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Original Research

Construction of a bivalent vaccine candidate against HAdV4/HAdV7 based on capsid-display strategy via Red-homologous recombination and counter-selection methodology

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ABSTRACT

Human adenoviruses (HAdVs) are major respiratory pathogens. Specifically, human adenovirus type 4 (HAdV4) and human adenovirus type 7 (HAdV7) are known for causing fever and pneumonia, with documented cases of fatalities among the population. In recent years, HAdV4/HAdV7 has been implicated in causing substantial outbreaks, leading to increased morbidity in multiple countries. Most HAdV4 and HAdV7 infections have been reported in North America, Asia, Europe, Africa, South America, Oceania, and the Middle East. Most fatalities occurred in North America (the United States) and Asia (China and Singapore). Engineered recombinant adenoviruses have played a crucial role as vaccine vectors. In this study, we constructed a recombinant adenovirus, Ad4ITRmut-Ad7E3, and evaluated it *in vitro* and *in vivo*. We observed that the replication rate of Ad4ITRmut-Ad7E3 was lower than that of the RI-67 strain, indicating that the mutation of inverted terminal repeats (ITRs) weakened the replication ability of HAdV4. Immunization of BALB/c mice with the bivalent Ad4ITRmut-Ad7E3 vaccine strain, administered by intraperitoneal injection and oral gavage, resulted in the elicitation of neutralizing antibodies targeting HAdV4 and HAdV7. This finding not only provides a novel method and technique for the efficient construction of a polyvalent recombinant adenovirus vaccine candidate against HAdV4 and HAdV7 but also against other prevalent adenovirus serotypes such as HAdV3, HAdV11, HAdV14, and HAdV55, from various regions.

1. Introduction

Human adenoviruses (HAdVs), initially isolated in 1953, are non-enveloped, double-stranded DNA viruses that belong to the family Adenoviridae that can cause respiratory, gastroenteritis, and ocular infections [1,2]. HAdVs are highly contagious pathogens that commonly lead to respiratory disease, encompassing common colds, bronchitis, tonsillitis, and severe pneumonia [3]. The lethality of untreated severe HAdVs pneumonia can be >50 % [4,5]. They can also trigger other medical conditions, including gastroenteritis, conjunctivitis, cystitis, carditis, and meningoencephalitis, depending on the type of infection [6,7]. HAdV infections can affect individuals of all age groups and vulnerable populations, including infants, school students, military recruits, and immunocompromised patients [6,8,9]. HAdVs are classified into 7 species (AdVA-G), encompassing 113 types [10]. Species B of HAdVs can be further divided into two subspecies: B1, which includes HAdV3, HAdV7, HAdV16, HAdV21, HAdV50, and B2, which encompasses HAdV11, HAdV14, HAdV34, HAdV35, and HAdV55. Among them, HAdV4 and HAdV7, categorized as species E and B, respectively, represent the major epidemic strains and show significant differences in sequence homology. Meanwhile, serotypes HAdV4 and HAdV7 have historically been the major cause of febrile respiratory illness (FRIs) in the United States (U.S.) military [11]. Initially, infections with HAdV4 and HAdV7 were primarily limited to the U.S. new military recruits, with rare occurrences among civilians [12]. However, over time, reports of infections among civilians have gradually increased [12,13]. The highest number of reported infections has occurred in North America, followed by Asia and Europe [14]. Most reports of fatalities have occurred in the United States, followed by China and Singapore

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HIGHLIGHTS

Scientific question

In recent years, human adenovirus type 4 (HAdV4) or human adenovirus type 7 (HAdV7) has been implicated in causing substantial outbreaks, leading to increased morbidity in multiple countries. However, no approved adenovirus vaccine or therapy for general use nor efficient antiviral therapy available for HAdV4 or HAdV7 has been developed.

Evidence before this study

The live oral vaccines of HAdV4 and HAdV7 can induce long-term immunity to protect the United States (U.S.) military from infection with HAdV4 and HAdV7 but have not been accessible to civilians. This vaccine is based on wild-type virus strains without artificial modifications, except for altering the route of viral invasion. Hence, administering this vaccine requires strict monitoring of vaccine users and their surroundings to prevent environmental contamination. Moreover, current vaccines exist in two forms, HAdV4 and HAdV7, necessitating two production lines for manufacturing. Currently, there is no bivalent vaccine available targeting both HAdV4 and HAdV7.

New findings

We developed a bivalent vaccine candidate, Ad4ITRmut-Ad7E3, using HAdV4 as the backbone and employing homologous recombination and counter-selection screening technology to incorporate genetic material from HAdV7. Our findings indicated that it can stimulate mice to produce neutralizing antibodies against HAdV4 and HAdV7. We observed a reduction in viral load in the lungs of these mice in challenge experiments, demonstrating the potential efficacy of Ad4ITRmut-Ad7E3 immunization.

Significance of the study

The bivalent vaccine candidate Ad4ITRmut-Ad7E3 can stimulate mice to produce neutralizing antibodies against both HAdV4 and HAdV7, and it has a distinct artificial site that is easy to identify. By constructing Ad4ITRmut-Ad7E3, we aim to reduce the cost of producing HAdV4 and HAdV7 vaccines while simplifying the administration method. This simplification may eventually enable a shift from a double-dose to a single-dose administration, thus minimizing environmental contamination associated with enteral adenovirus vaccine delivery. This study not only provides a novel method and technique for efficiently constructing a polyvalent recombinant adenovirus vaccine candidate against HAdV4 and HAdV7, but also against other prevalent adenovirus serotypes such as HAdV3, HAdV11, HAdV14, and HAdV55, from various regions.

[14–16]. HAdV4 and HAdV7 strains periodically resurface, contributing to localized infection outbreaks [12]. While HAdV4 and HAdV7 typically lead to respiratory and ocular diseases, infections can also lead to more severe lower respiratory tract illnesses, disseminated diseases, and even fatalities, particularly among infants and individuals with underlying immune or respiratory impairments [12,17]. Among other species B of HAdVs, HAdV3, HAdV14, and HAdV55 have been reported to trigger severe community-acquired pneumonia outbreaks in both military and civilian populations [18,19].

The live oral vaccines of HAdV4 and HAdV7 can induce long-term immunity to protect people from infection with HAdV4 and HAdV7 [20]. The U.S. military HAdV4 vaccine strain (AY594256) was derived from the epidemic strain RI67(AY594253); the two strains shared a high degree of genomic sequence similarity. Compared to RI-67, 6 mismatches and 4 gaps were detected in the genome of the HAdV4 vaccine strain used in the U.S. military, and the most critical mismatch was in the inverted terminal repeats (ITRs) [21]. The ITRs facilitate the replication of HAdVs from the origins of replication located within the ITRs and act as a jumping-back mechanism to initiate DNA synthesis [22,23]. Alteration of the 8 ITR bases may affect the rate of DNA replication of HAdVs. Hence, we constructed the infectious clones of the HAdV4 prototype strain with vaccine-like mutations in the ITRs and obtained a virus strain Ad4ITRmut. As for HAdV7, the construction of the vaccine strain is challenging due to the significant differences in genome between the U.S. military HAdV7 and the prototype strain. Therefore, we intended to replace the neutralizing epitope in the hexon of HAdV4 with that of HAdV7 to construct a bivalent vaccine candidate against HAdV4 / HAdV7.

