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Novel Toll-Like Receptor 9 Agonist Derived from *Cryptococcus neoformans* Attenuates Allergic Inflammation Leading to Asthma Onset in Mice

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Keywords
Asthma · *Cryptococcus neoformans* · Oligodeoxynucleotide · Th1 · Th2

Abstract
Introduction: The enhanced type 2 helper (Th2) immune response is responsible for the pathogenesis of allergic asthma. To suppress the enhanced Th2 immune response, activation of the Th1 immune response has been an alternative strategy for anti-asthma therapy. In this context, effective Th1-inducing adjuvants that inhibit the development of allergic asthma but do not flare the side effects of the primary agent are required in clinical treatment and preventive medicine. ***Objective:*** In this study, we aimed to determine the regulation of the Th2 type immune response in asthma by a novel immunostimulatory oligodeoxynucleotide (ODN) derived from *Cryptococcus neoformans*, termed ODN112, which contains a cytosine-guanine (CG) sequence but not canonical CpG motifs. ***Methods:*** Using an ovalbumin-induced asthma mouse model, we assessed the effect of ODN112 on prototypical asthma-related features in the lung and on the Th1/Th2 profile in the lymph nodes and lung of mice treated with ODN112 during sensitization. ***Results and Conclusion:*** ODN112 treatment attenuated asthma features in mice. In the bronchial lymph nodes of the lungs and in the spleen, ODN112 increased interferon-γ production and attenuated Th2 recall responses. In dendritic cells (DCs) after allergen sensitization, ODN112 enhanced cluster of differentiation

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(CD) 40 and CD80 expression but did not alter CD86 expression. Interleukin-12p40 production from DCs was also increased in a Th2-polarizing condition. Our results suggest that ODN112 is a potential Th1-inducing adjuvant during Th2 cell differentiation in the sensitization phase.

Introduction

Recent developments in clustering analysis report that the major clinical phenotype of asthma that is associated with eosinophilia presents as the type 2 (Th2/T2)-high inflammatory "allergic asthma" endotype [1]. The enhanced type 2 helper (Th2) immune response is mediated by antigen-specific Th cells and type 2 innate lymphoid cells that produce Th2 cytokines, such as interleukin (IL)-4, IL-5, and IL-13 [1]. Thus, Th2 cytokines play a critical role in the induction of type 2 inflammation in allergic asthma such as eosinophilia, allergen-specific IgE production, and IgE-mediated mast cell and basophil degranulation [1].

The conceptual balancing of Th1/Th2 as a therapeutic strategy for the clinical remission of allergic asthma led to the use of Th1-inducing adjuvants in realistic anti-asthma therapy [2]. Administration of live or heat-killed bacteria, such as *Mycobacterium bovis* [3], *Francisella tularensis* [4], or *Listeria monocytogenes* [5], suppresses the allergic Th2 responses through the induction of interferon (IFN)-γ-mediated Th1-like immune response. Pathogen-associated molecular patterns derived from microbes, such as cell wall components and purified proteins of mycobacteria [6], high levels of endotoxins [7], or immunostimulatory (ISS) oligodeoxynucleotide (ODN) [8], interact with pattern recognition receptors of immune cells, leading to a robust in vivo Th1 response.

Toll-like receptor (TLR) 9, a member of the TLR family, is expressed inside immune cells such as B cells and dendritic cells (DCs) of humans and mice and recognizes the unmethylated CpG DNA of bacteria and viruses [9]. TLR9 agonists have enormous potential as Th1-inducing adjuvants in protection against allergic disease [10]. TLR9 activation by specific ISS-DNA sequences rich in non-methylated CpG motifs such as 5′-purine-purine-cytosine-guanine-pyrimidine-pyrimidine-3′ (CpG-ODN) induces a strong Th1 immune response with IFN-γ induction [11]. The immunologic activities of CpG-ODN are dependent on the content of their palindromic hexamer [8]. Of the 3 major classes of CpG-ODN, the B-class CpG-ODN strongly induces DC maturation [12] and attenuates Th2 immune response through IL-12 induction, followed by IFN-γ production [13]. In some clinical trials, the B-class CpG-ODN attenuates Th2 immune response by balancing Th1/Th2 in allergy [14]. However, concerns exist about the undesirable side effects of repeated administrations of CpG-ODNs with a phosphorothioate backbone [15]. Heikenwalder et al. [15] reported that daily injection of CpG-ODN suppressed follicular DCs and germinal center B lymphocytes in lymphoid follicles and reduced primary humoral immune responses and immunoglobulin class switching. Therefore, there is a need to explore more practical Th1-inducing adjuvants that can inhibit the development of allergic asthma, without inducing side effects.

Cryptococcus neoformans is an opportunistic fungal pathogen, frequently associated with fatal meningoencephalitis in immunocompromised patients such as those with acquired immunodeficiency syndrome and organ transplantation [16]. The outcome of *C. neoformans* infection is dependent on the balance between Th1 and Th2 immune responses in vivo. A predominance of Th1 over Th2 type immune response leads to protection against *C. neoformans* infection [17], while Th2 immune predominance such as eosinophilia or elevated serum IgE increased susceptibility to cryptococcosis [18, 19]. Excess polarization of Th1 or Th2 might be associated with undesired effects in patients. In this context, adjuvants that have controllable and optimized effects on the Th1/Th2 balance for asthma prevention are required. Namely, ODNs having certain Th1-inducing activity as well as an unmodified backbone in order to not to leave it in vivo more than necessary could be a viable candidate.

In host defense against *C. neoformans*, the role of TLR9 in detecting the pathogenic DNA plays an important role in fungal clearance from the lungs through IL-12p40 induction [20]. A previous study by our group demonstrated that the presence of a certain CpG-independent mechanism is involved in TLR9-mediated immune activation by *C. neoformans* DNA [20]. We also demonstrated that a 24-base ODN fragment (termed ODN112) with an unmodified backbone of the *URA5* gene that encodes a virulent component of *C. neoformans* induces a robust IL-12p40 synthesis by DCs in a TLR9-dependent manner [21]. This evidence increases the possibility that a novel TLR9 agonist derived from *C. neoformans*, ODN112, could be a candidate Th1-inducing immune adjuvant for inhibiting Th2 in allergic asthma. In this study, we explore the potent inhibitory effect of ODN112 on allergic airway inflammation using the ovalbumin (OVA)-induced asthma mouse model.

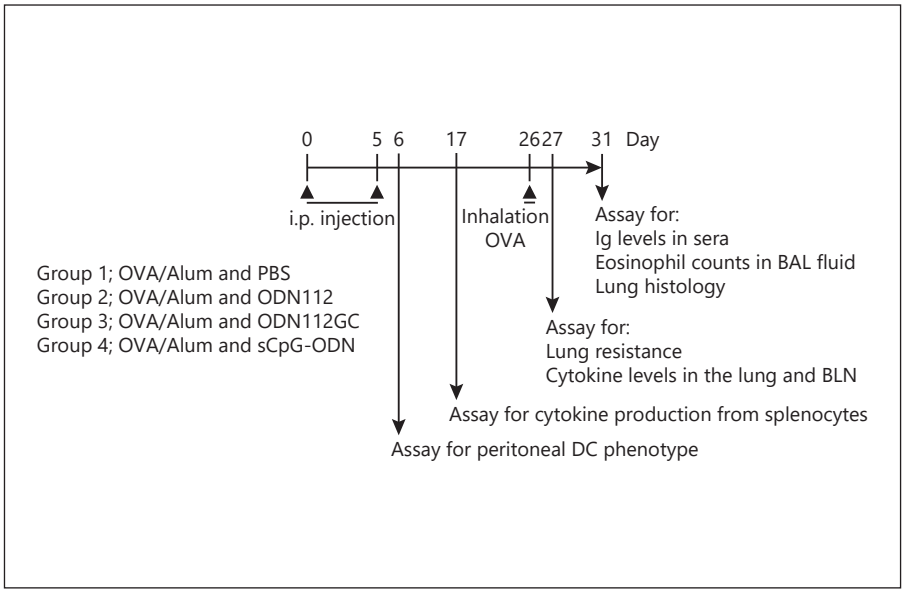

Fig. 1. Schematic figure illustrating the experimental design of the study. Mice were sensitized with intraperitoneal injections of OVA and ODNs adsorbed with aluminum hydroxide on days 0 and 5. On day 26, the mice were challenged with aerosolized OVA for 1 h on 2 occasions, 4 h apart. The phenotype of the DCs in the peritoneal cavity and the cytokine production from splenocytes were evaluated on days 6 and 17, respectively. After the OVA inhalation, lung resistance and cytokine levels in the lung and BLN were evaluated on day 27. On day 31, asthma-related features such as Ig levels in sera, eosinophil counts in BAL fluids, and lung histology in mice treated with PBS or ODNs were evaluated. ▲, time points of sensitization or inhalation; OVA, ovalbumin; ODN, oligodeoxynucleotide; DC, dendritic cell; BAL, bronchoalveolar lavage; BLN, bronchial lymph node.

