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Capsid containing virus like particle vaccine against Zika virus made from a stable cell line

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ABSTRACT

Zika virus infection during pregnancy is associated with severe birth defects including microcephaly in the new born. The lack of specific treatment calls for the development of a safe and effective vaccine for use in pregnant women. We recently tested the efficacy of a Virus Like Particle (VLP) vaccine for Zika virus in mice and found that Capsid-preMembrane-Env (CprME) VLPs generated a better neutralizing antibody response than preMembrane-Env (prME) VLPs. The superiority of CprME VLPs suggested that inclusion of capsid in the vaccine may enhance the immune response. However, production of CprME VLPs requires co-expression of NS2B-3 protease, which creates a major hurdle for generation of stable cell lines. To overcome this limitation, we generated a bicistronic vector that expresses CprME and NS2B-3 using an IRES sequence. This bicistronic expression cassette, in a lentiviral vector, was used to create a stable cell line that constitutively secretes CprME VLPs. The expression of NS2B-3, presence of capsid in the secreted VLPs, efficiency of VLP release, and stability of the cell line was extensively tested. Antigen sparing studies in mice using prME and CprME VLPs, both derived from stable cell lines, confirmed the superiority of CprME VLPs in generation of neutralizing antibody response. Capsid specific antibodies were detected in CprME VLP immunized mice providing mechanistic insights into the superiority of these VLPs. Challenge of CprME VLP immunized mice with Zika PRVABC59 showed complete protection against day 3 viremia further validating the efficacy of the vaccine. Our study is the first to generate a stable cell line secreting Zika CprME VLPs via natural NS2B-3 cleavage, demonstrate incorporation of capsid in CprME VLPs and complete protection in challenge studies. This is a major advancement for the Zika vaccine platform that is safe for use in pregnant women and readily scalable for use in developing countries.

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1. Introduction

As per the World Health Organization, Zika virus (ZIKV) infection and associated microcephaly remains a long term public health challenge. A recent decline in ZIKV infections should not be taken with complacence as flaviviruses have a history of re-emergence [1,2]. While the baseline incidence of microcephaly ranges from 0.02 to 0.12% (2–12 babies per 10,000) live births in the Unites States [3], the recent outbreaks of ZIKV infection have dramatically increased that incidence to 0.88–13.2% [3]. Moreover, data from Brazil, the hardest hit country with ZIKV suggests of a much higher percentage of microcephaly cases [4]. This devastating outcome of ZIKV infection in the unborn fetus calls for a safe and effective vaccine especially for use in pregnant women [5]. Interestingly, more recent data from Brazil is encouraging and suggests that there has been a decline in Zika virus associated birth defects. When looking into the mechanism, a case controlled study in Recife, Brazil reported that 64% of the study group showed positive serology for ZIKV [6]. Thus, it is plausible that the study population is now immune as a consequence of ZIKV exposure.

Currently there is no approved vaccine for ZIKV although there are several in the pipeline [7–21]. The ZIKV vaccines that have proceeded to phase I/II clinical trials include prME DNA or mRNA vaccines or Purified Inactivated whole Virus (PIV), all of which have their advantages [22]. However, there is no Virus Like Particle (VLP) vaccine candidate that is currently in clinical trials. VLP vaccines fare superior to PIV platforms due to their inherent non-infectious nature while generating a potent and long lasting immune response. As the structure of VLPs closely resembles the native virus particle, the antigens recognized by the immune

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system bear greater resemblance to a natural infection generating an effective immune response [23,24]. Moreover, for a vaccine to be commercially successful, it has to be economical enough for its receptivity in developing and underdeveloped countries where potential of Zika outbreaks remains high. Here again, VLP vaccines can be readily scaled up for cost effective production via use of stable cell lines.

We recently tested the efficacy of a VLP vaccine for Zika virus [15]. Immunization studies in mice showed that VLPs generated higher neutralizing antibody titers than DNA vaccine, with CprME VLPs showing a better neutralizing response than prME VLPs. The superiority of CprME VLPs warrants the inclusion of capsid in the vaccine for a better immune response. In the current study, we therefore developed a stable cell line releasing Zika CprME VLPs via a biscistronic IRES vector expressing CprME along with the NS2B-3. Co-expression of CprME and NS2B-3 protease allows cleavage of capsid C from prME [25–27] resulting in CprME VLP secretion. Cells transduced with the CprME-NS2B-3 lentiviral vector were single cell cloned and used to purify CPrME VLPs. Immunization studies were then conducted in mice using different antigenic dose of CprME VLPs and compared to a similar dose of prME VLPs using the clinically approved adjuvant, Alum. Our data demonstrates the superiority of CprME VLPs in generating a robust anti-ZIKV immune response at lower antigenic dose when compared to prME VLPs. Virus challenge studies showed that CprME VLP immunized mice were protected against viremia, validating the efficacy of the vaccine. Our study is the first to demonstrate incorporation of Capsid in CprME expressed Zika VLPs and generation of Capsid specific antibodies in CprME VLP immunized mice.

2. Results

2.1. Bicistronic CprME-IRES-NS2B-3 derived Zika VLPs show incorporation of capsid

Our recent study demonstrated that CprME VLPs are more immunogenic in mice with the generation of higher titers of neutralizing antibodies when compared to prME VLP immunized mice [15]. It is also well established that the cleavage of capsid by the NS2B-3 protease is essential for virion morphogenesis and release in flaviviruses [26]. We hence tested the efficiency of Zika versus WNV NS2B-3 protease in releasing Zika CprME VLPs. Expression of either Zika or WNV NS2B-3 protease resulted in release of Zika CprME VLPs into the supernatant (Fig. 1A). We next generated a vector that co-expressed Zika CprME and NS2B-3 to facilitate the generation of cell line stably expressing Zika CprME. For this Zika CprME and NS2B-3 were cloned into a vector by incorporation of an IRES sequence upstream of the NS2B-3 gene. We next tested the ability of this vector to produce CprME VLPs. Transfection with Zika CprME-IRES-NS2B-3 vector resulted in release of VLPs into the supernatants that showed incorporation of Capsid (Fig. 1B). Expression of CprME and NS2B-3 via a separate vector also resulted in VLP release although with lesser efficiency. As control, transfection with the prME expression construct resulted in high expression of VLPs that lacked incorporation of capsid while expression of CprME alone resulted in negligible VLP release. These data demonstrate that the flaviviral protease NS2B-3 is required for secretion of CprME VLPs and Zika VLPs derived using a bicistronic CprME-IRES-NS2B-3 vector show incorporation of capsid.

