理學博士學位請求論文

A study on the rotavirus ΔVP8* protein as a carrier protein of polysaccharide-conjugate vaccines

로타바이러스 ΔVP8* 단백질의
다당류 결합백신 운반체로서의 가능성 연구

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서울大學校 大學院
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A study on the rotavirus ΔVP8* protein as a carrier protein of polysaccharide-conjugate vaccines

by

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A Thesis for the Degree of **Doctor of Philosophy**

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ABSTRACT

A study on the rotavirus ΔVP8* protein as a carrier protein of polysaccharide-conjugate vaccines

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Rotaviruses are a primary cause of acute gastroenteritis in infants and children under five years of age, resulting in 24 million outpatient visits, 2.3 million hospitalizations, and 200,000 deaths annually. Although vaccination is one of the most powerful ways to protect children against rotavirus disease, the efficacy of currently available live-, attenuated oral rotavirus vaccines is impaired in low- and middle-income countries where rotavirus vaccines are mostly needed. Among vaccine technologies, a conjugate vaccine platform is a promising strategy to overcome the poor immunogenicity of bacterial polysaccharide antigens in infants and children. A carrier protein in conjugate vaccines works not only as an immune stimulator to polysaccharide, but also as an immunogen; with the latter generally not considered as a measured outcome in real world.

Here, I probed the potential advantage of the conjugate vaccine platform to enhance immunogenicity of a truncated rotavirus spike protein ΔVP8* and the application of ΔVP8* as a relevant carrier protein. ΔVP8* was covalently conjugated to capsular polysaccharides of *Salmonella* Typhi (Vi polysaccharide) and *Mycobacterium tuberculosis* (lipoarabinomannan). Different conjugation methods have been applied to augment the immunogenicity of both antigens in the conjugate vaccine platform.

I revealed that the conjugate vaccine platform induced specific immune responses

against both antigens in immunized mice. The conjugate vaccines elicit high antibody titers and functional antibodies against rotavirus when compared to immunization with a single antigen. Through these experiments, I propose rotavirus spike protein ΔVP8* as a relevant carrier protein for a conjugate vaccine platform with demonstrated antigenic potential with the use of bivalent vaccine.

Keyword :

Rotavirus spike protein ΔVP8*, conjugate vaccine platform, carrier protein, carrier-induced epitope suppression, Mycobacterium tuberculosis, Salmonella Typhi

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Abbreviations

ADH: adipic acid dihydrazide

AM: arabinomannan

ARRIVE: Animal Research: Reporting of *in vivo* Experiments

AEX: anion exchange resin

αGlu: α-glucan

BCG: Bacile Calmetter- Guérin

BCR: B cell receptor

BMPH: N-β-maleimidopropionic acid hydrazide

BSA: bovine serum albumin

CDAP: 1-cyano-4-dimethylaminopyridinum tetrafluoroborate

CEX: cation exchange chromatography

CIES: carrier-induced epitope suppression

CPS: capsular polysaccharide

CRM197: nontoxic diphtheria cross-reacting materials

dsRNA: double-stranded RNA

DT: diphtheria toxoid

ECL: enhanced chemiluminescence assay

E. coli: Escherichia coli

EDAC: *N*-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme linked immunosorbent assays

EPI: Expanded Program on Immunization

FliC: flagellin monomer

FPLC: fast protein liquid chromatography

HBGA: histo-blood group antigen

HBV: Hepatitis B virus

Hib: *Haemophilus influenzae* type b

HPAEC-PAD: high-performance anion-exchange chromatography with pulsed amperometric detection

IACUC: Institutional Animal Care and Use Committee

I.M.: Intramuscular

IPTG: isopropyl-β-D-thiogalactopyranoside

IVI: International Vaccine Institute

LAM: lipoarabinomannan

LB: Lysogeny broth

MAPS: multiple antigen presenting system

MES: 4-morpholine ethanesulfonic acid

M.tb: Mycobacterium tuberculosis

MWCO: molecular weight cut-off

NSP: non-structural protein

NMR: nuclear magnetic resonacne

OMPC: outer membrane protein complex

PBS: phosphate buffered saline

PD: non-typeable *Haemophilus influenzae* protein D

PRNT: plaque reduction neutralization test

rEPA: recombinant non-toxic form of *Pseudomonas aeruginosa* exotoxin A

RV: rotavirus

RVA: group A rotavirus

SATA: N-succinimidyl S-acetylthioacetate

SBA: Serum Bactericidal activity

S.C.: subcutaneous

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC-HPLC: size exclusion liquid chromatography

***S*. typhi: Salmonella enterica serovar Typhi**

TB: Tuberculosis

TBS: Tris-buffered saline

TBS-T: TBS with 0.1% Tween® 20

Tcarbs: carbohydrate-specific T cells

TFF: tangential flow filtration

TNBS: 2,4,6-trinitrobenzene sulfonic acid

TT: Tetanus toxoid

VP: viral protein

ΔVP8*: truncated rotavirus

Vi: virulence capsular polysaccharide

CHAPTER I. Literature Review

1. Rotavirus (RV)

Enteric and diarrheal diseases are one of the leading causes of illness and death among children under 5 years of age with more than half a million deaths per year worldwide (Collaborators, 2018). Before the 1970s, the aetiologic agent in many cases of infantile gastroenteritis was not identified, but in 1973 rotavirus was first identified by Bishop and her colleagues when they observed wheel-shaped particles in duodenal biopsy samples from children with severe diarrhea (Bishop et al., 1973) and in fecal samples from children with acute diarrhea (Flewett et al., 1973). The name rotavirus is derived from its distinctive morphological appearance (the Latin word 'rota', which translates to wheel). Rotaviruses were soon recognized as a primary cause of life-threatening diarrhea in infants and children under 5 years of age globally, attributed to in 24 million outpatient visits, 2.3 million hospitalizations, and 200,000 deaths annually (Tate et al., 2012); rotavirus is responsible for 25% - 30% of all severe diarrhea case in children in both developed and developing countries (Mortality and Causes of Death, 2016).

1.1. Virus Structure

Rotaviruses belong to the family *Reoviridae*, which are non-enveloped double-stranded RNA (dsRNA) viruses. The icosahedral, three concentric capsids with a diameter of approximately 100 nm enclose a genome of 11 segments of dsRNA encoding six structural viral proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and six non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6) (Fig. 1). The structural proteins are found in virus particles; however, nonstructural proteins are found in infected cells, not in the mature virion because NSPs are involved in viral replication, morphogenesis and assembly (Estes et al., 2001). The dsRNA is contained by the innermost layer which consists of the scaffolding protein VP2, the RNA-dependent RNA polymerase VP1, and VP3 (a guanylyltransferase and methylase); together these proteins comprise the innermost core of rotavirus. The intermediate layer is composed of VP6 which is added onto VP2 to form double-layered particles. The external layer is made up of the viral surface VP7 glycoprotein and the protease-sensitive VP4 spike protein. VP4 is essential for the virus life cycle because of its receptor-binding and cell penetration function.

Treatment of the virus with trypsin greatly enhances its infectivity; trypsin

cleavage of VP4 produces the polypeptides VP5* and VP8* and mediates conformational changes on the VP4, which is linked to membrane penetration (Rodriguez et al., 2014). VP7 is a calcium-binding protein that mediates cell attachment via its interaction with integrins after the initial attachment of the virus to the cell surface (Graham et al., 2003).

Both outer capsid proteins induce neutralizing, protective antibodies and are responsible for the virus attachment to the host. The nonstructural proteins function as an antagonist of the innate immune response and include the viral enterotoxin NSP4 (Estes, 2007).

1.2. RV-host cell binding and entry

Upon entry in the body, RV infects and replicates primarily in the mature enterocytes of the villi and in enteroendocrine cells of small intestine. The infection results in disruption of the epithelial cell homeostasis causing self-limiting illness. The early cell attachment of RV is sequentially mediated by interaction with several cell surface molecules, using the virus surface protein VP4 and VP7. Upon the first contact, the distally located VP8* domain of the VP4 plays a critical role in interaction with glycans including sialic acid, gangliosides, histo-blood group antigens (HBGAs), and mucin cores. Additionally, Rotavirus attachment is also mediated by the outer capsid protein VP5* and VP7 binding to integrins and the heat shock proteins on the host cell surface.

Following RV-cell entry, viral replication and assembly take place in cytoplasmic viroplasms whose main components are the viral protein NSP2 and NSP5 (Campagna et al., 2013). Mature virus particles are released from cells either by cell lysis or by Golgi-independent non-classical vesicular transport; in consequence, RV infection results in villus atrophy, enhanced epithelial cell turnover, and apoptosis (Crawford et al., 2017).

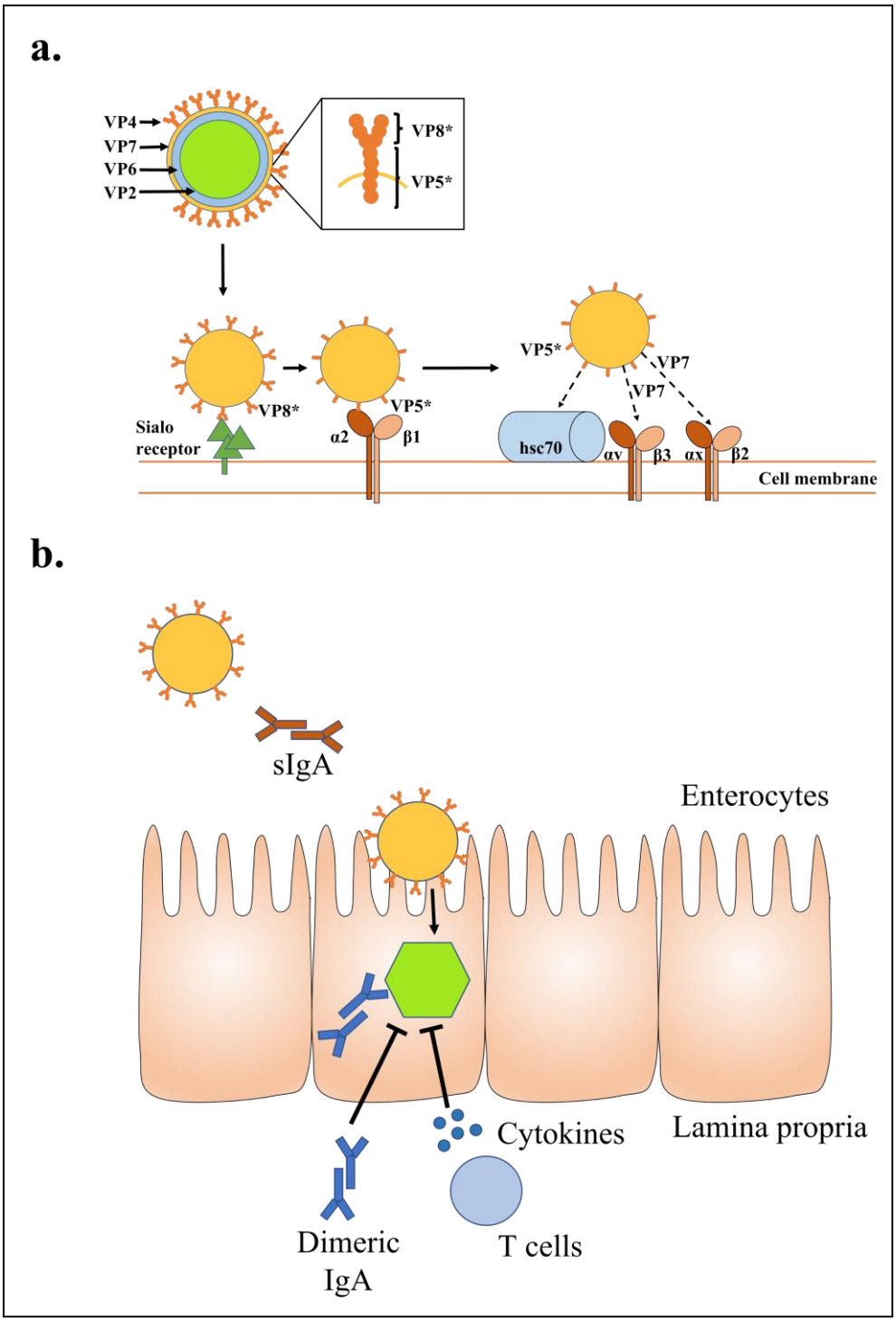


Figure 1. Schematic representation of a rotavirus virion. (a) A model for early rotavirus-cell interaction, (b) Potential mechanisms of rotavirus immunity (Angel et al., 2007; Lopez and Arias, 2004)

1.3. RV classification

Rotaviruses are classified into ten distinct groups (A to J) based on sequence and antigenic differences of the inner capsid protein VP6 (Fig. 2) (Banyai et al., 2017; Mihalov-Kovacs et al., 2015). Groups A-C are associated with acute gastroenteritis in both humans and animals, whereas groups D-J have been found only in animals (Saif and Jiang, 1994). Group A rotaviruses (RVA) are the most common cause of severe acute gastroenteritis in infants and young children. RVAs are further classified into different genotypes, based on sequence differences of two genes which encoded structural proteins, glycoprotein (G, or VP7) and protease-sensitive protein (P, or VP4) (Fig. 2). Currently, 36 G genotypes and 51 P genotypes of species RVA have been identified. P[8] (Wa strain) and P[4] (DS-1 strain) are the most common P types causing more than 91% of infections in both developed and developing countries (Santos and Hoshino, 2005). Geographical differences in RVA distribution have been observed; in Africa, P[6] (1076 strain) accounts for almost one-third of all P types (Ouermi et al., 2017). Human RVAs that show strong genetic homology with animal strains have been identified (Gentsch et al., 2005), and animal-to-human interspecies transmission can be predicted, particularly in low-income countries (Martella et al., 2010). Virus evolution is resulted from the accumulation of point mutations and genetic reassortments due to dual infection of individual cells by different species RVA.

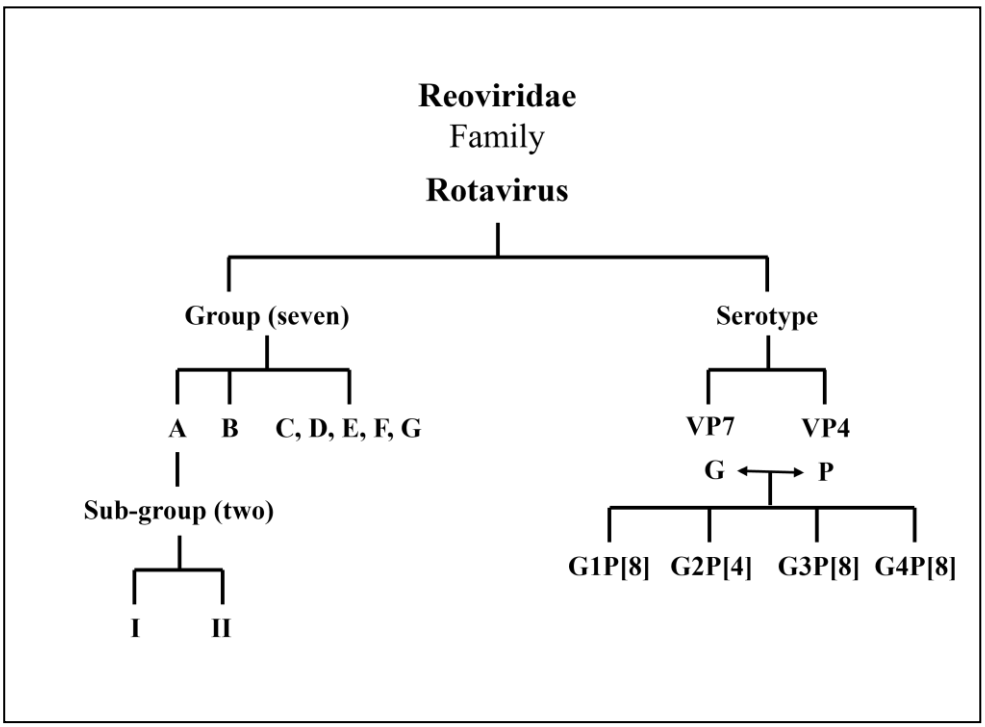


Figure 2. Rotavirus classification. Rotaviruses are classified into at least seven distinct groups (A to G) based on the antigenicity of the middle layer VP6. Group A rotaviruses are further subdivided into two subgroup (I and II). Rotavirus serotypes are based on neutralizing antibodies to VP7 glycoprotein (G) and VP4 protease cleaved protein (P).

