Live Zika virus chimeric vaccine candidate based on a yellow fever 17-D attenuated backbone

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Abstract

Zika virus (ZIKV) recently dispersed throughout the tropics and sub-tropics causing epidemics associated with congenital disease and neurological complications. There is currently no commercial vaccine for ZIKV. Here we describe the initial development of a chimeric virus containing the prM/E proteins of a ZIKV epidemic strain incorporated into a yellow fever 17-D attenuated backbone. Using the versatile and rapid ISA (Infectious Subgenomic Amplicons) reverse genetics method, we compared different constructs and confirmed the need to modify the cleavage site between the pre-peptide and prM protein. Using an immunocompromised mouse model, we demonstrate that mice infected with the chimeric virus produced levels of neutralizing antibodies close to those observed following infection with ZIKV and that pre-immunized mice were protected against subsequent viscerotropic and neuroinvasive virus following challenge with a heterologous strain of ZIKV. These data provide a sound basis for the future development of this ZIKV vaccine candidate.

Introduction

Zika virus (ZIKV; family *Flaviviridae*, genus *Flavivirus*) is a single-stranded positive-sense enveloped RNA virus. Its genome of 10.8 kb encodes a single polyprotein which is processed into three structural proteins (C, PrM and E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) by viral and host proteases [1]. Phylogenetic studies showed that all ZIKV strains characterized so far belonged among two distinct lineages (African and Asian) based on the initial geographic distribution of this virus[2]. ZIKV is a mosquito-borne flavivirus transmitted primarily by *Aedes spp*. mosquitoes [3]

Long considered to cause mild disease in humans, this arbovirus remained relatively unstudied until 2007, when it provoked a large outbreak in Micronesia [4]. Subsequently, several outbreaks occurred in different Pacific Ocean islands including French Polynesia in 2013, where it was associated with an increase of Guillain-Barré syndrome [5]. ZIKV then spread to the American continent causing major outbreaks in Central/South America and the Caribbean and was linked with an increase of congenital neurological complications. Sexual transmissions of ZIKV was also reported [6]. There is currently no commercial antiviral drug or vaccine for this virus [7].

Several approaches are now available with which to develop inactivated and recombinant (DNA- or RNA-based) vaccines [8–10]. However, live attenuated vaccines have several advantages including reduced costs and single dose induction of long-term immunity [11]. Recently, a chimeric ZIKV vaccine candidate based on the Japanese encephalitis virus live-attenuated train SA14-14-2 [12] was reported. The chimeric approach had been used since the late 1990s to develop vaccine candidates against several health-threatening flaviviruses including West-Nile virus, Japanese encephalitis virus and all serotypes of the dengue virus [13–15]. This approach consists of incorporating prM/E of a pathogenic flavivirus in a backbone of a licensed live-attenuated vaccine strain. Indeed, E protein is prominently exposed at the surface of viral particles and is *de facto* the major determinant of viral antigenicity [1]. In almost all cases, the well-characterized live attenuated 17-D strain used to prevent yellow fever virus (YFV) infections has been used as the genetic backbone. Some of these live-attenuated vaccines are currently commercially available [16,17].

Here we describe the development of a chimeric virus harboring the prM/E of an epidemic ZIKV (H/PF/2013) strain and the 17-D vaccine strain as the genetic backbone. The user-friendly and rapid ISA (Infectious Subgenomic Amplicons) reverse genetics method was used to generate this chimeric virus [18]. Finally, *in cellulo* and *in vivo* characterization of this strain demonstrated its potential to become a live-attenuated vaccine candidate.

Results

Design and rescue of chimeric viruses

The chimeric viruses were constructed using the yellow fever 17-D vaccine strain as a genetic backbone and by replacing the prM/E of this vaccine strain by those of the Asian ZIKV PF epidemic strains. Three different constructs, designated A, B and C, were constructed using variable sites flanking ZIKV prM/E coding sequences (Fig.1). Construct A harbored the pre-peptide and cleavage site before prM from the 17-D vaccine strain. Construct B harbored the pre-peptide from the 17-D vaccine strain and cleavage site before prM from ZIKV. Construct C harbored the pre-peptide and cleavage site before the prM from ZIKV. They all contained the cleavage site of the 17-D vaccine strain between E and NS1 proteins.

The ISA procedure was used to rescue the viruses as described previously: Three overlapping amplicons that contain the complete genome flanked at its 5' and 3' extremities respectively by the human cytomegalovirus promoter (pCMV) and the hepatitis delta ribozyme followed by the simian virus 40 polyadenylation signal (HDR/SV40pA) were transfected into a mix of HEK-293/BHK21 cells. Because the first amplicon contains the entire structural coding region, it was only necessary to exchange the first amplicon of our already functional yellow fever 17-D vaccine strain reverse genetic system to attempt replicative virus production.

For each construct, we performed two independent cell transfection experiments of five replicates. After an incubation of six days, cell supernatant media were passaged four times in Vero-E6 cells. Virus replication was assessed in cell supernatant medium at the last passage (called passage #4) using a real-time quantitative RT-PCR assay. No viral replication was detected for constructs A and B. In contrast, we detected virus replication only in one well (1/5) in both independent transfection experiments. These results highlighted the fact that the choice of the nature of the pre-peptide and cleavage site between the capsid and prM proteins is a crucial parameter when designing chimeric flaviviruses. During the first cell transfection experiment, we obtained at passage #4 high amounts of viral genome copies (1.78 e+9 Viral RNA copies/mL). This virus was designated CH-17-D/ZIKV and used for *in cellulo* and *in vivo* characterizations. Surprisingly, during the second experiment, we detected very low quantities of viral genome at passage #4 (3.57 e+3 Viral RNA copies/mL). This virus was designated CH-17-D/ZIKV*. We then performed four additional passages using the same procedure. Quantities of viral genomes in cell supernatants media were assessed from passage #1 to #8 and compared with that of CH-17-D/ZIKV (Fig.2). We found that amounts of viral genomes for CH-17-D/ZIKV reached a plateau at passage #2. In contrast, we observed an increase in the production of CH-17-D/ZIKV* from passage #6 to passage #8 where amounts of viral genome reached values similar to those observed with CH-17-D/ZIKV (2,67e+9 Viral RNA copies/mL) (Fig.2).