2.2. Generation of stable cell line secreting Zika CprME VLPs

Having established a vector that would allow production of CprME VLPs (Fig. 2A), we next sought to generate stable cell line that would constitutively secrete high levels of Zika VLPs. For this, we packaged the lentiviral vector in the presence of a helper construct and VSVG Env in 293 T cells. The lentiviral particles were used to transduce 293 T cells that were bulk selected by culturing in the presence of blasticidin (Fig. 2B). After 3 weeks and 6 passages of bulk selection, individual clones were selected by limiting dilution cloning in 96 well plate. The single cell stable clones were characterized for E protein expression using 4G2 antibody staining followed by flow cytometry (Fig. 2C). Several clones showed uniform cell population and high E protein expression and were selected for further characterization by testing release of VLPs in the culture supernatant. The cell clone CprME-NS2B-3-E2 showed the best VLP release followed by the bulk selected cell line (Fig. 2D). As the CprME-NS2B-3-E2 clone showed good E protein expression by flow cytometry as well as good VLP release by western blotting, this clone was selected for further analysis.

2.3. Characterization of CprME-NS2B-3-E2 cell line and its comparison with prME-F4 cell line

We recently generated the stable cell line PrME-F4 that constitutively releases high amounts of VLPs into the culture supernatants [15]. We hence compared the VLP production potential of the CprME-NS2B-3-E2 cell line with the prME-F4 cells. VLP production by the CprME-E2 cells was highly efficient and comparable to the PrME-F4 cells (Fig. 3A) suggesting that they could act as a good VLP source for vaccine production purposes. As expected, the prME VLPs lacked incorporation of the capsid when compared to VLPs produced from CprME-NS2B-3-E2 cells. To ascertain that we were detecting capsid incorporated into the virions and not free protein, we purified the CprME-NS2B-3-E2 derived VLPs on an Iodixanol gradient followed by western blotting of the individual fractions. Fraction 7 contained the most E protein that also correlated with the maximum band intensity for the capsid protein (Fig. 3B). Finally, we characterized the CprME-NS2B-3-E2 cell line for expression of HA-tagged NS2B-3 in the presence of appropriate controls. The CprME-F6, prME-F4 and CprME-NS2B-3-E2 cell lines showed expression of E protein via 4G2 staining but only the CprME-NS2B-3-E2 cell line showed NS2B-3 expression (Fig. 3C). Overall, these data demonstrate that the stable cell line CprME-NS2B-3-E2 secretes VLPs into the supernatant that incorporate the Capsid protein.

2.4. Consistency in VLP production and stability of cell line secreting Zika CprME VLPs

Use of stable cell lines that secrete consistent amounts of VLPs over a long period of time is essential for the VLP platform to be commercially successful. We hence confirmed the release of VLPs by the CprME-NS2B-3-E2 cell line over a period of several days and compared it to VLP production by the prME stable cell line F4 that we had generated previously and consistently produces high levels of VLPs. We found that the E protein released by the two cell lines was comparable (Fig. 4A). Interestingly, we confirmed that the VLPs released by the E2 cell line also contained the Capsid protein which was absent in the prME-F4 cells, as expected.

The stable nature of lentiviral integration is evident after repeated passage of transduced cells in the absence of drug selection pressure, in this case Blasticidin. We hence tested the stability of the CprME-NS2B-3-E2 cell line by passaging it in the absence or presence of Balsticidin for three weeks. At the end of the three week passage, culture supernatants were harvested for estimation of VLP release by western blotting and cells were stained for E protein expression and analyzed by flow cytometry. Supernatants collected from the cells cultured in the presence or absence of

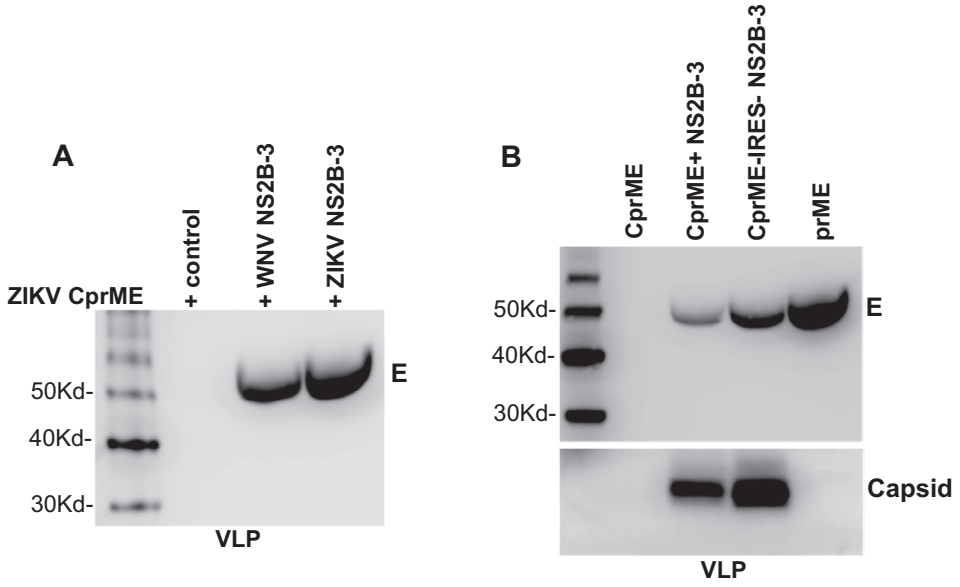


Fig. 1. Flaviviral protease NS2B-3 is required for secretion of CprME VLPs. 293 T cells were transfected with Zika virus CprME expression construct along with either WNV or ZIKV derived NS2B-3 expression plasmid. Equal volumes of culture supernatants were concentrated via ultracentrifugation, lysed and analyzed for Zika E protein expression via western blotting. (B) Bicistronic CprME-IRES-NS2B-3 derived Zika VLPs are released efficiently and show incorporation of capsid. 293 T cells were transfected with Zika CprME expression vector alone, CprME construct in the presence of NS2B-3 protease, the CprME-IRES-NS2B-3 or the prME expression vector. Equal volumes of culture supernatants were concentrated via ultracentrifugation, lysed and analyzed for Zika E and capsid protein expression by western blotting.

