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Research article

Characterization of protective immune responses against *Neisseria gonorrhoeae* induced by intranasal immunization with adhesion and penetration protein

Lingyin Xia [a,b,1], Qin Lu [a,b,1], Xiaosu Wang [a,b], Chengyi Jia [a,b], Yujie Zhao [a,b], Guangli Wang [a,b], Jianru Yang [a,b], Ningqing Zhang [a,b], Xun Min [a,b], Jian Huang [a,b,*], Meirong Huang [c,**]

[a] *Department of Laboratory Medicine, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou, China*
[b] *School of Laboratory Medicine, Zunyi Medical University, Zunyi, Guizhou, China*
[c] *Department of Blood Transfusion, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou, China*

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ABSTRACT

Drug-resistant *N. gonorrhoeae* is an urgent threat to global public health, and vaccine development is the best long-term strategy for controlling gonorrhea. We have previously shown that adhesion and penetration protein (App) play a role in the adhesion, invasion, and reproductive tract colonization of *N. gonorrhoeae*. Here, we describe the immune response induced by intranasal immunization with passenger and translocator fragments of App. The recombinant App passenger and translocator fragments induced high titers of IgG and IgA antibodies in serum and vaginal washes. Antibodies produced by App passenger and the combination of passenger and translocator mediated the killing of *N. gonorrhoeae* via serum bactericidal activity and opsonophagocytic activity, whereas antisera from translocator-immunized groups had lower bactericidal activity and opsonophagocytic activity. The antisera of the App passenger and translocator, alone and in combination, inhibited the adhesion of *N. gonorrhoeae* to cervical epithelial cells in a concentration-dependent manner. Nasal immunization with App passenger and translocator fragments alone or in combination induced high levels of IgG1, IgG2a, and IgG2b antibodies and stimulated mouse splenocytes to secrete cytokines IFN-γ and IL-17A, suggesting that Th1 and Th17 cellular immune responses were activated. In vivo experiments have shown that immune App passenger and transporter fragments can accelerate the clearance of *N. gonorrhoeae* in the vagina of mice. These data suggest that the App protein is a promising *N. gonorrhoeae* vaccine antigen.

1. Introduction

Neisseria gonorrhoeae is a specific human pathogen that causes sexually transmitted infections, namely, gonorrhea [1]. Gonorrhea is

* Corresponding author.Department of Laboratory Medicine, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou, China.
** Corresponding author.
E-mail addresses: 81537648@qq.com (J. Huang), 277792461@qq.com (M. Huang).
[1] These authors have contributed equally to this work.

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one of the most common sexually transmitted diseases worldwide, with approximately 87 million new cases diagnosed every year [2]. If left untreated, it can lead to serious complications, including pelvic inflammatory disease, infertility, ectopic pregnancy in females, urethritis in males, and blindness in newborn babies [3]. Untreated gonorrhea also increases the risk of HIV infection [4]. The rapid rise in antibiotic resistance has posed significant challenges for the treatment of gonorrhea [5]. The World Health Organization (WHO) has warned that the number of individuals with incurable gonorrhea is increasing and called for the development of new antibacterial drugs and vaccines [5]. Ideally, the vaccine antigen should have good conservation and immunogenicity, induce functional antibodies, mediate bactericidal activity or phagocytosis, and effectively inhibit pathogen adherence [6]. The development of *N. gonorrhoeae* vaccine has been limited for many years due to the fact that *N. gonorrhoeae* is a strictly human pathogen and is characterized by high surface antigenic variability [7]. Fortunately, Jerse et al. successfully established female mice treated with estradiol for *N. gonorrhoeae* infection of the reproductive tract, addressing the model constraints for gonococcal vaccine development [8]. Based on the establishment of genomic, proteomic, and bioinformatics-based vaccine target screening methods, potential *N. gonorrhoeae* vaccine targets were screened and identified, such as Neisseria heparin binding antigen (NHBA), multiple transferable resistance efflux pump protein (MtrE), adhesin complex protein (ACP), methionine sulfoxide reductase A and B domains (MsrA/B), and lipopolysaccharide (LOS) 2C7 peptide [6,9–13]. NHBA induces the production of bactericidal antibodies that inhibit the adhesion of *N. gonorrhoeae* to cervical epithelial cells [12]. MsrA/B, ACP, and MtrE induce antibodies with bactericidal activity [14–16]. A retrospective case-control study found that the incidence of gonorrhea in individuals vaccinated with the outer membrane vesicle meningococcal B vaccine (MeNZB) was significantly reduced, with a predicted vaccine efficacy of 31 % [17]. MeNZB vaccines can induce cross-reactive antibodies against *N. gonorrhoeae* in humans [18]. Antibodies directed against the LOS 2C7 peptide had bactericidal activity against *N. gonorrhoeae*. Furthermore, passive immunization with the LOS 2C7 peptide antibody protected against *N. gonorrhoeae* infection, and this study entered phase II clinical trials [19,20]. These advances have rekindled the interest in and hope for the development of *N. gonorrhoeae* vaccines. Further research and identification of highly conserved and effective vaccine targets are important for gonorrhea vaccine research.

Autotransporter proteins are widely spread in Gram-negative bacteria, and are a group of virulence factors secreted by a simple mechanism using type V or autotransporters. The protein family is mainly composed of an N-terminal signal peptide, a passenger domain, and a C-terminal translocator domain [21]. Most autotransporters are important virulence factors involving various functions such as bacterial adhesion, invasion, and cytotoxicity and are considered to be potential drug and vaccine research and development targets [22]. For example, the current commercially available *N. meningitidis* 4CMenB vaccine consists of a factor H binding protein (fHbp), NHBA, and Neisseria adhesin A (NadA). NadA is an autotransporter of *N. meningitidis*, but this protein does not exist in *N. gonorrhoeae* [23]. Eight autotransporters have been identified in *N. meningitidis*, but only two–IgA protease and adhesion and penetration protein (App)–are present in *N. gonorrhoeae* [24–26].

Our previous study found that App (also known as NGO2105 protein) is a conserved virulence factor of *N. gonorrhoeae* that is localized on the bacterial surface and is involved in bacterial adhesion, invasion, and colonization [27]. Fadi et al. proposed that App might be a candidate target for *N. gonorrhoeae* vaccine [28]. Intranasal immunization is a safe and simple non-invasive immunization modality that not only induces systemic and mucosal immune responses but also effectively induces genital tract-specific immune responses [29]. Cholera toxin subunit B (CTB) is a classical mucosal immunization vaccine adjuvant that generates mucosal antibody responses [30]. In this study, CTB was used as a vaccine adjuvant and mice were immunized with App passenger and translocator fragments via the intranasal route and study the titer, isotype, and functional activity of antibodies induced in response to evaluate the potential of the App protein as a candidate gonococcal vaccine.

