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Original article

Inhibition of allergic responses by CD40 gene silencing

Background: Gene silencing using small interfering RNA (siRNA) is a potent method of specifically knocking down molecular targets. Small interfering RNA is therapeutically promising, however, treatment of allergic diseases with siRNA has not been explored *in vivo*. The aim of this study was to evaluate therapeutic effects of CD40 siRNA on inhibition of allergic responses.

Methods: Mice sensitized with ovalbumin (OVA) and alum were treated with CD40 siRNA, scrambled siRNA, or phosphate buffer saline (PBS) alone, and then challenged intranasally with OVA.

Results: A significant reduction in nasal allergic symptoms was observed in the CD40 siRNA treated OVA-allergic mice compared to the controls of scrambled siRNA and PBS alone, which is correlated with the decrease of local eosinophil accumulation. CD40 siRNA treatment knocked down CD40 expression on dendritic cells (DCs) *in vivo* and impaired their antigen presenting function. Treatment with CD40 siRNA resulted in inhibition of OVA-specific T cell response and decrease of interleukin-4 (IL-4), IL-5, and interferon-γ production from T cells stimulated with OVA. Administration of CD40 siRNA also suppressed CD40 expression on B cells, resulting in down-regulation of OVA-specific immunoglobulin E (IgE), IgG1, and IgG2a levels. Additionally, increased regulatory T cells were observed in the CD40 siRNA treated mice.

Conclusions: The present study demonstrates a novel therapeutic use for siRNA in allergy. CD40 siRNA attenuated allergy through inhibition of DC and B cell functions and generation of regulatory T (Treg) cells.

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Generally speaking, two main strategies are being used for the patients with allergy: antigen-specific therapy and antigen-independent therapy. Antigen-specific therapy is not applicable for many allergy patients whose causative allergen is unknown or for the patients who have been sensitized to multiple allergens, making the antigen-specific tolerance induction hard to achieve. On the other hand, current antigen-independent treatments, such as antihistamines, leukotriene receptor antagonists, and inhaled corticosteroids, only target late events within the allergic cascade thus do not address the root cause. Therefore, a novel antigen-independent approach that targets upstream causative events is highly desired.

Gene silencing using small interfering RNA (siRNA) is a potent, selective, and widely-applicable method for specifically blocking expression of desired genes (1). Previous methods of blocking pathological genes have included administration of antisense oligonucleotides, but this approach has provided poor to mediocre clinical results (2–4). Small interfering RNA is more attractive due to its high specificity (5, 6), as well as superior potency when compared to antisense oligonucleotides (7–9). Recently siRNA-based therapeutics have entered clinical trials for macular degeneration and RSV infection (10). However, it has not been reported whether treatment with siRNA can be an effective alternative to the present medications, such as corticosteroids, for the clinical management of allergy after immunization *in vivo*. In this study, we explored the use of siRNA by silencing the CD40 molecule, which has previously been implicated in allergic responses.

It has been demonstrated that CD40/CD40 ligand (CD40L) interaction enhances Th2 cytokine production (11). Considering this, knockdown of CD40 expression may inhibit interleukin-4 (IL-4) production from T cells. On the other hand, knockdown of CD40 expression may also inhibit ability of B cells to undergo class switching and immunoglobulin E (IgE) synthesis (12).

In the present study, we investigated the feasibility of silencing CD40 expression by siRNA treatment as a means of controlling allergic disease *in vivo*. This is the first demonstration that CD40 siRNA is therapeutically useful for the control of allergic diseases even after allergic sensitization. The anti-allergy effects are associated with impaired function of dendritic cells (DCs) and B cells and generation of Treg cells.

Methods

Construction of CD40 siRNA-expressing vector

CD40 siRNA-expressing vectors were generated using the Silencer Express Kit (Ambion Inc., Autin, TX, USA). Sense (ACACTACACAAATGTTCCACTGGGCTGAGAACCGGTGTTTCG TCCTTTCCACAAG) and anti-sense (CGGCGAAGCTTTTTCCAAAAAATTCTCAG CCCAGTGGAACACTACACAAATG) hairpin siRNA template oligonucleotide, specific to CD40 mRNA, were used.

Immunization and treatment

Six to eight week-old male BALB/c mice (Charles River Canada, Saint-Constant, Canada) were injected intraperitoneally (i.p.) with 10 μg ovalbumin (OVA) and 4 mg $Al(OH_3)$ twice on days 0 and 14. Mice were also injected intravenously with phosphate buffer saline (PBS) alone, 60 μg control small hairpin siRNA expressing vector (control siRNA), 60 μg CD40 small hairpin siRNA expressing vector (CD40 siRNA) twice on days 27, 30, and 33. Each group consisted of five mice. The same mice were challenged intranasally (i.n.) on days 29 through 35 with OVA (600 μg). Immediately after the last nasal challenge, the number of sneezing and nasal rubbing movements was counted for 20 min according to the method previously described (13), and samples were collected on day 36. The protocols were approved by the Guidelines for Care and Use of Animals at University of Western Ontario.

Isolation of splenic DCs and B cells

Dendritic cells or B cells were isolated from spleens with anti-mouse CD11c or CD19 mAb-conjugated super-paramagnetic MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD11c^+$ or $CD19^+$ cells were isolated by a magnetic column. Purity checked by flow cytometry was usually >90%.

Reverse transcription polymerase chain reaction and real-time PCR

Total RNA was isolated from Treg cells or DCs after gene silencing, using Trizol (Invitrogen Life Technologies, Burlington, ON, Canada) according to the manufacturer's protocol. To generate the first-strand cDNA, the SuperScript Preamplification System was used. The CD40 primer used in this study was sense 5′-TCTAGAGTCCCGGATGCGAG-3′ and antisense 5′-GGATCCTCAAGGCTATGCTGTC G-3′. The primers of Foxp3 and GAPDH described before (14) were also used. The polymerase chain reactions (PCR) products were visualized by ethidium bromide staining.

