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Ad26.M.Env ZIKV vaccine protects pregnant rhesus macaques and fetuses against Zika virus infection

Amanda Martinot

Amanda.Martinot@tufts.edu

Tufts University https://orcid.org/0000-0001-6237-6191

Freek Cox

Janssen Vaccines & Prevention

Peter Abbink

Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, Harvard Medical School

Jonathon Hecht

Division of Anatomic Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School

Roderick Bronson

Harvard Medical School

Erica Borducchi

Beth Israel Deaconess Medical Center

William Rinaldi

Alphagenesis

Melissa Ferguson

Alpha Genesis Inc

Rafael De La Barrera

Walter Reed Army Institute of Research

Leslie van der Fits

Janssen Vaccines & Prevention B.V.

Dan Barouch

Beth Israel Deaconess Medical Center https://orcid.org/0000-0001-5127-4659

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Abstract

At the start of the Zika virus (ZIKV) epidemic in 2015, ZIKV spread across South and Central America, and reached parts of the southern United States placing pregnant women at risk for fetal microcephaly, fetal loss, and other adverse pregnancy outcomes associated with congenital ZIKA syndrome (CZS). For this reason, testing of a safe and efficacious ZIKV vaccine remains a global health priority. Here we report that a single immunization with Ad26.M.Env ZIKV vaccine, when administered prior to conception, fully protects pregnant rhesus macaques from ZIKV viremia in blood and tissues with no adverse effects in dams and fetuses. Furthermore, vaccination prevents ZIKV distribution in fetal tissues including brain. ZIKV associated neuropathology was absent in offspring of Ad26.M.Env vaccinated dams, although pathology was limited in sham vaccinated controls. Vaccine efficacy is associated with induction of ZIKV neutralizing antibodies in pregnant rhesus macaques. A Phase I trial showed that Ad26.M.Env (Ad26.ZIKV.001) was safe and immunogenic in people. These data suggest the feasibility of vaccine prevention of ZCS in humans.

Introduction

In late 2015, an epidemic of fetal microcephaly in Brazil associated with high levels of Zika virus (ZIKV), led to a global race to develop a ZIKV vaccine to protect women and unborn children from the potential devastating effects of congenital ZIKA syndrome (CZS) ([1–4]). ZIKV, a member of the *Flaviviridae* family, was first identified in Uganda in 1954, and while sharing a genus with other viruses that cause significant human disease such as dengue, yellow fever, Japanese encephalitis, and West Nile viruses, had only been associated with asymptomatic to mild flu-like symptoms prior to reports of CZS in Brazil in late 2015. Similar to other medically important flaviviruses, ZIKV is primarily acquired by *Aedes* mosquitos, but can be spread transplacentally in pregnant women ([5,6]), in blood transfusions ([7]), and through sexual contact ([8]). Recently, genetic polymorphisms have been associated with development of CZS ([9]).

The World Health Organization (WHO) declared an end to the ZIKV epidemic at the end of 2016 [1010]), and ZIKV transmission is currently at low levels world-wide, however vaccine development for emergency deployment remains a high priority by the WHO ([11]). ZIKV also remains a concern for individuals traveling to endemic areas and for individuals living in areas with continued transmission such as the Caribbean where over 15% of pregnant women continue to test positive for ZIKV ([12]). CDC guidance still recommends that pregnant women, partners of pregnant women, or those considering pregnancy to delay travel to areas with ZIKV outbreaks and to consult with medical providers before traveling to ZIKV endemic regions ([13]).

Preclinical studies in mice and non-human primates have shown that induction of neutralizing antibodies by a number of vaccine platforms is effective in preventing ZIKV viremia ([14–16]). A number of candidate ZIKV vaccines have completed safety studies in Phase I and II clinical trials ([17–19]). Current low levels of ZIKV world-wide limit the establishment of clinical trial sites and enrollment of participants in Phase III efficacy studies ([14,19–21]). Ideally a ZIKV vaccine could be deployed in the event of a ZIKV resurgence and administered safely to pregnant women. The macaque model has been a useful model for studying dynamics of viral replication and shedding during ZIKV infection ([22–26]). We and others have shown that the rhesus macaque model consistently reproduces features of ZIKV infection in pregnancy including prolonged

ZIKV viremia in blood and persistence of ZIKV in lymphoid tissues and the placenta ([23,25,27–33]). Although fetal microcephaly has not been reported as a fetal outcome of ZIKV infection in monkeys, experimental ZIKV challenge of pregnant non-human primates has recapitulated other adverse outcomes of ZIKV exposure observed in human pregnancy including fetal loss, fetal cerebral calcifications, gliosis, and long-term developmental alterations in infant macaques ([30,34–36]). As in humans, these events represent a small proportion of overall pregnancy outcomes. Although we and others have shown that DNA vaccination can protect against ZIKV viremia, break-through viral replication was seen in subsets of animals ([14]) and DNA vaccination was only partially protective against viremia in pregnant macaques ([37]). Attempts to treat pregnant monkeys with cocktails of neutralizing antibodies also failed to prevent ZIKV viremia and adverse fetal outcomes were observed including fetal loss ([27]). An Ad26.M.Env vaccine (Ad26.ZIKV.001) has been shown to be immunogenic in animal models[16]) and has been tested in a human Phase I study ([38]). In addition, it was shown to be protective against fetal demise in pregnant interferon alpha/beta receptor knock-out mice, a highly susceptible model where ZIKV infection leads to high levels of ZIKV plasma viremia and placental ZIKV viral replication and fetal loss ([39,40]). We opted to test this vaccine for safety and efficacy in pregnant rhesus macaques that were ZIKV challenged during the critical equivalent of human first trimester pregnancy. Here we show that Ad26.M.Env prevents ZIKV peripheral blood viremia and tissue viremia in pregnant macaques and fetuses with no evidence of ZIKV-associated fetal pathology in rhesus monkeys.

Results

Ad26.M.Env vaccination induced potent anti-ZIKV neutralizing antibodies and prior vaccination did not impact conception in female macaques.

Thirteen female macaques were immunized with 10^{11} vp of Ad26.M.Env expressing ZIKV M protein transmembrane domain lacking the peptide precursor (PR) and the envelope (Env) antigens (Ad26.M.Env) and were returned to the breeding colony 17 days post-vaccination (Fig. 1a). Dams were vaccinated a minimum of 2 months and a maximum of 7 months prior to challenge. An additional 13 females were selected that received sham vaccination that were placed in the breeding colony at the same time as the vaccine group. No adverse events were noted by clinical staff immediately following vaccination. In the weeks following vaccination, some animals had reports of weight loss, diarrhea, and trauma, but these clinical observations did not correlate temporarily with vaccine administration. A peak in total white blood cells was seen in vaccinated animals at four weeks post-vaccination likely due to vaccine immune stimulation **(Suppl Fig. 1)**.

Females in all groups were monitored bi-weekly by ultrasound for pregnancy. Pregnant females in the Ad26.M.Env vaccine and sham groups were infected with $1x10^6$ vp of ZIKV via the subcutaneous route ZIKV 6 weeks post-conception (10–30 weeks post-vaccination based on timing of confirmed pregnancy, **Suppl. Table 1**). Three pregnant sham animals were not infected to serve as normal pregnant controls. Plasma, sera, cerebral spinal fluid, urine, colorectal, cervical, and saliva samples were collected during pregnancy from all dams as indicated (Fig. 1a).