2. Materials and methods

2.1. Bacteria and plasmids

N. gonorrhoeae FA1090 (ATCC700825) and FA19 (BAA-1838) were purchased from the American Type Culture Collection. The two clinical strains were collected from the laboratory of the Affiliated Hospital of Zunyi Medical University. These *N. gonorrhoeae* strains were cultured on gonococcal base (GCB) agar (Oxoid, United Kingdom) or gonococcal base liquid (GCBL) at 37 °C with 5 % CO_2, for detailed preparation as described above [31].

The coding gene of the recombinant App passenger fragment (43–325aa) was amplified by polymerase chain reaction (PCR) from the *N. gonorrhoeae* FA1090 strain using the primers 5′-CCGGAATTCGGACACACTTATTTCGGCATCAACT-3′ and 5′-CCCAAGCTTATGTGGTTCGTAGAATACTGAATGG-3’. The coding gene of the recombinant App translocator fragment (660–1468aa, including the full-length translocator and part of the passenger fragment) was amplified by PCR from the *N. gonorrhoeae* FA1090 strain using the primers 5′-ATGGAAGGTATCCCACAAGGAGAAA-3′ and 5′-TGCGGTTGCGGCAAATCCC-3’. These PCR amplification products were cloned into the pCold-TF plasmid to construct the pCold-TF-*passenger* and pCold-TF-*translocator* recombinant plasmids.

2.2. Expression, purification of passenger and translocator recombinant proteins

Recombinant pCold-TF-*passenger*, pCold-TF-*translocator* and pCold-TF plasmids were transformed into *E. coli* BL21(DE3) cells. All recombinant expression strains were cultured in Luria-Bertani (LB) medium containing ampicillin (100 μg/mL) at 37 °C. When the bacteria grew to an optical density of 600 nm (OD_{600}) of 0.60, 0.05 mM isopropyl-β-*d*-thiogalactoside (IPTG) was added to induce the expression of recombinant protein at 15 °C for 8 h. Recombinant proteins were purified using a Ni^{2+}-nitrotriacetic acid (Ni-NTA) column. After purification, the residual imidazole was removed from the protein. And LPS in recombinant proteins was removed using

the EtEraser™ Endotoxin Removal Kit (Xiamen Bioendo Technology Co., Ltd.). Protein purity was assessed by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE gels were stained using Coomassie Blue Fast Staining and No-decoloring Solution (Epizyme, China). The protein concentration was measured using a BCA kit (Solarbio, China) for use in subsequent experiments.

2.3. Immunization of mice

Six-to-eight-week-old female BALB/c mice of specific pathogen-free (SPF) grade, weighing 18–20 g, were purchased from the Experimental Animal Center of Zunyi Medical University. All animal experiments were performed in accordance with the guidelines and codes of conduct established by the Ethics Committee of Zunyi Medical University (ZMU21-2202-048). Mice were randomly divided into five groups (eight animals per group): passenger fragment (Pa), translocator fragment (Tr), passenger and translocator fragment mixture (Pa + Tr), pCold-TF + CTB, and phosphate-buffered saline (PBS + CTB). A total of 50 μg of Pa fragment or Tr fragment or pCold-TF protein, or 25 μg Pa and 25 μg Tr mixture (Pa + Tr) was suspended in 20 μl of PBS and then mixed with 1 μg of cholera toxin subunit B (CTB) adjuvant (Absin, China) to immunize mice by the nasal mucosal route on days 0, 14, and 28, respectively. 20 μl of the prepared protein antigen mixture was slowly instilled into the nasal cavity of the mice with a sterile pipette until complete inhalation. An intraperitoneal booster immunization was performed once on day 42. Antibody titers were determined from tail vein blood and vaginal secretions collected seven days after each immunization.

2.4. Antibody examination by ELISA

Serum and vaginal washing antibody titers were measured using ELISA. Nunc-Immuno plates (96-well) were coated with purified antigen (Pa, Tr or pCold-TF) at a concentration of 10 μg/mL in PBS. The wrapped Nunc-Immuno plates were incubated at 4 °C overnight and washed with PBS-T (PBS containing 0.1 % Tween 20) and blocked with 2 % BSA at 37 °C for 2 h. Antisera from different immune groups were diluted with blocking buffer and transferred to the well (100 μl/well), followed by incubation at 37 °C for 1 h. The plates were washed thrice with PBS-T, then horseradish peroxidase (HRP)-labeled goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, and IgA were diluted 1:5000 and added to the plate for 1 h at 37 °C. The plate was washed thrice, and 3,3′, 5,5′-tetramethylbenzidine (TMB) solution (Solarbio, China) was added for the color reaction for 30 min. Subsequently, a termination solution (Solarbio, China) was added to terminate the reaction. The absorbance was recorded at 450 nm using a Victor 3 plate reader (PerkinElmer, USA). If the light absorbance value of the sample is 2.1 times higher than that of the negative control, the dilution of the sample would be chosen as the endpoint titer [32].

2.5. Analysis of the expression of native App in different N. gonorrhoeae

Bacterial concentrations of cultured *N. gonorrhoeae* FA1090 and FA19 as well as two clinical strains were adjusted to 0.5 McFarland units with GCBL. Take 1 mL of the above bacterial solution and transferred to a centrifuge tube at 4 °C and 12000 *rpm* for 2 min, the supernatant was discarded, 1 × loading buffer was added, and the samples were boiled at 100 °C for 15 min for later use. Whole bacterial lysates were separated by SDS-PAGE and transferred to PVDF membranes. Western blotting was performed using anti-passenger or anti-translocator antisera as primary antibodies (1:1000), and HRP-labeled goat anti-mouse IgG (ZSGB-Bio, China) was used as a secondary antibody (1:5000) to analyze the expression of App in different *N. gonorrhoeae*.