Real-time polymerase chain reactions (PCRs) were performed using SYBR Green PCR Master mix (Stratagene, La Jolla, CA, USA) and 100 nM of gene-specific primers. The PCR reaction conditions were 95°C for 10 min, 95°C for 30 s, 58°C for 1 min, and 72°C for 30 s (40 cycles). Mouse GAPDH mRNA was used for normalization to ensure equal amounts of starting RNA.

OVA-specific T cell response

T cells were isolated from lymph node by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). The unfractionated lymph node cells were cultured in 96-well plates at a concentration of 4 × 105 cells/well for 72 h in the presence or absence of OVA antigen. Cells were cultured for 2 days, and then cells were pulsed with 1 μCi of $[^3H]$thymidine (Amersham Pharmacia Biotech) for the last 16 h of culture. Cells were harvested onto glass fibre filters, and incorporated radioactivity was quantitated using a Wallac Betaplate liquid scintillation counter.

Measurement of OVA-specific IgE, IgG1, and IgG2a in sera

Sera were collected on days of 26, and 36, and stored at −80°C before use. Mouse serum titres of OVA-specific IgE were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates were coated with anti-mouse IgE monoclonal antibody. Nonspecific binding was blocked, and five-fold diluted sera were added to the plate. After adding biotinylated OVA, the plates were incubated with avidin-peroxidase. After washing, the TMB microwell peroxidase substrate system (BDbioscience, Mississuga, Canada) was applied, and optical density (O.D.) was measured at 450 nm. OVA-specific IgG1 and IgG2a were also measured by ELISA. Enzyme-linked immunosorbent assay plates were coated with OVA. Nonspecific binding was blocked, and 10-fold diluted serum samples were added to the plates. The plates were washed, and biotin-labelled anti-mouse IgG1 or IgG2a was added. The plates were incubated with avidin-peroxidase. After washing, the same substrate was applied, as described above.

Measurement of IgE and IgG1 secreted from B cells

B cells were cultured for 2 days in complete medium: RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 50 μM 2-mercaptoethanol, and 10% foetal calf serum (all from Life Technologies, ON, Canada) supplemented with recombinant IL-4 (50 ng/ml) and CD40L (5 μg/ml) using 96-well plates. Quantities of IgE and IgG1 released from B cells were determined using a sandwich ELISA with monoclonal antibodies specific for each antibody. Plates were coated with anti-mouse IgE, or IgG1. Then the culture supernatant was added, and plates were incubated with the second antibody of biotinylated anti-mouse IgE, or IgG1. Standard curves were generated using recombinant cytokines.

Mixed lymphocyte reaction

T cells were purified from C57BL/6 splenocytes and used as responders (10^5/well). Isolated DCs ($1–10 \times 10^4$/well) from BALB/c mice were used as stimulators. Cells were cultured for 2 days and an $[^3H]$-thymidine incorporation assay was performed as described for B cell proliferarion.

Generation of bone marrow-derived DCs

Dendritic cells were generated from bone marrow progenitor cells, as previously described (14). Briefly, bone marrow cells were flushed from the femurs and tibias of BALB/c mice (Charles River Canada, Saint-Constant, Canada), then washed and cultured in 24-well plates (2×10^6 cells/ml) in 2 ml of complete medium supplemented with recombinant GM-CSF, 10 ng/ml (Peprotech, Rocky Hill, NJ, USA) and recombinant mouse IL-4, 10 ng/ml (Peprotech). All cultures were incubated at 37°C in 5% humidified CO_2.

Gene silencing

Transfection was carried out according to the method described previously (14). Briefly, 1 μg vector that expresses CD40 siRNA or control siRNA was incubated with 5 μl of Geneporter (Gene

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Therapy Systems, San Diego, CA, USA) for 15 min. This then was added to 400 μl of DC cell culture. After 4 h of incubation, an equal volume of RPMI-1640, supplemented with 20% FCS, was added to the cells.

Pathology

After sacrificing, heads were fixed in 10% buffered formalin, and then the heads were decalcified and sectioned. Three micrometer thick cryosections of nasal tissue were stained with Luna staining. The number of eosinophils in the nasal septum or olfactory mucosa was evaluated microscopically using a high power field (10 × 40).

Statistical analysis

Data are expressed as means ± SEM. Statistical comparisons between groups were performed using Student's *t*-test or one-way ANOVA followed by the Newman–Keuls test. Differences with *P*-values less than 0.05 were considered significant.

Results

CD40 siRNA treatment suppresses allergic symptoms and eosinophilia

Gene silencing strategies using siRNA have been successfully tested in animal models of human diseases (10, 14). The therapeutic potential of siRNA for allergy has not yet been developed. CD40 is not only a costimulatory factor involved in numerous immune responses, but is also critical in development of allergy (15–17). Thus, we explored whether systemic CD40 siRNA can be an alternative treatment for allergy. Mice immunized with OVA and alum were injected with CD40 siRNA, control siRNA, or PBS alone, and challenged with intranasal OVA. According to established protocols (13, 18), sneezing and pruritus of the nose were assessed as major clinical symptoms of allergic rhinitis. Assessment of sneezing and nasal rubbing was performed immediately after the last nasal challenge. The number of sneezes in mice treated with CD40 siRNA was significantly fewer than in mice having received control siRNA or PBS alone (Fig. 1A, $P < 0.01$). The frequency of nasal rubbing in mice treated with CD40 siRNA was also significantly reduced compared with mice that received control siRNA or PBS alone (Fig. 1B, $P < 0.01$). These data suggest that CD40 siRNA treatment attenuates allergic symptoms in a postsensitization setting.