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CH-17-D/ZIKV genotypic characterization

In order to find the genotypic determinants associated with the difference of viral replication observed between CH-17-D/ZIKV and CH-17-D/ZIKV*, the complete genome of CH-17-D/ZIKV was obtained at passage #2 and #4 and compared with the sequence of the original construct. Only five substitutions were detected at passage #2 of which two were non-synonymous confirming the genome integrity of this strain (Table 1). Four of them were already fixed or almost fixed. At passage #4, all these mutations were fixed and no additional mutations were found. Interestingly, both non-synonymous mutations are located in domain II of E protein, respectively at residues E255 and E285 [28]. Consequently, the sequence of the 5' region of the viral genome of CH-17-D/ZIKV* (until NS1 coding region) was obtained at passage #4 and #8 (Table 1). While only one transitory substitution was detected at passage #4, four of the five mutations found with CH-17-D/ZIKV were detected including the two non-synonymous mutations located in E coding region. This high level of parallel evolution associated with the chronology of events strongly suggests that these four mutations are associated with the increase of replicative fitness observed with both viruses.

CH-17-D/ZIKV *in cellulo* characterization

To confirm the presence of the ZIKV E protein in Vero-E6 cells infected by the chimeric virus, we performed an indirect immunofluorescence assay using as primary antibody a specific ZIKV immune serum (Fig.3A). ZIKV PF and the 17-D vaccine strains were used as positive and negative controls. As expected, no fluorescence was observed with the 17-D vaccine strain and positive cells were observed with both chimeric and ZIKV strains confirming that the ZIKV E protein is expressed in infected cells. Since a cytopathic effect was observed with the ZIKV strain during these experiments, the number of positive cells was lower with this virus. Viability assays in Vero-E6 cells confirmed this observation: the CH-17-D/ZIKV virus is less cytopathic (mean value: 73% of cell viability) at day 5 post-infection than the ZIKV (mean value :49% of cell viability) (Fig.3B). We then performed comparative growth kinetics of these viruses in three different cell lines (HUH7.5, Vero-E6 and HEK-293). Cell supernatant media were harvested at different time points after infection to assess the amount of viral RNA (Fig.3 C,D and E). Similar growth kinetics curves were observed for all viruses in HUH7.5. In Vero-E6 cells higher amounts of viral genome were found in cell supernatants until five days post-infection with the chimeric virus. In HEK-293 cells, the chimeric virus had similar behavior to that of the 17-D vaccine strain.

CH-17-D/ZIKV *in vivo* characterization

Because ZIKV and 17-D vaccine strain do not replicate in immunocompetent mice, we used immunocompromised mice as the model to study the chimeric virus *in vivo*. Each time animals were immunized or infected, they were transiently immunocompromised following a two-step inoculation of anti-IFNAR antibody [26,27,29] as described in the Methods section.

Six groups of four mice were inoculated with two doses of CH-17-D/ZIKV, ZIKV and the 17-D vaccine strain to assess antibody production. Twenty-eight days after immunization, sera from mice were tested for the presence of antibodies to ZIKV (Fig.4) using a viral RNA Yield Reduction Neutralization Test (YRNT; see Methods, results were normalized using control group non-infected, non-immunized mouse sera). As a result, we demonstrated that immunization with the chimeric virus induced production of neutralizing antibodies against ZIKV confirming the starting hypothesis of this study. We detected a slightly higher level of neutralizing antibodies when mice were infected with ZIKV. In all cases, both doses used induced comparable neutralizing titers. As expected, mice immunized with the 17-D vaccine strain did not produce antibodies against ZIKV. We also attempted to isolate chimeric virus from animal blood samples to assess the ability of the chimeric virus to replicate *in vivo*: 2/16 were positive (Supplemental Fig.1) suggesting that this strain is able to replicate in mice since comparable neutralizing titers were measured with all mice immunized.

We assessed protection against subsequent infection by wild-type ZIKV. Four groups of four mice were immunized with two doses of CH-17-D/ZIKV and 17-D vaccine strain twenty-eight days prior to challenge with a ZIKV African strain (Dak84). Two control groups of four mice were also used: one immunized with PBS and then challenged (unvaccinated), and one immunized and challenged with PBS (mock). The protection was evaluated by determining the proportion of mice (i) viremic at day 2 and 3 post-challenge and (ii) with organs (brain and spleen) positive for the presence of ZIKV at day 10 post-challenge. At day 2 and 3 post-infection, respectively 12.5% and 50% of the mice immunized with the chimeric virus (both groups) had detectable viremia (**Table 2**). In contrast, 75% and 100% of the mice immunized with the 17-D vaccine strain (both groups) had detectable viremia respectively at days 2 and 3 post-infection. This difference is significant only at day 2 post-infection (p value =0.04; Fisher exact test). Viremia was found in 100% of the mice in the unvaccinated group. When viral RNA yields, measured in positive blood samples, were compared, no clear trend was observed except that higher values were measured at day 3 (Fig.4). At day 10 post-infection, 12.5% of the spleens and brains from mice immunized with the chimeric virus (both groups) were positive (**Table 2**). In contrast, respectively 100% and 87.5% of the spleens and brains from mice immunized with the 17-D vaccine strain (both groups) were positive (p value = 0.001 for spleens and 0.01 for brains; Fisher exact test). All the organs from mice of the

unvaccinated group were positive. Viral RNA yields found in the organs were quite variable in all positive samples (Fig. 5). These results demonstrated that immunization with the chimeric virus significantly protected mice against the systemic and brain infection induced by a heterologous ZIKV strain.

