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Generation of a thermostable, oral Zika vaccine that protects against virus challenge in non-human primates

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ABSTRACT

Here we report the development of a thermally stable, orally administered, candidate Zika vaccine using human serotype 5 adenovirus (AdHu5). We engineered AdHu5 to express the genes for the envelope and NS1 proteins of Zika virus. AdHu5 was formulated using a proprietary platform, OraPro, comprising a mix of sugars and modified amino acids that can overcome elevated temperatures (37 C), and an enteric coated capsule that protects the integrity of the AdHu5 from the acid in the stomach. This enables the delivery AdHu5 to the immune system of the small intestine. We show that oral delivery of AdHu5 elicited antigen-specific serum IgG immune responses in a mouse model and in a non-human primate model. Importantly, these immune responses were able reduce viral counts in mice and to prevent detectable viraemia in the non-human primates on challenge with live Zika virus. This candidate vaccine has significant advantages over many current vaccines that are maintained in a cold or ultra-cold chain and require parenteral administration.

1. Introduction

Zika is a member of the *Flaviviridae* virus family and of the same genus as the West Nile, yellow fever, dengue, and Japanese encephalitis viruses[1]. In 2015–2016 a Zika epidemic was declared by the World Health Organisation (WHO) after a rapid increase in cases was observed in Brazil and other countries. It was estimated that 1.5 million people were infected resulting in 3500 cases of infant microencephaly and cases of Guillain-Barré syndrome in adults [2,3]. Although the virus is spread mainly by mosquitoes, it is also transmitted by sexual contact and in several cases after blood transfusion [4,5].

Immunisation remains the most cost-effective strategy for the prevention of infectious diseases including Zika[6]. As such there is a continuing need to design safe, effective vaccines targeting emerging diseases.

Previous studies by Martinez et al 2020[7] using mouse models. have shown that rectal inoculation with Zika virus results in viremia with subclinical infection. In this setting Zika virus infects the mucosal epithelium and submucosal dendritic cells, inducing immune and inflammatory cell infiltration. Rectal transmission of Zika virus resulted in the generation of serum-neutralizing antibody responses. Furthermore, immunological priming through the rectal mucosa with an attenuated Zika virus strain resulted in significant protection from lethal subcutaneous Zika virus challenge, further eliciting robust memory CD4-positive ($CD4^+$) and $CD8^+$ T-cell and Zika virus-specific serum-neutralizing antibody responses. This highlights a rational strategy for mucosal immunization.

The WHO recommends where possible, non-parenteral (non-injected) route as preferred for ease of rapid administration[8]. Orally administered vaccines have advantages compared to traditional injection-based formulations[9], including improved safety, ease of distribution and reduced administration costs. The first orally administered vaccine was the Bacillus Calmette-Guérin (BCG) vaccine developed in the 1920 s for the prevention of tuberculosis[10] and the first orally administered adenovirus vaccine was a live non-attenuated virus vaccine developed in the 1950 s which demonstrated 95% efficacy in preventing adenovirus types 4 and 7 respiratory illness in USA military personnel with no reported significant adverse events.

Studies with SARS-COV2 have shown that oral vaccine administration stimulates humoral and cellular immune responses at both systemic and mucosal sites, leading to a broader immune response [11]. Despite the appeal of oral vaccination multiple hurdles exist

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such as successful delivery of intact and active antigen to the intestine, transport across the mucosal barrier; and subsequent activation of antigen-presenting cells [12,13,14] This makes oral delivery challenging, as formulations must overcome the harsh gastrointestinal environment, and avoid immune tolerance, to achieve adequate protection[15]. Furthermore, thermal stability and the removal of the need of a cold chain are key factors for successful vaccine dissemination.

In the following study we report on the development of an oral vaccine against Zika by engineering a replication-deficient adenovirus 5 vector (AdHu5) with genes expressing the envelope (Env) and non-structural (NS1) proteins of the Zika virus. AdHu5 was formulated into a thermostable dry powder and packaged into capsules with an enteric coating forming a new oral vaccine delivery platform, OraPro. The vaccine, OraPro-Zika was thermally stable and provided a robust immune response as measured by antigen-specific ELISA. Importantly, in mice, the vaccine reduced infection and disease and prevented viraemia in a non-human primate challenge model.

Taken together our data suggests this platform may be able to negate the requirement for a cold chain and parenteral administration simplifying the logistics for transporting vaccines to all areas of the world.

2. Materials & methods

2.1. Vaccine construction

The orally administered Zika virus vaccine (OraPro-Zika) was based on a non-replicating human adenovirus type 5 (AdHu5) (E1/E3 deleted) expressing Zika transgenes encoding the Envelope (Env) and non-structural protein 1 (NS1) under control of a constitutive cytomegalovirus (CMV) promoter (hAdV5-CMV-Env-NS1). The vector contained a synthetic full-length Env and NS1 fusion protein sequence (GenBank: KJ776791.2), separated by a signalase cleavage site, with added Kozak consensus sequence, spliced into the Gateway AdHu5 backbone (Thermofisher).

Synthetic Zika transgene cassette containing Env-NS1 genes under a pMA backbone was purchased from GeneArt. PCR was performed to amplify Kozak-Env-NS1 transgene using infusion primers:

_IF Forward 5′-AAAAAAGCAGGCTTTAGGAGGTACCCACCATGAT CAG-3′

_IF Reverse 5′-AAGCTGGGTCTAGATCTAGGATCCTGCAGTCAC CATTG-3′

and ligated into the Gateway shuttle plasmid pENTR 1A (Thermofisher) using In-Fusion® HD Cloning Kit (Takara Bio). Preparation of vaccine plasmids was conducted by recombination of the shuttle vector into an equivalent pAd/CMV/V5-DEST plasmid resulting in pAd5-FP-GW (Supplementary Fig. 1). The authenticity of the construct was confirmed by full length sequencing.

2.2. Recovery of recombinant Ad5- expressing ZIKV antigens

Recovery of recombinant Ad5-FP-GW from the infectious clone was carried out by transfection of a plasmid encoding E-NS1 genes into HEK293 cells. Post-transfection (96 h), cell culture medium was collected and filtered through a 0.2-μm filter, and the cell pellet was extracted via freeze/thaw in liquid nitrogen. The recombinant virus Ad-Zika was further amplified in HEK293 cells for seed stocks. Viral titre was determined by a median tissue culture infectious dose ($TCID_{50}$) assay performed in HEK293 cells.

