

# A novel allergen-specific therapy with regulatory T cells induced by CD40-silenced dendritic cells

Motohiko Suzuki, Makoto Yokota, Shinya Ozaki, Yoshihisa Nakamura

## Abstract

**Background:** We previously reported that dendritic cells (DCs) transfected with CD40 siRNA and pulsed by ovalbumin (OVA) (CD40-silenced OVA DCs) inhibited allergic responses through facilitation of regulatory T cells (Tregs). However, to our knowledge, no prior study has examined allergen-specific therapy by administration of siRNA-induced Tregs for the control of allergy.

**Objective:** We aimed to investigate the effect of Tregs induced in vitro on allergic responses and symptoms in vivo.

**Methods:** Mice were treated with Tregs (OVA DCs-induced Tregs) induced by CD40-silenced OVA DCs or Tregs (nonantigen DCs-induced Tregs) induced by DCs transfected with CD40 siRNA and pulsed with no antigen, and the effects of these Tregs on allergic responses were estimated.

**Results:** Administration of nonantigen DCs-induced Tregs prevented not only OVA-induced allergy but also keyhole limpet hemocyanin-induced allergy. Administration of OVA DCs-induced Tregs significantly reduced the number of sneezes and nasal rubbing movements, eosinophilia in the nasal mucosa, and the level of OVA-specific IgE in mice with OVA-induced allergy, compared with CD40-silenced nonantigen DC-induced Tregs in numbers 20 times greater, even in mice with established allergic rhinitis. Furthermore, Tregs induced by CD40-silenced DCs pulsed with Cry j 1, a major allergen of Japanese cedar pollen, inhibited Japanese cedar-induced allergy.

**Conclusions:** This study shows for the first time that both antigen-independent Tregs and antigen-specific Tregs can be induced by siRNA, and that therapy with siRNA-induced Tregs inhibits allergic responses and symptoms. It also shows that antigen-specific Tregs have more potent effects in inhibiting allergic responses than antigen-nonspecific Tregs.

**Key words:** Regulatory T cells, Allergy, CD40, siRNA, Dendritic cells.

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## Introduction

CD40 is an integral membrane protein in dendritic cells (DCs) that activates T cells. Blockade of the CD40-CD40L interaction is a potent tolerance-inducing strategy,<sup>1,2</sup> while the inhibition of this interaction suppresses T cell responses<sup>3</sup> and generates regulatory T cells (Tregs).<sup>4</sup>

RNA interference using small interfering RNA (siRNA) induces specific silencing of gene expression, and is a potent, selective, and easy method.<sup>5</sup> Andrew Fire and Craig Mello received the Nobel Prize in Medicine for this discovery. Silencing gene expression by siRNA is more useful and promising than conventional silencing strategies by gene or antibody, such as

blocking antibody, blocking protein, antisense oligonucleotide, and ribozymes.<sup>6-8</sup>

We previously reported that vector expressing siRNA specific for CD40 (CD40 siRNA) inhibits allergic responses not only as a means of prevention<sup>9</sup> but also as treatment.<sup>10</sup> However, direct administration of vector expressing siRNA may induce complications, because it is an antigen-nonspecific therapy and the vector or siRNA may change immune responses in vivo. We also showed that administration of CD40-silenced antigen-specific dendritic cells (DCs), transfected with CD40 siRNA but not vector CD40 siRNA and pulsed by antigen in vitro,

inhibited allergic responses and symptoms antigen-specifically.<sup>11</sup> However, CD40-silenced antigen-specific DCs may lead to unexpected complications in vivo, since siRNA in CD40-silenced DCs may cause unexpected problems. We additionally documented that CD40-silenced DCs induce facilitation of CD4<sup>+</sup>CD25<sup>+</sup> Tregs in vivo.<sup>11</sup> Furthermore, induction of Tregs by CD40-silenced DCs is not always the same by the conditions in vivo. Considering this, direct administration of antigen-specific CD4<sup>+</sup>CD25<sup>+</sup> Tregs, induced by siRNA in vitro, is an attractive strategy for safer and more effective control of allergic diseases. To our knowledge, however, therapy with antigen-specific CD4<sup>+</sup>CD25<sup>+</sup> Tregs induced by siRNA in vitro has not been reported for the control of allergy, and its usefulness is not known.

The generation of Tregs with anti-CD3/CD28 antibodies in vitro has been reported.<sup>12</sup> However, these are not antigen-specific Tregs. Antigen-specific Tregs are attractive for the treatment of allergy, since antigen-nonspecific Tregs may affect various immune responses and contribute to a range of diseases, including cancer.<sup>13</sup> It has been also reported that induced-Tregs generated by anti-CD3/CD28 antibodies differ from those induced by physiological-like activation with antigen/APC.<sup>14</sup>

In this study, we examined the effect on allergic diseases of CD4<sup>+</sup>CD25<sup>+</sup> Tregs induced by antigen-specific DCs transfected with siRNA in vitro. The results showed that administration of ovalbumin (OVA)-specific CD4<sup>+</sup>CD25<sup>+</sup> Tregs, induced by DCs transfected with CD40 siRNA and pulsed with OVA in vitro, inhibited allergic responses and symptoms in mice with allergic rhinitis, and that CD40-silenced DCs pulsed without antigen induced antigen-nonspecific Tregs. It was also shown that antigen-specific Tregs were more potent in inhibiting allergic responses and symptoms than antigen-nonspecific Tregs.

