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Development of a bivalent conjugate vaccine candidate against rotaviral diarrhea and tuberculosis using polysaccharide from *Mycobacterium tuberculosis* conjugated to ΔVP8* protein from rotavirus

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ABSTRACT

Conjugation of carbohydrate antigens with a carrier protein is a clinically proven strategy to overcome the poor immunogenicity of bacterial polysaccharide. In addition to its primary role, which is to help generate a T cell-mediate long-lasting immune response directed against the carbohydrate antigen, the carrier protein in a glycoconjugate vaccine can also play an important role as a protective antigen. Among carrier proteins currently used in licensed conjugate vaccines, non-typeable *Haemophilus influenzae* protein D has been used as an antigenically active carrier protein. Our previous studies also indicate that some carrier proteins provide B cell epitopes, along with T cell helper epitopes.

Herein we investigated the dual role of truncated rotavirus spike protein ΔVP8* as a carrier and a protective antigen. Capsular polysaccharide lipoarabinomannan (LAM), purified from *Mycobacterium tuberculosis* (M.tb), was chemically conjugated with ΔVP8*. Mouse immunization experiments showed that the resultant conjugates elicited strong and specific immune responses against the polysaccharide antigen, and the responses were comparable to those induced by Diphtheria toxoid (DT)-based conjugates. The conjugate vaccine induced enhanced antibody titers and functional antibodies against ΔVP8* when compared to immunization with the unconjugated ΔVP8*. Thus, these results indicate that ΔVP8* can be a relevant carrier protein for glycoconjugate vaccine and the glycoconjugates consisting of ΔVP8* with LAM are effective bivalent vaccine candidates against rotavirus and tuberculosis.

1. Introduction

Rotaviruses (RV) are among the most important enteric pathogens causing sever acute gastroenteritis in infants and young children under five years of age. RV infection led to 24 million outpatient visits, 2.3 million hospitalizations, and 200,000 deaths annually [1–3]. At present, RotaTeq®, Rotarix®, ROTAVAC®, and ROTASIIL® are the most widely used live attenuated oral vaccines, demonstrating 80%-90% vaccine efficacy especially in developed countries [4]. These vaccines are currently included in national immunization schedules in > 100 countries worldwide [5]. However, the live attenuated oral vaccines showed impaired protection efficacy in developing countries where rotavirus vaccines are mostly needed [6–9]. Poor nutrition, micronutrient deficiencies, and concurrent infection with other enteric pathogens may contribute to the diminished effectiveness of those attenuated vaccines [10]. In addition, there is a small increased risk of intussusception in vaccinated infants [11].

Tuberculosis (TB), caused by Mycobacterium tuberculosis (M. tb), is the leading killer among infectious diseases, and is responsible for 1.4 million deaths each year including 205.000 children in the world [12]. At present, Bacille Calmette-Guérin (BCG) vaccine, an attenuated strain of *Mycobacterium bovis*, is the only licensed vaccine available for TB. Although BCG vaccine has been extensively used as a part of national vaccination programs in countries with high TB rates, its effectiveness is variable against the development of TB both in pediatric and adult populations [13,14]. In parts, BCG vaccination has little effect on protection against adult pulmonary TB [13]. Due to the global burden attributable to RV and TB in the pediatric population, the development of an effective vac-

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cine against both diseases is urgently needed to control and prevent these diseases.

Rotavirus spike protein VP4 consists of its stalk (VP5*) and globular head (VP8*) [15]. A truncated VP8* protein, termed ΔVP8*, was reported to induce heterotypic neutralizing antibodies upon parenteral immunization in small animal models [16]. Furthermore, ΔVP8* has been used in several vaccine platforms including recombinant fusion proteins (ΔVP8* fused to a universal tetanus toxin $CD4^+$ T cell epitope P2) and nanoparticles (ΔVP8*inserted in the surface loops of the Norovirus P or S particle) [17–19]. Those vaccine candidates showed enhanced efficacy in resource-deprived countries as compared with live attenuated vaccines because non-replicating vaccines are not directly affected by microbiome composition or gut enteropathy [20,21].

Bacterial pathogens often express high-molecular weight capsular polysaccharides (CPSs), which serve as a protective external layer for the bacteria. When used as a vaccine, CPSs, as a type of T cell-independent antigen, are poorly immunogenic and fail to induce immunological memory in infants under two years of age [22–25]. However, covalent linkage of the carbohydrate antigens to immunogenic carrier proteins results in glycoconjugates which elicit booster response to the carbohydrate antigens [26,27]. Mycobacterial capsular polysaccharides are optimal targets for subunit vaccines due to their location at the outermost layer of cell and their differences from human glycans [28,29]. The major capsular polysaccharides of Mycobacterium Tuberculosis (M.tb) are α-glucan and lipoarabinomannan (LAM), respectively accounting up to 80% and 15% of the extracellular polysaccharide [30–33]. Chemical conjugation of those polysaccharides to relevant carrier protein can elicit antibody-mediated responses, characterized by IgM-to-IgG switching, a booster response, and sustained T cell memory to the polysaccharides [34–37]. Although conjugate vaccine platform was developed to enhance the immunogenicity of carbohydrate antigens, a carrier protein may also induce an immune response against itself [38–40]. For example, Affinivax's novel vaccine platform, the Multiple Antigen Presentation System (MAPS), uses pneumolysin as a carrier protein, resulting in comprehensive B- and T-cell immunity against both polysaccharides and protein components. In addition, *Salmonella* Typhimurium flagellin monomer (FliC) is also used as a self-adjuvating carrier protein in conjugate vaccine platform. Therefore, the conjugate vaccine platform may induce protective immunity against the pathogen not only from which the polysaccharide is derived, but also from which the carrier protein is derived [38].

