

Novel Vaccination for Allergy through Gene Silencing of CD40 Using Small Interfering RNA¹

Motohiko Suzuki,* Xiufen Zheng,* Xusheng Zhang,* Mu Li,* Costin Vladau,* Thomas E. Ichim,[§] Hongtao Sun,* Lisa R. Min,* Bertha Garcia,* and Wei-Ping Min^{2*†‡}

Small interfering RNA (siRNA) is a potent means of inducing gene-specific silencing. Gene silencing strategies using siRNA have demonstrated therapeutic benefits in animal models of various diseases, and are currently in clinical trials. However, the utility of gene silencing as a treatment for allergic diseases has not yet been reported. In this study, we report a novel therapy for allergy through gene silencing of CD40, a critical costimulatory molecule and a key factor in allergic immune responses. Silencing CD40 resulted in generation of immunoregulatory dendritic cells (DCs). Administration of CD40 siRNA remarkably reduced nasal allergic symptoms and local eosinophil accumulation in the OVA-induced allergic mice. The OVA-specific T cell response was inhibited after the CD40 siRNA treatment. Additionally, anti-OVA specific IgE and production of IL-4 and IL-5 of T cells stimulated by OVA were significantly decreased in CD40 siRNA-treated mice. Furthermore, we demonstrated that the therapeutic effects by CD40 siRNA were associated with impaired Ag-presenting functions of DCs and B cells, and generation of regulatory T cells. The present study highlights a therapeutic potential of siRNA-based treatment for allergic diseases. *The Journal of Immunology*, 2008, 180: 8461–8469.

Allergic diseases caused by excessive Th2 response are widespread all over the world (1–4). Despite the increased understanding of the pathophysiology of allergic responses, current therapy targets late events within the allergic cascade. Therefore, a novel approach that addresses upstream causative events is highly desired. RNA interference is a cellular defense mechanism against viral dsRNA in which the host cell selectively inactivates endogenous mRNA transcripts that are homologous to exogenous dsRNA (5). Gene silencing using small interfering RNA (siRNA)³ is a potent, selective, and easily inducible method for specifically blocking expression of desired genes (6). The use of siRNA is also more efficient than other gene-specific targeting approaches such as antisense oligonucleotides (7–9). Gene silencing strategies using siRNA have been successful in inducing therapeutic benefits in animal models of various diseases and are currently in clinical trials (10). It has been also reported that siRNA can inhibit Th2 responses in vitro (11, 12). However, the utility of gene silencing as a treatment for allergic diseases has not yet been reported.

CD40 is an integral membrane protein belonging to the TNFR superfamily that is inducible upon APC maturation and provides additional maturation signal to the APC (13). In addition, CD40 on dendritic cells (DCs) also activates T cells through “outside-in” signaling of the CD40L (CD154), which is expressed on T cells (14). CD40 cross-linking on isolated T cells has been demonstrated to increase Th2 cytokines and stimulate proliferation (15). To date, blockade of the CD40-CD40L interaction is being aggressively pursued as a tolerance-inducing strategy (16). Inhibition of this bidirectional interaction not only suppresses T cell responses (17) and Th2 cytokines (18–20) but also actively generates regulatory T (Treg) cells (21). Although Treg cells are classically thought of as Th1 inhibitory cells due to their activity against Th1-mediated diseases such as rheumatoid arthritis and type 1 diabetes (22), Treg cells have also been demonstrated to inhibit Th2 responses (23). Importantly, lower numbers of Treg cells are found in patients with allergic rhinitis in comparison to controls (24). Furthermore, B cells play a critical role in IgE synthesis, and require two signals to synthesis IgE (25–27). The first signal is provided by cytokines, such as IL-4. The second signal is provided by ligation of CD40.

Thus, the blockade of the CD40-CD40L interaction appears to be a promising method of inhibiting allergic disease. Accordingly, a recent study described the ability of anti-CD40L blocking Ab to suppress sensitization in a murine model of pollen allergy, although administration of anti-CD40L Ab after sensitization did not inhibit allergy (28). Unfortunately, such approaches are not clinically translatable because humans express CD40L on platelets. Therefore, ligation of this molecule causes thrombosis (29). Additionally, because the effects of anti-CD40 and CD40L Ab are transient, prolonged administration is often required (30). siRNA targeting CD40 prevented leukocyte adhesion on endothelial cells in vitro (31). Therefore, specifically knocking down CD40 before immunization using siRNA would be a clinically attractive strategy.

There are two main anti-allergy strategies being used for the patients with allergy: Ag-specific therapy and Ag-independent therapy. Ag-specific immune therapy is not applicable for many

*Department of Surgery, Department of Pathology, and Department of Microbiology and Immunology, University of Western Ontario, [†]Multi-Organ Transplant Program, University Hospital, and [‡]Transplantation and Regenerative Medicine, Lawson Health Research Institute, London, Ontario, Canada; and [§]Medistem Laboratories, San Diego, CA 92122

Received for publication September 4, 2007. Accepted for publication April 14, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This study is partially supported by the Canadian Institutes of Health Research and a grant from London Health Sciences Centre to the Multi-Organ Transplant Program.

