An Examination of the Safety and Efficacy of Aripo-Zika as a Zika Virus Vaccine Candidate

Manette Tanelus

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A. Jonathan Auguste, Chair

Sally Paulson

Andrea Bertke

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General Audience Abstract

Vaccines are one of the best tools available since their initial conception. Vaccines have collectively increased human lifespan and reduced the burden of disease in humans and animals worldwide. Vaccine research aims to create vaccines that have a perfect balance of safety and efficacy. The goal is to produce a vaccine that can generate a strong immune response against the virus(es) of interest, while causing the least harm or side effects from the vaccine. Insect-specific viruses are known to infect insects and replicate in insect cells but are unable to replicate in humans. The Auguste Lab has created a chimeric vaccine using the genome of an insect-specific virus called Aripo-Zika virus (AZ) that is genetically related to Zika virus. A person vaccinated with AZ is expected to develop an immune response against Zika but would not have any disease or side effects associated with a Zika infection or virus replication. In order to determine if this vaccine would be safe and effective enough to advance to clinical trials in humans, we must first determine if it is safe in smaller animal models. My studies have five central aims. First, determine the lowest dose of AZ that can be given and still be protective against Zika disease in mouse models. Second, determine if boosters are necessary and if they increase protection. Third, determine if immunity derived from vaccination can be passed down from mother to pups. Fourth, determine if Zika virus and AZ can co-exist in the same cell line without AZ replication occurring. Lastly, determine if mice can be vaccinated with AZ and subsequently with another similar Aripo virus-based vaccine (i.e., Aripo-West Nile)

without changing the effectiveness of the subsequent immunization. Our results showed that AZ is able to be passed from mother to pup, 10^{11} genome copies is the minimum protective dose, and boosters can increase the effectiveness of AZ. We also found that AZ does not replicate in vertebrate cells when it co-exists with ZIKV and subsequent vaccination with Aripo-West Nile does not seem affect the effectiveness of either vaccine.

An Examination of the Safety and Efficacy of Aripo-Zika as a Zika virus Vaccine Candidate

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Academic Abstract

Flaviviruses are a genus of vector-transmitted viruses that are nearly globally distributed, and flavivirus infections can result in life threatening diseases. Dengue, West Nile, yellow fever and Zika are among the most well-known and widely distributed flaviviruses. Zika virus (ZIKV) is a single strand positive-sense RNA virus. Zika induced disease has been linked to Guillain Barré Syndrome (i.e., an autoimmune illness that affects the nerves) in adults and congenital birth defects including microcephaly (i.e., a neurodevelopmental disorder due to impaired neural cell proliferation) in newborns. Insect-specific flaviviruses (ISFVs) are understudied given there apathogenic characteristics to humans and animals. However, given their close genetic relationship to vertebrate infectious flaviviruses, ISFVs can serve as a delivery system (i.e., vector) for flavivirus antigenic proteins. Aripo virus (ARPV) is a recently discovered ISFV isolated in Trinidad. We developed a chimeric Zika vaccine, Aripo-Zika, by substituting the pre-membrane and envelope genes of ZIKV into the ARPV genome. Here, we explored (i) the efficacy of Aripo-Zika (AZ) vaccination by evaluating passive transfer of maternal antibodies, (ii) the optimal dosage regimen, (iii) anti-vector immunity to the ARPV backbone, and (iv) the effects of boosters on vaccine efficacy. We also evaluated AZ safety via a co-infection study. Our results show a near linear relationship between increased dose and immunogenicity, with 10^{11} genome copies being the most effective minimum dose administered. Inclusion of boosters further increased the immunogenicity of AZ. Additionally, prior immunization with AZ

showed minimal effects on subsequent immunization with an ARPV-West Nile virus (AWN) vaccine candidate, confirming the applicability of the ARPV backbone to multiple flavivirus vaccine candidates. *In vitro* co-infection of ZIKV with ARPV, and ZIKV with AZ in African green monkey kidney cells (i.e., Vero-76) indicated ARPV and AZ remain incapable of replication in vertebrate cells, even in the presence of active ZIKV replication. Altogether, our data suggests that the ARPV platform is a safe and effective strategy for the development of flavivirus vaccines.

Acknowledgements

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nothing else matters. With the help of these people, I have been able to preserve my social life and remember to enjoy the small things in life as I try to maintain a work life balance.

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Chapter 1: Literature Review

Introduction to *Flaviviridae*.

The genus *Flavivirus* contains vector-transmitted viruses that have the potential to cause life threatening diseases[1-9]. Many flaviviruses (e.g., Dengue virus (DENV), West Nile Virus (WNV), yellow fever virus (YFV), Japanese encephalitis virus (JEV)) pose a global threat, and present very diverse disease presentations from mild flu-like symptoms to major neurological and/or hemorrhagic complications[5,6,8,10-13]. The *Flaviviridae* family includes several host-associated groups of viruses[7,14,15]. Most notably, it contains insect-specific flaviviruses and classical vertebrate infectious flaviviruses[6,14,16-19]. The most noteworthy flaviviruses include YFV, WNV, DENV, and JEV[5,6,20,21]. These viruses are known to be highly pathogenic and cause significant morbidity and mortality[5,6,17,22]. YFV causes yellow fever, and during the 18th and 19th century caused high mortality in Africa and the Americas [1,5]. Presently, YFV affects 200,000 people annually[5,6]. WNV and JEV are categorized by febrile illness and have been known to lead to neurological disease[6].

Flavivirus genome organization and replication.

The flavivirus virion is ~50nm in diameter and encapsidates an ~11kb single-stranded positive-sense RNA genome that encodes ten proteins within a single open reading frame (Figure 1B)[1,2,6,23,24]. This open reading frame is flanked by two untranslated regions (5'- and 3'-UTRs) which play a role in viral replication and immune modulation. There are three structural proteins: the nucleocapsid, envelope protein, and prM protein. There are seven non-structural

proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, that are responsible for replication and assembly[1,2,6,23,24]. Flaviviruses enter cells via receptor-mediated endocytosis (Figure 1A) where the acidic environment in the endosome causes fusion to occur releasing the genome[25]. Replication occurs in the endoplasmic reticulum followed by envelope rearrangement, glycosylation and proteolytic cleavage of prM in the Golgi[25]. The mature virion is then released from the cell[1,2,6,23,24,25].

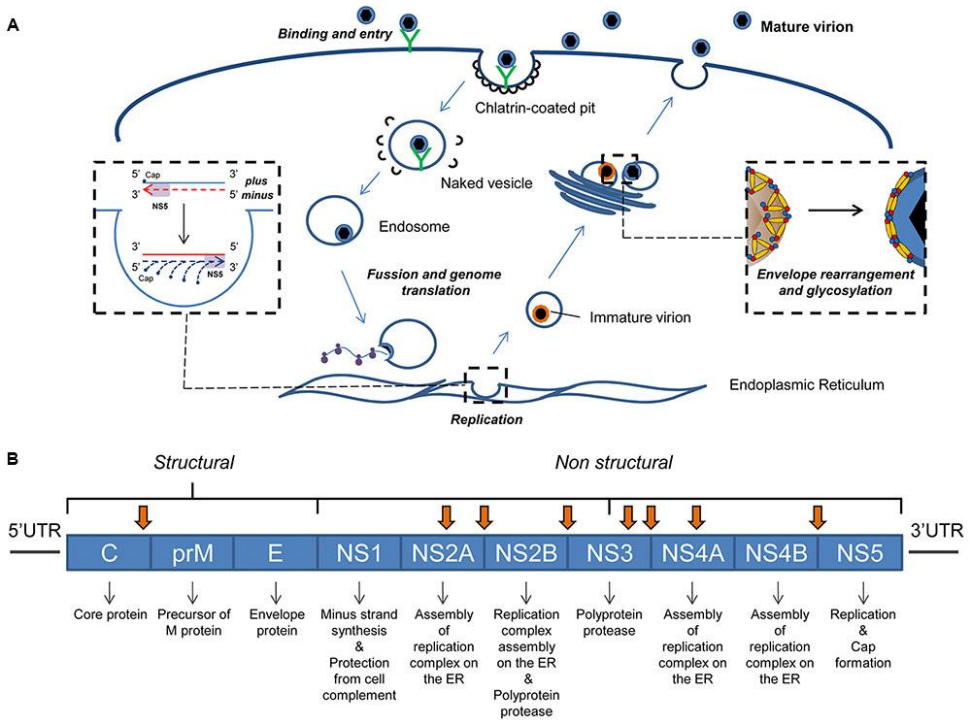

Figure 1. "Flavivirus infectious cycle and genome. (A) Diagram of flavivirus infectious cycle. (B) Genetic organization of flavivirus genomes. These genomes code for three structural and seven non-structural proteins, the main functions of which are shown. Arrows indicate the cleavage sites for the viral protease NS2B/NS3"[25].

Flavivirus phylogenetics and evolution

The genus *Flavivirus* comprises of 70 positive-sense single-stranded RNA viruses with many replicating in both vertebrate and invertebrate cells[6,8,14,19,26,27]. Vertebrate hosts are infected

with flaviviruses via the bite of hematophagous arthropods[5,6,8]. Flavivirus clades within a phylogeny are often formed based on the mode of transmission, or the host and vector associations[7,15,28]. Flaviviruses may be mosquito-borne (MBFV), tick-borne (TBFV), insect-specific (ISFV), and some have no-known vector (NKV)[7,14,15,27]. The NKV group are primarily associated with bats and rodents. The pathogenic flaviviruses within the TBFVs are

Yet, among the TBFVs there are pathogenic flaviviruses associated with *Ixodes spp*. and apathogenic flaviviruses associated with *Ornithodorus spp*[7]. Among the mosquito-borne flaviviruses (MBFV), there are many viruses associated with *Culex spp.* (ornithophilic) mosquitoes and those that are primarily associated with *Aedes spp.* (mammalophilic) mosquitoes[7,8,29]. *Aedes* associated flaviviruses include the Kedougou virus (KEDV) group, yellow fever virus (YFV) group, and dengue virus (DENV) group[7]. Phylogenetic analyses have shown that MBFVs have been introduced from the Old World (i.e., Africa, Asia, and Europe) to the New World (i.e. North America, Central America, and South America) 11 times[7].

Arbovirus interventions.

Given the various modes of transmission of arboviruses, several intervention strategies exist for preventing infection or reducing disease[10,16,30,31]. Vector population control and vaccine development are the prevalent strategies used to combat arbovirus spread[10,16,30-33]. Vaccines are among the most effective long-term preventive strategies because they protect human health and can be a dependable strategy to prevent the emergence of infectious diseases[34-36] . Unlike other interventions, vaccines have an expansive reach: they protect individuals, communities, and entire populations[34-36]. The impact of most vaccines on communities and

populations is almost immediate [34-36]. Live-attenuated vaccines, subunit vaccines, and inactivated vaccines have been among the most popular arbovirus vaccine platforms previously used, but RNA vaccines are rapidly emerging as a safe and effective platform[34-36]. Live-attenuated vaccines induce a strong immune response and closely mimics a natural infection, yet they carry the risk of residual virulence or reversion to virulence, especially in immune-compromised individuals[2,35]. Subunit vaccines are typically safe and well-tolerated even in immune-compromised individuals, yet they have reduced immunogenicity, and often requires inclusion of adjuvants or boosters to increase immunogenicity[35]. Inactivated virus vaccines remain among the safest vaccine platforms available, but they require specific processes for inactivation that are often difficult and expensive to reproduce and maintain[25,35]. RNA/DNA vaccines have become increasingly popular due to ease of production[35]. These nucleic -acid based vaccines employ DNA or RNA sequences that encode antigens to stimulate the immune response but do not retain the ability to replicate or revert to virulence[35]. However, RNA vaccines do have some limitations, and often have a low immunogenicity profile, and may require low temperature storage and transportation[35]. Recombinant live-attenuated chimeric vaccines have been developed for dengue and yellow fever virus and are now commercially available. However, there is currently no approved vaccine or treatment available for ZIKV. [1,2,10,24,25].