Herein, we developed glycoconjugates, employing a chemical conjugation of ΔVP8* to Mycobacterium capsular polysaccharide LAM. The following conjugates were developed, using two different chemical strategies: LAM-ΔVP8* and LAM-$\Delta VP8^*_{AH}$. In addition, we also generated the following Diphtheria Toxin (DT)-based conjugates: LAM-DT and LAM-DT_{AH} to evaluate the intrinsic value of ΔVP8* in comparison with DT, a benchmark carrier protein. Each conjugate was physico-chemically characterized. Antigen-specific antibodies in serum and virus-neutralizing antibody against RV determined the immunologic properties of LAM-ΔVP8* conjugates in mice. The results showed that LAM-ΔVP8* conjugates induced strong immune responses against polysaccharide and protein antigens, and the effect of ΔVP8* as a carrier was comparable to the one of DT, a benchmark carrier protein. Therefore, our study sheds light on an additional potential of conjugate vaccine platform for enhancement of the immune response to rotavirus spike protein ΔVP8*, and supports further evaluation of the M.tb polysaccharide-rotavirus protein conjugate vaccine towards clinical development.

2. Material and methods

2.1. Strains and reagents

Virulent M.tb strain H37Rv (ATCC27294) was cultured in Sauton's media as previously described [41]. In brief, the mycobacteria were incubated in the L-J medium until the inoculated mycobacteria formed colonies. The mycobacteria colony was incubated in Sauton-potato medium until the formation of the mycobacteria surface pellicles. Then, the pellicles were transferred to Sauton's media.

DNA sequences encoded for amino acids 65 to 223 of VP8*-P[8] were synthesized by Bioneer (Daejeon, Korea) and cloned into the expression vector pET28a (Novagen, USA) for protein expression. Amplification of the recombinant plasmids was conducted by transformation into *Escherichia coli* (*E. coli*) (strain BL21, DE3).

DT was obtained from PT Bio Farma (Persero). The 1-cyano-4-dimethylaminopyridinum tetrafluoroborate (CDAP), adipic acid dihydrazide (ADH), *N*-(3-dimethylaminopropyl)-N'-ethylcarbodii mide hydrochloride (EDAC) and 4-morpholine ethanesulfonic acid (MES) was purchased from Sigma. The CS-35 monoclonal antibody, which is known to recognize only the arabinan component of LAM, was procured from BEI resources (Manassa, VA).

2.2. Polysaccharide isolation

LAM was purified from the cell pellet by biphasic extraction with Triton X-114 followed by size exclusion chromatography with Sephacryl S-200 on XK 16/100 (GE Healthcare, USA) as previously described [42]. The cell pellet was suspended in phosphate buffered saline (PBS) containing Triton X-114 (8%, v/v) and disrupted by high pressure homogenizer (Panda Plus 2000, GEA Niro Soavi). Mycobacterial lysate was separated by centrifugation at 27,000 × g for 1hr to remove insoluble debris and the supernatant was subsequently placed in 37 °C water bath for at least 2hr. The biphase was clarified by centrifugation at 27,000 × g for 15 min (25 °C). LAM at the lower detergent layers was precipitated by adding 10 volumes of ice-cold 95% ethanol, followed by incubation at −20 °C for 16hr. The precipitate was harvested by centrifugation at 27,000 × g for 1hr (4 °C) and resuspended in endotoxin free water followed by addition of proteinase K. The digest was dialyzed for 24hr in running distilled water using a dialysis membrane tube (Spectra/Por® MWCO 3.5 kDa; Spectrum LAB., USA). It was initially freeze-dried and subsequently resuspended in 10 mM Tris (pH8.0), 0.2 M NaCl, 0.25% deoxycholate, 1 mM EDTA and 0.02% sodium azide (LPS running buffer). The mixture was further separated by size exclusion chromatography on Sephacryl S-200 on XK 16/100 (GE Healthcare, USA). Elution profile of the fractions were monitored by SDS-PAGE and silver staining with a periodic acid oxidation step. The fractions containing LAM were pooled and dialyzed against LPS running buffer without deoxycholate at 37 °C followed by 1 M NaCl and water for several days. The purified LAM was identified by SDS-PAGE and western blot with monoclonal antibody CS-35. The carbohydrate composition of the LAM was determined by HPAEC-PAD. The residual protein was measured by Bradford method (Thermo Scientific, USA) using bovine serum albumin (BSA) as a standard. Endotoxin levels and DNA quantification were performed using Endosafe nexgen-PTS spectrophotometer (Charles River, USA) and Quant-iT Picogreen dsDNA assay kit (Invitrogen, USA), respectively.

