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Original Article

Sublingual administration of liposomes enclosing alpha-galactosylceramide as an effective adjuvant of allergen immunotherapy in a murine model of allergic rhinitis

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AIT, allergen immunotherapy; AR, allergic rhinitis; APC, antigen-presenting cell; α -GalCer, alpha-galactosylceramide; α -GC-liposome, liposome enclosing α -GalCer; BMDC, bone marrow-derived dendritic cell; cDNA, complementary deoxyribonucleic acid; CLN, cervical lymph node; iNKT, invariant natural killer T cell; RNA, ribonucleic acid; ROR- γ t, retinoic acid receptor-related orphan receptor- γ t; T-bet, T-box transcription factor Tbx21; TCR, T cell receptor; TGF- β , transforming growth factor beta

ABSTRACT

Background: Sublingual immunotherapy (SLIT) is an established efficacious approach for the treatment of allergic rhinitis (AR). However, SLIT requires a long administration period to establish stable and adequate responses. This study investigated the efficacy of the sublingual administration of an allergen with liposomes enclosing α -GalCer (α -GC-liposome) as a potential adjuvant in mice with AR.

Methods: Mice with AR induced by OVA received the sublingual administration of OVA, α -GC-liposomes, or OVA plus α -GC-liposomes for 7 days. After nasal re-challenge with OVA, nasal symptoms were evaluated. The serum levels of OVA-specific Ig, the cytokine production of CD4⁺ T cells in the cultures of cervical lymph node (CLN) cells, and the gene expression of CLNs were analyzed.

Results: Although IL-4, IL-5 and IL-13 production from CD4⁺ T cells in CLN cells was significantly inhibited by the sublingual administration of OVA alone in mice with AR induced by OVA, their nasal symptoms were not significantly diminished. However, the combined sublingual administration of α -GC-liposomes and OVA completely suppressed nasal symptoms, downregulated Th2 and Th17 type cytokine production in CD4⁺ T cells as well as Th2 and Th17 gene expressions, and upregulated Th1 type cytokine production as well as Th1 gene expressions in CLN cells. Additionally, the serum levels of specific IgG2a were promoted, and specific IgE and IgG1 were inhibited.

Conclusions: Our findings suggest that the sublingual administration of an allergen with α -GC-liposomes as an adjuvant might increase the therapeutic efficacy and effectiveness of this treatment method.

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Introduction

Allergen immunotherapy (AIT) is considered the only effective fundamental therapy with the potential to alter the natural history of IgE-mediated type I allergic diseases such as allergic rhinitis (AR) and asthma.^{1–3} Traditionally, AIT has been administered by subcutaneous injection, termed subcutaneous immunotherapy (SCIT).⁴ Recently,

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sublingual immunotherapy (SLIT) has been introduced in many countries for the treatment of AR as an alternative to SCIT with improved safety and ease of administration. However, AIT including SLIT requires a long administration period of about 3 years to establish stable and adequate responses, and some patients with AR do not show significantly improved symptoms even after long-term therapy.^{1,2,5} Therefore, further improvements in AIT to enhance its effectiveness and shorten the treatment duration are required.

Alpha-galactosylceramide (α -GalCer), which is currently in clinical use, is a ligand of invariant natural killer T cells (iNKT cells) that is restricted by the non-classical MHC class I molecule CD1d on antigen-presenting cells (APCs).^{6,7} iNKT cells are characterized by the expression of an invariant T cell receptor (TCR) with a $V\alpha 14$ -J $\alpha 18$ chain paired with $V\beta 8$, $V\beta 7$ or $V\beta 2$ in mice⁸ and the $V\alpha 24$ -J $\alpha 18$ chain paired with $V\beta 11$ in humans.⁶ iNKT cells also express receptors associated with NK cells, such as NK1.1.⁷ Although iNKT cells are a small population of thymus derived T cells,⁹ α -GalCer-activated iNKT cells regulate immune responses during autoimmunity and tumor immunology by the rapid secretion of large amounts of cytokines, such as interferon- γ (IFN- γ), interleukin-2 (IL-2), IL-4, IL-13, IL-17, IL-21, IL-22, and tumor necrosis factor alpha (TNF- α),^{7,9–13} which also stimulate other immune cells to produce cytokines after interacting with CD1d-expressing APCs, such as macrophages, B cells, NK cells and dendritic cells.¹³ When activated, iNKT cells exert their effector functions within hours.¹⁴ They have important roles that bridge between innate and adaptive immunity^{15,16} as well as having a tolerogenic role in allergic airway inflammation.^{17,18}

In our previous study, the direct administration of α -GalCer, which is not water soluble, was poorly absorbed through the oral mucosa; therefore, an injection of bone marrow-derived dendritic cells (BMDCs) pulsed with α -GalCer was used as an adjuvant.¹⁹ However, this injection method is cumbersome and has some disadvantages for clinical applications. The current study aimed to improve the safety and enhance the therapeutic efficacy of the sublingual administration of an allergen. To achieve this, we co-administered liposomes enclosing α -GalCer (α -GC-liposomes) sublingually to the oral submucosa as an adjuvant with antigen in a murine model of OVA-induced nasal allergy. Liposomes are nano-sized spheres formed by a plurality of layers of phospholipid and are easily taken up into living tissues. Because the main constituent of liposomes is a phospholipid found in normal tissues, it has an excellent resolution capability *in vivo*. These liposome formulations have already been developed for anticancer drugs, and their safety was also confirmed when administered to humans.²⁰

In this study, we examined the influence of the concurrent sublingual administration of α -GC-liposome as an adjuvant with antigen for 1 week on the symptomatic improvement and local and systemic immunological change in mice after the development of AR to investigate its applicability in human patients with AR.

Methods

Mice and reagents

Six-week-old female C57BL/6 mice were purchased from SLC Inc. (Hamamatsu, Japan). CD1d-deficient ($CD1d^{-/-}$) mice kindly provided by Toshinori Nakayama from the Department of Immunology at Chiba University were established by the specific deletion of the CD1d gene segment and back-crossing 10 times on a C57BL/6 background.²¹ The animal experiments were approved by the Chiba University Institutional Animal Care and Use Committee and conducted in accordance with the guidelines of the committee. α -GC-

liposomes were provided by REGiMMUNE (Tokyo, Japan),²² which had been prepared as described previously.²³

Sensitization, sublingual administration, and nasal challenge

The protocols used for immunization, nasal challenge and treatment in this study were based on previously described protocols with some modifications for the treatment model.^{19,24} Briefly, mice were randomly allocated to one of five groups: a control group administered phosphate-buffered saline (PBS) and PBS sublingually (PBS/PBS), a placebo group administered OVA and PBS sublingually (OVA/PBS), and treatment groups administered OVA and either OVA alone (OVA/OVA), α -GC-liposomes alone (OVA/ α -GC-liposome), or OVA with α -GC-liposomes (OVA/OVA+ α -GC-liposome).

