Safety and immunogenicity of a new glycoengineered vaccine against Acinetobacter baumannii in mice

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Summary

Acinetobacter baumannii poses a serious threat to human health, mainly because of its widespread distribution and severe drug resistance. However, no licensed vaccines exist for this pathogen. In this study, we created a conjugate vaccine against A. baumannii by introducing an O-linked glycosylation system into the host strain. After demonstrating the ability of the vaccine to elicit Th1 and Th2 immune responses and observing its good safety in mouse models, the strong in vitro bactericidal activity and prophylactic effects of the conjugate vaccine against infection were further demonstrated by evaluating post-infection tissue bacterial loads, observing suppressed serum pro-inflammatory cytokine levels. Additionally, the broad protection from the vaccine was further proved via lethal challenge with A. baumannii. Overall, these results indicated that the conjugate vaccine could elicit an efficient immune response and provide good protection against A. baumannii infection in murine sepsis models. Thus, the conjugate vaccine can be considered as a promising candidate vaccine for preventing A. baumannii infection.

Introduction

Acinetobacter baumannii is a gram-negative opportunistic bacterial pathogen that has become a serious public health problem in hospitals globally. It can cause a variety of infections as a nosocomial pathogen including pneumonia, sepsis, meningitis, infection after trauma and urinary tract infection (Ayoub Moubareck and Hamoudi Halat, 2020). With the extensive use of antibiotics, multidrug-resistant A. baumannii has emerged (Zilberberg et al., 2016). In 2017, the World Health Organization published a global priority list of antibiotic-resistant bacteria and mentioned A. baumannii as a critically dangerous pathogen (World Health Organization, 2017). Improper antibiotic treatment can increase the synthesis of capsular polysaccharide (CPS) and further enhance the virulence of A. baumannii (Geisinger and Isberg, 2015), increasing the difficulty of clinical treatment. To manage the urgent and serious threat of drug resistance in A. baumannii, the implementation of prophylactic vaccination may be a cost-effective approach for preventing A. baumannii infection.

Many A. baumannii vaccines remain in the preclinical research stage. Some multicomponent antigens candidates such as inactivated whole cells (McConnell and Pachon, 2010, Zeng et al., 2019), outer membrane complexes (OMCs) (McConnell et al., 2011a), outer membrane vesicles (OMVs) (McConnell et al., 2011a; Huang et al., 2014) and live attenuated cells (Cabral et al., 2017) have been explored, and the elicited antibodies could provide protection against A. baumannii infection in murine models of severe sepsis. Although the aforementioned vaccines could induce strong immune responses and they are relatively easy to prepare, safety problems associated with the complexity of the components and the presence of lipopolysaccharide (LPS) must be resolved. To solve these problems, McConnell’s team prepared LPS-free OMCs and OMVs by deleting the lpxD gene in A. baumannii. The survival rates of mice immunized with LPS-free OMCs and OMVs were 62.5% and 75%, respectively (Pulido et al., 2018, 2020). Additionally, some single-antigen candidates including outer membrane proteins (Luo et al., 2012; Huang et al., 2016; Singh et al., 2016), biofilm-related protein (Fatibian et al., 2011; Bentancor et al., 2012a) and surface polysaccharides (Russo et al., 2013; Yang et al., 2017) can also elicit immune responses and protect against A. baumannii.
Infection. Although these subunit vaccines have overcome the safety problems, they require further exploration. For example, protein antigens are usually obstructed by surface polysaccharides, thereby hindering their recognition by antibodies.

