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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 a model, 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.**

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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 Hammoudi 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 (Fattahian *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 (termed 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), PglL 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 PglL 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 PglL.

In this study, we prepared a conjugate vaccine against *A. baumannii* by introducing an O-linked glycosylation system (PglL) into the *A. baumannii* ATCC 17978 strain. CPS of *A. baumannii* could be combined directly with the recombinant cholera toxin B subunit (CTB^{4573C}) under catalysis by PglL *in vivo*. The use of the glycosylation system in the host stain 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, PglL of *A. baumannii* could not transform CPS onto a protein (Lees-Miller *et al*., 2013). Thus, we introduced a broader O-linked glycosylation system (PglL from *Neisseria*) into *A. baumannii* to prepare the conjugate vaccine. First, the recombinant plasmids pMM-CTB^{4573C} and pET-pglL-CTB^{4573C} (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 PglL and CTB^{4573C} were co-expressed, whereas the molecular weight of CTB^{4573C} 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 CTB^{4573C}-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 immunoactivator 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

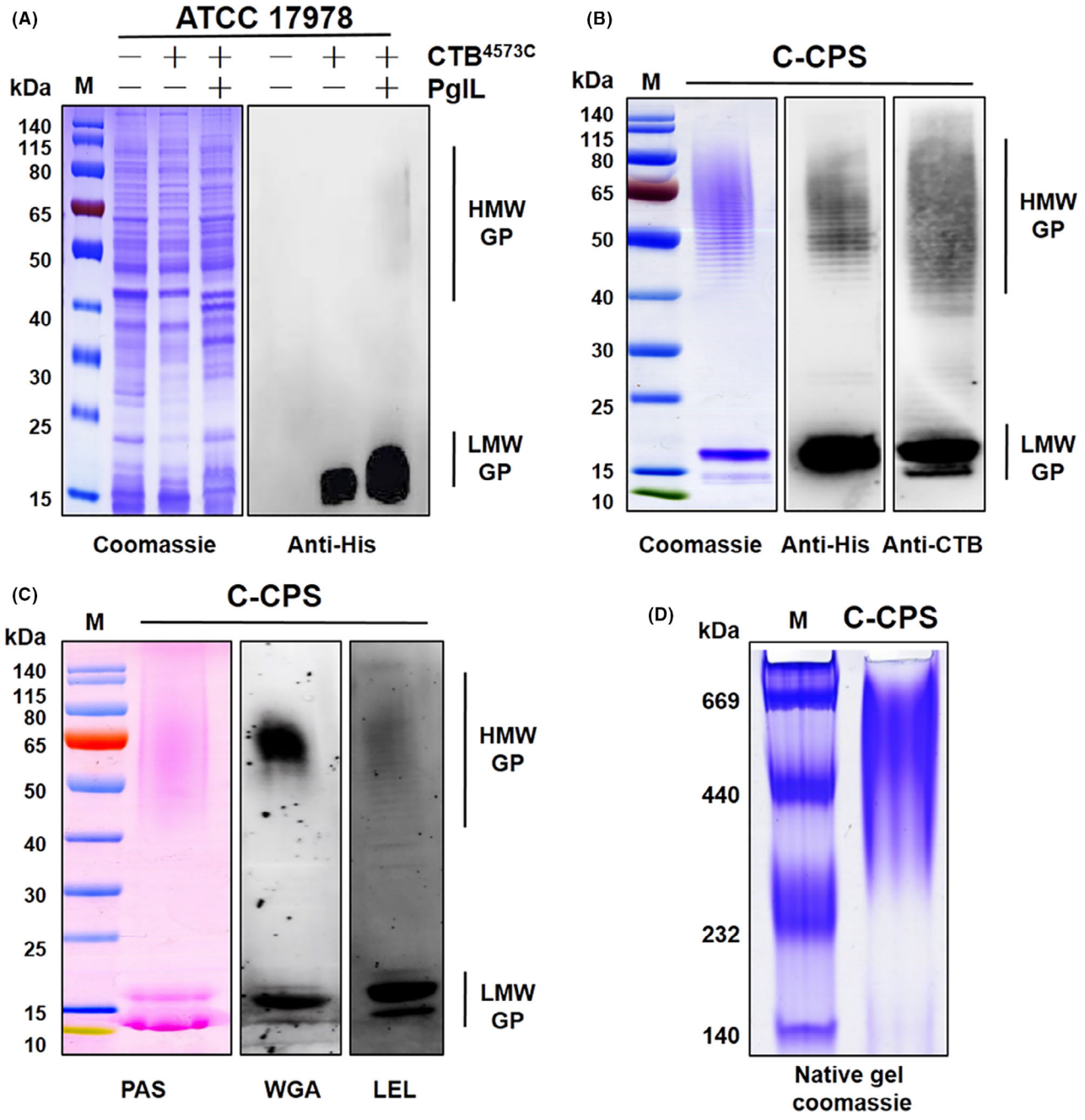

Fig. 1. Establishment of an O-linked glycosylation system in *Acinetobacter baumannii* ATCC 17978.
A. The recombinant plasmids pMM-CTB^{4573C} and pET-pgIL-CTB^{4573C} were transformed into *A. baumannii* ATCC 17978, followed by induction with IPTG. Coomassie blue staining (left) and western blotting (right) were used to analyse the glycosylation of the CTB^{4573C} protein. LMW GP refers to a glycoprotein containing short-chain CPS, HMW GP refers to a glycoprotein containing long-chain CPS.
B. The purified glycoprotein CTB^{4573C}-CPS (C-CPS) was separated via 12% SDS-PAGE, and C-CPS was then detected using Coomassie blue staining and Western blotting with antibodies against both 6 × His-tag and CTB.
C. C-CPS was separated via 12% SDS-PAGE, and the polysaccharide specificity of C-CPS was detected using periodic acid–Schiff staining and Lectins (LEL and WGA).
D. Native gel electrophoresis analysis of C-CPS.

