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A rapid strategy for constructing novel simian adenovirus vectors with high viral titer and expressing highly antigenic proteins applicable for vaccine development

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ABSTRACT

Adenoviral vectors have been widely used for the development of infectious disease vaccines. However, the challenge of human adenoviral vector rooted from the predominant adenovirus serotype 5 strain limiting its usefulness by the widespread pre-existing neutralizing antibodies in recipients. To circumvent this obstacle, we generated an ad-hoc adenovirus vector in human or primates. Here, a chimeric simian adenoviral vector Sad23 was constructed consisting in deleting of E1 and E3 regions of the full-length simian adenovirus serotype 23 genome (SAdV23) by Gibson assembly. To improve Sad23 virus propagating efficiency, the E4 region open reading frame 6 (orf6) was replaced by the corresponding element of human adenovirus type 5 (Ad5), designated Sad23L. The procedure for cloning this novel vector took a single week, and recombinant adenovirus was packaged with high titer in HEK293 cells. To verify the ability of this novel adenoviral vector to deliver foreign genes, Zika virus (ZIKV) prM-E genes were used as target genes for antigen expression. Recombinant adenoviruses Sad23L-prM-E, Sad23-prM-E and Ad5-prM-E were intramuscularly inoculated into Ad5-eGFP none pre-exposed or pre-exposed mice, and the immune response to ZIKV prM-E was compared between vectors. Sad23L-prM-E induced a fairly robust immune response and maintained immunogenicity in Ad5 pre-exposed mice, which suggested that Ad5 pre-existing immunity did not affect Sad23L-prM-E immunization. These preliminary results suggest that the proposed rapid strategy was effective in constructing a new adenoviral vector platform (Sad23 L) usable for the development of human vaccines.

1. Introduction

Recombinant adenovirus vectors have a good safety profile and induce broad and strong humoral and cellular immune response (Carnathan et al., 2015; von Delft et al., 2018; Xiang et al., 2014). Therefore, they are widely used in the development of infectious disease and cancer vaccines (Croyle et al., 2005; Liu and Muruve, 2003; Zhang et al., 2016). To overcome the widespread preexisting immunity of common adenoviruses, a range of rare human and chimpanzee adenovirus vectors have been developed (Abbink et al., 2015; Alonso-Padilla et al., 2016; Cheng et al., 2016; Colloca et al., 2012; Liu et al., 2009). Currently human adenovirus serotype 5 (Ad5) or Ad2 are widely applied in human populations, in which preexisting or induced neutralizing antibodies limit the longevity of the inserted gene expression and may increase unwanted side effects such as vector-toxicity (Buchbinder et al., 2008; McElrath et al., 2008). In addition, some replication-incompetent adenovirus vectors unsuccessfully rescue virus or propagate inefficiently in Ad5 E1-complementing cell lines. To overcome these problems, the open reading frame (orf) 6 of rare human or chimpanzee adenoviral E4 region (E4orf6) is replaced by the corresponding Ad5 E4orf6 (Lemckert et al., 2006; Silke and Matthias, 2000).

The construction of adenovirus vector is challenging because its genome is very large (36 kb) and has few available restriction sites. Classical methods are based on homologous recombination or rely on the rare restriction sites, but these methods are time consuming and difficult to control (Di et al., 2012; Jager et al., 2009). However, Gibson assembly ligation specifically enables multiple gene fragments to be ligated into a vector plasmid presenting independent restriction sites

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(Gibson et al., 2009).

In this article, we describe a rapid method of Gibson assembly to construct chimeric adenoviral vector Sad23L based on simian adenovirus type 23 (SAdV23) that is low-seroprevalence in humans (Ersching et al., 2010; Xiang et al., 2006). We also evaluated the efficiency of Sad23L, a newly developed vector, in terms of viral production, ZIKV prM-E protein expression, antigen delivery and immunogenicity in Ad5 none pre-exposed and Ad5 pre-exposed mice in comparison with the basic Sad23 and the commercial Ad5 vector, respectively.

2. Materials and methods

2.1. Cell line, virus, animal and plasmid

HEK293 cell line (ATCC-CRL-1573) was cultured in complete Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) and incubated at 37 °C in 5% CO_2. The wild simian adenovirus type 23 (SAdV23/AdC6/Pan6, ATCC-VR-592) was purchased from American Type Culture Collection (ATCC) and propagated in HEK293 cells. pShuttle-Flag-XBP1u plasmid was purchased from Addgene (Addgene, cat.no.63679). The commercial pBHGloxdeltaE13Cre plasmid was provided by Dr. JH Zhou (State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences), which was initial generated by Parks et al. (2000) (Bett et al., 1994). The pDC315-prM-E-ZIKV plasmid was kindly provided by Dr. Wei Zhao (School of Public Health, Southern Medical University, China). In-Fusion® HD Cloning Plus kit was purchased from Takara (Takara Biomedical Technology, Beijing, China, cat.no.638909). Zika virus (ZIKV) envelope (E) protein was purchased from Fitzgerald (Fitzgerald, Acton, MA, USA, cat.no.30-1932).

C57BL/6 mice were purchased from the Animal Experimental Centre of Southern Medical University, Guangzhou, China. All animal care and experimental procedures were in accordance with national and institutional policies for animal health and well-being. The study design was approved by laboratory Anima Ethics Committee of Southern Medical University, China.

2.2. Construction of E1-deleted SAdV23 plasmid pUC19-DNA12

The plasmid pUC19-DNA12 was constructed as follows. First, using simian adenovirus type 23 full genomic DNA (SAdV23) as template, PCRs were performed to amplify the DNA0 fragment (location nucleotide 1–450; see GenBank accession no. AY530877.1), DNA02 fragment (location 3414–9022) and DNA2 fragment (location 9007-15111) via primers F0, R0, F1, R1, F2 and R2 (Table 1). Second, using pUC57-Linker (synthesized by Beijing Genomics Institute, Beijing, China) plasmid as template, the DNA01 fragment was amplified by PCR with 5′ and 3′ primers F0-1 and R0-1. The DNA0, DNA01 and DNA02 fragments were mixed and jointed into a DNA1 fragment using overlapping PCR with primers F0 and R1. Third, pUC19 plasmid was digested with *EcoR*I and *Hind*III, and Gibson assembly of pUC19-DNA12 plasmid was performed by mixing DNA1, DNA2 and digested pUC19. The constructed plasmid was designated pUC19-DNA12 in which E1 was deleted.

