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Research paper

Live vaccine infection burden elicits adaptive humoral and cellular immunity required to prevent Zika virus infection

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ABSTRACT

Background: The emergence of Zika virus (ZIKV) as an important cause of congenital and childhood developmental disorders presents another challenge to global health. Efforts to develop a Zika vaccine have begun although vaccine development against flaviviruses, of which ZIKV belongs to, has proven to be time-consuming and challenging. Defining the vaccine attributes that elicit adaptive immune response necessary for preventing ZIKV infection could provide an evidence-based guide to Zika vaccine development.

Methods: We used a previously described attenuated ZIKV DN-2 strain in a type-I interferon receptor deficient mouse model and tested the hypothesis that duration of vaccine burden rather than peak level of infection, is a determinant of immunogenicity. We quantified both humoral and cellular responses against ZIKV using plaque reduction neutralisation test and flow cytometry with ELISPOT assays, respectively. Vaccinated mice were challenged with wild-type ZIKV (H/PF/2013 strain) to determine the level of protection against infection.

Findings: We found that the overall vaccine burden is directly correlated with neutralising antibody titres. Reduced duration of vaccine burden lowered neutralising antibody titres that resulted in subclinical infection, despite unchanged peak vaccine viraemia levels. We also found that sterilising immunity is dependant on both neutralising antibody and CD8+ T cell responses; depletion of CD8+ T cells in vaccinated animals led to wild-type ZIKV infection, especially in the male reproductive tract.

Interpretation: Our findings indicate that duration of attenuated virus vaccine burden is a determinant of humoral and cellular immunity and also suggest that vaccines that elicit both arms of the adaptive immune response are needed to fully prevent ZIKV transmission.

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1. Introduction

Arthropod-transmitted flaviviruses are major global health scourges. The prototypic flavivirus, yellow fever virus (YFV), periodically spills out from the sylvatic transmission cycle to cause epidemics in Africa and South America [1]. Dengue virus (DENV) causes an estimated 100 million cases of acute disease annually [2]. The Japanese encephalitis (JE) serocomplex group of viruses, including West Nile virus, cause occasional outbreaks of viral encephalitis in various parts of the world [3]. More recently, the emergence of Zika virus (ZIKV) as a novel cause of congenital and childhood developmental disorders adds to the conundrum [4−6]. As vector control to prevent flaviviral transmission has mostly proven to lack sustainability and is ineffective, as is implemented in many countries, prevention of such flaviviral diseases will need to rely on effective vaccines [7].

Flaviviral vaccine development has had mixed outcomes. The live attenuated YF vaccine is arguably one of the best vaccines in the world − a single dose elicits immunity that lasts for 10 years or more

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Research in context

Evidence before this study

Vaccine development against flaviviruses such as Zika virus has had mixed success despite the identification of numerous potential candidates. A major limitation is a lack of detailed understanding of the vaccine-inherent attributes that shape the development of adaptive immunity against infection. Studies on the successful yellow fever live attenuated vaccine (YF17D) have found that the magnitude of the innate immune response as well as longer duration of vaccine burden correlated with eventual neutralising antibody titre against yellow fever virus. However, whether duration of vaccine burden functionally determines vaccine immunogenicity and efficacy remain to be determined.

Added value of this study

This study highlights the critical role of vaccine burden in the development of adaptive immune responses against Zika virus infection. The study also demonstrates in an A129 mouse model that a successful Zika virus vaccine must elicit humoral and cellular immunity in concert to confer sterilising immunity and prevent Zika virus transmission.

Implications of all the available evidence

Our findings can guide the future development of vaccines against Zika virus and other flaviviruses. Vaccine burden may be useful as a determinant of immunogenicity, allowing for better down-selection of potential candidates. Moreover, vaccines that elicit both humoral and cellular immunity are needed to prevent infection of certain organs such as the testes, highlighting their possible necessity in preventing silent transmission from vaccinated males to unvaccinated females.

[8]. Likewise, both attenuated and inactivated vaccines against JEV as well as the inactivated vaccine against tick-borne encephalitis have also shown useful efficacy [9,10]. However, dengue vaccine development has not been met with similar success. The only licensed dengue vaccine, Dengvaxia®, was found to elicit imbalanced protection against the four dengue virus serotypes. Moreover, Dengvaxia® vaccination in those without prior dengue infection also resulted in increased risk of severe dengue [11,12]. Vaccine development against ZIKV has proceeded, with at least two vaccine candidates having completed phase 1 clinical trial [13,14]. However, whether these Zika vaccine candidates will meet with similar success as YF vaccine or present with problems that prevent widespread use, like Dengvaxia®, is uncertain as the properties that shape vaccine immunogenicity and hence efficacy has remained undefined.

Studies on YF vaccine have found that vaccination activates the innate immune response, including type-I interferon signalling, that shape the magnitude of antibody response [15,16]. We have also recently observed that viremia at day 7 but not day 3 post-vaccination correlated with neutralising antibody titres [17]. These findings suggest that the duration rather than peak level of vaccine burden determines shape vaccine-induced adaptive immunity. We tested this suggestion using a previously reported attenuated ZIKV strain. We found that truncation of vaccine burden in A129 type I interferon receptor-deficient mice reduced both vaccine immunogenicity and efficacy despite unchanged peak vaccine viremia [18]. With lowered neutralising antibody titres from vaccine viremia truncation, challenge with wild-type ZIKV led to four-fold or greater boost in

neutralising antibody titres; no antibody boosting was seen in animals with higher post-vaccination neutralising antibody titres. However, even unperturbed post-vaccination antibody titres were insufficient to prevent ZIKV infection without cellular immunity. Indeed, depletion of CD8$^+$ T cells in vaccinated animals led to detectable infection in the testes and epididymis but not the central nervous system upon wild-type ZIKV challenge. Our findings thus reveal insights on live viral vaccine duration shapes adaptive immunity and that both humoral and cellular immunity are needed to prevent ZIKV infection.