Neutralizing antibody titers were determined by both Immunospot focus reduction neutralization (FRNT) and by microneutralization assay (MN50 VNA). Before immunization, animals (OBF, 079 and 437) showed very low neutralization titers whereas the other six animals showed no neutralization titers when assayed with FRNT (Fig. 1b). Four weeks after immunization, all animals developed a neutralizing response that was maintained or only marginally decreased 8 weeks after immunization and in pre-challenge serum which was obtained 10 to 30 weeks after immunization (Fig. 1b,d). Four weeks after challenge, all animals of the Ad26.M.Env immunized group had increased ZIKV neutralization titers (Fig. 1b). Serum of animals of the non-immunized control group were assayed pre- and post- challenge. Pre-challenge, no neutralization titers were detected with FRNT analysis, whereas ZIKV neutralization titers developed after challenge (Fig. 1c). These FRNT results were confirmed by the MN50 neutralization assay (**Suppl. Figure 2)**. Post-challenge neutralizing titers were comparable between Ad26.M.Env and sham vaccinated animals, consistent with minimal amnestic antibody responses in vaccinated animals (Fig. 1b, c, **Suppl. Figure 2**). Next, antibody responses against ZIKV NS1 protein were measured by ELISA. Ad26.M.Env does not contain a NS1 antigen. In accordance, Ad26.M.Env vaccinated or sham vaccinated animals had low (079) or undetectable NS1 binding antibody responses in pre-challenge samples. Four weeks after challenge, all 5 non-immunized dams developed high NS1-specific antibody titers (mean titer of 3.69 log10). Eight out of 9 dams that received Ad26.M.Env also developed NS1-specific titers after challenge although the group mean NS1 titer (1.75log10) was approximately 100-fold (2 log10) lower compared to the group mean NS1-titer in the non-immunized animals (**Suppl. Figure 3**).

Ad26.M.Env vaccination induced anti-Env cellular immune responses in macaques.

Env and prM directed cellular immune responses were measured by IFNγ ELISPOT on frozen PBMC's isolated pre- immunization, post-immunization, pre-challenge, and post-challenge. Ad26.M.Env vaccination resulted in induction of ZIKV specific cellular responses (Fig. 2). The geometric mean Env-specific cellular immune responses in the group that received Ad26.M.Env was above 50 SFU per 10^6 PBMCs at week 4 and 8 after immunization, and at the pre-challenge timepoint (geomean SFU 55.03, 55.92, and 97.41, respectively). The Env-specific cellular immune responses after immunization were higher when compared to the Env-specific cellular immune responses pre-immunization, or in non-immunized animals which were both below the limit of detection (Fig. 2a,b). The prM-specific cellular responses were generally low and geometric mean responses do not exceed the cut-off of 50 SFU per 10^6 PBMCs (Fig. 2c,d). Notably, Env and prM cellular responses did not increase after challenge as compared to the pre-challenge timepoint indicating a lack of amnestic cellular responses in Ad26.M.Env vaccinated animals (Fig. 2a,c).

Ad26.M.Env vaccinated pregnant females were completely protected against ZIKV viremia in blood and tissues.

Pregnant females in the vaccine and sham groups were infected with $1x10^3$ PFU Zika virus from the 2015 Brazilian epidemic at 6 weeks post-conception (being 10 to 30 weeks post vaccination). Vaccinated dams had no detectable virus in plasma post-ZIKV challenge even though animals were challenged from 10 to 30 weeks after vaccination, depending on the timepoint of conceiving (Fig. 3a). In contrast, sham vaccinated pregnant macaques all had detectable viral load, with a mean peak viremia of 5.5 log10 on day 7 post-

challenge (Fig. 3b) consistent with peak viremia reported for ZIKV infected non-pregnant and pregnant macaques ([22,32,33,36]). On average, sham vaccinated animals had detectable virus for 42 days with a range of 7 to 56 days consistent with previous reports that pregnancy prolongs ZIKV viremia in rhesus monkeys ([32,36]). All colorectal, vaginal, saliva, and amniocentesis samples were negative for ZIKV for all ZIKV-challenged dams irrespective of vaccination status.

After confirmation of pregnancy, dams were monitored bi-weekly for fetal biometric analyses including measurements of biparietal diameter, occipitofrontal length, head circumference, and femur length by ultrasonography. No abnormalities were noted in fetal biometric parameters between study groups (**Suppl. Figure 4**) and fetal brain weights and brain:fetal body weight ratios at necropsy were similar across groups (**Suppl. Figure 5**).

Dams had scheduled Cesarian sections and euthanasia when fetuses were term, approximately 2 weeks prior to estimated delivery date. Maternal tissues previously shown to have detectable virus throughout pregnancy were collected for evaluation by RT-PCR for ZIKV viral RNA (vRNA). None of the nine Ad26.M.Env vaccinated dams had detectable vRNA in any tissues surveyed (Fig. 3c). All sham vaccinated dams had detectable vRNA in at least one of the analyzed tissues. 4/5 animals showed positive vRNA in maternal spleen, consistent with previous reports([23,32,34]), and one dam had vRNA detected in the axillary LN. One dam had detectable vRNA in the uterus, and 3/5 dams had virus detectable in the placenta (Fig. 3d). Placenta pathology was evaluated for dams in all groups and evaluated by both a veterinary pathologist and a human gynecological pathologist specializing in placental histopathology. Histopathological placental findings in all groups were typical of near-term/term placentas in macaques with evidence of maternal thrombosis and infarction in all groups (Table 1)([41]). Fetal: placental ratios were within the expected limits for term fetuses and did not vary significantly between groups (**Suppl. Figure 5**).

Table 1
Summary of gross and histopathological findings in dam and neonate tissues.

Animal ID	Condition	Group	Sex	Gross Findings	Histopathology- Fetus	Histopathology- Placenta
78	Control	3	F	NSF	Hemorrhage in lateral ventricle	moderate calcifications
583	Control	3	M	NSF	Cortical microcalcification	NSF
541	Control	3	M	NSF	Increased meningeal cellularity	NSF
05K	ZIKV	2	F	Hard, white lesion noted in liver	Cortical/neuroprogenitor dysplasia (mild); focal gliosis	placental thinning
560	ZIKV	2	F	absent occipital gyrus (L); focal proliferation of neuropil on the right cerebellar lateral hemisphere	Cortical/neuroprogenitor dysplasia (mild); Cortical and periventricular microcalcification; multifocal microhemorrhage; mild neuropil vacuolation/rarefication; multifocal meningeal proliferation; increased meningeal cellularity	NSF
05L	ZIKV	2	M	Dilated lateral ventricle upon examination of fixed specimens; asymmetry L parietal lobe	Cortical microcalcification	thrombosis maternal vessel
02X	ZIKV	2	M	Absent gyrus in parietal cortex; cloudy CSF	Microcalcification within neuroprogenitor clusters, perivascular edema, and necrosis; mild neuropil vacuolation/rarefication; focal gliosis	NSF
558	ZIKV	2	M	Enlarged ventricle noted upon examination of fixed specimens	Cortical/neuroprogenitor dysplasia (mild); cortical microcalcification; hemorrhage in lateral ventricle; multifocal gliosis; increased meningeal cellularity; multifocal spinal cord microhemorrhage	Moderate infarction/necrosis chorionic plate; thrombosis maternal vessel

