

still in the initial treatment phase or did not yet complete the tapering schedule. Therefore these data cannot be analyzed at this time. Of these 24 patients, 14 (58%) remained symptom free until the present (mean relapse-free period, 208 weeks). Ten patients experienced a relapse after treatment with omalizumab was discontinued (42%). In 7 of the patients with relapses, symptoms returned after approximately 12 weeks. In these patients omalizumab was restarted with different intervals but eventually similar outcomes, namely a continued symptom-free dosing interval of 12 weeks. Three patients had a relapse that was most likely related to exposure of a known trigger for urticaria. Patients restarted omalizumab treatment with a 4-week dosing interval, which was prolonged directly after the first dose until the interval of 8 weeks was reached before stopping treatment again. All patients who restarted treatment reached CR within 4 weeks after a single 300-mg omalizumab administration.

To summarize, the overall efficacy rates in this study (77.8% CR and 17% PR) are considerably higher than the previously reported combined efficacy rates of 5 randomized controlled trials (38.1% CR and 55.1% PR).<sup>7</sup> CR rates after our tapering schedule (58%) were greater than reported before; only 12% of the patients in a retrospective analysis by Metz et al<sup>8</sup> remained in CR in the 4- to 16-month follow-up period. Another retrospective analysis by Turk et al<sup>9</sup> reported 39% of the patients remained symptom free.

In conclusion, this study demonstrates the clinical benefit of dose optimization of omalizumab with our therapeutic algorithm in patients with refractory CSU. Larger studies are warranted to confirm our findings and to formally evaluate the cost-effectiveness of this personalized approach.

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## A novel allergen-specific therapy with CD40-silenced B cells and dendritic cells



### To the Editor:

Recently, regulatory B (Breg) cells were shown to contribute to inhibition of allergic responses.<sup>1,2</sup> However, the Breg cells in these studies were not antigen specific. Although IgE is generated from B cells, CD40 is required for germinal center formation, IgE production, and memory B-cell development.<sup>3-5</sup> Blockade of the CD40-CD40 ligand interaction also induces generation of regulatory T (Treg) cells.<sup>6</sup> Considering the above, CD40-silenced B cells would seem to be attractive for use in therapeutic approaches to allergic diseases.

RNA interference using small interfering RNA (siRNA) is a potent, selective, and easy method for specific blocking of gene expression. We previously reported that CD40-silenced antigen-specific dendritic cells (DCs), which were induced by siRNA, inhibited allergic responses and symptoms.<sup>6</sup> However, to our knowledge, there has been no prior study examining allergen-specific therapy with Breg cells induced by siRNA. In this study we investigated the effect of CD40-silenced allergen-specific B cells on allergic responses and examined the similarities and differences of CD40-silenced DCs and CD40-silenced B cells.

As shown in Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org), siRNA-specific CD40 (CD40 siRNA) suppressed CD40 expression on B cells, the proliferative response of B cells, and IgE synthesis by B cells. Next, the effect of CD40-silenced ovalbumin (OVA) B cells, which were transfected with CD40 siRNA and pulsed with OVA, on T-cell response was examined. CD40-silenced OVA B cells significantly inhibited OVA-specific T-cell proliferation (Fig 1, A). However, these cells did not inhibit keyhole limpet hemocyanin (KLH)-specific T-cell responses (Fig 1, B).

CD40-silenced B cells significantly upregulated the rate of forkhead box protein 3 (Foxp3)<sup>+</sup> cells in CD4<sup>+</sup>CD25<sup>+</sup> cells and *Foxp3* gene expression (Fig 1, C and D). CD4<sup>+</sup>CD25<sup>+</sup> cells generated by CD40-silenced OVA B cells significantly suppressed the ongoing OVA-specific T-cell response (Fig 1, E). However, these CD4<sup>+</sup>CD25<sup>+</sup> cells did not significantly inhibit the KLH-specific T-cell response (Fig 1, F). On the other hand, CD4<sup>+</sup>CD25<sup>+</sup> cells induced by CD40-silenced B cells pulsed with KLH did not significantly inhibit OVA-specific T-cell responses, although these cells significantly inhibited KLH-specific T-cell responses (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

The effect of CD40-silenced B cells *in vivo* was also investigated (see this article's Methods section in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). Mice that had received

