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MAJOR ARTICLE

Immunization With a Novel Human Type 5 Adenovirus-Vectored Vaccine Expressing the Premembrane and Envelope Proteins of Zika Virus Provides Consistent and Sterilizing Protection in Multiple Immunocompetent and Immunocompromised Animal Models

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Background. Zika virus (ZIKV) infection may be associated with severe complications and disseminated via both vector-borne and nonvector-borne routes. Adenovirus-vectored vaccines represent a favorable controlling measure for the ZIKV epidemic because they have been shown to be safe, immunogenic, and rapidly generable for other emerging viral infections. Evaluations of 2 previously reported adenovirus-vectored ZIKV vaccines were performed using nonlethal animal models and/or nonepidemic ZIKV strain.

Methods. We constructed 2 novel human adenovirus 5 (Ad5)-vectored vaccines containing the ZIKV premembrane-envelope (Ad5-Sig-prM-Env) and envelope (Ad5-Env) proteins, respectively, and evaluated them in multiple nonlethal and lethal animal models using epidemic ZIKV strains.

Results. Both vaccines elicited robust humoral and cellular immune responses in immunocompetent BALB/c mice. Dexamethasone-immunosuppressed mice vaccinated with either vaccine demonstrated robust and durable antibody responses and significantly lower blood and tissue viral loads than controls ($P < .05$). Similar findings were also observed in interferon-α/β receptor-deficient A129 mice. In both of these immunocompromised animal models, Ad5-Sig-prM-Env-vaccinated mice had significantly ($P < .05$) higher titers of anti-ZIKV-specific neutralizing antibody titers and lower (undetectable) viral loads than Ad5-Env-vaccinated mice. The close correlation between the neutralizing antibody titer and viral load helped to explain the better protective effect of Ad5-Sig-prM-Env than Ad5-Env. Anamnestic response was absent in Ad5-Sig-prM-Env-vaccinated A129 mice.

Conclusions. Ad5-Sig-prM-Env provided sterilizing protection against ZIKV infection in mice.

Keywords. adenovirus; envelope; premembrane; vaccine; Zika.

Zika virus (ZIKV) is a human-pathogenic flavivirus that has emerged from obscurity to cause epidemics in the Americas and Asia in recent years [1, 2]. Although most ZIKV-infected patients are asymptomatic or have self-limiting symptoms, some develop severe complications with long-term sequelae [3]. The major complications of ZIKV infection include congenital microcephaly and anomalies, severe neurological diseases such as Guillain-Barré syndrome and meningoencephalitis, and possibly epididymo-orchitis with potential long-term effects on fertility [2–4]. Unlike other emerging viral outbreaks involving patients with respiratory tract infections or viral hemorrhagic fever who are usually easily identifiable by their symptomatology, ZIKV can be transmitted from patients with minimal symptoms to others through both mosquito-borne and nonvector-borne routes, such as sexual and vertical transmissions [5, 6]. This makes it extra difficult to control the epidemic and highlights the urgency of developing a safe and effective vaccine.

In the past 2 years, a number of ZIKV candidate vaccines have been developed and evaluated in animal models, and some are now undergoing phase I and II clinical trials [7–23]. These include live-attenuated virus, purified inactivated virus, deoxyribonucleic acid (DNA) or messenger ribonucleic acid (mRNA), subunit, virus-like particles, and virus-vectored vaccines [24].

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Each vaccine type has its distinct advantages and limitations. For example, although a live-attenuated yellow fever virus vaccine has been used for a long time, there are concerns regarding the safety and potentially severe side effects of this type of flavivirus vaccines in severely immunocompromised patients, especially those with thymus disorders [25]. Structural alterations induced by inactivation processes for producing inactivated virus vaccines may render the vaccine's immunogenicity to be suboptimal, and multiple dosing is usually required to elicit adequate immune response [7, 8, 15, 24]. Zika virus DNA vaccines have been shown to be immunogenic in some animal studies, but efficient delivery of DNA vaccines in vivo remains unreliable and usually requires electroporation [7–9, 13]. Zika virus-like particle vaccines require multiple dosing for achieving optimal immunogenicity [16–18]. The more recently described RNA nanoparticles and modified mRNA vaccines were immunogenic in animal studies, but clinical data in human remain limited [12, 14, 19]. More importantly, some of these reported ZIKV vaccines were only evaluated in nonlethal mouse (adult BALB/c and C57BL/6 mice) and/or nonhuman primate (rhesus macaques) models that have self-limiting ZIKV infection [7, 8, 11, 12, 16, 17, 19]. This makes it more difficult to interpret the data regarding the vaccines' protective effects.

In addition to being safe, immunogenic, and protective, vaccines for emerging viral infections must also be easily producible in high quantities for rapid distribution. Adenovirus-vectored vaccines can be produced rapidly and may take as short as ~60 days to complete the processes of antigen-coding sequence synthesis, shuttle vector construction, virus packaging, and small-scale virus production. Adenovirus-vectored vaccines have been shown to be safe and capable of eliciting robust humoral and cellular immune responses in vitro, in animal models, and in clinical trials [8, 11, 24, 26, 27]. Moreover, nonhuman adenovirus-vectored vaccines may have the additional advantage of overcoming the concerns of pre-existing immunity to human adenovirus-based vaccines [8]. Unlike other types of vaccine that require multiple dosing, adenovirus-vectored vaccines can usually induce robust and durable immune response rapidly after a single dose. These advantages of adenovirus-vectored vaccines and the relevant clinical experiences prompted us to construct and evaluate 2 novel adenovirus 5 (Ad5)-vectored vaccines containing ZIKV premembrane-envelope (prM-Env) and Env proteins, respectively, in multiple nonlethal and lethal animal models.

MATERIALS AND METHODS

Phylogenetic and Amino Acid Sequence Conservation Analyses

The amino acid sequences of Env of 136 different ZIKV strains with complete genome sequences available in GenBank were analyzed. The evolutionary distances were computed using the Poisson correction method. All positions containing gaps and missing data were eliminated. There were a total of 495 positions in the final dataset. Phylogenetic analyses were performed using MEGA6.0 as previously described [28]. The ZIKV-Env amino acid sequence comparisons were performed using National Center for Biotechnology Information Blastp suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Construction of Recombinant Adenovirus 5 Containing Codon-Optimized Zika Virus-Premembrane-Envelope and/or Zika Virus-Envelope

Ad5-Env and Ad5-Sig-prM-Env were generated according to our previously described protocol with modifications [26]. First, prM and Env genes of an epidemic ZIKV strain (MRS_OPY_Martinique_PaRi_2015; accession number KU647676) were produced synthetically. Codon optimization was used to enhance transgene expression. Second, ZIKV-Env was cloned into the shuttle vector pDC316 of AdMax adenovirus vector system [29]. A Kozak sequence and tPA signal peptide sequence were included. The resulting recombinant vector was named pDC316-Env. ZIKV-prM-Env was cloned into pDC316. A Kozak sequence was included with (pDC316-Sig-prM-Env) or without (pDC316-prM-Env) the last 54-nucleotide sequence of ZIKV-capsid (C) gene. All 3 recombinant vectors were confirmed by sequencing. Third, pDC316-Env or pDC316-Sig-prM-Env was mixed with pBHGlox_E1,3_Cre, and the mixture was transformed into HEK293 cells to generate Ad5-Env and Ad5-Sig-prM-Env. The recombinant Ad5 was amplified in 293F cells, purified by SOURCE30Q ion exchange chromatography (GE Healthcare, Little Chalfont, United Kingdom), and concentrated by ultrafiltration with Amicon centrifugal ultrafiltration 100K unit (Merck Millipore, Burlington, MA). Virus solution was supplemented with 10% glycerol and stored in aliquots at −80°C until use. Virus titration was performed in HEK293 cells using Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. The expression of ZIKV-Env by recombinant shuttle plasmid and Ad5 vectors was detected by Western blot (Supplementary Methods).

