Passive Transfer of Immune Sera Induced by a Zika Virus-Like Particle Vaccine Protects AG129 Mice Against Lethal Zika Virus Challenge

Diego Espinosa a, Jason Mendy a, Darly Manayani a, Lo Vang b, Chunling Wang b, Tiffany Richard a, Ben Guenther a, Jayavani Aruri a, Jenny Avanzinia a, Fermin Garduno a, Peggy Farness a, Marc Gurwithe a, Jon Smith a, Eva Harris b,*, Jeff Alexander a,*, Diego Espinosa b, Jason Mendy a, Darly Manayani a, Lo Vang b, Chunling Wang b, Tiffany Richard a, Ben Guenther a, Jayavani Aruri a, Jenny Avanzinia a, Fermin Garduno a, Peggy Farness a, Marc Gurwithe a, Jon Smith a, Eva Harris b,*, Jeff Alexander a,*, Diego Espinosa b, Jason Mendy a, Darly Manayani a, Lo Vang b, Chunling Wang b, Tiffany Richard a, Ben Guenther a, Jayavani Aruri a, Jenny Avanzinia a, Fermin Garduno a, Peggy Farness a, Marc Gurwithe a, Jon Smith a, Eva Harris b,*, Jeff Alexander a,*, Diego Espinosa b, Jason Mendy a, Darly Manayani a, Lo Vang b, Chunling Wang b, Tiffany Richard a, Ben Guenther a, Jayavani Aruri a, Jenny Avanzinia a, Fermin Garduno a, Peggy Farness a, Marc Gurwithe a, Jon Smith a, Eva Harris b,*, Jeff Alexander a,*, Diego Espinosa b, Jason Mendy a, Darly Manayani a, Lo Vang b, Chunling Wang b, Tiffany Richard a, Ben Guenther a, Jayavani Aruri a, Jenny Avanzinia a, Fermin Garduno a, Peggy Farness a, Marc Gurwithe a, Jon Smith a, Eva Harris b,*, Jeff Alexander a,*, Diego Espinosa b, Jason Mendy a, Darly Manayani a, Lo Vang b, Chunling Wang b, Tiffany Richard a, Ben Guenther a, Jayavani Aruri a, Jenny Avanzinia a, Fermin Garduno a, Peggy Farness a, Marc Gurwithe a, Jon Smith a, Eva Harris b,*, Jeff Alexander a,*, Diego Espinosa b, Jason Mendy a, Darly Manayani a, Lo Vang b, Chunling Wang b, Tiffany Richard a, Ben Guenther a, Jayavani Aruri a, Jenny Avanzinia a, Fermin Garduno a, Peggy Farness a, Marc Gurwithe a, Jon Smith a, Eva Harris b,*, Jeff Alexander a,*.  

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1. Introduction

Zika virus (ZIKV) is a flavivirus closely related to human pathogens such as yellow fever, West Nile, Japanese encephalitis, tick-borne encephalitis, and dengue viruses (Pierson and Diamond, 2012). ZIKV was initially discovered in the Zika Forest of Uganda in 1947 in a sentinel Rhesus monkey that developed viremia (Dick et al., 1952). ZIKV is transmitted to humans by Aedes mosquito species, which are prevalent in African, Asian, and American tropical regions (Faye et al., 2014; Haddow et al., 2012; Hayes, 2009). ZIKV, apparently uniquely among arboviruses, is also transmitted by sexual activity. High viral loads have been detected in semen from infected patients (Atkinson et al., 2016; Foy et al., 2011; Hills et al., 2016; Musso et al., 2015), and sexual transmission from infected men and women to their partners has been reported (Hastings and Fikrig, 2017). It has been reported in mice that ZIKV infection damages the testis and leads to male infertility (Govero et al., 2016; Ma et al., 2016). Because of the usual benign course of disease and high percentage of subclinical infections, ZIKV was initially discounted as a significant human pathogen until a major outbreak occurred in 2007 on Yap Island, Micronesia (Duffy et al., 2009; Haddow et al., 2012; Lanciotti et al., 2008), followed by an outbreak in French Polynesia from 2013 to 2014 (Cao-Lormeau et al., 2016), and subsequent spread into many countries throughout the Western Hemisphere (Hennessey et al., 2016; Petersen et al., 2016). Brazil reported an estimated 500,000 to 1,500,000 human cases of ZIKV infection in 2015 (Bogoch et al., 2016) and it is likely that Zika will ultimately spread throughout most areas that have significant populations of vector mosquitoes.

As the geographic range of ZIKV increased, so did appreciation that ZIKV could cause serious human disease (Chan et al., 2016; Ioso et al., 2014). Guillain–Barré syndrome linked to ZIKV infection was detected in the 2013 outbreak in French Polynesia (Ansar and Valadi, 2015, Cao-Lormeau et al., 2016, Oehler et al., 2014). Concern was also amplified with the observation of an approximate 20-fold increase in incidence of congenital microcephaly in the 2015 outbreak in Brazil (Vogel, 2016). Evidence that ZIKV infection is associated with fetal microcephaly is, in part, based on the observation that microcephaly coincided temporarily with the ZIKV outbreak (off set by ~6 months) and subsequently, the detection of ZIKV in microcephalic fetal brain tissues (Besnard et al., 2014, Driggers et al., 2016, Marrs et al., 2016, Martines et al., 2016, Mlakar et al., 2016, Schuler-Faccini et al., 2016, Tang et al., 2016, Ventura et al., 2016). Association with neurologic disorders is
also supported by an animal model in which ZIKV infects neural progenitor cells leading to microcephaly in mice (Li et al., 2016). Further studies demonstrated that ZIKV targets and infects human embryonic stem cell-derived cerebral organoids (Dang et al., 2016). From the accumulated evidence to date, it is likely that ZIKV infection during pregnancy can cause microcephaly and associated congenital defects.

