

Impacts of CD40- and CD86-Silenced Antigen-Specific B Cells on the Control of Allergies

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Abstract

Background: We previously reported that CD40-silenced B cells inhibited allergic responses and symptoms. However, more potent therapies are needed. To our knowledge, the effects of CD86-silenced B cells and synergic effects of gene silencing in B cells by 2 small interfering RNAs (siRNAs) on allergic disease control have not been reported.

Objective: To investigate the effects of CD86-silenced B cells and synergic effects of gene silencing in B cells on allergic responses and symptoms.

Methods: Mice were treated with CD40- and/or CD86-silenced B cells transfected with siRNAs and pulsed with ovalbumin (OVA). And the effects of these B cells were estimated.

Results: CD86-silenced OVA-pulsed B cells significantly inhibited OVA-induced allergies. Treatment with CD40-/CD86-silenced OVA-pulsed B cells led to a significantly fewer sneezes and nasal rubbing movements, as well as lower OVA-specific immunoglobulin E (IgE) levels, than that with CD40-silenced or CD86-silenced OVA-pulsed B cells alone. These inhibitory effects were observed prior to sensitization as well as after the establishment of allergic rhinitis. CD40-/CD86-silenced OVA-pulsed B cells did not inhibit keyhole limpet hemocyanin-induced allergies. CD40-/CD86-silenced OVA-pulsed B cells also significantly inhibited allergic symptoms and OVA-specific IgE level in sera compared with CD40-/CD86-silenced OVA-pulsed dendritic cells (DCs). In addition, CD19⁺CD40⁺ B cells significantly increased in the nasal tissue after intravenous administration of these cells. Furthermore, CD40-/CD86-silenced B cells inhibited allergic symptoms caused by Cry j I, a major aeroallergen of Japanese cedar pollen, and Cry j I-specific IgE in sera.

Conclusion: This study showed, for the first time, that siRNA-induced CD86-silenced B cells significantly inhibited allergic responses and symptoms antigen-specifically, and that siRNA-induced CD40-/CD86-silenced antigen-specific B cells are a more useful antigen-specific therapy than CD40- or CD86-silenced B cells alone for the control of allergies. Furthermore, it was shown that CD40-/CD86-silenced B cells have stronger inhibition of IgE production and allergic symptoms than CD40-/CD86-silenced DCs.

Keywords

allergy, B cell, CD40, CD86, siRNA

Introduction

B cells are the source of immunoglobulin E (IgE), which is responsible for allergic responses. B cells also inhibit inflammation. Recently, regulatory B cells (Bregs) were shown to contribute to tolerance and the inhibition of excessive immune and allergic responses.^{1–4} However, there are very few reports of Bregs in relation to allergic diseases.

RNA interference using small interfering RNA (siRNA) induces specific silencing of gene expression and is a potent, selective, and easy method.⁵ Andrew

Fire and Craig Mello received the 2006 Nobel Prize in Medicine for their discovery of this technique.⁶

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We previously reported that the administration of siRNA-induced CD40-silenced antigen-specific dendritic cells (DCs) suppressed allergic responses.⁷ We also reported that CD40-silenced B cells inhibited allergic responses and symptoms.⁸ However, more potent therapies are needed.

T cells, which play important roles in allergic diseases, require costimulatory signals, which are delivered by costimulatory molecules (eg, CD40, CD80, and CD86), for activation, proliferation, and cytokine production. Blocking of CD40/CD80/CD86 with monoclonal antibodies reduces T-cell activity.⁹ We previously showed synergic effects of CD40 and CD86 silencing in DCs on the control of allergic diseases.⁸ However, it is controversy whether synergic effects of CD40 and CD86 silencing in B cells exists on the control of allergic diseases, and whether CD40- and CD86-silenced B cells has more potent inhibitory effects on allergic symptoms than CD40- and CD86-silenced DCs.

To the best of our knowledge, there has been no prior study examining allergen-specific therapy with siRNA-induced CD86-silenced or CD40-/CD86-silenced B cells. In this study, we estimated the effect of CD86-silenced OVA-pulsed B cells and CD40-/CD86-silenced OVA-pulsed B cells on allergic diseases. Consequently, this study showed that the administration of CD86-silenced OVA-pulsed B cells or CD40-/CD86-silenced OVA-pulsed B cells inhibited allergic responses and symptoms after the establishment of allergic rhinitis as well as before sensitization. In addition, CD40-/CD86-silenced B cells had stronger inhibition of allergic responses and symptoms, with synergic effects, than those of CD40- or CD86-silenced B cells alone. Furthermore, CD40-/CD86-silenced B cells had more potent inhibition of IgE and allergic symptoms than CD40-/CD86-silenced DCs.

Methods

Preparation of B Cells and DCs From Bone Marrow

We purified B cells as previously described.¹⁰ Bone marrow-derived DCs were also generated as previously reported.⁷

siRNA and Gene Silencing

Transfection of siRNA specific to CD40 (CD40 siRNA, 0.01 μ g, 0.1 μ g, or 2 μ g) into B cells was conducted as previously reported.¹⁰ Transfection of siRNA specific to CD86 (CD86 siRNA, 0.01 μ g, 0.1 μ g, or 2 μ g, CGUUGUGUGUGUUCUGGAA) into B cells was also performed as previously described.^{10,11} In addition, B cells were transfected with both of CD40 (1 μ g) and CD86 siRNA (1 μ g). B cells, transfected with CD40 siRNA (CD40-silenced OVA B cells), CD86 siRNA

(CD86-silenced OVA B cells), or both CD40 and CD86 siRNA (CD40-/CD86-silenced OVA B cells), were pulsed with ovalbumin (OVA; grade V, Sigma-Aldrich, St. Louis, MO). B cells were also transfected with siRNA (control siRNA) specific to the Luciferase gene GL2 Duplex and pulsed with OVA (control B cells). Furthermore, B cells transfected with CD40 and CD86 siRNA were pulsed with Cry j 1, a major allergen of Japanese cedar (*Cryptomeria japonica*) pollen which causes pollinosis frequently in Japan,¹² (CD40-/CD86-silenced Cry j 1 B cells) by the same method. Cry j 1 was purified by the method previously reported.^{13,14}

Transfection of CD40 siRNA (1 μ g) and CD86 siRNA (1 μ g) into DCs was also performed as previously described.^{8,15,16}

Immunization and Treatment

CD40-silenced OVA B cells (5×10^6 cells/mouse), CD86-silenced OVA B cells (5×10^6 cells/mouse), CD40-/CD86-silenced OVA B cells (5×10^6 cells/mouse), CD40-/CD86-silenced OVA DCs (5×10^6 cells/mouse), control B cells (CD40-expressing B cells, 5×10^6 cells/mouse), or phosphate-buffered saline (PBS) alone were injected intravenously into 7-week-old male BALB/c mice on day 1. These mice were sensitized with intraperitoneal OVA (10 μ g) and 2 mg Al (OH)₃ on days 2 and 16. The mice were also challenged intranasally (i.n.) with OVA (200 μ g in PBS) on days 21 through 27. Immediately after the nasal challenge, the number of sneezes and nasal rubbing movements was counted. Samples were collected on day 28 (Figure 1(A)).