2. Methods

2.1. Viruses

Wild-type H/PF/2013 ZIKV (KJ776791, from European Virus Archive) was passaged on C6/36 cell line. Infectious clone of the attenuated DN-2 was produced as described previously and the rescued virus was passaged in Vero cells but limited to 3 passages [18]. Sanger sequencing of PCR-amplified viral fragments was used to ensure that no mutation was introduced to DN-2 in the course of in vitro passaging. Sequence data was analysed using the Geneious software and compared against an available wild-type ZIKV sequence from NCBI (KJ776791). Viral titres were evaluated using plaque assay as previously described [19].

2.2. Animals

All studies were performed in accordance with guidelines provided by the National Advisory Committee for Laboratory Animal Research (NACLAR) in Singapore. All protocols in this study has been approved by the Institutional Animal Care and Use Committee at Singapore Health Services (Protocol #2016/SHS/1197).

Type I IFN receptor-deficient 129 SvEv (A129) mice were purchased from B&K Universal Ltd and housed in Duke-NUS Medical School. Six to 12-week-old mice were used for all experiments. Littermates were randomly assigned to each group.

Male mice were injected intraperitoneally (i.p.) with the indicated dosages of virus in 200 μL of PBS vehicle for DN-2 vaccination or H/PF/2013 challenge. Serum was acquired through centrifugation of submandibular blood collected on the specified days post-vaccination and challenge. Mice exhibiting a weight loss of greater than 20% of original body weight or at experimental endpoint were sacrificed via CO_2 asphyxiation and cervical dislocation. At termination, animal organs were harvested and stored at $-80\,^\circ$C.

2.3. Plaque reduction neutralization test

Plaque reduction neutralization test (PRNT) was performed on BHK-21 cells as previously described [18]. Briefly, serial two-fold dilutions were performed on serum samples after heat inactivation at 56 $^\circ$C for 30 min. Serum dilutions were incubated with 40 pfu of H/PF/2013 ZIKV for one hour at 37 $^\circ$C in RPMI medium supplemented with 2% foetal calf serum before inoculation onto BHK-21 cells. This mixture was then removed after an hour of incubation at 37 $^\circ$C upon which 1% carboxymethyl cellulose overlay was added. Cells were washed, fixed with 20% formalin and stained with 1% crystal violet five days later. Viral plaques were then enumerated visually. Plaque counts were plotted in a sigmoidal dose-response fit curve to calculate PRNT50 values and presented as reciprocal values.

2.4. ELISA

To quantify anti-ZIKV IgG in mouse serum, MaxiSorp™ plates (ThermoFisher Cat#44−2404−21) were coated with 2×10^6 PFU of H/PF/2013 ZIKV in coating buffer (79.5 mg $NaCO_3$+147 mg $NaHCO_3$ in

50 mL H$_2$0; pH 9.6) overnight at 4 °C. Plates were blocked with 5% BSA solution the following day for 2 h at room temperature. Mouse serum was added and incubated for 1.5 h at room temperature. Anti-ZIKV antibodies were then detected through the addition of HRP-conjugated goat anti-mouse secondary antibodies (Dako Cat#P0447, 1:2000 in PBST). TMB substrate (KPL Cat#50−76−00) was added for 7 min at room temperature in the dark before cessation of the reaction with 1 N H$_2$SO$_4$. Plates were read at an absorbence of 450 nm using a plate reader (Tecan Infinite M200) for quantification of anti-ZIKV antibodies.

2.5. CD8$^+$ T cell depletion

Optimization experiments were performed to determine the appropriate dosages required to achieve CD8$^+$ T cell depletions. A129 mice were administered intraperitoneally (i.p.) with anti-CD8a antibody (Bio X Cell #BE0061). A second antibody injection at the same doses was given 2 days later, as previously described [20]. Flow cytometry was performed one day after the second injection on submandibular blood stained with APC-conjugated anti-mouse CD3 (BD Cat#565643) and BUV395-conjugated anti-mouse CD8b antibodies (BD Cat#740278) to evaluate the optimal dose for successful CD8$^+$ T cell depletion.

For CD8 T-cell depletion, 10 μg of anti-CD8a antibody diluted in 200 μL PBS was injected i.p. at one and three days before challenge with H/PF/2013. Successful depletion of CD8$^+$ cells was confirmed via flow cytometry.

2.6. Flow cytometry

Flow cytometry analysis was performed using the BD Fortessa and FlowJo 10 software to evaluate depletion of CD8$^+$ T cells after administration of anti-CD8a monoclonal antibodies. Whole blood was subjected to red blood cell lysis using BD Pharm Lyse (BD Cat#555899) and fluorescently labelled with a combination of conjugated anti-B220 (BD Cat#557669), anti-CD3 (BD Cat#565643) and anti-CD8b (BD Cat#740278) antibodies at 1:400 dilution for analysis.

Spleens from vaccinated mice were collected to evaluate B and T cell populations in response to alterations to ZIKV DN-2 viraemia duration. Briefly, spleens were passed through a 70 μm cell strainer to obtain a splenocyte suspension. The splenocytes were then fluorescently labelled with conjugated anti-CD3, anti-CD8b, anti-CD44 (BD Cat#560570) and anti-CD62L (BD Cat#563252) antibodies at 1:400 dilution before subsequent analysis with the BD Fortessa.

2.7. ELISPOT

CD8 magnetic positive selection was performed on splenocyte suspensions acquired from vaccinated mice using CD8a (Ly-2) Microbeads (Miltenyl Biotec 130−117−044) according to manufacturer's instructions. Isolated CD8$^+$ cells were added to 96-well plates pre-coated with AN18 anti-mouse IFN-γ antibodies (i-DNA 3321−3−250) and stimulated with the ZIKV E$_{294-302}$ peptide IGVSNRDFV (Mimotopes) or PMA/Ionomycin as previously described [21]. Stimulations were performed in RPMI media overnight. Secondary biotnylated anti-IFNγ antibodies (iDNA 3321−6−250) were added after stimulation followed by streptavidin. Spot-forming units were then counted using ImmunoSpot S6 and analysed with the accompanying software as per manufacturer's protocol.

