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The mechanism of *Alcaligenes* Lipid A as a potent mucosal adjuvant

粘膜アジュバントとしてのアルカリゲネス菌リピド A の作用機序の解明

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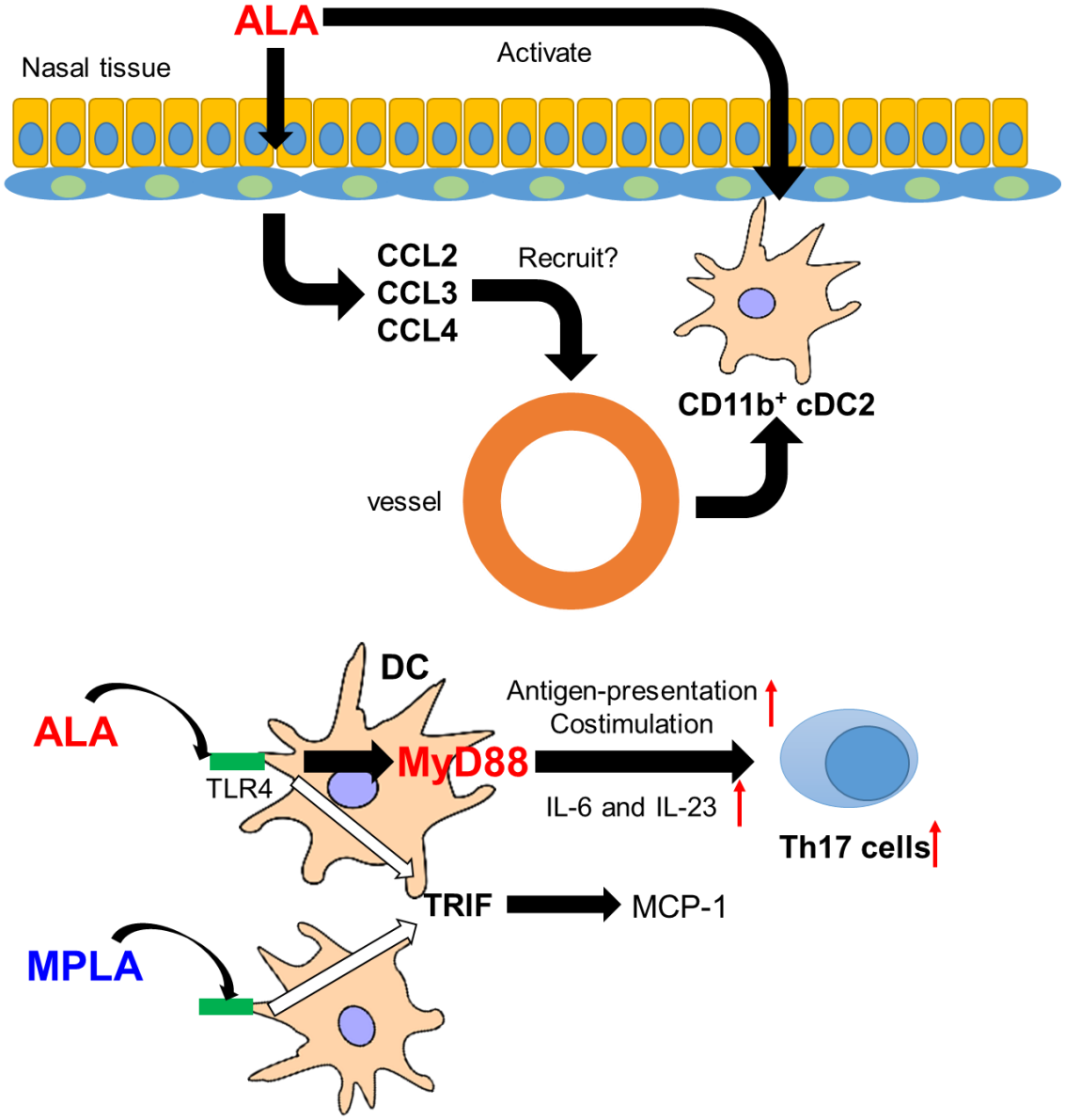
Abstract

Alcaligenes faecalis was previously identified as an intestinal lymphoid tissue-resident commensal bacteria, and our subsequent studies showed that lipopolysaccharide and its core active element (i.e., lipid A) have a potent adjuvant activity to promote preferentially antigen-specific Th17 response and antibody production. Here, I compared *A. faecalis* lipid A (ALA) with Salmonella-derived modified monophosphoryl lipid A (MPLA), a licensed lipid A–based adjuvant, to elucidate the immunological mechanism underlying the adjuvant properties of ALA. Compared with MPLA, ALA induced a higher level of antigen-specific antibody secretion in subcutaneous and intranasal administration. especially, superior in intranasal vaccination. Meanwhile, ALA showed enough safety, which is similar to MPLA. As an explanation of the better adjuvanticity of ALA than MPLA, ALA induced higher levels of MHC class II molecules and costimulatory CD40, CD80, and CD86 on dendritic cells (DCs), which in turn resulted in strong T cell activation. Moreover, ALA more effectively promoted the production of IL-6 and IL-23 from DCs than did MPLA, thus leading to preferential induction of Th17 cells. As underlying mechanisms, I found that the ALA–TLR4 axis stimulated both MyD88- and TRIF-mediated signaling pathways, whereas MPLA was biased toward TRIF signaling. These findings revealed the effects of ALA on DCs and T cells and its induction pattern on signaling pathways. Related to the adjuvanticity of nasal vaccine, ALA recruited activated $CD11b^{+}$ cDC2 into nasal tissues through the promoted production of chemokines (such as CCL2, CCL3, and CCL4). These results

reveal the uniqueness and superiority of ALA as a systemic and nasal adjuvant from both in vivo and in vitro perspectives.



Keywords: *Alcaligenes*; lipid A; MPLA; MyD88; TRIF; Th17; mucosal vaccine; cDC2

1. Introduction

Currently, most vaccines are administrated by subcutaneous or intramuscular, which induce potent systemic immunity [1]. However, some systemic and local side effects will occur, and only weak mucosal immunity is observed in these vaccines [2]. Since the mucus is the first line barrier against the external environment, mucosal immunity induced by mucosal vaccines is superior to systemic vaccines in the protection from some specific pathogens [3], such as influenza [4], COVID-19 [5], and HIV [6]. Furthermore, a mucosal vaccine can induce not only mucosal immunity but also systemic immunity, while almost none of the systemic side effects are raised [3]. These properties of mucosal vaccines make them more advantageous than the other administration types of vaccine. Currently, eight kinds of oral vaccines are licensed for the prevention of cholera, salmonella, poliovirus, and rotavirus, and one kind of intranasal vaccine against influenza is approved for clinical use [7] (Table 1). Additionally, several mucosal vaccines are in development or clinical trials [8], [9].

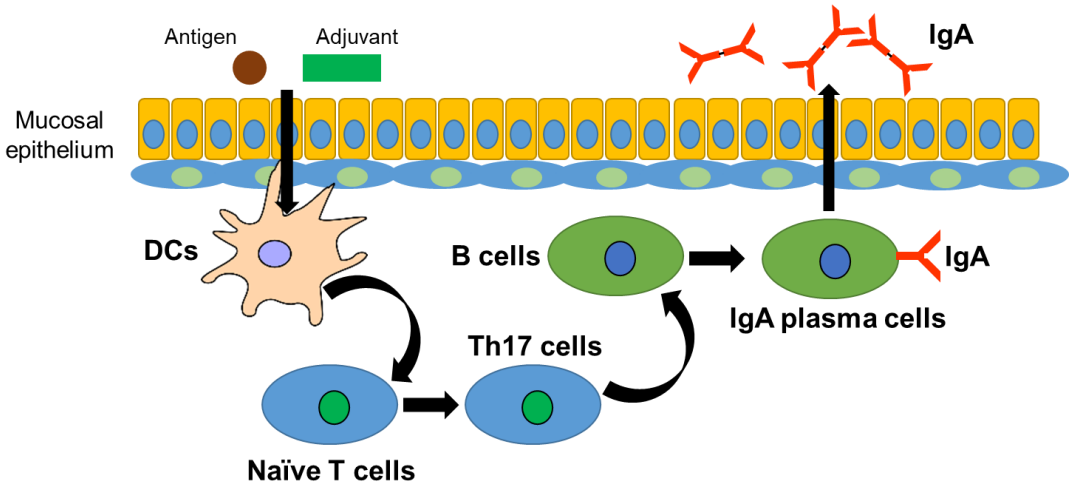


Figure 1. The immunity response induced by mucosal vaccines.

In the process of vaccination, the adjuvant usually activates the antigen presentation cells (APCs) to recognize antigens and activate the adaptive immunity for an antigen-specific response. This response not only occurred in lymphoid tissue but also in non-lymphoid tissue, such as mucus [10]. In the mucosal vaccination, the effect of adjuvant on the dendritic cells (DCs) resident in mucosal tissue, shows different phenotypes and responses toward adjuvant and antigen from the DCs in lymphoid tissue [11], [12]. The mucosal DCs are also affected by the other kinds of cells in mucosal tissue [13]. Furthermore, the response of Th17 cells is specifically indispensable in mucosal immunity and the class-switch and secretion of antigen-specific IgA on mucus [14]. These unique properties of mucosal immunity suggest the potential of the Th17 cell-response adjuvant to utilize in a mucosal vaccine. However, currently, there is no licensed Th17 cell-response adjuvant.

Table 1. Licensed mucosal vaccines

Pathogen	Trade name	Composition	Dosage
Rotavirus	Rotarix	Monovalent: culture passage attenuated	Oral aqueous, 2 doses
	Rotateq	Pentavalent: five human-bovine reassortant rotaviruses	Oral aqueous, 3 doses
Poliovirus	Biopolio (bOPV)	Culture passage attenuated polioviruses 1 and 3 serotypes	Oral aqueous, 3 doses
	mOPV and tOPV	Culture passage attenuated polioviruses 1, 2, and 3 serotypes	Oral aqueous, 3 doses
Salmonella Typhi	Typhi Vivotif	Live attenuated Ty21a strain, mutagenesis in LPS synthesis, and Vi polysaccharide genes	Oral capsule, 4 doses
Vibrio cholera	Dukoral	Heat and formaldehyde-inactivated O1 serogroups + CTB	Oral aqueous, 2 doses
	Euvichol, Shanchol	Heat and formaldehyde-inactivated O1 serogroups + 0139	Oral aqueous, 2 doses
	Vaxchora	Live attenuated 01 serogroup – ctxA attenuation	Oral aqueous, 1 dose
Influenza type A and B virus	FluMist/Fluenz	Quadrivalent antigens from circulating strains incorporated into live attenuated, cold-adapted donor influenza vector	Nasal spray, 1 dose

In the innate immune system, pathogen-associated molecular patterns (PAMPs) activate DCs through PRRs on their surface. These factors activate innate lymphoid cells through specific receptors to facilitate antigen presentation and consequently efficiently induce adaptive immunity. For example, CpG oligodeoxynucleotide (CpG-ODN) is a toll-like receptor 9 (TLR9) ligand, and the monophosphoryl lipid A from *Salmonella minnesota* R595 (MPL[R]) is a TLR4 ligand. This activation typically is associated with the subsequently increased expression of several molecules, such as

major histocompatibility complex (MHC) molecules for antigen presentation and various costimulatory molecules (i.e., CD40, CD80, and CD86). Activated DCs then prime T cells, thus instructing them for their differentiation into antigen-specific effector T cells, one of the most important parts of adaptive immunity [15]. Therefore, PAMPs and their related molecules are considered to be promising vaccine adjuvants for activating innate and adaptive immunity to enhance vaccine-induced immunity [16]. One component of the cell membrane of gram-negative bacteria is the well-known PAMP lipopolysaccharide (LPS), which can activate DCs and thus lead to the induction and enhancement of antigen-specific immune responses [17]. The active part of LPS, lipid A is responsible for the activity of LPS by binding to its receptor, TLR4 [18], [19]. However, because pathogen-derived LPS and lipid A cause severe toxicity in humans, including liver damage, fever, and septic shock, these agents are unsuitable as vaccine adjuvants [20], [21]. In one solution to this problem, chemical modification of lipid A from *Salmonella minnesota* R595 led to the derivative monophosphoryl lipid A (MPLA) [22], which was shown to be a safe adjuvant that led to induction of a T helper type 1 (Th1) response [23]. Currently, MPLA is widely used as an adjuvant in the human papillomavirus vaccine Cervarix [24] and the hepatitis B virus vaccine Fendrix [25]. MPLA is also an efficacious adjuvant in vaccines against rabies and leishmaniasis [26], [27], for which it induces potent antigen-specific Th1 responses.

