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Immunization with phage virus-like particles displaying Zika virus potential B-cell epitopes neutralizes Zika virus infection of monkey kidney cells

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ABSTRACT

Zika virus (ZIKV) is a mosquito-borne flavivirus that has re-emerged and is associated with many debilitating clinical manifestations. Research is currently being conducted to develop a prophylactic vaccine against the virus; however, there has not been any licensed ZIKV vaccine. Recent studies have identified potential B-cell epitopes (amino acids 241–259, 294–315, 317–327, 346–361, 377–388 and 421–437) on the envelope protein of ZIKV, which could be explored to develop peptide vaccines against ZIKV infection. Nevertheless, the immunogenicity of these epitopes has never been assessed. Here, we displayed these epitopes on highly immunogenic bacteriophage virus-like particles (VLPs; MS2, PP7 and Qβ) platforms and assessed their immunogenicity in mice. Mice immunized with a mixture of VLPs displaying ZIKV envelope B-cell epitopes elicited anti-ZIKV antibodies. Although, immunized mice were not protected against a high challenge dose of ZIKV, sera – albeit at low titers – from immunized mice neutralized (*in vitro*) a low dose of ZIKV. Taken together, these results show that these epitopes are B-cell epitopes and they are immunogenic when displayed on a Qβ VLP platform. Furthermore, the results also show that immunization with VLPs displaying a single B-cell epitope minimally reduces ZIKV infection whereas immunization with a mixture of VLPs displaying a combination of the B-cell epitopes neutralizes ZIKV infection. Thus, immunization with a mixture of VLPs displaying multiple ZIKV B-cell epitopes is a good strategy to enhance ZIKV neutralization.

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1. Introduction

The re-emergence of Zika virus (ZIKV) in the last decade caught the attention of the world. A ZIKV outbreak started in French Polynesia in 2013, spread to Brazil in 2015, and rapidly spread (in 2016) to many countries around the world especially the western hemisphere (reviewed in [1–5]). ZIKV causes ZIKV-related congenital syndrome (microcephaly, brain calcifications, congenital central nervous system anomalies, stillbirths, hypertonia) [6,7], miscarriages [8] as well as Guillain-Barre syndrome [9]. Zika is a positive-sense single-stranded RNA virus with a genome size of ~10.7 Kb. The genome is surrounded by an icosahedral capsid, which is enclosed by an envelope. ZIKV is transmitted primarily by *Aedes* spp. mosquitoes and less commonly through sexual contact, blood transfusion, and through vertical transmission from mother to fetus [1,10,11].

Currently, there are no approved therapies or vaccines to prevent against ZIKV infections. Different approaches are currently being explored to develop a ZIKV vaccine; for example, immunizations with an adenovirus vector carrying ZIKV envelope gene [12], ZIKV virus-like particles (VLPs) [13], a live-attenuated ZIKV [14], and immunization with an inactivated ZIKV [15] protect mice from ZIKV infections. While most of these strategies look promising, there may be safety issues associated with some of these strategies that could limit their use. Live-attenuated vaccines may infect and cause disease(s) in patients with a compromised immune system or cause disease(s) in the fetuses of pregnant women. They are therefore not recommended for these particularly vulnerable individuals [16,17]. Thus, there is an urgent need to develop a safe and effective ZIKV vaccine. Bioinformatics studies have predicted potential B-cell epitopes (amino acids 241–259, 294–315, 317–327, 346–361, 377–388 and 421–437) on the envelope protein of ZIKVs [18–21]. All epitopes were predicted as linear B-cell epitopes on the envelope, with epitope 241–259 overlapping part of a conformational epitope (amino acid 235–255). Despite these predictions, no study has assessed the immunogenicity of these epitopes or assessed their

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neutralization potentials. Assessing the immunogenicity of B-cell epitopes (peptide antigens) is very challenging because the peptides are very short. As such, they are very unstable in serum, are rapidly degraded following immunization, and are therefore less immunogenic [22,23]. To assess the immunogenicity as well as the neutralization potentials of the potential ZIKV B-cell epitopes, we displayed some of these epitopes on highly immunogenic stable bacteriophage VLP platforms (MS2, PP7, and Qβ).

MS2 and PP7 VLPs are derived from two fused monomers (single-chain dimer) of the coat proteins of MS2 and PP7 RNA bacteriophages, respectively [24,25]. Overexpression of the single-chain dimer coat proteins in bacteria allows the coat proteins to spontaneously assemble into VLPs; ninety copies of the single-chain dimer assemble to form VLPs. Thus, a genetic insertion of a foreign epitope on the single-chain dimer allows 90 copies of the epitope to be displayed on the VLPs. Qβ VLPs, on the other hand, are derived from monomers of the coat proteins of Qβ (an RNA bacteriophage as well). Overexpression of the coat protein in bacteria allows 180 copies of the monomers to assemble to form VLPs and its surface chemistry is most amenable to chemical conjugation. Chemical conjugation of a foreign epitope on the Qβ VLPs allows for at least 180 copies of the epitope to be displayed on the VLPs. We have used these three display platforms, in the past, to enhance the immunogenicity of less-immunogenic B-cell epitopes human papillomaviruses [26–28].The platforms do not contain the viral genome (cannot replicate) and as such they can be explored to develop safe ZIKV vaccines.