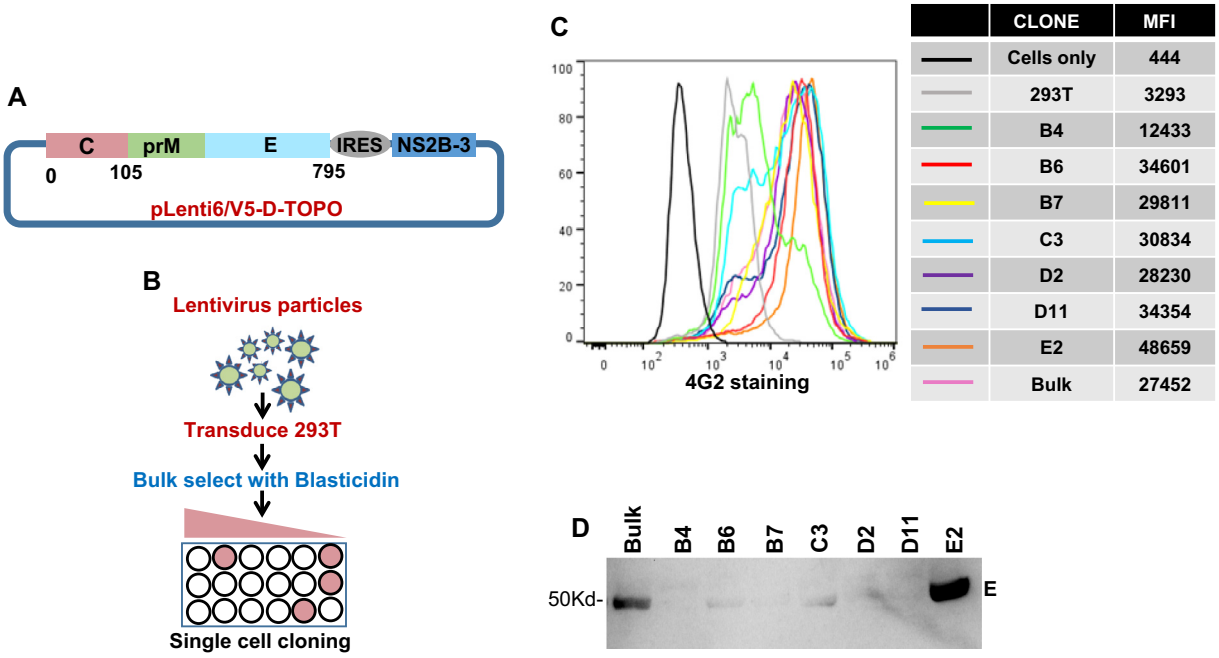


Fig. 2. Generation and characterization of stable cell lines secreting Zika CprME VLPs. (A) Schematic of lentiviral vector expressing the bicistronic Zika CprME-IRES-NS2B-3. (B) Lentiviral vector expressing ZIKV CprME-IRES-NS2B-3 was packaged in 293 T cells by transfecting with the vector along with the helper construct php-dl-NA and VSVG. The viral supernatants were harvested and used to transduce 293 T cells. Transduced cells were bulk selected using Blasticidin. Subsequently, single cell clones were generated from the bulk selected cells by limiting dilution cloning in 96 well plates. (C) Each single cell clone was characterized for ZIKV E protein expression using immunostaining followed by flow cytometry. Mean Fluorescence Intensity (MFI) of each single cell clone is indicated in the table. Cells only represents 293 T cells not stained with any antibody. (D) Equal volumes of culture supernatants from selected single cell clones were concentrated by ultracentrifugation. E protein expression was determined by western blotting.

Blasticidin for 21 days showed comparable VLP release as determined by Western blotting for the E and capsid protein (Fig. 4B). Moreover, cells passaged with or without Blasticidin showed similar staining for E protein at the end of the experiment (Fig. 4C) validating the stability of the integrated gene after multiple passages and in the absence of Blasticidin. These data demonstrate stable VLP production by the E2 cell line long-term after multiple passages in the absence of selection pressure.