Discussion

We present here the initial development of a chimeric ZIKV live-attenuated vaccine candidate based on a yellow fever attenuated 17-D genetic backbone. Using the ISA reverse genetics method, we were able to rapidly test several associations of subgenomic amplicons starting from a pre-existing 17-D vaccine strain reverse genetics system. This method was recently applied to Asian and African strains of ZIKV [20].

Three different designs were tested to incorporate the prM/E of ZIKV in the 17-D vaccine genetic backbone. Our results highlighted the necessity of modifying the cleavage site between the pre-peptide and prM protein for the construction of chimeric viruses as already described during development of chimeric ZIKV/DENV and DENV/ZIKV strains [30].

Nevertheless, we also demonstrated that chimeric viruses needed to acquire adaptive mutations to properly replicate in mammalian cells. Indeed, we observed low-percentage of virus recovery during cell transfection experiments and both replicative viruses rescued the shared four substitutions of which two were non-synonymous and located in domain II of the E protein. Interestingly, mutations located in this particular domain of the E protein were previously described *in cellulo* with 17-D vaccine strain based chimeric flaviviruses (DENV type 1/2 and Japanese encephalitis virus) [31,32]. These findings suggest that emergence of compensatory mutations in the E protein is probably necessary to restore the replicative fitness of the virus following exchange of two of its structural proteins.

By comparing the growth properties of our chimeric virus with its two parental strains in different mammalian cells, we found that this new synthetic virus had its own biological properties probably in relation to the nature of this new association of viral proteins. In fact, we observed than this strain is fitter than parental strains in Vero-E6 cells and close to 17-D vaccine strain in HEK-293 cells.

We used transitory immunocompromised mice as an animal model system to characterize *in vivo* the chimeric virus. We demonstrated that mice infected with this virus produced levels of neutralizing antibodies close to those observed following infection by ZIKV. Our results also showed that immunization using the chimeric strain significantly protected mice against brain and spleen invasion induced by a heterologous strain of ZIKV. In addition, the proportion of viremic mice was significantly reduced at day 2 post-challenge. Altogether, these results

provide evidence that this chimeric strain had all the prerequisites to be tested in a more relevant animal model such as the microcephalic sensitive mouse model [33].

In conclusion, our data provide sound basis for the future development of this vaccine candidate. Furthermore, the approach used in this study to rescue the chimeric virus showed that significant advances in the development of reverse genetics methods now offer the possibility of drastically reducing the timeframe between the emergence of a novel viral pathogen and the availability of a live-attenuated vaccine candidate.

Materials and Methods

Cell lines

All cells were grown at 37 °C in 5□% CO2 with 1□% Penicillin/Streptomycin (PS; 5000U.mL-1 and 5000µg.mL-1; Life Technologies) and supplemented with 1□% non-essential amino acids (Life Technologies) in media as specified below.

Baby hamster kidney (BHK-21; ATCC number CCL-10), human hepatocellular carcinoma (HUH7.5; [19]) and human embryonic kidney (HEK-293; ATCC number CCL-1573) cells were grown in Dulbecco's Modified Eagle's Medium High glucose (4500 mg/l) (Life Technologies) with 7□.5% heat-inactivated fetal bovine serum (FBS; Life Technologies). Vero-E6 (ATCC number CRL-1586) cells were grown in minimal essential medium (Life Technologies) with 7□.5% FBS.

Viruses

ZIKV Asian lineage strains PF (H/PF/2013, GenBank accession number: KJ776791) and Mart2015 (MRS_OPY_Martinique_PaRi_2015, GenBank accession number: KU647676), YFV 17-D (Produced by reverse genetics as described below; GenBank accession number: EU074025) and ZIKV African lineage strain Dak84 (A.taylori-tc/SEN/1984/41662-DAK ; GenBank accession number: KU955592) were used in this study. All these viral strains are available for the scientific community via the European Virus Archive goes Global (EVAg) project, a non-profit organization (https://www.european-virus-archive.com).

For each viral strain, we prepared a stock solution of clarified cell culture medium which was then used for all analyses. Briefly, a 25 cm2 culture flask of confluent Vero-E6 cells containing 667µL medium with 2.5% FBS (Life Technologies) was inoculated with 333µL of clarified infectious medium, incubated for 6 hours, washed once with Hank's Balanced Salt Solution (HBSS, Life Technologies) and incubated for 3 days with 7mL of fresh medium. Cell supernatant medium was harvested, clarified by centrifugation, supplemented with HEPES buffer (Final concentration of 25mM; Sigma), aliquoted and stored at -80°C.

All experiments using replicating viruses were performed in BSL3 facilities.

ISA procedure

Chimeric viruses and the YFV 17-D vaccine strain were rescued using the ISA (Infectious Subgenomic Amplicons) reverse genetics method as previously described [18,20,21].