Regarding the treatment regimen, 6-week-old female C57BL/6 or $CD1d^{-/-}$ mice were intraperitoneally (i.p.) injected with 100 μ g of chicken ovalbumin (OVA; grade V; Sigma, St. Louis, MO, USA), emulsified in 2 mg of aluminum hydroxide (Thermo Scientific Pierce, Rockford, IL, USA) in 200 μ L of PBS on Days 0, 7 and 14 (Fig. 1). The serum levels of OVA-specific IgG1 were measured to confirm sensitization.²⁵ For challenge, the mice received 20 μ L of PBS alone or containing 50 mg/ml of OVA into the nostrils by nasal instillation from Days 21–27 for 7 consecutive days; 10 μ L of solution was administered into each side of the nostrils. Sublingual administration was then performed as a treatment model after the nasal challenge. These mice were sublingually administered either 2 μ g of α -GC-liposomes and 1 mg of OVA in 20 μ L of PBS, 2 μ g of α -GC-liposomes in 20 μ L of PBS, 1 mg of OVA in 20 μ L of PBS, or 20 μ L of PBS alone from Days 28–34 for 7 consecutive days. Mice were held on their back for 1 min to prevent immediate swallowing of the allergen.²⁵ Subsequently, they were intranasally administered 50 mg/ml of OVA in 20 μ L of PBS or PBS alone from Days 35–41 for 7 consecutive days.

Evaluation of nasal symptoms

The allergic symptoms of mice were evaluated with a video recording device on Day 41 of the re-challenge period, and the numbers of sneezing and nasal-rubbing events every 5 min from 10 min after re-challenge were counted by an investigator who was blinded to the treatment.

Sampling of cervical lymph nodes and spleen

Blood samples were obtained from the tail vein of mice on Days 21, 28 and 41. The mice were then sacrificed, and their cervical lymph nodes (CLNs) and spleens were collected for further immunological analyses.

Detection of α -GC-liposome uptake in lymph node cells after sublingual administration

Two μ g of rhodamine-labeled α -GC-liposomes in 20 μ L of PBS or 20 μ L of PBS alone was administered sublingually to C57BL/6 mice. The mice were then sacrificed and their CLNs were removed at 3 h after sublingual administration. Lymph node cells incorporating rhodamine-labeled α -GC-liposomes were analyzed by FACScalibur® (BD Biosciences, San Diego, CA, USA). Briefly, cells were resuspended in FACS buffer, pre-incubated with 10 mg/ml of unlabeled anti-CD16/32 antibody (24G2) (BD Biosciences), and then stained on ice with a combination of Abs. We used combinations of FITC-conjugated mouse anti-CD11c antibody (N418) (eBioscience,

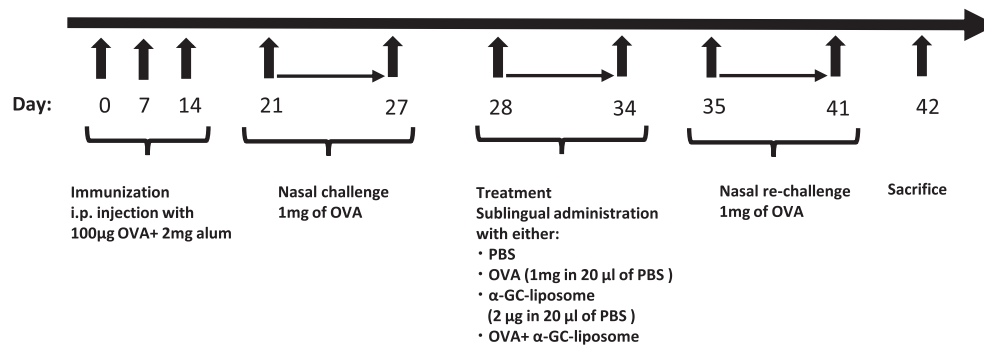


Fig. 1. Experimental design. Six-week-old female C57BL/6 mice were sensitized intraperitoneally (i.p.) with 100 µg of OVA and 2 mg of aluminum hydroxide (alum) at Days 0, 7 and 14. For the development of disease, 1 mg of OVA in 100 µl of PBS or PBS alone was administered intranasally from Day 21 for 7 consecutive days. Mice were then divided into four groups and administered either PBS, 1 mg of OVA, 2 µg of α-GC-liposomes or OVA plus α-GC-liposomes in 20 µl of PBS under the tongue for 7 consecutive days, followed by intranasal re-challenge with 1 mg of OVA in PBS or PBS alone for 7 consecutive days.

San Diego, CA, USA) with APC-conjugated mouse anti-CD11b antibody (M1/70) (eBioscience). Data were analyzed using CellQuest software (Becton Dickinson).

Measurement of serum OVA-specific immunoglobulins

The amounts of OVA-specific IgA, IgE, IgG1 and IgG2a were measured by ELISA according to previous reports.^{26,27} Briefly, ELISA plates were coated with either OVA (250 µg/ml) or rat anti-mouse IgA, IgE, IgG1, and IgG2a (BD Biosciences) for reference curves and incubated overnight at 4 °C. After washing 3 times with PBS containing 0.05% Tween 20 (PBST), the plates were blocked with PBS containing 2% bovine serum albumin (BSA; Sigma Chemical). Serum samples were added to the wells after 1:1000 or 1:3000 dilution in 2% BSA-PBS. After a 4-h incubation, the wells were washed with PBST, and IgA, IgE, IgG1, or IgG2a (BD Biosciences) were added at 2 µg/ml to the blocking buffer and incubated for 1 h. After washing, the wells were incubated with 100 µl of ExtrAvidin alkaline phosphatase (1:2000 dilution; Sigma Chemical) for 45 min, washed with PBST, and the reaction was developed with p-nitrophenyl phosphate (Sigma Chemical).