Eosinophilia is a typical phenomenon of allergic diseases and is associated with allergic symptoms and reactions (19). To investigate the effect of CD40 siRNA on eosinophilia, the number of eosinophils in the nasal septum and olfactory mucosa were quantified. The number of eosinophils infiltrating the nasal septum and olfactory mucosa per field (10 × 40) was significantly reduced in the CD40 siRNA treatment group, compared with control groups (Fig. 1C, $P < 0.01$). These data suggested that CD40 siRNA treatment inhibited eosinophilia in the nasal and

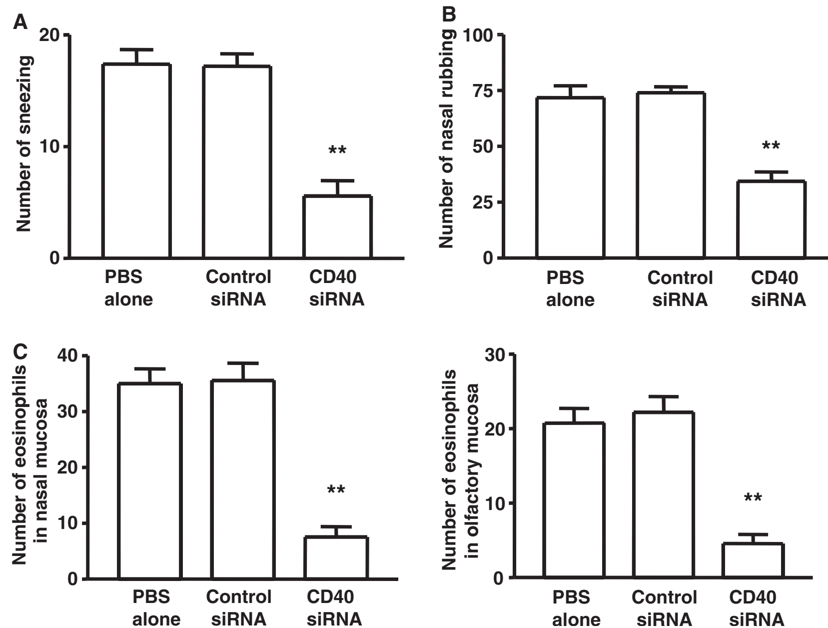

Figure 1. Reduction of allergic nasal symptom and eosinophilia by CD40 siRNA treatment. Mice were treated with PBS alone, control siRNA, and CD40 siRNA after immunization with OVA. And then, mice were challenged intranasally with OVA. The number of sneezing (A) or nasal rubbing (B) was counted for 20 min after the last nasal challenge. The numbers of eosinophils of nasal septum or olfactory mucosa per high power field (10 × 40) was also counted microscopically (C). Data represent mean + SEM and are representative of three experiments. $^{**}P < 0.01$ *vs* group of PBS alone or control siRNA.

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olfactory mucosa, resulting in the reduction of allergic symptoms and allergic reactions.

CD40 siRNA treatment inhibits IgE, IgG1, and IgG2a antibodies

Antigen-specific IgE and IgG1 are strongly associated with allergic responses (20). To determine the ability of CD40 siRNA in preventing antibody production to OVA, sera were collected before treatment and after intranasal challenge with OVA. Serum levels of OVA-specific IgE, and IgG1 in mice were measured by ELISA. Ovalbumin-specific IgE was detected before treatment, suggesting that mice were sensitized with OVA before treatment. After treatment, mice administered CD40 siRNA produced significantly lower OVA-specific IgE than mice given control siRNA or PBS alone, although there was no difference before treatment (Fig. 2A, $P < 0.01$). Intranasal OVA challenge did not increase IgE level in mice treated with CD40 siRNA, although intranasal challenge significantly increased IgE in the control groups ($P < 0.01$). Ovalbumin-specific IgG1 in mice treated with CD40 siRNA was also lower than that of mice given control siRNA or PBS alone after treatment, although there were no differences before treatment (Fig. 2B, $P < 0.01$). Intranasal OVA challenge significantly increased IgG1 in mice received PBS alone or control siRNA ($P < 0.01$). However, intranasal challenge did not significantly increase IgG1 level in mice treated with CD40 siRNA. This suggests that CD40 siRNA treatment inhibits antigen-specific IgE and IgG1 that induce allergic responses.

Next, we measured OVA-specific IgG2a in sera, which is associated with Th1 responses. Before treatment, there was no significant difference in the level of OVA-specific IgG2a among three groups, PBS alone, control siRNA, and CD40 siRNA. However, OVA-specific IgG2a in mice treated with CD40 siRNA was significantly lower than control groups after treatment (Fig. 2C).

Immune modulation of B cells *in vivo* by CD40 siRNA

It is well known that B cells produce IgE via immunoglobulin class-switching, which is a CD40-dependent process (12). Therefore, we investigated the effects of *in vivo* CD40 siRNA on the B cell compartment. We initially isolated B cells from spleens of allergic mice treated with PBS alone, control siRNA, or CD40 siRNA, and analysed CD40 expression on B cells by FACS. B cells isolated from the CD40 siRNA treated group expressed lower CD40 compared to control groups (Fig. 3A). The silencing effects of CD40 siRNA on B cells were also detected at the mRNA level by reverse transcription polymerase chain reaction (RT-PCR) and real time PCR (Fig. 3B,C). Next, we examined synthesis of total IgE and OVA-specific IgE by B cells isolated from mice treated with PBS alone, control siRNA, or CD40 siRNA after stimulation with CD40L and IL-4. Total and