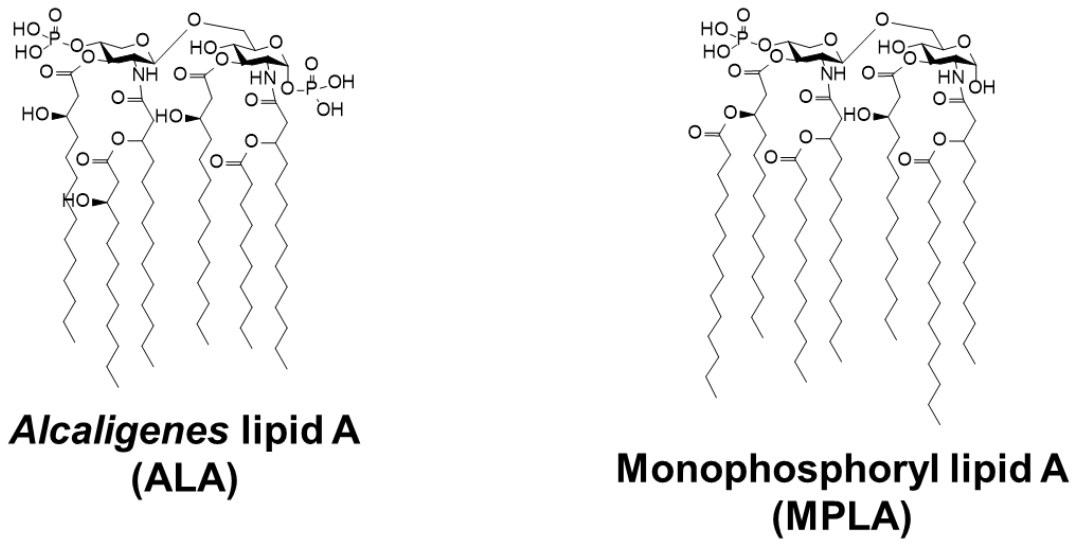


Figure 2. The molecular structure of *Alcaligenes* lipid A (ALA) and monophosphoryl lipid A (MPLA).

Unlike LPS and lipid A derived from pathogens, LPS from commensal bacteria is applicable as a vaccine adjuvant. As we reported previously, *Alcaligenes* spp. is a gut tissue–associated commensal bacteria that resides in Peyer's patches [28]. Although this symbiotic relationship of *Alcaligenes* and DCs in Peyer's patches did not induce pathogenic inflammation, *Alcaligenes* can activate the host immune system and enhance antigen-specific immune responses; this unique immunological phenomenon was achieved through the weak agonistic activity of *Alcaligenes* LPS on TLR4 [29], [30]. Through related experiments designed to explain the mild activity of *Alcaligenes* LPS, we recently revealed the unique molecular structure of its lipid A [31], [32] and the potential of *Alcaligenes* lipid A (ALA) as a promising candidate adjuvant. Indeed, ALA showed safe, potent adjuvanticity during intranasal and subcutaneous co-administration

with antigen in murine models [33], [34], [35]. In our evaluation of the adjuvanticity of ALA as a systemic adjuvant with the model antigen of ovalbumin (OVA), ALA induced OVA-specific IgG antibody production and preferentially enhanced OVA-specific Th17 responses over Th1/2 responses [33]. In addition, ALA was an effective mucosal adjuvant when it was nasally administered with pneumococcal surface protein A (PspA), a universal vaccine antigen of *Streptococcus pneumonia* [34]. ALA enhanced PspA-specific nasal IgA antibody production and a PspA-specific Th17 response, which was protective against *Streptococcus pneumoniae* infection [34].

Whereas the above, ALA had the potential as a mucosal adjuvant according to the induction of antigen-specific Th17 response, but not MPLA. Moreover, the mechanism of the differences between ALA and MPLA is not clear. One possibility is the unique effect of ALA on the DCs in mucosal tissue. Therefore, in the current study, I focused on the ability of ALA to activate DCs and on the interaction between DCs and T cells, and the specific effect of ALA on the DCs in nasal tissue to reveal its mechanism of action as a mucosal adjuvant.

2. Materials and Methods

2.1 Mice

BALB/cAJcl mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). C57BL/6JJmsSlc mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). $TLR4^{-/-}$ and $TLR4^{+/-}$ mice were generated on a C57BL/6 background, as previously described [36]. $MyD88^{-/-}$ and $MyD88^{+/+}$ mice were generated on a C57BL/6 background, as previously described [37]. Mice were raised under specific pathogen-free conditions at the National Institutes of Biomedical Innovation, Health, and Nutrition (Osaka, Japan). All experiments were approved by the Animal Care and Use Committee of the National Institutes of Biomedical Innovation, Health, and Nutrition and were conducted following their guidelines.

2.2 Lipid A

Alcaligenes lipid A was chemically synthesized as previously described [31]. monophosphoryl lipid A from *Salmonella minnesota* R595 (MPLA) was purchased from InvivoGen (San Diego, CA). Both were reconstituted in dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan) at a concentration of 1 mg/mL and stored at –30ºC.

2.3 Vaccination

For the intranasal vaccination, mice were immunized with 5 μg of OVA with or without 1 μg of *Alcaligenes* Lipid A or MPLA in 15 μL of PBS on day 0, 7, and 14. One

week after the last immunization, mice were sampled for serum, nasal wash fluid, and bronchoalveolar lavage fluid (BALF) for OVA-specific IgA and IgG ELISA measurement. For the subcutaneous vaccination, 0.1 μg OVA with or without 1μg of *Alcaligenes* Lipid A or MPLA in 200 μL of PBS were injected on day 0 and 10. One week after the last immunization, mice were sampled for the serum for OVA-specific IgG ELISA.

2.4 Intranasal safety of ALA

Mice were immunized with PBS as control, or 5 μg of OVA with 1 μg of *Alcaligenes* Lipid A or MPLA in 15 μL of PBS on day 0, 7, 14, and 21. On day 25, mice were sampled for serum and dissected and fixed in 37% formalin solution. The body weight was measured on day 0, 1, 7, 8, 14, 15, 21, 22, and 28. Body temperature was measured after the first and third administration every 2 h. The blood lymphocytes number were calculated with VetScan HM2 on day 0, 1, 14, 15, and 25.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

The flat-bottom 96-well immunoplates (Thermo Fisher Scientific Inc., Waltham, MA, USA) were coated with 1 mg/ml of OVA diluted in PBS at 4°C overnight. 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 three times with PBS containing 0.05% (v/v) Tween 20 (Nacalai Tesque, Inc.). Samples were serially diluted with 1% (w/v) BSA, containing 0.05% (v/v) Tween

20 in PBS, and added to the plates, following with the incubation for 2 h at room temperature. After washing with PBS containing 0.05% Tween 20 for three times, horseradish peroxidase-conjugated goat anti-mouse IgG or IgA (Southern Biotech, Inc., Birmingham, AL, USA) diluted with 1% (w/v) BSA, containing 0.05% (v/v) Tween 20 in PBS was added to the plates and left to react for 1 h at room temperature. After washing with PBS containing 0.05% Tween 20 for three times, tetramethylbenzidine peroxidase substrate (SeraCare Life Sciences Inc., Milford, MA, USA) was then added to react for 2 min at room temperature; 0.5 N HCl (Nacalai Tesque, Inc.) was added to stop the reaction. Absorbance was measured at 450 nm by the iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.6 Generation of BMDCs

Bone marrow cells were flushed from femurs and tibias collected from BALB/c or C57BL/6 mice, treated with red blood cell lysis buffer (1.5 M NH_4Cl, 100 mM $KHCO_3$, and 10 mM EDTA-2Na [all from Nacalai Tesque]) for 1 min and washed with complete RPMI-1640 medium (GIBCO, Invitrogen, Waltham, MA), which contained 10% fetal bovine serum, 1% penicillin-streptomycin (Nacalai Tesque), 50 μM 2-mercaptoethanol (GIBCO), and 1% 100 mM sodium pyruvate solution (100×; Nacalai Tesque). Cells were resuspended and cultured in the complete RPMI-1640 medium supplemented with 20 ng/mL GM-CSF (PEPROTECH, East Windsor, NJ). The culture medium was changed every 2 days. Cells were collected on day 6 or 7 for the isolation of BMDCs by

using a MACS separator (Miltenyi Biotec, Auburn, CA) and CD11c Microbeads UltraPure (Miltenyi Biotec) according to the manufacturer's instructions. The $CD11c^+$ BMDCs were used for subsequent experiments.

2.7 Detection of cytokines produced by BMDCs and cell-surface markers on BMDCs

For the analysis of cytokine production, 1×10^5 cells/well BMDCs were seeded in 96-well plates (ThermoFisher, Waltham, MA) and treated with various concentrations of ALA or MPLA for 48 h, after which the supernatants were collected for analysis. IL-6, MCP-1, and IL-12 were measured by using CBA Mouse Inflammation Kit (BD Biosciences. Franklin Lakes, NJ) according to the manufacturer's instructions. IL-23 was measured by using the IL-23 Mouse Uncoated ELISA Kit (Invitrogen) according to the manufacturer's instructions.