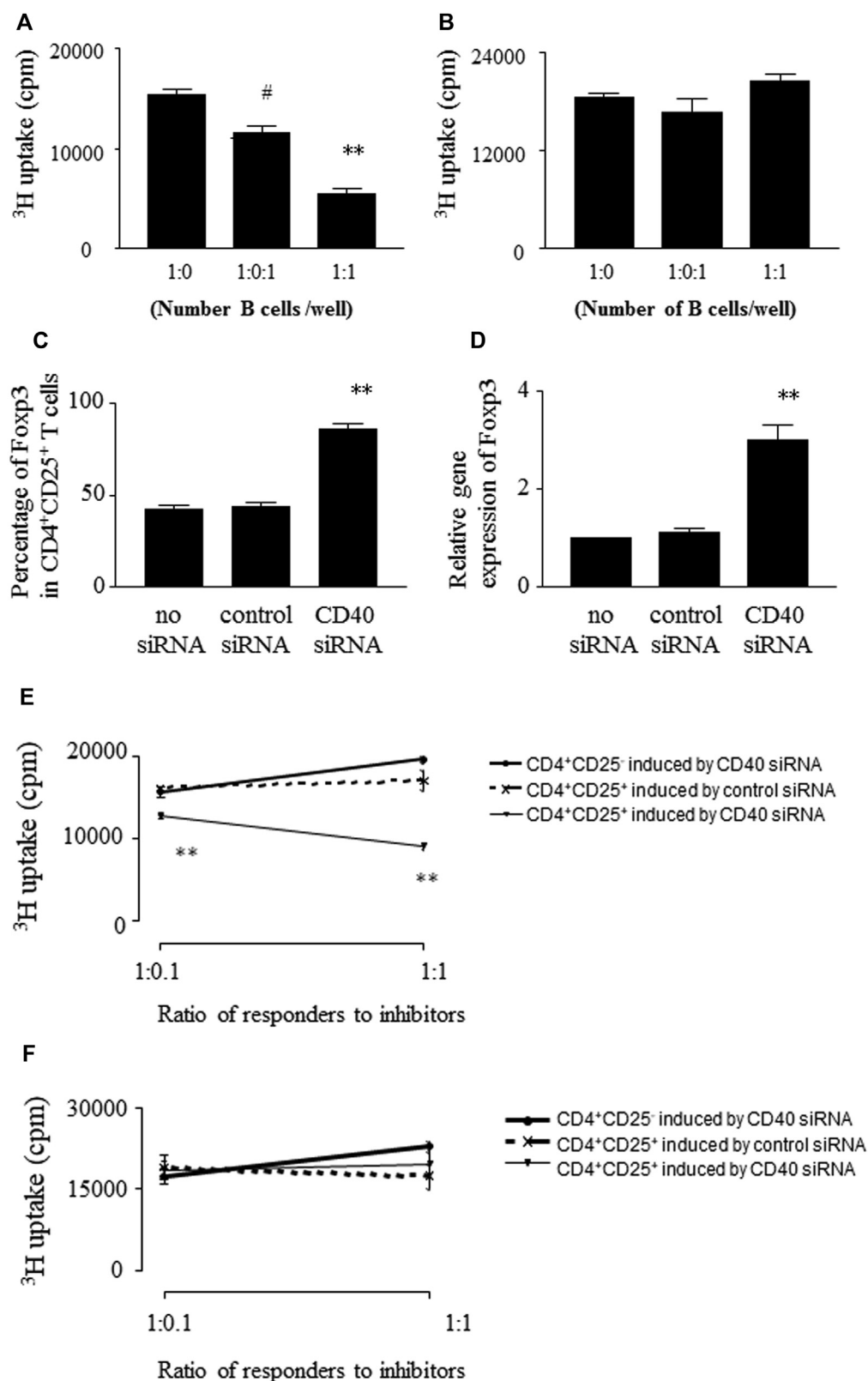


**FIG 1.** Immune modulation by CD40-silenced B cells *in vitro*. **A** and **B**, CD40-silenced B cells were added as inhibitors of OVA-specific (Fig 1, A) or KLH-specific (Fig 1, B) T-cell responses. <sup>#</sup>*P* < .05 versus 1:0 group. **C**, Percentage of Foxp3<sup>+</sup> cells in CD4<sup>+</sup>CD25<sup>+</sup> cells after coculture with B cells transfected with reagent alone, control siRNA, or CD40 siRNA. <sup>\*\*</sup>*P* < .01 versus no siRNA and control siRNA. **D**, *Foxp3* gene expression determined by using real-time quantitative PCR. <sup>\*\*</sup>*P* < .01 versus no siRNA and control siRNA. **E** and **F**, CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from coculture with CD40-silenced OVA B cells were added to OVA-specific (Fig 1, E) or KLH-specific (Fig 1, F) T-cell response. <sup>\*\*</sup>*P* < .01 versus CD4<sup>+</sup>CD25<sup>+</sup> induced by control siRNA and CD4<sup>+</sup>CD25<sup>+</sup>. <sup>\*\*</sup>*P* < .01 versus CD4<sup>+</sup>CD25<sup>+</sup> induced by CD40 siRNA and CD4<sup>+</sup>CD25<sup>+</sup> induced by control siRNA.