2.8. Viral rna extraction

Viral RNA was extracted from the serum acquired from submandibular bleeds using the QIAamp Viral RNA Mini Kit (Qiagen Cat# 52906) according to manufacturer's instructions. Harvested organs were homogenized with silica carbide using the FastPrep-24^{TM} 5 G instrument (MPBio). RNA was then extracted from the homogenate using a TRIzol LS (ThermoFisher #10296010) and chloroform method to induce phase separation. Organ RNA concentration was measured using the NanoDrop 2000 Spectrophotometer (Thermo-Fisher).

2.9. qRT-PCR

ZIKV RNA levels in serum and organs were quantified using the qScript One-Step RT-qPCR Kit (QuantaBio Cat# 96057-200) with ZIKV 1086 and ZIKV 1162c primers with ZIKV 1107 probe [](5′-6-FAM- AGCCTACCTTGACAAGCAGTCAGACAC TCAA-3′-BHQ-1 from Integrated DNA Technologies) as described previously [18]. RNA standards produced from in vitro transcription of primer and probe target regions were used to generate a standard curve for quantification. All qRT-PCR was performed and analysed using a LightCycler 480 RT-qPCR system (Roche) and LightCycler 480 software (Roche) respectively.

2.10. Nanostring analysis

Peripheral blood mononuclear cells (PBMCs) from A129 mice were isolated from submandibular blood collected at days 0,1, and 3 post-challenge as previously described [19]. RNA was extracted from PBMCs using the TRIzol and chloroform method described above. 50 ng of extracted RNA was then hybridized to the Nanostring nCounter Mouse Immunology panel at 65 °C for 24 h. 50 ng of brain or testes RNA extracted as described above was hybridized to the NanoString nCounter Mouse Inflammation v2 panel (NanoString Technologies) at 65 °C for 24 h.

Hybridized samples were quantified using the nCounter Sprint profiler (NanoString Technologies). Data was analysed using the nSolver Analysis Software (NanoString Technologies). Spearman correlation analysis was performed to identify genes significantly correlated with boosts in neutralising antibody titres. Subsequent pathway analysis was performed using GO Enrichment analysis. Significantly correlated pathways were clustered and summarized using REVIGO as described previously [22].

2.11. Statistical analyses

Unpaired t tests were performed for comparisons between datasets. Statistical significance was achieved if p-values were less than 0.05. All numerical data is presented as the mean $\pm$ SD (standard deviation). Graphs were generated with GraphPad Prism 8.3.1.

Correlation data was produced using nonparametric Spearman correlation analysis in GraphPad Prism 8.3.1 with significance achieved at p-values less than 0.05. Significantly correlated genes were analysed against PANTHER classification system using Gene Ontology (GO) Enrichment Analysis for biological processes with Fisher's exact test with FDR correction to identify significantly enriched GO terms.

2.12. Role of the funding source

3. Results

3.1. Truncation of DN-2 burden using a monoclonal antibody treatment reduced anti-ZIKV neutralising antibody titres without affecting CD8$^+$ T cell responses

We have previously shown that inoculation of DN-2 in A129 mice produced approximately 5 days of detectable viraemia [18]. This feature enabled us to use the area under the curve (AUC) of total duration of DN-2 viraemia as a surrogate measure of vaccine burden. To investigate the impact of vaccine burden on immunogenicity and efficacy in protection from wild-type virus infection, we capitalize on a previously reported anti-ZIKV monoclonal antibody, ZAb_FLEP, which targets a quaternary epitope near the ZIKV fusion loop to reduce DN-2 vaccine burden [18,23,24]. Intraperitoneal ZAb_FLEP treatment was instituted at days 3 and 6 after inoculation with DN-2, during or after viraemia peak respectively. DN-2 vaccination without ZAb-FLEP treatment and ZAb_FLEP only treatment served as controls for the experiment (Fig. 1a). Indeed, ZAb_FLEP treatment at day 3 but not day 6 post-vaccination successfully reduced the duration of both DN-2 viraemia and RNAemia compared to control mice without reducing peak viraemia or RNAemia levels (Fig. 1b and 1c). RNAemia was well-correlated to viremia levels, serving as an effective surrogate measurement (Supplementary Figure 1a). Area under the curve (AUC) of RNAemia measured throughout the period of monitoring was significantly decreased in mice that received ZAb_FLEP treatment 3 days post infection compared to mice without ZAb_FLEP treatment (Fig. 1d).

The impact of vaccine burden truncation on antibody response was evaluated at 21 days post-vaccination. ZAb_FLEP treatment at day 3 resulted in reduced antibody titre that neutralized 50% of ZIKV on a plaque reduction neutralization test (PRNT$_{50}$) compared to either delayed or no ZAb-FLEP treatment (Fig. 1e). Meanwhile, levels of anti-ZIKV IgG, as measured on an ELISA, were unaffected (Fig. 1f). These findings suggest that greater vaccine burden in untreated mice could have led to greater levels of affinity maturation in the B cells than mice with truncated DN-2 burden. Furthermore, significant positive correlation between RNAemia AUC and neutralising antibody titres was observed (Supplementary Figure 1b). No correlation, however, was found between peak RNAemia and neutralising antibody titres (Supplementary Figure 1c). These findings collectively suggest that the total vaccine burden is important for shaping the quality of neutralising antibody response.