ZIKV epidemiology.

ZIKV is an important member of the flavivirus genus. ZIKV was originally isolated in 1947 in Uganda, and has emerged within the last decade to cause significant outbreaks worldwide[8,11-13,23,32,37]. In 2007, there was the first report of a symptomatic outbreak of ZIKV virus on Yap

Island, Micronesia which was further confirmed by reverse transcriptase polymerase chain reaction (RT-PCR)[8,11,17] . Approximately 73% of Yap residents 3 years of age and older were infected with Zika virus, with 80% being subclinical[8,11]. In 2013-2014, an epidemic caused by ZIKV occurred in French Polynesia[8,12,17,23,37]. There were 19,000 cases of a dengue-like syndrome by December of that year followed by confirmed ZIKV circulation in New Caledonia, Cook Islands, and Easter Island (Chile) in 2014[8,12,23]. In 2016, ZIKV spread to Brazil which ultimately led to explosive outbreaks throughout Latin America and the Caribbean[11,17,23,37]. In Brazil, ZIKV infection was found to be associated with Guillain-Barré syndrome in adults and congenital Zika syndrome in newborns[8,11,37]. Guillain-Barré syndrome causes the body's immune system to attack the nerves; symptoms begin as weakness and tingling at the extremities that eventually leads to paralysis[8,11,32]. When Zika infection occurs during pregnancy, congenital Zika syndrome often occurs, characterized by malformations of the head and brain (microcephaly), hearing impairment, and seizures in newborns[8,11,23]. This has been shown to be due to the impact of Zika virus on fetal neural progenitor cell development[8,11,23,37]. In 2016, 15 cases of ZIKV were reported in Texas and Florida leading to 277 locally acquired cases[23]. Due to endemic transmission more than 500,000 cases were reported at the peak of the pandemic in the Americas in 2016[23]. As of 2018, over 3700 cases of congenital birth defects associated with ZIKV have been reported in the Americas, and ~30,000 suspected, probable, and confirmed cases of ZIKV were reported to the WHO worldwide[17,23,32]. Since then, approximately 86 countries worldwide have reported ZIKV infections[8,9,11]. A metanalysis study found the Americas have the highest seroprevalence (34%) among all WHO (World Health Organization) regions[17].

ZIKV transmission.

Unlike most flaviviruses, ZIKV has several modes of transmission[8,32]. Mosquito-borne transmission involves a number of different species[8,12,18,23,38]. For example, *Aedes Stegomyia, Aedes Diceromyia, and Aedes Fredwardsius* subgenera, including *Aedes africanus, Aedes luteocephalus, Aedes furcifer,* and *Aedes vittatus* are enzootic vectors of ZIKV in Africa with nonhuman primates (NHPs) playing a critical role in transmission as amplification hosts[12,32]. However, *Aedes aegypti* are widely distributed in the tropics and has been linked to urban Zika outbreaks[12,17,23]. Specifically, *Aedes aegypti* is presumed to have high vectoral capacity because they take several blood meals in a day[17]. Possible sexual transmission of ZIKV has also been documented in the United States and French Polynesia[12,23,32]. Additionally, isolation of cultured virus was found in the semen of patients exhibiting hematospermia[12]. While it remains unclear which components of the semen carry ZIKV, ZIKV RNA has been visualized via immunofluorescent staining and detected via *in situ* hybridization[12]. Vertical transmission has been documented in ZIKV patients especially during the Brazilian epidemic[8,23,32]. ZIKV is also transmitted from mother to fetus leading to a congenital infection[8,11,32]. ZIKV then replicates in macrophages and fetal endothelial cells, leading to microcephaly and other impairments[8,9,11,12,23] . Blood-borne transmission has also been reported via donated blood[12,32].

Insect-Specific Flaviviruses (ISFVs).

Insect-specific viruses (ISVs) are understudied members of their genus because their pathogenicity is not directly related to human or veterinary health[14,18,19,26,38-41]. Since their discovery in 1975, ISFVs have been discovered throughout the world and classified within two lineages, lineage I (classical) or lineage II (dual-host affiliated) ISFVs[14,27,38,42]. ISFVs are only able to infect mosquito cells and do not replicate in vertebrate cells[14,18,19,26,38,39,41]. ISFVs have

however, given their close phylogenic relationship to medically relevant flaviviruses, serve as viral vectors for developing chimeric flavivirus vaccines[39,40,42]. These chimeric vaccines are typically made up of genetic components of more than one virus, in many cases an insect-specific virus and a related pathogenic virus have been used to produce chimeric vaccines[1,2,4,20,24,41,42]. Chimeric vaccines are safe because they are host restricted yet have the ability to elicit strong immune responses due to their phylogenetic relationship to pathogenic viruses[1,2,4,20,24,41,42].

Chimeric Flavivirus vaccines.

Chimeric flaviviruses have been explored as a vaccine platform for providing protection against pathogenic flaviviruses because they contain structural proteins of Vertebrate Infectious Flaviviruses (VIFs) and the nonstructural proteins of ISFVs[4,34] (Figure 2). For example, BinJ/VIF-prME chimeric vaccine was found to be highly effective in low doses against two strains of WNV (NSW_{2011} strain and WNV_{NY99} strain)[4]. The immunogenicity of ISV-based chimeric vaccines has been explored by many researchers[1,2,4,20,42]. One possible justification is the fact that chimeric flaviviruses are able to enter vertebrate cells via surface receptors and trigger RIG-I and mediates the production of inflammatory cytokines which in turn enhances the humoral response[24,42]. Yet, replication does not occur because ISFVs are host restricted making these chimeras a safe and highly immunogenic option as vaccine antigens for pathogenic flaviviruses[24,42]. Thus, these chimeras do not replicate in vertebrate cells, however, they replicate to high titers in mosquito cells, and remain antigenically undifferentiated from pathogenic flaviviruses[10,24,40,42].

Aripo virus

Aripo virus (ARPV) is an ISFV isolated from a pool of *Psorophora albipes* mosquitoes collected in Trinidad in 2008 and is vertically transmitted in *Aedes aegypti* mosquitoes. ARPV has showed no replication in vertebrate cells or pathogenesis in mouse models[24,39]. However, ARPV has demonstrated the unique ability to enter vertebrate cells via clathrin-mediated endocytosis much like pathogenic flaviviruses[24,39]. Also, ARPV infection of macrophages induced significant levels of cytokines (i.e., TNF- α, IL-6, IL-12, IFN-γ, IL-10, and IL-17) commonly associated with pathogenic flavivirus induced disease, suggesting it is immunomodulatory in vertebrate cells[24,39]. Using prior knowledge of chimeric vaccines, my group recently developed the Aripo-Zika vaccine platform as a vaccine candidate for Zika virus. This chimeric vaccine candidate contains the surface proteins of Zika virus (prM-E proteins) substituted into the genome of ARPV (Figure 2)[24]. This resulting vaccine candidate resembles a pseudo-inactivated vaccine because it is fully replication competent in mosquito cells but is replication deficient in vertebrate cells[24]. Due to the intrinsic inability of ARPV to replicate in vertebrate cells, Aripo-Zika (AZ) presents comparable safety to an inactivated virus vaccine and can be administered at high doses safely[24]. The surface proteins of AZ retain the authentic native conformation of ZIKV prM and E proteins and are not affected by the inactivation processes used for inactivated virus vaccines[24]. Additionally, the insect-specific genomic constitution of AZ allows it to be reproduced at low contamination level in less expensive facilities unlike some inactivated and activated virus vaccines[24]. While much is known about AZ's immunogenicity[24], little is known about the dosage regimen, booster efficacy, and the impact of maternally transferred

antibodies on offspring immunity. **Our central goal is to determine the safety and efficacy of AZ**, and this overall goal will be addressed using five hypotheses and presented in two chapters.

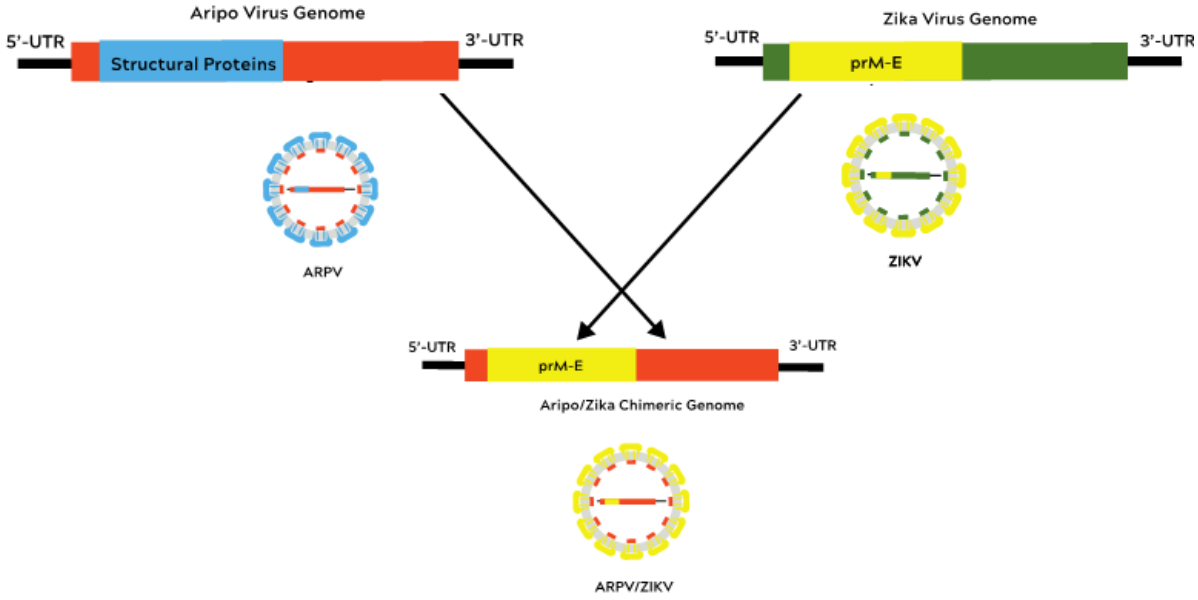

Figure 2. Schematic of the Aripo-Zika genome containing the prM-E proteins of Zika virus and the structural proteins of an insect-specific flavivirus (Aripo virus.

Chapter 2: Evaluate the optimal dose, booster regimen and maternal passively transferred immunity on vaccine-elicited immunogenicity and efficacy.