Table 1. Sequences of synthesized ODNs

ODN (length [bases])	Sequence (5′–3′)
ODN112 (24)	CTATGGTCGGTGCGCCTCTCAAGG
ODN112GC (24)	CTATGGTGCGTGCGCCTCTCAAGG
sCpG-ODN (20)	TCCATGACGTTCCTGACGTT

ODN, oligonucleotides; sCpG, CpG phosphorothioate-ODN1826.

Materials and Methods

Mice

Female C57BL/6 mice (CLEA Japan, Inc., Osaka, Japan) were maintained in specific pathogen-free conditions at the Institute for Animal Experimentation, Tohoku Medical and Pharmaceutical University (Sendai, Japan).

Oligonucleotides

ODN112, a 24-base ODN fragment of the *URA5* gene from *C. neoformans*, designated Cap67 (a kind gift from Stuart M. Levitz, Boston University, Boston, MA, USA), and a prototypic phosphorothioated CpG1826 ODN (CpG-ODN), as shown in Table 1, were synthesized and purified by high-performance liquid chromatography at Hokkaido System Science (Sapporo, Japan). A modified ODN112 derivative, in which CG was replaced by GC within 5′-GTCGGT-3′, termed ODN112GC, was also synthesized at Hokkaido System Science (Table 1). In the present study, CpG-ODN was used as a positive control for the treatment. In addition, ODN112GC was used as a negative control for the treatment with ODN112. We evaluated the effects of ODN treatment, compared to vehicle treatment.

Sensitization and Antigen Challenge

Six-week-old mice were sensitized with intraperitoneal injections of 8 μg OVA (Grade V; Sigma-Aldrich, St Louis, MO, USA) and oligonucleotides adsorbed with 4 mg aluminum hydroxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 500 μL saline on days 0 and 5. On day 26, the mice were challenged with aerosolized OVA (0.5% in saline) for 1 h on 2 occasions, 4 h apart [22]. The experimental design of the study, including the time points of sensitization, inhalation, and sampling, is indicated in Figure 1.

Measurement of Airway Hyperresponsiveness

Lung resistance was measured for 3 min under each condition by the Resistance and Compliance System (Finepoint; Buxco Electronics, Sharon, CT, USA) [23]. The conditions analyzed were baseline response to aerosolized saline and increasing doses (1.25, 2.5, 5, 10, and 20 mg/mL) of acetyl-β-methylcholine (methacholine; Sigma-Aldrich).

Measurement of OVA-Specific Antibodies

Serum levels of OVA-specific IgE and IgG1 antibodies were measured by ELISA [24]. In brief, microtiter plates (Nunc A/S Roskilde; Thermo Fisher Scientific, Denmark) were coated with 10 μg/mL OVA in 0.05 M bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After blocking with PBS containing 1% bovine serum albumin, serum samples diluted with 1% bovine serum albumin PBS were added to the well. Pooled serum of sensitized C57BL/6 female mice was used as a reference. Horseradish peroxidase-conjugated goat anti-mouse IgE (Bethyl Laboratories, Owing Mills, MD, USA) and IgG1 antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) diluted to 1:2,500 were used as detection antibodies. The concentrations of IgE and IgG1 were determined based on the absorbance at 450 nm.

Preparation of the BAL Fluids

Bronchoalveolar lavage (BAL) fluids were prepared as previously described [25]. Briefly, BAL samples collected on day 5 after

OVA inhalation (with 2 × 0.25 mL chilled PBS through a cannula inserted in the trachea) were centrifuged at 450 *g* for 10 min at 4°C. Cells (2×10^5) were stained with Diff-Quik solution (Sysmex Co., Kobe, Japan), and cell differential percentage was determined by counting a minimum of 200 cells by light microscopy.

Lung Histology

Lungs were isolated from mice at the indicated time points after OVA challenge, fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Sections were cut at a thickness of 4 μm and then stained with hematoxylin and eosin, periodic acid-Schiff (PAS), or Masson's trichrome staining. Eosinophil counts were estimated as the number of eosinophils per square millimeter, closely surrounding the bronchus. Mucin production was estimated as the proportion of PAS-positive cells in the total airway epithelium of bronchioles by PAS staining. The proportions of collagen fibers in peribronchial regions were evaluated by Masson's trichrome staining. The number of eosinophils and PAS-positive cells were calculated in each of the 5 random bronchioles in 3 lung sections from each mouse [26].

Preparation of the Lung Homogenate

For RT-PCR, the entire lungs were excised from sensitized mice 1 day after OVA challenge and homogenized in buffer RLT (QIAGEN, Valencia, CA, USA) supplemented with 1% 2-mercaptoethanol. For cytokine assay, the entire lungs were excised 1 day after OVA inhalation and homogenized in chilled 0.1% Triton-X PBS with 1% protease inhibitor (Sigma-Aldrich). After centrifugation at 15,000 *g* for 15 min at 4°C, the supernatants were stored at −80°C [25].

RT-PCR Analysis

Total RNA was extracted from entire lung homogenates using a ReliaPrep RNA Cell Miniprep system (Promega Corporation, Madison, WI, USA) or RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa Bio Inc., Otsu, Japan). Real-time RT-PCR was performed using gene-specific primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and a StepOnePlus Real-Time PCR System (Applied Biosystems) [25]. The primer sequences used for amplification are shown in online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000508535. The expression levels of target genes and hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) as a reference gene were calculated for each sample using the reaction efficiency, as determined by performing amplifications using standards.

Measurement of Cytokine Concentration

Levels of IL-4, IL-5, IL-13, and IFN-γ were assayed using ELISA Kits (eBioscience, San Diego, CA, USA). The detection limits were 4 pg/mL for IL-4, IL-5, and IL-13; and 15 pg/mL for IFN-γ. Total protein levels of the lung homogenates were assayed using a detergent-compatible protein assay kit (Bio-Rad Laboratory, Hercules, CA, USA). The cytokine and chemokine concentrations in the lung were adjusted for the protein level of each lung [25].

Preparation of Lung WBCs

Mice were sacrificed 1 day after OVA challenge. Pulmonary leukocytes were prepared as previously described [27]. Briefly, the lung vascular bed was flushed with 5 mL chilled saline that was injected into the right ventricle. The entire lungs were teased through a 40-μm cell strainer (BD Falcon, Bedford, MA, USA) and incubated in RPMI 1640 medium (Nakarai Tesque, Kyoto, Japan) with 10% fetal calf serum (FCS; Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin G, 100 μg/mL streptomycin, 10 mM HEPES, and 2 mM L-glutamine, containing 20 U/mL collagenase D and 1 μg/mL DNase I (Roche Diagnostics GmbH, Mannheim, Germany). After incubation for 60 min at 37°C with vigorous shaking, cells were resuspended in 4 mL of 40% (v/v) Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 mL of 80% (v/v) Percoll. After centrifugation at 600 *g* for 20 min at 15°C, cells at the interface were collected.

Preparation of Peritoneal Exudate Cells

One day after sensitization, peritoneal exudate cells were collected by washing the peritoneal cavity with 10 mL of cold 10% FCS RPMI medium, as previously described [28].