We next investigated the impact of truncating DN-2 burden on CD8$^+$ T cell responses, which could also play important roles in controlling ZIKV infection [25]. ZAb_FLEP was again administered to truncate vaccine burden in assigned groups in a separate cohort of A129 mice (Supplementary Figure 1d and 1e). Flow cytometry analysis of splenocytes harvested 21 days post-vaccination revealed no change in overall CD3$^+$ or CD3$^+$/CD8$^+$T cell percentage (Supplementary Figure 1f and 1 g). The percentages of total central CD44$^+$/CD62L$^+$central memory and CD44$^+$/CD62L$^-$ effector memory CD8$^+$ T cells were also unaffected DN-2 burden truncation (Figs. 1g and 1h, Supplementary Figure 1 h). Quantification of antigen-specific CD8$^+$ T cells by stimulating splenocytes with a known immunodominant ZIKV peptide epitope E$_{294-302}$ also revealed no significant difference in IFN-γ production in an ELISPOT assay (Fig. 1i) [21]. These findings suggest that ZAb_FLEP treatment did not affect the development of ZIKV antigen specific CD8$^+$ T cells. This finding is consistent with a recent clinical observation that antibodies neutralize extracellular virions but are unable to eliminate infected cells that continue to stimulate CD8$^+$ T cell response through MHC class I antigen [26]. The ZAb_FLEP treatment model thus allowed us to examine the role of humoral immunity on ZIKV immunity without being confounded by cellular immunity.

3.2. Truncation of DN-2 burden resulted in loss of sterilising immunity upon wild-type challenge

To determine how baseline neutralising antibody titres affect immunity, we challenged these mice with a lethal 10^4 PFU (i.p.) dose of the wild-type ZIKV (French Polynesian H/PF/2013 strain) (Fig. 2a) [27]. Unvaccinated control animals all showed the expected wild-type ZIKV viraemia that peaked at on day 2 post-challenge (Fig. 2b), reduction in body weights (Fig. 2c) and 100% mortality rate (Fig. 2d). Vaccinated animals, with or without DN-2 burden truncation, were negative for wild-type ZIKV RNAemia except for one animal with reduced DN-2 viraemia AUC (Fig. 2b). There was neither weight loss (Fig. 2c) nor mortality (Fig. 2d) in these vaccinated animals. However, animals with lower DN-2 vaccine burden showed a four-fold or greater boost in post-challenge antibody titres (Fig. 2e). Animals with higher DN-2 burden did not show such a similar post-challenge antibody titre boost (Fig. 2e). The post-challenge boost in neutralising antibody titres thus suggests a loss in sterilising immunity in animals with reduced DN-2 burden. However, we were unable to detect wild-type ZIKV infection in the brain, testes, liver, kidneys, epididymis, and eyes of these animals (Supplementary Figure 2) [28].

3.3. Gene expression signatures in peripheral blood mononuclear cells (PBMCs) suggests that sterilising immunity against ZIKV requires both humoral and cellular immunity

To explore the possibility of sub-detectable infection in animals with reduced vaccine burden, we profiled the gene expression in peripheral blood mononuclear cells (PBMCs) of these animals collected on days 1 and 3 post-challenge. Expression of immune genes was quantified using the NanoString nCounter Mouse Immunology panel. Spearman correlation analysis was used to identify genes significantly correlated with the magnitude of post-challenge neutralising antibody titre boost. Expectedly, Gene ontology (GO) enrichment analysis highlighted STAT phosphorylation and B cell development as the top pathways positively correlated with neutralising antibody boost (Fig. 3a), at both days 1 (Fig. 3b and 3c) and 3 post challenge (Supplementary Figure 3a and 3b). The transcriptional changes are thus consistent with the notion of subclinical infection in these animals. Conversely, in animals without boost in antibody titres following wild-type ZIKV challenge, the top expressed pathways were granulocyte recruitment and T cell proliferation (Fig. 3d). Level of expression of key genes in these pathways at both days 1 (Figs. 3e and 3f) and 3 (Supplementary Figure 3c and 3d) post challenge were also significantly and negatively correlated with antibody-fold change (Figs. 3e and 3f). Indeed, CD81 and Ceacam1 (Fig. 3f) have been previously shown as important co-stimulatory and proliferation markers, respectively, of CD8$^+$ T cells [29–31]. These findings suggest that CD8$^+$ T cell immunity may be needed to complement B cell immunity, despite maximal DN-2 vaccine burden-induced neutralising antibody titres.

