Suzuki et al. World Allergy Organization Journal (2020) 13:100447
http://doi.org/10.1016/j.waojou.2020.100447

WORLD ALLERGY ORGANIZATION JOURNAL

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Intranasal administration of regulatory dendritic cells is useful for the induction of nasal mucosal tolerance in a mice model of allergic rhinitis

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ABSTRACT

Background: Intranasally administered dendritic cells (DCs) migrate into blood and thymus to induce immune responses. Regulatory dendritic cells (DCs) are also useful agents for allergy control. However, to the best of our knowledge, the effects of intranasal administration of regulatory DCs on allergy have not been reported until now. Therefore, we examined the effects of intranasal route of administration of CD40-silenced DCs on allergic responses and compared these with the effects of other administration routes, based on our previous findings on the inhibitory effects of CD40-silenced DCs on allergic responses.

Methods: Mice with allergic rhinitis were treated intranasally, subcutaneously, intraperitoneally, or intravenously with CD40-silenced ovalbumin (OVA)-pulsed DCs that were transfected with CD40 siRNAs and pulsed with OVA antigen. The effects of these DCs on allergic reactions and symptoms were estimated.

Results: Intranasal, subcutaneous, intraperitoneal, or intravenous administration of OVA-pulsed CD40-silenced DCs inhibited allergic responses and symptoms in mice. Furthermore, intranasal administration of OVA-pulsed CD40-silenced DCs significantly reduced allergic symptoms and the number of eosinophils in the nasal mucosa compared with subcutaneous, intraperitoneal, or intravenous administration of these DCs. Intranasal administration of OVA-pulsed CD40-silenced DCs resulted in significantly up-regulated IL-10, IL-35, and *Foxp3* expression, and enhanced the percentage of $CD11c^{+}CD40^{-}$ and $CD4^{+}CD25^{+}$ cells within the cervical lymph nodes compared to subcutaneous, intraperitoneal, or intravenous routes of administration.

Conclusions: We believe that this is the first report to demonstrate that regulatory DCs infiltrate into the cervical lymph nodes after intranasal administration of these cells and that intranasal administration of regulatory DCs is more effective for the induction of tolerance in the nasal mucosa than subcutaneous, intraperitoneal, or intravenous administration.

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http://doi.org/10.1016/j.waojou.2020.100447

Received 30 March 2020; Received in revised from 12 July 2020; Accepted 15 July 2020

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Keywords: Intranasal administration, Allergic rhinitis, Dendritic cells, Cervical lymph nodes, Regulatory T cells

INTRODUCTION

Allergen immunotherapy, which involves the direct administration of increasing doses of allergens, has been used for the treatment of allergic rhinitis. Although this is an attractive therapy for desensitization or hyposensitization, some major problems are encountered, for example, side effects such as asthma exacerbation or anaphylaxis, frequent visits to doctors, and incomplete efficacy in some individuals. Subcutaneous or sublingual allergen-specific immunotherapy has been used clinically. Although intranasal allergen immunotherapy is an attractive local treatment for the control of allergic rhinitis, direct intranasal administration of allergen causes nasal allergic symptoms. Local intranasal allergen immunotherapy is also reported to be associated with very poor compliance because of the major side effects produced as a result of repeated nasal reactions to allergens.[2] Consequently, the use of nasal immunotherapy is decreasing in recent times.[3,4]

Dendritic cells (DCs) play an important role in the induction of immune responses. It was shown that intranasally administered DCs migrated into blood, lung, and thymus, and that lymphocytes generated effector memory T cell population.[5] Awasthi et al[6] also demonstrated that intranasal administration of DCs transfected with cDNA encoding the protective epitope of *Coccidioides species*, which are fungi, induced protective immunity against *C. posadasii* in mice. Additionally, DCs are able to inhibit allergic responses.[7,8] Regulatory DCs induce anergy, promote regulatory T-cell differentiation, and induce T-cell death (deletion).[9] Regulatory DCs have been regarded as potentially useful agents for managing allergic diseases.[10,11]

It has been reported that efficacy of drugs is dependent on their route of administration.[12,13] The effects of allergen immunotherapy also differ based on the routes of administration.[1] It must be considered that the efficacy of therapies depends on the administration route. Studies demonstrated that tight junctions, cell-cell junctional complexes in nasal epithelial cells, were disrupted in allergic rhinitis, suggesting that DCs easily enter the body through the mucosal membranes.[14,15] Considering these data, intranasal administration of regulatory DCs may serve as an attractive local immunotherapy for managing allergic rhinitis. However, to the best of our knowledge, the effect on efficacy of regulatory DCs with respect to the administration route has not been examined.

RNA interference is a simple, rapid, and selective method for silencing gene expression using small interfering RNAs (siRNAs).[16,17] Andrew Fire and Craig Mello received the 2006 Nobel Prize in Medicine for the discovery of this technique.[18] It was previously reported that intraperitoneal administration of siRNA-induced CD40-silenced antigen-specific DCs inhibited allergic responses and symptoms in an antigen-specific manner.[11] However, the effects of intranasal administration of regulatory DCs on allergy have not been reported till date. It is also unclear as to which route of administration related to regulatory DCs is the best for controlling allergic rhinitis. Therefore, we investigated differences in allergic responses and symptoms of mice administered with DCs via different routes.

METHODS

Gene silencing in bone marrow-derived DCs by siRNAs

DCs were generated from bone marrow progenitor cells of 6-to 8-week-old male BALB/c mice (Japan SLC, Shizuoka, Japan), as previously reported.[11,19-21] Briefly, bone marrow cells were flushed from the femurs and tibias and cultured. DCs (CD40-silenced DCs) were transfected with siRNA against CD40 (CD40 siRNA, UUCUCAGCCCAGUGGAACA). DCs (control DCs) were also transfected with siRNA (control siRNA) against

the luciferase GL2 Duplex. Briefly, CD40 siRNA (2 μg) or control siRNA (2 μg) were incubated with 20 μL of GeneSilencer reagent for 30 min. The mixture was then added to DCs cultured in 12-well plates. After incubation, RPMI 1640 medium supplemented with foetal bovine serum, murine GM-CSF, IL-4, and ovalbumin (OVA) was added to the cell suspension. DCs were pulsed with 100 μg/mL OVA for 24 h at 37 °C and subsequently washed 3 times with phosphate-buffered saline (PBS).

Co-culture of T cell and DCs transfected with or without CD40 siRNA

Six-to eight-week-old male BALB/c mice (Japan SLC) were sensitized with OVA (10 μg) and 2 mg of $Al(OH)_3$ intraperitoneally on days 1 and 15, and these mice were euthanized on day 29. $CD4^+CD25^-$ T cells were isolated from spleen using MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany). $CD4^+CD25^-$ T cell (2×10^6 cells/mL) and OVA-pulsed DCs (2×10^5 cells/mL) transfected with or without CD40 siRNA were co-cultured for 72 h.