2.3. Thermal stability of adenovirus vectors

The thermostabilised Ad-Zika vaccine was generated as previously described[16] forming the OraPro-Zika vaccine. In summary, the excipient formulation was filtered through a 0.22-μm filter and then admixed with high titre Ad-Zika (vaccine) or Ad-GFP (placebo) ($>10^{10}$ infectious forming units (IFU)/ml) measured using an Adeno X™ assay according to the manufacturer's instructions (Takara Bio Inc)). Once mixed, the solutions were lyophilised according to an optimised lyophilisation cycle. Post lyophilisation powders were recovered with a sterile micro spatula. $TCID_{50}$ assays were conducted on the post lyophilised powders for each adenovirus preparation. To assess thermostability different batches of post lyophilisation powders were assessed for moisture content by Karl Fisher titration (MT -V30). The mass of powder in each vial was determined prior to storage at two temperature ranges (2–8 and 25 °C) in thermal challenge chambers. Samples were then removed at time intervals (days 0, 41, 92 and 105), rehydrated using water for injection (WFI) and assessed for IFU/mg using an Adeno X™ assay according to the manufacturer's instructions (Takara, Bio Inc). IFU/ml results were normalised to IFU/mg of vaccine powder based on the rehydrated mass (mg) to effectively compare the results.

2.4. Enteric coating dissolution

Capsule doses for the non-human primate studies described below were prepared using size 5 gelatine capsules which were powder-filled and coated with an enteric protective polymer Eudragit L100-55 (Rohm Pharma GMBH, Germany) to give a coat density 8 mg/cm^2. To assess capsule integrity, coated capsules were filled with Coomassie Brilliant Blue R250 dye and dissolution evaluated using a type 2 paddle dissolution apparatus with a sinker at 37 °C to model the digestive tract. Capsules were exposed to 1 M hydrochloric acid, pH 1.0 for 4 h, and 1hr in phosphate buffer (pH 6.8). Dye release was measured by spectrophotometric analysis (595 nm) of the dissolution medium, sampled over time. The 4-hour acid dissolution phase was selected as an extreme model of stomach residency and was aligned to human Heidelberg Capsule analysis[17].

2.5. Production of live Zika virus

A low passage clinical isolate of ZIKV (HS-2015-BA-01) from a viraemic patient with symptomatic ZIKV infection in Bahia State/Brazil in 2015 was used in this study. The complete genome of the virus is under access KX520666 in GenBank. ZIKV stocks were propagated in C6/36 Aedes Albopictus cells after inoculating at a multiplicity of infection (MOI) of 0.1 and harvesting supernatants after 96 h. Virus stocks were titrated by plaque assay. The plaque assay consisted of serial dilution of samples for adsorption in Vero cell monolayers, for an hour. Samples were removed, following the addition of an overlay media containing 1.5% w/v carboxymethylcellulose (Synth, São Paulo, Brazil) in DMEM containing 2% (v/v) foetal bovine serum (FBS). After 5 days, plates were fixed, washed, and stained with Methylene blue (Synth, São Paulo, Brazil) 1% w/v. Results were expressed as plaque forming units (PFU)/ mL of supernatant or serum.

2.6. Animal studies

Animal studies were carried out in strict accordance with the regulations on ethical and animal experiments of the Brazilian Government (law 11794/2008). The experimental protocol was approved by the Committee on Animal Ethics of the Universidade Federal de Minas Gerais (CEUA/UFMG, permit protocol (CT no).

023 & 092 /2017). All surgeries were performed under ketamine xylazine (15 mg/kg) anaesthesia, and all efforts were made to minimize animal suffering. Studies with Zika virus were conducted under biosafety level 2 (BCL2) containment at Laboratorio de Interação Microorganismo-Hospedeiro and Laboratório de Imunofarmacologia from Instituto de Ciências Biológicas (ICB) at Universidade Federal de Minas Gerais with Institutional Biosafety Committee approval.

Two *in vivo* murine Zika viral challenge studies were conducted together with a non-human primate model (NHP) as previously described [18,19,20].

2.6.1. OraPro-Zika vaccine in murine models SV129 and A129(ABR)

For *in vivo* experiments, wild-type (WT) mice (SV129) and mice deficient in type I interferon receptor (IFN-$\alpha/\beta R^{-/-}$) on the SV129 background, were used. IFN-$\alpha/\beta R^{-/-}$ mice (ABR) and their congenic WT controls (SV129/Ev) were originally purchased from B & K Universal Limited UK. Animals were pre-dosed with sodium bicarbonate for 30 min to neutralise stomach acid prior to oral gavage with the lyophilised vaccine (1×10^8 IFU) or placebo, rehydrated in sterile WFI (Gibco UK) (n = 7 per treatment arm) [21,22]. Mice were vaccinated on days 0, 14 and 28. Blood serum samples were taken pre-vaccination and 12, 26 and 35 days post vaccination by superficial tail bleed. Viral challenge was performed by intravenous (tail vein) administration of 4×10^5 PFU of Zika virus (strain HS-2015-BA-01, Genbank Accession Number KX52066-A) 14 days after the last vaccination. Blood sampling by tail vein was conducted during the study and vena cava exsanguination post-viral challenge. Adverse effects characterised by the appearance of clinical signs such as ruffled fur, ataxia, partial or complete hind limb weakness or paralysis, and excessive body weight loss (>20%) were monitored daily. All experiments using ABR mice were terminated on day 7 post challenge[19].

2.6.2. Assessment of changes in intraocular pressure

During the study intraocular pressure (IOP) was used as biomarker of productive Zika virus infection and performed on mice at days 0, 3, and 5 after Zika virus challenge using a Tono-Pen Vet applanation tonometer (Reichert Technologies, NY) as previously described[23].

2.6.3. Histopathology to determine Zika infection

Brains from control and Zika virus-infected mice were collected at post-mortem and processed for haematoxylin and eosin (H&E). Histopathological scoring was performed as previously described[19]. Scoring was blinded to remove subjective bias. Histopathological scoring was performed in cerebral cortex and hippocampus. Each area was graded as follows: 0, no damage; 1, minimal tissue destruction and/or mild inflammation/gliosis; 2, mild tissue destruction and/or moderate inflammation/gliosis; 3, definite tissue destruction (neuronal loss and parenchymal damage) and intense inflammation; 4, necrosis (complete loss of all tissue elements with associated cellular debris). Meningeal inflammation was graded following a 0- to 4-, with 0 representing no inflammation and 1 to 4 corresponding to 1 to 4 cell layers of inflammation, respectively. The final score was calculated as a sum of cerebral cortex and hippocampus scores added to the score obtained from the meningeal inflammation analysis, totalizing a maximum of 12 points.