1.4. Rotavirus vaccine

The introduction of rotavirus vaccines has reduced the burden of rotavirus diseases in many countries. In the early 1980s, live attenuated oral rotavirus vaccines were tested for proof-of-concept. The first vaccine, Rotashield (Wyeth, USA), was introduced into routine immunization programs of US children in 1998; however, the first licensed vaccine was withdrawn 9 months after approval because it was associated with a rare but life-threatening intestinal obstruction known as intussusception. Seven years and several million deaths later, live attenuated oral rotavirus vaccines, Rotarix (RV1; GSK) and RotaTeq (RV5; Merck), were introduced on the market in 2006. Rotarix and RotaTeq were developed from human monovalent G1P[8] and pentavalent human-bovine reassortment G1-G4 and P[8], respectively. These vaccines have led to a 90% reduction in hospitalization for diarrhea (Sartorio et al., 2020) and have been associated with a lower risk of intussusception than RotaShield. In 2018, two additional vaccines from India, Rotavac (Bharat) and Rotasiil (Serum Institute), were recommended by WHO for all children. Rotavac and Rotasiil were based on a naturally attenuated G9P[11] neonatal human rotavirus strain and a different rotavirus strain containing five single-gene reassortants with VP7 protein for G1-G4 and G9, respectively. Today, these vaccines are currently included in national immunization schedule in > 100 countries and have led to marked decreases in hospitalizations and deaths from diarrhea. While these vaccines have been highly effective with 80 – 90% efficacy in developed countries, their efficacies are impaired in low- and middle-income countries where rotavirus vaccines are mostly needed (Glass et al., 2021).

The incidence of rotavirus diarrhea is similar in developed and developing countries suggesting that improvement in sanitation and drinking water facilities is not sufficient to prevent transmission of RV. Malnutrition, micronutrient deficiencies, and enteric co-infection may contribute to the diminished immunogenicity and effectiveness of those attenuated vaccines (Desselberger, 2017).

1.5. Vaccine development to improve the immunogenicity of ΔVP8*

Although current rotavirus vaccination has been highly effective with 80–90% efficacy against severe rotavirus disease in high-income countries, the effectiveness

of rotavirus vaccines is impaired in low-income countries (Armah et al., 2010; Jonesteller et al., 2017; Madhi et al., 2010; Yen et al., 2011; Zaman et al., 2010). The cause of the difference remains incompletely understood; malnutrition, micronutrient deficiencies, and enteric co-infection may contribute to the diminished effectiveness of the attenuated vaccines (Desselberger, 2017). In addition, changes in rotavirus epidemiology may be another possible reason for the reduced vaccine outcomes (Jiang et al., 2017). Furthermore, the live attenuated vaccines may be associated with a low-level risk of intussusception (Yen et al., 2016). Accordingly, the development of more effective next-generation vaccines is urgently needed to control and prevent the disease.

Subunit vaccines contain only the antigenic parts of the pathogen, such as polysaccharide or protein that are necessary to elicit a protective immune response. In comparison to their whole-pathogen counterparts, subunit vaccines have demonstrated enhanced efficacy and minimal adverse effects in resource-deprived countries because the vaccines are not directly affected by microbiome composition or gut enteropathy (Jiang et al., 2008; Kirkwood et al., 2019). In addition, subunit vaccines offer advantages over live attenuated vaccines because those vaccines do not require complex storage or transport conditions and have large-production capacity (Lu et al., 2021). However, subunit vaccines are less prone to elicit strong immune responses and consequently require for the application of nanotechnology or molecular adjuvants to boost the immunity (Wang and Xu, 2020; Zhao et al., 2017).

Multiple studies have reported that a truncated rotavirus VP8* (ΔVP8*) subunit protein is the target antigen due to its ability to induce highly potent neutralizing antibodies, which confer strong protection against RV (Wen et al., 2012; Xia et al., 2016). However, many defined neutralizing antigens, including ΔVP8*, possess low immunogenicity due to their small sizes with low valences. To this end, a large, multivalent vaccine platform has been developed that could make it possible for small viral or bacterial antigens to be more immunogenic (Kaumaya et al., 1993; Nassal et al., 2005; Wang et al., 2013; Wang et al., 2014). For example, 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)

(Tan et al., 2011; Wen et al., 2014; Xia et al., 2018).

1.6. **Model for studying Rotavirus infection and immunity**

The understanding of the pathogenesis of both human and animal rotaviruses is based primarily on the use of animal models of rotavirus infection. Cow, pig, sheep, rabbit, and mouse are the animal model used to explain rotavirus infection, pathology, diseases, immune response, and vaccine efficacy. Although much of the early work on rotavirus pathogenesis was defined by large animals (cow, pig, and sheep), small animal models have been beneficial to explain the mechanisms through which the microbiota interacts with RV (Ciarlet et al., 2002). Although mice are one of the infection animal models for studying the immune response during rotavirus infection, only neonatal mice (≤ 2 weeks of age) are suitable as diarrhea model with symptoms of diarrhea and dehydration (Du et al., 2017). Most studies have been conducted with the inbred BALB/c strain because the strain shows 1,000 times more susceptibility to infection than C57BL/6 background.

2. *Salmonella* Typhi

Typhoid fever is a systemic bacterial infection caused by Salmonella enterica serovar typhi (*S*. typhi), usually through ingestion of contaminated food or water. Typhoid fever remains a major health problem with global incidence of an estimated 12 to 27 million cases and 200,000 typhoid-fever-related deaths per year. Current control strategies become increasingly difficult due to the emergence of multidrug resistance.

S. typhi is a human-restricted intracellular pathogen and has become a major threat to the society, particularly in low- and middle-income countries where proper water, sanitation and hygiene (WASH) are required. The ability of *S*. typhi to replicate inside human macrophages is key to its pathogenicity; it is spread systemically via infected macrophage, resulting in typhoid fever.

Vaccination appears the most promising strategy to control the disease. The virulence capsular polysaccharide (Vi) of *Salmonella* serovar Typhi is the target of a protective humoral immune response (Fig. 3). Conjugation of Vi to a carrier protein can effectively convert a T cell-independent immune response to a T cell-dependent immune response, characterized by IgM-to-IgG switching, a booster response, and sustained T cell memory to Vi (An et al., 2011; Costantino et al., 2011). Vi-recombinant non-toxic form of *Pseudomonas aeruginosa* exotoxin A (rEPA) and Vi-TT (Tetanus Toxoid) conjugate vaccines have been licensed and marketed in China and India (MacLennan et al., 2014; Wain et al., 2015), while Vi-DT (Diphtheria Toxoid) and Vi- nontoxic diphtheria cross-reacting material (CRM_{197}) are undergoing clinical trials for use in infants and children (An et al., 2012; Bhutta et al., 2014).

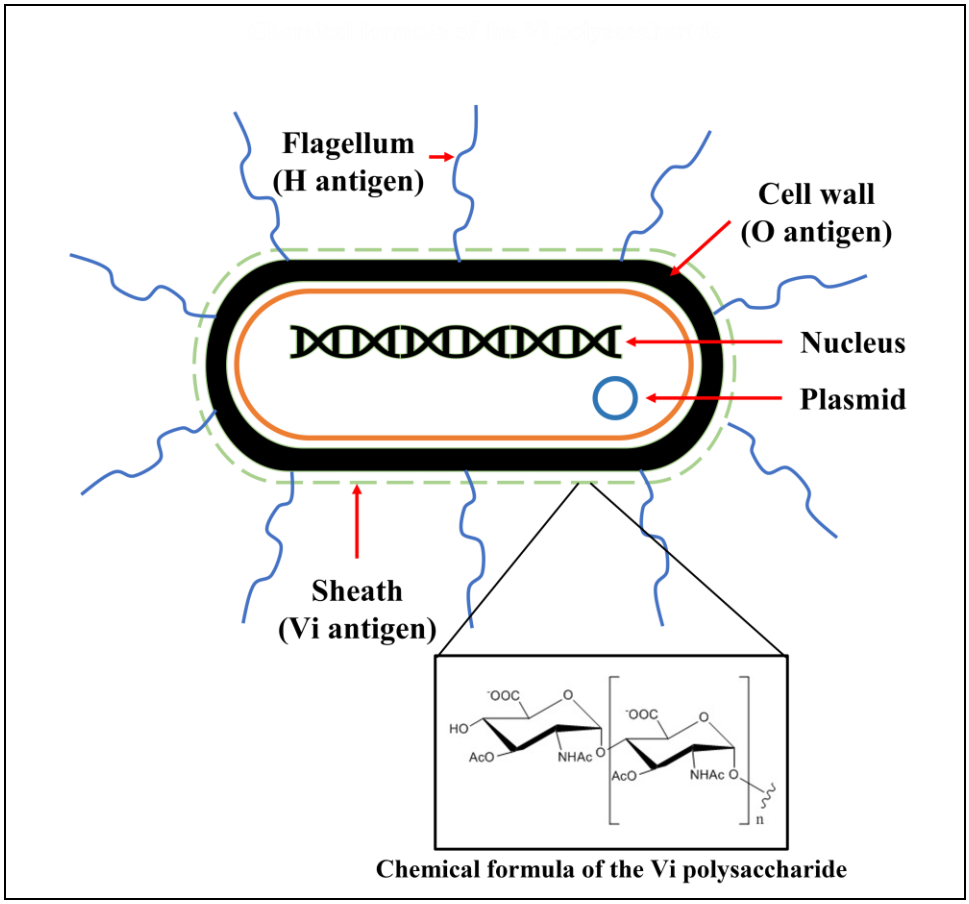


Figure 3. The structure of *Salmonella* typhi and chemical formula of the Vi polysaccharide (Hessel et al., 1999)

3. *Mycobacterium Tuberculosis*

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 (Dodd et al., 2017). 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 (Fine, 1995; Rodrigues et al., 1993). In parts, BCG vaccination has little effect on protection against adult pulmonary TB (Fine, 1995). Due to the global burden attributable to RV and TB in the pediatric population, the development of an effective vaccine against both pathogens is urgently needed to control and prevent the diseases.

Strategies for novel immunotherapy and vaccine development can be improved by a better understanding of all immune components involved in protecting against M.tb infection. Cell-mediated immunity is an important component to control mycobacterial infection (Cooper, 2009). However, recent immunological and genetic studies support that antibody-mediated immunity also plays a protective role against TB by enhancing both innate and cell-mediated immune response against TB (Achkar and Casadevall, 2013; Achkar and Prados-Rosales, 2018).

Antibody-mediated protection against M.tb can be established by: (1) passive transfer with antibodies against mycobacterial antigens shows the improved outcomes of experimental mycobacterial infections including prolongation in survival time, reduced dissemination, reduced tissue pathology, and reduced mycobacterial burden as measured by colony forming units; (2) the presence of antibodies against M.tb results in decreased susceptibility to infection and disease; and (3) deficits in humoral immunity and B-cell function cause increased susceptibility to disease (Achkar and Casadevall, 2013; Achkar et al., 2014).

Several mechanisms of humoral immunity to enhance the host defense against M.tb have been proposed. Vaccine-induced protective antibodies can be expected to promote ingestion by phagocytic cells and intracellular killing through FcR-mediated phagocytosis. Along these lines, antibodies to lipoarabinomannan (LAM) of M.tb promote bacterial opsonization and restrain intracellular growth (Chen et

al., 2016; de Valliere et al., 2005). Beyond opsonization, antibodies can also contribute to the host defense by promoting the clearance of polysaccharide or lipopolysaccharide which induces impaired immune responses through their immunomodulatory properties. Finally, many antibodies elicited by M.tb can contribute to host defense against TB through their dual nature as pro- and anti-inflammatory molecules. Antibodies mediating pro-inflammatory could induce bactericidal inflammatory response, while those that are anti-inflammatory could reduce tissue destruction (Achkar and Casadevall, 2013; Achkar et al., 2014).

Importantly, antibody-mediated immunity can provide protection against intracellular pathogens such as Salmonella, Chlamydia, Cryptococcus, and Histoplasma spp.; vaccines inducing humoral immunity have been developed and licensed for some of these pathogens such as Salmonella (Collins, 1974). These data could have important potential implications that humoral immune response may contribute to the control of TB disease.

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 (Fig. 4) (Daffe and Etienne, 1999; Kalscheuer et al., 2019). The major capsular polysaccharides of M.tb are α-glucan (αGlu) and LAM, respectively accounting up to 80% and 15% of the extracellular polysaccharide (Chatterjee et al., 1991; Chatterjee et al., 1992; Chatterjee and Khoo, 1998; Lemassu and Daffe, 1994). Mycobacterial capsular LAM and arabinomannan (AM) can each elicit high antibody responses that play a significant role in the defense against M.tb infection. The antibody-mediated immunity elicited by AM conjugates provides moderate protection in M.tb-infected mice (Prados-Rosales et al., 2017).

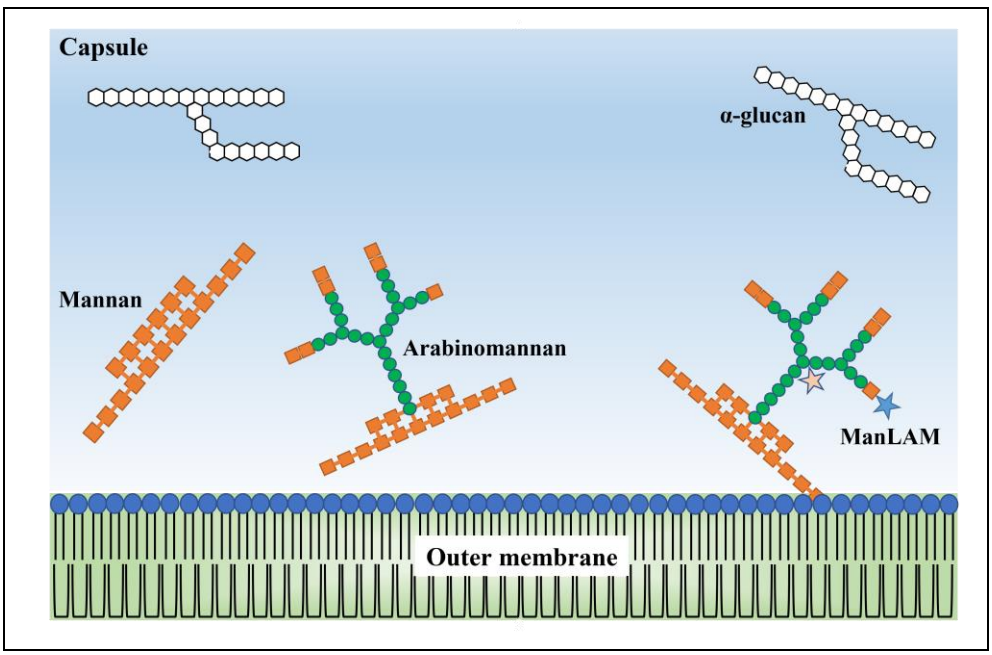


Figure 4. A current perspective of the cell envelope of M. tuberculosis (Angala et al., 2018)

4. **Conjugate vaccine**

The surface of bacterial pathogens is constituted with cell-surface polysaccharides that have very high molecular weights (often ≥ 100 kDa) and serve as a protective external layer for many bacterial. The capsular polysaccharides are important vaccine target because of not only their localization in bacteria, but also their roles in cellular recognition and signaling, including immune recognition and activation. Vaccines containing bacterial polysaccharides have been developed to induce a robust immune response against many pathogenic bacteria, such as meningococcus, *Haemophilus influenzae* type b, and *Streptococcus pneumococcus*. Although polysaccharide vaccines are immunogenic and protective in adults against serious infectious diseases, they fail to induce an antibody response and immunological memory in infants and children under two years of age, the population for whom the vaccines are mostly needed. Polysaccharides are defined as T-cell independent antigens which can directly stimulate B-cell differentiation into plasma cells by cross-linking B-cell receptors (Fig. 5a) (Pollard et al., 2009).