Animal ID	Condition	Group	Sex	Gross Findings	Histopathology- Fetus	Histopathology- Placenta
07G	Vaccinated, ZIKV	1	M	NSF	Cortical/neuroprogenitor dysplasia (mild); mild neuropil vacuolation/rarefication; focal gliosis	thrombosis maternal vessel
0BF	Vaccinated, ZIKV	1	M	NSF	NSF	Mild abruption; thrombosis maternal vessel
437	Vaccinated, ZIKV	1	M	NSF	NSF	Mild infarction/necrosis chorionic plate; placental thinning; thrombosis maternal vessel
07M	Vaccinated, ZIKV	1	F	NSF	Cortical microcalcification; multifocal microhemorrhage; mild neuropil vacuolation/rarefication	NSF
OC1	Vaccinated, ZIKV	1	F	NSF	NSF	NSF
OBE	Vaccinated, ZIKV	1	F	NSF	NSF	Not evaluated; Vaginal delivery infant
79	Vaccinated, ZIKV	1	F	NSF	NSF	NSF
32	Vaccinated, ZIKV	1	F	NSF	Mild neuropil vacuolation/rarefication	Mild infarction/necrosis chorionic plate; thrombosis maternal vessels

Neonates born to Ad26.M.Env vaccinated dams were negative for ZIKV virus and had no ZIKV-associated histopathological abnormalities.

Following Cesarian section and euthanasia, fetuses were inspected for gross abnormalities and fetal tissues collected for histopathology and evaluation of vRNA. Fetuses of vaccinated dams had no evidence of viral replication in tissues (Fig. 4a, **upper**) while 2/5 fetuses from sham vaccinated dams had detectable virus in tissues, one of which (Fetus 560) had extensive detection of ZIKV in the brain **(**Fig. 4a, **lower).** Histopathologic evaluation of brain from fetuses born to Ad26.M.Env vaccinated dams showed no evidence of previously reported ZIKV neuropathology including microcalcifications and perivascular edema (Fig. 4b-e, Fig. 5). Sham vaccinated fetuses had a constellation of abnormal findings **(**Table 1**)** including a gross cerebellar malformation (Fig. 4f), asymmetry of the left parietal lobe (**Suppl. Figure 6**, Table 1), and a gross dystrophic calcification on the liver (Table 1), focal edema **(**Fig. 4g**)**, microcalcification **(**Fig. 4h**)**, and

meningeal proliferation (Fig. 4i) However, overall the sham group had fewer histopathological findings than previously reported (Fig. 5)([32]) and gross and histological abnormalities in sham vaccinated, challenged fetuses could not be definitively linked to ZIKV viral replication.

Discussion

ZIKV viral infections world-wide resulted in a pandemic of fetal malformations and fetal loss in pregnant women in 2016. The repercussions of this devasting disease will continue to manifest itself as cohorts of exposed women and children continue to be followed. Although massive ZIKV exposure across endemic regions of South America has likely resulted in elevated neutralizing antibodies in exposed populations, waning of immunity over time will likely lead to re-emergence of ZIKV cyclically as seen with previous outbreaks in Malaysia. Furthermore, questions remain regarding the potential role for previous infection with other related viruses such as Dengue fever virus (DENV) in predisposing to development of ZCS([42]). Recent studies in both pregnant macaques and marmosets have shown evidence of enhanced neuropathology and placental pathology in previously DENV exposed, ZIKV infected animals ([43,44]). The United States is especially at risk for the re-emergence of a future ZIKV pandemic since the Aedes egypti mosquito vector is endemic to the southern United States. The lack of exposure of the US population to ZIKV during the 2016 epidemic makes US individuals vulnerable to future ZIKV epidemics. Development of a well-tolerated and safe vaccine that can be used pre-and perinatally is critical to protecting naïve individuals during future ZIKV outbreaks. The Ad26.M.Env (Ad26.ZIKV.001) vaccine was tested in a Phase I clinical trial and all regimens tested were well tolerated, with no safety concerns identified and induction of robust ZIKV neutralizing titers([38]). In addition, transfer of immune sera from vaccinated study participants to mice protected mice against ZIKV viremia ([38]). Here we show that an Ad26.M.Env vaccine is safe and efficacious against preventing ZIKV viremia in sera and tissue of pregnant rhesus macaques. While fewer neuropathological findings were detected in sham vaccinated animals in the current study as compared to our previous report, here we show that vaccination with Ad26.M.Env prevents ZIKV replication in both placenta and fetal tissue including brain in the non-human primate model. These data combined with the Phase I safety data in people suggests that the Ad26.M.Env is likely to be efficacious against maternal ZIKV infection.

Methods

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Outbred, healthy Indian-origin female rhesus monkeys (*Macaca mulatta*) were housed at Alphagenesis, Yemassee, SC. Animals selected for this study were research naive. Upon arrival and standard quarantine procedures, animals were tested for tuberculosis (TB) at least three times at intervals of two weeks. Animals were also screened for Herpes B, Simian retrovirus (SRV), Simian Immunodeficiency Virus (SIV), Simian T-cell Leukemia Virus (STLV), and Measles. All animals were Herpes B, SRV, SIV, and STLV negative. Study animals were selected based on age. Females were all aged 4–8 years old with similar age and weight distribution per study group **(Suppl. Table 1)**. The study protocol was reviewed and approved by the Alphagenesis Institutional Care and Use Committee (IACUC). All experiments conformed to regulatory standards outlined

by the American Veterinary Medical Association (AVMA) and American Association of Laboratory Animal Medicine (AALAM).

Breeding, immunization, and ZIKV challenge

Seven weeks prior to immunization, females were removed from their breeding group. For Group 1 (immunized, ZIKV-challenge), nine dams that were not pregnant according to ultrasound were intramuscularly immunized with $1x10^{11}$ vp Ad26.M.Env. The nine immunized dams were reintroduced to their breeding groups 17 days later. All animals were provided enrichment according to recommended guidelines. Dams were monitored for pregnancy every 2 weeks by ultrasound until confirmed pregnant. After confirmed pregnancy, dams were monitored by ultrasound every 4 weeks. For Group 1, all nine dams had confirmed pregnancy. For Group 2 (non-immunized ZIKV-challenge controls), 7 dams were included in the study, of which 5 were confirmed pregnant. Pregnant control dams (Group 3, no immunization, no ZIKV-challenge) were included from the breeding colony.

Approximately six weeks after calculated date of conception (based on ultrasound results), equivalent to human 1st trimester of pregnancy, dams from groups 1 and 2 were challenged with $1x10^6$ vp (10^3 PFU) of ZIKV-BR via the subcutaneous route. During pregnancy, blood and PBMCs were isolated for immunogenicity readouts. Plasma, cerebrospinal fluid (CFS), urine, colorectal biopsies, inguinal/axillary lymph node (LN) biopsies, rectal, vaginal and saliva secretion were taken to monitor viremia. At approximately week 21–23 of pregnancy (≈ week 16 following challenge) fetuses of all groups were delivered by Cesarian section except of one (OBE) from the vaccine group that was born naturally due to earlier than predicted delivery. One pregnancy was lost in the vaccine group due to culture confirmed staphylococcal placentitis (539) and this dam/infant pair was removed from the study. Reproductive failure or preterm delivery is significant among primates, and the effects observed in this study are within the historical control range for the testing facility (AGI), and within expected outcomes for pregnancies in rhesus monkeys.