2.3. Protein purification

Recombinant proteins were expressed in *E. coli* BL21 (DE3) as described previously [16]. Briefly, *E. coli* BL21 (DE3) cells harboring the expression vectors were grown at 37 °C until absorbance at 600 nm reached 0.6. The expression of tag-free ΔVP8* proteins were induced by the addition of 0.5 mM isopropyl-β-D-thiogalacto pyranoside (IPTG) at 18 °C overnight. The recombinant *E. coli* cells harboring ΔVP8* proteins were collected by centrifugation and lysed by sonication into 20 mM Tris buffer (pH7.6). Ion exchange chromatography using anion exchange resin (AEX), Fractogel EMD DEAE (Merck, Germany) resin in XK16/20 column with 16 cm bed height, was performed in negative (flow-through) mode to separate the ΔVP8* proteins from impurities such as host cell proteins and DNA. The unbound proteins were concentrated and then diafiltered into 20 mM Sodium Acetate (pH5) buffer by Tangential Flow Filtration (TFF) using 5 kDa molecular weight cut-off (MWCO) membrane cassettes for further purification. Subsequently, 5 kDa retentate was loaded on the cation exchange chromatography (CEX) using Eshmuno CPX (Merck, Germany) resin in XK16/20 column with 15 cm bed height, followed by washing of the column using 5 CVs of 20 mM Sodium Acetate (pH5) buffer to remove unbound proteins. The bound proteins were eluted using 10 CVs of 20 mM Sodium Acetate buffer with 1 M NaCl (pH5). For further application, the eluted fractions containing ΔVP8* proteins were concentrated and diafiltered into phosphate buffered saline (PBS, pH7.4) by TFF using 5 kDa membrane cassettes. The protein concentration of the samples was determined by Bradford assay using BSA as a standard. The protein was identified to contain ΔVP8* by immunoblotting with anti-Rotavirus antibody (AB-1129, Merck). The average size of the purified ΔVP8* proteins was determined by High performance size exclusion liquid chromatograph (SEC-HPLC; Agilent 1260 LC system, USA) equipped with a OHpak SB-804 HQ column (Shodex, Japan). Endotoxin levels and DNA quantification were performed using Endosafe nexgen-PTS spectrophotometer (Charles River, USA) and Quant-iT Picogreen dsDNA assay kit (Invitrogen, USA), respectively.

2.4. Conjugates

LAM were conjugated to ΔVP8* and DT with and without ADH linker, resulting in different ΔVP8*-based conjugates (LAM-ΔVP8* and LAM-ΔVP8*$_{AH}$) and other different DT-based conjugates (LAM-DT and LAM-DT$_{AH}$).

2.5. Carrier proteins derivatized with ADH linker

A solution containing 5 mg of carrier proteins (DT or ΔVP8*) was equilibrated in 80 mM MES buffer (pH5.0) at room temperature. EDAC and ADH were added to the solution with agitation for 90 min. The mixture was immediately dialyzed against 80 mM MES (pH5.6) using a dialysis membrane tube (Spectra/Por® MWCO 3.5 kDa; Spectrum LAB., USA) for 2 days to remove unbound EDAC and ADH. The hydrazide level on the modified proteins was determined by a trinitrobenzene sulfonic acid (TNBS) assay using acetylcholine chloride as a standard.

2.6. Conjugation of mycobacterial polysaccharides to carrier proteins

Conjugation of mycobacterial polysaccharides to carrier proteins was prepared as described previously [43–45]. Briefly, 4 mg of mycobacterial polysaccharides (LAM: 2.3 mg/mL) were dissolved in distilled water and mixed with agitation at 4 °C overnight. At t = 0, CDAP was added. Activation of random polysaccharide hydroxyls was achieved by addition of 100 mg/mL CDAP in acetonitrile at a ratio of 1 mg of CDAP for 1 mg of LAM. At 30 s, 0.1 M NaOH was slowly added to raise pH from 5.6 to 9, and the pH level was stably maintained. At 10 min, 4 mg of ΔVP8* protein (1 mg/mL), ΔVP8*$_{AH}$ (2.7 mg/mL), DT (5.9 mg/mL), or DT$_{AH}$ (2.3 mg/mL) in 0.1 M sodium borate (pH9.0) was added. After 16 h, the reaction was terminated by adding 0.5 mL of 2 M glycine (pH9.0) and incubated for 1 h. The conjugates were separated from the un-bound polysaccharide or carrier protein by FPLC on a Sephacryl 200 (GE Healthcare, USA) in PBS. Elution was carried out at 0.5 mL/min, monitoring at 206 nm and 280 nm. Each fraction was then assayed for polysaccharide and protein content by Anthrone assay and Lowry assay, respectively. The fractions containing the conjugate were pooled, concentrated by Amicon Ultra-15 (50 kDa MWCO, Millipore, Germany), and filtered through 0.2 μm filter.

2.7. Immunization

Immunization study is described in detail in Fig. 1a. Female 7-week-old BALC/c mice were purchased from KOATECH (Korea). Groups of 10 mice were injected with one of the following conjugates: (1) the LAM-ΔVP8* at 10 μg/dose of LAM and 8.1 μg/dose of ΔVP8*; (2) the LAM-ΔVP8*$_{AH}$ at 10 μg/dose of LAM and 9.9 μg/dose of ΔVP8*$_{AH}$; (3) the LAM-DT at 10 μg/dose of LAM and 7.2 μg/dose of DT; (4) the LAM-DT$_{AH}$ at 10 μg/dose of LAM and 7.4 μg/dose of DT$_{AH}$. Mice received injections of PBS as vaccine diluent control, 10 μg/dose of LAM, or 5 μg/dose of each carrier proteins. Immunogens in 200 μL volumes were intramuscularly administered four times at 2-week intervals (Fig. 2a). On Days 14, 28, 42 and 56, blood samples were taken from *retro*-orbital bleeding, allowed to clot for 30 min, and centrifuged at 1,200 × g at 4 °C to obtain sera. All animal studies were carried out as per the guidelines and following approval from Institutional Animal Care and Use Committee (IACUC) at International Vaccine Institute (Seoul, Korea).

