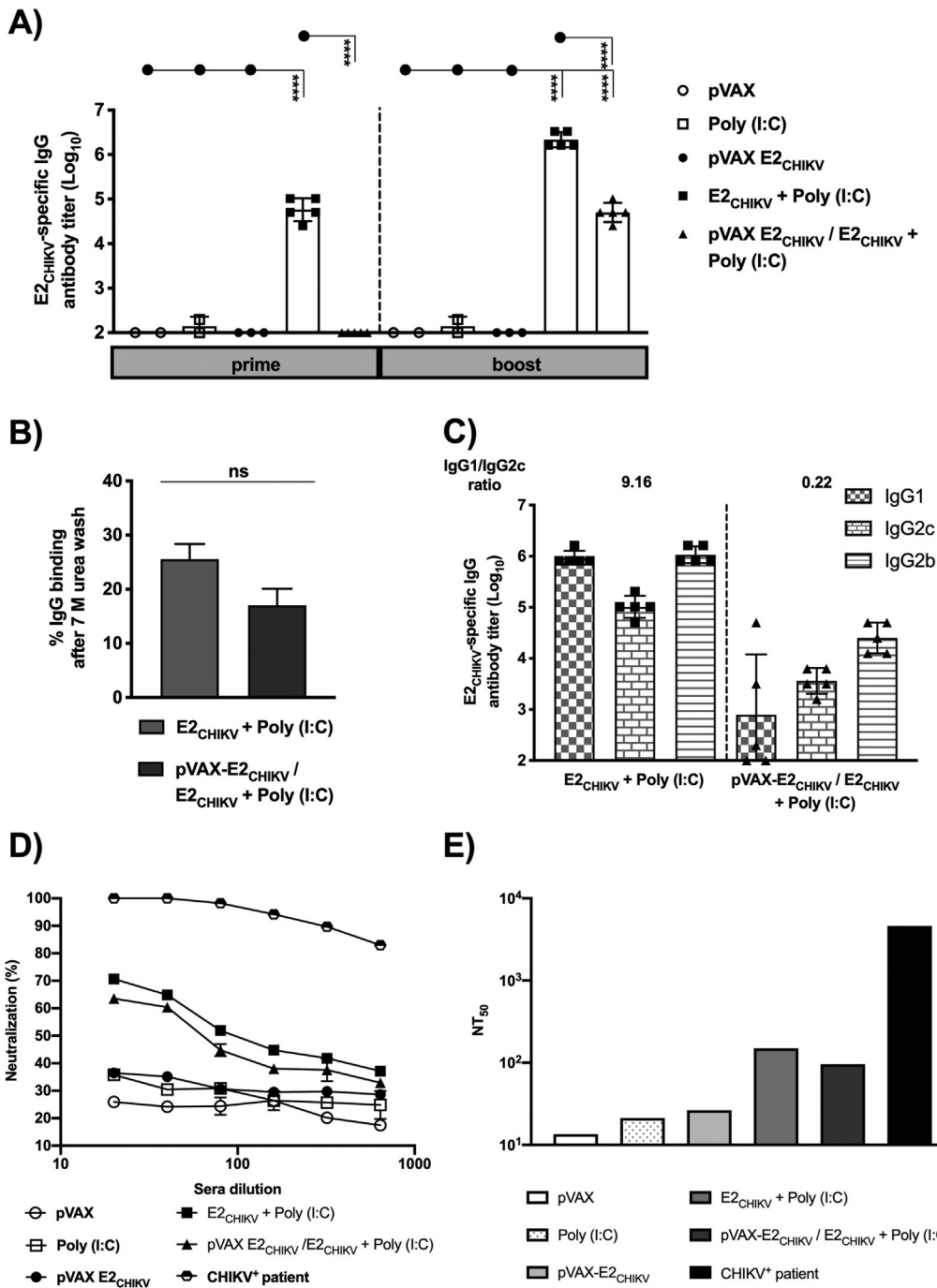
cells (Fig. 2C) and ASC (Fig. 2D), and also the highest frequency of GC B cells (Fig. 2E) and Tfh (Fig. 2F). Mice immunized with E2<sub>CHIKV</sub> + CpG ODN or E2<sub>CHIKV</sub> + Imiquimod presented lower cellular immune responses. In contrast, control groups showed negligible responses.