2.6. Flow cytometry analysis of antibody affinity to N. gonorrhoeae

N. gonorrhoeae FA1090 was cultured on GCB agar at 37 °C with 5 % CO_2, and after overnight culture, colonies were scraped and resuspended in PBS. The pellets were washed and resuspended in PBS containing 1 % BSA and 0.01 % Tween 20 till an OD_{600} of 0.5 was reached. Cell suspensions were incubated with anti-Pa, anti-Tr, anti-pCold-TF, or anti-Pa + Tr serum as primary antibodies (1:200) for 1 h at room temperature. These cell suspensions were washed thrice with PBS, incubated with FITC-labeled anti-mouse IgG antibody (1:200, Proteintech, USA) at room temperature for 1 h, and finally washed thrice with PBS. The cells were then fixed with 1 % formaldehyde. Using forward and side scattering to exclude debris and aggregates and by flow cytometry, data were collected on 20,000 gating events and the binding of antisera to bacteria was examined separately for the pCold-TF, Pa, Tr, and Pa + Tr groups. At the threshold set, untreated *N. gonorrhoeae* FA1090 was used as blank control and *N. gonorrhoeae* was co-incubated with FITC-labeled goat anti-mouse IgG as a negative control. The mean fluorescence intensity (MFI) values of each sample was analyzed, and fluorescence detection was performed using a BD FACS Canto II (Beckman Coulter).

2.7. Opsonophagocytic killing assay (OPA)

OPA assays were performed as previously described [33]. Fresh venous blood was collected from healthy subjects, shaken, and mixed well; polymorphonuclear leukocytes (PMNs) were extracted according to the manufacturer's instructions with Polymorphprep™ (Axis-Shield, Norway) separation solution, and the cells were diluted to 1×10^5 cells/mL in RPMI 1640 medium. *N. gonorrhoeae* FA1090 was inoculated onto GCB plates and incubated at 37 °C in an incubator containing 5 % CO_2 for 16–18 h. Colonies were picked to adjust the colony number to 1×10^5 CFU/40 μl, then 40 μl of heat-inactivated antisera at different dilution ratios were added and incubated at 37 °C in an incubator containing 5 % CO_2 for 15 min. Then, 10 % of human serum (as a complement

source) and 10 μl of 1×10^5 PMNs were added to reaction tubes, respectively. This was followed by incubation for 90 min at 37 °C in an incubator containing 5 % CO_2. Diluted reaction solutions were spread on GCB plates, and colony-forming units were counted the following day. The OPA titer was the highest antibody dilution that induced more than 50 % cell death. Human PMNs and serum were collected from healthy adult male and female volunteers with the approval of the Ethics Committee of Zunyi Medical University, and written informed consent was obtained from the donors (Grant No. KLLY-2022-083).

2.8. Serum bactericidal assay (SBA)

SBA assays were performed as previously described [33]. *N. gonorrhoeae* FA1090 was inoculated onto GCB plates and incubated at 37 °C in an incubator containing 5 % CO_2 for 16–18 h. Colonies were picked to adjust the colony number to 4×10^6 CFU/40 μl. Subsequently, 40 μl of heat-inactivated antisera at different dilution ratios were added and incubated at 37 °C in an incubator containing 5 % CO_2 for 15 min. Add 20 % fresh human plasma as a source of complement and incubate for 30 min at 37 °C and 5 % CO_2. Diluted reaction solutions were spread on GCB plates, and colony-forming units were counted the following day. The SBA titer was characterized as the highest antibody dilution that induced more than 50 % killing.

2.9. Adherence inhibition assay

Antibody-mediated adhesion inhibition assays were performed using human cervical cancer (ME-180) cells (ATCC HTB33) as previously described [34]. ME-180 cells were purchased from Fenghui Biotechnology Co, Ltd (Hunan, China). ME-180 cells were cultured in MEM (10 % FBS) in 24-well tissue culture plates for 24 h until confluent cell monolayers were formed. Gonococcal cells were inoculated onto GCB plates and cultured overnight, and colonies with pili were selected to prepare bacterial suspensions. Bacteria were preincubated with heat-inactivated antisera in MEM for 30 min at room temperature. ME-180 cells were washed thrice with PBS, and an antiserum-pretreated bacterial suspension was added to ME-180 cells at an MOI of 10:1. This was followed by incubation at 37 °C in 5 % CO_2 for 1 h. The solution was subsequently washed thrice with PBS to remove non-adherent bacteria. ME-180 cells were lysed with 1 % saponin, and the lysate was serially diluted and spread onto GCB plates to count the colony-forming units of the bacteria. Adhesion rates were calculated as the ratio of CFU adhered to the cells to the initial CFU.

2.10. Detection of cytokine levels

One week after the last immunization. BALB/c male mice (n = 3/group) were sacrificed and spleens were aseptically removed and homogenized by passing through a sterile plastic strainer under aseptic conditions. Single-cell solutions resuspended in RPMI 1640 (HyClone, USA), and supplemented with 10 % fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cell concentration was adjusted to 5×10^6 cells/mL, and the cells were added to a 24-well cell culture plate (1 mL/well). Finally, splenocytes derived from mice corresponding to immunized groups were stimulated by adding 10 μg of purified Pa or Tr or Pa + Tr or pClod-TF protein in PBS and cultured for 72 h at 37 °C in 5 % CO_2. Media supernatants were then collected, and the levels of IL-17A, IL-4, and IFN-γ in the supernatants were measured using ELISA according to the instructions of the cytokine assay kit (Proteintech, USA).

2.11. Immunizations and challenge experiments

Six-to-eight-week-old female BALB/c mice of specific pathogen-free (SPF) grade, weighing 18–20 g, were purchased from the Experimental Animal Center of Zunyi Medical University. Mice were randomly divided into five groups (eight animals per group): passenger fragment (Pa), translocator fragment (Tr), passenger and translocator fragment mixture (Pa + Tr), and phosphate-buffered saline (PBS). A total of 50 μg of Pa fragment or Tr fragment, or 25 μg Pa and 25 μg Tr mixture (Pa + Tr) was suspended in 20 μl of PBS and then mixed with 1 μg of CTB adjuvant (Absin, China) to immunize mice by the nasal mucosal route on days 0, 14, and 28, respectively. An intraperitoneal booster immunization was performed once on day 42. Two weeks after the last immunization, mice in proestrus stage were subcutaneously injected with 0.5 mg sesame oil-soluble estradiol on days −2, 0 (day of bacterial challenge), and +2 of challenge to increase susceptibility to *N. gonorrhoeae* [35]. Antibiotics are used to prevent overgrowth of commensal flora as described [35]. Mice in each group (n = 8/group) were inoculated vaginally with 5×10^7 CFU of piliated *N. gonorrhoeae* FA1090. Vaginal washes (the vaginal rinse with 50 μl of normal saline was repeated twice) were collected for quantitative culture of *N. gonorrhoeae* for 9 consecutive days post-challenge [19].

2.12. Statistical analysis

All experimental data were plotted and statistically analyzed using the GraphPad Prism software (version 8.0). Student's t-test was used to compare two independent groups, and analysis of variance (ANOVA) was used to compare multiple groups. Antibody titers were analyzed using the Šídák's multiple comparisons test. Kaplan-Meier analysis of time to clearance of infection. Group comparison was done using log-rank (Mantel-Cox) test.