Bacterial surface polysaccharides often possess the desirable characteristics of a candidate antigen, such as surface exposure, immunogenicity, and a role in the pathogenesis of diseases (Safadi et al., 2015). However, polysaccharides alone are T-cell-independent antigens, and they failed to elicit adequate immune responses and provide long-term protection, especially in high-risk groups such as elderly patients and children younger than 2 years. When a polysaccharide was combined with a carrier protein to create a conjugate vaccine, the antigen could be transformed into a T-cell-dependent antigen, and both B and T cells were involved in the immune response (Avci et al., 2011; Costantino et al., 2011). Therefore, conjugate vaccines are considered the most successful vaccine types. With the discovery of the bacterial protein glycosylation system, the preparation of conjugate vaccines using a biosynthetic method (terminated protein glycan coupling technology) has displayed obvious advantages compared with the traditional chemical method with a multistep process, leading to intense research (Frasch, 2009; Kay et al., 2019). To date, several glycosyltransferases have been successfully used for vaccine preparation, such as PglB from Campylobacter jejuni (N-linked) (Wacker et al., 2002; Reglinski et al., 2018), PglI from Neisseria (O-linked) (Gebhart et al., 2012; Pan et al., 2016) and PglS from Acinetobacter spp. (O-linked) (Harding et al., 2015; Harding et al., 2019), among which PglI has proven to have broader substrate specificity (Faridmoayer et al., 2008; Sun et al., 2018). Although A. baumannii has its own O-glycosylation system, the glycosyltransferase only could recognize a single pentasaccharide, which limits its application (Lees-Miller et al., 2013). There is no report on the application of the biosynthetic method to A. baumannii vaccine development. In fact, CPS of A. baumannii is synthesized using Wzx/Wzy-dependent processes. This pathway is compatible with current technologies, and the terminal sugar GalNAc can be recognized by PglI.

In this study, we prepared a conjugate vaccine against A. baumannii by introducing an O-linked glycosylation system (PglI) into the A. baumannii ATCC 17978 strain. CPS of A. baumannii could be combined directly with the recombinant cholera toxin B subunit (CTB4573C) under catalysis by PglI in vivo. The use of the glycosylation system in the host strain prevented the need to clone the polysaccharide gene cluster for vector construction and ensured the natural configuration of the polysaccharide antigen. After demonstrating the safety of the conjugate vaccine in a mouse model, animal experiments revealed that the vaccine could elicit robust antibody responses that reduced post-infection bacterial loads in tissues and protected against lethal challenge by A. baumannii in murine sepsis models. Thus, the conjugate vaccine could be effective vaccine candidate against A. baumannii infection.

Results

Glycoengineering a bioconjugate vaccine against A. baumannii

Previous studies revealed that A. baumannii ATCC 17978 has its own O-glycosylation pathway. However, PgL of A. baumannii could not transform CPS onto a protein (Lees-Miller et al., 2013). Thus, we introduced a broader O-linked glycosylation system (PglI from Neisseria) into A. baumannii to prepare the conjugate vaccine. First, the recombinant plasmids pMM-CTB4573C and pET-pglI-CTB4573C (Pan et al., 2016) were transformed into the host strain A. baumannii ATCC 17978. Coomassie blue staining and Western blot analysis revealed that the typical extended ladder appeared when PgL and CTB4573C were co-expressed, whereas the molecular weight of CTB4573C without glycosylation was approximately 15 kDa (Fig. 1A). The results indicated that the heterologous O-glycosylation system was efficiently expressed in A. baumannii ATCC 17978.

Then, the glycoprotein CTB4573C-CPS (C-CPS) was purified via affinity chromatography and size-exclusion chromatography. The purity reached approximately 95% (Fig. 1B). Staining of SDS gels with Coomassie blue and Western blotting using antibodies against both the 6×His-tag and CTB revealed the same typical extended ladder form (Fig. 1B). To further confirm the polysaccharide specificity of C-CPS, periodic acid–Schiff staining (Pierce glycoprotein staining kit; Thermo Fisher Scientific) and immunoblotting using Lycopersicon esculentum lectin (LEL) and wheat germ agglutinin (WGA) revealed that the bands also presented a typical ladder (Fig. 1C and Fig. S1), and each band in the ladder corresponded to a different number of repeated units (pentasaccharides). Cholera toxin B subunit (CTB) is an immunoadjuvant mainly in its pentameric form in nature (Sun et al., 2010). To confirm whether the glycosylation of CTB altered its polymer formation (Baldauf et al., 2015), native PAGE was performed, and the results illustrated that the molecular weight of C-CPS exceeded 400 kDa (Fig. 1D), compared with 50–100 kDa for the glycosylated C-CPS monomer (Fig. 1B). Furthermore, the results of competitive binding experiments illustrated that glycosylation did not significantly affect the ability of CTB to bind with its known target monosialotetrahexosylganglioside (Fig. S2). Meanwhile, the better stability of...
C-CPS was further confirmed at different times and temperatures (Figs. S3 and S4). The yield of C-CPS was approximately 450 µg per litre of culture medium at the experimental stage.