## Methods

### Generation of bone marrow-derived DCs and gene silencing by siRNA

DCs were generated from bone marrow progenitor cells, as previously described.<sup>9,10,11</sup> These DCs were transfected with transfection reagent alone (No siRNA DCs), siRNA (Control siRNA) specific to the Luciferase gene GL2 Duplex siRNA (Control DCs), or siRNA (CD40 siRNA, UUCUCAGCCCAG UGGAACA) specific to CD40. DCs transfected with CD40 siRNA were pulsed with OVA (CD40-silenced OVA DCs) or without OVA (CD40-silenced nonantigen DCs), as described previously.<sup>9,10,11</sup> DCs transfected with CD40 siRNA were also pulsed with Cry j 1, a major allergen of Japanese cedar (*Cryptomeria japonica*) pollen, (CD40-silenced Cry j 1 DCs) by the same method. Cry j 1 was purified by the method previously reported.<sup>15,16</sup>

### Generation of Tregs in vitro

Mouse naïve CD4<sup>+</sup> T cells were isolated from splenic cells of six to eight week-old male BALB/c mice using a Mouse Naïve CD4<sup>+</sup> T Cell Isolation Kit (R&D Systems, CA). Mouse naïve CD4<sup>+</sup> T cells ( $3 \times 10^5$ /mL) were co-cultured with  $6 \times 10^5$ /mL No siRNA DCs, Control DCs, CD40-silenced nonantigen DCs, CD40-silenced OVA DCs, or CD40-silenced Cry j 1 DCs for 5

days in 2 mL of complete medium, RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-ME, and 10% FCS supplemented with TGF-β (5 ng/mL) and IL-2 (50 IU/mL). CD4<sup>+</sup>CD25<sup>+</sup> T cells were collected using a MACS negative CD4 isolation kit and anti-CD25 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany).<sup>9</sup>

### Immunization and Treatment

Six to eight week-old male BALB/c mice (Japan SLC Inc., Shizuoka, Japan) were injected intravenously with PBS alone, Tregs ( $4 \times 10^5$ ,  $4 \times 10^6$ , or  $8 \times 10^6$  cells/mouse) induced by CD40-silenced nonantigen DCs, or Tregs ( $4 \times 10^5$  cells/mouse) induced by CD40-silenced OVA DCs on day 1. Mice were also injected intraperitoneally (i.p.) with 4 mg Al(OH)<sub>3</sub> and 10 µg ovalbumin (OVA) twice on days 2 and 15. Each group consisted of five mice. The same mice were challenged intranasally (i.n.) on days 21 through 27 with OVA (100 µg). Samples were collected on day 28.

In the second experiment, the protocol was the same as in the above experiment except that mice received PBS alone, Tregs ( $4 \times 10^5$  or  $4 \times 10^6$  cells/mouse) induced by CD40-silenced nonantigen DCs, or Tregs ( $4 \times 10^5$  or  $4 \times 10^6$  cells/mouse) induced by CD40-silenced OVA DCs and that mice were injected i.p. with 4 mg Al(OH)<sub>3</sub> and keyhole limpet hemocyanin (KLH), but not OVA, on days 2 and 15 and challenged i.n. on days 21 through 27 with KLH.

In the third experiment, mice were sensitized with OVA (10 µg) and 2 mg Al(OH)<sub>3</sub> intraperitoneally on days 1 and 14, and then the same mice were challenged intranasally with OVA (100 µg) on days 18 through 24. Intravenous administration of PBS alone, Tregs induced by CD40-silenced nonantigen DCs ( $4 \times 10^6$  or  $8 \times 10^6$  cells/mouse), or Tregs by CD40-silenced OVA DCs ( $4 \times 10^5$  cells/mouse), was performed on day 26. These mice were then re-challenged intranasally on days 27 through 32 with OVA (100 µg).

In the fourth experiment, mice were sensitized with Cry j 1 (3 µg) and 2 mg Al(OH)<sub>3</sub> intraperitoneally on days 1 and 14, and then the same mice were challenged intranasally with Cry j 1 (2 µg) on days 18 through 24. Intravenous administration of PBS alone, Tregs induced by CD40-silenced nonantigen DCs ( $8 \times 10^6$  cells/mouse), or Tregs by CD40-silenced Cry j 1 DCs ( $4 \times 10^5$  cells/mouse), was performed on day 26. These mice were then re-challenged intranasally on days 27 through 32 with Cry j 1 (3 µg).

This study was approved by Research Ethics Committee in Nagoya City University. Mice were housed in an environmentally-controlled animal facility at Nagoya City University in Japan. The protocols were in accordance with the Guidelines for Care and Use of Animals of Nagoya City University. Every effort was made to minimize the discomfort of the animals.

### Cry j 1-specific T cell response

CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD11c cells were isolated from spleen using MACS beads (Miltenyi Biotec). Spleen CD4<sup>+</sup>CD25<sup>+</sup> T cell ( $2 \times 10^6$  cells/mL) and DC ( $2 \times 10^5$  cells/mL) suspensions were cultured for 72 h and stimulated with 10 µg/mL Cry j 1 antigen.

**OVA-specific T cell response**

Splenic cells isolated by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) were cultured in 96-well plates at a concentration of  $4 \times 10^5$  cells/well for 72 h in the presence of 100  $\mu\text{g/mL}$  OVA antigen.

**Measurement of IL-2 production**

Spleen  $\text{CD4}^+\text{CD25}^-$  T cell ( $2 \times 10^6$  cells/mL) and DC ( $2 \times 10^5$  cells/mL) transfected with or without CD40 siRNA suspensions were cultured for 72 hours, stimulated with 10  $\mu\text{g/mL}$  Cry j 1. Quantities of IL-2 cytokines in the culture supernatants were determined by using a sandwich ELISA. Plates were coated with anti-mouse IL-2 (BioLegend, San Diego, CA). The culture supernatant was then added, and the plates were incubated with the second antibody of biotinylated anti-mouse IL-2 (BioLegend). Standard curves were generated by using recombinant cytokines.

**Measurement of OVA-specific, KLH-specific, and Cry j 1-specific IgE in sera**

Titers of specific IgE were measured by ELISA. Briefly, ELISA plates were coated with anti-mouse IgE monoclonal antibody (Yamasa, Tokyo, Japan). Non-specific binding was blocked and sera were added. After washing with wash buffer, biotinylated OVA, KLH, or Cry j 1 was added to the well. The plates were then incubated with avidin-peroxidase at 37°C for an hour after washing. The TMB microwell peroxidase substrate system (KPL, Gaithersburg, MD) was used, and optical density (O.D.) was measured at 450 nm.

**Nasal allergic symptoms**

Immediately after the last nasal challenge, the number of sneezes and nasal rubbing movements was counted for 20 min according to the method previously reported.<sup>11</sup>

**Pathology**

The heads were decalcified and sectioned. Three micrometer thick sections of nasal tissue were stained with Luna staining. The number of eosinophils in the nasal mucosa of the

nasal septum was counted microscopically in a field of view at 400 $\times$  magnification. The observer was blinded to treatment when counting the number of eosinophils.

