パイエル板組織内共生菌アルカリゲネス由来 LPS/lipid A のアジュバント応用に関する研究

The study on adjuvant application of LPS/lipid A derived from *Alcaligenes* spp, lymphoid tissue resident symbiotic bacterium in the Peyer's patches

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Abstract

Alcaligenes spp., including *A. faecalis*, is a gram-negative facultative bacterium uniquely residing inside the Peyer's patches. We previously showed that *A. faecalis*-derived lipopolysaccharides (*Alcaligenes* LPS) acts as a weak agonist of toll-like receptor 4 (TLR4) to activate dendritic cells (DCs) and shows adjuvant activity by enhancing IgG and Th17 responses to systemic vaccination. Here, I examined the efficacy of *Alcaligenes* LPS as a nasal vaccine adjuvant. Nasal immunization with ovalbumin (OVA) plus *Alcaligenes* LPS induced follicular T helper cells (Tfh cells) and germinal center (GC) formation in the nasopharynx-associated lymphoid tissue (NALT) and cervical lymph nodes (CLNs), and consequently enhanced OVA-specific IgA and IgG responses in the respiratory tract and serum. In addition, nasal immunization with OVA plus *Alcaligenes* LPS induced OVA-specific T cells producing IL-17 and/or IL-10, whereas nasal immunization with OVA plus cholera toxin (CT) induced OVA-specific T cells producing IFN-γ and IL-17, which are recognized as pathogenic type of Th17 cells. In addition, CT, but not *Alcaligenes* LPS, promoted the production of TNF-α and IL-5 by T cells. Nasal immunization with OVA plus CT, but not *Alcaligenes* LPS, led to increased numbers of neutrophils and eosinophils in the nasal cavity. Together, these findings indicate the benign nature of *Alcaligenes* LPS is an effective nasal vaccine adjuvant that induces antigen-specific mucosal and systemic immune responses without activation of inflammatory cascade after nasal administration.

Lipid A is responsible for biological effect of LPS and has been applied to adjuvant. Here, I also examined adjuvant activity and safety of chemically synthesized *Alcaligenes* lipid A. Mice subcutaneously immunized with OVA plus *Alcaligenes* lipid A showed increased levels of OVA-specific serum IgG antibody, comparing to immunization with OVA alone. In addition, *Alcaligenes* lipid A induced high levels of IL-17 production from splenic $CD4^+$ T cells, suggesting that *Alcaligenes* lipid A promoted antigen-specific Th17 cell responses as well

as *Alcaligenes* LPS did in nasal immunization. Moreover, *Alcaligenes* lipid A had little side effects, such as weight loss and fever, reducing number of lymphocytes and platelets. *In vitro* stimulation with *Alcaligenes* lipid A upregulated expression of MHCII, CD40, CD80 and CD86 and enhanced the production of cytokine, IL-6, which are involved in inducing antibody production and Th17 cell responses, from murine bone marrow-derived dendritic cells (BMDCs). Also, in human peripheral blood mononuclear cells (PBMCs), stimulation with *Alcaligenes* lipid A induced the production of cytokines, including IL-6 and IL-1β. These findings suggest that *Alcaligenes* lipid A is also a safe and applicable synthetic adjuvant for systemic vaccination.

1. Introduction

Commensal bacteria in the gut are involved in the regulation of host immunity; therefore, are expected to play important roles not only in host immune responses to immunization but also in host responses to pathogenic infection. Accumulating evidence have indicated the involvement of certain commensal bacteria in the regulation of specific immunity. For instance, *Klebsiella* spp. have been shown to induce Th1 cell polarization, and segmented filamentous bacteria have been shown to drive Th17 cell responses to pathogenic infection (1,2). Similarly, *Clostridium* spp. have been shown to induce regulatory T cells for the control of allergic diseases (3).

Previously, we have demonstrated that commensal bacteria are present not only in the intestinal lumen but also inside intestinal tissues such as Peyer's patches (PPs) and the colonic lamina propria (4). For the first time, we find that the gram-negative bacterium *Alcaligenes* spp. including *A. faecalis*, is a representative bacterium that symbiotically resides in PPs. Our previous study have shown that *A. faecalis* promotes the production of several cytokines (e.g., transforming growth factor beta [TGF-β], B-cell activating factor [BAFF], and interleukin 6 [IL-6]) by dendrtic cells (DCs) to enhance the production of IgA in the intestine (4,5). A subsequent study reveals that *A. faecalis* increases IL-10 producing DCs, which contributes to establish the symbiotic environment in the gut (6). A more recent study by our group using *A. faecalis* reveals that lipopolysaccharides (LPS) derived from *A. faecalis* (*Alcaligenes* LPS) possesses unique immunomodulatory activity. Indeed, *Alcaligenes* LPS enhances the production of IL-6 from DCs by acting as a weak agonist of toll-like receptor 4 (TLR4) (7). It is worth noting that the biological activity of *Alcaligenes* LPS is lower than that of E. coli-derived LPS (*E. coli* LPS) when *Alcaligenes* LPS or *E. coli* LPS is injected subcutaneously into mice together with OVA. In addition, *Alcaligenes* LPS can enhance both antigen-specific IgG production and Th17 cell responses without inducing excessive inflammation.

These findings suggest the potential of *Alcaligenes* LPS as a novel vaccine adjuvant (7).

Although subcutaneous or intramuscular injection is a commonly accepted and practiced vaccination, mucosal vaccination (e.g., nasal and oral vaccines) has currently attracted attention due to several advantages, including reduced fear and pain, decreased medical waste such as syringe and needle, and abatement of the work of medical staff responsible for vaccination. In addition, mucosal vaccination has the benefit of inducing both systemic and mucosal immune responses (8,9). And the licensed mucosal vaccine has been used in preventing or helping against diseases. For example, Rotarix is an oral vaccine against diarrhea caused by rotavirus (10). FluMist/Fluenz is a nasal vaccine against influenza caused by influenza A and influenza B viruses (11).

Oral or nasal administration is a major route for mucosal vaccine. In case of nasal immunization, nasopharynx-associated lymphoid tissue (NALT) is one of the responsible sites for inducing antigen-specific immune responses. NALT is located at the bottom edge of nasal cavity in rodents (12), and the human tonsils known as Waldeyer's tonsillar ring are considered as equivalent lymphoid tissues to rodent NALT (13). NALT has all the necessary immunocompetent cells, such as B cells, T cells, DCs, and M cells, to initiate antigen-specific immune responses (14). M cells located in the NALT epithelium act as antigen uptake cells to deliver antigens to DCs (14). The DCs then process and present the antigens to T cells and B cells in germinal center (GC) located in the NALT to initiate antigen-specific IgA responses (14,15). IgA class switching recombination is a critical step for promoting IgA^+ B cell development in the GC of NALT with the essential support by follicular T helper cells (Tfh cells) (15,16). The antigen-specific IgA produced by IgA^+ B cells is secreted through the epithelium into the nasal cavity, where it binds to antigens to prevent the invasions of pathogens from nasal cavity (14).

Although mucosal vaccination has these advantages, one of the issues to be solved includes the induction of immune tolerance to cause immune non-responses (17). Mucosal adjuvants are required to overcome the issues such as inducing immune tolerance by inducing mucosal antigen-specific immune responses. Recently, some adjuvant candidates for nasal vaccines have been developed by using microbial components (15). For example, when the TLR5 agonist, flagellin of *Salmonella* typhimurium, is used as a nasal adjuvant for the H7N9 influenza subunit vaccine, it can induce effective IgG and IgA antibody responses, Th1 and Th2 cell responses (18). Also, intranasal co-administration of adenylate cyclase toxin of *Bordetella pertussis* and pertactin elicits robust IgG and IgA antibody responses and has a protective effect when challenged with *B. pertussis* intranasally (19).

LPS is composed of lipid A, core oligosaccharide, and O-antigens (20). The chemical structure of lipid A varies among bacteria and shows different biological activities (20,21). Here, based on our previous findings on the adjuvant activity of *Alcaligenes* LPS (7), I evaluate the efficacy of *Alcaligenes* LPS and lipid A as an adjuvant when administered to mice by nasal immunization and subcutaneously injection, respectively. I find that *Alcaligenes* LPS as nasal adjuvant induced both systemic and mucosal immune responses, including antigen-specific IgG and IgA antibody production as well as Th17 cell responses. In addition, *Alcaligenes* lipid A as adjuvant shows antigen-specific IgG and Th17 cell responses. Both of *Alcaligenes* LPS and lipid A induce immune responses without local inflammation, which confirms the potential of *Alcaligenes* LPS and lipid A to function effectively as adjuvants.

2. Materials and Methods

2.1. Mice

Female BALB/c mice (age 8–9 weeks) were used for subcutaneous or nasal immunization and female BALB/c mice (age 4–5 weeks) were used for

preparation of bone marrow cells. The mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and were kept in a specific-pathogen-free (SPF) environment on a 12/12-h light/dark cycle at the National Institutes of Biomedical Innovation, Health, and Nutrition (Osaka, Japan). All experimental procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of the National Institutes of Biomedical Innovation, Health, and Nutrition (Approval Nos. DS27-47R13 and DS27-48R13).

2.2. Preparation of LPS and lipid A

Alcaligenes LPS and chemically synthesized *Alcaligenes* lipid A were provided by Professors Fukase and Shimoyama of Osaka University. *Alcaligenes* LPS was extracted from heat-killed (60°C for 30 min) *A. faecalis* (13111T, Biological Resource Center, NITE [NBRC], Japan) by using an LPS Extraction Kit (iNtRON Biotechnology, Inc., Sangdaewon-Dong, Korea). After extraction, *Alcaligenes* LPS is lyophilized and stored as a powder at -30°C. And *Alcaligenes* lipid A was chemically synthesized as previously described (22) For stock solution, *Alcaligenes* LPS was added to phosphate-buffered saline (PBS; Nacalai Tesque, Inc., Kyoto, Japan) and *Alcaligenes* lipid A was dissolved with dimethyl sulfoxide (DMSO; Nacalai Tesque, Inc.). The stock solution of the LPS or lipid A was prepared to a concentration of 1 mg/mL, sonicated for 5 min, and then stored at −30°C until use.