**Safety estimation of the conjugate vaccine**

Before animal immune experiments, the safety of C-CPS was evaluated. The mice were immunized...
subcutaneously with 20µg of polysaccharide (5-fold higher than the immune dose in the vaccine group) and continuously monitored for 10 days. After 1 h, rectal temperature and weight were measured at different time points. Blood was collected from the tail vein to detect pro-inflammatory cytokines. The results revealed no abnormalities in weight and temperature in the C-CPS group compared with the findings in the control group. Cytokine profiles (IL-6, TNF-α, IFN-γ and IL-1β) at different time points after immunization. There was no difference between the C-CPS and phosphate-buffered saline (PBS) groups, which indicates that C-CPS had no systemic toxicity.

Detection of serum biochemical indices after immunization. The levels of ALT, AST, ALP, LDH and BUN were evaluated. The results were all within normal ranges, indicating the excellent biocompatibility of C-CPS. The results are expressed as the means of the measured values (n = 4).

Immune evaluation of the conjugate vaccine

Female 6-week-old BALB/c mice were immunized subcutaneously with one of five treatments [aluminium hydroxide adjuvant (Al, 100µg per mouse, InvivoGen), CTB + Al, CPS + Al, C-CPS + Al or C-CPS] on days 0, 14 and 28. Thirteen days after each injection (Fig. 3A), blood was collected from the tail veins of mice, and antibody levels were determined using enzyme-linked
Conjugate Vaccine against Acinetobacter baumannii

The effectiveness of immunization was further determined by the bacterial load and cytokines levels after an intraperitoneal injection of a sub-lethal dose of A. baumannii. Fourteen days after the third immunization (day 42), mice in each group were intraperitoneally injected with $2 \times 10^7$ colony-forming units (CFUs, 0.8-fold of a half-lethal dose) of A. baumannii ATCC 17978. After 12 h, the lungs, spleen and blood were collected from each mouse for bacterial load testing. Mice in the C-CPS + Al and C-CPS groups exhibited significantly reduced bacterial burdens (approximately 100-fold reductions at 12 h after infection) in the lungs, spleen and blood compared with the findings in the CTB + Al, CPS + Al and Al groups (Fig. 4A).

Previous studies illustrated that serum pro-inflammatory cytokine levels were increased at 12 h after A. baumannii infection (Garcia-Quintanilla et al., 2014). To further determine whether immunization with C-CPS + Al or C-CPS could reduce the inflammatory response induced by A. baumannii ATCC 17978, serum levels of inflammatory factors were detected at 12 h after infection. Compared with the control levels, the levels of the pro-inflammatory cytokines tumour necrosis factor (TNF)-α, IL-6, IL-1β and IFN-γ were significantly lower in the C-CPS + Al and C-CPS groups (Fig. 4B). Meanwhile, the weights of the immunized mice exhibited less fluctuation (Fig. S7), which indicates that vaccination could prevent the pro-inflammatory cytokine release associated with the development of septic shock.

Additionally, the histological results at 12 h after infection indicated that mice in the C-CPS + Al and C-CPS groups had a normal structure and clearer alveoli. However, the control mice exhibited extensive lung lesions, inflammatory cell infiltration, alveolar oedema and structural damage (Fig. 4C), which indicates that C-CPS immunization could decrease inflammatory responses in the lungs. Thus, the pathological damage of tissues could be slowed by immunization.

Conjugate vaccine immunization elicited long-term protection

To further investigate the long-term protection ability of vaccines against A. baumannii, the mice were subcutaneously injected with Al, CPS + Al, C-CPS + Al and C-CPS at days 0, 14 and 28. Blood was collected on days 14, 28, 42, 56 and 70 from tail veins to facilitate quantitation of antibodies against A. baumannii ATCC 17978.
CPS (Fig. 5A). We found that the titres of CPS-specific antibodies in C-CPS + Al and C-CPS groups reached peak at two weeks post third injection. As time goes on, the C-CPS + Al group still remained a high level, although the antibody level of other groups gradually decreased (Fig. 5B). We further evaluated its long-term protective efficacy by intraperitoneal challenge with 4.9 × 10^7 CFUs (2.0 × LD₅₀) of A. baumannii ATCC 17978. The survival rate of mice immunized with C-CPS + Al still remained 90%, and while the CPS group and Al group was 20%, indicating that C-CPS vaccine provided a good long-term protection (Fig. 5C).