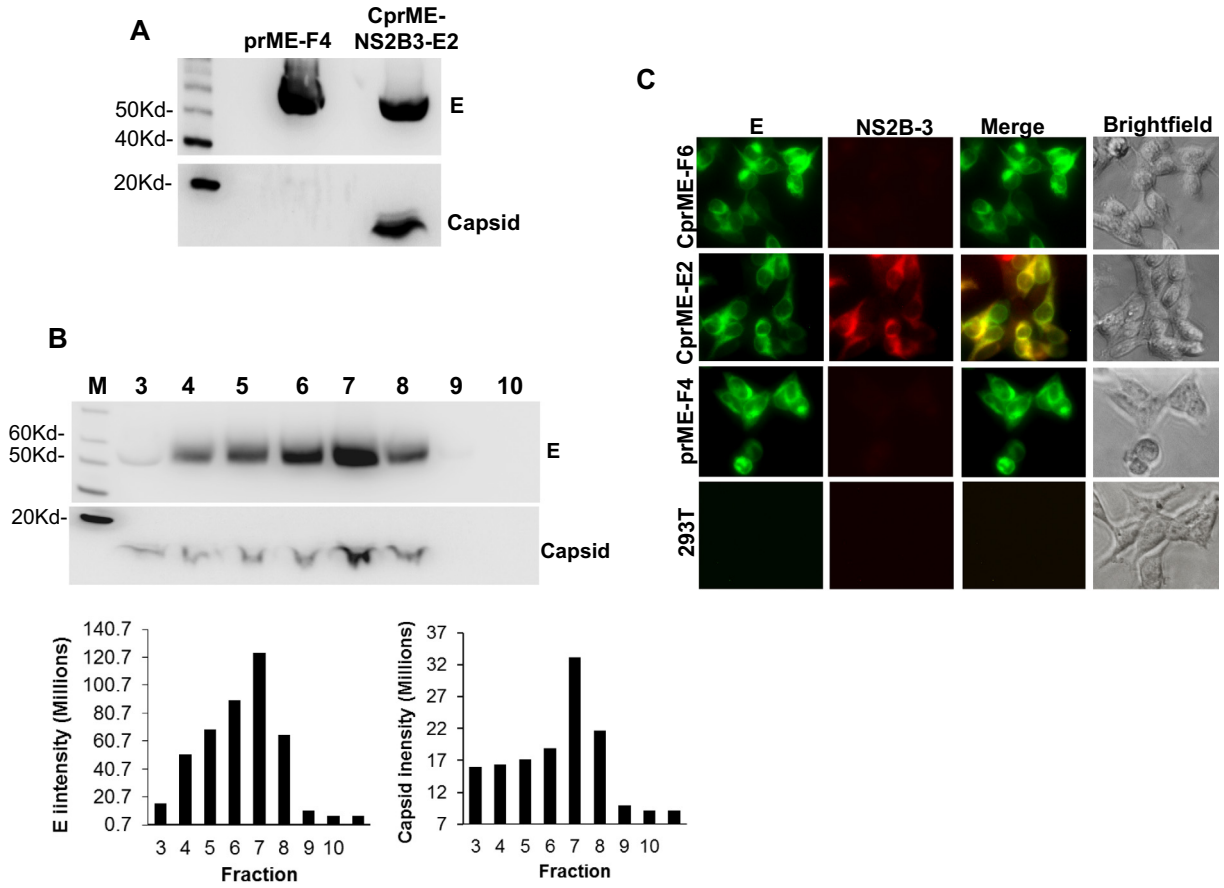


Fig. 3. Characterization of CprME-NS2B-3-E2 cell line and its comparison with prME-F4 cell line. (A) The stable cell line secreting CprME (CprME-E2) or prME (prME-F4) VLPs were seeded in equal numbers (5×10^6 cells) in 75 cm^2 tissue culture flasks and culture supernatants analyzed for Zika E and capsid protein expression by western blotting. (B) CprME-E2 derived VLPs were layered onto an Optiprep gradient, ultracentrifuged and 1 ml fractions collected starting from top. The individual fractions (3–10) were further concentrated by ultracentrifugation and analyzed for E and Capsid protein expression by western blotting. Graph represents densitometric analysis of the E and Capsid expression. (C) Stable cell lines CprME-F6, CprME-NS2B3-E2, prME-F4 or control 293 T cells were analyzed for expression of Zika E protein and HA-NS2B-3 expression after antibody staining followed by fluorescence microscopy.

2.5. Antigen sparing studies to test the efficacy of immune response elicited by Zika CprME versus prME VLPs

Determining the optimal antigen amount capable of eliciting a protective immune response is key to effective vaccine design. We hence conducted further analysis of the prME and CprME VLP vaccine via antigen sparing studies to determine the optimal dose that provides a saturating immune response. In our previous study we had compared prME and CprME VLPs in immunogenicity studies in mice and found differences in neutralizing antibody titers. Hence, in the current study we conducted antigen sparing studies using both prME and CprME VLPs. We used alum as the adjuvant that is most commonly used adjuvant approved for human use. The E protein content in the VLP preps was quantified by western blotting after loading a known concentration of purified baculovirus derived E protein (Fig. 5A).

Thereafter, groups of mice (N = 8) were immunized intramuscularly with 12, 6 or 3 μg of either CprME of prME VLPs in conjunction with alum adjuvant. The control group was mock immunized with PBS. A booster dose of respective VLP amount was administered at 2 weeks after the primary immunization. Five weeks post second immunization, mice were sacrificed (Fig. 5B) and serum samples analyzed for neutralizing antibody titers using the RVP assay. Mice immunized with different VLPs showed a dose dependent response with 12 μg (E protein) VLP dose showing the highest EC50 (Fig. 6A) and EC90 (Fig. 6B) antibody titers. Interestingly, and consistent with our previous study [15], CprME VLPs were more potent than prME VLPs at the same dose at inducing higher titers of neutralizing antibodies. This difference became apparent at a lower non-saturating VLP dose of 3 μg than a saturating VLP dose of 12 μg (Fig. 6C). We further compared neutralizing antibody titers in mice to convalescent sera from humans that were previously naturally exposed to Zika virus infection. Mice immunized with 12 μg CprME VLPs showed comparable EC50 titers to those seen in Zika infected human subjects (Fig. 6A). However, there was a significant difference in EC90 titers between CprME immunized mice and convalescent human subjects (Fig. 6B). Thus, the VLP vaccine can induce neutralizing antibody titers in a range comparable to those induced upon natural ZIKV infection. To determine whether capsid specific antibodies are generated in mice immunized with CprME VLPs, we conducted Radio Immunoprecipitation Assay (RIPA) with [^{35}S]Met/Cys labelled CprME cell lysates that were immunoprecipitated with pooled sera obtained from CprME or prME VLP immunized mice. As clearly evident, capsid antibodies are detected only in mice immunized by CprME VLPs but not PrME VLPs (Fig. 6D). Moreover, the E antibody levels were higher in CprME immunized group consistent with our previous findings [15]. The generation of anti-capsid antibodies in CprME but not prME VLP immunized mice also demonstrates a mechanism for higher immunogenicity of CprME VLPs.