2.3. Immunization

For nasal immunization, mice were immunized equally into the two nostrils of mice without anesthesia on day 1, 7, and 17 (23). One week after the final immunization, nasal wash, bronchoalveolar lavage fluid (BALF), serum, nasal passage, NALT, CLNs, and spleen were collected as previously described (23,24) and used for analysis. The groups were divided as follows: 5 μg of ovalbumin (OVA) (Sigma-Aldrich) alone; 5 μg of OVA with 10 μg of *Alcaligenes*

LPS or 1 μg of cholera toxin isolated from *Vibrio cholerae* (List Biological Laboratories, Campbell, CA, USA)

For subcutaneous injection, mice were immunized on day 1 and 10 (7). One week after the final immunization, serum and spleen were collected for analysis. The groups were divided as follows: 10 μg of OVA and 1 μg of OVA alone or plus 1 μg of *Alcaligenes* lipid A.

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as follows (25). The bottom of flat-bottom 96-well immunoplates (Thermo Fisher Scientific Inc., Waltham, MA, USA) were coated with OVA diluted in PBS to a concentration of 1 mg/mL and then the plates were incubated overnight at 4°C. After incubation, the plates were blocked with 1% (w/v) bovine serum albumin (BSA; Nacalai Tesque, Inc.) in PBS for 2 h at room temperature. After blocking, the plates were washed 3 times with PBS containing 0.05% (v/v) Tween 20 (Nacalai Tesque, Inc.).

Next, serum, nasal wash, or BALF samples were serially diluted with 1% (w/v) BSA, containing 0.05% (v/v) Tween 20 in PBS and seeded into the plates; the plates were then incubated for 2 h at room temperature and washed 3 times with PBS containing 0.05% Tween 20. After washing, horseradish peroxidase-conjugated goat anti-mouse IgG and IgA (Southern Biotech, Inc., Birmingham, AL, USA) diluted with 1% (w/v) BSA containing 0.05% (v/v) Tween 20 in PBS were added to the plates and left to react for 1 h at room temperature. After being left to react, the plates were washed 3 times with PBS containing 0.05% Tween 20. Tetramethylbenzidine peroxidase substrate (SeraCare Life Sciences Inc., Milford, MA, USA) was then added, and the plates were left to react for 2 min at room temperature; 0.5 N HCl (Nacalai Tesque, Inc.) was added to stop the reaction. Absorbance at 450 nm was measured by using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

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205 **2.5. Immunohistochemistry**

206 Immunohistological analysis was performed as follows (23). NALT and
207 CLNs were embedded into Tissue-Tek O.C.T. Compound (Sakura Finetek
208 Japan Co., Ltd., Tokyo, Japan) to make frozen blocks. Blocks were frozen by
209 liquid nitrogen and stored at −80°C until use. Sections (6-μm-thick) of NALT and
210 CLNs were cut at −20°C by using a Leica CM3050 S cryostat (Leica Biosystems,
211 Nussloch, Germany). Then, the sections of NALT and CLNs were air-dried,
212 fixed with 100% acetone (Nacalai Tesque, Inc.) for 1 min, and washed 2 times
213 with PBS for 5 min each time. After washing, the sections were blocked with
214 2% Newborn Calf Serum (NCS; Equitech-Bio, Kerrville, TX, USA) -PBS for 30
215 min, stained with Purified anti-mouse/human CD45R/B220 Antibody
216 (BioLegend, San Diego, CA, USA; clone: RA3-6B2) and biotin-PNA (Vector
217 Laboratories, Inc., Burlingame, CA, USA) and incubated overnight at 4°C. After
218 incubation, the sections were washed 2 times with PBS for 5 min each time,
219 stained with anti-hamster Cy3 (Jackson ImmunoResearch Inc., West Grove, PA,
220 USA) and Alexa Fluor 488/Streptavidin Conjugate (Invitrogen, Thermo Fisher
221 Scientific Inc.) for 30 min, washed 2 times with PBS for 5 min each time, stained
222 with DAPI (AAT Bioquest, Inc., Sunnyvale, CA, USA) for 10 min, and washed 2
223 times with PBS for 5 min each time. Finally, each section was covered with one
224 drop of Fluoromount (Diagnostic BioSystems, Pleasanton, CA, USA) followed
225 by a 24 × 36-mm-thick cover glass (Matsunami Glass USA Inc., Bellingham,
226 WA, USA) and observed under a BZ-9000 BioRevo fluorescence microscope
227 (Keyence Corp., Osaka, Japan).

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229 **2.6. T-cell assay**

230 T-cell assay was performed as follows (23). Spleen from subcutaneously
231 injected mice; spleen and CLNs from nasal immunized mice were collected to
232 make cell suspension. The cell suspension was passed through a 100-μm cell

filter (Thermo Fisher Scientific Inc.); then mixed with red blood cell lysis buffer (1.5 M NH_4Cl, 100 mM $KHCO_3$, and 10 mM EDTA-2Na [all Nacalai Tesque, Inc.]) for 1 min at room temperature; the resulting suspension was passed through a 100-μm cell filter (Thermo Fisher Scientific Inc.) again, and the filtrate was retained. $CD4^+$ T cells were purified from the filtrate by using CD4 (L3T4) MicroBeads and a magnetic cell separation system (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany). Splenic cells were treated with 30 Gy of ionizing radiation and used as antigen-presenting cells. The $CD4^+$ T cells (2×10^5 cells/well) and antigen-presenting cells (1×10^4 cells/well) were suspended in RPMI1640 medium (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS; Life Technologies, Thermo Fisher Scientific Inc.), 1 mM sodium pyruvate solution (Nacalai Tesque, Inc.), 1% penicillin–streptomycin mixed solution (Nacalai Tesque, Inc.), and 0.5 mM 2-mercaptoethanol (Gibco, Thermo Fisher Scientific Inc.); seeded in Nunc™ 96-Well, Nunclon Delta-Treated, U-Shaped-Bottom Microplates (Thermo Fisher Scientific Inc.); and cultured with or without 1 mg/mL OVA for 72 h. The number of viable cells was determined by using a CyQUANT Cell Proliferation Assay kit (Invitrogen, Thermo Fisher Scientific Inc.), and the absorbance of the cells was measured at 485/535 nm with an ARVO X2 (PerkinElmer, Yokohama, Japan) fluorescence microplate reader. The culture supernatant was collected and used for the measurement of the concentrations of the cytokines as follows: interferon gamma (IFN-γ), IL-4, IL-17, IL-10, and TNF-α. The cytokines were determined by using a BD CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA), and the concentration of IL-5 was determined by using IL-5-specific ELISA kit (BioLegend).

2.7. Preparation of bone marrow-derived dendritic cells

Mice bone marrow-derived dendritic cells (BMDCs) were cultured as previously described (7). Bone marrow cells were harvested from femurs of

mice and were treated with red blood cell lysis buffer (1.5 M NH4Cl [Nacalai Tesque, Inc.], 100 mM KHCO3 [Nacalai Tesque, Inc.], and 10 mM EDTA-2Na [Nacalai Tesque, Inc.]) for 5 minutes, then washed by RPMI1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Life Technologies, Thermo Fisher Scientific Inc.), 1% 100mM-sodium pyruvate solution (100×) (Nacalai Tesque, Inc.), 1% penicillin-streptomycin mixed solution (Nacalai Tesque, Inc.), and 0.1% 2-Mercaptoethanol (gibco, Thermo Fisher Scientific). BMDCs were cultured in the medium which is contained the concentration of 20 ng/mL GM-CSF (PeproTech, Rocky Hill, USA). Half of the culture medium was replaced with fresh medium containing GM-CSF in every 2 days. On day 6, BMDCs were sorted by using CD11c MicroBeads UltraPure, mouse and LS Columns from Magnetic Cell Separation System (Miltenyi Biotec). The experience procedure was according to the instruction of manufacturer.

2.8. Flow Cytometric Analysis

Flow cytometry was performed as follows (26). Cells of NALT, CLNs and nasal passage; BMDCs were incubated with 5 μg/mL anti-CD16/32 antibody (TruStain FcX; BioLegend) for avoiding non-specific staining and 7AAD viability staining solution (BioLegend) to detect dead cells, for 15 minutes at room temperature.

The cells were then stained with fluorescently labeled antibodies for 30 min at 4°C. For analyzation of NALT and CLNs from nasal immunized mice, GC and IgM^- IgA^+ B cells were stained with FITC-IgA (BD Biosciences; clone: C10-3), PE-Cy7-IgM (BioLegend; clone: RMM-1), AF647-GL7 (BioLegend; clone: GL7), and BV421-B220 (BioLegend; clone: RA3-6B2). Tfh cells were stained with FITC-CD3ε (BD Biosciences; clone: 145-2C11), PE-PD-1 (BioLegend; clone: 29F.1A12), APC-Cy7-CD8α (BioLegend; clone: 53-6.7), and BV421-CD4 (BioLegend; clone: RM4-5). Nasal passage from nasal immunizaed mice was used for analyzation of neutrophils and eosinophils and the cell were stained

with FITC-Ly6G^{+} (BioLegend; clone: 1A8), APC-Cy7-CD11b (BioLegend; clone: M1/70), BV421-Siglec-F (BD Biosciences; clone: E50-2440), and APC-CD45 (BioLegend; clone: 30-F11). And BMDCs were stained with FITCI-A^{d} (BD Biosciences; clone: AMS-32.1), PE-CD80 (BioLegend; clone: 16-10A1), APC-Cy7-CD86 (BioLegend; clone: GL-1) and PE-Cy7-CD40 (BioLegend; clone: 3/23). Samples were analyzed by MACSQuant® Analyzer (Miltenyi Biotec). Data were analyzed by using FlowJo, LLC Software 10.2 (BD Biosciences)

Intracellular cytokine staining was performed as previously described with modification (27,28). Cells collected from nasal immunized mice spleen were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 750 ng/mL ionomycin (Sigma-Aldrich) for 4 h at 37°C; 5 ng/mL brefeldin A (BioLegend) was added at around 3rd hour. After incubation, the cells were stained with NIR-zombie (BioLegend), FITC-TCR-β (BioLegend; clone: H57-597), PerCP-CD4 (BioLegend; clone: GK1.5), and BV421-CD45 (BioLegend; clone: 30-F11). The cells were fixed and permeabilized by using BD Cytofix/Cytoperm plus (BD Biosciences) and then stained with PE-IFN-γ (BioLegend; clone: XMG1.2) and AF647-IL-17A (BD Biosciences; clone: TC11-18H10). Samples were analyzed by MACSQuant® Analyzer (Miltenyi Biotec) and the data were analyzed using FlowJo software v.10.2 (BD Biosciences).