2.1: Evaluate Transmission Rates of Maternal Antibodies and Protective Efficacy Conferred to Offspring.

Hypothesis: Passive transfer of maternally derived antibodies will protect offspring from a lethal challenge of ZIKV. We used an immune-competent murine model (C57BL/6J) to evaluate transmission rates and efficacies of maternally-derived AZ antibodies to offspring. Briefly, dams were vaccinated with saline (PBS), ARPV, attenuated ZIKV, and AZ at six weeks old, mated at 8

weeks old, and litters challenged at 4 weeks old. ZIKV-specific neutralizing antibodies (nAbs) were measured by plaque reduction neutralization tests and protective efficacy was evaluated based on several outcomes, including survival, weight loss, viremia, and disease progression in mice.

2.2: Determine the Optimal Dosage Regimen to Achieve Sterilizing Immunity. *Hypothesis: Increasing the quantity of AZ administered in a single dose will increase AZ immunogenicity and lead to sterilizing immunity.* We inoculated immune-competent mice (C57BL/6J) with 10-fold dilutions of the vaccine ranging from 10^8 to 10^{12}genome copies (GC) to determine the optimal dose for achieving protective and/or sterilizing immunity. Efficacy was evaluated based on survival, weight loss, viremia, and disease progression in mice.

2.3: Determine if Boosters are Required to Achieve Sterilizing Immunity. Hypothesis: Boosters will increase immunogenicity of AZ. Mice (C57BL/6J) were divided into group 1 (AZ prime only), group 2 (AZ boosted at 2 weeks post prime), and group 3 (AZ boosted at weeks 2 and 4 post prime). ZIKV-specific neutralizing antibodies (nAbs) were measured by plaque reduction neutralization tests in Vero-76 cells. Efficacy was determined based on survival, weight loss, viremia, and disease progression in mice, and compared to control groups receiving ARPV, ZIKV and PBS immunizations.

Chapter 3: Investigate Vaccine Safety using Co-Infection and Anti-Vector Immunity studies with ARPV and AZ

3.1: Explore AZ Host Restriction during Co-Infection with VIFs. *Hypothesis: AZ will retain its natural host restriction and remain incapable of replication during co-infection with ZIKV in*

vertebrate cells. A co-infection study was conducted in Vero-76 cells to determine the effects of the presence of a vertebrate replication-competent flavivirus on the replication kinetics of ARPV and AZ. Growth curves were used to evaluate each virus' replication kinetics over time in both intra- and extra-cellular culture fractions.

3.2: Evaluate anti-vector immunity to the ARPV backbone and its impact on the immunogenicity and efficacy of subsequent ARPV-based vaccines. *Hypothesis: Prior immunization with AZ will have no effect on the immunogenicity and efficacy of a subsequent AWN immunization.* We explored anti-vector immunity by vaccinating mice with AZ, followed by immunization with AWN to determine the effects of vaccinating mice with vaccines derived from the same vector backbone. $PRNT_{50}$s were used to assess differences in immunogenicity.

Chapter 2: Evaluate the optimal dose, booster regimen and maternal passively transferred immunity on vaccine-elicited immunogenicity and efficacy.

Introduction

Vaccination and vector control are often the best defense strategies against the spread of vector-borne diseases[10,30,31,33,35,43]. Within vaccination programs, there are direct and indirect protections[44]. Direct protection is defined as lowering the probability of infection of vaccinated individuals as well as reducing the infectiousness of the pathogen[44]. Indirect protection may be described as diminishing transmission rates among the affected population containing both vaccinated and unvaccinated persons[44]. Direct protection often relates to protection for the vaccinated individual only, unless the individual is female, in which case passive transfer of antibodies can occur[44]. In most mammals, maternal transfer of antibodies during the gestational period provides passive immunity to offspring[44-48]. In humans, maternal antibody transfer can occur from 13 weeks in gestation via the neonatal Fc receptor (FcRn)[44,49]. During gestation, the level of IgG in fetal circulation is initially 5-10% of maternal levels and can exceed maternal levels by birth[44,49]. As the immune system of the newborn becomes more developed, the maternally derived protection wanes over time in a relatively linear fashion[44,49]. Herein, we used a murine model to explore this passive transfer transmission system. Our previous studies show that a single dose of AZ completely protected pregnant dams and prevented *in utero* transmission of ZIKV to neonates[24]. Specifically, AZ vaccinated dams produced neonates that showed no significant decrease in weight and the complete absence of ZIKV in neonatal brain tissue[24]. There was also an absence of infectious ZIKV in brain, spleen, and placenta tissues of dams[24]. However, antibody levels in pups were not measured. Our primary goals are to

determine if maternal antibody transfer is occurring among our vaccinated dams (i.e., AZ), compare fetal and maternal antibody levels, and evaluate the degree of protection afforded to offspring. We hypothesize that AZ vaccinated dams will transfer maternal antibodies to their pups, and pups will be completely protected from a lethal challenge of ZIKV. Next, we conducted a dose dependent study.

A "dose" may be defined as the amount of vaccine or antigen administered to an individual during immunization[50]. We recently showed that a single dose of AZ confers complete protection from all aspects of ZIKV-induced disease among vaccinated mice[24]. Specifically, a single dose of AZ provided complete protection from viremia, weight loss, and mortality in immune-competent (C57BL/6J) and immune-compromised murine models (IFN $\alpha\beta^{-/-}$)[24]. However, this protection was observed at the maximum dose possible and dose de-escalation studies have not been performed to date[24]. Our second goal is to explore vaccine efficacy at various doses and ultimately identify the minimal and optimal dose for achieving complete protection from ZIKV-induced disease.

Although we have shown complete protection from ZIKV-induced disease after a single immunization, ZIKV-specific neutralizing antibody (nAb) titers increased post-challenge, suggesting the absence of sterilizing immunity[24]. Here, we also aim to assess the effects of boosters on immunogenicity and the likelihood of achieving sterilizing immunity with this platform. A booster can be described as an additional immunization given to an individual after prime vaccination and is often employed to increase vaccine-elicited immunity, ultimately increasing efficacy[50]. Different vaccination strategies have unique advantages and disadvantages and often vary in their requirement for boosters[10,35,50]. Live-attenuated vaccines

have been known to confer rapid and robust long-lasting immunity with a single dose, while inactivated vaccines provide short-term immunity that may need to be re-established via the administration of boosters[35,50]. In some cases, suboptimal vaccines can lead to increased disease due to antibody dependent enhancement (ADE) of viral infections, and boosters are needed to surpass this threshold for ADE[51,52]. ADE is a phenomenon in which the binding of antibodies to a virion is suboptimal and neutralization does not occur[51,52]. The virions then create virus-immune complexes via the Fcγ receptors of target immune cells and replication occurs[51,52]. Given that our vaccine effectively represents a pseudo-inactivated vaccine, it is important that we determine if boosters will significantly increase immunogenicity, with the overall goal of achieving sterilizing immunity.

Materials & Methods:

a. Cell Lines and Viruses

Mosquito (C6/36) and African green monkey kidney cells (Vero-76) were purchased from ATCC (Manassas, VA, USA) and maintained according to ATCC guidelines. Zika virus strains PRVABC59 and DakAr D 41524 were obtained from Dr. Nisha Duggal (Virginia Tech, Blacksburg, VA, USA). West Nile virus strain NY-99 was obtained from the University of Texas Medical Branch at Galveston Texas. ARPV was originally isolated from *Psorophora albipes* mosquitoes collected from the Aripo savannahs on the Caribbean Island of Trinidad. The nucleic acid sequence for the novel ARPV was deposited in GenBank (accession number: MZ358890). The AZ chimera was constructed as described above. AWN was constructed similarly using the prM and E genes of WNV strain NY-99 from an infectious clone.

b. Animal Experiments

b1.1: Maternal Antibody Transfer

Six-week-old female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were inoculated subcutaneously (s.c.) with 2.7 X 10^8 GC ARPV (n=3), 1.7 X 10^{12} GC AZ (n=4), 3.3 X10^8 GC ZIKV PRVABC59 (n=5), or PBS (n=3; Table 1). Mice were bled retro orbitally via venous sinus post immunization (DPI) and serum was stored for ZIKV plaque reduction neutralization tests (PRNTs). At 30 DPI, dams were mated with twelve-week-old C57BL/6J males (Jackson Laboratory, Bar Harbor, ME, USA). Males were housed 24 hours in clean cages before mating. Males were euthanized after three weeks of co-housing with females.

Table 1. Mouse study group designations and their corresponding immunizations and challenge agents employed.

Group	Vaccine	Challenge
PBS	PBS	PBS
ARPV[1]	ARPV[1]	ZIKV Dakar
AZ[2]	AZ[2]	ZIKV Dakar
ZIKV PRVABC59	ZIKV PRVABC59	ZIKV Dakar
SHAM	PBS	ZIKV Dakar

Dams were monitored for signs of pregnancy and birth for four weeks. A small number of pups were sacrificed at two and three weeks old to collect sera for PRNTs when available. Pups were weaned at 21 days old, and dams were euthanized within 1 week of weaning. Pups were also bled at 4 weeks old immediately preceding ZIKV challenge. One day prior to challenge, 2.75 mg of MAR1-5A3 anti-mouse IFNAR-1 (Lot #: 0521L245; Leinco Technologies; St. Louis, MO, USA) was administered intraperitoneally (i.p.) to transiently inhibit Type I IFN (IFN-α/β) prior to ZIKV or PBS challenge (Table 1). Mice were challenged 30 DPI with a lethal dose of 1.3 X 10^5 PFU ZIKV DakAr D41524 administered subcutaneously. Following infection, mice were administered two additional 1.10 mg doses of MAR1-5A3 at 1- and 4-days post challenge (DPC). Bleeds were performed daily from alternating mice on days 1- 4 post challenge to quantify viremia. Weight change, disease progression, and survival were measured for 14 DPC (Figure 1). Birth dates were spread across two weeks, mice were challenged independently at 4 weeks ± 4 days of age.