Hexon is the most abundant capsid protein, with 720 copies within the HAdVs particle. Each hexon contains 7 highly variable regions (HVRs) that harbor type-specific epitopes of HAdVs [24]. Incorporation of the antigenic capsid into HAdVs has been developed as a novel vaccine strategy [25]. Some studies reported that the Ad7E3 epitope (DGREAADAFSPEIVLYTEN) located in the HVR5 region of HAdV7 is an effective virus-neutralizing epitope that stimulates the body to produce neutralizing antibodies (Nabs) specific to HAdV7 [26]. In addition to the HAdV4 and HAdV7 bivalent attenuated vaccine strains mentioned above, other bivalent and multivalent vaccines against adenoviruses have also been reported in recent years [27]. Some researchers have also utilized HAdV3 as part of the vaccine or vector framework, substituting the predicted epitopes within the HAdV3 hexon with the corresponding epitopes from the HAdV7 hexon.

Additionally, they have inserted the hexon of HAdV55 into the E3 region of HAdV3 to construct a multivalent adenovirus vaccine strain [19,28]. In addition, using adenovirus as a vector to construct a multivalent adenovirus vaccine, some researchers used the N.A. region of influenza vaccine to express hexon epitopes of HAdVs [29]. However, in the above studies, most of the bivalent adenovirus vaccine strains are based on the complete expression of foreign hexon, fiber knob, or person genes. There are two limiting factors *in situ* gene replacements or in E1 / E3 region expression of foreign genes: First, *in situ,* gene replacements are limited by the adenovirus group because the adenovirus genomes of different groups are quite different. Second, when the foreign antigen is expressed in the E1 region or E3 region, it can not be fully displayed because it affects the typical conformation of the antigen, thus reducing the immune effect.

The capsid-display strategy for the construction of bivalent HAdV4 / HAdV7 vaccines in our study is based on the genome of the existing HAdV4 vaccine with only a replacement of epitope-related sequences to obtain a recombinant virus capable of eliciting dual antibodies. The highlights of this study are presented in three aspects: first, we constructed and evaluated a bivalent vaccine candidate strain by hexon epitope substitution between two different adenovirus groups, group E (HAdV4) and group B (HAdV7). At the same time, previous studies had been conducted between the same group of different serotypes (e.g., HAdV7, HAdV3, HAdV55). The booming packaging of this bivalent adenovirus vaccine strain and the activation of neutralizing antibodies against HAdV4 and HAdV7 in mice provided the approach and idea for developing a polyvalent adenovirus vaccine. Second, functional mutations on the terminal 8 bases at the ITRs of HAdV4 virus reduced the recombinant virus's replication rate and cytotoxic effect and achieved specific attenuated effects. Third, traditional adenovirus genome modification requires the combination of shuttle plasmid modification and recombination function.

In contrast, the KansacB counter-selection cassette is introduced into the modification of the skeleton plasmid, which no longer relies on shuttle plasmids and is accessible from recognition sites of restriction enzymes and genome sequence. This approach to developing bivalent adenovirus vaccines is convenient, flexible, and particularly

suitable for areas where multiple serotypes are prevalent. Since the vaccine strain is wild, it is a critical issue to distinguish and identify the possible environmental contamination caused by the vaccine, and the recombinant bivalent vaccine constructed in this study has a distinct artificial site that is easy to identify, thus allowing this issue to be addressed to some extent.

2. Materials and methods

2.1. Strains, plasmid and cells

Escherichia coli (*E. coli*) strains DH10B and DY380 were preserved by our laboratory and kept as a laboratory strain. *E. coli* BJ5183 was purchased from Stratagene (C.A., USA) and kept in our laboratory. Plasmid pBR322 was purchased from Addgene and maintained in our laboratory. Adenoviral strain RI67 was purchased from ATCC. Plasmid pUC19-KanSacB was constructed and preserved in our lab. Cell lines AD293, 293T, and A549 were purchased from the National Infrastructure of Cell Line Resource. Recombinant adenovirus was cultured in AD293 cells, which were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1 % penicillin–streptomycin and 10 % fetal bovine serum (FBS).

2.2. Homology modeling

Homology modeling of the HAdV4 hexon was performed using the web-based SWISS-MODEL server (https://swissmodel.expasy.org/). The sequence of the HAdV4 hexon was used to search against the SWISS-MODEL template library for structures that matched the target sequence using BLAST (46) and HHBlits (47) [30]. Sequences of the chimpanzee adenovirus (Type 68/Simian 25) were then selected, with 89.7 % of the sequences matching the HAdV4 hexon. Pymol v0.99 (Robert-Guroff 2007) was utilized to generate the space-filling representation of the HAdV4 hexon structure and mimic 19 amino acid replacements at position HVR5 of the HAdV4 replacement with the HAdV7 epitope.

2.3. Construction of the modified HAdV4 complete genome plasmid by modifying the ITRs terminal sequences

The plasmid containing the complete HAdV4 genome was constructed using homologous recombination facilitated by RecBCD in *E. coli* strain BJ5183 [24,31,32]. First, the shuttle plasmid pBR4VAd4LR, containing the left (AdL) and right (AdR, which also included mutations within the 8 bases at the end of the ITRs region) ends of the HAdV4 genome, was constructed. The pBR4VAd4LR shuttle plasmid was then linearized using the *Hind*III restriction enzyme and dephosphorylated using Calf Intestinal Alkaline Phosphatase (New England Biolabs). *E. coli* BJ5183 cells were co-transformed with the purified HAdV4 genomic DNA (200 ng) and the linearized shuttle plasmid pBR4VAd4LR (200 ng). The recombinant plasmid DNA (pBRAd4ITRmut) was extracted from the bacterial broth using the Plasmid Midiprep Kit (Qiagen) and digested with appropriate restriction enzymes (*Not*I, *Eco*RV, *Kpn*I, and *Bam*HI) to confirm the integrity of the HAdV4 genome.

2.4. Construction of the recombinant HAdV4 plasmid pBRAd4ITRmut-Ad7E3

The linear DNA fragments E3-Kan-sacB for plasmid construction were generated through polymerase chain reaction (PCR) amplification. *E. coli* DY380 were co-transformed with the purified E3-KansacB (200 ng) and pBRAd4ITRmut (500 ng). The presence of the kansacB cassette insertion was confirmed by PCR and by digesting the recombinant plasmid DNA (pBRAd4ITRmut-E3kansacB) with *Bam*HI. The double-stranded DNA fragment intended to replace the kansacB cassette with the Ad7E3 sequence was generated through overlapping PCR. *E. coli* DY380 cells were co-transformed with 50 ng of PCR product Ad7E3 and 500 ng of pBRAd4ITRmut-E3kansacB. The correct KanSacB recombinant colonies were identified using PCR and digesting with *Hga*I. Appropriate restriction enzymes (*Bam*HI, *Eco*RV, *Kpn*I, and *Not*I) were also employed to detect possible rearrangements within the HAdV4 genomes.

2.5. Generation, amplification, purification, and titration of the recombinant virus Ad4ITRmut-Ad7E3

The Ad4ITRmut-Ad7E3 was reconstituted, amplified, purified, and titrated as described previously [33]. The experimental procedure is briefly described below: First, the adenovirus genome was released from the plasmid backbone using the restriction enzyme *AsisI*, followed by phenol–chloroform extraction. The genetically modified plasmids were transfected into 293T cells using Lipofectamine 3000 (Life Tech, USA) to rescue the recombinant bivalent vaccine strain Ad4ITRmut-Ad7E3. The successful rescue of recombinant viruses was confirmed by observing the typical cytopathic effects (CPE) in the cells. Viral supernatants were collected through three repeated freeze–thaw cycles and used to infect A549 cells. The viral genome was subsequently extracted from the supernatants using a purification kit, and the presence of Ad4ITRmut-Ad7E3 was confirmed through PCR. The infectious doses (TCID50) of the virus were determined following routine procedures.