Recombinant ZIKV-Env$_{1\text{-}409}$ and ZIKV-prM$_{1\text{-}110}$ Protein Preparation

Recombinant ZIKV proteins were prepared as previously described with modifications [30]. The synthetic coding sequences of ZIKV-Env and ZIKV-prM deletion mutants lacking the transmembrane and stem regions were separately cloned into *pET21a* vector with an N-terminal 6×His-tag. The resulting recombinant plasmids were transformed into *Escherichia coli* strain *BL21(DE3)*. Both rEnv$_{1\text{-}409}$ and rprM$_{1\text{-}110}$ were expressed as inclusion bodies and then purified in an AKTA Pure System by QXL and HisTrap HP (GE Healthcare). The purified inclusion bodies were refolded by 100-fold dilution into refolding buffer (100 mM Tris, 10 mM EDTA, 0.5 mol/L L-arginine, 1.5 mmol/L reduced glutathione, and 1.5 mmol/L oxidized glutathione) for overnight refolding at 4°C.

Immunocompetent BALB/c Mouse Model for Evaluation of Vaccine Immunogenicity

To evaluate the immunogenicity of the Ad5-vectored vaccines, 4- to 6-week-old female BALB/c mice (Wei Tong Li Hua Company, Beijing, China) were randomly divided into 3 groups to receive a

single dose of Ad5-Sig-prM-Env, Ad5-Env, or Ad5-Luc (controls) intramuscularly ($1 \times 10^{6-8}$ infectious units [ifu]) (Supplementary Table 1). Mouse sera were collected at 1 week and 4 weeks postvaccination for antibody detection (n = 6–15/group). For cell-mediated immune response evaluation, the mice were sacrificed at 2 weeks postvaccination (n = 10/group) and their spleens were excised. The splenocytes were extracted into Gibco Roswell Park Memorial Institute 1640 medium (10% fetal bovine serum) for intracellular cytokine staining and enzyme-linked immunospot (ELISPOT) assay (Supplementary Methods).

Dexamethasone-Immunosuppressed BALB/c Mouse Model for Evaluation of Serial Antibody Response Elicited by the Adenovirus 5-Vectored Vaccines

Patients with immunosuppression, including those on corticosteroid therapy, may develop severe ZIKV infection [3]. To investigate whether the antibody responses elicited by our Ad5-vectored vaccines were robust and lasting despite the use of corticosteroid, we evaluated the serial antibody response and viral loads in dexamethasone-immunosuppressed mice before and after challenge with an epidemic ZIKV strain (Puerto Rico strain PRVABC59; accession number KU501215; ZIKV-PR) as previously described with some modifications (Supplementary Methods) [31, 32]. ZIKV-PR was used because it has previously been thoroughly evaluated in the dexamethasone-immunosuppressed mouse model and its Env amino acid sequence was 100% homologous with that of ZIKV-MRS_OPY_Martinique_PaRi_2015.

Survival Studies With A129 Mice Receiving Active or Passive Immunization With Adenovirus 5-Vectored Vaccines

Survival studies using the type I interferon (IFN) receptor-deficient A129 mouse model for ZIKV infection were performed as previously described with slight modifications [33] (Supplementary Methods). The mice's clinical parameters were serially recorded, and their organ tissues were collected at necropsy for viral load studies and immunohistochemistry staining as previously described (Supplementary Methods) [31, 34]. For both the dexamethasone-immunosuppressed and A129 mouse models, each mouse received a single dose of vaccine intramuscularly (1×10^8 ifu) at day 0.

Detection of Binding and Neutralizing Antibodies in Mouse Sera

Serum anti-ZIKV-Env-specific and anti-ZIKV-prM-specific binding antibodies were detected by enzyme-linked immunosorbent assay, and serum neutralizing antibodies (nAb) were detected by plaque reduction neutralization test as previously described with modifications [35, 36] (Supplementary Methods).

Ethical Considerations

All the animal experiments were approved by the Animal Care and Use Committee of the Beijing Institute of Biotechnology (immunocompetent BALB/c mouse model) and the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong (dexamethasone-immunosuppressed and A129 mouse models).

Statistical Analyses

All data were analyzed with GraphPad Prism software (GraphPad Software, Inc.) as we previously described [31]. Kaplan-Meier survival curves were analyzed by the log rank test, and weight losses were compared using 2-way analysis of variance. Student's *t* test was used to determine significant differences in viral loads, and Tukey-Kramer post hoc test was used to discern differences among individual vaccine groups. Spearman rank correlation test was used to determine the correlation between antibody titers and viral loads. $P < .05$ was considered statistically significant.

RESULTS

Zika Virus-Envelope Amino Acid Sequences Are Highly Conserved Among Zika Virus Strains

Bioinformatic analysis was performed to identify the changes and differences in the amino acid sequence of the major immunogenic antigen ZIKV-Env among the different ZIKV strains. All ZIKV-Env sequences were categorized into 2 main groups that represented the Asian/American and African lineages of ZIKV (Supplementary Figure 1A). The ZIKV-Env amino acid sequence of ZIKV-MRS_OPY_Martinique_PaRi_2015 showed high similarity (≥99.2%) with most other ZIKV strains (121 of 135, 89.6%). A relatively lower similarity (98.0%) was seen in 1 Asian/American lineage strain (AMK79469.1, 1 of 135, 0.7%) and the 13 African lineage strains (13 of 135, 9.6%) (Supplementary Figure 1B). Overall, these findings corroborated with our previous report that the ZIKV-Env is highly conserved among different ZIKV strains and is therefore a suitable target for constructing vaccine candidates to provide protection against most epidemic ZIKV strains [28].

Construction and Characterization of Recombinant Adenovirus 5 Vectors Containing ZIKV-prM and/or ZIKV-Env Genes

Based on the bioinformatic analysis results, we designed synthetic codon-optimized ZIKV-Env and ZIKV-prM genes based on ZIKV-MRS_OPY_Martinique_PaRi_2015 (Figure 1A). All open reading frames were placed between the murine cytomegalovirus promoter and the polyadenylation signal of simian virus 40. pDC316-Env was designed to express ZIKV-Env directed by tPA signal peptide. To mimic the natural production process of ZIKV-Env protein, we also designed pDC316-prM-Env and pDC316-Sig-prM-Env. For the latter, the last 18Aa of C protein was placed on the N-terminus of ZIKV-prM-Env fusion peptide to act as a signal peptide.

To detect the expression of ZIKV-Env protein by the open reading frames, cell lysates of 293T cells transfected with pDC316-Env, pDC316-prM-Env, or pDC316-Sig-prM-Env