Overall, Poly (I:C) was selected as the adjuvant to be combined with E2<sub>CHIKV</sub> protein, since it was able to induce high E2<sub>CHIKV</sub>-specific IgG titers, IFN- $\gamma$  producing cells, ASC, and higher frequency of GC B cells and Tfh cells.

Recombinant E2<sub>CHIKV</sub> + Poly (I:C) immunization induces higher humoral response than the DNA vaccine.

In order to compare the immune response induced by the recombinant protein and the DNA vaccine, we performed homologous and

heterologous prime-boost immunizations. C57BL/6 mice were immunized twice with the homologous DNA vaccine pVAX-E2<sub>CHIKV</sub> or the E2<sub>CHIKV</sub> + Poly (I:C). Besides, mice were also immunized with the heterologous protocol, in which they received a prime with pVAX-E2<sub>CHIKV</sub> followed by a boost with E2<sub>CHIKV</sub> + Poly (I:C). After prime, only mice immunized with E2<sub>CHIKV</sub> + Poly (I:C) presented specific IgG titers (Fig. 3A). After boost, the heterologous group (pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C)) developed specific IgG titers that were statistically lower when compared to homologous E2<sub>CHIKV</sub> + Poly (I:C) group. The homologous DNA vaccine pVAX-E2<sub>CHIKV</sub>, as well as the control groups, were not able to induce significant antibody titers after prime or boost. These results were also confirmed by immunoblotting (Fig. S1). After the boost, only



**Fig. 3.** Analysis of specific humoral response after homologous or heterologous prime-boost immunization. C57BL/6 mice (n = 2 for control groups and n = 3 or n = 5 for experimental groups) were immunized twice with 100 µg of the DNA vaccine pVAX-E2<sub>CHIKV</sub> or with 100 µg of empty pVAX, as a control (i.m.); 10 µg of the E2<sub>CHIKV</sub> combined with Poly (I:C), or only Poly (I:C) as a control group (s.c.). Alternatively, mice were immunized with the heterologous protocol: the first dose consisted in 100 µg of the DNA vaccine pVAX-E2<sub>CHIKV</sub> (i.m.) and the second dose 10 µg of E2<sub>CHIKV</sub> combined with Poly (I:C) (s.c.). Mice were bled 14 days after each dose to evaluate the specific humoral response. **A)** anti-E2<sub>CHIKV</sub> IgG antibody titers on a logarithmic scale (Log<sub>10</sub>). **B)** The percentage of IgG anti-E2<sub>CHIKV</sub> pooled mice sera avidity was determined by adding an extra step of urea 7 M for 5 min. **C)** anti-E2<sub>CHIKV</sub> IgG subclasses antibody titers (Log<sub>10</sub>) were evaluated 14 days after the last dose. \*\*\*\*p < 0.0001, ns: not significant. Data represent mean ± SD and are representative of 3 independent experiments. **D)** Percentage of neutralization and **E)** 50% neutralization titers (NT<sub>50</sub>) of anti-CHIKV antibodies from immunized mice or convalescent patient serum were determined by PRNT. NT<sub>50</sub> values were calculated by non-linear regression. i.m.: intramuscular; s.c.: subcutaneous.

pooled sera from E2<sub>CHIKV</sub> + Poly (I:C) and pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C) groups were able to recognize, with different intensities, the E2<sub>CHIKV</sub> recombinant protein. In contrast, sera from mice immunized with pVAX, Poly (I:C) or pVAX-E2<sub>CHIKV</sub> did not recognize E2<sub>CHIKV</sub> (Fig. 3A).

Next, we analyzed the avidity of the induced E2<sub>CHIKV</sub>-specific antibodies, by including an extra step of incubation with urea, a chaotropic agent. Fig. 3B shows that antibodies from homologous E2<sub>CHIKV</sub> + Poly (I:C) and heterologous pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C) immunized groups displayed similar avidity. In addition, we characterized the specific IgG subclasses induced after the boost. Both immunization strategies were able to induce all tested IgG subclasses (IgG1, IgG2c and IgG2b) with different magnitudes. We also calculated the IgG1/IgG2c ratio as a surrogate for Th1 or Th2 driven immune responses, as Th1 cells secrete IFN-γ and promote IgG2c, while Th2 produce IL-4 and

regulate IgG1 secretion by B cells (Snapper and Paul, 1987; Stevens et al., 1988). In this way, this ratio is commonly used to evaluate if a certain vaccine formulation favors Th1 or Th2 immune responses. The IgG1/IgG2c ratio was lower in the group that received the heterologous pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C), suggesting a Th1 biased response (Fig. 3C).