**Statistical analysis**

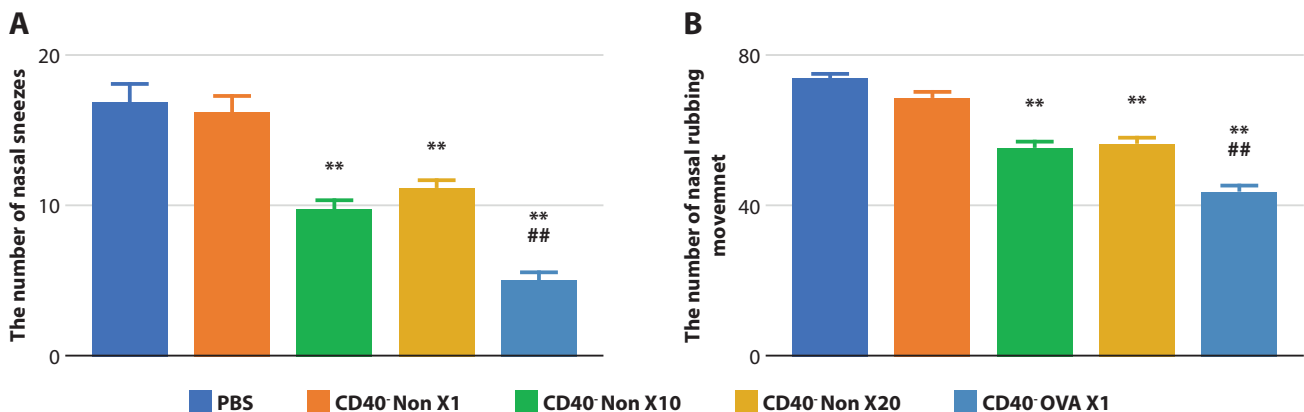
Data are expressed as means  $\pm$  SEM. Statistical comparisons between groups were performed using one-way ANOVA followed by the Newman-Keuls Test. Differences with *P*-values less than 0.05 were considered significant.

**Results**

**Prevention of OVA-induced allergy with CD40-silenced DC-induced OVA Tregs**

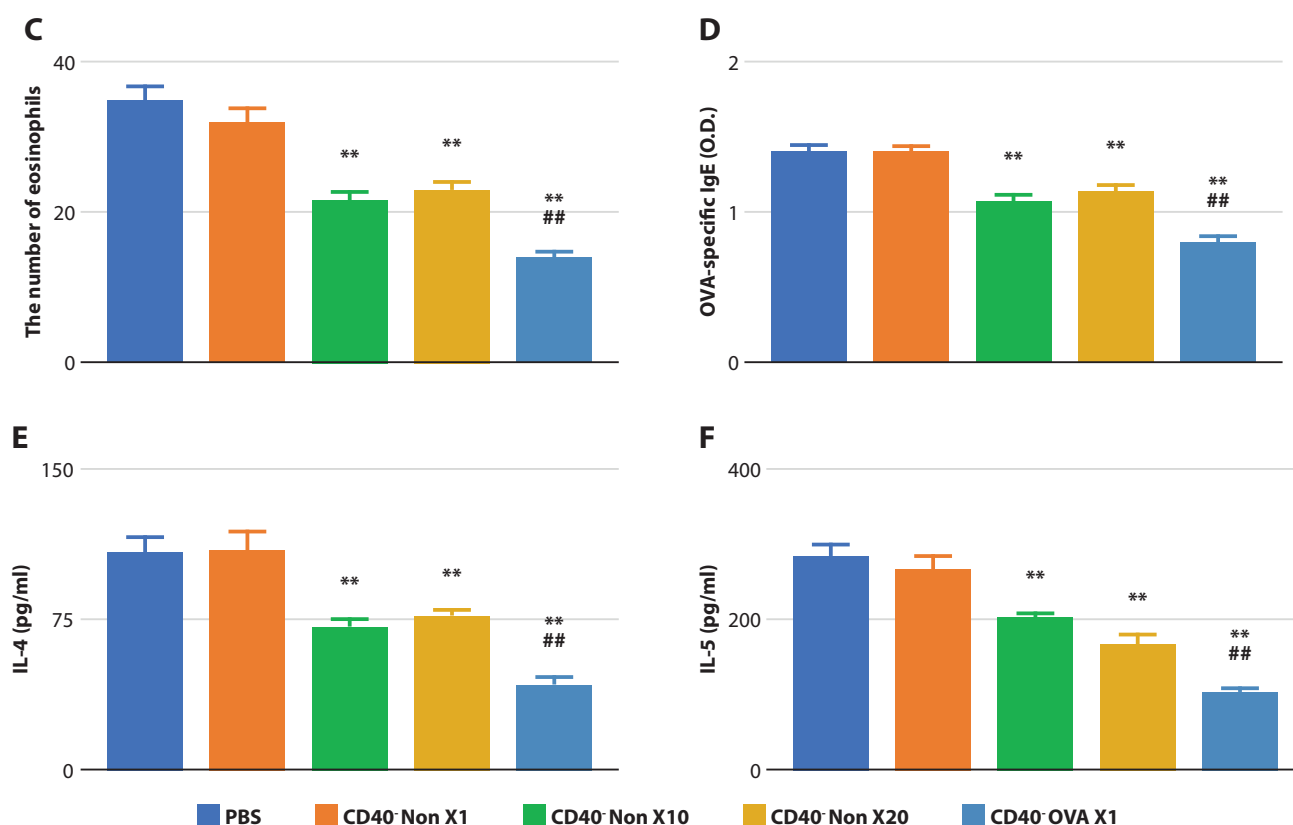
We investigated whether Tregs induced by CD40-silenced OVA DCs in vitro could prevent OVA-induced allergy. Mice that received PBS, CD40-silenced nonantigen DC-induced  $\text{CD4}^+\text{CD25}^+$  cells, or CD40-silenced OVA DC-induced  $\text{CD4}^+\text{CD25}^+$  cells were sensitized and challenged with OVA as described in Methods (treatment on day 1, sensitization on days 2 & 15, challenge on days 21-27, sample collection on day 28). The number of sneezes and nasal rubbing movements was counted immediately after the last nasal challenge to examine the effect of these T cells on nasal allergic symptoms. CD40-silenced OVA DC-induced Tregs significantly decreased the number of sneezes and nasal rubbing movements compared with the other groups (Figure 1A and B). Although CD40-silenced nonantigen DC-induced T cells at a concentration of  $4 \times 10^5$  cells/mouse did not reduce these symptoms, CD40-silenced nonantigen DC-induced T cells at levels 10 times greater and more ( $4 \times 10^6$  cells/mouse and  $8 \times 10^6$  cells/mouse) significantly inhibited these symptoms. However, there were no significant differences in symptom inhibition between CD40-silenced nonantigen DC-induced Tregs at levels of  $4 \times 10^6$  cells/mouse and  $8 \times 10^6$  cells/mouse.

