



Title	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
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1 パイエル板組織内共生菌アルカリゲネス由来
2 **LPS/lipid A** のアジュvant応用に関する研究
3 **The study on adjuvant application of LPS/lipid A**
4 **derived from *Alcaligenes* spp, lymphoid tissue**
5 **resident symbiotic bacterium in the Peyer's**
6 **patches**



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20 **2022**

21 **Abstract**

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

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

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

60 **1. Introduction**

61 Commensal bacteria in the gut are involved in the regulation of host immunity;
62 therefore, are expected to play important roles not only in host immune
63 responses to immunization but also in host responses to pathogenic infection.
64 Accumulating evidence have indicated the involvement of certain commensal
65 bacteria in the regulation of specific immunity. For instance, *Klebsiella* spp.
66 have been shown to induce Th1 cell polarization, and segmented filamentous
67 bacteria have been shown to drive Th17 cell responses to pathogenic infection
68 (1,2). Similarly, *Clostridium* spp. have been shown to induce regulatory T cells
69 for the control of allergic diseases (3).

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

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

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

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

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

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

141

142 **2. Materials and Methods**

143 **2.1. Mice**

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

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

153

154 **2.2. Preparation of LPS and lipid A**

155 *Alcaligenes* LPS and chemically synthesized *Alcaligenes* lipid A were
156 provided by Professors Fukase and Shimoyama of Osaka University.
157 *Alcaligenes* LPS was extracted from heat-killed (60°C for 30 min) *A. faecalis*
158 (13111T, Biological Resource Center, NITE [NBRC], Japan) by using an LPS
159 Extraction Kit (iNtRON Biotechnology, Inc., Sangdaewon-Dong, Korea). After
160 extraction, *Alcaligenes* LPS is lyophilized and stored as a powder at -30°C. And
161 *Alcaligenes* lipid A was chemically synthesized as previously described (22) For
162 stock solution, *Alcaligenes* LPS was added to phosphate-buffered saline (PBS;
163 Nacalai Tesque, Inc., Kyoto, Japan) and *Alcaligenes* lipid A was dissolved with
164 dimethyl sulfoxide (DMSO; Nacalai Tesque, Inc.). The stock solution of the LPS
165 or lipid A was prepared to a concentration of 1 mg/mL, sonicated for 5 min, and
166 then stored at -30°C until use.

167

168 **2.3. Immunization**

169 For nasal immunization, mice were immunized equally into the two nostrils of
170 mice without anesthesia on day 1, 7, and 17 (23). One week after the final
171 immunization, nasal wash, bronchoalveolar lavage fluid (BALF), serum, nasal
172 passage, NALT, CLNs, and spleen were collected as previously described
173 (23,24) and used for analysis. The groups were divided as follows: 5 µg of
174 ovalbumin (OVA) (Sigma-Aldrich) alone; 5 µg of OVA with 10 µg of *Alcaligenes*

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

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

181

182 **2.4. Enzyme-linked immunosorbent assay (ELISA)**

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

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

204

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).

228

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

233 filter (Thermo Fisher Scientific Inc.); then mixed with red blood cell lysis buffer
234 (1.5 M NH₄Cl, 100 mM KHCO₃, and 10 mM EDTA-2Na [all Nacalai Tesque, Inc.])
235 for 1 min at room temperature; the resulting suspension was passed through a
236 100- μ m cell filter (Thermo Fisher Scientific Inc.) again, and the filtrate was
237 retained. CD4⁺ T cells were purified from the filtrate by using CD4 (L3T4)
238 MicroBeads and a magnetic cell separation system (Miltenyi Biotec, Bergisch
239 Gladbach, North Rhine-Westphalia, Germany). Splenic cells were treated with
240 30 Gy of ionizing radiation and used as antigen-presenting cells. The CD4⁺ T
241 cells (2×10^5 cells/well) and antigen-presenting cells (1×10^4 cells/well) were
242 suspended in RPMI1640 medium (Sigma-Aldrich) supplemented with 10%
243 Fetal Bovine Serum (FBS; Life Technologies, Thermo Fisher Scientific Inc.), 1
244 mM sodium pyruvate solution (Nacalai Tesque, Inc.), 1% penicillin–
245 streptomycin mixed solution (Nacalai Tesque, Inc.), and 0.5 mM 2-
246 mercaptoethanol (Gibco, Thermo Fisher Scientific Inc.); seeded in Nunc™ 96-
247 Well, Nunclon Delta-Treated, U-Shaped-Bottom Microplates (Thermo Fisher
248 Scientific Inc.); and cultured with or without 1 mg/mL OVA for 72 h. The number
249 of viable cells was determined by using a CyQUANT Cell Proliferation Assay kit
250 (Invitrogen, Thermo Fisher Scientific Inc.), and the absorbance of the cells was
251 measured at 485/535 nm with an ARVO X2 (PerkinElmer, Yokohama, Japan)
252 fluorescence microplate reader. The culture supernatant was collected and
253 used for the measurement of the concentrations of the cytokines as follows:
254 interferon gamma (IFN- γ), IL-4, IL-17, IL-10, and TNF- α . The cytokines were
255 determined by using a BD CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD
256 Biosciences, San Jose, CA, USA), and the concentration of IL-5 was
257 determined by using IL-5-specific ELISA kit (BioLegend).

258

259 **2.7. Preparation of bone marrow-derived dendritic cells**

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

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

275

276 **2.8. Flow Cytometric Analysis**

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

282 The cells were then stained with fluorescently labeled antibodies for 30 min
283 at 4°C. For analyzation of NALT and CLNs from nasal immunized mice, GC and
284 IgM⁻ IgA⁺ B cells were stained with FITC-IgA (BD Biosciences; clone: C10-3),
285 PE-Cy7-IgM (BioLegend; clone: RMM-1), AF647-GL7 (BioLegend; clone: GL7),
286 and BV421-B220 (BioLegend; clone: RA3-6B2). Tfh cells were stained with
287 FITC-CD3 ϵ (BD Biosciences; clone: 145-2C11), PE-PD-1 (BioLegend; clone:
288 29F.1A12), APC-Cy7-CD8 α (BioLegend; clone: 53-6.7), and BV421-CD4
289 (BioLegend; clone: RM4-5). Nasal passage from nasal immunized mice was
290 used for analyzation of neutrophils and eosinophils and the cell were stained

291 with FITC-Ly6G⁺ (BioLegend; clone: 1A8), APC-Cy7-CD11b (BioLegend; clone:
292 M1/70), BV421-Siglec-F (BD Biosciences; clone: E50-2440), and APC-CD45
293 (BioLegend; clone: 30-F11). And BMDCs were stained with FITC-A^d (BD
294 Biosciences; clone: AMS-32.1), PE-CD80 (BioLegend; clone: 16-10A1), APC-
295 Cy7-CD86 (BioLegend; clone: GL-1) and PE-Cy7-CD40 (BioLegend; clone:
296 3/23). Samples were analyzed by MACSQuant[®] Analyzer (Miltenyi Biotec).
297 Data were analyzed by using FlowJo, LLC Software 10.2 (BD Biosciences)