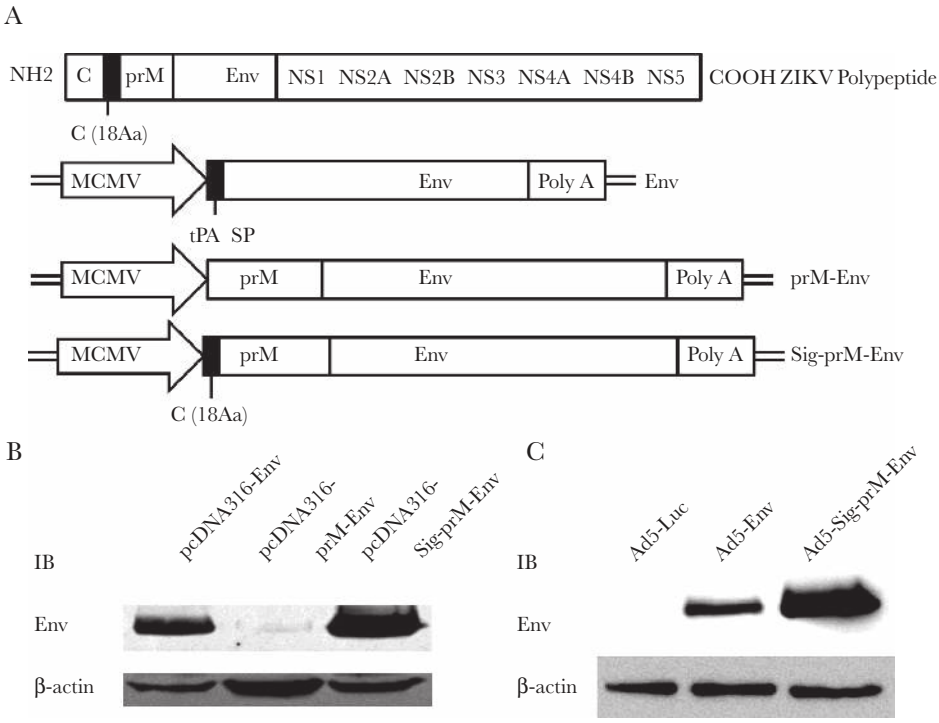

Figure 1. Construction and characterization of recombinant adenovirus 5 (Ad5) vectors expressing Zika virus (ZIKV)-prM and/or ZIKV-Env proteins. (A) Schematic representation of the ZIKV polyprotein and the 3 expression cassettes for expression of ZIKV-Env. In the first construct, the tPA SP sequence was included to direct the secreted expression of ZIKV-Env (amino acids 291–794 of the polyprotein). In the second and third constructs, the ZIKV-prM-coding sequence (amino acids 123–290 of the polyprotein) was fused to ZIKV-Env without (second construct) or with (third construct) the C (18Aa) (amino acids 105–122 of the polyprotein) added to the N-terminus of the ZIKV-prM-Env fusion peptide. (B) Detection of ZIKV-Env protein expression in 293T cells transfected with recombinant shuttle vectors pDC316-Env, pDC316-prM-Env, or pDC316-Sig-prM-Env. (C) Detection of ZIKV-Env expression in HEK293 cells infected with equal quantities of Ad5-Luc, Ad5-Env, or Ad5-Sig-prM-Env. Abbreviations: C, capsid; C (18Aa), capsid C-terminal 18-amino acid signal peptide; Env, envelope; MCMV, murine cytomegalovirus promoter; NS, nonstructural protein; PolyA, the polyadenylation signal of simian virus 40; prM, premembrane; tPA SP, tissue plasminogen activator signal peptide sequence used to direct the secreted expression of ZIKV-Env.

were probed by Western blot using ZIKV-Env-specific polyclonal antibody. Expression of ZIKV-Env protein was demonstrated in 293T cells transfected with either pDC316-Env or pDC316-Sig-prM-Env, but not in cells transfected with pDC316-prM-Env (Figure 1B). This suggested that the signal peptide present in pDC316-Sig-prM-Env but not pDC316-prM-Env was essential for the endoplasmic reticulum, Golgi, and membrane trafficking of the prM-Env peptide. pDC316-Env and pDC316-Sig-prM-Env were therefore used to generate Ad5-Env and Ad5-Sig-prM-Env. ZIKV-Env protein expression was examined by Western blot analysis of cell lysates of HEK293 cells infected with equal quantities of Ad5-Luc (luciferase), Ad5-Env, or Ad5-Sig-prM-Env. Protein expression of ZIKV-Env was detected in Ad5-Env-infected and Ad5-Sig-prM-Env-infected HEK293 cells, with the expression level being higher in Ad5-Sig-prM-Env-infected than Ad5-Env-infected cells (Figure 1C).

Vaccination With Ad5-Sig-prM-Env or Ad5-Env Elicited Potent Humoral and Cellular Immune Responses in Immunocompetent Mice

As the first step to evaluate the immunogenicity of the 2 vaccines, we evaluated the humoral and cellular immune responses of immunocompetent BALB/c mice vaccinated with Ad5-Sig-prM-Env, Ad5-Env, or Ad5-Luc (control). Vaccination with either Ad5-Sig-prM-Env or Ad5-Env induced anti-ZIKV-Env-specific binding antibodies in a dose-dependent manner (Figure 2A and B). The anti-ZIKV-Env-specific binding antibody response in mice receiving either vaccine was evident at as early as 1 week postvaccination, and the antibody titer became significantly higher at 4 weeks postvaccination ($P < .001$). The antibody titer at 1 week postvaccination was generally 0.5- to 1-log higher in the Ad5-Sig-prM-Env-vaccinated mice than the Ad5-Env-vaccinated mice receiving the same dose of vaccine ($P < .05$ for 1×10^8 ifu), whereas the antibody titer of the 2 groups were not significantly different at 4 weeks postvaccination (Figure 2C). Both groups had significantly higher antibody titer than the Ad5-Luc-vaccinated mice. Lower titers of serum anti-ZIKV-prM-specific binding antibody was also detected in the mice vaccinated with Ad5-Sig-prM-Env at 1 week and 4 weeks postvaccination (Figure 2D).

To evaluate the cellular immune response elicited by Ad5-Sig-prM-Env and Ad5-Env, the levels of anti-ZIKV-Env-specific IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-2, and CD107a were measured with multiparameter intracellular cytokine staining and ELISPOT assays. At 2 weeks postvaccination with either vaccine, the mice developed strong

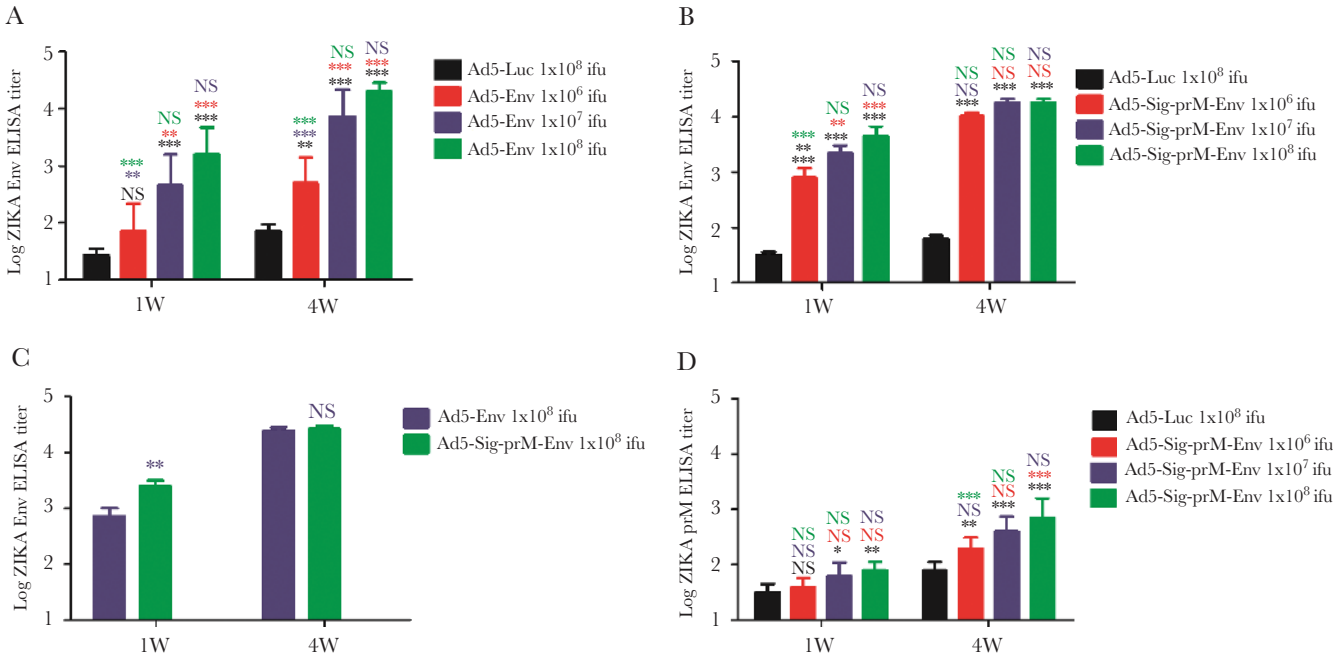

Figure 2. The humoral immune responses elicited by the adenovirus 5 (Ad5)-vectored Zika virus (ZIKV) vaccines in immunocompetent BALB/c mice. Each 4- to 6-week-old BALB/c mouse was vaccinated intramuscularly with a single dose of Ad5-Luc, Ad5-Env, or Ad5-Sig-prM-Env at different dosages (n = 6 [A, B, and D] to 15 [C] per group). The mice included in (C) were different from those included in (A) and (B). The anti-ZIKV-Env-specific (A, B, and C) and anti-ZIKV-prM-specific (D) antibody titers were measured at 1 week (1W) and 4 weeks (4W) postvaccination by enzyme-linked immunosorbent assay (ELISA). Statically significant differences (Student's *t* test) were marked by bars and asterisks (*, $P < .05$; **, $P < .005$; ***, $P < .001$). Abbreviations: Env, envelope; ifu, infectious units; NS, no significant difference; prM, premembrane.