Immunization and treatment

Six-to eight-week-old male BALB/c mice (Japan SLC) were sensitized with OVA (10 μg) and 2 mg of $Al(OH)_3$ intraperitoneally on days 1 and 15 and were subsequently challenged intranasally with OVA (100 μg) on days 21–27. Intranasal, subcutaneous, intraperitoneal, or intravenous administration of OVA-pulsed CD40-silenced DCs (7×10^6 cells/mouse) was performed on day 28. As a control, mice received OVA-pulsed control DCs (DCs transfected with control siRNA and pulsed with OVA, 7×10^6 cells/mouse) or PBS alone, intranasally, subcutaneously, intraperitoneally, or intravenously. These mice were then re-challenged on days 29–35 with OVA (100 μg). The number of sneezes and nasal rubbing movements were counted immediately after the last nasal challenge, ie, on day 35. On day 36, mice were anesthetized, and blood samples were collected by cardiac puncture. Mice were euthanized by cervical dislocation, and cervical lymph nodes and nose were obtained on day 36 (Fig. 1, sensitization on days 1 and 15, challenge on days 21–27, treatment on day 28, re-challenge on days 29–35, and sample collection on day 36). Five mice were used in each group; total 12 groups (intranasal, subcutaneous, intraperitoneal, or intravenous administration of intranasal administration of PBS alone, OVA-pulsed control DCs, or OVA-pulsed CD40-silenced DCs) were used for the study. Each experiment was repeated in triplicate.

Mice were housed in an environmentally controlled animal facility. Efforts were taken to minimize animal discomfort.

Real-time PCR

Total RNA was isolated from T cells using TRIzol, and real-time PCR was performed, as previously described.[11,19,22] The primers for *Foxp3* were as follows: sense 5′-CAGCTGCCTACAGTGCCCCTA G-3′ and antisense 5′-CATTTGCCAGCAGTG GGTAG CTG-3′. The primers for *GAPDH* were sense 5′-TGATGACATCAAGAAGGTGGTGAA-3′ and antisense 5′-TCCTTGGAGGCCAT GTAGG CCAT-3′.

Flow cytometry

Phenotypic analysis of T cells and DCs was performed on a FACScan according to the method previously described.[11,19,22] T cells were harvested and stained with anti-mouse CD4 and anti-mouse CD25 monoclonal antibodies, and DCs were stained with anti-mouse CD11c and anti-mouse CD40 monoclonal antibodies.

OVA-specific T-cell responses

OVA-specific T-cell responses were estimated as previously reported.[21] Lymphoid cells (2×10^6 cells/mL) from the cervical lymph nodes and spleen were cultured with OVA (100 μg/mL) at 37 °C for 72 h, after which the culture supernatants were collected.

Measurement of cytokine production

The levels of cytokines (IL-4, IL-5, IL-10, and IL-35) in the culture supernatants were measured using sandwich ELISA. Plates were coated with anti-mouse IL-4, IL-5, and IL-10 (PeproTech, Rocky Hill, NJ, USA), or IL-35 (BioLegend, San Diego, CA, USA). After addition of the culture supernatants, the plates were incubated with biotinylated anti-mouse IL-4, IL-5, and IL-10 (PeproTech), or IL-35 (BioLegend) antibodies. Standard curves were generated using

recombinant cytokines. The detection limits of these cytokines were 10–20 pg/mL.

Measurement of OVA-specific IgE in the sera

Titres of OVA-specific IgE were measured using ELISA. Briefly, ELISA plates were coated with anti-mouse IgE monoclonal antibodies (Yamasa, Tokyo, Japan). After non-specific binding was blocked, sera were added to the wells. After the addition of biotinylated OVA, plates were incubated with avidin-peroxidase. Using the TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD, USA), optical density (O.D.) was measured at 450 nm.

Nasal allergic symptoms

Sneezes and nasal rubbing movements were counted for 20 min immediately after the last nasal challenge, according to a previously described method.[11,19,22]

Pathology

Heads of mice were decalcified, sectioned, and fixed in formalin. Sections of nasal tissue were also evaluated via Luna staining. The number of eosinophils in the nasal septum was counted microscopically at 400 × magnification in six randomly selected locations across the anterior, middle, and posterior areas on the right and left sides of each mouse. The 6 counts were averaged for each mouse and results were expressed as means ± SEM of five mice, as previously reported.[11,19,22] The observer who counted the number of eosinophils was blinded to the treatment.

Statistical analysis

Results are expressed as means ± SEM. Statistical comparisons between groups were performed using one-way ANOVA followed by Newman–Keuls tests. Differences with p-values of less than 0.05 were considered significant.

RESULTS

Effect of OVA-pulsed DCs transfected with or without CD40 siRNA on production of IL-4 and IL-5

In order to examine the effect of OVA-pulsed control DCs and OVA-pulsed CD40-silenced DCs on Th2 cytokine (IL-4 and IL-5) production, either of these DCs and splenic $CD4^{+}CD25^{-}$ T cells from mice sensitized with OVA were co-cultured. Consequently, OVA-pulsed control DCs induced the production of IL-4 and IL-5 by T cells, although IL-4 and IL-5 were not detected in the co-culture of

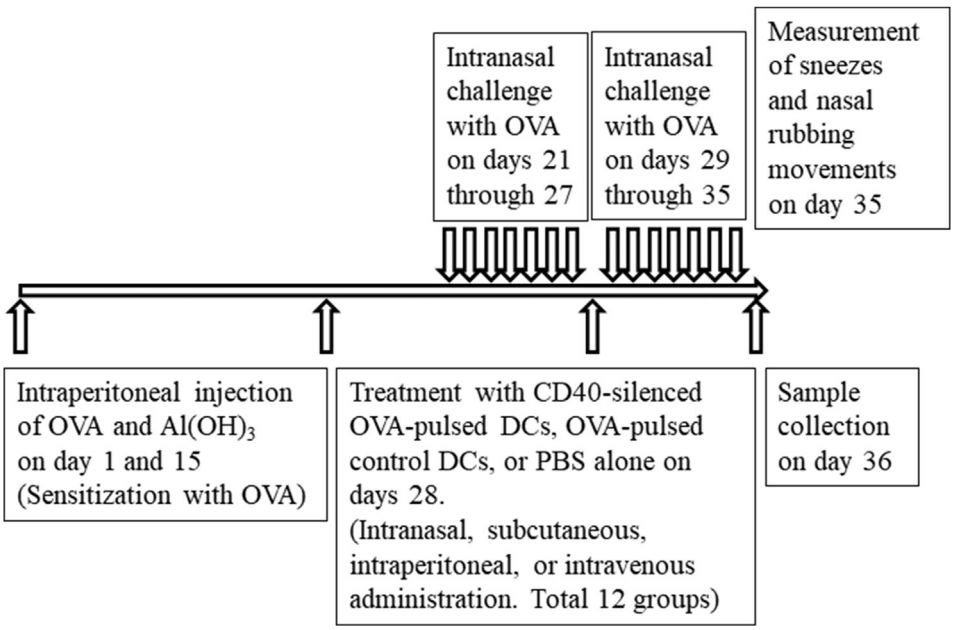

Fig. 1 Schematic protocol of the experiment. Mice were administered ovalbumin (OVA)-pulsed CD40-silenced dendritic cells (DCs) via intranasal, subcutaneous, intraperitoneal, or intravenous routes. As a control, mice received OVA-pulsed control DCs, or PBS alone via these four routes of administration