Discussion

Considering the threat of A. baumannii to human health, especially the emergence of multidrug-resistant A. baumannii because of high antibiotic use (Nasr, 2020), an effective vaccine to prevent A. baumannii infection is urgently needed. In this study, we described a simpler A. baumannii conjugate vaccine production strategy using an O-linked glycosylation system in vivo. This conjugate vaccine effectively promoted Th1 and Th2 immune responses after immunization, significantly reduced post-infection tissue bacterial loads, suppressed serum pro-inflammator cytokine levels and protected against lethal challenge by A. baumannii in a murine sepsis model.

The surface polysaccharides of A. baumannii are mainly CPS and lipooligosaccharide (LOS), and more than 20 A. baumannii CPS structures have been identified (Giguere, 2015). Immunization with CPS can induce the production of protective antibodies (Fedson et al., 2011). For example, a monoclonal antibody against the K1 capsule was used to increase the clearance of A. baumannii in a soft tissue infection model (Russo et al., 2013). Additionally, antibodies against CPS from the drug-resistant clinical strain SK44 reduced post-infection bacterial loads and provided 55% protection against A. baumannii infection in a murine pneumonia model (Yang et al., 2017). However, there is no T-cell participation after CPS immunization, and CPS vaccines cannot elicit adequate protection in children 2 years old and younger (Feldman et al., 2019).

Therefore, we prepared an A. baumannii conjugate vaccine by introducing an exogenous O-glycosylation system for the first time and observed good safety and effectiveness, making this vaccine superior to CPS-based vaccines. In A. baumannii ATCC 17978, both CPS and O-linked protein glycosylation share a common pathway (Lees-Miller et al., 2013). After reaching the periplasmic side, the UndPP oligosaccharides can be directly transferred to proteins by an oligosaccharyltransferase or further processed into CPS with repeated glycosylation steps. However, the existing O-glycosylation transferase of A. baumannii, which catalyses the synthesis of CPS, is a heterologous glycosylation system that is compatible with the synthetic pathway of A. baumannii CPS. Note that CPS of A. baumannii ATCC 17978 is identical to those of strains MAL34, S40, LUH5537 and ATCC 17961 (Giguere, 2015), indicating the possibility of cross-protection against different A. baumannii stains. Encouragingly, it has been reported that 62% of a group of 554 A. baumannii clinical isolates strains reacted with anti-SK44 CPS antibody, which suggests that our vaccine has potentially broad applicability (Yang et al., 2017). In fact, immunization with C-CPS provided partial protection against a lethal dose of A. baumannii MDR-ZJ06. The reason for the cross-protection may be the effect of the existence of the same monosaccharide structure in the CPSs of those strains. Although the antibodies against C-CPS have a significant immunoprotective effect, its yield is not high. The yield of C-CPS was approximately 450 μg per litre of culture medium at the experimental stage. To further improve the efficiency of protein glycosylation, we will delete the Wza transferase in future research to improve the utilization of polysaccharides. A. baumannii ATCC 17978 carries CPS and LOS on its surface. After several
rounds of ultracentrifugation, dialysis and mild acid hydrolysis during the extraction process, low-molecular-weight LOS can be effectively removed, permitting CPS of Acinetobacter baumannii to be extracted for ELISA coating and immunization.