2.9. Measurement of cytokines in supernatant of BMDCs or PBMCs

BMDCs (1×10^5 cells/well) or peripheral blood mononuclear cells (PBMCs) (2×10^5 cells/well, FUJIFILM Wako Pure Chemical, Osaka, Japan) were seeded into the Nunc™ 96-Well, Nunclon Delta-Treated, U-Shaped-Bottom Microplate (Thermo Fisher Scientific, Inc.). 0.1 or 1 ng/mL of *Alcaligenes* lipid A were used to incubate with BMDCs for 48 hours or PBMCs for 24 hours at 37°C. The culture supernatant from BMDCs and PBMCs were collected for measurement of cytokines. For detection of IL-6 and IL-23 in supernatant of BMDCs, BD cytometric bead array Mouse Inflammation Kit (BD Biosciences) and LEGEND

MAX™ Mouse IL-23 (p19/p40) ELISA Kit (BioLegend) were used. For detection of IL-6 and IL-1β in supernatant of PBMCs, BD™ CBA Human Inflammation Kit (BD Biosciences) and LEGEND MAX™ Human IL-1β ELISA Kit (BioLegend) were used. Samples were analyzed by MACSQuant® Analyzer (Miltenyi Biotec). The experience procedure was according to the instruction of manufacturer.

2.10. Measurement of blood cells and body temperature in mice

Blood samples (100 μL) mixed with 1.5 μL of 10 mM EDTA-2Na (Nacalai Tesque, Inc.) were diluted 1:6 with saline solution (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for measuring the number of lymphocytes and platelets with Vet Scan HMII hematology analyzer (Abaxis, Union City, CA, USA). Body temperature was measured from the rectal temperature of the subcutaneously injected mice.

2.11. Statistical analysis

Statistical analyses were performed by using PRISM 6 software (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SD. Statistical significance was determined by one-way ANOVA with the Bonferroni post-hoc test (*$p < 0.05$; **$p < 0.01$; n.s., not significant).

3. Results

3.1. Nasally co-administered *Alcaligenes* LPS promoted respiratory antigen-specific IgA antibody production

Previously, we demonstrated that *Alcaligenes* LPS enhanced IgA production by B cells co-cultured with DCs *in vitro* (7). I extended our previous study by investigating the efficacy of *Alcaligenes* LPS as an adjuvant for nasal vaccination *in vivo*. To determine the optimal dose of *Alcaligenes* LPS, mice were nasally immunized with OVA alone (Mock group), OVA plus 1 or 10 μg of *Alcaligenes* LPS and OVA plus 1 or 10 μg of *E. coli* LPS. The results showed

that nasal immunization with 10 μg of *Alcaligenes* LPS induced higher levels of nasal IgA responses than 1 μg of *Alcaligenes* LPS (Fig. 1A). And 10 μg of *E. coli* LPS showed comparable levels of nasal IgA responses to 10 μg of *Alcaligenes* LPS (Fig. 1A). However, 10 μg of *E. coli* LPS induced severe side effect such as lymphopenia (Fig. 1B). This result has been shown in our previous research (7). Therefore, I determined the 10 μg of *Alcaligenes* LPS for the nasal immunization in this study and employed cholera toxin (CT), a gold standard experimental mucosal adjuvant as control.

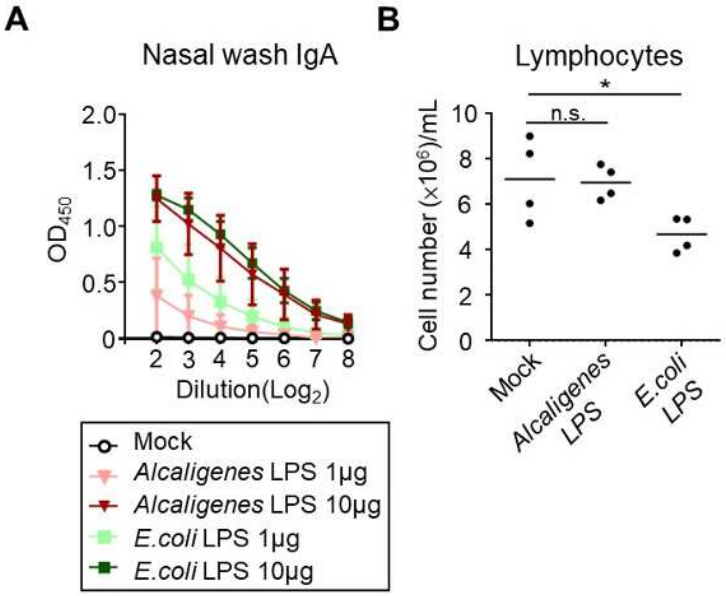


Fig. 1. *E. coli* LPS induced dose-dependent nasal IgA responses and lymphopenia in the blood. Mice were nasally immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS (1 or 10 μg) or *E. coli* LPS (1 or 10 μg); one week after the final immunization, (A) Nasal wash were collected to determine levels of OVA-specific IgA by ELISA (n = 4 per group). Mice were nasally immunized with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS (10 μg) or *E. coli* LPS (10 μg). 24 hours after immunization, (B) blood samples were collected to measure the number of lymphocytes (*n* = 4 per group). Data are representative of two independent experiments and analyzed by one-way ANOVA (*p < 0.05; n.s.: not significant).

To examine OVA-specific IgA production in the nasal wash and BALF, mice were nasally immunized with OVA alone (Mock group), OVA plus 10 μg of *Alcaligenes* LPS (*Alcaligenes* LPS group) or 1 μg of CT (CT group). I found that the nasal wash and BALF from Mock group showed undetectable levels of

OVA-specific IgA (Fig. 2). In contrast, the nasal wash and BALF from *Alcaligenes* LPS group contained substantial levels of OVA-specific IgA antibody, which were comparable to the levels of the CT group (Fig. 2).

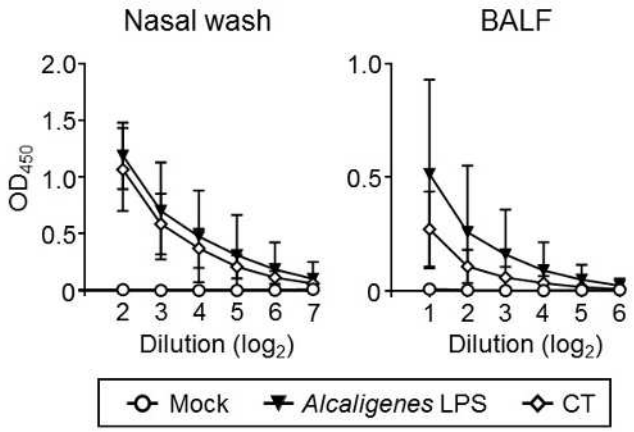


Fig. 2. *Alcaligenes* LPS promoted mucosal OVA-specific IgA antibody production upon nasal immunization. Mice were nasally immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT; one week after the final immunization, Nasal wash and BALF were collected to determine levels of OVA-specific IgA by ELISA (*n* = 5 or 6 per group).

The production of IgA antibody is associated with B cell class-switch recombination from IgM to IgA in the GC of NALT, which is supported by Tfh cells. Immunohistological analysis revealed that Mock group did not induce the formation of GC, while *Alcaligenes* LPS or CT group induced the formation of GC in the NALT (Fig. 3A). Consistent with this finding, flow cytometry analysis demonstrated that the percentage of GC $GL7^+$ B cells increased in both *Alcaligenes* LPS and CT groups when compared to Mock group (Fig. 3B). In addition, the percentage of PD-1^+ Tfh cells (Fig. 3B) and IgM^- IgA^+ B cells (Fig. 3C) were significantly increased in the NALT from *Alcaligenes* LPS group compared with that in Mock group. Together, these results indicate that *Alcaligenes* LPS promoted the formation of GC in the NALT with Tfh cells and IgA^+ B cells for the subsequent IgA antibody production in the respiratory tract.