In the second experiment (Figure 2(B)), BALB/c mice were injected intravenously with CD40-silenced OVA B cells (5×10^6 cells/mouse), CD86-silenced OVA B cells (5×10^6 cells/mouse), CD40-/CD86-silenced OVA B cells (5×10^6 cells/mouse), control B cells (CD40-expressing B cells, 5×10^6 cells/mouse), or PBS alone on day 1. These mice were sensitized with intraperitoneal keyhole limpet hemocyanin (KLH) (10 μ g) and 2 mg Al (OH)₃ on days 2 and 16 and then challenged i.n. with KLH (30 μ g) on days 21 through 27.

In the third experiment (Figure 1(C)), mice were sensitized with OVA (10 μ g) and 2 mg Al (OH)₃ intraperitoneally on days 1 and 15. All mice were challenged i.n. with OVA (200 μ g) on days 21 through 27. Intravenous administration of CD40-silenced OVA B cells (5×10^6 cells/mouse), CD86-silenced OVA B cells (5×10^6 cells/mouse), CD40-/CD86-silenced OVA B cells (5×10^6 cells/mouse), CD40-/CD86-silenced OVA DCs (5×10^6 cells/mouse), control OVA B cells (5×10^6 cells/mouse), or PBS alone was performed on day 28. These mice were then rechallenged i.n. on days 29 through 35 with OVA (200 μ g). The number of sneezes and nasal rubbing movements was counted

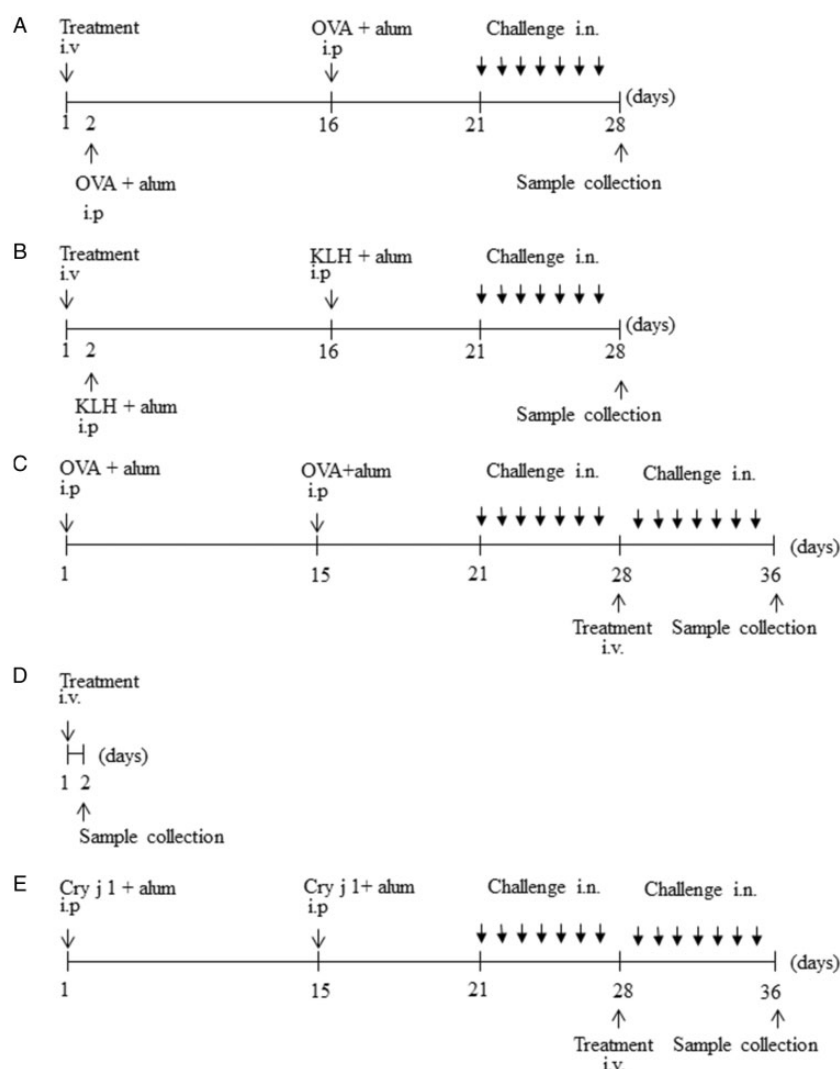


Figure 1. Schematic protocol of experiment. A, The first experiment treatment on day 1, sensitization with OVA on days 2 and 16, intranasal challenge with OVA on days 21–27, and sample collection on day 28. B, The second experiment. Treatment on day 1, sensitization with KLH on days 2 and 16, intranasal challenge with KLH on days 21–27, and sample collection on day 28. C, The third experiment. Sensitization on days 1 and 15, intranasal challenge with OVA on days 21–27, treatment on day 28, intranasal re-challenge with OVA on days 29–35, and sample collection on day 36. D, The fourth experiment. Treatment on day 1 and sample collection on day 2. E, The fifth experiment. Sensitization on days 1 and 15, intranasal challenge with Cry j 1 on days 21–27, treatment on day 28, intranasal re-challenge with Cry j 1 on days 29–35, and sample collection on day 36. i.n., intranasal administration; i.p., intraperitoneal administration; i.v., intravenous administration; KLH, keyhole limpet hemocyanin; OVA, ovalbumin.

immediately after the nasal challenge. Samples were collected on day 36.