Acinetobacter baumannii can cause a variety of infections, including pneumonia, meningitis, sepsis, post-traumatic infection and urinary tract infection, among which sepsis is one of the major clinical manifestations of Acinetobacter baumannii infection (Harris et al., 2019). To monitor the immunoprotective efficacy of C-CPS, an Acinetobacter baumannii-associated murine sepsis model was developed and applied recently. McConnell et al. successfully constructed a murine sepsis model via an intraperitoneal injection of the Acinetobacter baumannii ATCC 19606 strain and evaluated immunoprotective effects of an inactivated bacterial whole-cell vaccine and OMVs (McConnell et al., 2011b). Compared with other models, this model is typified by changes of physiological indices, multiple-organ infection, changes of inflammatory factor levels in

Fig. 4. Immune responses of mice against low-dose Acinetobacter baumannii challenge. Two weeks after the third immunization (day 42), the mice were intraperitoneally challenged with $2 \times 10^7$ colony-forming units of Acinetobacter baumannii ATCC 17978. A. Bacterial burdens in the lungs, spleen and blood were calculated at 12 h after infection. $P$ values were calculated using the one-way ANOVA test ($***P < 0.001$, $****P < 0.0001$). The data are presented as the mean ± SD ($n = 5$). B. Serum levels of TNF-$\alpha$, IL-6, IL-1$\beta$ and IFN-$\gamma$ were determined in samples obtained at 12 h after infection. $P$ value was determined by the one-way ANOVA test ($****P < 0.0001$). The data are presented as the mean ± SD ($n = 5$). C. H&E staining of lungs from immunized and control mice. Representative histopathological sections are shown (×10 magnification, bar = 100 $\mu$m). All data were collected at 12 h after challenge.

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serum and eventually death of the animals. During the infection period, bacterial virulence factors cause inflammation, resulted in increased levels of pro-inflammatory cytokines. By analysing pro-inflammatory cytokine levels, we can study the occurrence of infection and inflammation. Our results illustrated that C-CPS enhanced the resistance of mice to infection by *A. baumannii* ATCC 17978, reduced *A. baumannii* counts in blood and suppressed the expression of inflammatory factors, thereby inhibiting the development of sepsis and improving the survival of mice. To further prove the immunoprotective effect of the antibody, we evaluated the opsonophagocytic effects of the antisera on *A. baumannii* in vitro. The results demonstrated that C-CPS-specific antibodies promoted *A. baumannii* clearance in the presence of complement, thereby facilitating neutrophil killing. However, serum did not exhibit any bactericidal activity without neutrophils (date not shown), which indicates that the bactericidal effects of serum were neutrophil dependent.

Previous studies found that the biosynthesis pathway of capsule/O-linked glycan has similar characteristics in all *A. baumannii* strains despite the structural differences of their polysaccharides (Scott et al., 2014). Thus, this strategy could be easily adapted to other serotypes of *A. baumannii* to prepare multivalent conjugate vaccines. We recognize that a thorough serotyping study of many clinical isolates was urgently needed to define the seroprevalence more clearly, providing a reference for the development of multivalent conjugate vaccines. In addition to saccharide antigens, protein antigens could also be introduced into our vaccine in the future. The ideal protein subunit antigen is a virulence-related outer membrane protein that is exposed on the bacterial cell surface, present in most *A. baumannii* strains and highly conserved at the amino acid level (Pachon and McCon nell, 2014). For example, *Acinetobacter* trimeric autotransporter is a good candidate for preparing a multicomponent conjugate vaccine because of its virulence-associated ability to produce anti-adhesive, opsonophagocytic and bactericidal activity and enhance resistance to *A. baumannii* infection (Bentancor et al., 2012b).

In conclusion, the results of this study confirmed that the heterologous O-linked glycosylation system is feasible for the preparation of *A. baumannii* conjugate vaccines. We have not applied additional interventions in the process of polysaccharide synthesis, which can ensure the natural configuration of the polysaccharide.
antigen. Additionally, it is reported for the first time that a biocjugate vaccine of *A. baumannii* had a significant immunoprotective effect. Thus, C-CPS can be considered a promising candidate vaccine for preventing *A. baumannii* infection.

**Experimental procedures**

**Ethics statement**

The animal experimental procedures were approved by the Laboratory Animal Center of the Academy of Military Medical Sciences. Pathogen-free female BALB/c mice (6–8 weeks old) were housed in the center. All animal experiments were performed in accordance with the guidelines of the Academy of Military Medical Sciences Institutional Animal Care and Use Committee (IACUC-DWZX-2019-401).