Flow Cytometric Analysis

Lung WBCs were diluted to a density of 2×10^5/100 μL and cultured with 5 ng/mL of phorbol 12-myristate 13-acetate (Sigma-Aldrich), 500 ng/mL of ionomycin (Calbiochem, San Diego, CA, USA), and 2 μM of monensin (Sigma-Aldrich) for 4 h at 37°C before the cell surface was stained. Then, cells were pre-incubated with anti-FcγRII and III mAb (Clone 93; BioLegend, San Diego, CA, USA) on ice for 15 min in PBS containing 1% FCS and 0.1% sodium azide and stained with allophycocyanin (APC)/Cy7 or peridinin-chlorophyll protein complex-conjugated anti-cluster of differentiation (CD) 3 (Clone 17A2; BioLegend), phycoerythrin (PE) or fluorescein isothiocyanate-conjugated anti-CD4 (Clone GK1.5; BD Biosciences, San Jose, CA, USA), peridinin-chlorophyll protein complex -conjugated anti-CD8α (Clone 53-6.7; BioLegend), and APC-conjugated anti-CD25 (Clone 3C7; BioLegend). Cells were then incubated in the presence of Cytofix/Cytoperm (BD Biosciences Pharmingen), washed twice in BD perm/wash solution, and stained with PE-conjugated anti-IL-4 (Clone 11B11; BioLegend) or Foxp3 (Clone FJK-16s; Thermo Fisher Scientific). Cells in peritoneal lavage fluid were pre-incubated with anti-FcγRII/ III mAb (BioLegend) and stained with APC-conjugated anti-CD11c (Clone N418; BioLegend), PE-conjugated anti-I-A/I-E (Clone M5/114.15.2; BioLegend), PE/Cy7-conjugated anti-CD40 (Clone 3/23; BioLegend), fluorescein isothiocyanate-conjugated anti-CD80 (Clone 16-10A1; BioLegend), and APC/Cy7-conjugated anti-CD86 (Clone GL-1; BioLegend). Dead cells were excluded by 7-AAD staining (BioLegend) and viable cells were gated. The positive populations were defined based on isotype-matched control IgG for each antibody. Peritoneal DCs were gated as $CD11c^{high}$ $I\text{-}A/I\text{-}E^{high}$ cells. The stained cells were analyzed using a BD FACSAria II cell sorter (BD Biosciences) or BD FACSCant II flow cytometer (BD Biosciences).

Cell Preparation and Stimulation

Bronchial lymph nodes (BLNs) were obtained from mice 1 day after OVA challenge, as previously described [29]. To evaluate T-cell responses induced by sensitization, spleens were excised from sensitized mice before OVA inhalation. BLNs and spleens were teased apart between 2 ground glass slides and washed. BLN cells (4×10^5 cells/well) were cultured in the presence of 10 μg/mL OVA for 3 days. Spleen cells (4×10^5 cells/well) were cultured in the presence of 100 μg/mL OVA for 2 days.

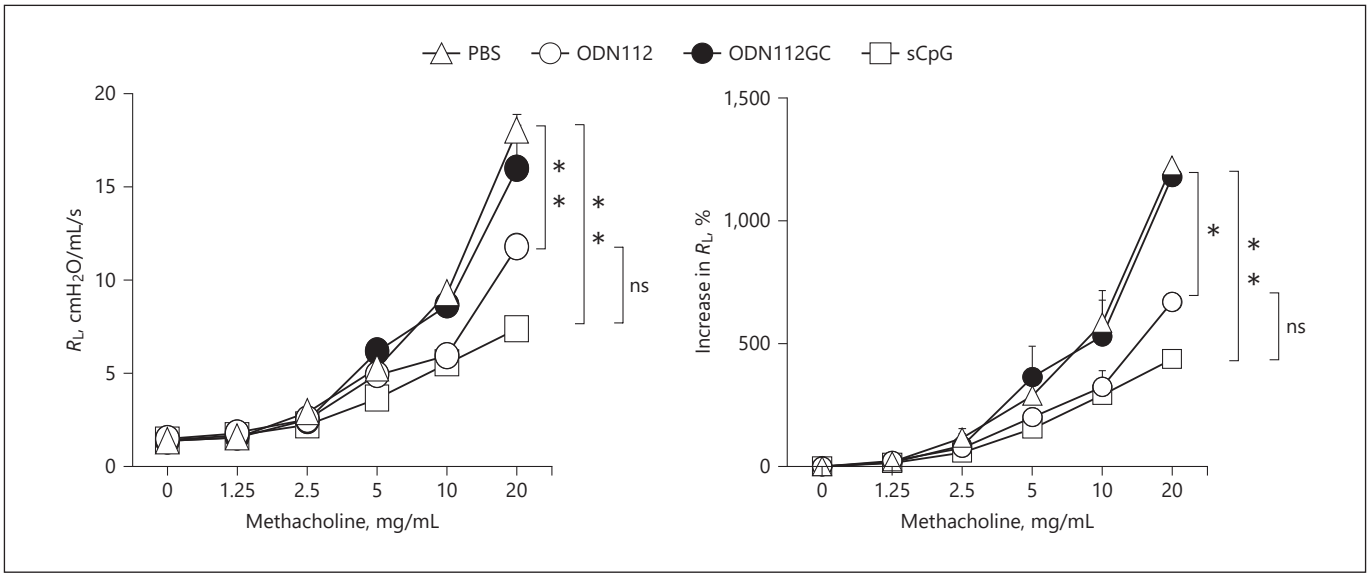

Fig. 2. ODN112 reduces lung resistance in asthmatic mice. The lung resistance was estimated by maximum values of R_L in response to inhaled methacholine or vehicle (left), and the change from baseline values of R_L in response to methacholine (right) in mice treated with vehicle or 10 μg ODNs was measured 1 day after OVA challenge. Data are shown as the mean ± SEM based on at least 3 independent experiments (n = 5–14). Δ, vehicle-treated mice; ○, ODN112-treated mice; ●, ODN112GC-treated mice; □, CpG-ODN-treated mice. * $p < 0.05$, ** $p < 0.01$ compared to vehicle-treated mice; ns, not significant; ODN, oligodeoxynucleotide; OVA, ovalbumin.

Preparation and Culture of DCs

Bone marrow-derived DCs (BM-DCs) were prepared as described previously [20]. In brief, bone marrow cells from WT mice were cultured at a density of 2×10^5 cells/mL in 10 mL RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin G, 100 μg/mL streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol, containing 20 ng/mL murine granulocyte-macrophage colony-stimulating factor (Wako Pure Chemical Industries). On day 8, the non-adherent cells were harvested and used as BM-DCs. The BM-DCs were stimulated at 1×10^5 cells/mL for 24 h at 37°C in 5% CO_2 with oligonucleotides in the presence of maturation factors such as IL-1β (10 ng/mL; PeproTech Inc., Rocky Hill, NJ, USA), tumor necrosis factor-α (50 ng/mL; PeproTech Inc.), and prostaglandin E_2 (10^{-6} M; Sigma-Aldrich) for the induction of Th2-oriented immune responses in DCs [30]. LPS prepared from *Escherichia coli* O-111 (Sigma-Aldrich) was used as a control for the stimulation of BM-DCs, and polymyxin B (Sigma-Aldrich) was used to neutralize the effects of LPS.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Differences between 2 groups were tested using a 2-tailed analysis and an unpaired Student's t test. Differences among 3 groups or more were tested using ANOVA with a post hoc analysis (Tukey's multiple comparison test). A p value of <0.05 was considered significant.