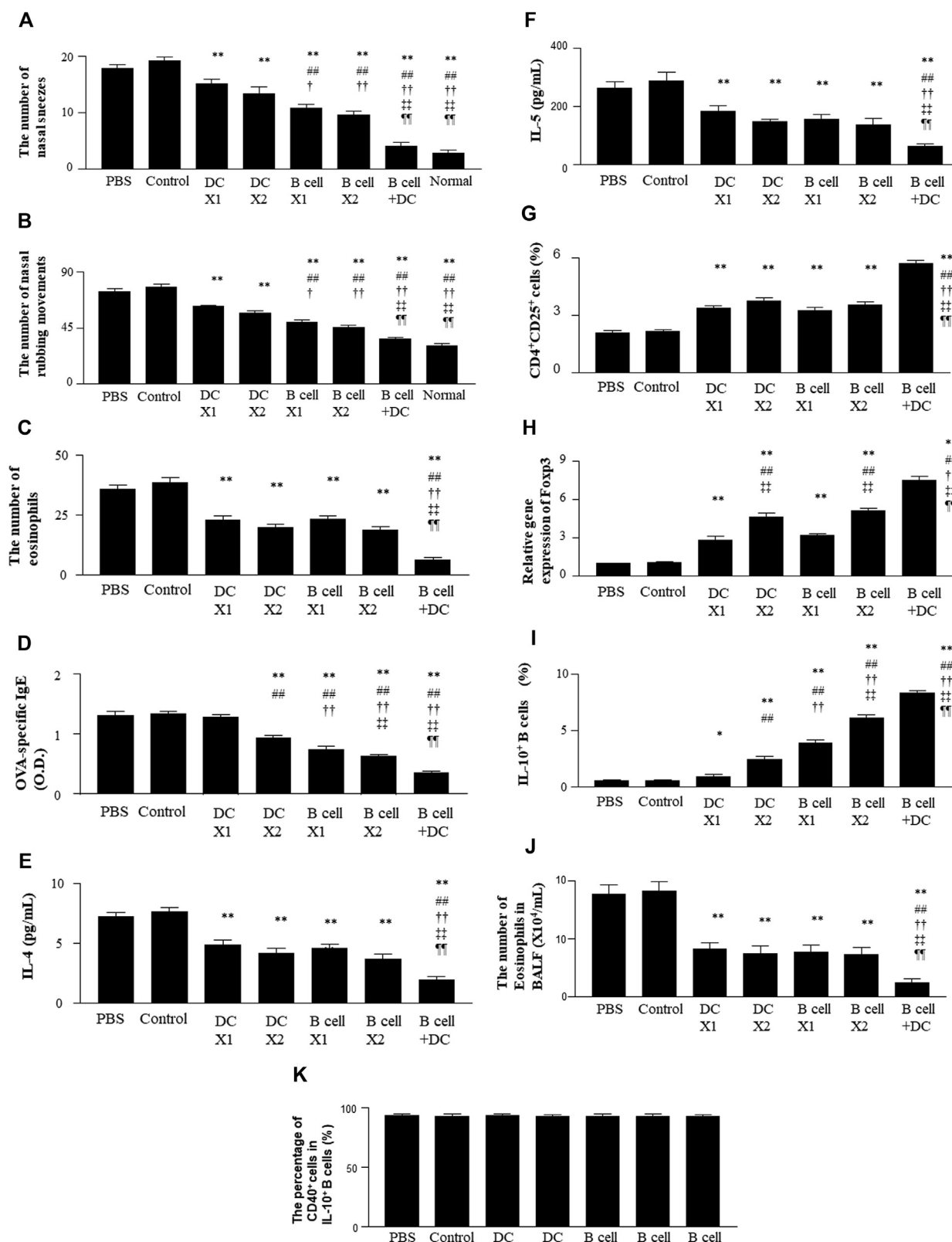


**FIG 2.** Effects of CD40-silenced OVA B cells on established allergic rhinitis. **A** and **B**, Numbers of sneezes (Fig 2, A) and rubbing movements (Fig 2, B). **C**, Number of eosinophils. **D**, OVA-specific IgE. **E**, Frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the spleen. **F**, *Foxp3* gene expression in the spleen. **G** and **H**, Production of IL-4 (Fig 2, G) and IL-5 (Fig 2, H) by splenocytes stimulated with OVA. **I**, Percentage of IL-10<sup>+</sup> B cells. **J**, Number of eosinophils in BALF. **K**, Percentage of CD40<sup>+</sup> cells in IL-10<sup>+</sup> B cells. *B cell* × 1, 1 × 10<sup>7</sup> CD40-silenced B cells; *B cell* × 2, 2 × 10<sup>7</sup> CD40-silenced B cells; *B cell* + DC, mixture of CD40-silenced B cells and DCs; *Control*, control B cells; *DC* × 1, 1 × 10<sup>7</sup> CD40-silenced DCs; *DC* × 2, 2 × 10<sup>7</sup> CD40-silenced DCs; *Normal*, no sensitization and treatment. \**P* < .05 and \*\**P* < .01 versus PBS alone and control groups. ##*P* < .01 versus DC × 1. †*P* < .05 and ††*P* < .01 versus DC × 2. ‡*P* < .01 versus *B cell* × 1. ¶*P* < .01 versus *B cell* × 2.

CD40-silenced OVA B cells were sensitized and challenged with OVA (Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). CD40-silenced OVA B cells significantly suppressed the number of sneezes, nasal rubbing movements, and eosinophils infiltrating the nasal septum, as well as levels of OVA-specific IgE and IgG<sub>1</sub>. Also, CD40-silenced OVA B cells increased IL-10 and IL-35 production. However, CD40-silenced OVA B cells did not significantly change nasal symptoms, eosinophilia in nasal septum, and levels of KLH-specific IgE in mice sensitized and challenged with KLH (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Next, we examined the therapeutic effects of CD40-silenced OVA B cells on established OVA-induced allergic rhinitis (Fig 2). CD40-silenced OVA B cells significantly inhibited the number of sneezes, the number of rubbing movements, the number of eosinophils, the level of OVA-specific IgE, the production of IL-4 and IL-5, and the number of eosinophils in bronchoalveolar lavage fluid (BALF). Numbers of CD40-silenced OVA B cells increased the rate of CD4<sup>+</sup>CD25<sup>+</sup> cells, Foxp3 gene expression, and the frequency of IL-10<sup>+</sup> B cells. Additionally, the percentage of CD40<sup>+</sup> cells in IL-10<sup>+</sup> B cells did not significantly differ, suggesting that CD40-silenced B cells induced IL-10<sup>+</sup> B cells. However, the mechanism by which CD40-silenced OVA B cells induced IL-10<sup>+</sup> B cells is unclear.

CD40-silenced OVA B cells also significantly inhibited nasal symptoms and levels of OVA-specific IgE compared with CD40-silenced OVA DCs. Also, the frequency of IL-10<sup>+</sup> B cells in the CD40-silenced OVA B-cell group was significantly greater than that in the CD40-silenced OVA DC group. Furthermore, combination therapy of CD40-silenced OVA B cells ( $1 \times 10^7$  cells per mouse) and CD40-silenced OVA DCs ( $1 \times 10^7$  cells per mouse) significantly suppressed the number of sneezes, the number of rubbing movements, the number of eosinophils, the level of OVA-specific IgE, and the production of IL-4 and IL-5 compared with values in CD40-silenced OVA B cells alone ( $2 \times 10^7$  cells per mouse) or CD40-silenced OVA DCs alone ( $2 \times 10^7$  cells per mouse). Although mice were challenged with antigen dissolved in PBS, sneezes and rubbing movements were seen even after challenge with PBS alone in the normal mice (mice without sensitization and treatment), which were injected and challenged with PBS alone. There were also no significant differences between the numbers of sneezes and rubbing movements in mice that received combination therapy and normal mice.

The rate of CD4<sup>+</sup>CD25<sup>+</sup> cells in spleen, Foxp3 gene expression, and the frequency of IL-10<sup>+</sup> B cells in mice that received combination therapy were significantly greater than those in the other groups. These findings suggest that CD40-silenced OVA B cells are therapeutically useful, even for mice with established allergic rhinitis.

It was also suggested that CD40-silenced OVA B cells are more useful for inhibiting symptoms and antigen-specific IgE than CD40-silenced OVA DCs. Additionally, combination therapy of CD40-silenced B cells and DCs is more beneficial, with synergic effects, although how this occurs is not apparent. Breg (B10) and Treg cells have independent roles.<sup>7</sup> B cell–B cell interaction<sup>8</sup> and DC-independent B cell activation<sup>9</sup> have been reported also.

These are all possible mechanisms for the difference between CD40-silenced OVA DCs and B cells. On the other hand, CD40-silenced OVA B cells did not significantly inhibit KLH-induced responses and symptoms in mice with established allergic rhinitis (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Furthermore, nasal symptoms were significantly inhibited by CD40-silenced B cells, CD40-silenced DCs, or combination therapy of CD40-silenced B cells and DCs even 56 days after administration (see Fig E6 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), suggesting long-term effects of CD40-silenced OVA B cells and DCs.

In conclusion, this study showed that CD40-silenced OVA B cells inhibited allergic responses and symptoms antigen specifically not only before antigen sensitization but also after establishment of allergic rhinitis.

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## METHODS

### Generation of DCs from bone marrow

DCs were generated from bone marrow progenitor cells, as previously described.<sup>E1</sup>

### Purification of bone marrow CD19<sup>+</sup> B cells

CD19<sup>+</sup> cells were purified from bone marrow of male BALB/c mice (Japan SLC, Shizuoka, Japan) that were not sensitized with OVA by using an anti-CD19 mAb-coated microbead B cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). B cells were cultured for 2 days in culture medium (OptiMEM supplemented with 10% FCS, 50  $\mu$ mol/L 2-mercaptoethanol, 100  $\mu$ g/mL streptomycin, and 100 U/mL penicillin) after transfection and then stimulated with recombinant IL-4 (50 ng/mL), recombinant B cell-activating factor of the TNF family (100 ng/mL), and LPS (10  $\mu$ g/mL) by using 96-well plates. All cultures were incubated at 37°C in a 5% humidified CO<sub>2</sub> atmosphere.

### siRNA and gene silencing

Transfection of siRNA specific to CD40 (CD40 siRNA, UUCUCAGCC CAGUGGAACA) into DCs or B cells was conducted, as described previously.<sup>E1</sup> CD40 siRNA (2  $\mu$ g) was incubated with 20  $\mu$ L of GeneSilencer reagent in 50  $\mu$ L of serum free RPMI 1640 medium at room temperature for 30 minutes. The mixture was then added to 400  $\mu$ L of DCs or B cells and incubated for 4 hours. Control cells were transfected with transfection reagent alone or siRNA specific to the Luciferase gene GL2 Duplex, representing a sham silencing control.