anti-ZIKV-Env-specific cellular immune responses (Figure 3A) as evidenced by the significantly higher ($P < .001$) mean percentage of IFN-γ-, TNF-α-, IL-2-, and CD107a-positive $CD8^+$ T cells and IFN-γ- and TNF-α-positive $CD4^+$ T cells in the vaccinated mice than the Ad5-Luc-vaccinated control mice. There was no significant difference between the mean percentages of anti-ZIKV-Env-specific cytokine-positive $CD8^+$ and $CD4^+$ T cells in the Ad5-Env-vaccinated mice and those in the Ad5-Sig-prM-Env-vaccinated mice. In contrast, the Ad5-Sig-prM-Env-vaccinated mice demonstrated significantly higher mean percentages of anti-ZIKV-prM-specific IFN-γ-positive $CD8^+$ and $CD4^+$ T cells than both the Ad5-Env-vaccinated and Ad5-Luc-vaccinated mice. The anti-ZIKV-Env-specific and anti-ZIKV-prM-specific IFN-γ and IL-2 ELISPOT assay results corroborated with those of the intracellular cytokine staining assays (Figure 3B). Overall, these findings suggested that both Ad5-Sig-prM-Env and Ad5-Env elicited robust anti-ZIKV-Env-specific $CD8^+$ and $CD4^+$ T-cell immune responses, whereas Ad5-Sig-prM-Env additionally elicited anti-ZIKV-prM-specific cellular immune response.

Dexamethasone-Immunosuppressed Mice Vaccinated With Ad5-Sig-prM-Env Developed Rapid-Onset, Robust, and Lasting Antibody Responses That Protected Them From Zika Virus Infection

The dexamethasone-immunosuppressed mouse model provided a platform for evaluating the vaccines' ability to elicit robust and durable antibody responses in hosts receiving corticosteroids. Both Ad5-Sig-prM-Env and Ad5-Env elicited similarly high levels of serum anti-ZIKV-Env-specific binding antibodies (~3.0 logs) at as early as 2 weeks postvaccination (Supplementary Figure 2A). The antibody titers peaked at 6 weeks postvaccination (~4.0 logs) and persisted at similarly high levels at 8 weeks postvaccination. It is interesting to note that Ad5-Sig-prM-Env elicited significantly higher titers of anti-ZIKV-specific nAb (≥1-log higher, $P < .001$) at 4–8 weeks postvaccination (Figure 4A). There was no detectable ZIKV RNA in the serum and tissues of the Ad5-Sig-prM-Env-vaccianted mice ($P < .05$), and there was detectable but significantly lower viral loads in the Ad5-Env-vaccinated mice than in the control mice ($P < .05$) (Figure 4B). These findings illustrated that the antibody response elicited by Ad5-Sig-prM-Env was rapid-onset, robust, and durable even in the presence of high-dose dexamethasone immunosuppression.

Active Immunization of A129 Mice With Ad5-Sig-prM-Env Provided Sterilizing Protection Against Zika Virus Infection

Interferon receptor-deficient mice develop fatal ZIKV infection and are frequently used for evaluation of antivirals and vaccines for ZIKV infection. To further evaluate the protective effects and immunogenicity of our Ad5-vectored vaccines, we vaccinated A129 mice with our Ad5-vectored or control vaccines. The Ad5-Sig-prM-Env-vaccinated mice developed minimal

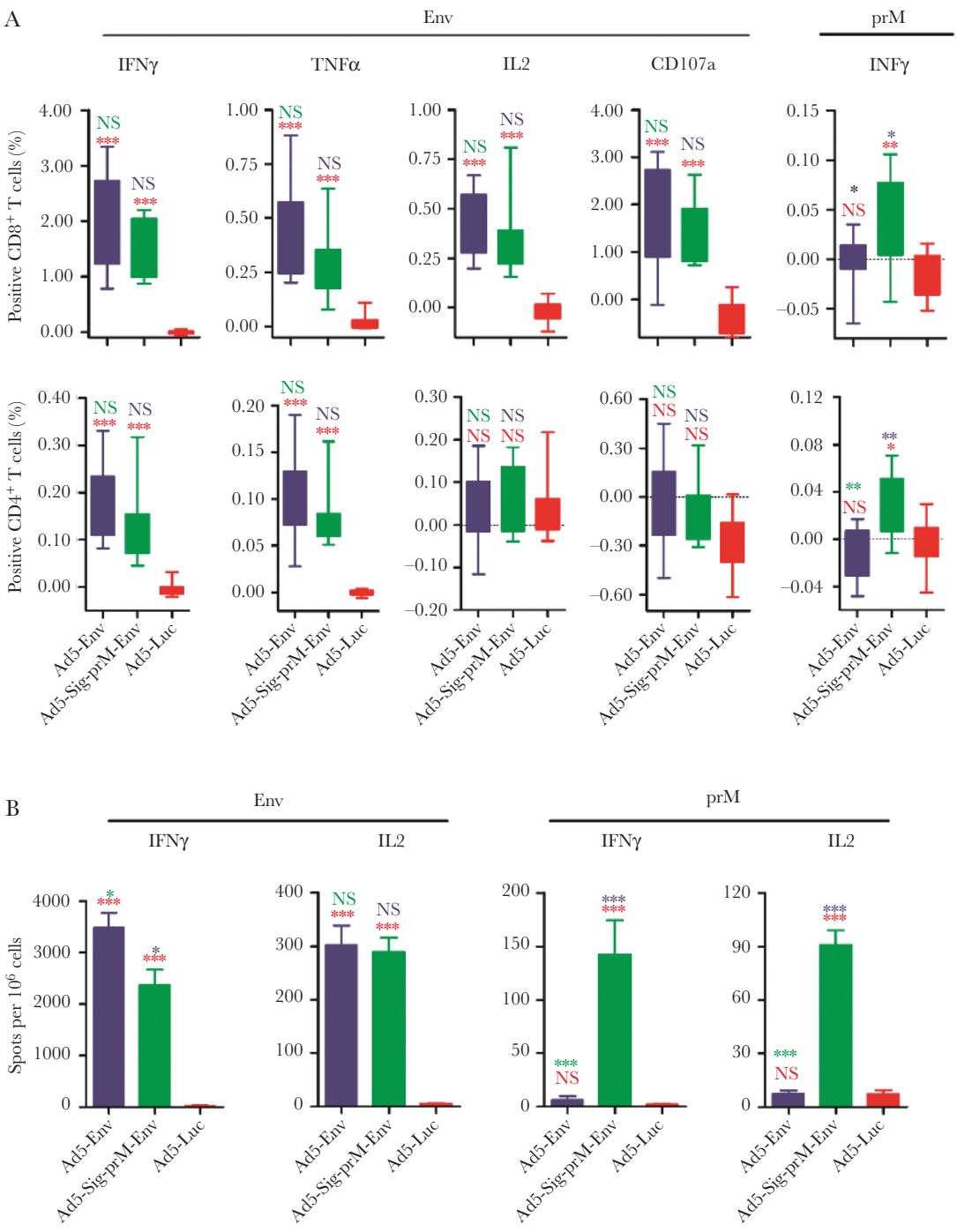

Figure 3. The cellular immune responses elicited by the adenovirus 5 (Ad5)-vectored Zika virus (ZIKV) vaccines in immunocompetent BALB/c mice. Each 4- to 6-week-old BALB/c mouse was vaccinated intramuscularly with a single dose of Ad5-Luc, Ad5-Env, or Ad5-Sig-prM-Env (n = 10 per group). The murine splenocytes were collected during necropsy at 2 weeks after vaccination. The cell-mediated immune responses were assessed by (A) multiparameter intracellular cytokine staining and (B) enzyme-linked immunospot assays. Statically significant differences (Student's *t* test) were marked by bars and asterisks (*, $P < .05$; **, $P < .005$; ***, $P < .001$). Abbreviations: CD107a, cluster of differentiation 107a; Env, envelope; IFN, interferon; IL, interleukin; NS, no significant difference; prM, premembrane; TNF, tumor necrosis factor.