Results

ODN112 Attenuates Prototypical Asthma-Related Features

Airway hyperresponsiveness (AHR) was estimated by maximum values of R_L in response to inhaled methacholine or vehicle and the change from baseline values of R_L. While the lung resistance in response to vehicle was not significantly different among the groups, the lung resistance in response to inhaled methacholine 1 day after OVA inhalation was attenuated by the treatment with ODN112 and CpG-ODN, whereas ODN112GC did not alter the increased lung resistance (Fig. 2). ODN112 did not significantly alter the expression of M1 and M3 muscarinic acetylcholine receptors and β2-adrenergic receptors, which are directly related to airway contraction and relaxation in the lung (online suppl. Fig. 1). We next evaluated the effect of ODN treatment on IL-4-directed Ig class switching. The treatment with ODN112 and CpG-ODN significantly reduced allergen-specific IgE (Fig. 3a) and IgG1 (Fig. 3b) levels in sera compared to those in vehicle-treated mice; however, ODN112GC did not significantly change the sera levels post OVA inhalation (Fig. 3).

We investigated the effect of ODN112 treatment on eosinophilic inflammation following allergen challenge

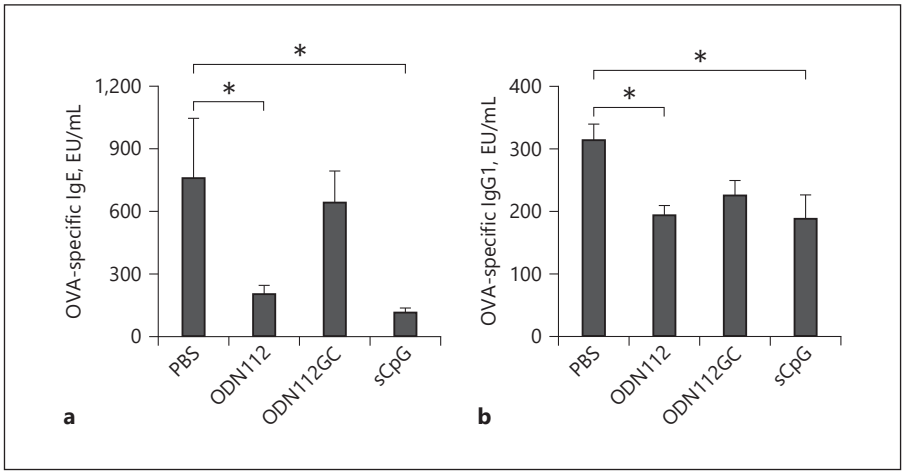

Fig. 3. ODN112 attenuates antigen-specific Th2-type immunoglobulin production in asthmatic mice. Sera were collected from mice treated with vehicle or 10 μg ODNs, 5 days after OVA challenge. OVA-specific IgE (**a**) and IgG1 (**b**) levels in sera were measured by ELISA. Data are shown as the mean ± SEM based on 2–3 independent experiments ($n = 11–13$). * $p < 0.05$ compared to vehicle-treated mice. Th2, type 2 helper; ODN, oligodeoxynucleotide; OVA, ovalbumin.

by comparing the number of inflammatory cells in BAL fluid of mice 5 days after OVA challenge. The number of eosinophils in BAL fluid was significantly lower in mice treated with 10 μg ODN112, 100 μg ODN112, and CpG-ODN, but not with ODN112GC, than that in mice treated with vehicle. The number of total cells, mononuclear cells, neutrophils, and lymphocytes did not change significantly among each treated group (Fig. 4a, b). In accordance with this observation, eosinophil infiltration in the peribronchial area was reduced in mice treated with ODN112 and CpG-ODN, but not with ODN112GC, compared to mice treated with vehicle (Fig. 4c). Although the eosinophil number in the BAL fluid of 100 μg ODN112-treated mice was lower than that in mice treated with 10 μg ODN112 (Fig. 4b), RBCs were observed in the BAL fluid of mice treated with 100 μg ODN112 (data not shown).

ODN112 Attenuates MUC5AC mRNA Production but Not Goblet Cell Hyperplasia in Asthma

We next evaluated goblet cell hyperplasia in airway epithelial cells in mice at various time intervals after OVA inhalation. The goblet cell number slightly increased on day 1 and significantly increased on days 3 and 5 (Fig. 5a). We measured the number of goblet cells in the airway epithelium on day 5 post OVA inhalation to assess mucus production by ODN112 treatment during the sensitization phase. Treatment with CpG-ODN significantly reduced mucus production in asthmatic mice (Fig. 5b). A similar tendency was observed in mice treated with ODN112, but not ODN112GC, although the difference between vehicle- and ODN112-treated mice did not reach significance at the time points examined (Fig. 5b).

MUC5AC mRNA expression in the lung 1 day after OVA inhalation was significantly reduced in mice treated with ODN112 and CpG-ODN, but not ODN112GC (Fig. 5c). MUC5B and MUC2 expression in the lung was not statistically different between vehicle- and ODN112-treated mice, or vehicle- and ODN112GC-treated mice after allergen inhalation (Fig. 5c). CpG-ODN significantly enhanced MUC5B mRNA expression but did not alter MUC2 expression in the lung after allergen inhalation (Fig. 5c). Thus, altered MUC5AC and MUC5B levels in mice treated with ODNs may reflect the histological goblet cell hyperplasia in the airway epithelium. On the other hand, the volume proportions of collagen fibers in the airway walls of mice treated with ODN112, ODN112GC, or CpG-ODN were not largely different when compared with the control group (Fig. 5d).

ODN112 Suppresses Th2 Cytokine Production and Enhances IFN-γ Production in Lungs and BLN

We further compared Th1 and Th2 cytokine levels in the lung among ODN-treated mice. ODN112 and CpG-ODN significantly reduced IL-4, IL-5, and IL-13 production in the lung compared with that in vehicle-treated mice, whereas the treatment with ODN112GC did not (Fig. 6a). In contrast, treatment with ODN112 and CpG-ODN, but not ODN112GC, enhanced IFN-γ production in the lung (Fig. 6a). Although the total number of $CD4^+$ T cells significantly increased in the lung of mice treated with ODN112 and CpG-ODN (Fig. 6b), the number of IL-4^+ $CD4^+$ and IL-4^+ $CD8^+$ T cells was significantly reduced with ODN112 and CpG-ODN 1 day after OVA inhalation (Fig. 6c). In contrast, the number of regulatory T cells, defined as $CD3^+CD4^+CD25^+$Foxp3 cells, was not signifi-