3. Results

3.1. Expression and purification of the recombinant fragments and conservation analysis of the App protein

To obtain the recombinant passenger and translocator fragments of App, we expressed the recombinant protein with the pCold-TF plasmid in *E. coli* BL21 (DE3) and purified the protein using Ni-NTA. High-purity recombinant translocator and passenger fragments were obtained while expressing the pCold-TF tag protein as a control (Fig. 1A). Mice were immunized by intranasal and intraperitoneal routes, and the experimental setup and experimental timeline are shown in Fig. 1B. Western blot analysis showed that the corresponding bands of the native App protein were specifically recognized by anti-passenger and anti-translocator antibodies in whole-cell lysates from *N. gonorrhoeae* FA1090, FA19, and the two clinical strains (Fig. 1C).

3.2. Passenger and translocator fragments of App induced the production of IgG and IgA antibodies

We evaluated the immunogenicity of the purified recombinant App passenger and translocator antigens in mice. Serum and vaginal secretions of mice were collected seven days after each immunization, and IgG and IgA antibody titers were detected by ELISA. As shown in Fig. 2A and B, elevated IgG antibody titers were detected the passenger (Pa), translocator (Tr), and passenger and translocator mixtures (Pa + Tr) groups of mice on day 7, and on day 49 the lgG antibody titers in Pa group reached 1.28×10^5, the lgG antibody titers in Tr group reached 1.02×10^7, and the anti-Pa and anti-Tr lgG antibody titers in Pa + Tr group reached up to 1.02×10^7, 1.02×10^7, respectively. Compared with the third immunization (day 35), IgG titers in vaginal secretions of mice in the Pa group and Tr group were increased 37.92 and 12.28 times after peritoneal enhanced immunization (day 47), and anti-Pa IgG and anti-Tr lgG titers in the Pa + Tr group were also increased 23.77 and 14.25 times, respectively (Fig. 2C and D). Compared with the third intranasal immunization, after the fourth intraperitoneal booster immunization, IgA antibody levels in the serum of mice in the Pa and Tr groups were elevated by 9.14-fold and 1.75-fold, respectively, and the titers of anti-Pa IgA and anti-Tr lgA in the Pa + Tr group were increased by 6.75-fold and 8.10-fold, respectively (Fig. 2E and F). Compared with the third intranasal immunization, after the fourth intraperitoneal booster immunization, the levels of IgA antibodies in vaginal secretions of mice in the Pa and Tr groups increased 1.95-fold and 1.63-fold, respectively, and the titers of anti-Pa IgA and anti-Tr lgA in the Pa + Tr group increased 12.95-fold and 5-fold, respectively (Fig. 2G and H).

Next, we detected specific IgG isotype in the mouse serum. The results showed that higher titers of IgG1, IgG2a, and IgG2b were induced in the Pa, Tr, and Pa + Tr groups, whereas IgG3 antibody titers were relatively lower (Fig. 3A and B).

3.3. Flow cytometric analysis of the binding of polyclonal antibodies with N. gonorrhoeae

The three experimental groups were compared with the blank control, negative control and pCold-TF control (Fig. 4A–C), and it was found that the curves of the Pa, Tr and Pa + Tr groups showed obvious positive peaks, and the mean fluorescence intensity values were 4993, 1635, and 5604, respectively (Fig. 4D and E). The mean fluorescence intensity values of the Pa + Tr group were 1.12 and 3.42 times higher than those of the Pa and Tr groups, respectively.

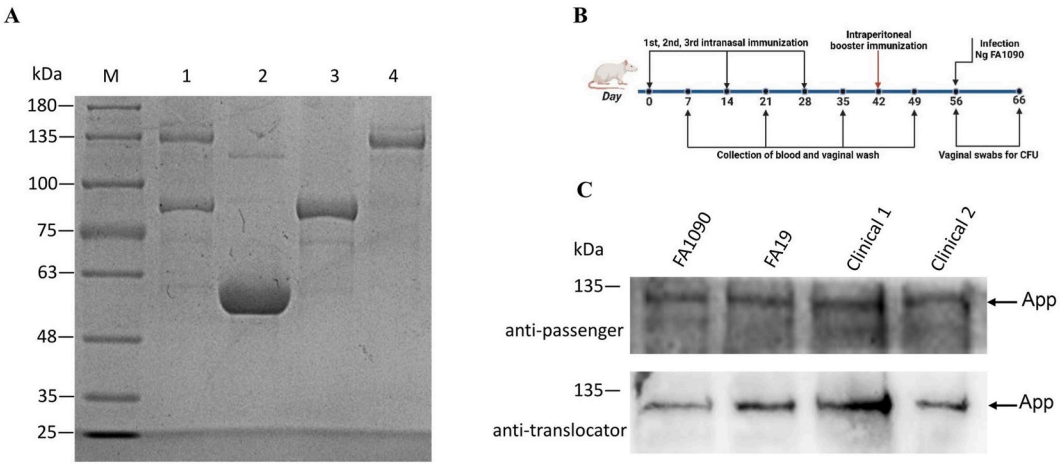

Fig. 1. Expression, purification, and conservation analysis of adhesion and penetration protein. (A) Purified App passenger and translocator fragments and pCold-TF tag protein were separated by 10 % SDS-PAGE. M: protein marker; lane 1: passenger and translocator fragment mixtures (90 kDa and 130 kDa); lane 2: pCold-TF tag protein (59 kDa); lane 3: Passenger fragment protein (90 kDa); lane 4: Translocator fragment protein (130 kDa). (B) Experimental setup and timeline of the mouse immunogenicity experiment. (C) Western blot analysis of native App in *N. gonorrhoeae* using passenger and translocator antiserum. SDS-PAGE gel-loaded (10 %) cell lysates from *N. gonorrhoeae* FA1090, FA19 and two clinical strains (lanes 1 to 4, respectively). The full and non-adjusted images can be found in supplementary material.