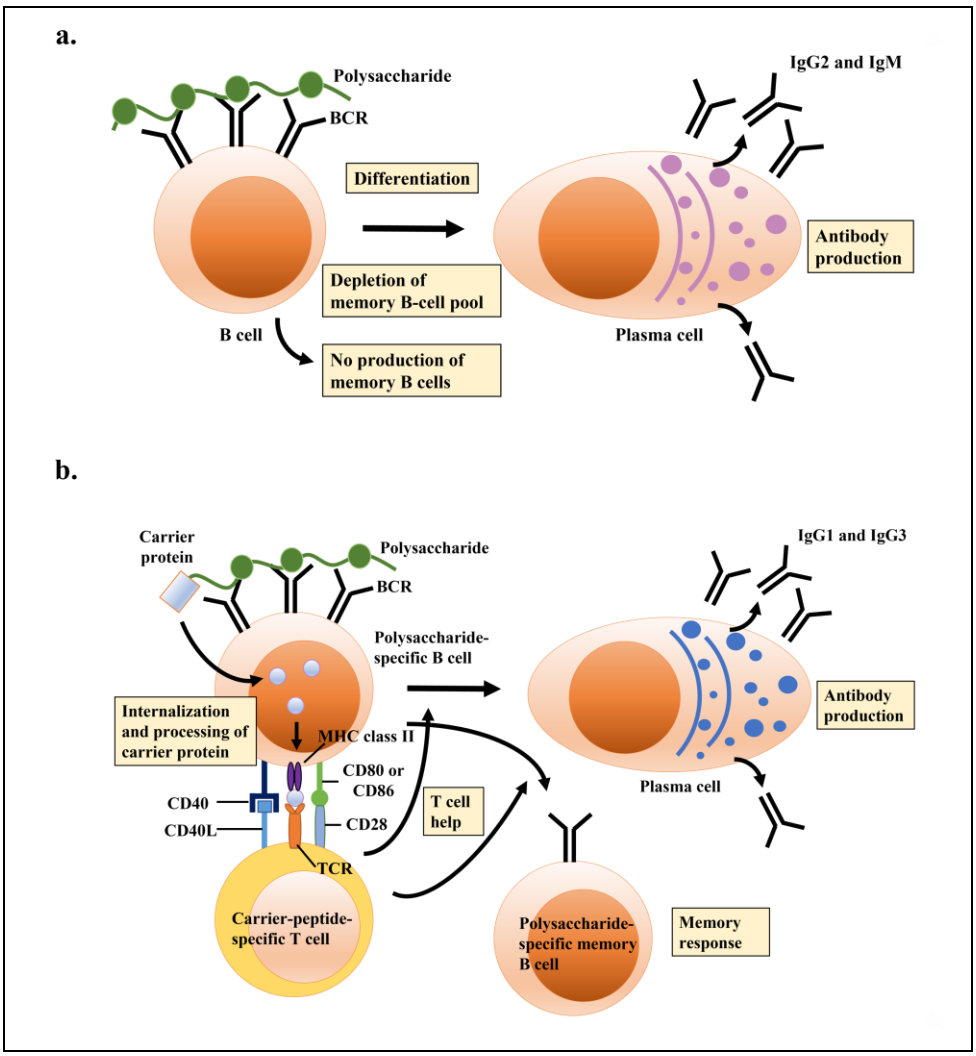


Figure 5. The immune response to polysaccharide and protein-polysaccharide conjugate vaccines. (a) Polysaccharides from the encapsulated bacteria that cause disease in early childhood stimulate B cell receptor (BCR) and drive the production of immunoglobulins. This process results in a lack of production of new memory B cells and a depletion of the memory B-cell pool, such that subsequent immune responses are decreased. (b) The carrier protein from protein-polysaccharide conjugate vaccines is processed by the polysaccharide-specific B cell, and peptides are presented to carrier-peptide-specific T cells, resulting in T-cell help for the production of both plasma cells and memory B cells (Pollard et al., 2009)

In contrast, protein-based, subunit vaccines can induce robust immune response, affinity maturation, and immunological memory through T cell-dependent immune response of B cells (Fig. 5b). In addition, CD4+ T cells can recognize the polysaccharide and acquire a memory response to the polysaccharide when the polysaccharide is conjugated to a carrier protein. Therefore, the approach of conjugating polysaccharides to proteins was used to overcome the low immunogenicity of polysaccharides in infants (Micoli et al., 2018). Classically, the antigen processing mechanism responsible for polysaccharide-protein conjugates was considered to be similar to the one for hapten-carrier conjugates. When hapten-carrier conjugates are introduced to the immune system, the carrier protein is digested by protease in the endosome and presented to the T cell receptor on classical peptide-recognizing $CD4^+$ T cells. However, an alternative mechanism has been recently proposed for $CD4^+$ T cell activation to the polysaccharide-protein conjugates. The polysaccharide-protein conjugates are processed as glycopeptide in the endosome of antigen-presenting cells. The peptide portion of the glycopeptide is presented via MHCII, resulting in the presentation of the covalently linked polysaccharide epitope. The polysaccharide stimulates a subset of $CD4^+$ T cell clones, designated carbohydrate-specific T cells (Tcarbs), and induces carbohydrate mediated $CD4^+$ T cell dependent immune response, regulating adaptive immune responses. This eventually leads to clonal activation of the cognate B cells and their differentiation into plasma cells producing antibodies, with isotype switching to IgG and affinity maturation, and subsequently into memory B cells (Avci et al., 2011).

TT, DT, or CRM_{197} as carrier proteins have shown to induce immune response against carrier proteins. The previous studies exploring the conjugate vaccine platform using Vi polysaccharide showed that some carrier proteins play the dual role as a carrier protein and a protective antigen (An et al., 2018; Kothari et al., 2015). Therefore, conjugate vaccine platform can lead to immune response against the pathogen not only from which the polysaccharide is derived but also from which the carrier protein is derived.

Currently licensed conjugate vaccines use a few carrier proteins [*i.e.* TT, DT, CRM_{197}, Outer Membrane Protein Complex (OMPC)] (Table 1). The limited number of carrier proteins implies repeated exposure to the same carrier, causing

various immune interference such as carrier-induced epitope suppression (CIES), carrier-specific enhancement of T cell help, and bystander interference (Avci et al., 2019; Pollabauer et al., 2009). In light of this, there is a need for alternative carrier proteins to overcome the impediments related to immune interference. Among several candidates, rEPA and a rationally designed recombinant protein containing strings of universal $CD4^+$ T-cell epitopes proved to be good candidates as carrier proteins (Tontini et al., 2016).

Tetanus Toxoid (TT)	Diphtheria Toxoid (DT)	Cross-Reactive Material 197 (CRM_{197})	*N. meningitidis* Outer Membrane Protein (OMP)	Non-Typeable *H. Influenzae* Derived Protein D (PD)
• Derived from Clostridium tetani • Inactivated with formalin • Purified with ammonium sulfate and filter sterilized prior to conjugation process	• Derived from Corynebacterium diphtheriae • Detoxified with formaldehyde • Purified by ammonium sulfate fractionation and diafiltration	• Enzymatically inactive, nontoxic mutant of diphtheria toxin • Requires no formaldehyde detoxification • Obtained at near 100% purity	• Outer membrane protein complex derived from N. meningitidis serogroup B strain 11 • Purified by detergent extraction, ultracentrifugation, diafiltration, and sterile filtration	• Antigenically conserved surface lipoprotein found in H. influenzae • Used in a nonacylated, antigenically active form
140 kDa	63 kDa	63 kDa	37 kDa	42 kDa

Table 1. List of current licensed carrier proteins (Knuf et al., 2011)

5. The aims of this study

Conjugate vaccine platform is a promising strategy to overcome the poor immunogenicity of bacterial polysaccharide antigens in infants and children. A carrier protein in conjugate vaccine works not only as an immune stimulator to polysaccharide, but also as an immunogen; with the latter generally not considered as a measured outcome in real world. For example, Affinivax's novel vaccine platform, the Multiple Antigen Presentation System (MAPS), uses pneumolysin as a carrier protein. The MAPS vaccine technology leads to comprehensive B- and T-cell immunity against both polysaccharides and protein components (Zhang et al., 2013). In addition, *Salmonella* Typhimurium flagellin monomer (FliC) is also used as a self-adjuvating carrier protein in conjugate vaccine platform (Chiu et al., 2020). This study aims to assess the potential of a conjugate vaccine platform to improve the immunogenicity of a truncated rotavirus spike protein ΔVP8*; this work opens possibilities for the use of ΔVP8* as a carrier protein of polysaccharide-conjugate vaccines.

In addition, optimal and efficient process development is required for safe, effective and affordable vaccines. Here, I aim to develop the optimal purification strategy for ΔVP8* and chemical conjugate strategies for the development of ΔVP8*-based conjugate vaccine.

Chapter II. Method and Material

1. Bacterial strains, plasmids and culture conditions

Escherichia coli (*E. coli*) strains were cultured in Lysogeny broth (LB) at 37°C, unless otherwise indicated. The following supplements were added if necessary: Kanamycie, 50 μg ml^{-1}; isopropyl-b-D-1-thiogalactopyranosie (IPTG), 1 mM. DNA sequences encoded for amino acids 64 to 223 of VP8*-P[4], 64 to 223 of VP8*-P[6], or 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 *E. coli* (strain BL21, DE3). Virulent M.tb strain H37Rv (ATCC27294) was cultured in Sauton's media as previously described (Dinadayala et al., 2008). 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.

2. Recombinant protein production

Recombinant proteins were expressed in *E. coli* BL21 (DE3) as described previously (Wen et al., 2012). Briefly, *E. coli* BL21 (DE3) cells harboring the expression vectors were grown at 37°C until absorbance at 600nm reached 0.6. The expression of tag-free ΔVP8* proteins were induced by the addition of 1 mM IPTG at 18°C overnight. The recombinant *E. coli* cells harboring ΔVP8* proteins were collected by centrifugation and lysed by sonication into 20mM Tris buffer (pH7.6). Purification of the proteins was carried out using resins of ion exchangers (Merck) according to manufacturer's instructions.

3. SDS-PAGE and immunoblot

Quality of the purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% separating gels, followed by immunoblotting. The gels were stained with SimplyBlue™ Safestain Solution (Invitrogen, USA). For immunoblotting, the proteins were transferred to a 0.2μm nitrocellulose membrane using Trans-Blot Turbo Transfer pack and Trans-Blot Turbo Blotting System (Bio-Rad, USA). The membrane was block with Tris-buffered saline (TBS) containing 5% non-fat milk (w/v) and incubated at room

temperature for 1h followed by rinsing three times with TBS with 0.1% Tween® 20 (TBS-T). The membrane was applied at 4°C overnight with anti-rotavirus polyclonal antibody (AB1129; Merck, Germany) and followed by rabbit anti-goat IgG conjugate (Southern Biotech, USA). Immunoreactive proteins were detected using enhanced chemiluminescence assay (ECL) (Thermo Scientific, USA).

4. Purification of recombinant proteins

Ion exchange chromatography using anion exchange resin (AEX), Fractogel EMD DEAE (Merck, Germany) resin in XK16/20 column with 16cm 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 20mM Sodium Acetate (pH5) buffer by Tangential Flow Filtration (TFF) using 5kDa molecular weight cut-off (MWCO) membrane cassettes for further purification. Subsequently, 5kDa retentate was loaded on the cation exchange chromatography (CEX) using Eshmuno CPX (Merck, Germany) resin in XK16/20 column with 15cm bed height, followed by washing of the column using 5 CVs of 20mM Sodium Acetate (pH5) buffer to remove unbound proteins. The bound proteins were eluted using 10 CVs of 20mM Sodium Acetate buffer with 1M 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 5kDa membrane cassettes. The protein concentration of the samples was determined by Bradford assay. 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). Quantity of the purified protein was measured by Bradford method 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.

5. Polysaccharide isolation

Capsular αGlu was isolated from liquid culture supernatant (Sauton's media). Briefly, 750mL of culture supernatants were filter-sterilized using a 0.2 μm filter

(Sartopore®2 Midicaps®, Sartorius) to remove intact bacteria. The solution was subsequently concentrated to a retentate volume of 50mL and diafiltrated against 0.01M NH_4Cl (pH8.3) using 100kDa MWCO cellulose membrane (Sartocon® Slice Hydrosart® Cassette, Sartorius). Ion exchange chromatography using a AEX, Fractogel EMD DEAE (Merck, Germany) resin in XK16/20 column with 16cm bed height, was performed in negative (flow-through) mode to separate αGlu from impurities. The fractions in flow-through were pooled, concentrated, and diafiltrated initially against 0.01M NH_4Cl (pH8.3) and subsequently against distilled water using 100kDa MWCO cellulose membrane. The purified αGlu was checked for the carbohydrate composition and purity using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and ^1H nuclear magnetic resonance (NMR) analysis, respectively. 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.

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 (Nigou et al., 1997). The cell pellet was suspended in 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 15min (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 3.5kDa; Spectrum LAB., USA). It was initially freeze-dried and subsequently resuspended in 10 mM Tris (pH8.0), 0.2M 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 1M NaCl and water for several days. The purifed 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 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.

6. Conjugation

Vi polysaccharide were conjugated to three different serotypes of ΔVP8* with ADH linker (Vi-ΔVP8*-P[4], P[6] and P[8]). αGlu and LAM were conjugated to ΔVP8* and DT with and without ADH linker, resulting in 4 different ΔVP8*-based conjugates (αGlu-ΔVP8*, αGlu-ΔVP8*$_{AH}$, LAM-ΔVP8*, or LAM-ΔVP8*$_{AH}$) and other 4 different DT-based conjugates (Glu-DT, αGlu-DT$_{AH}$, LAM-DT, or LAM-DT$_{AH}$).

6.1. Carrier proteins derivatized with ADH linker

Briefly, aspartic or glutamic acid residues of the purified ΔVP8* were modified with adipic acid dihydrazide (ADH) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) to achieve an efficient coupling of ΔVP8* (Kossaczka et al., 1997). The reaction was performed in 4-morpholine ethanesulfonic acid (MES) buffer (80mM, pH5.1) at room temperature for 1h, and then the excess linkers were removed by spin desalting column. The derivatized VP8* (ΔVP8*$_{AH}$) was filter sterilized through 0.2μm syringe filter. protein content and free hydrazide content were determined by Bradford assay and 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay, respectively.

6.2. Conjugation of Vi polysaccharide to ΔVP8*

Vi-ΔVP8* conjugates were prepared as previously described (An et al., 2018;

Kothari et al., 2014). 8mg of Vi was dissolved in 4mL of MES buffer (80mM, pH 4.9), and 250μL of 50 mg/mL EDAC was added and mixed with agitation for 5min at room temperature. Then 8mg of ΔVP8* was added to the mixture, and the reaction was performed with agitation at room temperature for 2h. The mixture was then dialyzed in PBS for 2 days. To isolate the conjugates from the free Vi or ΔVP8*$_{AH}$, the dialyzed mixture was separated using Fast protein liquid chromatography (FPLC) on a Sephacryl S-1000 (GE Healthcare, USA) resin in XK16/100 column with 90cm bed height. Each fraction was then assayed for polysaccharide and protein contents by Hesterin and Bradford assays, respectively. The average size of the Vi-ΔVP8* conjugates was determined using SEC-HPLC (Agilent 1260 LC system, USA) equipped with OHpak SB-804 HQ and OHpak SB-806 HQ columns in series (Shodex, Japan).

6.3. Conjugation of mycobacterial polysaccharides to carrier proteins

Conjugation of mycobacterial polysaccharides to carrier proteins was prepared as described previously (Lees et al., 2020; Lees et al., 1996; Shafer et al., 2000). Briefly, 4 mg of mycobacterial polysaccharides (αGlu: 4.5 mg/ml; LAM: 2.3 mg/mL) were dissolved in distilled water and mixed with agitation at 4°C overnight. At t = 0, 1-cyano-4-dimethylaminopyridinum tetrafluoroborate (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 1mg of LAM. At 30 s, 0.1M 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 (1mg/mL), ΔVP8*$_{AH}$ (2.7 mg/mL), DT (5.9 mg/mL), or DT$_{AH}$ (2.3 mg/mL) in 0.1M 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 206nm and 280nm. 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 (50kDa MWCO, Millipore, Germany), and filtered through 0.2 μm filter.

