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Design and immune characterization of a novel *Neisseria gonorrhoeae* DNA vaccine using bacterial ghosts as vector and adjuvant

Hongmei Jiao [a,b,c,*,1], Hui Yang [a,d,1], Dan Zhao [a], Jin Chen [a], Qianyun Zhang [a], Jiankun Liang [a], Yinyan Yin [a], Guimei Kong [a], Guocai Li [a,b,c,*]

[a] School of Medicine, Institute of Translational Medicine, Yangzhou University, Jiangsu Key Laboratory of Experimental & Translational Non-coding RNA Research, Yangzhou 225001, China
[b] Jiangsu Key Laboratory of Zoonosis/Joint International Research Laboratory of Agriculture and Agri-Product Safety, Yangzhou 225009, China
[c] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, China
[d] The Third People's Hospital of Changzhou, Changzhou 213001, China

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ABSTRACT

Gonorrhea, an important sexually transmitted disease, is becoming a growing public health problem around the globe. Vaccination is considered the best long-term approach for control of infection. In this study, we designed a novel *Neisseria gonorrhoeae* (*N. gonorrhoeae*) DNA vaccine delivered by bacterial ghosts and characterized its immune responses *in vitro* and *in vivo*. Our results demonstrate that bacterial ghosts greatly promoted BMDCs maturation and activation. Bacterial ghosts loaded with *N. gonorrhoeae* DNA vaccine were efficiently taken up by mouse macrophage RAW264.7 cells. Furthermore, oral immunization with the ghost vaccine candidate elicited greater $CD4^+$ and $CD8^+$ T cell responses and induced higher IgG responses than *N. gonorrhoeae* DNA vaccine alone. In addition, mice immunized with the vaccine candidate responded with a significant rise in bactericidal antibody titer. These results suggest that bacterial ghosts may function as a vaccine adjuvant by promoting BMDCs maturation, which in turn enhances the immune responses to the vaccine antigens. This study also highlights the potential of using bacterial ghosts as antigen delivery system in the development of an efficacious gonorrhea vaccine.

1. Introduction

Gonorrhea, caused by *Neisseria gonorrhoeae* (*N. gonorrhoeae*), is one of important sexually transmitted infections (STI) throughout the world with significant immediate and long-term morbidity and mortality. The World Health Organization (WHO) estimates that there are over 106 million new *N. gonorrhoeae* infections annually [1,2]. *N. gonorrhoeae* infections are associated with the development of pelvic inflammatory disease (PID), ectopic pregnancy and infertility in women, congenital blindness in offspring and epidimitis in men. *N. gonorrhoeae* infections significantly impact human health and can enhance the risk of HIV transmission and acquisition. Gonorrhea is becoming the second most common STI of bacterial origin [3–5].

Currently, inadequate control measures and rapid emergence of antibiotic resistance are becoming the main reasons for public concern. There is no effective vaccine or vaccine candidates for protection against *N. gonorrhoeae* infections in advanced stages of clinical development. The increasing threat of difficult-to-treat *N. gonorrhoeae* promote interest in developing effective vaccines for control of gonorrhea [1,6,7]. With respect to the choice of vaccine candidates, early human trials of crude killed whole cell vaccine showed no evidence for protection, even the vaccine contained highly inflammatory LOS. The whole cell vaccine was not developed further. A subsequent trial that showed no protection using isolated and purified pili vaccine. The probable reason for failure was antigenic variation of expressed pili lead to escaping specific immune responses in the naturally acquired infections [8].

Recent studies have shown that several potentially protective surface molecules have been identified that elicit immune responses in animal model. These antigens include transferrin binding proteins (TbpA/TbpB), lipooligosaccharide (LOS) and porin (PorB). Among them, the major outer membrane protein PorB has been identified that elicit bactericidal antibodies and/or play key roles in pathogenesis that could be targeted by a vaccine-induced response [1,9,10]. Nevertheless, newer vaccines such as DNA and subunit vaccines are poorly immunogenic in nature as compared

* Corresponding authors at: School of Medicine, Yangzhou University, 11 Huaihai Road, Yangzhou, Jiangsu 225001, China.
E-mail addresses: hmjiao@yzu.edu.cn (H. Jiao), gcli@yzu.edu.cn (G. Li).
[1] These authors contributed equally to this work.

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to traditional vaccines, and therefore necessitate an appropriate adjuvant or delivery system in the vaccine formulation [11,12].

Bacterial ghosts are empty bacterial cell envelopes, while still retaining all of the antigenic determinants of the envelopes in the same quality as in their living counterparts, and represent a potential platform which not only acts as potent candidate vaccines but also provide a tool for efficient adjuvant and vaccine delivery systems for proteins or DNA vaccines [13–15].

In the current study, we have developed a novel *N. gonorrhoeae* DNA vaccine delivered by *Salmonella enteritidis* (*S. enteritidis*) ghosts and assessed its immune responses *in vitro* and *in vivo*. It is the first time to demonstrate that the bacterial ghosts elicited an increased immune responses to *N. gonorrhoeae* DNA vaccine as delivery vector and novel adjuvant.