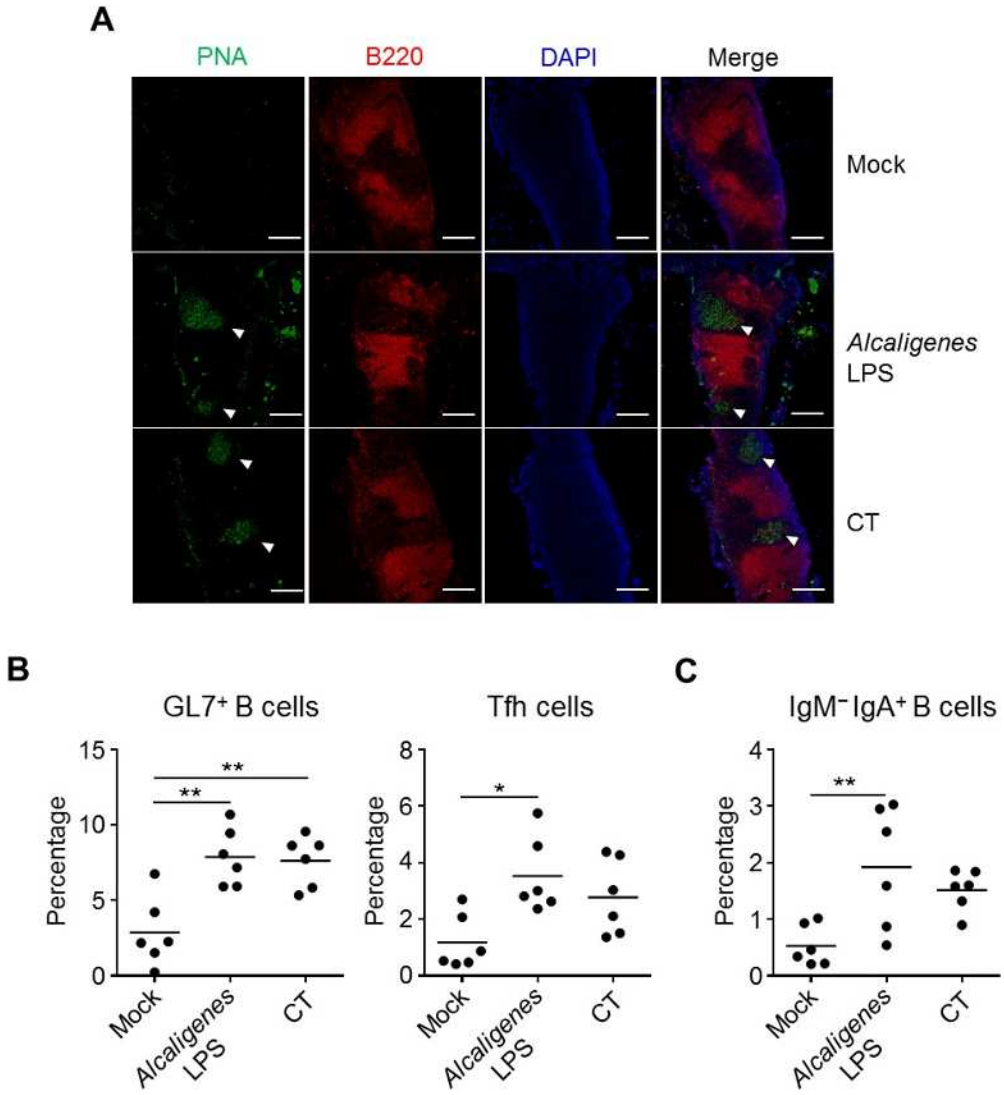


Fig. 3. *Alcaligenes* LPS induced GC formation in NALT. Mice were nasally immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT; one week after the final immunization, (A) formation of GC in NALT were observed by analysis of Immunohistochemistry. PNA: GC marker; B220: B cell marker; arrow heads: GC location. (B) Induction of GC GL7$^+$ B cells (gated on: CD3ε$^-$ B220$^+$ GL7$^+$) and Tfh cells (gated on: CD3ε$^+$ CD8α$^-$ CD4$^+$ PD-1$^+$) and (C) IgM$^-$ IgA$^+$ B cells (gated on: CD3ε$^-$ B220$^+$ GL7$^+$ IgM$^-$ IgA$^+$) in NALT were analyzed by flow cytometry analysis (n = 6 per group). Data are representative of two independent experiments and were analyzed by one-way ANOVA (*p < 0.05; **p < 0.01).

3.2. *Alcaligenes* LPS promoted systemic antibody responses

Next, I examined the immune responses in the CLNs, which are the lymph nodes that drain to the nose. As in the NALT, GC formation and significantly

increased or higher induction of GL7$^+$ B cells, Tfh cells, and IgM$^-$ IgA$^+$ B cells were detected in the CLNs from *Alcaligenes* LPS or CT group when compared to the Mock group (Fig. 4A–C).

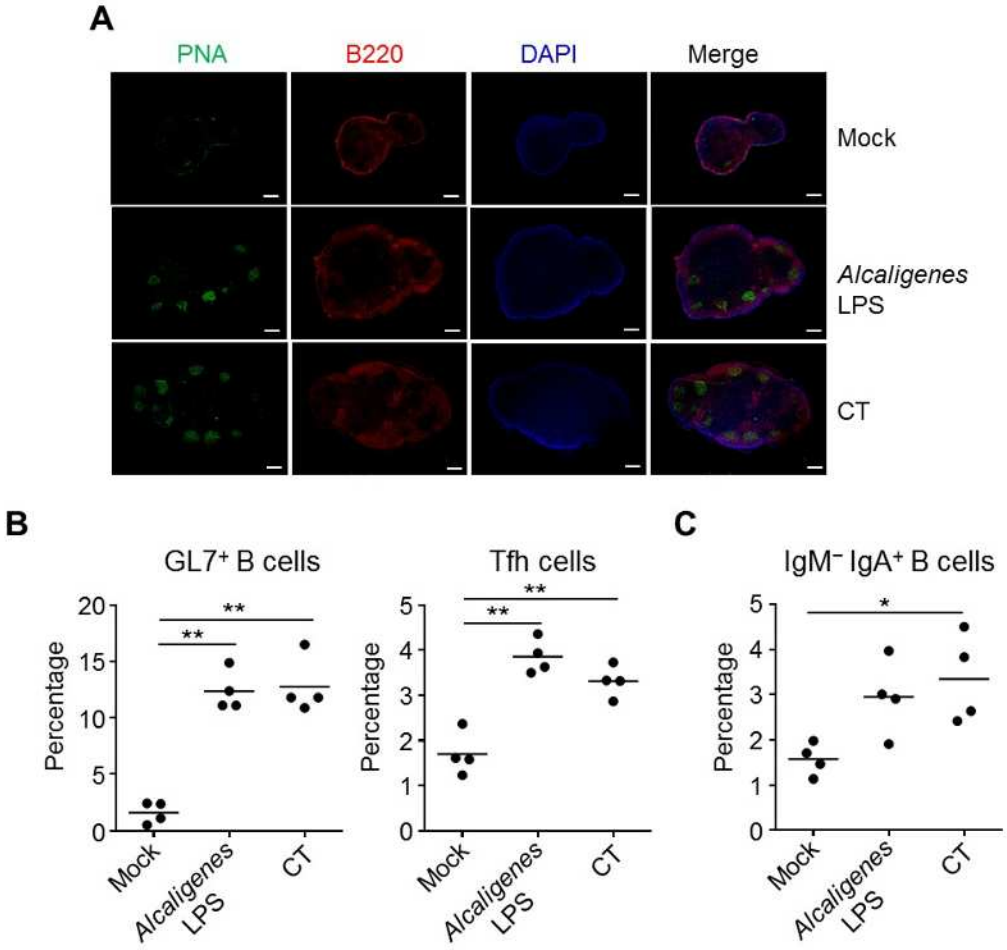


Fig. 4. *Alcaligenes* LPS induced GC formation in CLNs. Mice were nasally immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT; one week after the final immunization, (A) GC formation (PNA: GC marker; B220: B cell marker), and the induction of (B) GC GL7$^+$ B cells (gated on: CD3ε$^-$ B220$^+$ GL7$^+$) and Tfh cells (gated on: CD3ε$^+$ CD8α$^-$ CD4$^+$ PD-1$^+$) and (C) IgM$^-$ IgA$^+$ B cells (gated on: CD3ε$^-$ B220$^+$ GL7$^+$ IgM$^-$ IgA$^+$) in CLNs were examined by flow cytometry analysis. (n = 4 per group). Data are representative of two independent experiments and were analyzed by one-way ANOVA (*p < 0.05; **p < 0.01).

To further assess whether nasally co-administered *Alcaligenes* LPS also supports the induction of antigen-specific systemic antibody responses or not, I examined serum antibodies. Negligible levels of OVA-specific IgG and IgA responses were observed in Mock group. While higher levels of OVA-specific

IgG and IgA responses were noted in *Alcaligenes* LPS group, which were almost comparable to the levels of CT group (Fig. 5). Thus, co-administered *Alcaligenes* LPS could support the elevated antigen-specific systemic antibody responses upon nasal vaccination.

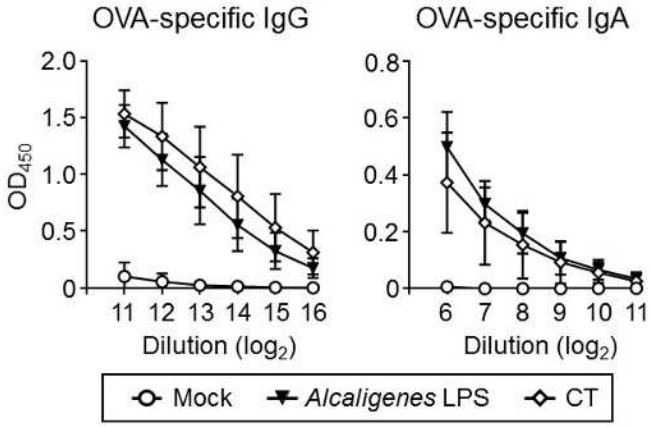


Fig. 5. *Alcaligenes* LPS promoted systemic antibody responses. Mice were nasally immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT; one week after the final immunization, serum was collected to determine OVA-specific IgG and IgA by ELISA (*n* = 5 per group). Data are representative of two independent experiments.

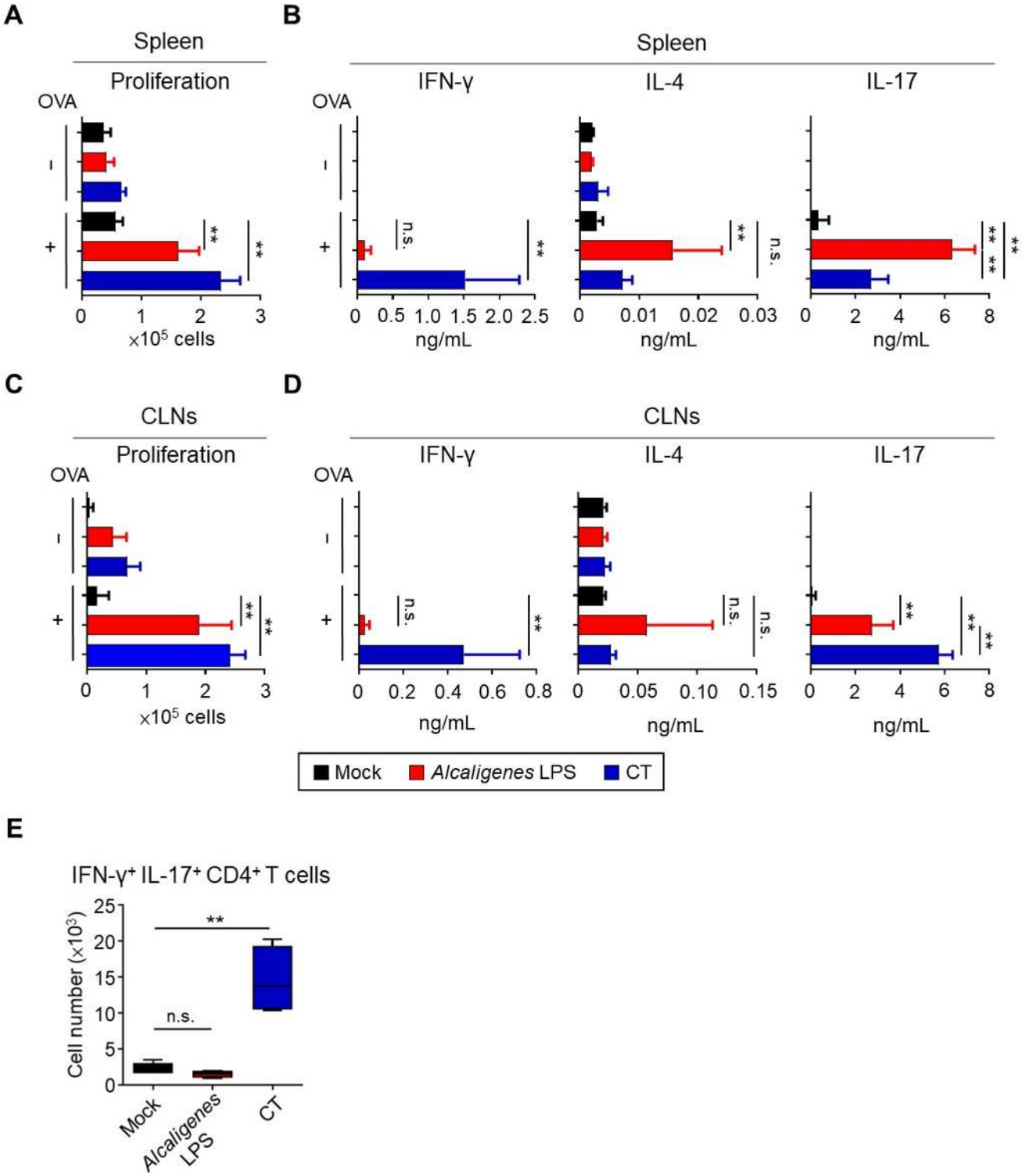
3.3. *Alcaligenes* LPS promoted an OVA-specific Th17 cell response