² Address correspondence and reprint requests to Dr. Wei-Ping Min, Multi-Organ Transplant Program, C9-136, London Health Sciences Centre, University Hospital, 339 Windermere Road, London, Ontario N6A 5A5, Canada. E-mail address: weiping.min@uwo.ca

³ Abbreviations used in this paper: siRNA, small interfering RNA; DC, dendritic cell; Treg, regulatory T.

patients when the causative allergen is unknown. Additionally, there is an increased incidence of patients with allergies that are sensitized to multiple allergens (2), making the Ag-specific tolerance induction hard to achieve. Especially in the case of therapy before sensitization, Ag-specific therapy is difficult because it is very difficult to forecast with which Ag patients will be sensitized.

In the present study, we investigated the feasibility of silencing CD40 expression by siRNA treatment as a means of controlling allergic disease in vivo. We discovered, for the first time, that CD40 siRNA is useful for the control of allergic diseases. The therapeutic effects are associated with impaired APC function of DCs and generation of Treg cells.

Materials and Methods

Generation of bone marrow-derived DCs

DCs were generated from bone marrow progenitor cells, as previously described (32). Briefly, bone marrow cells were flushed from the femurs and tibias of BALB/c mice (Charles River Breeding Laboratories Canada), then washed and cultured in 24-well plates (2×10^6 cells/ml) in 2 ml of complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-ME, and 10% FCS (all from Invitrogen) supplemented with recombinant GM-CSF (10 ng/ml; PeproTech) and recombinant mouse IL-4 (10 ng/ml; PeproTech). All cultures were incubated at 37°C in 5% humidified CO₂.

Construction of CD40 siRNA-expressing vector

CD40 siRNA-expressing vectors were generated using the Silencer Express kit (Ambion). Sense (ACACTACACAAATGTTCCACTGGGCTGAGAACC GGTTTCGTCCTTTCCACAAG) and antisense (CGGC GAAGCTTTTCCAAAAAATTTCTCAGCCCAAGTGGAACTACACA ATGTT) hairpin siRNA template oligonucleotide, specific to CD40 mRNA, were used.

Gene silencing

Transfection was conducted according to the method previously described (32). Briefly, 1 μ g of vector that expresses no siRNA, CD40 siRNA, scramble siRNA (control siRNA), or transfection reagent alone was incubated with 5 μ l of Geneporter (Gene Therapy Systems) for 15 min. This then was added to 400 μ l of DC or B cell culture. After 4 h of incubation, an equal volume of RPMI 1640, supplemented with 20% FCS, was added to the cells.

Flow cytometry

A phenotypic analysis of DCs or T cells was performed on a FACScan as previously described (32). At 72 h after transfection, DCs were harvested and stained with PE-conjugated anti-CD40 mAb. For T cells, we used CyChrome-conjugated anti-mouse CD4 and PE-conjugated CD25 mAb (eBioscience). Foxp3 expression was assessed by intracellular staining using a cell permeabilization kit (eBioscience).

RT-PCR and real-time PCR

Total RNA was isolated from Treg cells or DCs after gene silencing, using TRIzol (Invitrogen) according to the manufacturer's protocol. Briefly, 20 μ g of RNA was digested with 10 U of DNase I at 37°C for 30 min, extracted with phenol:chloroform (3:1), precipitated with ethanol, washed with 70% ethanol, and finally dissolved in 20 μ l of RNase-free water. To generate the first-strand cDNA, the SuperScript Preamplification System (Invitrogen) was used. For PCR amplification, the primers used in this study were: CD40 (150 bp) sense 5'-TGATTGTGCCAGCCAG GAAGC-3' and antisense 5'-CCCTTGATTGGGTTTCACAGTGTCT-3'; Foxp3 (382 bp) sense 5'-CAGCTGCCTACAGTCCCCCTAG-3' and antisense 5'-CATTTGCCAGCAGTGGGTAG-3'; GAPDH (249 bp) sense 5'-TGATGACATCAAGAAGGTGGTGA-3' and antisense 5'-TCCTTG GAGGCCATGTAGGCCAT-3'. The PCR products were resolved by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Real-time PCRs were performed using SYBR Green PCR Master mix (Stratagene) and 100 nM gene-specific primers. The PCR 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.

Isolation of splenic DCs and Treg cells

DCs were isolated from spleens by gradient centrifugation over Ficoll-Paque (Amersham Biosciences) and labeled with anti-mouse CD11c mAb-conjugated super-paramagnetic MicroBeads (Miltenyi Biotec). CD11c⁺ cells were isolated by a magnetic column.

CD4⁺CD25⁺ T cells were isolated from coculture of DCs and T cells and spleen of mice using MACS system according to the manufacturer's instruction. CD4⁺ T cells were negatively selected with a MACS negative selection CD4 isolation kit (Miltenyi Biotec), and CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were then purified using anti-CD25 MACS beads (Miltenyi Biotec).