2.3. Construction of E3-deleted SAdV23 plasmid pUC19-DNA345 and pUC19-DNA34678-Ad5-E4orf6

E3-deleted SAdV23 plasmid pUC19-DNA345 was generated as follows. First, using SAdV23 genomic DNA as template, the DNA3 (nucleotides 15111 to 23111), DNA4 (nt 23096-27111) and DNA5 (nt 31864-36604) fragments were amplified by PCR with primers F3, R3, F4, R4, F5 and R5 (Table 1). Second, pUC19-DNA345 plasmid was constructed by Gibson assembly with a mixture of DNA3, DNA4, DNA5 and *EcoR*I and *Hind*III digested pUC19. The new plasmid was called pUC19-DNA345 in which E3 was deleted.

By using SAdV23 genomic DNA as template, DNA3 (location 15111 to 23111), DNA4 (location 23096 to 27111), DNA6 (location from 31864 to 33841) and DNA7 (location from 34746 to 36604) fragments were amplified by PCR with primers F3, R3, F4, R4, F5, R6, F7 and R5. DNA8 (involving the 884bp Ad5-E4orf6) fragment was amplified from pBHGloxdeltaE13Cre plasmid by PCR using primers F8 and R8 (Table 1). The pUC19-DNA34678-Ad5-E4orf6 plasmid was constructed by Gibson assembly with a mixture of DNA3, DNA4, DNA6, DNA7, DNA8 and *EcoR*I and *Hind*III digested pUC19. The new plasmid was called pUC19-DNA34678 in which E3 was deleted and E4orf6 was replaced by the corresponding Ad5 element.

2.4. Construction of plasmids Sad23L-prM-E and Sad23-prM-E

Plasmids pUC19-DNA12 and pUC19-DNA34678 were separately cut by restriction enzymes *Pme*I and *Mlu*I. Digested DNA12 fragment was ligated to the linearized pUC19-DNA34678, and the new construct was called Sad23 L (L was taken from the initial of three major contributors surnames, indicating the improved Sad23 vector). The vector was sequenced (Beijing Genomics Institute, Beijing, China), and no mutation was found. The protocol for constructing Sad23 (E1/E3 deleted) vector was as described above without Ad5-E4orf6 element replacement.

ZIKV-prM-E genes in plasmid pDC315-prM-E-ZIKV were cloned into pShuttle-Flag-XBP1u plasmid by *EcoR*I and *BamH*I, and was called pShuttle2-prM-E. The construct was digested with *I-Ceu*I and *PI-Sce*I, and ligated to the Sad23 L or Sad23 plasmid digested with same enzymes. The expressing adenoviral vector was called Sad23L-prM-E or Sad23-prM-E. Sad23L-eGFP and Sad23-eGFP vectors were constructed and used as controls.

2.5. Rescuing of recombinant adenovirus Sad23L-prM-E, Sad23-prM-E and Ad5-prM-E

HEK293 cells were transfected with *Pac*I linearized Sad23L-prM-E, Sad23-prM-E or Ad5-prM-E (pBHGloxdeltaE13Cre and pDC315-prM-E) plasmids in X-tremeGENE HP DNA transfection solution. Adenovirus plaques became visible 8–10 days post transfection.

To test genetic stability of Sad23L-prM-E vector, the recombinant adenoviruses were serially passaged in HEK293 cell culture when full cytopathic effect appeared. Cells were frozen and thawed three times from 37°C to -80°C. The recombinant adenoviruses were passaged for 12 generations (P12). The size consistency of genomic DNAs from P1 and P12 were identified by digestion with *Hind*III and *Age*I. The digested DNA fragments were analyzed by agarose gel electrophoresis.

2.6. Determination of adenoviral vector expression

HEK293 cells were infected with recombinant adenoviruses Sad23L-prM-E, Sad23-prM-E and Ad5-prM-E, respectively, and the cells were harvested after 48 h. Cell lysates were prepared for Western blotting analysis by 12% SDS-PAGE and anti-ZIKV E antibody (Xu et al., 2018a). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as internal control.

2.7. Adenovirus propagation and purification

Recombinant adenoviruses Sad23L-prM-E, Sad23-prM-E and Ad5-prM-E were separately cultivated to 90% confluent monolayer of HEK293 cells in T75 flasks. When viral plaques became visible, cells were harvested and re-suspended in 10 ml of 1 × PBS (Phosphate-buffered saline). Viral particles were released by the freeze-thaw procedure and the suspensions were cryopreserved. The recombinant adenoviruses were purified from the suspensions by CsCl density-gradient centrifugation (Holkers et al., 2014). Titers of purified viruses were calculated in PFU/ml by standard plaque-forming assay on HEK293

cells. The concentration of virus particles per milliliter (vp/ml) after purification was determined by measuring UV absorbance at 260 nm (A_{260}) using a spectrophotometer. The virus particle (vp) was calculated as: vp = OD_{260} × dilution × 1.1 × 10^{12} × (wildtype genome/recombinant virus genome) (Zhou et al., 2010). Rescuing and purifying of Sad23L-eGFP, Sad23-eGFP and Ad5-eGFP control viruses were carried out according to the same protocol.

2.8. Testing of immune response to ZIKV E in adenovirus Sad23L-prM-E, Sad23-prM-E or Ad5-prM-E inoculated mice

Female C57BL/6 mice (5–6 weeks of age, n = 5 each group) were immunized with 5 × 10^7 PFU Sad23L-prM-E, Sad23-prM-E or Ad5-prM-E adenoviruses, respectively. PBS was administered as a mock control group (n = 5). Titer of antibody to ZIKV E was quantified 4 weeks post vaccination. Enzyme-linked immunosorbent assay (ELISA) plates were coated overnight with 7 μg/ml of ZIKV E protein (Fitzgerald.30-1932) and blocked with 1% bovine serum albumin (BSA) PBS. Sera were 3-fold serially diluted. Plates were incubated at 37 ℃ for 1 h and washed 5 times. After adding anti-mouse IgG-HRP antibody, the plates were incubated for 30 min at 37 ℃, then washed 5 times and developed with TMB substrate, stopping the reaction with 2 M hydrochloric acid and measuring OD at 450 nm. Log 10 endpoint titer was defined as the highest reciprocal serum sample dilution with absorbance greater > 2-folds the background value.