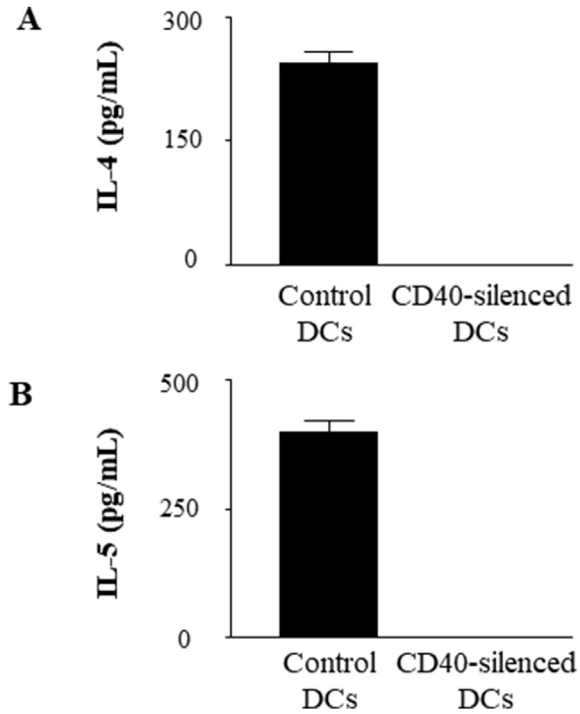

Fig. 2 Effect of OVA-pulsed DCs transfected with or without CD40 siRNA on cytokine production. Effect of OVA-pulsed DCs, transfected with or without CD40 siRNA, on cytokines released from T cells. $CD4^{+}CD25^{-}$ T cells and OVA-pulsed DCs transfected with (CD40-silenced DCs) or without (Control DCs) CD40 siRNA were cocultured. Production of IL-4 (A) and IL-5 (B) was measured by ELISA

OVA-pulsed CD40-silenced DCs and $CD4^{+}CD25^{-}$ T cells (Fig. 2A and B).

Therapeutic effects of intranasal administration of OVA-pulsed CD40-silenced DCs on mice with established allergic rhinitis

Mice were sensitized and challenged with OVA, as described in the methods section (Fig. 1). The number of sneezes and nasal rubbing movements on day 20 were significantly fewer than those on day 28 (data not shown). Although eosinophilia was not observed on day 20, it was observed on day 28 (data not shown). These findings suggest that allergic rhinitis had been established in these mice by day 28. After allergic rhinitis had been established, all the mice were administered either PBS alone, OVA-pulsed control DCs, or CD40-silenced OVA-pulsed DC via the intranasal, subcutaneous, intraperitoneal, or intravenous routes, as described in the Methods section (Fig. 1), to identify the most optimal route with respect to their effects. To evaluate the effect of these DCs on allergic symptoms, the numbers of sneezes and nasal rub movements were examined following the last challenge. The number of sneezes and nasal rub movements did not significantly differ among mice that had received PBS alone intranasally, subcutaneously, intraperitoneally, or intravenously (Fig. 3A). There were also no significant differences in the numbers of sneezes and nasal rub movements among mice that had received OVA-pulsed control DCs via these four routes of administration (Fig. 3B). However, the number of sneezes and nasal rub movements in mice that had received OVA-pulsed CD40-silenced DCs intranasally, subcutaneously, intraperitoneally, or intravenously, were significantly fewer than those in mice that received OVA-pulsed control DCs and PBS alone via these routes (Fig. 3A and B; $p < 0.01$). Further, the number of sneezes and nasal rub movements in mice that had received OVA-pulsed CD40-silenced DCs intranasally were significantly fewer than those in mice that had received OVA-pulsed CD40-silenced DCs subcutaneously, intraperitoneally, or intravenously ($p < 0.01$).

The number of eosinophils in the nasal mucosa, which are associated with allergic symptoms and allergic responses, were counted to evaluate the occurrence of eosinophilia in the different mice groups. The eosinophil counts in the nasal mucosa did not significantly differ among the mice that received PBS alone or OVA-pulsed control DCs intranasally, subcutaneously, intraperitoneally, or intravenously (Fig. 3C). However, significantly fewer eosinophils were observed in the nasal mucosa of mice that had received OVA-pulsed CD40-silenced DCs intranasally, subcutaneously, intraperitoneally, or intravenously compared with those treated with OVA-pulsed control DCs or PBS alone via these 4 routes of administration (Fig. 3 C-E; $p < 0.01$). Interestingly, the number of eosinophils in mice that had received OVA-pulsed CD40-silenced DCs intranasally was significantly smaller than that in mice which had received OVA-pulsed CD40-silenced DCs subcutaneously, intraperitoneally, or intravenously (Fig. 3 C; $p < 0.01$).

Effects of OVA-pulsed CD40-silenced DCs on cytokine production by cervical lymph node cells of mice with established allergic rhinitis

We measured the levels of Th2 cytokines (IL-4 and IL-5)—which are associated with allergic