Treatment and immunization

Six- to 8-wk-old male BALB/c mice were purchased from Charles River Breeding Laboratories Canada. Mice were injected i.p. with 50 μ g of CD40 siRNA, 50 μ g of control siRNA, or PBS alone twice on days 0 and 14. Mice were also injected i.p. with 10 μ g of OVA and 4 mg of Al(OH)₃ twice on days 2 and 16. Each group consisted of six mice. The same mice were challenged intranasally on day 21 through day 27 with OVA (600 μ g). 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 (33), and samples were collected on day 28. The observer assessed sneezing and nasal rubbing blinded to the treatment of the mice. All mice were housed in an environmentally controlled animal facility at the University of Western Ontario. The protocols were approved by the Guidelines for the Care and Use of Animals at University of Western Ontario. Every effort was made to minimize any discomfort of the animals.

MLR analysis

MLR cultures were performed in triplicate in 96-well plates. T cells were purified from C57BL/6 splenocytes and used as responder (1×10^5 /well). Control siRNA or CD40 siRNA-treated DCs ($1-10 \times 10^4$ /well from BALB/c) were used as stimulators. Cells were cultured for 3 days and pulsed with 1 μ Ci of [³H]thymidine (Amersham Biosciences) for the last 16 h of culture. Cells were harvested onto glass fiber filters, and incorporated radioactivity was quantitated using a Wallac Betaplate liquid scintillation counter. Results were expressed as mean cpm $\pm$ SEM of triplicate cultures.

To determine the ability of Treg cells to inhibit an MLR, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were added, at various ratios, to an MLR using normal C57BL/6 T cells as responders (5×10^4 /well) and irradiated (3000 rad) BALB/c spleen cells as stimulators (5×10^4 /well). The experimental procedure of incubating and harvesting cells was the same as described. Results were expressed as a mean cpm $\pm$ SEM of triplicate cultures.

Measurement of OVA-specific IgE and IgG1

Mouse serum titers of OVA-specific IgE were measured by ELISA. Briefly, ELISA plates were coated with anti-mouse IgE mAb (Zymed Laboratories). Nonspecific binding was blocked, and 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 (BD Biosciences) was applied according to the manufacturer's instructions, and OD was measured at 450 nm. OVA-specific IgG1 was also measured by ELISA. ELISA plates were coated with OVA. Nonspecific binding was blocked, and serum samples were added to the plates. The plates were washed, and biotin-labeled anti-mouse IgG1 (The Binding Site) was added. The plates were incubated with avidin-peroxidase. After washing, the same substrate was applied, as described.

Measurement of IL-4 and IL-5 release by in vitro splenocytes

Spleen cell suspensions (4×10^6 cells/ml) were cultured for 72 h in complete medium and 100 μ g/ml OVA. Quantities of IL-4 and IL-5 in the culture supernatants were determined using a sandwich ELISA with mAbs specific for each cytokine. Plates were coated with anti-mouse IL-4 (Endogen), or anti-mouse IL-5 (eBioscience). Then, the culture supernatant was added, and plates were incubated with the second Ab of biotinylated anti-mouse IL-4 (Endogen) or biotinylated anti-mouse IL-5 (eBioscience). Standard curves were generated using recombinant cytokines.

OVA-specific T cell response

T cells were isolated from spleens in allergic model mice with control siRNA or CD40 siRNA treatment by gradient centrifugation over Ficoll-Paque (Amersham Biosciences). All cells were cultured in 96-well plates at a concentration of 4×10^5 cells/well for 72 h in the presence or absence of OVA Ag.

To assess the inhibitory capacity of Treg cells, the isolated splenic cells of allergic mice injected with PBS alone were also cultured in 96-well plates at a concentration of 5×10^5 cells/well with 400 $\mu\text{g/ml}$ OVA Ag in the presence of $\text{CD4}^+\text{CD25}^+$ T cells or $\text{CD4}^+\text{CD25}^-$ T cells from the spleen of mice injected with control siRNA or CD40 siRNA for 72 h. An [^3H]thymidine incorporation assay was performed as described for MLR.

Measurement of B cell proliferation

B cells were cultured for 2 days in complete medium after transfection. B cells were then stimulated with recombinant IL-4 (50 ng/ml), IL-5 (5 ng/ml), and CD40L (5 $\mu\text{g/ml}$) using 96-well plates. A [^3H]thymidine incorporation assay was performed as described for MLR.

Measurement of IgE secreted from B cells

Quantities of IgE released from B cells were determined using a sandwich ELISA with mAbs specific for each Ab. Plates were coated with anti-mouse IgE (BD Pharmingen). Then, the culture supernatant was added, and plates were incubated with the second Ab of biotinylated anti-mouse IgE (BD Pharmingen). Standard curves were generated using recombinant cytokines.

Pathology

After being fixed in 10% buffered formalin, the heads were decalcified and sectioned. A 3- μm thick cryosection of nasal tissue was stained with Luna staining. The number of eosinophils in the bone marrow and nasal mucosa of the nasal septum was evaluated microscopically using a high power field (10×40). The observer assessed eosinophilia blinded to the treatment of the mice.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical comparisons between groups were performed using Student's *t* test or one-way ANOVA followed by the Newman-Keuls Test. Differences with values for *p* < 0.05 were considered significant.