7. Mouse immunization

BALB/c mice at about six weeks of age were purchased from KOATECH (Korea) and maintained under specific pathogen-free conditions at the Animal facility of International Vaccine Institute (IVI). For Vi-ΔVP8* conjugate study, they were randomly divided into 9 groups of 10 mice each (N = 10), and each group was immunized with one of the following immunogens: (1) a mixture of the three Vi-ΔVP8* conjugates, representing P[4], P[6] and P[8] rotaviruses, in equal amount of Vi (5 μg each) as a multivalent vaccine at 3.6 μg total ΔVP8*/mouse/dose; (2) the Vi-ΔVP8* of P[4] type at 1.0 μg ΔVP8*/mouse/dose; (3) the Vi-ΔVP8* of P[6] type at 1.1 μg ΔVP8*/mouse/dose; (4) the Vi-ΔVP8* of P[8] type at 1.5 μg ΔVP8*/mouse/dose; (5) ΔVP8* of P[4] type at 1.0 μg/mouse/dose; (6) ΔVP8* of P[6] type at 1.1 μg/mouse/dose; (7) ΔVP8* of P[8] type at 1.5 μg/mouse/dose; (8) the Vi polysaccharide without the ΔVP8* antigens at 5 μg/mouse/dose as a control; and (9) PBS as vaccine diluent control. Immunogens in 150μL volumes were administered subcutaneously (S.C.) three times at 2-week intervals. Blood samples were taken at 14, 28, and 42 days from retro-orbital bleeding, allowing to clot for 30min., and centrifuged at 1,200xg at 4°C.

For mycobacterium polysaccharide-ΔVP8* conjugate study, Groups of 10 mice were injected with one of the following conjugates: (1) the αGlu-ΔVP8* at 10 μg/dose of αGlu and 1.3 μg/dose of ΔVP8*; (2) the αGlu-ΔVP8*$_{AH}$ at 10 μg/dose of αGlu and 1.8 μg/dose of ΔVP8*$_{AH}$; (3) the αGlu-DT at 10 μg/dose of αGlu and 1.0 μg/dose of DT; (4) the αGlu-DT$_{AH}$ at 10 μg/dose of αGlu and 1.9 μg/dose of DT$_{AH}$; (5) the LAM-ΔVP8* at 10 μg/dose of LAM and 8.1 μg/dose of ΔVP8*; (6) the LAM-ΔVP8*$_{AH}$ at 10 μg/dose of LAM and 9.9 μg/dose of ΔVP8*$_{AH}$; (7) the LAM-DT at 10 μg/dose of LAM and 7.2 μg/dose of DT; (8) 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 αGlu, 10 μg/dose of LAM or 5 μg/dose of each carrier proteins. Immunogens in 200μL volumes were intramuscularly (I.M) 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 (IVI; Seoul, Korea). Experiments were designed and reported with reference to the Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines (Kilkenny et al., 2010).

8. Enzyme linked immunosorbent assays (ELISA)

ELISA was utilized to define Vi- or ΔVP8*-specific antibody titers using the method previously described (Cui et al., 2010). Briefly, Vi polysaccharide or ΔVP8* proteins of P[4], P[6] and P[8] rotaviruses were coated on 96-well plates at 1 μg/mL. After blocking with 1% (w/v) BSA, plates were incubated with mouse sera at serial 2-fold dilutions. Bound antibodies were measured by goat-anti-mouse IgG-Alkaline phosphate conjugate (1:5,000; Southern Biotechnologies, USA). Antibody titers were described as the maximum dilution of sera that exhibited at least cut-off signals of OD_{450} = 0.15.

9. Plaque reduction neutralization test (PRNT)

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 1h 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 cells compared with non-neutralized, serum free control as previously described (Hoshino et al., 1984).

10.Statistical analysis

Statistical differences among data sets were determined by GraphPad Prism 6 (GraphPad Software, Inc, USA) through a paired *t* test. Differences were considered non-significant (ns) for p-values >0.05 (marked as “ns”), significant for

p-values <0.05 (marked as *), highly significant for p-values <0.01 (marked as **) and extremely significant for p-values <0.001 (marked as ***), respectively.

Chapter III. Antigen purification and derivatization of ΔVP8* with ADH

1. Purification and specificity of Mycobacterial capsular polysaccharide

αGlu and LAM are capsular polysaccharides that can be found abundantly in the culture filtrate and cell envelope of M.tb H37Rv, respectively (Daffe et al., 2014). αGlu is the dominant capsular polysaccharide, composed of linear α-(1-4)-D linked glucose unit and having an apparent molecular mass of 1.3×10^{7} Da (Figure 6a) (Dinadayala et al., 2008; Geurtsen et al., 2009). 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 purified αGlu was checked for purity using HPAEC-PAD and ^{1}H NMR analysis. Mannose or arabinose was not detected by HPAEC-PAD analysis of purified αGlu (Figure 6b). Subsequently, ^{1}H NMR spectra of the purified αGlu showed two anomeric signals at 5.4 and 4.99 ppm, typical of α-(1-4)-glucosyl and α-(1-6)-glucosyl linkages, respectively (Figure 6c). The purified αGlu was identified by immunoblotting with CS-35 antibody (Figure 6d) (Dinadayala et al., 2004; Geurtsen et al., 2009; Ortalo-Magne et al., 1995). The molecular mass of LAM was estimated to be around 13kDa (Figure 7a) (Correia-Neves et al., 2019; Kaur et al., 2008; Shi et al., 2009). HPAEC-PAD profile of acid hydrolyzed LAM indicated that Man and Ara were the dominant products (Figure 7b) (Khoo et al., 2001). The purified LAM was identified by immunoblotting with CS-35 antibody (Figure 7c and d)

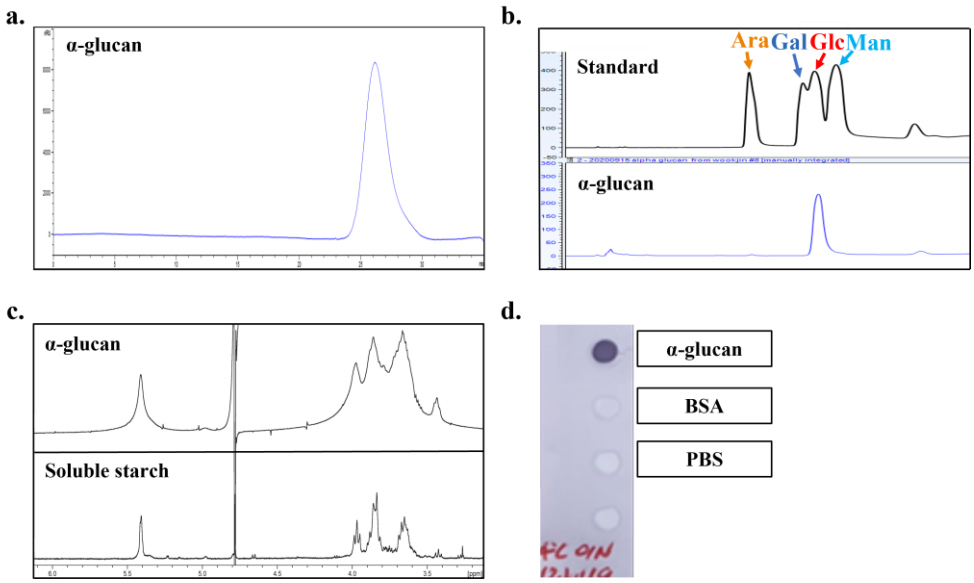


Figure 6. The physicochemical properties of αGlu from M.tb. The purified αGlu was analyzed using (a) SEC-HPLC with Shodex SB-806 column (100mM NaH_2PO_4, 100mM NaCl, 5% CH_3CN, pH7.2; 0.3mL/min), (b) HPAEC-PAD for carbohydrate analysis (Arabinose: Ara, Galactose: Gal, Glucose: Glu and Mannose: Man), (c) ^{1}H-NMR spectrum of the αGlu and starch and (d) Dot-blot followed by immunoblotting with CS-35 antibody

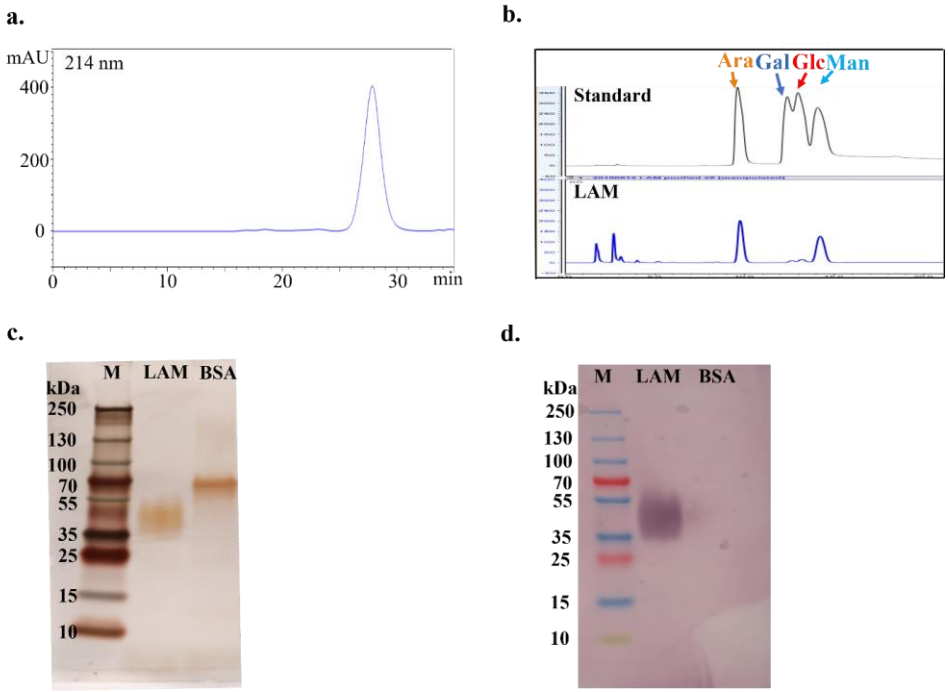


Figure 7. The physicochemical properties of LAM from M.tb. The purified LAM was analyzed using (a) SEC-HPLC with TSK gel 3000 PW_{XL} column (100mM NaH_2PO_4, 100mM NaCl, 5% CH_3CN, pH7.2; 0.3mL/min), (b) HPAEC-PAD for carbohydrate analysis (Arabinose: Ara, Galactose: Gal, Glucose: Glu and Mannose: Man), (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).

2. Expression, purification, and specificity of ΔVP8*

The recombinant ΔVP8* protein with P[4], P[6] or P[8] specificity was expressed in the E.coli system. The IPTG-induced bacteria were then collected by centrifugation and lysed to release soluble proteins. The ΔVP8* proteins were purified by two-step ion exchange chromatography. The yields of three recombinant proteins reached ~50 mg/L of culture. After purification, all the ΔVP8* proteins showed a single band of 18-20 kDa on SDS-PAGE and was eluted as a single peak in SEC-HPLC (Figure 8). 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.

Aspartic or glutamic acid residues of the purified ΔVP8* were modified with ADH in the presence of EDAC to achieve an efficient coupling of ΔVP8* to Vi (Figure 9a). $\Delta VP8^*_{AH}$ showed a broad peak at similar retention time on SEC-HPLC as the ΔVP8*, indicating no cross-linking of the VP8* had occurred (Figure 9b). Derivatization of ΔVP8* ($\Delta VP8^*_{AH}$) resulted in four to six ADH linkers per ΔVP8* (Figure 9c). The levels of modification were determined by TNBS assay.

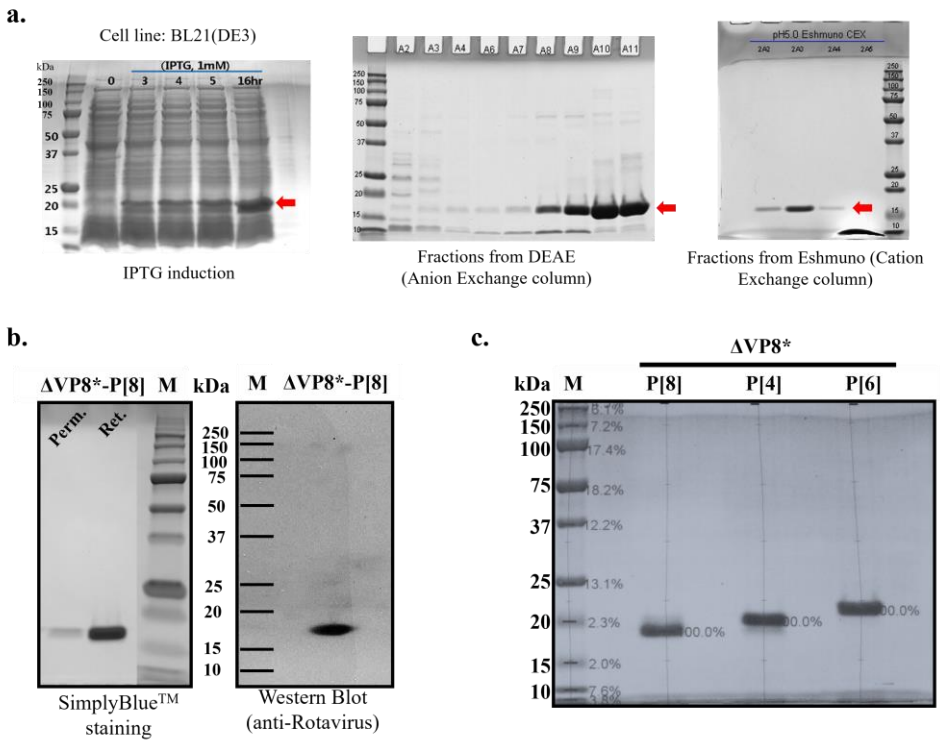
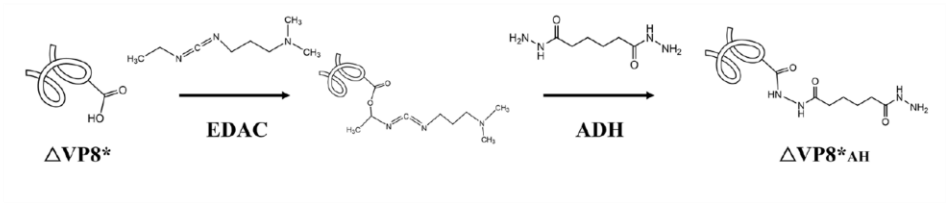
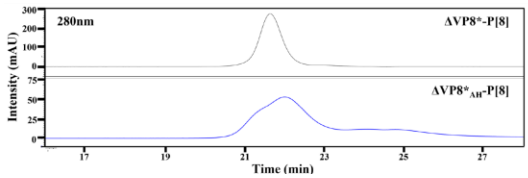


Figure 8. Expression and purification of the recombinant ΔVP8* proteins. (a) SDS-PAGE profiles of the expression of ΔVP8* upon IPTG induction, SDS-PAGE result of the anion exchange chromatography and the cation exchange chromatography, (b) SimplyBlue™ stained SDS-PAGE (Ret.: Retentate from 5 kDa TFF, Perm.: Permeate from 5 kDa TFF, and M: Marker) and Western blot analysis of ΔVP8*-P[8] probed with anti-Rotavirus antibody (AB1129), (c) SimplyBlue™ stained SDS-PAGE of the three purified ΔVP8*

a.



b.



c.

P types	ADH linkers per ΔVP8*
VP8*-P[4]	4
VP8*-P[6]	6
VP8*-P[8]	5

Figure 9. Preparation of ADH derivatized ΔVP8*-P[8] and its physicochemical characteristics. (a) Schematic diagram of ADH derivatization; EDAC activates ΔVP8* by reacting with carbonyl groups on amino acids, (b) SEC-HPLC profile of ΔVP8* before and after ADH derivatization. OHpak SB-804 HQ column, 0.1M NaCl, 0.1M NaH_2PO_4, 5% ACN, pH 7.2; 0.3 mL/min. flow rate, (c) The average number of ADH linkers on ΔVP8*. The concentration of protein and primary amine was measured by Bradford assay and TNBS assay, respectively.

Chapter IV. Development of a bivalent conjugate vaccine candidate against rotavirus and tuberculosis

1. 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. Because the consecutive administration of the same carrier protein may interfere the immune response to carbohydrate in conjugate vaccine platform, the identification of novel carrier proteins is important for the development of a glycoconjugate vaccine.

Among carrier proteins currently used in licensed conjugate vaccines, non-typeable *Haemophilus influenzae* protein D has been used as an antigenically active carrier protein. Several studies also indicate that some carrier proteins provide B cell epitopes, along with T cell helper epitopes.

Herein I investigated the dual role of truncated rotavirus spike protein ΔVP8* as a carrier and a protective antigen. Capsular polysaccharides, α-glucan (αGlu) and lipoarabinomannan (LAM) purified from *Mycobacterium tuberculosis* (M.tb), were 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* could be a relevant carrier protein for glycoconjugate vaccine and the glycoconjugates consisting of ΔVP8* with αGlu or LAM are effective bivalent vaccine candidates against rotavirus and tuberculosis.