### Measurement of B-cell proliferation

B cells were cultured for 2 days in complete medium after transfection. They were then stimulated with recombinant IL-4 (50 ng/mL), IL-5 (5 ng/mL), and CD40 ligand (5  $\mu$ g/mL) by using 96-well plates and cultured in a humidified 5% CO<sub>2</sub> atmosphere in air at 37°C. Cells were pulsed with 1  $\mu$ Ci of tritiated thymidine, cultured for an additional 16 hours, and collected onto glass-fiber filters. Tritiated thymidine incorporation was quantified with a scintillation counter (Hitachi, Tokyo, Japan). Results are expressed with a stimulation index.

### Measurement of levels of IgE secreted from B cells

Quantities of IgE released from B cells were determined, as described previously.<sup>E2</sup> Plates were coated with anti-mouse IgE. Culture supernatants were then added, and plates were incubated with biotinylated anti-mouse IgE. Standard curves were generated by using recombinant cytokines.

### Immunization and treatment

B cells were transfected with CD40 siRNA, as described above, and pulsed with OVA (grade V, Sigma-Aldrich, St Louis, MO; CD40-silenced OVA B cells). B cells were also transfected with GL2 control siRNA and pulsed with OVA (control B cells). CD40-silenced OVA B cells (1  $\times$  10<sup>7</sup> cells per mouse), control B cells (CD40-expressing B cells, 1  $\times$  10<sup>7</sup> cells per mouse), or PBS alone were injected intravenously on day 1 into 7-week-old male BALB/c mice purchased from Japan SLC. Mice were sensitized twice on days 2 and 16 with intraperitoneal OVA (10  $\mu$ g) and 2 mg of Al(OH)<sub>3</sub>. These mice were also challenged intranasally with OVA (200  $\mu$ g in PBS) on days 21 through 27. Immediately after the last nasal challenge on day 27, numbers of sneezes and nasal rubbing movements were counted. Samples were collected on day 28 (treatment with DCs on day 1, priming on days 2 and 16, challenge on days 21-27, and sample collection on day 28).

In the second experiment BALB/c mice were injected intravenously with CD40-silenced OVA B cells (1  $\times$  10<sup>7</sup> cells per mouse), control B cells (CD40-expressing B cells, 1  $\times$  10<sup>7</sup> cells/mouse), or PBS alone on day 1. All mice were sensitized twice on days 2 and 16 with intraperitoneal KLH (10  $\mu$ g) and 2 mg of Al(OH)<sub>3</sub> and then also challenged intranasally

with KLH (30  $\mu$ g) on days 21 through 27 (treatment with DCs on day 1, priming on days 2 and 16, challenge on days 21-27, and sample collection on day 28).

In the third experiment mice were sensitized with OVA (10  $\mu$ g) and 2 mg of Al(OH)<sub>3</sub> intraperitoneally on days 1 and 15, and then the same mice were challenged intranasally with OVA (200  $\mu$ g) on days 21 through 27. Intravenous administration of CD40-silenced OVA B cells (1  $\times$  10<sup>7</sup> cells per mouse [B cell $\times$ 1] or 2  $\times$  10<sup>7</sup> cells per mouse [B cell $\times$ 2]), control OVA B cells (1  $\times$  10<sup>7</sup> cells/mouse [control]), PBS alone, CD40-silenced OVA DCs (1  $\times$  10<sup>7</sup> cells per mouse [DC $\times$ 1] or 2  $\times$  10<sup>7</sup> cells per mouse [DC $\times$ 2]), or a mixture (B cell+DC) of CD40-silenced OVA B cells (1  $\times$  10<sup>7</sup> cells per mouse) plus CD40-silenced OVA B cells (1  $\times$  10<sup>7</sup> cells per mouse) was performed on day 28. These mice were then rechallenged intranasally on days 29 through 35 with OVA (200  $\mu$ g). Immediately after the last nasal challenge on day 35, numbers of sneezes and nasal rubbing movements were counted. Samples were collected on day 36 (sensitization on days 1 and 15, challenge on days 21-27, treatment on day 28, rechallenge on days 29-35, and sample collection on day 36). Additionally, normal mice without sensitization and treatment were established. These mice were injected and challenged with PBS only. Mice were injected with PBS alone intraperitoneally on days 1 and 15, and then the same mice were challenged intranasally with PBS only on days 21 through 27. Intravenous administration of PBS only was performed on day 28. These mice were then rechallenged intranasally on days 29 through 35 with PBS only. Immediately after the last nasal challenge on day 35, numbers of sneezes and nasal rubbing movements were counted (injection on days 1 and 15, challenge on days 21-27, injection on day 28, and rechallenge on days 29-35).

In the fourth experiment mice were sensitized with KLH (10  $\mu$ g) and 2 mg of Al(OH)<sub>3</sub> intraperitoneally on days 1 and 15. These mice were also challenged intranasally on days 21 through 27 with KLH (30  $\mu$ g). Intravenous administration of CD40-silenced OVA B cells (1  $\times$  10<sup>7</sup> cells per mouse), control B cells (1  $\times$  10<sup>7</sup> cells per mouse), or PBS alone was performed on day 28. All mice were rechallenged intranasally on days 29 through 35 with KLH (30  $\mu$ g; sensitization on days 1 and 15, challenge on days 21-27, treatment on day 28, rechallenge on days 29-35, and sample collection on day 36).

In the fifth experiment mice were sensitized with OVA (10  $\mu$ g) and 2 mg of Al(OH)<sub>3</sub> intraperitoneally on days 1 and 15, and then the same mice were challenged intranasally with OVA (200  $\mu$ g) on days 21 through 27. Intravenous administration of CD40-silenced OVA B cells (2  $\times$  10<sup>7</sup> cells per mouse), control OVA B cells (2  $\times$  10<sup>7</sup> cells per mouse), CD40-silenced OVA DCs (2  $\times$  10<sup>7</sup> cells per mouse), mixture of CD40-silenced OVA B cells (1  $\times$  10<sup>7</sup> cells/mouse) and CD40-silenced OVA DCs (1  $\times$  10<sup>7</sup> cells/mouse), or PBS alone was performed on day 28. These mice were then rechallenged intranasally on days 70 through 83 with OVA (200  $\mu$ g). Immediately after the last nasal challenge on day 83, numbers of sneezes and nasal rubbing movements were counted. Samples were collected on day 84 (sensitization on days 1 and 15, challenge on days 21-27, treatment on day 28, rechallenge on days 70-83, and sample collection on day 84).