In the fourth experiment (Figure 1(D)), intravenous administration of PBS alone or CD40-silenced OVA B cells (5×10^6 cells/mouse) was performed on day 1. Nasal lymphocytes were collected on day 2.

In the fifth experiment (Figure 1(E)), mice were sensitized with Cry j 1 (10 μ g) and 2 mg Al (OH)₃ intraperitoneally on days 1 and 15. All mice were challenged i.n. with Cry j 1 (20 μ g) on days 21 through 27. Intravenous administration of CD40-/CD86-silenced Cry j 1 B cells (5×10^6 cells/mouse) or PBS alone was performed on

day 28. These mice were then rechallenged i.n. on days 29 through 35 with OVA (20 μ g). The number of sneezes and nasal rubbing movements was counted immediately after the nasal challenge. Samples were collected on day 36.

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. Each group consisted of 5 mice. Experiments were repeated 3 times.

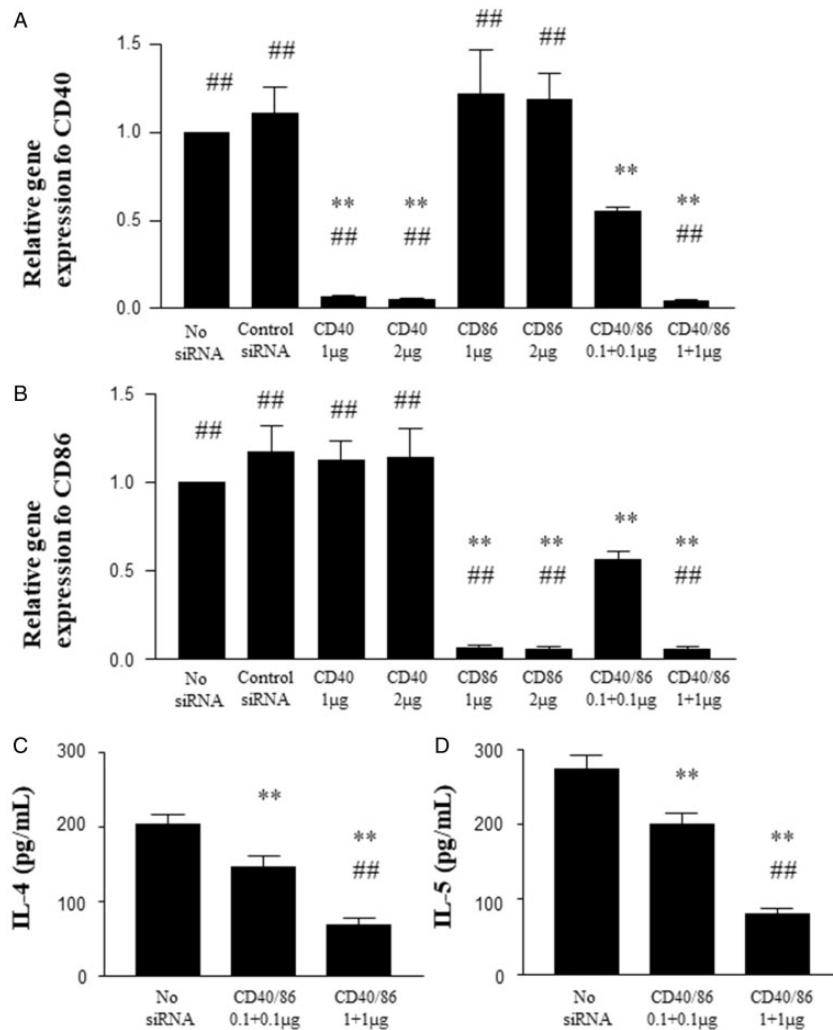


Figure 2. Gene silencing by siRNAs and inhibition of IL-4 and IL-5 by siRNA-transfected B cells in vitro. A, CD40 gene expression by real-time quantitative PCR. B cells were transfected with reagent alone (No siRNA), GL2 siRNA (Control siRNA), 1 μg or 2 μg of CD40 siRNA (CD40 1 μg or 2 μg), 1 μg or 2 μg of CD86 siRNA (CD40 1 μg or 2 μg), or CD40 and CD86 siRNA (CD40/CD86 1 + 1 μg or 0.1 + 0.1 μg). B, CD86 gene expression by real-time PCR. C, B cells transfected with reagent alone (No siRNA) or CD40/CD86 siRNA (CD40/CD86 1 + 1 μg or CD40/CD86 0.1 + 0.1 μg) were added to the ongoing OVA-specific T-cell responses. Quantities of IL-4 (D) and IL-5 (E) in culture supernatants were measured using ELISA. N = 5. ***P* < .01 versus No siRNA, Control siRNA, and CD86 groups (A). ***P* < .01 versus No siRNA, Control siRNA, and CD40 groups (B). ***P* < .01 versus No siRNA (C and D). ****P* < .01 versus CD40/CD86 0.1 + 0.1 μg siRNA, small interfering RNA.

Nasal Allergic Symptoms

Immediately after the nasal challenge, the number of sneezes and nasal rubbing movements was counted for 20 minutes according to the method described previously.^{7,15,17,18}

Measurement of Antigen-Specific IgE

Titers of antigen-specific IgE in sera were measured using ELISA. In brief, ELISA plates were coated with anti-mouse IgE monoclonal antibody (Yamasa, Tokyo, Japan). After nonspecific binding was blocked, sera were added to the plate. Biotinylated OVA was added, and

then the plates were incubated with avidin-peroxidase. 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 protocol. The optical density (O.D.) was measured at 450 nm.

Real-time Polymerase Chain Reaction

Total RNA was isolated from splenic samples using TRIzol (Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions.

Real-time polymerase chain reaction (PCR) was performed with SYBR Green PCR Master mix and gene-

specific primers as described previously.^{7,15,18} The PCR reaction was performed for 40 cycles. Mouse GAPDH mRNA was used for normalization to ensure equal amounts of starting RNA.

The primers used in this study were as follows:

- CD40: sense 5'-TCTAGAGTCCCGGATGCGAG-3' and antisense 5'-GGATCCTCAAGGCTATGCTGTC G-3'.
- CD86: sense 5'-ATGGACCCCAGATGCACCAT-3' and antisense 5'-TTTCCTCAGGCTCTCACTGC-3'.
- GAPDH: sense 5'-TGATGACATCAAGAAGGTGTGAA-3' and antisense 5'-TCCTTGGAGGCCATGTAGGCCAT-3'.