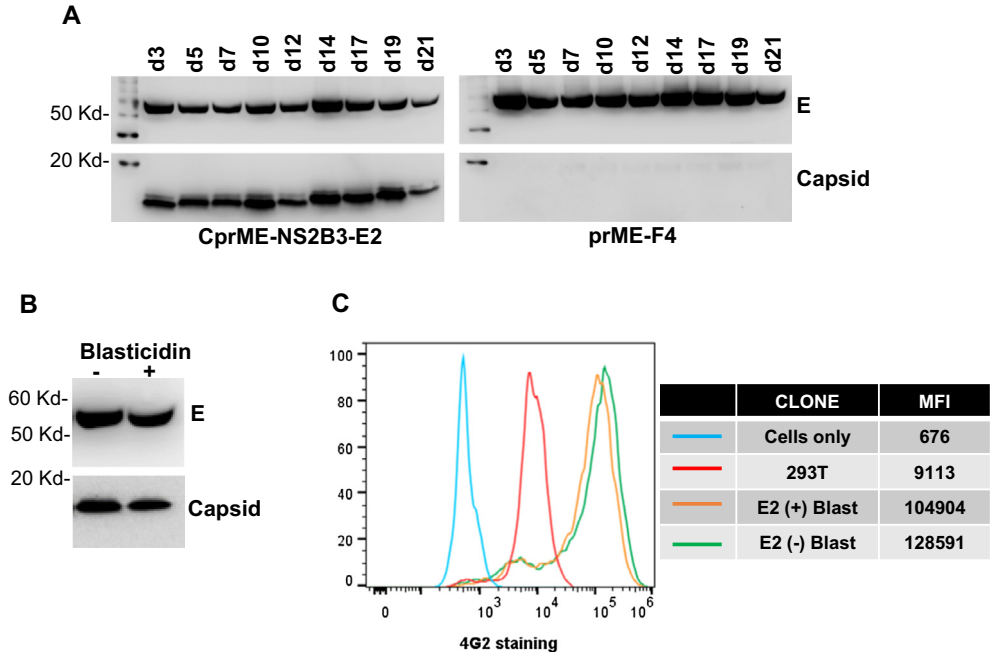
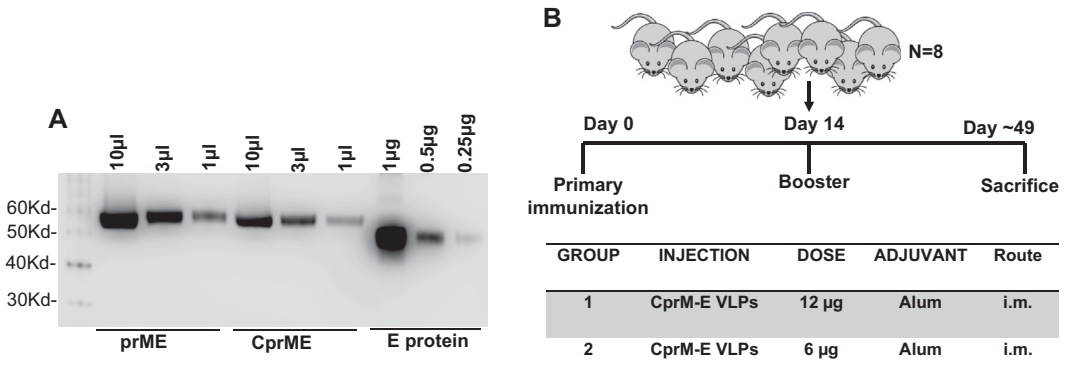


Fig. 4. CprME-NS2B3-E2 cell line consistently produces VLPs even in the absence of selection antibiotic blasticidin. (A) CprME-NS2B3-E2 and prME-F4 cells were seeded in equal numbers, and culture supernatants harvested every 2–3 days and cells sub-cultured. VLPs were pelleted by ultracentrifugation and analyzed for E and Capsid protein expression by western blotting. (B) CprME-NS2B3-E2 cell line was cultured in the presence or absence of blasticidin for 3 weeks. At the end of 3 weeks, culture supernatants were harvested, ultracentrifuged and assayed for E and Capsid protein expression by western blotting. (C) CprME-NS2B3-E2 cells cultured in the presence or absence of blasticidin for 3 weeks were analyzed for E protein expression after immunostaining followed by flow cytometry analysis. MFI of expression is indicated in the table.



GROUP	INJECTION	DOSE	ADJUVANT	Route
1	CprM-E VLPs	12 µg	Alum	i.m.
2	CprM-E VLPs	6 µg	Alum	i.m.
3	CprM-E VLPs	3 µg	Alum	i.m.
4	prM-E VLPs	12 µg	Alum	i.m.
5	prM-E VLPs	6 µg	Alum	i.m.
6	prM-E VLPs	3 µg	Alum	i.m.
7	Control	PBS	NA	i.m.

Fig. 5. Schematic of immunization studies in mice. (A) VLPs derived from CprME-NS2B3-E2 or prME-F4 cells were loaded on an SDS PAGE gel at 10, 3 and 1 µl. Baculovirus derived Zika E protein standard was loaded alongside at a known concentration of 1, 0.5 and 0.25 µg. E protein amounts were calculated in the VLP preps after quantitation of the E protein bands. (B) Balb/c mice (N = 8/group) were immunized with CprME or prME VLPs in conjunction with Alum adjuvant. Each group of mice received 12, 6 or 3 µg equivalent of E protein at day 0 followed by a single booster of the respective VLP dose at day 14. Mice were sacrificed on day 49 and serum collected and stored for further analysis.

2.6. Immunization with Zika CprME VLPs protects mice against ZIKV challenge

Infection of immunocompetent mice with Zika virus is limited in pathology due to strong interferon response that controls the virus. However, challenge studies with high titer virus injected via intravenous route can provide transient viremia upto day 7 that peaks at day 3 and can be used to access vaccine efficacy. To determine whether our CprME VLPs could protect mice against infectious virus challenge, mice were immunized with ~12 µg CprME VLPs complexed with alum followed by a booster VLP dose. At week 6, mice were bled by retro-orbital puncture and sera saved for neutralizing antibody determination. Twelve days after the bleed, mice were challenged with Zika virus PRVABC59 by i.v injection. Mice were sacrificed on day 3 post infection and plasma and spleen harvested for determination of viral load by real time RT-PCR. CprME VLP