Analysis of gene expression by quantitative real-time PCR

To analyze the gene expression of lymph node cells, total RNA was extracted from CLNs using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) and reverse transcribed into cDNA by

oligonucleotide priming using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The real-time PCR analysis of the *T-box transcription factor tbx21* (*T-bet*) (Mn00450960_m1), *Retinoic acid receptor-related orphan receptor-γt* (*Ror-γt*) (Mn01261022_m1) and *Transcription factor signal transducer and activator of transcription 3* (*Stat3*) (Mn01219775_m1) gene expressions was conducted with Predesigned TaqMan Gene Expression Assays and reagents (Applied Biosystems) in accordance with the manufacturer's specifications. Analysis using a StepOnePlus™ PCR system (Applied Biosystems) of *Gata3* (forward, 5'-CCTCTGGAGGAGGAACGCTAAT-3, reverse, 5'-GTTTCGGGTCTGGATGCCTTCT-3'), *Va14* (forward, 5'-CACTGCCACC-TACATCTGTGT-3, reverse, 5'-AGTCCCAGCTCCAAATGCA-3') and *Foxp3* (forward, 5'-CCTGGTTGTGAGAAGGTCTTCG-3, reverse, 5'-TGCTCCAGAGACTGCACCACTT-3') gene expressions was conducted with Europeans Operon MWG (Ebersberg, Germany). Forty cycles of amplification were performed as follows: 50 °C, 2 min; 95 °C, 20 s; 95 °C, 1 s and 60 °C, 20 s. The mRNA levels for each gene were normalized to the amount of β-actin mRNA (Mn00607939_m1, forward, 5'-CCAGCCTTCCTTCTTGGGTAT-3, reverse, 5'-TGGCATA-GAGGTCTTTACGGATGT-3').

Cell culture and cytokine measurements

Single-cell suspensions were prepared from the CLNs of treated mice as described previously,^{19,24} and CD4⁺ T cells were sorted by MACS with biotinylated anti-CD4 antibody (GK1.5; BioLegend, San

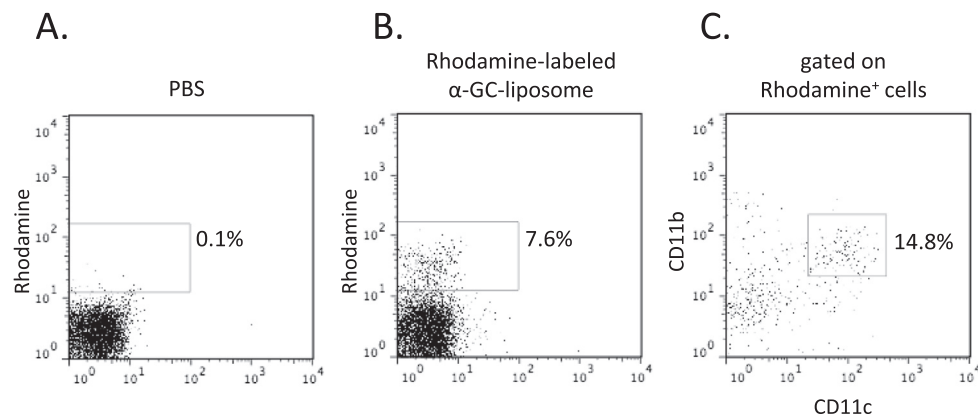


Fig. 2. Uptake of α-GC-liposomes into CLN cells. Rhodamine-labeled α-GC-liposomes or PBS were administrated sublingually to mice and CLN cells were collected after 3 h. Rhodamine fluorescing cells in the CLN cells of control mice administered PBS (A) or mice administered rhodamine-labeled α-GC-liposomes (B) are shown. CD11b and CD11c expressing cells in rhodamine fluorescing CLN cells are shown (C).

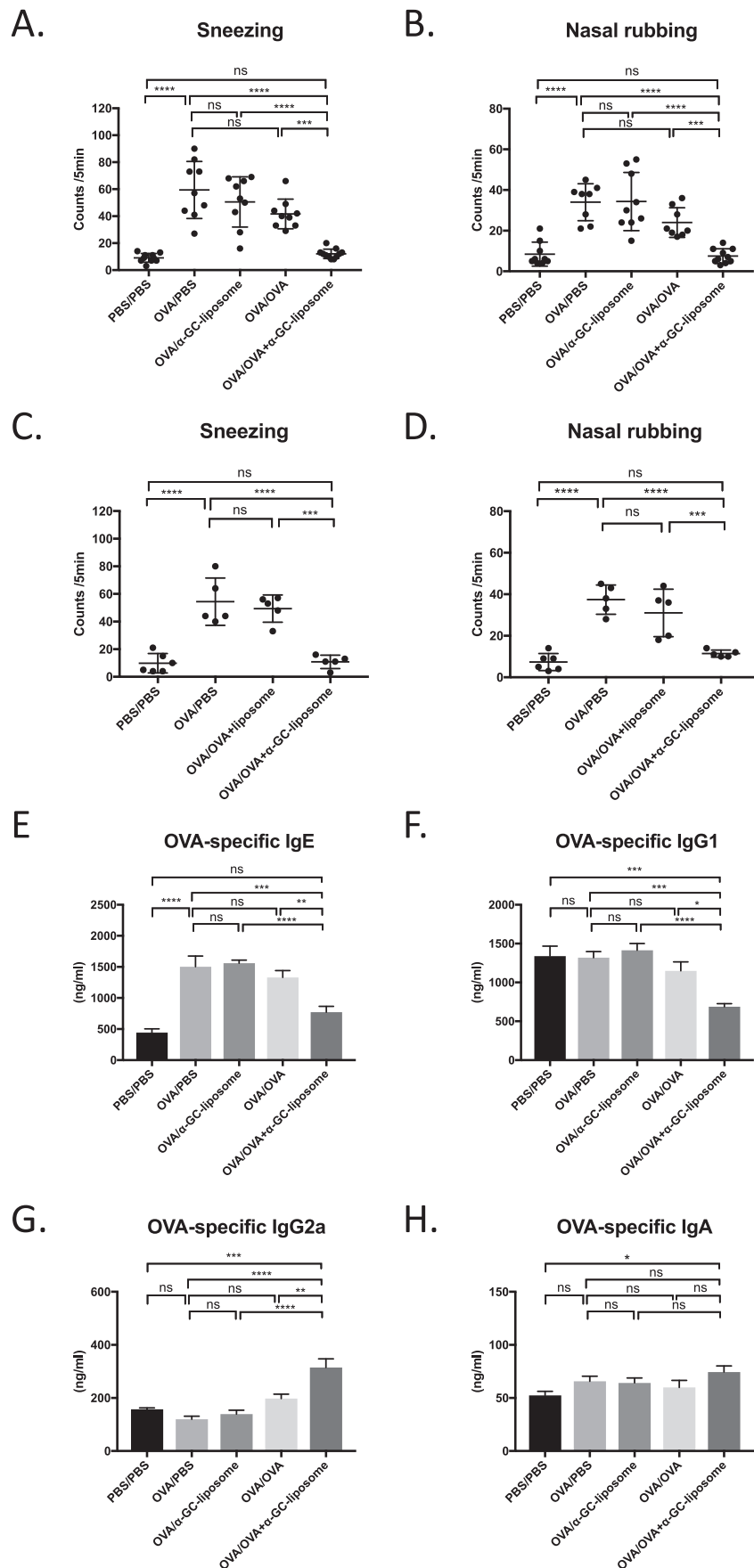


Fig. 3. Nasal symptoms and serum immunoglobulin levels after re-challenge. The numbers of sneezing (A) and nasal rubbing (B) events were counted every 5 min from 10 min after re-challenge. The numbers of sneezing (C) and nasal rubbing (D) events were counted every 5 min from 10 min after re-challenge in another setting including OVA/OVA + vector