Little is known about the nature and duration of protective immunity following natural ZIKV infection. To address this issue, the search for natural correlates of protection (CoP) relies on in vitro studies of post-infection immune responses and animal models of ZIKV infection. For several licensed vaccines, correlates of human protection rely on accepted levels of antibody titers, e.g., measles, influenza, pneumococcal and Hepatitis A (Plotkin et al., 2013). Specifically, for licensed flavivirus vaccines such as yellow fever and Japanese encephalitis, neutralizing antibody (nAb) immune responses are strongly correlated with protection (Belmusto-Worn et al., 2005; Hombach et al., 2005; Julander et al., 2011; Markoff, 2000; Van Gessel et al., 2011). Knowledge of CoP for ZIKV may provide a defined path to vaccine design and ultimate licensure. In contrast, clinical field trials to demonstrate vaccine efficacy are challenging to design and execute due to the fact that epidemics are difficult to predict, often sporadic, leading to a decline of ZIKV incidence in a given population before the clinical trial sites are functional.

The envelope (E) protein of flaviruses is involved in binding cellular receptors, enabling viral entry, and is the major target for nAbs (Modis et al., 2003). Isolation of monoclonal antibodies from ZIKV-infected donors has shown that the majority of the humoral immune response is directed to the E protein, with the most potent nAbs directed against the DIII lateral ridge and complex quaternary epitopes including more than one E domain, such as the DII dimer interface (Hasan et al., 2017; Sapparapu et al., 2016; Stettler et al., 2016; Wang et al., 2016). In contrast, flavivirus-specific antibodies recognizing the DII fusion loop tend to be cross-reactive and neutralize the virus less effectively (Cherrier et al., 2009; Dai et al., 2016; Sapparapu et al., 2016; Stettler et al., 2016; Stiasny et al., 2006; Vogt et al., 2011). In addition, several animal studies have demonstrated that ZIKV-specific E antibodies confer protection against ZIKV infection and pathology (Abbink et al., 2016; Dowd et al., 2016; Larocca et al., 2016; Richner et al., 2017; Sapparapu et al., 2016; Zhao et al., 2016). For these reasons, and because of the inherent safety of VLP vaccines, we have chosen a VLP vaccine development approach that efficiently generates antibodies against the ZIKV E protein. As shown here, neutralizing antibodies induced by this VLP vaccine, upon transfer to AG129 mice, provided significantly protection against subsequent ZIKV challenge.

2. Materials & Methods

2.1. ZIKV VLP DNA Design, Production, Purification, and Characterization of VLPs

The ZIKV cassette used to generate the VLP consisted of: 1) the human IL2 signal sequence (SS) (MYRMQLLSCIALSLALVTNS); 2) the human IL2 signal sequence (SS) (MYRMQLLSCIALSLALVTNS); 2) the GM-CSF signal sequence (SS) (VSGALQFA); 3) the mouse IL2 signal sequence (SS) (MYRMQLLSCIALSLALVTNS); 4) and the mouse IL2 signal sequence (SS) (MYRMQLLSCIALSLALVTNS). The cassette containing the structural genes was inserted downstream from a human CMV IE enhancer/promoter in the CMV/R plasmid described by Akahata et al. (2010). This plasmid was transiently transfected into HEK293 cells, and the ZIKV VLPs were concentrated and purified from media supernatant for C57Bl/6 x Balb/c (C6F1) mouse immunogenicity studies.

Briefly, HEK293 cells in serum-free suspension culture were transfected with 25 μg/mL of DNA in a 1:2 ratio (by weight) of plasmid to polyethylenimine (PEI) (Polysciences, PA). At 96 hour post-transfection, cell supernatant was harvested and clarified by centrifugation at 10,000 × g for 10 min at room temperature, then pooled and filtered through a Sartopore 2 XL 0.8 μm/0.2 μm filter (Sartorius, Germany) to produce a clarified harvest. The clarified harvest was concentrated and dialyzed into load buffer (25 mM Tris and 100 mM Sodium Citrate at pH 8.3) using a 500 kDa hollow fiber (Spectrum Labs, CA) prior to column chromatography.

The ZIKV VLPs were purified using a two-column chromatography process. ZIKV VLPs from the initial tangential flow filtration process were loaded onto single-step, tandem chromatography columns comprised of mix-mode chromatography resin, Capto Core 700 (GE, PA), and a Sartobind salt-tolerant interaction chromatography resin (STIC-PA). This process used both columns in a negative capture mode by using 25 mM Tris and 100 mM Sodium Citrate at pH 8.3. Chromatography experiments were performed using AKTA Start, operated with the UNICORN software (GE Healthcare, PA).

