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Research Article

A quadrivalent norovirus vaccine based on a chimpanzee adenovirus vector induces potent immunity in mice

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ABSTRACT

Norovirus (NoV) infection is a major cause of gastroenteritis worldwide. The virus poses great challenges in developing vaccines with broad immune protection due to its genetic and antigenic diversity. To date, there are no approved NoV vaccines for clinical use. Here, we aimed to develop a broad-acting quadrivalent NoV vaccine based on a chimpanzee adenovirus vector, AdC68, carrying the major capsid protein (VP1) of noroviral GI and GII genotypes. Compared to intramuscular (i.m.), intranasal (i.n.), or other prime-boost immunization regimens (i.m. + i.m., i.m. + i.n., i.n. + i.m.), AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3), administered via i.n. + i.n. induced higher titers of serum IgG antibodies and higher IgA antibodies in bronchoalveolar lavage fluid (BALF) and saliva against the four homologous VP1s in mice. It also significantly stimulated the production of blocking antibodies against the four genotypes. In response to re-stimulation with virus-like particles (VLP)-GI.1, VLP-GII.3, VLP-GII.4, and VLP-GII.17, the quadrivalent vaccine administered according to the i.n. + i.n. regimen effectively triggered specific cell-mediated immune responses, primarily characterized by IFN-γ secretion. Furthermore, the preparation of this novel quadrivalent NoV vaccine requires only a single recombinant adenovirus to provide broad preventive immunity against the major GI/GII epidemic strains, making it a promising vaccine candidate for further development.

1. Introduction

NoV is a major pathogen that causes acute gastroenteritis (AGE) outbreaks and epidemics in all age groups worldwide, posing serious health threats and significant economic losses to the public (Moore et al., 2015). Based on its gene structure characteristics, NoV is classified into ten genogroups (GI–GX) (Chhabra et al., 2019), with GI and GII being the main genogroups causing AGE in humans (de Graaf et al., 2016). Currently, the top five prevalent genotypes worldwide are GII.4, GII.3, GII.2, GII.17, and GII.6 (Kendra et al., 2022; Li et al., 2023). Infections caused by GI viruses mainly originate from the GI.1 prototype NoV strain (van Beek et al., 2018). Therefore, a desired vaccine should provide protection against both GI and GII genogroups.

In 2016, the World Health Organization stated that the development of NoV vaccines should be a major priority. Due to the lack of an *in vitro* culture system for NoVs and an effective animal model, vaccine research has been hindered for a long time. Currently, no licensed NoV vaccines are available. Vaccine candidates under clinical evaluation are mainly bivalent or monovalent (Kim et al., 2018; Leroux-Roels et al., 2018, 2022), and some in preclinical stages are nearly generated from recombinant virus-like particles (VLP) (Guo et al., 2008; Ma and Li, 2011; Bansal et al., 2013; Tamminen et al., 2013; Mathew et al., 2014; Wang et al., 2015; Verma et al., 2016). However, the production process of VLP-based vaccines is complex and costly compared to viral-vectored vaccines.

Concerning vaccine preparation, adenovirus vectors have become an important technical platform owing to their significant peculiarities, including easy operation, broad cell infection range, high safety, and the ability to simultaneously activate both humoral and cellular immune responses. Several excellent vaccine candidates based on adenovirus

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vectors have been developed (Milligan et al., 2016; Liu et al., 2021, 2023). The major capsid protein (VP1) of NoV contains a receptor-binding site, strain-specific antigenic determinants, and potential neutralizing antibody targeting sites (Todd and Tripp, 2019), which always be selected as the NoV vaccine candidate targeted antigen.

Here, we aimed to develop a novel quadrivalent NoV vaccine that co-expresses VP1s from four selected NoV genotypes (GI.1, GII.3, GII.4, and GII.17), utilizing chimpanzee adenovirus serotype 68 (AdC68) as the vector. We assessed the immunogenicity of this novel quadrivalent NoV vaccine, named AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3), by detecting the humoral, mucosal, and cellular immune responses it induced, thereby providing new ideas and strategies for the development of broad-spectrum NoV vaccines.

2. Materials and methods

2.1. Vaccine construction

Recombinant AdC68-empty vectors were developed as previously described (Guo et al., 2018). The VP1 genes of four strains of NoV: NoV GI.1, TF1/USA/2008 (GenBank: KT943503.1), NoV GII.3, GII/Hu/HKG/2014/GII.3/CUHK-NS-227 (GenBank: KJ499444.1), NoV GII.4, NoV Hu/GII.4/Hong Kong/CUHK6080/2012/CHN (GenBank: KC631827.1), and NoV GII.17 GII/Hu/HKG/2014/GII.17/CUHK-NS-491 (GenBank: KP698928.1) were codon-optimized for human expression and synthesized by Kingsray Biotechnology Co., Ltd., Nanjing, China. After being connected by the F2A linker, the *VP1* genes of GI.1, GII.3, GII.4, and GII.17 were cloned into the E1 or E3 region of the AdC68-empty vector via homologous recombination and inserted into an ORF made up of the cytomegalovirus (CMV) promoter and bovine growth hormone polyadenylation (BGH PolyA).

Recombinant adenoviruses were packaged, propagated, and purified in human embryonic kidney (HEK) 293 cells using cesium chloride gradient ultracentrifugation. QuickTiter™ adenovirus titer was used to titrate the infectious viral units per milliliter (infectious units, IFU/mL).

2.2. Virus-like particle (VLP) preparation

VLPs were prepared as previously described (Hou et al., 2022). Briefly, approximately 5–10 μg of selected recombinant plasmids (pPink–HC–GI.1, pPink–HC–GII.3, pPink–HC–GII.4, and pPink–HC–GII.17) were linearized using the restriction enzyme AflII (NEB, Illinois, LF, USA) and transfected into *Pichia pastries* strains (Invitrogen, Waltham, MA, USA) by electroporation at 1.5 kV for 5 ms.

Positive clones were selected from *Pichia* adenine dropout agar plates and cultured in 5 mL buffered glycerol-complex medium (BMGY) at 250 ×*g* and 28 °C for approximately 30 h. Next, 1–2 mL culture was transferred to 500 mL BMGY and inoculated mixtures under the same conditions until the optical density OD_{600} reached 2–6. The yeast cells were harvested, and BMMY (buffered methanol-complex) medium was added up to 100–150 mL. After 3–5 days, yeast was collected by centrifugation and resuspended in 0.15 mol/L phosphate-buffered saline (PBS) containing 1 mmol/L phenylmethylsulfonyl fluoride, homogenized at 4 °C, 1500 bar, and the supernatants were collected. Polyethylene glycol 6000 (7%) and 0.2 mol/L NaCl solution were added overnight to concentrate the supernatant. Concentrated supernatants were centrifuged, and the precipitate was resuspended in 0.15 mol/L PBS and dissolved at 4 °C overnight. The next day, the pellets were centrifuged at 14,000 ×*g* for 30 min, and the supernatants were collected. The yeast extract supernatant containing VLPs was subjected to 20 % sucrose cushion ultracentrifugation at 130,000 ×*g* for 4 h. Pellets were collected and resuspended in 0.15 mol/L PBS and layered onto 10%–50% sucrose gradients for ultracentrifugation at 270,000 ×*g* for 3 h (Beckman, Indianapolis, IN, USA). Twelve fractions were collected from the top to the bottom of the ultracentrifuge tube and VLP content was analyzed using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The pellet buffer was replaced with PBS using an Amicon Ultra-15 centrifuge (Millipore, Massachusetts, BSN, USA). Purified VLPs were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Eugene, OR, USA) and stored at −80 °C until use.