**Strains, growth conditions and plasmids**

The *A. baumannii* ATCC 17978 and MDR-ZJ06 strains were provided by Zhejiang University (Hangzhou, China). The bacterial strains were grown in Luria–Bertani (LB) broth or on solid medium containing 1.5% agar. *Escherichia coli* DH5α was used to clone plasmids. The glycosylated expression plasmids pMM-CTB4573C and pET-pglL-CTB4573C were constructed in our previous work (Pan et al., 2016). To induce glycosylation, cells were grown in LB broth at 37°C to an OD₆₀₀ of 0.6. Protein expression was induced by incubating cells with 1.0 mM IPTG at 30°C for 12 h.

**Western blot analyses**

Western blotting was performed as described previously (Pan et al., 2016). Whole-cell lysates were separated using 12% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes (GE Healthcare). Anti-6 × His antibodies conjugated to horseradish peroxidase (HRP, 1:3000, Abmart, Shanghai, China) were used to detect His-tag–fused proteins. Anti-CTB antibody (1:200, Abcam, Cambridge, MA, USA) was used to detect CTB and glycoproteins. LEL and WGA (10 µg ml⁻¹, Vector Labs, Burlingame, CA, USA) were used to detect the polysaccharides of glycoproteins. HRP-labelled anti-rabbit IgG (1:8000, TransGen Biotech, Beijing, China) and HRP-labelled streptavidin (1:2000, Vector Labs) were used as the secondary antibodies.

**Expression and purification of glycosylated proteins**

*Acinetobacter baumannii* cells were harvested via centrifugation at 8000 g for 10 min at 4°C and then dissolved in buffer A1 (20 mM Tris-HCl [pH 7.5], 10 mM imidazole, 500 mM NaCl). Cells were broken by homogenizer and centrifuged at 10 000 g at 4°C for 30 min to remove insoluble material. The glycosylated protein was purified using a chelating column (1.6 × 15 cm², GE Healthcare), that was pre-equilibrated with buffer A1. Bound protein was eluted with 100% buffer A2 (20 mM Tris-HCl [pH 7.5], 500 mM imidazole, 500 mM NaCl). The fraction containing glycoprotein was further purified using a Sephadex G-200 (1.6 × 90 cm², GE Healthcare) column, exchanging the buffer to PBS buffer. Fractions containing glycoprotein were collected and analysed via SDS-PAGE. The protein level of C-CPS was measured using the micro-bicinchoninic acid method (MicroBCA protein assay kit; Thermo Fisher Scientific). The carbohydrate level of C-CPS was measured using the phenol–sulfate method (Dubois, 1956).

**Vaccine safety evaluation**

For vaccine safety evaluation, C-CPS was subcutaneously injected into healthy BALB/c mice with 20 µg of polysaccharide. After 1 h, the mouse body weight and rectal temperature were measured at different time points, and the sera were collected at the same time. Cytokines (IL-1β, TNF-α, IL-6 and IFN-γ) were detected using commercially available ELISA kits (Dakewe, Shenzhen, China) according to the manufacturer’s instructions. The serum levels of alanine aminotransferase, aspartate transaminase, alkaline phosphatase, lactate dehydrogenase and blood urea nitrogen were detected using a biochemical autoanalyser (BS-350E, Mindray).

**Mouse immunization**

Groups of female BALB/c mice were used in immunization experiments, and the mice were divided into five groups via block randomization. CPS extraction was performed as described previously (Fregolini et al., 2011). The vaccines for *A. baumannii* including Al (100 µg per mouse), CTB + Al (40 µg per mouse), CPS + Al, CPS + Al and C-CPS were diluted with PBS. The polysaccharide dose was 4.0 µg per injection. The mice were injected three times with the same dosage at 2-week intervals. Thirteen days after each immunization, blood samples were taken from the tail vein, and the serum was collected by centrifugation and stored at 4°C.