The neutralizing capacity of the antibodies was determined by PRNT assay using sera obtained 14 days after the boost. As shown in Fig. 3D–E, sera from mice immunized with E2<sub>CHIKV</sub> + Poly (I:C) or pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C) partially neutralized the CHIKV isolate. We also observed that neutralization titers 50% (NT<sub>50</sub>) were higher in the sera from E2<sub>CHIKV</sub> + Poly (I:C) group than in the pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C). In contrast, sera from pVAX-E2<sub>CHIKV</sub> group, as well as the control groups, presented a much lower neutralizing capacity.

### 3.3. E2<sub>CHIKV</sub> + poly (I:C) elicits the highest cellular response

As we observed significant differences in the humoral responses between the homologous and heterologous groups, we then compared the cellular immune responses after this protocol. For this purpose, 15 days after the boost, we removed the spleen and dLNs to evaluate E2<sub>CHIKV</sub>-specific IFN- $\gamma$  producing cells and ASC, respectively.

Mice immunized with homologous E2<sub>CHIKV</sub> + Poly (I:C) or pVAX-E2<sub>CHIKV</sub> or the heterologous pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C) presented similar numbers of IFN- $\gamma$  producing cells (Fig. 4A). Of note, although the homologous pVAX-E2<sub>CHIKV</sub> group did not produce specific antibodies, it was able to induce specific IFN- $\gamma$  producing cells, similar to the other experimental groups. In contrast, splenocytes from the control groups produced negligible numbers of IFN- $\gamma$  producing cells. dLNs from homologous E2<sub>CHIKV</sub> + Poly (I:C) group presented the highest number of ASC (Fig. 4B), statistically different from the other groups. On the other hand, mice immunized with pVAX, Poly (I:C) or pVAX-E2<sub>CHIKV</sub> exhibited negligible responses.

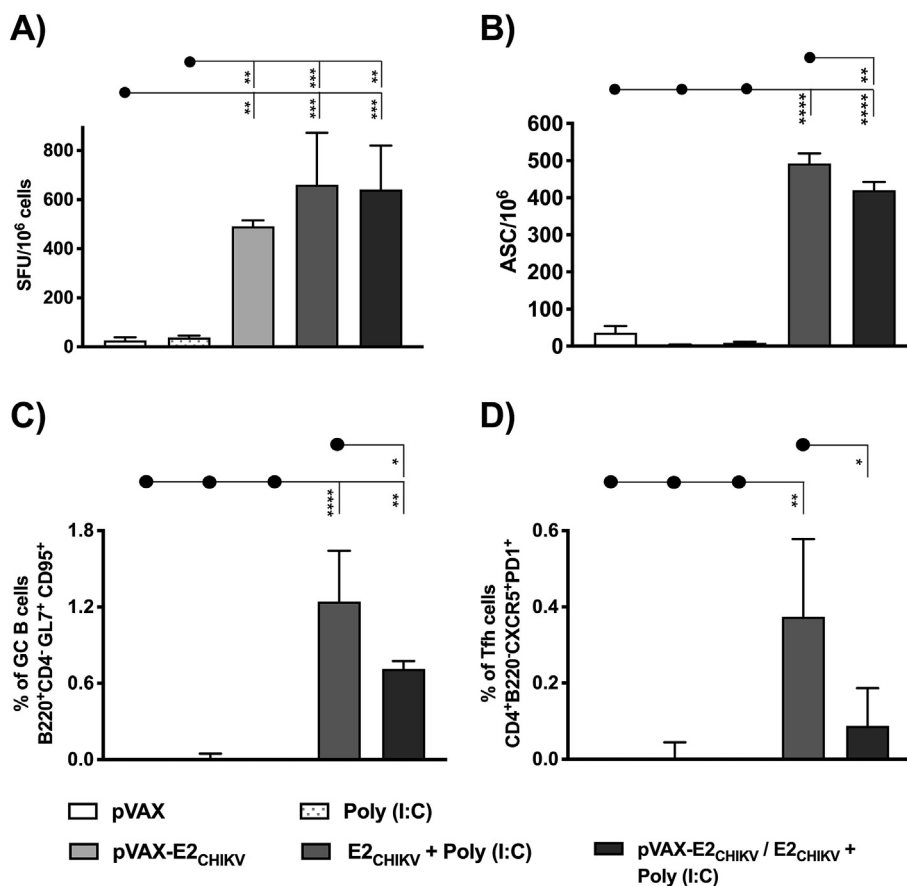
Finally, we analyzed the frequency of GC B cells and Tfh cells. dLNs from mice that received the homologous E2<sub>CHIKV</sub> + Poly (I:C) immunization displayed the highest frequency of both cell populations, followed by the heterologous group (Fig. 4C and D, respectively). Mice immunized with pVAX, Poly (I:C) or pVAX-E2<sub>CHIKV</sub> presented undetectable frequencies of GC B cells or Tfh cells.