Five live male infants and three live female infants were delivered among the vaccinated, challenged group. Three live male infants and two female infants were delivered to the non-vaccinated, challenged group. Two live males and one female infant were delivered to the control group (non-vaccinated, non-ZIKV challenged; Table 1). Dams and fetuses were euthanized for post-mortem gross pathology, histopathology, and virologic assessments.

METHOD DETAILS

ZIKV Challenge Stock Preparation

ZIKV-BR (Brazil ZKV2015) was propagated in Vero cells (World Health Organization, NICSC-011038011038) that were maintained in EMEM media supplemented with 10%FBS, 6mM L-glutamine and 1x pen/strep. Cells were passaged twice a week and incubated at 37°C, 10% CO_2.

Ultrasonography

Ultrasounds were performed bi-weekly in the ZIKV-infected pregnant rhesus monkeys as well as in 3 uninfected pregnant rhesus monkeys in the same breeding facility. Animals were sedated with Telazol (5mg/kg), and a GE Logic E with an 8CRS Micro-convex transducer (FOV 132, 3.6-10MHz) was used for multiparameter biometric measurements, including biparietal diameter (BPD), occipitofrontal diameter (OFD), head circumference (HC), crown-rump length (CRL), abdominal circumference (AC), and femur length (FL).

Amniocentesis

Animals were sedated with Telazol HCL (4–7 mg/kg IM). The area on the abdomen was clipped and sterilely prepped with triple alternating applications of betadine and alcohol. Using sterile technique, a 22-gauge 3.34-inch needle on a 3-cc syringe was inserted into the ventral abdomen to the amniotic sac with ultrasound guidance. 2 cc of amniotic fluid was collected and frozen immediately.

RT-PCR

RT-PCR assays were utilized to monitor viral loads in plasma, CSF, lymph node biopsies, colorectal biopsies, colorectal weck samples, and urine longitudinally every 2–4 weeks as indicated in the experimental design (see Fig. 1A) and amniotic fluid collected by amniocentesis at day 14 post-ZIKV infection, and from tissues collected at necropsy, essentially as previously described ([14,15,32,33]). RNA was extracted with a QIAcube HT (Qiagen, Germany). Liquid samples were extracted using the Qiacube 96 Cador pathogen HT, and tissue samples were lysed in Qiazol, using the Tissuelyser II (Qiagen, Germany), chloroform treated and extracted with the Qiacube 96 RNeasy HT kit. The wildtype ZIKV BeH815744 Cap gene was utilized as a standard. RNA standards were generated using the AmpliCap-Max T 7 High Yield Message Maker Kit (Cell Script) and purified with RNA clean and concentrator kit (Zymo Research, CA, USA). RNA quality and concentration was assessed by the BIDMC Molecular Core Facility. Log dilutions of the RNA standard were reverse transcribed and included with each RT-PCR assay. Viral loads were calculated as virus particles (VP) per microgram of total RNA as measured on the NanoDrop (Thermo Scientific, Waltham, MA, USA) or as VP per million cells, as shown in Figs. 3–4. Assay sensitivity was > 100 copies/mL, > 100 copies per million cells, and > 3 copies/mg total RNA.

Neutralization Assays

ZIKV-specific neutralizing antibodies were measured by fold reduction neutralization (FRNT) and microneutralization (MN) assays, as previously described ([14,15,32,33]). For FRNT assay Vero cells were seeded at a concentration of $2x10^4$ cells/well in 96-well plates 24 hours prior to the assay initiation. Heat inactivated serum samples were serially diluted prior to being mixed and incubated with input virus ZIKV-PR (PRVABC59) for 1 hour at 37°C. Cell-seeded 96-well plates were infected with 100 µL of the virus/serum mixtures for 1 hour before the addition of overlay media. Each serum dilution was tested in triplicate wells. Approximately 24 hours after infection, ZIKV foci were detected using an antiflavivirus detection antibody, a horseradish peroxidase (HRP)-conjugated secondary antibody and True-Blue peroxidase substrate. ZIKV foci were visualized and counted using an ImmunoSpot analyzer and software. Each assay run included virus input and media-only control wells, as well as negative and positive control serum samples. Neutralizing antibody

titers were reported as the inverse of the serum dilution estimated to reduce the number of input virus by 50% (FRNT50) as shown in Fig. 1.

For MN, serum samples were serially diluted three-fold in 96-well micro-plates, in a total volume of 100uL. 10^2 PFU ZIKV-PR (PRVABC59) in a total volume of 100uL was added and incubated at 35°C for 2 hours. Serum/virus mixtures were then transferred to microtiter plates containing confluent Vero cell monolayers (World Health Organization, NICSC-011038011038). After incubation for 4 d, cells were fixed with absolute ethanol: methanol for 1 hr at − 20° C and washed three times with PBS. The pan-flavivirus monoclonal antibody 6B6-C1 conjugated to HRP (6B6-C1 was a gift from JT Roehrig, CDC) was then added to each well, incubated at 35° C for 2 h, and washed with PBS. Plates were washed, developed with 3,3’,5,5’–tetramethylbenzidine (TMB) for 50 min at room temperature, stopped with 1:25 phosphoric acid, and absorbance was read at 450 nm. For a valid assay, the average absorbance at 450 nm of three non-infected control wells had to be % 0.5, and virus-only control wells had to be R 0.9. Normalized absorbance values were calculated, the MN50 titer was determined by a log mid-point linear regression model. The MN50 titer was calculated as the reciprocal of the serum dilution that neutralized R 50% of ZIKV, and seropositivity was defined as a titer R10, with the maximum measurable titer 7,290, as shown in **Suppl. Figure 2**.

Tissue Collection and Histopathology

Within 14 days of estimated term gestation (26 weeks), dams and fetuses were euthanized with intravenous sodium pentobarbital, and delivery was by caesarian section. Complete necropsies were performed by a veterinarian on fetuses immediately following euthanasia, utilizing standard necropsy procedures with standard sterile surgical grade necropsy instruments and dissection blades. Briefly, peripheral lymphoid tissues were collected, followed by the gastrointestinal tract and abdominal organs. The pleural cavity was opened and the tongue, pharynx, trachea, esophagus, heart, and lungs (“pluck”) were removed *en masse.* Reproductive organs were collected, followed by brain, spinal cord, and eyes. Ruskin-Liston bone cutting forceps were used to expose the spinal cord to the level of the *cauda equina*. Limited necropsies were performed on dams for tissues previously shown to harbor viral RNA including reproductive organs, lymphoid tissues, spleen, and placenta. Fresh tissues were collected utilizing sterile blades for viral RT-PCR in RNAlater (Ambion). Frozen tissue for histopathology was prepared by trimming tissue, placing tissue samples into cryomolds with optimal cutting temperature medium (OCT, Tissue-Tek), and flash freezing on-site. Additional tissues were fixed in 10% neutral buffered formalin (NBF) for histopathology. Formalin-fixed tissues were trimmed, processed, and embedded in paraffin, sectioned, and stained with hematoxylin and eosin, and evaluated independently by two veterinary pathologists (A.J.M., R.B.) and gynecologic pathologist (J.L.H).