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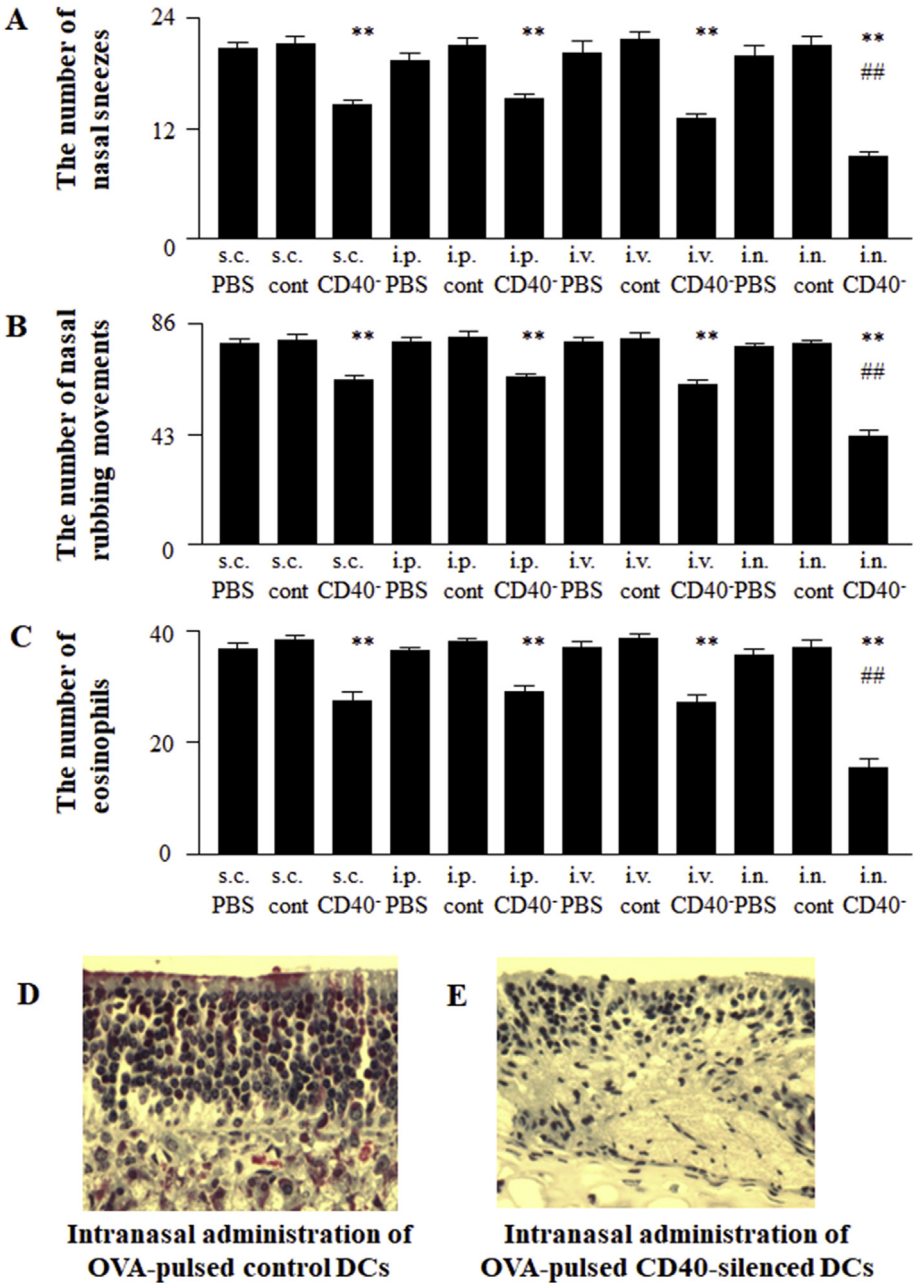

Fig. 3 Therapeutic effects of CD40-silenced ovalbumin (OVA)-pulsed dendritic cells (DCs) on established allergic rhinitis. Mice with OVA-induced allergic rhinitis were administered PBS alone (PBS), OVA-pulsed DCs (cont), or OVA-pulsed CD40-silenced DCs ($CD40^{-}$) via the intranasal (i.n.), subcutaneous (s.c.), intraperitoneal (i.p.), or intravenous (i.v.) routes (Fig. 1). The number of sneezes (A) and nasal rubbing movements (B) following the last nasal challenge are shown. (C) Eosinophilia of the nasal septum. Nasal tissue was stained with Luna staining. Typical sections of the nasal septum of mice that received OVA-pulsed control DCs (D) or OVA-pulsed CD40-silenced DCs intranasally (E) are shown. **$p < 0.01$ *versus* all PBS and cont groups. ##$p < 0.01$ *versus* s.c. $CD40^{-}$, i.p. $CD40^{-}$, and i.v. $CD40^{-}$ groups

responses—produced by the cervical lymph node cells following stimulation with OVA. The production of IL-4 and IL-5 did not significantly differ among mice that had received PBS alone or OVA-pulsed control DCs intranasally, subcutaneously, intraperitoneally, or intravenously (Fig. 4 A and B). However, the production of IL-4 and IL-5, in mice that had received OVA-pulsed CD40-silenced DCs intranasally, subcutaneously, intraperitoneally, or intravenously was significantly lower than that in mice that had received OVA-pulsed control DCs and PBS alone through these four routes (Fig. 4 A and B; $p < 0.01$). In addition, intranasal administration of OVA-pulsed CD40-silenced DCs resulted in significantly inhibited production of IL-4 and IL-5 compared to that observed upon