Preparation of subgenomic DNA fragments

The complete viral genome was amplified by PCR as three overlapping DNA fragments. The first and last fragments were flanked respectively by the 5' and 3' termini and included the human cytomegalovirus promoter (pCMV) and the hepatitis delta ribozyme followed by the simian virus 40 polyadenylation signal (HDR/SV40pA). We started by using a reverse genetics system designed for the YFV 17-D strain (available on supplemental material). Because the first DNA fragment contained all regions encoding structural genes, only this fragment was modified to design chimeric viruses (the primers are listed in supplemental table 2).

DNA fragments were generated by PCR using *de novo* synthesized genes (Genscript) as templates. The sequence of the primers used is listed in supplemental table 1. PCR mixes were prepared using the Platinum PCR SuperMix High Fidelity kit (Life Technologies) following the manufacturer's instructions. Amplifications were performed using ABI 2720 thermal cycler (Applied Biosytems) with the following conditions: 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 60°C for 30 s, 68°C for 5 min plus 10 min final elongation at 68°C. PCR product size and quality were controlled by running gel electrophoresis and DNA fragments were purified using the High pure PCR product purification kit (Roche).

Cell transfection

Mixtures of BHK-21 and HEK-293 cells were seeded into PureCoat amine 6-well cell culture plates (Corning) one-day prior to transfection. Cells were transfected with 2µg of an equimolar mix of the three DNA fragments using lipofectamine 3000 (Life Technologies) and following the manufacturer's instructions. Each transfection was performed in 5 replicates. After 24h of incubation, the cell supernatant media were removed and replaced by fresh cell culture medium. Six days post-transfection, cell supernatant media were passaged four times using 6-well cell culture plates of confluent Vero-E6 cells: cells were inoculated with 100µL of diluted (1/3) cell supernatant media, incubated 2h, washed with HBSS and incubated six days with 3mL of medium. Remaining cell supernatant media were stored at -80°C and called passage #1, #2, # and #4. To ensure the complete removing of DNA used during the transfection, the passage #4 was used to assess virus replication: 100µL of cell supernatant medium was collected to detect viral RNA using a qRT-PCR assay (see below). Passage #3 was used to produce virus stock solutions of YFV 17-D and chimeric viruses.

RNA extraction and real-time quantitative PCR assays

RNA extraction was performed using the Qiacube HT and the Cador pathogen extraction kit (both from Qiagen) following the manufacturer's instructions. Briefly, 100µl of cell supernatant medium was collected into an Sblock containing recommended quantities of VXL, proteinase K and RNA carrier. A DNAse digestion step (QIAGEN) was performed to remove the DNA used during cell transfection. The quantity of viral RNA was quantified by real-time quantitative RT-PCR (qRT-PCR; EXPRESS One-Step Superscript™ qRT-PCR Kit, universal, Life Technologies). The sequence of the primers used to detect ZIKVs, YFV 17-D and chimeric viruses is listed in supplemental table 3. 3.5 µL of RNA was used for each reaction (final volume of 10µL). Amplifications were performed using the QuantStudio 12K Flex Real-Time PCR System (Applied Biosytems) with the following conditions: 10 min at 50°C, 2 min at 95°C, and 40 amplification cycles (95°C for 3 sec followed by 30sec at 60°C). Amounts of viral RNA were calculated from standard curves (quantified T7-generated synthetic RNA standards were used).

Complete Genome sequencing

Complete and partial genome sequencing of chimeric virus were performed as previously described [22]. Viral RNA extraction was performed as described above. A set of specific primer pairs (supplemental table 4) was used to generate amplicons by RT-PCR using the Superscript III One-Step RT-PCR Platinum TaqHifi kit (Life Technologies). For each passage sequenced, purified PCR products were pooled and analyzed using the Ion PGM Sequencer (Life technologies) according to the manufacturer's instructions. Resulting reads were analyzed using the CLC Genomics Workbench 6 software (CLC Bio). They were trimmed using quality score, by removing the primer sequences at their termini and by finally systematically removing 6 nt at the 5′ and 3′ termini. Remaining reads with a length greater than 29 were mapped using the designed sequence of the chimeric virus as reference. Mutation frequency for each position was calculated as the number of mutated reads divided by the total number of reads at that site. Only substitutions with a frequency ≥10% were taken into account for analysis. All sequences are available in the Supplemental Material.

Tissue culture infectious dose 50 (TCID50) assay

96-well cell culture plate of confluent Vero-E6 containing 100µL/well of media was inoculated (50µL/well) with 10-fold serial dilutions of centrifugation clarified cell culture

supernatant medium. Each dilution was repeated 6 times. The plate was incubated for 7 days and read for absence or presence of CPE in each well. TCID50 titers were then calculated using the Reed-Muench method [23].

Cell Viability assay.

Confluent cells were inoculated at an MOI (multiplicity of infection) of 0.01 in a 96-well cell culture plate in triplicate for each measurement day. Every day for a period of 5 days we performed the cell titer blue viability assay (Promega) following the manufacturer's instructions.

Virus growth kinetics

Confluent cells were inoculated at an MOI of 0.01 in a 6-well cell culture plate in triplicate. Every day for a period of 7 days, 100µL of cell supernatant medium was collected to measure amounts of viral RNA by qRT-PCR (see above).

Indirect immunofluorescence assay

Confluent Vero-E6 cells were inoculated at an MOI of 0.01 in an 8-well cell culture in Lab-Tek II Chamber Slide System in duplicate. At 5 days post-infection, cells were washed twice with HBSS and fixed with 4% paraformaldehyde for 2 hours. Detection of viral antigens was performed as previously described [18,24] using as primary antibody a ZIKV-specific immune serum (dilution: 1/50) collected from a Syrian Hamster immunized with the ZIKV strain Mart2015 (see below). This serum was shown to neutralize more than 90 % of ZIKV PF replication until 1/3000 dilution (data not shown). The secondary antibody used was a Goat anti-hamster Alexa 488 antibody (Invitrogen) at 1/500 dilution. Slides were observed using a Eurostar II fluorescence microscope with the Europicture software (Euroimmune).