## 4. Discussion

CHIKV infection is associated with long-term arthralgia and morbidity. Despite half a century of pre-clinical studies and some clinical trials, there is no licensed vaccine against CHIKV yet. Some vaccine candidates against CHIKV have entered clinical trials, such as formalin-inactivated, live attenuated, recombinant viral vectors and virus-like

particles. Two candidates that have moved to phase II trials were based on CHIKV structural components, including the E2 protein (Reyes-Sandoval, 2019). Studies have demonstrated that neutralizing monoclonal antibodies against E2 protein inhibit CHIKV cell entry and protect mice after challenge (Fox et al., 2015; Selvarajah et al., 2013). Furthermore, the CHIKV E2 glycoprotein has been shown to be the most dominant target for neutralizing antibodies in infected patients (Kam et al., 2012b, 2012c), while IgG3 anti-E2 is the main isotype observed in the sera of convalescent patients (Kam et al., 2012a, 2012c). Besides, these are neutralizing antibodies, and their removal reduced in approximately 80% the specific antibody titer (Kam et al., 2012a). Subunit vaccines have several advantages over traditional vaccines approaches, being safety and few side effects the most remarkable. Here, we describe the pre-clinical evaluation of synthetic subunit candidates based on the E2<sub>CHIKV</sub> surface antigen.

An essential issue for the development of efficacious subunit vaccine candidates is the selection of a suitable adjuvant. Adjuvants are ideal components to be part of a subunit vaccine as they potentiate the immune responses, lower the amount of antigen, reduce the number of doses to induce protection, and increase seroconversion in special populations (Apostólico et al., 2016). Here, we tested three promising adjuvants that are also being evaluated in clinical trials: CpG ODN 1826, Imiquimod R837 and Poly (I:C) (Apostólico et al., 2016). CpG oligodeoxynucleotides (ODN) are synthetic unmethylated CpG motifs recognized by TLR9, that have been used in clinical trials as adjuvants for vaccines targeting infectious diseases and cancer (Scheiermann and Klinman, 2014). On the other hand, Imiquimod R837 (1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine) mimics ssRNAs, is sensed by TLR7/8, and has been widely used in clinical trials specially for cancer treatment (Kamath et al., 2018). Finally, Polyinosinic:polycytidylic acid (Poly (I:C)) is a synthetic analog of double-stranded RNAs (dsRNAs) that is capable of activating TLR3 and RIG-I-like receptors (retinoic acid-inducible gene -I- like



**Fig. 4.** Assessment of cellular immune response after homologous and heterologous prime-boost immunizations. C57BL/6 mice ( $n = 2$  for control groups and  $n = 3$  or  $n = 5$  for experimental groups) were immunized as in Fig. 2. A) Fifteen days after the last dose, pooled splenocytes were cultured in the presence of recombinant E2<sub>CHIKV</sub> for 18 h to evaluate the number of IFN- $\gamma$  producing cells by ELISpot assay. SFU: spot forming units. Alternatively, cells from draining lymph nodes (dLNs) (B) were placed in culture for 16 h to evaluate the number of E2<sub>CHIKV</sub> specific antibody secreting cells (ASC) by ELISpot; or immediately labeled with fluorescent antibodies to determine the frequency of (C) germinal center (GC) B cells (B220<sup>+</sup>CD4<sup>-</sup>GL7<sup>+</sup>CD95<sup>+</sup>) or (D) T follicular helper (Tfh) cells (CD4<sup>+</sup>B220<sup>-</sup>CXCR5<sup>+</sup>PD1<sup>+</sup>). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Data represent mean  $\pm$  SD and are representative of 2 independent experiments.