298 Intracellular cytokine staining was performed as previously described with
299 modification (27,28). Cells collected from nasal immunized mice spleen were
300 stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich)
301 and 750 ng/mL ionomycin (Sigma-Aldrich) for 4 h at 37°C; 5 ng/mL brefeldin A
302 (BioLegend) was added at around 3rd hour. After incubation, the cells were
303 stained with NIR-zombie (BioLegend), FITC-TCR-β (BioLegend; clone: H57-
304 597), PerCP-CD4 (BioLegend; clone: GK1.5), and BV421-CD45 (BioLegend;
305 clone: 30-F11). The cells were fixed and permeabilized by using BD
306 Cytofix/Cytoperm plus (BD Biosciences) and then stained with PE-IFN-γ
307 (BioLegend; clone: XMG1.2) and AF647-IL-17A (BD Biosciences; clone: TC11-
308 18H10). Samples were analyzed by MACSQuant[®] Analyzer (Miltenyi Biotec)
309 and the data were analyzed using FlowJo software v.10.2 (BD Biosciences).
310

311 **2.9. Measurement of cytokines in supernatant of BMDCs or PBMCs**

312 BMDCs (1×10^5 cells/well) or peripheral blood mononuclear cells (PBMCs)
313 (2×10^5 cells/well, FUJIFILM Wako Pure Chemical, Osaka, Japan) were seeded
314 into the Nunc[™] 96-Well, Nunclon Delta-Treated, U-Shaped-Bottom Microplate
315 (Thermo Fisher Scientific, Inc.). 0.1 or 1 ng/mL of *Alcaligenes* lipid A were used
316 to incubate with BMDCs for 48 hours or PBMCs for 24 hours at 37°C. The
317 culture supernatant from BMDCs and PBMCs were collected for measurement
318 of cytokines. For detection of IL-6 and IL-23 in supernatant of BMDCs, BD
319 cytometric bead array Mouse Inflammation Kit (BD Biosciences) and LEGEND

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

325

326 **2.10. Measurement of blood cells and body temperature in mice**

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

333

334 **2.11. Statistical analysis**

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

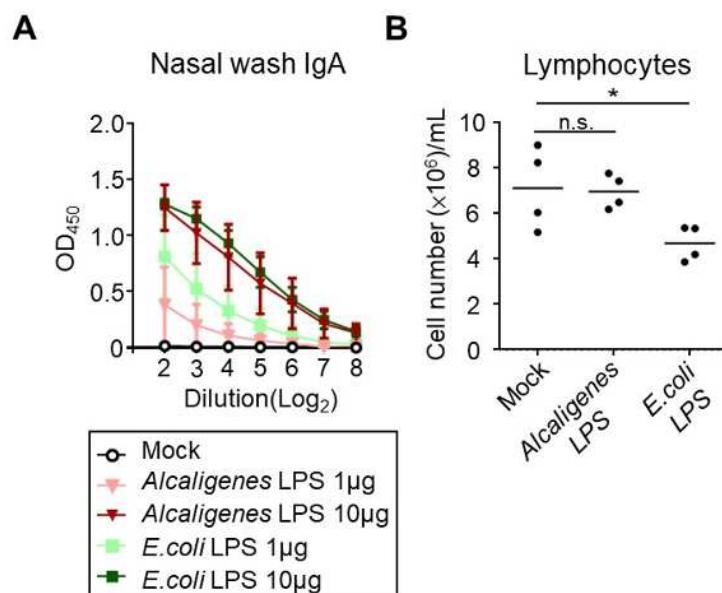
339

340 **3. Results**

341 **3.1. Nasally co-administered *Alcaligenes* LPS promoted respiratory
342 antigen-specific IgA antibody production**

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

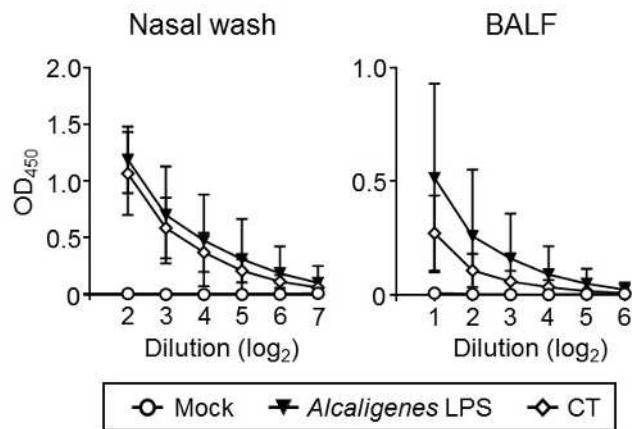
349 that nasal immunization with 10 μ g of *Alcaligenes* LPS induced higher levels of
 350 nasal IgA responses than 1 μ g of *Alcaligenes* LPS (Fig. 1A). And 10 μ g of *E.*
 351 *coli* LPS showed comparable levels of nasal IgA responses to 10 μ g of
 352 *Alcaligenes* LPS (Fig. 1A). However, 10 μ g of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



357
 358 **Fig. 1.** *E. coli* LPS induced dose-dependent nasal IgA responses and lymphopenia in the
 359 blood. Mice were nasally immunized 3 times with OVA alone (Mock) or with OVA plus
 360 *Alcaligenes* LPS (1 or 10 μ g) or *E. coli* LPS (1 or 10 μ g); one week after the final
 361 immunization, (A) Nasal wash were collected to determine levels of OVA-specific IgA by
 362 ELISA ($n = 4$ per group). Mice were nasally immunized with OVA alone (Mock) or with OVA
 363 plus *Alcaligenes* LPS (10 μ g) or *E. coli* LPS (10 μ g). 24 hours after immunization, (B) blood
 364 samples were collected to measure the number of lymphocytes ($n = 4$ per group). Data are
 365 representative of two independent experiments and analyzed by one-way ANOVA (* $p <$
 366 0.05; n.s.: not significant).