2.6.4. OraPro-Zika vaccine in non-human primates

Three groups of *Callithrix Penicillata* (Black-Tufted Marmosets) were used. Each group had two subjects one male, one female. Groups one and two contained Zika naive animals that were orally dosed with enteric-coated capsules, negating the need for stomach acid neutralization, and containing 1.5×10^8 IFU of OraPro-Zika or placebo (hAdV5-GFP) on Days 1 and 13. The third group consisted of convalescent animals previously infected with zika virus that acted as a positive control. All animals were challenged subcutaneously with 5×10^5 plaque forming units (PFU) of Zika virus (clinical isolate HS-2015-BA-01) on Day 23.

2.6.5. Collection and storage of non-human primate biological samples

Blood samples were collected from the femoral vein in EDTA containing tubes on day 0, 13 and 23, prior to zika virus challenge, and on days 3,4,5,6,9,10 post challenge. Samples were divided in two. Half was stored in −70 and half was destined to RNA extraction which was made on the same day of collection. Transaminase analysis (ALT) levels were determined on day 0 and day 23 (post vaccination) and day 3, 4, 5, 6 and 9 post challenge with zika virus.

2.6.6. Analysis of copy number by PCR amplification in non-human primates

RNA was extracted from the serum of the infected marmosets using the viral RNeasy Mini Kit® (Qiagen, Heiden, Germany) following the manufacturer's recommendations. One-step qRT-PCR One-step qRT-PCR was performed using QuantiNova Probe RT-PCR Kit (Qiagen, Heiden, Germany). Primers/probe sets specific for ZIKV were designed as described by Lanciotti et al. 2008 (ZIKV 835 5′- TTGGTCATGATACTGCTGATTGC-3′; ZIKV 911c 5′-CCTTCCACAAAGTCCCTATTGC-3′; ZIKV 860-FAM 5'-FAM CGGCATACAGCATCAGGTGCATAGGAG-NFQ/MGB-3' - Integrated DNA technologies®). Amplification was performed on an ABI 7500 System (Applied Biosystems, Foster City, CA) using the following procedure: 45 °C for 10 min, 95 °C for 5 min, and 45 cycles at 95 °C for 5 s and 60 °C for 30 s. All PCR reactions were accompanied by a standard curve of viral RNA copies and a no-template control. Results were expressed as relative number of genome copies of ZIKV per sample. Viremia was considered when at least half of the infected animals had detectable viremia.

2.7. ELISA assays

2.7.1. Murine

Antigen specific, total IgG responses were measured by ELISA. Recombinant Zika NS1 or Env proteins in phosphate buffered saline (PBS) were coated on Maxisorb plates for 1 h at room temperature. Each plate was washed 3 times with PBS containing Tween 20 (0.05% (v/v)) and then blocked with 2.5% (w/v) BSA for 2 h before being rewashed. The murine serum samples were diluted 1:1000 in 10% BSA (w/v) and applied to the plates for 15 mins at room temperature. After further washing, plates were incubated for 2 h with polyclonal rabbit anti-mouse immunoglobulin – Peroxidase antibody (P0260 Dako, 1:2000). Plates were washed and tetramethylbenzabene (TMB) (50 µl) (Dako) added as a substrate for horse radish peroxidase (HRP) development. The reaction was stopped after 15 min by addition of 0.2 M NaOH (100 µl). Absorbance at 450 nm was measured for each sample.

2.7.2. Non-human primate

IgG titres in the serum of the infected marmosets were estimated by indirect ELISA. Briefly, Zika virus (HS-2015-BA-01) was UV-inactivated and diluted in 0.01 M carbonate buffer (pH 9.6) to a concentration of 5×10^5 IFU per well and incubated overnight at 4 °C. The plate was washed 3 times and blocked with PBS containing 1% non-fat milk powder for 2 h, then washed as before. The serum was diluted (1:100), plated in duplicate, and incubated for 3 h at room temperature, followed by a further wash step. Plates were subsequently incubated for 2 h with an anti-human IgG (Fc specific) – Peroxidase antibody (A0170 Sigma) (1/2000), washed, and developed as described above.

2.8. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8.3.0 The analyses used for each dataset are described in the figure caption. IOP, infectious virus titres (assessed by PFU or IFU) and virus levels upon challenge by qRT-PCR were compared using Mann-Whitney U. The significance values compared to the placebo or control group are shown in each figure. One way ANOVA with Dunnett's test for multiple comparison was used to assess change in virus specific IgG. Percentage weight changes in mice post-Zika virus challenge were compared by two-way ANOVA, comparing vaccinated to placebo control group. P values are shown in each Figure. All p values are 2-tailed, and significance levels are denoted as: **** P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, or not significant (ns).

3. Results

3.1. OraPro-Zika after lyophilisation and thermal challenge remains infectious

To assess the thermal stability of OraPro-Zika, lyophilised samples were challenged at 2–8 °C and 25 °C over a time course of 109 days. Vaccine infectivity was measured by IFU assays. A high degree of thermal tolerance was evident (Fig. 1). No significant difference was observed in infectious virus after storage at 2–8 °C over the time course. A minimal but statistically significant drop was evident after 109 days at 25 °C (one way ANOVA with Dunnett's test for multiple comparison p = 0.0012) but this was well below the pre-requisite 1 log loss cut off. Taken together these data show OraPro-Zika exhibited good thermal stability.