For the analysis of cell-surface markers, BMDCs were detached from the plates and stained with 5 μg/mL anti-CD16/32 antibody (Fc Block, clone 93; BioLegend, San Diego, CA) and 7-AAD Viability Staining Solution (BioLegend) for 15 min at room temperature. Cells were then stained with BV420-anti-CD11c (BioLegend; clone N418; dilution 1:100), FITC-anti-I-Ad (BioLegend; clone 39-10-8; dilution 1:100), PE-anti-CD80 (BioLegend; clone 16-10A1; dilution 1:100), PE-Cy7-anti-CD40 (BioLegend; clone 3/23; dilution 1:100), APC-anti-CD86 (BioLegend; clone GL-1; dilution 1:100), and APC-Cy7-anti-CD11b (BioLegend; clone M1/70; dilution 1:100) for 30 min on ice.

Samples were analyzed by using a MACSQuant Analyzer (Miltenyi Biotec). Flow cytometry data were analyzed by using FlowJo software version 10.2 (BD Biosciences).

For inhibitor experiments, BMDCs were cultured for 48 h in the presence of 10 ng/mL ALA or MPLA with or without the addition of takinib (Sigma-Aldrich) or GSK8612 (Sigma-Aldrich) in 2 μM. The supernatants and BMDCs were collected and analyzed as described earlier.

2.8 Antigen processing and presentation assay

OT-IIZ cells, provided by Dr. N. Shastri (University of California, Berkeley, CA), were cultured in the complete RPMI-1640 medium. BMDCs from C57BL/6 were used as antigen-presenting cells. BMDCs were first pulsed overnight in 0.1 mg/mL OVA (Sigma-Aldrich, St Louis, MO), followed by treatment with ALA or MPLA for 24 h. Then, BMDCs were co-cultured with OT-IIZ cells at a 1:1 ratio for 24 h. To assess β-galactosidase production stimulated through antigen presentation, cells were incubated in CPRG buffer (0.6 mM CPRG [Sigma-Aldrich], 1 mM $MgCl_2$ [Nacalai Tesque], 100 mM 2-mercaptoethanol [Sigma-Aldrich], and 0.125% Nonidet P-40 [Nacalai Tesque] in PBS) for 3 h at 37ºC. The reaction between CPRG and β-galactosidase was measured at 570 nm (reference, 655 nm).

2.9 Cocultures of BMDCs and T cells

First, 96-well plates were coated with 0.5 μg/mL anti-CD3ε antibody (Biolegend)

at 4ºC overnight and washed 3 times with PBS. Naïve $CD4^+$ T cells were isolated by using Naïve $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec) and MACS separator (Miltenyi Biotec) according to the manufacturer's instructions. For the T cell activation and proliferation assay, naïve $CD4^+$ T cells were stained with CFSE (Sigma-Aldrich) before being seeded in the coated plate wells in 1×10^5 cells/well. ALA- or MPLA-treated BMDCs were co-cultured at 1×10^4 cells/well with T cells for 2 days. T cell activation markers and T cell proliferation were evaluated by flow cytometry. Cells were stained with Fc block and 7-AAD as mentioned earlier, treated with BV421-anti-CD11c (BioLegend; clone N418), PE-Cy7-anti-CD69 (BD; clone H1.2F3), and APC-Cy7-anti-CD4 (BioLegend; clone: RM4-5) for 30 min on ice, and analyzed.

For the T cell polarization assay, T cells were seeded into coated plates without CFSE staining. Untreated BMDCs were co-cultured with T cells with or without the addition of various concentrations of ALA or MPLA. For CBA assays, culture supernatants were collected after 5 days; cytokines in the supernatants were measured by using Mouse Th1/Th2/Th17 CBA Kit (BD Biosciences) according to the manufacturer's instructions.

2.10 Preparation of cell lysates and western blotting

BMDCs were stimulated with a series of concentrations of ALA or MPLA for 20 min (for p65 and c-jun) or 2 h (for IRF3), washed once with PBS, and lysed by incubating for 30 min on ice in RIPA buffer (Millipore, Millipore, MA) supplemented

with protease and phosphatase inhibitors. The total protein concentration was quantified by BCA assay and separated on NuPAGE 4% to 12% Bis-Tris mini gels (Invitrogen). The gels were transferred onto the PVDF membrane; reacted with primary antibodies (dilution, 1:1000) to β-actin (Biolegend; clone 2F1-1), p65 (clone D14E12), p-p65 (clone 93H1), c-jun (clone 60A8), p-c-jun (clone E6I7P), IRF3 (clone D83B9), and p-IRF3 (clone E6F7Q; all from Cell Signaling); and subsequently incubated with secondary antibodies of goat anti-mouse IgG (Southern Biotech, Birmingham, AL) for β-actin and donkey anti-rabbit IgG (Biolegend) for the others. The membranes were analyzed by using a LAS-4000 (GE Healthcare, Chicago, IL).

2.11 NF-κB/AP-1 reporter assay

HEK-Blue hTLR4 cells (Invivogen) were cultured according to the manufacturer's instructions. Cells were stimulated with ALA or MPLA at 2.5×10^4 cells/well for 24 h. Supernatants were collected for analysis using QUANTI-Blue solution (Invivogen) according to the manufacturer's instructions.

2.12 Analysis of DC in the nasal tissue

PBS or 1 μg of ALA or MPLA was administrated nasally. After 24 h, the nasal tissue was collected and treated with 2 mg/mL Collagenase I for 60 min. Cells were stained with Fc block and 7-AAD as mentioned earlier, treated with BV421-anti-CD11c (BioLegend; clone N418), FITC-anti-I-A^d (BD; clone AMS-32.1), PE-anti-CD103 (BD;

clone M290), PE-Cy7-anti-CD40 (BioLegend; clone 3/23), PE-Cy7-streptavidin (BD), APC-anti-CD11b (BioLegend; clone M1/70), APC-Cy7-anti-CD8α (BioLegend; clone 53-6.7), Biotin-anti-CD80 (BD; clone 16-10A1), Biotin-anti-CD86 (BD; clone GL-1) for 30 min on ice, and analyzed.

2.13 RT-PCR

RNA samples were extracted with Sepasol-RNA I super G (Nacalai Tesque) according to the manufacturer's instructions. Then, DNase I and SuperScript VILO (Invitrogen) were used according to the manufacturer's instructions to make the cDNA. RT-PCR occurred on CFX Opus 96 (Bio-Rad).

2.14 Statistics

Data are shown as mean ± 1 SD and analyzed by one or two-way ANOVA; $P < 0.05$ was considered significant.

3. Results

3.1. ALA induces potent antigen-specific IgA and IgG when nasally administrated with OVA

I first compared the adjuvanticity of ALA and MPLA by co-administration with OVA subcutaneously and nasally in mice. When subcutaneously administrated, the level of OVA-specific IgG in serum in the ALA group was higher than MPLA group (Figure 3A). Meanwhile, during the intranasal administration, the secretion of OVA-specific IgA in the nasal wash and BALF was potently induced in the ALA group, while almost none of the response was induced in the OVA group and MPLA group (Figure 3B). Moreover, both ALA and MPLA groups induced the secretion of OVA-specific IgG in serum, but the level of the ALA group was higher than the MPLA group in intranasal administration (Figure 3C). These results indicated that ALA showed better adjuvanticity than MPLA in subcutaneous and intranasal administration. Especially, ALA was superior to MPLA as a mucosal adjuvant, which can induce both mucosal and systemic responses.

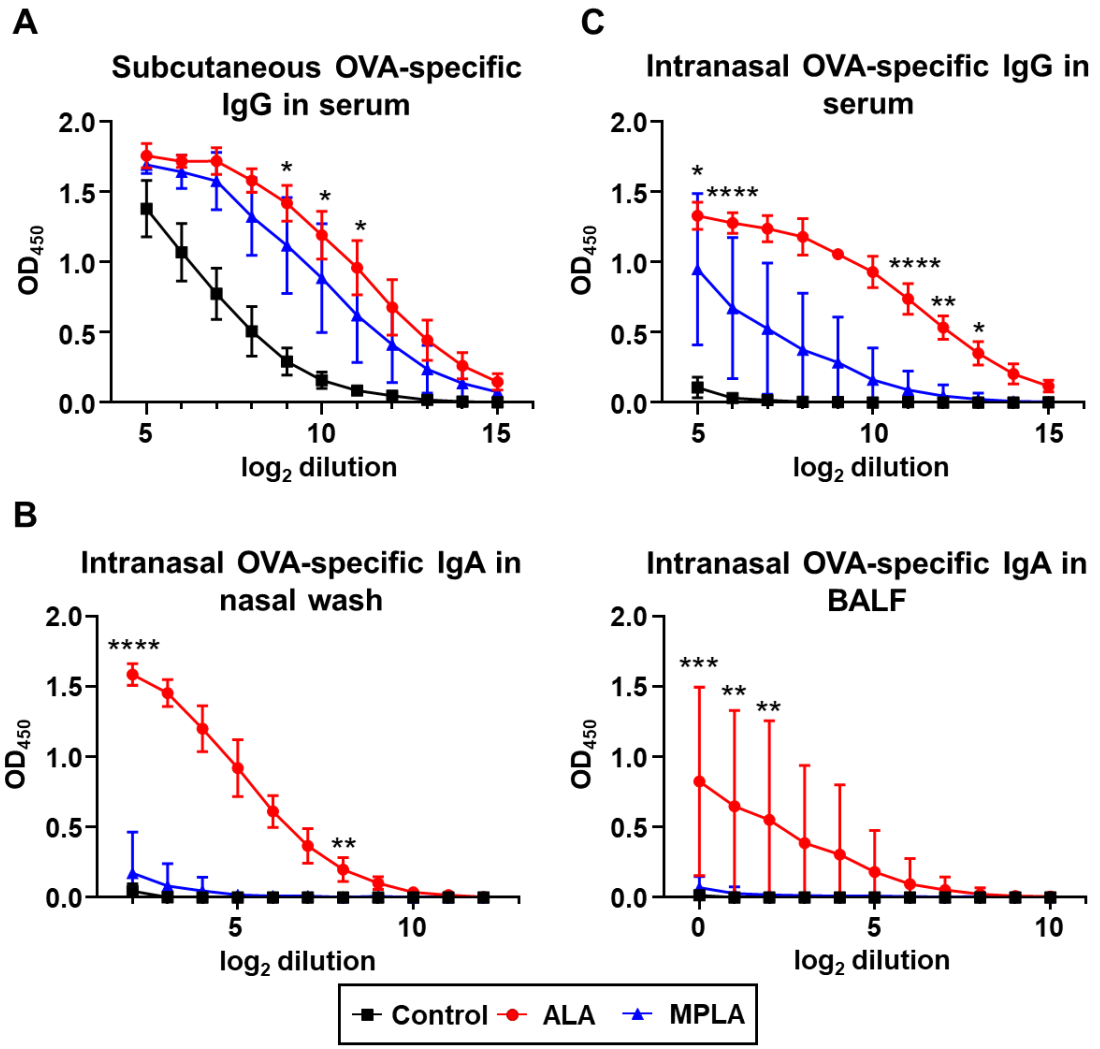


Figure 3. OVA-specific antibody induced by ALA or MPLA in subcutaneous or intranasal administration. (A) OVA-specific IgG in serum when subcutaneously administrated. (B) OVA-specific IgA in the nasal wash and BALF and (C) OVA-specific IgG in serum when intranasal vaccinated (n = 4 per group). The data are shown as mean ± 1 SD from a representative of two independent experiments; differences were analyzed by two-way ANOVA (*P<0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). OD, optical density

3.2. ALA does not occur side effects

I next compared the safety of ALA and MPLA in intranasal administration. Compare with the control group, the body weight and the number of lymphocytes in blood during the vaccination had no obvious difference in ALA or MPLA group (Figure 4A and 4C); and the body temperature after injection is similar to ALA and MPLA groups (Figure 4B). Furthermore, for concern of the side effects on the lung and liver from lipid A, one week after the final vaccination, mice were dissected. Compared with the control group, there was no obvious lesion on the lung and liver from ALA or MPLA group (Figure 4D). Moreover, no side effects were observed in the tissue of the nasal cavity (data were not shown).

