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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Tests in mice of a dengue vaccine candidate made of chimeric Junin virus-like particles and conserved dengue virus envelope sequences

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Abstract Two new vaccine candidates against dengue virus (DENV) infection were generated by fusing the coding sequences of the self-budding Z protein from Junin virus (Z-JUNV) to those of two cryptic peptides (Z/DENV-P1 and Z/DENV-P2) conserved on the envelope protein of all serotypes of DENV. The capacity of these chimeras to generate virus-like particles (VLPs) and to induce virus-neutralizing antibodies in mice was determined. First, recombinant proteins that displayed reactivity with a Z-JUNV-specific serum by immunofluorescence were detected in HEK-293 cells transfected with each of the two plasmids and VLP formation was also observed by transmission electron microscopy. Next, we determined the presence of antibodies against the envelope peptides of DENV in the sera of immunized C57BL/6 mice. Results showed that those animals that received Z/DENV-P2 DNA coding sequences followed by a boost with DENV-P2 synthetic peptides elicited significant specific antibody titers (≥6.400). Finally, DENV plaque-reduction neutralization tests (PRNT) were performed. Although no significant protective effect was observed when using sera of Z/DENV-P1-immunized animals, antibodies raised against vaccine candidate Z/DENV-P2 (diluted 1:320) were able to reduce in over 50 % the number of viral plaques generated by infectious DENV particles. This reduction was comparable to that of the 4G2 DENV-specific monoclonal cross-reactive (all serotypes) neutralizing antibody. We conclude that Z-JUNV-VLP is a valid carrier to induce antibody-mediated immune responses in mice and that Z/DENV-P2 is not only immunogenic but also protective in vitro against infection of cells with DENV, deserving further studies. On the other side, DENV's fusion peptide-derived chimera Z/DENV-P1 did not display similar protective properties.

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Introduction

Dengue is a mosquito-borne disease caused by a Flavivirus that has become a global public health threat in recent years, due to its high prevalence (over a hundred countries worldwide) (WHO/TDR 2009). In 2013, a study made by cartographic approaches estimated that there are 390 million dengue infections per year, of which only 96 million are apparent (Bhatt et al. 2013). At present, there are officially four dengue virus serotypes (DENV 1–4) that infect humans, although a fifth serotype was found in Malaysian primates in 2013 (Normile 2013). There is no current vaccine available to cope with this health problem, although enormous research efforts have been deployed and several candidates have been tested (Capeding et al. 2014; George et al. 2015; Guy et al. 2011; Kanesa-thasan et al. 2001; Kirkpatrick et al. 2015; Kitchener et al. 2006; Osorio et al. 2014; Thomas et al. 2013; Villar et al. 2015).

The envelope protein (E) of Flaviviruses is responsible for virion assembly, receptor binding, and fusion to the host cell membrane, and it is also the main target for neutralizing antibodies (Chambers et al. 1990). Protein E is associated with its cell receptor before virus endocytosis is triggered. After that, fusion of DENV envelope to the target cell membrane takes place because of an E protein rearrangement under the low endosomal pH. Low pH exposes the *fusion loop* of E, inserting the fusion peptide into the membrane of the host cell and induces a subsequent rearrangement of lateral monomers. Consequently, there is a conformational change of the E homodimer to a trimer state. The trimeric structure folds, forms a hairpin, and generates the fusion pore that allows RNA release in the cytoplasm and continuation of the virus cycle (Alen and Schols 2012; Modis et al. 2004). These conformational changes also contribute to displaying other highly conserved cryptic regions present in E. The high degree of conservation observed in some of these cryptic regions is mainly due to the fact that they are not constantly submitted to the host's immune pressure that will repeatedly select sequence variants.

Virus-like particles (VLPs) are self-mounting tri-dimensional empty structures that are generated by some virus proteins. These particulate antigens are efficiently detected by our immune system which rapidly initiates specific immune responses against them. Good examples are the hepatitis B virus (HBV) or papillomavirus (HPV) vaccines, for which a single antigen with these features, i.e., the HBsAg in the case of HBV or the L1 protein for HPV, is preventive (Wheeler et al. 2008). Another example described in the literature is the Z protein from Junin virus (Z-JUNV). Junin virus is transmitted by rodents in rural Argentina, causing Argentine hemorrhagic fever (AHF), a severe disease that can cause neurological manifestations. The Z protein from JUNV is considered a matrix viral protein with relevant properties, such as interactions with cellular factors or induction of viral budding (Urata and de la Torre 2011). Studies conducted by researchers of our group in the past demonstrated the ability of Z to generate VLPs (Goni et al. 2010), suggesting a possible use of Z as a carrier system for antigenic peptides (Borio et al. 2012).

Our hypothesis for this study was that fusing relevant DENV peptides to the C-terminal region of Z-JUNV would generate VLP structures that could induce significant antibody-mediated immune responses, which might interact with the DENV infection process. We chose two highly conserved peptides (99–100 % conservation at amino acid level among all DENV serotypes) previously identified by members of our group within domain II of the E protein (Fleith et al., manuscript in preparation). Our previous experience with prime-boost protocols suggested that we might be successful in our quest by administering these peptide sequences as two different immunogenic formulations, which turned out to be the case. We show here (i) the steps followed for generating the molecular constructions, (ii) the adapted immunization protocol used to immunize animals, and (iii) the preliminary results obtained using sera of immunized animals to neutralize DENV and prevent infection of live cells.