Viral RNA Yield Reduction Neutralization Test (YRNT)

Vero-E6 cells were seeded into a 96-well cell culture plate one day prior to infection (5×10^4 cells in 100µL of medium containing 2.5% FBS per well). The next day, twofold serial dilutions of sera (from 1/20 to 2560; diluted with medium containing 2.5% FBS) were mixed (50:50; v/v) with appropriate amounts of viral stock (diluted in medium containing 2.5% FBS), incubated 1h 30 min. at 37°C/5%CO2 and then added to cells (50µL/well). The amount of virus added had been calibrated to ensure that virus production in cell supernatant medium did not reach a plateau at the readout time [25]. Cells were incubated for 3 days and 100µL of cell supernatant medium was harvested to perform nucleic acid extraction and to quantify amounts of viral RNA using a real-time qRT-PCR assay (see above). Each serum dilution was tested in triplicate. For each experiment, a virus replication control (VC) was performed: serial dilution of a negative serum (from control group of mice; from 1/20 to 2560) was tested in duplicate with the same virus stock dilution. For each serum dilution, viral RNA yield

reduction (% of viral inhibition) was calculated using as reference the mean amount of viral RNA obtained with VC at the corresponding dilution. 50% and 90% viral inhibition cut-offs were used to estimate 'viral RNA Yield Reduction Neutralization 50% and 90% (YRNT50 ; YRNT90) titers using the method of Reed and Muench [23].

***In vivo* experiments**

Ethics statement

Animal protocols had been approved by the local ethics committee (Comité d'éthique en expérimentation animale de Marseille - C2EA -14; protocol number #9327). All *in vivo* experiments were performed in accordance with the European legislation covering the use of animals for scientific purposes (Directive 210/63/EU) and French national guidelines.

Animal handling

Animals were maintained in ISOcage P Bioexclusion System (Techniplast) with unlimited access to food and water and 12h-light/12h-dark cycle. Animals were individually monitored every day to detect appearance of any clinical sign of illness/suffering. Virus/Antibody inoculation, blood collection as well as euthanasia (cervical dislocation) were performed under general anesthesia (isofluorane).

Golden hamster immunization

One 4-weeks-old female Syrian Hamster (Janvier) was intra-peritoneally immunized with 100µL containing **10^5 TCID50** of ZIKV strain Mart2015. 21 days later the Hamster was re-injected with the same dose. The hamster did not show any sign of illness or weight loss.

15 days later, the hamster was euthanized and a blood sample (intracardiac puncture) was collected. After centrifugation, serum was collected and stored at -80°C.

Mice immunization and challenge experiments

Six groups of eight 3-weeks-old female C57/bl6 mice (Charles River) were intraperitoneally inoculated with 100µL of virus: two groups were immunized with the YFV 17-D strain (two doses: 10^4 and 10^5 TCID50), two groups were immunized with the ZIKV PF strain (two doses: 10^5 and 10^6 TCID50) and two groups were immunized with the CH-17-D/ZIKV strain (two doses: 10^4 and 10^5 TCID50). Two control groups of 4 mice were used to perform (i) negative control group (non-immunized mice; used to performed virus replication control (VC); see above) and (i) positive control group (non-immunized mice; challenged and used as positive control during challenge experiments.

All the mice used were immunocompromised following a two-step inoculation of anti-IFNAR antibody (clone MAR1-5A3; Interchim; intraperitoneal injection ; 120µL) [26,27]: 1mg one day prior and 1 mg one after each infection/immunization (*i.e.* the mice challenged were immunocompromised twice with this two-step procedure).

Blood collection (10µL) from the tail vein was performed at days 2 and 3 post-infection (4 animals per group) to assess viremia by qRT-PCR and detect infectious virus by cell culture isolation. Immediately after its collection, all the blood was inoculated into a 12-well cell culture plate containing confluent Vero-E6 cells and 150µL of medium/well. After an incubation of 2 hours, 100µL of the inoculum was harvested to perform nucleic acid extraction and to quantify amounts of viral RNA using a real-time qRT-PCR assay as described above (viremia). The cells were washed (HBSS), 1.5mL/well of fresh medium was added to the cells which were incubated for 6 days. Finally, 100µL of cell supernatant medium was harvested to perform nucleic acid extraction and to quantify amounts of viral RNA using a real-time qRT-PCR assay as described above.

At day 28 post-infection, 4 animals per group (including the negative control group) were euthanized. Blood samples were then collected (intracardiac puncture) from euthanized animals. After blood centrifugation, sera were stored at -80°C before being used to performe the neutralization tests.

All the remaining animals (4 per groups immunized and the positive control group) were then challenged with 10^6 TCI50 of ZIKV Dak84. Blood collection (10µL) from the tail vein was performed at days 2 and 3 post-challenge to assess viremia by qRT-PCR and detect infectious virus by cell culture isolation as described above. At day 10 post-challenge, all the animals were euthanized. Organs (spleen, brain and one lobe of liver) were then collected in 1 mL of HBSS supplemented with 10% of FBS, and crushed 10 minutes at 30 cycles per second with one bead of tungsten using the Tissue Lyser machine (Retsch MM400). After centrifugation at 5000g during 10 min, the supernatant medium was collected centrifuged again at 10,000g for 10 min. 50 µL of the supernatant medium was used to perform nucleic acid extraction and to quantify amounts of viral RNA using a real-time qRT-PCR assay (see above).