3.4. CD8$^+$ t cells are crucial for the complete clearance of ZIKV infection in the testes and epididymis of mice

To test the possibility that sterilising immunity in animals with maximal DN-2 vaccine burden induced neutralising antibody responses required CD8$^+$ T cell responses, we included selective CD8$^+$ T cell depletion into our animal model. We utilized anti-CD8a monoclonal antibodies (i.p.) to selectively deplete the CD8$^+$ lymphocyte population in ZIKV DN-2 vaccinated A129 mice (without any burden truncation) before wild-type ZIKV challenge (Fig. 4a) [20]. A corresponding IgG2b isotype antibody was used as a control in a separate group of animals. An optimal dosage of 10μg of anti-CD8a monoclonal antibody for CD8$^+$ depletion was determined through a series of titrations (Supplementary Figure 4a). Flow cytometric analysis of
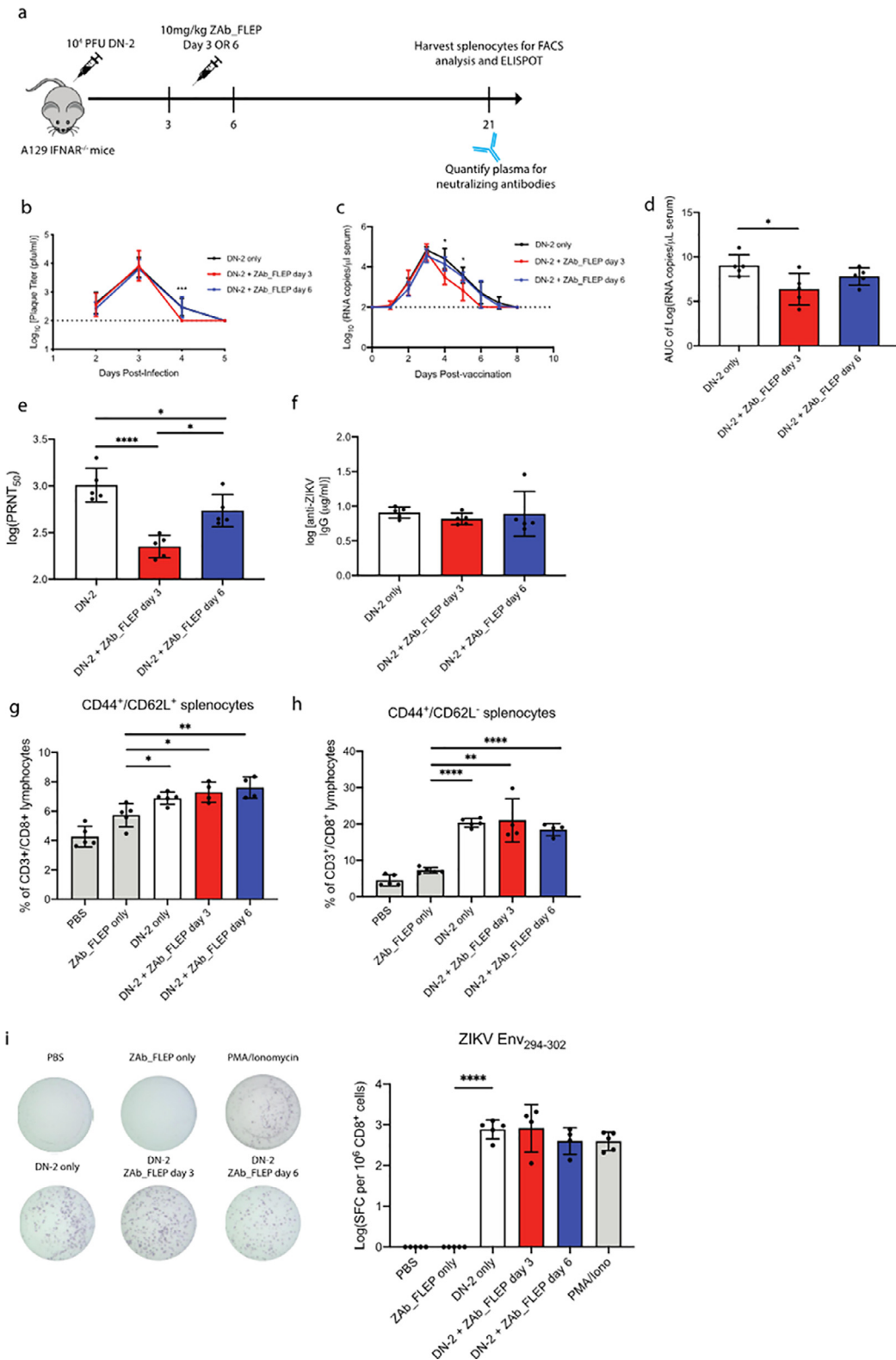
Fig. 1. Truncation of DN-2 burden using a monoclonal antibody treatment reduced anti-ZIKV neutralising antibody titres without affecting CD8^{+} T cell responses. (a) Experimental layout of vaccine burden truncation of A129 mice vaccinated with 10^4 pfu of DN-2 (i.p.) using 10 mg/kg of ZAb_FLEP (i.p.). Serum extracted from submandibular blood was quantified via plaque assay (b) and qRT-PCR (c) for ZIKV infectious particles and genomic copies respectively. Dotted lines indicate limits of detection. (d) ZIKV RNAemia area under the curve (AUC) was significantly lower in animals administered with ZAb_FLEP on day 3 post-vaccination. (e) Neutralising antibody titres measured on day 21 post-vaccination were significantly lower in animals administered ZAb_FLEP on day 3 post-vaccination. (f) Anti-ZIKV IgG titres in serum measured on day 21 post-vaccination via ELISA show no differences despite truncation of DN-2 burden. (g-h) Central memory CD8^{+} *T* cells denoted by CD3^{+}/CD8^{+}/CD44^{+}/CD62L^{+} expression (g) and effector memory CD8^{+} *T* cells denoted by CD3^{+}/CD8^{+}/CD44^{+}/CD62L^{−} expression (h) were quantified via flow cytometry analysis of splenocytes harvested 21 days post-vaccination. (i) CD8^{+} cells magnetically isolated from splenocytes harvested 21 days post-vaccination were stimulated with the ZIKV $Env_{294-302}$ peptide and stained for IFNγ-producing cells. Two independent experiments were performed with five animals in each group. One flow cytometry data point from the day 3 cohort was excluded due to poor sample quality. Data are presented as mean±SD. Statistical analyses were performed using the unpaired t-test. *$p \leq 0.05$, **$p \leq 0.01$ and ****$p \leq 0.0001$.
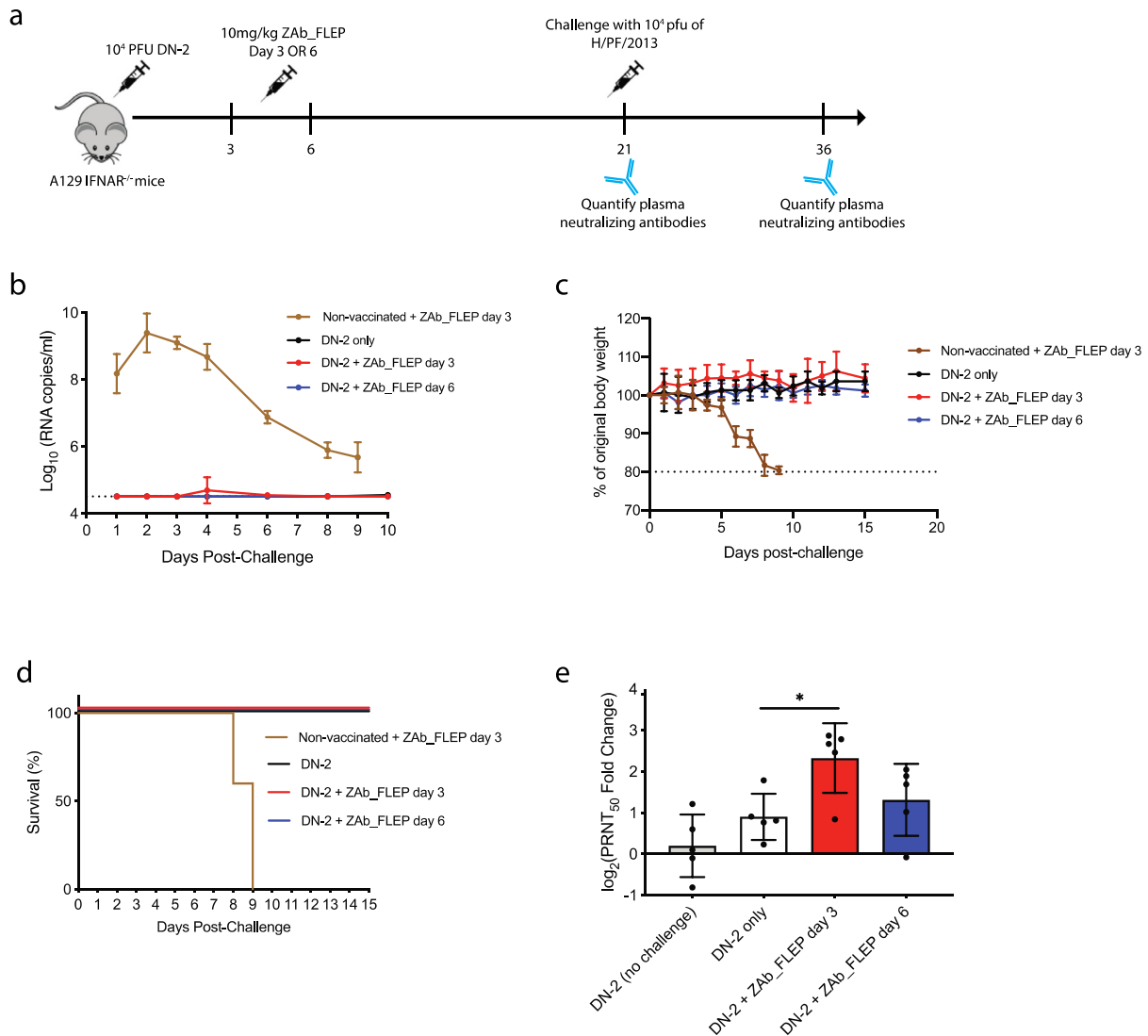
Fig. 2. Truncation of DN-2 burden resulted in loss of sterilising immunity upon wild-type challenge. (a) Experimental layout of vaccine burden truncation using 10 mg/kg ZAb_FLEP (i.p.) in A129 mice vaccinated with 10^4 pfu of DN-2 and subsequent challenge with 10^4 pfu of H/PF/2013 (i.p.). (b) ZIKV genomic copies were quantified using qRT-PCR on serum extracted from submandibular blood. The dotted line indicates limit of detection. Mice were (c) weighed daily post-challenge and (d) survival was tracked to 15 days post-challenge. (e) Neutralising antibody titres were quantified from serum obtained pre-challenge and 15 days post-challenge via plaque reduction neutralization tests (PRNT) to obtain neutralising antibody titre fold-change. Two independent experiments were performed with five animals in each group. Data are presented as mean±SD. Statistical analysis was performed using unpaired t-test. *$p \leq 0.05$.