Materials and methods

Plasmids A plasmid (pZ/GFP) containing Z-JUNV and GFP (green fluorescent protein) coding sequences was used as control in all experiments and has been previously described (Borio et al. 2012). To generate the DENV immunogens, pZ/GFP was digested with *Bam*HI and *Not*I to remove the GFP open-reading frame. Long synthetic oligonucleotides with partial *Bam*HI or *Not*I flanking sites encoding the conserved peptides DENV-P1 (sense 5′-GATCCGGTGTTGACCGTGGTTGGGGTAACGGTTGCGGTCTGTTCGG-TAAAGGTGGTTAGGC-3′ and antisense 5′GGCCGCCTAACCACCTTTACCGAACAGACCGCAACCGTTA-CCCCAACCACGGTCAACACCG3′) and DENV-P2 (sense 5′GATCCGTTGTTGTTCTGGGTTCTCAGGAAGGTGCGATGCACACCGCGCTGACCGGTGCGAC-CGAAATCTAGGC3' and antisense 5′-GGCCGCCTAGATTTCGGTCGCACCGGTCAGCGCGGTGTGCAT-CGCACCTTCCTGAGAACCCAGAACAACAACG-3′) were annealed in vitro and used to replace the GFP coding sequence. The size of Z-JUNV (291 bp) was increased to 342 and 364 bp, respectively, because of these long oligonucleotide additions. After gel and sequencing confirmation, the new plasmids, pZ/DENV-P1 and pZ/DENV-P2, were purified using a cesium chloride gradient to obtain endotoxin-free DNA for cell transfection and immunization. DNAs were

quantified in a Picodrop® equipment and the purity was determined by calculating their absorbance ratios 260/280 and A260/230.

Synthetic peptides, vaccine formulations, and immunization protocol This project was approved by the Ethics Committee on Animal Experimentation. Eight weeks old male C57BL/6 mice (4 animals per group) were immunized using a heterologous prime-boost protocol. The protocol consisted of a priming DNA dose (50 μg) in *Bordetella pertussis* monophosphoryl lipid A (MPLA; kind gift of Dr. Isaias Raw and Dr. Flavia S. Kubrusly, Butantan Institute, Sao Paulo, Brazil) by intramuscular injection (posterior muscles of the thigh: biceps femoris and/or abductor) followed 6 weeks later by a booster subcutaneous inoculation of 20 μg of synthetic peptides DENV-P1 (VDRGWGNGCGLFGKGG) and DENV-P2 (KLH-CLGSQEGAMHTALTGATEI, with the three initial valine residues exchanged by a cysteine for synthesis viability) in incomplete Freund's adjuvant (IFA). Fifteen days after, the last immunization blood was collected for serologic assays.

Cell culture and fluorescence microscopy HEK 293 (ATCC® CRL-1573™) human kidney cells were grown in aseptic conditions, in six-well cell culture plates using DMEM medium supplemented with 10 % fetal bovine serum (Gibco, Life Technologies). Low-confluence (30–50 %) cells were transfected using Lipofectamine 2000 CD (Invitrogen, Life Technologies) with 1 μg DNA/well of the Z-VLPs encoding plasmids (control, Z-VLP/GFP; samples, Z/DENV-P1 or Z/DENV-P2). Forty-eight hours later, these cells were analyzed by indirect immunofluorescence assay. Cells were washed three times with phosphate-buffered saline (PBS), fixed with 4 % formaldehyde in PBS for 20 min, washed and permeabilized with 0.1 % Triton X-100 in PBS for 10 min before a new wash was performed, and the primary antibody (polyclonal anti-Z-JUNV produced in rabbit, kindly provided by Dr. Mario Enrique Lozano) diluted 1:2000 in PBS/1 % BSA/0.1 % Triton X-100 was added to the wells for 1 h at room temperature. After extensive washes, the anti-rabbit secondary antibody conjugated to Alexa Fluor 555 (Molecular Probes) diluted 1:500 in the same buffer was added for a 1-h final incubation at room temperature. After another round of washes, pictures were taken in an inverted fluorescence microscope (Olympus IX71).

Virus-like particle purification and transmission electronic microscopy For purification of VLPs, transfection was carried out using mixtures of plasmids and Lipofectamine 2000 CD (Invitrogen)-DNA/Lipofectamine ratio 1:4, each of them being added to three 150-cm^2 cell culture flasks containing 70–80 % confluent HEK 293 cells. Seventy-two hours later, transfected cell culture supernatants were collected and clarified (2800 g/20 min, room temperature) and VLPs in the supernatant were purified by ultracentrifugation on a sucrose cushion (30 % sucrose—96,000*g*/2 h/4 °C) in polyallomer tubes placed in a Sw41.Ti rotor of an Optima XE-100 Beckman Coulter® centrifuge. Pellets were resuspended in PBS and stored at 4 °C for a maximum of 7–10 days. A drop of 25 μL of the purified VLPs was placed on nickel grids covered with parlodion and coated with carbon. The contrast was made with uranyl acetate (2 %) for 1 min in the dark and dried with filter paper. These grids were analyzed on a JEM-1011 TEM microscope the following day.