2.6. Western blotting analysis

Western blotting analysis was performed to confirm the expression of the hexon protein of the recombinant adenovirus. The wild-type HAdV4-infected cells served as the positive control, while uninfected cells were used as blank controls. Cell extracts were collected and added to the loading buffer, then boiled for 10 min, separated using a 10 % SDS-PAGE gel, and transferred onto a nitrocellulose membrane (at 100 V for 120 min). The membrane was probed with a mouse polyclonal antibody A7nH (Guangzhou Institute of Respiratory Diseases) and amplified using a goat anti-mouse IgG-HRP antibody (Sigma-Aldrich Corp.). The signal was further enhanced with HRP-labeled goat anti-rabbit IgG conjugate (Sigma-Aldrich Corp.) before chromogenic detection using 3,3′Diaminobenzidine (DAB).

2.7. Transmission electron microscopy (TEM) analysis

The AD293 cells infected with Ad4ITRmut and Ad4ITRmut-Ad7E3 were cultured and harvested 72 h post-infection (h p.i.). Subsequently, these cells were prepared according to the standard protocols[34], and the ultrathin section samples were examined using transmission electron microscopy (TEM).

2.8. Indirect immunofluorescence (IF) assay

Immunofluorescence (IF) assays were conducted to detect the hexon protein expression of the recombinant adenovirus Ad4ITRmut-Ad7E3 in AD293 cells. AD293 cells were infected with Ad4ITRmut-Ad7E3. After 72 h p.i., the cells were fixed with methanol at −20 °C for 10 min and subsequently incubated with 1 % BSA at 37 °C for 30 min. Following this, they were incubated at 37 °C for 1 h with the mouse monoclonal antibody Mab 3G5 (Guangzhou Institute of Respiratory Diseases). Afterward, the fixed monolayers were thoroughly washed, and FITC-conjugated goat anti-mouse IgG (KPL Co. Ltd, USA) was added and incubated at 37 °C for 30 min. Finally, the monolayers were covered with glycerol and examined under a fluorescence microscope (Olympus Corp., Japan).

2.9. Growth curve of recombinant adenovirus

293 T cells (5×10^5 cells/mL) were seeded into 12-well plates and infected with Ad4ITRmut-Ad7E3 at a multiplicity of infection (MOI) of

1 and 10. The cells were washed 3 times with DMEM containing 2 % FBS after 2 h infection. Subsequently, 2 mL of DMEM containing 2 % FBS was added to each well of the 12-well plates and incubated at 37 ° C. At 0, 24, 48, 72, and 96 h after infection, the contents of the wells were collected to detect the viral genome copy number, and the growth curve of the recombinant adenovirus was plotted.

2.10. Animal immunization

Thirty specific pathogen-free (SPF) female BALB/c mice, aged 6–8 weeks, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. These BALB/c mice were inoculated with Ad4ITRmut-Ad7E3, wild-type HAdV4, and wild-type HAdV7 via oral and intraperitoneal immunization routes with a dosage of 1.0×10^7 inclusion-forming units (IFU) per mouse. Control mice were immunized with 0.2 mL of phosphate buffer saline (PBS). As a result, there were 8 groups of mice in the animal experiment, with each group consisting of 3 mice (n = 3). All immunizations were conducted at two-week intervals, totaling 3 times. Blood samples were collected from the caudal vein before and at two-week intervals following immunization for colorimetric microneutralization (CMN) assays.

2.11. Virus titration assay

Experimental methods for viral titers have been previously reported [35,36] and are summarized as follows: 500 μl of A549 cells (5×10^5 cells/mL) were seeded into 24-well plates and cultured in DMEM with 10 % fetal calf serum (FCS). After the cells were plated for one hour, adenovirus was added to the cell culture medium and incubated for 48 h after being mixed by gently shaking. Media and cells were removed or overlaid with DMEM containing 0.6 % low melting agarose and 2 % FBS. At 72 h p.i., cells were fixed with 10 % formaldehyde and stained with crystal violet.

2.12. Antibody neutralization assays

Antibody neutralization assays were used to examine the neutralizing abilities of different sera against HAdV4/HAdV7, as previously described [35,37]. The experimental method is briefly described as follows. The blood obtained from the tail veins of immunized mice in two-week intervals was centrifuged at 5,000 g for 30 min to separate serum. 5 μL of serum from each mouse was heat-inactivated at 56 ˚C for 30 min, diluted 1:10 in 45 μL DMEM. Moreover, 50 μL of each dilution was mixed with 50 μL of HAdV4/HAdV7 (200 TCID50) at 37 ˚C for one hour in 5 % CO_2. They were then added dropwise onto confluent A549 cell monolayers in six-well plates. After incubation at 37 ˚C, 5 % (v/v) CO_2 for one hour, 2 ml of 0.1 % (w/v) immunodiffusion, agarose in DMEM supplemented with 10 % (v/v) FBS and 1 × penstrep was added to each well. After incubation at 37 ˚C, 5 % (v/v) CO_2 for 72 h, the agarose overlay was removed, and the cell monolayer was fixed with 1 ml/well formaldehyde [37 % (w/v) formaldehyde stabilized with 10 % - 15 % (v/v) methanol] for 20 min at room temperature. The fixative was discarded, and 1 ml of 1 % (w/v) crystal violet in 10 % (v/v) methanol was added per well. Plates were then incubated at room temperature for 20 min, rinsed thoroughly with water, and the plaques were subsequently enumerated.

2.13. Challenge experiments

Immunization mice were further divided into a challenge group and a non-challenge group (control group). After the last immunization, immunization groups were challenged intranasally with 50 μL (about 1×10^7 IFU) of wild-type HAdV4 / HAdV7 and sacrificed on day 3 post-challenge. The individual lungs were collected aseptically and homogenized in 500 μL of PBS, and 50 μL of the lung homogenates were used for copy number detection. The copy numbers of HAdV4 and HAdV7 were assessed using real-time PCR detection.

2.14. Statistical analysis

Statistical analyses were performed with Prism 7 (GraphPad Software; RRID: SCR_002798). Comparisons of several independent test series were evaluated using two-way ANOVA. An unpaired *t*-test was used for the statistical analysis of other experiments (P value < 0.05).

3. Results

3.1. The replacement of the HAdV4 hexon with the corresponding sequence of HAdV7 did not affect the overall structure of the hexon

We replaced the DSKTIVANYDPDIVM epitope of the hexon region of HAdV4 with the E3 sequence DGREAADAFSPEIVL of the HAdV7 hexon and named Ad4/Ad7E3. The three-dimensional (3D) structure simulation of the AdV4 / AdV7 hexon trimer using Pymol software revealed that the replacement of the HAdV4 E3 hexon with the corresponding sequence of HAdV7 did not affect the overall structure of the hexon and the replaced epitope regions were still displayed outside of the hexon (Fig. 1).