2.3. Mouse experiments

Female C57BL/6 mice (6–8 weeks) were purchased from SPF Biotechnology (Vital River Laboratory Animal Technology, Beijing, China) and fed in specific pathogen-free (SPF) animal facilities at Tianjin Medical University, Tianjin, China. For the single-shot strategy, C57BL/6J mice (6–8 weeks) were vaccinated with 1×10^8 IFU of AdC68-empty, AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3), AdC68-GI.1-GII.3 (E1), or AdC68-GII.4-GII.17 (E3) at week 0 and serums were collected at week 6.

For the prime-boost immunization strategy, C57BL/6J mice (6–8 weeks) were vaccinated intramuscularly (i.m.) or intranasally (i.n.) with 1×10^8 IFU of AdC68-empty, AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3), AdC68-GI.1-GII.3 (E1), or AdC68-GII.4-GII.17 (E3) at week 0 and were vaccinated intramuscularly or intranasally at the same dose at week 6. At week 10, serum, bronchoalveolar lavage fluid (BALF), and saliva were collected and analyzed using enzyme-linked immunosorbent assay (ELISA) to measure the antibody levels. These recombinant adenoviral vector NoV vaccine candidates were diluted in PBS without the addition of any adjuvant.

2.4. Western blotting assay

HEK 293 cells were grown and seeded in 6-well plates and infected at a dose of 5×10^6 IFU with AdC68-empty, AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3), AdC68-GI.1-GII.3 (E1), AdC68-GII.4-GII.17 (E1), or AdC68-GII.4-GII.17 (E3). After incubation for 24 h at 37 °C, cells were collected and lysed in 200 μL of RIPA (Beyotime Biotechnology, Shanghai, China) buffer with protease inhibitors (Roche, Basel, Switzerland). Each cell lysate was subjected to 10 % SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and labeled respectively with four mouse anti-NoV-VP1 monoclonal antibodies at 1:5000 dilution overnight at 4 °C, including GII.4, and GII.17 purchased from GeneTex (Texas, SA, USA) and GI.1, and GII.3 customized by GenScript Biotech Corporation (Nanjing, China). Next, the membranes were incubated with goat anti-mouse IgG H&L (horseradish peroxidase [HRP]) at a 1:10000 dilution (Sigma-Aldrich, Shanghai, China) for 1 h at room temperature (20–25 °C).

2.5. ELISA

Binding IgG and IgA antibody levels in mouse serum, BALF, and saliva were measured using ELISA. Briefly, ELISA plates were coated overnight at 4 °C with VLP-GI.1, VLP-GII.3, VLP-GII.4, or VLP-GII.17 (50 ng per well). For IgG detection, serum samples were serially diluted two-fold from 1:100 starting dilution. For IgA detection, BALF and saliva samples were two-fold serially diluted from 1:5 starting dilution while serum samples were serially diluted two-fold from 1:50. After incubation for 1 h at 37 °C, the secondary antibodies, HRP-conjugated goat anti-mouse IgG antibody (1:10000) (Abcam, Cambridge, UK) and HRP-conjugated goat anti-mouse IgA (1:7000) (SouthernBiotech, Alabama, BHAM, USA), were added. Plates were further incubated for 1 h at 37 °C followed by the addition of TMB substrate (NCM Biotech, Suzhou, China). Sulfuric acid solution (2 M H_2SO_4) was used to stop the reaction. The $OD_{450–630}$ was recorded using a microplate reader (Tecan, Männedorf, Switzerland). The binding antibody endpoint titer was defined as the reciprocal of the highest serum dilution that yielded an absorbance greater than or equal to 0.1 OD unit above the absorbance of the control samples.

2.6. Blocking antibody assay

Briefly, ELISA plates were coated with 100 μL porcine stomach mucin (Yuanye Bio-Technology, Shanghai, China) at a concentration of

10 μg/mL. The serum samples were two-fold serially diluted with a 1:100 starting dilution and an equal-volume mixture of NoV VLPs. The samples were then incubated for 1.5 h at room temperature (20–25 °C) and added to the pre-coated plates. The bound VLPs were detected using a NoV VLP type-specific antibody, followed by incubation with HRP-conjugated goat anti-rabbit IgG (Abcam, Cambridge, UK) and TMB substrate. OD_{450} was measured using a microplate reader (Tecan, Männedorf, Switzerland). Maximum binding was determined using VLPs without mouse sera. The 50% blocking antibody titer (BT50) was defined as the reciprocal of the highest serum dilution that blocked 50% of maximum VLP binding.

2.7. Cytokine ELISAs

Splenocytes were isolated from mice, and cytokine levels were measured using ELISA. Briefly, newly isolated splenocytes were seeded in 96-well plates at a density of 1×10^6 cells/well. The cells were then separately stimulated with VLP-GI.1, VLP-GII.3, VLP-GII.4, or VLP-GII.17 (10 μg/mL) for 48 h at 37 °C with 5% CO_2. Concanavalin A (10 μg/mL) and PBS served as positive and negative controls, respectively. The supernatant was collected and used to measure cytokines using the IFN-γ, TNF-α, IL-2, and IL-4 ELISA Kits (Invitrogen, Waltham, MA, USA), following the manufacturer's instructions.

2.8. IFN-γ ELISPOT assay

The ELISPOT assay was performed using freshly isolated mouse splenocytes. Briefly, ELISPOT plates (Millipore, Massachusetts, BSN, USA) were activated by adding 50 μL 70% ethanol per well for 2 min, washed with sterile water, and coated with capture antibody AN18 (Mabtech, Stockholm, Sweden) diluted in sterile PBS (15 μg/mL) at 4 °C overnight. The pre-coated plates were blocked with complete RPMI medium for at least 30 min on the next day before seeding 1×10^6 splenocytes into each well and separately stimulated with VLP-GI.1, VLP-GII.3, VLP-GII.4, and VLP-GII.17 (10 μg/mL) for 48 h at 37 °C with 5% CO_2, cell stimulation cocktails (Invitrogen, Waltham, MA, USA) and complete RPMI medium were used as positive and negative controls, respectively. IFN-γ spot forming units were detected by staining of PVDF membranes with detection antibody R4-6A2-biotin (Mabtech, Stockholm, Sweden) at 1 μg/mL for 2 h at room temperature. Subsequently, the membranes were incubated with streptavidin-HRP diluted (1:1000) in PBS containing 0.5% fetal bovine serum for 1 h at room temperature and developed using TMB substrate solution (Mabtech, Stockholm, Sweden). The spots were scanned and quantified using an ImmunoSpot reader (CTL, Ohio, CLE, USA).

2.9. Statistical analysis

All data are presented as means ± standard error of the mean. Groups were compared using a one-way analysis of variance with Tukey's multiple comparison test according to the distribution of data. All tests were performed using GraphPad Prism 8.2.4 software.