[1] Aripo virus
[2] Aripo-Zika virus

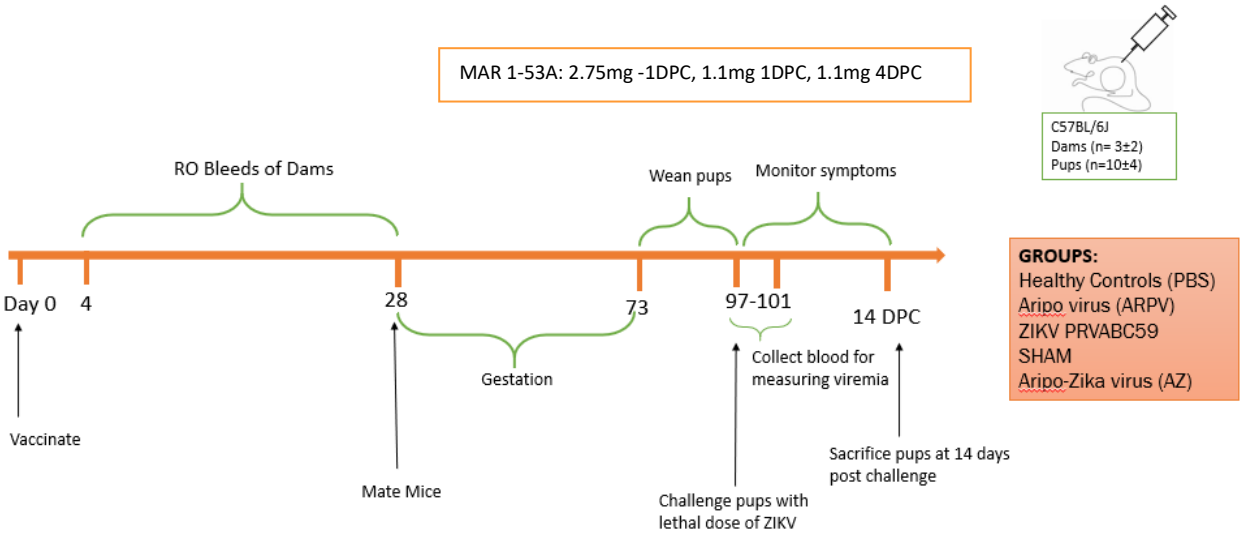

Figure 3. Schematic depicting maternal antibody study. Immune competent mice were subcutaneously inoculated at D0 with 2.7 X 10^8 GC (Genome Copies) ARPV, 1.7 X 10^{12} GC AZ, 3.3 X 10^8 GC ZIKV PRVABC59, or PBS as indicated by the groups. MAR1-5A3 was administered intraperitoneally to transiently inhibit Type I IFN (IFN-α/β) prior to ZIKV challenge of 1.3 X 10^5 PFU ZIKV DakAr D41524 subcutaneously.

b1.2: Dose Dependent Study

Four-week-old C57BL/6J mice were divided into nine groups (n = 6/group; Jackson Laboratory, Bar Harbor, ME, USA) and inoculated subcutaneously (s.c.) with 1.3 X 10^7 GC ARPV, 3.1 X 10^{12} GC–1.5 X 10^8 GC AZ, 3.3 X10^8 GC ZIKV PRVABC59, or PBS (Table 1). Mice were bled weekly post immunization and serum were stored for ZIKV plaque reduction neutralization tests ($PRNT_{50}$).

Mice were challenged 30 DPI with a lethal dose of 2.2 X10^5 PFU ZIKV DakAr D41524 or PBS administered subcutaneously. One day prior to inoculation, 2.75 mg of MAR1-5A3 (Lot #: 0521L245; Leinco Technologies; St. Louis, MO, USA) was administered intraperitoneally to

transiently inhibit Type I IFN (IFN-α/β) prior to ZIKV challenge. Additional MAR1-5A3 injections and bleeds were performed as described above. Weight changes, disease progression, and survival were assessed for 14 DPC.

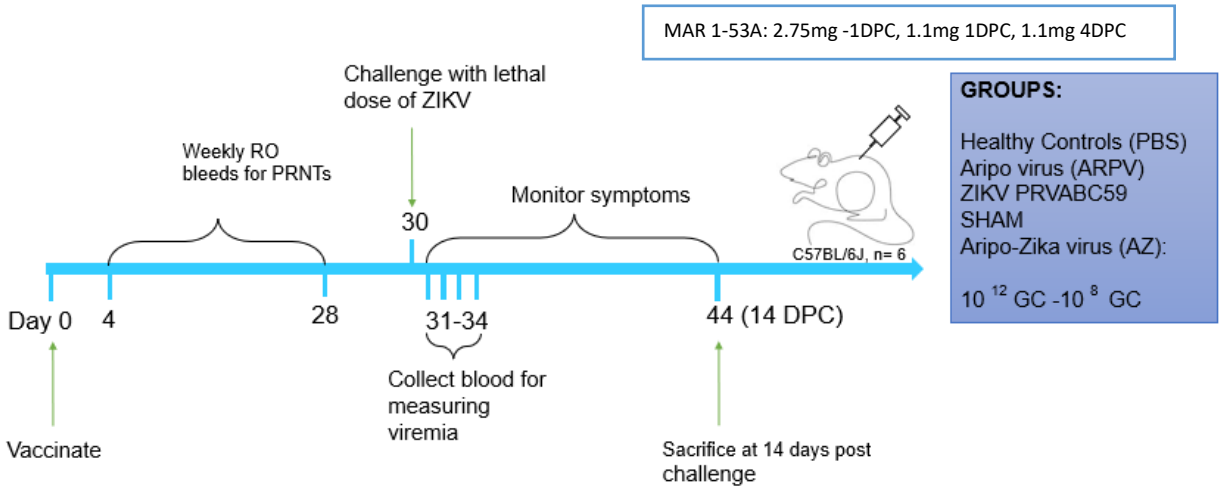

Figure 4. Schematic depicting dose dependent study. Immune competent mice were subcutaneously inoculated at D0 with 1.3 X 10^7 GC (Genome Copies) ARPV, 3.1 X 10^{12} GC–1.5 X 10^8 GC AZ, 3.3 X 10^8 GC ZIKV PRVABC59, or PBS. MAR1-5A3 was administered intraperitoneally to transiently inhibit Type I IFN (IFN-α/β) prior to ZIKV challenge of 2.2 X10^5 PFU ZIKV DakAr D41524 subcutaneously.

b1.3: Booster Study

Four-week-old C57BL/6J mice divided into seven groups (n = 6/group; Jackson Laboratory, Bar Harbor, ME, USA) were inoculated subcutaneously (s.c.). The control groups were inoculated with 2.2 X 10^9 GC ARPV, 3.3 X10^8 GC ZIKV PRVABC59, or PBS (Table 1). AZ groups are: Prime immunization only (NB), four-week booster (1B), two- and four-week booster (2B). Inoculations are as follows: 5.6 X 10^{11} GC AZ (initial/prime), 3.6 X 10^{11} GC AZ (2 week & 4-week boosters). Mice were bled weekly for ZIKV $PRNT_{50}$. ZIKV PRVABC59 and ARPV were boosted at four weeks. One day prior to challenge, 2.75 mg of MAR1-5A3 at 11mg/mL (Lot #: 0521L245; Leinco Technologies; St. Louis, MO, USA) was administered intraperitoneally to transiently inhibit Type I IFN (IFN-α/β) prior to ZIKV challenge. Mice were then subcutaneously inoculated with 2.1 X 10^5 PFU of Zika DakAr 41524 or PBS. Following challenge, mice were given two additional 1.10 mg doses of MAR1-5A3 on 1 and 4 DPC. Alternating retro-orbital bleeds were performed daily for 4 DPC to assess viremia. Weight change, disease progression, and survival were assessed for 14 DPC as described above.

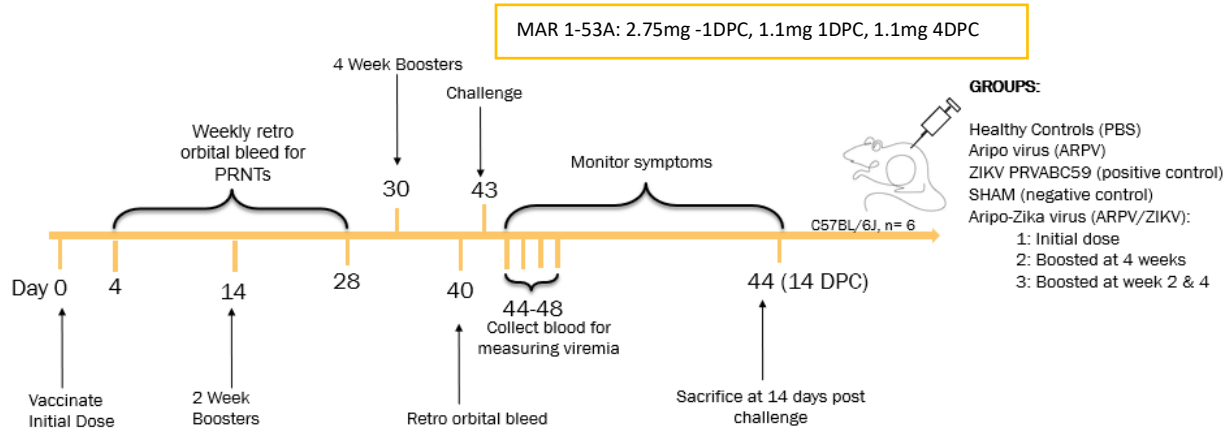

Figure 5. Schematic depicting Booster study. Immune competent mice were subcutaneously inoculated at D0 with 2.2 X 10^9 GC (Genome Copies) ARPV, 3.3 X10^8 GC ZIKV PRVABC59, 5.6 X 10^{11} GC AZ (initial/prime), 3.6 X 10^{11} GC AZ (2 week & 4-week boosters), or PBS. MAR1-5A3 was administered intraperitoneally to transiently inhibit Type I IFN (IFN-α/β) prior to ZIKV challenge of 2.1 X 10^5 PFU of Zika DakAr 41524 subcutaneously.

c. RNA Extraction and Reverse Transcription Quantitative PCR (RT-qPCR)

RNA extractions were performed using QIAmp Viral RNA Mini kits (QIAGEN) according to the manufacturer's instructions. RT-qPCR was performed using iTaq^{TM} Universal Probes One-Step kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's guidelines, using primers and probes listed in Table 2.

Table 2. Primers and Probes used for RT-qPCR of ZIKV, AWN, ARPV, and AZ throughout studies.

Virus	Primers	Probes
ARPV, AZ & AWN	ARPV-7562F (5'-CGGTGTTCATTGAGGATGAC-3'), ARPV-7714R (5'-TGATACGTCCAGGTTCGGTA-3')	TR9096-P2-7680F (5'-6FAMCGCTGCCTCATGGCAATTCG-BHQ1-3')
ZIKV	ZIKV-1086F (5'-CCGCTGCCCAACACAAG-3'), ZIKV-1162cR (5'-CCACTAACGTTCTTTTGCAGACAT-3')	ZIKV-1107-FAM (5'-6FAM-AGCCTACCTTGACAAGCAGTCAGACACTCAA-BHQ1-3')

d. Plaque Assays

Samples were diluted in serial 1:10 dilutions. Samples were mixed thoroughly by repeated pipetting. 100 µL of diluted virus was plated on 12-well plates containing 70% confluent Vero-76 cells. Cells were incubated for one hour at 37 °C and 5% CO_2 and plates were rocked every 10 minutes to evenly distribute inoculum. After infection, cells were overlayed with 0.4% agarose (SeaKem ® LE Agarose) diluted in DMEM half media containing 2% FBS (fetal bovine serum) and 1% penicillin G and streptomycin. Plates were incubated at 37 °C and 5% CO_2 for 4 days, and then fixed with 10% formaldehyde for at least one hour. Plates were then stained with 1% crystal violet for 5 minutes, washed with distilled water, and plaques were counted.

e. Plaque Reduction Neutralizing Assays

135 µL of diluent was placed into column A 1-12 of a U-bottom 96-well plate, and 75 µL was placed into B-H (1-12). Sera for all groups was heat inactivated at 56°C for one hour. Plate was labeled and 15µL of undiluted antibody containing sera sample was added to corresponding well of row A. Sample was mixed thoroughly and 75 µL was transferred down 96-well until desired dilutions were met. 75 µL of desired virus (e.g., ZIKV) PRNT stock was added to each well containing sample dilutions as well as 12 viruses only wells containing only diluent. Once stock virus was added to all wells, the 96-well plate was incubated for one hour. Twelve well plates were labeled and 100 µL of diluted virus and sera was added to the corresponding wells on a 12-well plate containing confluent Vero-76 cells. 12 well plates were then incubated for one hour at 37 °C and 5% CO_2 rocking every 10 minutes to evenly distribute inoculum. Cells were overlayed with 0.4% agarose (SeaKem ® LE Agarose) and DMEM half media containing 2% FBS (fetal bovine serum) and 1% penicillin G and streptomycin. Plates were incubated at 37 °C and 5% CO_2 for 4 days. Plates were fixed in 10% Formaldehyde for at least one hour then stained with 1% crystal violet for 5 minutes and washed with DI water before counting plaques.

f. Statistical analysis

Data normality was assessed using a combination of Q-Q plot, and box-plot analyses. Data were normalized by log10 transformation when necessary. One-way ANOVAs, two-way ANOVAS, and mixed-effects analyses were performed to establish significance. T-tests were used to evaluate statistical significance among groups. Kaplan–Meier survival plot of pups post challenge, with significance analyzed by log-rank (Mantel–Cox) test. All statistical analyses performed on Prism 9.3 (GraphPad).