2.8. ELISA assay

An ELISA was used to determine the serum antibody titers against mycobacterial polysaccharides or carrier proteins. Briefly, 96-well microtiter plates (MaxiSorp, Nunc) were coated with 10 μg of mycobacterial polysaccharide or 1 μg of carrier proteins at 4 °C overnight. After blocking with 1% Bovine Serum Albumin (BSA) for 1 h, the plates were washed with 0.05% PBS-T (Tween 20 in PBS). Serum samples from immunized mice were added to the plates, serially diluted, and then incubated for 2 h at 37 °C. The plates were again washed with 0.05% PBS-T, followed by incubation with secondary antibody for 1 h at 37 °C. The plates were washed with 0.05% PBS-T and developed by using 4-Nitrophenyl phosphate substrate (Sigma). The optical density at 405 nm was measured on ELISA plate reader (SpectraMax 190, Molecular Device).

2.9. Plaque reduction neutralization assays

A virus neutralization assay based on plaque reduction was performed to determine neutralizing antibodies elicited in vaccinated mice. Briefly, MA104 cells were cultivated in six-well plates. After a treatment with trypsin (10 μg/mL in Medium 199), rotaviruses of P [8] (Wa strain, G1P8) were incubated with mouse sera at given dilutions, and then added to MA104 cell monolayers in 6-well plates and incubated for 1 h at 37 °C on a rocking platform. The cells were washed with prewarmed, serum-free Medium 199, gently overlaid with 0.7% agarose and incubated at 37 °C. The cells were fixed with 10% formalin and stained with 0.5% crystal violet. The neutralization titers of the sera were defined as the maximum dilutions of the mouse sera that can reduce at least 50% of infected