3. Results

3.1. VP1 antigen was successfully expressed in the newly designed AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) vaccine

By removing both the E1 and E3 sections from the AdC68 vector, we retained 8 kb space to load exogenous genes. To create a quadrivalent NoV vaccine AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) (Fig. 1A), we selected the VP1s from four genotypes of NoV, namely the GI.1 strain of the NoV GI gene cluster and the most common strains, GII.3, 4, and 17. In addition, we also prepared bivalent vaccines as controls: GI.1-GII.3 were cloned into the E1 region of the adenoviral vector to obtain AdC68-GI.1-GII.3 (E1), GII.4-GII.17 were cloned into the E3 region to obtain AdC68-GII.4-GII.17 (E3). Concurrently, we prepared recombinant adenovirus AdC68-GII.4-GII.17 (E1) to investigate whether there is a difference in the expression of exogenous genes between the E1 and E3 regions.

Western blotting was performed to detect the expression of antigen genes. The results showed that all *VP1* genes in the various vaccine formulations exhibited specific bands between 55 and 70 kDa (Fig. 1B–E), which was consistent with the theoretical size of the VP1 protein, demonstrating that the VP1 antigens of the four genotypes were successfully expressed. There was no difference in the expression of GII.4 or GII.17 VP1 protein between the E1 and E3 regions (Fig. 1D and E, Supplementary Fig. S1). We selected the bivalent vaccine AdC68-GI.1-GII.3 (E1) and AdC68-GII.4-GII.17 (E3) as the control in the subsequent animal experiments.

3.2. The i.n. immunization induced higher levels of serum lgG than i.m. immunization

To detect the effects of the inoculation route on immunization efficacy, mice were immunized via i.m. or i.n. with a single dose of 1×10^8 IFU NoV vaccine. AdC68-empty served as the sham control (Fig. 2A). Six weeks after inoculation, serum was collected to measure the levels of specific IgG against VLPs of each NoV genotype (Fig. 2B–E). The quadrivalent vaccine simultaneously induced specific IgG against all four VLPs via the i.m. or i.n. route. Specifically, after i.m. vaccination, the geometric mean titers (GMT) of lgG induced by the quadrivalent vaccine against VLP-GI.1, VLP-GII.3, VLP-GII.4, and VLP-GII.17 were 6,400, 14,703, 3,676, and 3,676, respectively. In i.n. vaccination group, the GMT of IgG were 61,187, 102,400, 11,143, and 22,286, respectively. The antibody titers induced by the i.n. route were significantly higher than those induced by i.m.

After i.n. vaccination, the GMT of lgG against VLP-GI.1 and VLP-GII.3 induced by the bivalent vaccine AdC68-GI.1-GII.3 (E1) were 2.0 and 1.4 times (GMT: 122,880 and 143,360) higher than the corresponding values of the quadrivalent vaccine (GMT: 61,187 and 102,400) (Fig. 2B and C). The GMT of lgG against VLP-GII.4 and VLP-GII.17 induced by the bivalent vaccine AdC68-GII.4-GII.17 (E3) were 5.2 and 8 times (GMT: 58,813 and 178,289) higher than the corresponding values of the quadrivalent vaccine group (GMT:11,143 and 22,286) (Fig. 2D and E). These results indicated that i.n. inoculation with the quadrivalent vaccine fostered substantially higher production of serum IgG against the four genotypes of VLP than i.m. immunization.

3.3. The i.n. + i.n. regimen induced superior serum lgG antibody responses

In this study, we adopted intramuscular vaccination (i.m.) to immunize the mice in a single-shot strategy, then detected IgG antibodies in serum at 2, 4, 6, and 8 weeks (Supplementary Fig. S2). We found that IgG antibody titers were increasing, and there were no significant differences between 6 and 8 weeks in AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3).

Therefore, to improve the level of antibody responses induced by the quadrivalent vaccine, we used four different prime-boost strategies: i.m. + i.m., i.m. + i.n., i.n. + i.m., and i.n. + i.n. Mice were boosted 6 weeks after primary immunization (Fig. 3A). Four weeks after the last vaccination, serum IgG levels against the VLPs of the four NoV genotypes were measured (Fig. 3B–E). The results showed that the GMT of GI.1-specific IgG induced by the quadrivalent vaccine after i.n. + i.n. inoculation was 135,118, which was significantly higher than that of the i.m. + i.m. and i.n. + i.m. strategies. The titers were numerically higher than those of the i.m. + i.n. regimen, although the difference was not significant. Similarly, the GMT of GII.3-specific IgG induced by the quadrivalent vaccine administered via i.n. + i.n. was 327,680, which was significantly higher than that induced by the other three prime-boost regimens. The GMTs of GI.1- and GII.3-specific IgGs induced by the quadrivalent vaccine were comparable to those induced by the bivalent vaccine AdC68-GI.1-GII.3 (E1) via the i.n. + i.n. regimen.