Results

Silencing CD40 in vitro and in vivo using siRNA

Mature DCs express high levels of CD40 (34). To assess the efficacy of gene silencing by CD40 siRNA, we performed standard 6-day DC cultures in the presence of GM-CSF and IL-4, followed by transfecting DC with CD40 siRNA, control siRNA, empty vector, or transfection reagent alone. Gene-silenced DCs were subsequently induced to maturation by stimulation with LPS. As shown in Fig. 1A, mature DCs expressed high levels of CD40, which was not altered by treatment with the transfection reagent alone, empty vector, or control siRNA. CD40 siRNA treatment resulted in potent inhibition of CD40 expression (Fig. 1A). The silencing effects of CD40 siRNA were also detected at the mRNA level by RT-PCR and real-time PCR as compared with control siRNA (Fig. 1, B and C). Transfection of DCs with siRNA did not alter the viability or ability to respond to maturation stimuli (data not shown). Altogether, these in vitro data demonstrate the feasibility and efficacy of CD40 siRNA to silence the expression of CD40 in DCs.

Next, we investigated the ability of CD40 siRNA to suppress CD40 expression in splenic DCs in vivo. Mice were injected i.p. with 200 μl of CD40 siRNA (50 μg per each mouse), control siRNA (50 μg per each mouse), or PBS alone. Seven days after injection, CD40 expression of splenic DCs was analyzed by flow cytometry. CD40 siRNA treatment knocked down CD40 expression of splenic DCs compared with the control siRNA or PBS alone (Fig. 1D). This result suggests that CD40 siRNA treatment knocked down CD40 expression in DCs in vivo.

Alteration of immune response of DCs by gene silencing of CD40

DCs are the most potent APCs that play a substantial role in initiating protective immune responses (35) as well as immune tolerance. Because CD40 expression is known to be involved in the

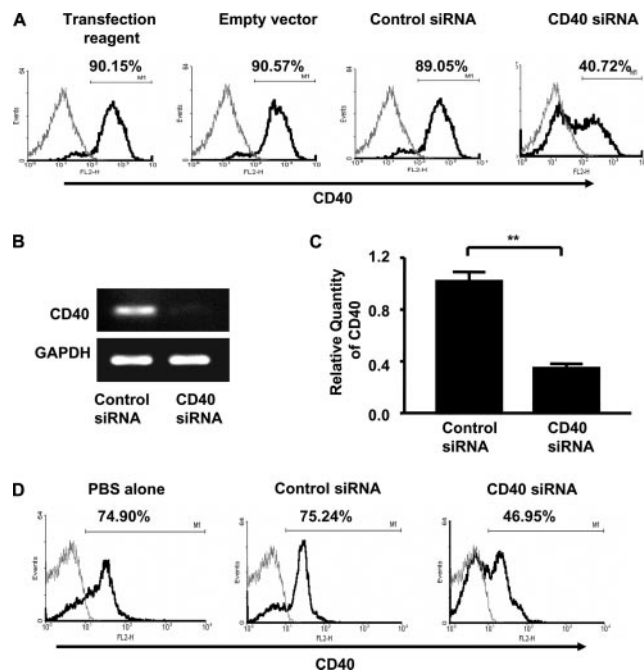


FIGURE 1. CD40 silencing DCs with CD40 siRNA. *A*, Phenotypic analysis of DCs transfected with CD40 siRNA in vitro. Bone marrow-derived DCs from BALB/c mice were cultured for 6 days in the presence of GM-CSF and IL-4. DCs (1×10^6) were transfected with 1 μg of empty vector, control siRNA, CD40 siRNA, or transfection reagent alone on day 6. At 48 h after transfection, CD40 expression was assessed by flow cytometry using PE-conjugated Abs (black line histogram) and isotype controls (gray line histogram). *B*, CD40 expression determined by RT-PCR. At day 6, cultured DCs were transfected with control siRNA or CD40 siRNA in vitro. At 48 h after gene silencing, total RNAs were extracted from DCs transfected 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*, CD40 expression was determined by real-time quantitative PCR. DC culture and gene silencing using the same protocols described in *B* were used. Real-time quantitative PCR was performed as described in *Materials and Methods*. **, *p* < 0.01 by Student's *t* test. *D*, Silencing CD40 expression of splenic DCs in vivo. BALB/c mice were injected i.p. with 50 μg of CD40 siRNA, 50 μg of control siRNA, or PBS alone. DCs were isolated from the spleen 7 days after injection, and cells were stained with PE-labeled anti-CD40 mAb. DCs were analyzed by flow cytometry.

activation of immune responses by DCs, we sought to investigate whether DCs with knockdown of CD40 would display immune regulatory properties. We assessed T cell proliferation, $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ Treg subsets, and cytokine production from T cells that were stimulated by CD40-silenced DCs. First, to evaluate the capacity of DCs to stimulate T cell response after CD40 silencing, an allogeneic MLR was performed. DCs transfected with control siRNA initiated vigorous proliferative responses. In contrast, CD40-silenced DCs failed to stimulate allogeneic T cells (Fig. 2A), suggesting that CD40 silencing caused a state of immune privilege or hyporesponsiveness.