Pathology

The heads of the mice were decalcified and sectioned; 3- μ m-thick sections of nasal tissue were stained using Luna staining. The number of eosinophils in the nasal mucosa of the nasal septum was counted microscopically in a single field of view at 400 $\times$ magnification. The number of eosinophils were counted in 6 randomly selected locations in the anterior, middle, and posterior areas on the right and left sides, and the mean was calculated for each mouse as previously reported.^{7,15,17} The observer was blinded to treatment when counting the number of eosinophils.

Flow Cytometry

Nasal lymphocytes were prepared by enzyme extraction with collagenase, as previously described.^{19,20} An analysis of nasal lymphocytes was performed by flow cytometry according to the method previously described.^{7,16} Nasal lymphocytes were stained with anti-mouse CD19 and anti-mouse CD40 monoclonal antibody to examine nasal mucosal distribution of intravenous administrated B cells.

Measurement of Cytokine Release

Mice were sensitized with OVA (10 μ g) and 2 mg Al (OH)₃ intraperitoneally on days 1 and 7. Spleens were collected on day 21. OVA-specific T-cell responses were performed as previously reported.⁸ Splenic CD4⁺CD25⁻ T cells (2×10^6 cells/mL) and DCs (2×10^5 cells/mL) were cultured with OVA (100 mg/mL). To assess inhibitory effects, siRNA-induced B cells were added to the ongoing responses. Cells were cultured for 72 hours. Quantities of IL-4 and IL-5 in culture supernatants were measured using a sandwich ELISA. Plates were coated with anti-mouse IL-4 or IL-5 (PeproTech, Rocky Hill, NJ). Supernatant was added, and plates were incubated with peroxidase-linked anti-mouse IL-4

or IL-5 (PeproTech). Standard curves were generated using recombinant cytokines.

Statistics

Data are indicated as mean $\pm$ standard error of the mean. Data were statistically analyzed using 1-way analysis of variance followed by the Newman–Keuls test. Differences (Figures 2 to 5) or unpaired *t* test (Figures 6 and 7) were considered statistically significant at $P < .05$.

Results

Silencing of CD40 and CD86 on B Cells In Vitro

We estimated the effects of CD40 and CD86 siRNAs on B cells gene expression. Bone marrow-derived B cells were cultured and transfected with transfection reagent alone (no siRNA), GL2 siRNA (control siRNA), CD40 siRNA (1 μ g or 2 μ g), CD86 siRNA (1 μ g or 2 μ g), a mixture of CD40 siRNA (0.01 μ g) and CD86 siRNA (0.01 μ g), a mixture of CD40 siRNA (0.1 μ g) and CD86 siRNA (0.1 μ g), or a mixture of CD40 siRNA (1 μ g) and CD86 siRNA (1 μ g). The results of real-time PCR showed that 1 μ g or 2 μ g CD40 siRNA significantly suppressed CD40 gene expression (Figure 2 (A); $P < .01$). However, CD86 siRNA did not reduced CD40 expression. On the other hand, 1 μ g or 2 μ g CD86 siRNA inhibited CD86 gene expression (Figure 2(B); $P < .01$), although CD40 siRNA did not reduced CD86 gene expression. The mixture of CD40 siRNA (0.01 μ g) and CD86 siRNA (0.01 μ g) did not significantly suppressed gene expression of CD40 and CD86 on B cells (data not shown). In addition, the mixture of CD40 siRNA (0.1 μ g) and CD86 siRNA (0.1 μ g) significantly reduced gene expressions of CD40 and CD86 on B cells. Furthermore, the mixture of CD40 (1 μ g) and CD86 (1 μ g) siRNA had more potent inhibitory effects on gene expression than the mixture of CD40 (0.1 μ g) and CD86 (0.1 μ g) siRNA.

Inhibition of Releases of IL-4 and IL-5

We investigated whether B cells transfected with the mixture of CD40 and CD86 siRNA could inhibit productions of IL-4 and IL-5 released from splenic T cells. B cells transfected with the mixture of CD40 siRNA (0.1 μ g) and CD86 siRNA (0.1 μ g) could suppressed productions of IL-4 and IL-5 compared with B cells transfected with no siRNA (Figure 1(C) and (D)). Furthermore, B cells transfected with 1 μ g siRNA had more potent inhibitory effects on productions of IL-4 and IL-5 than those transfected with 0.1 μ g siRNA, suggesting siRNA-dependent mechanisms of these inhibitory effects.