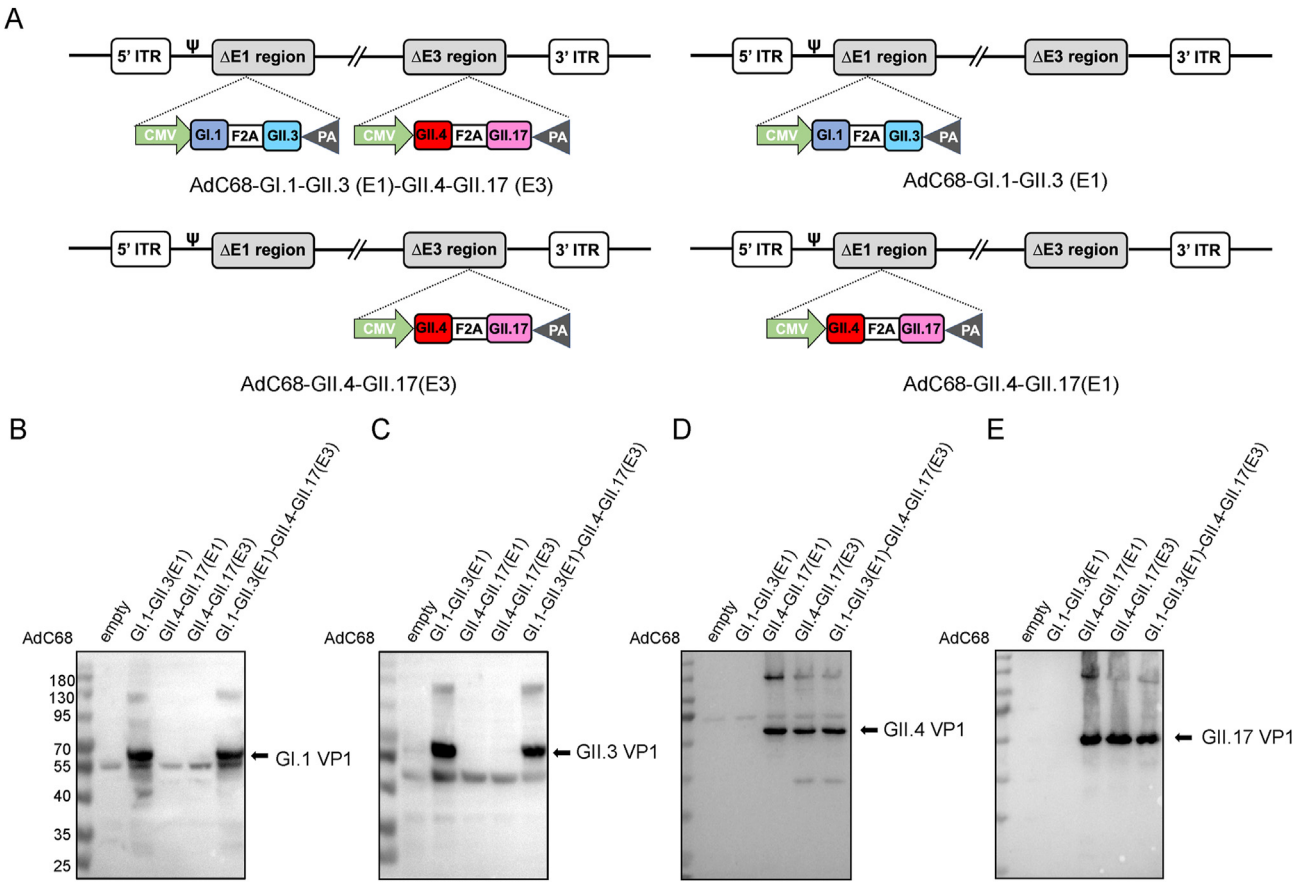

Fig. 1. Design and characterization of AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3). **A** Schematic diagrams of quadrivalent and bivalent vaccines. **B**–**E** Expression of VP1s analyzed by Western blot. The HEK 293 cells were infected with different NoV vaccines at 5×10^6 IFU. After 24 h, the cells were collected to check the expression of VP1s using mouse monoclonal antibodies against NoV-GI.1-VP1 (**B**), anti-NoV-GII.3-VP1 (**C**), anti-NoV-GII.4-VP1 (**D**), and anti- NoV-GII.17-VP1 (**E**). AdC68-empty was used as the control. The protein size marker was indicated in the left panel.

Taken together, the prime-boost regimen strongly improved the serum IgG levels, and the i.n. + i.n. regimen outperformed the i.m. + i.m. and i.n. + i.m. regimens.

3.4. The i.n. + i.n. regimen induced superior mucosal responses

To assess the ability of NoV vaccines to induce mucosal responses, we simultaneously tested specific IgA titers against the four VLPs in the serum, saliva, and BALF of mice 4 weeks after the last immunization with the i.m. + i.n. and i.n. + i.n. regimens.

The GMTs of serum IgA against the four VLPs induced by the quadrivalent vaccine after i.m. + i.n. inoculation were 280, 360, 180, and 320, respectively, and were 360, 640, 480, and 550, respectively, with the i.n. + i.n. regimen, indicating that the i.n. + i.n. regimen could produce higher titers of serum-specific IgA than did the i.m. + i.n. regimen (Fig. 4A). For the quadrivalent vaccine, there were no significant differences in IgA levels against corresponding NoV compared with those induced by the bivalent vaccines [AdC68-GI.1-GII.3 (E1) and AdC68-GII.4-GII.17 (E3)] with the i.n. + i.n. regimen (Fig. 4A) except for the GII.17-specific and GII.4-specific IgA titers.

GMTs of specific IgA antibodies against the four VLPs in the BALF after the quadrivalent vaccine vaccination with i.m. + i.n. regimen were 30, 114, 42, and 138, respectively, and 114, 234, 114, and 126, respectively, with i.n. + i.n. regimen, indicating that the i.n. + i.n. regimen produced more robust BALF IgA responses than did the i.m. + i.n. regimen (Fig. 4B). The ability of the quadrivalent vaccine to trigger GI.1- and GII.3-specific IgA was comparable to that of the bivalent vaccine AdC68-GI.1-GII.3 (E1), whereas that to induce GII.4- and GII.17-specific IgA was less than that of AdC68-GII.4-GII.17 (E3).

The GMTs of specific IgA antibodies against GI.1, GII.3, GII.4, and GII.17 in the saliva induced by the quadrivalent vaccine with the i.m. + i.n. regimen were 32, 32, 13, and 25, respectively, and 32, 61, 25, and 90, respectively, with the i.n. + i.n. regimen (Fig. 4C), indicating that the i.n. + i.n. regimen outperformed the i.m.+i.n. regimen. No significant differences in salivary IgA titers were observed between the quadrivalent and bivalent vaccines with the i.n. + i.n. regimen.

Taken together, although the quadrivalent vaccine AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) evoked specific IgA against the four VLPs of NoV in both regimens, the i.n. + i.n. regimen induced a higher magnitude of IgA responses than did the i.m. + i.n. regimen. Compared to the bivalent vaccines, the quadrivalent vaccine induced comparable levels of GI.1- and GII.3-specific IgA, but lower levels of GII.4- and GII.17-specific IgA. Generally, the quadrivalent vaccine was superior to the bivalent vaccines in inducing specific IgA antibody responses against multiple NoVs.

3.5. The i.n. + i.n. regimen triggered potent blocking antibodies

We examined the BT50 against the VLPs of four genotypes of NoV in mouse sera 4 weeks after i.n. + i.n. boosting. The BT50 values against GI.1 and GII.3 induced by the bivalent vaccine AdC68-GI.1-GII.3 (E1), were 897 and 456, respectively, and those induced by the quadrivalent vaccine were 740 and 424, respectively, indicating that the two vaccines did not differ in their ability to induce blocking antibodies (Fig. 5A and B). The quadrivalent vaccine induced similar levels of GII.4-blocking