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

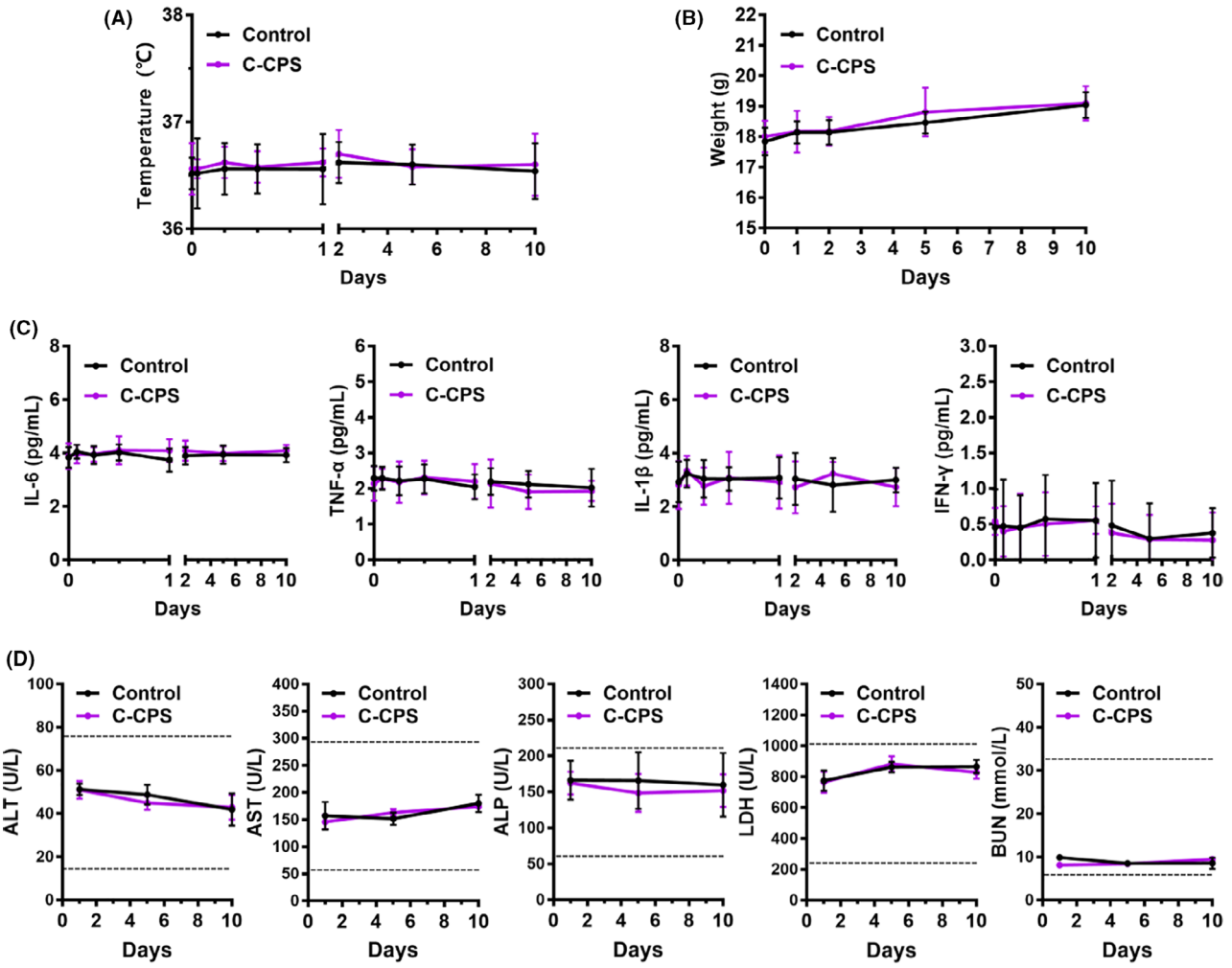

Fig. 2. Safety estimation of the conjugate vaccine.
A and B. Weight and temperature changes in mice immunized with CTB^{4573C}-CPS (C-CPS) containing 20 μg of polysaccharide. No changes in body weight and temperature were found in the C-CPS group compared with the findings in the control group.
C. Cytokine 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.
D. 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).

subcutaneously with 20 μg 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 control group findings (Fig. 2A). Similarly, there was no difference between the C-CPS and phosphate-buffered saline (PBS) groups concerning serum inflammatory factor levels, which indicates that C-CPS had no systemic toxicity (Fig. 2B). Finally, serum biochemical indices (alanine aminotransferase, aminotransferase, alanine aminotransferase, lactate dehydrogenase and blood urea nitrogen) were detected at 1, 5 and 10 days after immunization, and all indicators were within normal ranges (Fig. 2C), indicating the excellent biocompatibility of C-CPS.

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, 100 μg 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

immunosorbent assay (ELISA). ELISA-based measurement of the titres of the IgG antibody against *A. baumannii* ATCC 17978 polysaccharide in serum samples revealed increases upon both C-CPS + Al and C-CPS immunization, and the addition of an aluminium adjuvant together with free polysaccharide