Diego, CA, USA) and anti-biotin beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and resuspended in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% FCS, L-glutamine (2 μ M), penicillin (100 U/mL), streptomycin (100 μ g/mL), HEPES; [4- (2-hydroxyethyl) piperazine-1-ethanesulfonic acid] (10 mM), 2-mercaptoethanol (55 μ M), 1% non-essential amino acids and 1 mM sodium pyruvate (all from Gibco BRL, Grand Island, NY, USA). CD4⁺ T cells (3.0×10^6 cells/well) were restimulated with 1 mg/ml OVA and irradiated splenic feeder cells (1.0×10^7 cells/well) obtained from naïve mice in a round-bottomed 24-well microculture plate. The culture supernatants were then harvested at 72 h, and the amounts of IL-4, IL-5, IL-10, IL-13, IL-17A, TGF- β and IFN- γ were determined using an ELISA Ready-SET-Go! Kit (eBio-science) in accordance with the manufacturer's instructions.

Statistical analyses

Data were analyzed with GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Differences between multiple groups were analyzed by one-way analysis of variance followed by Tukey's post-test. Differences between two groups were analyzed by unpaired Student's *t*-test. *P* values less than 0.05 were considered significant. NS indicates “not significant”.

Results

Uptake of α -GC-liposomes into CLNs after sublingual administration

α -GC-liposomes were labeled with rhodamine to evaluate their uptake into mouse CLNs following sublingual administration. Rhodamine fluorescing cells in lymph node cells were detected in mice administered α -GC-liposomes but not PBS, and these cells were positive for CD11b and CD11c. Representative FACS profiles are shown in Figure 2A–C. Similar results were reproduced in another two experiments.

Symptomatic improvements in mice with AR receiving the combined sublingual administration of α -GC-liposomes and OVA

Increased numbers of sneezes and nasal rubbing events were observed after nasal re-challenge with OVA in OVA/PBS mice. The nasal symptoms were completely abrogated after re-challenge in the OVA/OVA+ α -GC-liposome mice, similar to the PBS/PBS mice. In contrast, no significant improvement in symptoms was found in OVA/OVA mice after re-challenge with OVA (Fig. 3A, B). Furthermore, in another experiment, no significant improvement of symptoms was observed in OVA/OVA + vector control liposome (not containing α GC) mice compared with OVA/PBS mice (Fig. 3C, D).

Effects of combined sublingual administration of α -GC-liposomes and OVA on OVA-specific IgE, IgG1 and IgG2a production

There were no significant differences in the serum levels of OVA-specific IgE at Day 28 among any groups (data not shown). The serum levels of OVA-specific IgE in OVA/PBS mice and OVA/ α -GC-liposome mice were increased at Day 42 (Fig. 3E). The levels of OVA-specific IgE and IgG1 in OVA/OVA+ α -GC-liposome mice were

significantly lower than in OVA/OVA, OVA/ α -GC-liposome and OVA/PBS mice at Day 42 (Fig. 3E, F). In contrast, the serum levels of OVA-specific IgG2a were significantly increased in the OVA/OVA+ α -GC-liposome mice compared with OVA/OVA, OVA/ α -GC-liposome and OVA/PBS mice (Fig. 3G). The serum levels of OVA-specific IgA were significantly increased in the OVA/OVA+ α -GC-liposome mice compared with PBS/PBS, but there were no significant differences in the levels of OVA-specific IgA between the remaining groups (Fig. 3H).

Upregulated Th1 and downregulated Th2 and Th17 type cytokine profiles of CD4⁺ T cells in the CLNs of mice with AR receiving combined sublingual administration of α -GC-liposomes and OVA

The levels of IL-4, IL-5 and IL-13 in the supernatants of CD4⁺ T cells from CLNs were significantly lower in OVA/OVA+ α -GC-liposome and the OVA/OVA mice than in OVA/PBS mice (Fig. 4A–C). The levels of IL-4 and IL-5 in OVA/OVA+ α -GC-liposome mice were significantly lower than in OVA/OVA mice (Fig. 4A, B). In contrast, the levels of IFN- γ in OVA/OVA+ α -GC-liposome mice were significantly higher than in any other group (Fig. 4D). The levels of IL-17A in OVA/OVA+ α -GC-liposome mice were significantly lower than in OVA/OVA, OVA/ α -GC-liposome and OVA/PBS mice (Fig. 4E). The levels of IL-10 in OVA/OVA+ α -GC-liposome mice were significantly lower than in OVA/PBS and OVA/ α -GC-liposome mice; however, there was no significant difference in the levels between OVA/OVA+ α -GC-liposome and OVA/OVA mice (Fig. 4F). There were no significant differences in the levels of TGF- β in CD4⁺ T cells from CLNs among any mice sensitized with OVA (Fig. 4G).

Upregulated Th1 and downregulated Th2 and Th17 type gene profiles of CD4⁺ T cells in the CLNs of mice with AR receiving combined sublingual administration of α -GC-liposomes and OVA

The expressions of various genes encoding transcription factors and cytokines associated with either Th1, Th2, Th17 or Treg cells in CLNs were examined. The expressions of GATA3 and ROR- γ t genes in OVA/OVA+ α -GC-liposome mice were significantly lower than in OVA/OVA, OVA/ α -GC-liposome and OVA/PBS mice (Fig. 5A, C). In contrast, the expression of the T-bet gene in CLNs from OVA/OVA+ α -GC-liposome mice was significantly higher than in any other group (Fig. 5B). No significant differences in the expressions of Foxp3 and TGF- β genes were observed among any groups (Fig. 5D, E). Expression of the V α -14 gene in CLNs was significantly higher in OVA/OVA+ α -GC-liposome and OVA/ α -GC-liposome mice than in OVA/OVA mice and OVA/PBS mice (Fig. 5F).