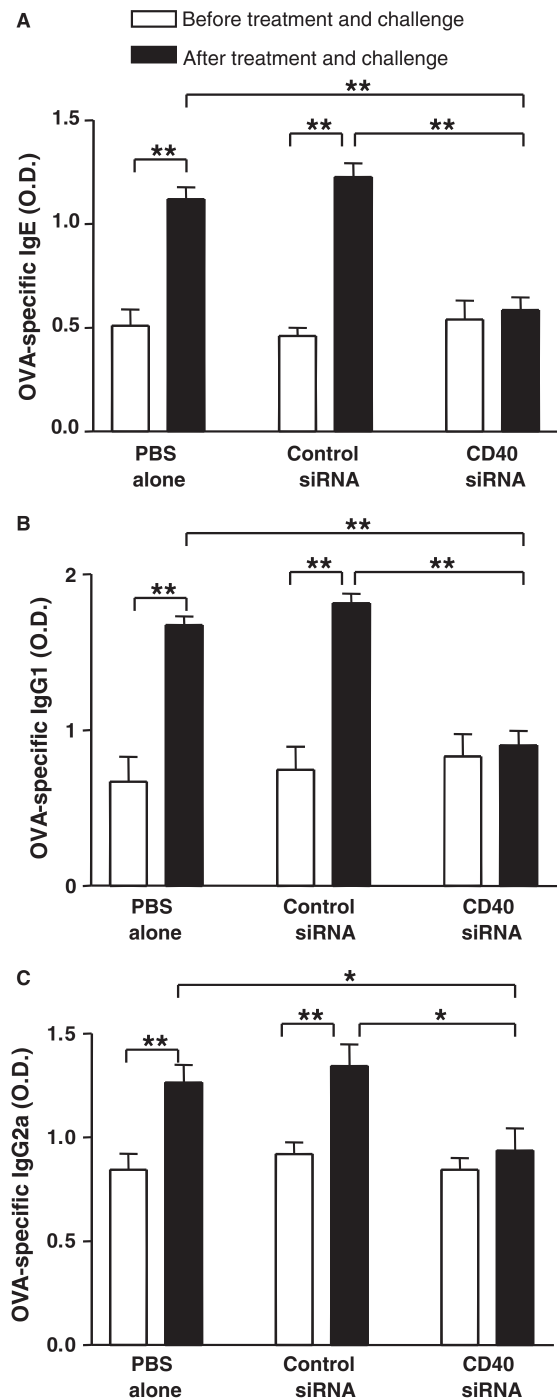

Figure 2. Modulation of OVA-specific antibody by CD40 siRNA. Mice were immunized with OVA. And then, mice were treated with PBS alone, control siRNA, and CD40 siRNA, followed by intranasal challenge with OVA. Sera were collected before and after treatment and challenge. Levels of OVA-specific IgE, IgG1, and IgG2a in sera were measured by ELISA. Levels of antibodies before and after treatment and challenge were determined in the same ELISA. (A) OVA-specific IgE. (B) OVA-specific IgG1. (C) OVA-specific IgG2a. Data represents mean + SEM and are representative of three experiments. $*P < 0.05$, $**P < 0.01$.

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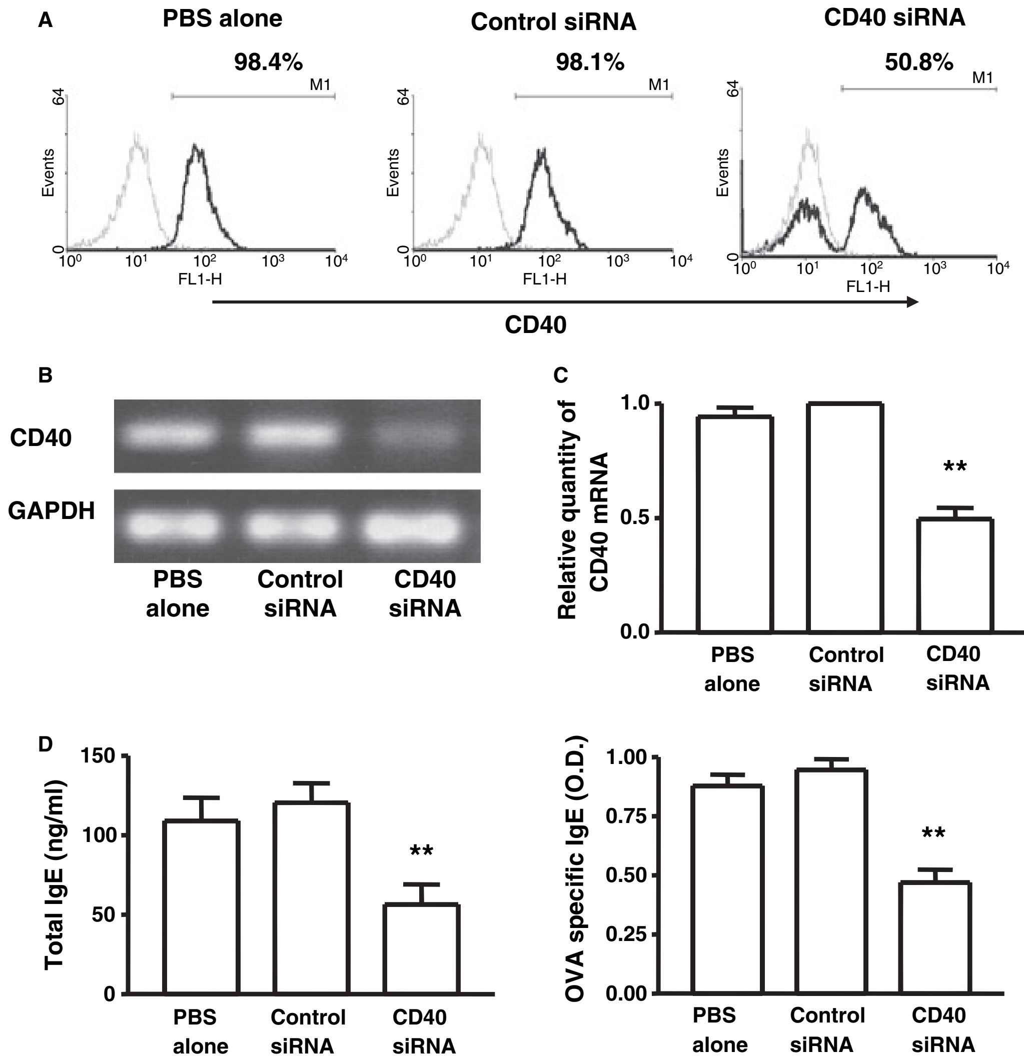

Figure 3. Modulation of B cell response. Mice were treated with PBS alone, control siRNA, and CD40 siRNA after immunization with OVA. And then, mice were challenged intranasally with OVA. (A) Silencing CD40 expression of splenic B cells *in vivo*. B cells were isolated from the spleen, and cells were stained with FITC-labelled anti-CD40 mAb. B cells were analysed by flow cytometry. (B) CD40 expression determined by RT-PCR. Total RNAs were extracted from B cells in mice injected with control siRNA or CD40 siRNA, respectively. RT-PCR was performed using primers specific to CD40 and GAPDH, as described in Materials and Methods. (C) Real time quantitative PCR performed as described in *Materials and Methods*. (D) Reduction of total IgE and OVA-specific IgE productions from B cells. Total IgE and OVA-specific IgE produced by B cells were measured by ELISA. Data represents mean + SEM and are representative of three experiments. $^{**}P < 0.01$ *vs* group of PBS alone or control siRNA.