resulted in no significant increase in antibody titres (Fig. 3B). Additionally, antibody subtypes (IgG1 and IgG2a) were further detected, and the results indicated that C-CPS + Al and C-CPS could stimulate the simultaneous production of IgG1 and IgG2a antibodies (Fig. 3C), revealing that the IgG isotypes associated with T-dependent immunity were induced. For further verification, the concentrations of interferon (IFN)-γ, interleukin (IL)-2 and IL-4 in the supernatant of splenocytes cultured from immunized mice were measured on day 42. Compared with the control group findings, the splenocytes of mice immunized with C-CPS + Al or C-CPS secreted higher levels of IFN-γ, IL-2 and IL-4 (Fig. 3D). These results demonstrated that CTB-based conjugate vaccines could effectively elicit T-cell-dependent immune responses *in vivo*.

To further prove the immunoprotective effect of the antibody, we evaluated the opsonophagocytic effects of the antisera on *A. baumannii in vitro*. In the presence of HL60 phagocytic cells and complement, serum raised against C-CPS exhibited opsonophagocytic activity against *A. baumannii*. As presented in Fig. 3E, the results revealed that the specific bactericidal activities of C-CPS + Al and C-CPS reached 88.98 $\pm$ 3.80 and 85.55 $\pm$ 2.98%, respectively. Meanwhile, with the dilution of serum, there was no difference in the bactericidal rate between the two groups (50% bactericidal rate). These findings suggested that high levels of functional antibodies could be produced following immunization with conjugate vaccines.