To understand how *Alcaligenes* LPS inducing antigen-specific antibody responses, I examined T cell responses such as cell proliferation and cytokine production in the spleen and CLNs. $CD4^+$ T cells from the spleen and CLNs of mice nasally immunized with OVA plus *Alcaligenes* LPS or CT proliferated vigorously upon the *ex vivo* stimulation with OVA, when compared with those from the Mock group (Fig. 6A, 6C). The finding suggests that *Alcaligenes* LPS is a potent nasal adjuvant for the enhancement of $CD4^+$ T cell responses.

Next, I examined the production of cytokines from OVA-specific $CD4^+$ T cells, especially related to the Th1 (IFN-γ), Th2 (IL-4), and Th17 (IL-17). Consistent with low OVA-induced $CD4^+$ T cell proliferation activity of the Mock group from spleen and CLNs, it was noted that the production of cytokines in Mock group is negligible or low. (Fig. 6B, 6D). In contrast, splenic and CLNs $CD4^+$ T cells

445 from the *Alcaligenes* LPS group preferentially produced IL-17 with significant
446 increase of IL-4 and little production of IFN-γ, whereas the CT group showed
447 significantly increased production of both IFN-γ and IL-17 with less production
448 of IL-4 (Fig. 6B, 6D).

449 It has been considered that T cells secreting IL-17 alone are considered non-
450 pathogenic and contribute to immunological defense against extracellular
451 pathogens, whereas T cells producing both IL-17 and IFN-γ are pathogenic to
452 cause inflammation and autoimmunity. One of the differences between
453 *Alcaligenes* LPS and CT groups was the significantly higher IFN-γ production
454 in the CT group. Base on this result, I found that splenic $CD4^+$ T cells from the
455 CT group contained significantly higher numbers of IFN-γ^+ IL-17^+ $CD4^+$ T cells
456 compared with that in the Mock or *Alcaligenes* LPS group by performing flow
457 cytometry analysis (Fig. 6E). These results indicate that nasally co-
458 administered *Alcaligenes* LPS primarily induced Th17 cell-mediated non-
459 pathogenic responses, whereas nasally co-administered CT induced
460 pathogenic Th17 cell responses.



461 **Fig. 6.** *Alcaligenes* LPS promoted OVA-specific Th17 cell responses. Mice were nasally
462 immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT; one
463 week after the final immunization, splenic or CLNs $CD4^+$ T cells were collected and
464 stimulated with OVA by *ex vivo*. (A) Proliferation activity of splenic $CD4^+$ T cells were

determined by CyQUANT® Cell Proliferation Assays Kits and fluorescence microplate reader, ARVO X2 with measuring at 485/535 nm. (B) Production of cytokines: IFN-γ, IL-4 and IL-17 in the supernatant of splenic $CD4^+$ T cell culture was collected and measured by the CBA kit. (C) Proliferation activity of CLNs $CD4^+$ T cells. (D) Production of cytokines: IFN-γ, IL-4 and IL-17 in the supernatant of CLNs $CD4^+$ T cell culture. (E) Number of IFN-γ^+ IL-17^+ $CD4^+$ T cells (gated on: zombie- $CD45^+$ TCR-β^+ $CD4^+$ IFN-γ^+ IL-17^+) in mice spleen were analyzed by intracellular flow cytometry analysis (*n* = 4 or 6 per group). Data are representative of two independent experiments and were analyzed by one-way ANOVA (**$p < 0.01$; n.s.: not significant).

3.4. *Alcaligenes* LPS had low Inflammatory but high regulatory properties

In addition to classical T helper cell subsets associated with cytokines examined above, T cells are also known to produce various inflammatory and regulatory cytokines (29). Therefore, I applied T-cell assay to examined other cytokine production profiles (e.g., TNF-α, IL-5, and IL-10) by OVA-specific $CD4^+$ T cells from CLNs and spleen of *Alcaligenes* LPS group. TNF-α is a cytokine related to inflammation such as neutrophilia in tissue and can be secreted by Th1 cells (30,31). IL-5 is involved in inducing tissue eosinophilia and is produced by Th2 cells (32). IL-10 is the cytokine that regulate immune responses and is produced by $CD4^+$ T cells such as T regulatory cells (33).

The results revealed that CT group significantly induced TNF-α and IL-5 production in the supernatant of splenic $CD4^+$ T cell culture and significantly induced TNF-α production from CLNs $CD4^+$ T cell culture, but not the Mock and *Alcaligenes* LPS groups. It is interesting to note that the production of IL-10 was preferentially heightened in the supernatant of splenic and CLNs $CD4^+$ T cell culture from the *Alcaligenes* LPS group (Fig. 7A–B).

Considering that the cytokines produced by T cells can cause neutrophilia and eosinophilia (32,34), which can lead to local inflammation, I examined the numbers of neutrophils and eosinophils in the nasal cavity of the nasally immunized mice. Consistent with the cytokine profiles, flow cytometry analysis

revealed the increased numbers of neutrophils and eosinophils in the nasal cavity of CT group compared with the Mock or *Alcaligenes* LPS group (Fig. 8). These results indicate that, unlike CT, *Alcaligenes* LPS did not induce local inflammation in the nasal cavity.

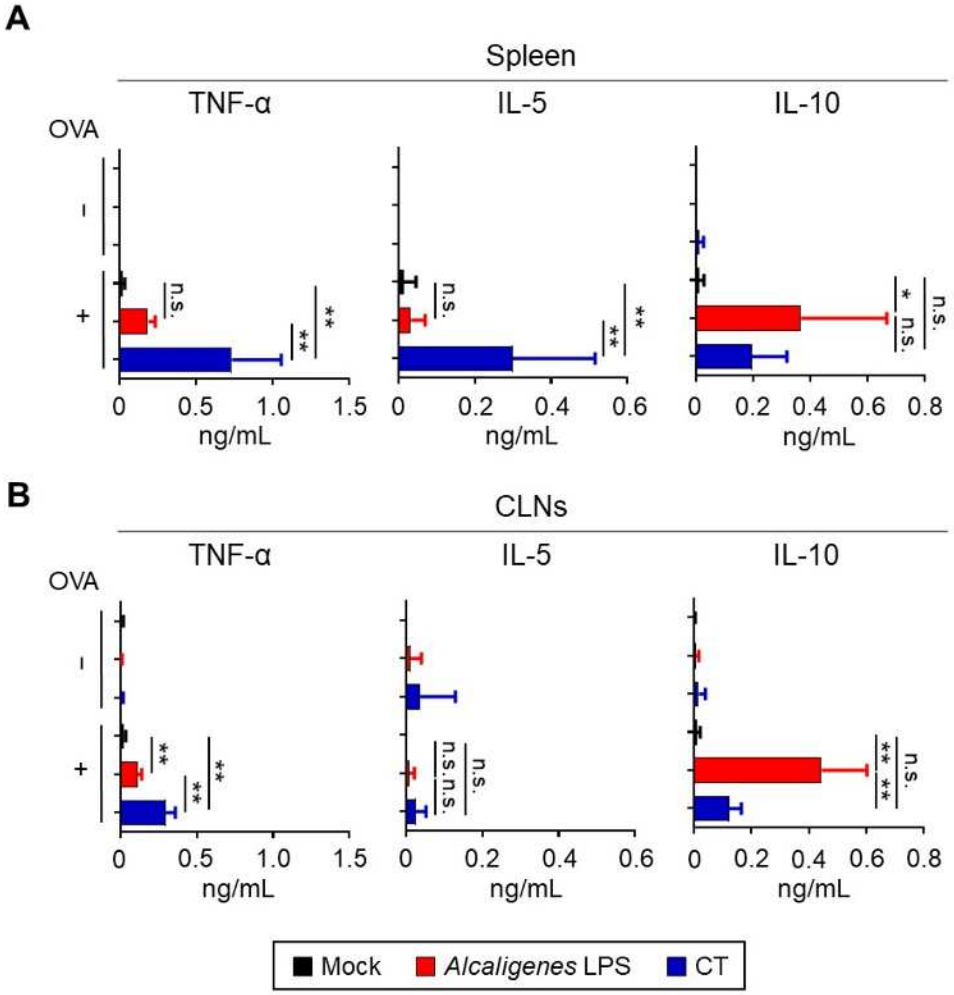


Fig. 7. *Alcaligenes* LPS had low Inflammatory but high regulatory properties. Mice were nasally immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT; one week after the final immunization, the production of cytokines: TNF-α, IL-5, and IL-10 in the supernatant of (A) splenic $CD4^+$ T cell culture and of the (B) CLNs $CD4^+$ T cell culture were surveyed after *ex vivo* stimulation by OVA (n = 6 per group). Data are representative of two independent experiments and were analyzed by one-way ANOVA (*p < 0.05; **p < 0.01; n.s.: not significant).