2. 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 (Collaborators, 2018; Parashar et al., 2006; Tate et al., 2012). At present, RotaTeq®, Rotarix®, ROTAVAC®, and ROTASIIL® are the most widely used live attenuated oral vaccines, owing to 80%-90% vaccine efficacy especially in developed countries (Yen et al., 2011). These vaccines are currently included in national immunization schedules in >100 countries worldwide (WHO, 2020). However, the live attenuated oral vaccines showed impaired protection efficacy in developing countries where rotavirus vaccines are mostly needed (Armah et al., 2010; Jonesteller et al., 2017; Madhi et al., 2016; Zaman et al., 2010). Poor nutrition, micronutrient deficiencies, and concurrent infection with other enteric pathogens may contribute to the diminished effectiveness of those attenuated vaccines (Desselberger, 2017). In addition, there is a small increased risk of intussusception in vaccinated infants (Yen et al., 2016).

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 (Dodd et al., 2017). 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 population (Fine, 1995; Rodrigues et al., 1993). In parts, BCG vaccination has little effects on protection against adult pulmonary TB (Fine, 1995). Due to the global burden attributable to RV and TB in the pediatric population, the development of an effective vaccine 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*) (Prasad et al., 1990). A truncated VP8* protein, termed ΔVP8*, was reported to induce heterotypic neutralizing antibodies upon parenteral immunization in small animal models (Wen et al., 2012). 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) (Tan et al., 2011; Wen et al., 2014; Xia et al., 2018). 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 (Groome et al., 2020; Groome et al., 2017).

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 infant under two years of age (Astronomo and Burton, 2010; Avci and Kasper, 2010; Barrett, 1985; Coutinho and Moller, 1973). However, covalent linkage of the carbohydrate antigens to immunogenic carrier proteins results in glycoconjugates which elicit booster response to the carbohydrate antigens (Avery and Goebel, 1929; Goebel and Avery, 1929). 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 (Daffe and Etienne, 1999; Kalscheuer et al., 2019). The major capsular polysaccharides of Mycobacterium Tuberculosis (M.tb) are α-glucan (αGlu) and lipoarabinomannan (LAM), respectively accounting up to 80% and 15% of the extracellular polysaccharide (Chatterjee et al., 1991; Chatterjee et al., 1992; Chatterjee and Khoo, 1998; Lemassu and Daffe, 1994). 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 (Chen et al., 2020; Hamasur et al., 1999; Prados-Rosales et al., 2017; Schwebach et al., 2002). Although conjugate vaccine platform was developed to enhance the immunogenicity of carbohydrate antigens, a carrier protein may also induce an immune response against itself (Avci et al., 2011; Broker et al., 2017; Lees et al., 1994). 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 (Broker et al., 2017).

Herein, I developed glycoconjugates, employing a chemical conjugation of ΔVP8* to Mycobacterium capsular polysaccharide, αGlu and LAM. The following conjugates were developed, using two different chemical strategies: direct conjugation using EDAC (αGlu-ΔVP8* and LAM-ΔVP8*) and indirect conjugation using ADH (αGlu-ΔVP8*$_{AH}$ and LAM-ΔVP8*$_{AH}$). In addition, I also generated the following DT-based conjugates: αGlu-DT, LAM-DT, αGlu-DT$_{AH}$ 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 ΔVP8*-based conjugates in mice. The results showed that ΔVP8*-based 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. Therefore, this 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.

3. Results

3.1. Mycobacterial polysaccharides conjugates.

As illustrated in Figure 10a, glycoconjugates were synthesize with 4 mg of αGlu (4.5 mg/mL) and LAM (2.3 mg/mL) at a ratio polysaccharide/protein of 1:1 (w/w). The chromatogram profile showed that conjugates eluted earlier than either unconjugated polysaccharide or unconjugated ΔVP8*, confirming the larger size of the conjugate with respect to unconjugated components (Figure 10b and c). The large size of the conjugates resulted in a typical smear at the top of 12% SDS-PAGE (Figure 10b and c) and the decreased retention time in SEC-HPLC (Figure 10d and e). The amount of polysaccharide and protein in the ΔVP8*-based conjugates and the DT-based conjugates is shown in Table 2.

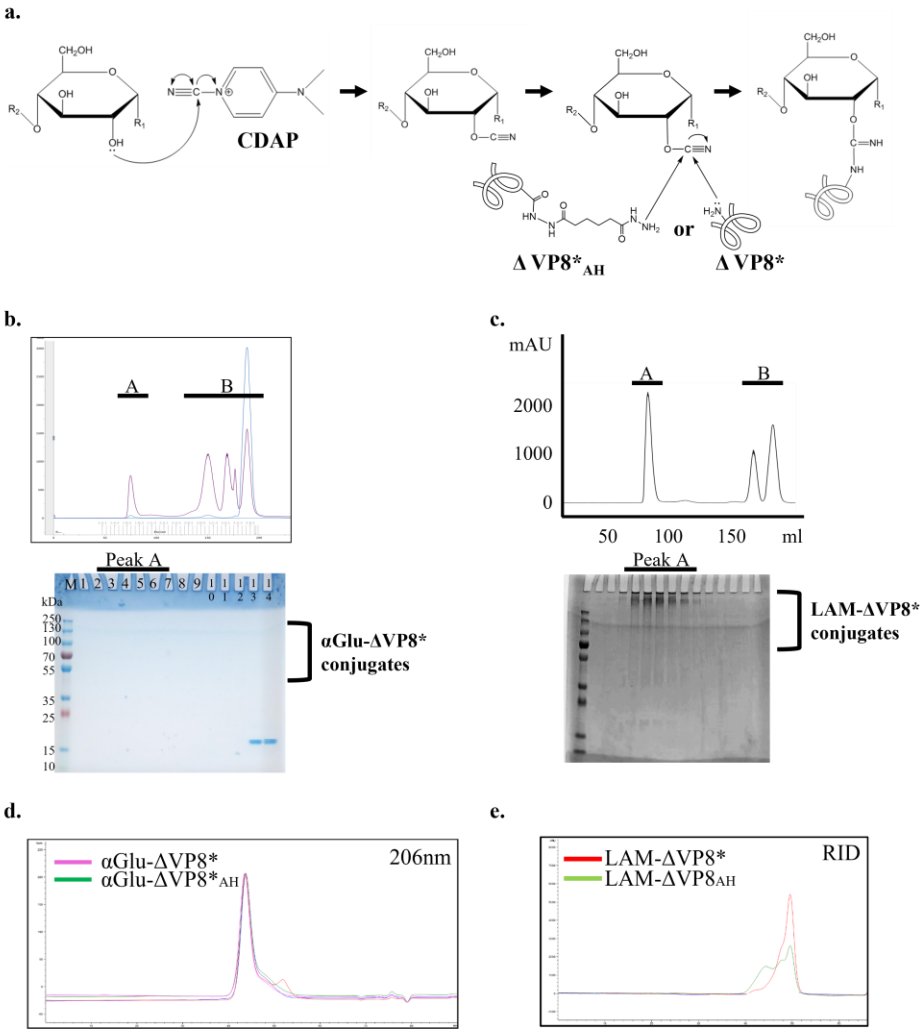


Figure 10. Preparation of M.tb polysaccharide-ΔVP8* conjugate and its physicochemical properties. (a) Schematic diagram of ΔVP8*-based conjugates; CDAP activates LAM by reacting with hydroxyl groups on carbohydrates, (b and c) Separation of ΔVP8*-based conjugates by size-exclusion chromatography. After conjugate reaction, the mixture was applied onto a Sepharcyl S200 column (XK16/100). ΔVP8*-based conjugates were eluted in early fractions (peak A) owing to their high molecular weight, whereas the excess αGlu, 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, (d and e) SEC-HPLC profile of ΔVP8*-based conjugates. OHpak SB-806 and 804 column 100mM NaH_2PO_4, 100mM NaCl, 5% CH_3CN, pH7.2; 0.3mL/min

Table 2. Characteristics of M.tb polysaccharide conjugates

Saccharide	Carrier protein	Linker	Polysaccharide (mg)	Protein (mg)	Protein/Polysaccharide (w/w)
α-Glu	ΔVP8*	-	2.9	0.3	0.1
		ADH	2.1	0.2	0.1
	DT	-	2.7	0.3	0.1
		ADH	2.4	0.5	0.2
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]αGlu and LAM concentration was based on Anthrone assay
[2]ΔVP8* and DT protein concentration was based on Bradford assay

3.2. αGlu-ΔVP8*$_{AH}$ conjugates can elicit the immunogenicity of αGlu and ΔVP8* antigens

To assess the carrier effect of ΔVP8* in a glycoconjugate platform, the immunogenicity of the glycoconjugates αGlu-ΔVP8*$_{AH}$ and αGlu-DT$_{AH}$ was tested in mice and compared to the unconjugated αGlu. Four doses (10 μg/dose) of αGlu-ΔVP8*$_{AH}$ and αGlu-DT$_{AH}$ were administrated intramuscularly at two-week intervals (Figure 11a). αGlu, ΔVP8* and DT were immunized as single antigen controls by the same protocol. An additional group of mice received PBS as a control. In immunogenicity study, mice were administrated without an adjuvant to focus exclusively on the intrinsic value of ΔVP8* as a carrier.

αGlu specific IgG levels were elicited after the 3rd inoculation in mice immunized with αGlu-ΔVP8*$_{AH}$ (Figure 11b). The αGlu specific antibody response induced by αGlu-ΔVP8*$_{AH}$ was lower than the one induced by αGlu-DT$_{AH}$ at day 28 and 42, and comparable to that of αGlu-DT$_{AH}$ at day 56 (Figure 11b), and IgG1 was the most abundant IgG subclass (Figure 11d). Administration of αGlu-ΔVP8*$_{AH}$ elicited significantly higher levels of ΔVP8* specific IgG antibodies than unconjugated ΔVP8* and PBS (Figure 11c), and IgG1 was the most abundant IgG subclass (Figure 11e).

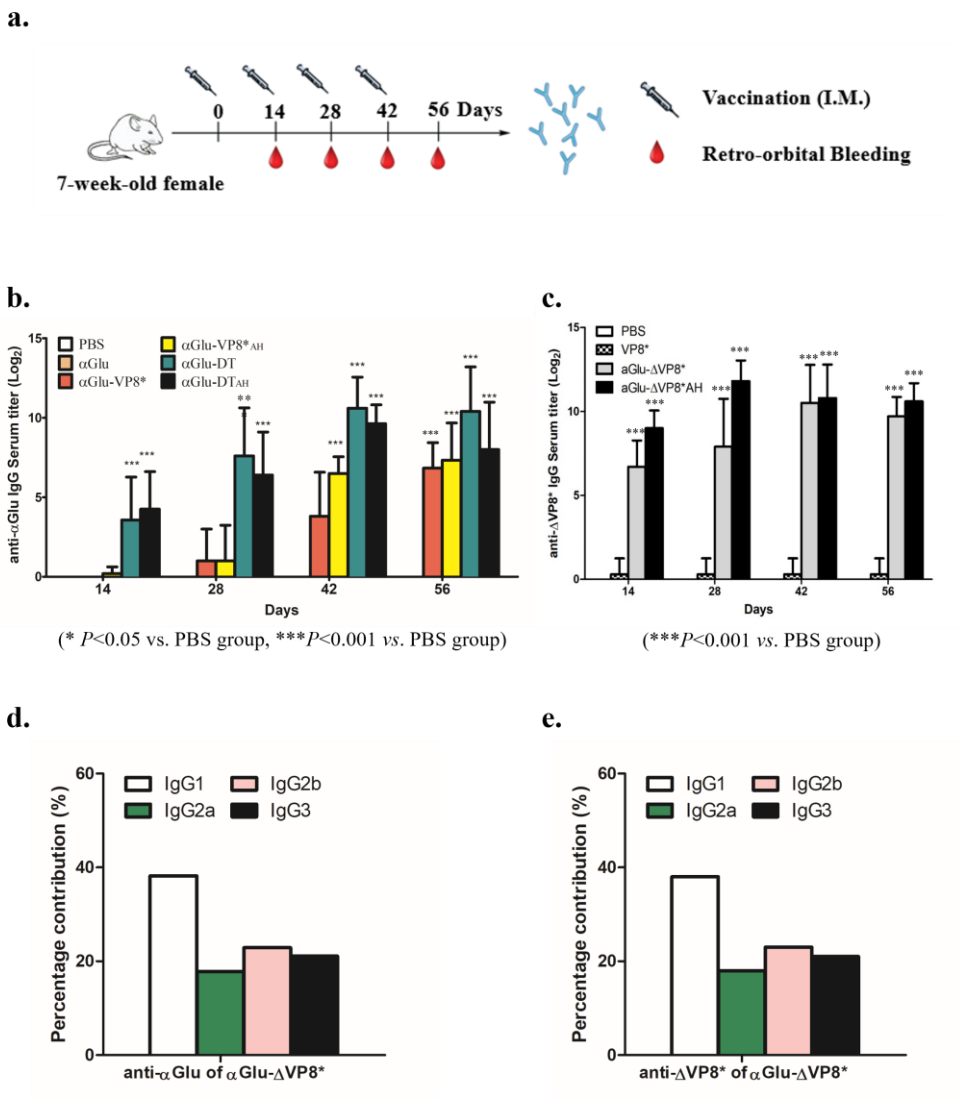


Figure 11. Immunogenicity study with *α*Glu-conjugates. (a) A schematic diagram for intramuscular vaccination and bleeding, (b) Anti-*α*Glu and (c) Anti-ΔVP8* antibody titer of conjugates, (d) Percentage contribution of IgG subclasses to total IgG against (d) *α*Glu and (e) ΔVP8*; data are mean ± s.d.; two sample *t*-test; *P <0.05, **P< 0.01, *** P <0.001.

3.3. Immunogenicity of LAM-ΔVP8* conjugates in mice

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 Figure 12a, 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-ΔVP8*$_{AH}$ at 10 μg of LAM and 9.9 μg of ΔVP8*$_{AH}$ for mouse/dose; (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$_{AH}$. Controls were injected with PBS as vaccine diluent control, 10 μg/dose of LAM, or 5 μg/dose of each carrier proteins. Immunogens in 200μL 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 (Figure 12b). Administration of ΔVP8*-based conjugates elicited significantly higher levels of ΔVP8* specific IgG antibodies than unconjugated ΔVP8* (Figure 12c), and IgG1 was the most abundant IgG subclass (Fig. 12d).

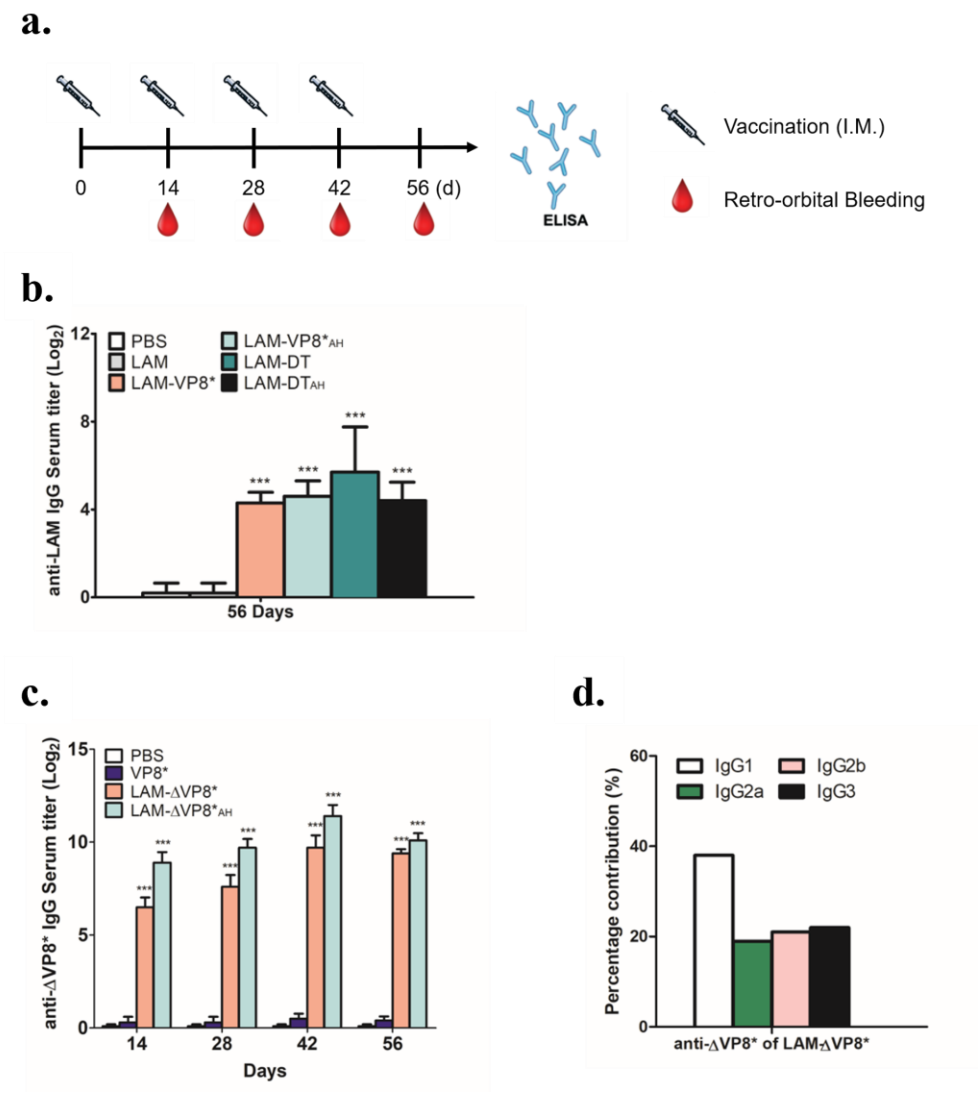


Figure 12. Immunogenicity study with LAM-conjugates. (a) A schematic diagram for intramuscular vaccination and bleeding, (b) Anti-LAM and (c) Anti-ΔVP8* antibody titer of conjugates, and (d) Neutralization analysis of sera from immunized mice; Days 56 immune sera were used; data are mean ± s.d.; two sample *t*-test; *P <0.05, **P< 0.01, *** P <0.001.