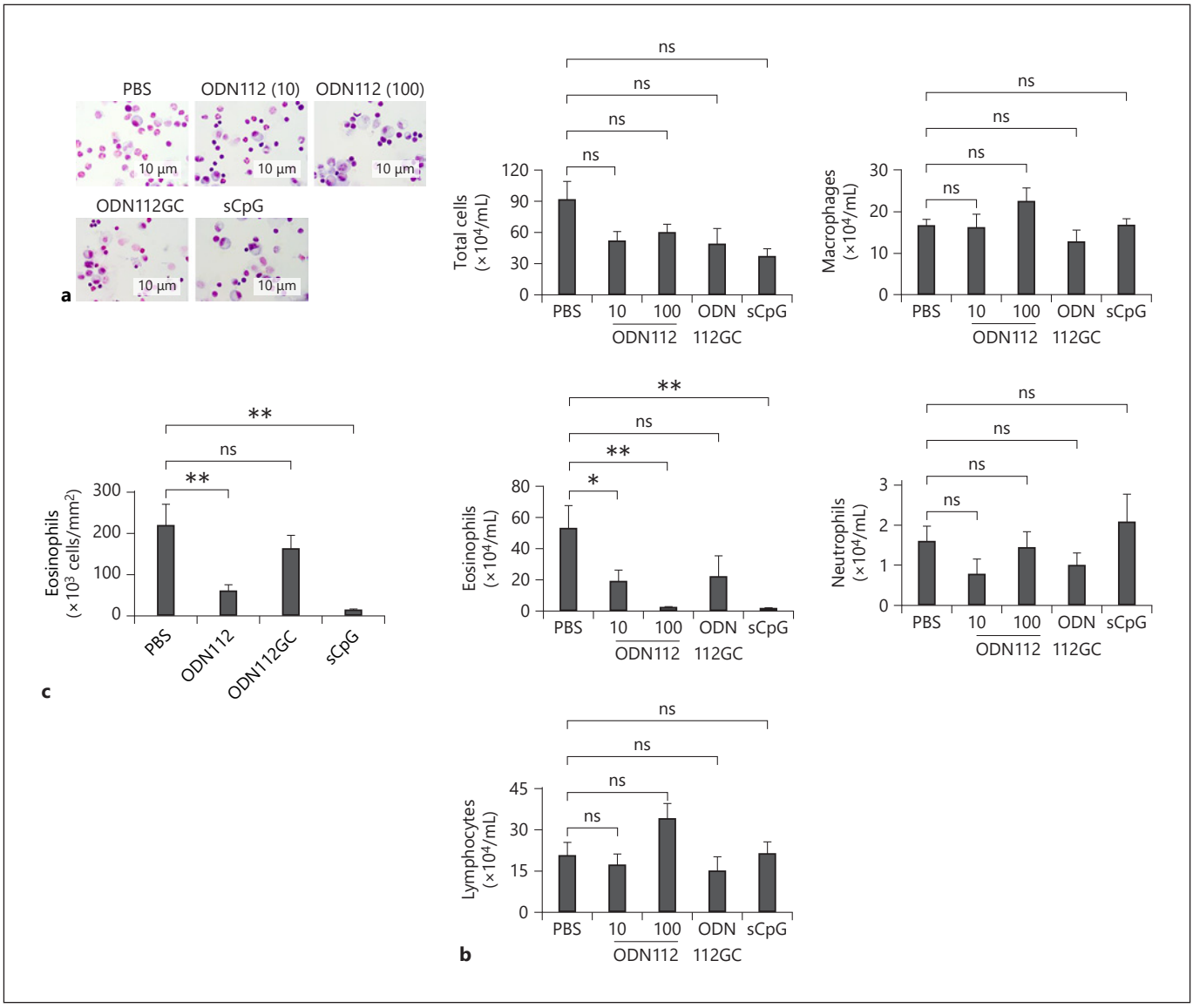

Fig. 4. ODN112 attenuates eosinophilic infiltration in the lungs of asthmatic mice. BAL and lung samples were collected on day 5 after OVA inhalation from mice treated with vehicle or 10 μg or 100 μg of ODNs. Cells in the BAL fluid were stained with Diff-Quik solution, and cell composition was determined under light microscopy. **a** Representative microscope photograph of cells in the BAL fluid of mice (original magnification ×1,000). Scale bar indicates 10 μm. **b** Cell composition in BAL fluid of mice. **c** The number of eosinophils per square millimeter closely surrounding the bronchi in mice. Data are shown as the mean ± SEM (n = 6–13 mice/group). * $p < 0.05$, ** $p < 0.01$ compared to vehicle-treated mice. ns, not significant; BAL, bronchoalveolar lavage; OVA, ovalbumin; ODN, oligodeoxynucleotide.

cantly different between mice treated with PBS and mice treated with ODNs (Fig. 6d). In BLN, IL-4, IL-5, and IL-13 were significantly reduced 1 day after OVA inhalation in mice treated with ODN112 and CpG-ODN, compared with vehicle. IFN-γ increased in the BLN of mice treated with ODN112 and CpG-ODN compared with vehicle (Fig. 6e). These results suggest that the attenuated Th2 cytokine production associated with increased IFN-γ in the mice lung treated with ODN112 may be responsible for the attenuated prototypical asthma-related features.

ODN112 Suppresses Allergen Sensitization

We measured Th2 cytokine production in the spleen to evaluate the T cell phenotypes produced during the

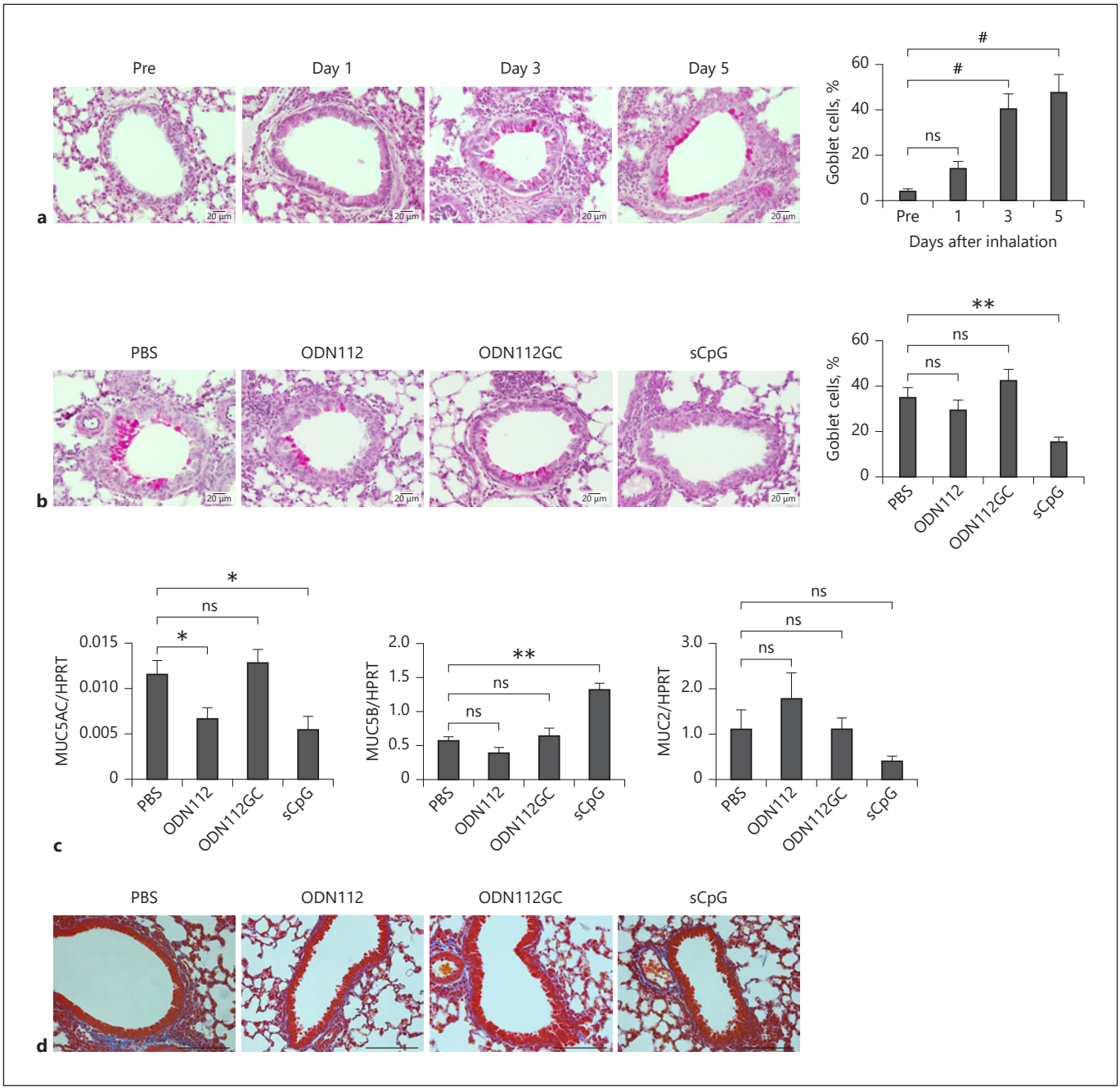

Fig. 5. ODN112 attenuates MUC5AC expression in the lung, but not the percentage of goblet cells in epithelial cells. **a** The lungs were excised from mice following OVA challenge. The percentage of goblet cells were evaluated by the proportion of PAS-positive cells in the total airway epithelium of the bronchioles. The photomicrographs were taken at ×400. Data are shown as the mean ± SEM of 5 random bronchioles in the 3 lung sections from each mouse (n = 4/group). **b** The lungs were collected from mice treated with vehicle or 10 μg ODNs 5 days after OVA challenge. The percentage of goblet cells in mice treated with 10 μg ODNs were compared to those in mice treated with vehicle. The photomicrographs were taken at ×400. Data are shown as the mean ± SEM of 5 random bronchioles in the 3 lung sections from each mouse (n = 9/group). **c** The lung was collected from mice treated with vehicle or 10 μg ODNs 24 h after OVA challenge. Gene expression levels were measured by qRT-PCR. Data are shown as the mean ± SEM of 4 mice. Experiments were repeated twice with similar results. **d** The volume of collagen fibers in the airway wall was evaluated by Masson's trichrome staining. Representative microscope photographs (original magnification ×400) of the staining are shown (n = 4/group). Experiments were repeated twice with similar results. $^{\#}p < 0.05$ compared to the levels before OVA inhalation. * $p < 0.05$, ** $p < 0.01$ compared to vehicle-treated mice. ns, not significant; ODN, oligodeoxynucleotide; OVA, ovalbumin.