Harvest supernatant containing VLPs or column purified VLPs were resolved by NuPAGE 4–12% Bis-Tris precast protein gels (Invitrogen, CA) and stained with InstantBlue Coomassie stain reagent (Expedeon) or transferred onto a nitrocellulose membrane using iBlot dry blotting system (Invitrogen, CA). The nitrocellulose membrane with transferred protein was blocked with 5% milk (Labscientific, Inc. NJ) in phosphate buffered saline containing 0.05% Tween 20 (PBS-T) and incubated with a mouse monoclonal antibody (MyBioSource, CA) to recombinant (generated in insect cells) E protein (MyBioSource) at 1:1000 dilution. The membrane was washed three times with PBS-T and probed with goat anti-mouse HRP-conjugated antibody (Invitrogen) at 1:10,000 dilution. The western image was developed by using an enhanced chemiluminescent (ECL) substrate kit Supersignal West Femto (ThermoFisher Scientific).

ZIKV VLP morphology was analyzed by negative stained electron microscopy at The Scripps Research Institute Microscopy Core Facility (La Jolla, CA). Briefly, purified ZIKV VLPs were absorbed on to the glow-discharged carbon-coated 200 mesh copper grid. The grid was washed and stained with 1% uranyl acetate and visualized by a Philips CM100 Transmission Electron Microscope with Soft Imaging Systems MegaView III CCD camera.

2.2. ZIKV Challenge Strain

Prototype Zika Nica 2-16 strain used for challenge was originally isolated from a ZIKV-infected Nicaraguan patient in 2016 by the National Virology Laboratory, Ministry of Health, Managua, Nicaragua (Tabata et al., 2016). The virus was propagated in C6/36 cells to generate the virus stock and titered by focus-forming assay on BHK cells (Kostyuchenko et al., 2016).

2.3. Mouse Experiments

Immune sera for passive transfer studies were generated by immunizing C6F1 mice via the intramuscular (IM) route on days 0 and 32 using a dose of 1, 5, and 25 μg of ZIKV VLP vaccine formulated with or without 100 μg alum (Alhydrogel) adjuvant. For the IM immunization, 100 μl of vaccine was injected into the hind limbs (bilateral quadriceps) at 50 μl per hind limb using a 1 ml tuberculin syringe and 27 gauge needle. On day 68 following the booster ZIKV VLP immunization, 200 μl of blood was collected from the orbital sinus plexus using non-heparinized Natelson tubes and dispensed into serum separation tubes for serum collection. The pooled mouse immune sera were tested for nAb activity against Zika Reporter Virus Particles (RVPs) expressing luciferase.
Five-to-seven week old AG129 mice on the 129/Sv background and deficient in interferon (IFN)-α/β and IFN-γ receptors, were used for this study and were bred under specific pathogen-free conditions in an animal facility of the University of California, Berkeley. Pool murine immune sera generated by ZIKV VLP immunization in CB6F1 mice were passively transferred into groups of 5 AG129 mice at 250 μL per mouse using the intravenous (IV) route of administration. Within 1 h post-transfer of immune sera, mice were challenged with 1000 focus-forming units (FFU) of ZIKV strain Nica 2-16 in 50 μL of PBS by subcutaneous (SC) injection in the left hind foot pad. Control animals included naïve (ZIKV challenge only), untreated, and mice treated with ZKA190 human mAb (1 mg/kg) previously shown to be protective against ZIKV challenge (Wang et al., 2017). Blood specimens were collected at 12-hour, 2-day, and 4-day time-points, post-infection, via the facial (submandibular) vein and allowed to clot at room temperature prior to collection of sera by centrifugation. Terminal bleeds on surviving animals were performed at 18 days post-challenge. Following virus challenge, mouse weight, morbidity, and mortality were monitored daily. A 1–to-5 morbidity scale was adapted from (Orozco et al., 2012): 1) Healthy; 2) Displaying mild signs of lethargy, some fur ruffling, and no hunched posture; 3) Fur ruffling, mild signs of lethargy, early signs of hind leg paralysis possible; 4) Fur ruffling, increased lethargy and limited mobility, and signs of paralysis common; and 5) Moribund, minimal mobility consistent with inability to reach food or water, neurological signs evident (paralysis/seizures). Mice were euthanized if weight loss was equal to or >20% of their original weight and/or if they scored “5” on the morbidity scale. All animal experiments comply with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and are accredited by the American Association for the Accreditation for Laboratory Animal Care (AAALAC) and carry appropriate US government assurances (NIH Assurance A4282-01, USDA 93-R-0444). Absorption Systems conducts an IACUC review for all proposed animal studies and approved the mouse study. Mice were allowed to acclimate to the environment for 3 days prior to initiation of the study. The CB6F1 mouse experiments comply with the National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.4. Quantitative Reverse Transcription PCR (RT-qPCR)

As a surrogate measurement for viremia, ZIKV RNA copy levels in serum were determined by RT-qPCR from blood samples collected 2 and 4 days post-ZIKV challenge. ZIKV RNA was extracted from serum samples using the QiAamp Viral RNA Mini Kit (Qiagen, MD) and RNA levels were determined by TaqMan one-step RT-qPCR using a standard curve with 10-fold dilutions of ZIKV Nica 2–16 RNA. Primer sets used for this assay were modified from Lanciotti et al. (Lanciotti et al., 2008) as follows: ZIKV_1086_Fwd, 5′-CCGCTGCCCAACCAACAAG-3′; ZIKV_1162c_Rev, 5′-CCACTAAGTTCTTTGAGCACT-3′; Probe ZIKV_1107-FAM_UG&Nica 5′-AGGCTACCTGAAAGCAATGACACTA-3′. Viral loads in RT-qPCR assays are expressed on a log10 scale as viral RNA genome equivalents/ml after comparison with a standard curve produced using serial 10-fold dilutions of ZIKV RNA. The detection limit of the qRT-PCR assay was determined by assaying serial dilutions of purified ZIKV RNA in the range of 10^3–10^6 genome equivalents/ml. A value of 100 GE/mL was established as the limit of detection of the assay as this dilution consistently resulted in detectable PCR amplification above background (no template control) levels.

