Extended application of *Alcaligenes* lipid A as a vaccine adjuvant

アルカリゲネスリピド A のワクチンアジュバントとしての応用展開

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Abstract

Research from ours and other laboratories previously identified *Alcaligenes* spp. as a commensal bacterium that resides in lymphoid tissues, including Peyer's patches. We found that *Alcaligenes*-derived lipopolysaccharide acted as a weak agonist of Toll-like receptor 4 due to the unique structure of lipid A, which lies in the core of lipopolysaccharide. This feature allowed the use of chemically synthesized *Alcaligenes* lipid A as a safe synthetic vaccine adjuvant that induces Th17 polarization to enhance systemic IgG and respiratory IgA responses to T-cell–dependent antigens (e.g., ovalbumin and pneumococcal surface protein A) without excessive inflammation. Here, I conducted two investigations aimed at expanding the adjuvant functionality of *Alcaligenes* lipid A.

The first investigation focused on its adjuvant effect on T-cell–independent antigens, which has not been previously examined. For this purpose, I examined the adjuvant activity of *Alcaligenes* lipid A on a *Haemophilus influenzae* B conjugate vaccine that contains capsular polysaccharide polyribosyl ribitol phosphate (PRP), a T-cell–independent antigen, conjugated with the T-cell–dependent tetanus toxoid (TT) antigen (i.e., PRP-TT). When mice were subcutaneously immunized with PRP alone or mixed with TT, *Alcaligenes* lipid A did not affect PRP-specific IgG production. In contrast, PRP-specific serum IgG responses were enhanced when mice were immunized with PRP-TT, but these responses were impaired in similarly immunized T-cell–deficient nude mice. Furthermore, TT-specific—but not PRP-specific—T-cell activation occurred in mice immunized with PRP-TT together with *Alcaligenes* lipid A. In addition, coculture with *Alcaligenes* lipid A promoted significant proliferation of and enhanced antibody production by B cells. Together, these findings suggest that *Alcaligenes* lipid A exerts an adjuvant activity on thymus-independent Hib polysaccharide antigen in the presence of a T-cell–dependent conjugate carrier antigen.

Second purpose of my study is to verify the application of *Alcaligenes* lipid A as a mucosal vaccine adjuvant. Mucosal vaccination is an ideal method to induce protective immunity against various pathogens. However, antigens alone are insufficient to elicit robust mucosal immune responses, necessitating the development of effective adjuvants. In this study, I also evaluated the effectiveness of *Alcaligenes*-derived lipid A as an adjuvant for sublingual immunization, a novel vaccination route garnering significant attention. Comparing to nasal administration as we previously examined, sublingual administration is able to not only induce immune responses in respiratory tracts but also in intestinal tracts with fewer safety issues. When mice were sublingually immunized with *Alcaligenes* lipid A and ovalbumin (OVA), a model antigen, an enhanced production of OVA-specific IgA was detected in both the respiratory and intestinal tracts, along with increased OVA-specific IgA and IgG

antibodies in serum. Additionally, sublingual immunization with cholera toxin B subunit (CTB) and lipid A resulted in elevated levels of CTB-specific IgG and IgA responses in the intestinal tract and systemic compartments, leading to the suppression of diarrhea induced by oral challenge with cholera toxin. Furthermore, immunization with pneumococcal surface protein A (PspA) plus *Alcaligenes* lipid A induced high levels of PspA-specific Th17 responses, as well as IgA and IgG responses, in both the respiratory tract and systemic compartments, providing protection against *Streptococcus pneumoniae* infection. These findings suggest that *Alcaligenes*-derived lipid A is a potent sublingual vaccine adjuvant with potential efficacy against both respiratory and intestinal infectious diseases.

As a conclusion, *Alcaligenes* lipid A could enhance immune responses against both T cell-dependent antigens and T cell-independent antigens and furtherly exert a role as a suitable sublingual vaccine adjuvant to help protect infection pathogens.

1. General Introduction

Vaccines must use the host immune sequence of innate and adaptive phases for effectively promote the induction of an antigen-specific defense especially during the adaptive immune response (Messina et al., 2019). The activation of adaptive immunity involves antigen-presenting cells (APCs), such as dendritic cells (DCs), a key immune cell bridging the innate and adaptive phases of host immunity. For example, DCs can recognize microbial components (e.g., lipopolysaccharide [LPS]) through pattern-recognition receptors such as Toll-like receptors (Lipscomb and Masten, 2002), which induce the secretion of immune enhancing cytokines and promote antigen processing and presentation for the initiation and enhancement of antigen-specific immune responses (Lee and Iwasaki, 2007).

Adjuvants can enhance immune responses induced by vaccines due to the poor immunogenicity of vaccine antigens themselves, especially for mucosal vaccines to ensure that the vaccination induces protective immunity rather than immune tolerance, which can prevent excessive and harmful immune responses (Lycke, 2012). Various studies have verified that certain microbial components and metabolites can influence host immunity, like toll-like receptors (TLRs) and LPS as mentioned before. However, the vaccine adjuvants especially mucosal vaccine adjuvants are numbered, such as monophosphoryl-lipid A (MPLA). The development of new effective and safe adjuvants for mucosal vaccines are necessary.

We previously showed that the commensal bacterium *Alcaligenes* specifically resides within Peyer's patches, a well characterized mucosa-associated lymphoid tissue for the initiation of antigen-specific immune responses in the intestine (Obata et al., 2010; Kunisawa and Kiyono, 2012). *Alcaligenes* organisms are taken up by dendritic cells (DCs) and promote the production of antibody-enhancing cytokines, including interleukin 6 (IL-6), thus leading to an elevated IgA antibody response in the intestine (Obata et al., 2010; Sato et al., 2013). In addition, compared with non-symbiotic *Escherichia coli*, symbiotic *Alcaligenes* have low inflammatory activity, which is explained at least partly by the unique features of its LPS (Fung et al., 2016; Shibata et al., 2018; Hosomi et al., 2020).

Several lines of evidence suggest that the structure of lipid A, which lies within the core of the LPS molecule, is related to its activity as a TLR4 ligand (Chandler and Ernst, 2017; Shimoyama et al., 2021). Compared with *E. coli*-derived lipid A, *Alcaligenes*-derived lipid A has shorter acyl chains that are modified with several functional groups, leading to appropriate activation of host immunity without excessive inflammation (Shimoyama et al., 2021). These characteristics prompted us to evaluate *Alcaligenes*-derived LPS and lipid A as a new and safe adjuvant candidate. Indeed, we found that both purified *Alcaligenes* LPS and chemically synthesized lipid A enhanced antibody production and Th17 responses to systemically or nasally immunized antigens (i.e., ovalbumin and pneumococcal surface protein A [PspA], a surface virulence factor of *Streptococcus pneumoniae*) (Wang et al., 2020; Yoshii et al., 2020; Wang et al., 2021).

As a conclusion, the *Alcaligenes* lipid A is supposed to be one ideal adjuvant. In this study, I, based on previous research, expended the examination of *Alcaligenes* lipid A and provided more information to support the application of lipid A as a vaccine adjuvant.

2. Materials and Methods

Mice

Because Hib vaccines are mainly used in infants, female BALB/c and nu/nu BALB/c mice were obtained after finishing lactation (age, 4 weeks, CLEA Japan) and kept for 1 week before experiments were initiated.

Female BALB/c mice (age, 8 weeks, CLEA Japan) were purchased and kept for 1 week before experiments were initiated. All animal experiments were conducted in accordance with the Animal Care and Use Committee guidelines of the National Institutes of Biomedical Innovation, Health, and Nutrition (NIBIOHN) and the Committee on the Ethics of Animal Experiments of NIBIOHN (approval nos. DSR04-37R7 and DSR04-38R7).

Preparation of *Alcaligenes* lipid A and PRP-tyramine

Alcaligenes lipid A (PEPTIDE INSTITUTE inc; or chemically synthesized as previously described (Shimoyama et al., 2021) was dissolved in dimethyl sulfoxide (Nacalai Tesque), and stored at −30 °C.

For use as the coating antigen in enzyme-linked immunosorbent assays (ELISAs), PRP (National Institute for Biological Standards and Control) was coupled to tyramine as follows. Briefly, 5 mg of PRP was dissolved in 10 mL of 0.01 N NaOH (Nacalai Tesque); 65 μL of acetonitrile (FUJIFILM) containing 65 mg of cyanogen bromide (FUJIFILM) was then added to the NaOH solution. The pH of the solution was maintained at 10.8 with 0.1 N NaOH and incubated at room temperature for 10 min. After PRP was activated, 1 mL of 0.5 M $NaHCO_3$ (Nacalai Tesque) containing 50 mg of tyramine hydrochloride (FUJIFILM) was added to the solution, and the pH was adjusted to 8.5 with 0.1 N HCl (FUJIFILM). The solution was transferred into dialysis bags (Sigma-Aldrich) and dialyzed against distilled water at 4 °C for 24 h followed by phosphate-buffered saline (PBS) at 4 °C for 24 h (Kaplan et al., 1983; Barra et al., 1988). The coupled PRP-tyramine was stored at –80 °C until use.

Preparation of PspA protein and endotoxin removal

The PspA gene was amplified by polymerase chain reaction (PCR) and cloned into pET16b plasmid (Novagen), as previously described, to yield pET16b-PspA plasmid (Suzuki et al., 2015). To obtain PspA recombinant proteins, the plasmids

were transformed into *E. coli* strain BL21 (DE3) (Takara). The expression of recombinant protein was induced by adding isopropyl-β-D-thiogalactopyranoside (Nacalai Tesque). The pellets were sonicated for a minute three times in buffer A (10 mM Tris-HCl [pH 8.0] (Nippon Gene), 400 mM NaCl (Nacalai Tesque), 5 mM MgCl2 (Nacalai Tesque), 0.1 mM PMSF (Nacalai Tesque), 1 mM 2-mercaptoethanol (Nacalai Tesque), and 10 % glycerol (Nacalai Tesque)). After centrifugation of the mixture at 4 ℃ and 17,800 × g for 15 min, the supernatants were filtered through a 0.45 μm Millex-HV filter unit (Merck Millipore) , and the recombinant protein was purified by using an NGC chromatography system (Bio-Rad, Hercules, California, USA) with a HisTrap HP columns (Cytiva). PspA was eluted with buffer A containing 100 to 500 mM imidazole (Nacalai Tesque). The eluted protein was loaded into a 30K centrifugal filter units (Merck Millipore) for concentration and exchange with PBS (Nacalai Tesque). After concentration, Triton X-114 (Nacalai Tesque) was added into the eluted protein to make a 1 % of concentration. After vortex, the protein was kept on ice for 5 min. Then, the protein was kept on 37 ℃ water bath for 5 min. Finally, after centrifugation at 25 ℃ and 3000 × g for 3 min, the supernatant was collected. After repeating for 3 times, the concentration of purified protein was measured by using a BCA protein assay kit (Thermo Fisher) and the concentration of endotoxin was measured by using LAL Endotoxin Assay Kit, Chromogenic, ToxinSensor (Funakoshi). The purity of the eluted protein was confirmed in a NuPAGE electrophoresis system (Invitrogen) followed by staining with Coomassie brilliant blue (ATTO).