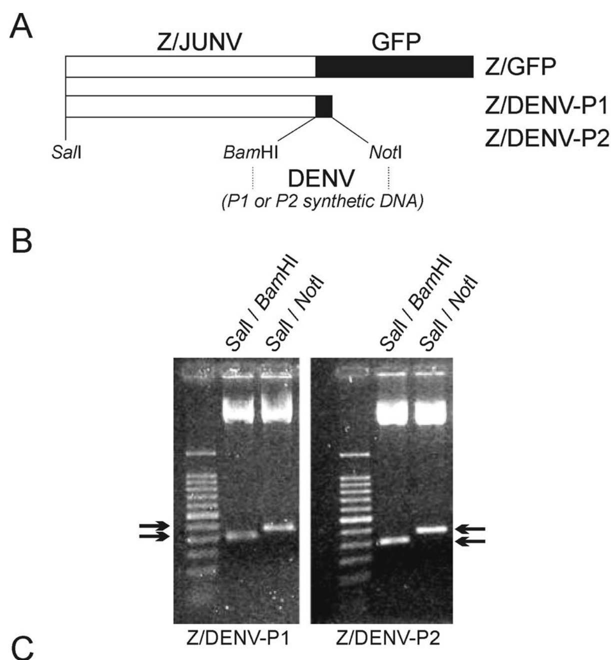
ELISA assay and statistical analysis Ninety six-well plates (MAXISORP, NUNC, Thermo Scientific®) were pretreated with 0.25 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 5.8 (4 h/room temperature), washed three times with the same sodium phosphate buffer, and then sensitized at 4 °C/overnight with the relevant synthetic peptides (0.5 μg/well) in borate buffered saline (0.1 M boric acid, 25 mM sodium tetraborate, 0.15 M sodium chloride pH = 8.4). The following day, the liquid was removed from the plates, which were washed three times with 0.1 % Tween 20 in PBS. Pools of sera obtained from immunized mice were used as source of primary antibodies. Sera were diluted 1:200, 1:400, 1:800, 1:1600, or 1:3200 in PBS/0.1 % Tween20/1 % BSA before incubation for 1 h and 30 min at 37 °C. An anti-mouse IgG, HRP conjugated and produced in goat (Sigma-Aldrich®), was used at a dilution of 1:10.000 as secondary antibody. OPD (ImunoPierce, Thermo Scientific®) was used as a chromogen for 20 min in the dark, followed by a stop solution (2.5 M sulfuric acid), and reading at 490 nm (TECAN® model infinite M200). Statistical analysis was performed using a one-way ANOVA, p value ≤0.05.

Plaque reduction neutralization tests Plaque reduction neutralization tests (PRNT) were performed according to WHO recommendations, with minor modifications. Different dilutions (1:40, 1:80, 1:160, and 1:320) of inactivated sera (56 °C, 30 min), obtained 2 weeks after the booster dose, were incubated with the DENV-2 virus at 37 °C for 1 h. The mouse monoclonal flavivirus group-specific antibody 4G2 (hybridoma D1-4G2-4–15, ATCC HB-112, (Falconar 1999) was used at the same dilutions as a positive neutralizing control. After that, HuH7.5 cells (ATCC PTA-8561) were incubated at 37 °C for 1 h and 30 min in a 5 % CO_2 atmosphere with the mixture of serum plus virus. Then, the mix was removed and a 1.6 % carboxymethylcellulose/DMEM/10 % fetal bovine serum solution was added to the wells. Five days later, the cells were fixed with 3 % paraformaldehyde and stained with crystal violet. The plaque-forming units (p.f.u/well) were then counted using a stereomicroscope.

Results

Design of the DENV candidate vaccines

Two conserved peptides present on the envelope of 480 available sequences of DENV analyzed, regardless of their serotype, were used in our study to test their behavior as hypothetical cryptic B-cell epitopes. Two chimerical vaccine candidates were designed combining the 3D structure and self-budding capacities of the Z protein of JUNV and each of these two peptide sequences. Figure 1a shows a schematic view of the two constructs as well as some molecular details of the constructions, including an experimental control construct that comprises Z-JUNV fused to the green fluorescent protein (GFP). The new recombinant plasmid constructs were analyzed by restriction enzyme digestion as well as by DNA sequencing. Figure 1b shows the results of the restriction enzyme analyses of the clones. Plasmids were digested with *Bam*HI/*Sal*I or *Bam*HI/*Not*I and were subsequently separated by agarose gel electrophoresis. Bands of the expected sizes were observed for Z-JUNV alone (291 bp), Z/DENV-P1 (342 bp), or Z/DENV-P2 (364 bp). Figure 1c shows the complete amino acid sequence of both antigens, as deduced from the sequenced DNAs, confirming the adequacy to the design planned. Two small amino acids (one glycine and one serine) were artificially included at the fusion site between both sequences in an attempt to generate some flexibility (Wegner et al. 2002) and thus permit better antibody recognition of the DENV peptides.