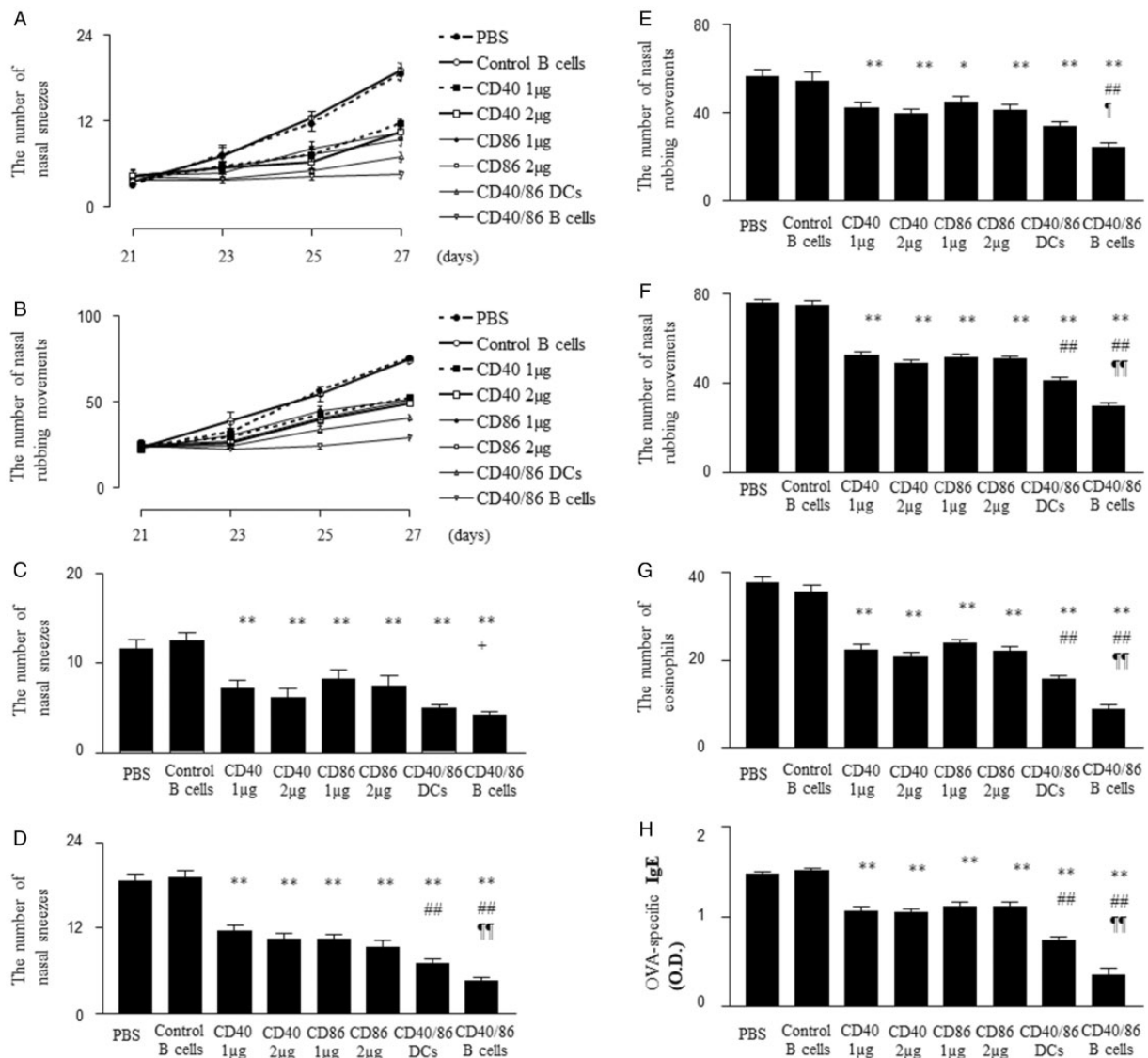


Figure 3. Prevention of OVA-induced allergies by siRNA-transfected B cells. Five mice were treated with PBS alone, Control B cells, CD40-silenced OVA B cells (1 μg or 2 μg of CD40 siRNA), CD86-silenced OVA B cells (1 μg or 2 μg of CD86 siRNA), CD40-/CD86-silenced OVA-pulsed dendritic cells (CD40/CD86 DCs, 1 μg CD40 and 1 μg CD86), and then sensitized and challenged with OVA. Sneezes (A) and nasal rubbing movements (B) were counted. Sneezes on days 25 (C) and 27 (D). Nasal rubbing movements on days 25 (E) and 27 (F). G, The number of eosinophils in the nasal septum. H, The level of serum OVA-specific IgE. * $P < .05$ versus PBS and Control B cells groups. ** $P < .01$ versus the PBS and Control B cells groups. ### $P < .01$ versus the CD40-silenced groups and CD86-silenced groups. + $P < .05$ versus CD86 1 μg group. ¶ $P < .05$ versus CD40/CD86 DCs group. ¶¶ $P < .01$ versus CD40/CD86 DCs group. OVA, ovalbumin; PBS, phosphate-buffered saline.

Prevention of OVA-Induced Allergy With CD40-/CD86-Silenced OVA B Cells

We estimated the preventative effect of CD86- or CD40-/CD86-silenced OVA B cells on OVA-induced allergies. We also compared the effects of CD40-/CD86-silenced OVA B cells and CD40-/CD86-silenced OVA DCs. Mice were treated with PBS alone, control B cells, or CD40-silenced OVA B cells, CD86-silenced OVA B cells, CD40-/CD86-silenced OVA DCs, or

CD40-/CD86-silenced OVA B cells. These mice were sensitized and challenged with OVA, as described in the Methods. To evaluate the effect of these B cells on allergic symptoms, the number of sneezes and nasal rubbing movements was counted after OVA challenge. There were no significant differences of the numbers of sneezes and nasal rub movements on days 21 and 23 among groups of PBS alone, control B cells, CD40-silenced B cells, CD86-silenced B cells, CD40-/CD86-silenced B cells, or CD40-/CD86-silenced DCs

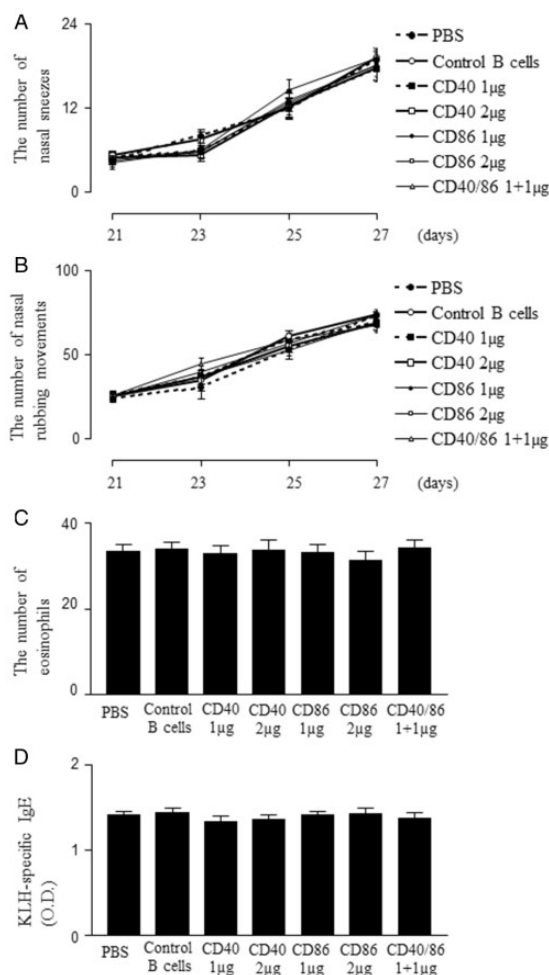


Figure 4. Lack of effect of siRNA-transfected B cells on the prevention of KLH-induced allergy. Five mice were treated with PBS alone, Control B cells, CD40-silenced OVA B cells (1 μg or 2 μg of CD40 siRNA), CD86-silenced OVA B cells (1 μg or 2 μg of CD86 siRNA), or CD40-/CD86-silenced OVA-pulsed B cells (CD40/CD86 1 + 1 μg), and then sensitized and challenged with KLH. Sneezes (A) and nasal rubbing movements (B) were counted after the last nasal challenge. C, The number of eosinophils in the nasal septum. D, The level of KLH-specific IgE in sera. KLH, key-hole limpet hemocyanin; PBS, phosphate-buffered saline.