Immunization

Mice were anesthetized with isoflurane (FUJIFILM) and then subcutaneously immunized with a total volume of 200 μL PBS containing either 0.01 μg of the Hib capsular polysaccharide PRP, 0.01 μg of PRP plus 0.024 μg of tetanus toxoid (TT) (EMD Millipore), Haemophilus B PRP–TT conjugate vaccine (ActHIB; Sanofi) equivalent to 0.01 μg of PRP or 1 μg of PRP with or without 1 μg of *Alcaligenes* lipid A (Wang et al., 2020), or PBS only.

Mice were anesthetized with isoflurane and then sublingually immunized with a total volume of 5 μL PBS containing either 5 μg of the OVA with or without 1 μg of *Alcaligenes* lipid A, or 5 μg of the PspA with or without 1 μg of *Alcaligenes* lipid A, or 2 μg of the Cholera toxin B subunit (CTB) (FUJIFILM) with or without 1 μg of *Alcaligenes* lipid A or PBS only. After immunization, mice were left forward bending under anesthetized for 30 min. Mice received three immunizations at 1-week intervals.

Mice received 3 immunizations at 1-week intervals. One week after the final immunization, blood was harvested from the mice and kept on ice until centrifuged at 4 ℃, 3000 × g for 10 min. The serum was transferred into a fresh tube and stored at –80 ℃.

Detection of antigen-specific antibodies by enzyme-linked immunosorbent assay (ELISA)

The production of PRP, TT, OVA, PspA and CT-specific antibodies was detected by ELISA. Briefly, 96-well immunoplates (Thermo Fisher Scientific) were coated with 100 μL of 5 μg/mL PRP-tyramine or 0.1 μg/mL TT or 1 mg/mL of OVA or 5 μg/mL of CT or 5 μg/mL of PspA in PBS at 4 °C overnight. After the coating solution was removed, the plates were saturated with 170 μL of 1 % bovine serum albumin (BSA, Nacalai Tesque) dissolved in PBS for 2 h at room temperature. Plates were then rinsed 3 times with 200 μL of wash buffer (PBS containing 0.05 % Tween 20 [Nacalai Tesque]). Each well then received mouse serum (2-fold serially diluted in PBS containing 0.05 % Tween 20 and 1 % BSA), and the plates were incubated at room temperature for 2 h. The plates were then again washed 3 times with 200μL of wash buffer; goat anti-mouse IgG, IgG1, IgG2a, IgG3, IgA antibody conjugated with horseradish peroxidase (SouthernBiotech; diluted 1:4000 in PBS containing 1 % BSA and 0.05 % Tween 20) was added to each well; and the plates were incubated at room temperature for 1 h. The plates again were washed 3 times with 200 μL of wash buffer; tetramethylbenzidine peroxidase substrate (SeraCare Life Sciences) was added to the plates; and the plates were incubated at room temperature for 2 min, after which 0.5 N HCl (Nacalai Tesque) was added to each well. The absorbance of samples at 450 nm (OD_{450}) was measured by using an iMarkTM Microplate Absorbance Reader (Bio-Rad Laboratories).

T-cell assay

At 1 week after the final immunization, the submandibular lymph nodes (SMLNs) and spleens from immunized mice were harvested, homogenized, and then filtered through 100-μm cell strainers (Corning) separately. These single-cell suspensions were treated with 1 mL of red blood cell lysis buffer (10 mM $NaHCO_3$ [Nacalai Tesque], 1 mM EDTA-2Na·$2H_2O$ [Dojindo Molecular Technologies], 0.15 M NH_4Cl [Nacalai Tesque]) for 1 min at room temperature. After washing with MACS Buffer (2 mM EDTA-2Na·$2H_2O$, 0.5 % BSA in PBS), $CD4^+$ T cells were purified by using a magnetic cell separation system and anti-mouse CD4 (L3T4) magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and MS columns (Miltenyi Biotec). Purified $CD4^+$ T cells were purified by using a magnetic cell separation system and anti-mouse CD4 (L3T4) magnetic beads (Miltenyi Biotec) and MS columns (Miltenyi Biotec). Purified $CD4^+$ T cells were resuspended in RPMI medium (Sigma) containing 10 % fetal bovine serum (Serana), 1 mM sodium pyruvate solution (Nacalai Tesque), 1 % penicillin–streptomycin mixed solution (Nacalai Tesque), and 0.1 % 2-mercaptoethanol (Gibco) and were seeded at a concentration of 2×10^5 cells/well into 96-well plates (Nunc 96-Well, Nunclon Delta-Treated, U-Shaped-Bottom Microplates, Thermo Fisher Scientific). Each well also received splenic APCs (2×10^4 cells/well) from unimmunized mice that had been treated with 30 Gy of ionizing radiation (MBR-1520R-4). The purified $CD4^+$ T cells mixed with APCs were incubated in the presence or absence of 1 mg/mL of OVA or 4

μg/mL of PspA at 37 °C in 5 % CO2. After 4 days of incubation, live T cells were counted by using CyQUANTTM Direct Cell Proliferation Assay Kits (Invitrogen). Cytokines in the supernatant was measured by the BD™ Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences) according to manufacturing instructions and analyzed with a MACSQuant® Analyzer (Miltenyi Biotec). Data analysis was performed using FlowJo 10.0.7 (Tree Star).

Coculture of B cells with *Alcaligenes* lipid A and measurement of IgG production

Spleens from naïve mice were homogenized and filtered through 100-μm cell strainers. The suspensions were treated with 1 mL of red blood cell lysis buffer for 1 min at room temperature. After washing with MACS Buffer, splenic $B220^+$ cells were purified by using a magnetic cell separation system with anti-mouse CD45R (B220) magnetic beads (Miltenyi Biotec) and LS Columns (Miltenyi Biotec). The B cells were then seeded (10^5 cells/well) into 96-well plates without or with 100 ng/mL of *Alcaligenes* lipid A and incubated at 37 °C in 5 % CO_2. After the 5-day incubation, live B cells were counted by using CyQUANTTM Direct Cell Proliferation Assay Kits.

The total IgG contents in the B-cell culture supernatant were measured by using antigen-specific ELISAs as mentioned before. Briefly, 96-well immunoplates were coated with 100μL of 2 μg/mL goat anti-mouse Ig (SouthernBiotech). After the plates were washed with wash buffer (3 × 200 μL/well), dilutions of culture supernatant and 2-fold serially diluted standard antibody (unconjugated mouse IgG, SouthernBiotech) were added to wells, and plates were incubated at room temperature for 2 h. The plates were washed again with wash buffer (3 × 200 μL/well); goat anti-mouse IgG antibody conjugated with horseradish peroxidase (diluted 1 : 4000 in PBS containing 1 % BSA and 0.05 % Tween 20) was added to each well; and the plates were incubated at room temperature for 1 h. The plates were washed again with wash buffer (3 × 200 μL/well); tetramethylbenzidine peroxidase substrate was added to the plates; and the plates were incubated at room temperature for 2 min, after which 0.5 N HCl was added to each well. The absorbance of samples at 450 nm was measured by using an iMarkTM Microplate Absorbance Reader.

***S. pneumoniae* culture and infection model**

S. pneumoniae Xen10 was cultured in brain-heart infusion broth (Becton) at 37 °C in 5 % CO_2 with no aeration overnight. *S. pneumoniae* was then collected by centrifugating for 15 min at 4 °C, 3000 × g and then washed twice with PBS for 3 min at 4 °C, 9100 × g. One week after the final immunization, mice were nasally challenged with 1.5×10^7 CFU (40 μL per mouse) of *S. pneumoniae* under anesthesia. The survival and body weight of the infected mice were monitored for 14 days.

CT challenging

One week after the final immunization, mice were fasted for 8 h and orally challenged with 50 μg CT in 200 μL. 16 h after CT challenging, the volume of water in cecum was measured.

Statistical analysis

Data are presented as mean ±1 SD. Statistical analyses were performed by using Student's t-test and one-way ANOVA with Tukey's multiple comparison test after ROUT outlier identification (PRISM 10.1.2, GraphPad Software, San Diego, CA, United States). Statistical significance was established at $p < 0.05$.

3. Chemically synthesized *Alcaligenes* Lipid A as an adjuvant to augment immune responses against *Haemophilus Influenzae* type B conjugate vaccine

3.1 Specific introduction

Host immunity includes both innate and adaptive phases for the induction of antigen-specific immune responses. In general, the innate phase, a beginning of immune response reacts foreign antigen or pathogen in prompt manner using the pattern-recognition system (e.g., TLRs), which leads to the activation of the adaptive immune response recognizes and eliminates pathogens specifically (Netea et al., 2019).

B-cell responses are divided into two types, which differ according to their need for T-cell involvement. T-cell–independent (TI) antigens induce rapid but short-lived production of IgM (Nutt et al., 2015). Because TI antigens, which include most polysaccharides, cannot be presented to T cells through major histocompatibility complex (MHC) class II (Mond et al., 1995), B-cell development and IgG class switching cannot be induced without input from T cells. In contrast, during T-cell–dependent (TD) B-cell responses, T cells are activated via their interaction with APCs through receptor pairing to MHC molecules and various costimulatory molecules (Mond et al., 1995).

Haemophilus influenzae type B (Hib), a gram-negative pathogenic bacterium, is a frequent cause of bacterial meningitis among children (Anderson et al., 1977). The polyribosyl ribitol phosphate (PRP) of Hib has been used as the antigen for vaccines against Hib. Because PRP is a TI antigen, commercially available vaccines (e.g., ActHib) include a modified PRP to which a TD carrier protein antigen (e.g., tetanus toxoid) has been conjugated, to enhance the immunogenicity of PRP (Guttormsen et al., 1999; Kelly et al., 2004).