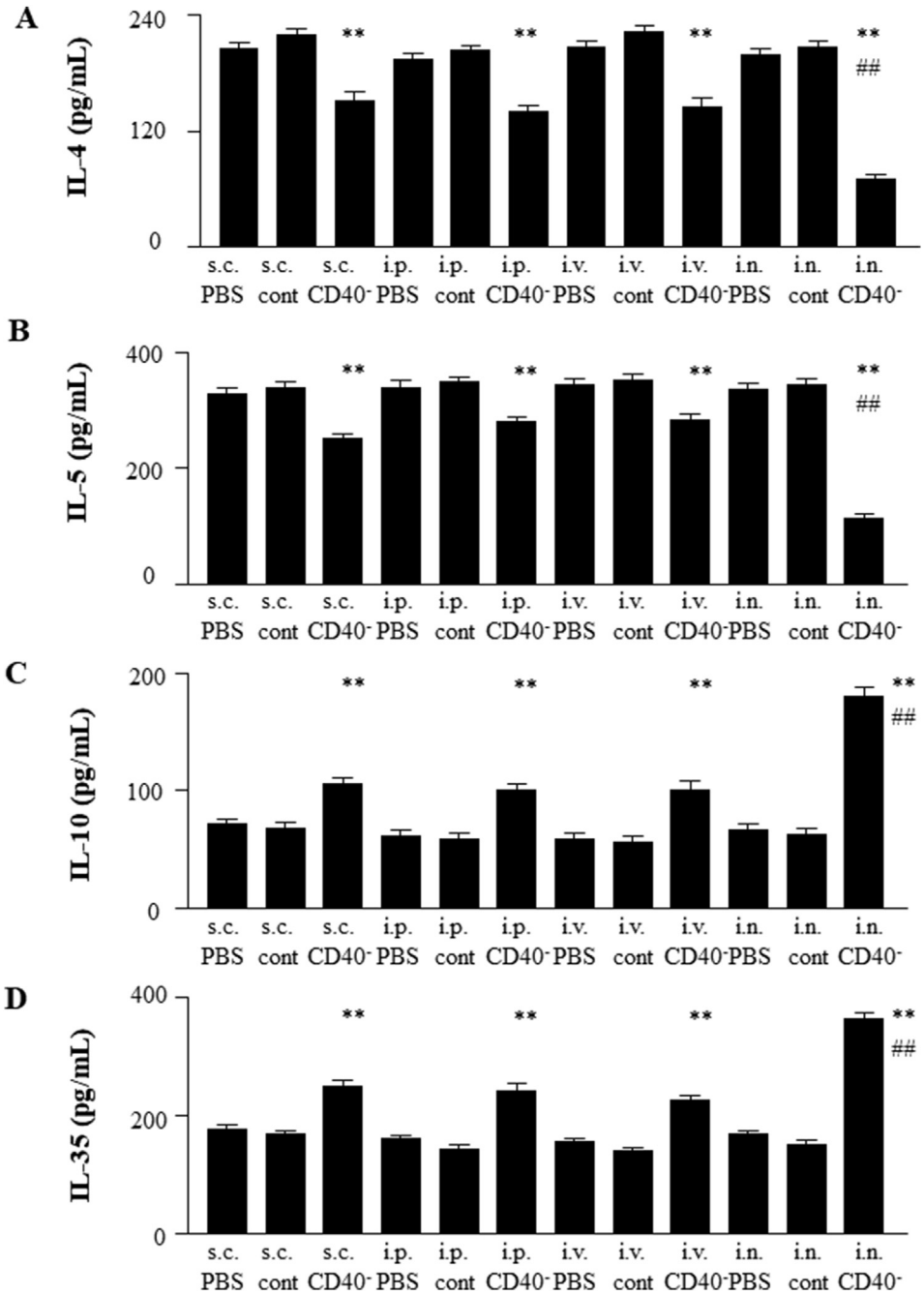

Fig. 4 Cytokine modulation by CD40-silenced ovalbumin (OVA)-pulsed dendritic cells (DCs) within the cervical lymph nodes. Mice with OVA-induced allergic rhinitis were administered PBS alone (PBS), OVA-pulsed DCs (cont), or OVA-pulsed CD40-silenced DCs ($CD40^-$) via the intranasal (i.n.), subcutaneous (s.c.), intraperitoneal (i.p.), or intravenous (i.v.) routes (Fig. 1). Cervical lymph nodes were collected. After lymphocytes in the cervical lymph nodes were stimulated with the OVA antigen for 72 h, cytokine production was measured by ELISA. Production of IL-4 (A), IL-5 (B), IL-10 (C), and IL-35 (D). **$p < 0.01$ *versus* all PBS and cont groups. ##$p < 0.01$ *versus* s.c. $CD40^-$, i.p. $CD40^-$, and i.v. $CD40^-$ groups

subcutaneous, intraperitoneal, or intravenous administration of OVA-pulsed CD40-silenced DCs ($p < 0.01$).

We also measured the levels of regulatory cytokines (IL-10 and IL-35) produced by cervical lymph node cells following stimulation with OVA. Production of IL-10 and IL-35 did not significantly differ among mice that had received PBS alone or OVA-pulsed control DCs intranasally, subcutaneously, intraperitoneally, or intravenously (Fig. 4 C and D). Nonetheless, IL-10 and IL-35 production in mice that had received OVA-pulsed CD40-

silenced DCs subcutaneously, intraperitoneally, or intravenously was significantly higher than that in mice that had received OVA-pulsed control DCs and PBS alone through these routes (Fig. 4 C and D; $p < 0.01$). Additionally, intranasal administration of OVA-pulsed CD40-silenced DCs resulted in significantly up-regulated production of IL-10 and IL-35 compared to that observed upon subcutaneous, intraperitoneal, or intravenous administration of OVA-pulsed CD40-silenced DCs ($p < 0.01$).

Effects of OVA-pulsed CD40-silenced DCs on the facilitation of regulatory T cells in the cervical lymph nodes of mice with established allergic rhinitis

Next, we evaluated whether the administration of OVA-pulsed CD40-silenced DCs increased *Foxp3* expression and the percentage of regulatory T cells within the cervical lymph nodes. No significant differences in *Foxp3* expression and $CD4^+CD25^+$ T cells percentage within the cervical lymph nodes were observed among mice that had received PBS alone or OVA-pulsed control DCs intranasally, subcutaneously, intraperitoneally, or intravenously (Fig. 5 A and B). The expression of *Foxp3* and percentage of $CD4^+CD25^+$ T cells in the cervical lymph nodes of mice that had received OVA-pulsed CD40-silenced DCs intranasally, subcutaneously, intraperitoneally, or intravenously were significantly higher than those in mice that had received OVA-pulsed control DCs and PBS alone via these four routes. (Fig. 5A and B; $p < 0.01$). Additionally, *Foxp3* expression and the percentage of $CD4^+CD25^+$ T cells within the cervical lymph nodes of mice that had received OVA-pulsed CD40-silenced DCs intranasally were significantly higher than those in mice that had received OVA-pulsed CD40-silenced DCs subcutaneously, intraperitoneally, or intravenously ($p < 0.01$).

Distribution of $CD11c^+CD40^-$ DCs in cervical lymph nodes after the administration of OVA-pulsed CD40-silenced DCs

We examined the distribution of $CD11c^+CD40^-$ DCs in cervical lymph nodes after the administration of OVA-pulsed CD40-silenced DCs using flow cytometry. The percentage of $CD11c^+CD40^-$ DCs after administration of OVA-pulsed CD40-silenced DCs via the intranasal, subcutaneous, intraperitoneal, or intravenous routes was significantly higher than that after administration of PBS alone or OVA-pulsed control DCs via these routes.