OVA-specific IgE in CD40 siRNA group were lower than control groups (Fig. 3D). These data suggest that CD40 siRNA suppresses CD40 expression on B cells and inhibits IgE synthesis from B cells *in vivo*.

CD40 siRNA treatment inhibits T cell response and Th2 immune response

Allergy is associated with antigen-specific T cell responses which support B cell class switching and IgE production (21). In order to assess the T cell response in the CD40 siRNA treated mice, we collected lymph node T cells from the mice after challenge with OVA following immunization with OVA and treatment of PBS alone, control siRNA, or CD40 siRNA. T cells were stimulated *in vitro* with OVA antigen to examine OVA antigen-specific T cell response. Ovalbumin-specific T cell response in the mice that received CD40 siRNA was lower than that in the mice that received control siRNA or PBS alone (Fig. 4A), suggesting that CD40 siRNA reduced OVA-specific response.

Th2 cytokines, such as IL-4 and IL-5, play important roles in allergy. Interleukin-4 is essential for the production of IgE and IgG1 (22). Interleukin-5 is associated with

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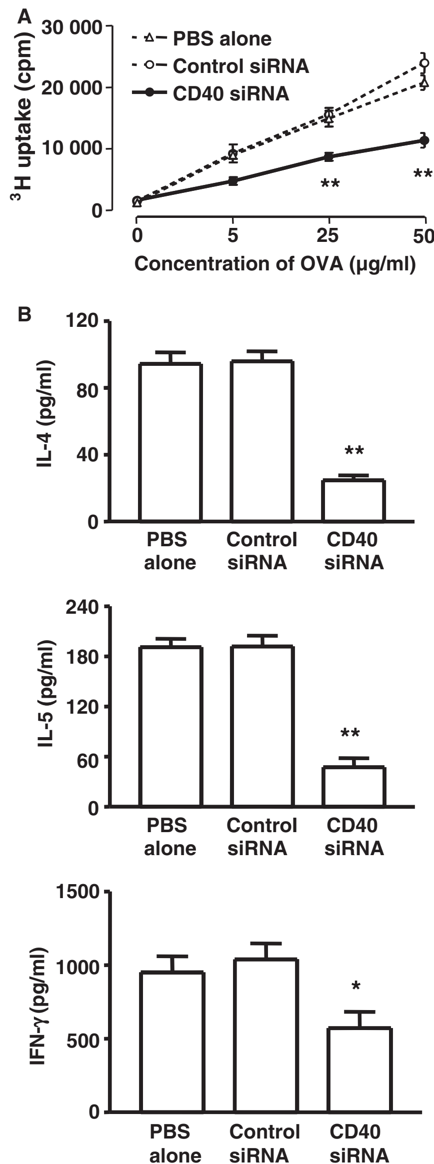

Figure 4. Immune modulation *in vivo* by CD40 siRNA treatment. Mice were treated with PBS alone, control siRNA, and CD40 siRNA after immunization with OVA. And then, mice were challenged intranasally with OVA. Lymphocytes were isolated from the spleen or lymph node. (A) Inhibition of OVA-specific T cell response. Unfractionated lymph node cells were cultured in 96-well plates at a concentration of 4×10^5 cells/well. Ag-specific recall responses were performed in the presence of OVA at indicated concentrations. (B) Modulation of IL-4, IL-5, and IFN-γ productions from splenic splenocytes by CD40 siRNA. Spleen cell suspensions (4×10^6 cells/ml) were cultured for 72 h in complete medium and 200 μg/ml OVA. Quantities of IL-4, L-5 and IFN-γ in the culture supernatants were determined using a sandwich ELISA. Data are representative of three experiments. $*P < 0.05$, $**P < 0.01$ *vs* group of PBS alone or control siRNA.

migration and activation of eosinophils (19). In order to investigate whether CD40 siRNA administration modulates cytokine productions, we measured cytokine production from T cells stimulated with OVA *in vitro*. T cells of mice injected with CD40 siRNA released significantly less IL-4 and IL-5 (Fig. 4B, $P < 0.01$) than T cells from mice that had received control siRNA or PBS alone. This suggests that CD40 siRNA treatment suppresses the production of Th2 cytokines in the mice immunized with OVA, which may contribute to therapeutic effects observed in allergy.

To detect whether cytokine suppression was selective towards Th2 effectors, we measured interferon-γ (IFN-γ), which is associated with Th1 responses, by splenic cells stimulated with OVA. Splenic cells of mice received CD40 siRNA produced significantly lower IFN-γ compared with control groups (Fig. 4B). Interferon-γ was reduced by less than 50% in the mice treated with CD40 siRNA, while IL-4 and IL-5 were reduced by more than 70% of control level. This may suggest that CD40 siRNA strongly suppressed Th2 cytokines in mice with allergy, compared with Th1 cytokines.

CD40 siRNA treatment inhibits DC-mediated T cell responses

Mature DCs potently activate naïve T cells, partly through high expression of CD40 (23). To assess the efficacy of gene silencing *in vivo* by CD40 siRNA, we isolated DCs from allergic mice treated with PBS alone, control siRNA, or CD40 siRNA, and investigated CD40 expression on DC cells by FACS. As shown in Fig. 5A, CD40 siRNA treatment resulted in inhibition of CD40 expression. The silencing effects of CD40 siRNA were also detected at the mRNA level by RT-PCR and real time PCR (Fig. 5B,C). In addition to their role in initiation of immune responses, DCs can also play a role in inhibiting immune responses and inducing tolerance (24). Since CD40 expression is known to be involved in the activation of immune responses by DCs, we investigated whether DCs in mice treated with CD40 siRNA would display immune regulatory properties. We assessed T cell proliferation and cytokine production from T cells that were stimulated by DCs in mice treated with PBS alone, control siRNA, or CD40 siRNA. In order to evaluate the capacity of DCs to stimulate T cell response after CD40 silencing, an allogeneic mixed lymphocyte reaction (MLR) was performed. Dendritic cells in mice which did not receive CD40 siRNA initiated vigorous proliferative responses. In contrast, DCs in mice treated with CD40 siRNA failed to stimulate allogeneic T cells (Fig 5D). These data suggest that CD40 siRNA can knockdown CD40 expression on DCs *in vivo*, and that CD40 silencing causes a state of immune privilege or hyporesponsiveness.