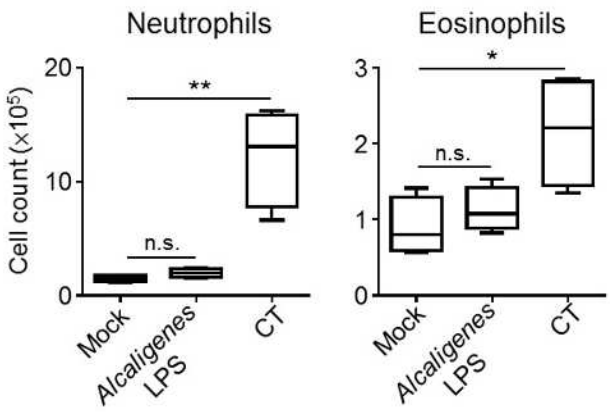


Fig. 8. *Alcaligenes* LPS induced little inflammation at the site of administration. Mice were nasally immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT; one week after the final immunization, the number of neutrophils (gated on: 7AAD$^-$ CD45$^+$ CD11c$^+$ Ly6G$^+$) and eosinophils (gated on: 7AAD$^-$ CD45$^+$ CD11c$^+$ Siglec-F$^+$) in the nasal passage were determined by flow cytometry analysis (n = 4 per group). Data are representative of two independent experiments and analyzed by one-way ANOVA (*p < 0.05; **p < 0.01; n.s.: not significant).

3.5. Subcutaneously co-administered *Alcaligenes* lipid A promoted systemic antibody responses

As described above, I showed that nasal immunization with OVA plus *Alcaligenes* LPS showed effective immune responses without severe side effects. In addition, a previous study by our group showed that subcutaneous immunization of OVA plus *Alcaligenes* LPS also induced the safe and effective immune responses (7). Based on these findings, I then focused on the adjuvant activity of lipid A, the activity site of LPS, to trigger the immune responses induced by subcutaneous immunization (20).

Mice were subcutaneously immunized with OVA alone (Mock group) or plus chemically synthesized *Alcaligenes* lipid A (*Alcaligenes* lipid A group) and serum antibodies were analyzed by ELISA. Mice subcutaneously immunized with 1 μg of OVA plus *Alcaligenes* lipid A showed increased levels of OVA-specific serum IgG when compared with mice immunized with 1 μg of OVA alone (Fig. 9A). Furthermore, mice immunized with 1 μg of OVA plus *Alcaligenes* lipid A showed higher levels of OVA-specific IgG than mice

immunized with 10 μg of OVA alone. Consistent with these results, mice immunized with 1 μg of OVA plus *Alcaligenes* lipid A showed higher levels of OVA–specific IgG1, IgG2b, and IgG3 than mice immunized with 1 μg of OVA (Fig. 9B). These results indicate that *Alcaligenes* lipid A could enhance antigen-specific systemic antibody responses upon subcutaneous injection.

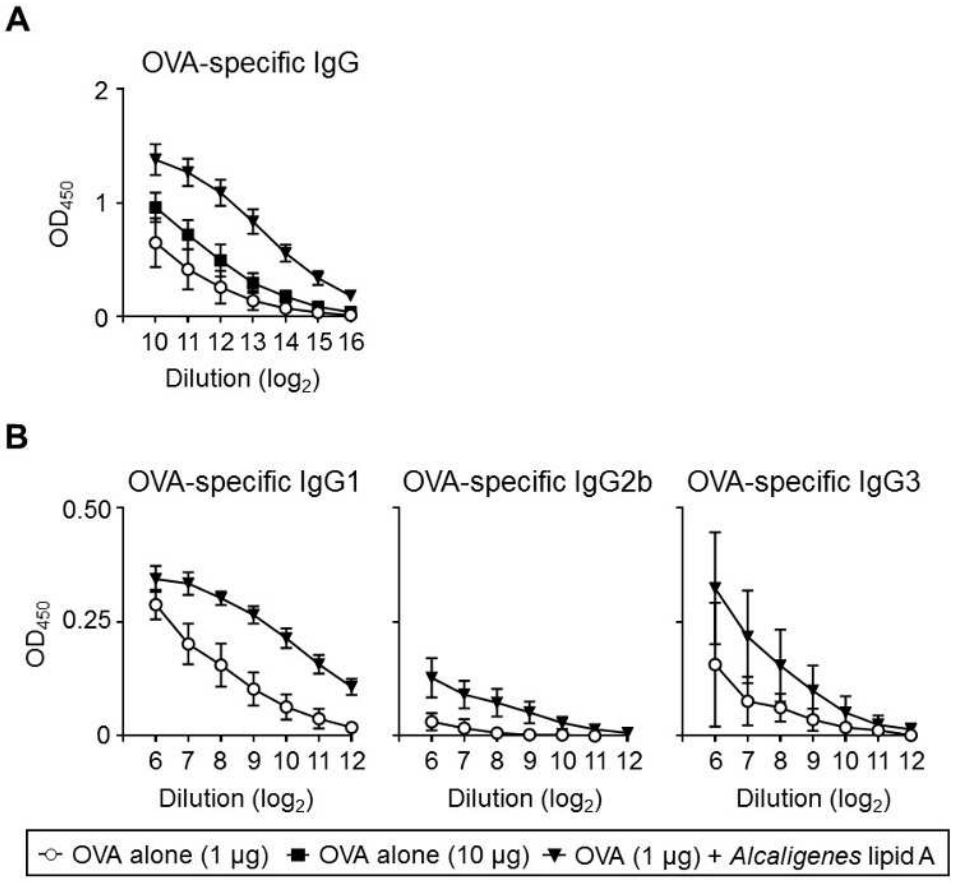


Fig. 9. *Alcaligenes* lipid A promoted OVA–specific systemic antibody responses. Mice were subcutaneously immunized 2 times with OVA plus *Alcaligenes* lipid A. One week after the last immunization, serum was collected to measure levels of (A) OVA-specific IgG, (B) IgG1, IgG2b and IgG3 by ELISA (n = 7 per group). Data are combined of two independent experiments.

3.6. *Alcaligenes* lipid A promoted an OVA-specific Th17 cell responses

To unveil the mechanism of *Alcaligenes* lipid A inducing antigen-specific systemic antibody responses, I examined effects of *Alcaligenes* lipid A on T cell responses such as cell proliferation and cytokine production in the spleen. $CD4^+$

T cells from the spleen of *Alcaligenes* lipid A group proliferated vigorously upon the *ex vivo* stimulation with OVA, when compared with those from the Mock group (Fig. 10A). The finding suggests that *Alcaligenes* lipid A has adjuvant activity to promote $CD4^+$ T cell responses.

Next, I examined the production of cytokines from OVA-specific $CD4^+$ T cells. Splenic $CD4^+$ T cells from the *Alcaligenes* lipid A group preferentially produced IL-17 with little production of IL-4 and IFN-γ. And *Alcaligenes* lipid A group showed the increased tendency of IFN-γ and the decreased tendency of IL-4 when compared with Mock group (Fig. 10B). These results collectively indicate that *Alcaligenes* lipid A preferentially induced Th17 cell responses.

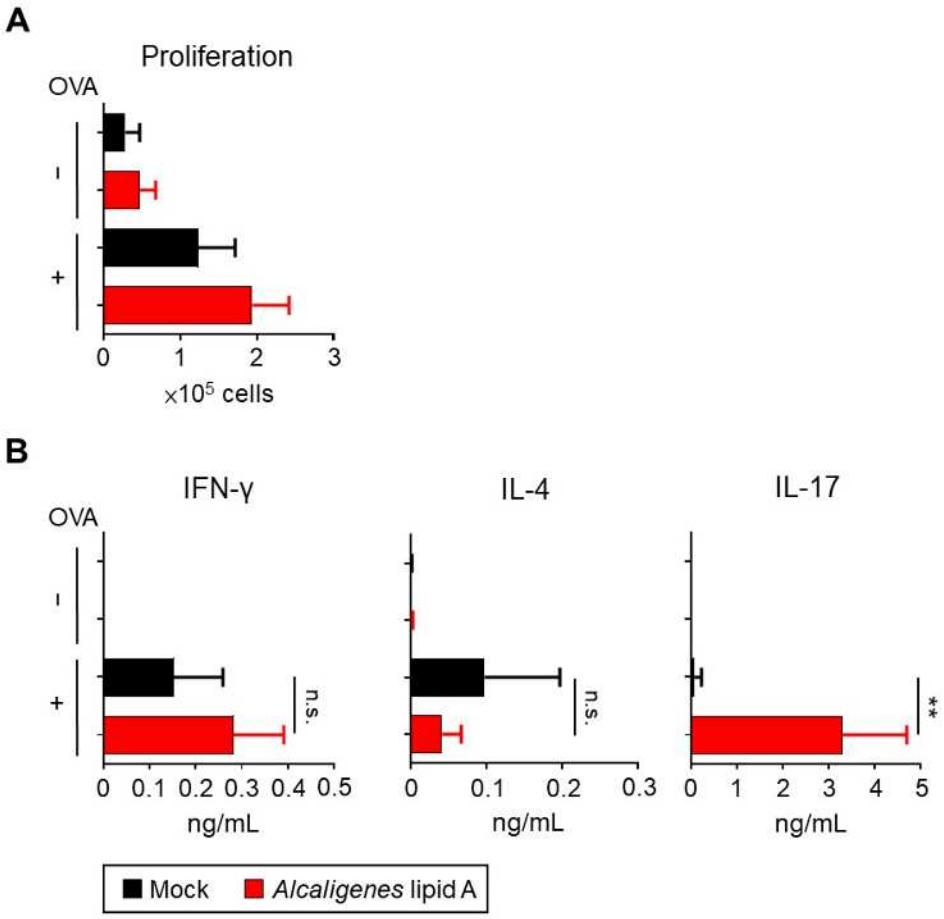


Fig. 10. *Alcaligenes* lipid A promoted OVA-specific Th17 cell responses. Mice were subcutaneously immunized 2 times with OVA plus *Alcaligenes* lipid A; one week after the final immunization, splenic $CD4^+$ T cells were collected and stimulated with OVA by *ex vivo*. (A) Proliferation activity of splenic $CD4^+$ T cells were determined by CyQUANT® Cell

Proliferation Assays Kits and fluorescence microplate reader, ARVO X2 with measuring at 485/535 nm. (B) Production of cytokines: IFN-γ, IL-4 and IL-17 in the supernatant of splenic $CD4^+$ T cell culture was collected and measured by the CBA kit (n = 6 per group). Data are combined of two independent experiments and analyzed by one-way ANOVA (**p < 0.01; n.s.: not significant).