blood collected after anti-CD8a antibody treatment and immediately before wild-type ZIKV challenge revealed significant ablation of the CD3$^+$/CD8$^+$lymphocyte population (Fig. 4b). Total B cells and pre-challenge neutralising antibody titres were unaffected with CD8$^+$ T cell depletion (Supplementary Figure 4b, 4c and 4d). Upon challenge, all mice remained healthy with no detectable ZIKV RNAemia (Supplementary Figure 4e and 4f). However, at the point of experiment termination on day 15 post-challenge, RT-PCR revealed wild-type ZIKV infection in several organs of CD8-depleted animals. While the brain and eyes tested negative (Fig. 4c and 4d), two out of the five animals showed detectable wild-type ZIKV in the livers and kidneys, although the rate of infection was not statistically significant compared to controls (Figs. 4e and 4f). In the testes and epididymis, however, 4 out of the 5 (80%) and all 5 (100%) CD8$^+$-depleted mice, respectively, tested positive for wild-type ZIKV (Fig. 4g and 4h); none of the control mice showed infection in these same organs. Moreover, inflammatory genes showed upregulated expression in infected compared to uninfected testes, suggesting that the RT-PCR

positive findings were not due to virus deposition but rather infection that stimulated inflammation. Consistent with this notion that the RT-PCR positivity represented a significant pathological finding, no difference in innate immune or inflammatory responses was found in the brain (Supplementary Figure 4 g and 4 h). These findings indicate that cellular immunity is critical in complementing baseline neutralising antibodies to prevent wild-type ZIKV infection and systemic dissemination.