2. Materials and methods

2.1. Genetic insertions of ZIKV B-cell epitopes on single-chain dimer of PP7 and MS2

Two expression plasmids (pDSP62 [24] and pDSP7K [25]) were used to genetically insert ZIKV B-cell epitopes on MS2 and PP7 bacteriophage coat proteins. Plasmids pDSP62 and pDSP7K express single-chain dimers of MS2 and PP7 coat proteins, respectively, which spontaneously assemble into VLPs. Polymerase chain reactions were used to insert different ZIKV B-cell epitopes (representing aa 241–259, 294–315, 317–327, 346–361, 377–388 and 421–437 from the envelope protein of ZIKV strain MR-766; Fig. 1A) on to the N-terminus of the single-chain dimer of MS2 and PP7 coat proteins as previously described [27]. Briefly, the forward PCR primer contained an NcoI restriction site followed by lysine, then the respective epitope sequences, a linker sequence (STGVGS) and a sequence complementary to MS2 or PP7 coat protein (in the order listed). A reverse primer, E3.2 (5′ CGGGCTTTGTTAGCAGCCGG 3′), which anneals downstream of a BamHI restriction site was used for all PCR reactions. PCR reactions were done using pDSP62 and pDSP7K plasmids as templates and PCR fragments were digested with the aforementioned restriction enzymes and cloned to their respective plasmids. The plasmids were then transformed into C41 *E. coli* cells and screened for recombinant protein (virus-like particles; VLPs) expression. All constructs were sequenced to confirm the insertion of ZIKV epitopes.

2.2. Recombinant VLPs expression, purification and transmission electron microscopy (TEM)

C41 cells expressing recombinant coat proteins (MS2-Zika-epitope or PP7-Zika-epitope) were grown at 37 °C until the cells reached an optical density $(OD)_{600}$ of 0.6. The bacteria were then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 5 h. The induced cells were then pelleted, lysed and VLPs were purified by gel filtration on a Sepharose CL-4B column [25]. To assess whether the expressed recombinant coat proteins (MS2-Zika-epitope or PP7-Zika-epitope) assembled into VLPs, we conducted TEM as previously published [28].

2.3. Conjugation of ZIKV B-cell epitopes to Qβ VLPs

ZIKV epitopes, which could not be successfully displayed by genetic insertion on MS2 or PP7 VLPs due to the failure of the recombinant coat proteins to assemble into VLPs, were displayed on Qβ bacteriophage VLPs by chemical conjugation as follows: First, Qβ VLPs were expressed and purified from C41 cells as described above for PP7 and MS2. ZIKV epitopes 294–315 and 421–437 – synthesized with an additional cysteine residue at the C-terminus – were conjugated to lysine residues on Qβ VLPs using a heterobifunctional cross-linker, SMPH [Succinimidyl 6-((beta-maleimidopropionamido)hexanoate)]. In addition to these, epitopes 241–259 and 346–361 were also synthesized and conjugated on Qβ VLPs. Conjugation efficiency was assessed on an SDS PAGE gel.

2.4. Immunoassays to assess the genetic display of epitopes on VLPs

To assess the display of the target ZIKV epitopes on VLPs, an enzyme-linked immunosorbent assay (ELISA) was conducted as previously published [28]. Briefly, 96-well plates were coated overnight with 1000 ng of MS2-Zika-epitope or PP7-Zika-epitope VLPs. The wells were blocked after which anti-ZIKV envelope serum at 4-fold dilutions was added and incubated for 2 h. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000 dilution) was added and the plates were incubated for 1 h. The wells were developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) solution. Bacteriophage MS2-Zika-epitope or PP7-Zika-epitope VLPs, which had more than twice OD_{450} values relative to control VLPs at highest serum dilution were considered to display ZIKV epitopes on the surface of the VLPs. VLPs that did not react with serum (i.e. confirmed the display of ZIKV epitopes on the surface) were tested in a Western blot to determine if ZIKV epitopes could be detected on the coat proteins. Western blotting was conducted as follows: one hundred ng or 500 ng of PP7-Zika-epitope VLPs, PP7 VLPs, and purified ZIKV envelope (Supplementary Materials and Fig. 1A) were resolved on an SDS PAGE gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked and 1:5000 dilutions of PP7 or anti-ZIKV envelope sera were added and incubated for 2 h. HRP-conjugated goat anti-mouse IgG antibodies (1:10,000 dilution) were added to the membranes and they were developed using SuperSignal West Pico (Luminol/Enhancer and stable peroxide) solutions.

2.5. Immunization of mice and ELISA

All animal work was done in accordance with Michigan Technological University animal care and use guidelines. Balb/c mice (4 per group) were intramuscularly (i.m.) immunized thrice (at two-week intervals) with 5 μg each of MS2-Zika-E377-388, PP7-Zika-E241-259, PP7-Zika-E346-361, Qβ-Zika-E241-259, Qβ-Zika-E294-315, Qβ-Zika-E346-361, Qβ-Zika-E421-437 VLPs, or 5 μg of control MS2, PP7, Qβ VLPs. Additionally, two other groups were immunized (using same route/schedule) with a mixture of VLPs that consisted of 5 μg each of MS2-Zika-E377-388, Qβ-Zika-E241-259, Qβ-Zika-E294-315, Qβ-Zika-E346-361 and Qβ-Zika-E421-437 or immunized with 10 μg of ZIKV envelope (aa 1–449; Supplementary Materials). All immunizations were done with alum hydroxide adjuvant. Two weeks after the third immunization, sera were collected for ELISAs and neutralization assay studies.

To assess antibody titers in sera, ELISAs were conducted as previously described [28]. Briefly, 96-well plates were coated with 500 ng of streptavidin-conjugated to the ZIKV epitopes (described

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