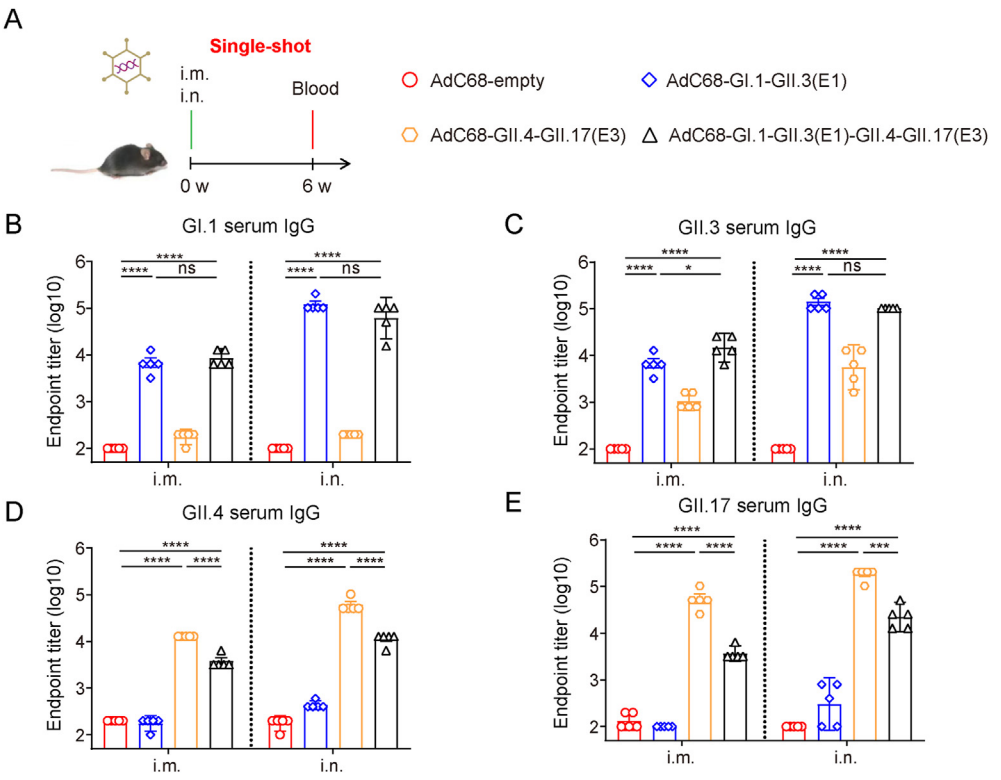

Fig. 2. Intranasal immunization induced higher levels of serum lgG than intramuscular immunization. **A** Schematic of the experiments. **B–E** Mice were immunized intramuscularly (i.m.), intranasally (i.n.) with the bivalent vaccine AdC68-GI.1-GII.3 (E1) or AdC68-GII.4-GII.17 (E3), or the quadrivalent vaccine AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) at a dose of 1×10^8 IFU (n = 5). After six weeks, the immunized mice were bled. Titers of lgG against VLP-GI.1 **(B)**, VLP-GII.3 **(C)**, VLP-GII.4 **(D)**, and VLP-GII.17 **(E)** were determined by ELISA assay. ns, no significance; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

antibodies but significantly lower levels of GII.17-blocking antibodies than did the bivalent vaccine AdC68-GII.4-GII.17 (E3) (Fig. 5C and D).

Overall, the quadrivalent vaccine AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) simultaneously induced blocking antibodies against the four genotypes of NoV in a balanced manner and was therefore superior to the bivalent vaccines in terms of the broad spectrum of induced blocking antibodies.

3.6. *The i.n. + i.n. strategy can trigger robust cellular immunity*

To investigate whether the quadrivalent vaccine with the i.n. + i.n. regimen could induce a cellular immune response, we measured the cytokines IL-2, IFN-γ, TNF-α, and IL-4 secreted by splenocytes under the stimulation of the four VLPs.

The results of ELISA showed that there was no significant difference in the levels of IL-2, TNF-α, or IL-4 cytokines produced in the bivalent and quadrivalent vaccine groups, compared with those in the AdC68-empty control group, whereas the levels of IFN-γ were significantly higher in the bivalent and quadrivalent vaccine groups than in the AdC68-empty control group (Fig. 6A–D).

When stimulated by VLP-GI.1 and VLP-GII.3 (Fig. 6A and B), the levels of IFN-γ secreted in the quadrivalent vaccine group were comparable to those secreted in the AdC68-GI.1-GII.3 (E1) bivalent vaccine group. Interestingly, when stimulated by VLP-GI.1 and VLP-GII.3, the AdC68-GII.4-GII.17 (E3) bivalent vaccine group could also secrete a certain amount of IFN-γ (Fig. 6A and B), suggesting that there might be a cross-cellular immune response. The levels of IFN-γ secreted in the quadrivalent and AdC68-GII.4-GII.17 (E3) bivalent vaccine groups under the stimulation of VLP-GII.4 and VLP-GII.17 were also comparable (Fig. 6C and D), which indicated that the quadrivalent vaccine was similar to the bivalent vaccine in terms of its ability to activate specific cellular immune responses. This suggests that the quadrivalent vaccine could simultaneously trigger specific cellular immune responses against the four selected NoV genotypes, with a response capacity comparable to that of the bivalent vaccines.

We further examined the number of splenocytes producing IFN-γ under the stimulation of the four VLPs by ELISPOT assay. Consistent with the ELISA assay, the ELISPOT results showed that all tested vaccine groups activated T cells to secret IFN-γ. With stimulation by VLP-GI.1 and VLP-GII.3, the number of IFN-γ secreting cells in the quadrivalent vaccine group was comparable to that in the AdC68-GI.1-GII.3 (E1) bivalent vaccine group (Fig. 6E and F); under the stimulation of VLP-GII.4 and VLP-GII.17, the number of IFN-γ-secreting cells was comparable between the quadrivalent and AdC68-GII.4-GII.17 (E3) bivalent vaccine groups (Fig. 6G and H).

Generally, the quadrivalent vaccine had a comparable ability to activate specific cellular immune responses as that of the bivalent vaccines. However, the quadrivalent vaccine was superior to the bivalent vaccines in inducing specific cellular responses against multiple NoV genotypes.

4. Discussion

GI and GII clusters are the major subgroups that infect humans, with GI.1 being the prevalent strain with in the GI subcluster. From 1995 to 2019, GII.4 was the globally dominant endemic strain, and the second most prevalent genotype was GII.3, followed by GII.2, GII.17, and GII.6 (Kendra et al., 2022). Notably, the GII.17 strain replaced the previously prevalent GII.4 Sydney 2012 strain and became the predominant strain in several Asian countries in 2014–2015 (Chan et al., 2015; de Graaf et al., 2016). This serves as a warning, indicating that future norovirus outbreaks may originate from genotypes other than those currently targeted