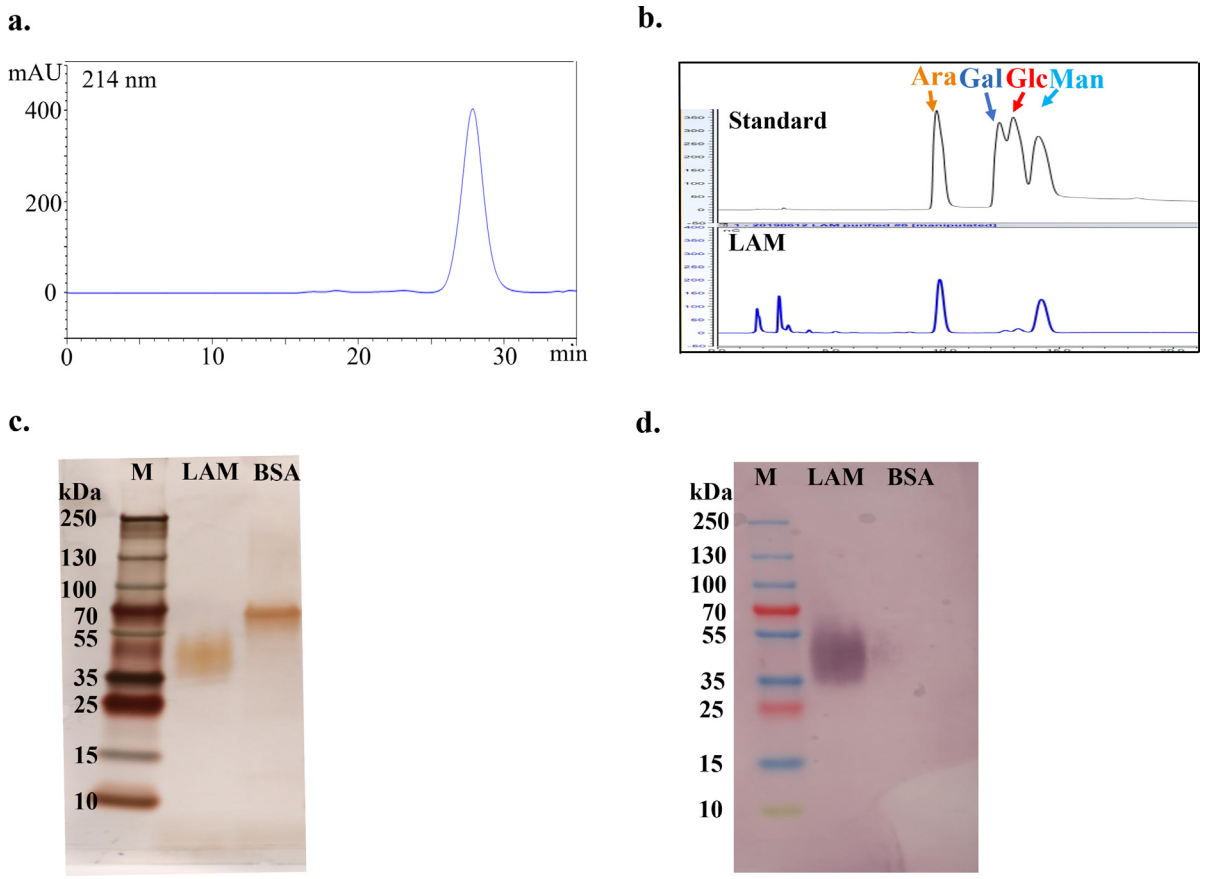

Fig. 1. The purified LAM was analyzed using (a) SEC-HPLC with TSK gel 3000 PW_{XL} column (100 mM NaH_2PO_4, 100 mM NaCl, 5% CH_3CN, pH7.2; 0.3 mL/min), (b) HPAEC-PAD for carbohydrate analysis (Arabinose: Ara, Galactose: Gal, Glucose: Glu and Mannose: Man), (c) SDS-PAGE followed by PAS staining and (d) SDS-PAGE followed by immunoblotting with CS-35 antibody (Lane 1: Marker, Lane 2: LAM, Lane 3: BSA).

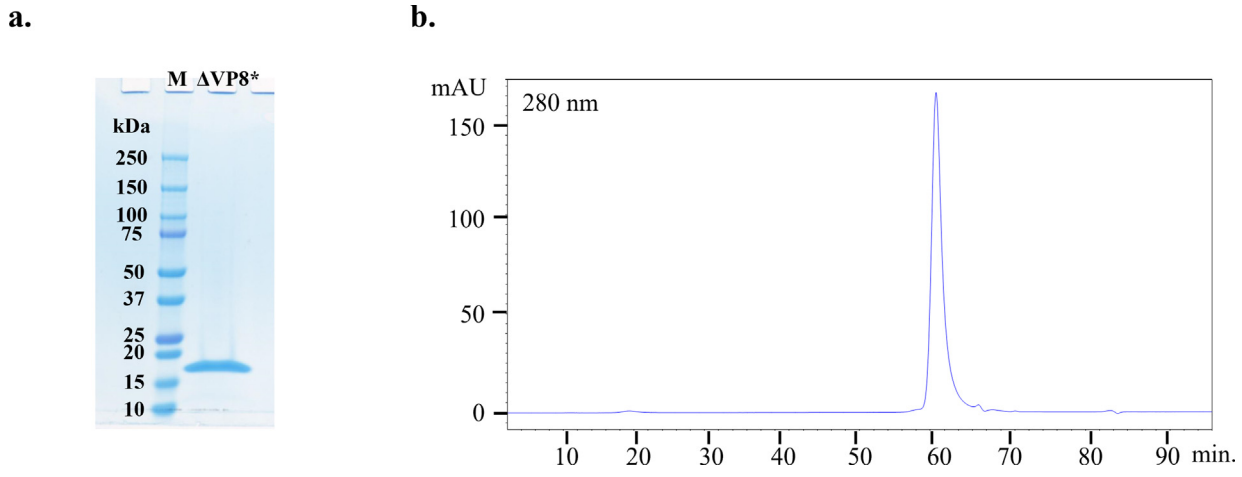

Fig.2. Production and physicochemical characteristics of the recombinant ΔVP8* proteins. (a) SimplyBlue™ stained SDS-PAGE (M: Marker) (b) SEC-HPLC analysis of purified ΔVP8*-P[8]. OHpak SB-804 HQ and OHpak SB-806 HQ columns in series, 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% ACN, pH 7.2; 0.3 mL/min. flow rate.

cells compared with non-neutralized, serum free control as previously described [46].

2.10. Statistical analysis

Student's *t*-test, Welch's *t*-test, or Kolmogorov-Smirnov test was used for the comparison of the anti-LAM, anti-ΔVP8*, and anti-DT IgG immune responses between the conjugates and controls depending on whether the variance of data was equal or not and the distribution of data. For all the analyses, P values<0.05 were considered to be significant. Log_2 transformations were applied to ELISA titers. Statistical analysis was performed using STATA software (StataCrop LP, Version 11.0).

3. Results

3.1. Purification and specificity of LAM and ΔVP8*

LAM is capsular polysaccharides that are found abundantly in cell envelope of M.tb H37Rv [47]. LAM is a glycolipid, consisting of a branched arabinan polymer attached to a mannan polysaccharide backbone that extends from a phosphatidylinositol mannoside anchor at the reducing end. The molecular mass of LAM was estimated to be around 15 kDa (Fig. 1a) [48–50]. HPAEC-PAD profile of acid hydrolyzed LAM indicated that Man and Ara were the dominant products (Fig. 1b) [51]. The purified LAM was identified by SDS-PAGE followed by PAS staining (Fig. 1c) and immunoblotting with CS-35 antibody (Fig. 1d). Residual protein level was less than the lower limit of detection (10 μg/mL). The residual nucleic acid and endotoxin levels were approximately 0.7 μg/mL and 0.2 Endotoxin unit (EU)/μg LAM, respectively, which are within the acceptable levels for recombinant subunit vaccines.

The ΔVP8* protein was purified by two-step ion exchange chromatography. The yield of the recombinant protein reached approximately 50 mg/L of culture. After purification, ΔVP8* protein showed a single band of 18–20 kDa on SDS-PAGE (Fig. 2a) and was eluted as a single peak in SEC-HPLC (Fig. 2b). The residual nucleic acid and endotoxin levels were 0.4 ng/mL and 1.84 EU/mL, respectively, which are within the acceptable levels for recombinant subunit vaccines.