Strongly encouraged by these obvious immune responses induced by the conjugate vaccine, we further established a murine sepsis model to evaluate its protective effect. First, the half-lethal doses (LD_{50}s) of *A. baumannii* ATCC 17978 and MDR-ZJ06 were determined in mice (Figs. S5 and S6). On day 42, mice were abdominally challenged with 4.9×10^7 CFUs ($2.0 \times LD_{50}$) of *A. baumannii* ATCC 17978 (Fig. 3F). The mice immunized with C-CPS + Al or C-CPS exhibited greater protection (100 and 80% survival, respectively) than those immunized with CTB + Al (20% survival), CPS + Al (30% survival) and Al (10% survival). To further prove that C-CPS can provide cross-protection, mice were intraperitoneal challenged with approximately 1.2×10^7 CFUs of *A. baumannii* MDR-ZJ06 ($2.0 \times LD_{50}$) at day 42. The 7-day survival rates of mice immunized with C-CPS + Al and C-CPS were 70% and 80%, respectively. All control mice died within 2 days, and the 7-day survival rates did not exceed 30% (Fig. 3G). These results indicated that the conjugate vaccine possessed significant ability to evoke protective immune responses.

Evaluation of vaccine-induced protection in mice challenged with a sub-lethal dose of A. 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 2×10^7 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 subcutaneous 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