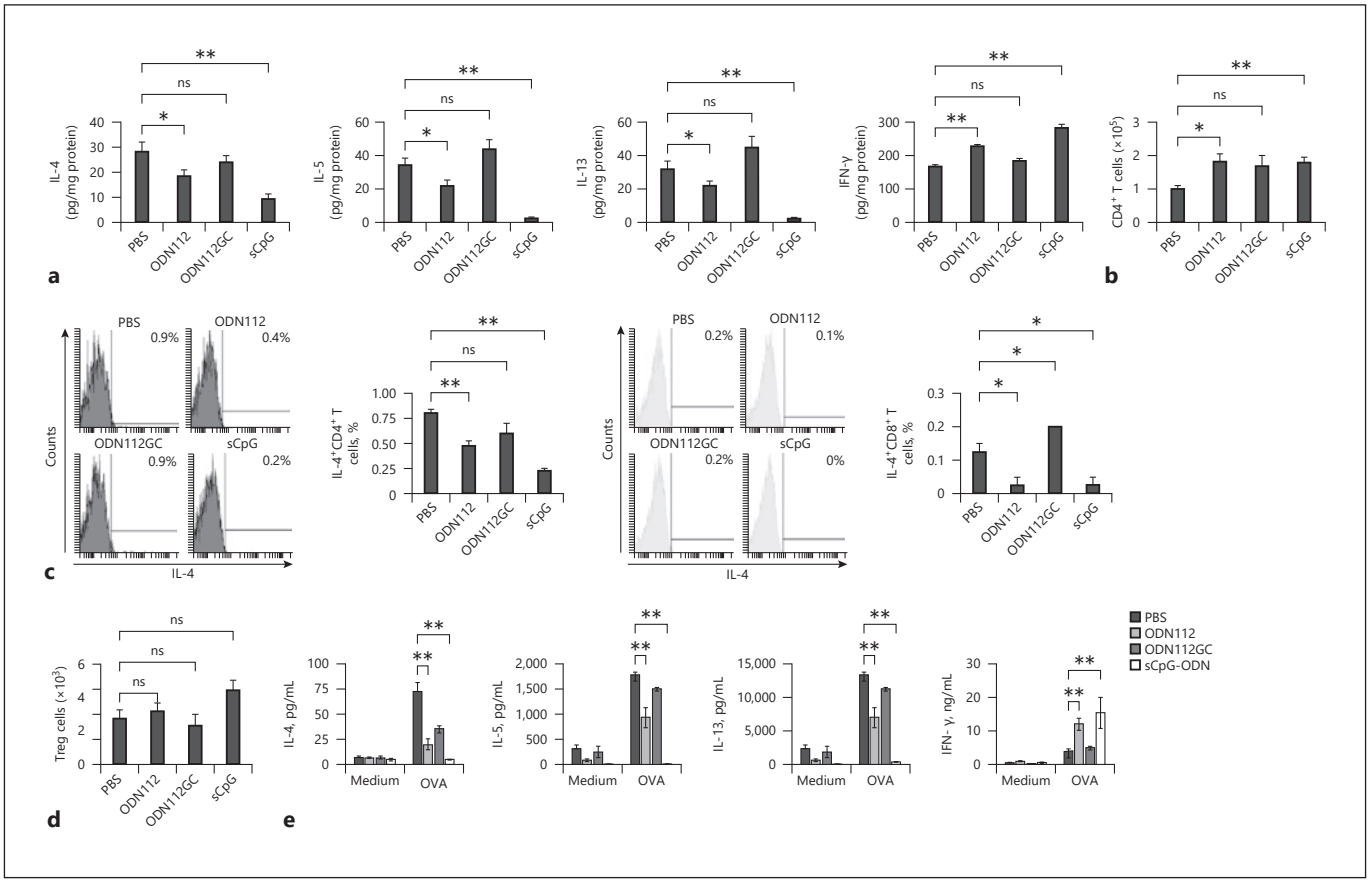

Fig. 6. ODN112 attenuates Th2 cytokine production and enhances IFN-γ production in the lung and BLNs. **a** The entire lungs from mice treated with vehicle or 10 μg ODNs were excised 1 day after OVA inhalation and homogenized. Cytokine levels in the lung were measured by ELISA. The number of $CD4^+$ T cells in the lung (**b**), representative profiles and the percentage of IL-4-producing T cells (**c**), and the number of regulatory T cells in the lung (**d**) were assayed by flow cytometry. Data are shown as the mean ± SEM based on 4–13 mice. **e** BLN cells were prepared from mice treated with vehicle or 10 μg ODNs 1 day after OVA challenge and stimulated with 10 μg/mL of OVA for 3 days. Cytokine levels in the culture supernatants were measured by ELISA. Data are shown as the mean ± SD based on triplicate cultures. Experiments were repeated twice with similar results. * $p < 0.05$, ** $p < 0.01$ compared to vehicle-treated mice. Th2, type 2 helper; IFN, interferon; BLN, bronchial lymph node; ODN, oligodeoxynucleotide; OVA, ovalbumin; IL, interleukin.

sensitization phase of ODN treatment. ODN112 and CpG-ODN significantly reduced IL-5 and IL-13 production from splenocytes stimulated with OVA, but not ODN112GC (Fig. 7). IL-4 production from splenocytes was undetectable level (<4 pg/mL, data not shown). In contrast, ODN112 and CpG-ODN significantly increased IFN-γ production from splenocytes stimulated with OVA. These results suggest that ODN112 and CpG-ODN may play an important role in the attenuation of Th2 cytokine production and the induction of IFN-γ production by modulating Th1/Th2 balance during Th cell differentiation in allergic sensitization.

ODN112 Increases CD40 and CD80 Expression, and IL-12p40 Production from DCs

DCs play a key regulatory role in the direction of T-cell differentiation through cytokine production and a specific co-stimulatory molecule expression. Therefore, to assess the effect of ODN112 on DC phenotype, we evaluated CD40, CD80, and CD86 expression on peritoneal DCs after the treatment and IL-12p40 production from Th2-oriented DCs stimulated with ODNs. CD40 and CD80 expression on DCs was significantly increased by the administration of ODN112 or CpG-ODN. In contrast, CD86 expression on peritoneal DCs was not altered by the coadministration of ODNs (Fig. 8a, b). ODN112