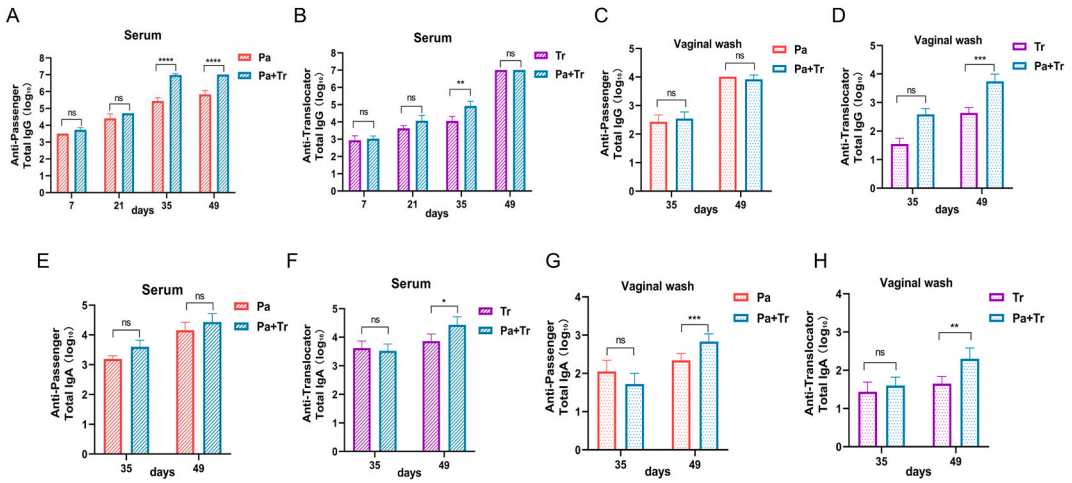

Fig. 2. The titers of IgG and IgA antibodies in serum and vaginal secretions of immunized mice were analyzed by ELISA. Serum anti-passenger IgG (A) and anti-translocator IgG (B) titers were measured in each group on day seven after each immunization. Anti-passenger IgG (C) and anti-translocator IgG (D) titers were measured in vaginal secretions on days 35 and 49 after immunization. Serum anti-passenger IgA (E) and anti-translocator IgA (F) titers were measured on days 35 and 49 after immunization. Anti-passenger IgA (G) and anti-translocator IgA (H) titers were measured in vaginal secretions on days 35 and 49 after immunization. Data are shown as mean ± standard deviation (n ≥ 3). ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, and ns, not significant.

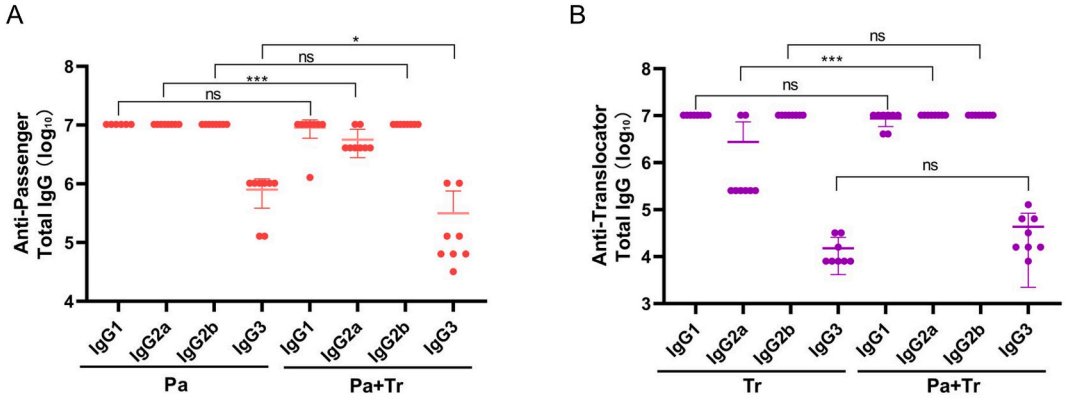

Fig. 3. The antibody isotype in serum were analyzed by ELISA. Anti-passenger (A) and anti-translocator (B) titers of IgG1, IgG2a, IgG2b, and IgG3 were analyzed on day 49 after immunization. Data were analyzed using Šídák's multiple comparisons test (n ≥ 3). ***$P < 0.001$, *$P < 0.05$, and ns, not significant.

3.4. Evaluation of opsonophagocytic and bactericidal activity of antisera

For OPA analysis, the results showed that the antisera of the Pa and Pa + Tr groups killed *N. gonorrhoeae* in a concentration-dependent manner, and their killing titers (>50 % bactericidal activity) were 1:80 (Fig. 5A and C). The antiserum of the Tr group showed some bactericidal activity but did not reach 50 % bactericidal activity (Fig. 5B). For SBA analysis, the results showed that antisera from the Pa and Pa + Tr groups showed concentration-dependent SBA killing, with bactericidal titers of 1:80 and 1:20, respectively (Fig. 5D and F). The antiserum of the Tr group showed some bactericidal activity but did not reach 50 % bactericidal activity (Fig. 5E).

3.5. Antiserum can inhibit the adhesion of gonococcus to cervical epithelial cells

Our previous studies have shown that the App is involved in mediating gonococcal adhesion to cervical epithelial cells [27]. Therefore, we evaluated whether antisera from the different immunization groups could inhibit App-mediated gonococcal adhesion. Antisera from the Pa, Tr, and Pa + Tr groups were diluted to ratios of 1/20, 1/40, and 1/80 and incubated with *N. gonorrhoeae* FA1090 and ME-180 cells. Adherent colonies were counted and compared with the number of colonies observed with the untreated group. The results showed that the antisera of the Pa and Pa + Tr groups could inhibit the adhesion of *N. gonorrhoeae* FA1090 strain to ME-180