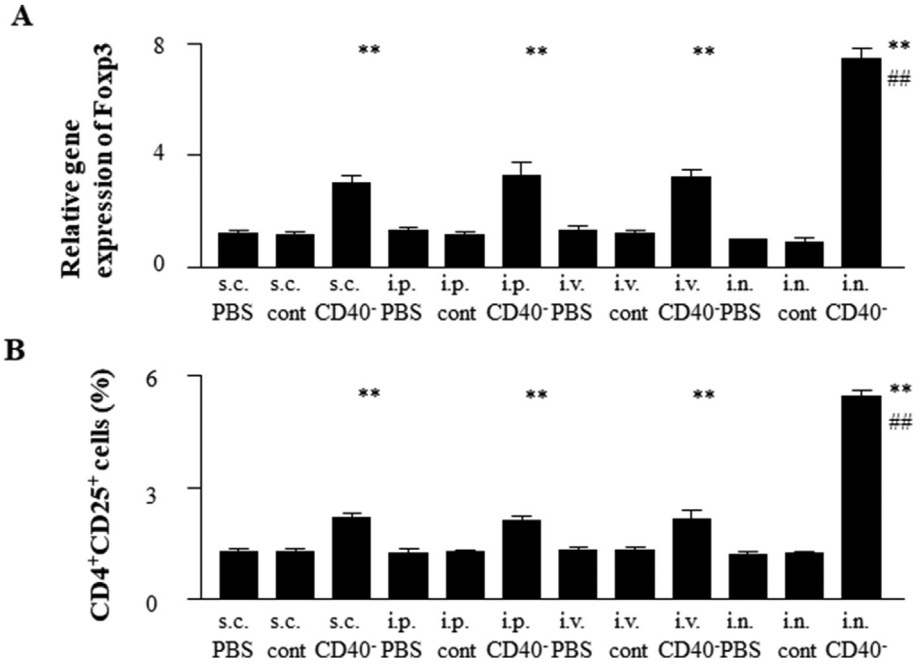

Fig. 5 Up-regulation of regulatory T cells by CD40-silenced ovalbumin (OVA)-pulsed dendritic cells (DCs) in the cervical lymph nodes. Mice with OVA-induced allergic rhinitis were administered PBS alone (PBS), OVA-pulsed DCs (cont), or OVA-pulsed CD40-silenced DCs ($CD40^-$) via the received intranasal (i.n.), subcutaneous (s.c.), intraperitoneal (i.p.), or intravenous (i.v.) routes.(A) *Foxp3* expression in cervical lymph nodes. (B) Percentage of $CD4^+CD25^+$ cells in cervical lymph nodes. **$p < 0.01$ *versus* all of PBS and cont groups. ##$p < 0.01$ *versus* s.c. $CD40^-$, i.p. $CD40^-$, and i.v. $CD40^-$ groups

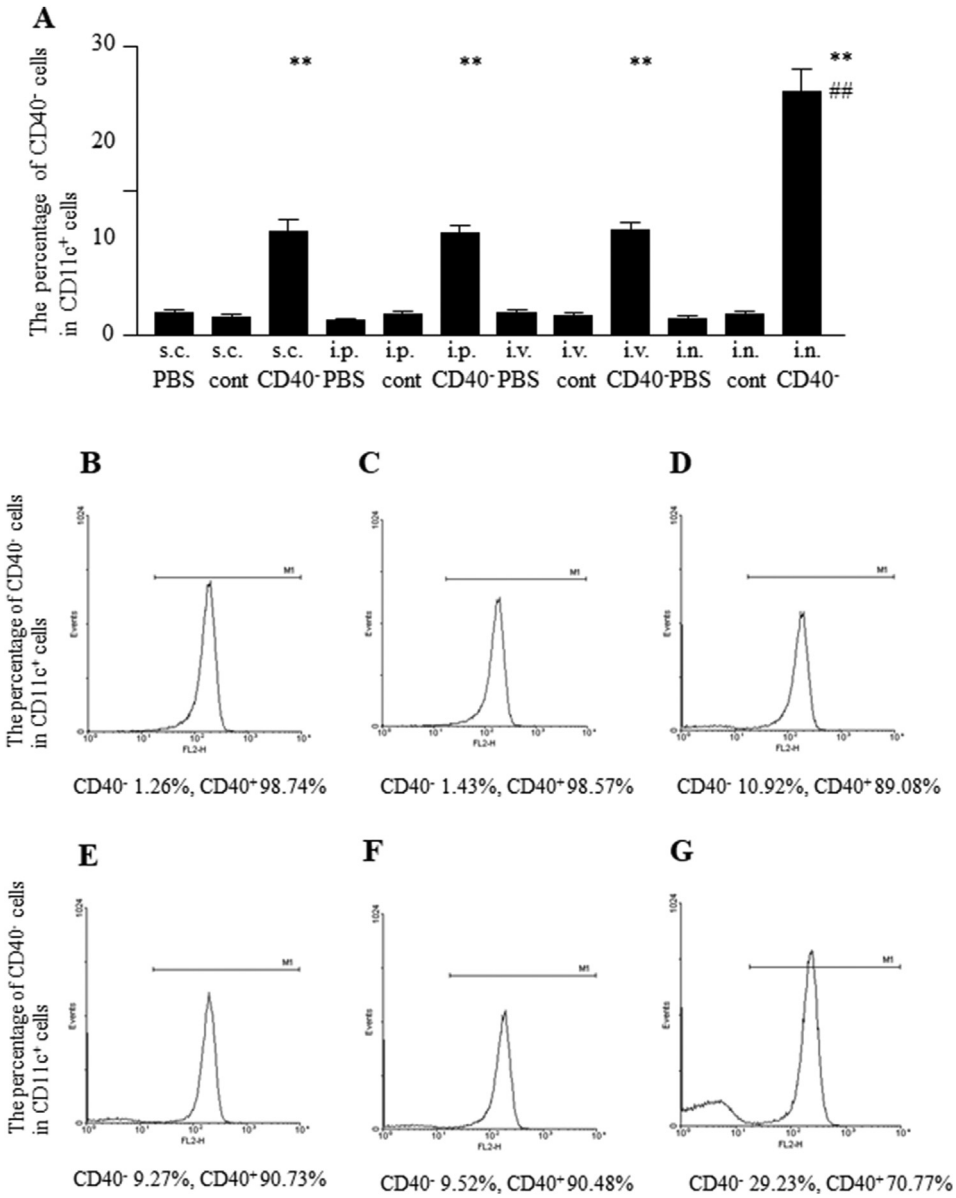

Fig. 6 Flow cytometry for the percentage of $CD11c^{+}CD40^{-}$dendritic cells (DCs) in the cervical lymph nodes after administration of OVA-pulsed CD40-silenced DCs. Mice with OVA-induced allergic rhinitis were administered PBS alone (PBS), OVA-pulsed DCs (cont), or OVA-pulsed CD40-silenced DCs ($CD40^{-}$) via the subcutaneous (s.c.), intraperitoneal (i.p.), intravenous (i.v.), or intranasal (i.n.) routes. First $CD11c^{+}$ cells were gated in the cervical lymph nodes. (A) The percentage of $CD40^{-}$ cells in $CD11c^{+}$ DCs of the cervical lymph nodes. Typical flow cytometric analysis of $CD11c^{+}CD40^{-}$ DCs in the cervical lymph nodes after intranasal administration of PBS alone (B) and OVA-pulsed control DCs (C). Typical flow cytometry analysis of $CD11c^{+}CD40^{-}$ DCs after administration of OVA-pulsed CD40-silenced DCs via the subcutaneous (D), intraperitoneal (E), intravenous (F), or intranasal (G) routes. **$p < 0.01$ *versus* all of PBS and cont groups. ##$p < 0.01$ *versus* s.c. $CD40^{-}$, i.p. $CD40^{-}$, and i.v. $CD40^{-}$ groups

(Fig. 6A–G). Additionally, the percentage of $CD11c^{+}CD40^{-}$ DCs after intranasal administration was significantly higher than that after subcutaneous, intraperitoneal, or intravenous administration. This suggests that the number of $CD11c^{+}CD40^{-}$ DCs in the cervical lymph nodes changes according to the route of administration.