4. Discussion

The development of flaviviral vaccines have relied on empirically derived strategies that can be challenging and time-consuming. Identification of determinants of vaccine-mediated immunity and protection against flaviviruses could allow for improved vaccine development strategies. Though A129 mice lack type I interferon receptor and would not elicit the full spectrum of innate immune response expected in immunocompetent human infection, the
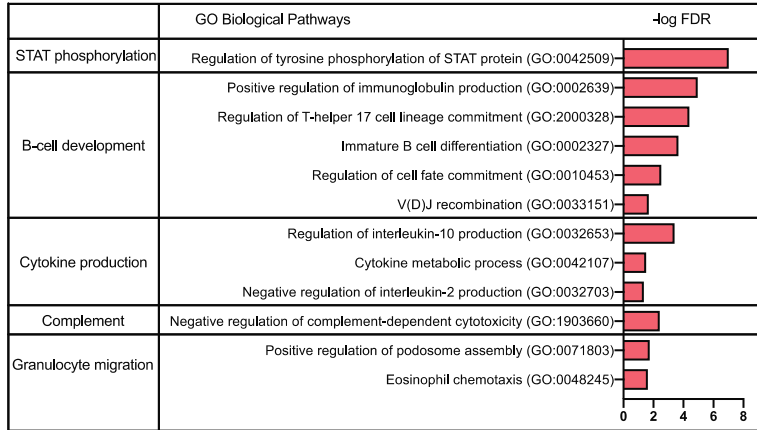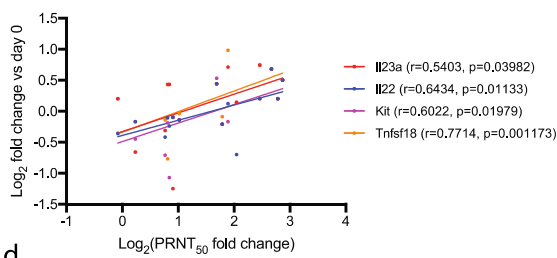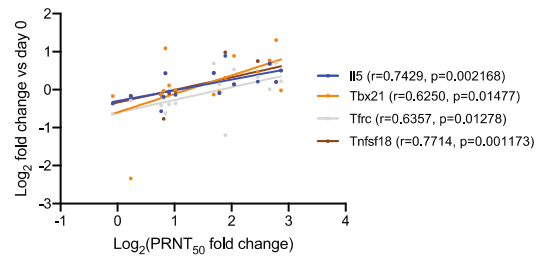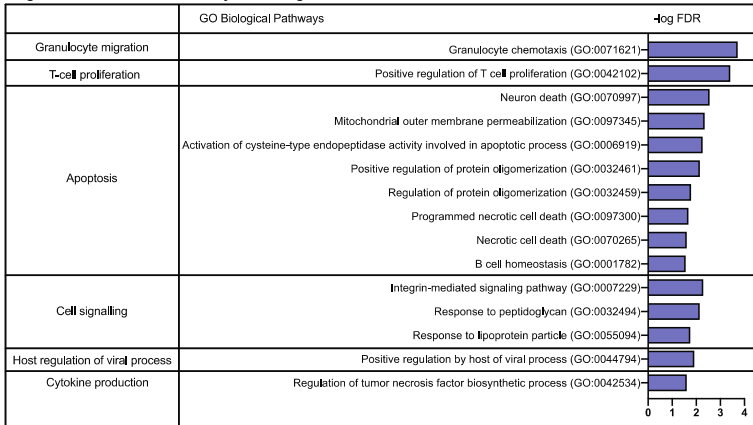
Fig. 3. Gene expression signatures in peripheral blood mononuclear cells (PBMCs) suggests that sterilising immunity against ZIKV requires both humoral and cellular immunity. Biological pathways (a) positively and (b) negatively correlated with the magnitude of neutralising antibody titre boosts post-challenge based on gene expression counts acquired through Nanostring analysis of PBMCs harvested 1- and 3-days post-challenge. Significantly correlated genes on day 1 post-challenge from the top positively regulated pathways of (c) STAT phosphorylation and (d) B cell development and the top negatively regulated pathways of (e) granulocyte migration and (f) T cell proliferation are shown. Significantly enriched pathways were identified through Fisher's exact test with False Discovery Rate (FDR) correction. Correlation analysis was performed using Spearman correlation with significance defined as $p \leq 0.05$.

Fig. 4. CD8^{+} T cells are crucial for the complete clearance of ZIKV infection in the testes and epididymis of mice. (a) Experimental layout to selectively deplete CD8^{+}cells by administering 10μg of 2.43 anti-CD8a monoclonal antibody (i.p.) to A129 mice vaccinated with 10[4] pfu of DN-2 and subsequently challenged with 10[4] pfu of H/PF/2013. (b) Flow cytometry analysis to evaluate depletion of CD8^{+}cells in peripheral blood after anti-CD8a monoclonal antibody treatment of mice. (c-h) Viral loads of organs harvested 15 days post-challenge assessed by qRT-PCR for (c) brain, (d) kidneys, (e) liver, (f) eyes, (g) testes, and (h) epididymis. Dotted lines indicate limit of detection. One isotype control mouse developed a testicular tumour and was omitted from subsequent analysis of the testes and epididymis. Two independent experiments were performed with five animals in each group. Data are presented as mean±SD. Statistical analyses were performed using unpaired t tests. $^*p \leq 0.05$ and $^{****}p \leq 0.0001$.

presence of type II IFN receptor allows for T cell signalling to support the development of adaptive immunity [24,32]. This mouse model has also been used previously to assess the efficacy of several ZIKV vaccine candidates [18,33−35]. Though findings in animal models may not fully translate into a human clinical setting, the A129 mouse model remains the most well-established model for our study.

We have used the attenuated ZIKV DN-2 strain in our investigation, which we have previously shown that vaccination with DN-2 fully protected A129 mice from wild-type H/PF/2013 ZIKV challenge [18]. Moreover, DN-2 infection reproducibly elicits viraemia without producing signs of illness in A129 mice [18]. This property is consistent with the yellow fever vaccine, where vaccine viraemia did not correlate with vaccine-associated adverse events [36,37]. We were thus able to explore, without compromising the health of vaccinated mice before wild-type ZIKV challenge, how the total live vaccine burden impacts immunogenicity and efficacy.