Results

2.1: Passive Transfer of AZ antibodies protects adolescent mice from ZIKV disease

There was no significant difference in weight change, mortality, and viremia between AZ and PBS (healthy controls) pups throughout all 14 DPC (multiple unpaired t tests). In stark contrast, there was a significant decrease in ZIKV PRVABC59 pup weights between 3 and 14 DPC (multiple unpaired t tests; $p \leq 0.033$). Our results also show a significant decrease in the weight of challenged ARPV pups between 6 and 14 DPC (≤ 0.033) and SHAM mice at 3 to 14 DPC (multiple unpaired t tests; Figure 6A; $p \leq 0.033$). There was significant mortality observed among the ZIKV PRVABC59, SHAM, and ARPV groups (log-rank (Mantel–Cox) tests), relative to the AZ and PBS healthy control group. ARPV experienced 60% mortality by 9 DPC and 80% overall mortality by 14 DPC (Figure 6B). Similarly, ZIKV PRVABC59 mice experienced 100% mortality by 10 DPC (Figure 6B), and SHAM mice experienced 80% mortality by 13 DPC. ZIKV PRVABC59 mice showed a significant increase in viremia when compared to PBS at 2 to 4 DPC (Multiple unpaired t tests; $p \leq 0.033$). SHAM and ARPV showed a significant increase in viremia between 1 and 4 DPC (Figure 6C; $p \leq 0.0002$). These efficacy results are supported by the $PRNT_{50}$ data which show AZ pups have the highest nAb titers peaking as high as 3.23±0.27 by three weeks after birth, and 2.77±0.18 at four weeks after birth. AZ-immunized dams produced nAb titers of 3.76±0.49 at 28DPI, while ZIKV PRVABC59 dams were significantly lower ($p \leq 0.033$ (*) at 2.91±0.17 at 28DPI. Three-week old AZ pups produced nAb titers at 2.77±0.18 while ZIKV PRVABC59 produced significantly lower nAb at 1.35±0.12. At four weeks nAb titers waned in AZ pups (2.77±0.18) and ZIKV PRVABC59 pups (1.48±0.21). In summary, pups derived from AZ-

immunized mice produced high nAb titers and presented no weight loss, mortality, or viremia when challenged at four weeks ±4 days of age.

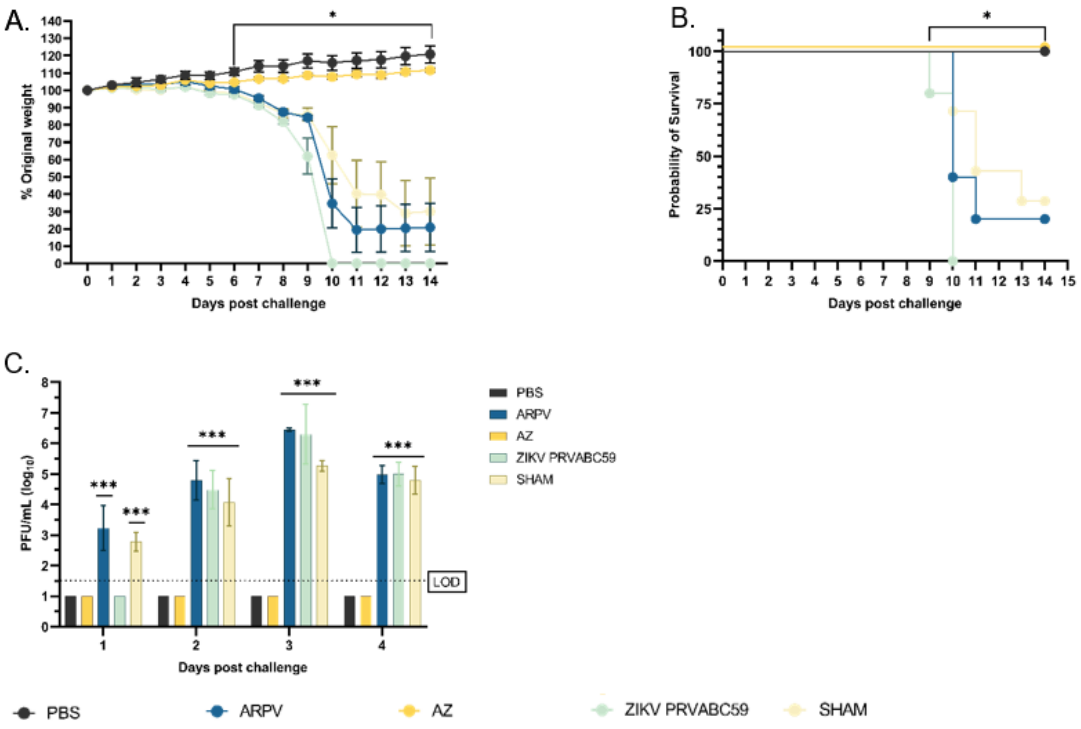

Figure 6. Passive transfer of maternal antibodies provide protective immunity in 4-week old AZ pups (n=6±4/group). Dams were immunized with the indicated agents as described in 2.1, or with saline as a negative control. Four-week-old pups were challenged with a lethal dose of 1.3 X 10^5 plaque forming units (PFU) ZIKV DakAr D41524 administered subcutaneously. (A) Changes in weight were measured daily for 14 days post-challenge. (B) Kaplan–Meier survival plot of pups post challenge, with significance analyzed by a log-rank (Mantel–Cox) test. (C) Viremia was measured on days 1-4 post-challenge. Data points represent mean values and error bars represent standard deviation. Significance was determined by two-way ANOVA and multiple t-tests. Unless otherwise marked, asterisks indicate significance compared to saline-inoculated negative controls (PBS): $p \leq 0.033$ (*), $p \leq 0.0002$ (***). Unless otherwise marked, there was no significant difference between PBS and AZ groups.

Table 3. $PRNT_{50}$ titers of dams and pups estimated during the maternal antibody transfer studies. Mean values signify log10 of the reciprocal of the highest serum dilution able to reduce WNV plaque formation by 50%. Two-way ANOVA: ($p < 0.0002$; Tukey Test: $p \leq 0.033$ (*). *Indicates significance when compared to each other within the same column as indicated by the corresponding colors.

	Dams D28 $PRNT_{50}$	**3-week pup $PRNT_{50}$**	**4-week pup $PRNT_{50}$**
PBS	1.30±0	1.30±0	1.30±0
ARPV	1.30±0	1.30±0	1.30±0
AZ	3.76±0.49*	3.23±0.27*	2.77±0.18
ZIKV PRVABC59	2.91±0.17*	1.35±0.12*	1.48±0.21
SHAM	1.30±0	1.30±0	1.30±0

2.2: AZ shows protective immunity at ≥10 ^{11} Genome Copies

Results of our dose de-escalation studies showed that there was a significant increase in ZIKV viremia at 3 and 4 DPC among the ARPV-immunized mice (Figure 7A, $p \leq 0.033$). Similarly, there was a significant increase in viremia at 2 and 3 DPC among the SHAM-immunized mice (Figure 7A, multiple unpaired t tests, $p \leq 0.033$). Results also indicate that there was no significant difference in viremia between PBS, and the AZ 10^{12} and AZ 10^{11} groups. There was a significant increase in viremia among the AZ 10^{9}-10^{8} at 2 DPC (Figure 7B, multiple unpaired t tests, $p \leq 0.033$), and there was a significant increase in viremia among the AZ 10^{10}-10^{8} groups between 3 and 4 DPC, in comparison to the PBS healthy controls (Figure 7B, multiple unpaired t tests, $p \leq 0.033$). There was no significant difference in weight change among PBS, ARPV, AZ 10^{12}- AZ 10^{8}, ZIKV PRVABC59, and SHAM groups throughout the study period (Figure 7C-D). AZ 10^{12} and 10^{11} showed a $PRNT_{50}$ significantly greater than PBS and ZIKV PRVABC59 as early as 7dpv (Table 4). Twenty-eight days post vaccination, AZ 10^{12} has a significantly greater nAb titer (3.41±0.27), when compared to ZIKV PRVABC59 at 3.00±0.16 (Table 4). AZ 10^{12} nAb did not change significantly post challenge while 10^{11}-AZ10^{10} significantly increased in neutralizing titer post

challenge (Table 3). AZ 10^9, AZ 10^8, SHAM, and ARPV groups had an average $PRNT_{50}$ of 3.71±0.50 post challenge (Table 3). Unfortunately, the ZIKV post-challenge nAb were unquantifiable and needs to be rerun to assess nAb titers.

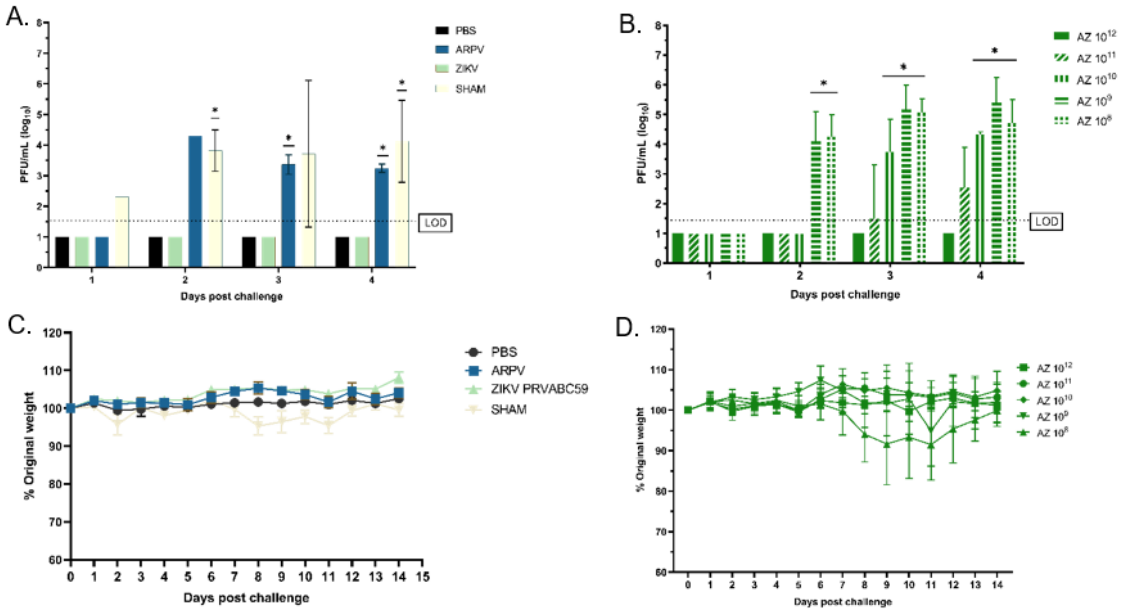

Figure 7. AZ shows protective immunity at ≥10 ^{11} Genome Copies (n=6/group). Mice were inoculated with 100µL of the indicated agents as in 2.2, or with saline. (A-B) Viremia was measured on days 1-4 post-challenge. (C-D) Changes in weight were measured daily for 14 days post-challenge. Data points represent mean values and error bars represent the standard deviation. Significance was determined by two-way ANOVA and multiple t-tests. Unless otherwise marked, asterisks indicate significance compared to saline-inoculated negative controls (PBS): $p \leq 0.033$ (*).