Next, the number of eosinophils in the nasal septum was counted to evaluate eosinophilia, which is associated with allergic symptoms and allergic responses in the nose. The number of eosinophils infiltrating the nasal mucosa in mice injected with Tregs induced by CD40-silenced OVA DCs was



**Figure 1. Prevention effects of allergy by CD4+CD25+ cells induced by CD40-silenced OVA DCs.**

Five mice were injected intraperitoneally and challenged intranasally with OVA after treatment of PBS alone, CD40-silenced nonantigen DC-induced  $\text{CD4}^+\text{CD25}^+$  cells (CD40- Non,  $4 \times 10^5$  “X1”,  $4 \times 10^6$  “X10”, or  $8 \times 10^6$  “X20”, cells/mouse), or CD40-silenced OVA DC-induced  $\text{CD4}^+\text{CD25}^+$  cells (CD40- OVA,  $4 \times 10^5$  cells/mouse). The number of sneezes (A) and nasal rubbing movements (B) was counted after the last nasal challenge.



**Figure 1. (Continued)**

(C) Eosinophilia of the nasal septum. (D) The level of OVA-specific IgE in sera. The level of IL-4 (E) and IL-5 (F) production from splenic splenocytes stimulated by OVA was measured by ELISA. \*\*  $P < 0.01$  versus groups of PBS alone and CD40- Non X1. ##  $P < 0.01$  versus groups of CD40- Non (X10, X20). Experiments were repeated 3 times with similar result.

significantly fewer than that in mice with PBS alone or Tregs induced by CD40-silenced nonantigen DCs (Figure 1C). CD40-silenced nonantigen DC-induced Tregs at levels of  $4 \times 10^6$  cells/mouse or  $8 \times 10^6$  cells/mouse also significantly inhibited this eosinophilia, whereas CD40-silenced nonantigen DC-induced Tregs at the level of  $4 \times 10^5$  cells/mouse did not (Figure 1C).

We also measured OVA-specific IgE in sera by ELISA, since IgE is associated with allergic reactions. CD40-silenced nonantigen DC-induced Tregs at levels of  $4 \times 10^6$  or  $8 \times 10^6$  cells/mouse also significantly suppressed the level of OVA-specific IgE, although CD40-silenced nonantigen DC-induced Tregs at the level of  $4 \times 10^5$  cells/mouse did not. Tregs produced by CD40-silenced OVA DCs inhibited OVA-specific IgE significantly more than the other groups (Figure 1D). These data suggest that Tregs induced by CD40-silenced OVA DCs prevent production of OVA-specific IgE.

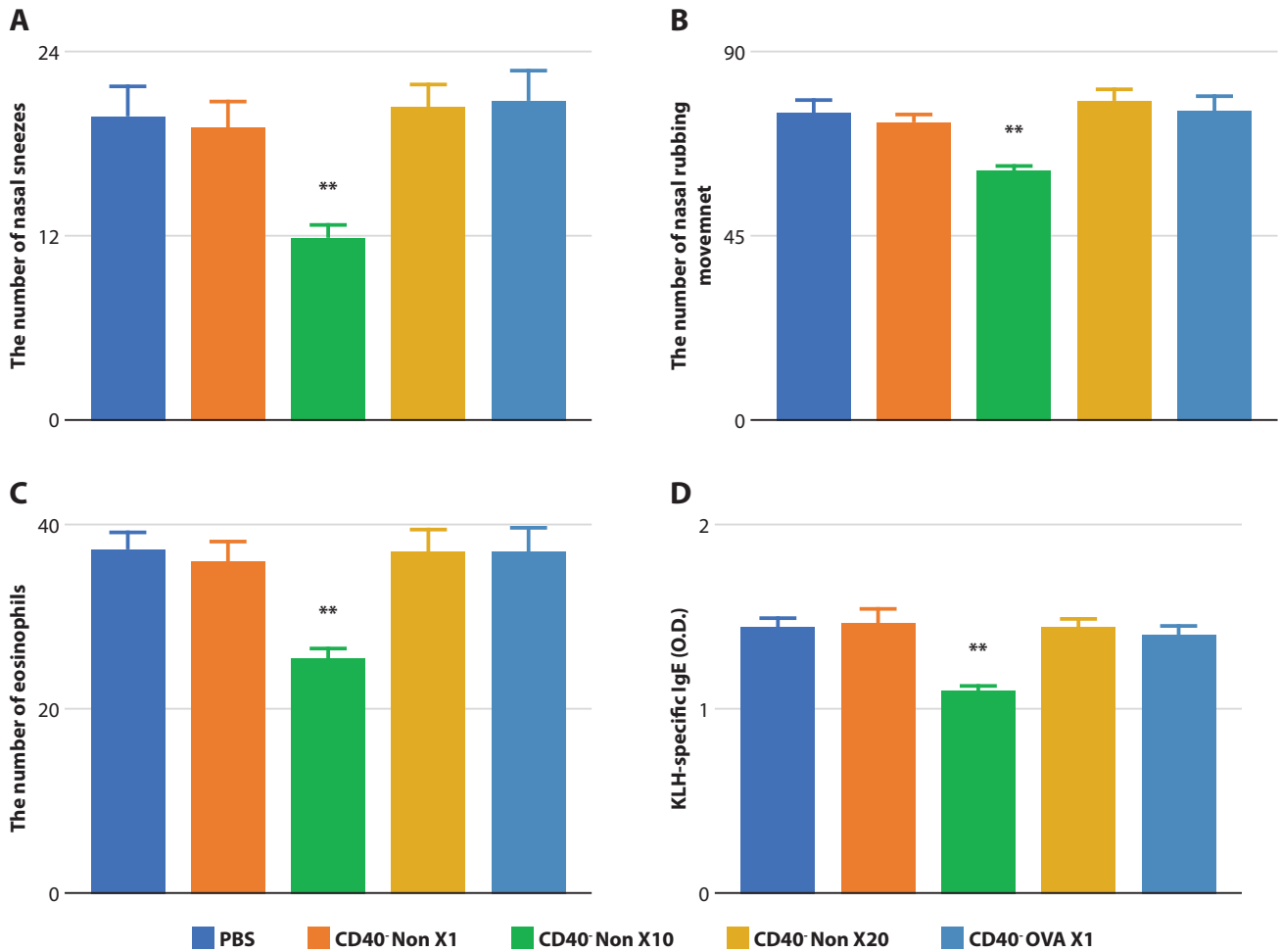
IL-4 and IL-5 play important roles in the development of allergic diseases. In order to investigate the effect of Tregs induced by CD40-silenced OVA DCs on cytokine production, we measured the production of IL-4 and IL-5 from splenic T cells stimulated with OVA in vitro. There were no significant differences between mice received PBS alone and CD40-silenced nonantigen DC-induced Tregs at levels of  $4 \times 10^5$  cells/mouse in the productions of IL-4 and IL-5. The levels of IL-4 and IL-5 produced in mice that received Tregs induced by CD40-silenced OVA DCs were significantly lower than those