Statistical analysis

All data obtained were analyzed using Graph pad prism 7 software (Graph pad software). All graphical representations and statistical analyses were also performed on Graph pad prism 7 software.

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Table 1 Mutations detected during the passages that followed cell transfection of chimeric viruses.

Chimeric virus	Nucleotide position	Frequency at #P2	Frequency at #P4	Frequency at #P8	Region	Nucleotide change	aa change
CH-17-D/ZIKV	291	100%	100%	n.a	C	A>G	-
	1625	66%	90%	n.a	E	T>C	V>A
	1706	100%	100%	n.a	E	G>T	G>V
	2514	100%	100%	n.a	NS1	A>G	-
	4482	100%	100%	n.a	NS2B	A>G	-
CH-17-D/ZIKV*	291	n.a	n.d	70%	C	A>G	-
	1303	n.a	100%	n.d	E	C>T	H>Y
	1625	n.a	n.d	68%	E	T>C	V>A
	1706	n.a	n.d	69%	E	G>T	G>V
	2514	n.a	n.d	69%	NS1	A>G	-

Table 2 Protection of transitory immunocompromised mice challenged with a heterologous strain of ZIKV. Groups of four mice were immunized with two doses (10e4 and 10e5 $TCID_{50}$) of CH-17-D/ZIKV, the 17-D vaccine strain or PBS (unvaccinated). Twenty-eight days later, mice were challenged with 10e6 $TCID_{50}$ of a ZIKV African strain. The proportion of mice with detectable viremia at days 2 and 3 post-challenge and with positive spleen/brain at day 10 post-challenge was expressed as percentage. Results from both doses of viruses are pooled (results of each groups are in supplemental Table 1). Detection of viral RNA was assessed using a real time RT-PCR assay. Amounts of viral RNA detected in positive samples are represented in figure 5.

Viral strain	Viremia - day 2	Viremia - day 3	Spleens	Brains
CH-17D/ZIKV (both doses)	12,5 % (1/8)	50 % (4/8)	12,5 % (1/8)	12,5 % (1/8)
YFV 17D (both doses)	75% (6/8)	100 % (8/8)	100 % (8/8)	87,5 % (7/8)
Unvaccinated	100 % (2/2)	100 % (4/4)	100 % (4/4)	100 % (4/4)