4. Discussion

Cell-mediated immunity is an important component to control mycobacterial infection (Cooper, 2009). However, recent immunological and genetic studies support that antibody-mediated immunity also plays a protective role against TB (Achkar and Casadevall, 2013; Achkar and Prados-Rosales, 2018). Among many constituents of mycobacterial cell envelope, αGlu and LAM are the major component of cell wall-associated lipoglycans. LAM can induce high antibody responses in infected hosts (Yu et al., 2012); antibodies to LAM have moderate protective efficacy in M.tb infected mice (Chen et al., 2020; Correia-Neves et al., 2019; Prados-Rosales et al., 2017). αGlu and LAM, however, are T cell-independent antigens, which can activate B cells without the cooperation of helper T cells and are characterized by a lack of immunologic memory (Avci et al., 2019). 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 (Avci et al., 2011, 2013; Comstock and Kasper, 2006). 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 (Avci et al., 2019). However, the repeated use of carrier proteins causes immune interference such as carrier-induced epitope suppression and carrier-specific enhancement of T cell help (Avci et al., 2019; Pollabauer et al., 2009). Therefore, there is a significant need for novel carrier proteins.

Here, I demonstrated that not only did the conjugation of ΔVP8* to αGlu and 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 the previous findings in that the conjugate vaccine platform using Vi polysaccharide enhances immune responses to carrier protein in mice (An et al., 2018; Kothari et al., 2014). 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 (Snapper and Mond, 1996) and ΔVP8* is less immunogenic in mice (Xue et al., 2016). αGlu and LAM were 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 of the resulting conjugates (Lang and Huang, 2020). 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, this study demonstrates 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 (Avci et al., 2019). 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.

Chapter V. Rotavirus spike protein ΔVP8* as a novel carrier protein for conjugate vaccine platform with demonstrated antigenic potential for use as bivalent vaccine

1. Abstract

Conjugate vaccine platform is a promising strategy to overcome the poor immunogenicity of bacterial polysaccharide antigens in infants and children. A carrier protein in conjugate vaccines works not only as an immune stimulator to polysaccharide, but also as an immunogen; with the latter generally not considered as a measured outcome in real world. Here, I probed the potential of a conjugate vaccine platform to induce enhanced immunogenicity of a truncated rotavirus spike protein ΔVP8*. ΔVP8* was covalently conjugated to Vi capsular polysaccharide (Vi) of *Salmonella* Typhi to develop a bivalent vaccine, termed Vi-ΔVP8*. This study demonstrated that the Vi-ΔVP8* vaccine can induce specific immune responses against both antigens in immunized mice. The conjugate vaccine elicits high antibody titers and functional antibodies against *S*. Typhi and Rotavirus (RV) when compared to immunization with a single antigen. Together, these results indicate that Vi-ΔVP8* is a potent and immunogenic vaccine candidate, thus strengthening the potential of conjugate vaccine platform with enhanced immune responses to carrier protein, including ΔVP8*.

2. Introduction

Enteric and diarrheal diseases are major causes of death, especially in young children in the developing world (Crump and Mintz, 2010; Tate et al., 2016). The bacterial pathogen *Salmonella* enterica serovar Typhi (*S*. Typhi) and rotavirus (RV) are known to cause enteric fever and severe acute gastroenteritis, respectively (Crump and Mintz, 2010; Tate et al., 2012). Globally *S*. Typhi infected 21.7 million people, resulting in 200,000 deaths in 2014 (Mogasale et al., 2014). RV was responsible for the death of 128,000 children under the age of five in 2016 (Collaborators, 2018).

Currently, several vaccines are licensed and commercially available to protect against *S*. Typhi or RV infection. However, a live-attenuated oral vaccine Ty21a and parenteral Vi polysaccharide (Vi) vaccine fail to induce protective antibodies in the populations at high risk of enteric fever, especially infants and young children (Khan et al., 2012; Murphy et al., 1991). Only Vi-tetanus toxoid (Vi-TT) conjugated vaccine induces protective levels even in children less than 2 years old (Mitra et al., 2016). The vaccines against RV provide lower immune response and less clinical protection in developing countries as compared to more developed regions (Jonesteller et al., 2017; Yen et al., 2011), and there is a small risk of intussusception in infant from oral rotavirus vaccine administration (Yen et al., 2016). *S*. Typhi is transmitted through ingestion of contaminated food or water and RV through the fecal-oral route directly from person to person. The prevalence of typhoid fever and rotavirus diarrhea has significant regional overlap in many parts of the world (Crawford et al., 2017; DeRoeck et al., 2007); therefore, a combined and effective vaccine against both pathogens could improve availability and compliance.

Vi of *S*. Typhi is the target of a protective humoral immune response. The chemical conjugation of Vi to a carrier protein results in an enhanced antibody response and an explicit memory response by converting T-cell independent to T-cell dependent response (An et al., 2018; Costantino et al.,

2011). The glycoconjugate platforms also provide a unique antigen structure allowing internalization and antigen presentation of the immunogen by antigen-presenting cells (Avci et al., 2019). In addition, typhoid conjugate vaccines are highly efficacious for infants more than 6 months of age in endemic countries (Capeding et al., 2020; Mohan et al., 2015). Taking advantage from the availability of Vi in our lab, we have conducted a study to assess the ability of a truncated rotavirus spike protein ΔVP8* as a novel carrier protein to elicit satisfactory anti-Vi titers and its ability as an immunogen.

Current licensed glycoconjugate vaccines use only a few carrier proteins [*i.e.*, Tetanus Toxoid (TT), Diphtheria Toxoid (DT), CRM_{197}, or Outer Membrane Protein Complex (OMPC)] (Avci et al., 2019). The limited number of carriers implies repeated exposure to the same carrier, inducing various immune interference such as carrier-induced epitope suppression (CIES), carrier-specific enhancement of T cell help, and bystander interference (Avci et al., 2019; Pollabauer et al., 2009). Therefore, the need for an alternative carrier seems obvious despite the availability of several safe and effective carriers above. Among several candidates, the recombinant non-toxic form of *Pseudomonas aeruginosa* exotoxin A (rEPA) and a rationally designed recombinant protein containing strings of universal $CD4^+$ T-cell epitopes proved to be very good carriers (Tontini et al., 2016). A conjugate vaccine composed of Shigella O-specific polysaccharide or Vi bound to rEPA elicited protective anti-polysaccharide IgG and induction of immunologic memory. The protective efficacy of the conjugate vaccines was 74% against *Shigella sonnei* shigellosis and 90% against typhoid fever (Szu et al., 2013; Taylor et al., 1993). In conjugate platform, novel polyepitope carrier proteins, consisting of a sequential string of universal human $CD4^+$ T helper epitopes, exerts a strong carrier effect on inducing anti-polysaccharide serum antibody titers and bactericidal activity of antibodies elicited against *Neisseria meningitidis* (Baraldo et al., 2005;

Baraldo et al., 2004).

Rotavirus spike protein, VP4, consists of its stalk (VP5*) and globular head (VP8*). The VP8* fragment of VP4 has been reported to elicit primarily homotypic neutralizing antibodies although the protective immunity was not as effective as full length VP4. In particular, the truncated form of VP8*, termed ΔVP8*, could induce high levels of homotypic and varying levels of heterotypic virus neutralizing antibodies in immunized animals, which demonstrates the potential of a subunit vaccine (Wen et al., 2012; Wen et al., 2014).

P[8] RV strains (Wa strain) and P[4] RV strains (DS-1 strain) are the predominant human rotavirus strains, which are responsible for over 90% of the rotavirus infection among children worldwide (Santos and Hoshino, 2005). In Africa, however, P[6] RV strains (1076 strain) accounted for almost one third of all P types (Santos and Hoshino, 2005). Therefore, a multivalent vaccine formulated with P[4], P[6] and P[8] specificity could provide wider coverage and afford optimal protection against the common circulating rotavirus infections (Groome et al., 2017).

I developed purification steps for ΔVP8* antigens of P[4], P[6], and P[8] strains using two steps of ion-exchange chromatography. Different conjugation strategies were tested to improve vaccine design. The Vi-ΔVP8* conjugates were characterized physicochemically, and then mixed as a multivalent vaccine. Antigen-specific antibodies in serum, bactericidal activity against *S*. Typhi, and virus-neutralizing antibody response against RV determined the immunologic properties of Vi-ΔVP8* conjugates in mice. The results showed that the Vi-ΔVP8* conjugates induced significantly high immune response toward the ΔVP8* antigens and Vi polysaccharides, thus making it a promising multivalent vaccine candidate against multiple rotavirus P types and *S*. Typhi. Overall, the results data shed light on an additional potential of a conjugate vaccine platform for enhanced immunogenicity of ΔVP8*.

3. Results

3.1. Chemical conjugation of ΔVP8*$_{AH}$ to Vi

As illustrated in Figure 13, glycoconjugates were synthesized with 8mg of Vi (2 mg/mL) at a ratio Vi/ΔVP8*$_{AH}$ of 1:1 (w/w) and EDAC (50mg/mL) in MES buffer at pH 5.6. The chromatogram profile showed that Vi-ΔVP8* eluted earlier than either free Vi or free ΔVP8*, confirming the larger size of the conjugate with respect to free components (Figure 14a). The large size of the conjugates resulted in decreased retention time in SEC-HPLC (Figure 14b), and a typical smear appeared at the top of 12% SDS-PAGE (Figure 14c). The recoveries of Vi and ΔVP8* were ~40% and ~10%, respectively (Table 3). As illustrated in Figure 15, an attempt to directly link ΔVP8* to Vi was tried through the reaction between the primary amine in ΔVP8* and carboxylic groups along the Vi polysaccharide, previously activated with EDAC. However, the direct conjugation method was not successful. The limited number or low reactivity of primary amine in ΔVP8* could be one reason, and steric hindrance could be another. Alternatively, N-β-maleimidopropionic acid hydrazide (BMPH) was tried to facilitate coupling ΔVP8* to Vi. For this, ΔVP8* was firstly thiolated by N-succinimidyl S-acetylthioacetate (SATA) to introduce thiol groups, which were reacted with the maleimide groups in heterobifunctional linker, BMPH; the hydrazide groups left on the other side in the BMPH were further coupled with the carbonyls on Vi polysaccharide (Figure 15). However, the recovery of ΔVP8* in Vi-BMPH-ΔVP8* conjugates was lower (5%) than that obtained in Vi-ADH-ΔVP8* conjugates (10%) (Table 4).

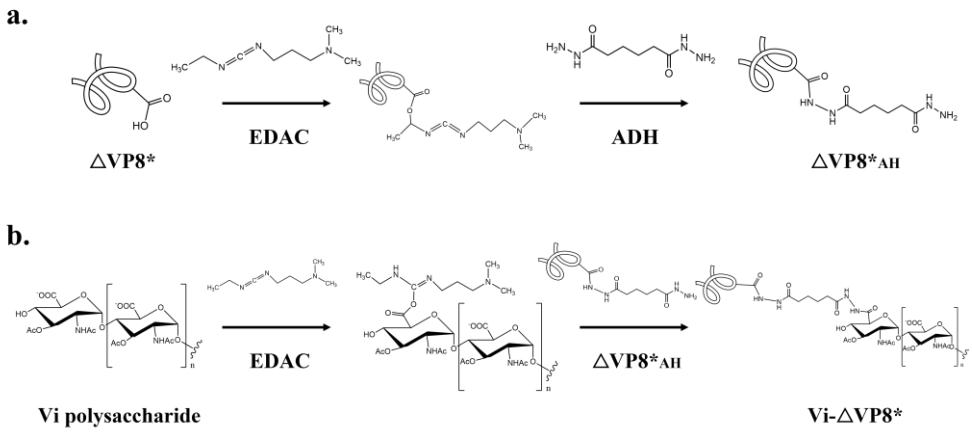


Figure 13. Schematic representation of the preparation of Vi-ΔVP8*. (a) ΔVP8* was derivatized with EDAC/ADH, (b) Vi polysaccharide was conjugated with $\Delta VP8^*_{AH}$.

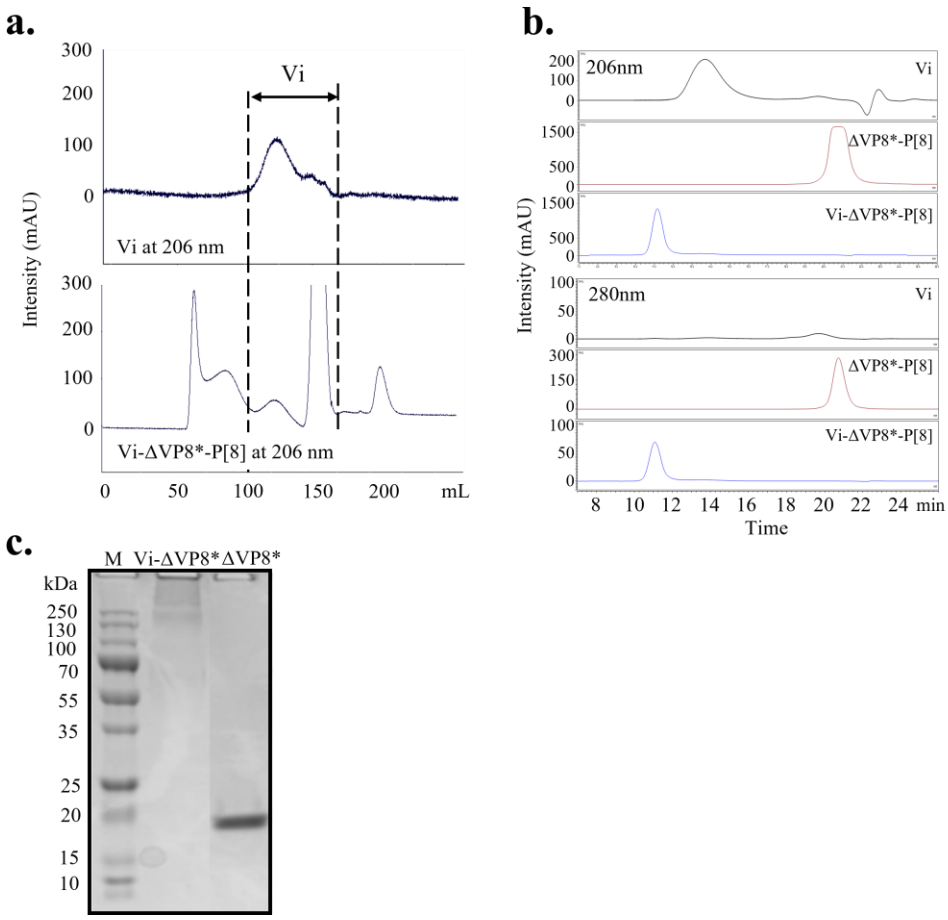


Figure 14. Physicochemical characteristics of the glycoconjugates. (a) Sephacryl S-1000 profile of Vi and Vi-ΔVP8*-P[8]. Vi-ΔVP8*-P[8] was purified on Sephacryl S-1000 (1.6cm × 90cm) eluting with 10mM NaH_2PO_4, 5mM NaCl, pH 7.0 at a flow rate of 0.2 mL/min. (b) SEC-HPLC profiles of Vi-ΔVP8*-P[8]. Tskgel G5000 PW_{XL} column, 0.1M NaCl, 0.1M NaH_2PO_4, 5% ACN, pH 7.2; 0.5 mL/min. flow rate. Vi polysaccharide, ΔVP8*-P[8], and Vi-ΔVP8*-P[8], (c) SDS-PAGE profile of Vi-ΔVP8*-P[8] (Lane 1: Marker, Lane 2: Vi-ΔVP8*-P[8] and Lane 3: ΔVP8*-P[8]). 12% gel, SimplyBlue™ staining. The grouping of gels cropped from different parts of the same gels.