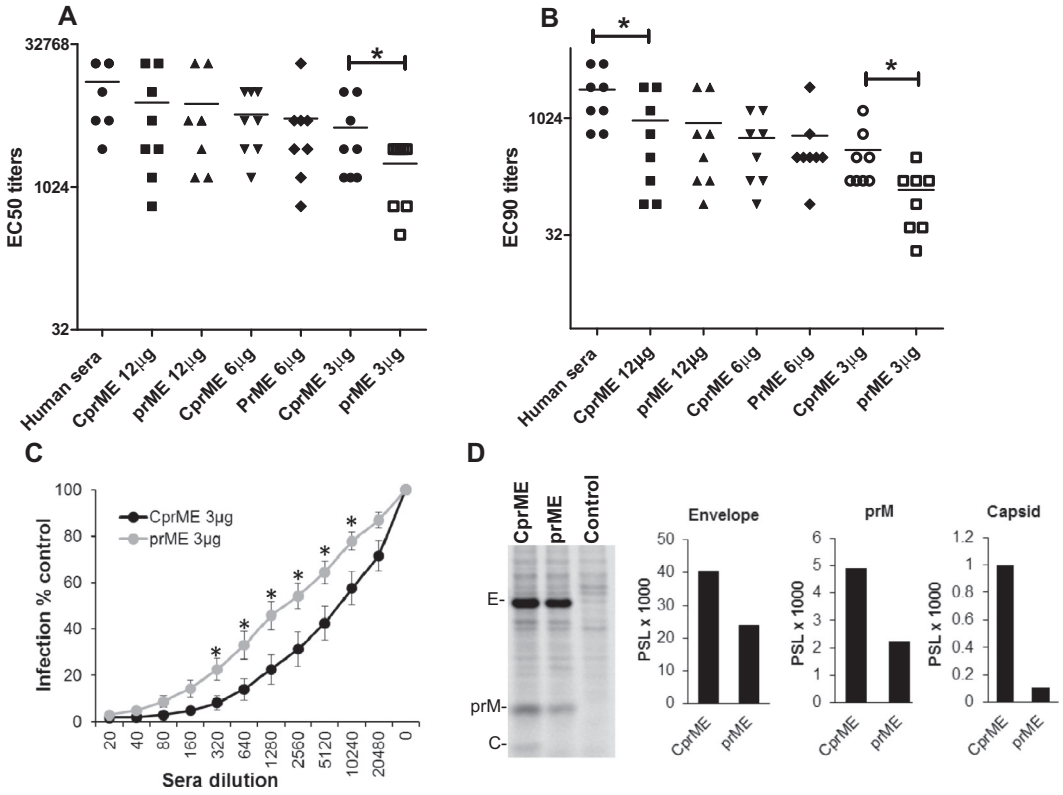


Fig. 6. EC50 and EC90 titers of mice immunized with different doses of CprME or prME VLPs. Groups of 8 mice each were immunized with the indicated doses of CprME or prME VLPs. Sera samples from the mice were analyzed for neutralization of Zika RVPs using quantitative microscopy. All samples were assayed in technical duplicates and (A) EC50 and (B) EC90 values calculated from neutralization curves. Human sera obtained from Zika infected convalescent subjects was used for comparing vaccine efficacy to a natural infection. *$p < 0.05$ student's *t* test. (C) Serial dilution of sera from mice groups immunized with 3 μg of CprME or prME VLPs was used for neutralization assay. Data are mean ± SE from 8 mice/group. *$p < 0.05$ student's *t* test. (D) CprME-NS2B-3-E2 cells were radiolabeled with [^{35}S]Met/Cys protein labeling mix. Cell lysates were immunoprecipitated with ProteinA beads coated with pooled sera samples from mice immunized with 3 μg of CprME or prME VLPs. Immunoprecipitated complexes were resolved on an SDS-PAGE gel flowed by PhosphorImager analysis. The Photo Stimulated Luminescence (PSL) values for the Envelope, prM and Capsid proteins is depicted in the adjacent graphs.

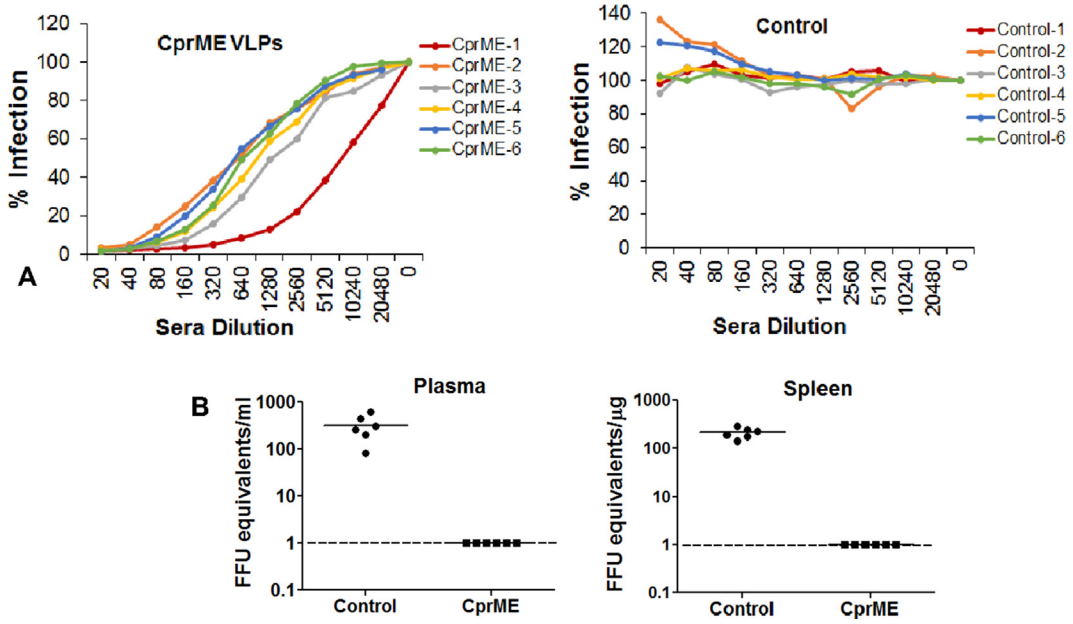


Fig. 7. Immunization with CprME VLPs protects mice against ZIKV challange. Balb/c mice (N = 6/group) were immunized with CprME VLPs in conjunction with Alum adjuvant or mock immunized with PBS and Alum (Control). (A) At week 6, mice were bled by retro-orbital puncture and sera analyzed for presence of neutralizing antibodies against ZIKV. Different line graph colors represent individual mice. (B) Mice were challenged with Zika virus PRVABC59 by intravenous injection. The animals were sacrificed on day 3 post infection and plasma and spleen samples assayed for viral load by real time RT-PCR. Dotted line represents the limit of detection for the assay.

immunized mice showed generation of high neutralizing antibody titers compared to control mice (Fig. 7A). This correlated with the fact that mice immunized with CprME VLPs were completely protected against Zika virus challenge while the control mice showed moderate levels of virus replication both in the plasma and spleen (Fig. 7B). This confirms that the CprME VLPs comprise a good vaccine candidate that results in high neutralizing antibody titers that are protective against live virus challenge.

3. Discussion

Recent outbreak of ZIKV infection and the associated neonatal birth defects has highlighted the need for a vaccine. Although the outbreak of 2015–2017 has subsided, past experiences with arboviruses suggest that the threat has diminished but not eliminated. The repeated outbreaks of other arboviruses like Dengue and Chikungunya that co-circulate in the same regions as Zika [6] suggests that Zika outbreaks are likely to reemerge. Based on these factors, the World Health Organization is recommending the development of a Zika vaccine for the target population of pregnant women and women of child bearing age. Hence, a safe and effective vaccine for use in pregnant women is urgently needed.