However, hyporesponsiveness does not necessarily equate with active elaboration of tolerance promotion. It has been documented that blockade of the CD40-CD40L interaction actively generates Treg cells (21), which may contribute to active immune suppression. Accordingly we examined whether CD40-silenced DCs were capable of generating Treg cells in vitro. Splenic T cells from C57BL/6 mice were incubated with DCs transfected with CD40 siRNA or control siRNA. After incubation for 6 days, cells were stained with Abs against CD4, CD25, and Foxp3 and analyzed by

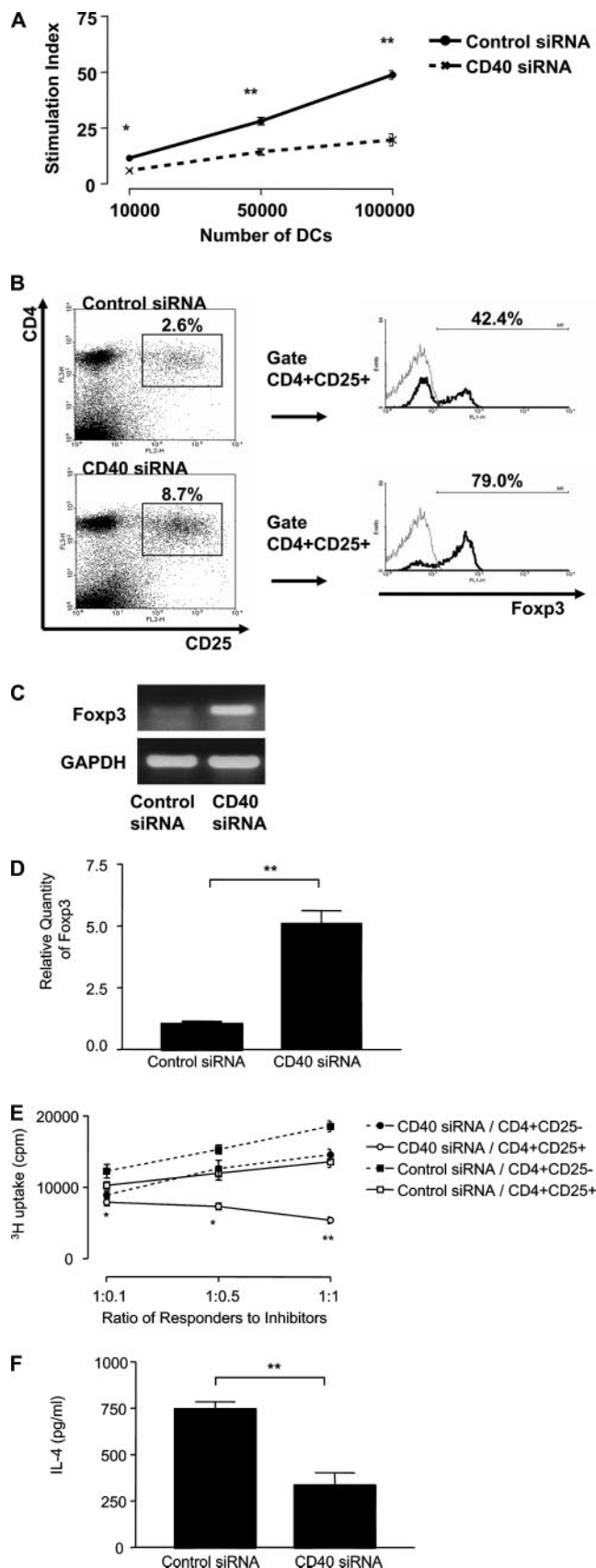


FIGURE 2. Immune modulation by CD40 silencing DCs. **A**, CD40 silencing inhibits DC allostimulatory ability. Bone marrow-derived DCs from BALB/c mice were cultured in the presence of GM-CSF and IL-4. DCs were transfected with control siRNA or CD40 siRNA on day 6. Allogeneic (C57BL/6) T cells (1×10^5 /well) were incubated with siRNA-treated DCs at the indicated concentration for 72 h. Proliferation was

flow cytometry. DCs transfected with CD40 siRNA stimulated a 3-fold increase in the CD4⁺CD25⁺ T cell population (Fig. 2B), which expressed higher levels of the Foxp3 compared with T cells stimulated with control siRNA transfected DCs (Fig. 2B). Furthermore, results of RT-PCR and real-time PCR confirmed that DCs transfected with CD40 siRNA increased the Foxp3 gene expression on T cells (Fig. 2, C and D). The T cells incubated with control siRNA-transfected DCs showed lower populations of CD4⁺CD25⁺ T cells that expressed low levels of Foxp3 (Fig. 2, B–D). These results suggest that CD40-silenced DCs facilitate Treg cell generation.