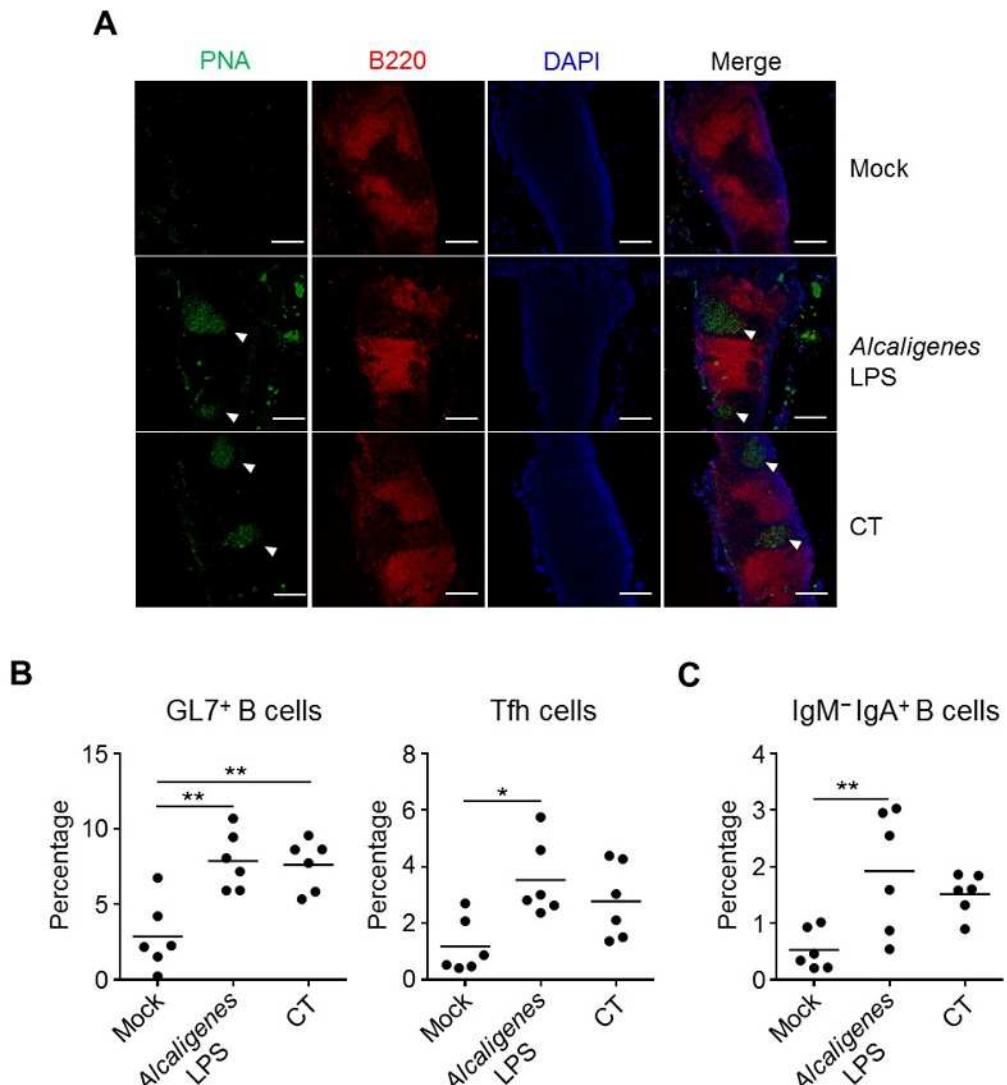
367 To examine OVA-specific IgA production in the nasal wash and BALF, mice
 368 were nasally immunized with OVA alone (Mock group), OVA plus 10 μ g of
 369 *Alcaligenes* LPS (*Alcaligenes* LPS group) or 1 μ g of CT (CT group). I found that
 370 the nasal wash and BALF from Mock group showed undetectable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

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



374
375 **Fig. 2.** *Alcaligenes* LPS promoted mucosal OVA-specific IgA antibody production upon
376 nasal immunization. Mice were nasally immunized 3 times with OVA alone (Mock) or with
377 OVA plus *Alcaligenes* LPS or CT; one week after the final immunization, Nasal wash and
378 BALF were collected to determine levels of OVA-specific IgA by ELISA ($n = 5$ or 6 per
379 group).

380 The production of IgA antibody is associated with B cell class-switch
381 recombination from IgM to IgA in the GC of NALT, which is supported by Tfh
382 cells. Immunohistological analysis revealed that Mock group did not induce the
383 formation of GC, while *Alcaligenes* LPS or CT group induced the formation of
384 GC in the NALT (Fig. 3A). Consistent with this finding, flow cytometry analysis
385 demonstrated that the percentage of GC GL7⁺ B cells increased in both
386 *Alcaligenes* LPS and CT groups when compared to Mock group (Fig. 3B). In
387 addition, the percentage of PD-1⁺ Tfh cells (Fig. 3B) and IgM⁻ IgA⁺ B cells (Fig.
388 3C) were significantly increased in the NALT from *Alcaligenes* LPS group
389 compared with that in Mock group. Together, these results indicate that
390 *Alcaligenes* LPS promoted the formation of GC in the NALT with Tfh cells and
391 IgA⁺ B cells for the subsequent IgA antibody production in the respiratory tract.



392

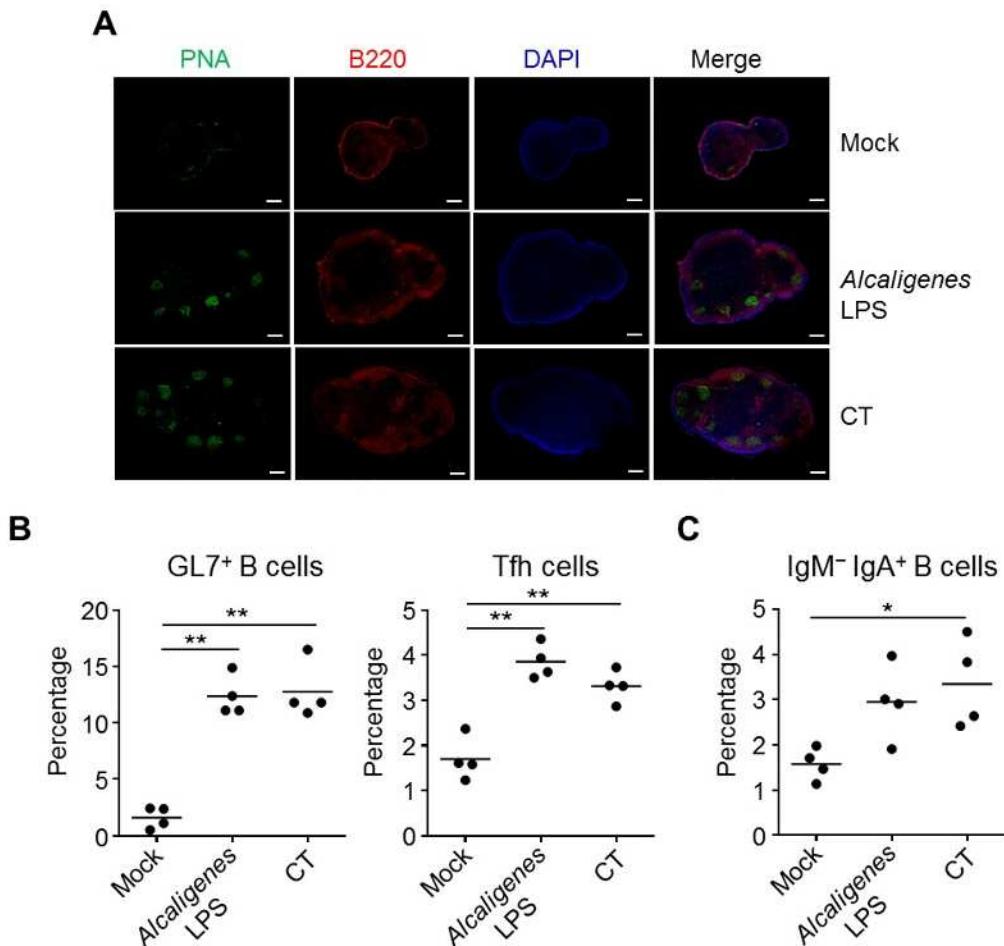
393 **Fig. 3. *Alcaligenes* LPS induced GC formation in NALT.** Mice were nasally immunized 3
 394 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT; one week after the
 395 final immunization, (A) formation of GC in NALT were observed by analysis of
 396 Immunohistochemistry. PNA: GC marker; B220: B cell marker; arrow heads: GC location.
 397 (B) Induction of GC GL7⁺ B cells (gated on: CD3 ϵ ⁻ B220⁺ GL7⁺) and Tfh cells (gated on:
 398 CD3 ϵ ⁺ CD8 α ⁻ CD4⁺ PD-1⁺) and (C) IgM⁻ IgA⁺ B cells (gated on: CD3 ϵ ⁻ B220⁺ GL7⁺ IgM⁻
 399 IgA⁺) in NALT were analyzed by flow cytometry analysis ($n = 6$ per group). Data are
 400 representative of two independent experiments and were analyzed by one-way ANOVA (* p
 401 < 0.05; ** p < 0.01).