MGNCNGASKSNQPDSSRATQPAAEFRRVAHSSLYGRYNCKCCWFA
DTNLITCNDHYLCLRCHQGMLRNSDLCNICWKPLPTTITVPVEPTAPP
P<u>GS</u>**VDRGWGNGCGLFGKGG**

MGNCNGASKSNQPDSSRATQPAAEFRRVAHSSLYGRYNCKCCWFA
DTNLITCNDHYLCLRCHQGMLRNSDLCNICWKPLPTTITVPVEPTAPP
P<u>GS</u>**VVVLGSQEGAMHTALTGATEI**

Fig. 1 Vaccine candidates used in our study. **a** Schematic representation of the chimeric constructs depicting the most relevant components. **b** Agarose gel eletrophoreses of restriction fragments obtained after digestion of the relevant plasmid constructs; (*left panel*) *lane 1*: 100-bp molecular weight marker; *lane 2*: Z/DENV-P1 digested with *Sal*I and *Bam*HI; *lane 3*: Z/DENV-P1 digested with *Sal*I and *Not*I (*right panel*) *lane 1*: 100-bp molecular weight marker; *lane 2*: Z/DENV-P2 digested with *Sal*I and *Bam*HI; *lane 3*: Z/DENV-P2 digested with *Sal*I and *Not*I. **c** Amino acid sequences of Z/DENV-P1 (*upper*) and Z/DENV-P2 (*lower*). *Underlines* indicated the residues inserted to give flexibility to the hinge region between Z-JUNV and DENV peptides. In *bold* are marked sequences of the DENV epitopes

In vitro characterization of the Z-JUNV chimeras

Once generated, the capacity of these constructs to generate VLPs was tested. To this aim, we transfected HEK 293 cells with each of the three Z-JUNV recombinant constructs. First, to determine whether or not the VLPs were being generated by the transfected cells, transmission electron microscopy analyses (TEM) of the cell supernatants concentrated by sucrose gradients was performed. Figure 2a shows representative micrographs of the structures observed after the analysis of each group of cells. According to the images obtained, the expression products generated by HEK 293 cells transfected with Z/GFP, Z/DENV-P1, and Z/DENV-P2 were able to self-mount into virus-like structures that displayed sizes compatible with those previously predicted for equivalent JUNV structures, e.g., 50–100 nm (Borio et al. 2012).

Once the Junin virus-like structures were observed by TEM, we next decided to study the reactivity of those structures with antibodies that specifically recognize Z-JUNV protein by immunofluorescence. Immune reactivity represents a strong indication of the correct identity of the protein and, in some cases, of its correct tridimensional structure as well. Figure 2b displays the results of the immunofluorescence assays with a highly specific anti-Z rabbit polyclonal serum. As can be seen in this figure, only HEK 293 cells that had been transfected with Z-JUNV recombinant chimeras were specifically recognized by the antibodies present in the serum of animals. Altogether, the above data suggests that VLPs based on the Z self-budding protein of JUNV were generated and correctly mounted by the transfected cells.

In vivo induction of a specific anti-DENV and anti-JUNV humoral immune response

As per our previous observations, Z-JUNV VLPs were being formed in the plasmid-transfected cells and were structurally correct. Subsequently, we analyzed by ELISA the capacity of these constructs to induce a humoral immune response against the JUNV-DENV chimera in mice. For this, we immunized