3.7. *Alcaligenes* lipid A induced BMDCs activation

DCs play the key role in the induction of acquired immunity, including antibody production and T cell responses through acting as antigen-presenting cells. To examine effects of *Alcaligenes* lipid A on DCs, I measured expression of MHC II and costimulatory molecules, including CD40, CD80 and CD86 when BMDCs were stimulated with *Alcaligenes* lipid A by flow cytometry analysis. The expression of MHC II, CD40, CD80 and CD86 was increased by *Alcaligenes* Lipid A in dose-dependent manner (Fig. 11A), indicating that *Alcaligenes* lipid A induces activation of DCs.

Cytokines profile of DCs determines class of T cell responses, thus I next examined cytokines produced from BMDCs. IL-6 and IL-23 produced from BMDCs showed increased levels by stimulation with *Alcaligenes* lipid A in dose-dependent manner (Fig. 11B), indicating that *Alcaligenes* lipid A activates DCs to induce production of Th17 polarization-associated cytokines, including IL-6.

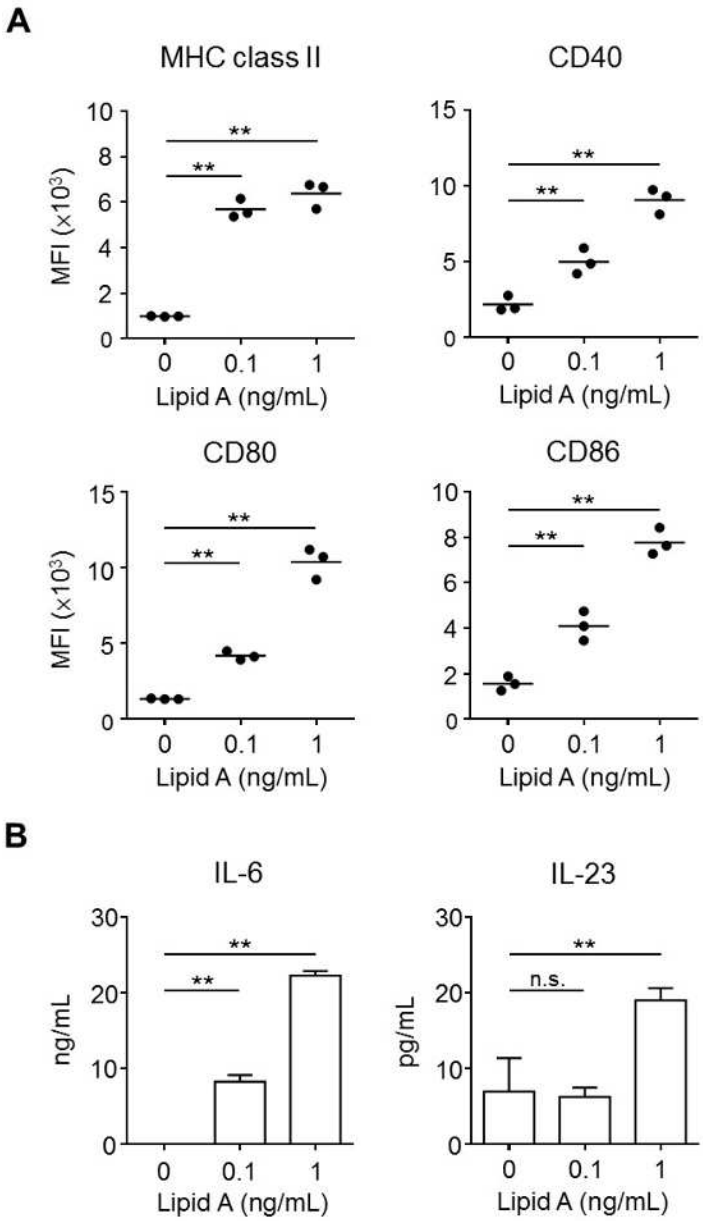


Fig. 11. *Alcaligenes* lipid A activated BMDCs. BMDCs were stimulated with *Alcaligenes* lipid A at the concentration of 0.1 and 1 ng/mL. After incubation for 48 hours, (A) expression of MHCII, CD80, CD86 and CD40 were analyzed by flow cytometry analysis. (B) Production of cytokines: IL-6 and IL-23 in the supernatant of BMDCs were collected and measured by the ELISA kit (n = 3-4 per group). Data are representative of two independent experiments and analyzed by one-way ANOVA (*p < 0.05; **p < 0.01; n.s.: not significant).

3.8. *Alcaligenes* lipid A activated human PBMCs

To consider effects of *Alcaligenes* lipid A on human, I examined cytokines produced from PBMCs stimulated with *Alcaligenes* lipid A. In individuals 1 and

2, stimulation of 1 ng/mL with *Alcaligenes* Lipid A increased levels of IL-6 and IL-1β (Fig. 12). These results indicated that *Alcaligenes* lipid A could activate human PBMCs. Furthermore, since IL-6 and IL-1β were required for Th17 cell-differentiation in human, these results also suggest that *Alcaligenes* lipid A could induce antigen-specific Th17 cell responses in human.

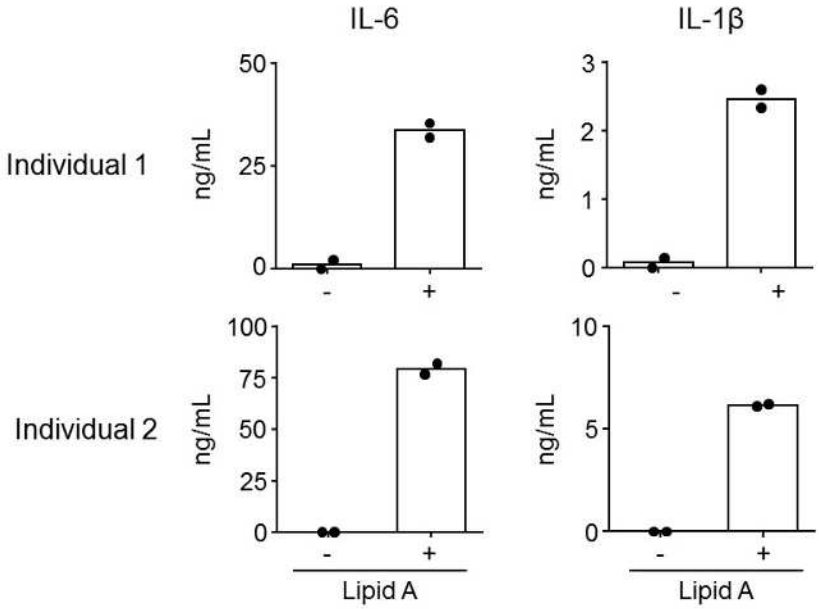


Fig. 12. *Alcaligenes* lipid A activated PBMCs. Human PBMCs from two independent individuals were stimulated with *Alcaligenes* lipid A at the concentration of 1 ng/mL. After incubation for 24 hours, production of cytokines: IL-6 and IL-1β in the supernatant of PBMCs were collected and measured by the CBA kit and the ELISA kit.

3.9. *Alcaligenes* lipid A showed no severe side effects.

Considering the safety of *Alcaligenes* lipid A as an adjuvant, I examined the number of lymphocytes and platelets in blood of mice. Lymphocytes and platelets were measured 24 hours after immunization. Mice immunized with OVA plus *Alcaligenes* lipid A showed lower numbers of lymphocytes and platelets, comparing with mice immunized with OVA alone, but the number of lymphocytes and platelets is within a reasonable physiological range of mice or slightly below the normal physiological range of mice. (Fig. 13A).

Body temperature was surveyed at different time points after the immunization (0, 1st to 8th and 24th hour) by measuring rectal temperature of mice. Immunization with *Alcaligenes* lipid A showed no effects on body temperature, comparing with Mock group (OVA alone), and body temperature of all mice are within a reasonable physiological range of mice (Fig. 13B). Consistent with this result, body weight was not changed before and after immunization with OVA with or without *Alcaligenes* lipid A (Fig. 13C). Collectively, these results indicate that immunization with *Alcaligenes* lipid A did not cause severe side effects on mice, suggesting application of *Alcaligenes* lipid A as an adjuvant is safe.

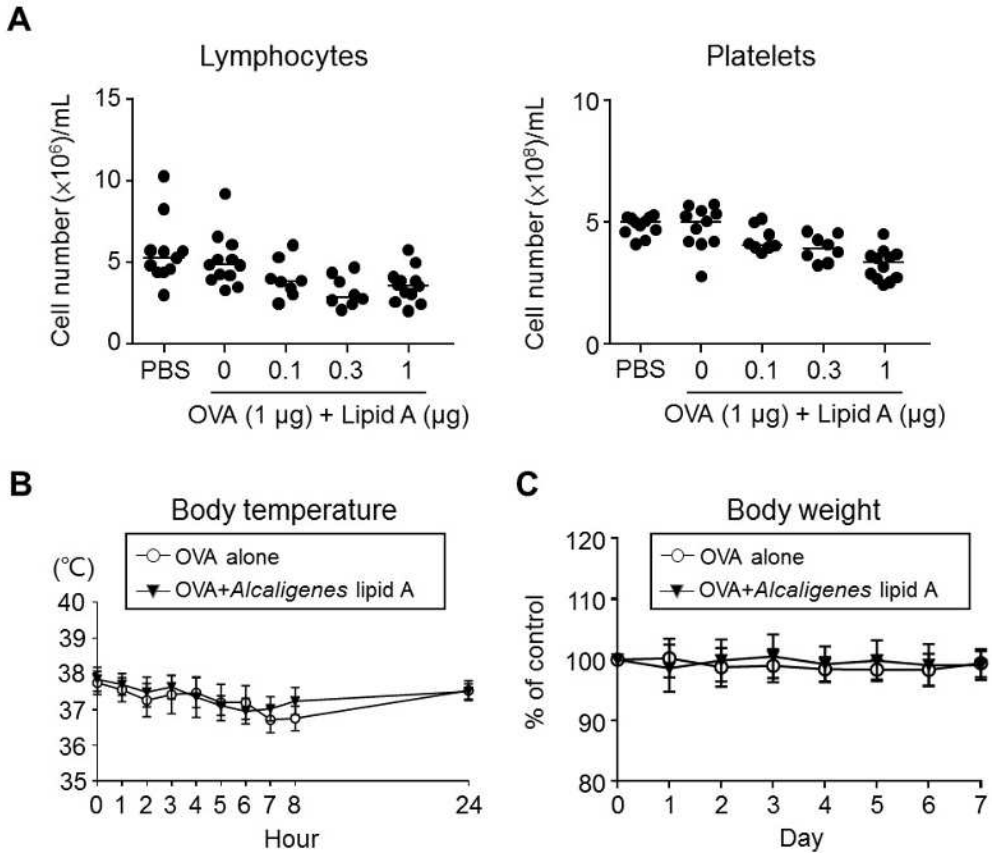


Fig. 13. *Alcaligenes* lipid A showed no severe side effects. Mice were subcutaneously immunized with OVA plus *Alcaligenes* lipid A. (A) The number of lymphocytes and platelets were measured in blood after 24 hours from immunization. (B) Body temperature at 0, 1st to 8th and 24th hour and (C) body weight in 7 days after the immunization were continuously monitored (n = 4 per group). Data are representative of two independent experiments.