(Figure 3(A) and (B)). The administration of CD40-silenced B cells, CD86-silenced B cells, CD40-/CD86-silenced DCs, or CD40-/CD86-silenced B cells led to a significantly lower number of sneezes and nasal rubbing movements than did administration of control B cells or PBS alone on days 25 and 27 (Figure 3(C) to (F)). CD40-/CD86-silenced OVA B cells also significantly suppressed the number of sneezes and nasal rubbing movements compared to CD40-silenced B cells, CD86-silenced B cells, and CD40-/CD86-silenced DCs on day 27.

Next, the number of eosinophils at the nasal septum was counted to estimate eosinophilia, which is associated with allergic symptoms. The administration of CD40-,

CD86-, or CD40-/CD86-silenced OVA B cells significantly reduced the number of eosinophils compared with the administration of PBS alone or control B cells (Figure 3(G); $P < .01$). The number of eosinophils in mice injected with CD40-/CD86-silenced OVA B cells was also significantly fewer than those injected with CD40-silenced B cells, CD86-silenced B cells, or CD40-/CD86-silenced DCs ($P < .01$).

The level of OVA-specific IgE in sera was examined using ELISA, because IgE is strongly associated with allergic reactions. CD40-, CD86-, and CD40-/CD86-silenced OVA B cells significantly reduced the levels of OVA-specific IgE compared to PBS alone and control B cells (Figure 3(H); $P < .01$). Furthermore, the level of OVA-specific IgE in mice that received CD40-/CD86-silenced OVA B cells was significantly lower than those that received CD40-silenced B cells, CD86-silenced B cells, or CD40-/CD86-silenced DCs ($P < .01$).

No Prevention of KLH-Caused Allergies by CD40-/CD86-Silenced OVA B Cells

We evaluated whether CD86- or CD40-/CD86-silenced OVA B cells could inhibit allergic responses caused by KLH. Mice were treated with PBS alone, control B cells, or CD40-, CD86-, or CD40-/CD86-silenced OVA B cells. The mice were also sensitized and challenged with KLH, as described in the Methods. No significant differences were observed in the number of nasal sneezes or nasal rubbing movements, the number of eosinophils at the nasal septum, or the level of KLH-specific IgE in sera among the groups (Figure 4(A) to (D)). These results suggested that CD86- and CD40-/CD86-silenced OVA B cells inhibit allergic responses and symptoms in an antigen-specific manner.

Therapeutic Effects of CD40-/CD86-Silenced OVA-Pulsed B Cells on Mice With OVA-Induced Allergic Rhinitis

We investigated whether CD86- or CD40-/CD86-silenced OVA B cells could inhibit allergic responses and symptoms even in mice with established allergy. We also compared the effects of CD40-/CD86-silenced OVA B cells and CD40-/CD86-silenced OVA DCs. Mice with allergic rhinitis, which were sensitized and challenged with OVA, were treated with PBS alone, CD40-/CD86-silenced OVA-DCs, or CD40-, CD86-, or CD40-/CD86-silenced OVA-B cells, as described in the Methods. The number of sneezes and nasal rubbing movements on day 27 was significantly higher than those on day 21 (data not shown). Eosinophils in the nasal cavity were not found on day 20, whereas they were found on day 27 (data not shown). These results suggested that allergic rhinitis was established on day 27.