Truncation of DN-2 burden was achieved through administration of the ZAb_FLEP anti-ZIKV antibody, allowing for investigation of the impact of live vaccine infection duration without affecting peak viremia. We found that truncation of vaccine and hence antigenic burden, as reflected by viremia AUC, led to lower levels of neutralising antibodies, despite no difference in overall IgG antibody levels. There is a possibility that the reduction in neutralizing antibody titres could have been due to antibody-mediated termination of germinal centres [38]. However, the high-affinity IgG that is required to disrupt B cell interactions with follicular dendritic cells should also affect antigens displayed on classic dendritic cells. As CD8$^+$ T cell responses are unaffected by ZAb_FLEP treatment (Supplementary Figure 1 g), it is more likely that the observed decrease in neutralizing antibody titres is due to the truncation of vaccine viraemia. The resultant reduction of neutralising antibodies proved insufficient to protect against wild-type ZIKV infection. Upon antigen encounter, B cells likely undergo extensive proliferation in germinal centres with eventual differentiation into memory or antibody-producing plasma B cells [39,40]. Antigen persistence, such as vaccine viraemia duration, may thus stimulate greater levels of affinity maturation of virus-specific antibodies and therefore be critical for the production of potent neutralising antibodies [41]. Indeed, a recent study found that the affinity of neutralising antibodies after YF17D vaccination continued to improve up to 6 to 9 months post-vaccination [42], suggesting that a relatively long antigen persistence may be needed in germinal centres for robust adaptive immunity [43].

That vaccine burden shapes adaptive immunity may also explain the efficacy of other flaviviral vaccines. Studies on Dengvaxia® vaccination have found proportionately more detectable viraemia with serotype 4 component of the vaccine, followed by serotypes 3, 1 and 2 [44]. Along similar trend, vaccine efficacy was highest against dengue virus (DENV) serotype 4, followed by serotypes 3, 1 and 2 [12]. The vaccine efficacy of TAK-003, another tetravalent dengue vaccine that has completed phase 3 clinical trial and is undergoing long-term follow up also showed correlation between vaccine burden and efficacy [45,46]. Finally, yellow fever vaccination elicits detectable viraemia that lasts for several days in well over 80% of subjects consistently [17,47]. Consequently, duration of vaccine burden could be a useful predictor of the quality and durability of immunity.

Our findings also show that sterilising immunity against ZIKV requires both humoral and cellular immunity. Reduced neutralising antibody titres, despite unperturbed CD8+ T cell counts, resulted in a loss of sterilising immunity. On the other hand, CD8+ T cell depletion resulted in infection and systemic dissemination of wild-type ZIKV despite unperturbed B cell count and baseline neutralising antibody titres. To demonstrate the role CD8$^+$ T cells play in sterilising immunity, we have chosen the CD8$^+$ T cell depletion approach instead of comparing passive serum infusion with adoptive CD8$^+$ T cell transfer from vaccinated to naïve mice, as has been previously applied to study immunity against DENV infection [48]. Passive serum infusion

lacks memory B cells that would be activated and expand upon antigen encounter. Presence of memory B cell activation that would be activated upon wild-type virus challenge would more accurately represent vaccine-induced immunity. The CD8$^+$ T cell depletion following live attenuated DN-2 vaccination thus allowed us to glean insights into the roles that B and CD8$^+$ T cells play, respectively, in protection against wild-type ZIKV infection.

Our finding showing wild-type infection in testes and epididymis of CD8$^+$ T cell depleted vaccinated mice is, to the best of our knowledge, unique. CD8$^+$ T cells could also have abrogated systemic spread of wild-type ZIKV in animals with lowered neutralising antibody titres through DN-2 viraemia truncation. Furthermore, previous work suggests that CD8$^+$ T cells may contribute to protection against congenital ZIKV infection [49]. This finding thus raises a safety concern with Zika vaccines that do not elicit sufficient levels of cellular immunity. Given that ZIKV can be transmitted through the sexual route, lack of ZIKV-specific CD8$^+$ T cells could result in silent virus transmission from vaccinated males to unvaccinated females [50,51]. This risk is especially important as testicular infection can last for much longer than ZIKV viraemia [52]. Any ZIKV vaccine candidate must thus be assessed for its ability to confer sterilising immunity, especially of the reproductive tract. There are currently several ZIKV vaccine candidates in clinical development, with a plasmid DNA-launched vaccine and a formalin-inactivated vaccine at the forefront, having completed Phase I trials [13,14]. Other candidates including mRNA and recombinant vaccines are in the midst of phase I clinical trials, having shown promise in preclinical models [53−55]. The quality of cellular immunity engendered by these vaccine candidates should thus be evaluated before further clinical development.

While CD8$^+$ T cell depletion reduced vaccine-induced protection against wild-type ZIKV in various organs, sites protected by the tight blood-brain barrier, namely the brain and the eyes, remained sterile. This outcome suggest that the type and level of immunity needed to protect against flaviviral infection is dependant on the anatomy of infection. Indeed, the brain and eyes are isolated from blood by endothelial cells linked by tight junctions, or the blood-brain barrier (BBB) [56]. This BBB may lower the immunity threshold needed to protect against CNS infection. This may explain why inactivated vaccines against Japanese encephalitis and tick-borne encephalitis have successfully prevented central nervous system infections [9,10]. In contrast, inactivated vaccines have mostly shown to be less immunogenic and likely insufficient in protecting against systemic DENV infection [57−59].

In conclusion, our findings show that vaccine burden is an important determinant of vaccine immunogenicity and that in the A129 Type I interferon-deficient mouse model, both B and T cell immune responses are necessary to confer protection against ZIKV infection.

Data sharing

Contributors

C.Y., E.S.G, S.S.K, L.R., S.V. and E.E.O conceptualized the study and designed the experiments. C.Y., E.S.G., S.S.K, and S.W. performed the *in vivo* experiments, with support from S.G.V. C.Y., E.S.G., S.S.K, E.Z.O and H.C.T performed the virological and serological experiments. C.Y, E.S.G, and N.Z.H. performed the T cell assays, with support from L.R. Host expression analyses were conducted by E.Z.O. and jointly analysed by C.Y and K.R.C. C.Y., E.S.G. K.R.C. and E.E.O wrote the manuscript. All authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

Acknowledgements

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.103028.

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