Mice were housed in an environmentally controlled animal facility at Nagoya City University in Japan. Protocols were in accordance with the "Guidelines for care and use of animals" of Nagoya City University. Every effort was made to minimize animal discomfort. Each group consisted of 5 mice. Experiments were repeated 3 times.

### Nasal allergic symptoms

Immediately after the last nasal challenge, numbers of sneezes and nasal rubbing movements were counted for 20 minutes, according to the method previously reported.<sup>E1</sup>

### BALF

BALF was collected, and numbers of eosinophils in BALF were measured by using the method previously reported.<sup>E3</sup>

## Antigen-specific T-cell response

Splenic CD4<sup>+</sup>CD25<sup>−</sup> T cells ( $2 \times 10^6$  cells/mL) and DCs ( $2 \times 10^5$  cells/mL) in mice with OVA sensitization were cultured with 100  $\mu$ g/mL antigen of OVA or KLH for 72 hours at 37°C in RPMI-1640 medium containing 10% FBS and penicillin G/streptomycin. Cells were pulsed with 1  $\mu$ Ci of tritiated thymidine (PerkinElmer, Billerica, Mass), and incorporated radioactivity was quantitated by using a liquid scintillation counter, as described previously.<sup>E2</sup> Results are expressed as mean  $\pm$  SEM counts per minute of triplicate cultures.

CD40-silenced OVA B cells were added at various ratios during measurement of OVA-specific or KLH-specific T-cell responses to determine the ability of CD40-silenced OVA B cells to inhibit an OVA-specific or KLH-specific T-cell response.

CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>−</sup> T cells were isolated from cocultures of splenic CD4<sup>+</sup> T and B cells transfected with control or CD40 siRNA to assess the effects of induced Treg cells. OVA- or KLH-specific T-cell responses were observed in 96-well plates. Purified CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>−</sup> T cells induced by CD40-silenced OVA B cells or control B cells were added to the ongoing OVA- or KLH-specific T-cell responses as inhibitors. A tritiated thymidine incorporation assay was performed.

## Measurement of antigen-specific IgE and IgG<sub>1</sub> levels

Mouse serum titers of antigen-specific IgE were measured by using ELISA. Briefly, ELISA plates were coated with anti-mouse IgE mAbs (Yamasa, Tokyo, Japan). Nonspecific binding was blocked, and sera were added to the plate. After adding biotinylated OVA or KLH, plates were incubated with avidin-peroxidase. After washing, the TMB microwell peroxidase substrate system (KPL, Gaithersburg, Md), which develops a blue color when reacted with peroxidase, was applied according to the manufacturer's instructions. OD was measured at 450 nm. Antigen-specific IgG<sub>1</sub> levels were also measured by using ELISA. ELISA plates were coated with OVA or KLH, nonspecific binding was blocked, and serum samples were added to the plates. Plates were washed, and horseradish peroxidase-labeled anti-mouse IgG<sub>1</sub> (Bio-Rad Laboratories, San Diego, Calif) was added. Plates were incubated with avidin-peroxidase. After washing, the same substrate was applied, and OD was measured at 450 nm.

## Measurement of *in vitro* cytokine release from splenocytes

Lymphoid cells ( $2 \times 10^6$  cells/mL) from spleens were cultured with OVA (100  $\mu$ g/mL) at 37°C. After incubation for 72 hours, culture supernatants were collected. Quantities of cytokines (IL-4, IL-5, IL-10, and IL-35) in culture supernatants were measured by using a sandwich ELISA. Plates were coated with anti-mouse IL-4, IL-5, IL-10 (PeproTech, Rocky Hill, NJ), or IL-35 (BioLegend, San Diego, Calif). Culture supernatant was then added, after which plates were incubated with the second antibody of biotinylated anti-mouse IL-4, IL-5, IL-10 (PeproTech), or IL-35 (BioLegend). Standard curves were generated by using recombinant cytokines.

## Real-time PCR

Total RNA was isolated from spleens with TRIzol (Thermo Fisher Scientific, Yokohama, Japan), according to the manufacturer's protocol. In brief, 20  $\mu$ g of RNA was digested by using DNase I, extracted with phenol/chloroform (3:1), precipitated with ethanol, washed with ethanol, and dissolved in RNase-free water. The first-strand cDNA was generated with the SuperScript Preamplification System.

Real-time PCR was performed with SYBR Green PCR Master mix and gene-specific primers. The PCR reaction was performed under these conditions for 40 cycles. Mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA was used for normalization to ensure equal amounts of starting RNA.

Primers used in this study were as follows: *Cd40*, sense 5'-TCTA-GAGTCCCGGATGCGAG-3' and antisense 5'-GGATCCTCAAGGCTATGC TGTC G-3'; *Foxp3*, sense 5'-CAGCTGCCTACAGTGCCCCCTAG-3' and antisense 5'-CATTTGCCAGCAGTGGGTAGCTG-3'; and *Gapdh*, sense 5'-TGATGACATCAAGAAGGTGGTGA-3' and antisense 5'-TCCTTGGAG GCCATGTAGGCCAT-3'.

## Flow cytometry

Phenotypic analysis of T and B cells was performed with a FACScan (BD Biosciences, San Jose, Calif), according to the method previously described.<sup>E1,E2</sup> T cells were harvested and stained with anti-mouse CD4, anti-mouse CD25 and anti-mouse Foxp3 mAbs. B cells were also stained with anti-mouse CD19, anti-mouse CD40, and anti-mouse IL-10 mAbs.

## Pathology

Heads of mice were decalcified and sectioned. Three-micrometer-thick sections of nasal tissue were stained with Luna staining. Numbers of eosinophils in the nasal mucosa of the nasal septum were counted microscopically in a single-field of view at  $\times 400$  magnification.<sup>E1</sup> The observer was blinded to treatment when counting eosinophil numbers.

## Statistics

Data are indicated as means  $\pm$  SEMs. Data were statistically analyzed by using 1-way ANOVA, followed by the Newman-Keuls Test. A *P* value of less than .05 was considered statistically significant.