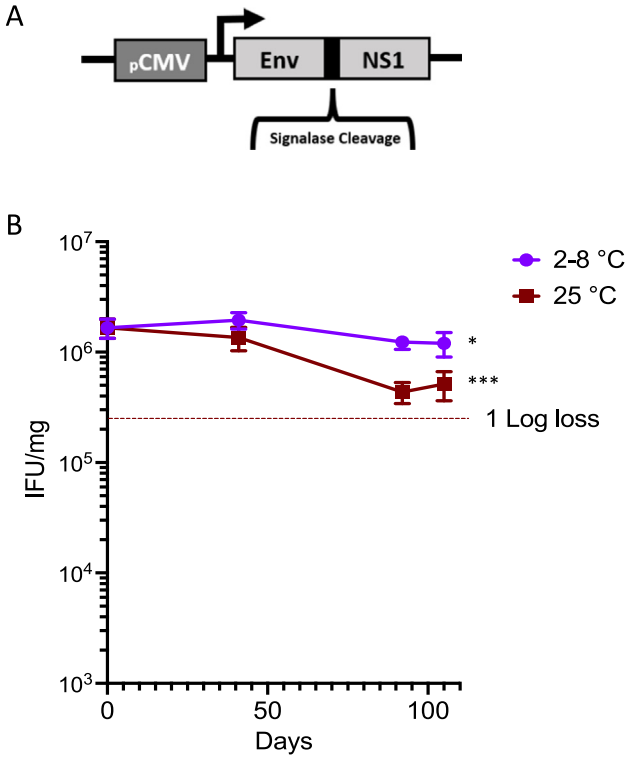


Fig. 1. Assessment of lyophilised adenovirus (Ad5) OraPro-Zika thermostability. **A** Schematic representation of the OraPro-Zika cassette within the Ad5 backbone. Genes encoding envelope (*ENV*) and non-structural protein 1(*NS1*) were cloned downstream of the CMV promoter. **B.** Lyophilised vaccine was temperature challenged at 2–8 °C and 25 °C for 109 days. Infectious integrity was assessed by AdenoX and expressed as infectious viral particles (IFU) per mg of lyophilised powder. Data show is the average of n = 3 biological replicates. Bars equal ± standard deviation (SD). Statistical analysis for significance was conducted using one way ANOVA with Dunnett's for multiple comparisons.

3.2. Enteric coated capsule dissolution modelling

To test the integrity of the OraPro-Zika vaccine for oral delivery, dissolution assays were conducted to model the transit of the vaccine through the stomach and release into the duodenum. Dissolution testing is widely used as an analytical technique for evaluating the drug release characteristics and consistency of a pharmaceutical product. Stabilised OraPro-Zika vaccine was lyophilised, packaged into gelatine capsules and coated with an enteric polymer. Dissolution assays were used according to United States Pharmacopeia (USP) guidelines (ref). After exposure to pH conditions associated with the stomach and duodenum (Fig. 2), the capsules remained intact and acid-resistant for over 250 min. Maximum release of capsule contents was evident within 30 min of exposure to pH conditions representative of the duodenum. Expression of each antigen was confirmed by dot blots of infected cell lysate (data not shown).

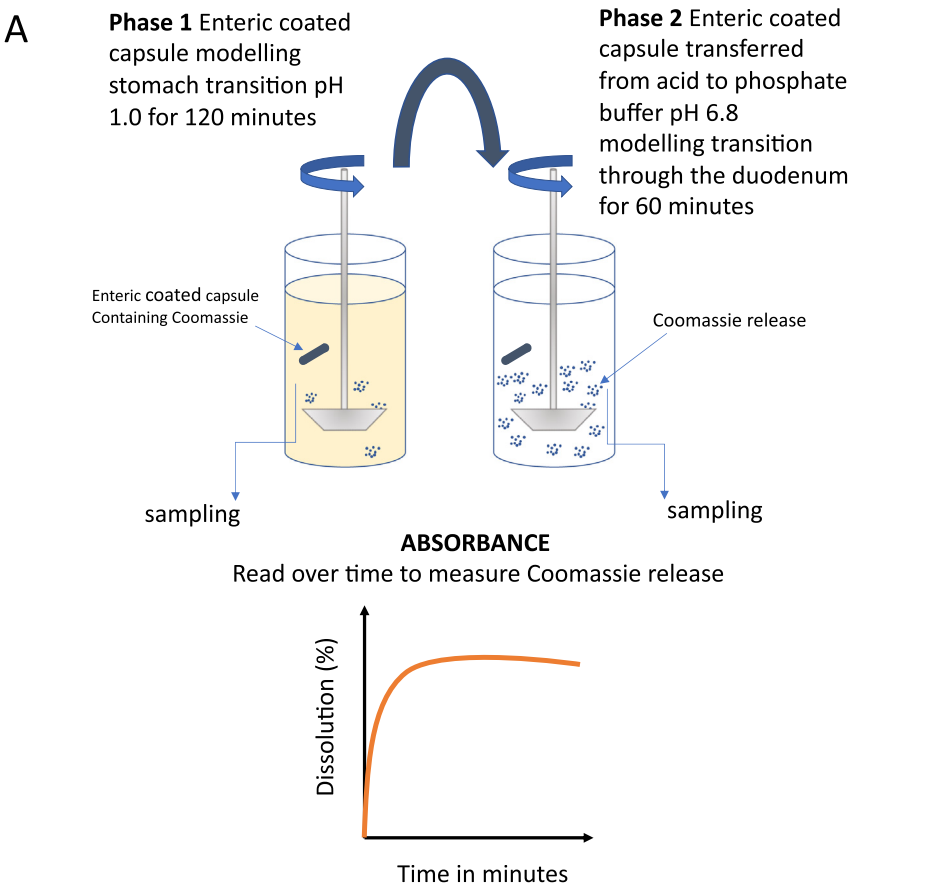
3.3. OraPro-Zika induces antibodies against Zika virus NS1 and Env antigens in mice

Immunocompetent mice (SV129) and their interferon deficient (IFN-α/βR$^{-/-}$) derivatives ABR were chosen as models to test the protective efficacy of the OraPro-Zika vaccine. In this setting SV129, which are non-permissive for Zika virus replication were used as a negative control whilst ABR (IFN-α/βR$^{-/-}$) which support replication of live Zika virus[45] was used to test vaccine utility upon challenge with live virus. Groups of mice (n = 7) received 3 doses of vaccine or placebo by oral gavage after sodium bicarbonate stomach acid neutralisation (Fig. 3A).