402

403 **3.2. *Alcaligenes* LPS promoted systemic antibody responses**

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

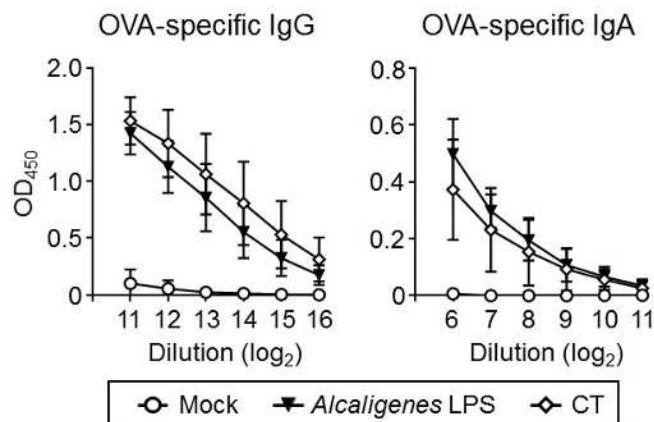
406 increased or higher induction of GL7⁺ B cells, Tfh cells, and IgM⁻ IgA⁺ B cells
 407 were detected in the CLNs from *Alcaligenes* LPS or CT group when compared
 408 to the Mock group (Fig. 4A–C).



409
 410 **Fig. 4.** *Alcaligenes* LPS induced GC formation in CLNs. Mice were nasally immunized 3
 411 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT; one week after the
 412 final immunization, (A) GC formation (PNA: GC marker; B220: B cell marker), and the
 413 induction of (B) GC GL7⁺ B cells (gated on: CD3 ϵ ⁻ B220⁺ GL7⁺) and Tfh cells (gated on:
 414 CD3 ϵ ⁺ CD8 α ⁻ CD4⁺ PD-1⁺) and (C) IgM⁻ IgA⁺ B cells (gated on: CD3 ϵ ⁻ B220⁺ GL7⁺ IgM⁻
 415 IgA⁺) in CLNs were examined by flow cytometry analysis. ($n = 4$ per group). Data are
 416 representative of two independent experiments and were analyzed by one-way ANOVA (* p
 417 < 0.05; ** p < 0.01).

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

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



426
427 **Fig. 5.** *Alcaligenes* LPS promoted systemic antibody responses. Mice were nasally
428 immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT; one
429 week after the final immunization, serum was collected to determine OVA-specific IgG and
430 IgA by ELISA ($n = 5$ per group). Data are representative of two independent experiments.

431

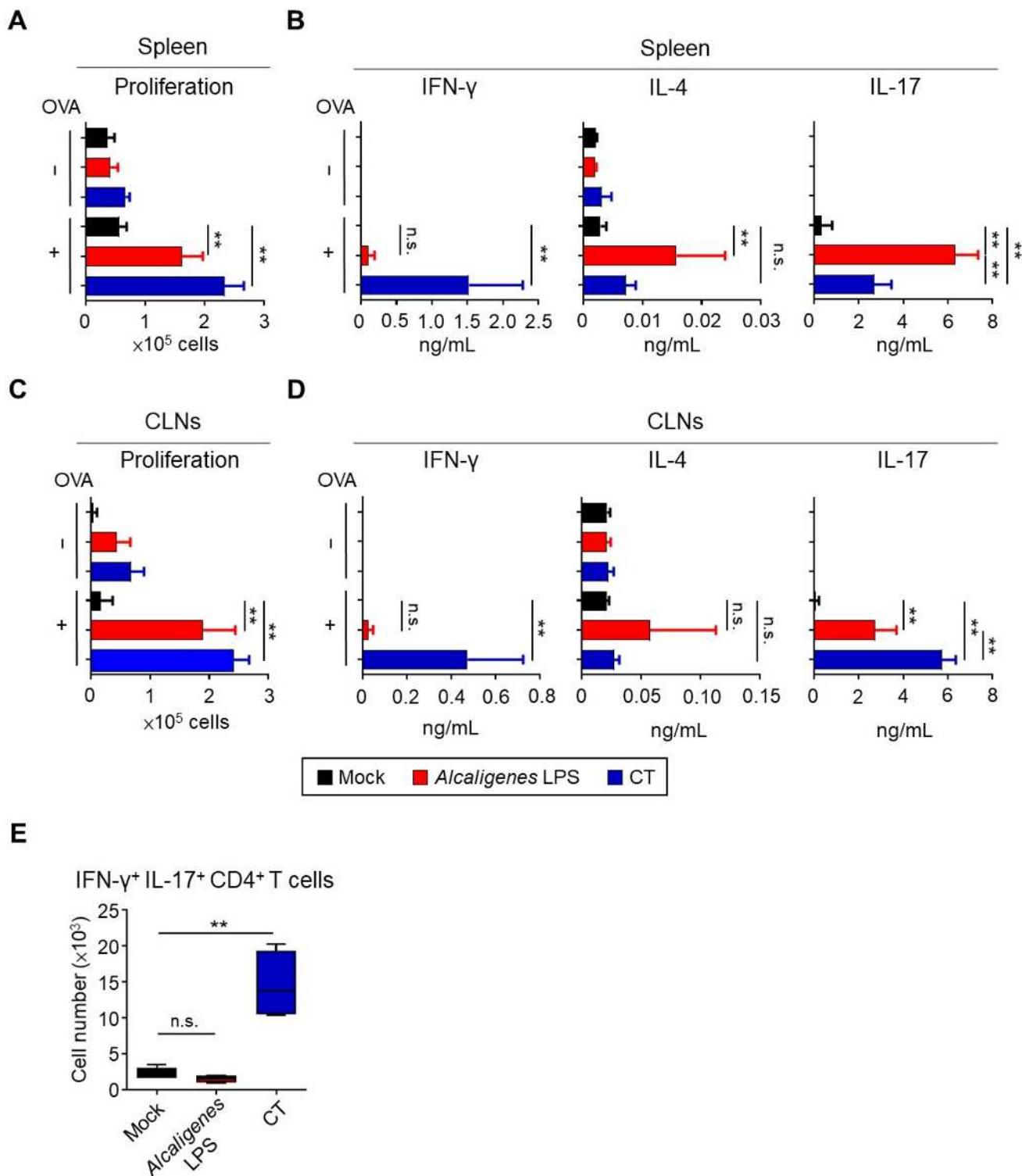
432 3.3. *Alcaligenes* LPS promoted an OVA-specific Th17 cell response