Next, we examined the inhibitory functions of Treg cells generated by CD40-silenced DCs. We isolated CD4⁺CD25⁺ T cells or CD4⁺CD25[−] T cells after the 6-day incubation with CD40-silenced DCs and nonsilenced DCs. CD4⁺CD25⁺ T cells generated by CD40-silenced DCs significantly suppressed ongoing MLR, whereas CD4⁺CD25⁺ T cells generated by control siRNA-treated DCs did not shown inhibitory effects (Fig. 2E). Additionally, CD4⁺CD25[−] T cells derived from incubation with silenced or nonsilenced DCs exhibited no suppression of ongoing MLR. These data suggest that CD4⁺CD25⁺ T cells generated by CD40-silenced DCs are functionally competent at inhibiting T cell responses.

Furthermore, we investigated cytokine production from splenic lymphocytes stimulated with CD40-silenced DCs. Because IL-4 is strongly associated with Th2 response and allergy (3), we measured IL-4 production in the supernatant after 48 h of incubation with CD40-silenced DCs and allogeneic T cells. IL-4 production from lymphocytes incubated with CD40-silenced DCs was lower ($p < 0.01$) than that from lymphocytes incubated with control DCs (Fig. 2F), suggesting that CD40-silencing DCs inhibited IL-4 production.

Modulation of B cells by CD40 siRNA

B cells play a critical role in IgE synthesis, and signal provided by ligation of CD40 is required for IgE synthesis by B cells (25–27). Therefore, we investigated the effect of siRNA on B cells in vitro.

determined using [³H]thymidine incorporation. Results are expressed as stimulation index. Triplicate samples were measured. *, $p < 0.05$; **, $p < 0.01$ by Student's *t* test. **B**, CD40-silenced DCs generate Treg in vitro. BALB/c-derived DCs were transfected with control siRNA or CD40 siRNA. T cells purified from C57BL/6 splenocytes were stimulated with tolerogenic DCs (DCs transfected with CD40 siRNA) or control DCs (DCs transfected with control siRNA) for 6 days. T cells were triple-stained with Cy5-labeled anti-CD4 mAb, PE-labeled anti-CD25 mAb, and FITC-labeled anti-Foxp3 mAb. Cells were analyzed by flow cytometry. **C**, Foxp3 expression was determined by RT-PCR. DC culture, gene silencing, and T cell stimulation were performed using the same protocols described in **B**. Total RNAs were extracted from T cells. RT-PCR was performed using primers specific for Foxp3 and GAPDH, as described in *Materials and Methods*. **D**, Foxp3 expression was determined by real-time quantitative PCR. *, $p < 0.01$ by Student's *t* test. **E**, The inhibitory function of Treg cells caused by CD40-silencing DCs. MLR was performed using BALB/c T cells as responders (5×10^4 cells/well) and C57BL/6 spleen cells (irradiated at 3000 rad) as stimulators (5×10^4 cells/well). CD4⁺CD25⁺ and CD4⁺CD25[−] T cells were isolated from coculture of spleen cells from C57BL/6 mice and DCs from BALB/c mice treated with CD40 siRNA or with control siRNA. CD4⁺CD25⁺ and CD4⁺CD25[−] T cells were added to each well as inhibitors (5 – 50×10^3 cells/well). *, $p < 0.05$, **, $p < 0.01$ vs counterpart control group determined by Student's *t* test. **F**, IL-4 production by lymphocytes. DC culture, gene silencing, and T cell stimulation were performed using the same protocols described in **B**. After 48 h of incubation, supernatants were collected. Titer of IL-4 was measured by ELISA. *, $p < 0.01$ by Student's *t* test.

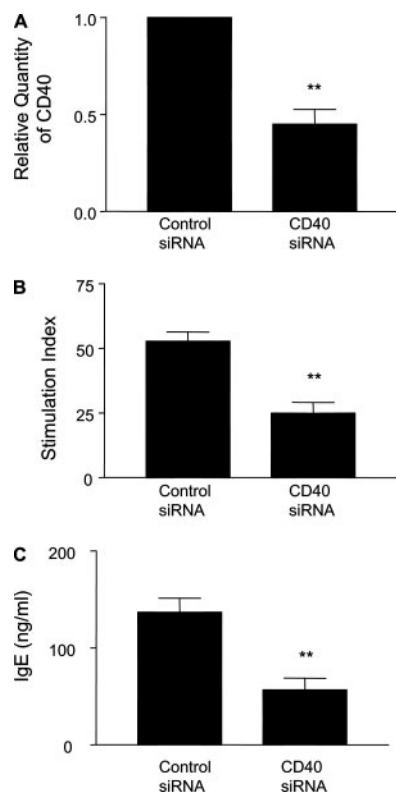


FIGURE 3. CD40 siRNA modulates B cells. *A*, CD40 siRNA induces CD40-silenced B cells. B cells were collected from spleen of normal mice, and isolated B cells were transfected with control siRNA or CD40 siRNA in vitro. After total RNAs were extracted from B cells, real-time PCR was performed using primers specific to CD40 and GAPDH, as described in *Materials and Methods*. **, $p < 0.01$ by Student's t test. *B*, Inhibition of B cell response by CD40 siRNA. After isolated B cells from spleen of normal mice were transfected with control siRNA or CD40 siRNA in vitro, B cells were stimulated with IL-4, IL-5, and CD40L. Proliferation was determined using [^{3} H]thymidine incorporation as described in *Materials and Methods*. Results were expressed as stimulation index. **, $p < 0.01$ by Student's t test. *C*, Inhibition of IgE synthesis by CD40 siRNA. After isolated B cells from spleen of normal mice were transfected with control siRNA or CD40 siRNA in vitro, B cells were stimulated with IL-4, IL-5, and CD40L. The level of IgE in supernatant was measured by ELISA. **, $p < 0.01$ by Student's t test.