ELISPOT

ZIKV-specific cellular immune responses were assessed by IFN-γ ELISPOT assays shown in Fig. 2 using pools of over- lapping 15-amino-acid peptides covering the prM and Env proteins (JPT, Berlin, Germany), essentially as we previously described ([14]). 96-well multiscreen plates (Millipore, MA, USA) were coated overnight with 100 mL/well of 5 mg/ml anti-human interferon-g (BD Biosciences, CA, USA) in endotoxin-free Dulbecco’s PBS (D-PBS). The plates were then washed three times with D-PBS containing 0.25% Tween 20

(D-PBS-Tween), blocked for 1–4 hr with D-PBS containing 5% FBS at 37°C, and incubated with 2 mg/ml of each peptide and $2x10^5$ monkey PBMC in triplicate in 100 mL reaction mixture volumes. Following an 18–24 hr incubation at 37°C, the plates were washed nine times with PBS-Tween and incubated for 3 min with distilled water. The plates were then incubated with 1 mg/ml biotinylated anti-human interferon-g (U-Cytech Biosciences, UT, NETH) for 2 hr at room temperature, washed six times with PBS-Tween, and incubated for 2 hr with streptavidin-alkaline phosphatase (Southern Biotechnology Associates, AL, USA). Following five washes with PBS-Tween and one with PBS, the plates were developed with nitroblue tetrazolium-5- bromo-4-chloro-3-indolyl-phosphate chromogen (Pierce, IL, USA), stopped by washing with tap water, air-dried, and read using an ELISPOT reader (Cellular Technology Ltd., OH, USA). The numbers of spot-forming cells (SFU) per 10^6 cells were calculated. The medium background levels were typically < 15 SFU per 10^6 cells. SFU per 10^6 PBMCs of unstimulated PBMCs was subtracted from specific responses of corresponding individual macaques. Specific responses that were at or below zero after background subtraction were set to 1.

ELISA

Monkey ZIKV NS1 ELISA kits (Alpha Diagnostic International, TX, USA) were used to determine endpoint binding antibody titers using a modified protocol ([14]). 96-well plates coated with ZIKV NS1 protein (RV-403310-1 Alpha Diagnostics) were first equilibrated at room temperature with 300 ml of kit working wash buffer for 5 min. 6 ml of monkey serum was added to the top row, and 3-fold serial dilutions were tested in the remaining rows. Serum samples were incubated at room temperature for 1 hr, and plates washed 4 times. 100 mL of anti-monkey IgG HRP-conjugate working solution was then added to each well and incubated for 30 min at room temperature. Plates were washed 5 times, developed for 15 min at room temperature with 100 ml of TMB substrate, and stopped by the addition of 100 ml of stop solution. Plates were analyzed at 450nm/550nm on a VersaMax microplate reader using Softmax Pro 6.0 software (Molecular Devices, CA, USA). ELISA endpoint titers were defined as the highest reciprocal serum dilution that yielded an absorbance > 2-fold over background values and plotted as Log10 endpoint titer.

Declarations

DATA AND SOFTWARE AVAILABILITY

All data generated and analyzed in this study are available from the Lead Contact upon reasonable request.

AUTHOR CONTRIBUTIONS

DHB, LF, FC conceptualized and designed the study; WJR, MJF, AJM, AC, and FC supervised and conducted the NHP studies; AJM, RB, and JLH performed the pathological analyses. RAB performed the neutralization assays. FC, ENB, LF, and AJM curated and analyzed the data; AJM wrote the paper; FC, LF, and DHB reviewed and edited the manuscript.

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COMPETING INTERESTS

L.F. and F.C. are or were employees of Janssen Vaccines & Prevention B.V. D.H.B. is a co-inventor on the patent PCT/US2017/036900 (Compositions and methods for preventing and treating ZIKA virus infection). All other authors declare no competing interests.

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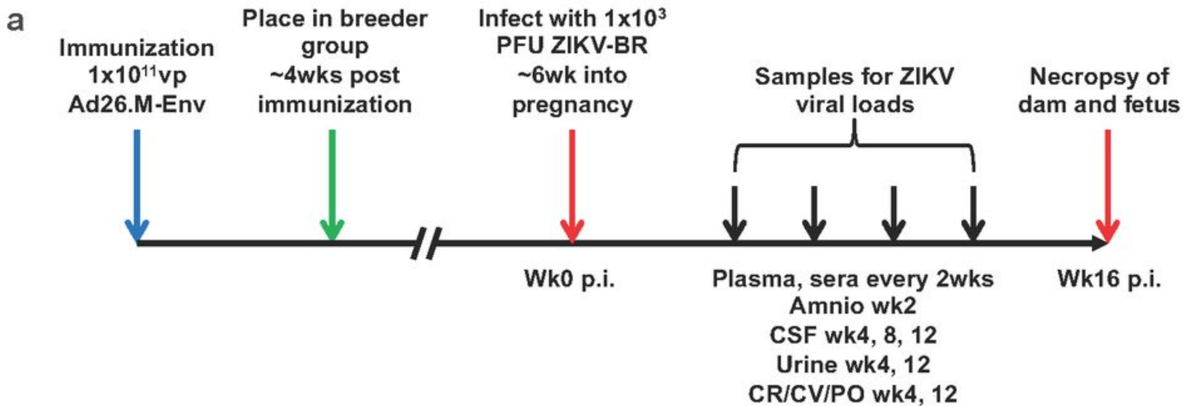
Figures