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

440 Next, I examined the production of cytokines from OVA-specific CD4⁺ T cells,
441 especially related to the Th1 (IFN- γ), Th2 (IL-4), and Th17 (IL-17). Consistent
442 with low OVA-induced CD4⁺ T cell proliferation activity of the Mock group from
443 spleen and CLNs, it was noted that the production of cytokines in Mock group
444 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

465 determined by CyQUANT® Cell Proliferation Assays Kits and fluorescence microplate
466 reader, ARVO X2 with measuring at 485/535 nm. (B) Production of cytokines: IFN- γ , IL-4
467 and IL-17 in the supernatant of splenic CD4 $^{+}$ T cell culture was collected and measured
468 by the CBA kit. (C) Proliferation activity of CLNs CD4 $^{+}$ T cells. (D) Production of
469 cytokines: IFN- γ , IL-4 and IL-17 in the supernatant of CLNs CD4 $^{+}$ T cell culture. (E)
470 Number of IFN- γ $^{+}$ IL-17 $^{+}$ CD4 $^{+}$ T cells (gated on: zombie- CD45 $^{+}$ TCR- β $^{+}$ CD4 $^{+}$ IFN- γ $^{+}$ IL-
471 17 $^{+}$) in mice spleen were analyzed by intracellular flow cytometry analysis ($n = 4$ or 6 per
472 group). Data are representative of two independent experiments and were analyzed by
473 one-way ANOVA (** $p < 0.01$; n.s.: not significant).

474

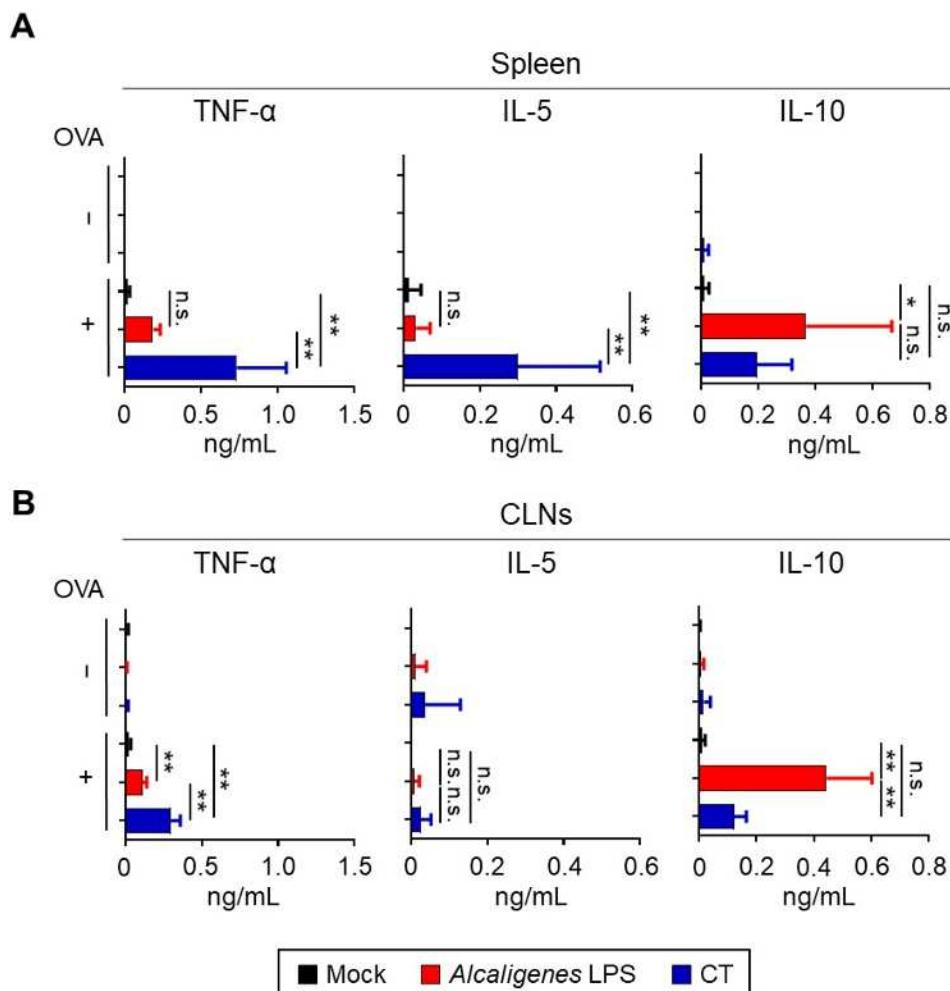
475 **3.4. *Alcaligenes* LPS had low Inflammatory but high regulatory properties**

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

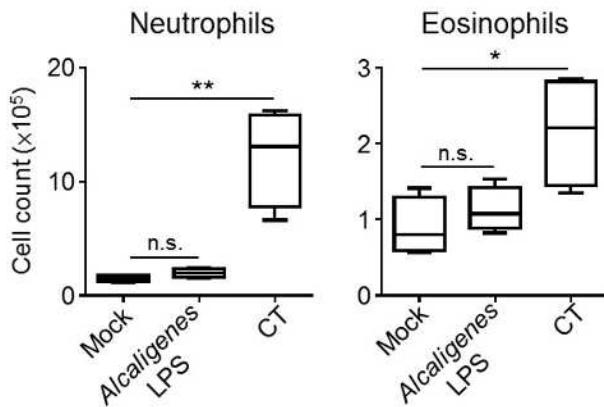
485 The results revealed that CT group significantly induced TNF- α and IL-5
486 production in the supernatant of splenic CD4 $^{+}$ T cell culture and significantly
487 induced TNF- α production from CLNs CD4 $^{+}$ T cell culture, but not the Mock and
488 *Alcaligenes* LPS groups. It is interesting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