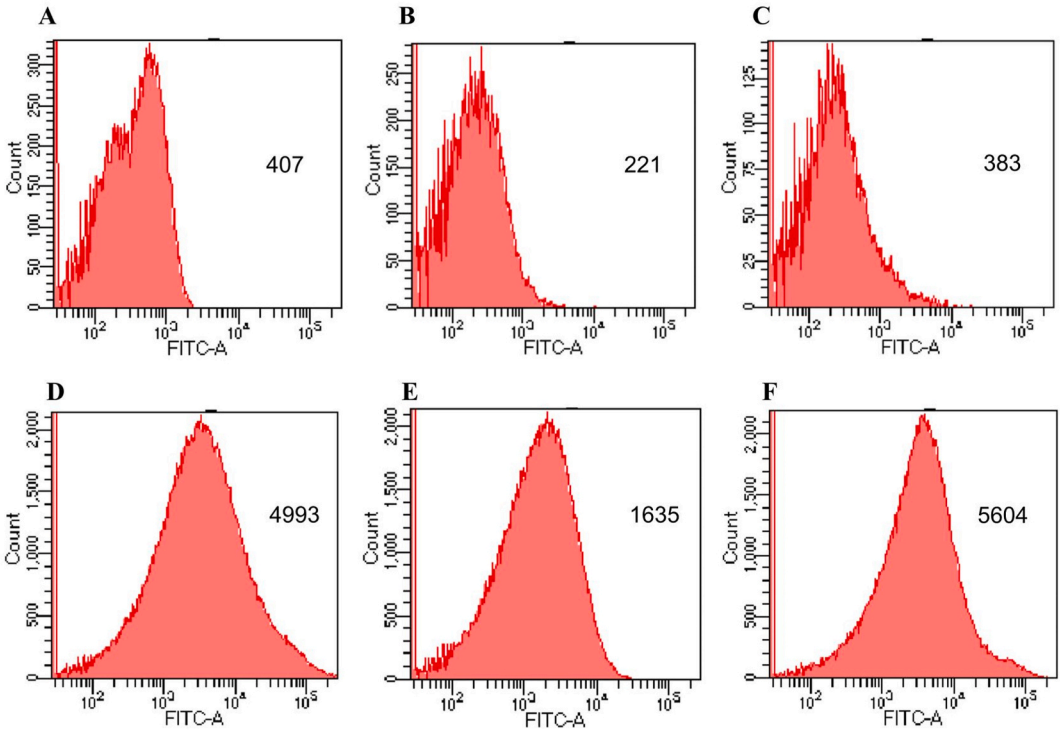

Fig. 4. Flow cytometry was performed to analyze the binding ability of antisera to *N. gonorrhoeae*. Untreated *N. gonorrhoeae* FA1090 (A) was used as a blank control. *N. gonorrhoeae* was co-incubated with FITC-labeled goat anti-mouse IgG (B) as a negative control. *N. gonorrhoeae* FA1090 was co-incubated with antisera from pCold-TF (C), Pa (D), Tr (E), and Pa + Tr (F) immunized groups and stained with FITC-labeled goat anti-mouse IgG antibody. Figures alongside the histogram represent the mean fluorescence intensity values, all experiments were conducted in triplicate.

cells in a concentration-dependent manner, and the inhibitory efficiency of the antisera of the Pa and Pa + Tr groups was similar and superior to that of the antiserum in the Tr group (Fig. 6A–C).

3.6. Immunization with Pa, Tr, and Pa + Tr induced Th1 and Th17 cell immune responses

Th1, Th2, and Th17 cells play crucial roles in the immune responses against *N. gonorrhoeae* [36]. To explore the cellular immune responses induced by the different immune groups, we used Pa, Tr, and Pa + Tr mixtures to stimulate the spleen cells of corresponding immunized mice in vitro for analysis of cytokine secretion. Compared with PBS and pCold-TF group, Pa, Tr, and Pa + Tr could mediate significantly increased IL-17A (Th17) and IFN-γ (Th1) production in mouse spleen cells (Fig. 7A and B). Tr-induced IL-17A and IFN-γ levels were higher than those of the Pa group ($P < 0.05$), but there was no significant difference in comparison to the levels of the Pa + Tr group ($P > 0.05$). Pa, Tr, and Pa + Tr did not significantly stimulate IL-4 secretion from splenocytes compared to pCold-TF controls (Fig. 7C).

3.7. Passenger and translocator fragment-immunized mice can accelerate the clearance of N. gonorrhoeae infection

To evaluate the protective effect of each immunization group against gonococcal infection, we challenged immunized (Pa, Tr, Pa + Tr) and control mice (PBS + CTB) with gonococcal FA1090 strain after the fourth intraperitoneal immunization and performed quantitative gonococcal culture of vaginal washings within 9 days. The median clearance time for *N. gonorrhoeae* infection in PBS mice was 9 days, while the median clearance time for Pa, Tr, and Pa + Tr immunized mice was 7.5, 7.5, and 7 days, respectively (Fig. 8). These results suggest that Pa, Tr and Pa + Tr protein can accelerate the clearance of *N. gonorrhoeae* in the mouse genital tract.

4. Discussion

Considering the threat posed by drug-resistant *N. gonorrhoeae*, there is an urgent need to screen and identify potential vaccine candidates to aid the development of gonococcal vaccines [37]. Here, we describe the immune response induced by intranasal immunization with App passenger and translocator fragments and evaluate the role of antibodies in bactericidal and opsonophagocytic activities and adhesion inhibition. These antibodies demonstrated some bactericidal and opsonophagocytic activity and could significantly reduce the adhesion of *N. gonorrhoeae* to human cervical epithelial cells, suggesting that App is a promising vaccine antigen for *N. gonorrhoeae*.

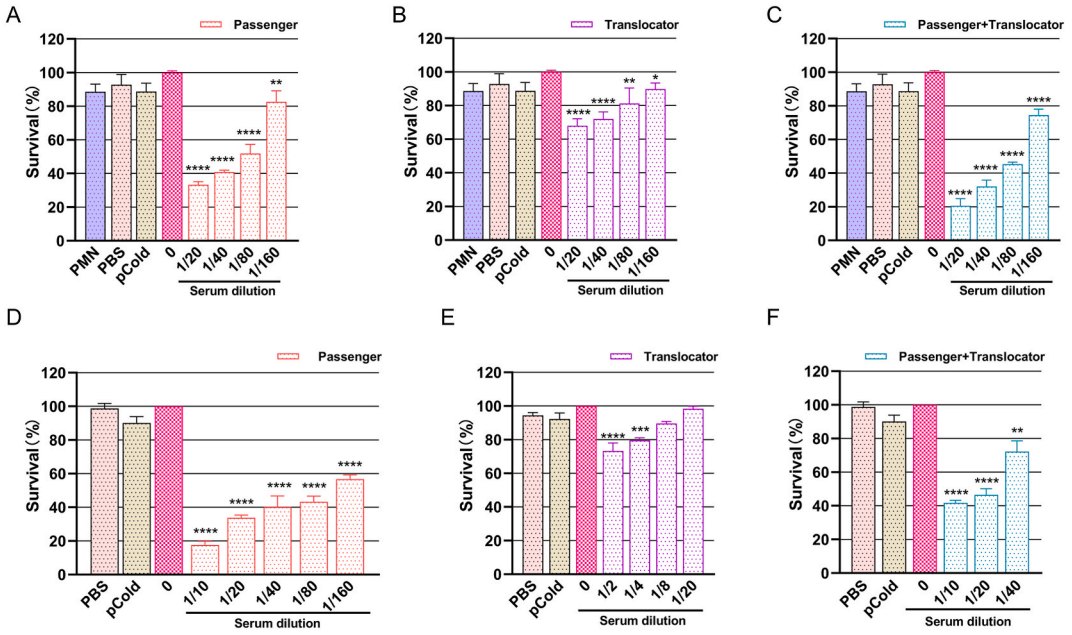

Fig. 5. Analysis of the opsonophagocytic and bactericidal activity of antiserum against *N. gonorrhoeae*. Opsonophagocytic activity was assayed by diluting heat-inactivated antisera from Pa (A), Tr (B) and Pa + Tr (C) immunized groups in different ratios. The survival of bacteria was analyzed. The *N. gonorrhoeae* FA1090 and human PMNs or PBS control group antisera or pCold-TF group antisera (pCold-TF) were used as control. Data are shown as mean ± standard deviation (n ≥ 3). Complement-mediated bactericidal activity was determined by diluting heat-inactivated antisera from Pa (D), Tr (E), and Pa + Tr (F) immunization groups in different ratios. The relative survival rate was calculated according to the survival rate of each sample relative to that observed with the untreated wild-type strain (0) (the survival rate of the untreated wild-type strain is set to 100 %). Data are shown as mean ± standard deviation (n ≥ 3). ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, relative to the untreated wild-type strain.