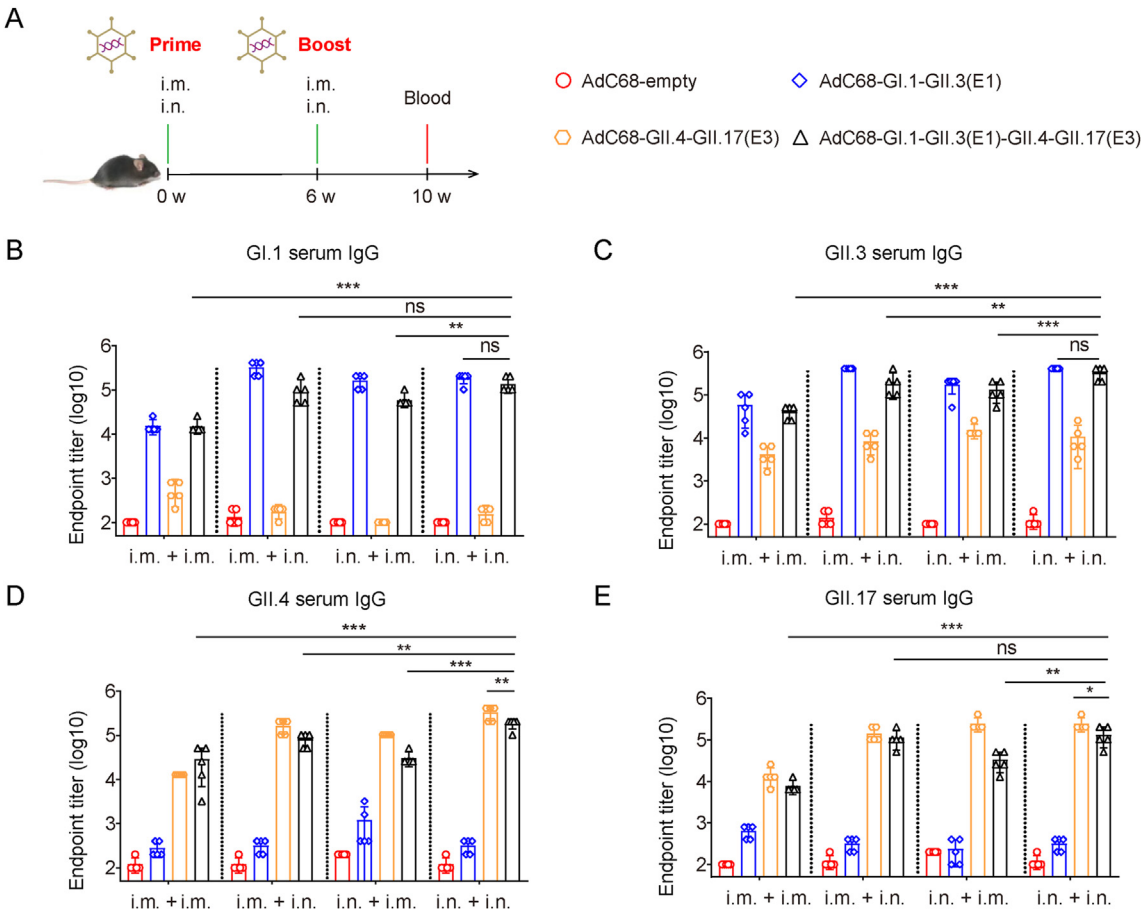

Fig. 3. The intranasal + intranasal regimen induced superior serum IgG antibody responses. **A** Schematic of the experiments. **B–E** The mice were primed intramuscularly and boosted intramuscularly (i.m. + i.m.) or primed intramuscularly and boosted intranasally (i.m. + i.n.) or primed intranasally and boosted intramuscularly (i.n. + i.m.) or primed intranasally and boosted intranasally (i.n. + i.n.) with the bivalent vaccine AdC68-GI.1-GII.3 (E1) or AdC68-GII.4-GII.17 (E3) or the quadrivalent vaccine AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) at a dose of 1×10^8 IFU (n = 5). Six weeks after priming, the mice were boosted according to the above strategies. Four weeks after the booster, the mice were bled. Titers of IgG against VLP-GI.1 **(B)**, VLP-GII.3 **(C)**, VLP-GII.4 **(D)**, and VLP-GII.17 **(E)** determined by ELISA assay. ns, no significance; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

by vaccine development. Therefore, we used the chimpanzee adenoviral platform to develop a novel quadrivalent NoV vaccine, AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3), which covers the most prevalent NoV genotypes worldwide.

Optimal vaccination strategies are crucial in the fight against infectious diseases. Studies suggest that the i.m. regimen can induce robust systemic humoral and cellular immune responses even without mucosal immunity, whereas the i.n. regimen fills this gap (Afkhami et al., 2022). The "prime-boost" vaccination strategy has shown great efficacy in boosting immune responses (Liu et al., 2021; Parys et al., 2022; Xing et al., 2023). We observed that AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) exhibits significant immunological advantages in systemic and mucosal antibody responses, as well as T cell responses, when using the i.n. + i.n. regimen.

Specific IgA and IgG activated by viral antigens are crucial immune effectors against NoV (Lindesmith et al., 2003; Atmar et al., 2011; Ramani et al., 2015; Dutch et al., 2016). Our vaccine, AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3), was shown to simultaneously provoke systemic and mucosal antibody responses against multiple prevalent NoV strains. Guo et al. assessed the immune responses to an AdHu5-based monovalent GII.4 vaccine in mice, which elicited a relatively weak GMT of IgG after post-priming immunization and increased to 632,978 at day 14 after the third immunization (Guo et al., 2008). In our preclinical study, AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) immunized with prime-boost regimens induced high titers of specific IgG against GI.1, GII.3, GII.4, and GII.17, which was significantly higher than that induced by i.n. administration alone (Figs. 2 and 3). Protection against enteric pathogens such as NoV, which can rapidly induce severe clinical symptoms, may depend on a rapid and local mucosal immune response at the site of infection. The Phase II clinical trial of HIL-214 revealed that the vaccination regimen of 15 μg GI.1/50 μg GII.4 + 500 μg $Al(OH)_3$ induced the optimal immunogenic balance in healthy adults, the GMT of vaccine-induced IgA was 811 (GI.1) and 579 (GII.4) at 4 weeks after prime-boost immunization (Leroux-Roels et al., 2018). In contrast, AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) induced a specific serum IgA GMT of up to 1200 four weeks after prime-boost immunization. Furthermore, the presence of pre-existing NoV-specific IgA in saliva is associated with gastroenteritis prevention (Ramani et al., 2015; Tamminen et al., 2018). In our study, both the bivalent and quadrivalent vaccines produced high titers of salivary IgA against various genotypes (Fig. 4).

Aerosols and droplets are generated when infected people vomit (Marks et al., 2000; Bonifait et al., 2015; Tan et al., 2024). The virus can be aerosolized when flushing toilets, leading to the inhalation of the virus and subsequent respiratory tract. Therefore, we tested the BALF IgA. The results showed that the AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) could increase the level of IgA in BALF to some extent, indicating the vaccine may have the potential to take precautions against aerosol transmission of NoV. Studies showed that intranasal immunization can induce mucosal immune response (Hartwell et al., 2022; Mao et al., 2022; Xing et al., 2024), and IgA class switching occurs in the nasal-associated lymphoid tissues (NALT) of mice (Harkema, 1990), along with the