3.2. The recombinant plasmid pBRAd4ITRmut-Ad7E3 with ITRs mutations was successfully obtained

The schematic diagram illustrating the construction of the recombinant plasmid pBR4VAd4ITRmut-Ad7E3 is shown in Fig. 2. The plasmid pBR4VAd4LR was used to construct the shuttle plasmid pBR4VAd4ITRmut-kansacB. The amplification product of the left and right arms of pBR4VAd4LR is 3,542 bp and 5,663 bp, respectively (Fig. S1A and B). The vector plasmid pBR4VAd4LR was linearized by *HindIII* (Fig. S1C), then the linear plasmid and the extracted HAdV4 gDNA co-transformed into *E.coli* strain BJ5183, obtained the recombinant plasmid containing the ITRs-modified HAdV4 genome and named pBR4VAd4ITRmut. The results of *AsisI* enzyme digestion and sequencing identification confirmed the successful construction of the recombinant plasmid pBR4VAd4ITRmut was confirmed (Fig. 2). We obtained the target cassette kansacB, which shares homology with the upstream and downstream E3 sequences of the HAdV4 hexon (Fig. S3A). After the first homologous recombination, the plasmid pBRAd4ITRmut-kansacB was obtained, and the successful insertion of kansacB was identified (Fig. S3B). We then obtained the targeting fragment of HAdV7 E3 containing the upstream and downstream homologous arms (Fig. S3C). The HVR5 epitope sequence of HAdV4 in the recombinant plasmid pBRAd4ITRmut was replaced with the E3 epitope sequence of HAdV7: The targeting fragments containing HAdV7 E3 epitope sequences were recombined with the plasmid pBRAd4ITRmut-kansacB. Colony clones containing pBRAd4ITRmut-Ad7E3 were screened out by reverse screening of genes using sacB. Following the second homologous recombination, colony clones with the kansacB gene replaced by HAdV7 E3 were screened and named pBRAd4ITRmut-Ad7E3 (Fig. S3D). The successful construction of the recombinant plasmid pBRAd4ITRmut-Ad7E3 was identified by sequencing (Fig. S3E). Moreover, the enzyme digestion results confirmed the integrity of the plasmid backbone (Fig. S3F).

3.3. The recombinant virus Ad4ITRmut-Ad7E3 successfully expressed the antigenic properties of HAdV7 E3

The recombinant plasmid pBRAd4ITRmut-Ad7E3 was transfected into 293 T cells, and within 24 - 48 h, typical cytopathic effects (CPE) were observed. These CPE were not significantly different from those induced by WT HAdV4 (Fig. 3A). The PCR results indicated that RI67 sequences of the recombinant virus Ad4ITRmut-Ad7E3 were maintained (Fig. 3B), and sequencing confirmed that the recombinant virus Ad4ITRmut-Ad7E3 did not lose any other specific sequences. As a result, the genome sequence of Ad4ITRmut remained consistent with that of WT HAdV4. The Western blot result indicates that the first to

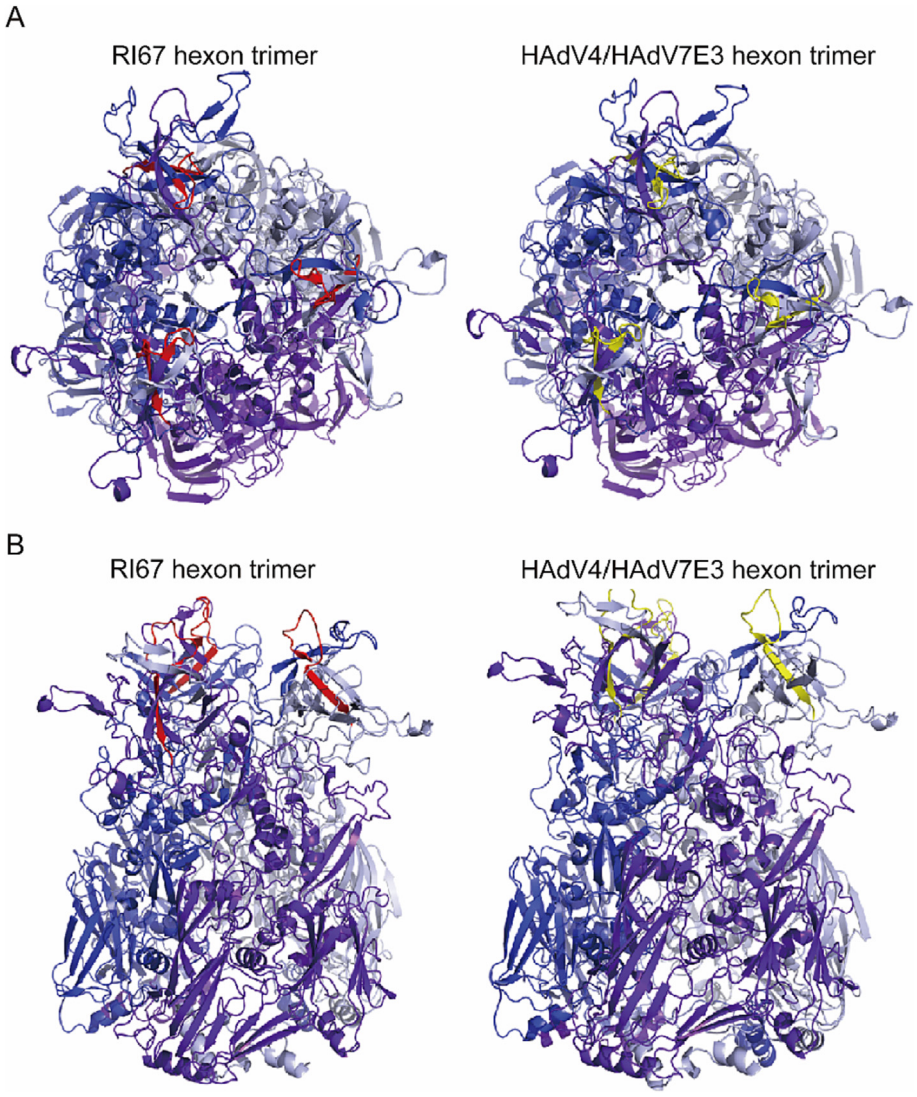

Fig.1. Molecular modeling of chimeric Ad4/Ad7E3 hexon. The Ad4E3 epitope is shown in red; the Ad7E3 epitope shown in yellow. A) Top view of molecular modeling of chimeric RI67 HAdV4 hexon and chimeric Ad4/Ad7E3 hexon. B) Side view of molecular modeling of RI67 HAdV4 hexon and chimeric Ad4/Ad7E3 hexon. Abbreviation: HAdV, human adenoviruse.

third generations of Ad4ITRmut-Ad7E3 adenovirus all showed the presence of an 83 kDa to 175 kDa band, consistent with the 104 kDa protein of the HAdV4 hexon protein. This result indicates that the recombinant virus Ad4ITRmut-Ad7E3 successfully expressed the antigenic properties of HAdV7 E3 (Fig. 3C). In addition, under equal protein loading and identical exposure times, the concentration of detected hexon protein bands in Ad4ITRmut-Ad7E3 increased with each generation, which indicates that the recombinant virus had initiated continuous proliferation in 293 T cells, as depicted in Fig. 3C. Under an electron microscope, the recombinant virus Ad4ITRmut-Ad7E3 particles exhibited a honeycomb-like arrangement with uniform size, similar to WT HAdV4 (Fig. 3D).

Cells and supernatants collected from A549 cells infected with the virus at various time points (0 h, 24 h, 48 h, 72 h, and 96 h) were analyzed to determine the virus genome copied number. The results revealed that regardless of the multiplicity of MOI (1 or 10), the replication rate of the recombinant virus Ad4ITRmut-Ad7E3 was slower than that of WT HAdV4 (Fig. 4A and Fig. 4B). To characterize further the recombinant virus Ad4ITRmut-Ad7E3, Ad4ITRmut, HAdV7, and the blank control group, an indirect immunofluorescence assay was conducted to assess their binding to the 3G5 monoclonal antibody. The results indicated that both the recombinant virus Ad4ITRmut-Ad7E3 and HAdV7 exhibited detectable fluorescence signals, whereas Ad4ITRmut and the blank control groups did not exhibit any detectable fluorescence (Fig. 4C). This observation demonstrates that the chimeric Ad7E3 epitope within the recombinant virus Ad4ITRmut-Ad7E3 expresses the antigenic characteristics of the E3 neutralizing epitope of HAdV7.