Mouse IFN-gamma ELISpotPLUS kits (MabTech, Sweden) were used to determine antigen specific T cell response. Spleens were removed 4 weeks post immunization and splenocytes were isolated to detect antigen specific T lymphocyte response by enzyme-linked immunospot (ELISpot) as previously described (Li et al., 2014; Zhu et al., 2016). Mouse splenocytes (3 × 10^5 cells/well) were stimulated with the ZIKV-E peptide pool (10 μg/ml). Cells were incubated with phorbol myristate acetate (PMA) or without peptide serving as positive and negative control, respectively. After 36 h, the cells were removed. Plates were incubated with 1 μg/ml of Biotin-Mouse IFN-γ detection antibody at room temperature for 2 h, followed by 1 h incubation with alkaline phosphatase-conjugated streptavidin. After washing 5 times, BCIP/NBT-plus substrate solution was added for color development and stopped with deionized water. The numbers of spots were determined as spot-forming cells (SFCs)/10^6 cells on a CTL Immunospot Reader.

2.9. Measurement of cross-reactive neutralizing antibodies between Sad23 L and Ad5 viruses

Female C57BL/6 mice (5–6 weeks of age, n = 5 each group) were inoculated with 10^8 PFU Ad5-eGFP. After 3 weeks, Ad5 pre-exposed mice were immunized with 5 × 10^7 PFU Sad23L-prM-E, Sad23-prM-E or Ad5-prM-E adenoviruses, respectively (Table 3). PBS was administered as a mock control group (n = 5). Specific antibodies and T cell response to ZIKV E antigen were tested 4 weeks post virus immunization.

Neutralizing antibodies (NAb) in plasmas of mice immunized with PBS, Sad23L-prM-E or Ad5-prM-E were tested on HEK293 cells for cross-reactive neutralization of Sad23L-eGFP and Ad5-eGFP by a plaque reduction assay as previously described (Farina et al., 2001).

3. Results

3.1. Generation of recombinant adenoviruses Sad23L-prM-E and Sad23-prM-E

The full-length genomic DNA of simian adenovirus serotype 23 strain (SAdV23) was purified. The novel plasmid Sad23 L (E1/E3 deleted and E4orf6 replaced by Ad5 element) and Sad23 vectors (E1/E3 deleted) were generated from SAdV23 genome by PCRs and Gibson assembly according to the above described methods (Fig. 1A). The fragment ZIKV prM-E was inserted into Sad23 L and Sad23 (dE1/E3) vectors, designated as Sad23L-prM-E and Sad23-prM-E, respectively (Fig. 1A). The empty Sad23 and Sad23L-prM-E vectors analyzed with *Age*I, *Hind*III and *Sph*I showed the right size pattern of DNA bands in agreement with the predicted molecular weights (Fig. 1B). The data indicated that these two recombinant simian adenoviral vectors were as intended.

3.2. Identification of recombinant adenoviruses Sad23L-prM-E, Sad23-prM-E and Ad5-prM-E

Plasmids Sad23-L-prM-E, Sad23-prM-E and Ad5-prM-E were transfected into HEK293 cells and the recombinant viruses were rescued when viral plaques became visible 8–10 days after transfection (Fig. 2A). Sad23L-prM-E, Sad23-prM-E and Ad5-prM-E adenoviruses were harvested and further inoculated to HEK293 cells. ZIKV-E proteins were detected by Western blotting with anti-ZIKV-E antibody (Fig. 2B). In comparison with the negative control, the Zika specific protein bands (55 kDa) were detected in Sad23L-prM-E, Sad23-prM-E and Ad5-prM-E adenovirus infected cells (Fig. 2B).

3.3. Adenovirus Sad23L-prM-E genomic stability and propagating efficiency

Genomic DNA of Sad23L-prM-E was extracted from passage 1 (P1) and passage 12 (P12) of HEK293 cell cultures and digested with *Hind*III or *Age*I. The generated DNA fragments were clearly seen by agarose gel electrophoresis (Fig. 2C) and were of the predicted length, suggesting that Sad23L-prM-E was genetically stable.

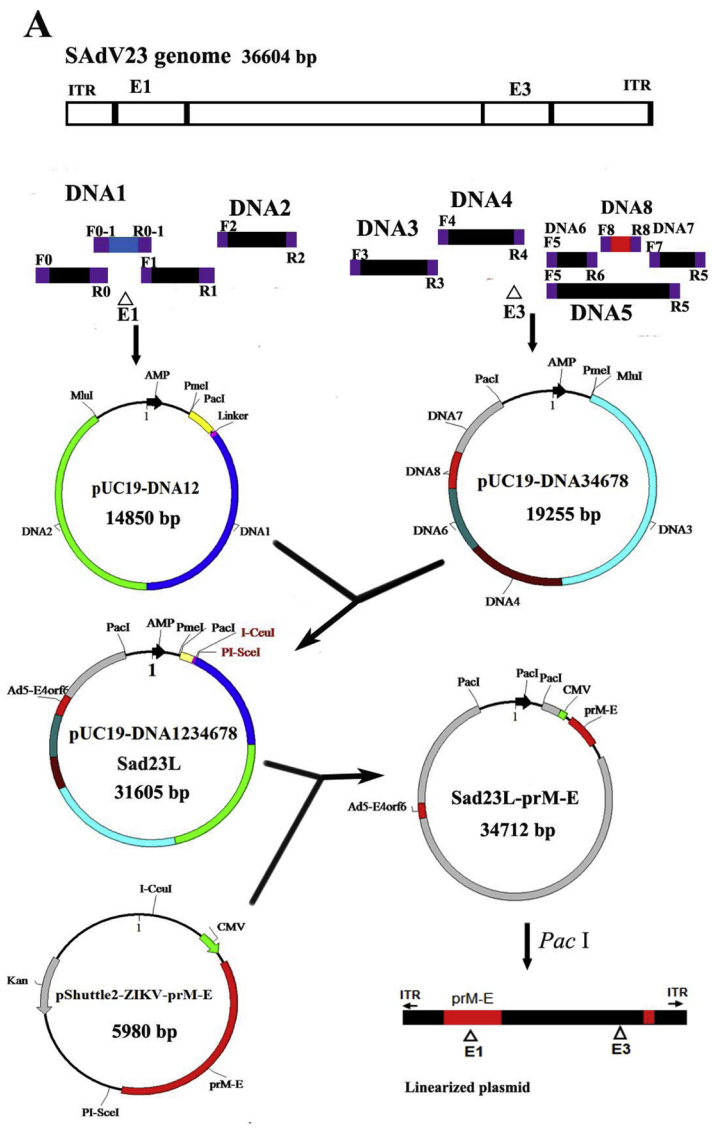
HEK293 cells at 90% confluence in T75 flasks were respectively infected with adenovirus Sad23L-prM-E, Sad23-prM-E or Ad5-prM-E. The viruses harvested from cell lysate supernatants were purified by CsCl density-gradient centrifugation (Fig. 2D). The visible, lower and stronger white layers were infectious recombinant adenoviruses, the top weaker white layers were defective viruses (Fig. 2D). After purification, the titers of infectious viruses were determined by standard plaque-forming assay on HEK293 cells. Sad23L-prM-E titer was 4.35 × 10^{11} PFU/ml (8.5 × 10^{12} vp/ml), Sad23-prM-E titer was 2.5 × 10^9 PFU/ml (3.6 × 10^{12} vp/ml) and Ad5-prM-E titer was 2.18 × 10^{12} PFU/ml (9.96 × 10^{12} vp/ml), respectively. The data showed that Sad23 L titer was 100 folds higher than Sad23, but similar to Ad5.