**ELISA**

For indirect ELISA, 96-well plates were coated with 100 µl of CPS (10.0 µg) from *A. baumannii* ATCC 17978 in carbonate coating buffer (50 mM Na₂CO₃-NaHCO₃, pH 9.6) via incubation at 4°C overnight. The wells were washed thrice with wash buffer (1× PBS containing...
0.05% Tween-20) and blocked with 5% milk in PBS for 2 h at 37°C. Plates were again washed and patted dry. Then, 100 µl of sera was serially diluted 2-fold in dilution buffer (PBS supplemented with 0.5% [w/v] milk) and added to each well, followed by incubation for 1 h at 37°C. After another washing and drying step, 100 µl of HRP-conjugated goat anti-mouse IgG, IgG1 or IgG2a antibody (1:15 000, Abcam, Cambridge, MA, USA) diluted in dilution buffer were added to each well and incubated at 37°C for 1 h. After four steps of washing and patted dry, 100 µl of TMB solution was added to each well, and the plates were reacted for 10 min at room temperature. The reaction was stopped by the addition of 50 µl of 2 M H₂SO₄, and the absorbance was measured at a wavelength of 450 nm using a microplate spectrophotometer.

**Splenocyte culturing and stimulation**

On day 42, the spleen tissues of BALB/c were aseptically isolated. The spleen of each animal was crushed through a 200-mesh cell strainer in RPMI 1640 medium and centrifuged at 300 g for 5 min. Afterwards, the contaminating red blood cells were lysed using erythrocyte lysis buffer (0.9% ammonium chloride, eBioscience) for 5 min at room temperature. The resulting cell pellet was washed three times with complete RPMI 1640 medium. Subsequently, the splenocytes (2.5 × 10⁵) were co-incubated with the indicated antigens at 37°C with 5% CO₂ and 95% humidity (5 µg of polysaccharide antigen in PBS or PBS alone). Cells were stimulated with concanavalin A as a positive control. After 72 h of incubation, culture supernatants were collected, and the concentrations of IFN-γ, IL-2 and IL-4 were determined using commercially available ELISA kits (Dakewe) according to the manufacturer’s instructions.

**Opsonophagocytic killing assay**

The assays were performed as previously described (Burton and Nahm, 2012). Briefly, HL60 cells (ATCC, CCL-240) were cultured in RPMI1640 medium containing 10% heat-inactivated foetal calf serum. After 3 weeks of continuous culture, HL60 cells (6 × 10⁵ cells ml⁻¹) were cultured in medium containing 0.8% N, N-dimethylformamide (Sigma, St. Louis, MO, USA) for 4 days. The serum of each group was heat-treated at 56°C for 30 min to inactivate endogenous complement components. HL60 cells (4.0 × 10⁸ cells per well), A. baumannii ATCC 17978 (1 × 10³ CFUs per well), complement (#31061-3, Pel-Freez) and immunized or control sera (diluted 1:1, 1:3, 1:9, 1:27 or 1:81) were mixed and incubated for 45 min at 37°C in 5% CO₂. The microtiter plates were placed on ice for 20 min to terminate the reaction. Finally, 10 µL of the reaction mixture from each well was plated in duplicate on LB plates. The plates were incubated overnight at 37°C, and colonies were counted the next day. Microtiter plates containing naïve mouse sera were used as negative controls. The percentage of opsonophagocytic killing was determined using the following formula: per cent bacteria killed = (1 – [bacteria surviving in sample tubes/bacteria survived in negative control tubes with naïve sera]) × 100.

**Serum cytokine levels and bacterial loads**

To prepare the inoculum, A. baumannii ATCC 17978 was cultured at 37°C at an OD₆₀₀ of 2.0 and then diluted with normal saline to approximately 2.0 × 10⁷ CFUs/200 µl. Bacterial loads and cytokine levels were determined in vaccinated and control mice 12 h after challenge (n = 5 per group). Blood samples were collected, and the serum levels of IL-1β, TNF-α, IL-6 and IFN-γ were determined using ELISA kits (Dakewe). Bacterial loads in blood were estimated as CFUs per ml via serial dilution and plating. Lungs and spleens were collected aseptically, weighed, washed and homogenized in 1 ml of sterile PBS. Homogenates were serially diluted and plated in duplicate on LB plates for bacterial enumeration. The plates were incubated overnight at 37°C, and colonies were counted the next day. The results were expressed as log CFU.