3.4. The recombinant virus Ad4ITRmut-Ad7E3 elicited antibodies against HAdV4 and HAdV7 in mice

The experimental design for the *in vivo* evaluation of Ad4ITRmut-Ad7E3 bivalent adenovirus is depicted in Fig. 5A. After three weeks of immunization, antibodies generated by the Ad4ITRmut-Ad7E3 oral administration group were found to neutralize 60 % of 5,000 IFU HAdV4, with 40 % of HAdV4 remaining unneutralized. In contrast, the Ad4ITRmut-Ad7E3 intraperitoneal injection group achieved 100 % neutralization of 5,000 IFU HAdV4 (Fig. 5B). This result indicates that intraperitoneal immunization of mice significantly elevated the levels of antiviral antibodies in the serum compared to oral immunization. *In vitro,* experiments assessing the neutralization of HAdV7 with serum antibodies revealed that the Ad4ITRmut-Ad7E3 group produced higher levels of virus-neutralizing antibodies than the HAdV4 and PBS groups after the first intraperitoneal immunization

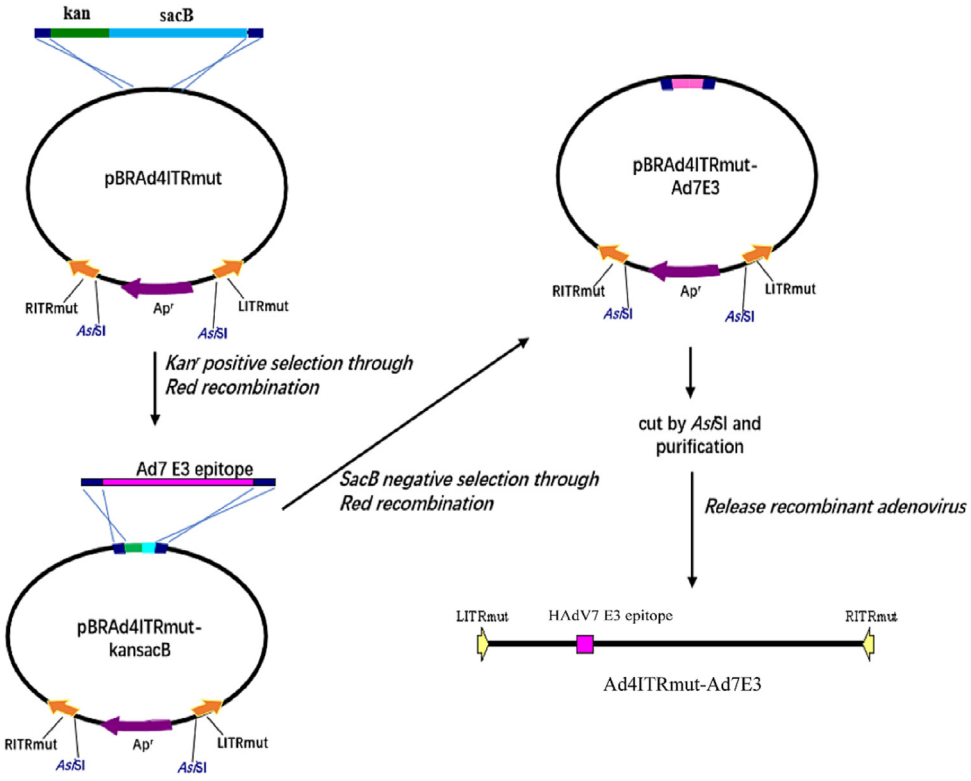

Fig.2. Schematic diagram of the recombinant plasmids pBR4VAd4ITRmut-Ad7E3 constructs. Abbreviation: HAdV, human adenoviruse.

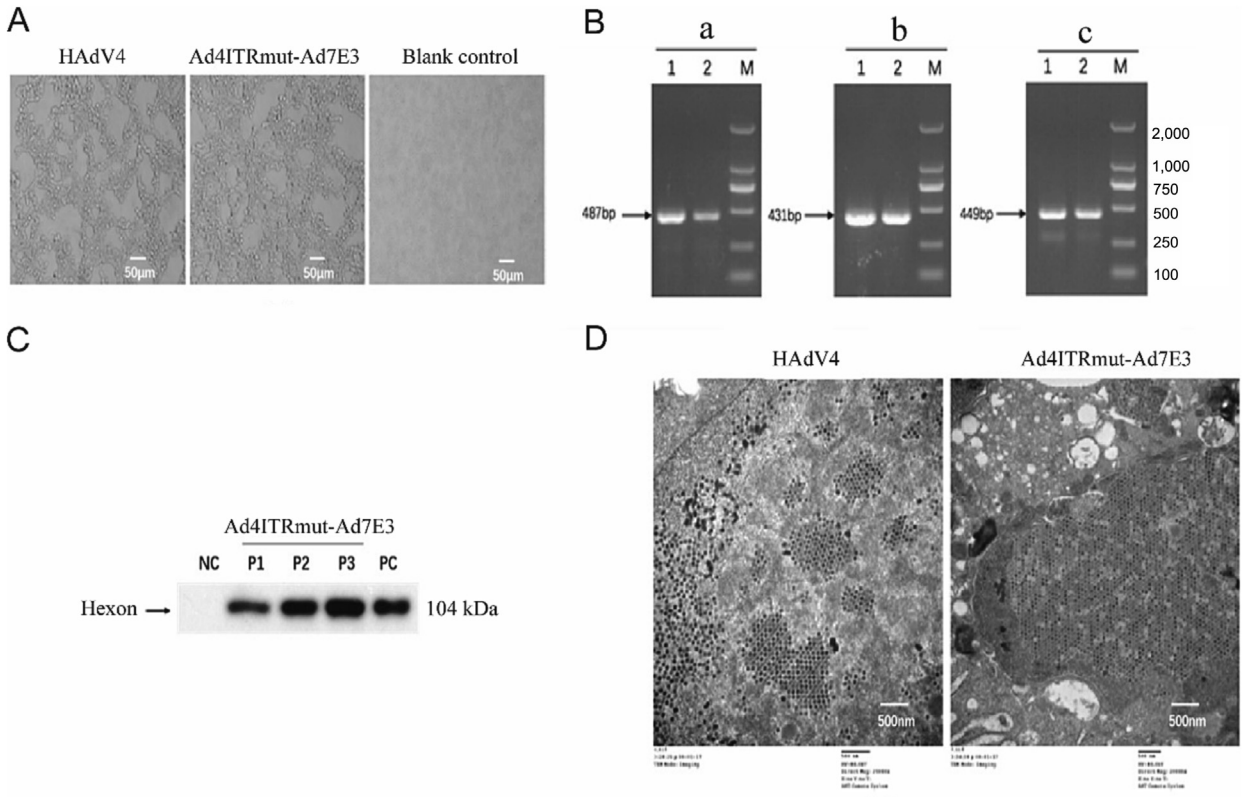

Fig.3. Evaluation of the antigenic characteristics of HAdV7 E3 in the recombinant virus Ad4ITRmut-Ad7E3. A) The CPE induced by the recombinant virus Ad4ITRmut-Ad7E3 in 293 T cells. B) Identification of the RI67 sequence of the recombinant virus Ad4ITRmut-Ad7E3 through PCR. a) PCR detection at the 5 k base pairs position of the Ad4ITRmut-Ad7E3 genome; b) PCR detection at the 10 k base pairs position of the Ad4ITRmut-Ad7E3 genome; c) PCR detection at the 25 k base pairs position of the Ad4ITRmut-Ad7E3 genome. C) Detection of the expression of HAdV7 E3 antigenic characteristics in the recombinant virus Ad4ITRmut-Ad7E3 through Western blot analysis. D) Electron microscopic visualization of AdV4ITRmut-Ad7E3 and WT HAdV4. Abbreviation: HAdV, human adenoviruse; CPE, cytopathic effects; PCR, polymerase chain reaction.