2. Materials and methods

2.1. Bacterial strains, media, ***N. gonorrhoeae*** *DNA vaccine and animal*

N. gonorrhoeae strain WHO-A was grown at 37 °C in fastidious broth (FB) agar (containing 35 g/L columbia broth base, 5 g/L glucose, 5 g/L yeast extract, 2 g/L neopeptone, 0.05% wt/vol hematin, 10% vol/vol Tween 80, 0.1% wt/vol pyridoxal and 1% wt/vol NAD) as described previously [16–18]. To produce *N. gonorrhoeae* DNA vaccine, full length *porB* gene was inserted into an eukaryotic expression vector (pVAX1) and subsequently transformed into *Escherichia coli* (*E. coli*). *E. coli* carrying *N. gonorrhoeae* DNA vaccine (pVAX1-porB) were identified and stored at −70 °C in LB growth medium. To produce recombinant PorB, full length *porB* gene was inserted into a prokaryotic expression vector (pET-30a) and subsequently transformed into *E. coli* BL21(DE3). Recombinant protein was induced with IPTG and purified using Ni-NTA Purification System under native conditions. BALB/c mice were obtained from the Animal Research Center of Yangzhou University (Yangzhou, China). All experimental protocols on mice were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals in China. All animal experiments were approved by Animal Ethics Committee of Yangzhou University.

2.2. Bacterial ghosts production

S. enteritidis ghosts were produced according to the method described previously [19]. Briefly, the lysis plasmid pBBR1MCS-E containing lysis gene E and thermo-sensitive λpL/pR-cI857 cassette was transformed in *S. enteritidis* by electroporation. For the preparation of *S. enteritidis* ghosts, *S. enteritidis* carrying the lysis plasmid was inoculated into LB broth containing 50 μg/ml of Kanamycin and cultured at 28 °C until the OD600 reached 0.3–0.4. E gene expression was induced by raising the culture temperature to 42 °C and the bacteriolysis was monitored by measuring the OD600. After completion of lysis (OD600 between 0.2 and 0.3), the ghosts were collected by centrifugation, washed and lyophilized for further use.

2.3. Preparation and delivery of DNA vaccine-loaded ***S. enteritidis*** *ghosts to murine macrophages*

N. gonorrhoeae DNA vaccine (pVAX1-porB) was loaded in *S. enteritidis* ghosts by diffusion through the lysis tunnel as previously described with a little modification [20,21]. Briefly, the plasmid pVAX1-porB was extracted by maxi preparation and the lyophilized *S. enteritidis* ghosts were suspended in various concentrations of DNA solution (containing 25 mM $CaCl_2$) at 37 °C with agitation for 2 h. Subsequently, the bacterial ghosts were centrifuged, washed and suspended with PBS (hereafter termed SE ghosts (pVAX1-porB)). The loading efficiency was determined by measurement of DNA concentration in supernatant at 260/280 nm and agarose gel electrophoresis. The loading efficiency (LE%) was calculated with the formula below:

$$LE\% = \frac{Total\ \ DNA - Free\ \ DNA\ \ in\ \ supernatant}{Total\ \ \mathrm{DNA}} \times 100\%$$

1×10^6 RAW264.7 cells were seeded into 6-well tissue culture plates. The SE ghosts (pVAX1-porB) were added to cells at a ratio of 1:200 (cells: bacterial ghosts). After 4 h, the cells were washed to remove excess SE ghosts (pVAX1-porB) and further incubated with culture medium for 24–48 h. The unloaded SE ghosts were used as the control.

2.4. RT-PCR

Transcription of the *porB* gene in transfected macrophages was determined using RT-PCR. Total RNA from macrophages was extracted using the Rneasy Mini Kit (Qiagen, Valencia, CA) and cDNA was generated using PrimeScript™ RT reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. PCR amplification was carried out using the following primers: P1: 5'-CGGCATATGAAAAAATCCCTGATTGCCCTG-3', P2: 5'-CCCCTCGAG GAATTTGTGGCGCAGAACGAC-3'. PCR products were then examined by agarose gel electrophoresis.

2.5. Western blot

Expression of the *porB* gene in transfected macrophages was determined using western blot. The proteins from cell pellets were collected and run on a SDS-PAGE. Separated proteins were then transferred to a PVDF membrane. Mouse antiserum to whole cell *N. gonorrhoeae* and HRP-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, USA) were used as primary and secondary antibodies, respectively.

2.6. Generation and treatment of bone marrow-derived DCs (BMDCs)

BMDCs were generated from the bone marrow cells of mice as our previously described with minor modifications [22]. Briefly, bone marrow cells were flushed from the femurs and suspended in cDMEM containing 20 ng/ml rmGM-CSF and 10 ng/ml rmIL-4 (PeproTech, Rocky Hill, NJ, USA), and then cultured at 37 °C with 5% CO_2. On day 4, the fresh cDMEM containing rmGM-CSF was added. On day 7, the BMDCs were harvested. 5×10^5 cells per well were subsequently seeded into 24-well tissue culture plates and rested overnight.

2.7. Expression of key surface molecules on BMDCs

The cultured BMDCs were treated with SE ghosts (pVAX1-porB) or SE ghosts and stained with Fluorochrome-conjugated antibodies (CD86-FITC, CD80-PE, CD40-PE, MHC-II-FITC or the respective isotypes) for 30 min at 4 °C. LPS and medium were used as controls. The cells were washed twice with PBS containing 2% FBS and analyzed with flow cytometry and FlowJo software (BD Biosciences, USA).

2.8. Immunization of mice

Four groups of 6–8 week-old mice were inoculated orally with one of the following: 1 mg SE ghosts (pVAX1-porB) containing 100 μg pVAX1-porB, 100 μg pVAX1-porB, 1 mg SE ghosts, or 100 μl PBS as a control. All groups of immunized mice were boosted with the same vaccine components twice at 2-week intervals.

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