B cells were collected from spleen of normal healthy mice. Isolated B cells were transfected with control siRNA or CD40 siRNA. After transfection, total RNA was extracted from B cells. Real-time PCR was performed to study the effect of CD40 siRNA on CD40 gene expression of B cells (Fig. 3A). CD40 siRNA significantly knocked down CD40 siRNA compared with control siRNA. Additionally, CD40 siRNA suppressed B cell proliferative response by stimulation with IL-4, IL-5, and CD40L (Fig. 3B). Furthermore, CD40 siRNA significantly inhibited IgE synthesis generated by B cells with stimulation of IL-4, IL-5, and CD40L (Fig. 3C). These data suggest that CD40 siRNA suppressed CD40 gene expression on B cells, and had an inhibitory effect on B cell function.

Treatment of OVA-induced allergy by CD40 siRNA

Gene silencing strategies using siRNA have been successfully tested in animal models of various diseases. However, the therapeutic potential of siRNA for allergy has not yet been developed. CD40 is a critical costimulatory factor in development of allergy (18–20, 36). Thus, we explored whether CD40 siRNA can be an

alternative treatment for allergy in a prophylactic setting. Mice that had been primed with CD40 siRNA, control siRNA, or PBS alone were immunized i.p. with OVA and alum, and challenged with OVA. Sneezing and pruritus of the nose are clinically major symptoms of allergic rhinitis. Nasal rubbing is a good parameter of pruritus of the nose. We investigated the effect of CD40 siRNA on nasal allergic symptoms by quantification of sneezing and nasal rubbing. 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 ($p < 0.01$) than in mice having received control siRNA or PBS alone (Fig. 4A). The frequency of nasal rubbing in mice treated with CD40 siRNA was also significantly reduced ($p < 0.01$) compared with the mice that received control siRNA or PBS alone (Fig. 4B). A ~35% reduction of sneezing number and ~33% reduction of nasal rubbing number were observed in the CD40 siRNA-treated mice, as compared with control mice. These data suggest that CD40 siRNA treatment attenuates allergic symptoms.

Eosinophilia is a typical phenomenon of allergic diseases and is associated with allergic symptoms and allergic reaction (4). To investigate the effect of CD40 siRNA on eosinophilia, we counted the number of eosinophils in the nasal septum, and bone marrow (Fig. 4C). The number of eosinophils infiltrating the nasal septum per field (10×40) was significantly reduced ($p < 0.01$) in the CD40 siRNA treatment group, as compared with control groups (Fig. 4D). To further understand the effect of CD40 siRNA on eosinophilia inflammation, we next examined bone marrow cavities in the skull base for eosinophilopoiesis (increased eosinophil production from bone marrow precursor cells). CD40 siRNA therapy significantly decreased ($p < 0.01$) bone marrow eosinophilia (Fig. 4E). The number of eosinophil counts was reduced by 55% in nasal septum and by 50% in bone marrow, respectively, after CD40 siRNA treatment. These data suggested that CD40 siRNA treatment inhibited eosinophilia in the nose and bone marrow, resulting in the reduction of allergic symptoms and allergic reactions.

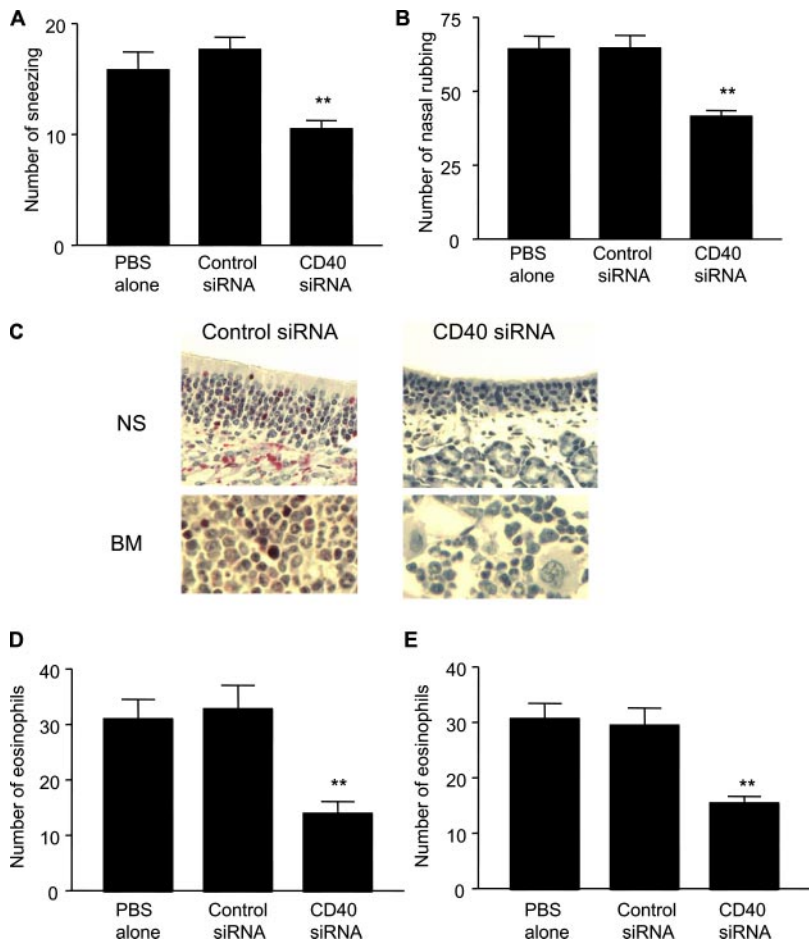
CD40 siRNA treatment decreases IgE and IgG1 Abs