Although our previous studies (Wang et al., 2020; Yoshii et al., 2020; Wang et al., 2021) demonstrated that *Alcaligenes* lipid A is an effective adjuvant for TD antigens such as ovalbumin and PspA, whether it also efficiently boosts the antigenicity of TI antigens remained unclear. Here we aimed to extend the application of *Alcaligenes* lipid A by determining its adjuvanticity on a *Haemophilus* B conjugate

vaccine as an example TI antigen–based conjugate vaccine.

3.2 Results

Alcaligenes lipid A enhances both PRP- and TT-specific IgG responses after immunization with *Haemophilus* B conjugate vaccine

When PRP is conjugated to a protein, such as TT, the complex acts as a TD antigen and thus induces PRP-specific IgG production (Guttormsen et al., 1999; Kelly et al., 2004). Therefore, whether *Alcaligenes* lipid A enhances the immune response against the conjugated PRP of the *Haemophilus* B conjugate vaccine was examined first. Consistent with a previous study (Schneerson et al., 1980), PRP-specific IgG production was induced in mice immunized with the *Haemophilus* B conjugate vaccine compared with PBS (as a control). Specifically, mice immunized with *Haemophilus* B conjugate vaccine in the presence of *Alcaligenes* lipid A had higher levels of PRP-specific serum IgG than did mice immunized with *Haemophilus* B conjugate vaccine alone at different antigen doses of 0.01 and 1 μg of PRP (Figure 1, 2A). Among IgG subtypes, higher levels of IgG3 were detected in groups immunized with *Alcaligenes* lipid A (Figure 3C). In addition, mice immunized with *Haemophilus* B conjugate vaccine plus *Alcaligenes* lipid A had higher levels of TT-specific serum IgG (Figure 2B). These results show that *Alcaligenes* lipid A can enhance the production of IgG against PRP, a TI antigen, when PRP is conjugated to TT.

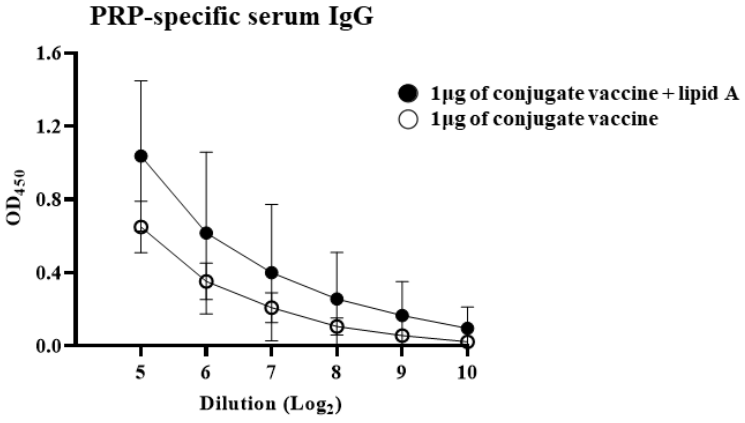

Figure 1. *Alcaligenes* lipid A enhanced PRP-specific IgG production in *Haemophilus* B conjugate vaccination at high dose antigen. Mice were immunized subcutaneously with *Haemophilus* B conjugate vaccine containing 1 μg of PRP with or without 1 μg of *Alcaligenes* lipid A. Serum was collected 1 week after the final immunization and PRP-specific IgG was measured by ELISA (n = 3/group). The data are presented as mean ± 1 SD.

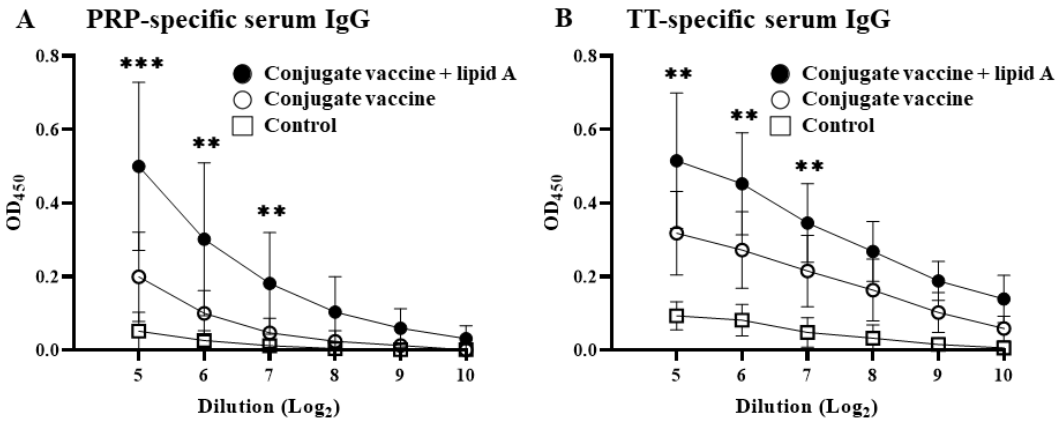

Figure 2. *Alcaligenes* lipid A enhanced antigen-specific IgG production in *Haemophilus* B conjugate vaccination. Mice were immunized subcutaneously with PBS (control group) or *Haemophilus* B conjugate vaccine containing 0.01 μg of PRP with or without 1 μg of *Alcaligenes* lipid A. Serum was collected 1 week after the final immunization, and the levels of **(A)** PRP-specific IgG and **(B)** TT-specific IgG were measured by ELISA (n = 11/group). The results shown are presented as mean ± 1 SD. Data are combined from two independent experiments, and statistical significance was evaluated by using one-way ANOVA (**, $P < 0.01$; ***, $P < 0.001$; the asterisks represent the significant difference between two experimental groups).

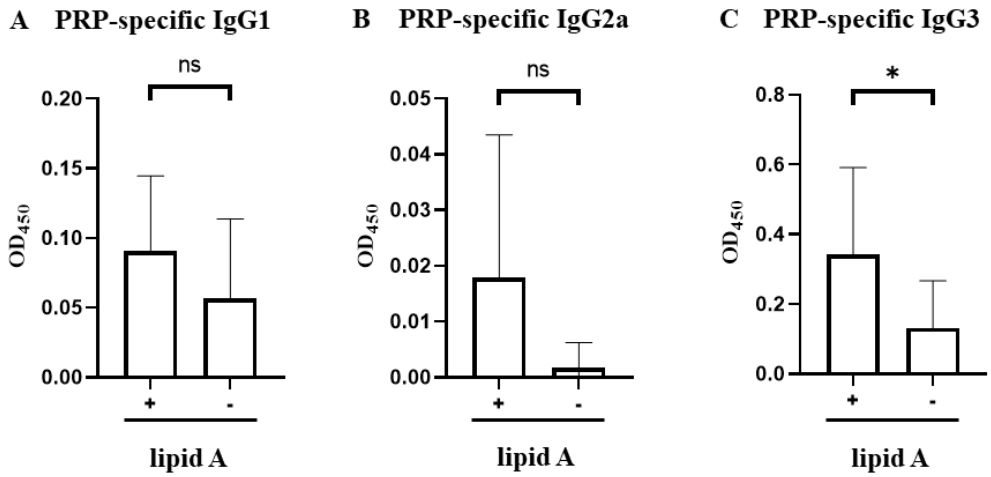

Figure 3. PRP-specific IgG3 is enhanced by *Alcaligenes* lipid A. Mice were immunized subcutaneously with *Haemophilus* B conjugate vaccine containing 0.01 μg of PRP with or without 1 μg of *Alcaligenes* lipid A. The subtypes of PRP-specific IgG were detected with ELISA. Low levels of IgG1 **(A)** and few IgG2a **(B)** were detected while significant enhancement on IgG3 **(C)** was observed, suggesting that the *Alcaligenes* lipid A may not influence T cells. Data are combined from two independent experiments and are presented as mean ± 1 SD. The statistical significance was evaluated by using Student's *t*-test (n = 11/group; ns, not significant; *, $P < 0.05$).

Conjugation of PRP to the TT carrier protein is necessary for *Alcaligenes* lipid A–mediated enhancement of a PRP-specific IgG response

To verify the importance of the conjugation of carbohydrate antigen PRP to protein career TT for the adjuvant activity of *Alcaligenes* lipid A in the enhanced PRP-specific IgG production, mice were next immunized with either PRP only or mixed (no physical coupling) with TT in the presence or absence with *Alcaligenes* lipid A. Neither immunization with PRP alone nor with PRP plus lipid A induced an IgG response (Figure 4A). Furthermore, no PRP-specific IgG response was detected in mice immunized with both antigens (PRP mixed with TT) even with *Alcaligenes* lipid A (Figure 4B); meanwhile, TT-specific IgG response was not enhanced (Figure 4C). These findings indicate that only when PRP is conjugated with TT, *Alcaligenes*

lipid A could enhance IgG production against PRP.