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

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



499
 500 **Fig. 7.** *Alcaligenes* LPS had low inflammatory but high regulatory properties. Mice were
 501 nasally immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT;
 502 one week after the final immunization, the production of cytokines: TNF- α , IL-5, and IL-10
 503 in the supernatant of (A) splenic CD4 $^{+}$ T cell culture and of the (B) CLNs CD4 $^{+}$ T cell culture
 504 were surveyed after *ex vivo* stimulation by OVA ($n = 6$ per group). Data are representative
 505 of two independent experiments and were analyzed by one-way ANOVA (* $p < 0.05$; ** $p <$
 506 0.01; n.s.: not significant).



507

508 **Fig. 8.** *Alcaligenes* LPS induced little inflammation at the site of administration. Mice were
 509 nasally immunized 3 times with OVA alone (Mock) or with OVA plus *Alcaligenes* LPS or CT;
 510 one week after the final immunization, the number of neutrophils (gated on: 7AAD⁻ CD45⁺
 511 CD11c⁺ Ly6G⁺) and eosinophils (gated on: 7AAD⁻ CD45⁺ CD11c⁺ Siglec-F⁺) in the nasal
 512 passage were determined by flow cytometry analysis ($n = 4$ per group). Data are
 513 representative of two independent experiments and analyzed by one-way ANOVA (* $p <$
 514 0.05; ** $p < 0.01$; n.s.: not significant).

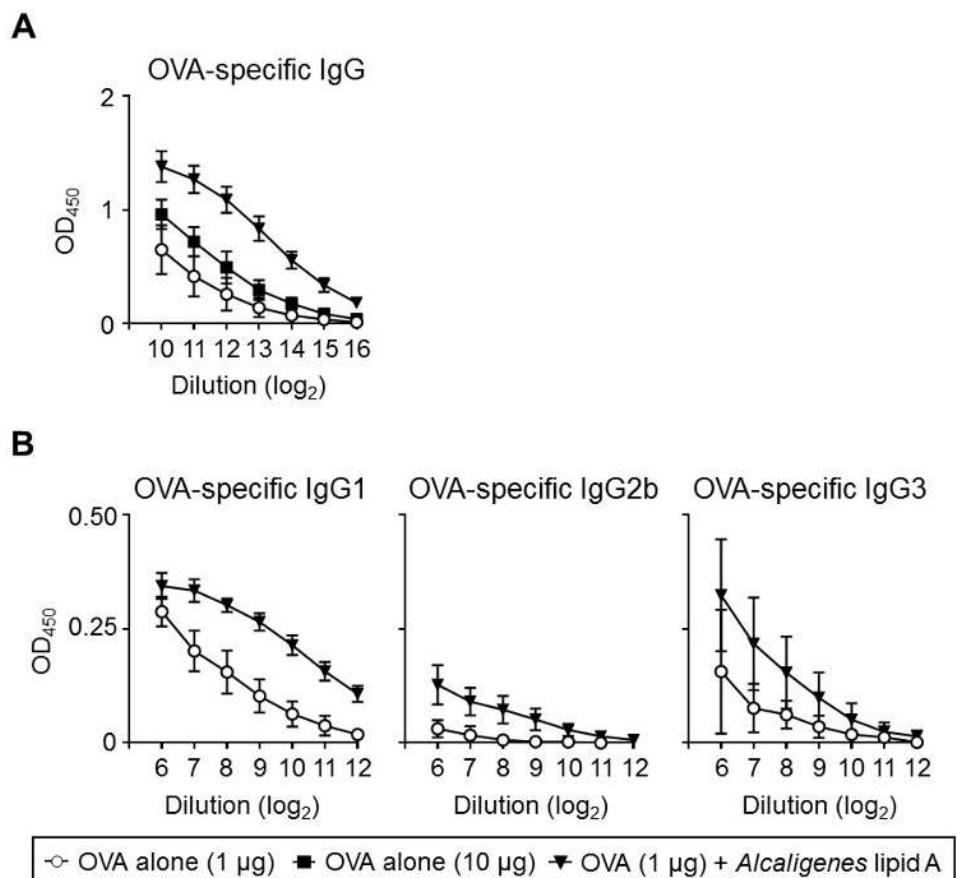
515

516 **3.5. Subcutaneously co-administered *Alcaligenes* lipid A promoted
 517 systemic antibody responses**