## REFERENCES

- E1. Suzuki M, Zheng X, Zhang X, Zhang ZX, Ichim TE, Sun H, et al. A novel allergen-specific therapy for allergy using CD40-silenced dendritic cells. *J Allergy Clin Immunol* 2010;125:737-43.e6.
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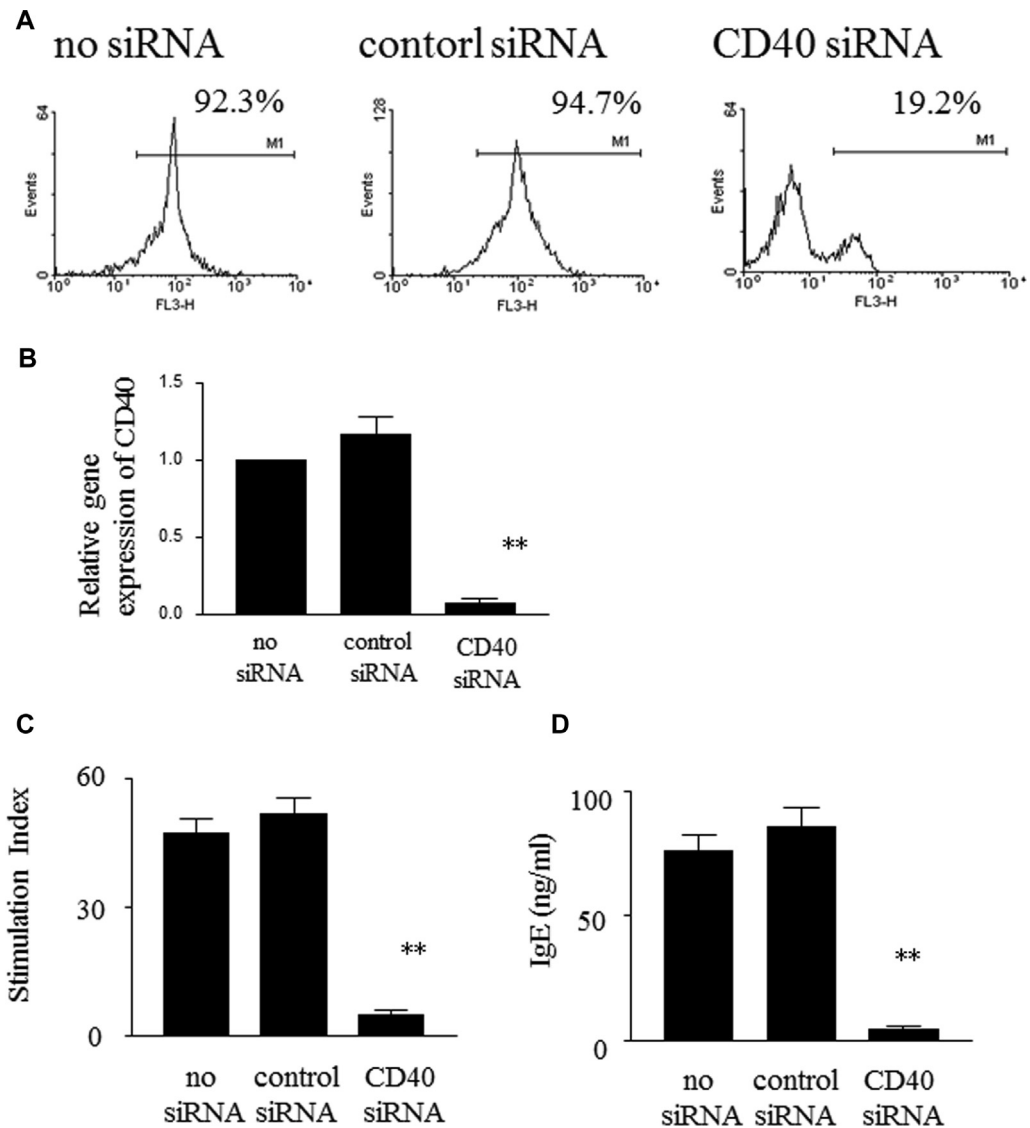


**FIG E1.** Modulation by CD40 siRNA. **A**, CD40 expression analyzed by using FACScan. B cells were transfected with reagent alone (no siRNA), GL2 siRNA (control siRNA), or CD40 siRNA. **B**, CD40 expression determined by using real-time PCR. **C**, B-cell proliferation. **D**, Quantities of IgE released from B cells. \*\* $P < .01$  versus no siRNA and control siRNA.

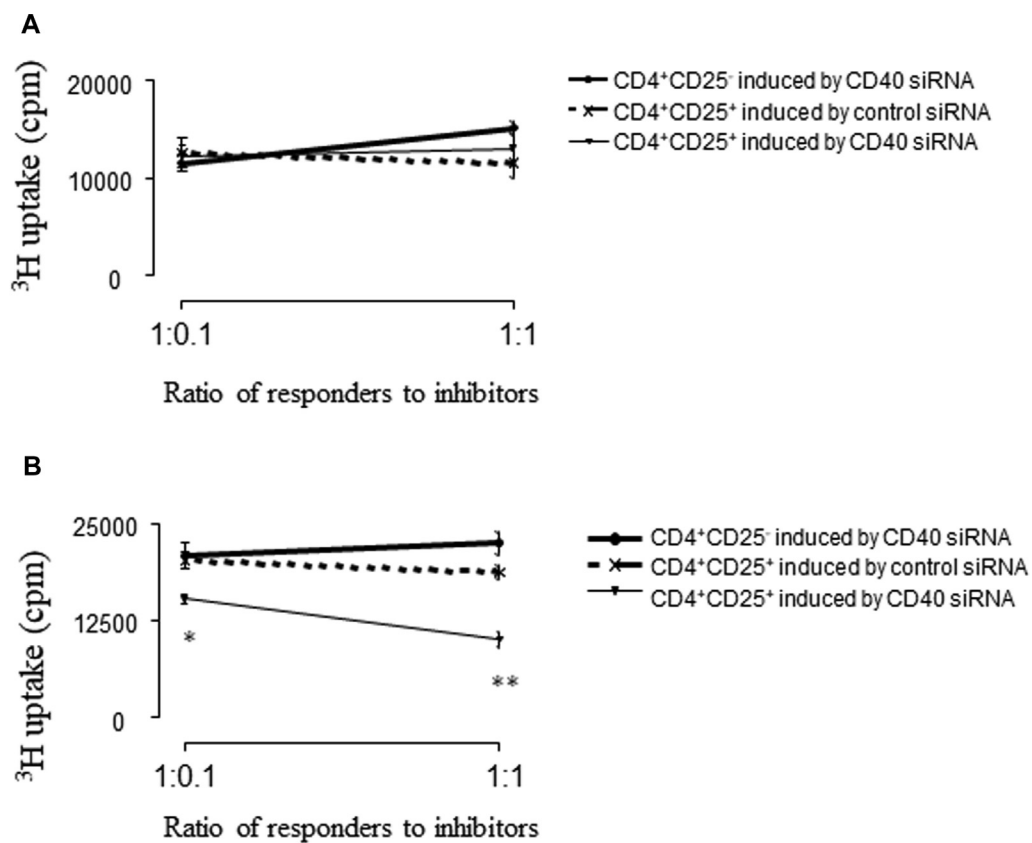


**FIG E2.** Immune modulation by CD40-silenced B cells pulsed with KLH (CD40-silenced KLH B cells) *in vitro*.  $\text{CD4}^+\text{CD25}^+$  or  $\text{CD4}^+\text{CD25}^-$  cells isolated from coculture with CD40-silenced KLH B cells were added to OVA-specific T-cell response (**A**) or KLH-specific T cell response (**B**). \* $P < .05$  and \*\* $P < .01$  versus  $\text{CD4}^+\text{CD25}^+$  induced by control siRNA and  $\text{CD4}^+\text{CD25}^-$ .