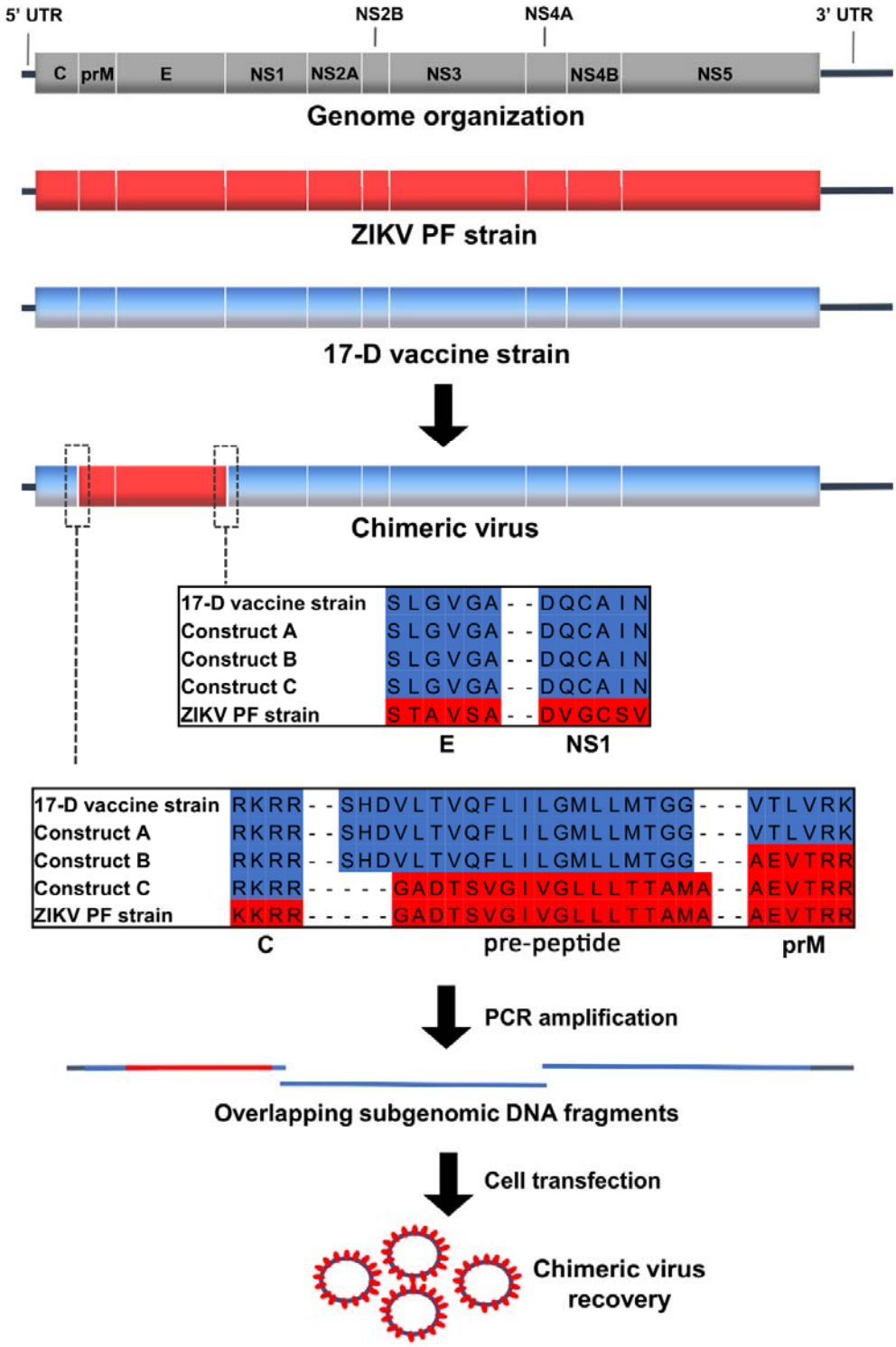

Figure 1 Schematic representation of design and recovery strategies used to generate chimeric viruses. We recovered infectious virus with construct C only.

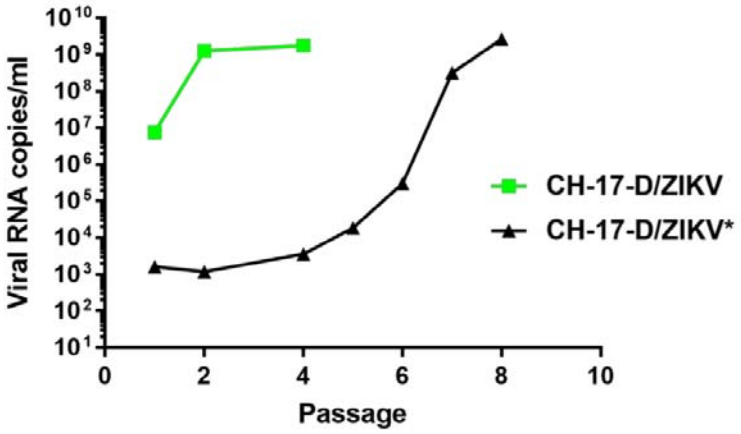

Figure 2 Evolution of viral production of chimeric viruses during serial passage that followed cell transfection. A mix of BHK-21/HEK-293 cells was transfected. Cell supernatant media were then passaged 4-8 times in Vero-E6 cells. Viral production in cell supernatant medium was assessed using a real time quantitative RT-PCR assay (expressed as mean ±SD).