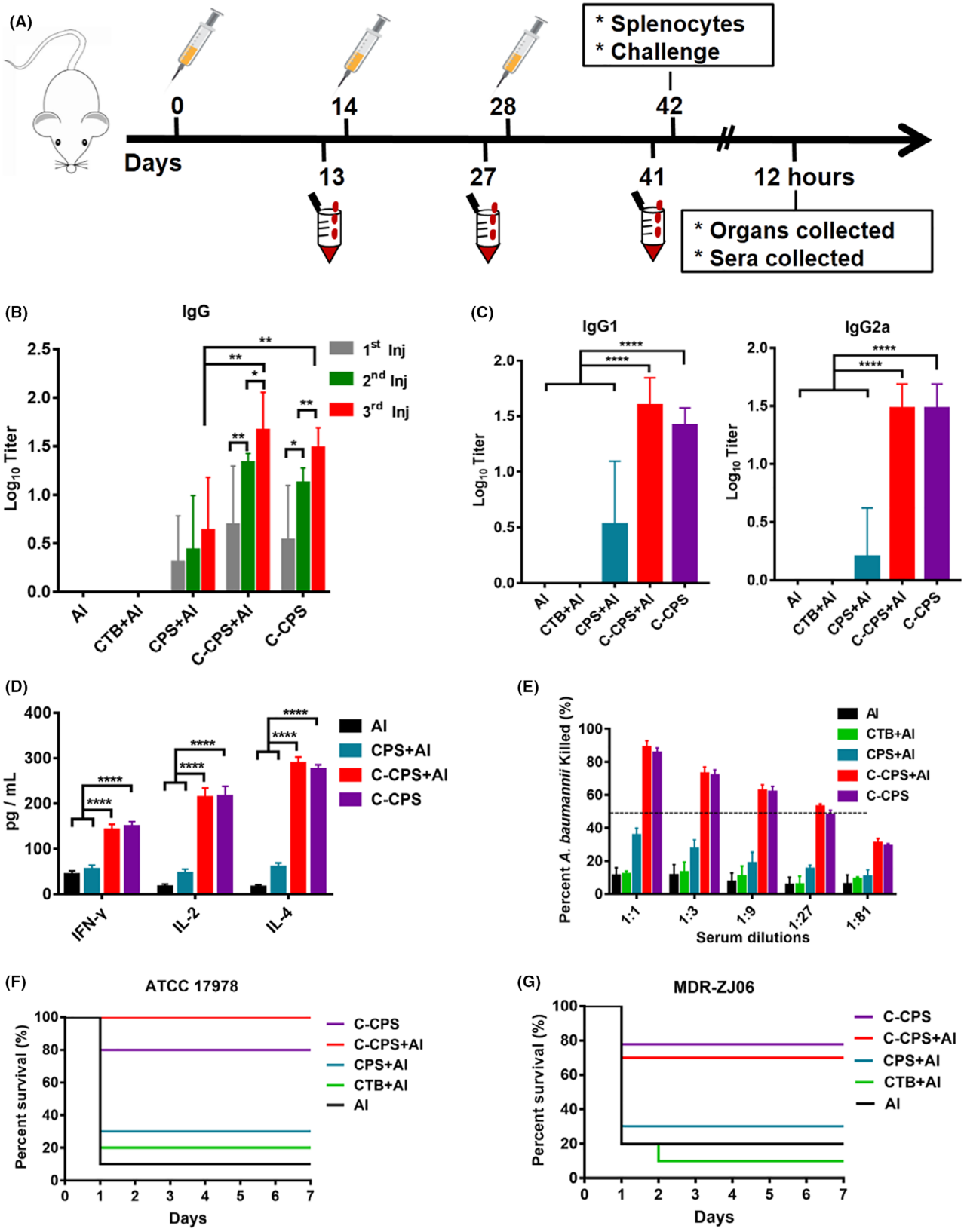

Fig. 3. Immune effects of CTB^{4573C}-CPS (C-CPS) in mice.
A. Schematic diagram of the immunization schedule and overall experimental design.
B. Groups of 10 mice were immunized on days 0, 14 and 28 with Al, CTB + Al, CPS + Al, C-CPS + Al or C-CPS. Total IgG titres in serum against the *A. baumannii* ATCC 17978 polysaccharide were determined in serum samples collected on days 13, 27 and 41.
C. IgG1 and IgG2a titres were determined in serum samples collected on day 41.
D. Two weeks following the last booster dose, splenocytes from mice ($n = 4$) were incubated with the corresponding antigen polysaccharide (5 $\mu g\ ml^{-1}$) for 3 days. The supernatants were harvested, and the IFN-γ, IL-2 and IL-4 levels were determined via enzyme-linked immunosorbent assay.
E. The results of opsonophagocytic assays are presented. Mouse serum (3-fold serial dilution), complement, HL60 cells and *A. baumannii* ATCC 17978 were incubated in round-bottom 96-well plates with shaking and plated in Luria–Bertani broth to measure bacterial survival using colony-forming units, and the percentage of killing was calculated.
F and G. Vaccinated and control mice were infected intraperitoneally with approximately 4.9×10^7 colony-forming units (CFUs, $2.0 \times LD_{50}$) of *A baumannii* ATCC 17978 (F) or 1.2×10^7 CFUs ($2.0 \times LD_{50}$) of *A. baumannii* MDR-ZJ06 (G), and survival was monitored over the subsequent 7 days ($n = 10$). Data are presented as the mean ± SD. Statistical analysis was performed using a Kruskal–Wallis test (serum titres) and one-way ANOVA with Dunn's multiple comparison test for the multiple-group comparation. (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

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 ($2.0 \times LD_{50}$) 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-inflammatory 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 polysaccharide units by Wzy polymerase, followed by transfer to the cell surface by Wza transferase. Because the endogenous O-oligosaccharyltransferase of *A. baumannii* cannot catalyse long sugar chains, we introduced a heterologous O-glycosylation system into *A. baumannii* 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 SMAL, SK44, 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