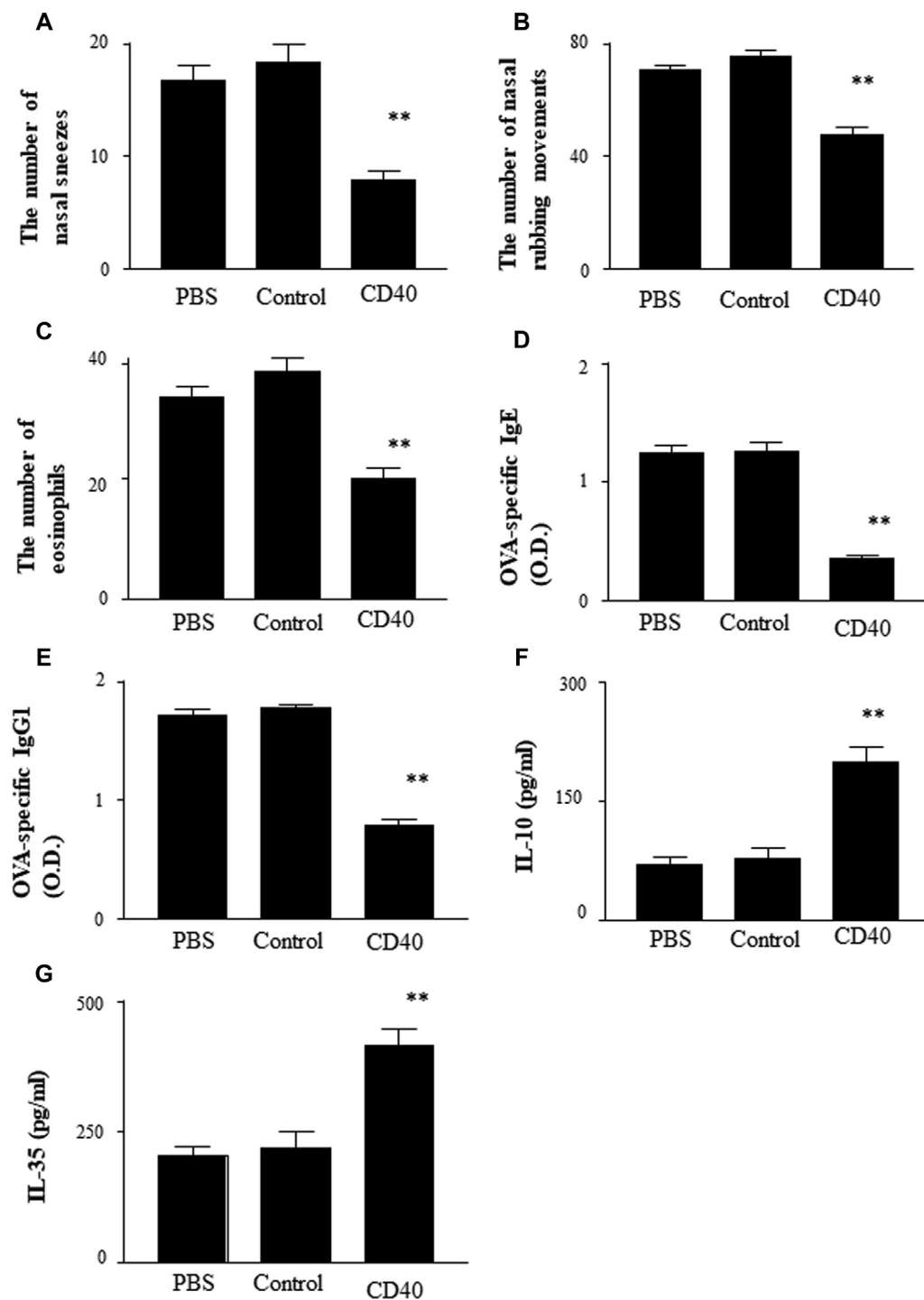


**FIG E3.** Prevention of OVA-induced allergy by administration of CD40-silenced OVA B cells. Mice that had been treated with intravenous injections of CD40-silenced OVA B cells (CD40), control B cells (Control), or PBS alone (PBS) were sensitized and challenged with OVA, as described in the [Methods](#) section. **A** and **B**, Numbers of sneezes (Fig E3, *A*) and nasal rubbing movement (Fig E3, *B*) were measured after OVA challenge. **C**, Eosinophilia in the nasal mucosa. **D**, OVA-specific IgE in sera. **E**, OVA-specific IgG<sub>1</sub> in sera. **F** and **G**, Production of IL-10 (Fig E3, *F*) and IL-35 (Fig E3, *G*) from splenocytes stimulated with OVA. \*\**P* < .01 versus group of PBS alone and control B cells.