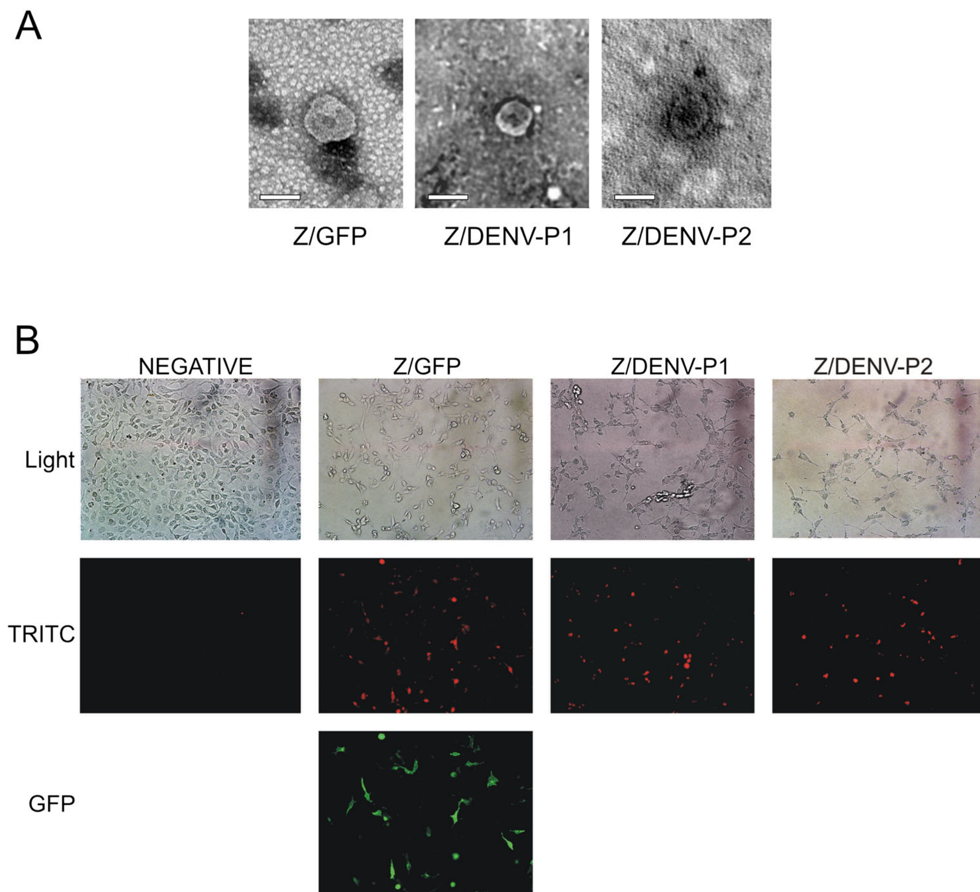


Fig. 2 *In vitro* analyses of the vaccine candidates. **a** Transmission electron microscopy micrographs representative of each of the sucrose gradient-purified Z/JUNV virus-like particles. **b** Light microscopy or immunofluorescence analyses of HEK 293 cells untransfected or transfected with pZ/GFP (positive control), or the vaccine candidates Z/DENV-P1 or Z/DENV/P2. A specific rabbit anti-Z/JUNV antiserum was used as source of primary antibodies for the recognition of Z, and TRITC was the fluorophore conjugated to the secondary anti-rabbit antiserum. GFP detection under a UV microscope was also performed for Z/GFP-transfected cells for positive confirmation of transfection efficiency. *White bars* in A represent a size of 100 nm

C57BL/6 mice i.m. with 50 μg/dose of each plasmid construct in *Bordetella pertussis* monophosphoryl-lipid A (MPLA) stable emulsion, to mimic previous reports that showed the beneficial effect of this adjuvant to induce broad immune responses after DNA immunization (Sasaki et al. 1997). Plasmids had been previously purified by cesium chloride isopycnic ultracentrifugation to eliminate *E. coli* endotoxin contents (LPS final content <2 ng/ml or 20 EU/ml). Six weeks after the priming dose, a 20 μg/mouse s.c. booster dose of synthetic DENV-P1 or DENV-P2 peptides in incomplete Freund's adjuvant was administered to the mice (Fig. 3a).

As expected, a single immunization with the recombinant plasmids displayed no significant antibody reactivity against P1 or P2 in either group of mice at the time of boost (data not shown). However, after boosting this initial immune response with synthetic peptides encoding the DENV E sequences under study, a different picture showed up. Figure 3b shows that, although no significant antibody reactivity could be observed in the sera of animals immunized with chimeras and peptides of the P1 sequence (left panel), titers of over 6.400 could be detected in all animals that had been immunized against the DENV E protein P2 sequence (middle panel, $p \leq 0.001$ in all cases when compared to control or P1-immunized mice). Furthermore, the right panel in Fig. 3b shows that these antibody levels were not generated by a single immunization after either DENV-P2 plasmid DNA or synthetic peptide administrations (nor DENV-P1 peptide either, data not shown) and were only elicited by the combined DNA/peptide prime-boost immunization protocol.

Neutralization of DENV infection by sera of immunized mice

Since we had detected a significant anti-DENV humoral immune response in those animals immunized with the DENV P2 products, we next wanted to determine whether the antibodies raised against the P2 sequence could have any effect on the virus particles. DENV mouse models are not widely available, and those that exist do not mimic the course of, or the immune responses risen against, natural human infections. Thus, we performed the traditional in vitro neutralization studies, since neutralizing the interaction between the virus particles and the host cell could represent a protective activity for the vaccinated human host. To this aim, we performed the plaque-reduction neutralization tests (PRNT) using sera of all immunized mice and controls.

Figure 4a shows representative pictures of the plaques generated by DENV 5 days after infecting HuH7 cells with