We investigated cytokine production from splenic lymphocytes stimulated with DCs in mice treated with PBS alone, control siRNA, or CD40 siRNA. We measured IL-4 and IFN-γ productions in the supernatant after 48 h of incubation using DCs and allogeneic T cells. Interleukin-4 and IFN-γ productions from lymphocytes incubated with DCs from mice which received CD40

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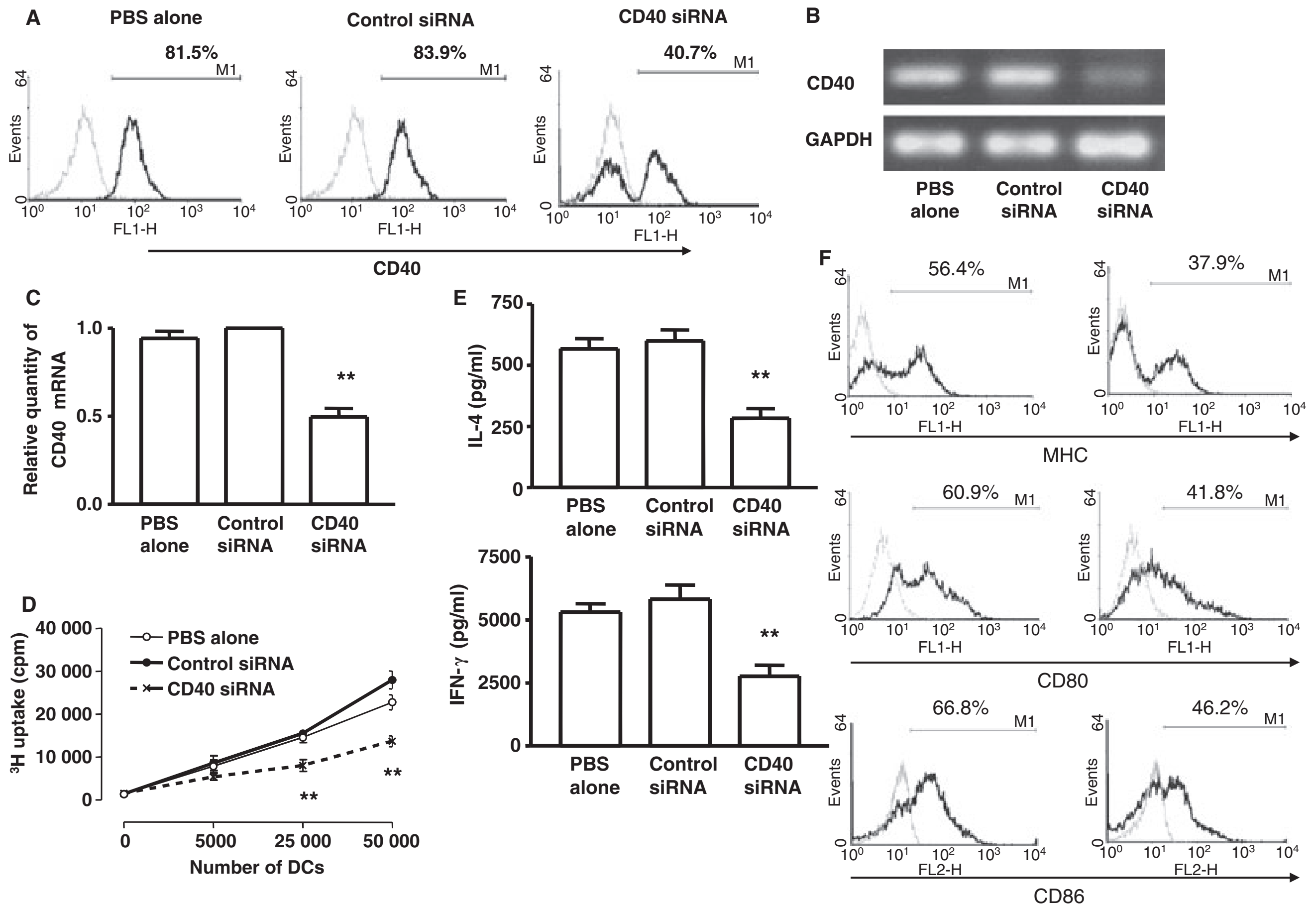

Figure 5. Modulation of DCs and DCs-mediated T cell response. Mice were treated with PBS alone, control siRNA, or CD40 siRNA after immunization with OVA. And then, mice were challenged intranasally with OVA. (A) Silencing CD40 expression of splenic DCs *in vivo*. DCs were isolated from the spleen, and cells were stained with FITC-labelled anti-CD40 mAb. DCs were analysed by flow cytometry. (B) CD40 expression determined by RT-PCR. Total RNAs were extracted from DCs in mice injected with PBS alone, control siRNA or CD40 siRNA, respectively. RT-PCR was performed using primers specific to CD40 and GAPDH, as described in Materials and Methods. (C) Real time quantitative PCR performed as described in *Materials and Methods*. (D) CD40 silencing inhibits DCs' allostimulatory ability. Isolated DCs from spleen in mice treated with PBS, control siRNA, or CD40 siRNA were incubated with allogeneic (C57BL/6) T cells (1×10^5/well). Proliferation was determined using [^{3}H]-thymidine incorporation. Triplicate samples were measured. (E) IL-4 and IFN-γ productions by lymphocytes. T cell stimulation was performed using the same protocols described in (D). After 48 h incubation, supernatants were collected. Titre of IL-4 and IFN-γ were measured by ELISA. **$P < 0.01$ *vs* counterpart group. (F) Silencing MHC II, CD80, and CD86 expressions on splenic DCs *in vivo*. Mice were treated with control siRNA or CD40 siRNA after immunization with OVA. And then, mice were challenged intranasally with OVA. DCs were isolated from the spleen, and cells were stained with anti-MHC II, CD80, or CD86 mAb. DCs were analysed by flow cytometry. Data are representative of three experiments.

siRNA was lower than that from lymphocytes incubated with DCs from mice which received control siRNA or PBS alone (Fig. 5E, $P < 0.01$). This suggests that CD40 siRNA treatment inhibited IL-4 and IFN-γ productions by impairing DC function.