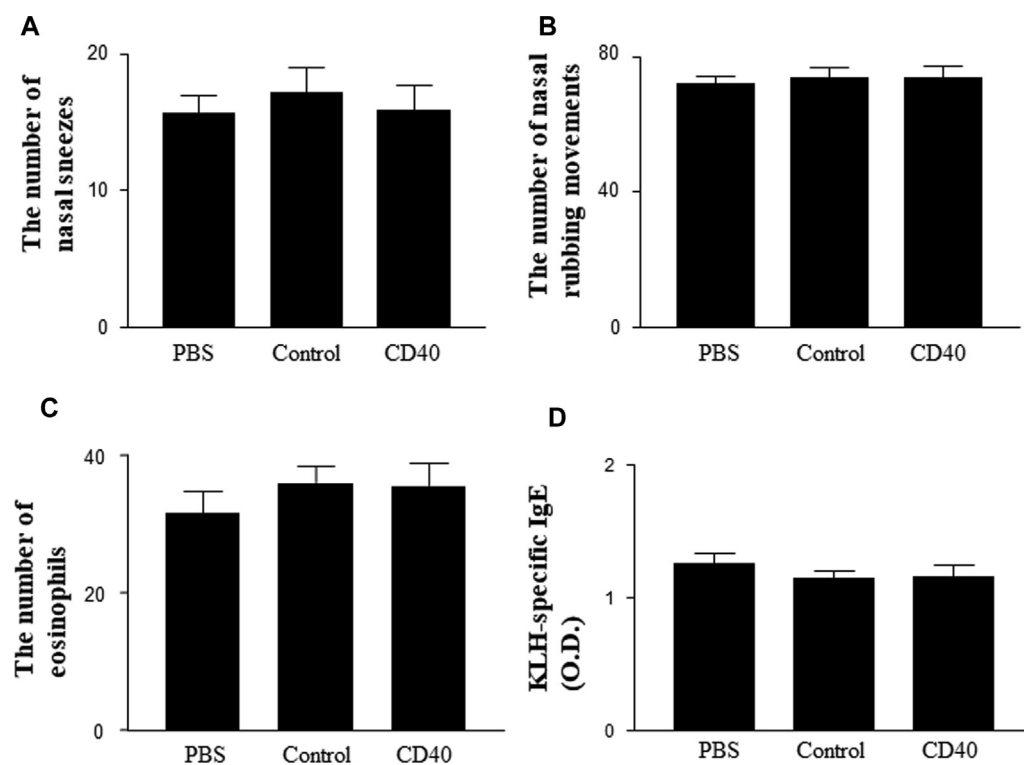


**FIG E4.** CD40-silenced OVA B cells did not prevent KLH-induced allergy. Mice that had been treated with intravenous injections of CD40-silenced OVA B cells (CD40), control B cells (control), or PBS alone (PBS) were sensitized and challenged with KLH, as described in the [Methods](#) section. **A** and **B**, Numbers of sneezes (Fig E4, A) and nasal rubbing movements (Fig E4, B) were counted after KLH challenge. **C**, Eosinophilia in the nasal mucosa. **D**, KLH-specific IgE in sera.

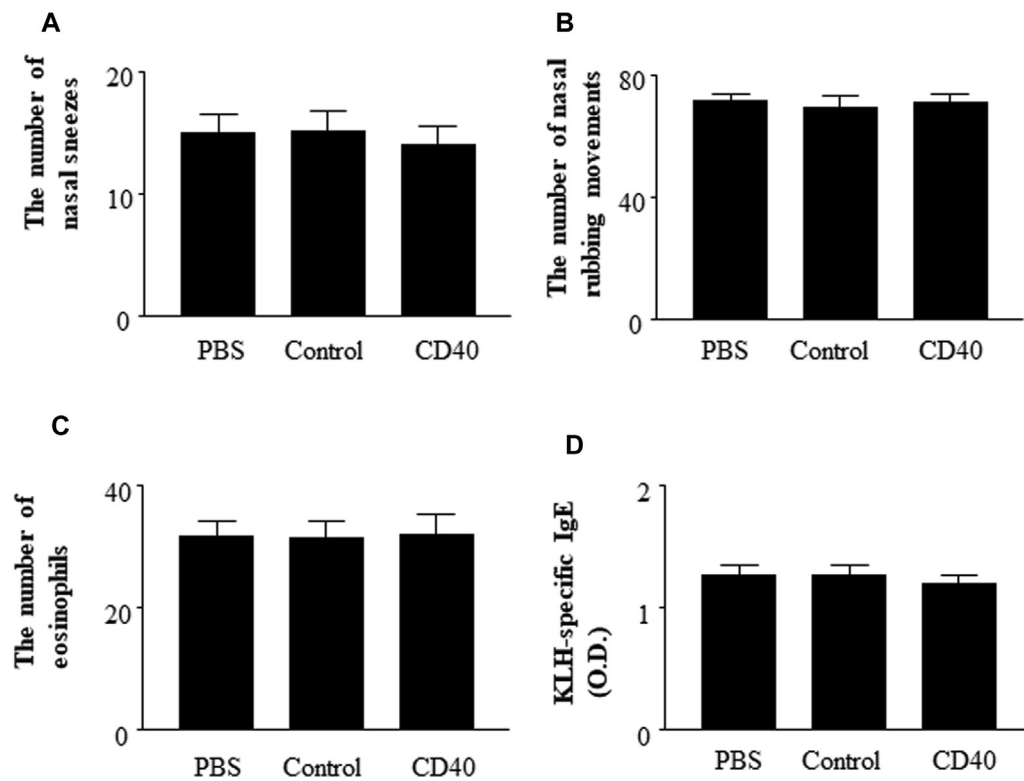


**FIG E5.** Effect of CD40-silenced OVA B cells on established allergic rhinitis caused by KLH. Mice with KLH-induced allergic rhinitis were treated with PBS alone (PBS), control OVA B cells (control), or CD40-silenced OVA B cells (CD40). **A** and **B**, Numbers of sneezes (Fig E5, A) and nasal rubbing movements (Fig E5, B) were counted after KLH challenge. **C**, Eosinophilia in the nasal mucosa. **D**, KLH-specific IgE in sera.

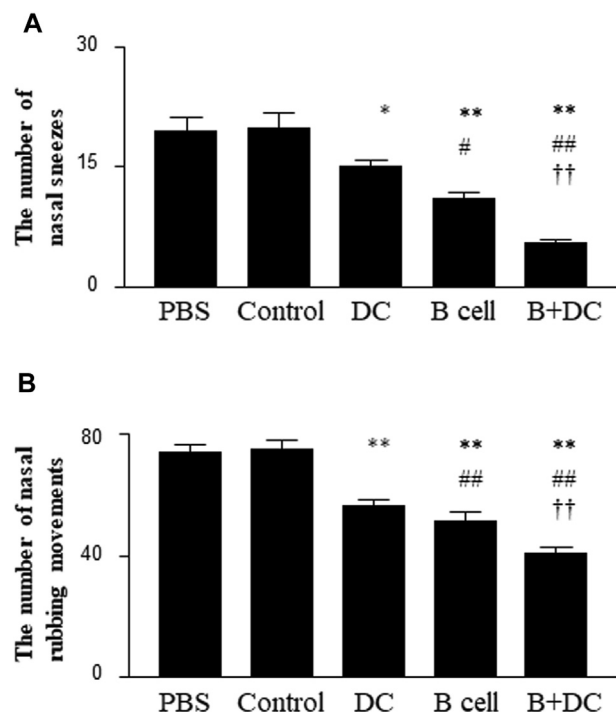


**FIG E6.** Long-term effect of CD40-silenced OVA B cells. Mice with OVA-induced allergic rhinitis were treated with PBS alone (PBS), control OVA B cells (control), CD40-silenced OVA DCs, CD40-silenced OVA B cells (B cell), or mixture (*DC+B*) of CD40-silenced OVA DCs and CD40-silenced OVA B cells. Numbers of sneezes (**A**) and nasal rubbing movements (**B**) were measured 56 days after treatment. \* $P < .05$  and \*\* $P < .01$  versus groups of PBS alone and control. # $P < .05$  ## $P < .01$  versus DCs. †† $P < .01$  versus B cells.