Reversed nasal symptoms and serum levels of specific immunoglobulins in OVA+ α -GC-liposome-treated CD1d^{-/-} mice

To investigate the contribution of iNKT cells to the symptomatic improvement and altered immunoglobulin profiles in OVA/OVA+ α -GC-liposome mice, CD1d^{-/-} mice were used for further experiments. We noted significantly increased sneezing and nasal-rubbing events in OVA/OVA+ α -GC-liposome CD1d^{-/-} mice than in OVA/OVA+ α -GC-liposome wild-type (WT) mice (Fig. 6A, B). The serum levels of OVA-specific IgE and IgG1 were increased, and the

control liposome mice instead of OVA/OVA mice. The serum levels of OVA-specific IgE (E), IgG1 (F), IgG2a (G), and IgA (H) were measured by ELISA. Data presented are the mean $\pm$ standard error of the mean of at least three independent experiments. N = 8–10 mice per group (A, B and E–H), N = 5–6 mice per group (C, D). ns: not significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001. PBS/PBS, mice received PBS and were administered PBS sublingually. OVA/PBS, mice received OVA and were administered PBS. OVA/OVA, mice received OVA and were administered OVA alone. OVA/ α -GC-liposome, mice received OVA and were administered α -GC-liposomes alone. OVA/OVA+ α -GC-liposome, mice received OVA and were administered OVA and α -GC-liposomes. OVA/OVA + vector control liposome, mice received OVA and were administered OVA and vector control liposomes (not containing α -GalCer).

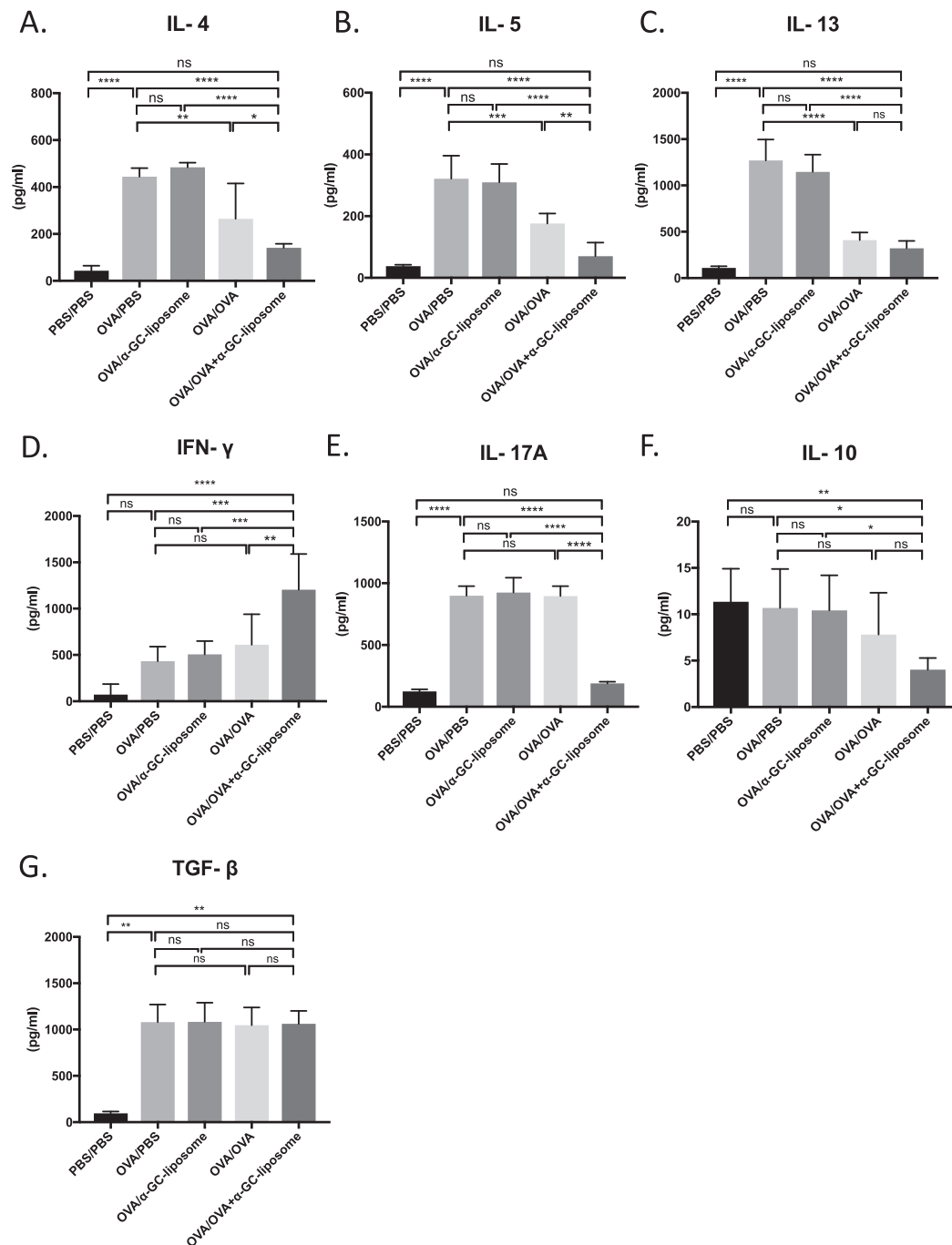


Fig. 4. Cytokine production by CLN cells. The levels of IL-4 (A), IL-5 (B), IL-13 (C), IFN-γ (D), IL-17A (E), IL-10 (F) and TGF-β (G) in the supernatants of CD4⁺ T cell cultures, sorted from CLN cells upon re-stimulation with 1 mg/ml of OVA. N = 8–10 mice per group. ns: not significant, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

levels of specific IgG2a were decreased, in the OVA/OVA+α-GC-liposome CD1d^{-/-} mice compared with OVA/OVA+α-GC-liposome WT mice (Fig. 6C–E). The difference in the levels of OVA-specific IgA between the two groups was not significant (Fig. 6F).

Reversed cytokine and gene expression profiles of CLN cells from OVA+α-GC-liposome-treated CD1d^{-/-} mice

The levels of IL-4, IL-5 and IL-17A in the culture supernatant of CD4⁺ T cells from CLNs were significantly higher in OVA/OVA+α-GC-liposome CD1d^{-/-} mice than in OVA/OVA+α-GC-liposome WT mice (Fig. 7A, B, E). The levels of IFN-γ in OVA/OVA+α-GC-

liposome CD1d^{-/-} mice were significantly lower than in OVA/OVA+α-GC-liposome WT mice (Fig. 7D). No significant differences in the levels of IL-13, IL-10, and TGF-β were observed between CD1d^{-/-} mice and WT mice (Fig. 7C, F, G). The gene expressions of GATA3 and ROR-γt in CLN cells obtained from OVA/OVA+α-GC-liposome CD1d^{-/-} mice were significantly higher than those in OVA/OVA+α-GC-liposome WT mice (Fig. 7H, J). The gene expressions of T-bet and Vα-14 in CD1d^{-/-} mice administered OVA/OVA+α-GC-liposomes were significantly lower than in WT mice (Fig. 7I, M). There were no significant differences in the gene expressions of Foxp3 and TGF-β between CD1d^{-/-} mice and WT mice (Fig. 7K, L).