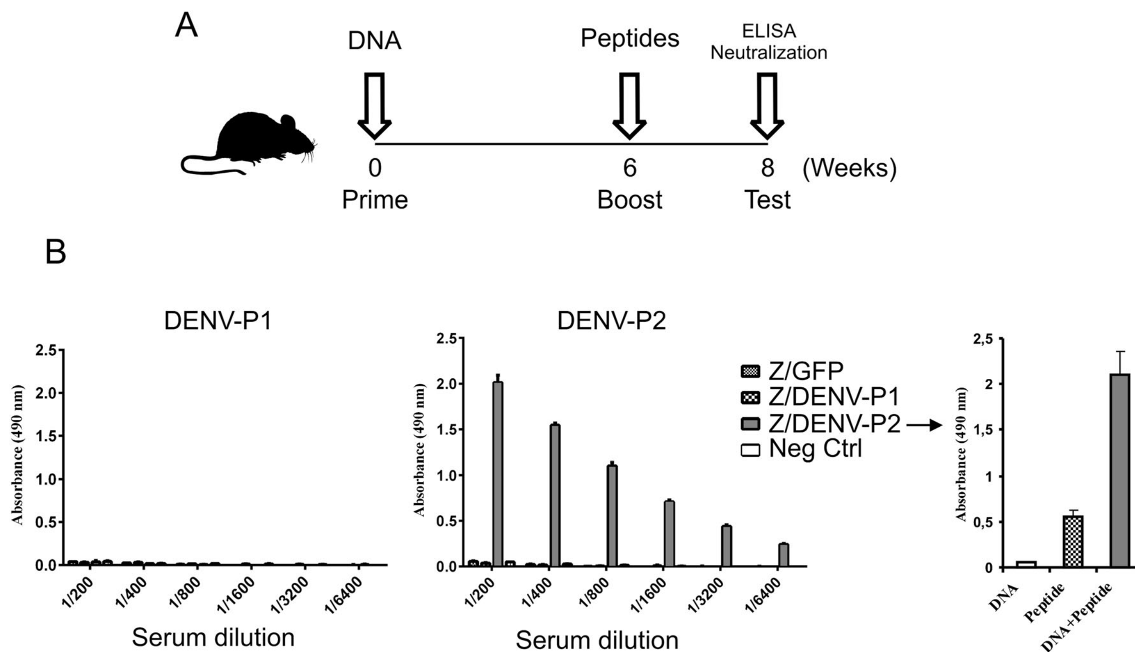


Fig. 3 *In vivo* immunogenicity studies of the vaccine candidates. **a** Prime-boost regime used in our experiments and tests performed using the resultant mice sera. **b** Enzyme-linked immunosorbent assays (ELISA) performed to detect the presence of reactive anti-DENV-P1 (*left*) or DENV-P2 (*middle*) antibodies in each group of mice immunized according to the protocol depicted in A. The level of antibodies elicited by each DENV-P2 immunogen when administered individually or combined in the prime-boost protocol is shown in the *right panel*. Results represent mean values and standard deviations of a pool of sera from four different animals (mean ± SD, $n = 8$)

clarified serotype 2 dengue viruses. As can be observed in that figure, when compared to the control cells or with cells that received the DENV suspensions pre-incubated with sera of mice immunized with DENV P1 constructs, a significant reduction in the number of lysis plaques (the cytopathic effect of DENV on HuH7 cells) could be observed in those cell monolayers that had received DENV pre-incubated with sera of animals containing antibodies against DENV P2, suggesting a significant neutralizing effect of the antibodies present in these mice sera. All control sera, including those elicited after immunization with individual immunogens, did not display a significant neutralizing activity.

In Fig. 4b, a graphical representation of the number of plaques detected in each group of wells at a dilution of 1:320 is shown. Additionally, the same figure shows the percentage of reduction of plaques by effect of the antibodies present in the relevant sera or after using the 4G2 neutralizing monoclonal antibody. A significant mean reduction of 62.5 % in the number of viral plaques was observed when using the sera of P2-immunized mice, only surpassed by the widely neutralizing monoclonal antibody.

Discussion

Several human trials of DENV vaccine formulations have been completed so far, with a majority based on attenuated DENV particles or DENV/yellow-fever virus (YFV) recombinant viruses. Those candidates displayed, at best, limited protective activities (Capeding et al. 2014; George et al. 2015; Guy et al. 2011; Kanesa-thasan et al. 2001; Kirkpatrick et al. 2015; Kitchener et al. 2006; Osorio et al. 2014; Thomas et al. 2013; Villar et al. 2015). Most studies reported no differences between placebo and immunized individuals regarding the number of serious adverse events (SAE) on the field. However, all of the vaccine candidates include in their formulation the Envelope (E) and pre-membrane (prM) proteins of the virus, which have recently been confirmed to be related to antibody-dependent enhancement (ADE) of the disease (de Alwis et al. 2014).

In an attempt to avoid ADE, we chose two cryptic epitopes within the E protein of DENV and engineered them to become part of a VLP. Two conserved peptides present on the envelope of 480 available sequences of DENV analyzed, regardless of their serotype, were identified by our collaborators (Fleith et al. manuscript in preparation) while studying conservation versus solvent exposition and tridimensional structure of the E protein. Both peptides are exposed on the surface of the E protein trimer formed during fusion, but not on the dimer present on the surface of the mature viral particles, suggesting that they could behave as cryptic epitopes for antibody recognition. Additionally, they display critical activities for the virus (P1 corresponds with the sequence of the fusion peptide and P2 has an unknown function but according to Fleith's manuscript blocks virus cycle progression when mutated). By fusing these peptides to a harmless JUNV-