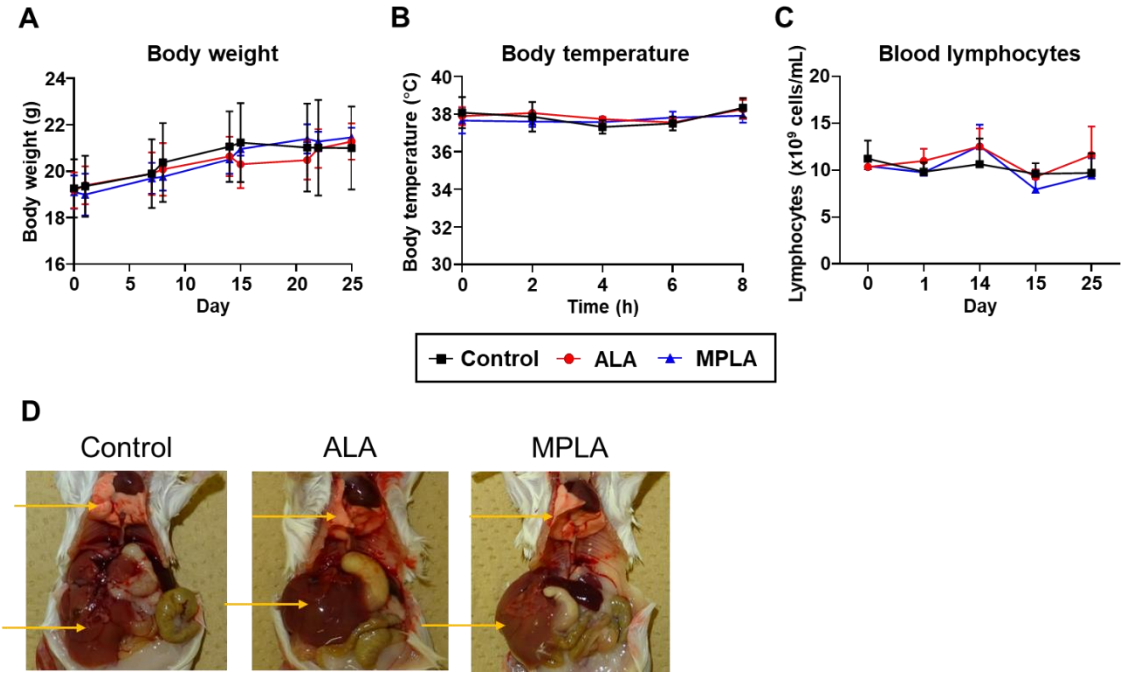


Figure 4. The safety of ALA or MPLA was compared when administrated nasally. (A) The body weight was measured during the administration. (B) The body temperature was measured from

the rectum after injection. (C) The number of lymphocytes in blood was measured with VetScan HM2. (D) Mice were sacrificed and dissected to observe the lung and liver, which were marked by yellow arrows (n = 4 per group). The data are shown as mean ± 1 SD from a representative of two independent experiments.

3.3. ALA upregulates the MHC class II expression and antigen presentation activity of DCs

To examine the mechanism of ALA as a potent mucosal adjuvant. I first put my emphasis on the effect of ALA on DCs. Given that microbial stimulation is known to increase the expression of MHC II, a key molecule in the presentation of antigen to $CD4^+$ T cells [38], I first compared the adjuvant activity of ALA and MPLA by evaluating the MHC II expression on bone marrow-derived DCs (BMDCs). Both ALA and MPLA upregulated MHC II expression on BMDCs, but the levels were higher for ALA than for MPLA (Figure 5A).

These findings led us to hypothesize that ALA also upregulates antigen presentation to $CD4^+$ T cells. To test this hypothesis, I used $CD4^+$ T cell hybridoma OT-IIZ cells, which specifically respond to OVA-derived peptide presented by MHC class II molecules [39], in an antigen-presenting reporter assay. Both ALA and MPLA increased the antigen-presenting activity of BMDCs compared with untreated BMDCs, and ALA-treated BMDCs showed greater antigen presentation to OT-IIZ cells than did MPLA-

treated BMDCs (Figure 5B). These results demonstrate that ALA more potently enhanced the antigen presentation ability of BMDCs toward T cells than MPLA.

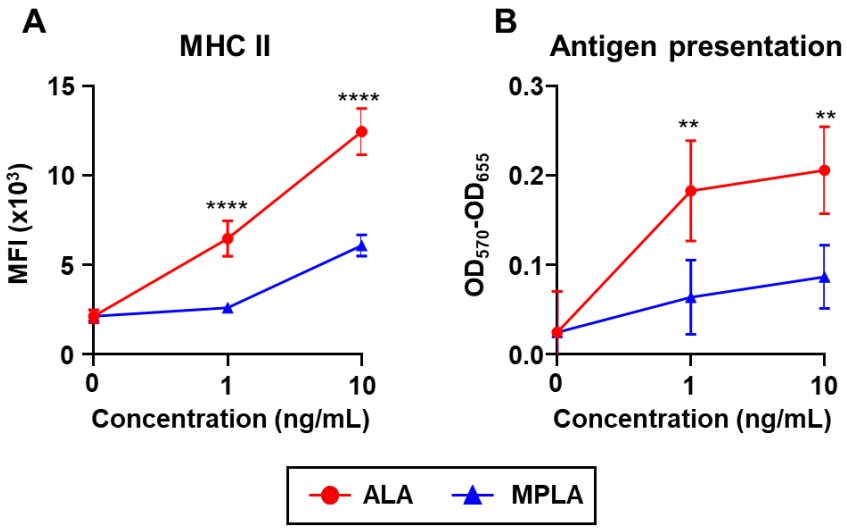


Figure 5. *Alcaligenes* lipid A (ALA) upregulated the expression of MHC II and improved antigen presentation on T cells. (A) BMDCs were treated for 48 h with ALA or MPLA at the indicated concentrations. The expression of MHC II on BMDCs was analyzed by flow cytometry (n = 3 per group). (B) BMDCs were preloaded with 0.1 mg/mL ovalbumin (OVA) for 16 h, treated with ALA or MPLA for 24 h, and co-cultured with OT-IIZ cells for 24 h. The response of OT-IIZ cells to antigen presentation was measured by CPRG assay (n = 5 per group). The data are shown as mean ± 1 SD from a representative of two independent experiments; differences were analyzed by two-way ANOVA (**P < 0.01, ****P < 0.0001). MFI, mean fluorescence intensity; OD, optical density

3.4. ALA induces increased expression of costimulatory molecules on BMDCs and enhanced T cell activation and proliferation

In addition to MHC II-mediated antigen presentation, costimulatory molecules on DCs (i.e., CD40, CD80, and CD86) are essential for T cell activation through their interaction with CD40L and CD28 [40]. In this regard, treatment of BMDCs with either ALA or MPLA increased the expression of CD40, CD80, and CD86, and ALA exerted a more potent effect than MPLA (Figure 6A).

I next aimed to stimulate DCs with either ALA or MPLA and examine their effects on T cell activation. As a marker of T cell activation, I measured the expression of CD69, a type II transmembrane glycoprotein related to the C-type lectins which are rapidly expressed on activated T cells [41]. Co-culture with ALA- or MPLA-treated BMDCs increased the expression of CD69 on T cells, and ALA-treated BMDCs accomplished greater T cell activation than MPLA-treated BMDCs (Figure 6B). I further analyzed T cell proliferation by staining with carboxyfluorescein succinimidyl ester (i.e., CFSE assay) and noted fewer undivided T cells (division 0) and more dividing cells (divisions 1, 2, and 3) when co-cultures contained ALA-treated BMDCs compared with MPLA-treated BMDCs (Figure 6C). Finally, the number of T cells co-cultured with ALA-treated BMDCs was larger than the MPLA-treated BMDCs (Figure 6D). These results indicate that, compared with MPLA, ALA enhanced the ability of BMDCs to induce T cell activation more potently than MPLA.

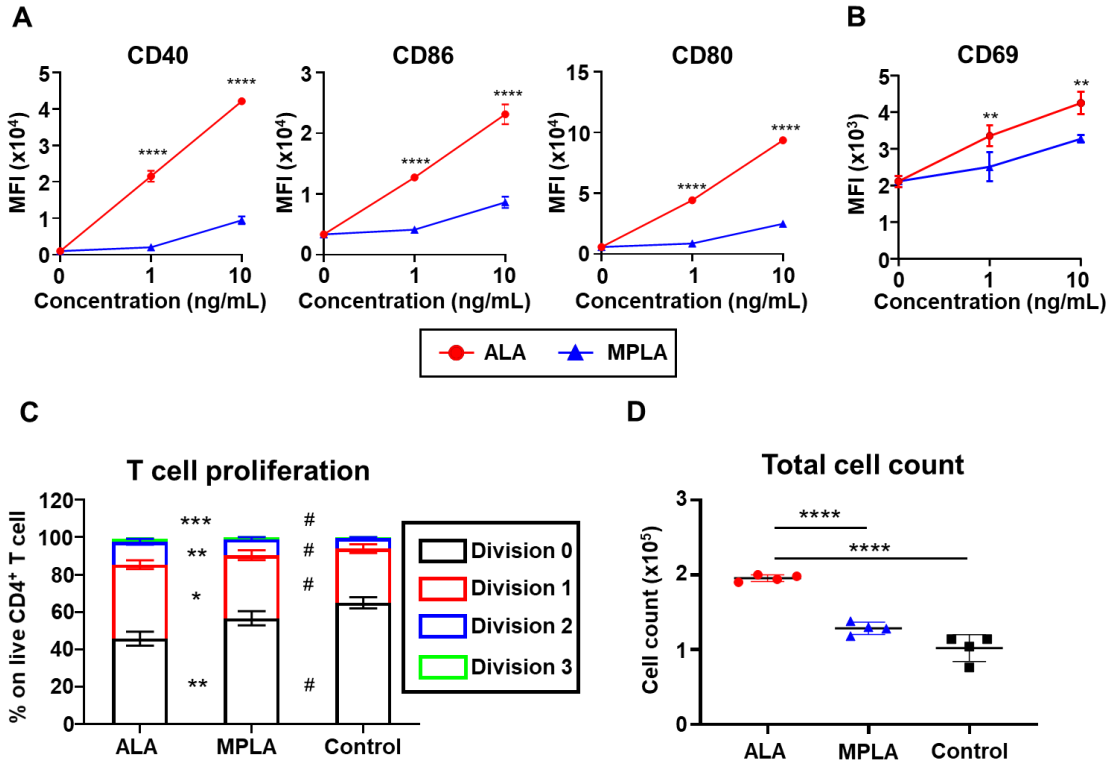


Figure 6. ALA upregulated the expression of co-stimulatory molecules and improved the activation and proliferation of T cells. (A) BMDCs were treated for 48 h with ALA or MPLA at the indicated concentrations. The expression of CD40, CD86, and CD80 on BMDCs was analyzed by flow cytometry (n = 3 per group). (B) ALA- or MPLA-treated BMDCs were co-cultured for 2 days with naïve $CD4^+$ T cells in anti-CD3ε-coated plates. The expression of CD69 on T cells was analyzed by flow cytometry (n = 4 per group). (C) Naïve $CD4^+$ T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured with ALA- or MPLA-treated BMDCs in anti-CD3ε-coated plates for 2 days. The proliferation of T cells was evaluated as the dilution of CFSE in flow cytometry (n = 4 per group). (D) The cell number in the experiment of (C) were counted by trypan blue. The data are shown as the mean ± 1 SD from a representative of two independent experiments; differences were analyzed by two-way ANOVA (*P < 0.05, **

$P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ for ALA vs. MPLA; # $P < 0.05$ for MPLA vs. control).