weight loss of <3% throughout the study period (Figure 5A). The mean body weight loss of the Ad5-Env-vaccinated mice was more than that of the Ad5-Sig-prM-Env-vaccinated mice, but it was generally still less than that of the controls. The Ad5-Sig-prM-Env-vaccinated and Ad5-Env-vaccinated mice had very low clinical scores (Figure 5B). The survival rates of the Ad5-Sig-prM-Env-vaccinated and Ad5-Env-vaccinated mice were 100% and 83.3%, respectively, which were significantly higher than that of the controls ($P < .05$) (Figure 5C). Corroborating with the clinical findings, the Ad5-Sig-prM-Env-vaccinated

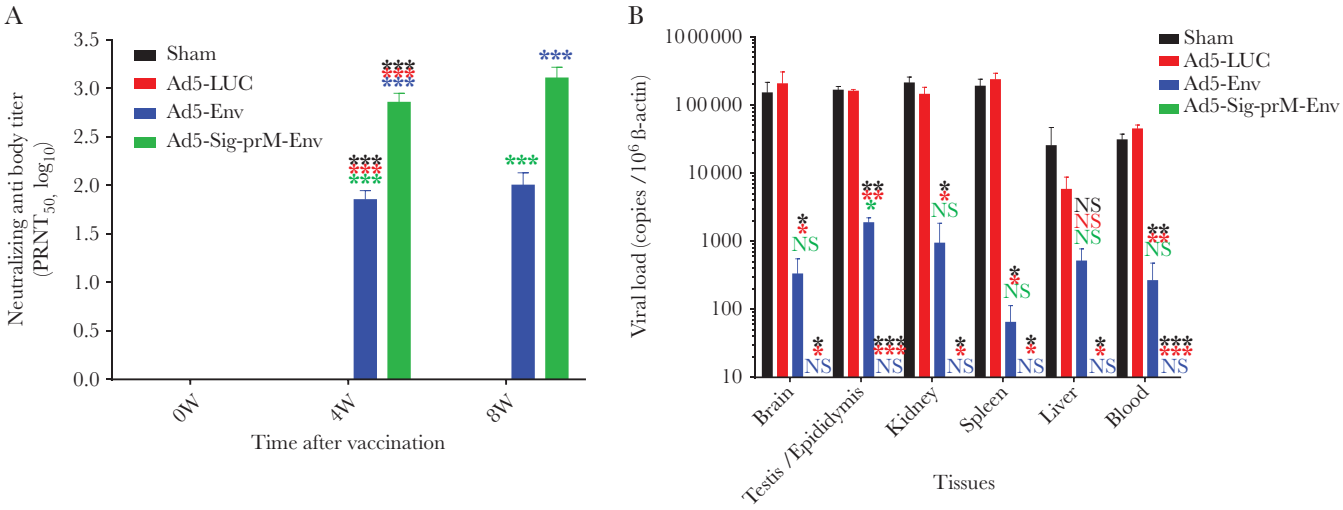

Figure 4. Serological and virological parameters of dexamethasone-immunosuppressed mice vaccinated with sham vaccine, Ad5-Luc, Ad5-Env, or Ad5-Sig-prM-Env. (A) Serum neutralizing antibody titers of the mice. To avoid unnecessary suffering, no additional mice were included in the 2 control groups for evaluation of antibody responses at 8 weeks postvaccination because they were not expected to survive virus challenge at 4 weeks postvaccination. (B) Viral loads in the blood and major organ tissues of the mice collected at 5 days post-Zika virus inoculation. Zika virus ribonucleic acid copies in the blood and tissues of the mice were determined by real-time reverse-transcription polymerase chain reaction and normalized by β-actin as described in the Supplementary Methods. *, $P < .05$; **, $P < .005$; ***, $P < .001$. The labels above each bar indicate the comparisons between the group represented by the bar and the groups that are represented by the same color of the "*" or "NS." Error bars represent standard error of the mean. Total n = 6 per group. Results were combined from 2 independent experiments. Abbreviations: Ad5, adenovirus 5; Env, envelope; NS, not significant; prM, premembrane; $PRNT_{50}$, plaque reduction neutralization test titer that showed 50% of plaque reduction.

mice had undetectable ZIKV RNA loads in serum (3 and 6 days postinfection [dpi]) (Figure 5D) and major organ tissues (6 dpi) (Figure 5E), whereas the Ad5-Env-vaccinated mice had detectable but significantly lower ZIKV RNA loads in serum and organ tissues than the controls ($P < .05$). ZIKV-NS1 antigen expression was undetectable in the brain and testis of the Ad5-Sig-prM-Env-vaccinated mice (Figure 6). Similar to the dexamethasone-immunosuppressed mice, the Ad5-Sig-prM-Env-vaccinated and Ad5-Env-vaccinated A129 mice had similar serum anti-ZIKV-Env-specific binding antibody titers (Supplementary Figure 2B), but the Ad5-Sig-prM-Env-vaccinated mice had significantly higher serum anti-ZIKV-specific nAb titers ($P < .001$) (Figure 5F). The serum anti-ZIKV-specific nAb titers were found to correlate with the differential viral loads ($R^2 = 0.845$). Given the undetectable ZIKV RNA load in the Ad5-Sig-prM-Env-vaccinated mice, we further compared their pre- and post-ZIKV challenge serum samples and found the lack of an anamnestic response (Figure 5G), thus confirming sterilizing immunity. Taken together, these findings demonstrated that Ad5-Sig-prM-Env elicited sterilizing protection against ZIKV infection in the severely immunocompromised IFN receptor-deficient A129 mice. The higher serum anti-ZIKV-specific nAb titer of the Ad5-Sig-prM-Env-vaccinated mice provided a likely explanation of the better protective effects observed in our A129 mouse model.

Passive Immunization With Serum of Mice Vaccinated With Ad5-Sig-prM-Env Protected A129 Mice Against Zika Virus Infection

To further ascertain the importance of ZIKV-specific antibody response for the observed protection against ZIKV infection, we performed adoptive transfer studies using the lethal A129 mouse model. After ZIKV inoculation, the A129 mice that received Ad5-Sig-prM-Env-vaccianted mouse antisera (nAb titer 3.11 logs) had the best clinical parameters with 100% survival, minimal weight loss, and no clinical symptoms (Figure 7A–C). Zika virus RNA was not detectable by quantitative reverse transcription-polymerase chain reaction in their sera (3 and 6 dpi) (Figure 7D) and tissues (6 dpi) (Figure 7E). Two (33.3%) mice that received Ad5-Env-vaccianted mouse antisera (nAb titer 1.60 logs) were euthanized at 5 dpi because they developed >10% weight loss with clinical symptoms, whereas the remaining 4 (66.7%) mice survived with mean body weight loss <10% and minimal clinical symptoms. Low levels of ZIKV RNA were detectable in serum (3 dpi) (Figure 7D) and organ tissues collected at necropsy (Figure 7E), but they were significantly lower than those of the control mice ($P < .05$). All (100%) of the control mice that received Ad5-Luc-vaccinated or sham-vaccinated mouse antisera died at 5–6 dpi with mean weight loss >15% and high clinical scores. The mean ZIKV RNA loads in their sera and tissues collected at necropsy (5–6 dpi) were significantly higher than those of the mice that received antisera of Ad5-Sig-prM-Env-vaccinated and Ad5-Env-vaccinated mice ($P < .001$).