Table 4. $PRNT_{50}$ titers of Dose Dependent study results post vaccination and post challenge. Mean values signify log10 of the reciprocal of the highest serum dilution able to reduce WNV plaque formation by 50%. Two-way ANOVA, p <0.0002. Tukey test, p ≤0.033 (*). *Indicates significance when compared to each other within the same column as indicated by corresponding colors.

	1 week post immunization Mean ± SD	**4 weeks post immunization Mean ± SD**	**2 weeks post challenge Mean ± SD**
PBS	1.30±0	1.30±0	1.30±0
ARPV	1.30±0	1.30±0	3.71±0
ZIKV PRVABC59	1.40±0.16	3.00±0.16*	NT
AZ 10^{12}	2.66±0.16*	3.41±0.27*	3.18±0.15
AZ 10^{11}	1.80±0.36*	1.75±0.46	4.19±0.59
AZ 10^{10}	1.45±0.25	1.38±0.15	3.51±0.24
AZ 10^{9}	1.45±0.37	1.30±0	3.81±0.16
AZ 10^{8}	1.40±0.24	1.30±0	3.71±0
SHAM	1.30±0	1.30±0	3.65±0.13

NT: not tested.

2.3: AZ-immunized mice receiving prime or prime-boosts are completely protected from ZIKV-induced disease

There were no statistically significant differences in weight change among PBS, ARPV, ZIKV PRVABC59, SHAM, and any of the AZ booster groups throughout the study (Figure 8A). However, there was a significant increase in viremia between 2 and 4 DPC in the ARPV- and SHAM-immunized groups (Figure 8B; multiple unpaired t test, p ≤ 0.002). Two weeks after the 2nd booster, AZ NB (no booster) showed nAb titer consistent with ZIKV PRVABC59 (3.26±0.17). While, the AZ 1B (4-week booster) and AZ 2B (2&4-week booster) produced higher nAb titer of 3.36±0.23 and 3.50±0.17 respectively. Post challenge, AZ NB, AZ 1B, and AZ 2B has an increased nAb titer of 3.61±0.16, 3.61±0.16, and 3.50±0.17 respectively. Post-challenge nAb titers were not significantly from pre-challenge nAb values among AZ NB, AZ 1B, and AZ 2B.

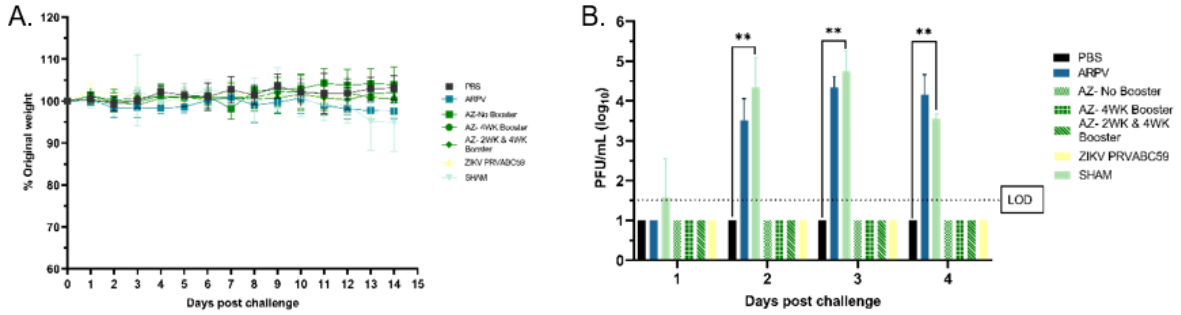

Figure 8. AZ booster mice are protected from weight loss and viremia (n=6/group). Mice were inoculated with 100µL of the indicated agents as in 2.3, or with saline as a negative control. (A) Changes in weight were measured daily for 14 days post-challenge. (B) Viremia was measured on days 1-4 post-challenge. Data points represent mean values and error bars represent the standard deviation. Significance was determined by two-way ANOVA and t-tests. Unless otherwise marked, asterisks indicate significance compared to saline-inoculated negative controls (PBS): $p \leq 0.002$(**).

Table 5. $PRNT_{50}$ titers etimated during the booster study from sera collected prior to ZIKV challenge. Mean values signify the log10 reciprocal of the highest serum dilution able to reduce ZIKV plaque formation by 50%.

Immunization group	6-week post immunization Mean ± SD	2 weeks post challenge Mean ± SD
PBS	1.30±0	NT
ARPV	1.30±0	NT
ZIKV PRVABC59	3.26±0.17	NT
AZ NB	3.25±0.25	3.61±0.17
AZ 1B (4 WEEKS)	3.36±0.23	3.61±0.16
AZ 2B (2&4 WEEKS)	3.50±0.17	3.76±0.61
SHAM	1.30±0	3.56±0.42

Discussion

This chapter employs a variety of *in vivo* studies to further define important characteristics related to AZ immunogenicity and efficacy, relative to ARPV and ZIKV controls. Altogether our results continue to support AZ as an effective and promising vaccine candidate. First, we sought to determine rates of maternal antibody transfer among immunized dams to offspring, compare fetal and maternal antibody levels, and evaluate the degree of protection afforded to offspring. Pups born to dams vaccinated with AZ 28 days before mating remained healthy after ZIKV challenge. Prior to challenge, ~4-week-old pups derived from AZ-immunized dams presented with the highest nAb titers, in excess of the $PRNT_{50}$ threshold of 100 required for protection. Passive transfer of nAbs were documented in 100% of AZ (n=10) and ZIKV (n=10) pups tested, and as expected, the levels of nAb titers estimated in pups correlated well with levels estimated in dams. ZIKV PRVABC59 is able to confer immunogenicity in immune-competent mice, but not at protective levels (Figure 6, Table 3). Interestingly, antibody titers rapidly waned after weaning, ultimately leading to the absence of any protection in the ZIKV-immunized group.

Further studies are needed to determine the longevity of protection afforded by passive transfer of maternal antibodies after AZ immunization. It will also be interesting to dissect the impact of milk-derived antibodies on efficacy. Considering mammals including humans can confer passive transfer of maternal antibodies, it could become beneficial to determine if passive transfer of maternal antibodies is effective in providing immunogenicity in non-human primate (NHP) models.

Next, we sought to further characterize the immunogenicity of ARPV/ZIKV vaccination by evaluating the minimal dose required to offer complete protection from a ZIKV challenge, and also explore the impact of boosters on immunogenicity. A significant advantage of the AZ platform is the high titers achieved at low biosafety containment in cell culture, which can accommodate easy production of large numbers of doses. As such, establishing a minimum effective dose is essential to understanding production capacity and vaccine delivery. Based on our results, there was no significant difference in viremia between PBS, AZ 10^{12}, and AZ 10^{11} groups. However, there was a significant increase in viremia at AZ 10^{10}-10^{8} at 3 and 4 DPC (Figure 7, Table 4). Thus, the minimal dose that facilitates complete protection from ZIKV-induced disease is ≥10^{11} GC. This data suggest that we can produce a total of 40 doses in a single 150cm^2 flask of cells. To avoid the likelihood of ADE, lower doses within the range of 10^{10}-10^{8} GC should not be used.

Finally, our booster study revealed that 1 or 2 AZ boosters did not significantly increase nAb titers when measured 6 weeks post prime. This data is very interesting and suggests boosters have no impact on short-term immunogenicity. Our data also shows that nAb titers in the 2-booster group did not significantly increase after ZIKV challenge, indicating the presence of sterilizing immunity in this group. These data suggest that two AZ boosters generate sufficient immunogenicity to completely prevent ZIKV infection, such that the challenge has no impact on circulating ZIKV-specific immune responses. ZIKV PRVABC59 was also boosted at four weeks post prime (Figure 8, Table 5) and showed comparable nAb titers to the NB and AZ 1-boost group. As expected, the 2-booster AZ group presented higher neutralizing antibody titers when compared to ZIKV PRVABC59 prime-boost group. Prime immunization and boosting with ARPV

presented no change in the presence of ZIKV-specific nAb responses. As observed in my earlier studies, and thus expected here, all AZ and ZIKV groups were completely protected upon ZIKV challenge. Further studies are nonetheless needed to determine the impact of boosters on long-term immunity and protective efficacy.

In summary, optimizing vaccine availability, immunogenicity and efficacy through an improved understanding of vaccine characteristics is critically important for the successful licensure, approval and dissemination to the public. This chapter highlights that a dose of 10^{11} GC will offer complete protection from ZIKV-induced disease, administration of a 3-dose vaccine series will provide short-term sterilizing immunity, and maternal antibodies are effectively transferred to newborns in protective quantities.

Chapter 3: Investigate Vaccine Safety using Co-Infection and Anti-Vector Immunity studies with ARPV and AZ

Introduction

Vaccine safety is among the most critical factors for licensure and is equally or even more important than efficacy[10,50,53]. Vaccines are meant to be immunogenic in patients while conferring little to no side effects, and safety profiles must be characterized in preclinical studies to avoid adverse events during clinical trials in humans[35,43]. Live-attenuated vaccines offer rapid, robust and long-lived immunity but may be less safe than other vaccine platforms, because in rare cases these can revert to their pathogenic form[2,35]. Our "pseudo-inactivated" ISFV vaccine platform is safe because of its inherent host restriction for replication in mosquito cells only[24]. As described above, the AZ chimera contains the surface proteins of ZIKV only, and the replication machinery of ARPV rendering the chimera incapable of replication in vertebrate cells[24]. Our recent studies on the AZ chimera and ARPV backbone show that these viruses are unable to replicate its genome in vertebrate cells, and also show no evidence of translation in a variety of vertebrate cell types[24,39]. We also showed that AZ was exceptionally safe *in vivo*, and no hematological effects, signs of illness or weight loss were observed post vaccination[24]. Additionally, we explored intracranial inoculation of AZ to suckling mice which is among the most permissive environments for arbovirus replication and pathogenesis[24]. Our studies showed no histopathological effects of AZ in suckling mice brain sections and no virus could be detected in these samples[24]. In order to completely explore this safety profile, we performed co-infection studies to determine what effects the presence of other vertebrate replication-

competent machinery derived from vertebrate-pathogenic viruses will have on our chimeric vaccine using an *in vitro* model.