3.2. Chemical conjugation of ΔVP8* to LAM

Aspartic or glutamic acid residues of the carrier proteins were modified with ADH in the presence of EDAC to achieve an efficient coupling. Based on TNBS assay, 6 ADH linkers and 12 ADH linkers were introduced to ΔVP8* and DT, respectively. $\Delta VP8^*_{AH}$ showed a broad peak at similar retention time on SEC-HPLC as the ΔVP8*, indicating no cross linking of the VP8* had occurred (Fig. S1). Hydroxyl groups along the PS chains were randomly activated with CDAP at a weight to weight (w/w) ratio of 1:1, and linked to the hydrazides of ADH linkers or lysines on the carrier protein. In order to assess the impact of ADH linkers on a polysaccharide-protein coupling, series of vaccine constructs was prepared from ΔVP8*AH or DTAH derivatives. Although derivatization of carrier proteins resulted in higher number of functional groups for linkage, conjugates made with different chemistries did not show any significant difference in terms of the protein recovery (Table 1).

As illustrated in Fig. 3a, glycoconjugates were synthesized with 4 mg of LAM (2.3 mg/mL) at a ratio polysaccharide/protein of 1:1 (w/w). The chromatogram profile showed that LAM-ΔVP8* eluted earlier than either unconjugated LAM or unconjugated ΔVP8*, confirming the larger size of the conjugate with respect to unconjugated components (Fig. 3b). The large size of the conjugates resulted in a typical smear at the top of 12% SDS-PAGE (Fig. 3b) and the decreased retention time in SEC-HPLC (Fig. 3c). The amount of polysaccharide and protein in the ΔVP8*-based conjugates (LAM-ΔVP8* and LAM-ΔVP8*AH) and the DT-based conjugates (LAM-DT and LAM-DTAH) is shown in Table 1.

3.3. LAM-ΔVP8* conjugates can elicit the immunogenicity of both antigens

In immunogenicity study, the conjugates were administrated to mice without an adjuvant to focus exclusively on the intrinsic value of ΔVP8* as a carrier. As illustrated in Fig. 4a, Four different groups of mice were intramuscularly immunized with each conjugate: (1) the LAM-ΔVP8* at 10 μg/dose of LAM and 8.1 μg/dose of ΔVP8*; (2) the $LAM\text{-}\Delta VP8^*_{AH}$ at 10 μg of LAM and 9.9 μg of $\Delta VP8^*_{AH}$ for mouse/dose; (3) the LAM-DT at 10 μg/dose of LAM and 7.2 μg/dose of DT; (4) the $LAM\text{-}DT_{AH}$ at 10 μg/dose of LAM and 7.4 μg/dose of DT_{AH}. Controls were injected with PBS as vaccine diluent control, 10 μg/dose of LAM, or 5 μg/dose of each carrier proteins. Immunogens in 200 μL volumes were administered four times at 2-week intervals. Immunization with the unconjugated LAM induced very low levels of LAM-specific IgG antibodies. In contrast, the glycoconjugates induced highly elevated titers of IgG antibodies to LAM; anti-LAM IgG levels induced with ΔVP8*-based conjugates were comparable to those levels administered with DT-based conjugates (Fig. 4b). Administration of ΔVP8*-based conjugates elicited significantly higher levels of ΔVP8* specific IgG antibodies than unconjugated ΔVP8* (Fig. 4c). The neutralizing capacity of serum antibodies was measured using the plaque reduction neutralizing test (PRNT). Rotavirus neutralizing activity was higher in conjugate-immunized mice, with 50% reduction in plaque count (PRNT50) titers ranging from 1:128 to 1:512 (Fig. 4d).

4. Discussion

Cell-mediated immunity is an important component to control mycobacterial infection [52]. However, recent immunological and genetic studies support that antibody-mediated immunity also plays a protective role against TB [53,54]. Among many constituents of mycobacterial cell envelope, LAM is the major component of cell wall-associated lipoglycans. LAM can induce high antibody responses in infected hosts [55]; antibodies to LAM have moderate protective efficacy in M.tb infected mice [34,37,50]. LAM, however, is a T cell-independent antigen, which can activate B cells without the cooperation of helper T cells and is characterized by a

Table 1
Characteristics of M.tb polysaccharide conjugates.

Saccharide	Carrier protein	Linker	Polysaccharide[1] (mg)	Protein[2] (mg)	Protein/Polysaccharide (w/w)
LAM	ΔVP8*	–	1.3	1.2	0.9
		ADH	1.2	1.3	1.1
	DT	–	2.0	1.2	0.6
		ADH	2.1	1.3	0.6

[1] LAM concentration was based on Anthrone assay
[2] ΔVP8* protein concentration was based on Bradford assay