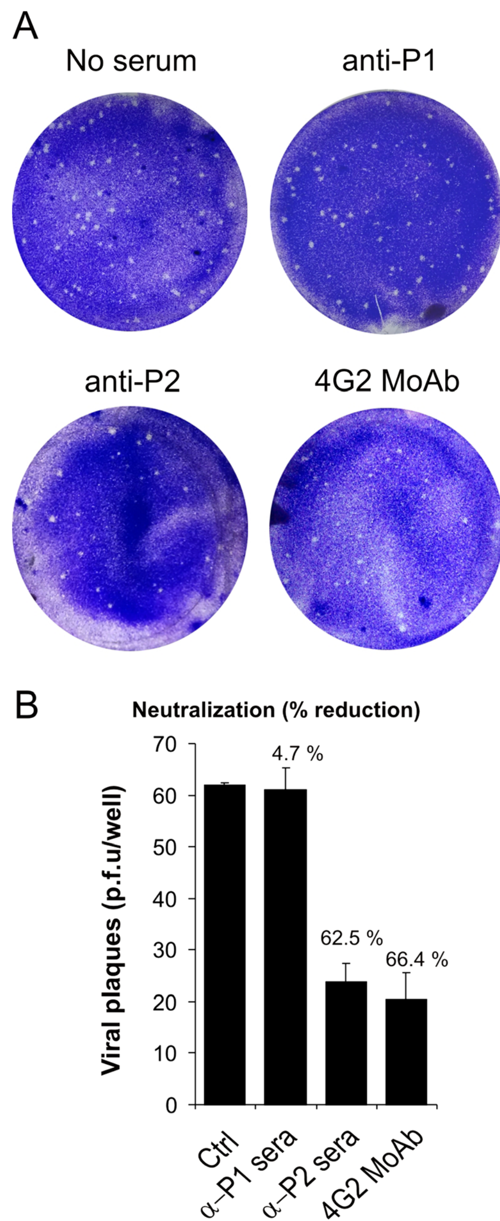


Fig. 4 Plaque-reduction neutralization tests (PRNTs) to detect antiviral immune responses in immunized mice. **a** Crystal violet stained HuH-7 cell monolayers cultured in six-well plates and incubated for 5 days with mixtures of DENV-2 viruses and sera of the different groups of mice (n = 4 per group) or with monoclonal antibody 4G2, as indicated. Sera were used at a dilution of 1:320. Plaques of cell lysis were counted with a stereomicroscope. **b** Graphical representation of the mean number (± SD) of plaque-forming units counted per well of a six-well plate incubated with the mixtures of sera (or monoclonal antibody 4G2) and DENV-2 particles, indicating also the percentage of reduction in the number of plaques detected at a serum dilution of 1:320

VLP, we generated a DENV vaccine candidate that is not only theoretically safer (not infectious and not inflammatory by itself) but also easier to produce in a large scale than those based on attenuated viral particles currently under evaluation as experimental human vaccines. Thus, our formulation includes only non-infectious DNA that encodes the VLPs fused—or subsequently boosted—with short DENV envelope peptides, but not the whole virion or the pre-membrane/envelope proteins of DENV included within the YFV vectors. In our candidate, each immunogenic component is precisely defined and the relevant DENV sequences represent conserved DENV epitopes that could possibly be part of a future universal vaccine.

Initially, in vitro analyses of the vaccine candidates showed that the constructions were molecularly correct and were able to generate particulate structures that looked like VLPs under electron microscopy. Indirect immunofluorescence assays also showed that Z protein had been successfully expressed in transfected HEK 293 cells. Therefore, when put altogether, the results of sequencing, immunofluorescence, and transmission electron microscopy did confirm the correct expression of Z/DENV-P1 and Z/DENV-P2 from the plasmid DNA vaccines. In this work, we preferred to use DNA vaccines because they are easier to obtain and purify, lowering the cost when compared to the Z-JUNV-purified VLPs. Administering DNA will also ensure that protein folding, glycosylation, or other post-translational modifications will mimic those of the natural Z-JUNV protein.

In vivo analyses of the humoral immune responses induced by the immunizations showed that the vaccine candidate based on P1 (the DENV fusion peptide) did not seem to induce any detectable humoral response. At first, we held the technology responsible for this, believing that the peptide might have not stacked to the plate. However, later negative data on the virus-neutralizing capacities of antibodies present in the sera of P1-immunized animals showed that, even in the case that antibodies would be present in those mice, those would not display any detectable effect on the viral particles, and thus, P1 could not be considered as a feasible vaccine candidate. The better response detected against Z/DENV-P2 may reflect that it is indeed a better immunogen, at least in our experimental setup. Besides, it is worth noting that we could not detect antibody reactivity (contrary to the NS1 protein tested side by side, data not shown) against this peptide in any of the dengue patients sera tested. This, together with the fact that this antibody epitope is displayed only on the surface of the envelope protein trimer during the last events of the invasion process, suggests that it may not be significantly presented to the immune system during natural infection. If that is the case, it is quite possible that it does not participate in ADE phenomena or other unwanted side effects linked to antibody recognition of the DENV infectious particles.