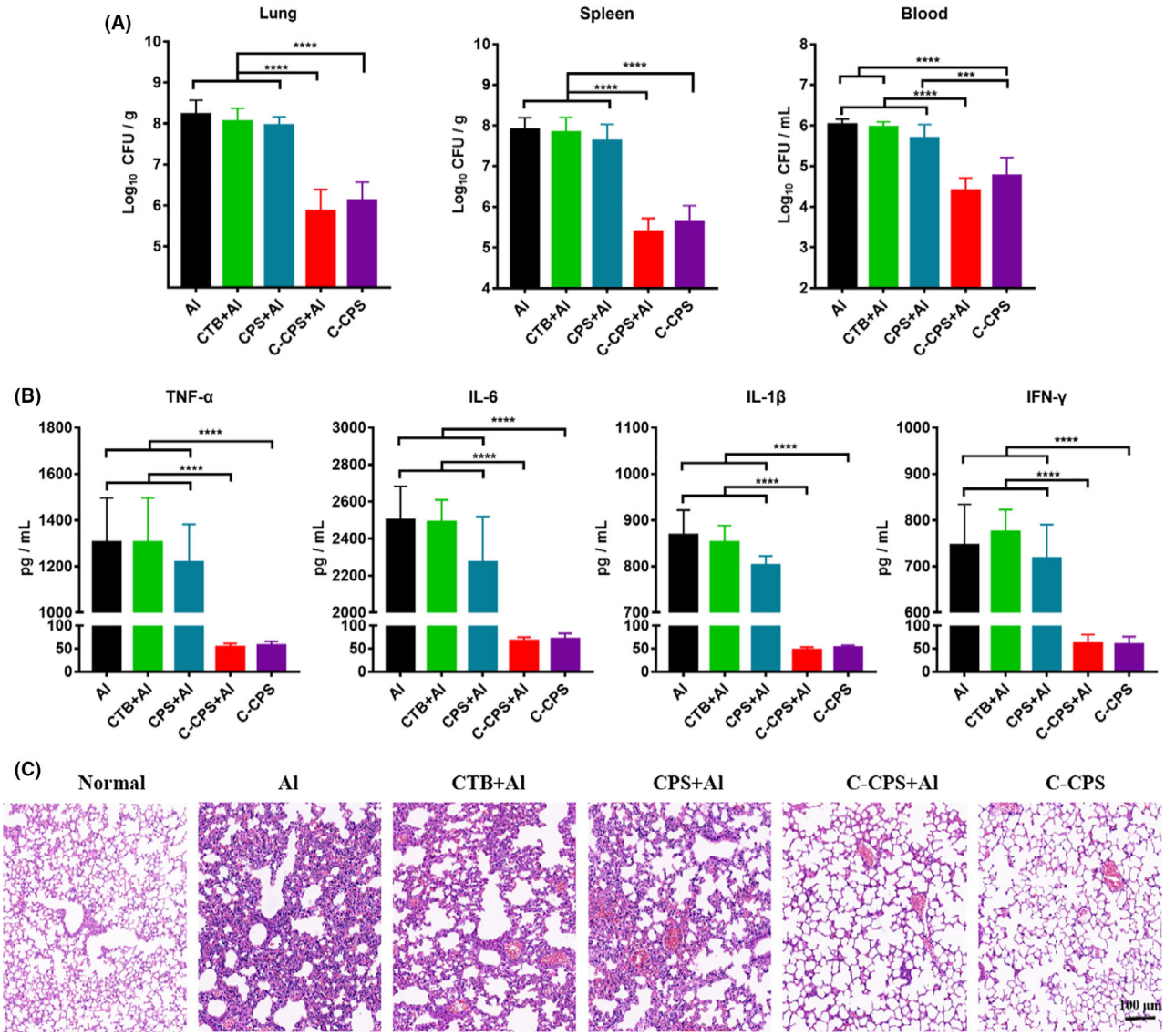

Fig. 4. Immune responses of mice against low-dose *Acinetobacter baumannii* challenge. Two weeks after the third immunization (day 42), the mice were intraperitoneally challenged with 2×10^7 colony-forming units of *A. baumannii* ATCC 17978.
A. Bacterial burdens in the lungs, spleen and blood were calculated 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-α, IL-6, IL-1β and IFN-γ 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 μm). All data were collected at 12 h after challenge.

rounds of ultracentrifugation, dialysis and mild acid hydrolysis during the extraction process, low-molecular-weight LOS can be effectively removed, permitting CPS of *A. 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 *A. baumannii* infection (Harris *et al.*, 2019). To monitor the immunoprotective efficacy of C-CPS, an *A. 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 *A. 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