3.5. ALA educates DCs to polarize activated $CD4^+$ T cells to Th17 cells

Lipid A is known to activate DCs and induce the consequent secretion of various cytokines and chemokines for the control of T cell differentiation [42], [43]. Whereas BMDCs yielded similar levels of MCP-1 expression regardless of treatment with ALA or MPLA (Figure 7A), BMDCs produced high levels of IL-6 and IL-23 after ALA treatment but only scant amounts after stimulation with MPLA (Figure 7B-C). In addition, neither ALA nor MPLA induced detectable production of IFN-γ, IL-10, or IL-12 from BMDCs (data not shown).

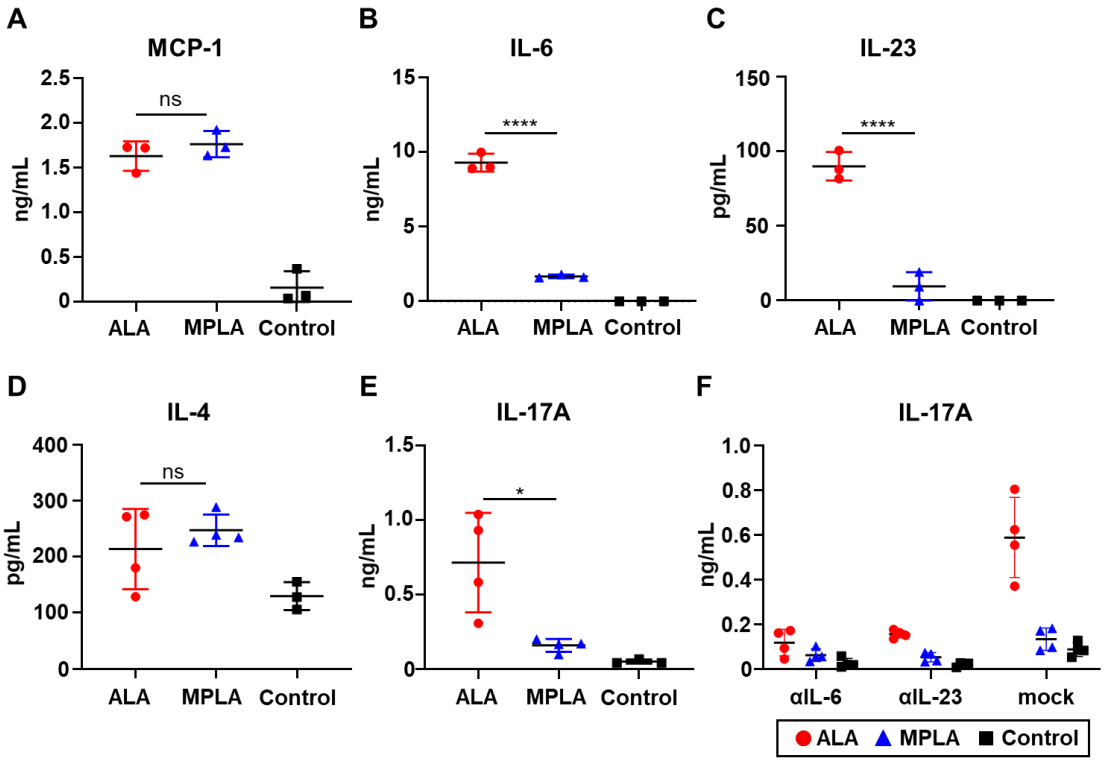


Figure 7. ALA induced the production of IL-6 and IL-23 and enhanced the differentiation of Th17 cells. (A–C) Cytokine concentrations in the supernatant of ALA- or MPLA-treated BMDCs were evaluated by using the CBA assay and ELISA (n = 3 per group). (D–E) BMDCs were co-cultured for 5 days with naïve $CD4^+$ T cells in anti-CD3ε-coated plates in the presence of ALA or MPLA. T cell cytokine concentrations in the supernatant were measured by using the CBA assay (n = 4 per group). (F) BMDCs and T cells were co-cultured in the presence of ALA or MPLA with or without the antibody for IL-6 or IL-23. The secretion of IL-17A was blocked by the addition of anti-IL-6 or anti-IL-23 (n = 4 per group). The data are shown as the mean ± 1 SD from a representative of two independent experiments; differences were analyzed by one-way ANOVA (*P < 0.05, **** P < 0.0001; ns, not significant).

MCP-1, IL-6, and IL-23 all play important roles in the control of differentiation of T helper cell subsets. Specifically, MCP-1 is indispensable in the process of Th2 cell differentiation [44], and IL-6 and IL-23 are key during Th17 cell differentiation [45]. Consistent with the respective effects on MCP-1 production, IL-4 production from activated T cells was weak and similar in the presence of ALA- with MPLA-treated BMDCs (Figure 7D). In addition, activated T cells showed greater IL-17A production in co-cultures containing BMDCs treated with ALA than with MPLA, consistent with the amounts of IL-6 and IL-23 secreted from BMDCs (Figure 7E). Meanwhile, by adding the antibody of IL-6 or IL-23, the production of IL-17A in the co-culture with ALA was blocked (Figure 7F). These results demonstrate that, unlike MPLA, ALA educates DCs

to dictate the preferential differentiation of T cells into Th17 cells through the production of the Th17-inducing cytokines IL-6 and IL-23.

3.6. ALA and MPLA utilize distinct TLR4 signaling pathways

TLR4 plays an essential role in the immunological responses to lipid A variants, including MPLA [46]. We previously confirmed that *Alcaligenes* LPS likewise requires the expression of TLR4 to activate BMDCs [30], but whether ALA also depends on the expression of TLR4 in BMDCs to achieve their activation has been unclear. Here, I evaluated the dependency of ALA on TLR4 expression by using $TLR4^{-/-}$ (KO) and $TLR4^{+/-}$ (HE) BMDCs. The upregulation of MHC II and co-stimulatory molecules (i.e., CD80, CD86, and CD40) and cytokines (IL-6 and MCP-1) in ALA-treated DCs was eliminated in TLR4 KO BMDCs (Figure 8A-B).

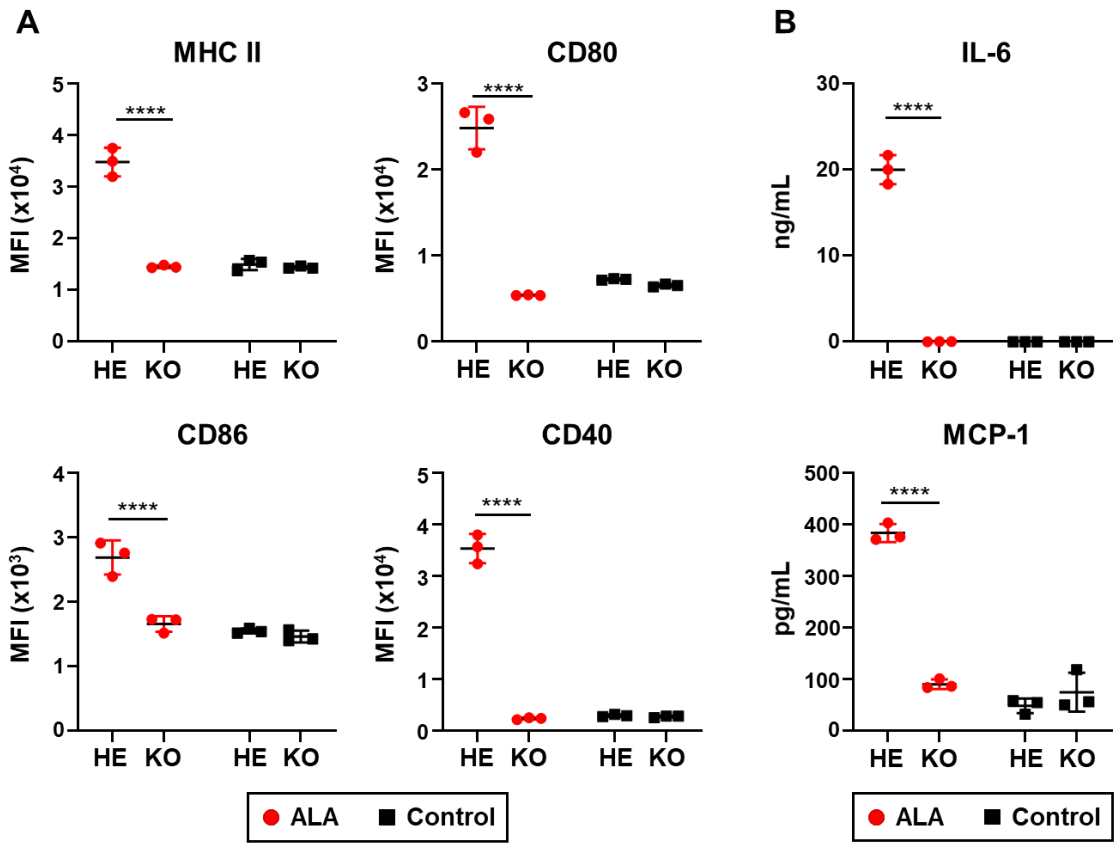


Figure 8. ALA is recognized by toll-like receptor 4 (TLR4). (A) BMDCs from TLR4 heterozygous (HE) and knock-out (KO) mice were treated with ALA for 48 h. The expression of MHC II, CD40, CD86, and CD80 on BMDCs was analyzed by flow cytometry. (B) Cytokine concentrations in the supernatant of ALA-treated BMDCs from TLR4 HE and KO mice were measured by using the CBA assay (n = 3 per group). The data are shown as the mean ± 1 SD from a representative of two independent experiments; differences were analyzed by one-way ANOVA (****$P < 0.0001$).