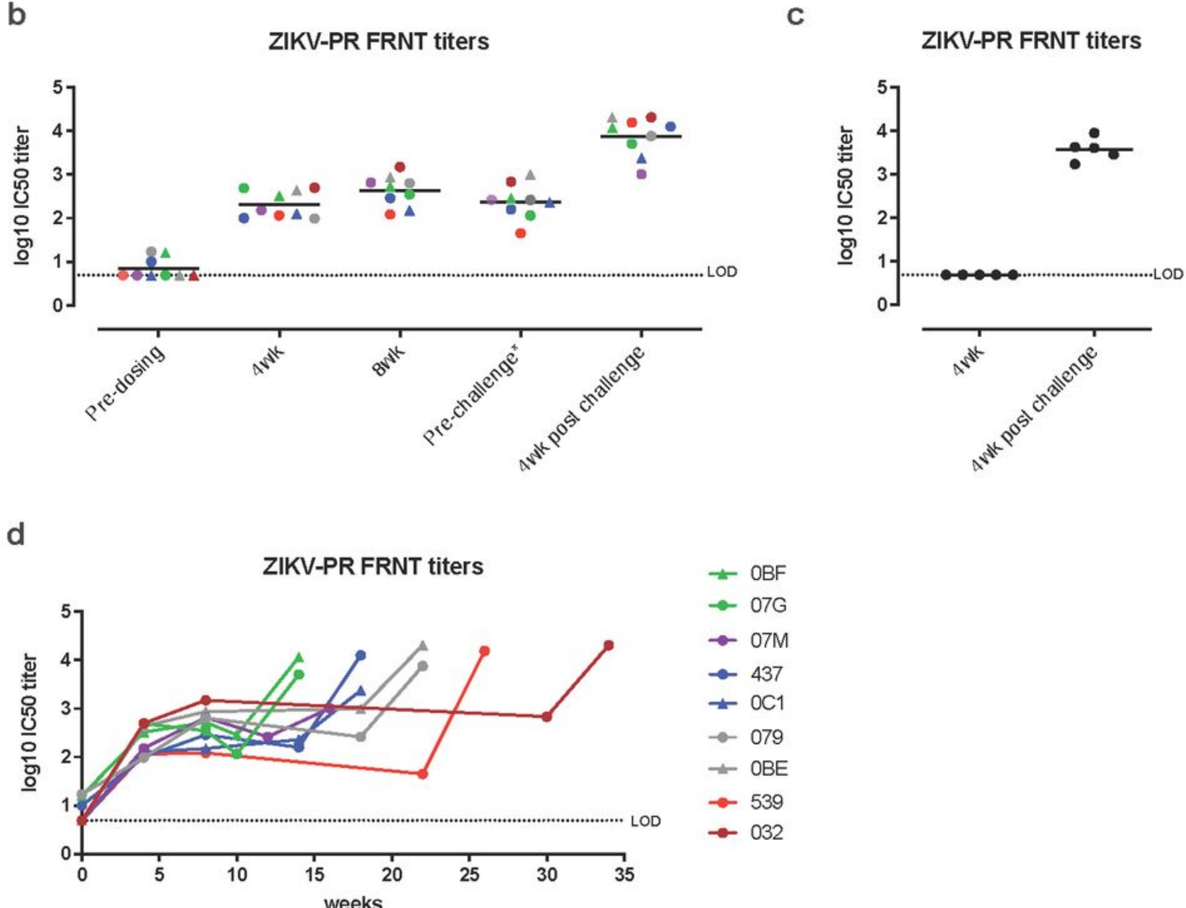


Figure 1

Ad26.M.Env induces robust neutralizing antibodies in sera prior in female breeding macaques. (a) Macaques were vaccinated 17 days prior to introduction into breeding groups, monitored for pregnancy every two weeks and challenged with ZIKV-Br six weeks post-conception. ZIKV-PR neutralizing antibody responses in sera of dams (n= 9) immunized with 10^{11} vp Ad26.M.Env **(b,d)** or non-immunized control animals (n=5) **(c)** determined by FRNT. Colored connective lines represent the neutralization response in time **(d)**. Neutralizing antibody titers are reported as the log10 of the inverse of the serum dilution that reduce the number of input

virus by 50% (IC50). The mean responses per group are indicated with a horizontal line. The dashed line shows the lower limit of detection (LOD) defined as the log10 of one dilution below the start dilution of the samples (0.70 log10 or 1.3 log10, dependent on the assay run). Individual animals of group 1 (Ad26.M.Env immunized) are color coded to represent number of weeks between immunization and challenge; green (10wk); purple (12 wk); blue (14 wk); gray (18 wk); red (22 wk); brown (30 wk).). Amino= amniocentesis; CSF=cerebral spinal fluid; CR=colorectal swab; CV= cervical swab; PO= oropharyngeal swab.

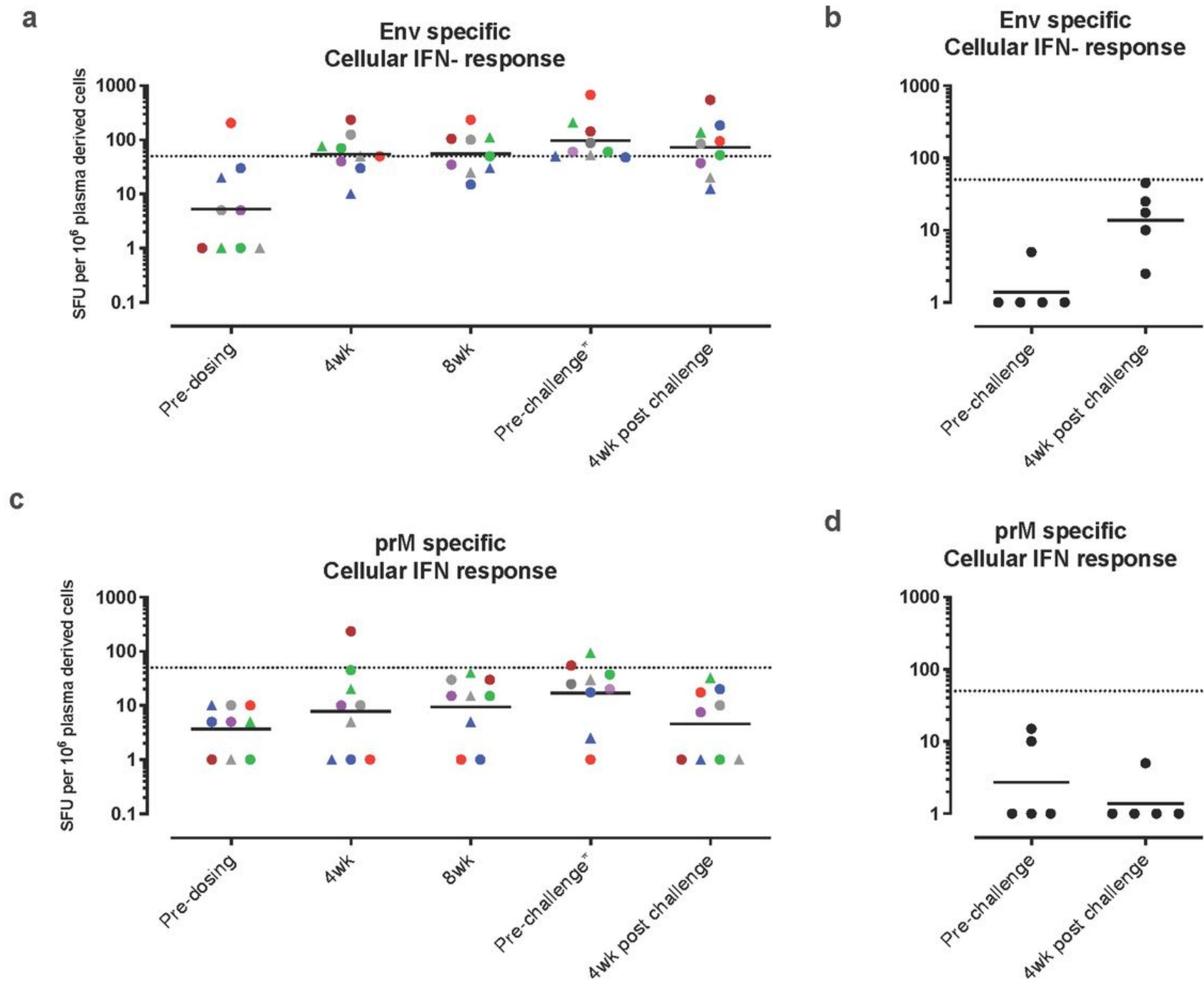


Figure 2

Ad26.M.Env vaccine induces low level cellular immune anti-Env responses in pregnant macaques. IFNγ ELISPOT responses in PBMCs of dams (n= 9) immunized with 10^{11} vp Ad26.M.Env (**a,c**) or non-immunized control animals (n=5) (**b,d**). (**a,b**) Env-specific responses and (**c,d**) PrM specific responses are shown. The geometric mean response per group is indicated with a horizontal line. Responses above 50 SFU per 10^6 PBMCs indicated by the dotted line are considered positive. Individual animals of group 1 (Ad26.M.Env

immunized) are color coded to represent number of weeks between immunization and challenge; green (10wk); purple (12 wk); blue (14 wk); gray (18 wk); red (22 wk); brown (30 wk).