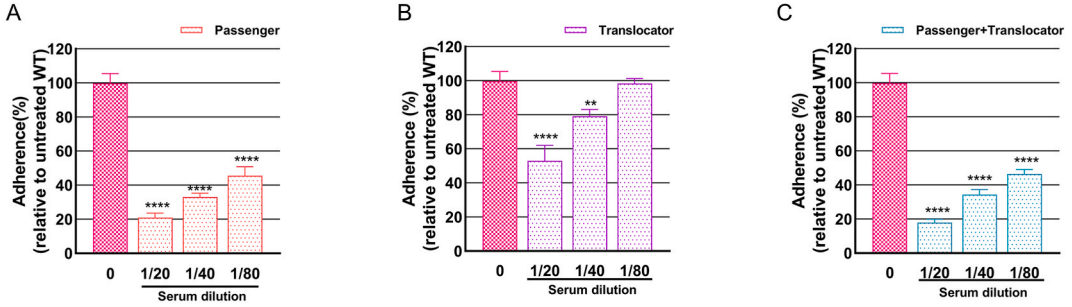

Fig. 6. Antibody-mediated inhibition of *N. gonorrhoeae* adhesion to human cervical epithelial cells (ME-180). *N. gonorrhoeae* FA1090 was pre-incubated with heat-inactivated Pa (A), Tr (B), and Pa + Tr (C) antisera followed by adhesion assays. Relative adherence was calculated from the adherence rate of each sample relative to that of the untreated wild-type strain (0) (adherence rate of the untreated wild-type strain was set to 100 %). Data are shown as mean relative percentages from three replicates. ****$P < 0.0001$, **$P < 0.01$, relative to the untreated wild-type strain.

Autotransporters are important virulence factors and potential vaccine targets for Gram-negative bacteria [21]. We have previously shown that the App protein plays a role in the adhesion, invasion, and reproductive tract colonization of *N. gonorrhoeae* and is a potential protein vaccine target [27]. Therefore, in this study, we expressed and purified App passenger and translocator fragments and then immunized mice via three nasal mucosal routes plus one intraperitoneal. Combined modes of immunization increase systemic and local antibody titers and enhance immunoprotection compared to other immunization methods [38]. As expected, the antibody titer results showed further increases in IgG and IgA levels in serum and vaginal secretions after intraperitoneal booster immunization. In some of these groups, it occurred that the corresponding antibody titers of the Pa + Tr group were higher than the Pa and Tr groups at the same time point, and we speculate that there is certain synergistic effect between Pa and Tr antigens in terms of immune activation thereby increasing the antibody titers. This is consistent with previous findings that subunit vaccines, constructed by mixing or fusing multiple proteins and combining them with adjuvants, resulted in increased antibody titers as well as functional antibody activity observed in mice [39,40]. Furthermore, The IgG antibody isotype results showed that the IgG1/IgG2a ratios were all greater than 1, suggesting a Th2 response bias [41]. This may be related to the use of CTB adjuvants, which can trigger characteristic Th2 immune

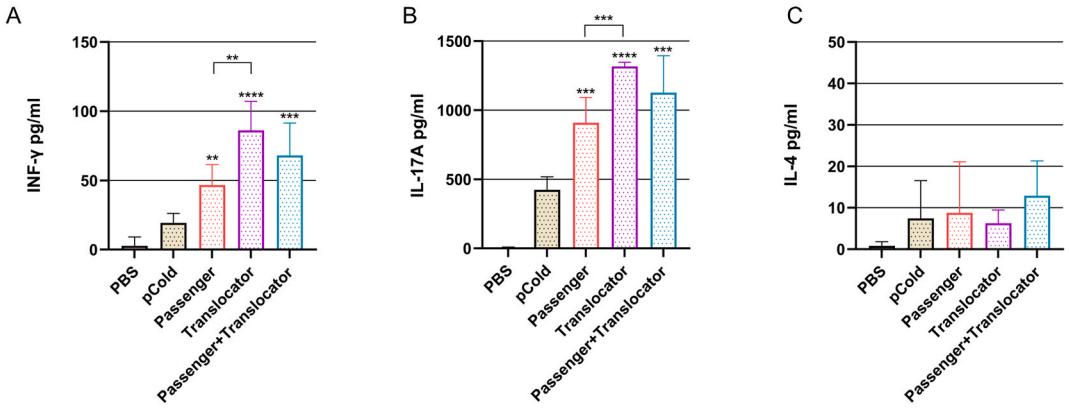

Fig. 7. Analysis of cytokines produced by specific antigen-stimulated mouse splenocytes. Seven days after the last immunization, 10 μg of recombinant pCold-TF, Pa, Tr, and Pa + Tr proteins were cultured with splenocytes from mice in the corresponding immunized groups at 37 °C for 72 h. Levels of IFN-γ (A), IL-17A (B), and IL-4 (C) in culture supernatants were measured by ELISA. Data are shown as mean ± standard deviation (n = 3). $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, relative to the pCold-TF group.