Although ALA and MPLA are both recognized by TLR4, their effects on cytokine and chemokine production from BMDCs and subsequent T cell differentiation differ

markedly. In particular, ALA more potently promoted IL-6 expression, which is dependent on myeloid differentiation factor 88 (MyD88) signaling [47], [48], whereas ALA and MPLA induced similar levels of MCP-1 production, which is mediated through a signaling pathway dependent on TIR-domain-containing adapter-inducing interferon-β (TRIF) [49], [50] (Figure 7A-B). Therefore, I supposed that ALA and MPLA activate different signaling pathways upon TLR4 binding.

To test this hypothesis, I evaluated the phosphorylation levels of transcription factors p65 and c-jun, which are both dependent on MyD88 and respectively mediate the NF-κB and AP-1 signaling pathways [51]. Compared with MPLA, ALA induced greater phosphorylation of p65 and c-jun (Figures 9A-B). Consistently, NF-κB/AP-1 reporter assay showed strong activation of the NF-κB/AP-1 pathway in ALA (Figure 9C). In contrast, the phosphorylation levels of TRIF-dependent IRF3 were nearly identical regardless of whether BMDCs were stimulated with ALA or MPLA (Figures 9A-B).

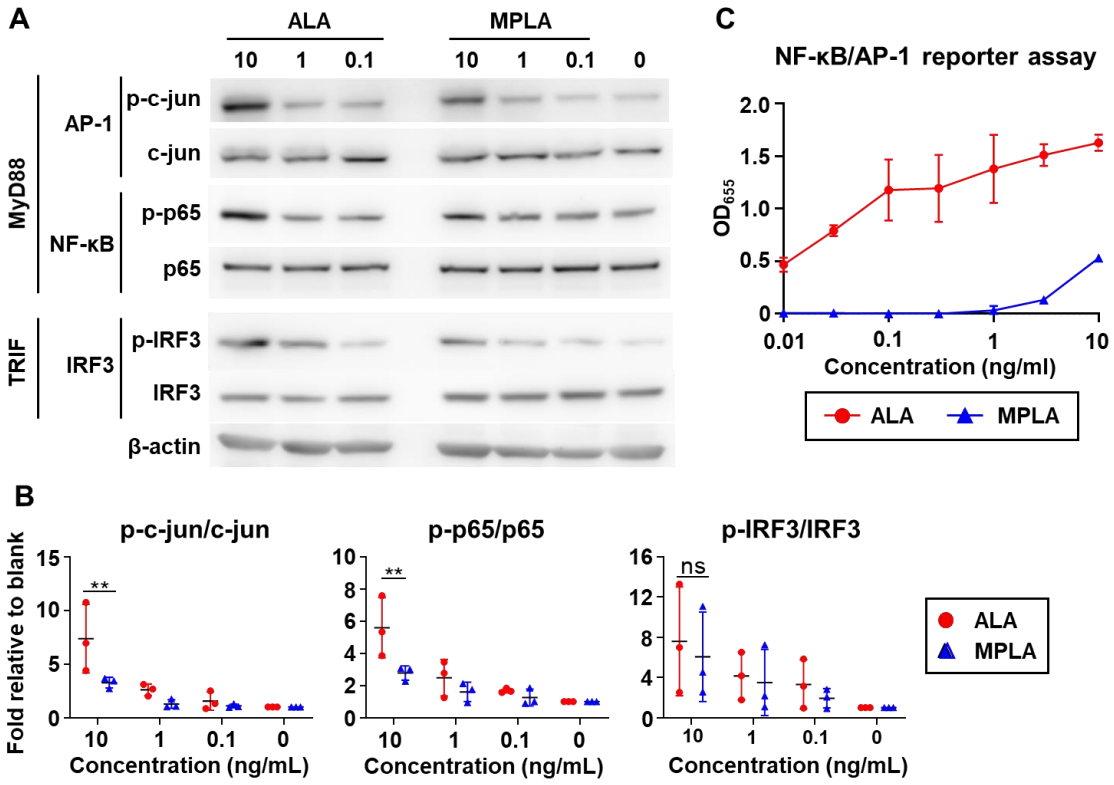


Figure 9. The induction of MyD88 and TRIF by ALA or MPLA. (A) ALA induced both the MyD88 and TRIF signaling pathways, but MPLA was TRIF-biased. BMDCs were treated with ALA or MPLA for 20 min (p65 and c-jun) or 2 h (IRF-3) and then lysed in RIPA buffer for western blotting. The results shown are from one of three representative experiments. (B) Quantification of the results in panel A by using ImageJ. (C) The reporter assay for NF-κB/AP-1 induction by ALA or MPLA through TLR4 (n = 4 per group). The data are shown as the mean ± 1 SD from two representative independent experiments; differences were analyzed by two-way ANOVA (**P < 0.01; ns, not significant).

Furthermore, the MyD88$^{-/-}$ (KO) and MyD88$^{+/+}$ (WT) BMDCs were utilized to evaluate the difference between ALA and MPLA on the MyD88 and its downstream

NF-κB and AP-1 signaling pathways. Compare with the WT, the phosphorylation of c-jun by ALA was completely blocked in KO (Figure 10A-B), and the phosphorylation of p65 by ALA and MPLA was downregulated in KO (Figure 10A and 10C).

These results suggest that whereas ALA was similar to MPLA in the induction of the TRIF signaling pathway, ALA was superior to MPLA in the MyD88 signaling pathway.

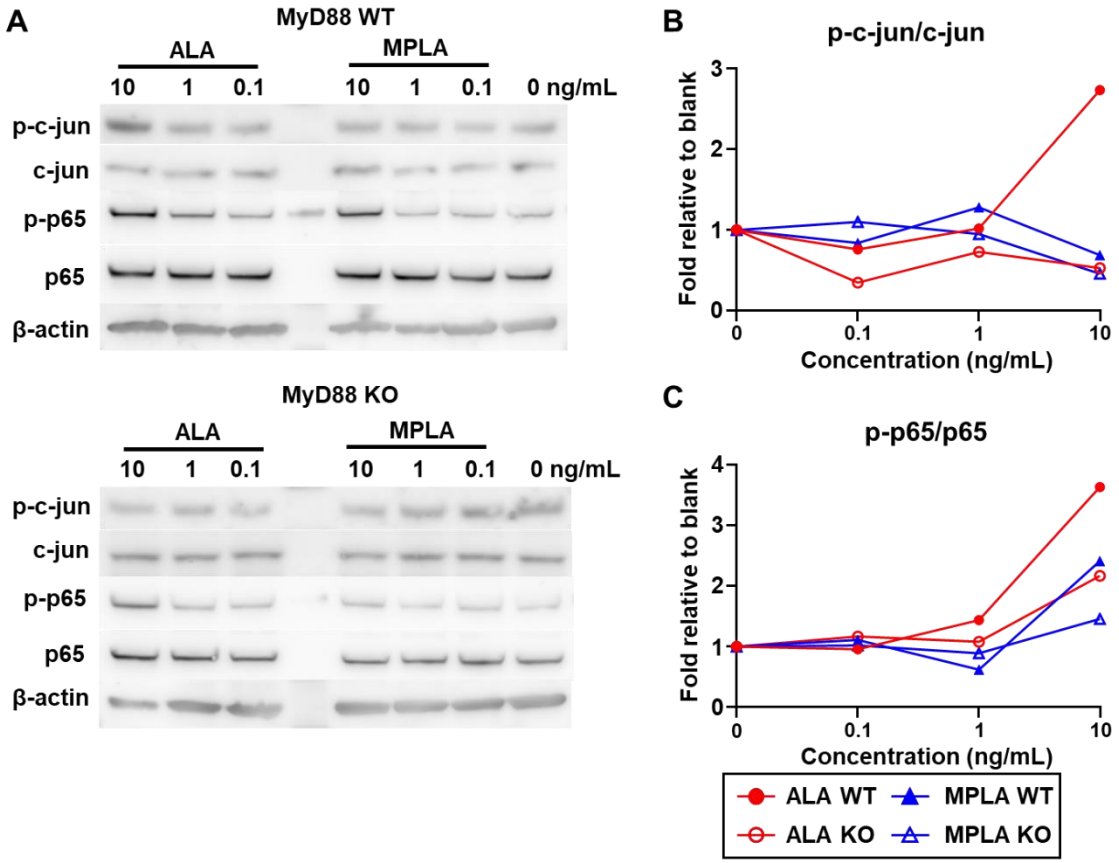


Figure 10. The induction of MyD88 by ALA or MPLA in MyD88 WT BMDCs, and block in MyD88 KO BMDCs. (A) BMDCs of MyD88 WT and KO were treated with ALA or MPLA for 20 min and lysed in RIPA buffer for western blotting. The phosphorylation of c-jun and p65

induced by ALA in MyD88 WT were blocked in MyD88 KO. (B) (C) Quantification of the phosphorylation of c-jun and p65, respectively, from the results in panel A by using ImageJ.

3.7. The MyD88-dependent signaling pathway is indispensable for ALA-induced upregulated expression of cytokines and costimulatory molecules in DCs

To verify that the effect of ALA on the expression of cytokine and costimulatory molecules in BMDCs is dependent on the MyD88-mediated signaling pathway, I used takinib [52], an inhibitor of TAK1 in the MyD88-mediated pathway, and GSK8612 [53], which inhibits IRF3 in the TRIF-mediated pathway.

Treatment with takinib—but not GSK8612—inhibited the ALA-induced enhancement of IL-6 production from BMDCs (Figure 11A). In contrast, GSK8612 completely prevented and takinib partially inhibited the expression of MCP-1 induced by either ALA or MPLA (Figures 11B). These results indicate that the expression of IL-6 after ALA treatment is predominantly dependent on the MyD88 signaling pathway, whereas the expression of MCP-1 after ALA or MPLA treatment is dependent mainly on the TRIF signaling pathway and only partially on the MyD88 signaling pathway. Furthermore, takinib—but not GSK8612—inhibited both ALA-induced and MPLA-increased expression of MHC II and costimulatory molecules (i.e., CD40, CD80, and CD86) (Figure 11C–F).