In order to observe the propagating efficiency of these three adenoviruses, the enhanced green fluorescent protein (eGFP) gene was cloned into Sad23L, Sad23 and Ad5 vectors, respectively. The 1 × 10^9 vp rescued viruses were inoculated to HEK293 cells in T25 flasks for 48 h incubation. The stronger fluorescence was observed with Sad23L-eGFP and Ad5-eGFP infected cells, while weaker fluorescence was seen in Sad23-eGFP infected cells (Fig. 2E). Virus plaque was only found in Sad23-eGFP infected cells, suggesting higher viral cell toxicity (Fig. 2E).

Infection ratio (vp-to-PFU) was 19.54 for Sad23L-prM-E, 1440 for Sad23-prM-E and 4.68 for Ad5-prM-E (Table 2). Infection ratio of Sad23L-prM-E was much lower than Sad23-prM-E, and close to Ad5-prM-E (Table 2). This data suggested that with a same amount of viral vps infected cells, Sad23L-prM-E and Ad5-prM-E were effectively replicating and with more infectious capacity than Sad23-prM-E.

3.4. Immunogenicity of recombinant adenoviruses

To assess the immunogenicity of recombinant adenoviruses, five mice were intramuscularly injected with 5 × 10^7 PFU of Sad23L-prM-E, Sad23-prM-E or Ad5-prM-E. PBS was injected as a mock five mice control group. Fig. 3**A** shows that all Sad23L-prM-E, Sad23-prM-E and Ad5-prM-E infected mice induced high levels of ZIK V E antibodies in comparison with control (P = 0.0002). The mean endpoint titer raised in the three groups of adenovirus-infected mice were $10^{3.324}$, $10^{3.203}$ and $10^{3.32}$, respectively (P > 0.05) (Fig. 3A).



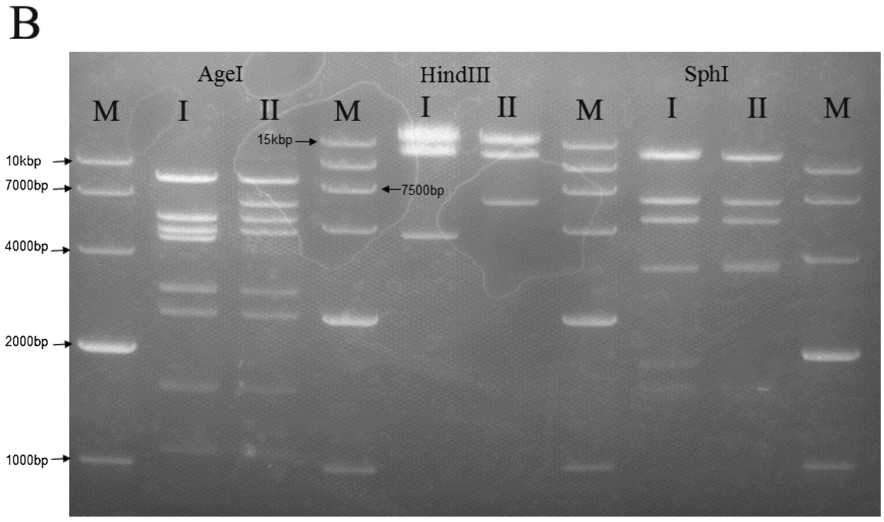


Fig. 1. Strategy for construction of a novel simian adenovirus expressing vector Sad23L-prM-E.
(**A**) Flow chart for construction of a novel simian adenoviral expressing vector. All adenoviral DNA segments were cloned into pUC19 plasmids, in which the E1 and E3 regions of SAd23V genome were deleted and E4orf6 was replaced with Ad5-E4orf6. Aimed genes (ZIKV prM-E) were inserted into pShuttle2-Flag, then cloned into Sad23 (dE1/E3) and Sad23L vectors. (**B**) Restriction analysis of empty Sad23 (dE1/E3) and Sad23L-prM-E plasmid DNAs with *Age*I, *Hind*III and *Sph*I. Digests were separated by electrophoresis in 1% agarose gel. **I**: Sad23 (dE1/E3) plasmid; **II**: Sad23L-prM-E plasmid; **M**: DNA molecular marker.