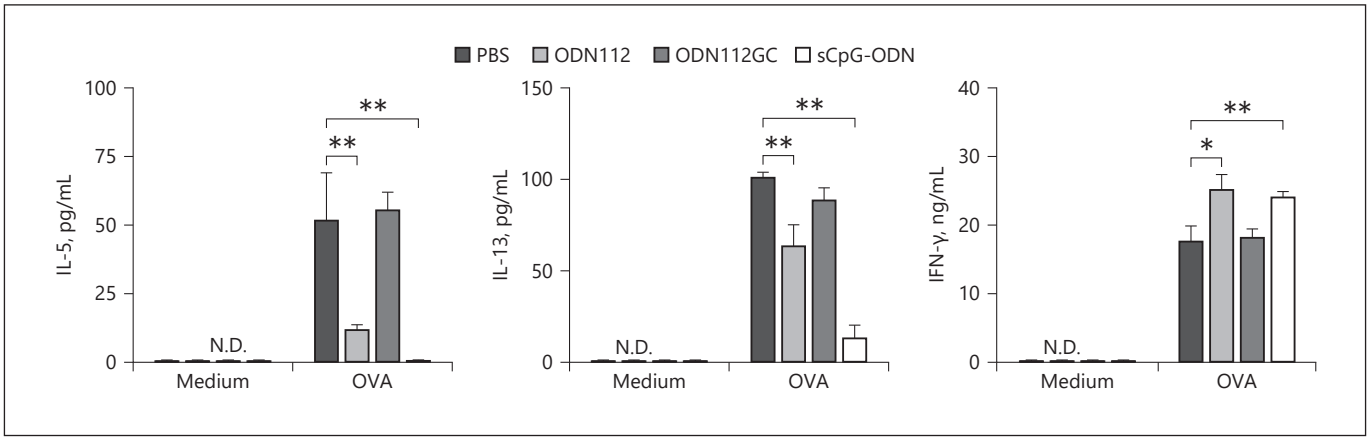

Fig. 7. ODN112 attenuates Th2 cytokine production and enhances IFN-γ production in the spleen. Mice were sensitized with OVA and aluminum hydroxide in the presence of vehicle or 10 μg ODNs. Twelve days after sensitization, splenocytes were prepared from each mouse and stimulated with 100 μg/mL of OVA for 2 days. Cytokine levels in the culture supernatants were measured by ELISA. Data are shown as the mean ± SD based on triplicate cultures. Experiments were repeated twice with similar results. * $p < 0.05$, ** $p < 0.01$ compared to vehicle-treated mice. Th2, type 2 helper; IFN, interferon; OVA, ovalbumin; ODN, oligodeoxynucleotide; IL, interleukin.

and CpG-ODN, but not ODN112GC, enhanced IL-12p40 synthesis from Th2-oriented BM-DCs (Fig. 8c). Such enhanced IL-12p40 production was not affected by the presence of polymyxin B, whereas the LPS-induced IL-12p40 production was significantly reduced in the presence of polymyxin B, suggesting that the IL-12p40 production after the stimulation with ODN112 or CpG-ODN was not induced by contaminated LPS in the ODNs (Fig. 8c).

Discussion

This study reports the first evidence of a novel eukaryotic TLR9 agonist containing a non-canonical CpG motif, 5′-GTCGGT-3′, in the suppression of allergic asthma. In the battle against *Cryptococcus* infection, the host innate immune system senses its DNA and induces a Th1 immune response for protection against the infection [20, 31], whereas the microorganism resists the host innate immune system by inducing a Th2 immune response to cryptococcal mannoproteins [32] or capsular polysaccharide glucuronoxylomannan [33]. In the present study, we used ODN112 derived from cryptococcal DNA as a tool for inducing a Th1 immune response in asthma. The main features of ODN112 treatment in our study are as follows: suppressed Th2 cytokine production by ODN112 administration during the sensitization phase; enhanced IFN-γ production in the lung and BLN after the onset of asthma-related features; significant reduction of IL-4$^+$ CD4$^+$ and IL-4$^+$ CD8$^+$ T cells in the lung; and attenuated allergen-induced asthmatic airway responses including AHR, mucus gene expression, antigen-specific immunoglobulin, and eosinophil accumulation in the airway. Furthermore, ODN112 also enhanced CD40 and CD80 expression and IL-12p40 synthesis by Th2-oriented DCs.

Enhanced AHR and airway remodeling including an increased volume of the airway smooth muscle, thickening of the basement membrane, and goblet cell hyperplasia are responsible for airway narrowing after allergen inhalation [34]. Of these features, ODN112 suppressed AHR but did not alter the other characteristics of airway remodeling, as evidenced by: (1) ODN112 attenuated the R_L value in response to inhaled methacholine after OVA inhalation, (2) ODN112 did not alter mRNA levels of M1 and M3 muscarinic acetylcholine receptors and β2-adrenergic receptors in the lung, and (3) ODN112 did not histologically alter the volume of collagen and mucus production in airway. Although further studies are required to determine whether ODN112 attenuates airway remodeling induced by repeated long-term allergen exposure, since the asthma mouse model is not sufficient for the evaluation of airway remodeling, our data suggest that attenuated AHR after treatment with ODN112 may not be attributable for its effect on relieving histological change of airways after allergen inhalation but rather its suppressive activity against Th2-type immune response. Therefore, verification of the treatment effect of ODN112 on the Th1/Th2 balance during the elicitation phase of asthmat-

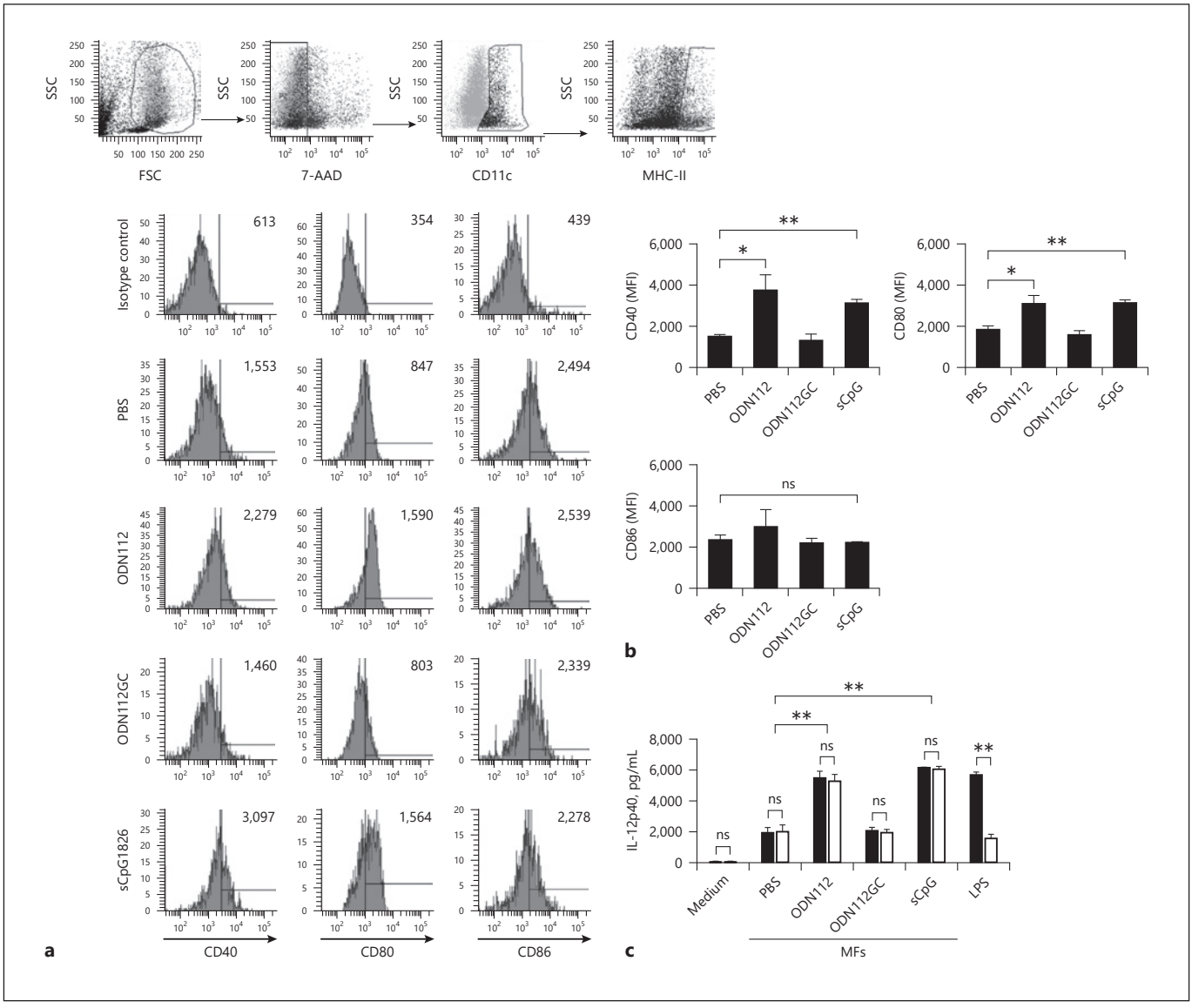

Fig. 8. ODN112 increases IL-12p40 production from Th2-oriented DCs. Mice were sensitized with OVA and aluminum hydroxide in the presence of vehicle or 10 μg ODNs. Peritoneal lavage fluid was collected 1 day after the sensitization, and the MFI of CD40, CD80, and CD86 on DCs was determined by flow cytometry analysis. **a** Representative profiles of co-stimulatory molecules on peritoneal dendritic cells. **b** MFI of co-stimulatory molecules was analyzed in each group. Data are shown as the mean ± SD of 3 mice. **c** Bone marrow-derived DCs were cultured with maturation factors and vehicle or ODNs in the presence or absence of polymyxin B. IL-12p40 level in the culture supernatants was measured by ELISA. Data are shown as the mean ± SD based on triplicate cultures. Experiments were repeated twice with similar results. ■, the cultures in the absence of polymyxin B; □, the cultures in the presence of polymyxin B. * $p < 0.05$, ** $p < 0.01$. ns, not significant; IL, interleukin; Th2, type 2 helper; DC, dendritic cell; OVA, ovalbumin; ODN, oligodeoxynucleotide; MFI, mean fluorescence intensity.