2.5. Antibody Neutralization Assay

ZIKV-specific nAb titers in mouse serum samples were determined using a Zika RVP neutralization assay with methodology similar to that previously described for dengue RVPs (Mattia et al., 2011). The assay is based on the capacity of Zika RVPs (Integral Molecular, Philadelphia, PA) to infect host Vero cells and express Renilla luciferase. The RVPs incorporate pR8.95 structural proteins of the Brazilian SPH2015 strain. Reductions in luciferase activity in the presence of serially diluted immune serum indicate nAb activity. A ZIKV RVP titration was performed to determine the dynamic range of luminescence detected from Vero infected cells. A maximum RLU of approximately 40,000 was achieved and assay background was ~130 RLU as measured in wells containing cells alone without virus infection. A dilution of virus in the upper end of the linear portion of the titration curve corresponding to a signal of ~30,000 was selected for performing the neutralization assays. This dose of virus and dynamic range were determined to be enough to permit the evaluation of the reduction of luminescence following neutralization. Since the starting dilution of test serum is 1:10 and antibody titers below this dilution cannot be quantified, the 1:10 titer is considered the limit of detection of the assay. To assess circulating ZIKV-specific nAbs, blood samples were collected at 12 h after transfer of immune sera and viral challenge, and from surviving animals, at 18 days after challenge. The Zika RVP neutralization assay was performed on serum from individual mice using 96-well plates. All test sera were heat-inactivated at 56 °C for 35 min prior to the assay. Serial 3-fold dilutions of serum (1:5 starting dilution) were prepared in assay medium (DMEM + 10% FBS + 10 mM HEPES, pH 8.0) and incubated with an equal volume of Zika RVPs previously determined to yield sufficiently high luciferase activity. Each sample was run in triplicate in a single experiment. Assays were repeated only if positive serum and virus only control values were out of range based on historical data. Final assay dilutions ranged from 1:10 to 1:590,490. Following a 1-hour incubation at 37 °C, Vero cells (2 × 10^4 cells per well) were added to the RVP-serum mixture. Assay plates were then incubated at 37 °C for 72 h. Luciferase activity was measured using the Renilla Luciferase Assay System (Promega, WI), and plates were read on a Spectramax M3 plate luminometer (Molecular Devices, CA). NT_{50} nAb titers were determined using non-linear regression analysis in GraphPad Prism 7, defined as the reciprocal of the dilution that neutralizes 50% of Zika RVP infection.

2.6. Statistical Analysis

The Student’s t-test was used to compare differences between experimental groups for resulting nAb titers and viral RNA levels. The Wilcoxon Mann-Whitney test was used to compare nAb titer differences between protected and non-protected mice. Mantel-Cox survival curves were analyzed by the log rank test. Results were considered statistically significant if the p-value was <0.05. For correlation, the coefficient of determination (R^2) was determined using linear regression analysis. All statistical analysis was performed using Graph Pad Prism version 7 software (San Diego, CA).

3. Results

3.1. ZIKV VLP Design, Production, Purification, and Characterization

The ZIKV VLP vaccine candidate is comprised of the prM and E structural proteins derived from the African MR766 strain but with the E ectodomain from the Brazilian SPH2015 strain (Fig. 1a). HEK293 cells were transfected with the ZIKV VLP expression plasmid complexed to PEI. Four days following transfection cell supernatant was harvested and clarified by centrifugation. Supernatant was subsequently purified using column chromatography and cell supernatants pre- and post-purification were evaluated by a SDS-PAGE Coomassie gel (panel b) and
Western blot (panel c). As shown in the Coomassie gel (panel b, lane 1), are multiple staining protein bands that when transferred to a Western Blot (panel c, lane 1) a 55 kDa band was detected using an anti-E antibody. Following purification, a highly purified protein migrating at the appropriate size is detected in the coomassie gel (panel b, lane 2) and identity is confirmed in the Western Blot (panel c, lane 2). Positive control, full-length recombinant E protein running at approximately 50 kDa is evident in panels b and c, lane 3. The faster migration of the recombinant E protein, which was generated in insect cells, maybe due to a less complex glycosylation pattern. The particle was further characterized by negative staining using uranyl acetate and visualized by electron microscopy (panel d). The population of particles is predominantly composed of two different sizes, 35 nm and 55 nm. Four random areas of the EM grid were counted and approximately 80% of the VLPs were 35 nm in size.