The cellular immune response to the ZIKV E peptide pool was measured from splenocytes of infected mice by ELISpot (Fig. 3B). All three adenoviruses were able to stimulate strong IFN-γ T cell responses > 400 SFCs/10^6 cells, while control mice had no response ($P < 0.0001$). Sad23L-prM-E showed a moderately higher response than Sad23-prM-E and lower than Ad5-prM-E but these differences were not significant ($P = 0.118$). However, the difference observed between Sad23-prM-E and Ad5-prM-E was significant ($P = 0.019$)

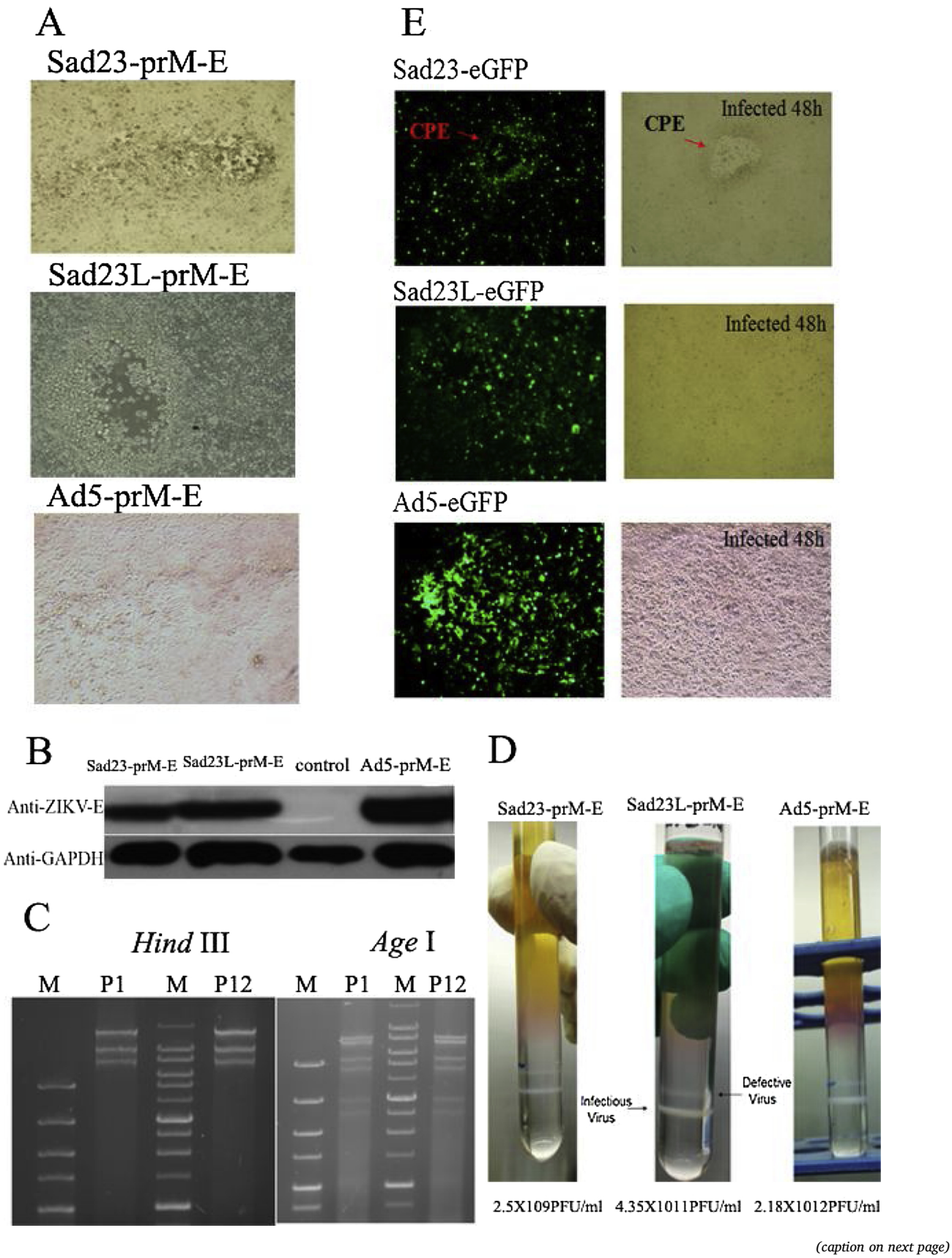


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Fig. 2. Rescue, purification and identification of recombinant adenoviruses.
(**A**) Visible cytopathic effect (CPE) of HEK293 cells in 8–10 days after transfection with linearized adenoviral vectors. (**B**) Protein expression of recombinant adenoviruses detected by Western blot. HEK293 cells were infected with Sad23L-prM-E, Sad23-prM-E or Ad5-prM-E virus, respectively. After 48 h, protein samples were analyzed by Western blot with 12% SDS-PAGE and antibodies to ZIKV E and GAPDH. Empty Sad23 (dE1/E3) virus was used as negative control. (**C**) Genomic DNAs of passage 1 (P1) and passage 12 (P12) of adenovirus Sad23L-prM-E were purified and digested with *Age*I and *Hind*III. (**D**) Adenoviruses Sad23L-prM-E, Sad23-prM-E and Ad5-prM-E were purified by isopycnic CsCl density-gradient ultracentrifugation. The lower white layer was infectious virus. Viral titer was determined by standard plaque-forming assay on HEK293 cells. (**E**) A dose of 1×10^9 vps Sad23L-eGFP, Sad23-eGFP or Ad5-eGFP adenovirus was inoculated to HEK293 cells in a T25 flask for incubation of 48 h. The infected cells were observed with a fluorescence microscope.

(Fig. 3B). Sad23L-prM-E was able to induce a robust and specific cellular immune response against ZIKV E antigen in mice.

3.5. No cross-reactivity of neutralizing antibodies between Sad23 L and Ad5 vectors

Plasmas of PBS, Sad23L-prM-E or Ad5-prM-E immunized mice (Ad5 none pre-exposed) were tested for the neutralizing antibodies (NAb) to Sad23L-eGFP and Ad5-eGFP viruses on HEK293 cells. Mice immunized with Ad5-prM-E induced high levels of neutralizing antibodies against Ad5-eGFP (mean NAb titer was 1:368), but could not neutralize Sad23L-eGFP virus (NAb titer < 1:10) (Fig. 4A). Mice immunized with Sad23L-prM-E elicited neutralizing antibodies against Sad23L-eGFP (NAb titer was 1:256) but not against Ad5-eGFP (NAb titer < 1:10). The results indicated that no cross-reactive neutralizing antibodies were elicited between Sad23L and Ad5 vector inoculated mice, suggesting the neutralizing antibodies to Ad5 were unable to neutralize Sad23 L virus *in vitro*.