Importantly no adverse reactions were evident during or after vaccine administration. Sera were obtained pre- (day 3) and post-oral administration of the vaccine on days 12, 26 and 35. Sera were assessed for the presence of IgG antibodies against Zika virus NS1 or Env proteins by ELISA. ABR (IFN-α/βR$^{-/-}$) mice showed a significant rise in antibody levels against the Env and NS1 proteins by day 12 (one way ANOVA with Dunnett's test for multiple comparisons' p > 0.0001) compared to pre-vaccination. However, levels declined after the second and third dosing, but continued to be higher than pre-immune samples (Fig. 3 B-C). Concordant with their immunocompetent phenotype SV129 mice showed a rise in antibody levels against Env after three doses (p = 0.0063). Anti-NS1 IgG peaked after the second dose (p = 0.0065) and remained elevated after dose three (p = 0.05) (Fig. 3 D-E). These data suggested the vaccine could elicit an authentic immune response.

OraPro-Zika vaccine helps prevent increases in intraocular pressure and weight loss after infectious Zika virus challenge.

Previous studies in ABR (IFN-α/βR$^{-/-}$) mice infected with Zika virus have shown substantial weight loss and alterations in intraocular pressure (IOP) can act as surrogate biomarkers of disease progression [24,25] In the following study both SV129 (immunocompetent) and ABR (IFN-α/βR$^{-/-}$) mice were challenged with Zika virus post vaccination and alterations in weight and ocular pressure measured at time points over 6 days. As expected, no significant weight loss was evident in either the SV129 placebo or OraPro-Zika vaccinated groups concordant with the inability of this model to support optimal Zika virus replication (Fig. 3F). Contrastingly, ABR (IFN-α/βR$^{-/-}$) mice which are capable of supporting Zika virus replication showed a significant loss of body mass in the placebo group compared to the vaccinated group over 6 days (two-way ANOVA p = 0.0002) (Fig. 3F).



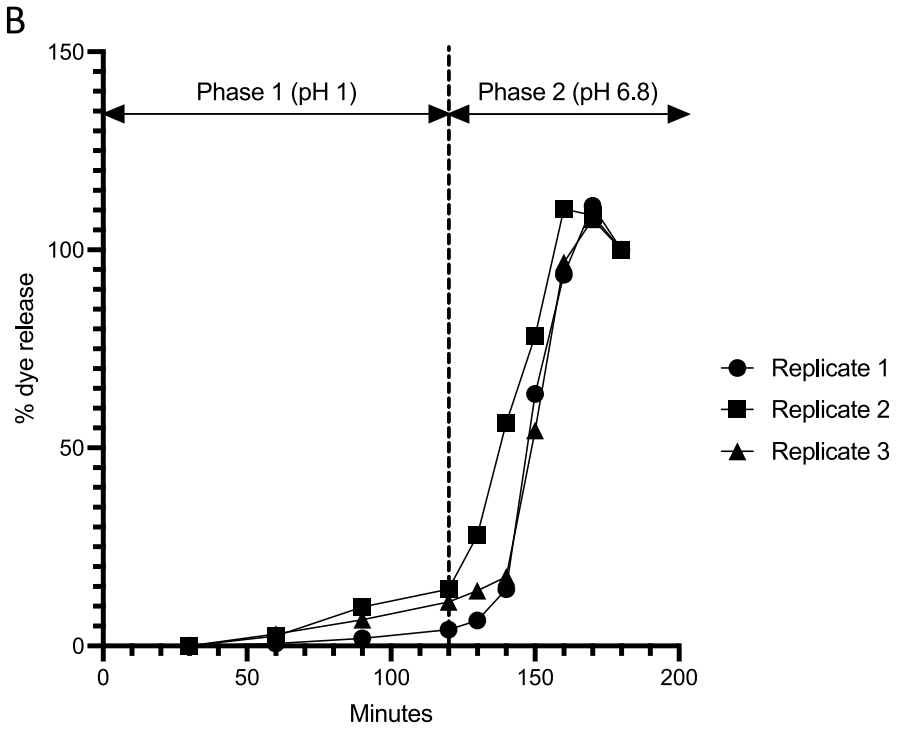
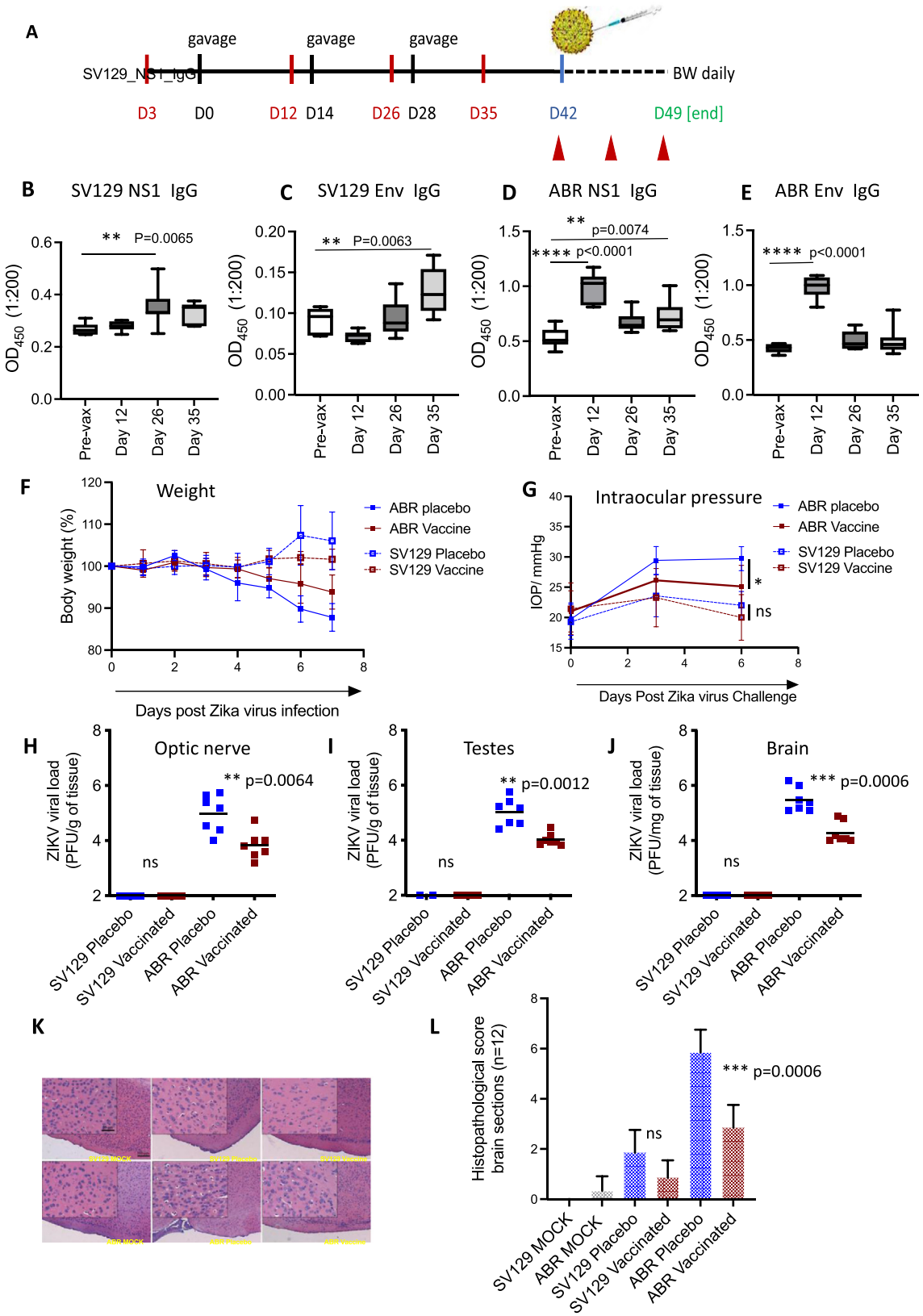