3.2. ZIKV VLP Immunogenicity in CB6F1 Mice

Immune sera obtained following one and two ZIKV VLP immunizations of CB6F1 mice indicated that the VLP candidate vaccine induced Zika viral particle neutralization in the 100 to 5000 NT50 titer range depending on dose, number of VLP immunizations, and presence of aluminum hydroxide (Alhydrogel; alum) as adjuvant (Fig. 2). The use of alum to augment the nAb titer is most evident at the lower doses where alum appeared to cause dose-sparing. Following one immunization (Panel a) the 1 µg VLP dose adsorbed to alum increased the immune response by approximately 5-fold, 100 vs. 500 NT50 geometric mean antibody titer (GMT). This is also evident following two VLP immunizations (Panel b) where the 1 µg VLP dose response was increased from 620 to 4200 GMT when the ZIKV VLPs were adsorbed to alum. The benefit of using alum is less evident at the higher antigen doses. It is interesting to note that there was no antigen dose response when the VLPs were adsorbed to alum. Following one immunization, the 5 µg VLP/alum dose was the most immunogenic (500 GMT) whereas following two VLP/alum immunizations, the 1 µg dose (4200 GMT) was the most immunogenic. Considering the extensive overlap of individual immune responses, there are no significant differences in immune responses generated using the 1, 5, and 25 µg doses when adsorbed to alum and a maximum response was achieved at the lowest immunogen dose tested. Alum provides a significant dose-sparing effect as 1 µg on alum was as immunogenic as 25 µg of free VLP. The immune sera from individual mice, 25 µg VLP vaccine alone and adsorbed to alum groups, were pooled for transfer to AG129 mice and the NT50 titers were evaluated (Panel c). The titers were as expected approximately 3000 (VLP) and 4000 (VLP + alum).

3.3. Experimental Challenge Study Design

Immune sera from ZIKV VLP-immunized CB6F1 mice were passively transferred to AG129 mice prior to challenge with 1000 FFU of ZIKV. Experimental groups and conditions are depicted in Table 1. The immune sera test articles were administered neat (undiluted) and diluted 1:5 in a volume of 250 µL. The control groups consisted of: 1) sera from CB6F1 mice immunized with Alum-only; 2) Naïve mice (no transfer of sera); 3) Untreated mice (no transfer of sera and no ZIKV challenge); and 4) Positive control (ZKA190 mAb) transfer.
Table 1

| Group | Passive transfer inoculum<sup>a</sup> | Study time points |
|-------|-------------------------------------|-------------------|
|       |                                     | (0 h) 1 h 12 h 2 days 4 days 18 days |
| 1     | VLP serum (1:5)                      | ZIKV nAb<sup>b</sup> Viral RNA<sup>c</sup> Viral RNA<sup>c</sup> nAb<sup>d</sup> |
| 2     | VLP serum                            | GA129 mice only 18 days GE = 5 per group, n = 5 for each group, male and female. |
| 3     | VLP + alum serum                     | GA129 mice only 18 days GE = 5 per group, n = 5 for each group, male and female. |
| 4     | VLP + alum serum (1:5)               | GA129 mice only 18 days GE = 5 per group, n = 5 for each group, male and female. |
| 5     | Alum only serum                      | GA129 mice only 18 days GE = 5 per group, n = 5 for each group, male and female. |
| 6     | Naïve (no serum)                     | GA129 mice only 18 days GE = 5 per group, n = 5 for each group, male and female. |
| 7     | Untreated (no serum, no ZIKV<sup>e</sup>) | GA129 mice only 18 days GE = 5 per group, n = 5 for each group, male and female. |
| 8     | Positive control ZKA190 mAb<sup>f</sup> | GA129 mice only 18 days GE = 5 per group, n = 5 for each group, male and female. |

Abbreviations: Alum, aluminum hydroxide adjuvant; ZIKV, Zika virus; nAb, neutralizing antibody

<sup>a</sup> Immune serum (250 μL) delivered by intravenous tail vein injection.
<sup>b</sup> The untreated group was not challenged with ZIKV.
<sup>c</sup> 1000 FFU of ZIKV strain Nica 2-16 injection into foot pad.
<sup>d</sup> Bleeds for serum were taken to measure nAb.
<sup>e</sup> Bleeds for serum were taken to measure viral genome copies by RT-qPCR.
<sup>f</sup> ZKA190 mAb transferred was 1 mg/kg in 250 μL.

3.4. ZIKV-specific nAb Titers

To evaluate the ZIKV-specific nAb titers achieved in the circulation and to help establish CoP, nAb titers were evaluated in individual mice during the 18-day study. For this purpose, a blood specimen was collected 12 h following antibody transfer as well as 18 days post-challenge in surviving mice. Fig. 3 depicts the titers measured in the mice following immune sera transfer and ZIKV challenge. It should be noted that prior to passive antibody transfer, the nAb titers measured for pooled immune sera were 2849 (ZIKV VLP-induced) and 3837 (ZIKV VLP + alum-induced). As shown in (Panel a), 12 h after the transfer of immune sera from mice immunized with VLP without alum, the GMT measured was 359 with a range of 293 to 484 (VLP-induced immune sera). The 1:5 diluted VLP sera attained a significantly lower GMT of 80 with a range of 66 to 96. Comparable nAbs titers were reached when the immune sera transferred were generated in mice immunized with ZIKV VLP formulated with alum adjuvant; a GMT of 394 with a range of 258 to 542 (neat) and a significantly lower GMT of 120 with a range of 93 to 141 (1:5 diluted VLP + alum sera). The positive control, ZKA190 mAb attained a GMT of 3870 with a range of 1989 to 11,347. No measurable antibody titers at the 12-hour time point were detected for the Alum, Naïve and Untreated control groups of mice.