in mice that received PBS or Tregs induced by CD40-silenced nonantigen DCs (Figure 1E and F). This suggests that OVA-specific Tregs suppress the production of Th2 cytokines, which may contribute to the prevention of allergy.

#### **No preventive effect of Tregs induced by CD40-silenced OVA DCs on KLH-induced allergy**

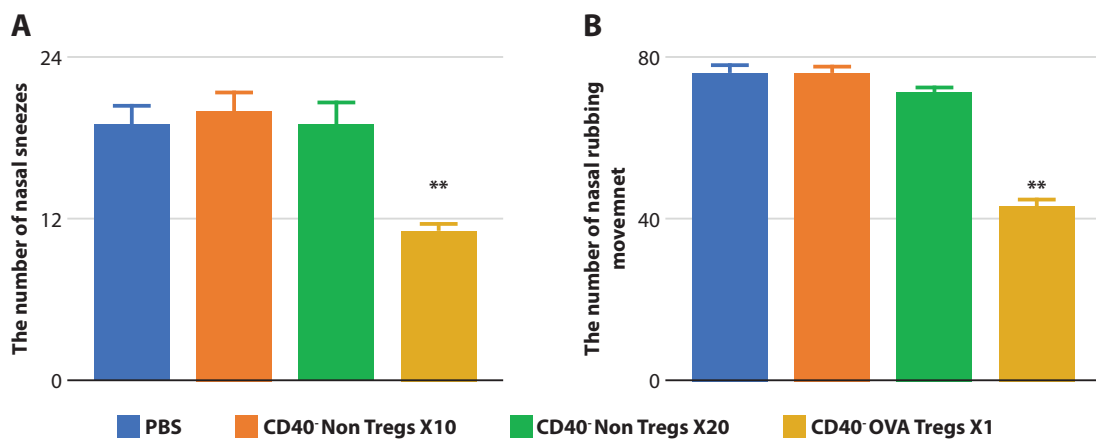
To investigate antigen specificity, we examined whether Tregs induced by CD40-silenced OVA DCs in vitro can inhibit allergic responses and symptoms caused by KLH. Mice received PBS, CD40-silenced nonantigen DC-induced Tregs, or CD40-silenced OVA DC-induced Tregs were sensitized and challenged with KLH as described in Methods (treatment on day 1, sensitization on days 2 & 15, challenge on days 21-27, sample collection on day 28). Administration of Tregs induced by CD40-silenced OVA DCs did not significantly inhibit the number of nasal sneezes, nasal rubbing movements, or eosinophils at the nasal septum and the level of KLH-specific IgE in sera compared with mice that received PBS alone (Figure 2A-D). These findings suggest that Tregs induced by CD40-silenced OVA DCs inhibit allergen reactions and symptoms in an antigen-specific manner.

Administration of CD40-silenced nonantigen DC-induced Tregs ( $4 \times 10^6$  cells/mouse) inhibited the number of nasal sneezes, nasal rubbing movements, and eosinophils at the nasal mucosa and KLH-specific IgE levels in sera compared with the other groups (Figure 2A-D). These results suggest



**Figure 2. No allergy prevention effect from CD4<sup>+</sup>CD25<sup>+</sup>Tregs induced by CD40-silenced OVA DCs.**

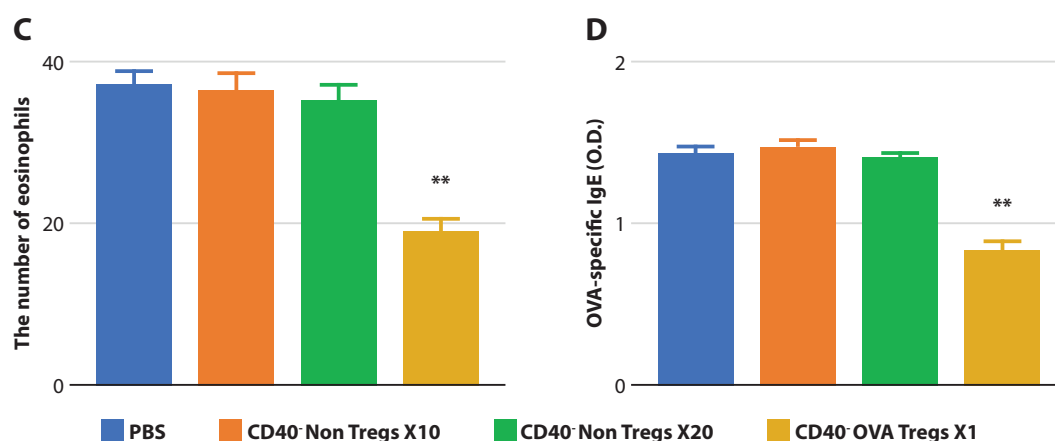
Five mice were injected intraperitoneally and challenged intranasally with KLH after treatment with PBS alone, CD40-silenced nonantigen DC-induced CD4<sup>+</sup>CD25<sup>+</sup> cells (CD40<sup>-</sup> Non, 4 × 10<sup>5</sup> “X1” or 4 × 10<sup>6</sup> “X10” cells/mouse), or CD40-silenced OVA DC-induced CD4<sup>+</sup>CD25<sup>+</sup> cells (CD40<sup>-</sup> OVA, 4 × 10<sup>5</sup> “X1” or 4 × 10<sup>6</sup> “X10” cells/mouse). The numbers of sneezes (A) and nasal rubbing movements (B) were counted after the last nasal challenge. (C) Eosinophilia of the nasal septum. (D) The level of KLH-specific IgE in sera. \*\* P < 0.01 versus groups of PBS alone, CD40<sup>-</sup> Non X1, and CD40<sup>-</sup> OVA (X1, X10). Experiments were repeated 3 times with similar result.