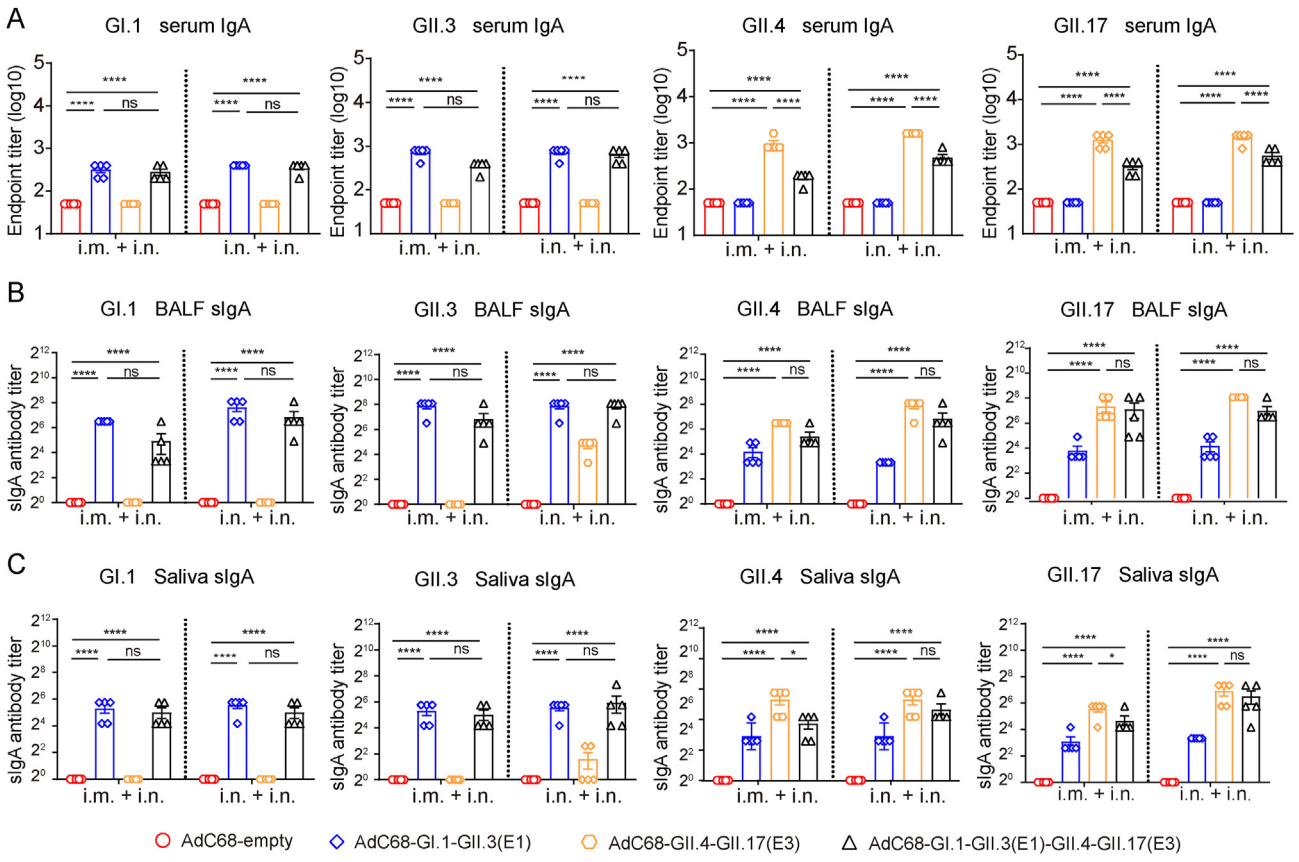

Fig. 4. The intranasal + intranasal regimen induced superior mucosal responses. The immunization schemes are identical to Fig. 3 (n = 5). Serum, bronchoalveolar lavage fluid (BALF), and saliva samples were collected four weeks after the last vaccination. **A** Serum titers of IgA against GI.1, GII.3, GII.4, and GII.17 determined by ELISA. **B** The titers of IgA in BALF against GI.1, GII.3, GII.4, and GII.17 were determined by ELISA. **C** Titers of IgA in saliva against GI.1, GII.3, GII.4, and GII.17 were determined by ELISA. ns, no significance; *, $P < 0.05$; ****, $P < 0.0001$.

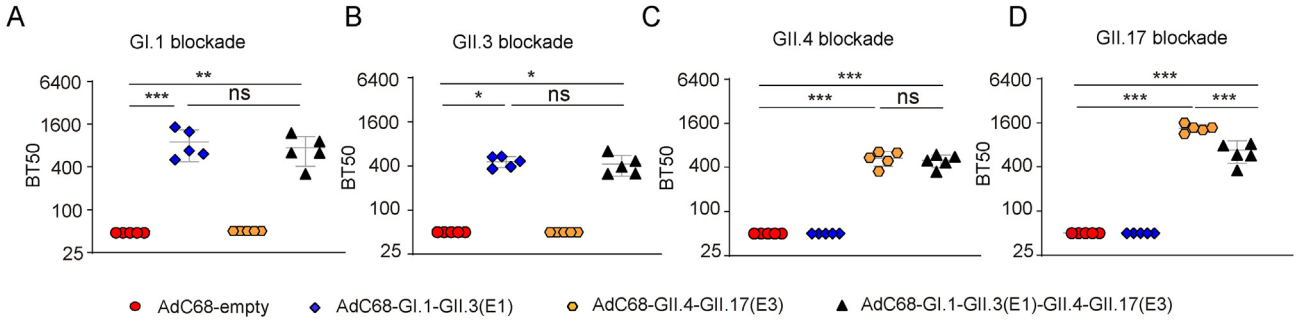

Fig. 5. The intranasal + intranasal regimens triggered potent blocking antibodies. **A**–**D** The mice were primed intranasally and boosted intranasally with the bivalent vaccine AdC68-GI.1-GII.3 (E1) or AdC68-GII.4-GII.17 (E3), or the quadrivalent vaccine AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) at a dose of 1×10^8 IFU (n = 5). Six weeks after priming, the mice were boosted. Four weeks after boosting, the mice were bled. The 50% blocking antibody titer (BT50) against GI.1 **(A)**, GII.3 **(B)**, GII.4 **(C)**, and GII.17 **(D)** were determined by ELISA assay. ns, no significance; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

coordinated activation of T/B cell responses within the NALT in a germinal-center-like manner (An et al., 2021). In our study, as shown in Fig. 4B and 4C, it was observed that the sIgA in BALF and saliva against GII.4 and GII.17 was improved in the AdC68-GII.4-GII.17 (E3) bivalent NoV vaccine group, which indicated that the i.n. + i.n. regimen has an advantage over i.m. + i.n. especially in enhancing mucosal sIgA immune response through intranasal prime and boost. Meanwhile, it can be clearly found that the bivalent NoV vaccine AdC68-GII.4-GII.17 (E3) was able to induce cross-reactive binding antibody against GII.3 (Fig. 3C), which may result from the similar epitope between GII.3 and GII.4 or GII.3 and GII.17. Thus, owing to the improved mucosal immune response, the specific sIgA antibodies against GII.4 and GII.17 which have cross-reaction with GII.3 was efficiently elevated. Consistently, a study conducted by Xing et al. (Xing et al., 2024) also revealed that the i.n prime-i.n boost regimen of AdC68-HATRBD induced a substantial increase in anti-RBD and -HA sIgA antibodies compared to the prime-only regimen. In conclusion, i.n. + i.n. regimen may provoke a more robust, protective immune response, especially mucosal immunity against NoV infection, better than i.m. + i.n.

Hhisto-blood group antigens (HBGAs) are the most distinctive host attachment factors for NoV (Ramani et al., 2015). Blocking antibodies that prevent the virus from binding to HBGA are the first identified correlation for NoV protection (Shanker et al., 2016). In the NCT02038907 trial, the peak BT50 of blocking antibodies induced by the