Fig. 2. Dissolution assay to test the integrity of enteric-coated capsules modelling transit through the gut. A. Schematic diagram of the assay. Enteric-coated gelatine capsules containing lyophilised OraPro-Zika and Coomassie blue were exposed to acidic conditions modelling the stomach (pH 1.0) for 120 min followed by 60 min exposure to phosphate buffer modelling the duodenum (pH 6.8). Samples were taken over time and Coomassie dye release measured by spectrophotometry. B. Data are presented as % dye release over time for n = 3 individual biological assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)


A
gavage
gavage
gavage
SV129_NS1_IgG
BW daily
D3
D0
D12
D14
D26
D28
D35
D42
D49 [end]
B
SV129 NS1 IgG
** P=0.0065
OD450 (1:200)
Pre-vax
Day 12
Day 26
Day 35
C
SV129 Env IgG
** P=0.0063
D
ABR NS1 IgG
**
p=0.0074
**** p<0.0001
E
ABR Env IgG
**** p<0.0001
F
Weight
Body weight (%)
Days post Zika virus infection
ABR placebo
ABR Vaccine
SV129 Placebo
SV129 Vaccine
G
Intraocular pressure
IOP/ mmHg
Days Post Zika virus Challenge
*
ns
H
Optic nerve
ZIKV viral load (PFU/g of tissue)
** p=0.0064
ns
SV129 Placebo
SV129 Vaccinated
ABR Placebo
ABR Vaccinated
I
Testes
** p=0.0012
J
Brain
ZIKV viral load (PFU/mg of tissue)
*** p=0.0006
K
L
Histopathological score brain sections (n=12)
*** p=0.0006
SV129 MOCK
ABR MOCK

Assessment of IOP in ABR (IFN-α/βR$^{-/-}$) mice after challenge showed those receiving placebo presented a significant rise in IOP over six days, compared to those vaccinated with OraPro-Zika (p = 0.01 by Mann-Whitney, Fig. 4G). Of note, although immunocompetent SV129 mice showed no significant difference in IOP between placebo and OraPro-Zika there was trend towards an increase in the placebo group. Taken together these data show OraPro-Zika provided protection against challenge with live virus in a highly permissive murine model.

OraPro-Zika reduces Viral titre in murine challenge models.

To further assess the ability of OraPro-Zika to prevent infection we conducted Zika virus titrations (expressed as plaque-forming units, PFU) on tissue samples from the brain, optic nerve, and testes of euthanised mice 6 days post-challenge. Placebo treated ABR (IFN-α/βR$^{-/-}$) mice showed significantly greater numbers of PFU in each post-mortem tissue on a weight basis compared to OraPro-Zika vaccinated animals (Mann-Whitney, brain p = 0.0006; optic nerve p = 0.0064 and testes p = 0.0012) (Fig. 3 H, I, J respectively). Non-permissive SV129 showed no difference in viral titre between the placebo and OraPro-Zika vaccinated animals. Lastly, histological analysis was conducted to assess brain lesions and aberration within the placebo and OraPro-Zika groups after challenge with live Zika virus in both SV129 and ABR (IFN-α/βR$^{-/-}$) mice. As expected, ABR (IFN-α/βR$^{-/-}$) mice that received OraPro-Zika showed significantly lower numbers of histological brain abnormalities (p = 0.0006) compared to placebo control (Fig. 3 K & L). As expected, immunocompetent SV129 mice showed no significant difference between vaccinated and placebo groups consistent with their non-permissive phenotype. Taken together these data suggest that OraPro-Zika may be effective at reducing the pathological effects of live Zika virus.

OraPro-Zika vaccine prevents viraemia in non-human primates challenged with Zika virus.

To model the likely clinical scenario, we assessed the efficacy of OraPro-Zika in non-human primates. Three groups of Black-Tufted Marmosets (*Callithrix Penicillata*) were used. The first two were naive to zika infection whilst the third were a convalescent group previously exposed to zika virus infection which acted as a positive control for the study. The naive animals were immunized orally with either placebo or OraPro-Zika using enteric coated capsules negating the need for stomach acid neutralization (Fig. 4A). Two animals per group (one male, one female) received two capsular doses, 12 days apart. Importantly, no adverse reactions were evident, including vomiting or diarrhoea, based upon daily observations. Additionally, transaminases analysis (ALT) levels in serum was similar to baseline in all experimental groups suggesting the absence of side effects of vaccination (Supplemental Figure S2). Serum samples were collected on days 1, 12 and 22, which on the timeline equated to pre-treatment, 12-days post first dose, 9-days post second dose followed by days 3 to 9 post challenge (Fig. 4A). Whole-inactivated virus specific IgG antibody concentrations were measured by ELISA (Fig. 4B). To compare the placebo and OraPro-Zika groups, the data was normalised and expressed as fold over pre-treatment levels. Eleven days after the primary administration of OraPro-Zika, anti-Zika IgG levels increased 4.6-fold compared to pre-vaccination. Nine days after administration of dose 2, a 5.6-fold increase was evident relative to pre-vaccination levels. The placebo group showed no substantial rise in anti-Zika IgG at any time after vaccination compared to pre-treatment, (Fig. 4B). To address the clinical significance of the 5.6-fold rise in IgG within the OraPro-Zika cohort we assessed absolute IgG serum levels versus those from convalescent animals previously exposed to Zika virus (Fig. 4C). As expected, no significant difference in IgG levels were noted over the time course in the convalescent animals which showed an average IgG level of 0.14 pg/ml. The vaccinated cohort showed a time-dependent increase in IgG levels reaching a maximum of 0.16 pg/ml. Taken together OraPro-Zika was able to elicit an IgG response in keeping with that seen in animals which had experienced a prior natural Zika infection.