**Figure 3. Therapeutic effects of CD4<sup>+</sup>CD25<sup>+</sup>Tregs induced by CD40-silenced OVA DCs in vitro on established allergic rhinitis.**

Five mice with OVA-induced allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced CD4<sup>+</sup>CD25<sup>+</sup> cells (CD40<sup>-</sup> Non, 4 × 10<sup>6</sup> “X10” or 8 × 10<sup>6</sup> “X20” cells/mouse), or CD40-silenced OVA DC-induced CD4<sup>+</sup>CD25<sup>+</sup> cells (4 × 10<sup>5</sup> “CD40-OVA X1” cells/mouse). The number of sneezes (A) and nasal rubbing movements (B) was counted after the last nasal challenge.





**Figure 3. (Continued)**

(C) Eosinophilia of the nasal septum. (D) The level of OVA-specific IgE in sera. \*\*  $P < 0.01$  versus group of PBS alone, CD40<sup>-</sup> Non X10, and CD40<sup>-</sup> Non X20. Experiments were repeated 3 times with similar result.

that CD40-silenced nonantigen DC-induced Tregs are not antigen-specific.

#### **Therapeutic effects of Tregs induced by CD40-silenced OVA DCs on mice with established OVA-induced allergic rhinitis**

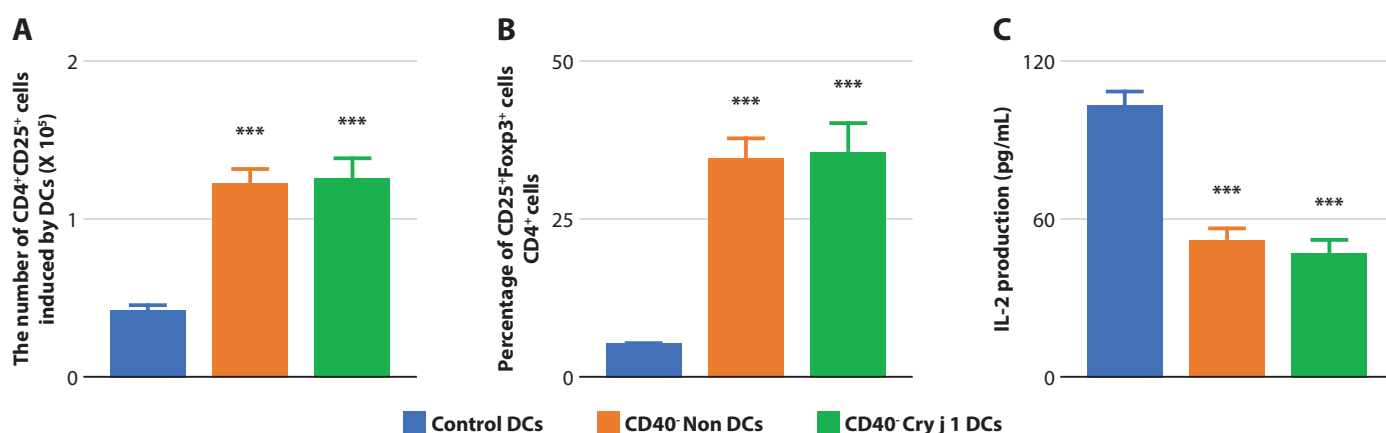
Mice with established allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced Tregs, or CD40-silenced OVA DC-induced Tregs. After treatment, nasal re-challenge with OVA was performed (sensitization on days 1 & 14, nasal challenge on days 18-24, treatment with Tregs on day 26, nasal re-challenge on days 27-32, sample collection on day 33). The number of sneezes and nasal rubbing movements on day 24 was significantly higher than on day 17 (data not shown). Eosinophils in the nasal septum were seen on day 24, although no eosinophilia was found on day 17 (data not shown). These results suggest that mice were suffering from allergic rhinitis on day 24. There were no significant effects on the number of sneezes, nasal rubbing movements, or eosinophils in the nasal mucosa, or the level of OVA-specific IgE in sera, even when CD40-silenced nonantigen DC-induced Tregs

( $8 \times 10^6$  cells/mouse) were injected (**Figure 3A-D**).

Tregs induced by CD40-silenced OVA DCs in vitro significantly reduced the number of sneezes, nasal rubbing movements, and eosinophils in the nasal mucosa, and the level of OVA-specific IgE in sera, compared with the other groups, PBS alone, and Tregs induced by CD40-silenced nonantigen DCs (**Figure 3A-D**). These findings suggest that Tregs induced by CD40-silenced OVA DCs are therapeutically useful even for mice with established allergic rhinitis.