receptors, or RLRs). Furthermore, Poly (I:C) and its derivatives have been tested in several clinical trials such as cancer therapeutics and influenza vaccination (Martins et al., 2015). Our immunization strategy with E2<sub>CHIKV</sub> combined with different adjuvants showed that Poly (I:C) was the best candidate. Mice immunized with E2<sub>CHIKV</sub> + Poly (I:C) presented the highest E2<sub>CHIKV</sub>-specific IgG titers, IFN- $\gamma$  producing cells, ASC, frequency of GC B cells and Tfh cells. Indeed, Poly (I:C) was a strong candidate since it not only mimics viral RNA and activates TLR3, but also matures dendritic cells and polarizes T cell responses to a Th1 profile (Martins et al., 2015). In agreement with these findings, Poly (I:C) was also the best adjuvant to use with the recombinant Zika virus envelope protein (E<sub>ZIKV</sub>) (Amaral et al., 2020). Other adjuvants also induced robust humoral response using E2<sub>CHIKV</sub> antigen. BALB/c mice immunized with E2<sub>CHIKV</sub> in the presence of alum or liposomes induced protective humoral response similar to the CHIKV inactivated vaccine (Kumar et al., 2012). Another work compared the humoral and cellular immune responses induced in BALB/c mice after immunization with E2<sub>CHIKV</sub> in the presence of Freund's complete adjuvant (FCA), alum or Montanide ISA 720 adjuvants (Khan et al., 2012). Although all tested adjuvants induced specific immune responses, FCA and Montanide ISA are not licensed for humans, and FCA is no longer recommended for animals (Apostólico et al., 2016).

Several studies have shown that the humoral response plays a central role against CHIKV infection. However, the correlates of protection have not yet been fully elucidated. Thus, in the absence of an effective vaccine against CHIKV, it is necessary to evaluate different immune parameters induced by a vaccine candidate. After administration of a vaccine, GC formation and the presence of Tfh cells are important to generate high affinity antibodies and memory cells (Linterman and Hill, 2016; Aloulou and Fazilleau, 2019). We analyzed these populations in the dLNs after the immunization with E2<sub>CHIKV</sub> in the presence of different adjuvants. We observed that mice immunized with E2<sub>CHIKV</sub> + Poly (I:C) displayed the highest frequency of GC B cells and Tfh cells. Until now some studies have shown the importance of GC and Tfh cells. For example, a vaccine against *Plasmodium vivax* using vivax malaria protein 1 (VMP001) with MPL adjuvant and nanoparticles was able to increase the specific humoral immune response, induce the formation of GC and expand Tfh cells (Moon et al., 2012).

The ability of a new vaccine candidate to induce IFN- $\gamma$  is important against intracellular pathogens. Here we showed that splenocytes from mice immunized with E2<sub>CHIKV</sub> + Poly (I:C) displayed the highest number of specific IFN- $\gamma$  producing cells when compared to other combinations using CpG or Imiquimod adjuvants. Our data is different from previous work (Khan et al., 2012), which did not observe IFN- $\gamma$  in splenocytes when BALB/c mice were immunized with recombinant E2<sub>CHIKV</sub> in the presence of FCA, alum or Montanide ISA720. However, another group showed that splenocytes from BALB/c mice immunized with E2<sub>CHIKV</sub> combined with liposomes induced higher IFN- $\gamma$  levels than mice immunized with E2<sub>CHIKV</sub> + alum (Kumar et al., 2012).

Some vaccines require more than one dose to be effective. The prime-boost regimen is traditionally administered as multiple homologous doses. However, heterologous prime-boost consists of different strategies using the same antigen. The heterologous prime-boost immunization broadly activates the immune system and can be an alternative to traditional approaches (Lu, 2009). Here, we used both immunization strategies to systematically compare the components of the humoral and cellular immune responses against E2<sub>CHIKV</sub> antigen. Of note, our DNA vaccine pVAX-E2<sub>CHIKV</sub> alone was not able to induce specific antibody titers, probably as result of its arrest in the cell cytoplasm. This result differs from two other studies in which BALB/c mice immunized with a DNA vaccine encoding the consensus sequence of E2<sub>CHIKV</sub> or all the envelope proteins (E1+E2+E3) followed by electroporation were able to produce specific antibody response (Muthumani et al., 2008; Mallilankaraman et al., 2011). Besides, the interconnected DNA vaccine induced neutralizing antibodies in rhesus macaques similar to those observed in convalescent patients (Mallilankaraman et al., 2011). The

difference may be due to the fact that both constructs included a leader sequence and were also administered *in vivo* via electroporation, well-known strategies that contribute to protein secretion and increase transfection rate, respectively (Kutzler and Weiner, 2008).