At the 18-day time point (Fig. 3, Panel b), the nAb titers were significantly increased vs. the 12-hour time point for each test article group, with GMTs achieved of 1699 (p = 0.0002), range 1414 to 2483 (VLP); 3160 (p ≤ 0.0001), range 3110 to 3210 (VLP 1:5); 1448 (p = 0.008), range 1079 to 2201 (VLP + alum); and 2509 (p = 0.002), range 2070 to 3040 (VLP + alum 1:5). It should be noted that only 2 out of the 5 mice that received 1:5 diluted immune sera survived ZIKV challenge. As expected, the Alum only and Naïve mice did not survive to day 18 following ZIKV challenge. No measurable antibody titers on day 18 were detected for the untreated control group. With regard to the ZKA190 mAb group, the day 18 nAb titers significantly dropped to a GMT of 293 (p = 0.0353), with a range of 146 to 943 compared to the 12-hour time point, likely indicating the transient nature of the transferred mAb. However, it is not known if any de novo synthesis of nAb occurred due to viral replication and contributed to the NT<sub>50</sub> value observed at 18 days post virus challenge.

3.5. Circulating Viral RNA

To evaluate viral RNA in circulation, blood samples were collected on days 2 and 4 post-ZIKV challenge and analyzed by RT-qPCR (Fig. 4a and b, respectively). Peak viral RNA in serum, measured in genome equivalents (GE)/mL, in the Alum and Naïve groups were reached on Day 2, attaining geomeans of 463,631 and 317,848, respectively. Compared to the Alum-only control group, a significant reduction in GE/mL (p ≤ 0.0001) was observed when VLP and VLP + alum-induced immune sera were transferred to AG129 mice prior to the ZIKV challenge. The VLP and VLP + alum groups had <100 GE/mL, a reduction of at least 3 logs relative to the Alum only group. Administration of 1:5 dilution of VLP-induced and VLP alum-induced immune sera also significantly reduced the GE/mL (p ≤ 0.0001), albeit to a lesser extent, with geomeans of 564 and 986, respectively. The positive control ZKA190 mAb, when passively transferred to AG129 mice, also significantly reduced GE/mL to below detectable limits (p ≤ 0.0001).

By Day 4, the amount of viral RNA in serum increased for all test article groups, with GE/mL detected of 3262 (VLP); 70,501 (VLP 1:5); 1481 (VLP + alum); and 18,627 (VLP + alum 1:5), Fig. 4b. The relative GE levels in the Alum and Naïve groups dropped by Day 4 with geomeans of 35,830 and 19,328, respectively. In spite of the increase in the amount of viral RNA observed in the test article groups (neat sera), the GE levels still remained significantly lower vs. the Alum control group, p = 0.012 (VLP) and p = 0.009 (VLP + alum). The transferred ZKA190 mAb was most protective, with 3 of 5 mice presenting significant reductions in GE/mL vs. the Alum only and Naïve groups, p = 0.008. This is predictable due to the higher ZIKV-specific nAb titers measured at 12 hour post-transfer compared to VLP-induced and VLP + alum-induced immune.
sera. Interestingly, with regard to the ZKA190 mAb group, there were two individual mice that did attain detectable levels of viral RNA in the serum, 240 and 2152 GE/mL, potentially indicating viral replication and lack of sterile immunity. In support of viral replication in these mice is the observation that the nAb titers 18 days post virus challenge for these mice were 716 (240 GE/mL) and 943 (2152 GE/mL). In contrast, the three mice with no measurable viral RNA levels in serum at Day 4 had low level NT50 titers of 146, 147, and 150 at the 18 day post challenge time point.

3.6. Level of Efficacy Achieved Following Passive Antibody Transfer: Body Weight, Morbidity, and Mortality

Protection against ZIKV challenge afforded by passively transferring immune sera to AG129 mice is shown in Fig. 5. AG129 mice that received undiluted sera from VLP immunized mice or VLP + alum immunized mice retained approximately 100% of their body weight over the course of the 18-day study (Fig. 5a). This observation was also the case when the positive control ZKA190 mAb was transferred. However, mice that received VLP and VLP + alum-induced sera diluted 1:5 prior to transfer experienced precipitous weight loss beginning at approximately Day 11. Severe weight loss was also evident in the Alum and Naïve control groups starting approximately Day 10 following ZIKV challenge.

Morbidity was evaluated on a 1 to 5 scale shown in Fig. 5b. The VLP- and VLP + alum-induced immune sera delivered neat, in addition to ZKA190 mAb, protected the AG129 mice against ZIKV-induced morbidity. An increase in morbidity was evident around Day 12 following ZIKV challenge for the VLP-induced sera (diluted 1:5), VLP + alum-induced sera (diluted 1:5), Alum, and Naïve groups.

As depicted in Fig. 5c, the mice in the VLP-induced immune sera, VLP + alum-induced immune sera, and ZKA190 mAb groups were significantly protected against mortality relative to the Alum (p = 0.0015) and Naïve control (p = 0.0021) groups. All mice in the Alum only and Naïve groups succumbed to infection by Day 16. Partial protection was afforded in the VLP (1:5 diluted sera transfer) group where only 2 of 5 mice survived. Similarly, only 2 mice survived in the VLP + alum (1:5 diluted sera transfer) group.