#### **Immune regulatory properties of Tregs induced by DCs (CD40-silenced *Cry j 1* DCs) transfected with CD40 siRNA and pulsed with *Cry j 1***

Next, we investigated Tregs induced by CD40-silenced DCs (CD40-silenced *Cry j 1* DCs) pulsed with *Cry j 1* but not OVA, because OVA is a food allergen but not aeroallergen. *Cry j 1* is one of the major allergens of Japanese cedar pollen which cause severe allergic diseases in Japan.<sup>15-19</sup> Bone marrow-derived DCs were transfected with CD40 siRNA or Control siRNA (Control DCs). DCs transfected with CD40 siRNA were pulsed



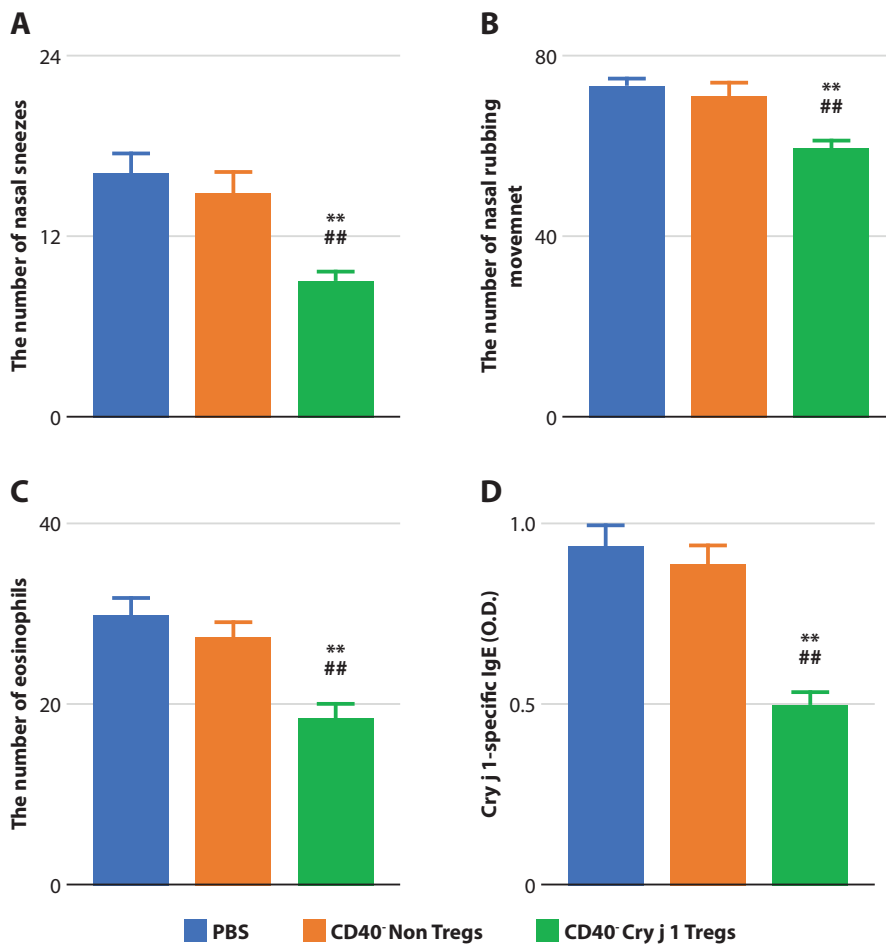
**Figure 4. Modulation by CD40 siRNA in vitro.** (A) DCs were transfected with Control siRNA (Control DCs) or CD40 siRNA. DCs transfected with CD40 siRNA were pulsed without *Cry j 1* (CD40<sup>-</sup> Non DCs) or with *Cry j 1* (CD40<sup>-</sup> *Cry j 1* DCs). The numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells induced from  $3 \times 10^5$  naïve CD4<sup>+</sup> cells by Control DCs, CD40<sup>-</sup> *Cry j 1* DCs, and CD40<sup>-</sup> Non DCs were examined. (B) The percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> T cells in CD4<sup>+</sup> T cells after co-culture of T cells and DCs. (C) Quantity of IL-2 production after co-culture of T cells and DCs. \*\*\* $P < 0.001$  versus group of Control DCs. Experiments were repeated 3 times with similar result.

with Cry j 1 (CD40-silenced Cry j 1 DCs) or no antigen (CD40-silenced nonantigen DCs). Naïve T cells, separated from splenic T cells in naïve mice as described in Methods, were co-cultured with Control DCs, CD40-silenced nonantigen DCs, or CD40-silenced Cry j 1 DCs. Although we assessed the number of CD4<sup>+</sup>CD25<sup>+</sup> cells were induced from 3 × 10<sup>5</sup> naïve CD4<sup>+</sup> cells, the number of CD4<sup>+</sup>CD25<sup>+</sup> cells induced by CD40-silenced Cry j 1 DCs or CD40-silenced nonantigen DCs were significantly higher than that by Control DCs. (Figure 4A). The percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> cells in CD4<sup>+</sup> T cells induced by CD40-silenced nonantigen DCs and CD40-silenced Cry j 1 DCs were significantly higher compared with those induced by Control DCs (Figure 4B). And we investigated whether CD4<sup>+</sup>CD25<sup>+</sup> cells induced by CD40-silenced Cry j 1 DCs could affect IL-2 production in order to examine the mechanism of Treg induction, since the association between IL-2 production and Treg expansion has been reported.<sup>20,21</sup> Cry j 1-specific T cell response was generated by a co-culture of DCs and CD4<sup>+</sup>CD25<sup>-</sup> T cells isolated from the spleen in mice sensitized with Cry j 1 antigen. Quantity of IL-2 in the supernatant was measured by ELISA. Consequently, IL-2 production

was significantly inhibited by CD40-silenced nonantigen DCs or CD40-silenced Cry j 1 DCs (Figure 4C).