In order to demonstrate no cross-reactive immunity between Ad5 and SadL23 *in vivo*, three groups of naïve mice were inoculated with Ad5-eGFP, and then tested for Ad5 neutralizing antibody in three weeks (Fig. 4B). All Ad5 pre-exposed mice developed high level of neutralizing antibodies against Ad5-eGFP (mean NAb titer was 1:53.3), but not in sham mice ($P = 0.0023$). Ad5 pre-exposed mice were then immunized with 5×10^7 PFU Sad23L-prM-E, Sad23-prM-E or Ad5-prM-E adenoviruses, respectively. Titer of antibodies to ZIKV E protein was detected by ELISA four weeks post immunization, and the mean endpoint titers raised in three adenoviruses infected mice were $10^{3.46}$, $10^{3.27}$ and $10^{2.90}$, respectively (Fig. 4C), but not in sham groups of controls ($P < 0.001$). Sad23L-prM-E had significantly higher E-specific antibody titer than Ad5-prM-E in Ad5 pre-exposed mice ($P = 0.017$).

Specific T cell response was measured by ELISpot four weeks post three adenovirus vector immunization for Ad5 pre-exposed mice (Fig. 4D). The results showed that E peptides induced IFN-γ response with 664.4 ± 43.6 SFCs/million cells in the Sad23L-prM-E group, 522.4 ± 18.6 in Sad23-prM-E, and 317.2 ± 49.5 in Ad5-prM-E, which were significantly higher than observed in the sham group of mice ($P < 0.001$). The T cell response of Sad23L-prM-E was significantly higher than Ad5-prM-E in Ad5 pre-exposed mice ($P = 0.004$).

The E-specific antibody titer and T cell response to ZIKV prM-E within three types of adenoviral vectors were compared in consideration of Ad5 none pre-exposed and pre-exposed mice. The E-specific antibody titers to three vectors between Ad5 none pre-exposed and pre-exposed mice were not statistically different ($P > 0.05$, Fig. 4E). The specific T cell response to Ad5-prM-E was significantly reduced between Ad5 none pre-exposed (688.5 ± 83.4 SFCs/million cells) and pre-exposed mice (317.2 ± 49.5 SFCs/million cells, $P = 0.005$, Fig. 4F), suggesting Ad5 pre-exposed immunity limited Ad5-prM-E immunization, but Sad23-prM-E and Sad23L-prM-E were not limited by Ad5 pre-exposed immunity (Fig. 4F).

Overall, Sad23L-prM-E immunization was not affected by Ad5 pre-exposed immunity, suggesting no cross-reactivity of neutralizing antibody between Sad23 L and Ad5 vectors.

4. Discussion

In previous reports, a number of rare human and simian adenoviral vectors utilized in the development of vaccine candidates for multiple pathogens, such as HIV (Tatsis et al., 2009), HCV (Swadling et al., 2014), Ebola virus (Kobinger et al., 2006), ZIKV (Abbink et al., 2016; Cox et al., 2018; Xu et al., 2018b) were tested against preexisting antibodies to common adenovirus (Abbink et al., 2018, 2015; Dakin et al., 2015). These data indicated that adenovirus vectors were a promising approach for vaccines and elicited specific immune response to carried antigens in mice and non-human primates (NHP). In addition, some adenovirus-delivered vaccine such as human adenovirus 6 and chimpanzee adenovirus 3 vectors encoding non-structural (NS) proteins of HCV went to clinical trial and induced sustained protective T cells responses (Barnes et al., 2012). Therefore, the development of novel adenovirus vectors may provide more option for clinical utilization.

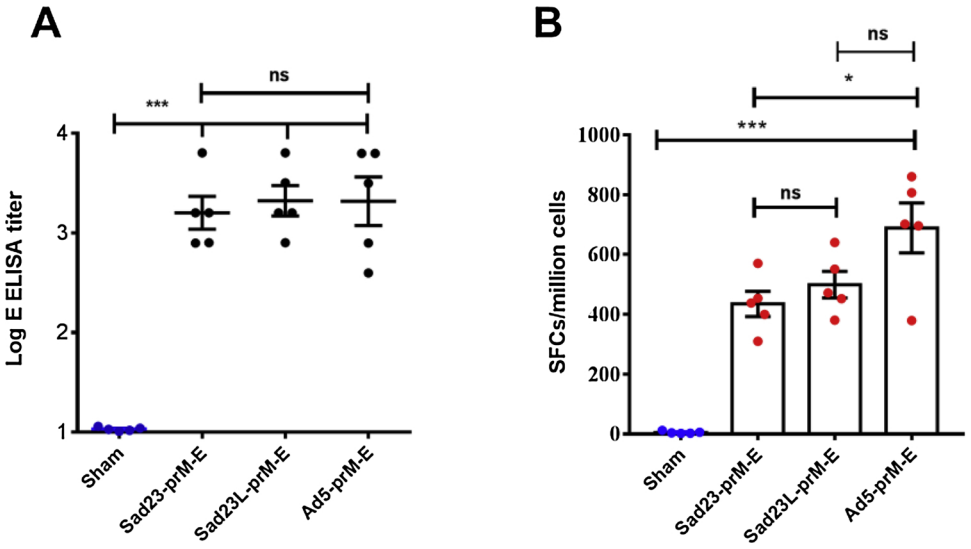

Fig. 3. Comparison of ZIKV E immunogenicity among Sad23L-prM-E, Sad23-prM-E or Ad5-prM-E immunized mice. Each group (n = 5) of C57BL/6 mice was inoculated intra-muscularly with 5×10^7 PFU viruses, respectively, with PBS as a mock control (n = 5). (**A**) Endpoint titers of ZIKV E-specific antibodies measured in the collected sera four weeks post immunization by ELISA. (**B**) Characterization of T lymphocyte response. Splenocytes were isolated for evaluation of IFN-γT cell response to a ZIKV E peptide pool by ELISpot. SFCs = IFN-γspot-forming cells. Data was collected as means ± SEM. *P* values were analyzed by one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, $P > 0.05$ or no significant difference.