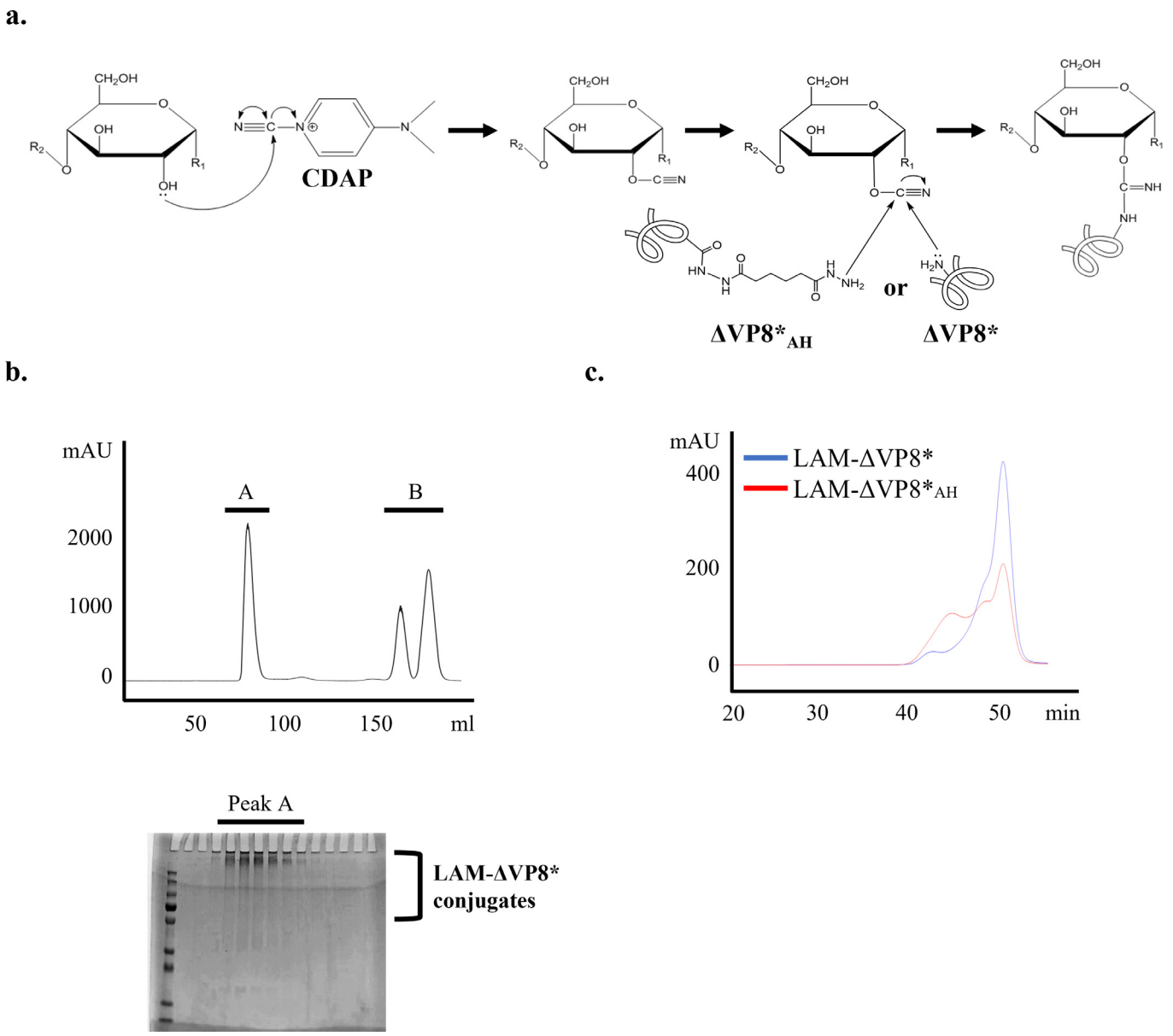

Fig. 3. Generation of LAM-ΔVP8* conjugates. a) Schematic diagram of LAM-ΔVP8* conjugates b) Separation of LAM-ΔVP8* by size-exclusion chromatography. After conjugate reaction, the mixture was applied onto a Sepharcyl S200 column (XK16/100). LAM-ΔVP8* conjugates were eluted in early fractions (peak A) owing to their high molecular weight, whereas the excess LAM and ΔVP8* were separated in the latter fractions (peak B). The peak A of the gel-filtration were analyzed by SDS-PAGE that is shown below the elution curve. c) SEC-HPLC profile of LAM-ΔVP8*$_{AH}$. OHpak SB-804 HQ and OHpak SB-806 HQ columns in series, 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% ACN, pH 7.2; 0.3 mL/min. flow rate.

lack of immunologic memory [56]. A conjugate vaccine platform is an established procedure to improve the immunogenicity of the polysaccharide antigen. Conjugation of the polysaccharide to a carrier protein can effectively convert a T cell-independent immune response to a T cell-dependent immune response, characterized by polysaccharide-specific isotype switching from IgM to IgG and generation of polysaccharide-specific memory B cells [39,57,58]. Traditionally, a few carrier proteins such as Tetanus toxoid (TT), DT, nontoxic diphtheria cross-reacting material (CRM_{197}), and meningococcal outer membrane complex (OMPC) have been used in a conjugate vaccine platform to optimize the immunogenicity of polysaccharide antigens [56]. However, the repeated use of carrier proteins causes immune interference such as carrier-induced epitope suppression and carrier-specific enhancement of T cell help [56,59]. Therefore, there is a significant need for novel carrier proteins.