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

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

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



537

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

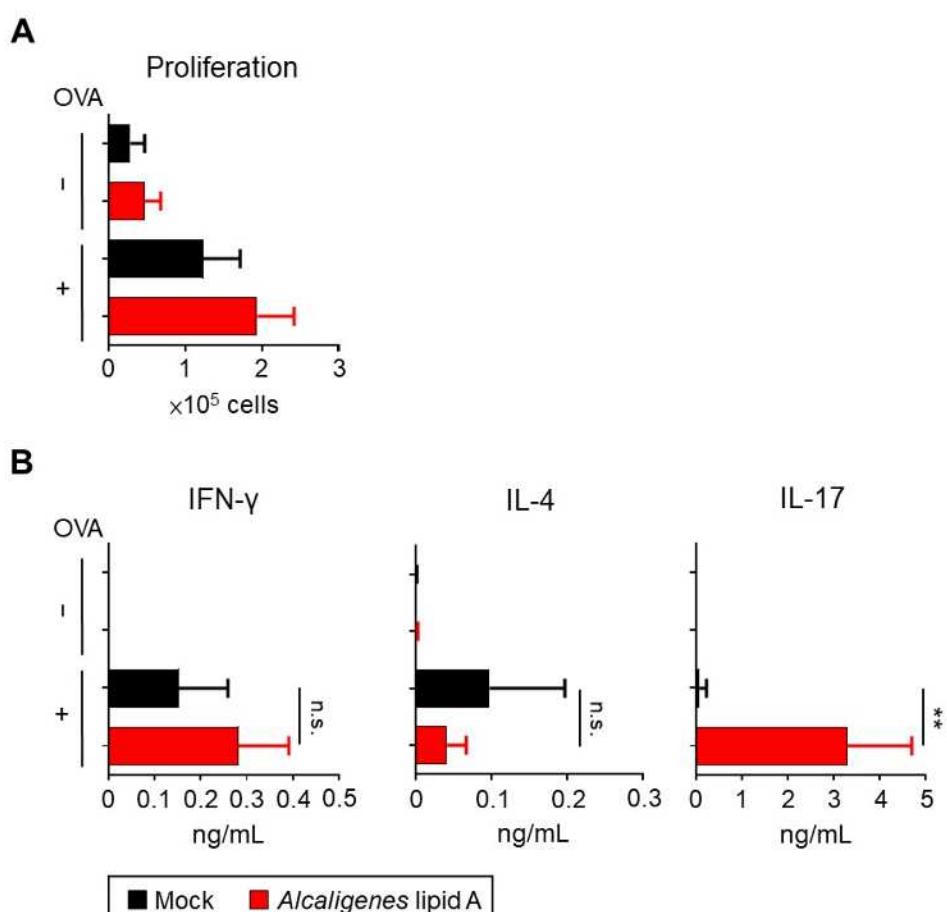
543

544 **3.6. *Alcaligenes* lipid A promoted an OVA-specific Th17 cell responses**

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

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

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



558
559 **Fig. 10.** *Alcaligenes* lipid A promoted OVA-specific Th17 cell responses. Mice were
560 subcutaneously immunized 2 times with OVA plus *Alcaligenes* lipid A; one week after the
561 final immunization, splenic CD4⁺ T cells were collected and stimulated with OVA by *ex vivo*.
562 (A) Proliferation activity of splenic CD4⁺ T cells were determined by CyQUANT® Cell

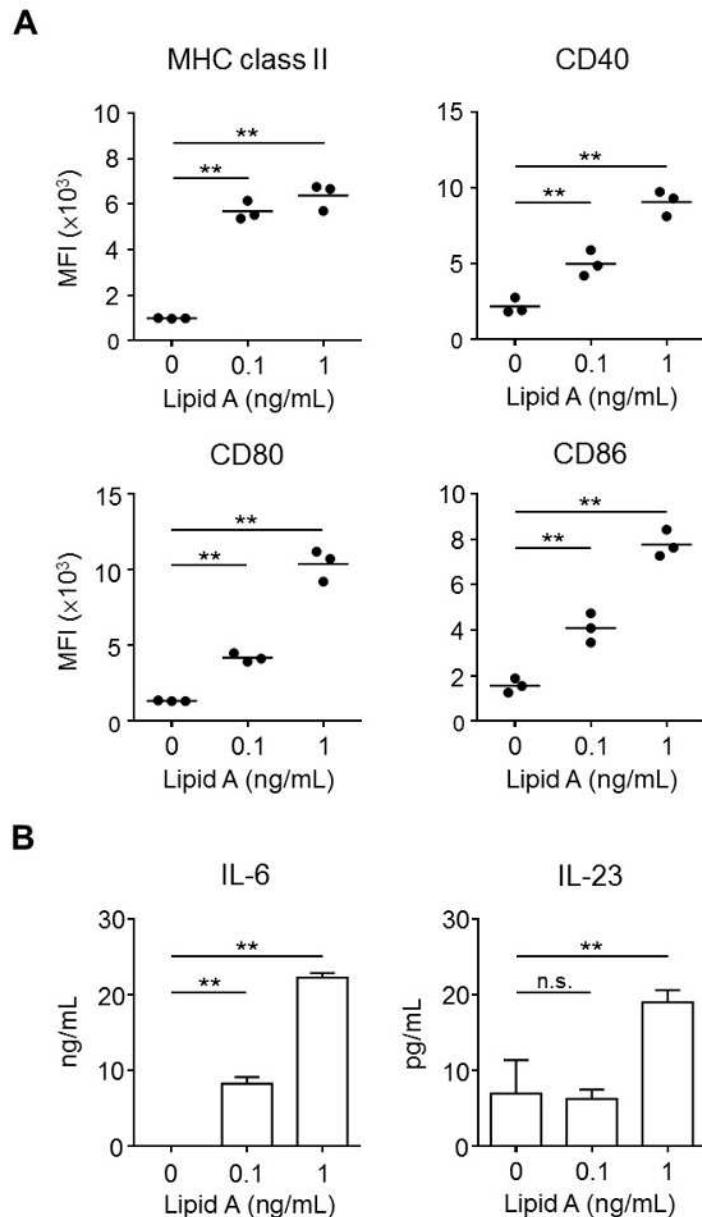
563 Proliferation Assays Kits and fluorescence microplate reader, ARVO X2 with measuring at
564 485/535 nm. (B) Production of cytokines: IFN- γ , IL-4 and IL-17 in the supernatant of splenic
565 CD4 $^{+}$ T cell culture was collected and measured by the CBA kit ($n = 6$ per group). Data are
566 combined of two independent experiments and analyzed by one-way ANOVA (** $p < 0.01$;
567 n.s.: not significant).

568

569 **3.7. *Alcaligenes* lipid A induced BMDCs activation**

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

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



583

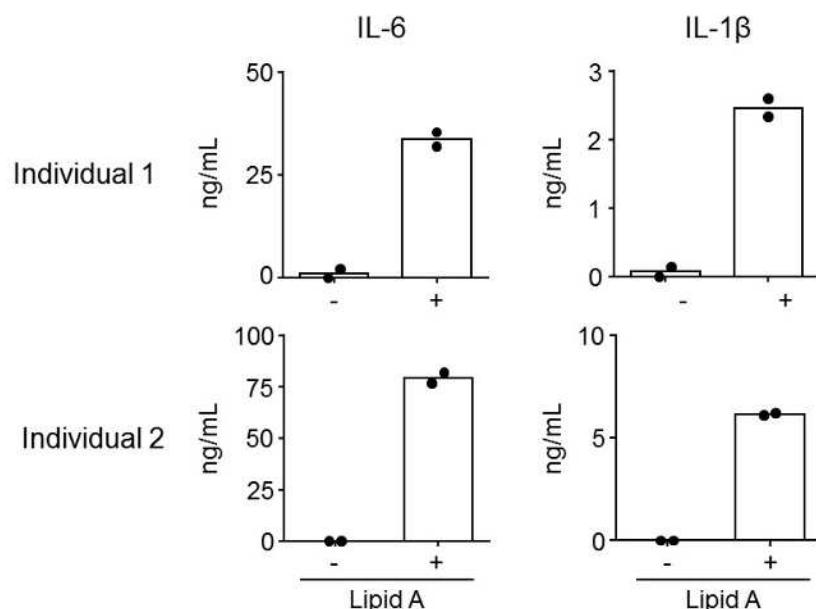
584 **Fig. 11.** *Alcaligenes* lipid A activated BMDCs. BMDCs were stimulated with *Alcaligenes*
 585 lipid A at the concentration of 0.1 and 1 ng/mL. After incubation for 48 hours, (A) expression
 586 of MHCII, CD80, CD86 and CD40 were analyzed by flow cytometry analysis. (B)
 587 Production of cytokines: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

590

591 3.8. *Alcaligenes* lipid A activated human PBMCs

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

594 2, stimulation of 1 ng/mL with *Alcaligenes* Lipid A increased levels of IL-6 and
595 IL-1 β (Fig. 12). These results indicated that *Alcaligenes* lipid A could activate
596 human PBMCs. Furthermore, since IL-6 and IL-1 β were required for Th17 cell-
597 differentiation in human, these results also suggest that *Alcaligenes* lipid A
598 could induce antigen-specific Th17 cell responses in human.