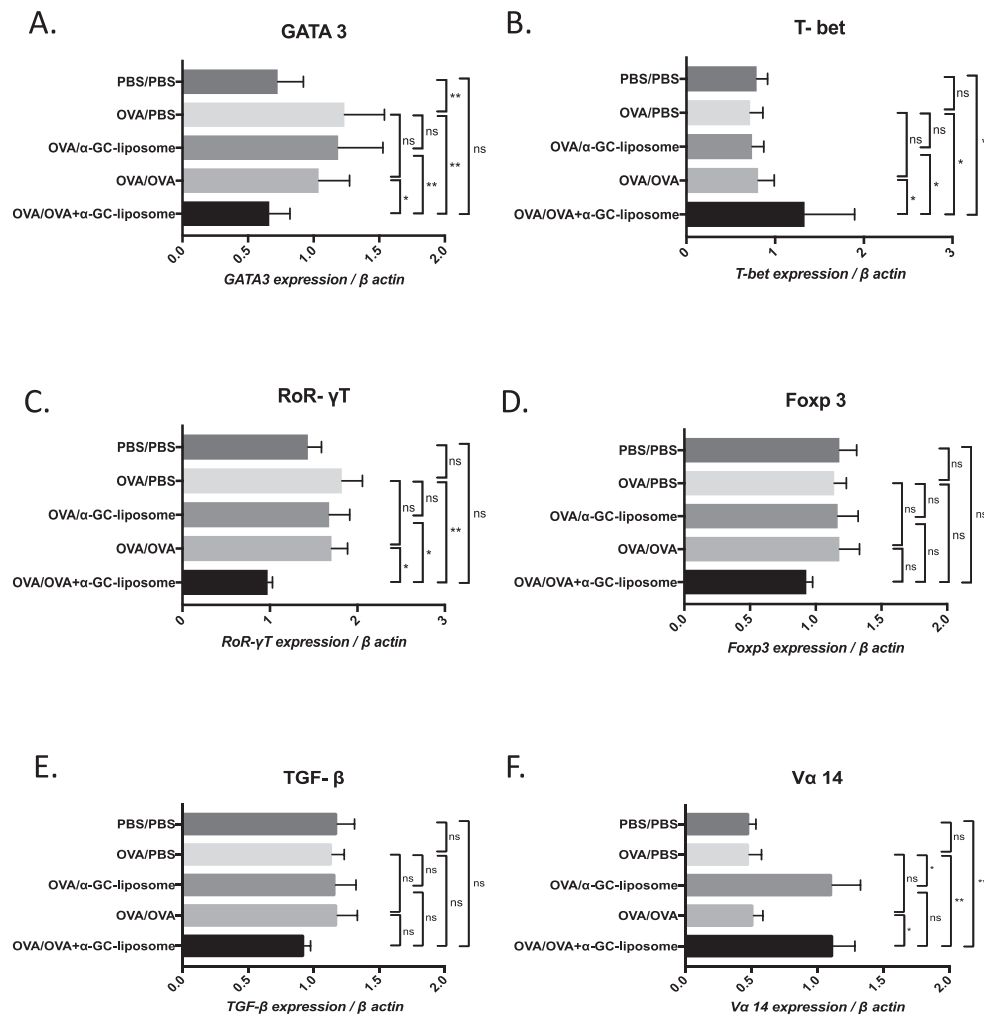


Fig. 5. Gene expressions of CLN cells. The relative gene expressions of GATA3 (A), T-bet (B), ROR- γ t (C), V α -14 (D), Foxp3 (E) and TGF- β (F) of the CLN cells after 72 h upon re-stimulation with of 1 mg/ml of OVA evaluated by quantitative RT-PCR. N = 8–10 mice per group. ns: not significant, *P < 0.05, **P < 0.01.

Discussion

This study was performed to develop an adjuvant capable of boosting the therapeutic efficiency and effectiveness of the sublingual administration of allergens. We examined the adjuvant effect of α -GC-liposomes via their concomitant administration with an oral mucosal administration of antigen after the onset of AR as a treatment model.

The sublingual administration of OVA alone for 1 week significantly inhibited the production of Th2 type cytokines from CD4⁺ T cells and the gene expression of GATA3 in CLN cells of mice with AR induced by OVA compared with those in non-treated AR mice; however, nasal symptoms were not significantly diminished. In contrast, the combined sublingual administration of α -GC-liposome as an adjuvant of OVA completely suppressed the nasal symptoms of AR mice, significantly upregulated IFN- γ production in CD4⁺ T cells and the gene expression of T-bet, and down-regulated IL-17A production and the gene expressions of ROR- γ t as well as Th2 type cytokine production and GATA3 expressions. The serum levels of specific IgE and IgG1 were inhibited, whereas specific IgG2a levels were increased. IgG2a is a Th1-associated immunoglobulin in mice and is associated with Th1-type inflammation, whereas IgG1 is a Th2-associated immunoglobulin.²⁸

Notably, the treatment with OVA plus vector control liposomes (not containing α GalCer) did not induce a significant improvement of symptoms and immunological parameters (data not shown), suggesting that liposomes (not containing α GalCer) alone do not have an adjuvant effect. Additionally, the nasal symptoms, cytokine production, immunoglobulin production and Th1-, Th2- and Th17-associated gene expressions were reversed in OVA plus α -GC-liposome-treated CD1d^{-/-} mice, showing that the sublingual administration of α -GC-liposomes as an adjuvant suppressed nasal allergic reactions by inducing Th1 cells and alleviating Th2 and Th17 cells via iNKT cell activation in the presence of antigen.