ic airway responses is further required. The salient difference between ODN112 and CpG-ODN is in its backbone. CpG-ODN contains a full phosphorothioate backbone, which prevents its degradation by DNase, thereby increasing the risk of excess immunological responses in vivo [15]. In contrast, the backbone of ODN112 is not modified because a phosphorothioate backbone completely abolished its effect on DC activation [21]. This feature might reduce the risk of excess immunological response. We found that ODN112 treatment with a dose 10 times higher than CpG-ODN is needed for similar suppressive effects on the eosinophil count in BAL fluid, suggesting that

unmodified ODN112 may be easily degraded in vivo. Furthermore, we previously showed that the concentration of IL-12p40 in the culture supernatant of BM-DCs stimulated with 30 μg/mL ODN112 was 2 times lower than that in the culture supernatant of BM-DCs stimulated with 1 μg/mL CpG-ODN [21]. Although the data regarding whether ODN112 is generally a weaker stimulator for TLR9 than sCpG-ODN are not adequately accumulated, the potency of ODN112 as a stimulator for TLR9 might be approximately 10–60 times lower than that of CpG-ODN. Further research regarding its delivery system and the degree of DNase resistance required for ODN112 stability in vivo is required. Horner and Raz [35] showed that ISS-ODN-conjugated allergen was more effective in inducing Th1-type immune response than ISS-ODN mixed with allergen. Encapsulating and sealing ISS-ODN inside nanoparticles may be also an effective method to protect ODN against breakdown by DNases [36]. Thus, improving intracellular delivery and binding of ODN112 with allergen are necessary to improve its efficacy as a Th1-inducing adjuvant in asthma treatment.

The sequence in ODN112 that differs from the sequence in CpG motif is unique and responsible for the suppression of asthmatic features in mice. Regarding the role of non-canonical CpG motif in the anti-Th2 immune response, Iliev et al. [37] have showed that the genomic DNA of *Lactobacillus rhamnosus* GG with a core sequence of TTTCGTTT motif potentially suppressed the OVA-specific IgE production in mice through TLR9-dependent activation of DCs and induction of IFN-γ production by $CD4^+$ T cells. In contrast, ISS-ODN containing a unique core sequence, 5′-ATTTTTAC-3′, and a 6-base secondary loop structure in *L. gasseri* JCM1131 genome enhanced immunostimulatory activity such as IL-12p70 and IFN-γ production in human peripheral blood mononuclear cells [38]. In the present study, ODN112 lacks canonical CpG motifs but contains a unique core sequence, 5′-GTCGGT-3′. In particular, a cytosine-guanine (CG) in the 6-base fragment in ODN112 is the key for the anti-allergic activity, although we could not rule out the possibility that the secondary loop structure of ODN112 might also play an important role in the induction of Th1 immunity in asthma. Within further limitation of the present study, we could not completely rule out the possibility of the involvement of other pathogen recognition receptors in the recognition of 5′-GTCGGT-3′ because we could not use TLR9KO mice in the present study. However, the results from the in vitro experiment strongly suggest that stimulatory activities of ODN112 are TLR9 dependent [21].

The suppressive effect of ODN112 on allergen sensitization implies the preventative effect of ODN112 on the development of Th2 cells in asthma. DCs, the most proficient antigen-presenting cells, play a critical role in adaptive immune responses by priming Th2 cells to respiratory allergens, which is a critical step for the development and exacerbation of allergic asthma [39]. Sustained IL-12 signaling induces STAT4 activation in T cells, which skew naive Th cells toward the Th1 phenotype as defined by IFN-γ expression [40, 41]. IFN-γ antagonizes the development of Th2 cells and also converts fully polarized Th2 cells into IFN-γ-producing Th1 cells by transduction of T-bet [42]. We showed that ODN112 significantly increased IL-12p40 production from both Th2-oriented DCs and immature DCs in our present and previous studies [21]. In the present study, although IL-12p40 and IFN-γ in the peritoneal lavage fluids at 1 and 3 days after sensitization were undetectable (<15 pg/mL, data not shown), the co-stimulatory molecular expression pattern of CD80 on peritoneal DCs following sensitization indicates that ODN112 and CpG-ODN induce the Th1-inducing capacity in Th2-biased DCs [43]. In addition, upregulation of CD40 and IL-12 may synergistically enhance IFN-γ production by T-cell receptor-stimulated T cells [44]. Several factors involved in the induction of co-stimulatory molecule expression on antigen-presenting cells are reported. Previously, it was demonstrated that IL-4 is an important cytokine for CD86 expression on macrophages [45]. In contrast, IFN-γ upregulates CD40 and CD80 in monocytes [46]. Furthermore, TLR agonists themselves, such as ODNs and LPS, induce co-stimulatory molecule expression on DCs [47]. Therefore, our data suggest that the Th1-type cytokine milieu regulated by ODNs is responsible for enhanced CD40/CD80 expression on DCs in the peritoneal cavity at the time of sensitization. The enhanced IFN-γ production, as well as the reduced Th2 cytokine production, was observed in the spleen of ODN112-treated mice before allergen inhalation. This suggests that ODN112 redirects immune responses from Th2 to Th1 during sensitization by changing the DC phenotype, which exhibits the suppressed prototypical asthma-related features after allergen inhalation. In clinical settings, the reduced allergen-induced Th1 response is an important factor related to ongoing severe atopic asthma [48]. In patients with allergic asthma, blood IL-12 levels are lower than those in healthy controls, which is associated with reduced IL-12-dependent IFN-γ production [49]. In addition, normalization of IFN-γ responses is important for resolution of inflammation in asthma [48].

In summary, our results indicate that the CD40/CD80/IL-12/IFN-γ axis activation induced by ODN112 during the sensitization phase suppressed asthmatic immune responses in the lungs followed by AHR after the development of asthma. Also, our data suggest the possibility that suppressive activity of ODN112 on Th2 cell differentiation in the sensitization phase maintains a long-term effect into the elicitation phase, which may not only be a benefit for the prevention of asthma onset but also for the prevention of asthma exacerbation.

Statement of Ethics

All experimental procedures involving animals were approved by the Committee of Animal Experiments at the Tohoku Medical and Pharmaceutical University (approval numbers: 15001-cn, 16002-cn, and 17004-cn). We took the utmost care to alleviate any pain and suffering of the mice.

Disclosure Statement

The authors have no financial conflicts of interest to declare.

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Author Contributions

Conceived and designed the experiments: I.O. and Kazuyoshi K. Performed the experiments: T.M., K.D.-O., K.S., K.I., C.M., S.S., and T.K. Analyzed the data: Kazuyoshi K., I.O., T.M., K.I., K.D.-O., Kaori K., J.K., H.Y., and D.T. Contributed reagents/materials/analysis tools: Kazuyoshi K., I.O., T.T., M.T., K.I., E.K., and H.T. Contributed to the writing of the manuscript: T.M., I.O., Kazuyoshi K., Kaori K., and J.K.

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