Ten days after the second dose the animals were challenged with a subcutaneous dose of 5×10^5 PFU of live Zika virus. Blood was collected according to the schedule in Fig. 4A. No detectable virus could be found in the blood of animals that received OraPro-Zika or had previously been exposed to zika virus infection and were convalescent after challenge (Limit of detection < 4 copy per ml). In contrast, in the placebo-cohort, Zika viraemia was detected post-challenge in the blood, with copy numbers between ~ 10 and 1000 per mL, increasing over days 2–5, with subsequent resolution by day 8 in keeping with the disease pathology of zika infection (Fig. 4D).

4. Discussion

In this study, we addressed several challenging features attributed to vaccinology, firstly, the development of a thermostable vaccine negating the need for a "cold chain", secondly the potential to deliver the vaccine orally and finally to address this with a clinically meaningful target virus, in this instance Zika. In summary we showed that an orally administered Ad5- vaccine encoding genes for the Zika envelope (Env) and NS1 proteins induced a specific immune response that reduced Zika infection in both murine and NHP models and remained thermally stable for over 109 days at 25 °C.

To demonstrate that our oral vaccine prevented Zika infection we initially undertook studies in an interferon knockout mouse ABR (IFN-α/β R-/-) designed to support active replication of Zika virus upon infection[26] and its corresponding immunocompetent background model SV129. Measurement of IgG against Zika NS1 and Env proteins post vaccination with OraPro-Zika showed a significant rise after preliminary dosing however, on subsequent administration while IgG remained significantly higher compared to pre-treatment the expected "boost" effect was not observed in ABR mice. Contrastingly, in the non-permissive SV129 the trend towards increased anti NS1 and Env IgG was evident. One of the principal concerns regarding the use of viral vectors in particular

Fig. 3. Assessment of the efficacy of the OraPro-Zika vaccine in murine models SV129 and ABR (IFN-α/β R-/-) A. Schematic diagram of the vaccination and sampling schedule. **B.** Assessment of anti-NS1 IgG levels after vaccination of SV129 mice **C.** Assessment of anti-Env IgG levels after vaccination of SV129 mice. **D.** Assessment of anti-NS1 IgG levels after vaccination of ABR mice **E.** Assessment of anti-Env IgG levels after vaccination. Data shown is from n = 7 animals per treatment group. Statistical significance was determined using one way ANOVA with Dunnett's test for multiple comparisons. **F.** SV129 and ABR mice were vaccinated with placebo or OraPro-Zika and challenged with infectious Zika virus. Alterations in weight were measured daily (n = 7 animals per treatment group). Statistical significance was determined by two-way ANOVA. **G.** SV129 and ABR mice vaccinated with placebo or OraPro-Zika and challenged with infectious Zika virus were assessed for changes in intraocular pressure on days 3 and 6 post-challenge. Bars represent ± SD. Statistical significance was determined by Mann-Whitney *U* test (two-tailed) comparing the placebo and vaccine groups in each case. To test the ability of OraPro-Zika vaccination to reduce viral replication after Zika virus challenge, tissue samples at post-mortem **H.** optic nerve, **I.** Testes and **J.** Brain were assessed for the presence of infectious virus using plaque forming assays (PFU) expressed per gram of tissue. Bars represent ± SD of n = 7 animals per treatment group. Statistical significance was assessed by Mann Whitney U (two-tailed) comparing placebo and OraPro-Zika for each murine model. **K.** Histological analysis of brain abnormalities after Zika virus challenge **L.** aberrations were given a cumulative score as described in the methods section totalling a maximum of 12. Data shown is representative of n = 7 animals per group. Bars represent ± SD. Statistical analysis compared vaccinated and placebo groups using Mann-Whitney U (two-tailed).