**Histopathologic examination**

Lung tissue from sepsis model mice was removed under sterile conditions 12 h after infection. All lung samples were fixed with 4% paraformaldehyde (Solarbio, Beijing, China), embedded in paraffin, sliced and stained with haematoxylin and eosin. Slices were examined under a microscope.

**Mouse model of A. baumannii infection**

The inoculum was prepared as previously mentioned. Each mouse was intraperitoneally injected with 4.9 × 10⁷ and 1.2 × 10⁷ CFUs of A. baumannii ATCC 17978 and MDR-ZJ06, respectively (n = 10 per group). Fourteen days and Forty-two days after the third immunization, the mice were intraperitoneally injected with 200 µL of the bacterial suspension respectively, and survival was monitored continuously for 7 days.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software Inc., San Diego, CA, 2021). The authors publish for Society for Applied Microbiology and John Wiley & Sons Ltd.
CA, USA). Data were presented as the mean ± SD. Data were analysed using the Kruskal–Wallis test and one-way ANOVA for the multiple-group comparisons. Differences were considered significant at $P < 0.05$.

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Conflict of interest
None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Polysaccharide specificity of CTB4573C-CPS (C-CPS) was detected using western blotting. The purified glycoprotein CPS-C and C-OPS (biosynthesis in S. flexneri 2a strain 301 whose O-polysaccharide branch has no GlcNAC) was separated via 12% SDS-PAGE. Lycopersicon esculentum lectin (LEL) and wheat germ agglutinin (WGA) (Vector labs, Burlingham, CA, USA) was used to detect terminal GlcNAC in glycoproteins, and HRP labeled streptavidin (Vectorlabs, Burlingham, CA, USA) was used as the secondary antibody.

Fig. S2. Evaluation of the binding affinity of CTB4573C-CPS (C-CPS) for monosialotetrahexosylganglioside (GM1). GM1 (#G7641, Sigma) was diluted to 2 µg/mL with PBS to coat 96-well plates (at 4°C overnight). After being washed with PBST, 200 µL of blocking buffer containing 5% (w/v) milk in PBST were added to each well and then the plates were placed at 37°C for 2 h. After washing the plates with PBST again and patting dry, 100 µL rabbit anti-CTB antibody (provided from National Institutes for Food and Drug Control) (1:200) was added and incubated at 37°C for 1 h. After another washing and drying step, 100 µL HRP labeled goat anti-rabbit antibody (1:8000) was added and incubated at
37°C for 1 h. After four steps of washing and patted dry, 100 µL of TMB solution were added to each well, and the plates were reacted for 10 min at room temperature, and the reaction was stopped by adding 50 µL of stop solution. The absorbance was measured at a wavelength of 450 nm using a microplate spectrophotometer. The binding data are presented as the mean ± SD (n = 3), which indicates that the binding ability of cholera toxin B (CTB) to GM1 was not significantly affected by the glycosylation of CTB.

Fig. S3. Analysis of CTB4573C-CPS (C-CPS) degradation. Native gel electrophoresis was performed to analyze the degradation of C-CPS. The result illustrated that glycosylated recombinant cholera toxin B subunit (CTB4573C) had no obvious change after incubation at room temperature for 48 h, which indicates that the glycosylated protein skeleton was stable.

Fig. S4. Thermal stability of CTB4573C-CPS (C-CPS) was detected using a protein thermal shift assay. When the dye bound to hydrophobic amino acid residues, it emitted a fluorescence signal. C-CPS was depolymerized gradually with increasing temperature from 25 to 99°C, and the depolymerization temperature was approximately 69°C.

Fig. S5. The determination of the half-lethal dose (LD50) of *Acinetobacter baumannii* ATCC 17978 in mice. Mice were changed via intraperitoneal injection with different doses of an *A. baumannii* ATCC 17978 suspension, and survival was monitored continuously for 7 days.

Fig. S6. The determination of the half-lethal dose (LD50) of *Acinetobacter baumannii* MDR-ZJ06 in mice. Mice were changed via intraperitoneal injection with different doses of an *A. baumannii* MDR-ZJ06 suspension, and survival was monitored continuously for 7 days.

Fig. S7. The weight of mice were determined for 60 h after infection. The data are presented as the mean ± SD (n = 5).