DISCUSSION

Adenovirus-vectored vaccines have the advantages of being safe, highly immunogenic even with single-dose regimens, and easily producible. We and others have previously evaluated the safety and immunogenicity of (1) adenovirus-vectored vaccines for

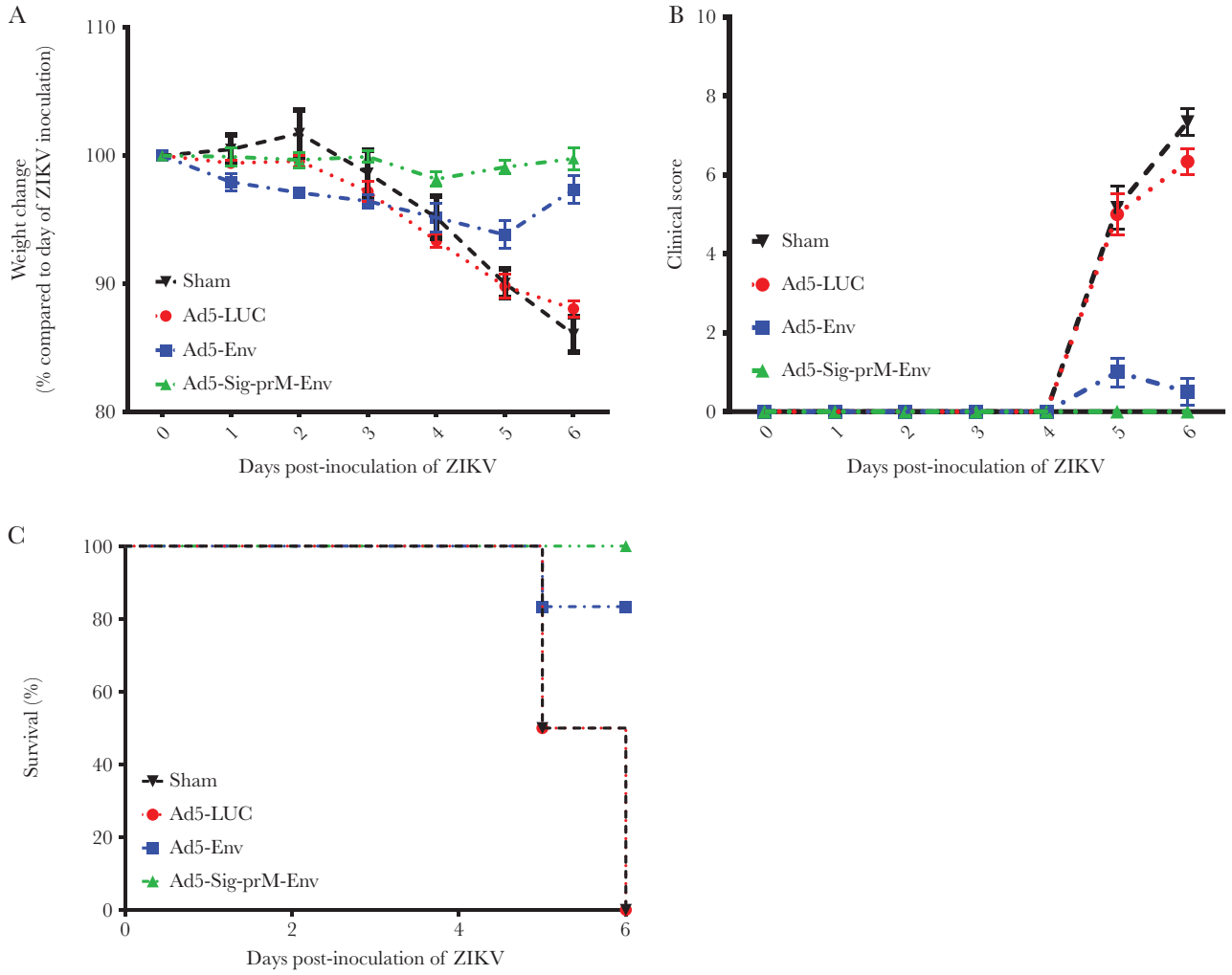

Figure 5. Clinical, virological, and serological parameters of A129 mice vaccinated with sham vaccine, Ad5-Luc, Ad5-Env, or Ad5-Sig-prM-Env. (A) Body weights, (B) clinical scores, and (C) survival times and rates of the mice were monitored for 6 days (survived mice) or until euthanasia. Clinical scores: normal = 0; ruffled fur = 2; lethargy, pinched, hunched, wasp waisted = 3; labored breathing, rapid breathing, inactive, neurological = 5; and immobile = 10. (D) Viral loads in the blood of the mice collected at 3 days post-Zika virus (ZIKV)-inoculation, and (E) viral loads in the blood and major organ tissues of the mice collected at 6 days post-ZIKV-inoculation. Zika virus ribonucleic acid copies in the blood and tissues of the mice were determined by real-time reverse-transcription polymerase chain reaction and normalized by β-actin as described in the Supplementary Methods. (F) Serum neutralizing antibody titers of the mice at 0 and 4 weeks. (G) Serum neutralizing antibody titers of the Ad5-Sig-prM-Env-vaccinated mice at 1 week before (Pre) and 1 week after (Post) ZIKV challenge. Red line indicates the median value. The number of animals showing a 4-fold increase in neutralizing antibody titer (positive anamnestic response) at 1 week after ZIKV challenge is indicated at the bottom of the graph. *, $P < .05$; **, $P < .005$; ***, $P < .001$. The labels above each bar indicate the comparisons between the group represented by the bar and the groups that are represented by the same color of the "*" or "NS." Error bars represent standard error of the mean. Total n = 6 per group. Results were combined from 2 independent experiments. Abbreviations: Ad5, adenovirus 5; Env, envelope; NS, not significant; prM, premembrane; $PRNT_{50}$, plaque reduction neutralization test titer that showed 50% of plaque reduction; W, weeks postvaccination.

Ebola virus, human immunodeficiency virus, and respiratory syncytial virus infections and malaria in clinical trials [26, 27, 37–42]. GamEvac-Combi, a heterologous vesicular stomatitis virus-/Ad5-vectored Ebola vaccine, and our Ad5-vectored Ebola vaccine have recently been approved in Russia (registration number: LP-003390) and China, respectively [43, 44]. In this study, we developed and evaluated 2 novel human Ad5-vectored vaccines expressing the ZIKV-Sig-prM-Env and ZIKV-Env proteins, respectively, in mice with varying degrees of immunodeficiency.

Two other adenovirus-vectored vaccines using different ZIKV polypeptide fragments have recently been reported, but neither has demonstrated convincing evidence of sterilizing protection. Abbink et al [8] showed that a single-dose intramuscular injection of a rhesus adenovirus type-52 (RhAd52)-vectored vaccine containing the ZIKV-M-Env proteins could induce ZIKV-specific nAb response and ZIKV-Env-specific cellular immune response in rhesus macaques. It is noteworthy that unlike the immunosuppressed mouse models used in the current study, ZIKV-infected rhesus macaques generally develop mild, self-limiting symptoms and do not die, which make the protective effects of the vaccines more difficult to interpret in the short term than in the long term [45].