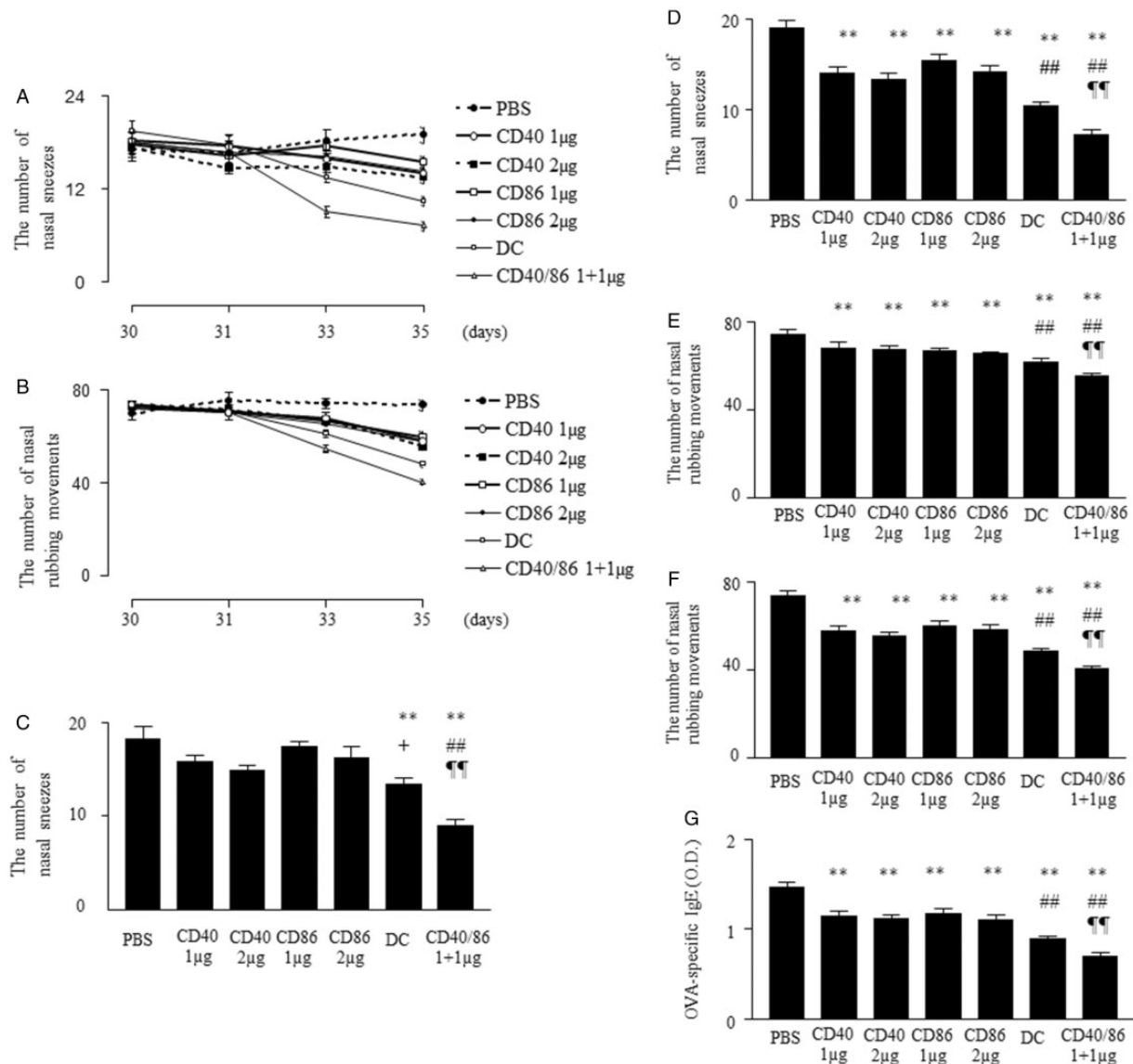


Figure 5. Therapeutic effects of siRNA-induced OVA-pulsed B cells on established allergic rhinitis. Five mice with OVA-induced allergic rhinitis were treated with PBS alone, CD40-silenced OVA-pulsed B cells (1 μg or 2 μg of CD40 siRNA), CD86-silenced OVA-pulsed B cells (1 μg or 2 μg of CD86 siRNA), CD40-/CD86-silenced OVA-pulsed B cells (CD40/CD86 1 + 1 μg), or CD40-/CD86-silenced OVA-pulsed dendritic cells (DC). Sneezes (A) and nasal rubbing movements (B) were counted. Sneezes on days 25 (C) and 27 (D). Nasal rubbing movements on days 25 (E) and 27 (F). G, The level of serum OVA-specific IgE. ** $P < .01$ versus PBS groups. ### $P < .01$ versus the CD40-silenced B cells and CD86-silenced B cells. + $P < .05$ versus CD86 1 μg group. ¶¶ $P < .01$ versus DC. OVA, ovalbumin; PBS, phosphate-buffered saline.

There were no significant differences of the numbers of sneezes and nasal rub movements on days 30 and 31 among groups of PBS alone, CD40-silenced B cells, CD86-silenced B cells, CD40-/CD86-silenced B cells, or CD40-/CD86-silenced DCs (Figure 5(A) and (B)). CD40-, CD86-, or CD40-/CD86-silenced OVA B cells significantly suppressed the number of sneezes and nasal rubbing movements compared with that of treatment with PBS alone on days 33 and 35 (Figure 5(C) to (F)). The number of sneezes and nasal rubbing

movements in mice that received CD40-/CD86-silenced OVA B cells was also significantly lower than those that received CD40-silenced B cells, CD86-silenced B cells, or CD40-/CD86-silenced DCs on days 33 and 35 ($P < .01$). Furthermore, CD40-/CD86-silenced OVA B cells significantly inhibited the levels of serum OVA-specific IgE compared with that of treatment with PBS alone, CD40-silenced B cells, CD86-silenced B cells, CD40-/CD86-silenced OVA DCs (Figure 5 (G); $P < .01$).

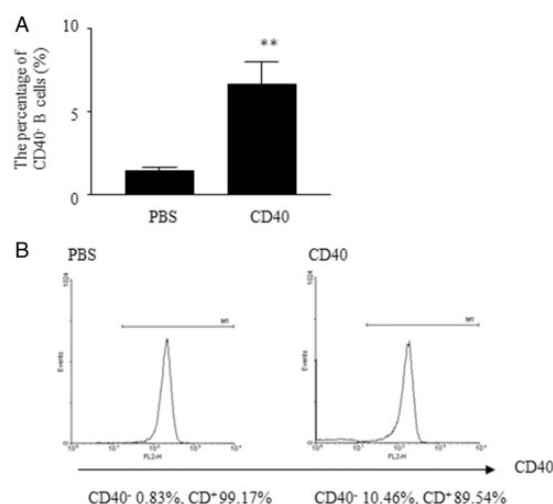


Figure 6. Nasal mucosal distribution of CD19⁺CD40⁻ B cells. Intravenous administration of PBS or CD40-silenced B cells was performed. The rate of CD40⁻ cells in CD19⁺ B cells of nasal lymphocytes was examined using flow cytometry. A, The percentage of CD40⁻ cells in CD19⁺ B cells in 5 mice. B, Results, which show the lowest percentage in mouse received PBS alone (PBS) and the highest percentage in mouse received CD40-silenced B cells (CD40), were shown. ** $P < .01$ versus PBS group. PBS, phosphate-buffered saline.

Nasal Mucosal Distribution of Intravenously Administered B Cells

We estimated nasal mucosal distribution of intravenously administered B cells. Mice received PBS alone or CD40-silenced B cells intravenously on day 1, as described in the “Methods” section. Nasal lymphocytes were collected on day 2. The rate of CD40⁻ cells in CD19⁺ B cells of nasal lymphocytes was examined by flow cytometry. Significant increase of CD40⁻ cells was seen in mice treated with intravenous administration of CD40-silenced B cells compared with treatment of PBS alone (Figure 6). This suggested that intravenous administration of CD40-silenced B cells appears in nasal tissue.