In our previous preliminary study, the direct administration of crude α -GalCer in addition to antigens to the sublingual mucosa failed to induce adjuvant effects in AR mice. We hypothesized that α -GalCer was being minimally taken up into the mucosa because it is not water soluble, so to effectively deliver α -GalCer into the mucosa and present it to iNKT cells, we administered α -GalCer-pulsed BMDCs with allergen by sublingual injection using a prophylactic model in our previous study.¹⁹ However, this injection method was considered not suitable for clinical use. Therefore, to improve the clinical usefulness of α -GC, the present study investigated the utility of α -GC-liposomes, which are easily dissolved in water, as an adjuvant for the sublingual administration of allergens

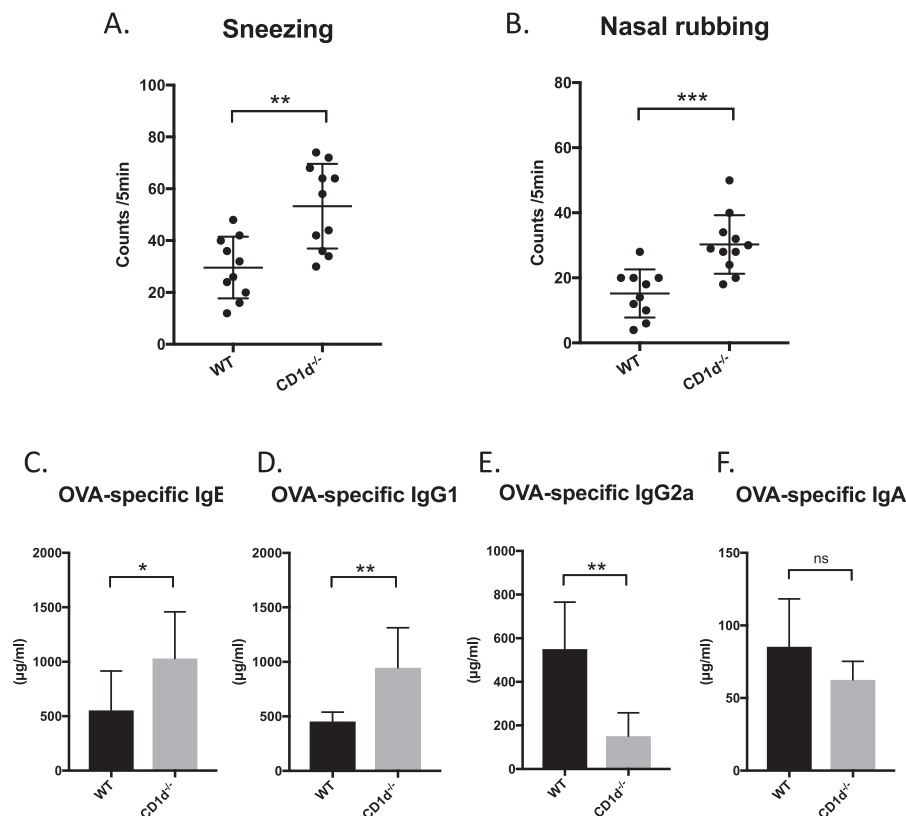


Fig. 6. Nasal symptoms and serum immunoglobulin levels in CD1d^{-/-} mice after re-challenge. The numbers of sneezing (A) and nasal-rubbing (B) events in wild type (WT) and CD1d^{-/-} mice were counted every 5 min from 10 min after re-challenge with OVA. The serum levels of OVA-specific IgE (C), IgG1 (D), IgG2a (E) and IgA (F) in WT and CD1d^{-/-} mice were measured by ELISA. N = 10–11 mice per group. ns: not significant, *P < 0.05, **P < 0.01.

in the treatment model. We originally tried to enclose the cedar pollen antigen into liposomes as well as α -GalCer for use in patients with AR induced by cedar pollen; however, this was unsuccessful because the purified antigen had a differential molecular weight and electrical charge from the allergen used in the current study, and thus might be not suitable for enclosing into liposomes. Therefore, α -GC-liposomes and antigen were separately and concurrently administered in this study.

The uptake of α -GC-liposomes following their sublingual administration was confirmed in CLN cells expressing CD11b and CD11c, indicating they were potential antigen presenting cells (APCs) including DCs. Immature APCs in the oral mucosa are thought to migrate to the regional CLNs.²⁴ Additionally, the induction of iNKT cells in CLNs was suggested by the increased V α 14 gene expression in CLN cells following the sublingual administration of α -GC-liposomes. However, no improvements in the nasal symptoms or any immunological changes were induced following the sublingual administration of α -GC-liposomes without allergen, suggesting that the adjuvant effects of α -GC-liposomes were exerted in the presence of an adequate concentration of allergen. The administration of α -GalCer and liposomes individually to humans have already been used.^{20,29,30} In the current study, we showed that the sublingual administration of α -GC-liposomes was a simpler and safer method compared with the injection of BMDCs pulsed with α -GalCer into the oral submucosa.

Although the precise immunological role of α -GalCer-activated iNKT cells as an adjuvant in allergic tolerance is still unclear, based on our current results and findings from previous work, we propose the following possible mechanisms. In oral mucosa, α -GC-liposomes might be taken up by DCs, after which the DCs move to

regional lymph nodes and present α -GalCer to iNKT cells. Stimulation by α -GalCer is thought to induce the massive and rapid production of IFN- γ and IL-12, induce the full maturation of DCs, upregulate the expressions of CD86, CD40 and MHC on DCs, induce strong Th1 responses to antigens, and exert a potent inhibitory effect on Th2 cell differentiation as well as on subsequent IgE production.^{7,10,13,31–33} It has also been suggested that iNKT cells can directly suppress Th2 cell function by inducing IFN- γ production and suppressing Th2 cytokine production in memory Th2 cells.³⁴ The current study showed that when α -GC-liposomes were used as an adjuvant with antigen, increased T-bet gene expression was observed, enhancing Th1-skewed responses. The transcription factor T-bet is required for Th1 differentiation and IFN- γ production, which inhibits Th2 cell recruitment^{35–37} and regulates IFN- γ -mediated IgG2a class switching in B cells.³⁸

IL-17 is involved in the development of autoimmunity³⁹ and the pathogenesis of allergic diseases such as asthma.⁴⁰ It was suggested that chronic airway inflammation was caused by Th2 cells and Th17 cells.^{41–43} The frequencies of IL-17⁺CD4⁺ cells or IL-17⁺ cells were significantly higher in the nasal mucosa of patients with AR than in control subjects⁴⁴ and correlated with the nasal symptoms.⁴⁴ The intranasal administration of anti-IL-17 Abs to mice with AR reduced the nasal symptoms and Th2 and Th17 responses in the nasal mucosa.⁴⁵ The orphan nuclear receptor (ROR- γ t) acts as a key transcription factor in the Th17 cell lineage-commitment process in mice.^{36,46} The current study showed that the production of IL-17A and the gene expression of ROR- γ t in CLN cells were inhibited in OVA/OVA+ α -GC-liposome mice. The combined administration of α -GC-liposomes with allergen induced IFN- γ production, which might inhibit the generation of Th17 cells.⁴⁷ To elucidate the precise