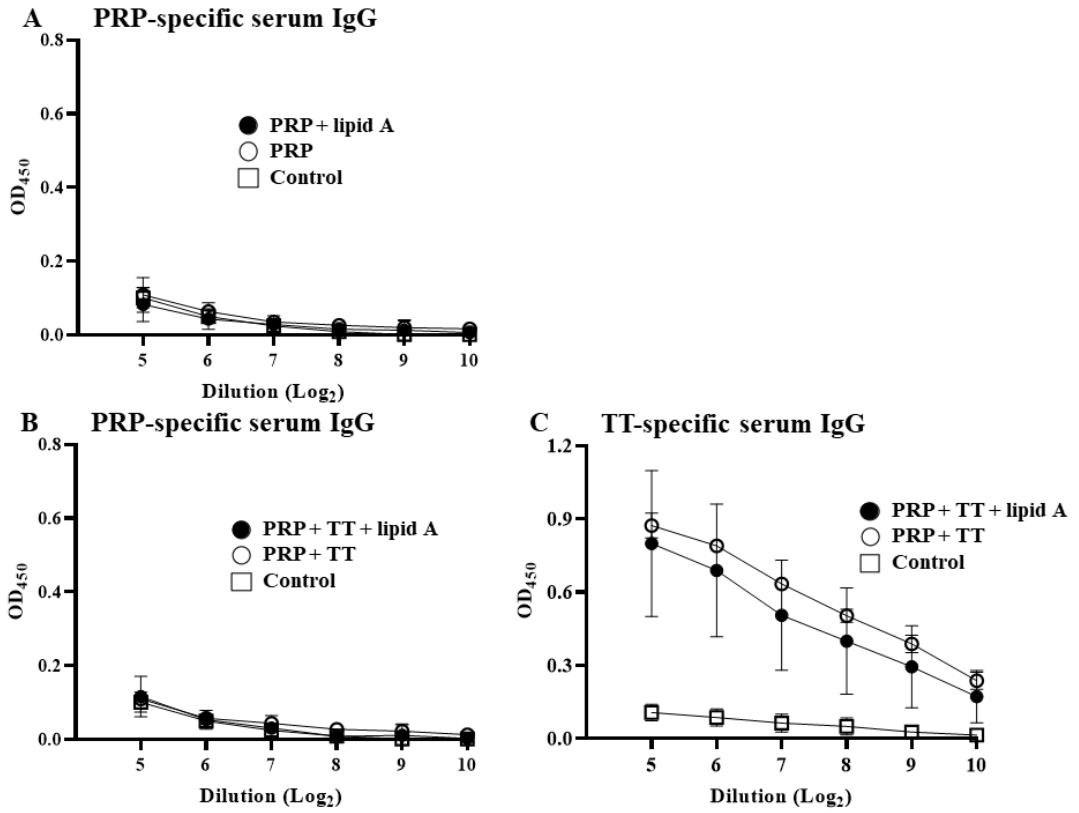

Figure 4. Conjugation of PRP to TT carrier protein is essential for enhancement of PRP-specific IgG production by *Alcaligenes* lipid A. **(A)** Mice were immunized subcutaneously with PBS (control group) or 0.01 μg of unconjugated PRP with or without 1 μg of *Alcaligenes* lipid A. **(B)** Mice were immunized subcutaneously with PBS (control group) or 0.01 μg of unconjugated PRP plus 0.024 μg of TT and with or without 1 μg of *Alcaligenes* lipid A. Serum was collected 1 week after the final immunization, and the level of PRP-specific IgG was measured by ELISA (experimental group, n = 5; control group, n = 4). Data are representative of two independent experiments and are presented as mean ± 1 SD.

T cells are required for the induction of a PRP-specific IgG response

It has been found that conjugation of PRP with TT is necessary for the induction and augmentation of PRP-specific IgG response by *Alcaligenes* lipid A. These findings led me to examine the importance of T cells in *Alcaligenes* lipid A-mediated enhancement of antigen-specific IgG production. Immunization of nude mice, which have a deteriorated or absent thymus and thus lack T cells, with *Haemophilus* B conjugate vaccine with or without *Alcaligenes* lipid A induced scant PRP-specific IgG production (Figure 5A) and no TT-specific IgG response (Figure 5B). These results show that *Haemophilus* B conjugate vaccine-induced IgG responses to either PRP or TT require T cells and that *Alcaligenes* lipid A cannot augment these IgG responses in

the absence of T cells.

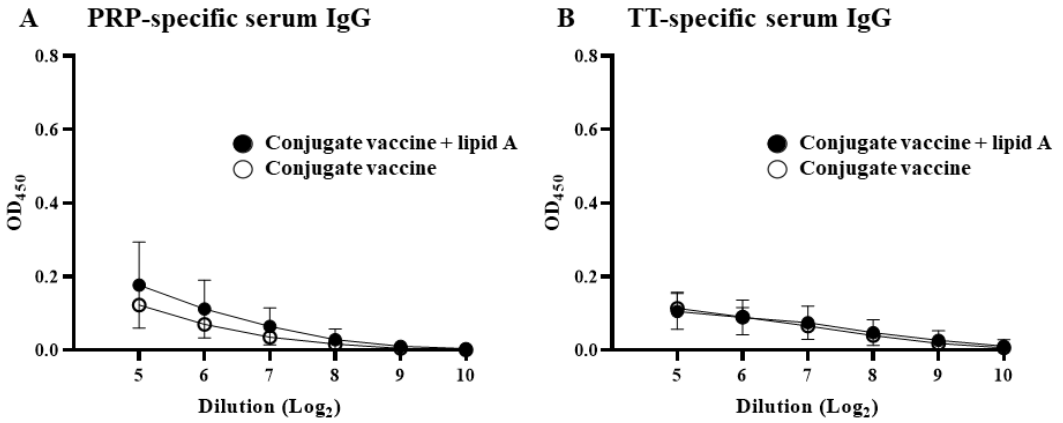

Figure 5. T cells are required for *Alcaligenes* lipid A–promoted PRP-specific IgG production. T cell–deficient nude mice were immunized subcutaneously with *Haemophilus* B conjugate vaccine with or without 1 μg of *Alcaligenes* lipid A. Serum was collected 1 week after the final immunization, and the levels of **(A)** PRP-specific IgG and **(B)** TT-specific IgG were measured by ELISA. Data are representative of two independent experiments and are presented as mean ± 1 SD. (n = 4/group).

T cells are induced by *Haemophilus* B conjugate vaccine but are not enhanced by *Alcaligenes* Lipid A

Since T cells are required for adjuvanticity of *Alcaligenes* lipid A to enhance antigen-specific antibody production in response to the *Haemophilus* B conjugate vaccine, next the effects of *Alcaligenes* lipid A on T cells were investigated. Splenic $CD4^+$ T cells were isolated from mice immunized with *Haemophilus* B conjugate vaccine with or without *Alcaligenes* lipid A and measured their ability to proliferate ex vivo upon stimulation with antigen (e.g., PRP and TT) in the presence of APCs. Stimulation with TT (Figure 4B)—but not PRP (Figure 6A)—increased T-cell counts in the immunized groups. However, including *Alcaligenes* lipid A at immunization did not further increase the number of T cells (Figure 6B). Cytokine analysis revealed that IL-17A was preferentially detected in the immunized groups (Figure 7C) while there were no significant differences between IFN-γ (Figure 7A) and IL-4 (Figure

7B). These results show that although the *Haemophilus* B conjugate vaccine induces a TT-specific T-cell response, concurrent immunization with *Alcaligenes* lipid A does not enhance it.

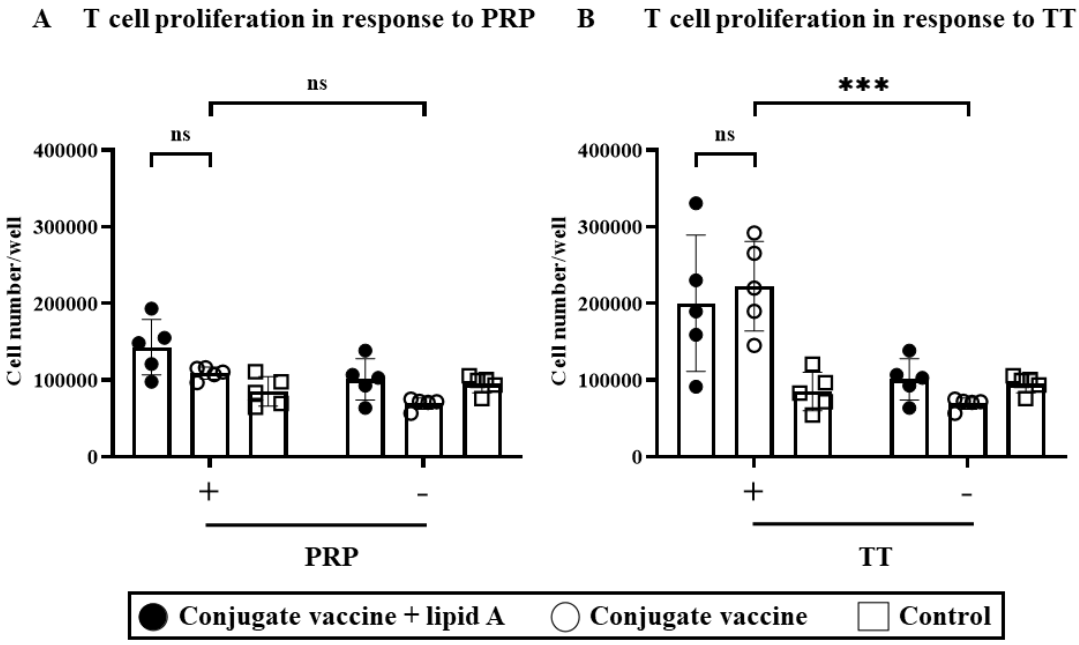

Figure 6. *Alcaligenes* lipid A has no effect on the TT-specific T-cell response. Mice were immunized subcutaneously with *Haemophilus* B conjugate vaccine with or without 1 μg of *Alcaligenes* lipid A; control mice were immunized with PBS. Splenic $CD4^+$ cells were collected 1 week after the final immunization and stimulated with (+) or without (–) **(A)** 2.08 μg/mL PRP or **(B)** 5 μg/mL TT. After stimulation for 4 days, live $CD4^+$ cells were counted. Data are representative of two independent experiments and are presented as mean ± 1 SD. (n = 5/group), and statistical significance was evaluated by using one-way ANOVA (ns, not significant; ***, $P < 0.001$).

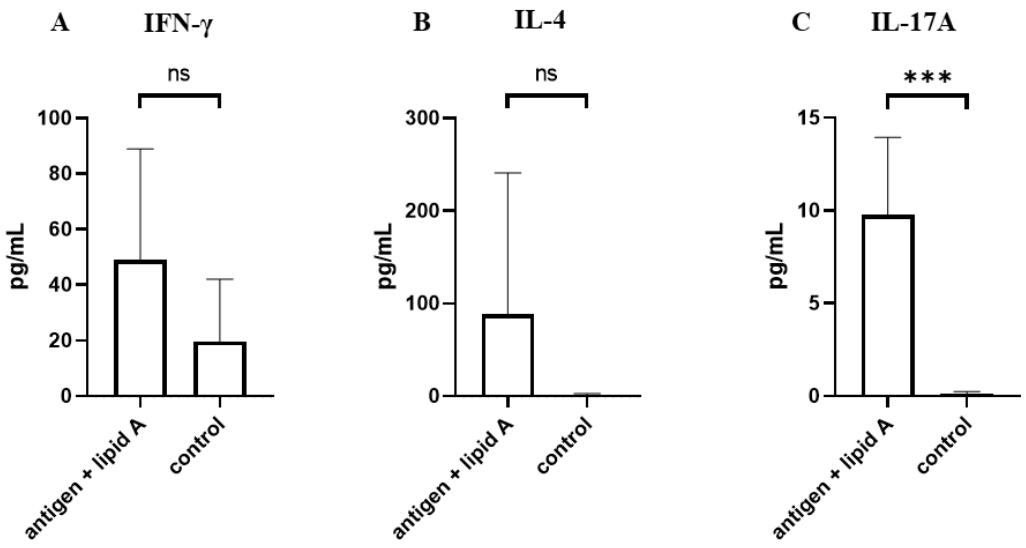

Figure 7. IL-17 is induced by *Alcaligenes* lipid A. Mice were immunized subcutaneously with *Haemophilus* B conjugate vaccine with or without 1 μg of *Alcaligenes* lipid A; control mice were immunized with PBS. Splenic $CD4^+$ cells were collected 1 week after the final immunization and stimulated with 5 μg/mL TT. After the stimulation, all the supernatant of T cells was collected to measure the IFN-γ **(A)**, IL-4 **(B)**, and IL-17 **(C)**. Data are representative of two independent experiments and are presented as mean ± 1 SD. The statistical significance was evaluated by using Student's *t*-test (n = 5/group; ns, not significant; ***, $P < 0.001$).