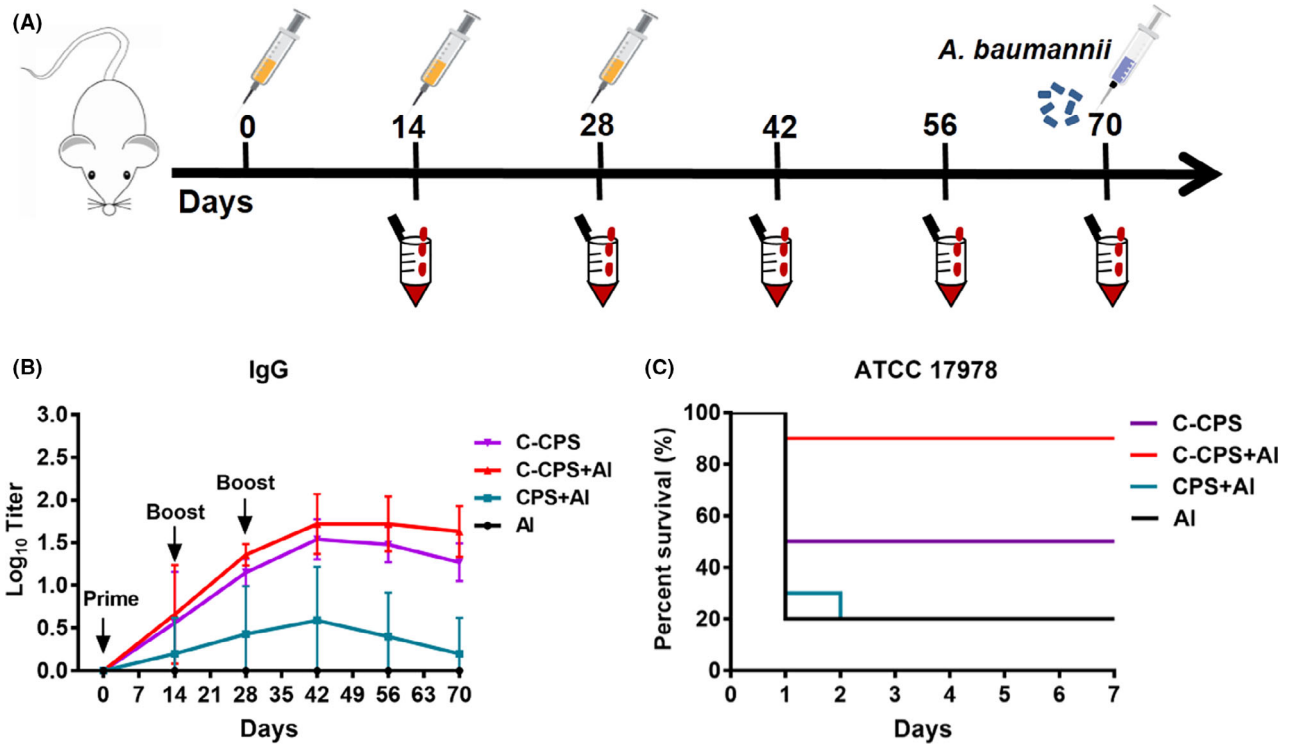

Fig. 5. Long-term protection evaluation of the conjugate vaccine.
A. Schematic illustration of immunization study. Groups of 10 mice were immunized on days 0, 14, and 28 with AI, CPS + AI, C-CPS + AI or C-CPS. Sera were collected every 2 weeks.
B. Serum CPS-specific IgG titres from individual mice were analysed by ELISA. C. Mice vaccinated were infected intraperitoneally with approximately 4.9×10^7 CFU mouse^{-1} of *A baumannii* ATCC 17978 at day 70, and their survival times were monitored ($n = 10$)

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 McConnell, 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 bioconjugate 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 centre. 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* DH5a was used to clone plasmids. The glycosylated expression plasmids pMM-CTB^{4573C} and pET-pglL-CTB^{4573C} were constructed in our previous work (Pan *et al*., 2016). To induce glycosylation, cells were grown in LB broth at 37°C to an OD_{600} 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 cm^2, 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 cm^2, 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 (Fregolino *et al*., 2011). The vaccines for *A. baumannii* including Al (100 μg per mouse), CTB + Al (40 μg per mouse), CPS + Al, C-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 Na_2CO_3-$NaHCO_3$, 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 H_2SO_4, 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 (2.5×10^6) were co-incubated with the indicated antigens at 37°C with 5% CO_2 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 (6×10^5 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 (4.0×10^5 cells per well), *A. baumannii* ATCC 17978 (1×10^3 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% CO_2. 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 OD_{600} of 2.0 and then diluted with normal saline to approximately 2.0×10^7 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 4.9×10^7 and 1.2×10^7 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, USA). Data were presented as the mean ± SD. Data were analysed using the Kruskal–Wallis test and one-way ANOVA for the multiple-group comparations. 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 CTB^{4573C}-CPS (C-CPS) was detected using western blotting. The purified glycoprotein C-CPS 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 CTB^{4573C}-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 CTB^{4573C}-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 (CTB^{4573C}) 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 CTB^{4573C}-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 (LD_{50}) 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 (LD_{50}) 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).