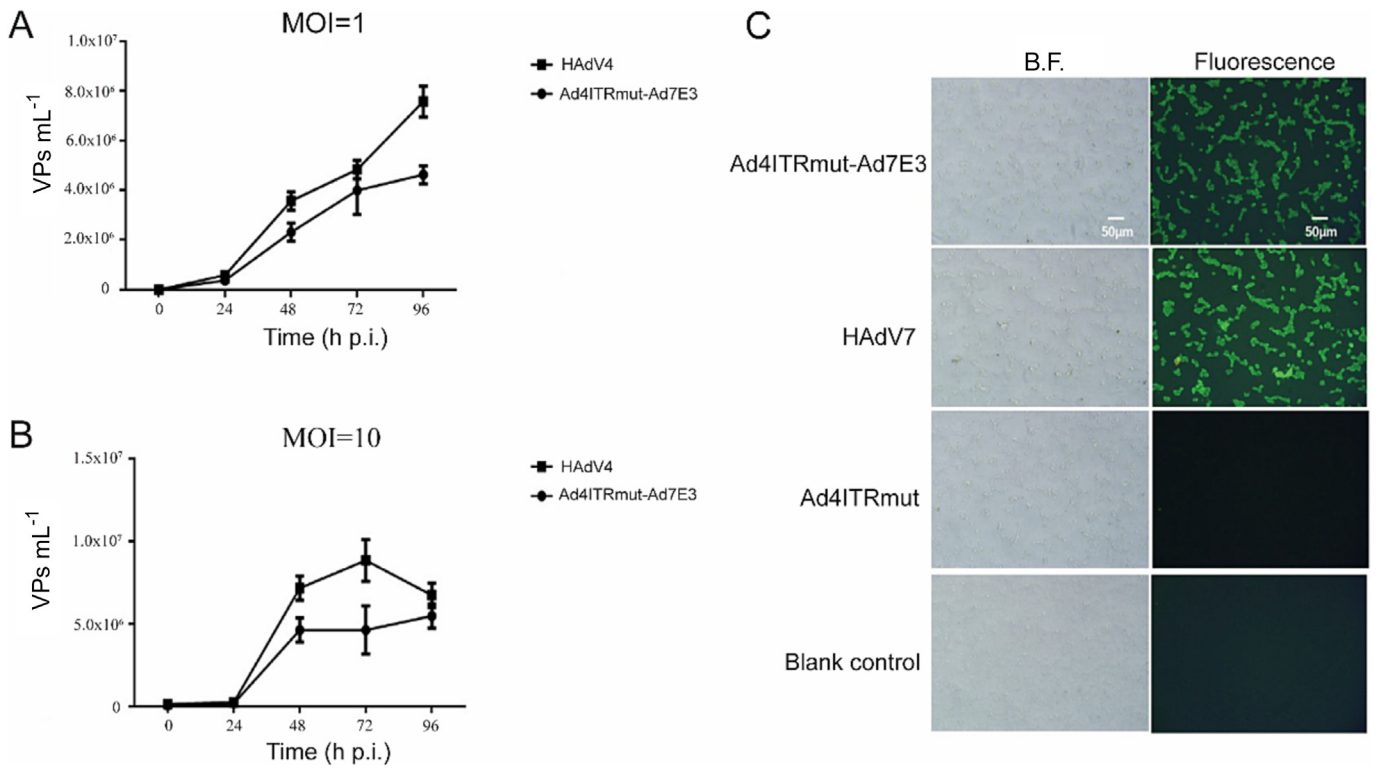

Fig.4. Detection of the replication rate of Ad4ITRmut-Ad7E3 and identification of HAdV7 E3 epitope expression. A) Determining the replication rate of the recombinant virus Ad4ITRmut-Ad7E3 at an MOI of 1. B) Determining the replication rate of the recombinant virus Ad4ITRmut-Ad7E3 at an MOI of 10. C) Detecting the expression of HAdV7 E3 antigenic characteristics in the recombinant virus Ad4ITRmut-Ad7E3 through indirect immunofluorescence assay. Abbreviations: B.F., bright field; Fluorescence, fluorescence field; HAdV, human adenoviruse; MOI, multiplicity of infection; h p.i, hour post infection.

(Fig. 5C). Following the second booster dose, the antibodies generated by the Ad4ITRmut-Ad7E3 group antibodies generated by the Ad4ITRmut-Ad7E3 group were able to neutralize 73 % of 5,000 IFU HAdV7. At the same time, 27 % of HAdV7 remained unneutralized. This neutralization rate was higher than that of the HAdV4 and PBS groups.

Moreover, the HAdV7 group achieved 100 % neutralization of serum antibodies against HAdV7 *in vitro* after the first immunization (Fig. 5C). The level of neutralizing viral antibodies increased with the number of immunizations. Additionally, the amount of neutralizing viral antibodies increased with the number of booster doses in the Ad4ITRmut-Ad7E3 group immunized by oral gavage. After the second booster immunization, the antibodies generated by the Ad4ITRmut-Ad7E3 group were able to neutralize 24 % of 5,000 IFU HAdV7, while 76 % of HAdV7 remained unneutralized. Although this neutralization rate was significantly higher than that of the HAdV4 and PBS groups, the level of antibody production against HAdV7 was notably lower than that achieved by the intraperitoneal injection group (Fig. 5D). Although Ad4ITRmut-Ad7E3 differs from WT HAdV4 in only one epitope, it is capable of eliciting an immune response against HAdV7 after initial immunization.

3.5. Intraperitoneal injection of Ad4ITRmut-Ad7E3 reduced the viral load of HAdV4 and HAdV7 in the lungs of immunized mice

The Ad4ITRmut-Ad7E3/HAdV4/HAdV7/PBS immunized groups in the HAdV4 challenge group were intranasally challenged with 1×10^7 IFU of HAdV4, respectively. Similarly, the Ad4ITRmut-Ad7E3/HAdV4/HAdV7/PBS immunized groups in the HAdV7 challenge group were intranasally challenged with 1×10^7 IFU of HAdV7. The results of the challenge-protection test revealed that the viral copy number of HAdV4 in the lungs of mice in the intraperitoneal injection group immunized with Ad4ITRmut-Ad7E3 and HAdV4, was lower than that in the PBS groups (Fig. 6A). This result confirmed that the antibodies produced by the mice injected intraperitoneally with Ad4ITRmut-Ad7E3 were protective against HAdV4 challenge. Additionally, the viral copy number of HAdV4 in the lungs of mice immunized with Ad4ITRmut-Ad7E3 was lower than that in the HAdV4 groups (Fig. 6A), demonstrating that the antibodies generated against HAdV4 in mice immunized with intraperitoneal injection of Ad4ITRmut-Ad7E3 provided more robust protection against HAdV4 challenge compared to the HAdV4 group. In the HAdV7 challenge group, the viral copy number of HAdV7 in the lungs of mice in the intraperitoneal injection group immunized with Ad4ITRmut-Ad7E3 was lower than that in the HAdV4 and PBS groups (Fig. 6B), further confirming that antibodies generated against the recombinant adenovirus play a protective role against HAdV7 challenge.