Next, we investigated timeframe of CD40 suppression after CD40 siRNA treatment. Mice were injected intravenously with CD40 siRNA. One week or two weeks after the injection, isolated splenic DC were assessed for CD40 protein by flow cytometry. CD40 expression on DCs was reduced one week after CD40 siRNA injection (data not shown). However, CD40 expression on DCs were not suppressed two weeks after CD40 siRNA injection (data not shown).

Activation of DCs through CD40 results in increase of MHC II, CD80, and CD86 expression (25), which are associated with allergy. Therefore, we examined whether CD40 siRNA can suppressed not only CD40 expression but also other accessory molecules such as MHC II, CD80, and CD86 by flow cytometry. Consequently, we found that CD40 siRNA suppressed MHC II, CD80, and CD86 expression on DCs *in vivo* (Fig. 5F). Furthermore, we investigated whether the same phenomena were seen *in vitro*. Bone marrow derived DC (BMDCs) were

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transfected with CD40 siRNA, and expression of CD40, MHC II, CD80, and CD86 was analysed with flow cytometry after stimulation with LPS. CD40 siRNA suppressed CD40 expression on DCs *in vitro* (data not shown) as *in vivo*. However, CD40 siRNA did not suppress MHC II, CD80, and CD86 expressions on DCs vitro (data not shown).

CD40 siRNA facilitated generation of OVA-specific Treg

It has been documented that blocking the CD40–CD40L interaction actively generates Treg cells (26) which may contribute to active immune suppression. Accordingly, we examined whether CD40 siRNA were capable of generating Treg *in vivo*. Mice that had been immunized with OVA and alum were injected with CD40 siRNA, control siRNA, or PBS alone, and challenged with OVA. After collecting lymph node mononuclear cells, real time PCR was performed. Results of real time PCR showed that CD40 siRNA treated animals possessed a population of cells with the Treg phenotype, determined by the significantly increased Foxp3 gene expression (Fig. 6A). Flow cytometry analysis also showed an increase of the $CD25^+$ $Foxp3^+$ subpopulation of $CD4^+$ T cells in mice treated with CD40-silenced DCs, as compared to mice treated with control-DCs or PBS alone (Fig. 6B).

Next, we examined antigen specificity of Treg generated by CD40 siRNA. Mice were immunized with OVA, bovine serum albumin (BSA), and alum. After sacrifice, splenocytes were collected and antigen-specific T cell response was performed by OVA or BSA stimulation. $CD4^+$ $CD25^+$ Treg were also isolated from spleen and lymph node in OVA-sensitized mice which received CD40 siRNA, and were added to OVA-specific or OVA-specific T cell response to evaluate antigen-specific suppression. Although $CD4^+$ $CD25^+$ T cells significantly inhibited OVA-specific T cell response, $CD4^+$ $CD25^+$ T cells did not inhibit BSA-specific T cell response (Fig. 6C). These findings suggest that CD40 siRNA administration gives rise to the expansion of an antigen-specific $CD4^+$ $CD25^+$ Treg population *in vivo*.