Significant progress has been made in the field of Zika vaccines with multiple platforms in different phases of clinical trial (NCT02963909, NCT02840487, NCT02887482, NCT02809443, NCT02952833). However, none of the platforms currently in testing in humans is a VLP based vaccine. There are obvious benefits of a VLP vaccine. Firstly, no infectious virus is involved in any stage of production of the VLP vaccine making them extremely safe. Secondly, unlike Purified Inactivated Virus vaccines, VLPs do not need inactivation with formalin which can result is loss of certain epitopes due to formalin mediated crosslinking. Our study shows that the commonly used alum adjuvant can provide a saturating immune response with the VLPs suggesting that it is possible to develop the vaccine for commercial use.

Although the VLP platform provides many advantages there are obvious limitations. For one, the cost of producing large quantities of VLPs can be a limiting factor. To overcome this, we have generated stable cell lines that constitutively secrete high concentrations of VLPs into the culture supernatant. This is achieved by careful selection of single cell clones and characterizing the cell line under rigorous conditions. Our study does indicate that not all single cell clones when selected under similar conditions show high levels of VLP secretion. Secondly, it is imperative that these cells do not lose the integrated flaviviral structural genes during expansion and vaccine production especially in the absence of selection agents like Blasticidin. Our study shows that these cell lines can be robust and retain the integrated flaviviral construct even when passaged repeatedly in the absence of Blasticidin.

Both previous studies by our group [15] and the current study show that presence of Capsid in the VLP vaccine candidate can provide better immune response than VLPs without capsid. To incorporate Capsid in the VLPs, co-expression of NS2B-3 is essential although other methods like expressing Capsid from a separate construct have been reported [28,29]. As in the case of natural infection, the CprME polyprotein is cleaved by NS2B-3 in the ER [25,26], we decided to create a single vector expressing CprME and NS2B-3 using an IRES sequence. With the help of this unique strategy, we were finally able to generate a stable cell line that secretes high levels of capsid containing VLPs. Previous studies were also unable to confirm the presence of Capsid in the VLP preps due to a lack of availability of reactive antibodies. With the availability of anti-Zika Capsid antibody from GeneTex we have demonstrated that the cprME-IRES-NS2B-3 construct and CprME-NS2B-3-E2 stable cell line produces VLPs in the supernatant that incorporate the capsid protein.

Challenge studies with ZIKV in immunocompetent mice have been difficult due to a robust IFN response that limits virus replication [30]. However, challenge with high titer virus intravenously can result in detectable viremia on day 3 that can be used as a surrogate for measuring vaccine efficacy [19]. We also found in our study that CprME VLP based vaccine can provide protection against intravenous challenge with ZIKV further validating the efficacy of this vaccine candidate. Furthermore, we found that antibodies produced in mice immunized with the highest dose of CprME VLPs were comparable to the EC50 titers of antibodies present in the serum of naturally Zika infected convalescent human subjects although the EC90 titers did show a significant difference. This is conceivable as a replicating virus infection would be capable of eliciting a stronger neutralizing antibody response compared to non-replicating VLPs. Although titers of antibodies present in different species are not a direct comparison and efficacy of the vaccine will need to be tested in humans, our findings do support further development of Capsid containing VLP platform for flaviviruses.

4. Conclusions

Overall, our study describes the development of a VLP platform for immunization and protection against Zika virus. Dose dependent response studies clearly proved the effectiveness of a better neutralizing antibody response for CprME versus prME VLPs at lower antigen dose. Reduction in viremia following virus challenge provides further evidence of vaccine effectiveness. This is a first report of development of a stable cell line secreting Zika CprME VLPs via natural NS2B-3 protease mediated cleavage and has implications for other flaviviruses dependent on protease dependent cleavage for particle release. Moreover, we also demonstrate here for the first time, the incorporation of capsid in CprME VLPs and generation of an immune response against the capsid protein in CprME VLP immunized mice. This data also provides insights into the superiority of CprME versus prME VLPs. Thus, the stable cell line secreting capsid containing VLPs provides a robust platform that is highly immunogenic making it ideal for use in resource limiting and undeveloped countries.

5. Methods

5.1. Cell culture and reagents

293 T and Vero cells were obtained from ATCC and cultured in DMEM supplemented with 10% FBS. All transfections were performed using Turbofect reagent (Thermo Fisher) as per the manufacturer's instructions. The WNV CprME and Rep/GFP plasmids have been described previously [31] and were kindly provided by Dr. Ted Pierson (NIAID). Plasmid containing the WNV NS2B-3 accessory fusion protein expressing the active protease has been described previously [32] and was a kind gift from Dr. Frank Scholle (NC State Univ.). The OptiPrep™ Density Gradient (Iodixanol) was from Sigma, the 2% Aluminum hydroxide solution (Alhydrogel) was from Invivogen and the baculovirus expressed recombinant ZIKV envelope protein was from Fitzgerald Industries International. Human sera samples from subjects naturally exposed to Zika virus infection were kindly provided by BEI Resources.

5.2. Generation of bicistronic vector expressing ZIKV C-prM-E and protease NS2B-3

ZIKV C-prM-E and prME constructs have been described previously [15]. For generation of a bicistronic vector expressing Zika CprME and NS2B-3, a codon optimized Zika NS2B-3 containing an HA Tag and IRES sequence was synthesized by GenScript Inc. This construct was then subcloned downstream of the CprME cassette in the lentiviral vector pLentiZikaCprME to generate pLentiZika CprME-IRES-NS2B-3. The NS2B-3 open reading frame was also PCR amplified from the lentivirus vector and cloned into pcDNA3.1+ vector using the directional pcDNA3.1 TOPO cloning kit (Invitrogen) to generate Zika-NS2B-3. The expression of NS2B-3 was confirmed by transient transfection and staining with anti-HA antibody (Sigma).