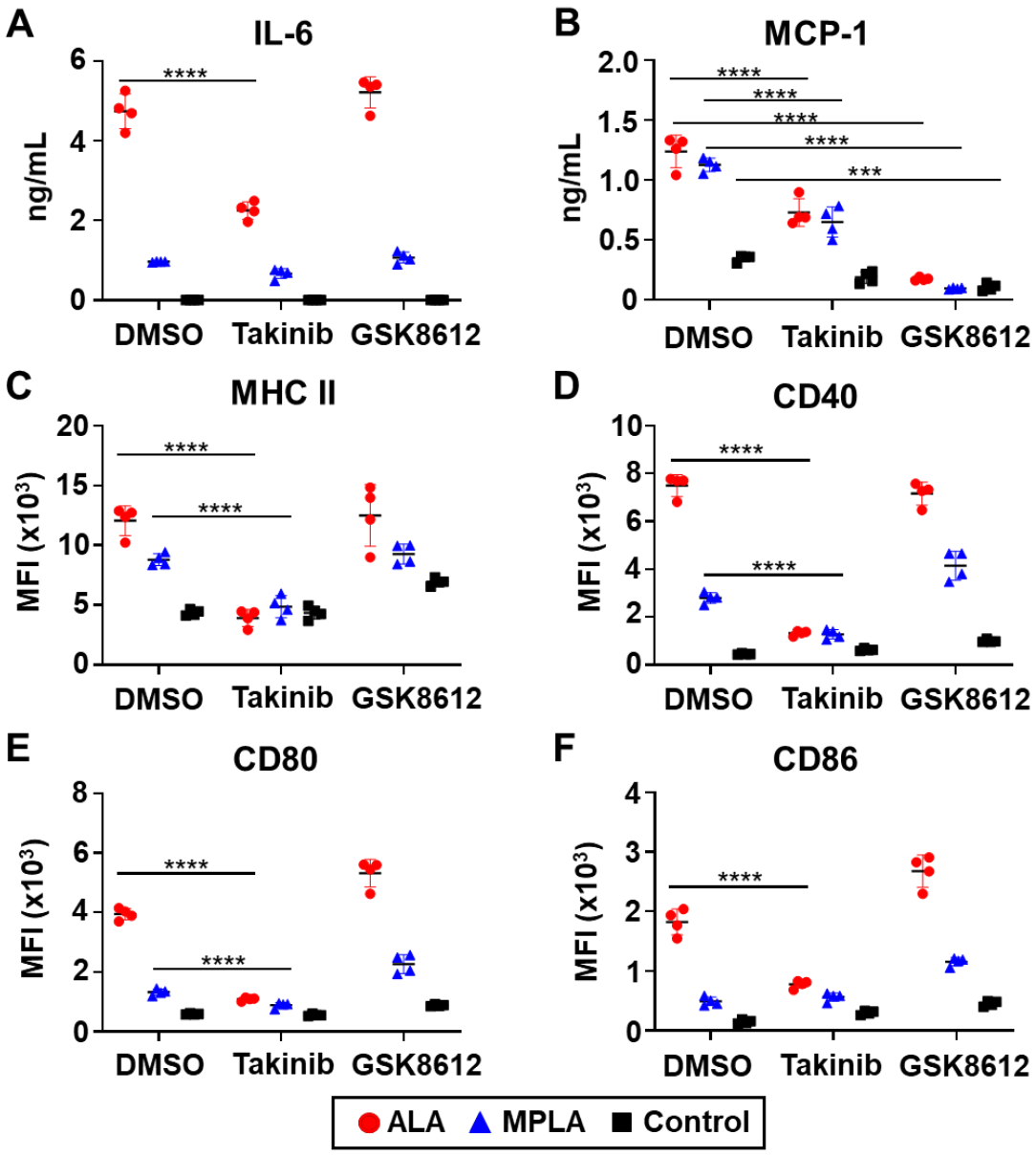


Figure 11. The MyD88-dependent production of IL-6 and expression of MHC II and co-stimulatory molecules after ALA stimulation. BMDCs were treated for 48 h with ALA or MPLA in the presence or absence of takinib or GSK8612. (A–B) The production of IL-6 and MCP-1 was measured by using the CBA assay. (C–F) The expression of MHC II and co-stimulatory molecules was analyzed by flow cytometry (n = 4 per group). The data are shown as the mean ± 1 SD from two representative independent experiments; differences were analyzed by two-way

ANOVA (***$P < 0.001$, ****$P < 0.0001$).

Together, these findings indicate that the upregulated expression levels of MHC II and costimulatory molecules after the treatment of DCs with either ALA or MPLA were all dependent on MyD88.

3.8. ALA induces the recruitment and activation of cDC2s in the nasal tissue

To reveal the in vivo mechanism of the superiority of ALA in the nasal vaccine, I analyzed the effect of ALA on the nasal tissue. I first quantified the numbers of DCs and the subtypes of DCs. After the nasal administration of ALA, a greater number of $CD11b^{+}CD103^{-}$ cDC2s infiltrated into the nasal tissue than MPLA (Figure 12A). Furthermore, the activation of DCs was evaluated by the expression level of co-stimulatory molecules (CD40, CD86, and CD80) on DCs. The expression of CD40 and CD80 were upregulated on the DCs of the ALA group higher than in the MPLA group, but both groups had little change in the expression of CD86 (Figure 12B). These results demonstrated that intranasal administrated ALA induced the infiltration and activation of cDC2 in nasal tissue but little effect was observed in MPLA.

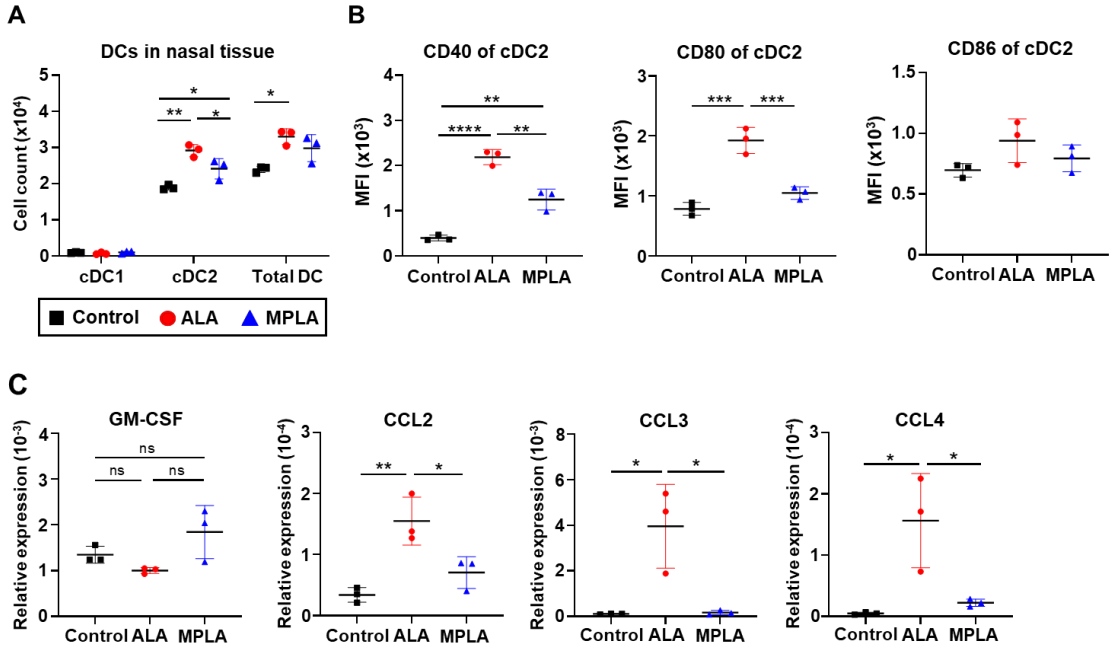


Figure 12. cDC2s were infiltrated in nasal tissue through the expression of chemokines by ALA. ALA or MPLA were administrated nasally. After 24 h, (A) the number of cDC1 and cDC2 were counted and (B) the expression of co-stimulatory molecules was analyzed with the flow cytometer. (C) The expression of GM-CSF, CCL2, CCL3, and CCL4 in the tissue was evaluated with RT-PCR (n = 3 per group). The data are shown as the mean ± 1 SD from two representative independent experiments; differences were analyzed by one-way ANOVA (*P<0.05, **P<0.01, ***P < 0.001, ****P < 0.0001; ns, not significant).

The increase of cDC2 in nasal tissue by ALA may be due to the proliferation by GM-CSF, recruitment by chemokines, or both. To test this issue, the expression of GM-CSF and chemokines (CCL2, CCL3, and CCL4) by ALA or MPLA in the nasal epithelial tissue was evaluated by RT-PCR. Compared with the control group, either ALA or MPLA did not enhance the expression of GM-CSF, which suggested that the increase of cDC2 was

not induced by the proliferation (Figure 12C). Meanwhile, compared with the control or MPLA group, the expression of CCL2, CCL3 and CCL4 were upregulated in the ALA group. These results suggested that the increase of cDC2 in the ALA group was due to the recruitment by chemokines.

4. Discussion

In this study, I demonstrated the superiority of ALA in inducing mucosal immunity and safety in an intranasal vaccine. To reveal the mechanism of ALA as a potent mucosal adjuvant, I compared ALA with MPLA in terms of their ability to activate cells and signaling pathways *in vitro*, and the specific effect of ALA on nasal tissue *ex vivo*. ALA induced both the MyD88 and TRIF signaling pathways to upregulate the activation of DCs and potently increased the interaction between DCs and $CD4^+$ T cells, thus explaining ALA's positive effect on acquired immunity when used as a vaccine adjuvant. By using inhibitors of various transcription factors in the MyD88 or TRIF signaling pathways, I learned that these activation effects from ALA on DCs were due to ALA-associated induction of both the MyD88 and TRIF signaling pathways. In contrast, MPLA was biased toward the TRIF signaling pathway and only weakly enhanced the activation of DCs and their interaction with T cells. These differences implicate ALA as a more effective adjuvant than MPLA. ALA induced the infiltration of cDC2s in the nasal tissue in the intranasal vaccine, and these cDC2s were activated in higher expression of co-stimulatory molecules. ALA also upregulated the expression of chemokine in the nasal tissue, which may recruit the cDC2s from circulation into the nasal tissue. In contrast, these effects were not observed in MPLA, which implies that ALA has advantages as an intranasal vaccine adjuvant that MLPA does not.

In a previous report, MPLA showed high safety benefits because of its bias toward the TRIF signaling pathway [50], which is dependent on the single phosphoryl group in

the structure of MPLA [54]. This single phosphoryl group leads to inefficient dimerization of the TLR4–MD-2 complex, which thus decreases the induction of MyD88 but has almost no effect on the TRIF signaling pathway [55]. In previous reports, pathogen-derived LPS induced a strong MyD88 cascade, which produced excessive inflammatory cytokines and inflammasomes and led to the liver and neuron damage [56], [57], thus disqualifying it as a safe adjuvant, whereas the TRIF-biased MPLA showed sufficient safety. Consequently, numerous chemical mimetic compounds based on the structure-activity relationship of MPLA have been designed and tested to obtain more effective and safer TRIF-bias adjuvants[58], [59]. We previously demonstrated *in vivo* the safety of both *Alcaligenes* LPS and lipid A as adjuvants [30], [33], [60]; these findings prompted us to suppose that ALA—like MPLA—is TRIF-biased. However, my current results from the signaling pathway experiments (Figure 9) refuted my hypothesis. Furthermore, my preliminary studies comparing ALA and MPLA showed that they had similar levels of safety *in vivo* (Figure 4). To explain the similarity, I thought that downmodulating the induction of the MyD88 signaling pathway might improve the safety of the adjuvant. In our previous study, *Alcaligenes* LPS induced much milder IL-6 expression and inflammation in the lung than *E. coli* LPS did [30], and more importantly, by upregulating the activation of DCs and T cells, the synergy between the MyD88 and TRIF signaling pathways may increase vaccine adjuvanticity [61]. Furthermore, in our previous study, *Alcaligenes* LPS induced only

IL-17^{+} T cells, which are considered protective Th17 cells, but not IFN-γ^{+}IL-17^{+} T cells [62], which—as pathogenic Th17 cells—cause autoimmunity diseases [60].