Table 3. Characterization of Vi-ΔVP8* conjugates.

Conjugates	ADH linkers per protein	Recovery[1] (%)		Average size (kDa)	Vaccine Formulation	
		Vi	ΔVP8*		Vi (μg)[2]	ΔVP8* (μg)[3]
Vi-ΔVP8*-P[4]	4	35	10	2665	5	1.0
Vi-ΔVP8*-P[6]	6	33	11	2583	5	1.1
Vi-ΔVP8*-P[8]	5	44	15	2773	5	1.5
Multivalent formulation[4]	—	—	—	—	15	3.6

[1]Recovery of each conjugate obtained was calculated on the basis of Vi concentration and protein concentration.
[2]Vi concentration was based on Hestrin assay
[3]ΔVP8* protein concentration was based on Bradford assay
[4]Multivalent formulation is the mixture of Vi-ΔVP8*-P[4], P[6], and P[8]

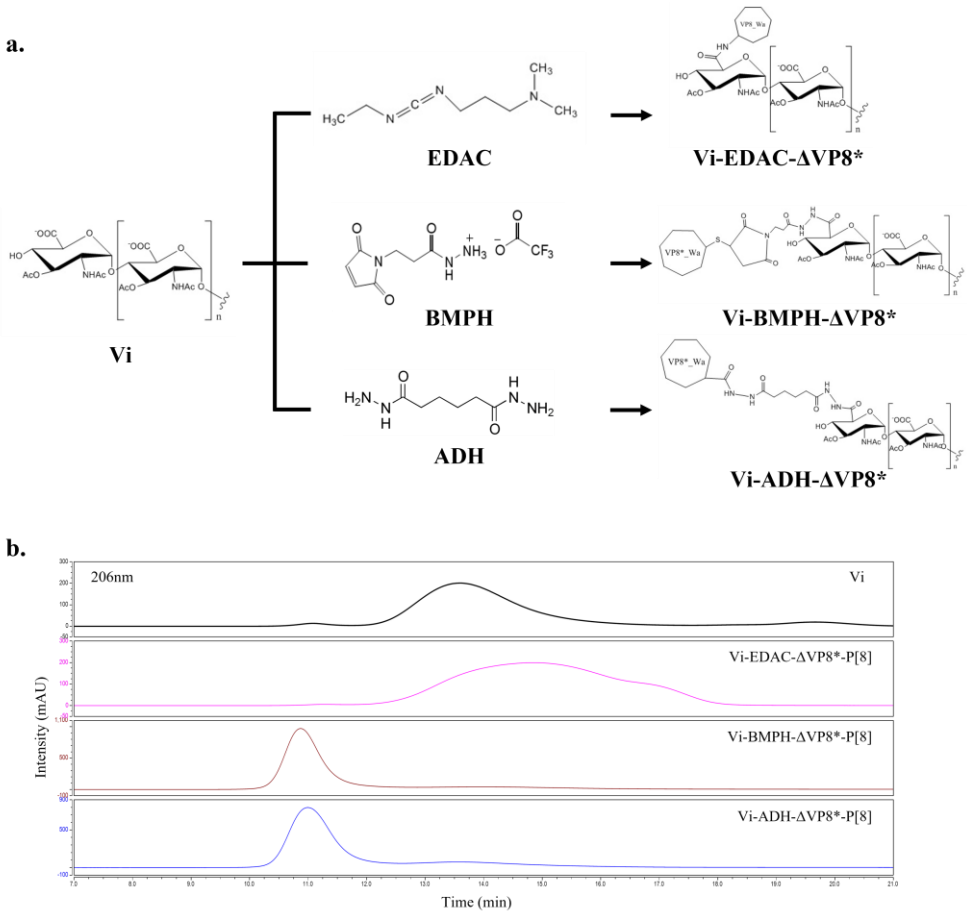


Figure 15. Conjugation scheme and physicochemical characteristics of Vi-ΔVP8* conjugates with different linkers. (a) Conjugation scheme for the synthesis of Vi-ΔVP8* conjugates, (b) SEC-HPLC analysis of Vi-ΔVP8*-P[8] conjugates

Table 4. Chemical analysis of conjugates prepared by different linkers.

Conjugate	Linker	Recovery (%)[1]	
		Polysaccharide[2]	Protein[3]
Vi-EDAC-ΔVP8*-P[8]	EDAC	0	0
Vi-BMPH-ΔVP8*-P[8]	BMPH	40	5
Vi-ADH-ΔVP8*-P[8]	ADH	44	15

[1]Recovery of each conjugate obtained was calculated on the basis of Vi concentration and protein concentration.
[2]Vi concentration was based on Hestrin assay.
[3]ΔVP8* protein concentration was based on Bradford assay.

3.2. Immunogenicity of Vi-ΔVP8* conjugates in mice

In immunogenicity study, Vi-ΔVP8* conjugates were administrated to mice without an adjuvant to focus exclusively on the intrinsic value of ΔVP8* as a carrier. As illustrated in Figure 16a, mice were subcutaneously injected with each conjugate (5 μg Vi/dose) or multivalent formulation (15 μg Vi/dose) on days 0, 14 and 28. Four different groups of mice were injected with 5 μg of Vi, 1.0 μg of ΔVP8*-P[4] , 1.1 μg of ΔVP8*-P[6] , 1.5 μg of ΔVP8*-P[8]. Controls were injected with PBS. Serum anti-Vi IgG titers and functional antibody levels were measured by ELISA and complement-mediated SBA, respectively. Immunization with the unconjugated Vi induced very low levels of Vi-specific IgG antibodies, and it was consistent with the previous results that antisera raised against Vi alone in PBS elicited low titers in ELISA (An et al., 2011; Kothari et al., 2014). In contrast, the glycoconjugates induced highly elevated titers of IgG antibodies to Vi after a single immunization (Figure 16b). The high titers observed on Day 14 persisted through Day 42. IgG1 was consistently the dominant IgG subclass against Vi in the immunized mice (Figure 16c). Diluted serum samples were incubated with S. Typhi to determine bactericidal activity of sera from vaccinated mice. The serum from mice immunized with Vi alone or Vi-VP8* conjugates showed a significant ability to prevent the growth of S. typhi more than 50% until reaching 1:200 dilution, while sera from VP8* group showed no SBA titer (Figure 16d and Figure 17). These data indicated that Vi-ΔVP8* conjugates elicited high level of anti-Vi IgG and complement-mediated SBA responses against S. Typhi isolate C6524.

I assessed the levels of IgG antibodies to ΔVP8* elicited by immunization, and neutralizing capacity of serum antibodies was measured using the PRNT. As previously reported (Wen et al., 2012; Xue et al., 2015), ΔVP8* induced low levels of serum anti-ΔVP8* IgG (Figure 18a). In contrast, the Vi-ΔVP8* induced high levels of IgG specific for ΔVP8*, and the levels consistently increased through Day 42, with IgG1 being the dominant isotype (Figure 18a and b). Rotavirus neutralizing activity was higher in conjugate-immunized mice, with 50% reduction in plaque count (PRNT50) titers ranging from 1:128 to 1:512 (Figure 18c).

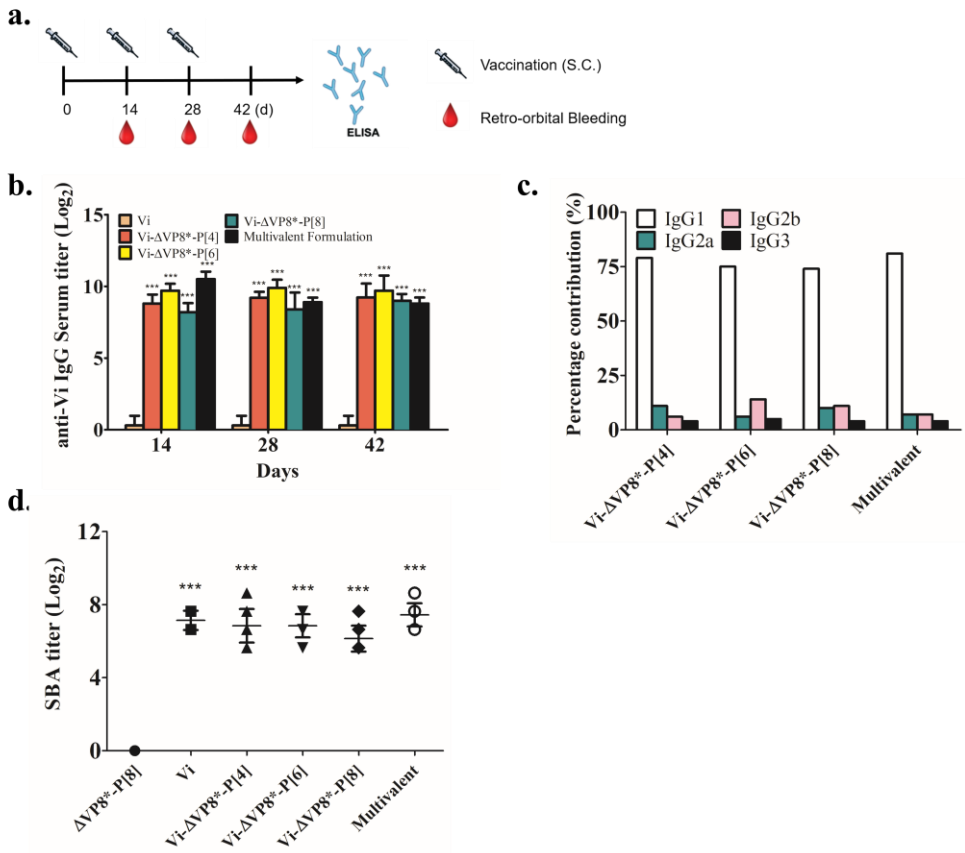


Figure 16. Immunogenicity study with Vi-ΔVP8* for anti-Vi. (a) A schematic diagram for subcutaneous vaccination and bleeding, (b) Anti-Vi antibody titer of conjugates, (c) Percentage contribution of IgG subclasses to total IgG against Vi, (d) Serum bactericidal activity (SBA) titers observed for sera from mice immunized with Vi-ΔVP8* conjugates. BALB/c mice were immunized SC with 5μg of Vi-ΔVP8*-P[4], P[6] or P[8], or 15μg of multivalent formulation based on Vi polysaccharide 3 times at 2-week intervals as described in Methods; data are mean ± s.d.; two sample *t*-test; *P <0.05, **P< 0.01, *** P <0.001

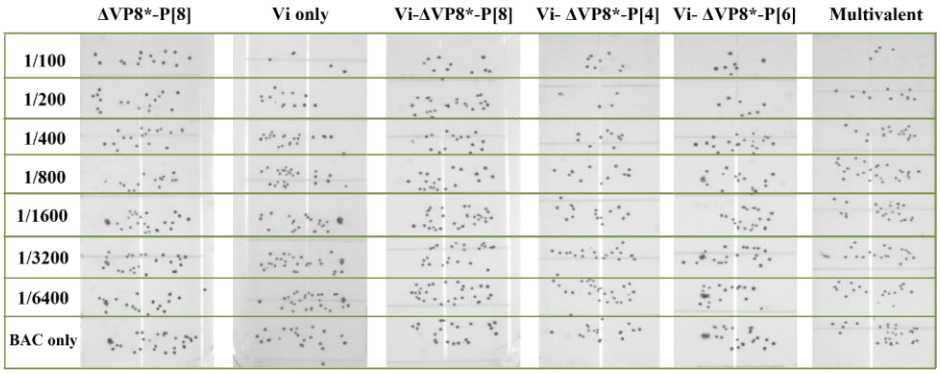


Figure 17. SBA measures functional *S*. Typhi specific antibodies capable of complement-mediated bacterial killing.

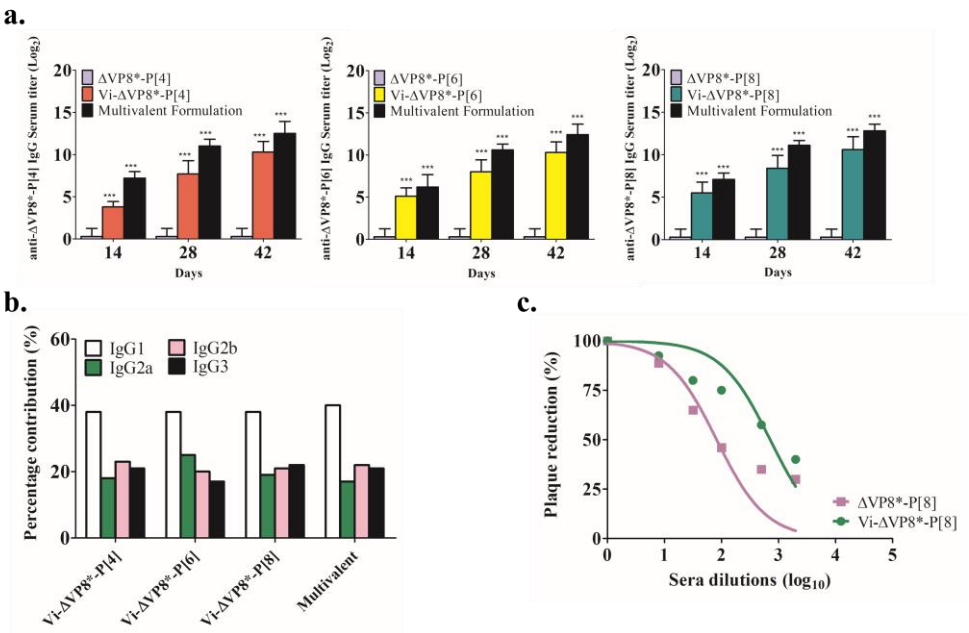


Figure 18. Immunogenicity study of Vi-ΔVP8* for anti-ΔVP8*. (a) IgG of mice immunized with ΔVP8* or Vi-ΔVP8* conjugates, (b) Percentage contribution of IgG subclasses to total IgG against ΔVP8*, (c) Neutralization analysis of sera from immunized mice; Days 42 immune sera for titers were used; BALB/c mice were immunized S.C. with 5μg of Vi-ΔVP8*-P[4], P[6] or P[8], or 15μg of multivalent formulation based on Vi polysaccharide, 3 times at 2-week intervals as described in Methods; data are mean ± s.d.; two sample *t*-test; **P* <0.05, ***P*< 0.01, *** *P* <0.001

4. Discussion

Previous studies exploring the conjugate vaccine platform using Vi conjugated to carrier proteins interestingly showed that some carrier proteins play the dual role as a carrier protein and a protective antigen (An et al., 2018; An et al., 2015; Kothari et al., 2014). In this study, I further demonstrated the potential of Vi conjugation to enhance immune responses in mice to carrier protein compared with those induced by the native protein. For this aim, I produced ΔVP8* antigens from three predominant P rotaviruses and developed Vi-ΔVP8* conjugates by using different linkers and coupling methods, with ADH being the linker of choice. The multiple activation sites of each Vi and multiple linkage points of ΔVP8* allowed us to produce conjugates of large molecular weight. The Vi-ΔVP8* conjugates were carefully characterized, including SEC-HPLC for its molecular size, Hestrin assay for Vi contents, and Bradford assay for protein contents. After analytical assay, three Vi-ΔVP8* conjugates displaying the ΔVP8* antigens of the P[4], P[6], and P[8] specificity were mixed as a multivalent vaccine. Immunization with the Vi-ΔVP8* conjugates in mice significantly increased the immunogenicity of ΔVP8* and induced the T cell-dependent humoral immune response to the Vi. Although multivalent formulated vaccines contained three times higher amount of Vi antigen than in each Vi-ΔVP8* conjugate, the anti-Vi IgG antibody titers were similar. This was similar to the data seen with the Vi-DT conjugate vaccine during its development where the relationship between antigen load and immune response was observed, and up to 10 μg per dose of Vi antigen led to increasing immune response, beyond which it was seen to decrease (Figure 19) (Cui et al., 2010). Meanwhile, the multivalent vaccine elicited higher IgG titers to all P[4], P[6], and P[8] ΔVP8*s as compared to each Vi-ΔVP8* conjugate, though not in a proportionate fashion. Although the saturation limit for ΔVP8 needs to be checked in further studies, this may be explained by the sequence alignments among three ΔVP8*s; VP8*-P[4] and P[8] share sequence identity of 82%, VP8*-P[6] and P[8] share sequence identity of 63%, and VP8*-P[4] and P[6] share sequence identity of 60%. In addition, the Vi-ΔVP8* conjugates were capable of eliciting functional antibodies against RV and *S*. Typhi. Increased titers of IgG antibodies and serum neutralizing antibodies may lead to protection against both pathogens through serum antibody transudation into the intestinal lumen (Westerman et al., 2005).