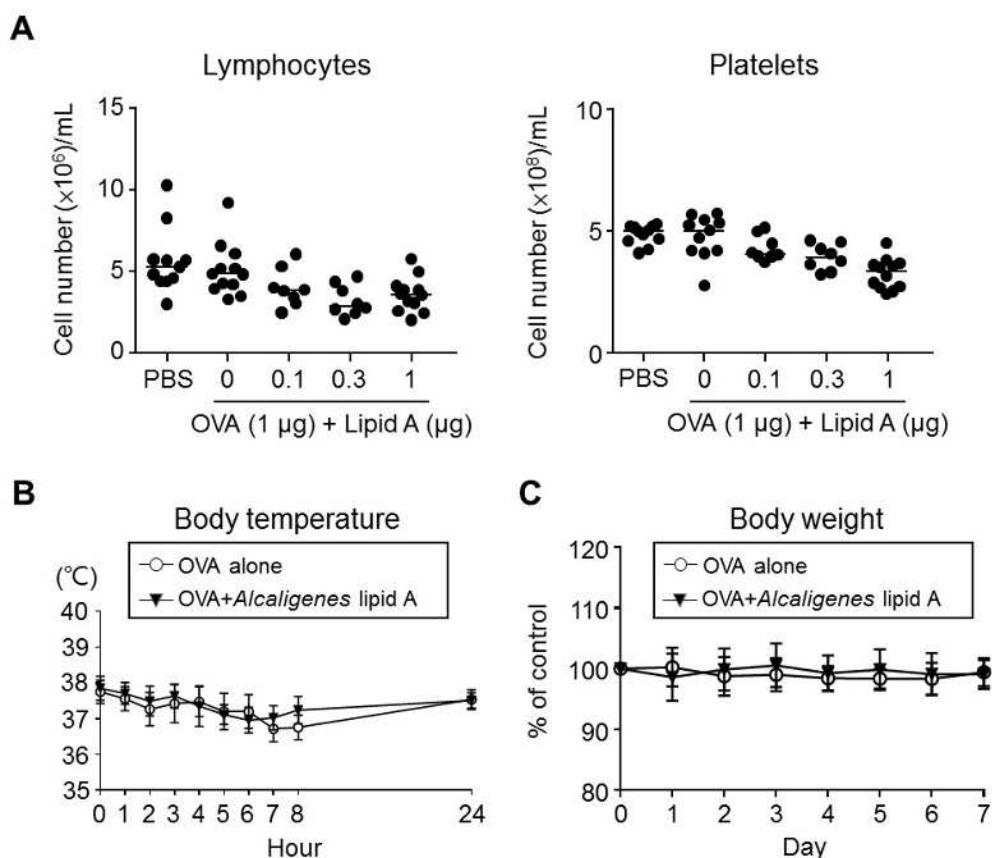
599
600 **Fig. 12.** *Alcaligenes* lipid A activated PBMCs. Human PBMCs from two independent
601 individuals were stimulated with *Alcaligenes* lipid A at the concentration of 1 ng/mL. After
602 incubation for 24 hours, production of cytokines: IL-6 and IL-1 β in the supernatant of
603 PBMCs were collected and measured by the CBA kit and the ELISA kit.

604

605 **3.9. *Alcaligenes* lipid A showed no severe side effects.**

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

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



623
 624 **Fig. 13.** *Alcaligenes* lipid A showed no severe side effects. Mice were subcutaneously
 625 immunized with OVA plus *Alcaligenes* lipid A. (A) The number of lymphocytes and platelets
 626 were measured in blood after 24 hours from immunization. (B) Body temperature at 0, 1st
 627 to 8th and 24th hour and (C) body weight in 7 days after the immunization were
 628 continuously monitored ($n = 4$ per group). Data are representative of two independent
 629 experiments.

630

631 **4. Discussion**

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

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

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

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

675 To understand the mechanism for the induction of Th17 cell responses and
676 the serum antibody production by *Alcaligenes* LPS and lipid A, I examined the
677 effects of *Alcaligenes* lipid A on DCs in this study. DCs present antigen to T cells
678 via MHC class II and costimulatory molecules, such as CD80 and CD86
679 (47,48), which are required for T cell activation, including T cell proliferation and
680 differentiation. In this regard, *Alcaligenes* lipid A enhances expression of MHCII,
681 CD80 and CD86 from BMDCs in this study. Moreover, differentiation of T cells
682 is determined by differences in cytokine environment produced from antigen-
683 presenting cells, including DCs. For example, transforming growth factor beta
684 (TGF- β) and IL-6 promote the differentiation of Th17 cells, and IL-23 promotes
685 the stabilization of Th17 cells in mice (49–51). Consistent with our previous
686 reports about *Alcaligenes*-mediated activation of DCs (4,5,7), *Alcaligenes* lipid
687 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-1 β , 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-1 β , 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

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

720 Regarding the immunological property of *Alcaligenes* LPS, our previous
721 studies indicated that *Alcaligenes* LPS has little cytotoxic activity. Indeed,
722 compared with *E. coli* LPS, *Alcaligenes* LPS showed lower endotoxin activity in
723 the limulus amebocyte lysate test and caused only limited inflammatory
724 reactions when intraperitoneally injected into mice, including lower levels of
725 serum IL-6, less change in body temperature, and less damage to lung tissue
726 with little infiltration of inflammatory cells such as neutrophils and eosinophils
727 (7).

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

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

745 As biochemical characteristics, the structure of LPS is mainly composed of
746 lipid A, core oligosaccharide, and O-antigens. Lipid A is the active center of LPS
747 and acts as an agonist of TLR4/MD-2 complex. The activity as a TLR4 agonist
748 is determined by several feature of lipid A structure. As for lipid A component in
749 *Alcaligenes* LPS, a mixture of tetra- to hexa-acylated species was identified,
750 and the lipid A with hexa-acylated species was composed of a
751 bisphosphorylated glucosamine disaccharide backbone carrying 14:0 (3-OH)
752 as primary and 12:0 (3-OH) and 10:0 as secondary fatty acids with distribution
753 in a 3 + 3 fashion with respect to the disaccharide backbone, which were
754 different with *E. coli* LPS whose lipid A has 4 + 2 symmetry and is composed of
755 14:0 (3-OH) as primary and 14:0 and 12:0 as secondary fatty acids (22,59).

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

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

771

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780

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