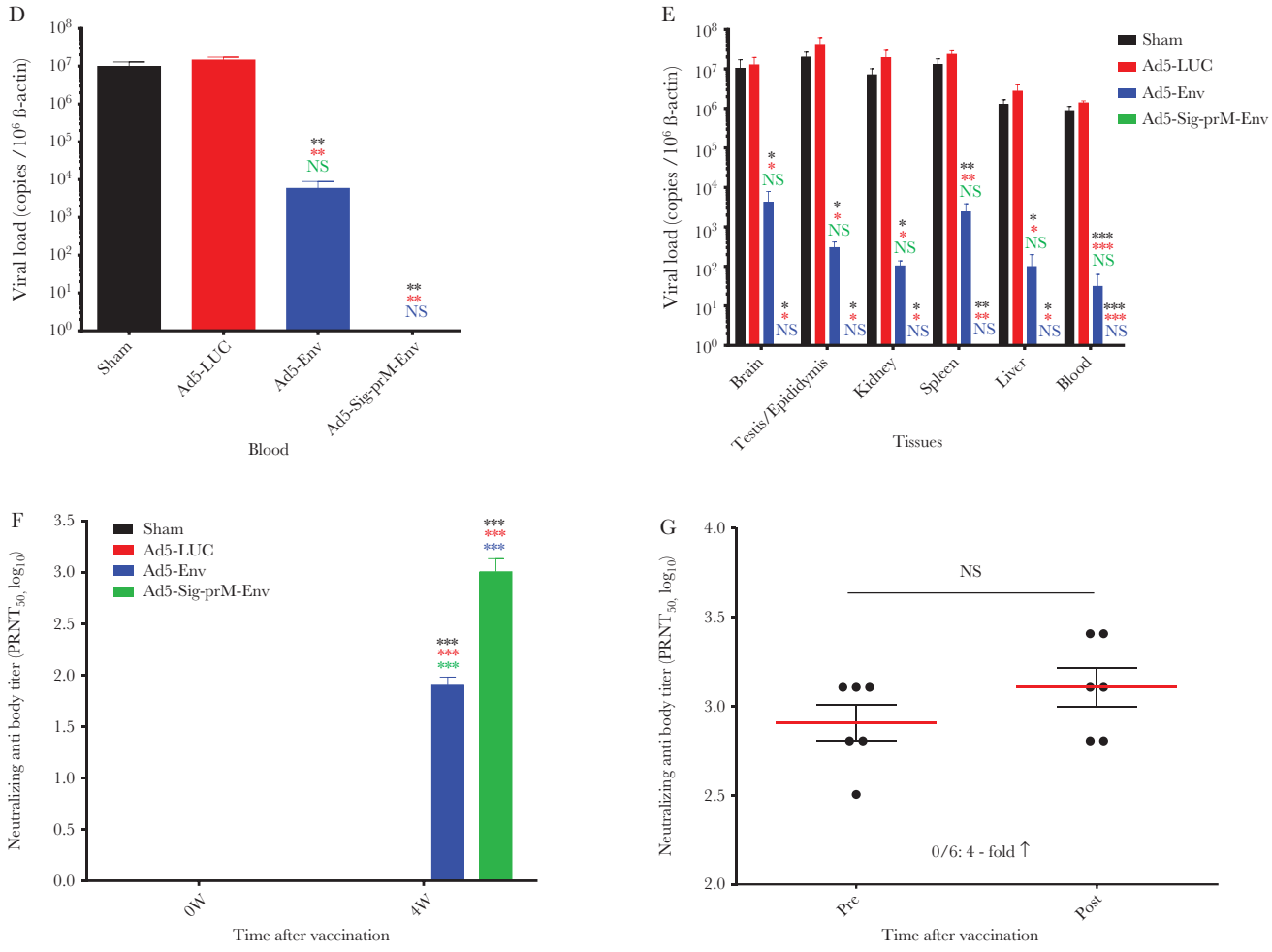

Figure 5. *Continued*

Kim et al [11] showed that subcutaneous administration of 2 doses of a recombinant adenovirus-vectored vaccine expressing codon-optimized ZIKV-M-Env (not prM-Env) antigens induced antibody response in adult C57BL/6 mice. The protective effects of the vaccine were indirectly assessed in suckling mice born to vaccinated female C57BL/6 mice infected with a ZIKV strain belonging to the African lineage, but not directly in an adult lethal mouse model using epidemic ZIKV strain. Moreover, whether the use of T4-fibritin foldon trimerization domain to replace the ZIKV-Env transmembrane domain would reduce the immunogenicity of this vaccine was not clearly investigated. Reduced immunogenicity has been reported for another ZIKV DNA vaccine that used Japanese encephalitis virus transmembrane domain to replace ZIKV-Env transmembrane domain [9]. During the revision of this manuscript, another nonhuman adenovirus-vectored vaccine expressing ZIKV-M/E glycoproteins was reported to protect against ZIKV infection in mice [46]. However, unlike the present study, data on the lack of anamnestic response in the vaccinated mice was not reported by Xu et al [46] to confirm sterilizing immunity of their vaccine. In view of these limitations, we used multiple immunocompetent and immunocompromised mouse models in this study to more clearly demonstrate the effects of our Ad5-vectored vaccines.

We first showed that both Ad5-vectored vaccines elicited robust humoral and cellular immune responses in immunocompetent BALB/c mice. Based on these results, we then evaluated the immunogenicity and protective effects of our vaccines in immunosuppressed mice with severe ZIKV infection. Because immunosuppression with steroid therapy is increasingly used in patients with various diseases, which may dampen the robustness and durability of vaccine-elicited antibody responses, we evaluated the serial antibody responses elicited by our vaccines in dexamethasone-immunosuppressed mice. We showed that both vaccines elicited rapid-onset, robust, and durable antibody responses in these mice, and Ad5-Sig-prM-Env elicited significantly higher titers of anti-ZIKV-specific nAb titers.

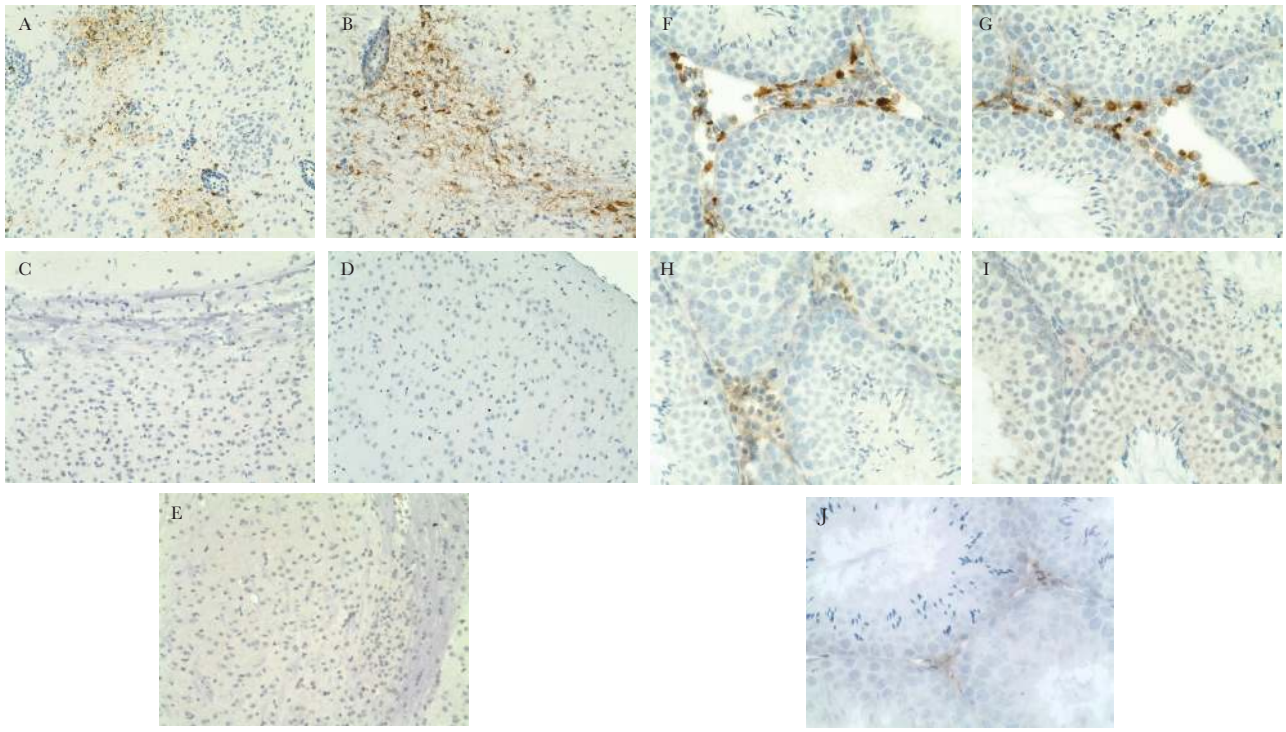

Figure 6. Representative immunohistochemistry findings in the brain and testis of A129 mice vaccinated with sham vaccine, Ad5-Luc, Ad5-Env, or Ad5-Sig-prM-Env. Immunohistochemistry staining of the brain and testicular tissue sections of the mice was performed as we previously described [31]. ZIKV-NS1 protein expression was abundantly observed in the brain and testis (Leydig cells) tissue sections of mice vaccinated with either sham vaccine (A and F) or Ad5-Luc (B and G), but this was minimal or absent in those of mice vaccinated with either Ad5-Env (C and H) or Ad5-Sig-prM-Env (D and I) and uninfected control mice (E and J). (A–E) Brain tissue sections (×200) of mice vaccinated with sham vaccine (A), Ad5-Luc (B), Ad5-Env (C), or Ad5-Sig-prM-Env (D), and uninfected control (E). (F–J) Testicular tissue sections (×400) of mice vaccinated with sham vaccine (F), Ad5-Luc (G), Ad5-Env (H), or Ad5-Sig-prM-Env (I), and uninfected control (J). The methodological details of vaccination and ZIKV inoculation are listed in the Supplementary Methods and Supplementary Table S1. Abbreviations: Ad5, adenovirus 5; Env, envelope; prM, premembrane; ZIKV, Zika virus.