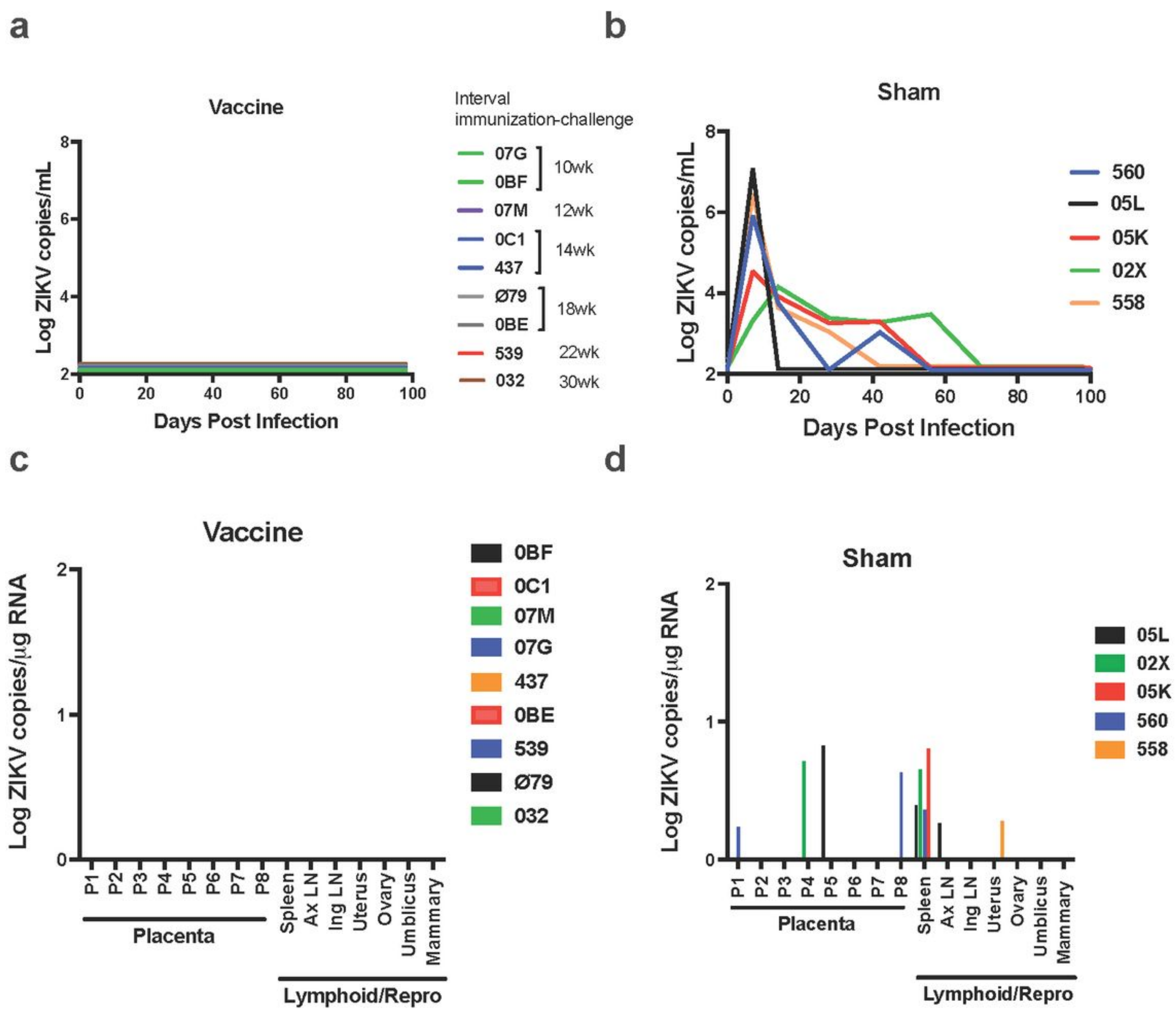


Figure 3

Ad26.M.Env vaccination prevents viral replication in blood and dissemination of ZIKV to tissues in pregnant dams. Pregnant Ad26.M.Env vaccinated (n=9) and sham vaccinated (n=5) macaques were challenged with $1x10^3$ PFU of ZIKV at 6 weeks following conception and sera was monitored longitudinally for 100 days following challenge. ZIKV log10 copies per mL of sera from (**a**) Ad26.M.Env vaccinated dams and (**b**) Sham vaccinated dams were determined by QRT-PCR and depicted as log10 ZIKV copies/mL sera. The limit of detection of this assay was 100 copies/mL sera (2Log10). Number of weeks in (**a**) indicate the time between immunization and challenge. Cesarian sections were performed 10-14 days prior to estimated full-term

delivery dates. Lymphoid and reproductive tissues were collected from dams and viral RNA determined by RT-PCR for (**c**) Ad26.M.Env vaccinated and (**d**) sham vaccinated pregnant macaques.