4. Discussion

HAdV4 is the sole adenovirus within group E known to cause respiratory tract infections, while HAdV7 in group B is responsible for acute respiratory infections (ARIs) in humans [38]. A comparative analysis of the complete genomes of the U.S. HAdV4 vaccine strain and its epidemic strain, RI67, has revealed significant differences in the first 8 bases of the ITRs between these two strains [20]. ITRs are vital *cis*-acting elements crucial for viral packaging, and those located in the reverse terminal repeat sequence play a pivotal role in adenovirus replication. DNA replication in adenovirus relies on the binding of dCMP to the 5′ end, with pTP acting as a primer and the 3′ end ITRs serving as the template for chain replacement synthesis [39]. These replaced single-strand molecules can self-anneal and form cyclized structures resembling handle-like ring molecules. Subsequently, this cyclized structure synthesizes double-stranded DNA offspring through the exact mechanism. In summary, the base composition and secondary structure of the ITRs terminus are critical determinants that influence the 'jumping-back' replication mechanism in adenovirus replication.

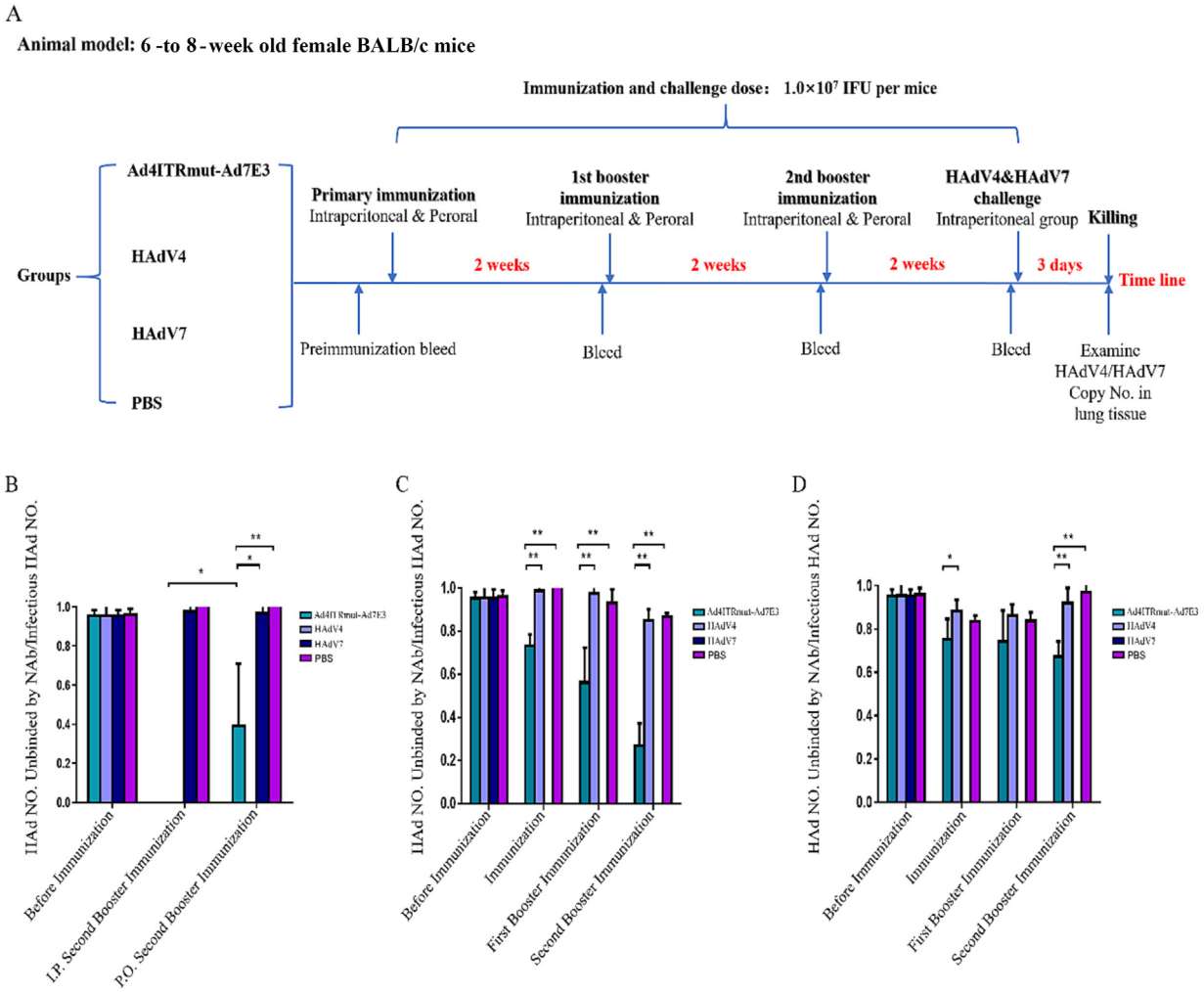

Fig.5. Neutralization activity of serum from mice administered Ad4ITRmut-Ad7E3 via intraperitoneal injection and oral administration against HAdV4 and HAdV7. A) Experimental design for the evaluation of the bivalent recombinant virus Ad4ITRmut-Ad7E3 in mice. B) Neutralization of HAdV4 in serum generated by intraperitoneal and oral immunization of mice. C) Neutralization of HAdV7 in the serum from mice immunized via intraperitoneal injection. D) Neutralization of HAdV7 in serum from mice immunized via oral administration. (Note: The serum dilution was 1:10; $*P < 0.05$; $**P < 0.01$). Abbreviations: HAdV, human adenoviruse; PBS, phosphate buffer saline; IFU, inclusion-forming unit

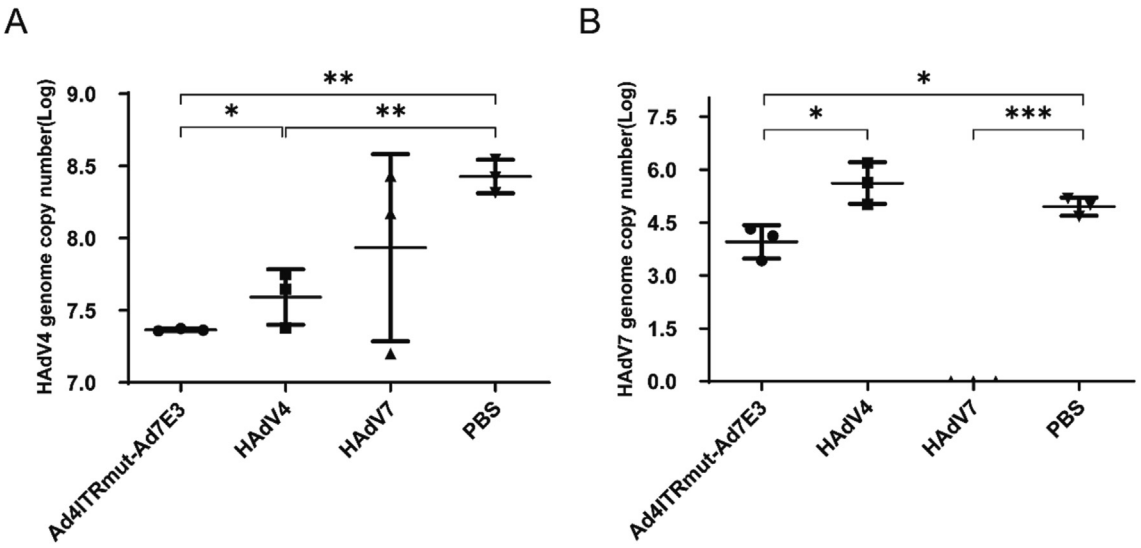

Fig.6. Assessment of the viral loads of HAdV4 and HAdV7 in the lungs of mice after intraperitoneal injection of Ad4ITRmut-Ad7E3. A) The HAdV4 genome copy number remains after the HAdV4 challenge. B) The HAdV7 genome copy number remains after the HAdV7 challenge. Note: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Abbreviations: HAdV, human adenoviruse; PBS, phosphate buffer saline.