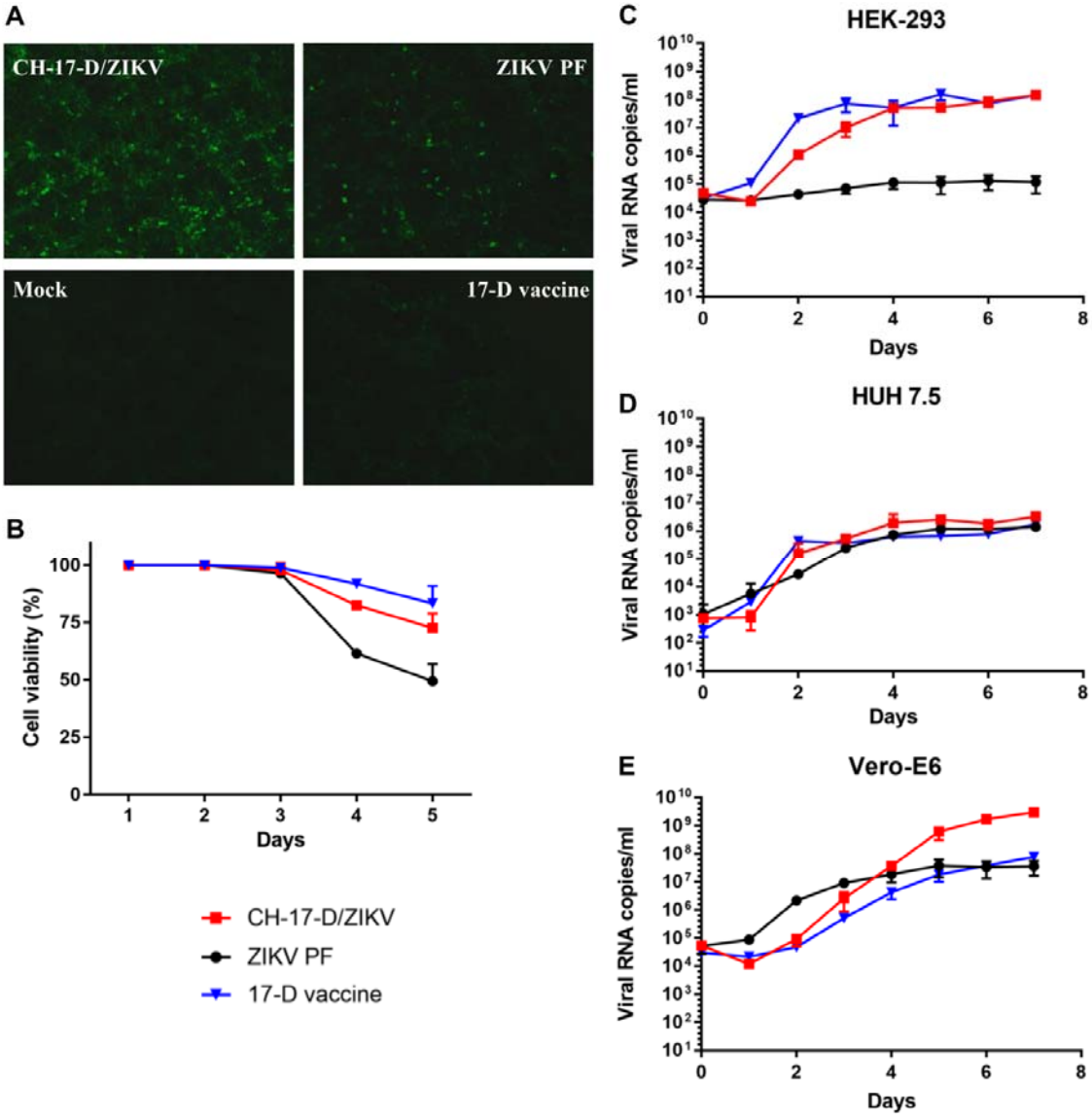

Figure 3 CH-17-D/ZIKV *in cellulo* characterization. **Panel A:** Expression of the ZIKV E protein in Vero-E6 was confirmed using an indirect immunofluorescence assay using as primary antibody a specific ZIKV immune serum. Uninfected cells (mock) and cells infected by ZIKV and the 17-D vaccine strain were used as controls. **Panel B:** Cell viability assay performed in Vero-E6 cells with CH-17-D/ZIKV, ZIKV and the 17-D vaccine strains (expressed as mean ±SD). **Pane C/D/E: C**omparative growth kinetics of CH-17-D/ZIKV, ZIKV 17-D vaccine strains HEK-293 (**C**), HUH 7.5 (**D**) and Vero E-6 (**E**). Cell supernatant media were harvested at different time points after infection to assess the amount of viral RNA using a real time quantitative RT-PCR assay (expressed as mean ±SD).

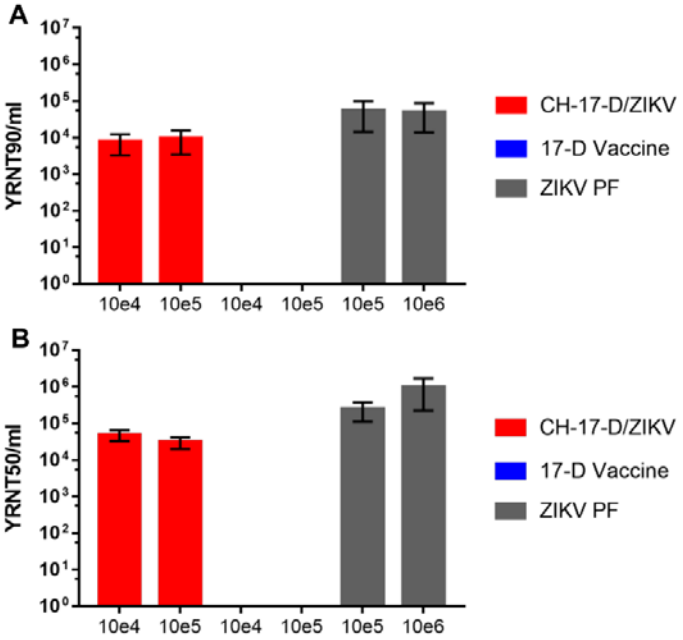

Figure 4 Neutralizing antibody titers of transitory immunocompromised mice at day 28 post-immunization. Groups of four mice were immunized with two doses of CH-17-D/ZIKV, ZIKV and the 17-D vaccine strain (from 10e4 to 10e6 $TCID_{50}$). Twenty-eight days later, sera from mice were tested for the presence of antibodies to ZIKV using a viral RNA Yield Reduction Neutralization Test expressed as mean ±SD of YRNT50 and YRNT90 titers.

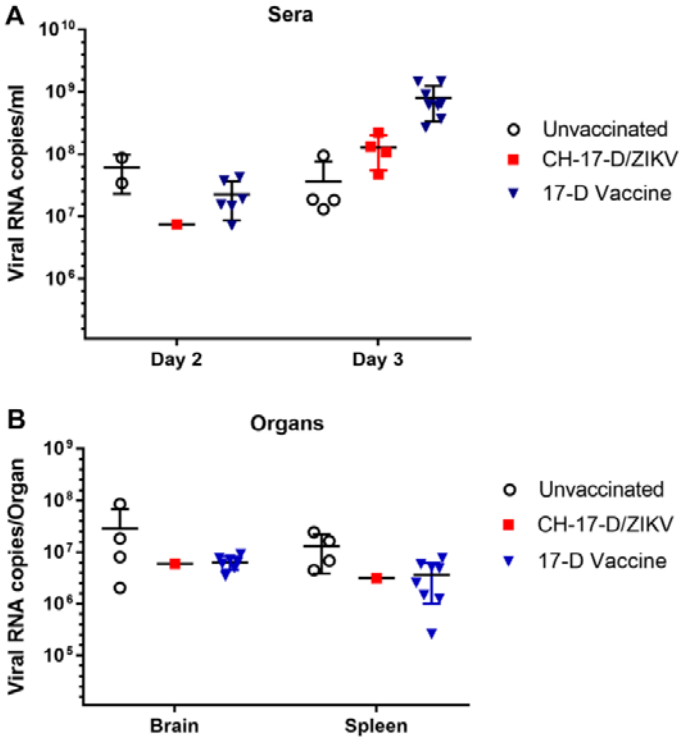

Figure 5 Amounts of viral RNA detected in positive blood, brain and spleen samples collected during challenge experiments. Amounts of viral RNA in positive samples collected during challenge experiments (cf. Table 2) using a real time quantitative RT-PCR assay. Mean values ±SD are represented respectively by black lines and error-bars. Results from both doses of viruses are pooled (results of each groups are in supplemental Figure 2)