**Therapeutic effects of Tregs induced by CD40-silenced Cry j 1 DCs on mice with established Cry j 1-induced allergic rhinitis**

We assessed the effects of siRNA-induced Tregs on allergic diseases caused by aeroallergen, Japanese cedar pollen. Mice with allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced Tregs, or CD40-silenced Cry j 1 DC-induced Tregs. After treatment, nasal re-challenge with Cry j 1 was performed (sensitization on days 1 & 14, nasal challenge on days 18-24, treatment with Tregs on day 26, nasal re-challenge on days 27-32, sample collection on day 33). No eosinophilia in the nasal septum was found on day 17, whereas eosinophilia was seen on day 24 (data not shown). The numbers of sneezes and nasal rubbing movements on day 24 were significantly higher than those on day 17 (data not shown). These suggest that allergic rhinitis was established on day 24. After treatment with CD40-silenced nonantigen DC-induced Tregs, there were no significant effects on the number of sneezes, nasal rubbing movements, eosinophilia in the nasal mucosa, and



**Figure 5. Therapeutic effects of CD4<sup>+</sup>CD25<sup>+</sup>Tregs induced by CD40-silenced Cry j 1 DCs in vitro on established allergic rhinitis.** Five mice with Cry j 1-induced allergic rhinitis were treated with PBS alone, CD40-silenced nonantigen DC-induced CD4<sup>+</sup>CD25<sup>+</sup> cells (8 × 10<sup>6</sup> cells/mouse, CD40<sup>-</sup> Non Tregs) or CD40-silenced Cry j 1 DC-induced CD4<sup>+</sup>CD25<sup>+</sup> cells (4 × 10<sup>5</sup> cells/mouse, CD40-Cry j 1 Tregs). The number of sneezes (A) and nasal rubbing movements (B) was counted after the last nasal challenge. (C) Eosinophilia of the nasal septum. (D) The level of Cry j 1-specific IgE in sera. \*\* P < 0.01 versus group of PBS alone, ## P < 0.01 versus group of CD40<sup>-</sup> Non Tregs. Experiments were repeated 3 times with similar result.

the level of Cry j 1-specific IgE in sera (**Figure 5A-D**). However, Tregs induced by CD40-silenced Cry j 1 DCs in vitro significantly reduced the number of sneezes, nasal rubbing movements, and eosinophilia in the nasal mucosa, and the level of Cry j 1-specific IgE in sera, compared with other groups, PBS alone, and Tregs induced by CD40-silenced nonantigen DCs (**Figure 5A-D**). These findings suggest that Tregs induced by CD40-silenced Cry j 1 DCs are therapeutically useful for mice with allergic rhinitis caused by Japanese cedar pollen.

## Discussion

Administration of Tregs induced by CD40-silenced nonantigen DCs before sensitization significantly reduced allergic responses and symptoms not only in OVA-induced allergy but also in KLH-induced allergy. These results suggest that Tregs induced by CD40-silenced nonantigen DCs are antigen-nonspecific Tregs. Patients who suffer from sensitization to multiple allergens are increasing.<sup>22</sup> Antigen-specific therapy for these patients is not easy, nor is it applicable for patients with an unknown causative allergen. Thus, CD40 silenced nonantigen DC-induced Tregs may be an alternative, antigen-independent therapy for the prevention of allergic diseases.

Although blockade of CD40-CD40L interaction induce Tregs,<sup>4,23</sup> the underlying mechanism of Treg expansion by blockade of CD40-CD40L is not known.<sup>24</sup> However, low-dose IL-2 expands CD4<sup>+</sup> regulatory T cells with a suppressive function in vitro.<sup>21</sup> Both blockade of B7-CD28 and CD40-CD40L also activated Foxp3<sup>+</sup> regulatory T cells and reduced IL-2 production.<sup>20</sup> When CD25<sup>+</sup> CD4<sup>+</sup> T cells compete with other cells for IL-2, CD4<sup>+</sup>CD25<sup>+</sup> T cells further up-regulate the CD25 (IL-2R alpha chain).<sup>25</sup> And Vogel et al.<sup>20</sup> assumed that the low amount of IL-2 is enough for the survival of CD4<sup>+</sup>Foxp3<sup>+</sup> cells, but not enough for the survival of CD4<sup>+</sup>Foxp3<sup>-</sup> cells. This study showed that blockade of only CD40-CD40L pathway inhibited IL-2 productions. These suggest that blockade of CD40-CD40L induces expansion of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs through reduction of IL-2 production.

We previously reported that CD40-silenced OVA DCs inhibited allergic reactions and symptoms. However, CD40-silenced OVA DCs may induce unexpected problems in vivo. CD40 siRNA may go out of DCs and induce problems such as inhibition of CD40 gene on other cells, interferon response, and off-target effect, although these have been not reported. If deficiency of CD40-CD40L interaction occurs in vivo, this may lead susceptibility to infection<sup>26,27</sup> like hyper IgM syndrome.<sup>28</sup> dsRNA, less than 30 bp in length, are generally believed to avoid interferon responses.<sup>29</sup> However, interferon response should be paid attention to even in siRNA, since siRNA could interfere with interferon response<sup>30,31</sup> and since the threshold of dsRNA length to induce interferon responses varies by cell types.<sup>29</sup> In future, various Treg phenotype may be revealed. Even if siRNA-induced Tregs include various Treg phenotype, it may be possible to collect only specific phenotype before administration in time to come. The advantages of this novel therapy with siRNA-induced Tregs presented herein include: 1) no interferon responses caused by siRNA; 2) no off-target effects by siRNA; 3) no inhibition of CD40 gene expression in vivo by CD40 siRNA; 4) no unexpected problems by siRNA or

siRNA-transfected DCs; 5) higher stability in the numbers of siRNA-induced Tregs administered (induction of Tregs by CD40-silenced DCs is not always the same by the conditions in vivo), and 6) possibility to select specific Treg phenotype before administration, compared with therapy with siRNA-transfected DCs. On the other hand, the advantages of therapy with siRNA-transfected DCs presented herein include: 1) less time for preparation in vitro, 2) less cost, and 3) possibilities of tolerance, anergy, and apoptosis by modified DCs,<sup>32-34</sup> compared with therapy with siRNA-induced Tregs.

In this study, we report a novel antigen-specific therapy for the control of allergic diseases, using Tregs induced by CD40-silenced antigen-specific DCs transfected with CD40 siRNA in vitro, and siRNA-induced antigen-nonspecific Tregs for the prevention of allergic diseases. Furthermore, antigen-specific Tregs induced by siRNA-modulated DCs are attractive since they have more potent inhibiting effects on allergic responses and symptoms than antigen non-specific Tregs.

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## Conflict of interest

None

## Authors' contributions

Motohiko Suzuki and Yoshihisa Nakamura designed the study. Motohiko Suzuki and Makoto Yokota wrote the manuscript. Makoto Yokota and Shinya Ozaki contributed to data collection. Shinya Ozaki and Yoshihisa Nakamura performed the statistical analysis and interpretation of the results. All authors read and approved the final manuscript.

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