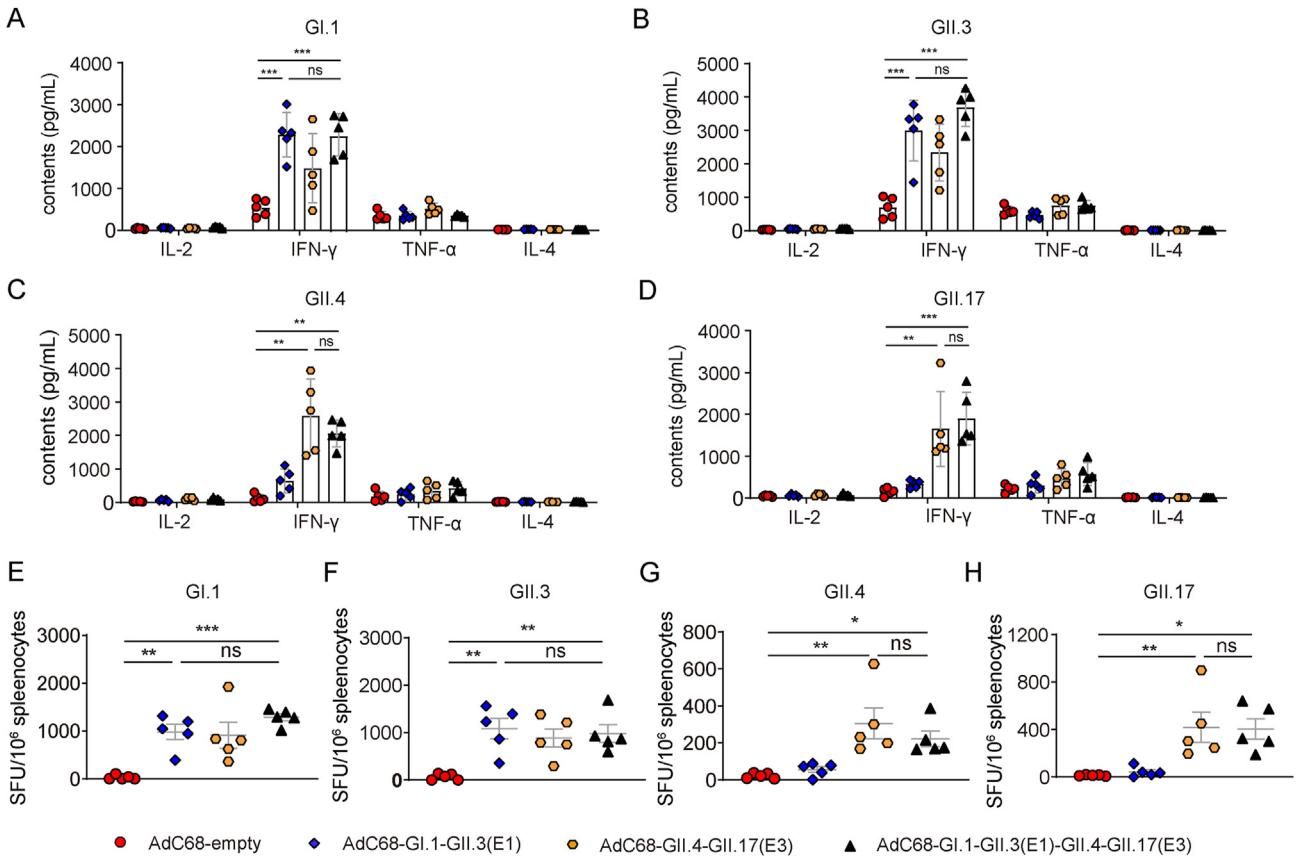

Fig. 6. The i.n. + i.n. strategy triggered robust cellular immunity. The mice were primed intranasally and boosted intranasally with the bivalent vaccine AdC68-GI.1-GII.3 (E1) or AdC68-GII.4-GII.17 (E3), or the quadrivalent vaccine AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) at a dose of 1×10^8 IFU (n = 5). Six weeks after priming, the mice were boosted. Four weeks after the boosting, the mice were euthanized. The splenocytes were collected and stimulated with the virus-like particles (VLPs). Contents of IL-2, IFN-γ, TNF-α, and IL-4 in the supernatants from the cells stimulated with VLP-GI.1 **(A),** VLP-GII.3 **(B)**, VLP-GII.4 **(C)**, and VLP-GII.17 **(D)** harvested after 48 h as determined by enzyme-linked immunosorbent assay. The IFN-γ produced by the cells was quantified by ELISA after stimulation with VLP-GI.1 **(E)**, VLP-GII.3 **(F)**, VLP-GII.4 **(G)**, and VLP-GII.17 **(H)**. ns, no significance; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

vaccine with 15 μg GI.1/50 μg GII.4 + 500 μg $Al(OH)_3$ were 413 (GI.1) and 843 (GII.4) and were 399 (GI.1) and 424 (GII.4) at 4 weeks after the prime-boost strategy (Leroux-Roels et al., 2018). Results from the Phase Ib clinical trial of the monovalent GI.1 tablet vaccine developed by Vaxart (NCT02868073) showed that the BT50 of blocking antibody induced in participants after 4 weeks with high-dose vaccination was 98.5 (Kim et al., 2018). In our experiment, AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) induced blocking antibodies against GI.1, GII.3, GII.4, and GII.17 VLPs after 4 weeks with i.n. + i.n. administration, with BT50 values of 740, 424, 493, and 678 respectively, and the induction ability was the same as that of the bivalent vaccine (Fig. 5). Clinical trial results (NCT1609257) have shown that a blocking antibody level >1:500 is associated with a lower incidence of severe vomiting and diarrhea in humans (Bernstein et al., 2015). These findings suggest that AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) could provide comparable protection against multiple NoV genotypes by eliciting high levels of blocking antibodies. Furthermore, it showed almost no inhibitory effects on the functionality or intensity of HBGA-blocking antibodies specific to NoV compared to the bivalent vaccine, which has significant implications for preventing clinical NoV infection.

Current assessment of NoV vaccine candidates has primarily focused on antibody-mediated immunity. However, humoral immunity is not the sole factor involved in clearing NoV. Insufficient induction of systemic T cell responses may lead to limited virus clearance (Pattekar et al., 2021). In this context, we explored whether AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) could also induce cellular immune responses. The results showed that the quadrivalent vaccine activated specific T cell immune responses comparable to those of the bivalent vaccine. Similar to the control of many other RNA viruses, interferon-mediated immune responses are central to controlling NoV infections (Atmar et al., 2015; Russell et al., 2017; Chen et al., 2021; Barman et al., 2022). Therefore, based on the observation that quadrivalent vaccine could induce T cells to secrete high levels of IFN-γ, AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3) may provide broad-spectrum cross-protection against multiple NoV genotypes that are not included in the vaccine as well.

Due to the limited exogenous gene-holding capacity of third-generation adenoviral vectors, our vaccine could not encompass enough NoV genotypes to produce broader protective spectrums. Using conserved neutralizing epitopes from various pandemic strains of NoVs selected by bioinformatics analysis as the targeted antigens may effectively overcome this limitation.

5. Conclusions

In summary, we developed a quadrivalent NoV vaccine with i.n. + i.n. regimen, which can provide preventive immunity against both the prototype strain GI.1 and the prevalent strains GII.3, GII.4, and GII.17, showing the potential to prevent diarrheal diseases and save lives worldwide. As an intranasal vaccine, AdC68-GI.1-GII.3 (E1)-GII.4-GII.17 (E3), only requiring the use of a single recombinant adenovirus, shows unique advantages not only in the prevention of mucosal infectious diseases but also in safety, easy storage, distribution, and easy acceptance by the population, making it a promising vaccine candidate against NoV for further development.

Data availability

All the data generated during the current study are included in the manuscript.

Ethics statement

All animal procedures were approved by the Committee on the Use and Care of Animals at Tianjin Medical University. The animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Institute of Microbiology, Chinese Academy of Sciences (IMCAS) and conducted in compliance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the IMCAS Ethics Committee.

Author contributions

Yihua Jiang: investigation, formal analysis, validation, writing-original draft; Lingjin Sun: investigation, formal analysis, validation, writing-original draft; Nan Qiao: data curation, investigation, formal analysis, visualization, writing-original draft; Xiang Wang: investigation and validation; Caihong Zhu: investigation and validation; Man Xing: methodology, data curation, investigation, formal analysis, validation; Hui Liu: project administration, conceptualization, methodology; Ping Zhou: project administration, conceptualization, methodology, investigation, visualization, formal analysis, validation; Dongming Zhou: project administration, conceptualization, methodology, funding acquisition, resources, supervision, writing-review and editing.

Declaration of competing interest

All authors declare that there are no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2024.07.002.

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