Effects of OVA-pulsed CD40-silenced DCs on IgE in sera and cytokine production in the spleen of mice with established allergic rhinitis

The levels of OVA-specific IgE in the sera—a phenomenon that is associated with allergic responses—was investigated using ELISA. No significant differences in OVA-specific IgE were seen in

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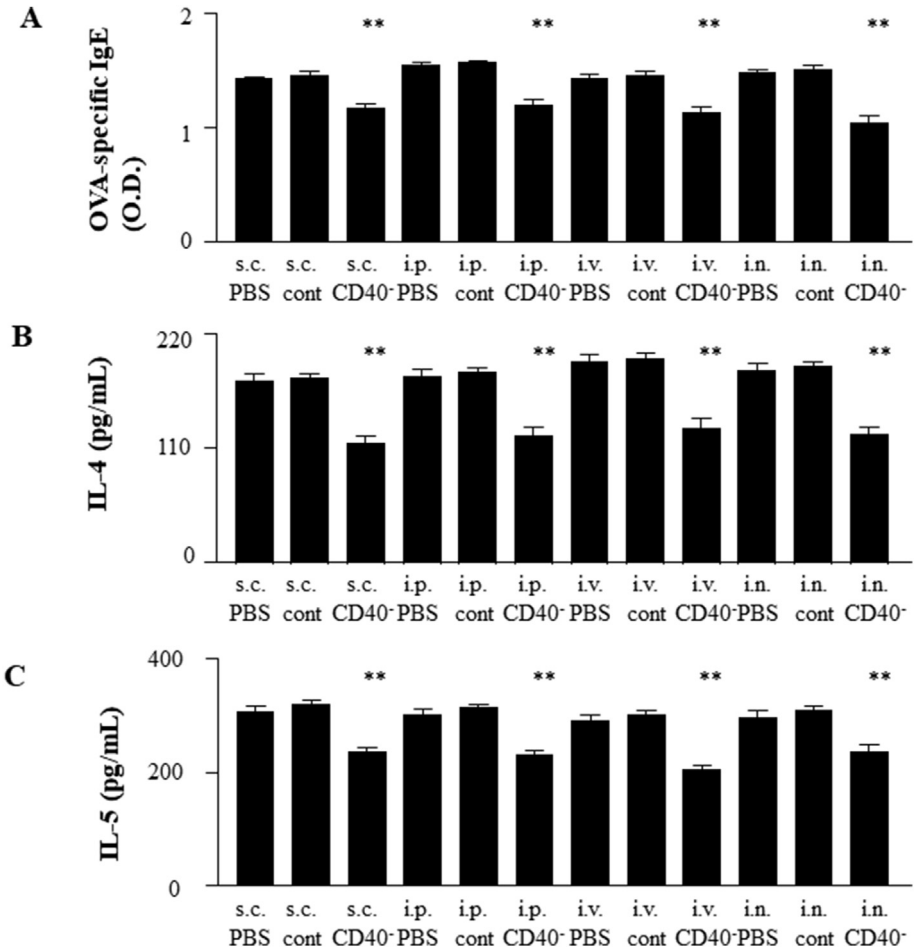

Fig. 7 Modulation of ovalbumin (OVA)-specific IgE in sera and cytokines released from the splenocytes in response to OVA-pulsed CD40-silenced dendritic cells (DCs). Mice were administered PBS alone (PBS), OVA-pulsed DCs (cont), or OVA-pulsed CD40-silenced DCs ($CD40^{-}$) via the intranasal (i.n.), subcutaneous (s.c.), intraperitoneal (i.p.), or intravenous (i.v.) routes. OVA-specific IgE in the sera was measured using ELISA (A). Splenocytes were collected and the release of IL-4 (A) and IL-5(B) by splenocytes in response to OVA stimulation was also estimated using ELISA. **$p < 0.01$ versus all PBS and cont groups

the sera among mice that had received PBS alone or OVA-pulsed control DCs intranasally, subcutaneously, intraperitoneally, or intravenously (Fig. 7 A). The levels of OVA-specific IgE in mice that had received OVA-pulsed CD40-silenced DCs intranasally, subcutaneously, intraperitoneally, or intravenously was significantly lower than that in mice that received OVA-pulsed control DCs or PBS alone via these routes of administration (Fig. 7 A; $p < 0.01$). However, the levels of OVA-specific IgE in the sera did not significantly differ among mice that had received OVA-pulsed CD40-silenced DCs intranasally, subcutaneously, intraperitoneally, or intravenously.

Furthermore, we examined the effect of CD40-silenced OVA DCs on the release of Th2 cytokines (IL-4 and IL-5) by splenic cells following stimulation with OVA. The release of IL-4 and IL-5 did not significantly differ among mice that had received PBS alone or OVA-pulsed control DCs intranasally, subcutaneously, intraperitoneally, or intravenously (Fig. 7 B andC). However, the release of IL-4 and IL-5 by splenic cells in mice that had received OVA-pulsed CD40-silenced DCs intranasally, subcutaneously, intraperitoneally, or intravenously was significantly lower than that in mice that had received OVA-pulsed control DCs or PBS alone through these four routes (Fig. 7 B and C; $p < 0.01$). Additionally, the release of IL-4 and IL-5 did not significantly differ among mice that had received OVA-pulsed CD40-silenced DCs intranasally, subcutaneously, intraperitoneally, or intravenously.

DISCUSSION

The present study showed that intranasal administration of OVA-pulsed CD40-silenced DCs significantly reduced the number of sneezes and nasal rub movements compared with subcutaneous, intraperitoneal, or intravenous modes of administration of OVA-pulsed CD40-silenced DCs. This suggests that intranasal administration of regulatory DCs is more effective for the control of allergic rhinitis than the subcutaneous, intraperitoneal, or intravenous modes for the administration of regulatory DCs.

Th2 cytokines, such as IL-4 and IL-13, induce the disruption of the tight junctions in the epithelium in asthma and allergic rhinitis.[15,23] This may allow the DCs to enter the body easily through the mucosa; this might be the reason why the intranasal DCs therapy exerts a beneficial effect on the condition. A higher degree of disruption of the tight junctions of the epithelium may be observed if the allergic inflammation is more intense. The greater disruption of the barriers allows the regulatory DCs to enter the body more easily through the mucosa.

Cervical lymph nodes are regional lymph nodes of the upper airways. Intranasal administration of antigens induces Th2-cell accumulation within the cervical lymph nodes, which in turn induces the production of IL-4 and IL-5. IL-4 is essential for the production of IgE,[24] and IL-5 induces the activation and migration of eosinophils.[25] Cervical lymph node cells play an important role in the onset and worsening of allergic rhinitis. This study showed that there was more efficient infiltration of regulatory DCs into the cervical lymph nodes upon using the intranasal mode of administration. The present study also suggested that regulatory DCs administered via the intranasal route inhibit allergic responses in the cervical lymph nodes more effectively, and induce regulatory T cells in cervical lymph nodes more effectively compared with those administered via other routes. These results suggest that intranasal administration of regulatory DCs inhibits allergic responses and symptoms through the inhibition of allergic responses in the cervical lymph nodes, and may also inhibit nasal allergic responses through the inhibition of nasal lymphocytes.