4. Discussion

In this study, I revealed that *Alcaligenes* LPS as a nasal vaccine adjuvant have the efficacy to enhance antigen-specific respiratory including nasal and BALF IgA antibody responses. Consistent with the elevation of IgA antibody responses, the data indicated that nasal immunization with OVA plus *Alcaligenes* LPS induced GC formation in the NALT and CLNs, where Tfh cells were also induced. According to our previous study, *Alcaligenes* LPS stimulated BMDCs or PP-derived DCs to produce IL-6 (7), a cytokine involved in the differentiation of Tfh cells, Th17 cells, and IgA^+ B cells (35–38). Collectively, these findings indicate that *Alcaligenes* LPS creates an immunological environment that promotes GC formation with Tfh cells and Th17 cells, which in turn induces antibody responses in the NALT and CLNs.

I also found that similar serum IgG antibody responses induced by *Alcaligenes* LPS immunized nasally or by *Alcaligenes* lipid A immunized subcutaneously. In this study, *Alcaligenes* lipid A immunized subcutaneously induced the subclasses antibody responses, including IgG1, IgG2b and IgG3. This evidence was further supported by that Th17 cell responses induce production of IgG1, IgG2b and IgG3 by secretion of IL-17 or IL-21 from Th17 cells (39). Moreover, IgG antibody contributes to activating complement and phagocytosis by macrophages and neutrophils to defense bacterial and viral infections (40). Especially, since IgG1 and IgG3 show high activities in binding to C1q to induce complement system, it is suggested that *Alcaligenes* lipid A is helpful to protect against pathogen invasion through these immune responses (40).

As mentioned above, *Alcaligenes* LPS as nasal adjuvant induced Th17 cell responses which is same as CT induced in this study, however, CT induces different Th17 cell type from *Alcaligenes* LPS. Both adjuvants induced T cells producing IL-17, but the T cells induced by CT also expressed IFN-γ, whereas those induced by *Alcaligenes* LPS did not. IL-17 and IFN-γ–producing T cells

are considered pathogenic because they induce severe inflammatory responses in autoimmune diseases (41,42). Consistent with our present findings, studies by other groups have shown that CT induces IL-6, IL-1β, and IL-23 from DCs (43), which creates an environment that promotes the differentiation of pathogenic Th17 cells (44,45). Regarding *Alcaligenes*, in our previous studies, we demonstrated that heat-killed *Alcaligenes* induces the production of IL-6, BAFF, TGF-β, and IL-10 when co-cultured with BMDCs, PP DCs, or murine PP cells (4,7). However, in our previous study, heat-killed *Alcaligenes* did not induce BMDCs to produce IL-12 (7), the cytokine that causes differentiation of Th1 cell. These characteristics plausibly led to the preferential differentiation of non-pathogenic Th17 cells by *Alcaligenes* LPS. Thus, it is likely that the production of IL-1β by antigen-presenting cells is the factor to determine which type of Th17 cells (pathogenic or non-pathogenic) is induced in our experimental condition. This is consistent with the results of a previous study, which showed that IL-1β is required for the pathogenicity of Th17 during intracellular bacterial infection (46).

To understand the mechanism for the induction of Th17 cell responses and the serum antibody production by *Alcaligenes* LPS and lipid A, I examined the effects of *Alcaligenes* lipid A on DCs in this study. DCs present antigen to T cells via MHC class II and costimulatory molecules, such as CD80 and CD86 (47,48) ,which are required for T cell activation, including T cell proliferation and differentiation. In this regard, *Alcaligenes* lipid A enhances expression of MHCII, CD80 and CD86 from BMDCs in this study. Moreover, differentiation of T cells is determined by differences in cytokine environment produced from antigen-presenting cells, including DCs. For example, transforming growth factor beta (TGF-β) and IL-6 promote the differentiation of Th17 cells, and IL-23 promotes the stabilization of Th17 cells in mice (49–51). Consistent with our previous reports about *Alcaligenes*-mediated activation of DCs (4,5,7), *Alcaligenes* lipid A increases IL-6 and IL-23 production from murine BMDCs. Furthermore,

688 human PBMCs stimulated by *Alcaligenes* lipid A had increased production of
689 IL-6 and IL-1β, which contribute to the differentiation and stabilization of human
690 Th17 cells (49–52). IL-23 was also produced by the PBMCs; however, unlike
691 IL-6 and IL-1β, the reactivity was different among the individuals. This
692 difference between individuals indicates that *Alcaligenes* lipid A may have
693 multiple pathways to induce human Th17 development (49–52).
694 In addition, *Alcaligenes* lipid A enhanced the expression of CD40 on BMDCs,
695 suggesting that *Alcaligenes* lipid A could induce antibody production via the T
696 cell independent pathway. CD40 expressed on DCs plays a role in regulating B
697 cell proliferation by the direct interaction via CD40L expressed on B cells,
698 leading to enhance IgG production (53).
699 In the present study, I also examined the safety of *Alcaligenes* LPS and lipid
700 A when being used as an adjuvant. I found that CT, but not *Alcaligenes* LPS,
701 induced inflammation in the nasal cavity, which was characterized by increased
702 infiltration of neutrophils and eosinophils with the increased levels of TNF-α and
703 IL-5 production from T cells. Consistent with the findings, our previous studies
704 showed that the production levels of TNF-α and nitric oxide (NO), an
705 inflammatory molecule that induces TNF-α production, were lower in BMDCs
706 treated with *Alcaligenes* LPS than in BMDCs treated with *E. coli* LPS (7,54).
707 TNF-α also upregulates vascular endothelial cell adhesion molecules such as
708 intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule
709 (VCAM-1), and E-selectin, thereby promoting the migration of neutrophils and
710 eosinophils to sites of inflammation (32,55). And IL-5 induces the differentiation
711 of eosinophils and interacts with IL-17 to promote the survival and
712 degranulation of eosinophils (32), leading to tissue inflammation and damage.
713 In addition, *Alcaligenes* LPS induced T cells secreting IL-10, which inhibits
714 neutrophil recruitment by regulating the secretion of chemokines such as
715 CXCL9 and 12 and CCL3–5, 11, and 17 (56). Together, these findings indicate
716 that *Alcaligenes* LPS did not induce inflammation because of lower production

of T cells producing TNF-α or IL-5 and higher production of T cells producing IL10 compared to CT, resulting in the migration of fewer eosinophils and neutrophils to the nasal cavity.

Regarding the immunological property of *Alcaligenes* LPS, our previous studies indicated that *Alcaligenes* LPS has little cytotoxic activity. Indeed, compared with *E. coli* LPS, *Alcaligenes* LPS showed lower endotoxin activity in the limulus amebocyte lysate test and caused only limited inflammatory reactions when intraperitoneally injected into mice, including lower levels of serum IL-6, less change in body temperature, and less damage to lung tissue with little infiltration of inflammatory cells such as neutrophils and eosinophils (7).

Alcaligenes lipid A did not show severe side effects on mice, when the number of lymphocytes and platelets, body temperature and body weight were examined as safe indicators for *Alcaligenes* lipid A as an adjuvant. However, the number of lymphocytes and platelets shows a decrease in *Alcaligenes* lipid A group, comparing with Mock group, but most of them were in normal range of mice, suggesting that *Alcaligenes* lipid A has a slight risk of causing leukopenia and thrombosis. I thought that it is still necessary to have more careful consideration such as setting a safety dose, for the clinical application of *Alcaligenes* lipid A.

In terms of IL-6 production from BMDCs, TLR4-deficient BMDCs did not respond to *Alcaligenes* LPS, whereas TLR2-deficient BMDCs produced comparable levels of IL-6 as wild type BMDCs (7). Further, *Alcaligenes* LPS did not act as a competitive inhibitor of *E. coli* LPS in the IL-6 production from BMDCs (7), collectively suggesting that *Alcaligenes* LPS acts as a weak agonist of TLR4, which is expressed in the nasal or lung tissues of mice (57,58). This suggests that *Alcaligenes* LPS induced the immune responses also through combination of TLR4.

As biochemical characteristics, the structure of LPS is mainly composed of lipid A, core oligosaccharide, and O-antigens. Lipid A is the active center of LPS and acts as an agonist of TLR4/MD-2 complex. The activity as a TLR4 agonist is determined by several feature of lipid A structure. As for lipid A component in *Alcaligenes* LPS, a mixture of tetra- to hexa-acylated species was identified, and the lipid A with hexa-acylated species 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 fashion with respect to the disaccharide backbone, which were different with *E. coli* LPS whose lipid A has 4 + 2 symmetry and is composed of 14:0 (3-OH) as primary and 14:0 and 12:0 as secondary fatty acids (22,59).

Although the other component of LPS such as O-antigen possibly plays some roles in the adjuvant activity of LPS (60), our previous studies implicated that the uniqueness of lipid A structure is the critical determinant of inflammatory activity. However, it is needed to study more details such as the relationship between the bioactivity and the structure of *Alcaligenes* LPS or lipid A to affect the signal pathway including MyD88 pathway after binding to TLR4 receptor to trigger the immune responses.

In conclusion, *Alcaligenes* LPS showed efficacy as a nasal vaccine adjuvant to induce respiratory and systemic immune responses without inducing local inflammation via the induction of non-pathogenic Th17 cell responses and GC formation. And chemically synthesized *Alcaligenes* lipid A as an adjuvant in systemic vaccination promoted both antigen-specific IgG antibody and Th17 responses in mice by directly stimulating DCs. Stimulation with *Alcaligenes* lipid A also induced the production of IL-6 and IL-1β in human PBMCs, suggesting a potency to be applied for use in human.

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