Epitopes within the HVRs serve a dual purpose: they act as diagnostic markers to distinguish different serotypes and are the primary sites for type-specific epitopes that stimulate neutralizing antibodies (Nabs) [31,40,41]. In the case of HAdV7, a pivotal type-specific epitope is located in HVR5, as highlighted in previous studies [26]. Conversely, for HAdV4, the essential type-specific epitope resides in HVR7, while the epitope in HVR5 contributes minimally to Nabs [32]. Therefore, the chimeric recombinant adenovirus stimulates the Nabs to HAdV7 but has little effect on the significant serotype epitopes of HAdV4 after replacing the HVR5 domain epitope of HAdV4 with the E3 epitope of HAdV7. Some studies utilized restriction endonucleases and shuttle plasmids to insert the HIV capsid antigen into HAdV5 hexon, which induced anti-HIV humoral immune responses in mice [42]. However, the present study uses Red homologous recombination, and counter-selection methodology was utilized to construct a bivalent recombinant adenovirus Ad4ITRmut-Ad7E3. This approach does not require a shuttle plasmid and is not limited to the endonuclease cleavage sites to replace DNA fragments, rendering it convenient.

Alterations in the ITRs sequence can impact the replication rate of adenoviruses. Consequently, we assessed the growth rate of WT HAdV4 and Ad4ITRmut-Ad7E3 adenoviruses, discovering that Ad4ITRmut-Ad7E3 displayed slower growth and weaker virulence compared to WT HAdV4. This phenomenon could be attributed to the disrupted interbase binding resulting from the 8-base mutation at the ITR's terminus, which subsequently affects adenovirus replication. CAR is the primary receptor for HAdV4 [43], and mice cells also express CAR. While mice are not the ideal model for studying adenovirus vaccines, vaccine efficacy can be roughly gauged by monitoring the viral load in the lungs. Intriguingly, in the case of human adenovirus infection, where the viral load of HAdV4 and HAdV7 in the lungs had no lethal effect on mice, Ad4ITRmutAd7E3-immunized mice exhibited a reduction in the number of HAdV4 and HAdV7 viruses in their lungs compared to the PBS control group.

FDA-approved live oral HAdV4 and HAdV7 vaccines have been highly effective in reducing the risk of respiratory HAdV infections within the U.S. military, but have not been available to civilians. However, adenovirus infections and outbreaks are global occurrences, necessitating ample vaccines for civilian populations. Developing new adenovirus vaccines must consider the following aspects: 1) Adenoviruses are widespread globally, with the predominant virus strains varying by region. 2) The current live oral vaccine used by the U.S. military is based on wild-type virus strains without artificial modifications, except for altering the route of viral invasion. Administering this vaccine requires strict monitoring of vaccine users and their surroundings to prevent environmental contamination. Thus, incorporating artificial markers to track the vaccine strain is necessary. 3) Oral live vaccines exist in two forms, HAdV4 and HAdV7, necessitating two production lines for manufacturing. Utilizing genetic engineering technology to obtain multivalent adenovirus vaccines could reduce the number of production lines, lower vaccine production costs, and enable efficient vaccine identification and tracking. Such an approach to developing recombinant multivalent vaccines can also meet the varying needs for different serotypes of major pathogenic adenoviruses in different countries and regions.

In this study, we developed a bivalent vaccine candidate using HAdV4 as the backbone and employing homologous recombination and counter-selection screening technology to incorporate genetic material from HAdV7. The vaccine design is rooted in the low variability E-group HAdV4 strain, while HAdV7 exhibits substantial genomic variability across different regions, yet crucial neutralizing epitopes remain conserved. To immunize mice, we employed both intraperitoneal injection and peroral administration routes. Our findings indicated that both routes stimulated mice to produce neutralizing antibodies against HAdV4 and HAdV7, although the intraperitoneal injection group showed higher antibody levels than the peroral administration group. Subsequently, mice from the intraperitoneal injection group, displaying more robust immune responses, underwent nasal challenge experiments. We observed a reduction in viral load in the lungs of these mice, demonstrating the potential efficacy of Ad4ITRmut-Ad7E3 immunization.

Interestingly, the titer of neutralizing antibodies produced by the bivalent vaccine strain was lower than that of the wild HAdV4 and HAdV7 strains, particularly in the oral administration group. The exact reason for this remains unclear, as the modifications made to the viral genome were relatively minor, including an 8-base change at the end of the ITRs and a 45-base epitope substitution in the L3 region. However, the reduced replication ability of the modified bivalent vaccine strain in animals may contribute to the attenuated immune response. It is important to note that this study represents an initial endeavor to construct a bivalent adenovirus vaccine across different groups. We acknowledge the limitations of this study and recognize the need for more extensive animal experiments to enhance the accuracy of results. Future work will involve in-depth analysis and validation of the experimental outcomes using more sensitive animal models.

Nevertheless, we are encouraged that even the presentation of 15 amino acid-neutralizing epitopes can stimulate the production of neutralizing antibodies and provide protective effects. By constructing Ad4ITRmut-Ad7E3, we aim to reduce the cost of producing HAdV4 and HAdV7 vaccines while simplifying the administration method. This simplification may eventually allow a shift from a double-dose to a single-dose administration, thus minimizing environmental contamination associated with enteral adenovirus vaccine delivery. In future research, we intend to explore the construction and validation of multivalent adenovirus vaccine candidates, incorporating additional serotypes such as HAdV3, HAdV11, HAdV14, and HAdV55. We will also utilize more effective animal models to conduct further *in vivo* evaluations of the recombinant bivalent adenovirus.

Ethics statement

This study was approved by the Committee on the Ethics of Animal Experiments of the Beijing Institute of Biotechnology (approval number: IACUC of AMMS-13-2017-013).

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Peng Wang: Conceptualization, Formal analysis , Resources, Writing – review & editing. **Yunting Shao:** Data curation, Investigation, Validation, Writing – original draft, Writing – review & editing. **Xichun Yang:** Investigation, Validation, Writing – original draft. **Wenning Zhang:** Visualization. **Jianguang Zhou:** Resources, Supervision. **Fang Huang:** Methodology, Supervision. **Shuang Liu:** Investigation. **Jiping Zheng:** Conceptualization, Resources, Writing – review & editing. **Chengjun Wu:** Conceptualization, Project administration, Resources, Writing – review & editing. **Shanhu Li:** Conceptualization, Project administration, Resources, Project administration and Software , Funding acquisition, Writing – review & editing.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bsheal.2024.02.001.

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