***Alcaligenes* lipid A activates B cells, leading to enhanced cell numbers and antibody production**

Given that *Alcaligenes* lipid A failed to enhance the T-cell response to the *Haemophilus* B conjugate vaccine, it is wondered whether direct stimulation of B cells might lead to upregulation of IgG secretion. Therefore, naïve splenic $B220^+$ B cells were then cocultured with *Alcaligenes* lipid A. Treatment with *Alcaligenes* lipid A significantly increased the number of B cells (Figure 8A) and the amounts of IgG (Figure 8B) in culture supernatants. These results indicate that *Alcaligenes* lipid A directly promotes B-cell proliferation and antibody production.

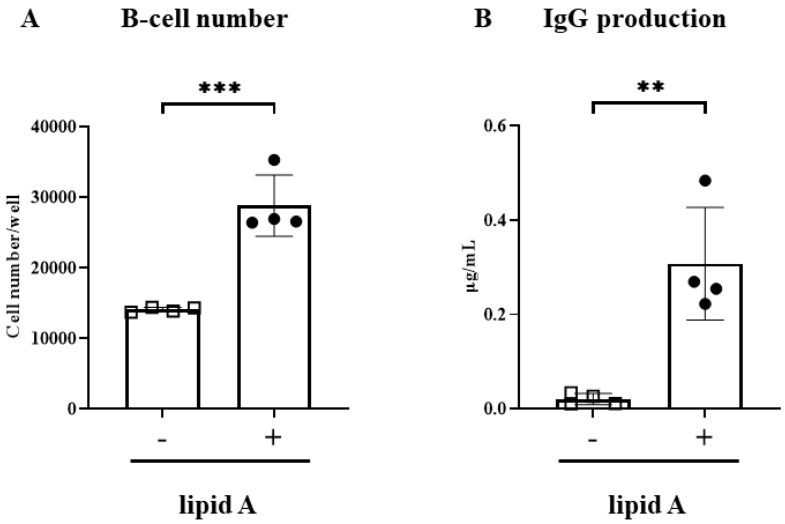

Figure 8. *Alcaligenes* lipid A directly activates B cells. Splenic $B220^{+}$ cells were isolated from naive mice. After 4 days of culture with (+) or without (–) *Alcaligenes* lipid A, **(A)** live B cells were counted, and the **(B)** IgG content in the culture supernatant was measured. Data are representative of two independent experiments and are presented as mean ± 1 SD, and statistical significance was evaluated by using Student's *t*-test (n = 4/group; **, $P < 0.01$; ***, $P < 0.001$).

3.3 Specific Discussion

An unexpected finding in the current study was that *Alcaligenes* lipid A did not enhance the T cell-response to immunization (Figure 6), suggesting that the pathway through which *Alcaligenes* lipid A increases the IgG response differs from that through which it activates DCs and thus influences the T-cell response. One possible reason might be the way through which antigens are presented to T cells. For TD antigens, DCs act as the major APCs and present antigens to T cells for activation together with costimulatory molecules such as CD80 (Tai et al., 2018). Meanwhile, cytokines secreted by DCs also sense T cells, inducing different types of response (Terhune et al., 2013), which then activate B cells through cell-contact. In contrast, B cells recognize glycoconjugate antigens because of their carbohydrate portion and thus retrieve the entire antigen through B-cell receptors (Popi et al., 2016; Avci et al., 2019). After being processed, the peptide portion is presented to T cells via MHC II, thus activating T cells, which then secrete cytokines to activate B cells (Avci and Kasper, 2010; Avci et al., 2011). During the first type of response, DCs produce T-

cell–activating cytokines, including IL-12 (which induces Th1 differentiation), IL-4 (Th2 differentiation), and IL-6, IL-23, and TGF-β (Th17 differentiation) (Kimura and Kishimoto, 2010; Terhune et al., 2013). In contrast, B cells, which might secrete only negligible amounts of other cytokines, produce considerable IL-6 (Chousterman and Swirski, 2015), suggesting that the T-cell response induced by B cells may differ from that induced by DCs. Hence, perhaps the way in which T cells are activated by APCs influences the subsequent T-cell response. Thus, *Alcaligenes* lipid A may preferentially activate B cells directly to yield adjuvanticity for the *Haemophilus* B conjugate vaccine.

TLR4 is expressed not only on APCs such as DCs (Vaure and Liu, 2014), which has been proved to be a target for *Alcaligenes* lipid A (Shibata et al., 2018) during responses induced by TD antigens such as PspA, but also on B cells (Vaure and Liu, 2014), thus suggesting at least two possible mechanisms through which *Alcaligenes* lipid A exerts its effects on antigen-specific IgG production. Regarding the first possibility of a direct effect of lipid A on B cells, coculture with *Alcaligenes* lipid A increased B-cell numbers and their ability to secrete IgG (Fig 8). These effects likely occurred through the TLR4 pathway. B-cell proliferation might involve the phosphatidylinositol 3-kinase signaling pathway (Venkataraman et al., 1999), which can be induced through TLR4 signaling (Dil and Marshall, 2009). In addition, due to upregulation of MyD88, B cells in germinal centers (GCs) show increased reactivity to TLR ligands, leading to enhanced proliferation and promotion of class-switching recombination; MyD88 facilitates B-cell differentiation into plasma cells (Rawlings et al., 2012). The second possible mechanism underlying the enhancement of IgG production in response to *Alcaligenes* lipid A is through effects on DC-mediated antigen-specific T-cell responses. Because PRP is a TI antigen (Guttormsen et al., 1999; Kelly et al., 2004), the induction of a PRP-specific IgG response required both T cells and conjugation of PRP with TT. Indeed, lacking the help from T cells, polysaccharide-activated B cells undergo apoptosis and thus fail to mount a PRP-specific IgG response (Rappuoli, 2018; Rappuoli et al., 2019; Cobb et al., 2004). The conjugation with TT may not only induce T cell-response but also affect the antigen uptake. It was reported that the TD portion of conjugate vaccine mediated their uptake by DCs, which will trigger the formation of GCs (Rappuoli, 2018). Meanwhile, our previous study demonstrated that *Alcaligenes* lipid A induced the formation of GCs (Yoshii et al., 2020) in which B cells activated through TLRs show higher viability (Rawlings et al., 2012). These findings collectively implicate a possibility that *Alcaligenes* lipid A may enhance the antibody production against conjugated vaccine directly through the contact to B cells and indirectly through the simultaneous induction of GCs. However, apart from the conjugation, the TT itself might also influence the response because, unexpectedly, when TT was co-administered with PRP together with *Alcaligenes* lipid A, TT-specific response was not enhanced. The mechanism has not been fully explained because the reaction of LPS-induced TT-specific response is not very representative (Mohammadi et al., 2014).

In general, vaccination with TI antigens induces IgM-mediated immunity only,

and long-lasting IgG-mediated immunity has been difficult to achieve. Conjugation of TD carrier proteins to some TI antigens can induce class-switching (Avci et al., 2011). The resulting induction of IgG production has increased the efficacy of immune responses against various TI antigens to provide sufficient protection and even prevention in some cases (Cochi et al., 1985; Granoff et al., 1993). In the current study, the adjuvant activity of the *Alcaligenes* lipid A on the TI antigen in the *Haemophilus* B conjugate vaccine (i.e., PRP) presumably was mediated through the direct activation of B cells instead of via enhancement of T-cell responses. In addition to creating a conjugate vaccine that induces a sufficient T-cell response for the induction of class-switching to IgG, enhancing the proliferation of B cells and their IgG secretion will enhance immune responses to TI antigens. As a result, *Alcaligenes* lipid A induced higher immune responses with a lower dose of antigens, reducing the required amount of antigens which can reduce the side effect of antigens themselves is one important characteristic of the adjuvant. Thus, our current findings support the use of *Alcaligenes* lipid A as an adjuvant to augment and accelerate vaccine-induced immune responses.

As a short summary, I showed that *Alcaligenes* lipid A successfully enhanced immune responses against T-cell independent antigens. For the application of *Alcaligenes* lipid A as an adjuvant in clinical in the future, I then focused on the effect of *Alcaligenes* lipid A on the sublingual vaccines, one potential mucosal vaccine.

4. Chemically synthesized *Alcaligenes* Lipid A as a sublingual adjuvant to augment protective immune responses in the respiratory and gastrointestinal tracts

4.1 Specific introduction

Mucosal tissues are capable of preventing the colonization and invasion of various microbes, due to their biological barriers (e.g., epithelium, mucus, and antimicrobial peptide) (Holmgren and Czerkinsky, 2005). Several types of immune-related cells such as M cells specialized antigen-uptake cells to transport antigens into gut-associated lymphoid tissue, DCs, T cells, and B cells, contribute to the creation of highly specialized mucosal immune systems (Holmgren and Czerkinsky, 2005; Takahashi et al., 2009). Especially, antigen-specific secreted IgA antibodies on the surface of the tissues play a predominant role in the humoral immune responses to protect the host from pathogens (Holmgren and Czerkinsky, 2005; Takahashi et al., 2009; Kunisawa et al., 2008). Mucosal vaccines are capable of inducing antigen-specific IgA antibody production, making them potentially more suitable for combating mucosa-related infectious diseases compared to the injection-type vaccines which primarily induce systemic immune responses, such as IgG antibody production in the blood.