Here, we demonstrated that not only did the conjugation of ΔVP8* to LAM induce enhanced anti-ΔVP8* IgG titers, but it also elicited polysaccharide specific IgG antibody responses in mice. ΔVP8*-based conjugates induced significantly elevated ΔVP8* antibody titers after the first dose; the result obtained is consistent with our previous findings in that the conjugate vaccine platform using Vi polysaccharide enhances immune responses to carrier protein in mice [60,61]. However, the same quantity of polysaccharide or ΔVP8* antigen alone did not mount an appreciable immune response even after booster doses because polysaccharides are T-independent antigens [62] and ΔVP8* is less immunogenic in mice [63]. LAM was linked to the proteins directly or with ADH to compare the effect of linker on conjugation and immunogenicity. Because cross-linking reagents possess different spacer lengths, binding specificities, flexibility/rigidity, and hydrophilicity, each of these linker characteristics can influence the immunogenicity

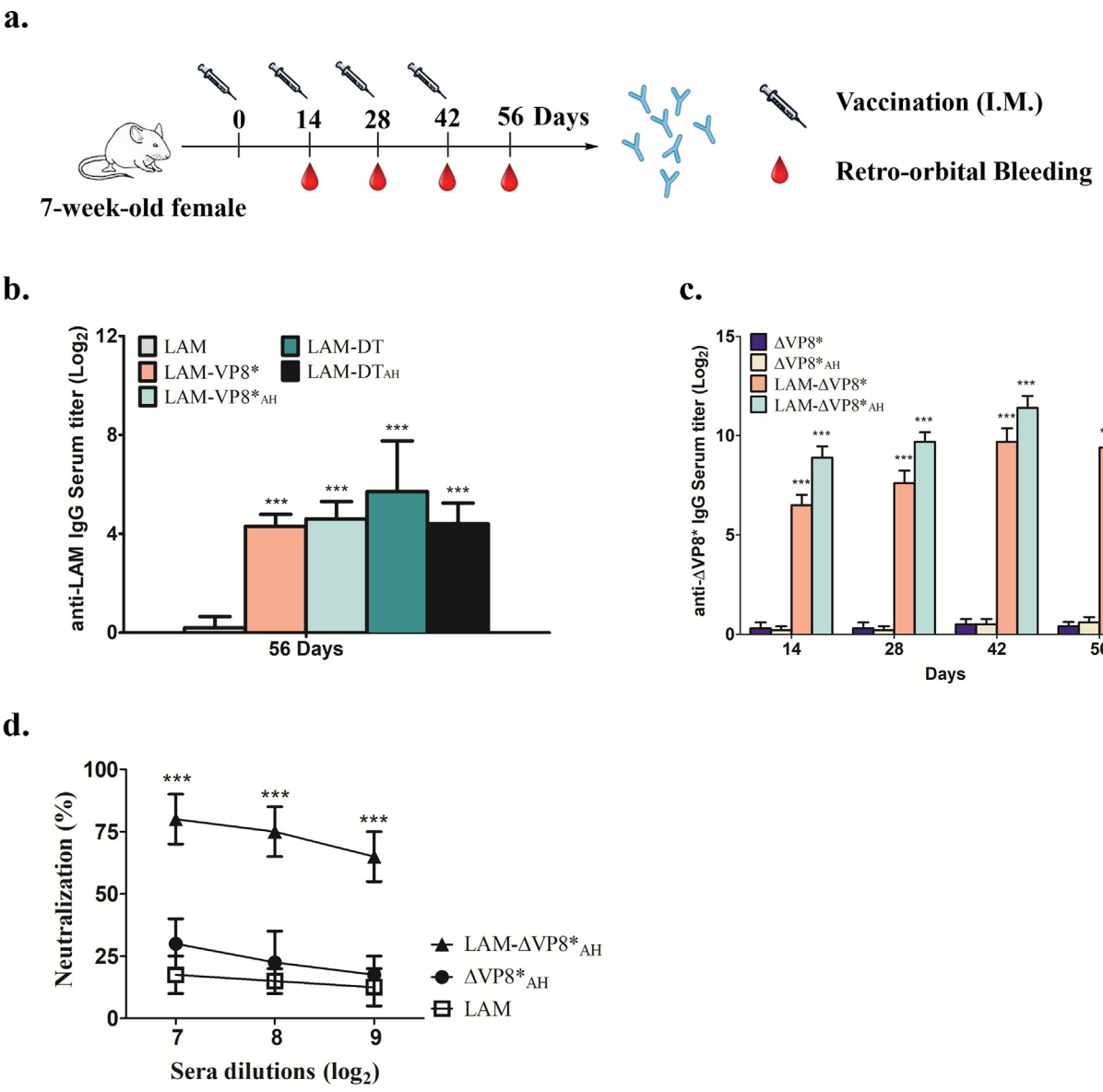

Fig. 4. Immunogenicity study with LAM-conjugates (a) A schematic diagram for intramuscular vaccination and bleeding. (b) Anti-LAM; (c) Anti-ΔVP8* antibody titer of conjugates; and (d) Neutralization analysis of sera from immunized mice; Days 56 immune sera were used; data are mean ± s.d.; two sample *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

of the resulting conjugates [64]. The protein recovery was not significantly altered by ADH derivatization (Table 1), and the comprehensive immunogenicity of the conjugate vaccines in mice was not significantly affected by the ADH derivatization.

In summary, our findings demonstrate that rotavirus spike protein ΔVP8* in glycoconjugate platform could play not only its traditional role as an immunogen, but also an additional role as a relevant carrier for carbohydrate antigens [56]. The data supports use of a viral peptide as a carrier protein in glycoconjugates and also further evaluation of the M.tb-rotavirus conjugate vaccine towards clinical development.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.09.067.

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