5.3. Detection of ZIKV E protein expression

Detection of ZIKV virus E protein was conducted either via immunofluorescence microspcopy, flow cytometry or western blotting. For fluorescence microscopy, the cells were fixed with 4% formaldehyde in PBS followed by permeabilization with 0.1% TritonX100/PBS. The cells were stained using E specific antibody MAB10216 (clone 4G2, Millipore, 1:200 dilution) that reacts with Flavivirus group specific antigen [33] followed by secondary antibody Alexa 488 conjugated goat anti-mouse IgG (Invitrogen, 1:200 dilution) and analyzed by fluorescence microscopy. For flow cytometry analysis, cells were trypsinized, washed with PBS, fixed and permeabilized using the Cytofix Cytoperm reagent (BD Biosciences) as per the manufacturer's instructions. Cells were stained using the primary antibody 4G2 at 1:500 dilution and secondary antibody Alexa 488 conjugated goat anti-mouse IgG (Invitrogen, 1:500 dilution). Cells were assayed by flow cytometry on a Gallios Flow Cytometer (Beckman Coulter) and at least 20,000 events for each sample were acquired. Data was analyzed using FlowJo software (Tree Star). For Western blotting, lysates were resolved on an SDS-PAGE gel, transferred onto PVDF membranes and probed with ZIKV E antibody (GTX133314, GeneTex, 1:3000) or ZIKV Capsid antibody (GTX 133317, 1:1000) followed by HRP conjugated anti-mouse secondary antibody and bands visualized via enhanced chemiluminescence using the super signal west femto maximum sensitivity substrate (Pierce). The protocol for radiolabeling of cells with [35S]Met/Cys protein labeling mix followed by immunoprecipitation of cell lysates has been described previously [34].

5.4. Production of reporter virus particles (RVP) and RVP based microneutralization assay

ZIKV RVPs were generated using protocol described previously [15,34]. 293 T cells were co-transfected with the ZIKV virus CprME construct along with plasmid containing the sub-genomic GFP expressing replicon derived from lineage II strain of WNV [31]. The RVPs were harvested 48 h post transfection, aliquoted and stored for future use. RVPs were titrated in Vero cells plated in 96 well clear bottom black plates at 5000 cells per well. Thereafter, cells were infected with serial two-fold dilutions of the RVPs and incubated for 72 h. The plates were fixed using 4% formalin/PBS, images acquired using the Cytation5 imaging system (BioTek) which provides a read out of the number of GFP positive cells per well. For neutralization assays, mouse sera or antibodies were serially diluted in DMEM and incubated with the RVPs for 1 h at room temperature. Subsequently, the virus:sera mix was added to Vero cells and number of GFP positive cells quantitated as described above.

5.5. Generation of stable cell line expressing ZIKV CprME-NS2B-3

Lentiviral vector expressing ZIKV CprME-IRES-NS2B-3 was packaged into lentiviral particles in 293 T cells by transfecting with the vector along with the helper construct php-dl-NA (NIH AIDS Reagent program) and VSVG Env. The viral supernatants were collected at 48 h post transfection, aliquoted and stored. To generate stable cell lines, 293 T cells were transduced with the lentiviral particles and the cells were selected using Blasticidin at a concentration of 10 μg/ml. Bulk selected cells were passaged 8–10 times and stained for ZIKV E protein expression using monoclonal antibody MAB10216 (4G2, Millipore) at regular intervals to confirm selection. Subsequently, single cell clones were generated from the bulk selected cells using limiting dilution cloning in 96 well plates. Each single cell clone was further characterized for ZIKV E protein expression using immunostaining followed by flow cytometry. The release of VLPs from selected cell lines was determined by western blotting and the clone most potent at VLP release was selected for further studies.

5.6. Production of VLPs for immunization

VLPs for immunization were generated and purified as follows. 293 T-PrME-F4 or 293 T-CPrME-NS2B-3-E2 stable cell lines were seeded in 150 cm^2 tissue culture flasks in the absence of blasticidin. Culture supernatants were harvested every 2–3 days and cells sub cultured. VLPs were concentrated as per the protocol of Brien et al [35]. Harvested supernatants (25–30 ml) were transferred into ultracentrifuge tubes and carefully underlayered with 5 ml of 25% glycerol in TNE buffer. VLPs were pelleted by centrifugation at 110,500*g* for 3 h at 4 °C. Thereafter, the supernatant was carefully removed and the VLP pellet resuspended in TNE buffer. The total protein content in the VLP preps was measured using the micro BCA kit (Pierce) and specific E protein in the preps was detected by western blotting using the GTX133314 antibody. Baculovirus derived recombinant Zika virus E protein (Fitzgerald Industries) expressed in an insect cell line was used to estimate the E protein concentration in VLP preps. For the 293 T-CPrME-NS2B-3-E2 cell line, 10^7 seeded cells yielded ~24 μg E protein. The VLP yield from the 293 T-PrME-F4 cell line was approximately two fold higher at ~48 μg E protein from 10^7 seeded cells.

5.7. Mice studies

All animal use was reviewed and approved by the TTUHSC El Paso Institutional Animal Care and Use Committee (IACUC). All experiments were performed in accordance with relevant guidelines and regulations. For immunization studies, 8 week old Balb/c mice were purchased from Jackson laboratory and housed in pathogen free animal facility at Texas Tech University Health Sciences Center, El Paso. Mice were divided into groups of eight mice each and immunized with different doses of prME or CprME VLP preparations using the 2% Alhydrogel adjuvant (InvivoGen). The VLP Alhydrogel mix was injected intramuscularly in each thigh in a volume of 50 μl. Mice received one respective booster VLP dose at week 2 after the primary immunization. Control mice were sham injected with PBS. Blood was collected at week ~7 post first immunization under terminal isoflurane anesthesia followed by intracardiac puncture. Blood samples were collected in serum separator tubes as per the manufacturer's recommendations. After coagulation, the tubes were centrifuged, sera harvested, aliquoted and stored at −80 °C until further use.

5.8. Virus challenge experiments

8 week old Balb/c mice (N = 6/group) were immunized with CprME VLPs (12 μg) as described above in the presence of 2% Alhydrogel adjuvant (InvivoGen). Control groups were mock injected with PBS in the presence of 2% Alhydrogel adjuvant. At week 6, mice were bled by retro-orbital puncture and sera saved for neutralizing antibody determination. Twelve days after the bleed, the mice were challenged with 200 PFU of Zika virus PRVABC59 by intravenous injection. Mice were sacrificed on day 3 post infection and plasma and spleen collected. Viremia was determined in plasma and spleen samples using the protocol described by Miner et al. 2016 [36]. Briefly, RNA was extracted from plasma samples using the Viral RNA Mini Kit and from splenic tissue using the RNeasy Mini kit (Qiagen). ZIKV RNA levels were determined by one step RT-PCR using the primers and probes described by Lanciotti et al. 2008 [37]. Viral burden is expressed as FFU equivalent per ml of plasma or per μg of RNA.

Declaration of Competing Interest

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

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Author contributions

AJ and HG conceived the study, performed experiments, analyzed data and wrote the manuscript. TMG and GR performed experiments and analyzed data.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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