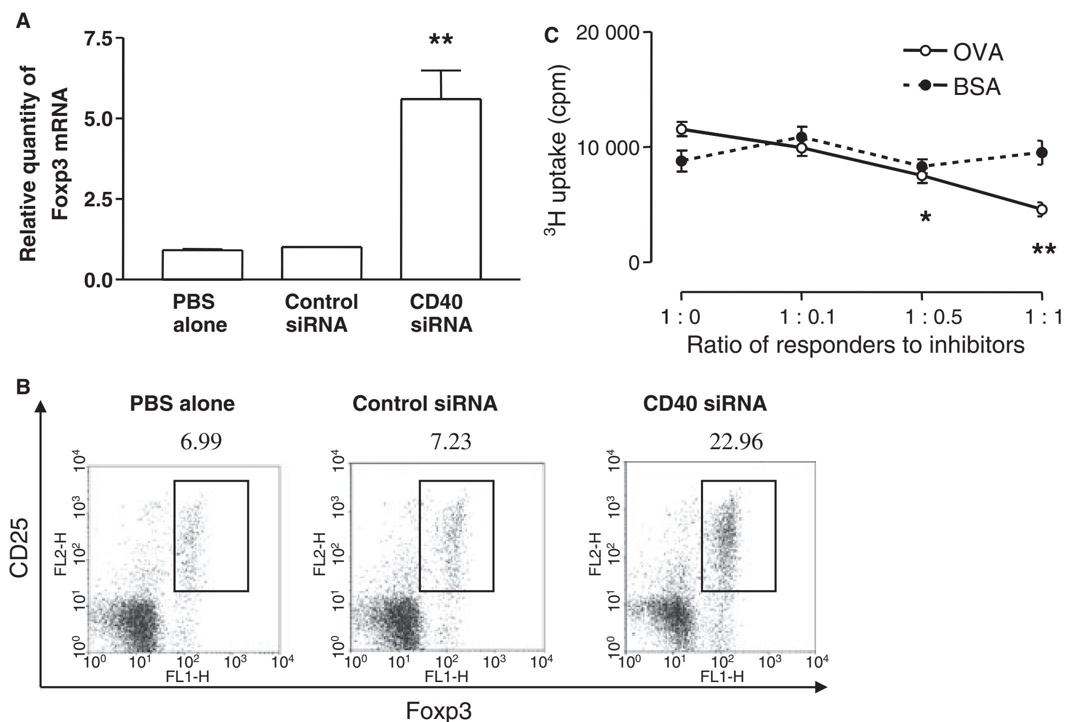

Figure 6. Increase of Foxp3 gene expression. Mice were treated with PBS alone, control siRNA, and CD40 siRNA after immunization with OVA. And then, mice were challenged intranasally with OVA. (A) Upregulated expression of Foxp3 genes. Total RNAs were extracted from lymph nodes in mice injected with PBS alone, control siRNA or CD40 siRNA, respectively. Real time quantitative PCR performed as described in Materials and Methods. $^{**}P < 0.01$ *vs* group of PBS alone or control siRNA. (B) Phenotypic analysis of *in vivo*-generated Treg. Splenic $CD4^+$ T cells were stained with PE-labelled anti-CD25 mAb and FITC-labelled anti-Foxp3 mAb and analysed by flow cytometry. (C) Inhibition of ongoing OVA-specific, but not BSA-specific, T cell response by Treg induced by CD40 siRNA. Mice were immunized with OVA, BSA, and alum. Splenocytes were collected and antigen-specific T cell response was performed by OVA or BSA stimulation. $CD4^+$ $CD25^+$ T cells as inhibitors, isolated from mice injected with CD40 siRNA, were added to OVA-specific or BSA-specific recall response. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* group of 1 : 0. Data represents mean ± SEM and are representative of three experiments.

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Discussion

Control of immune responses by APC, of which the DC is the most potent, is dependent on a variety of signals between antigen presenting cell (APC) and T cells. In addition to MHC and cytokine signals, costimulatory molecules are critical for activation/inhibition of T cell responses. Among the known costimulatory molecules, CD40 plays critical roles in T cell activation and differentiation (11, 27). Blocking CD40/CD40L interaction has been demonstrated to effectively induce tolerance in transplantation, autoimmunity, and allergy animal models (15–17, 26). Therefore, we questioned whether antigen-nonspecific inhibition of CD40 expression by systemic administration of siRNA would be effective at suppressing autoreactive responses. Using the OVA presensitization model of allergic rhinitis, we demonstrated that CD40 siRNA administration, but not control siRNA, was capable of: (i) inhibiting pathological symptoms of allergy; (ii) diminishing eosinophilia in the olfactory mucosa; (iii) suppressing production of cytokines and IgE antibody responses associated with allergic response; (iv) inhibiting APC expression of CD40 *in vivo*; and (v) stimulating a Foxp3 expressing population of cells with Treg cell properties.

Current approaches for treating allergy do not induce a long-term immune modulation. One possible explanation could be the difference between a short-term acting intervention that targets events downstream of the allergic cascade *vs* targeting initiating events. Specifically, glucocorticoids, antihistamins, and leukotriene inhibitors all target the effector phase of the allergic response (28). As with any biological response, intervening at the late phases of pathology does not produce long term effects or result in the subsequent tolerance. One possible explanation for the observed profound therapeutic effects is that by inhibiting the CD40–CD40L interaction, the current approach blocks an early activation event, thus inhibiting the allergic cascade upstream and preventing its maturation. Since the CD40–CD40L interaction itself is needed for class-switching to occur (12), and because we observed reduced levels of CD40 on B cells (Fig. 4), we can not rule out the possibility that siRNA-mediated effects were due to inhibition of costimulatory signals needed for class switching. Additionally, although we demonstrated inhibition of IgG2a, there remains a possibility that B cell proliferation in general was inhibited.

Generation of Treg cells has been associated with tolerization to allergens both in animal and clinical models. Numerous studies have documented the association between Treg cells and tolerance to allergens in clinical situations (29–31). Since we have observed increased Treg in mice that have been treated with CD40 siRNA, we believe that this Treg generation may be a means by which tolerance may be induced.

In this study, CD40 siRNA administration induced OVA-specific Treg *in vivo*. Previous studies have demonstrated that nonspecific immune modulation during the presence of an ongoing response may induce antigen-specific immune suppression. For example, in the case of type I diabetes, treatment with anti-CD3 monoclonal antibody has been demonstrated to induce Treg cells with antigen specificity (32). Blocking the CD40-CD40L interaction actively generates Treg cells. It may be possible that in the context of OVA-reactive T cells, the encounter of the preactivated cells with APC expressing low levels of costimulatory molecules may lead to selective apoptosis or generation of Treg specific only to the antigen which caused the initial clonal expansion. Such a scenario is known to occur in transplantation tolerance induced by anti-CD40L antibody, in which donor-specific tolerance has been observed (33).

It has been reported that CD40–CD40L interaction play an important role against infection, and that CD40-deficient mice have an increased susceptibility to infection (34). In this study, CD40 siRNA suppressed not only Th2 responses but also Th1 responses. Considering these, CD40 siRNA treatment may induce side-effects such as infection. Future experiments, for example siRNA conjugated with antigen, to minimize side-effect is expected.

siRNA mediates efficient gene silencing by RNA interference. However, usefulness of siRNA for *in vivo* gene therapy is limited because of instability *in vivo* (35). siRNA expressing vector (shRNA) is more versatile and stable *in vivo* than siRNA, therefore, we injected shRNA into mice in this study. Antigen-dependent therapy is not applicable for patients with an unknown causative allergen. There is increasing incidence of patients with allergies who suffer from sensitization to multiple allergens (36), making the antigen-specific tolerance induction hard to achieve. This study showed that administration of CD40 siRNA alone can effectively inhibit allergic responses and attenuate symptoms of allergy. Thus, CD40 siRNA-based treatment may be an alternative antigen-independent therapy, with potential for the patients having an unidentified causative allergen or with multiple causative allergens.

In summary, we report a novel immune therapy for allergy through silencing CD40 gene on DCs and B cells. Treatment with CD40 siRNA after sensitization significantly attenuates allergic symptoms and pathological changes, suggesting potential clinical use of CD40 siRNA.

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