Sublingual administration is recognized as an advantageous method for drug

delivery. Drugs administered sublingually can easily and directly enter the systemic circulation through various blood vessels located beneath the thin sublingual epithelium, bypassing the enterohepatic first-pass effect. This results in rapid and highly efficient absorption comparing to traditional oral delivery systems (Hua, 2019). Additionally, a network of lymphatic vessels under the sublingual epithelium facilitates the induction of immune responses in the sublingual mucosa. It has been reported that APCs, such as DCs in the sublingual mucosa, uptake antigens and migrate to regional lymph nodes before disseminating to distant lymph nodes, thereby inducing humoral responses at distant mucosal sites (Kraan et al., 2014; Paris et al., 2021). Considering the limitations of mucosal vaccines like oral vaccines which mainly induce a strong immune response in the intestinal tract, sublingual vaccines are potentially to be a better type of mucosal vaccine.

Focusing on the application of *Alcaligenes* lipid A, I also demonstrate that *Alcaligenes* lipid A effectively induces antigen-specific IgA responses in both the respiratory (PspA-specific) and intestinal (CT-specific) tracts, providing protection against *S. pneumoniae* infection and cholera toxin-induced diarrhea.

4.2 Results

Sublingual administration of OVA together with *Alcaligenes* lipid A enhanced antigen-specific immune responses at the respiratory and gastrointestinal tracts together with systemic compartments

In order to examine whether *Alcaligenes* lipid A can enhance immune responses in both respiratory and intestinal tracts, I co-administered mice sublingually with *Alcaligenes* lipid A and ovalbumin (OVA), a model antigen to analyze the humoral immune responses. In respiratory tract, OVA-specific antibody responses-evidenced by elevated by IgA levels in nasal wash fluids (Figure 9A) and IgG in bronchoalveolar lavage fluids (BALF) (Figure 9B) were enhanced in mice sublingually immunized with OVA together with *Alcaligenes* lipid A, compared to OVA alone. Notably, despite individual variations, a trend towards increased OVA-specific IgA in the BALF was also observed (data not shown). Meanwhile, the enhanced OVA-specific fecal IgA responses highlighted the effect of *Alcaligenes* lipid A on intestinal immunity (Figure 9C). Moreover, OVA-specific IgG in serum were enhanced in mice sublingually immunized with OVA alongside *Alcaligenes* lipid A (Figure 9D). Collectively, these findings demonstrated that sublingual immunization using *Alcaligenes* lipid A as an adjuvant robustly enhances antigen-specific antibody production not only in the respiratory and intestinal tracts but also systemically.

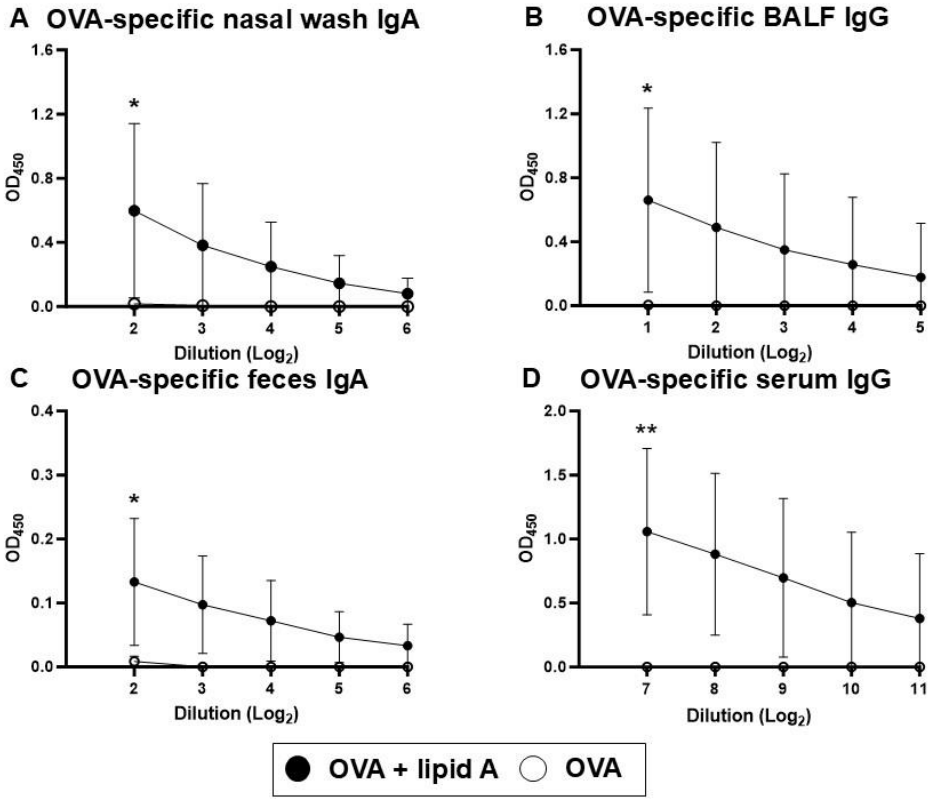

Figure 9. Sublingual administration of *Alcaligenes* lipid A enhanced the production of OVA-specific antibodies both in the mucosal and systemic compartments. Mice were immunized sublingually with OVA with or without *Alcaligenes* lipid A. Nasal wash, BALF, feces, serum were collected 1 week after the final immunization, and the levels of **(A)** OVA-specific nasal wash IgA (n = 6/group) and **(B)** OVA-specific BALF IgG (n = 6/group) and **(C)** OVA-specific feces IgA (n = 6/group) and **(D)** OVA-specific serum IgG (experimental group, n = 6/group; control group, n = 5/group). were measured by ELISA The results shown are presented as mean ± 1 SD. Data are a combination of two independent experiments, and statistical significance was evaluated by using Student's t-test (*, $P < 0.05$; **, $P < 0.01$).

Sublingual administration of *Alcaligenes* lipid A enhanced cholera toxin B subunit-specific mucosal and systemic humoral immune responses

Having verified that *Alcaligenes* lipid A could enhance antigen-specific antibody production in the intestinal tract, we next explored its potential as an adjuvant for sublingual vaccines against intestinal diseases. In this issue, CTB was employed as a model vaccine against Vibrio cholerae (Baldauf et al., 2015). Unlike OVA, mice immunized with CTB alone exhibited a measurable CT-specific IgA response in feces (Figure 10A) and IgG response in serum (Figure 10B). Notably, co-administration of CTB with *Alcaligenes* lipid A resulted in elevated levels of IgA responses in feces (Figure 10A) and IgG in serum (Figure 10B). These findings suggest that *Alcaligenes* lipid A is a potent enhancer of antigen-specific antibody responses against intestinal

pathogens and toxins.

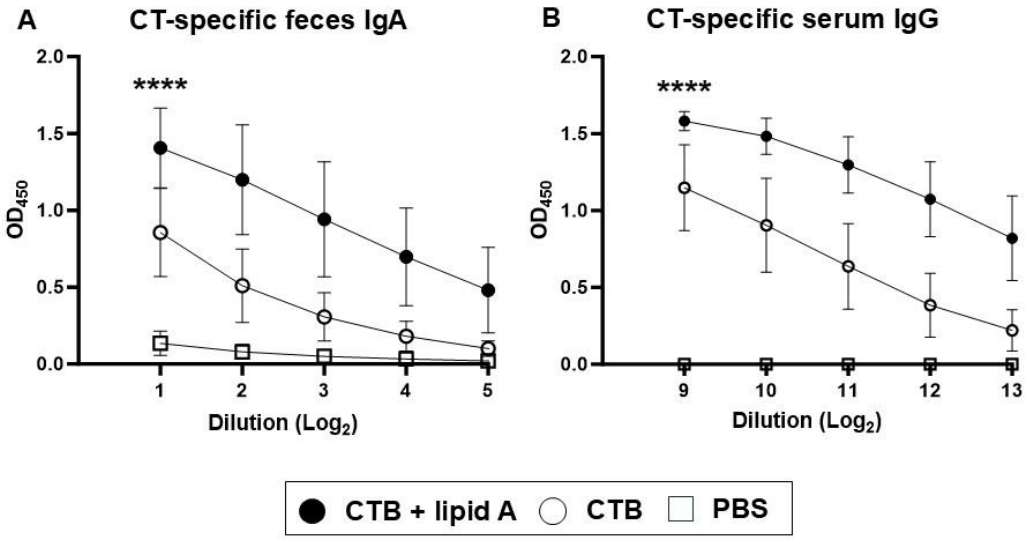

Figure 10. Sublingual administration of *Alcaligenes* lipid A enhanced the production of CTB-specific antibodies. Mice were immunized sublingually with PBS or CTB with or without *Alcaligenes* lipid A. Feces and serum were collected 1 week after the final immunization, and the levels of **(A)** CTB-specific feces IgA (n = 10/group) and **(B)** CTB-specific serum IgG (positive experimental group, n = 9/group; negative experimental group and control group, n = 10/group) were measured by ELISA. The results shown are presented as mean ± 1 SD. Data are a combination of two independent experiments, and statistical significance was evaluated by using one-way ANOVA (****, $P < 0.0001$; the asterisks represent the significant difference between two experimental groups).

Sublingual administration of CTB together with *Alcaligenes* lipid A prevented cholera toxin-induced diarrhea

Based on these results, we sought to determine whether sublingual administration of CTB with *Alcaligenes* lipid A could confer protective immunity against CT challenge. One week after final immunization, mice were orally challenged with CT, and water content in the cecum was collected and measured to assess diarrhea symptoms. As anticipated from the data on intestinal IgA production, mice immunized with CTB alone exhibited a trend toward reduced water content in the cecum, but the difference was not statistically significant, because CTB possesses high antigenicity (Figure 11). In contrast, mice immunized with CTB with

Alcaligenes lipid A demonstrated a significant reduction in cecal water content compared to unimmunized controls. These results indicate that *Alcaligenes* lipid A serves as a potent adjuvant for sublingual vaccines, offering protection against intestinal infections.