Co-infection occurs when there is simultaneous infection of cells or organisms by two or more viruses[54,55]. Co-infection can sometimes result in genetic exchange between viruses (i.e., recombination or reassortment)[55]. Given that AZ contains genomic material from ARPV and ZIKV, it is important to determine if co-infection of AZ with ZIKV can lead to replication of AZ in vertebrate cells, which can subsequently alter the overall pathogenesis of ZIKV or another co-infecting flavivirus pathogen. In order to elucidate this, we co-infected cells with ZIKV and ARPV, ZIKV and AZ, and performed growth curves to determine the impact of co-infection on each virus' growth kinetics as compared to controls: AZ, ARPV, and ZIKV. Next, we wanted to explore anti-vector immunity.

Anti-vector immunity is a concern for many viral-vectored vaccine platforms, especially those that may be used to deliver antigens for several pathogens[56,57]. Among viral vectored vaccines, immunity is often induced against the viral vector as well as the antigen of interest when conferring vaccine-induced immunity[56-58]. As previously stated, while ARPV does not replicate in vertebrate cells, it is closely related phylogenetically to pathogenic flaviviruses[24,39]. Previous studies have shown that ARPV is able to enter vertebrate cells and elicit an immune response suggesting immunity is induced to the backbone[24,39]. Several T-cell epitopes have also been recognized among the non-structural proteins of VIFs, suggesting that these may be targeted in our vaccine's backbone[24]. Here, we will explore the potential for and possible impact of anti-vector immunity to determine if immunization with two vaccines (i.e., Aripo-Zika and Aripo-

West Nile) containing an ARPV backbone impacts immunogenicity of the subsequent vaccine or boosts responses to the prime immunization.

Materials & Methods:

a. Intracellular and Extracellular Viral Replication Kinetics

3.1: Co-infection study

Vero-76 cells were infected as described in Table 3 in triplicate in six-well plates, and time points taken over 120 hours (Timepoints: 0HR, 3HR, 6HR, 12HR, 24HR, 36HR, 48HR, 72HR, 96HR, 120HR). Samples were harvested, RNA isolated and viral replication was investigated within the intracellular and extracellular fractions as previous described. Virus groups were inoculated as follows: (Table 6).

Table 6. Co-infection study groups

Groups	Virus Infected	MOI
ARPV only	ARPV	5.7 X 10^6 GC
AZ only	AZ	5.4 X 10^6 GC
ZIKV only	ZIKV Dakar	2.4 X 10^6 GC
ZIKV-ARPV	ZIKV Dakar & ARPV	ZIKV: 4.0 X 10^6 GC ARPV: 1.6 X 10^5 GC
ZIKV-AZ	ZIKV Dakar & AZ	ZIKV: 8.7 X 10^5 GC AZ: 3.2 X 10^4 GC

RNA extractions were performed using QIAmp Viral RNA Mini kits (QIAGEN) according to the manufacturer's instructions. RT-qPCR was performed using iTaqTM Universal Probes One-Step kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's guidelines. Co-infected groups were analyzed by RT-qPCR run in triplicates twice (once with each set of primers and probe). Probes and primers are listed in Table 7.

b. Anti-Vector immunity Animal Experiments

Four-week-old C57BL/6J mice divided into seven groups (n = 6/group; Jackson Laboratory, Bar Harbor, ME, USA) and inoculated subcutaneously (s.c.) with 2.7 X 10^8 GC ARPV, 1.7 X 10^{12} GC AZ, 3.3 X10^8 GC ZIKV PRVABC59, or PBS (Table 7, Figure 9). Mice were bled post immunization and serum were stored for PRNTs. ARPV/AWN, AZ/AWN, ZIKV/AWN, AWN only groups were immunized with 0.64-1.7 X 10^5 GC of AWN at 39 DPI.

Table 7. Anti-vector immunity study groups

Group	1st Vaccine	2nd Vaccine
PBS	PBS	PBS
ARPV[1]	ARPV[1]	-
ARPV/AWN[2]	ARPV[2]	AWN[3]
Aripo-Zika virus (AZ)	AZ	-
AZ/AWN	AZ	AWN
ZIKV	ZIKV PRVABC59	-
ZIKV/AWN	ZIKV PRVABC59	AWN
AWN	-	AWN

[3] Aripo-West Nile virus

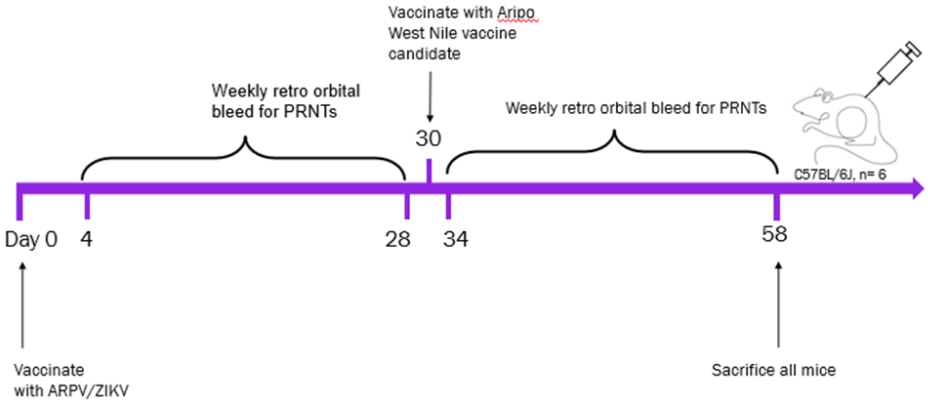

Figure 9. Schematic of anti-vector immunity study

Results

3.1: ARPV & AZ remain incapable of replication in vertebrate (Vero-76) cells when coinfected with ZIKV

As described above, we determined the growth kinetics of co-infecting viruses in both the intracellular and extracellular fractions of infected Vero-76 cells. Results from our intracellular data showed a significant difference in GC/mL between ZIKV and ARPV groups at 6-120 hours post infection (Figure 10: A; multiple unpaired t tests; $p \leq 0.03$). There was also a significant difference in GC/mL between ZIKV and AZ at 6-120 hours post infection (Figure 9: B; multiple unpaired t tests; $p \leq 0.03$). ARPV and AZ did not show replication in virus only and coinfected groups (Figure 10: C-D; multiple unpaired t tests; $p \leq 0.03$, Table 8). Extracellular factions showed similar growth kinetics (Figure 11: A-D).

3.2: Anti-Vector immunity studies show AZ immunization boosts nAb titers of AWN vaccination

Results of our anti-vector immunity studies showed that prior immunization with ARPV, PBS, or ZIKV did not significantly affect the immunogenicity of the subsequent AWN immunization. Mice were immunized with a low dose of AWN to help dissect differences in nAb responses. nAb titers against WNV were estimated at 1.3 Log_{10} $PRNT_{50}$ for the AWN only group, and this was similarly estimated for all of the groups studied, with the exception of AZ/AWN. Interestingly, the AZ/AWN group showed significantly increased WNV-specific nAb titers 28 days post AWN immunization in comparison to the AWN only group (Table 9). Additionally, although not statistically significant, ZIKV-specific nAbs were elevated in the AZ/AWN group when compared to AZ group, suggesting there is the likelihood of a boost in immunogenicity of the primary immunization and secondary (Table 9).

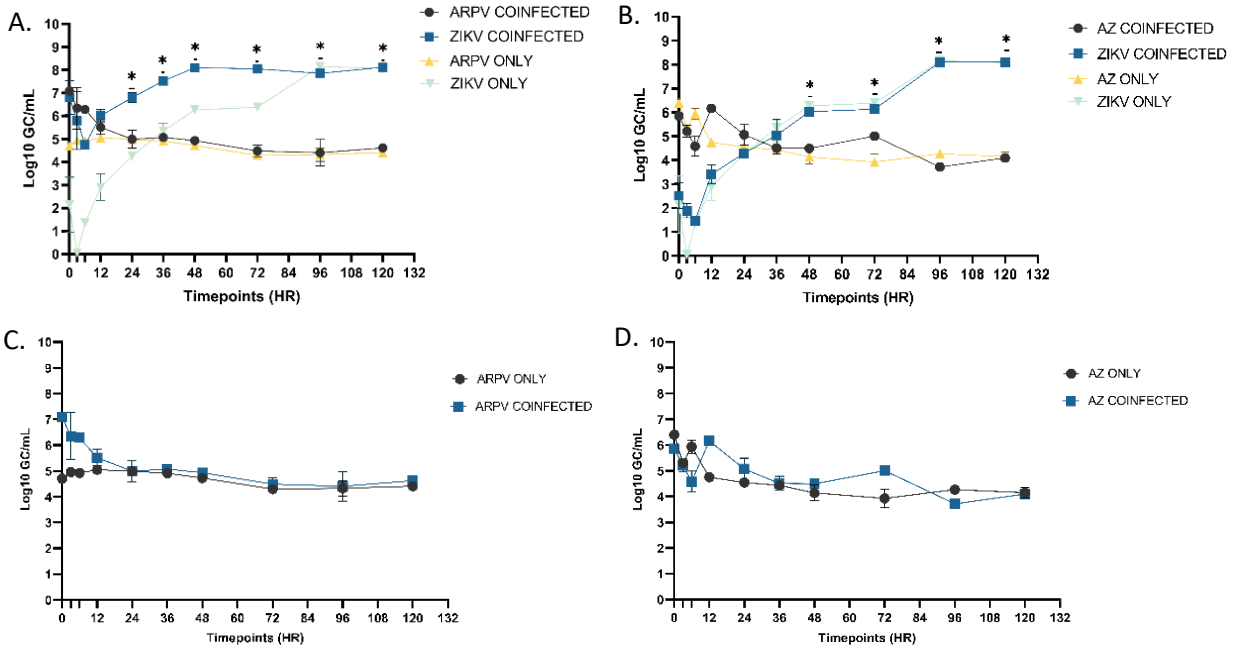

Figure 10. ARPV & AZ do not replicate in vertebrate (Vero-76) during co-infection in intracellular factions. ARPV (1.6 X 10^5 GC) & AZ (3.2 X 10^4 GC) were separately co-infected with ZIKV: 4.0 X 10^6 GC and ZIKV: 8.7 X 10^5 GC of Zika DakAr respectively in replicates of three. ARPV (5.7 X 10^6 GC), AZ (5.4 X 10^6 GC), and ZIKV (2.4 X 10^6 GC) contained virus only groups. Growth kinetics were compared for (A) ARPV vs. ZIKV, (B) AZ vs. ZIKV, (C) ARPV only vs ARPV coinfected, and (D) AZ only vs AZ coinfected. Data points represent mean values of intracellular genome copies and error bars represent the standard deviation. Significance was determined by one-way ANOVA and multiple t- tests. Asterisks indicate significance compared between two coinfected groups. P ≤ 0.033 (*).