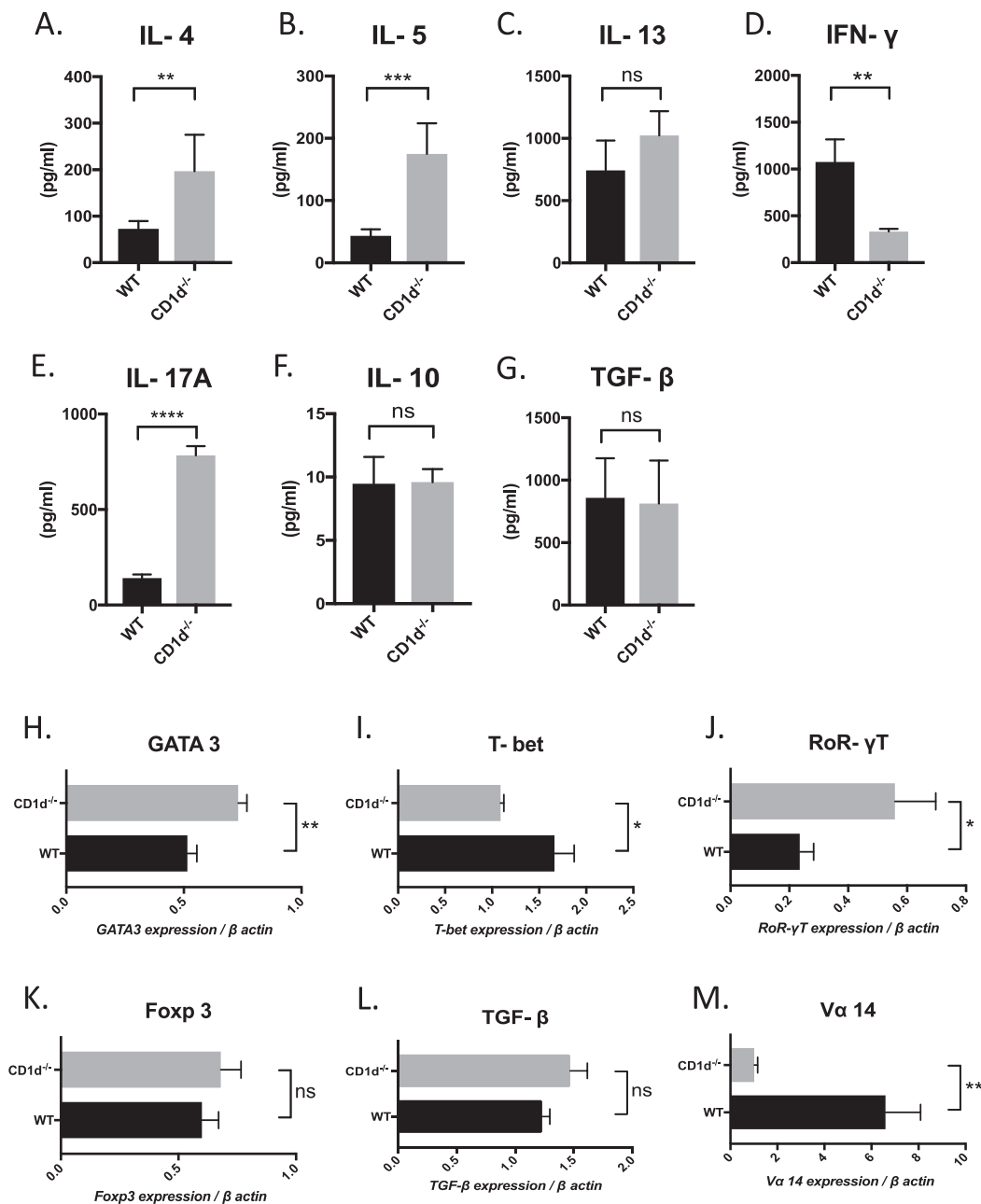


Fig. 7. Cytokine productions and gene expressions of CLN cells from CD1d^{-/-} mice. The levels of IL-4 (A), IL-5 (B), IL-13 (C), IFN-γ (D), IL-17A (E), IL-10 (F) and TGF-β (G) in the supernatants of CD4⁺ T cell cultures, sorted from the CLN cells of WT and CD1d^{-/-} mice, upon re-stimulation with 1 mg/ml of OVA. The relative gene expressions of GATA3 (H), T-bet (I), ROR-γt (J), Foxp3 (K), TGF-β (L) and Vα-14 (M) of CLN cells from WT and CD1d^{-/-} mice after 72 h of re-stimulation with 1 mg/ml of OVA evaluated by quantitative RT-PCR. N = 10–11 mice per group. ns: not significant, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

role of IL-17A in exerting a therapeutic effect and the mechanism of IL-17A suppression with the sublingual administration of α-GC-liposomes with antigens, further experiments using neutralizing antibodies or cytokine knockout mice will be needed.

It was reported that regulatory T cells (Tregs) involved in the efficacy of allergen immunotherapy.^{48–50} However, in this study, the increased expressions of immunosuppressive cytokines such as IL-10, TGF-β and genes such as Foxp3 and TGF-β were not observed in mice administered the combined administration of α-GC-liposomes and antigen as well as mice receiving antigen alone, suggesting that Tregs played no major role in the improvement of

symptoms and immunological changes at the early timepoint of the treatment or in this system.

Although this study was unable to detect a significant difference in pathological changes in the nasal mucosa between groups treated with or without α-GC-liposomes (data not shown), changes associated with the adjuvant effect of α-GC-liposomes were observed in the serum levels of immunoglobulins, gene expressions, and cytokine production profiles in CLN cells.

In conclusion, α-GC-liposomes are suitable for sublingual administration because they are readily taken up at mucosal sites and transferred into the regional lymph nodes to induce iNKT cell

activation. Our findings suggest that the sublingual administration of α -GalCer enclosed in liposomes, but not crude α -GalCer or liposomes alone, is a useful adjuvant for SLIT, characterized by the capacity to induce Th1 cells and to suppress Th2 and Th17 cells and the potential to relieve nasal allergic symptoms. This approach might increase the therapeutic efficacy and effectiveness of SLIT and could be a new therapeutic method. A clinical study should be performed to evaluate the safety and efficacy of sublingual immunotherapy with α -GC-liposomes in human subjects.

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Conflict of interest

EFK and YI are employees of REGIMMUNE. The rest of the authors have no conflict of interest.

Authors' contributions

SS, DS, TS, SY, YI and YOKA designed the study. SS and DS wrote the manuscript. TS, SY, YOKU, FI, TI, TH, EFK and TA checked data and performed the statistical analyses. TS, YOKU, FI, TI, TA, TH, EFK and YI contributed to data collection. YOKA reviewed the manuscript. All authors read and approved the final manuscript.

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