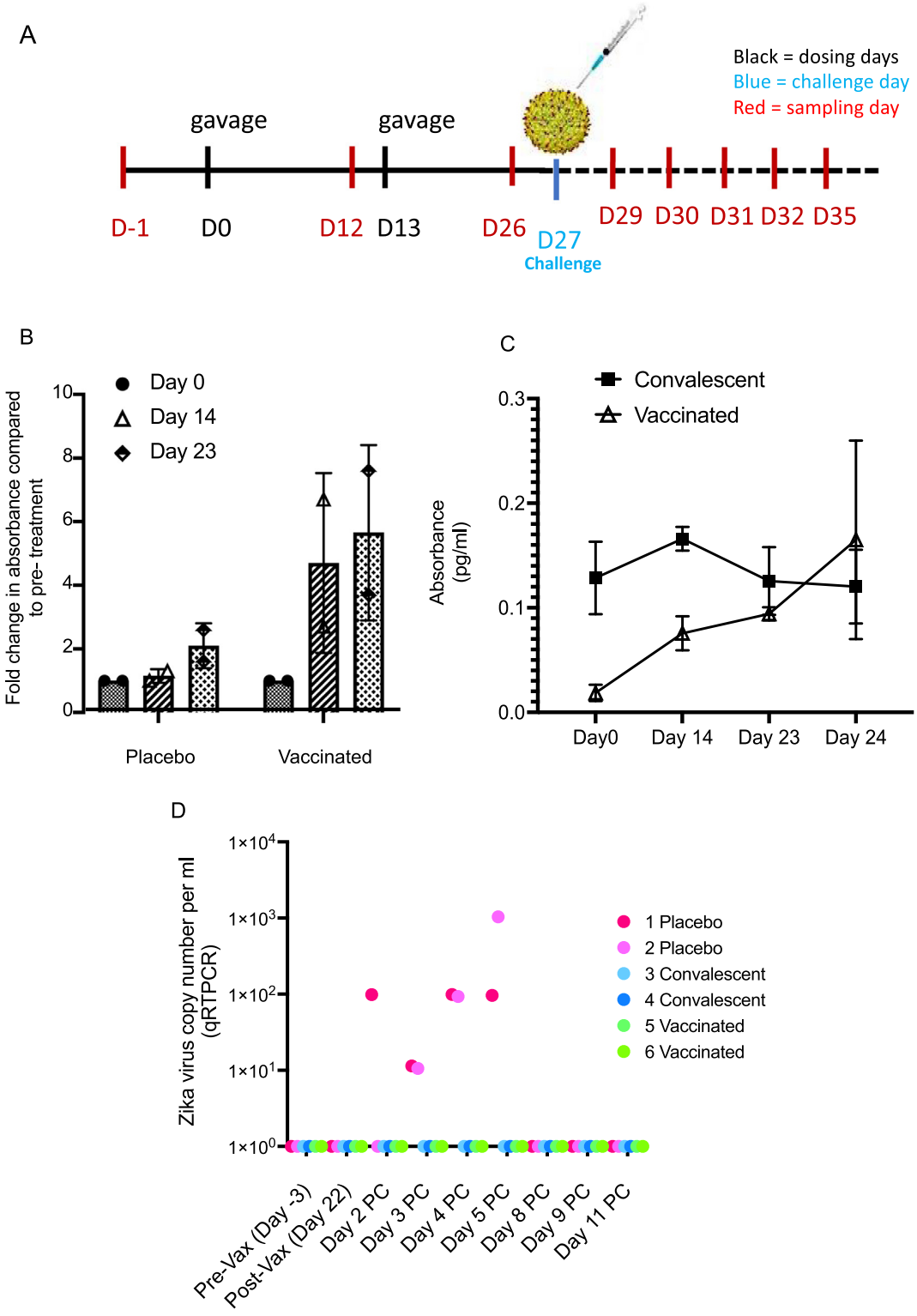


Fig. 4. Analysis of the efficacy of OraPro-Zika in a non-human primate challenge model A. Schematic representation of the vaccination and sampling timeline. **B.** Assessment of anti-Zika virus IgG levels post vaccination with placebo or OraPro-Zika. IgG levels were determined using ELISA against inactivated Zika virus. Data shown is expressed as fold change compared to pre-treatment to normalise for variation between animals (n = 2) Bars represent ± SD. **C.** Assessment of the clinical relevance of IgG levels in OraPro-Zika vaccinated animals compared to IgG levels in serum from convalescent NHP recovering from live Zika infection. D. Analysis of viremia by qRT-PCR in serum from NHP that were convalescent or vaccinated with placebo or OraPro-Zika and subsequently challenged with live Zika virus. Assessment was conducted over 8 days in keeping with Zika virus natural disease resolution.

adenovirus is pre-existing immunity or generation of immunity to the vector itself, limiting the re-use of the vector either as a platform or for boost dose(s). Several studies have shown that adenovirus vectored vaccine constructs induce immune responses after intranasal or intramuscular vaccination [27,28,29], with an accompanying immune reaction to the vector itself.

One study generated pre-existing anti-vector immunity in mice by mucosal delivery of wild-type replication-competent aden-

ovirus type 5[30]. These pre-immune mice were then immunised orally with Ad5 expressing a rabies glycoprotein at doses shown to induce rabies glycoprotein antibodies sufficient for protective immunity. Induction of a protective response was possible, even in the presence of vector-neutralising antibody titres. In addition, orally vaccinated mice had no reduction in anti-rabies glycoprotein titres relative to adenovirus-naive mice[31].

Delivery of a vectored vaccine via mucosal routes is not limited by pre-existing immunity to the vector[32], and this is likely one of the main benefits of using the oral route. The ability to elicit protective responses from ingestion of adenoviral vectors containing expression cassettes encoding antigens has been demonstrated in earlier studies[33].

One explanation for our observation of the lack of a boost in mice is that oral vaccination in the murine model relied on stomach acid neutralisation using sodium bicarbonate, and it is possible this may have been less effective impacting the vaccines integrity. Nonetheless, surrogate biomarkers used to assess protection against challenge with live Zika virus showed that even a single dose of vaccine was sufficient to significantly reduce adverse effects such as the rise of the intraocular pressure and loss of body weight. Similarly, although not statistically significant, the SV129 (immunocompetent) model which is non-permissive for effective Zika virus replication also showed a trend towards decreased intraocular pressure and reduced weight loss in the OraPro-Zika group compared to the placebo. This was also borne out in the histological assessment for abnormalities in post-mortem brain tissue where a reduction of abnormal histological scoring was strongly associated with the administration of OraPro-Zika.

Having demonstrated that OraPro-Zika provided a measurable efficacy in the murine models, we performed a challenge study in NHP to investigate the potential clinical utility. In this setting enteric coated capsules were given orally negating the need for stomach acid neutralisation. Upon vaccination a significant rise in anti-Zika IgG was evident after the first dose concordant with our murine model. Furthermore, the level of IgG was similar to that in convalescent serum from NHP previously infected with zika virus. Most strikingly, in challenge studies convalescent and OraPro-Zika vaccinated animals showed no evidence of replicative Zika virus, whilst the placebo vaccinated cohort showed full blown infection which modelled the disease pathology as it was rectified by day 8 of the study. Whilst we hypothesise the immune protection in our model systems is potentially via a mucosal root our study is limited and does not define cellular mechanisms or sIgA. Nonetheless, IgG and challenge studies remain the gold standard for vaccine efficacy.

Other adenovirus-vectored vaccines using different Zika polypeptide fragments have recently been reported, but unlike OraPro-Zika none have demonstrated convincing evidence of significant protection during live virus challenge following oral administration of the vaccine. (Abbink et al. (2016) [34] Kim et al. (2016) [35] Xu et al (2018) [36] and Guo et al. (2018)[37]).

Our preliminary study with OraPro-Zika suggests oral administration of a non-replicating adenovirus vector provides protection against challenge and warrants further investigation to establish mode of action.

CRediT authorship contribution statement

Jeffrey Drew: Conceptualisation, Writing – original draft, Writing – review & editing

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: JD, AB, JS and PB are employees of iosBio Ltd and hold share options. JD is founder of iosBio Ltd. MT, and VC declare no conflict.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2023.02.055.

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