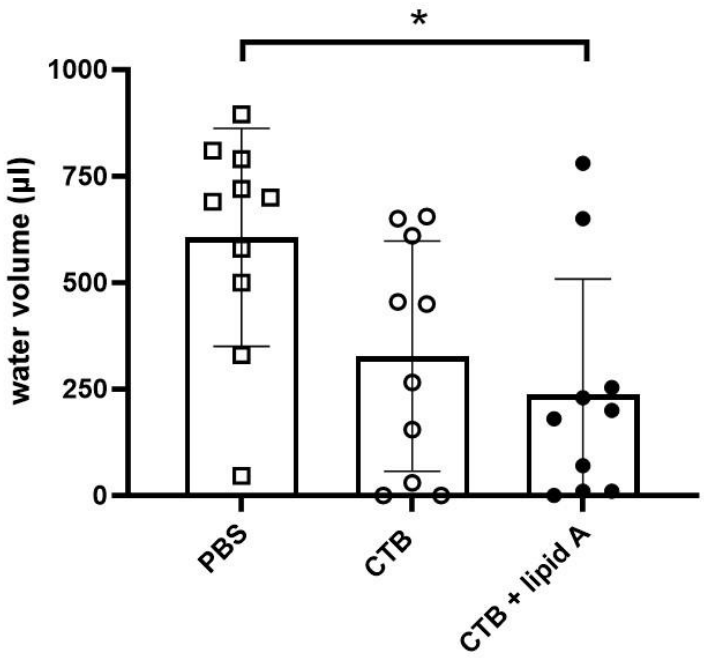

Figure 11. The enhancement of CT-specific immune responses by the sublingual administration of *Alcaligenes* lipid A suppressed CT-induced diarrhea. Mice were immunized sublingually with PBS or CTB with or without *Alcaligenes* lipid A. one week after the final immunization, mice were fasted and orally challenged with a high dose of CT. The cecum water volume was measured (n = 10/group). Data are a combination of two independent experiments, and statistical significance was evaluated by using one-way ANOVA (*, $P < 0.05$).

Sublingual administration of PspA combined with *Alcaligenes* lipid A enhanced PspA-specific mucosal and systemic humoral immune responses

To further assess the potential of *Alcaligenes* lipid A as an adjuvant for respiratory infections, we evaluated its effect using PspA, a broadly expressed antigen across most serotypes of *S. pneumoniae* and known for its protective role against pneumococcal infection in mice (Larry et al., 1991; Briles et al., 2000). In mice immunized with PspA alone, no PspA-specific antibody response was detected in any of the samples measured. In contrast, in mice sublingual immunization with PspA and *Alcaligenes* lipid A resulted in a robust enhancement of PspA-specific IgA antibodies in the nasal cavity and BALF (Figure 12A, and 12B), as well as increased PspA-

specific IgG responses in both BALF and serum (Figure 12C, and 12D). These findings suggested that *Alcaligenes* lipid A could effectively amplify antigen-specific humoral immune responses against respiratory infectious diseases.

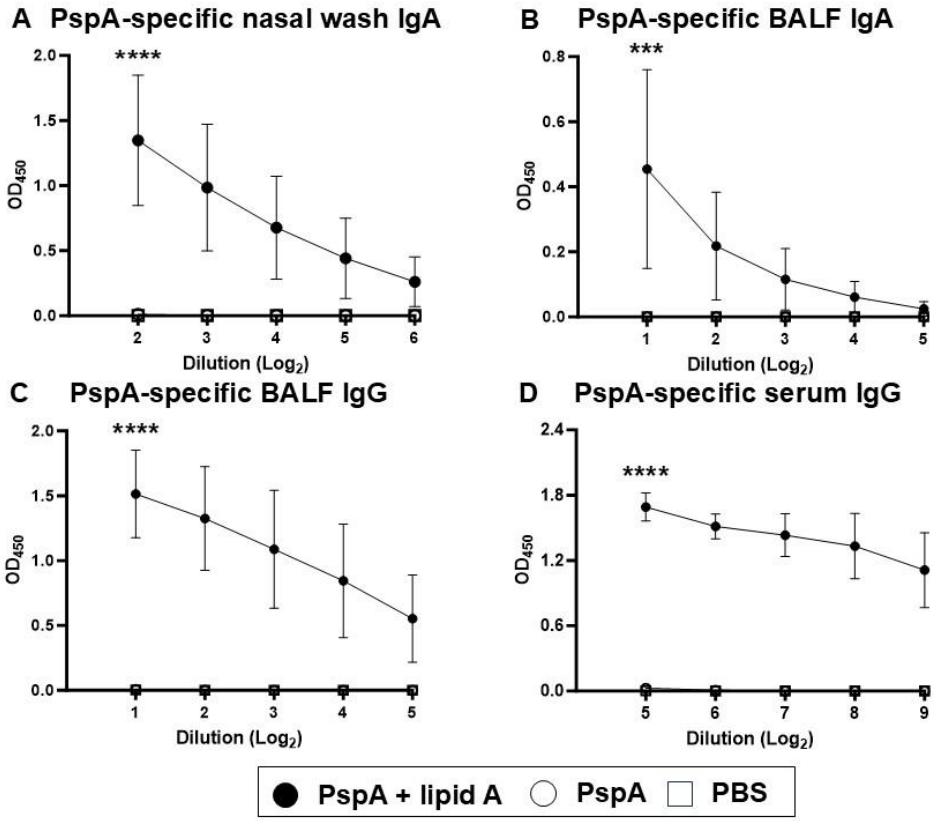

Figure 12. Sublingual administration of *Alcaligenes* lipid A enhanced the production of PspA-specific antibodies. Mice were immunized sublingually with PBS or PspA with or without *Alcaligenes* lipid A. Nasal wash, BALF, serum were collected 1 week after the final immunization, and the levels of **(A)** PspA-specific nasal wash IgA (positive experimental group, n = 7/group; negative experimental group, n = 8/group, control group, n = 6/group) and **(B)** PspA-specific BALF IgA (positive experimental group, n = 8/group; negative experimental group, n = 6/group, control group, n = 7/group) **(C)** PspA-specific BALF IgG (positive experimental group, n = 8/group; negative experimental group, n = 6/group, control group, n = 8/group) and **(D)** PspA-specific serum IgG (positive experimental group, n = 8/group; negative experimental group and control group, n = 6/group) were measured by ELISA. The results shown are presented as mean ± 1 SD. Data are a combination of two independent experiments, and statistical significance was evaluated by using one-way ANOVA (***, $P < 0.001$; ****, $P < 0.0001$; the asterisks represent the significant difference between two experimental groups).

Sublingual immunization of PspA in combination with *Alcaligenes* lipid A enhanced PspA-specific Th17 responses

Beyond humoral immunity, Th17 responses are critical in defending host against

extracellular bacteria such as *S. pneumoniae* (Curtis and Way, 2009). We therefore explored whether *Alcaligenes* lipid A could induce Th17 responses through sublingual immunization, as we previously reported in nasal or systemic immunization (Wang et al., 2020). To test this possibility, $CD4^+$ T cells isolated from SMLNs, a primary site for immune responses to sublingual antigen (Hervouet et al., 2014) (Figure 13A), and spleen (Figure 13B). Upon in vitro restimulation with antigen presenting cells plus PspA, $CD4^+$ T cells from both tissues of mice immunized with PspA plus *Alcaligenes* lipid A secreted higher levels of IL-17A comparing to those from naïve mice or mice immunized with PspA alone. These findings suggested that *Alcaligenes* lipid A could enhance antigen-specific Th17 immune responses, offering enhanced protection against respiratory infectious diseases.

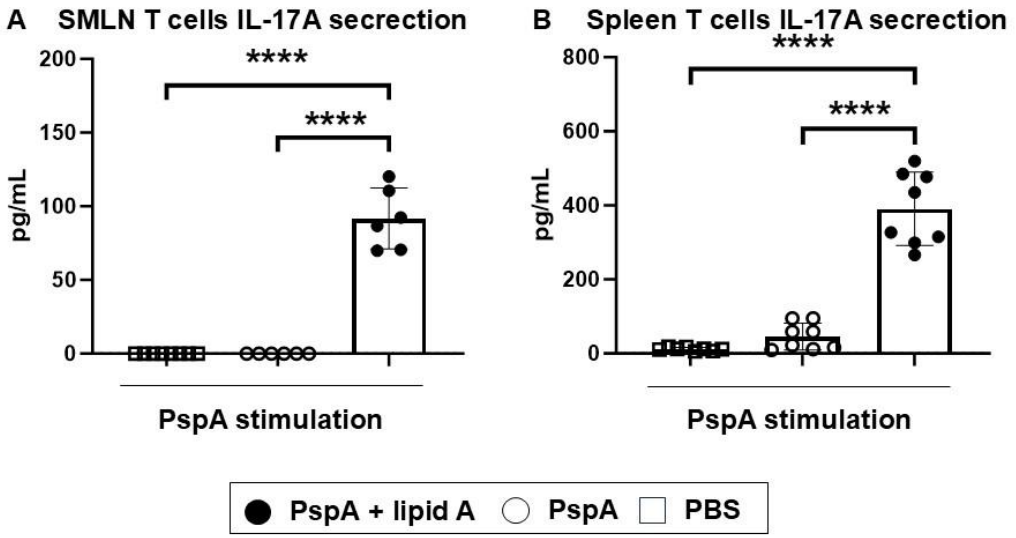

Figure 13. Sublingual administration of *Alcaligenes* lipid A enhanced PspA-specific Th17 responses. Mice were immunized sublingually with PBS or PspA with or without *Alcaligenes* lipid A. $CD4^+$ T cells from SMLNs and spleens were collected 1 week after the final immunization, and the levels of IL-17A from PspA-stimulated T cells of **(A)** SMLNs (positive experimental group, n = 6/group; negative experimental group, n = 7/group, control group, n = 8/group) and **(B)** spleens (n = 8/group) were measured by CBA kit. The results shown are presented as mean ± 1 SD. Data are a combination of two independent experiments, and statistical significance was evaluated by using one-way ANOVA (****, $P < 0.0001$).

Sublingual immunization of PspA together with *Alcaligenes* lipid A protects respiratory infection against *S. pneumoniae*

Our findings on humoral and Th17 responses allowed us to investigate whether sublingual immunization of PspA plus *Alcaligenes* lipid A could provide protective immunity against *S. pneumoniae* infection. One week after the last immunization, mice were challenged with *S. pneumoniae* via respiratory route, and their body weight was monitored for 14 days along with survival rates. The body weight of naïve mice and mice immunized with PspA alone rapidly declined (Figure 14A), and all mice succumbed before day 3 (Figure 14B). On the other hand, mice immunized with PspA together with *Alcaligenes* lipid A showed a rapid recovery in body weight from day 2, and all survived. These results indicate that *Alcaligenes* lipid A could be an effective adjuvant for sublingual vaccines, providing effective protection against respiratory infection by *S. pneumoniae* in mice.