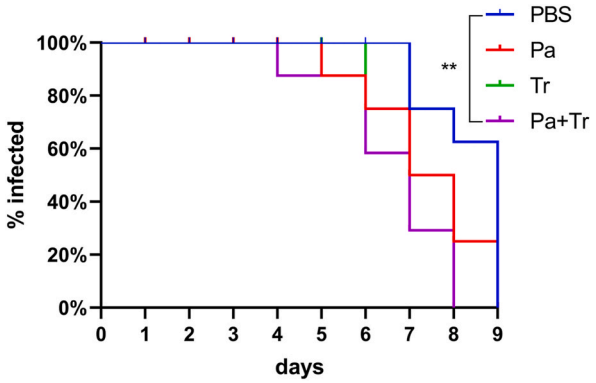

Fig. 8. Immunization with Passenger and Tr fragments decrease *N. gonorrhoeae* FA1090 infection in a mouse reproductive tract model. Mice (n = 8/group) were challenged vaginally with 5×10^7 CFU of piliated *N. gonorrhoeae* FA1090 two weeks after the last immunization and vaginal washings were collected for 9 consecutive days for quantitative gonococcal culture. The percentage of culture-positive mice over time was calculated. $^{**}P < 0.01$, relative to the PBS group.

responses [42]. Western blotting showed that both anti-passenger and anti-translocator antisera could recognize native App protein expressed in *N. gonorrhoeae* FA1090 strain, FA19 strain, and two randomly selected clinical isolates (Fig. 3C), indicating that these antisera had good immunoreactivity and that App was conserved among different *N. gonorrhoeae* strains (Fig. 1B). Flow cytometry analysis also showed that antisera from Pa, Tr and Pa + Tr groups bound well to *N. gonorrhoeae* FA1090 strain compared to untreated and pCold-TF groups. Humoral immunity plays an important role in resistance to *N. gonorrhoeae* infection, and vaccine-induced production of antibodies with neutralizing capacities is important for protection against gonorrhea [43]. For instance, antibodies against the *N. gonorrhoeae* candidate targeting MsrA/B and NHBA mediate the killing of *N. gonorrhoeae* via serum bactericidal and opsonophagocytic activity [12,14]. In our experiments, antisera from the Pa and Pa + Tr immunized groups showed bactericidal and opsonophagocytic effects. The bactericidal effect of antiserum in Pa + Tr immunized group was lower than that in Pa group, which may be due to the interference effect between anti-translocator antibody and anti-passenger antibody in Pa + Tr immunized group. However, the bactericidal and opsonophagocytic activity of the antisera in the Tr group were lower than those in the Pa and Pa + Tr groups. We speculate that this may be due to the fact that the translocator domain is mainly localized on the cell membrane and therefore fails to be fully exposed, resulting in lower binding efficiency of antibodies in Tr than in pa and Pa + Tr groups [44]. Indeed, the results of flow cytometric analysis also showed that the binding of anti-translocator antibodies to *N. gonorrhoeae* was less potent than that observed with Pa and Pa + Tr immunized group antisera. Adhesion is the first step in establishing *N. gonorrhoeae* infection and inhibiting adhesion to epithelial cells is an effective strategy for controlling gonorrhea [45]. For example, the gonococcal vaccine candidate target, MetQ, is involved in the adhesion of *N. gonorrhoeae* to cervical epithelial cells, and anti-MetQ can block the adhesion of *N. gonorrhoeae* to cervical epithelial cells [34]. Our previous study showed that App is involved in the adhesion and invasion of *N. gonorrhoeae* in human cervical epithelial cells [27]. The results of this study showed that antisera from the Pa, Tr, and Pa + Tr immunized groups inhibited the adhesion of *N. gonorrhoeae* to ME-180 cells in a concentration-dependent manner.

The Th1, Th2, and Th17 cell immune responses play important roles in *N. gonorrhoeae* infection [36]. Gonococcal-induced Th17 responses are mainly involved in the recruitment of neutrophils into the genital tract as well as other innate defense mechanisms [46]. The blockade of IL-17A or loss of the IL-17A receptor (IL-17RA) can lead to the inhibition of neutrophil influx and prolonged bacterial infection [36]. IL-17RA(KO) mice are more susceptible to *N. gonorrhoeae* infection, suggesting that Th17 cellular immunity plays an important role in resistance to *N. gonorrhoeae* infection [47]. Liu et al. found that treatment of *N. gonorrhoeae*-infected mice with anti-IL-10 antibodies resulted in faster clearance of *N. gonorrhoeae* infection and demonstrated protection against secondary infection, which was associated with enhanced Th1 (IFN-γ) and Th2 (IL-4) responses [48]. Our results showed that the levels of cytokines IL-17A and IFN-γ were significantly increased after in vitro stimulation of splenocytes derived from respective immunized mice with Pa, Tr, and Pa + Tr, suggesting that Th17 and Th1 immune responses were activated [49]. Compared to the pCold-TF control group, neither the Pa and Tr groups nor the Pa + Tr group showed significantly increased IL-4 secretion after immunization, suggesting that Th2 cellular immune responses may not be significantly activated, possibly because activation of Th17 responses is partially dependent on TGF-β expression, which suppresses Th1 and Th2 responses [7]. Although there was a Th2 response bias in our lgG isotype results, which may also be related to the fact that Th2 responses can be induced using CTB adjuvants. Currently, estradiol-treated mouse models are the only animal models to investigate the efficacy of gonorrhea vaccines in females [50]. In this study, we used estradiol-treated mouse models and observed that immunization Pa, Tr, and Pa + Tr accelerated the clearance of gonococcal infection to some extent compared with the PBS group. However, the accelerated clearance in mice in our study does not seem impressive. Most current studies have shown that Th1-driven immune response strategies are critical in the development of gonorrhea vaccines [51–53]. From our cytokine results, the Th1 immune response induced by mice in the three experimental groups did not seem to be satisfactory, which may have affected our in vivo protective efficacy.

5. Conclusions

In summary, we described several key features of the App protein passenger and translocator fragments, including their good immunogenicity that resulted in the induction of high titers of specific IgG and IgA antibodies, as well as their effective elicitation of Th1 and Th17 cell immune responses. These antibodies play a role in anti-gonococcal infection via serum bactericidal activity, opsonophagocytic activity, and inhibition of gonococcal adhesion to cervical epithelial cells. Immune App Pa, Tr, and Pa + Tr fragments can accelerate the clearance of *N. gonorrhoeae* in the mouse reproductive tract. These characteristics support the use of App protein as a candidate target for gonococcal vaccines.

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CRediT authorship contribution statement

Lingyin Xia: Writing – original draft, Methodology. **Qin Lu:** Writing – original draft, Methodology. **Xiaosu Wang:** Writing – original draft, Methodology. **Chengyi Jia:** Methodology. **Yujie Zhao:** Methodology. **Guangli Wang:** Formal analysis. **Jianru Yang:** Formal analysis. **Ningqing Zhang:** Data curation. **Xun Min:** Writing – review & editing, Conceptualization. **Jian Huang:** Writing – review & editing, Project administration, Funding acquisition, Data curation, Conceptualization. **Meirong Huang:** Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Meirong Huang reports financial support was provided by the National Natural Science Foundation of China. Jian Huang reports financial support was provided by the Science and Technology Project of Guizhou. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25733.

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