It has been reported that OVA-pulsed CD40-silenced DCs inhibited the allergic responses and symptoms in an antigen-specific manner;[11] therefore, intranasal administration of OVA-pulsed CD40-silenced DCs, which was the route of administration used in this study, may have inhibited the allergic responses and symptoms in an allergen-specific manner.

DCs can be isolated from the peripheral blood of patients with allergic diseases. Besides, DCs can also be generated *in vitro* using human monocytes isolated from the peripheral blood.[25,26] CD40 in these DCs can be silenced by siRNA and pulsed with antigens *in vitro*. In addition, intranasal administration of cells is also the preferred route of administration. Therefore, a strategy using intranasal administration of these CD40-silenced antigen-specific DCs can be easily translated into treatment for humans with allergic diseases.

DC therapy has been investigated for rheumatoid arthritis in humans. Benham et al[27] had reported a clinical trial using tolerogenic DCs deficient for CD40 and expressing high levels of CD86, which were generated by NF-κB inhibitors for managing rheumatoid arthritis. Eighteen patients received a single dose of the tolerogenic DCs, which were pulsed with a peptide antigen intradermally. This study showed an improvement in the prognosis of this disease, reduction of effector T cells, and increased ratio of regulatory to effector T cells. The reported adverse events were transient leukopenia or lymphopenia, transient anemia, transient elevation of liver transaminases, self-limited headache, and low blood sugar levels. All the adverse events were of grade 1—out of a maximum of 4 grades—and reactions at injection site and anaphylaxis were not seen. Bell et al[28] also reported an unblinded, randomized, controlled, dose escalation Phase I trial in which knee symptoms stabilized in 2 patients who had received autologous DCs, loaded with autologous synovial fluid as a source of autoantigens via the intra-articular route. However, 2 severe adverse events, ie, a flare-up of rheumatoid arthritis and pneumonia were reported in a patient with refractory rheumatoid arthritis. Both these events were considered to be unrelated to DC therapy. Other side effects were mild or moderate but not severe. On the other hand, to our knowledge, clinical trials of DC

therapy have not been performed for allergic diseases of humans. However, therapeutic effects of DC therapy on allergic diseases have been reported not only in mice but also in rats. Pettersson et al showed that subcutaneous injections of DCs exposed to estrogen *in vitro* exhibited therapeutic effects on acute experimental allergic encephalomyelitis in rats.[29] Li et al also showed that injections with DCs infected with adenoviral particles encoding a shRNA targeting CCR7 significantly reduced the numbers of white blood cells, neutrophil, and lymphocyte and the levels of IL-4 and IgE in bronchoalveolar lavage fluid in rats, suggesting the therapeutic effects of DC therapy on asthma.[30] Therefore, in the future, DC therapy may be used for clinically managing rheumatoid arthritis and allergic diseases. Clinical trials of DC therapy are necessary to develop it for the control of allergic diseases in humans. Especially, clinical trials on intranasal therapy with regulatory DCs are expected to be conducted for the management of allergic rhinitis in the future; such studies would provide information regarding the effects and safety of intranasal therapy with regulatory DCs.

In humans, direct administration of allergens, as a traditional allergen immunotherapy, has been used for the management of allergic diseases. In direct administration, not only regulatory DCs, but also non-regulatory DCs uptake allergens *in vivo*. Non-regulatory DCs induce allergic responses as shown in this study, although regulatory DCs inhibit the allergic responses. Additionally, allergens bind to mast cells and basophils, which induce allergic responses and anaphylaxis. Although intranasal allergen immunotherapy is an attractive local therapy for allergic rhinitis, direct intranasal administration of allergens causes allergic responses and adverse symptoms in the nose. In our study, allergens were already pulsed by DCs before the administration of DCs, which prevented allergen uptake by non-regulatory DCs, resulting in stronger inhibition of allergic responses and symptoms. Allergen uptake by DCs before administration also prevents allergen binding to mast cells and basophils, resulting in a reduction in side effects, such as injection-site reactions and anaphylaxis. Benham et al[27] demonstrated that intradermal administration of CD40-deficient and high CD86-expressing tolerogenic DCs did not cause injection-site reactions and anaphylaxis, although intradermal injection of allergens frequently caused injection-site reactions similar to that observed in skin tests. Additionally, the mechanism underlying allergen immunotherapy is complicated and unclear. The mechanism may differ based on the different cell types that take up allergens; in addition, directly administered allergen can be taken up by various cells (not only by DC but also by other antigen presenting cells, such as B cells and Langerhans cells). This suggests that the mechanism underlying DC therapy is simpler and safer than direct administration of allergen. Accordingly, direct administration of regulatory DCs transfected by allergens should result in higher efficiency and safety compared with traditional allergen immunotherapy in not only mice, but also in humans. However, several issues remain to be addressed before initiating clinical treatment. The first is that DC therapy is expensive. However, methods of treatment using DCs may become more advanced in the future, and the cost may also decrease. The second is that some patients may not be comfortable with intranasal DC therapy—that may be similar to the administration of nasal corticoids—because of local problems, such as nasal irritation/dryness, epistaxis, and odour of medicine and additives.[31] In this study, not only intranasal administration of CD40-silenced OVA DCs, but also subcutaneous, intraperitoneal, or intravenous administration of OVA-pulsed CD40-silenced DCs inhibited the allergic responses and symptoms in the nose, although no significant differences of the effects were seen among these three routes of administration. Considering this, subcutaneous, intraperitoneal, or intravenous administration of regulatory DCs can be used as methods of choice in patients who are not comfortable with intranasal administration. In conclusion, in this study, we have demonstrated the usefulness of intranasal administration of regulatory DCs for the management of allergic rhinitis in an animal model. Future studies, including clinical trials and animal experiments related to intranasal administration of regulatory DCs, will widen our understanding of this field of research.

Author contributions
MS: designed the study and wrote the paper. MY and YN: performed experiments and collected the data. YK: collected the data. WM: revised the manuscript. SO: analysed the data. All authors read and approved the final manuscript.

Ethics approval
This study was approved by the Research Ethics Committee in Nagoya City University.

Funding
This study is partially supported by Grants-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science, Grant in Aid from Suzuken Memorial Foundation, and Grant in Aid from Kobayashi Foundation.

Consent of publication
All authors read and approved the final manuscript to submit World Allergy Organization Journal.

Availability of data and materials
Not applicable.

Declaration of Competing Interest
None.

Acknowledgement
None.

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