Similar protective effects and antibody responses were observed in the well established lethal A129 mouse model for ZIKV infection. Immunization of these IFN receptor-deficient mice with Ad5-Sig-prM-Env elicited robust binding and nAb responses and resulted in 100% survival rate with minimal weight loss and undetectable serum and tissue ZIKV RNA loads. More importantly, there was a close correlation between the nAb titer and the viral load, providing an explanation of Ad5-Sig-prM-Env's better protective effects against ZIKV infection. It was previously reported that nAb titers >2-logs was considered protective [14]. This corroborated with our findings that the ~3-logs nAb titer elicited by Ad5-Sig-prM-Env was associated with complete protection, whereas the <2-log nAb titer elicited by Ad5-Env was associated with partial protection against ZIKV infection in A129 mice. The molecular mechanism of the differences between the 2 vaccines' immunogenicities is likely related to the biological functions of the Env and prM proteins. The Env protein is the major immunogenic antigen of flaviviruses [47]. It forms heterodimers on the viral surface with the membrane protein and plays key roles in cell entry [48]. The prM protein plays a critical role in the folding of Env protein of flaviviruses and the release of virus particles from infected cells [49]. Notably, the ZIKV peptide fragments used in the other 3 adenovirus-vectored vaccines were different from the ones we used. Abbink et al [8] did not express the first 93 amino acids of prM and only encoded the short M-peptide, which is the product of furin cleavage of prM during natural infection. Kim et al [11] expressed the extracellular portion of the ZIKV-Env [11]. Xu et al [46] used amino acids 216–794 of the ZIKV polyprotein to construct their vaccine. It would be important to directly compare the immunogenicities of these vaccines with Ad5-Sig-prM-Env in future studies.

CONCLUSIONS

We did not investigate our vaccines' potential to elicit antibody-dependent enhancement on other flaviviruses, because the in vitro and in vivo significance of this phenomenon remains controversial [50]. Given the excellent mouse model data of Ad5-Sig-prM-Env in the present study and the experiences from previously successful human trials indicating the distinct advantages of adenovirus-vectored vaccines, clinical trials should be considered for this urgently needed countermeasure among at-risk population groups once further preclinical data on the purity and safety of the vaccine become available.

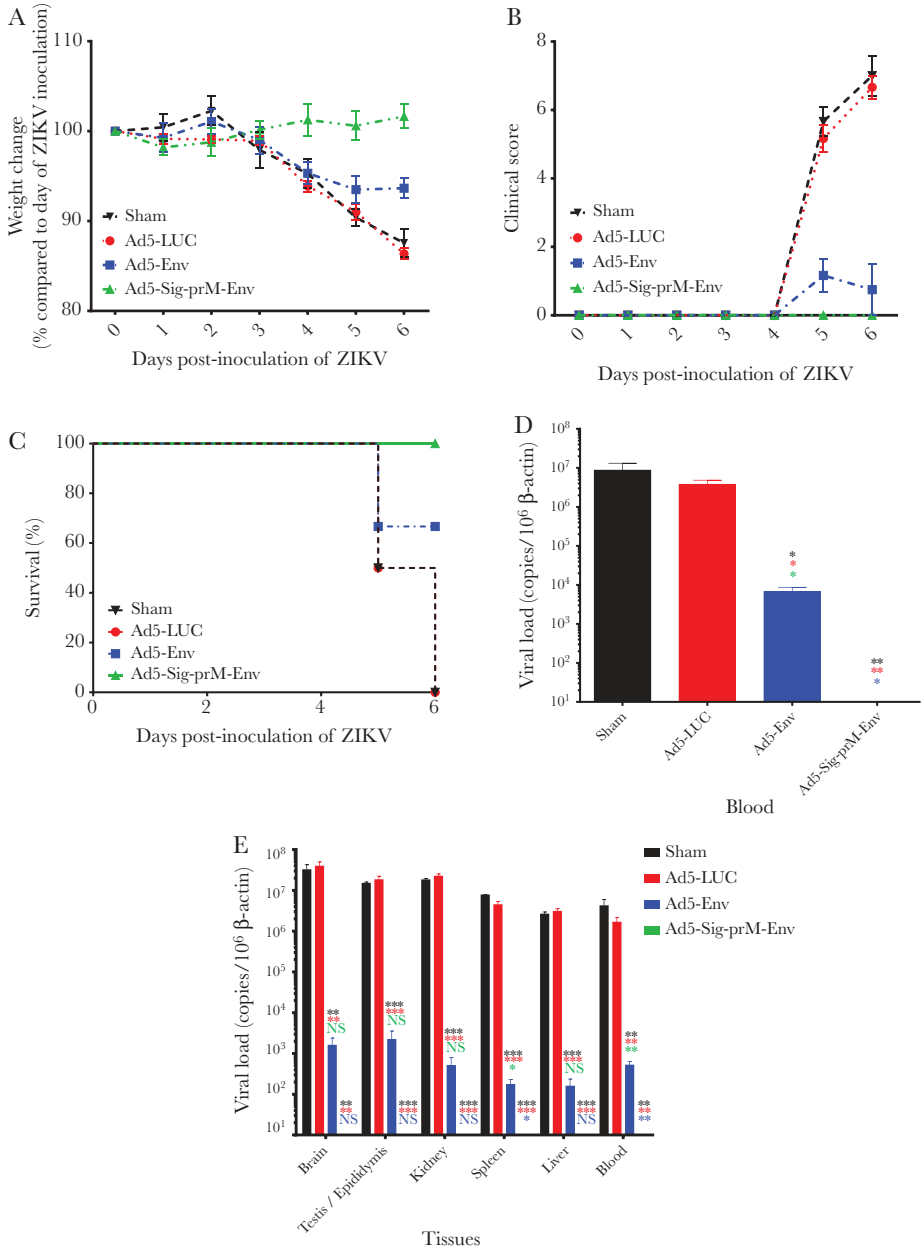

Figure 7. Clinical and virological parameters of A129 mice receiving antisera obtained from BALB/c mice vaccinated with sham vaccine, Ad5-Luc, Ad5-Env, or Ad5-Sig-prM-Env. (A) Body weights, (B) clinical scores, and (C) survival times and rates of the mice were monitored for 6 days (survived mice) or until euthanasia. Clinical scores: normal = 0; ruffled fur = 2; lethargy, pinched, hunched, wasp waisted = 3; labored breathing, rapid breathing, inactive, neurological = 5; and immobile = 10. (D) Viral loads in the blood of the mice collected at 3 days post-Zika virus (ZIKV)-inoculation, and (E) viral loads in the blood and major organ tissues of the mice collected at 6 days post-ZIKV-inoculation. Zika virus ribonucleic acid copies in the blood and tissues of the mice were determined by real-time reverse-transcription polymerase chain reaction and normalized by β-actin as described in the Supplementary Methods. *, $P < .05$; **, $P < .005$; ***, $P < .001$. The labels above each bar indicate the comparisons between the group represented by the bar and the groups that are represented by the same color of the "*" or "NS." Error bars represent standard error of the mean. Total n = 6 per group. Results were combined from 2 independent experiments. Abbreviations: Ad5, adenovirus 5; Env, envelope; NS, not significant; prM, premembrane.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Disclaimer. The sponsors had no role in the design and conduct of the study, in the collection, analysis and interpretation of data, or in the preparation, review, or approval of the manuscript.

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Potential conflicts of interest. J. F.-W. C. has received travel grants from Pfizer Corporation Hong Kong and Astellas Pharma Hong Kong Corporation Limited and was an invited speaker for Gilead Sciences Hong Kong Limited and Luminex Corporation. The corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for publication. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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