When looking at the neutralizing activity of Z/DENV-P2, contrary to Z/DENV-P1, we could observe that this immunogen potently neutralize virus entry. In 2014, members of our group carried out a study on the function of DENV-P2 peptide for the virus (Fleith et al., manuscript in preparation). The original P2 sequence (aa 250–270) was mutated and the resulting DENV genome obtained was sub-cloned into a

bacterial artificial chromosome (BAC). The infectious clone obtained by simultaneous substitution of residues 250–253 and 255 with alanines had a significant impact in reducing the replication and spread of the virus in insect and human cells. These results indicate the important role of this peptide for the virus and suggest that antibodies against it may interfere with the virus cycle irreversibly.

Other authors have pursued vaccination against dengue by using peptidic immunogens. Rocha and colleagues (2014) conducted a study using three conserved peptides present on the envelope protein: pep01 (MAKDKPTLDIELGGGGM AKMKPTLDIELGGGGMAKNKPTLDFELGG GGMAQGKPTLDFEL) in domain I, pep02 (WLVHKQ WFLDLPLPWGGGGWMVHRQWFFDLPLPWGGGGW-LVHRQWFLDLPLPWGG GGWLVHKQWFLDLPLW), and pep03 (LVTFKTAHAKKQEVGGGGLVTFK NAAHAKKQEVGGGGLVTF KNPHAKKQDVG GGGLVTFKVPHAKRQDV) included in domain II. Immunizations were performed in BALB/c mice with three immunizations of 50 μg of these peptides in incomplete Freund's adjuvant, and results showed significant humoral immunogenicity. Contrary to us, antibody reactivity of 16 human sera was observed against these three peptides, suggesting that none of them are cryptic sequences. None of the peptides tested coincide with P2 and, unfortunately, the antibodies induced were unable to neutralize dengue virus particles *in vitro* in a PRNT assay.

A more promising recent study characterized 145 monoclonal antibodies against dengue virus and detected two new conformational epitopes, the fusion loop epitope (FLE) and the envelope dimer epitope (EDE), the latter being broadly reactive across the dengue serocomplex and fully neutralizing against DENV (Dejnirattisai et al. 2015). The authors show that it is possible to completely neutralize DENV infectious particles using picomolar concentrations of one of these antibodies (EDE). However, despite describing epitopes in the same regions of our P1 (FLE) and P2 (EDE) peptides, they do not coincide, since FLE and EDE are formed by amino acid residues present in both units of the E dimer, but not in any of them separately.

Concerning the prime-boost immunization regime, other authors have also successfully used protocols similar to ours to test vaccine candidates against DENV infection. A report by Azevedo et al. (Azevedo et al. 2013) describes intense protection against a DENV-2 challenge after priming BALB/c mice with plasmid DNA construct encoding E and boosting those animals with a chimeric YFV with the prM and E genes replaced by those of DENV. Two doses of DNA, two doses of YFV recombinant, or both vectors admixed or given by different routes also protected the animals and promoted 100 % survival. No specific epitopes were defined for the antibodies detected in the PRNT assay, and the authors used a mouse brain-adapted DENV-2 strain inoculated intracerebrally, a model that is far from mimicking either natural infections or the protective mechanisms induced after an infected *Aedes* mosquito bite.

Some obstacles that we already envision for our P2 vaccine candidate include the fact that DNA vaccines, such as pZ/DENV-P2, although easier to obtain, do not immunize well larger animals or humans (Saade and Petrovsky 2012). The possibility of using purified Z-JUNV VLPs for immunization is however very attractive, since it could possibly be immunogenic during all stages of the pipeline of vaccine development. However, additional research has to be performed now to scale up the production of these VLPs, thus recovering large quantities of these structures in a purified form. Large-scale peptide synthesis, on the contrary, has been made broadly available nowadays, and it is thus feasible to believe that formulations based on synthetic peptides in adjuvants can become part of a near future widely used mass-vaccines development technologies. Other hurdles include for example the fact that, although in theory ADE should not be a concern, this possibility cannot be ruled out until specific tests have been performed.

This was a proof-of-concept study in which we demonstrated the immunogenicity of a DENV/JUNV chimera and protection against DENV-2. Although feasible to expect, because of the conservation of P2 sequence among all serotypes, we did not test protection against the other three DENV serotypes. This has to be performed in the near future. Finally, we have not determined the cellular immune responses that might have been induced after immunization or whether these would be mediated by CD4 or CD8. However, in preliminary tests run with the available on-line bioinformatics freeware, results did not reveal the presence of any CD4 or CD8 epitopes within P1 or P2 sequences, neither for mice nor for available human MHC haplotypes.

In conclusion, we have described here the use of a peptide sequence that is highly conserved among all DENV isolates and serotypes as a candidate vaccine formulation that induces antibodies with neutralizing activity against DENV-2 and that should therefore be considered for further tests in order to develop an antigen formulation that might serve as a universal DENV virus vaccine.

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The authors of this manuscript hereby declare that they are official inventors of a Patent, with application number 62147274, provisionally submitted on April 14th, 2015, to the United States Patent and Trademark Office (USPTO).

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