3.7. Immune Correlates of Protection

Immune CoP were evaluated based on the capacity of circulating nAb to protect against death and circulating viral RNA following ZIKV challenge in AG129 mice. Shown in Fig. 6a is the distribution of the
individual mice based on their level of circulating nAb titer at 12 hour post-infection vs. survival. There was a significant inverse relationship between level of circulating nAb and mortality \((p = 0.0001)\). Fifteen of eighteen mice that had a NT\(_{50}\) antibody level of approximately 125 or higher survived, whereas all mice that had a NT\(_{50}\) titer of <125 succumbed to infection. There was also a strong correlation \((R^2 = 0.79)\) between the level of circulating nAb titer measured at 12 hour post-transfer and level of circulating viral RNA 2 days post-challenge (Fig. 6b). The correlation was also conducted without the ZKA 190 mAb group to restrict analysis to polyclonal antibody responses. The correlation was determined to be somewhat higher \((R^2 = 0.88)\).

4. Discussion

The objective of this study was to determine whether passive transfer of ZIKV-specific nAbs generated by ZIKV VLP immunization in CB6F1 mice could protect AG129 mice upon ZIKV infection. ZIKV VLP particles were generated by transient PEI chemical transfection of HEK293 cells using an expression plasmid encoding ZIKV structural genes prM and E. VLPs in the cell supernatant harvest were processed by column chromatography resulting in highly purified VLP particles. Two particle sizes of 35 nm and 55 nm were evident by EM analysis. Similar particle sizes were observed for the flavivirus Tick-Borne Encephalitis (Allison et al., 2003). Allison and colleagues identified two major size particle classes, separated on sucrose gradients, stained by uranyl formate, and analyzed by EM that appeared to be distinct assembly products of 30 nm and 55 nm. The smaller particle corresponded in size to the mature wild-type mature subviral particles where the E proteins are arranged in a regular \(T = 1\) icosahedral lattice wherein the larger particles were similar in size to the native virus. Additionally, misshapen particles were evident that may represent damaged particles. In contrast, Boigard et al. generated a more uniform Zika VLP particle size distribution of 50 nm to 65 nm in diameter (mean 60 nm) when observed in electron micrographs (Boigard et al., 2017). A possible reason for this consistent size distribution of VLPs may be due to the addition of the capsid component to the structural gene cassette, i.e., CprME. They were unable to detect the capsid protein in the VLP transfected lysates using an anti-C antibody but assumed that due to detection of E and prM proteins that the capsid was cleaved.

The ZIKV VLP may have unexpected structural changes to the surface of the particle due to either its chimeric composition and/or production of two distinct particle sizes. We have not done extensive epitope mapping by antibody recognition of our VLP vs. the native virus. In preliminary studies, we do know that our VLP is recognized by the highly characterized antibody, ZIKV-117 (Sapparapu et al., 2016). Epitope mapping studies by this group revealed that ZIKV-117 antibody recognizes a unique quaternary epitope on the E protein dimer–dimer interface. Additional epitope mapping studies will be required to further understand any structural changes our ZIKV VLP may present vs. the native virus.

Immune sera generated in CB6F1 mice following VLP immunization, were transferred to AG129 mice and, as expected, nAb titers of 2849 (VLP-induced sera) and 3837 (VLP + alum-induced sera) were diluted in the circulation of recipient AG129 mice to levels of 359 to 394, respectively. This level of circulating antibody was sufficient to prevent weight loss, morbidity, and mortality following ZIKV challenge relative to unimmunized controls. However, immune sera diluted 1:5 prior to transfer resulted in circulating nAb titers of only 80 and 120 and provided minimal protection. Only 2 out of 5 mice survived in each of the groups receiving a 1:5 diluted sera, VLP and VLP + alum groups.

Although passive transfer of nAbs was protective in adult mice, this level of protection did not provide sterilizing immunity. As determined on Day 2 post-infection, viral RNA levels in serum for the VLP-induced and VLP + alum-induced immune sera (near) groups were below the assay’s threshold of detection with the exception of one mouse, in the VLP + alum group, which had a low level of detectable viral RNA. However, by Day 4, VLP and VLP + alum-induced immune sera groups registered viral RNA levels of 3262 and 1481 GE/mL, respectively. Whether this extent of viral RNA reduction in human subjects would be sufficient to protect a developing fetus is unknown, but we assume even low levels of viral RNA may be a potential risk. Further, the fact that nAb titers increased from the 12-hour to the 18-day time point suggests that virus replication likely induced de novo nAb production. The circulating nAb titer 12-hour post-transfer for the positive control ZKA190 mAb group diminished by day 18 (3870 to 293), suggesting that ZIKV replication was sufficiently reduced to prevent de novo antibody production and that the drop of nAb titer was likely due to tissue binding and the half-life of the antibody in vivo. However, it should be noted that the ZKA190 monoclonal antibody group had two outliers regarding viral RNA levels on Day 4. Two out of 5 mice achieved detectable RNA levels of 240 and 2152 GE/mL potentially signifying low levels of viral replication in these mice.

Several animal studies demonstrated that nAb, induced by vaccination or passively transferred in serum, is a correlate of protection against flavivirus challenge (Ben-Nathan et al., 2003; Diamond et al., 2003; Engle and Diamond, 2003; Hammon and Sather, 1973; Julander et al., 2011; Kreil et al., 1997; Kreil et al., 1998; Lubiniecki et al., 1973; Swanstrom et al., 2016; Tesh et al., 2002). Specifically, in the case of
ZIKV infection, recent studies evaluating multiple vaccine delivery formats have also provided compelling evidence that nAb is a likely CoP (Abbink et al., 2016; Dowd et al., 2016; Larocca et al., 2016; Richner et al., 2017; Sapparapu et al., 2016; Zhao et al., 2016).