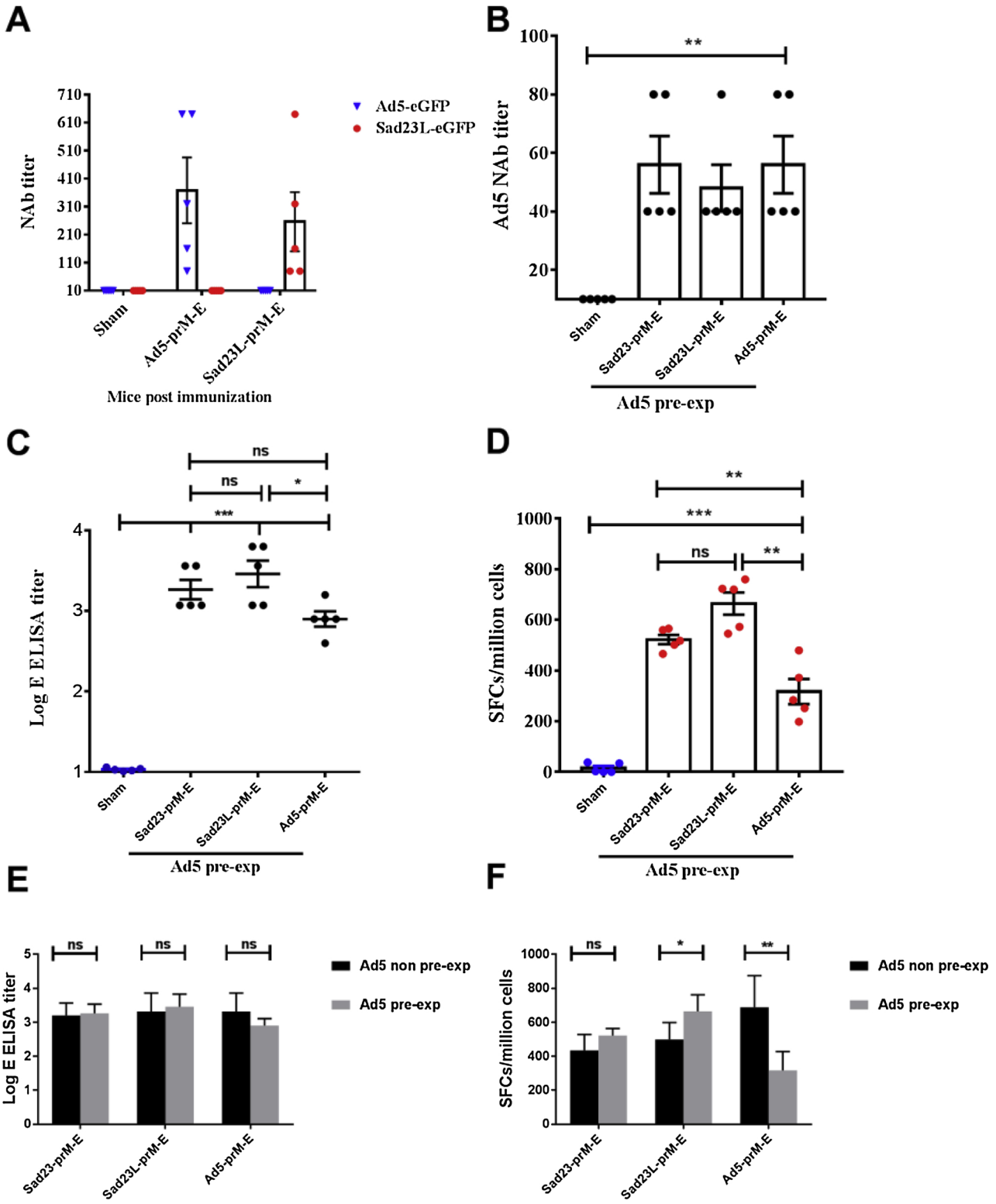

Fig. 4. Measurement of cross-reactive neutralizing antibodies between Sad23 L and Ad5 vectors. (**A**) Plasmas of PBS, Sad23L-prM-E or Ad5-prM-E immunized mice were test for neutralization antibodies to Sad23L-eGFP and Ad5-eGFP viruses on HEK293 cells. Sham group was PBS injected mice as negative control. (**B**) Neutralizing antibody titer to Ad5 vector at 3 weeks after inoculation with Ad5-eGFP virus. (**C**) Endpoint titer of ZIKV E-specific antibody measured by ELISA in the collected plasma of Ad5 pre-exposed mice four weeks post immunization with three types of adenoviral vectors. (**D**) Splenocytes were isolated from three type adenoviral vector immunized Ad5 pre-exposed mice after 4 weeks of immunization. Specific IFN-γT cell response to a ZIKV E peptide pool was measured by ELISpot. (**E**) ZIKV E-specific antibody titers to Ad5-prM-E, Sad23-prM-E or Sad23L-prM-E were detected between Ad5 none pre-exposed and pre-exposed mice, respectively. (**F**) Specific T cell response to Ad5-prM-E, Sad23-prM-E or Sad23L-prM-E were quantified between Ad5 none pre-exposed and pre-exposed mice, respectively. Data was collected as means ± SEM. *P* values were analyzed by one-way ANOVA or two-tailed *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, $P > 0.05$ or no significant difference.

Table 1
Primer and linker sequences for constructing simian adenoviral vectors.