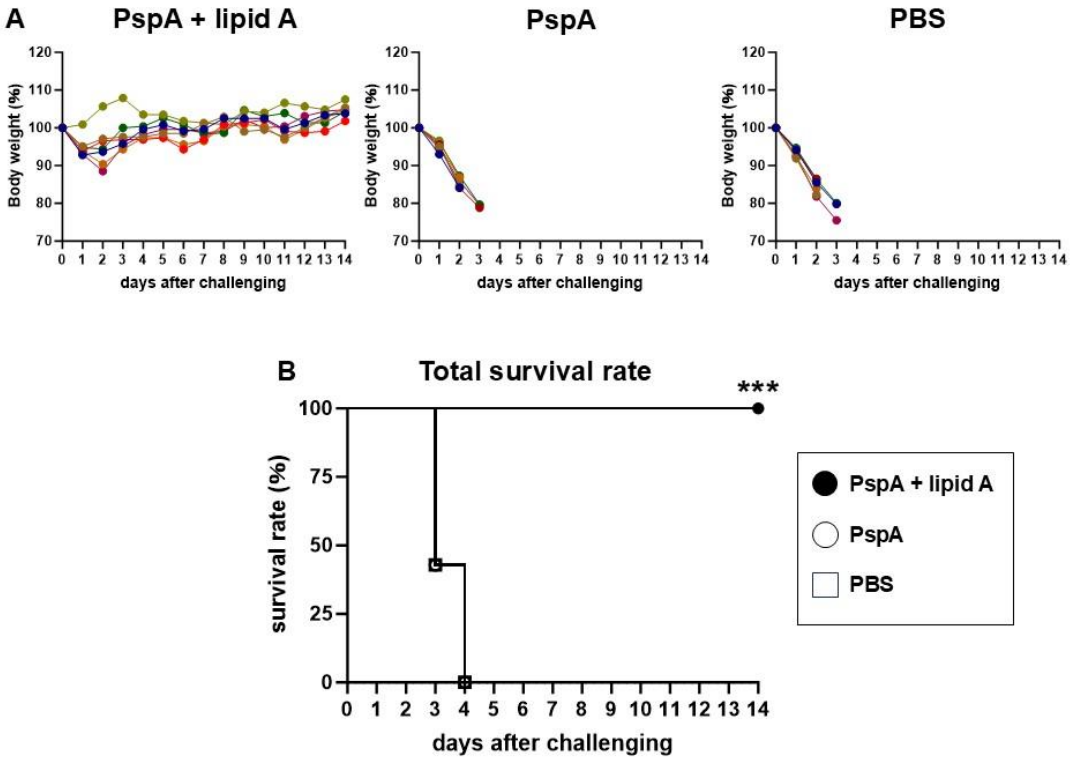

Figure 14. *Alcaligenes* lipid A-enhanced PspA-specific immune responses protected mice from *S. pneumoniae* infection. Mice were immunized sublingually with PBS or PspA with or without *Alcaligenes* lipid A. one week after the final immunization and after one more day for recovery of sampling, mice were nasally challenged with a lethal dose of *S. pneumoniae*. The **(A)** body weights and **(B)** survival rates were calculated each day for 2 weeks (n = 7/group). Data are a combination of two independent experiments, and statistical significance was evaluated by using Kaplan-Meier survival analysis (***, $P < 0.001$; the asterisks represent the significant difference between two experimental groups).

4.3 Specific Discussion

During sublingual immunization, the migration of immune cells was reported to play a significant role. For example, CCL19 and CCL21 expression level were significantly enhanced in lymph nodes comparing with activation of antigen-specific $CD4^+$ T and B cell responses induced by DCs (Song et al., 2009). After activation, DCs, T and B cells are recruited to distant mucosal tissues (e.g., nasopharynx associated lymphoid tissues and Peyer's patches) through the axis of CCL19/CCL21-CCR7, resulting in the protection both of respiratory and intestinal infection.

In this study, sublingual immunization with CTB together with *Alcaligenes* lipid A induced CTB-specific IgA antibody production to suppress CT-induced diarrhea as expected. Meanwhile, co-administration of PspA and *Alcaligenes* lipid A induced PspA-specific IgA helped protect mice from invasion of *S. pneumoniae*. Considering our previous studies reported that the *Alcaligenes*-driven LPS and lipid A could activate DCs with enhanced T cell-responses and antibody responses, the direct activation of DCs of *Alcaligenes* lipid A is thought to be main mechanism to enhance protective immune responses during sublingual administration of *Alcaligenes* lipid A.

A few numbers of adjuvants have been examined for mucosal vaccines including MPLA, which has been reported to act as a TLR4 agonist (Mata-Haro et al., 2007) and enhance humoral immune responses (Chen et al., 2019). Focusing on the application of *Alcaligenes* lipid A as an adjuvant, we previously compared the effect and potential mechanism of MPLA and *Alcaligenes* lipid A. Results of intranasal administration to mice showed the *Alcaligenes* lipid A induced higher levels of the production of antigen-specific IgA antibodies in nasal wash fluids, compared to MPLA (Sun et al., 2024). Mechanistically, it has been previously reported that MPLA is a TRIF-biased TLR4 agonist (Mata-Haro et al., 2007), while our previous study demonstrated that the *Alcaligenes* lipid A could activate DCs through both MyD88 and TRIF signaling pathways and the enhanced secretion of IL-6 and expression of costimulatory molecules were related to the MyD88 pathway (Sun et al., 2023). Another study also verified that *Alcaligenes* lipid A upregulated the expression of CCL2 on stromal cells and CCL3 on $CD45^+$ immune cells and thus enhanced the recruitment and infiltration of DCs into nasal tissues (Sun et al., 2024). These results might furtherly express how the *Alcaligenes* lipid A activate DCs during sublingual administration. It is known that the structure of lipid A differs among bacteria, and it has been reported recently that *Alcaligenes* lipid A possesses hexa-acylated species that was composed of a bisphosphorylated glucosamine disaccharide backbone carrying 14:0 (3-OH) as primary and 12:0 (3-OH) and 10:0 as secondary fatty acids with distribution in a 3 + 3 symmetric fashion with respect to the disaccharide backbone, which were different from *E. coli* lipid A that has 4 + 2 asymmetry and is composed of 14:0 (3-OH) as primary and 14:0 and 12:0 as secondary fatty acids (Shimoyama et al., 2021) and could be used to enhance immune responses against T cell-dependent antigens (Wang et al., 2020 and Yoshii et al., 2020). Although further studies are required to consummate the theory, it is suggested that the differences between the *Alcaligenes*-driven lipid A and *E. coli*-driven lipid A contribute to the safety of *Alcaligenes*-driven lipid A. And as an example, the different structure of

MPLA from *Alcaligenes* lipid A, one single phosphoryl group, is thought to lead to an inefficient dimerization of the TLR4–MD-2 complex and thus decrease the activation of MyD88 (Sun et al., 2023). The unique structure of *Alcaligenes* lipid A might contribute to its application as a suitable sublingual vaccine adjuvant.

Also, in the past, one of our previous studies showed that nasal administration of PspA together with *Alcaligenes* lipid A to mice recruited neutrophils to inoculation site and prevented bacterial growth in the lung tissues, resulting in the protection of *S. pneumoniae* infection (Yoshii et al., 2020). However, the side effects of nasal vaccination itself related to the transition to the central nervous system should be considered (Lemiale et al., 2003). Indeed, accumulation of antigens was observed in the olfactory bulbs and brain in mice administered intranasally while antigens remained undetectable in the olfactory bulbs and brain after sublingual administration, theoretically suggesting the sublingual administration a safer alternative comparing to nasal administration (Song et al., 2008). Fortunately, we obtained the same results in this study when we applied *Alcaligenes* lipid A sublingually without the same risk (Song et al., 2008). Furtherly, the sublingual administration of *Alcaligenes* lipid A induced a strong immune response in intestinal tract and successfully helped suppress the damage caused by antigen covered the shortage that administration of *Alcaligenes* lipid A as an oral vaccine adjuvant did not enhance immune responses in intestinal tract (data not shown). It can be concluded that sublingual vaccines are more suitable for the application of *Alcaligenes* lipid A as a mucosal adjuvant, though the sublingual route is now wildly used for sublingual immunotherapy for allergy responses (Paris et al., 2021) instead of vaccination. Finally, a suitable adjuvant requires high effect and safety. Apart from MPLA, when comparing to CT which can be used to enhance immune responses in noses and intestines but will induce excessive inflammation like recruiting extra neutrophils (Yoshii et al., 2020), *Alcaligenes* lipid A showed higher safety like stable body weight and body temperature (Wang et al., 2020). Thus, *Alcaligenes* lipid A could be an ideal adjuvant with high efficiency and high safety for sublingual vaccines.

5. General Discussion

We previously reported that *Alcaligenes* LPS acts as an TLR4 agonist, thereby enhancing antigen-specific immune responses without excessive inflammation and leading to the possibility of its use as a safe adjuvant (Shibata et al., 2018 and Wang et al., 2021). My current study extended our research. Besides, we are planning for non-clinical trials to examine the effect of *Alcaligenes* lipid A in the future.

In conclusion, the first research demonstrated the efficacy of *Alcaligenes* lipid A as an adjuvant for a Hib vaccine that includes the TI antigen PRP. Specifically, *Alcaligenes* lipid A enhanced the PRP-specific IgG response when Hib PRP was conjugated to a TD antigen (i.e., TT) as a carrier protein. Together, current findings indicate that *Alcaligenes* lipid A exerted adjuvant activity for a TI polysaccharide

antigen only when it was conjugated to a TD carrier protein. The induction pathway for the TI antigen did not include enhancement of T-cell responses and thus differs from that of TD antigens.

Furtherly, the second research showed that *Alcaligenes* lipid A enhanced both PspA- and CT-specific humoral responses both in the respiratory and intestinal tracts as well as systemic compartments and thus protected both respiratory and intestinal infections, indicating that *Alcaligenes* lipid A could exert a role as a sublingual vaccine adjuvant to induce strong antigen-specific Th17 responses and antibody production in both respiratory and intestinal tracts, resulting in the protection from extracellular bacteria and toxins.

6. References

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