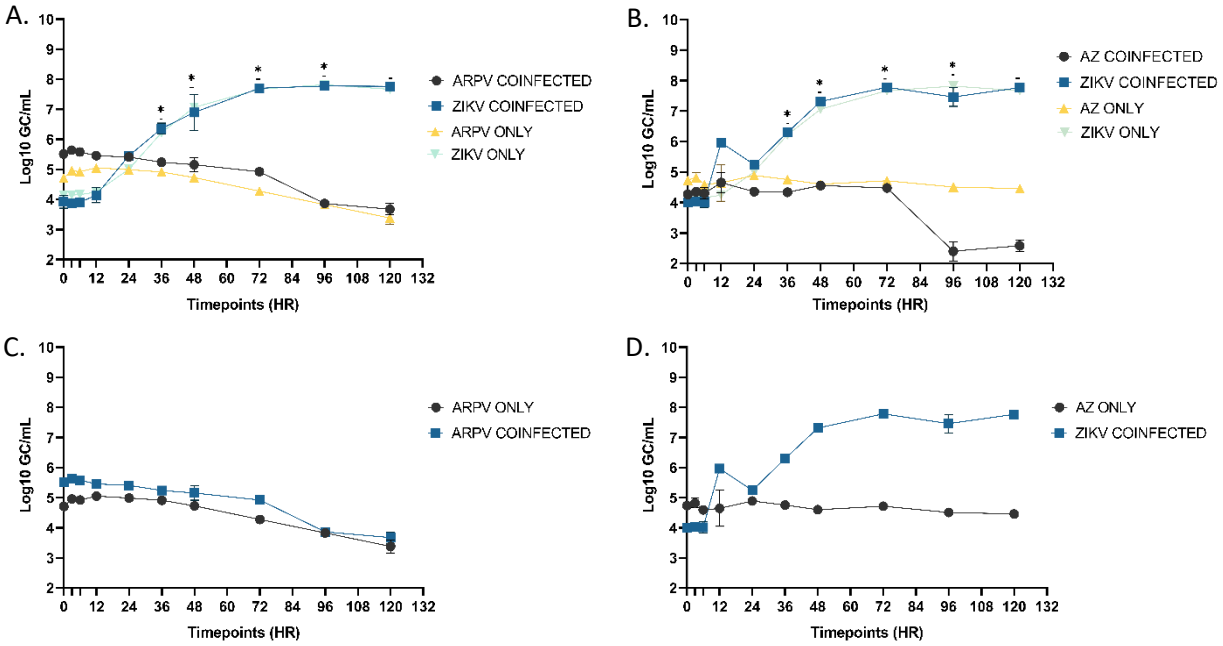

Figure 11. ARPV & AZ do not replicate in vertebrate (Vero-76) during co-infection in extracellular factions. ARPV (1.6 X 10^5 GC) & AZ (3.2 X 10^4 GC) were separately co-infected with ZIKV: 4.0 X 10^6 GC and ZIKV: 8.7 X 10^5 GC of Zika DakAr respectively in replicates of three. ARPV (5.7 X 10^6 GC), AZ (5.4 X 10^6 GC), and ZIKV (2.4 X 10^6 GC) contained virus only groups. Growth kinetics were compared for (A) ARPV vs. ZIKV, (B) AZ vs. ZIKV, (C) ARPV only vs ARPV coinfected, and (D) AZ only vs AZ coinfected. Data points represent mean values of intracellular genome copies and error bars represent the standard deviation. Significance was determined by one-way ANOVA and multiple t- tests. Asterisks indicate significance compared between two coinfected groups. P≤ 0.033 (*).

Table 8: Co-infection GC/mL initial and final timepoints. Significance was determined by two-way ANOVA and Tukey test $p \leq 0.033$ (*). Virus only groups were compared to corresponding coinfected groups.

	0HR Log_{10}GC/mL	**120 HR Log_{10}GC/mL**
Intracellular		
ARPV only	4.71±0.06	4.41±0.06
ARPV co-infected	7.09±0.03*	4.62±0.14
ZIKV coinfected	6.81±0.69*	8.12±0.09
ZIKV only	2.14±1.20	8.07±0.04
Extracellular		
AZ only	6.42±0.13*	4.15±0.20
AZ co-infected	5.88±0.13	4.10±0.04
ZIKV coinfected	2.49±0.54	8.10±0.04
ZIKV only	2.14±1.20	8.07±0.04

Table 9: $PRNT_{50}$ titers estimated using anti-vector study cardiac sera.
Mean values signify log10 of the reciprocal of the highest serum dilution able to reduce ZIKV plaque formation by 50%. Significance was determined by two-way ANOVA and Tukey test $p \leq 0.033$ (*).

	WNV Mean ±SD	**ZIKV Mean ±SD**
PBS	1.30±0	1.30±0
AWN	1.30±0	1.30±0
ARPV	1.30±0	1.30±0
ARPV/AWN	1.30±0	1.30±0
AZ	1.30±0	3.60±0.35
AZ/AWN	1.70 ±0.36*	3.81±0.35
ZIKV	1.30±0	NT
ZIKV/AWN	1.30±0	NT

NT: Not tested.

Discussion

Based on our results, AZ and ARPV remain incapable of replication in vertebrate cell culture (Figure 9C-D; Figure 10C-D). In fact, ZIKV titers increased significantly in Vero-76 cells while ARPV and AZ did not grow over time in both intracellular and extracellular factions (Figure 9A-B; Figure 10A-B, Table 8). Previous studies have shown that AZ and ARPV are able to enter vertebrate cells and induce immune responses[24,39]. Here we see evidence of AZ and ARPV in both intracellular and extracellular fractions indicating this immunologic advantage is still present. However, they remain unable to replicate and egress from cells via exocytosis as seen with ZIKV (Figure 10A-B). Thus, AZ remains a safe chimeric vaccine that can be administered to vertebrates without the fear of gaining replication ability in Zika endemic localities. Overall, it would be interesting to explore co-infection of AZ with other pathogenic flaviviruses such as DENV and WNV.

Next, we examined the potential for and possible impact of anti-vector immunity to determine if immunization with two vaccines (i.e., Aripo-Zika (AZ) and Aripo-West Nile (AWN)) containing an ARPV backbone impacts immunogenicity of the subsequent vaccine, and/or boosts responses to the prime immunization. Sera collected 58 DPI produced significantly higher WNV nAb titers in the AZ/AWN group (1.70 ±0.36), but the remaining groups were consistently low at 1.30±0. The small increase observed in WNV-specific nAbs post AZ immunization suggests the absence of anti-vector immunity, and rather a small boost in immunogenicity to AWN, such that an antibody response could be detected, in contrast to other groups studied where no response was detected. The low or undetectable WNV nAb titers in most groups observed are likely due to the low dose of AWN 2.37-1.68 X 10^5 GC) administered compared to AZ (1.7 X 10^{12}

GC). It would be interesting to repeat this study using a higher dose of AWN to generate meaningful, more robust nAb responses. Further studies are therefore necessary to increase the titer of AWN via serial passaging of the virus in C6/36 cells before this anti-vector immunity study can be repeated. Although ARPV and ZIKV PRVABC59 were administered at lower doses than AZ which may have impacted immunogenicity, ZIKV was administered at a similar dose in the booster and dose dependent studies, where it maintained its immunogenicity and protective efficacy.

Chapter 4: Conclusions

The work presented in Chapter One was conducted to determine the optimal dose, effects of boosters, and effects of maternally transferred antibodies on vaccine-mediated immunogenicity and protection. Previous studies have shown that chimeric ISFV-based vaccines have the capability of inducing immunogenicity at low doses[1,4,14,20,31,42]. Specifically, our chimeric Zika vaccine has been shown to be highly immunogenic with evidence of a robust humoral response[24]. Our studies performed here further confirm AZ's robust immunogenicity profile and safety in an immune-competent mouse model. Namely, these studies indicate AZ when administered at 10^{11} GC provides protective immunity in immune-competent murine models, doses of $\geq 10^{12}$ GC or the use of a three-dose series can help achieve sterilizing immunity, and maternal AZ derived antibodies are passed from dams to pups at protective levels up to four weeks after birth. In humans, IgG antibodies are known to transfer to neonates via the placenta[45,47,48,59]. However, humans no longer absorb macromolecules to transfer after birth, while rodents transfer antibodies up to 21 days after birth via suckling[46]. While murine model studies have been successful, mice are not able to replicate human immune response to Zika infections. Thus, it would be beneficial to assess the impact of various dosage regimens, boosters, and maternal transfer of antibodies in non-human primates (NHPs) in order to assess the efficacy of AZ in a model more closely related to humans. These data would determine the phenotypic advantages of an AZ vaccine for ZIKV as compared to other projected vaccines for ZIKV.

Despite multiple vaccines being explored for ZIKV vaccine development, with several in clinical trials[10,60], our vaccine platform has many advantages that set it apart. AZ is easy to produce at

high titers in flasks over four days at a BSL2 facility and does not require heat inactivation. Also, AZ can be produced, stabilized, and transported at room temperature. Lastly, AZ can be transported, reconstituted and reproduced in BSL2 labs worldwide during a pandemic.

There is a Zika vaccine (VRC5283) that is currently in phase 2 of clinical trials that can be compared to our vaccine [60,61]. This vaccine is made up of JEV signal sequences (SS) inserted in the place of ZIKV prM SS at the amino-terminal region in the plasmid[60,61]. Unlike our vaccine, this is a DNA vaccine which comes with many disadvantages that our vaccine platform does not have. DNA vaccine disadvantages include relative instability, integration into the host genome, inflammatory responses in the host, and toxicity[35,61]. While our vaccine faces some hurdles they are not as pronounced as the hurdles faced with nucleic acid vaccine platforms.

One of the hurdles our vaccine faces is FDA approval. Namely, the C6/36 mosquito cells used to grow AZ are not an FDA-approved substrate. In order to avoid this hurdle, further studies are necessary to produce ARPV in an FDA approved cell substrate at a high enough titer. Blind serial passaging of AZ in an FDA approved cell substrate could allow us to establish a high enough titer of AZ for vaccine production. For example, Sf9s are an armyworm cell substrate that is FDA approved and has been used in many therapeutics and vaccines[53,62,63]. Specifically, Sf9 cells have been explored in recombinant vaccine production of various flaviviruses[64-67]. Alternatively, work could be done to gain FDA approval of a mosquito cell line. However, AZ retains the ability to replicate in mosquito cells which indicates AZ could circulate in mosquito populations which may be of concern to the public.

These studies also indicated that AZ remains a safe vaccine for vertebrates. This is highlighted by ARPV and AZ's continued inability to replicate in vertebrate cells, even during co-infection with ZIKV. Our data also suggests anti-vector immunity to the ARPV backbone may not be of significant concern. This is especially important because Zika has been shown to cocirculate with other flaviviruses in the Americas and Africa[6,7,17]. Zika, like many other tropical diseases impacts developing countries that do not have funding or the capability to eradicate disease using conventional approaches. Multivalent vaccines have become popular in these areas due to their abilities to target multiple viruses endemic in a population simultaneously. Given the impact of Zika, yellow fever and West Nile virus worldwide, a multivalent or combination vaccine that targets combinations of these virus would be beneficial. Thus, upon immunization, the vaccine could develop a strong immune response to multiple related flavivirus pathogens that co-circulate in endemic regions.

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