Therapeutic Effects of CD40-/CD86-Silenced Cry j 1-Pulsed B Cells on Mice With Cry j 1-Induced Allergic Rhinitis

Aeroallergen often causes allergic rhinitis and asthma, whereas OVA is not aeroallergen. It is important to examine the effect of CD40-/CD86-silenced B cells on aeroallergen-induced allergy. Therefore, we examined whether CD40-/CD86-silenced Cry j 1 B cells could inhibit allergic responses and symptoms even in mice with allergic rhinitis caused by not OVA but Cry j 1, a major aeroallergen of Japanese cedar (*Cryptomeria japonica*) pollen which causes pollinosis frequently in Japan.¹² Mice with allergic rhinitis, which were

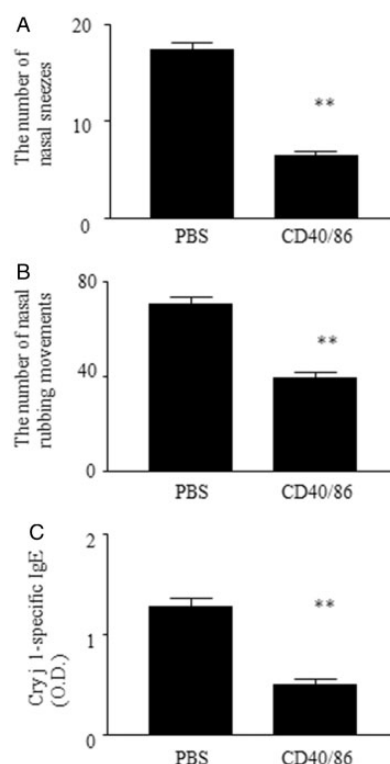


Figure 7. Therapeutic effects of siRNA-induced Cry j 1-pulsed B cells on established allergic rhinitis. Five mice with Cry j 1-induced allergic rhinitis were treated with PBS alone (PBS) or CD40-/CD86-silenced Cry j 1-pulsed B cells (CD40/CD86). Sneezes (A) and nasal rubbing movements (B) were counted after the last nasal challenge. C, The level of serum Cry j 1-specific IgE. ** $P < .01$ versus PBS group. PBS, phosphate-buffered saline.

sensitized and challenged with Cry j 1, were treated with PBS alone or CD40-/CD86-silenced Cry j 1-B cells, as described in the Methods. CD40-/CD86-silenced Cry j 1 B cells significantly inhibited the number of sneezes and nasal rubbing movements, and the levels of serum Cry j 1-specific IgE compared with that of treatment with PBS alone (Figure 7(A) to (C); $P < .01$).

Discussion

In this study, the administration of CD86-silenced OVA B cells inhibited eosinophilia in the nasal cavity and OVA-specific IgE in sera. This suggested that CD86-silenced B cells can suppress allergic responses in vivo. In addition, CD86-silenced B cells inhibited allergic symptoms. Considering these results, use of CD86-silenced B cells seems to be a useful approach for the control of allergic diseases. And the number of sneezes and nasal rubbing movements, the number of eosinophils in the nasal mucosa, and the level of OVA-specific IgE was significantly inhibited in mice that received CD40-/CD86-silenced OVA B cells compared to those that received CD40- or CD86-silenced

OVA B cells. This result suggested a synergic effect of CD40 and CD86 siRNA.

CD40 siRNA suppressed gene expression of CD40, but not CD86, on B cells in this study. In contrast, CD86 siRNA suppressed gene expression of CD86, but not CD40, on B cells. This suggested that siRNA can knock down gene expression specifically even in B cells.

The administration of CD86- or CD40-/CD86-silenced OVA-pulsed B cells suppressed allergic responses and symptoms after the establishment of allergic rhinitis as well as before allergen-sensitization. Considering these, therapy with CD86- and CD40-/CD86-silenced OVA-pulsed B cells may not only be useful as a vaccine therapy (prevention) of allergic diseases but also as a treatment of allergic diseases.

The present study showed that intravenous administration of CD40-/CD86-silenced OVA B cell significantly inhibited eosinophilia in the nasal mucosa and OVA-specific IgE in sera compared with intravenous administration of CD40-/CD86-silenced OVA DCs in both experiments of prevention and treatment. The numbers of nasal sneezes and nasal rubbing movement in mice received CD40-/CD86-silenced B cells were also significantly fewer than those in mice received CD40-/CD86-silenced DCs in both experiments of prevention and treatment. Considering these, CD40-/CD86-silenced B cell may have more potent inhibitory effects on allergic responses and symptoms than CD40-/CD86-silenced DCs not only as a vaccine therapy but also as a treatment. The mechanism of the more potent inhibitory effect of CD40-/CD86-silenced B cells on allergic symptoms may be also associated with more potent inhibition of eosinophilia in the nasal mucosa and antigen-specific IgE. Furthermore, these suggested that CD40 and CD86 on B cells are critical than those on DCs for forming allergic inflammation in mouse nasal mucosa, and B cells are more critical than DCs in pathogenesis of allergic diseases. In addition, we previously reported synergic effects of CD40-silenced OVAs and CD40-silenced B cells for the control of allergic diseases, suggesting synergic effects of CD40-/CD86-silenced DCs and CD40-/CD86-silenced B cells.

We showed CD40-/CD86-silenced B cells reduced allergic responses caused by OVA and Cry j 1. On the other hand, Snelgrove et al.²¹ showed that alternaria-derived serine protease induced rapid release of IL-33 that is different from acquired allergic response, although aeroallergen, such as cat dander, house dust mite, and ragweed, did not. IL-33 also plays an important role in the pathogenesis of allergic diseases.²² In addition, Japanese cedar pollen has serine protease activity.²³ However, it is considered that Cry j 1 is peptidylase but not serine protease.²⁴ Considering this, the mechanism of Japanese cedar-derived serine protease may be different from that of OVA and Cry j 1, although

it seems that the mechanism of OVA and Cry j 1 is same with each other. Therefore, CD40-/CD86-silenced OVA B cells may not inhibit allergic responses caused by Japanese cedar-derived serine protease like that caused by OVA and Cry j 1. Further experiments are necessary to resolve it.

In this study, we showed that siRNA-induced CD86-silenced B cells or siRNA-induced CD40-/CD86-silenced B cells are useful therapies for the control of allergic diseases. However, B cells play important roles in various immune diseases as well as allergic diseases. In addition, CD40 and CD86 are necessary to develop those diseases. Therefore, this study may contribute to the development of a novel therapeutic approach not only for allergic diseases but also other immune diseases. However, studies of regulatory B cells just begun. We expect further investigation to develop novel therapies with regulatory B cells.

Conclusion

This study showed that siRNA-induced CD86-silenced B cells significantly inhibited allergic responses and symptoms antigen-specifically, and that therapy with siRNA-induced CD40-/CD86-silenced B cells have stronger inhibition of allergic responses and symptoms than therapy with CD40-silenced B cells, CD86-silenced B cells, or CD40-/CD86-silenced DCs. In addition, CD40-/CD86-silenced B cells inhibited aeroallergen-induced allergy. Furthermore, it was shown that regulatory B cells significantly increased in the nasal tissue after intravenous administration of these cells.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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