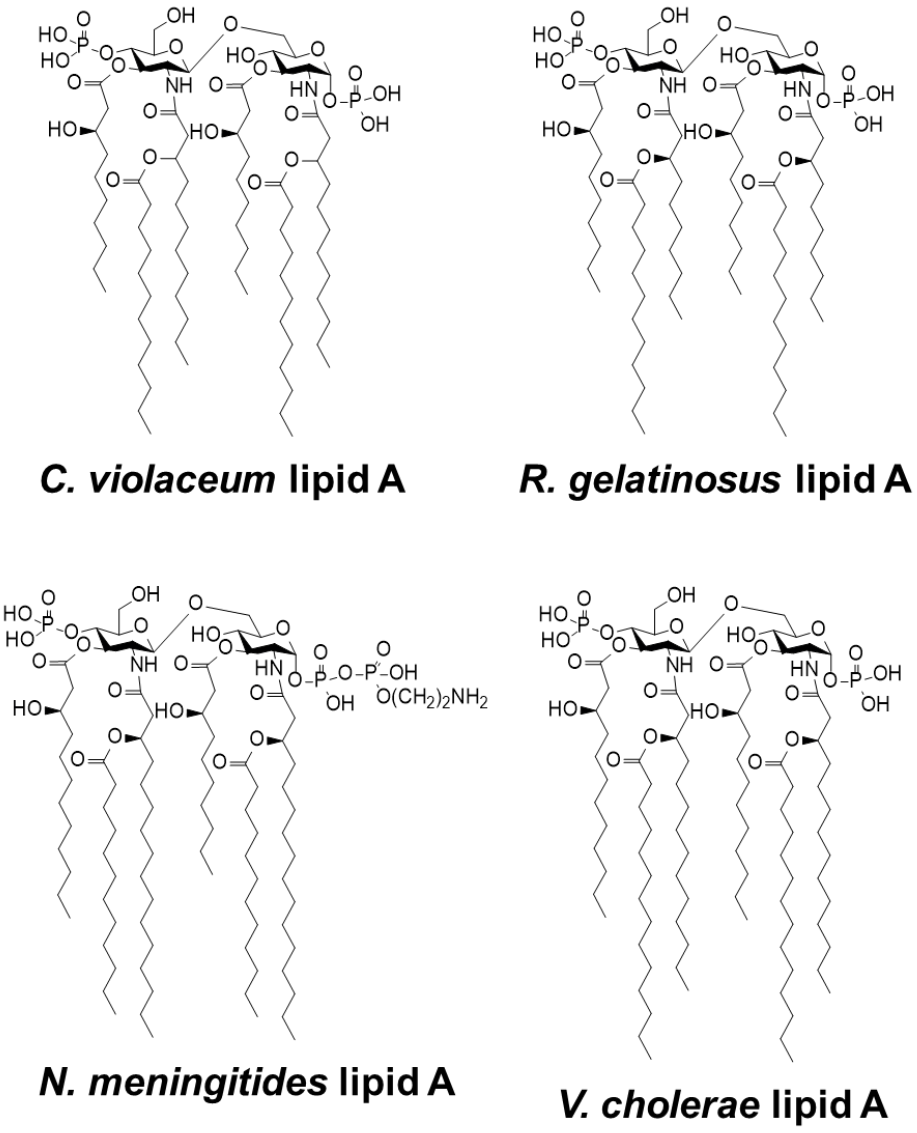


Figure 13. The molecular structure of lipid A variants.

Moreover, ALA has a unique structure in terms of the location, length, and modification of its acyl groups; these features may contribute to the ability of ALA—

compared with other varieties of lipid A—to immunomodulate without toxicity by appropriately activating the MyD88 signaling pathway [63]. For most hexaacylated lipid A molecules, the distribution of the acyl groups on the backbone is asymmetric (4+2), whereas other lipid A molecules—including ALA—have a symmetric (3+3) distribution of acyl groups [64]. The volume of the hydrophobic part of lipid A that is derived from the length of these acyl chains and their distribution is closely related to the TLR4/MD2 activation potential. That is, five of the six acyl chains of the canonical hexaacylated lipid A are positioned in the hydrophobic MD-2 pocket, and the remaining acyl chain interacts with the hydrophobic surface of the neighboring TLR4 [64]. These interactions contribute to TLR4/MD-2 complex dimerization, which triggers the activation of innate immunity [19]. Therefore, lipid A that is hypo-acylated or has relatively short acyl groups cannot induce TLR4/MD-2 complex dimerization and tends to show antagonistic activity. In previous studies, the symmetric lipid A variants from *C. violaceum* and *R. gelatinosus*, which have relatively short acyl chains (i.e., 10- or 12-carbon acyl groups), were immunoantagonistic[64], [65]. In contrast, the symmetric lipid A molecules from *N. meningitides* and *V. cholerae*, which have 12- or 14-carbon acyl groups and thus are similar to the lipid A of *E coli*, have highly similar immunostimulatory effects [66]. The structural properties of ALA are similar to those of *N. meningitides* and *V. cholera*, but ALA has a short, 10-carbon acyl chain—this intermediate hydrophobicity may be the key to low-toxicity immune activation. In addition, the secondary acyl chain of ALA carries a hydroxyl group [31], which is

relatively rare. These unique structures may contribute to the low-toxicity immunomodulatory properties of ALA.

In the evaluation of the phosphorylation in MyD88 KO and WT BMDCs, the phosphorylation of c-jun was completely blocked while the phosphorylation of p65 was partially blocked. In some recent studies, TLR4 induces not only the MyD88 and TRIF signaling pathway, but also the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway [67]. The PI3K/Akt signaling pathway can activate the NF-κB but has no influence on AP-1, which explains the partial block in the phosphorylation of p65 when MyD88 was knockout.

The clinical application of ALA in vaccines is an important issue. One of the properties of ALA is that it induces potent Th17 cell responses *in vivo* and *in vitro* but almost no Th1 cell response [33], [34]. My current study revealed that this property was because ALA induced BMDCs to produce IL-6 and IL-23 but not IL-12. Furthermore, ALA could recruit the cDC2s into the nasal tissue, which had the potential to polarize a Th17 response. Currently, almost none of the adjuvants currently in use are Th17-specific [68]. In mucosal immunity, although it is unclear just how Th17 cells promote IgA class switching on B cells, the help from Th17 cells is indispensable in the expression of antigen-specific high-affinity IgA [69], [14]. In addition to the induction of IgA, the Th17 cell itself can effectively protect the host from respiratory infections [70]. In previous reports, the intranasal administration of the outer membrane vesicle pertussis vaccine (omvPV) can protect mice from the infection of *B. pertussis* [71].

Intranasal boost administration of SARS-CoV-2 spike ectodomain with the adjuvant CFA01 induced higher antigen-specific IgA secretion in the upper respiratory tract than subcutaneous administration [72]. In our previous study, the intranasal administration of ALA by adding PspA can efficiently protect the mice from the infection of *Streptococcus pneumonia* [34]. The vaccines adding the ALA as an adjuvant have the potential to protect from respiratory pathogens.

The experiments in which I used takinib and GSK8612 to reveal the dependency of cytokine expression on MyD88 and TRIF yielded some unexpected results. First, takinib partially decreased but did not completely inhibit the expression of MCP-1 (Figure 11B). Most previous reports have claimed that MCP-1 is either a TRIF-dependent or MyD88-independent cytokine [49], [54], [73]. However, the presence of κB sequences in MCP-1 and other TRIF-dependent genes (i.e., IP-10, RANTES, and IFN-β) [74] suggests that NF-κB might participate in the regulation of TRIF-dependent genes. One previous study [75] clearly explains the special regulation of TRIF-dependent genes in TLR signaling; specifically, p65 of NF-κB is an indispensable adaptor in the IRF3-induced transcription of TLR4 and TLR9 signaling but not TLR3 signaling. Although the differences between TRIF from TLR4 and TRIF from TLR3 remain unclear, they could perhaps explain apparent contradictions between my results and those of other reports. Second, GSK8612 upregulated the expression of MHC II, CD80, and CD86 (Figure 11C–F), suggesting an inhibitory effect of TBK1—or other, unknown GSK8612 targets—on the MyD88 signaling pathway [76], [77].

As a limitation of the current study, I discussed the effects of ALA and MPLA on BMDCs, however, BMDCs are not the best representation of DCs. Both in mice and humans, the subtypes of DCs are divided into conventional DC1 (cDC1), cDC2, plasmacytoid DC (pDC), and monocyte-derived DC (moDC), which have different phenotypes and immunity functions [78]. According to their expression of TLR4, high-level expression was reported on moDC, while the expression on pDC, cDC1, and cDC2 is relatively low, which suggested a more potent response to ALA on moDC [79]. To their immunity function, cDC2 and moDC had the potential to induce Th17 cell response [80], like the ALA-educated BMDC. The specific target cells of ALA *in vivo* is still needed to discover.

Finally, in the intranasal administration of ALA or MPLA, ALA is superior to MPLA more than in the subcutaneous administration (Figure 3). The enhancement of ALA in subcutaneous administration can be attributed to the potent activation on DCs and T cells by ALA, which we discussed on BMDC and BMDC-T cell co-culture in vitro; however, the superiority of ALA in intranasal administration has a relationship with the specific effect from ALA in the nasal cavity as well. The intranasal administration of ALA induced activated cDC2 infiltration in the nasal tissue (Figure 12A-B), but the same effect was not observed in the subcutaneous administration of ALA (data not shown). The cDC2 is an important inducer in the T follicular helper cell (T_{fh}) –dependent antibody response [81] and polarizes the Th17 response in the airway [82]. As the previous report, cDC2 can be recruited by the chemokine (such as CCL2,

CCL3, and CCL4) to peripheral tissue in inflammation [83]. The expression of CCL2, CCL3, and CCL4 was evaluated in the nasal tissue induced by ALA, which suggests the source of cDC2 (Figure 12C). Besides the cDC2 itself, several kinds of cells can express chemokines under stimulation. Other kinds of APCs, such as macrophages can secrete CCL2, CCL3, and CCL4 [84], as well as the stromal cells [85]. Moreover, the epithelial cells and mast cells can be the source of CCL2 as well [86], [87]. The particular source of chemokine still needs to be discovered.

In conclusion, ALA showed potent adjuvanticity and enough safety on the intranasal vaccine. As the mechanism, ALA induced both the MyD88 and TRIF signaling pathways, whereas induction by MPLA was TRIF-biased. In particular, ALA-induced BMDCs expressed higher levels of MHC II, costimulatory molecules, and cytokines such as IL-6 but the same level of MCP-1 expression as BMDCs treated with MPLA. Consequently, ALA-educated BMDCs had better antigen presentation and Th17 cell differentiation than those primed by using MPLA. Moreover, ALA stimulated the expression of chemokines in the nasal tissue, which recruited the $CD11b^{+}$ cDC2 and induced the Th17 response in vivo.

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