Primer	Sequence (5'-3')
F0	CAGCTATGACCATGATTACGCCAAGCTTGTTTAAACCCTTAATT
	AAGCATCATCAATAATATACCTCAAAC
R0	ACGTAAAAACACCGGACTTTG
F0-1	CAAAGTCCGGTGTTTTTACGTACGATATCATTTC
R0-1	CCCGTT GCAGGACAAC A GATCGATATCTGACCGATTCAT
F1	TGTTGTCCTG CAACGGGACG G
R1	TCCGCCGCTGGATCATGT ACTTCTTCGT C
F2	AG TACATGATCC AGCGGCGGA
R2	CGTTGTAAAACGACGGCCAGTGAATTCACGCGTGGAGCGGAAGGTGACCGGGT
F3	CAGCTATGACCATGATTACGCCAAGCTTGTTTAAACAAGACCCG
	GTCACCTTCCGCTCCACGCGTCAAGT
R3	CGCAAGACCA AGCATGTGCC TTTGC
F4	GCAAAGGCAC ATGCTTGGTC TTGCG
R4	CCATGGGACA TTCAATCGTA
F5	TACGATTGAA TGTCCCATGG ATCACCCCCT TATCCAGTGA AATA
R5	CGTTGTAAAACGACGGCCAGTGAATTCTTAATTAACATCATCAATAATATACCTC AAAC
R6	ATGCACGATTATGACTCTACCCCCATGTAGCCGTGATCCCGACCACCGA
F7	GCCAAATGGAACGCCGGACGTAGTCATTCGTACTTGCTGTAGCAGAACC
F8	GAGGATTGTCCTCGGTGGTCGGGATCACGGCTACATGGGGGTAGAGTCA
R8	GGACCAGGTTCTGCTACAGCAAGTACGAAAATGACTACGTCCGGCGTTCCATTTGGC
Linker[a]	CGTACGATATCATTTCCCCGAAAGTGC
	CACCTGACCGTAACTATAACGGTCCTAAGGTAGCGAAGCATCTATGT
	CGGGTGCGGAGAAAGAGGTAATGAAATGGCATTATGGGTATTATG
	GGTCTGCATTAATGAATCGGTCAGATATCGACAT

[a] Restriction enzyme: *I-Ceu*I and *PI-Sce*I.

Table 2
Capacity for producing recombinant adenoviruses in HEK293 cells.

Virus type	Input viruses	Output viruses		Infection ratio[c]
	(vp/cell)	(PFU/cell)[a]	(vp/cell)[b]	(vp/PFU)
Sad23L-prM-E	1	8.7×10^{2}	1.7×10^{4}	19.54
Sad23L-eGFP	1	9.6×10^{2}	1.32×10^{4}	13.75
Sad23-prM-E	1	2.5	3.6×10^{3}	1440
Sad23-eGFP	1	7.5	7.6×10^{3}	1013
Ad5-prM-E	1	4.7×10^{3}	2.2×10^{4}	4.68
Ad5-eGFP	1	4.36×10^{3}	1.55×10^{4}	3.44

[a] Viral PFU as determined by standard plaque-forming assay on HEK293 cells.
[b] Viral particle concentration (vp) was measured by UV absorbance at 260 nm (A_{260}) after purification.
[c] The infection ratio was defined as vp over PFU from the purified viruses. The viral titer (PFU) or viral particle (vp) was calculated as mean value from four independent experiments.

Table 3
Mice immunization regimen.

group	number	Prime	Titer (PFU)	Boost	Titer (PFU)
Sham	5	PBS	100μl	PBS	100μl
Vaccination	5	Ad5-eGFP	10^{8}	Ad5-prM-E	5×10^{7}
	5	Ad5-eGFP	10^{8}	Sad23-prM-E	5×10^{7}
	5	Ad5-eGFP	10^{8}	Sad23L-prM-E	5×10^{7}

Approximately 30%–100% of the adult population carried specific neutralizing antibodies to AdHu5 in Europeans, North Americans, Asians or Africans (Zhang et al., 2013). As expected, reporting seroprevalence rates of SAdV23 were approximately 20% in Brazil, 1.5%–4% in the United States and Thailand (Ersching et al., 2010; Xiang et al., 2006), indicating that chimpanzee adenoviruses might be a better vaccine vector candidate than human adenoviruses, considering the problem of NAb pre-existence. In this study, we developed the rare chimpanzee adenovirus vector Sad23L, which showed low seroprevalence, no cross-reactivity with Ad5 and high propagating efficiency. Therefore, Sad23L vector could overcome the preexisting NAb of common human adenoviruses. In Sad23L construct, the original E1/E3 regions of Sad23 genome were deleted (dE1/E3) and E4orf6 was replaced by Ad5 elements (Ad5-E4orf6). The entire procedure was completed over a single week by PCR amplification and Gibson assembly methods. This strategy allows the construction of a novel recombinant adenoviral vector rapidly independent of restriction enzyme sites. In addition, simian adenoviral E4orf6 was replaced with the corresponding Ad5 E4orf6, which improved the propagating efficiency of the recombinant virus in propagating cells. Sad23L vector had a

higher propagation level than Sad23 (E1/E3 deleted only) in HEK293 cells, because of a better interaction between the Ad5-E1 and Ad5-E4orf6 proteins than between Ad5-E1 and Sad23-E4orf6 proteins. As previously described, the complex between the E1B-55 K and E4orf6 protein mediated necessary and efficient transportation of viral mRNA for late virus protein synthesis and viral DNA replication (Silke and Matthias, 2000). In this study, HEK293 cells infected with Sad23L-eGFP displayed stronger fluorescence rate than Sad23-eGFP confirming this hypothesis. The infection ratio (vp/PFU) of Sad23L-prM-E was much lower than Sad23-prM-E, and presented characteristics similar to Ad5-prM-E. The Sad23L (dE1/E3-Ad5E4orf6) and Sad23 (dE1/E3) as well as Ad5 (dE1/E3) vectors all expressed ZIKV prM-E proteins and elicited strong humoral and cellular immune response in mice. Hence Sad23L vector had higher propagating efficiency leading to the production of high titer of viruses consistent with vaccine production.

In conclusion, a week was sufficient to successfully generate a novel recombinant simian adenoviral vector Sad23L using the rapid Gibson assembly method. This strategy is simple and restriction enzyme-independent. The Sad23L vector has low seroprevalence and its consistent immunogenicity could not be neutralized by sera of preexisting neutralizing antibodies against Ad5. The novel simian adenoviral vector Sad23L shows high potential capacity as a vector for vaccine development or gene transfer in clinical practice.

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

T.L. and C.L. designed research; S.L., P.Z., X.M., Q.W., B.L., J.L. and W.Z. performed research; S.L., T.L., C.L. and J.P.A analyzed data; and S.L., T.L., C.L., and J.P.A. wrote the paper.

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