In our study, the level of nAb necessary to protect against detectable circulating viral RNA may be only addressed by evaluating the ZKA190 mAb positive control group where 3 of the 5 mice exhibited no detectable viral RNA in the serum. Levels of nAb achieved in these mice 12 h after passive transfer ranged from approximately 2000 to 11,000. The specific 3 mice with no detectable viral RNA on day 4 had nAb levels 12 h after passive transfer of 2379, 3202, and 11,347 while the two mice with detectable viral RNA had nAb levels of 1989 and 5049. Therefore, nAb levels above approximately 2000 were required to provide sterilizing immunity for 2 of the 3 mice with the outlier being a nAb titer of 5049 that was not sufficient to prevent the detection of viral RNA. Other groups have provided evidence that sterilizing immunity can be achieved in the mouse model. Richner et al., using a modified mRNA vaccine expressing prM-E, protected AG129, C57Bl/6, and Balb/c mice against death (Richner et al., 2017). Specifically, in the case of Balb/c mice treated with 2 mg of anti-ifnar1 blocking antibody and challenged with the mouse-adapted ZIKV Dakar 41519 strain, an EC50 nAb titer of approximately 10,000 was sufficient to prevent viremia and tissue dissemination. Sumathy et al., using inactivated ZIKV adsorbed to alum, immunized rabbits and passively transferred the immune sera to Balb/c mice which were subsequently challenged with the African MR766 ZIKV strain. No viremia was detected in these mice when circulating levels of nAb were as low as approximately 100 PRNT50 (Sumathy et al., 2017).

Zhao et al. studied the structural basis of antibody protection by passively transferring mouse-derived ZIKV-specific mAbs to C57Bl/6 mice prior to ZIKV challenge (Zhao et al., 2016). They observed reduced viremia (by ~1–2 logs) and complete clinical protection as measured by weight loss and mortality. Sapparapu et al. isolated a panel of human mAbs isolated from subjects previously infected with ZIKV (Sapparapu et al., 2016). One of the mAbs, ZIKV-117, recognized a quaternary epitope on the E protein dimer-dimer interface. This specific mAb when administered to pregnant mouse dams, prior to ZIKV challenge, significantly reduced virus recovery from low to undetectable levels in placenta and fetal brain vs. dams receiving a control mAb. Our study described herein is in line with the Zhao and Sapparapu studies in that transferred ZIKV-specific antibody significantly protects against viral infection but may not provide sterilizing immunity. Taken together, these studies support ZIKV-specific antibody as a CoP against ZIKV infection and pathogenesis. The necessary level of ZIKV-specific nAb that must be achieved to prevent viral replication and protection of the developing fetus, even in animal models, is unknown. In part, it is difficult to directly compare animal study results due to different strains of mice used, different strains of ZIKV used for challenge, and the measure of neutralizing antibody by different assay methods. In our study, a log NT50 serum antibody titer of approximately 125 was required for protection against weight loss, morbidity, and mortality. Studies are in progress to compare the ZIKV RVP luciferase-based assay used in this study to the standard Plaque Reduction Neutralization Test (PRNT) to determine correlation and bias between the assays. Shan et al., has developed a high throughput assay for ZIKV and dengue virus diagnosis that indirectly addresses this issue (Shan et al., 2017). Their assay utilizes luciferase viruses to quantify the neutralizing antibody titers in a 96-well format and found that neutralization titers derived from the ZIKV luciferase-based assay were on the average 2.5-fold higher than those derived from the corresponding ZIKV plaque assay. Further animal studies will be informative when evaluating the level of viremia the fetus can be exposed to vs. clinical outcome, but it may be that a vaccine strategy will be required that prevents any viremia to be truly effective against fetal injury.

In summary, we evaluated the protective capacity of passively transferred VLP-induced immune sera to AG129 mice. The immune sera were able to protect mice against weight-loss, morbidity, and mortality. Although circulating viral RNA levels following ZIKV challenge was reduced by at least 3-logs vs. control mice, a reduction that would likely prevent infection of mosquitos, sterilizing immunity was not achieved. Additional studies evaluating this ZIKV VLP vaccine candidate’s capacity to protect against ZIKV challenge in mouse and NHP models will be instructive. In our specific case, VLP immunization induced high ZIKV-specific nAb titers in the range of 2849 (VLP-induced sera) to 3837 (VLP + alum-induced sera). It will be of interest to evaluate the level of ZIKV-specific nAb that is maintained over an extended period of time to define the duration of VLP vaccine-induced nAb immune response. We currently have an ongoing VLP active immunization study whereby the AG129 mice were immunized with a dose titration of the VLP vaccine formulated with alum. It will be of importance to determine to what extent mice will be protected against viral challenge when higher ZIKV-specific nAbs are achieved.

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Conflict of Interest Statement

All members of PaxVax (JM, DM, LV, TR, BG, Jayavani A, Jenny A, FG, MG, JS, Jeff A) are current paid employees of PaxVax and also own PaxVax stock. The other authors declare no conflict of interests. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Author Contributions

JM, LV, MG, JS, DE, EH, Jeff A designed the study. DE, JM, DM, LV, CW, TR, BG, Jayavani A, Jenny A, FG, JS, Jeff A generated the ZIKV VLP-induced mouse immune sera, performed animal experiments, conducted immunological assays, and analyzed data. JM, DE, Jeff A wrote the manuscript. MG, JS, EH reviewed and edited the manuscript.

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