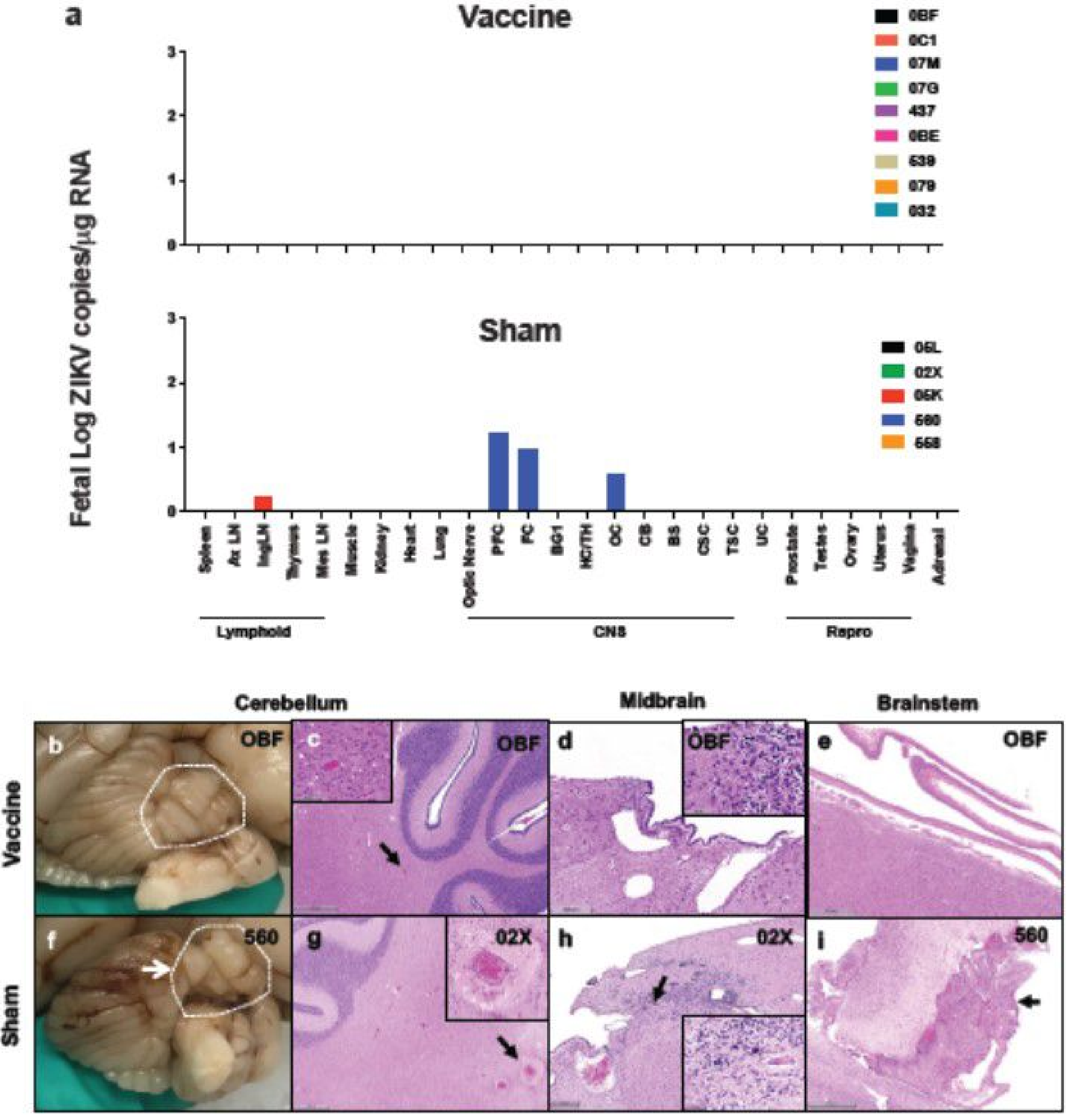


Figure 4

Ad26.M.Env vaccination prevents ZIKV dissemination and tissue pathology in fetuses following ZIKV challenge of pregnant dams. Necropsy and collection of fetal tissues were performed on fetuses following euthanasia after term Cesarian delivery. **(a)**RT-PCR was performed on fetal tissues in fetuses from

Ad26.M.Env vaccinated and ZIKV-challenged dams **(upper)** and sham vaccinated and ZIKV-challenged dams **(lower)**. Gross and histopathological changes in fetuses from Ad26.M.Env vaccinated and ZIKV challenged dams **(b-e)** as compared to sham vaccinated and ZIKV challenged dams **(f-i)** showing normal cerebellar folio **(b)**, vasculature within cerebellum **(c)**, midbrain progenitor cells **(d)**, and meninges **(e)** as compared to cerebellar dysplasia **(f)**, perivascular edema of cerebellar vessel **(g)**, microcalcification within midbrain progenitor cells **(h)**, and unusual thickening of the meninges **(i)**in macaque infants from sham vaccinated dams. Ax LN= axillary lymph node; Mes LN= mesenteric lymph node; PFC=prefrontal cortex; FC=frontal cortex; BG1= basal ganglia section 1; HC/TH= hippocampus, thalamus; OC= occipital lobe; CB=cerebellum; BS= brain stem; CSC=cervical spinal cord; TSC= thoracic spinal cord; UC=umbilical cord.

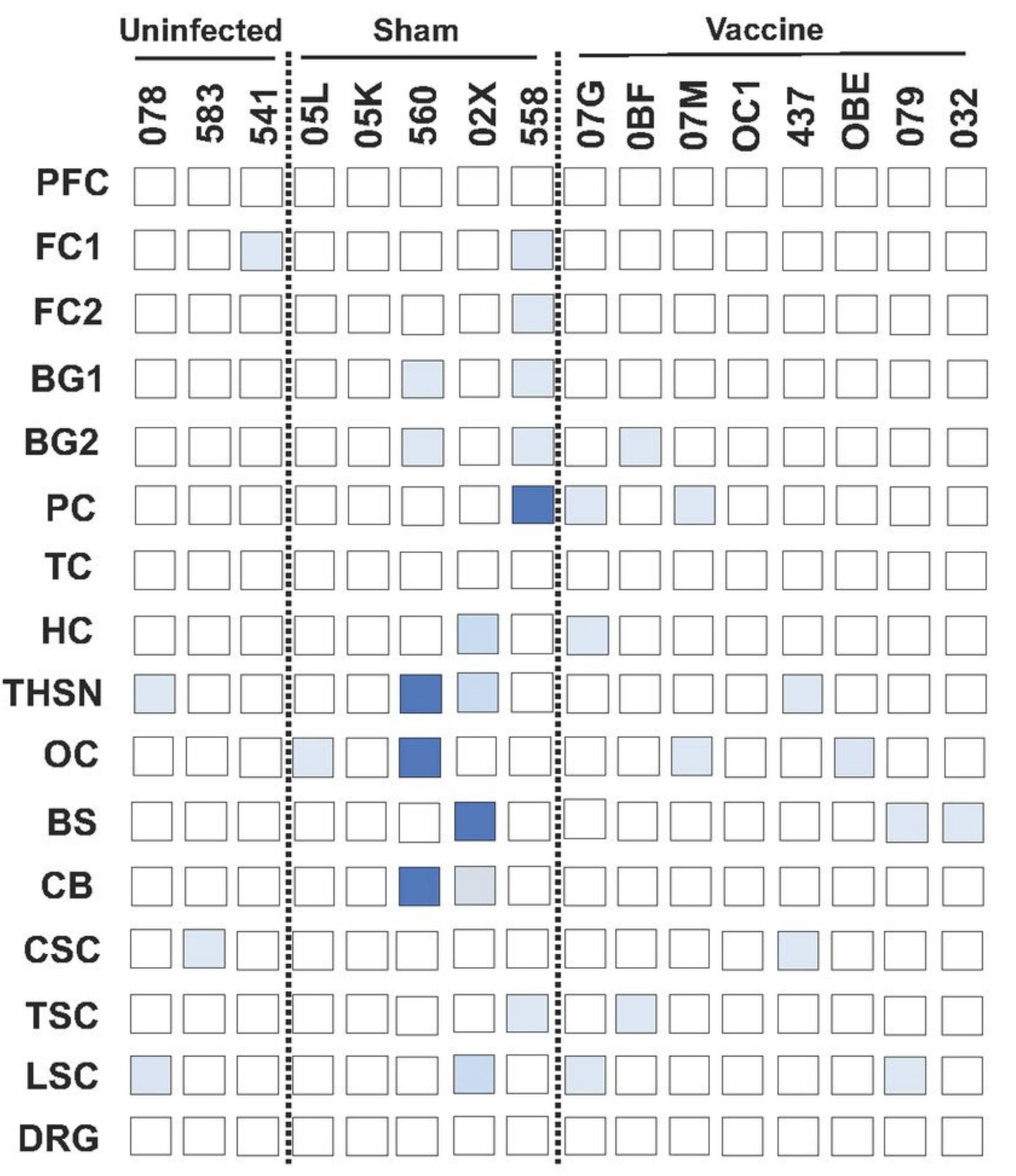


Figure 5

Histopathologic scoring for neuropathology in macaque infants. Summary of neuropathologic lesions in the CNS (see also **Table 1**). Scoring system is defined as previously described (). PFC, prefrontal cortex; FC, frontal cortex; BG, basal ganglia; TH, thalamus; SN, substantia nigra; HC, hippocampus; OC, occipital cortex; PC, parietal cortex; TC, temporal cortex; CB, cerebellum; BS, brain stem; CSC, cervical spinal cord; TSC, thoracic spinal cord.

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Supplementary Files

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