On the other hand, homologous immunization with E2<sub>CHIKV</sub> + Poly (I:C) induced the highest specific antibody titers after prime and boost than the other strategies. Heterologous prime-boost regimen has been shown to enhance immune responses against antigens derived from several intracellular pathogens (Lu, 2009). Here, we first immunized C57BL/6 mice with a DNA vaccine and then with the recombinant protein E2<sub>CHIKV</sub> in the presence of Poly (I:C). Mice immunized with the heterologous protocol (pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C)) only presented specific antibody titer against E2<sub>CHIKV</sub> after the boost. This was expected, since our DNA vaccine pVAX-E2<sub>CHIKV</sub> did not induce specific antibody titers. Furthermore, both homologous E2<sub>CHIKV</sub> + Poly (I:C) and heterologous pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C) induced neutralizing antibodies against a CHIKV isolate, a desired feature for disease control that is associated with CHIKV clearance in humans (Kam et al., 2012a). Similarly, some studies have also demonstrated the induction of neutralizing antibodies in BALB/c mice immunized with bacteria-expressed E2<sub>CHIKV</sub> recombinant protein (Kumar et al., 2012; Khan et al., 2012; Weber et al., 2015).

Regarding cellular immune responses, splenocytes from mice immunized with homologous E2<sub>CHIKV</sub> + Poly (I:C) or heterologous pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C) presented similar number of specific IFN- $\gamma$  producing cells. The same was observed for homologous immunization with pVAX-E2<sub>CHIKV</sub>. This is in line with a previous work that used a DNA vaccine encoding the consensus sequence of E2<sub>CHIKV</sub> followed by electroporation (Muthumani et al., 2008). Later, the same authors showed that mice and non-human primates immunized with a DNA vaccine encoding the CHIKV envelope proteins (E1+E2+E3) displayed specific humoral and cellular immune responses, including IFN- $\gamma$  production (Mallilankaraman et al., 2011). An elegant study compared the immunogenicity of heterologous prime-boost vaccination using DNA replicon (DREP), recombinant protein and modified vaccinia virus Ankara (MVA) (Hallengard et al., 2014). The highest humoral response was observed when mice first received DREP or MVA followed by the recombinant protein in the presence of Matrix-M adjuvant. For the cellular immune response, the best strategy was prime with DREP then boost with MVA or recombinant protein + Matrix-M (Hallengard et al., 2014). However, we believe that our work is the first to analyze the heterologous prime-boost strategy with DNA and recombinant protein using solely E2<sub>CHIKV</sub> as antigen.

Collectively, our results showed that mice immunized with homologous E2<sub>CHIKV</sub> + Poly (I:C) developed the highest E2<sub>CHIKV</sub>-specific humoral and cellular immune responses.

## 5. Conclusions

In conclusion, our study investigated the E2<sub>CHIKV</sub> specific humoral and cellular immune responses using different vaccine platforms (DNA and recombinant protein), adjuvants (CpG ODN, Imiquimod and Poly (I:C)) and strategies (homologous and heterologous). Altogether, our results suggest that two doses of E2<sub>CHIKV</sub> + Poly (I:C) were able to induce stronger immune responses than homologous pVAX-E2<sub>CHIKV</sub> or heterologous pVAX-E2<sub>CHIKV</sub>/E2<sub>CHIKV</sub> + Poly (I:C) strategies. Moreover, recombinant E2<sub>CHIKV</sub> + Poly (I:C) immunized mice presented the highest sera neutralization capacity. Our results indicate that poly (I:C) is a promising adjuvant to be included in a subunit vaccine formulation against CHIKV infection.

## Ethics statement

All animal protocols used in this study were approved by the IACUC (protocol number #5759150416) and carried out in accordance with the recommendations of the Brazilian 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.

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### CRedit authorship contribution statement

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

### Declaration of competing interest

The authors declare that they have no conflicts of interest.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crimmu.2021.03.001>.

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