Affinity-based purification of recombinant proteins has become the method of choice for high-throughput protein production due to potential advantages of affinity tags including ease of use, the high level of purity, and the ability to scale up production (Ecker et al., 2020). Removal of affinity tags from a purified recombinant protein is generally required for vaccine applications because the presence of the affinity tags may interfere with physicochemical properties and immunogenicity of the recombinant protein (Waugh, 2005). However, the complete removal of affinity tags from the target protein is difficult to achieve. To eliminate problems related to them, it is desirable to develop purification methods for tag-free recombinant proteins. I produced the three types of tag free-ΔVP8* by two steps of ion-exchange chromatography to maintain high purity and high levels of consistency between runs. Immunoblotting and SEC-HPLC analysis revealed that the purified ΔVP8* are similar to the results of previous studies (Dormitzer et al., 2001; Wen et al., 2012).

Parenterally administered Vi-ΔVP8* vaccines may have advantages over currently licensed live oral vaccines due to their potential for concomitant use with other vaccines. Specifically, coformulation of Vi-ΔVP8* vaccines with Expanded Program on Immunization (EPI) vaccines may lower the delivery cost and increase compliance of the vaccination in endemic countries. For example, Vi-CRM_{197} can be given to infants concomitantly with Diphtheria-Tetanus-Pertussis (DTP) vaccine starting at 6 weeks or with measles vaccine at 9 months. The Clinical trial of a Vi-CRM_{197} vaccine in infants demonstrated that the vaccinated individuals showed higher levels of anti-Vi antibodies as compared to the pre-immune level of anti-Vi antibodies (Bhutta et al., 2014). In addition, studies of the Vi-rEPA vaccine showed that the conjugate vaccine was safe and induced a protective immunogenic response when it was used concomitantly with EPI vaccines (Thiem et al., 2011).

Additional investigations are required to explore the full potential of multivalent Vi-ΔVP8* vaccine efficacy. Firstly, scale-up from shake flasks to large-scale fermentations allows for high-yield production of ΔVP8* in *E. coli*. In addition, further optimization of conjugation methods can lead to high conjugation efficiency and product consistency with target characteristics. These involve multiple strategies which will result in production of conjugate vaccines at low cost and eventually support its technology transfer to manufacturers in developing

countries. Secondly, the mouse sera after immunization with the Vi-ΔVP8*-P[8] showed high neutralizing titers against Wa strain rotavirus in cell culture. Ideally, I should examine the mouse sera from Vi-ΔVP8*-P[4], Vi-ΔVP8*-P[6], or multivalent Vi-ΔVP8* immunized mice to determine neutralizing activity and cross-neutralizing activity against homologous and heterologous P types of rotaviruses for a comprehensive understanding of the further broad efficacy of the multivalent vaccine. This could be part of further evaluation of the vaccine candidates during post-optimization of their manufacturing process. In addition, use of adjuvants in the formulations of conjugate vaccines is also being planned to potentiate the immune response to the vaccine, as adjuvants have shown to induce an enhanced, prolonged, and sustainable immune response (HogenEsch et al., 2018)

This work highlights the potential of a conjugate vaccine platform to enhance the immunogenicity of a truncated rotavirus spike protein ΔVP8* that generally induces low immune responses. As a proof of concept, Vi is conjugated to ΔVP8* of P[4], P[6], and P[8] rotaviruses. Mouse immunization studies demonstrated the significantly improved immune responses to ΔVP8* of Vi-ΔVP8* vaccines. Immune responses induced by Vi-ΔVP8* include high titers of Vi-specific serum IgG antibodies and functional antibodies. The results collectively indicated that highly immunogenic Vi-ΔVP8* conjugates show excellent promise as vaccine candidates which can tackle two significant enteric pathogens and that a conjugate vaccine platform using Vi has the potential to enhance the immunogenicity of carrier proteins.

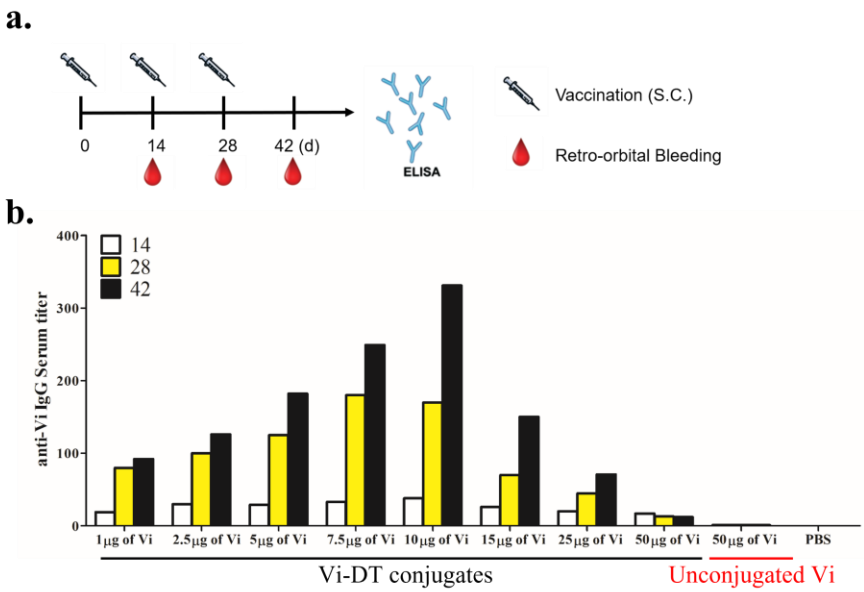


Figure 19. Influence of Vi amount on the immunogencity of Vi conjugates in mice. (a) A schematic diagram for subcutaneous vaccination and bleeding, (b) Anti-Vi IgG antibody titer. Mice were immunized with three doses of Vi-DT conjugates at 1 μg, 2.5 μg, 5 μg, 7.5 μg, 10 μg, 15 μg, 25 μg, or 50 μg Vi/does. Mice were injected with 50 μg of Vi. Controls were injected with PBS.

Chapter VI. Overall Discussion and Prospective

Vaccination is the most successful strategy to control many infectious diseases. Improvement of biomaterials through bioengineering and insights into immunological mechanisms have facilitated modern subunit vaccine to be rationally designed and more precisely targeted. However, some of antigens are still less immunogenic; therefore, they require an adjuvant or a complex formulation such as recombinant fusion proteins consisting of the antigen and a universal T cell epitope to increase the magnitude and duration of the protective immunity based on T cell response and humoral immunity.

Among several vaccine platforms, conjugate vaccines have dramatically reduced bacterial infections in high-risk groups such as infants and children under two years of age (Rappuoli et al., 2019; Thanawastien et al., 2015). A carrier protein in conjugate vaccines serves as an immune stimulator to a covalently linked polysaccharide antigen, resulting in better immune response in infants and children (Avery and Goebel, 1929; Goebel and Avery, 1929). Among carrier proteins currently used in licensed conjugate vaccines, non-typeable *Haemophilus influenzae* protein D (PD) induces antibody responses not only to a polysaccharide antigen but also to itself. For example, PD in the pneumococcal vaccine Synflorix (GSK) showed a 35.6% protection rate against acute otitis media caused by *Haemophilus influenzae* (Agency, 2009). Though additional advantages of carrier protein as an immunogen were reported in numerous literatures, sufficient data for its immunogenic potential has not been accumulated (Broker et al., 2017).

I was interested in elucidating the potential application of conjugate vaccine platform in enhancing the immunogenicity of carrier proteins and giving insight into the full potential of ΔVP8* as a relevant carrier protein. First, to investigate the influence of conjugate vaccine platform on the carrier protein, I needed to find proper immunogenic carrier with low immunogenicity. ΔVP8* is known to induce highly potent neutralizing antibodies. However, its low immunogenicity owing to the small size with low valency is the limitation to use it as vaccine target antigen. Based on the published works, a large, multivalent vaccine platform can make ΔVP8* more immunogenic. For example, nanoparticle platforms enable polyvalent antigenic presentation, allowing enhanced immunogenicity to antigens for vaccine development (Xia et al., 2018).

It has drawn my attention that conjugate vaccine platform provides polyvalent

formation of antigens in which multiple proteins are linked to polysaccharide as beads on a string. The common immunological principle suggests that larger, polyvalent antigens, particularly those retaining more pathogen-associated molecular patterns (PAMPs), elicit higher immune responses compared with those induced by a smaller, single or low valent antigen. The conjugate vaccine platform enables the codelivery of polysaccharide and protein; several copies of protein antigens can be displayed on one polysaccharide to achieve cluster effect. The protein antigen that is displayed by a polyvalent platform provides enhanced immune response over that elicited by its free monovalent components due to immune cell uptake, processing, and immunomodulation (Plummer and Manchester, 2011; Zhao et al., 2013).

Additionally, based on the published observation in the field, carbohydrates typically have immunomodulatory activities triggering the innate immune system. LAM has been found to induce pro-inflammatory response by modulating the cytokine response via interaction with TLR2 and CLRs on antigen-presenting cells. αGlu activates NF-κB and drives pro-inflammatory responses cytokine production. In addition, zwitterionic polysaccharides are potent immune modulators eliciting T cell-derived chemokines and cytokines that influence the immune response. Furthermore, there are number of natural and synthetic carbohydrates which have been used as adjuvants in human vaccines (Lang and Huang, 2020; Pifferi et al., 2021). Therefore, the carbohydrate in conjugate vaccine platform may play a role as an adjuvant inducing better immune response to protein antigens.

The effect of homologous carrier proteins on the immunogenicity of the conjugate vaccines is ambiguous. In one study, infants who were previously immunized with TT showed higher levels of protection against *Haemophilus influenzae* type b (Hib) upon a single dose of Hib-TT (Booy et al., 1997); however, in another study, priming infants with DT and TT did not develop enhanced immune response against Hib even after two booster doses of the Hib conjugates (Lieberman et al., 1995). In addition, several studies have reported that administration of multivalent conjugate vaccines with the same carrier can result in immune interference and impaired immune response to the polysaccharide due to carrier overload or carrier epitope suppression (Dagan et al., 2010; Micoli et al., 2018). As an example of carrier epitope suppression, tetravalent pneumococcal TT

conjugates were found to suppress the anti-polysaccharide immune response of Hib-TT conjugates (Dagan et al., 1998). Bystander interference is another negative implication of homologous carrier proteins. For example, the reduced immune response of Hib and hepatitis B virus (HBV) were reported in Hexavac® (Sanofi Pasteur) consisting of DT, TT, HBV, inactivated poliovirus, Hib polysaccharide conjugated to TT, and acellular pertussis (Boot and Schipper, 2009). Therefore, the need for a novel carrier protein seems obvious despite the availability of carrier proteins currently used in licensed vaccines.

ΔVP8* plays key roles in rotavirus immune response, inducing effective levels of homotypic and varying titers of heterotypic rotavirus neutralizing antibodies (Feng et al., 2019; Nair et al., 2017). In addition, ΔVP8* significantly improved immunogenicity of the antigen components in the fusion of two or three proteins, thus demonstrating its high potential as a subunit vaccine (Wen et al., 2014; Xia et al., 2016). These data strengthen my conviction to use ΔVP8* as a novel carrier protein in a conjugate vaccine platform.

From this study, I propose that conjugate vaccine platform can enhance the immunogenicity of ΔVP8* that generally induces low immune responses. Additionally, ΔVP8*-based conjugates can effectively convert a T cell-independent immune response to a T cell-dependent immune response, characterized by IgM-to-IgG switching, a booster response and sustained T cell memory to polysaccharide.

In another perspective, this work opens possibilities for the use of ΔVP8* as a carrier protein in a conjugate platform. Specifically, I am considering conjugation of ΔVP8* to O-specific polysaccharide from *Shigella*, because RV and *Shigella* are invasive enteric pathogens that are strongly associated with acute diarrhea in infants subjected to malnutrition (Ferdous et al., 2013; Petri et al., 2008). Since there is no licensed vaccine currently available to prevent *Shigella*, the development of a conjugate vaccine could be a significant step towards a safe and efficacious vaccine against both pathogens.

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국문 초록

영 · 유아에서 심한 장염을 일으키는 원인 중에서 로타바이러스 감염이 가장 흔하고 매년 전 세계 5세 이하 어린이 2천만명 이상이 로타바이러스에 감염되며 이 중 약 20만명이 사망한다. 백신 접종으로 로타바이러스 감염에 의한 입원이나 사망을 예방할 수 있지만, 개발도상국에서 경구용 로타바이러스 백신의 유효성은 50% 정도로 선진국 (85%)에 비해 상대적으로 낮다. 또한 현재 사용되는 경구용 로타바이러스 백신은 장중첩증을 야기시킨다고 알려져 있다. 이러한 이유로 기존 상용화된 백신의 광범위한 사용에도 불구하고 새로운 로타바이러스 백신의 개발은 계속 진행되고 있다. 임상 단계에 도달한 다수의 백신 가운데 재조합 단백질 백신인 P2-VP8*의 임상연구가 가장 앞서 있으며, 불활성화 바이러스형, 로타바이러스 VLP 외 다양한 백신 개발 플랫폼이 적용되고 있다.

다양한 백신 개발 플랫폼 중에서, 접합백신 플랫폼은 영유아에서 다당체에 대한 면역원성을 향상시키기 위해 개발되었다. 접합 백신의 경우, 다당체 표적 항원에 연결된 운반단백질을 구성하는 펩타이드가 항원제시세포의 주조직접합성 분자에 제시될 수 있고 T 세포가 활성화될 수 있다. 이는 T세포가 더 강력한 면역 반응을 자극하고 더 빠르고 오래 지속되는 면역 기억을 촉진하기 때문에 다당체에 대한 면역반응을 향상시킨다. 접합백신에 사용되는 운반단백질은 다당체의 면역원성을 증가시키는 역할뿐만 아니라 항원으로서의 역할도 한다고 보고되었다.

본 연구에서는 로타바이러스 스파이크 단백질 ΔVP8*을 접합백신 플랫폼의 운반단백질로 사용할 경우, 다당체와 ΔVP8*의 면역원성이 향상되는 것을 알아보고자 하였다. ΔVP8*은 공유결합에 의해 *Salmonella* Typhi의 Vi polysaccharide와 *Mycobacterium tuberculosis*의 lipoarabinomannan에 접합되었고, 각 항원에 대한 면역원성을 향상시키기 위해 다른 종류의 접합체(linker)가 이용되었다. 다양한 접합체 중, ADH를 사용한 경우에 ΔVP8*과 다당체가 가장 효율적으로 결합되었고 생쥐에서 접종실험을 통해 ΔVP8*과 다당체의 항체의 생성이 증가함을

확인할 수 있었다. 또한, 플라크 억제 시험법을 통해 로타바이러스에 대한 중화항체가 형성되는 것을 확인할 수 있었다. 결론적으로 ΔVP8*을 이용한 접합 백신 기술은 ΔVP8*의 면역원성 뿐만 아니라 다양한 다당체의 면역효과를 향상시킬 수 있었다. ΔVP8*을 이용한 접합백신 개발은 대량 생산이 가능하고 주사제로서 사용되기에 안정성면에서도 우수하기 때문에 다양한 감염병 예방에 크게 도움이 될 수 있다고 생각된다.

주요어: 로타바이러스 스파이크 단백질 ΔVP8*, 접합백신 플랫폼, 운반단백질, 운반단백질 연계 결정인자 억제 효과, 결핵, 장티푸스

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