Ag-specific IgE and IgG1 are strongly associated with allergic responses (37). To determine the ability of CD40 siRNA in preventing Ab production to OVA, serum levels of OVA-specific IgE, and IgG1 in mice were measured by ELISA. Mice treated with CD40 siRNA produced significantly lower OVA-specific IgE than mice given control siRNA or PBS alone (Fig. 5A). OVA-specific IgG1 in mice treated with CD40 siRNA was also lower than IgG1 in mice given control siRNA or PBS alone (Fig. 5B). This suggests that CD40 siRNA treatment inhibits Ag-specific IgE and IgG1 that induce allergic responses.

CD40 siRNA treatment inhibits Th2 immune response

Th2 cytokines, such as IL-4 and IL-5, play important roles in allergy. IL-4 is essential for the production of IgE and IgG1 (3). IL-5 is associated with migration and activation of eosinophil (4, 38–40). To investigate whether therapy with CD40 siRNA modulates cytokine productions, we measured cytokine production from T cells stimulated with OVA in vitro. Fig. 6 shows the levels of IL-4 and IL-5 released from splenic T cells of mice treated with CD40 siRNA, control siRNA, or PBS alone before immunization. T cells from mice injected with CD40 siRNA released significantly lower levels ($p < 0.01$) of IL-4 than T cells from mice that had received control siRNA or PBS alone (Fig. 6A). T cells from mice that received CD40 siRNA also released significantly lower ($p < 0.01$)

FIGURE 4. Reduction of allergic nasal symptom and eosinophilia by CD40 siRNA treatment. *A*, Reduction of the number of sneezes. Mice were immunized i.p. and challenged intranasally with OVA after treatment of PBS alone, control siRNA, and CD40 siRNA. The number of sneezing episodes was counted for 20 min after the last nasal challenge. **, $p < 0.01$ vs group of PBS alone or control siRNA. *B*, Reduction of the number of nasal rubbing events. The number of nasal rubbing episodes was counted for 20 min after the last nasal challenge. **, $p < 0.01$ vs group of PBS alone or control siRNA. *C*, Eosinophilia of the nasal septum, and bone marrow. After sacrificing, nasal sections were fixed and decalcified, and Luna staining was performed. Typical sections of the nasal septum (NS) and bone marrow (BM) in mice treated with control siRNA or CD40 siRNA are shown. The number of eosinophils per high power field (10×40) was counted microscopically in the nasal septum (*D*), and bone marrow (*E*). **, $p < 0.01$ vs group of PBS alone or control siRNA.



IL-5 than the T cells from mice that received control siRNA or PBS alone (Fig. 6B). IL-5 was reduced by 70% of control level, whereas IL-4 was reduced by 67% in the mice treated with CD40

siRNA. This suggests that CD40 siRNA treatment suppresses the production of Th2 cytokines in the mice immunized with OVA, which may contribute to therapeutic effects in allergy.

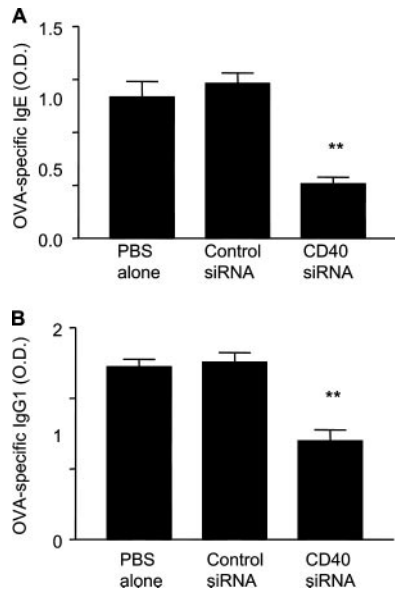


FIGURE 5. Modulation of OVA-specific Ab by CD40 siRNA. Mice were immunized and challenged with OVA after treatment of PBS alone, control siRNA, and CD40 siRNA. The level of OVA-specific IgE and IgG1 in sera was measured by ELISA. Data show the normalized OD by subtracting the background OD. *A*, OVA-specific IgE. **, $p < 0.01$ vs group of PBS alone or control siRNA. *B*, OVA-specific IgG1. **, $p < 0.01$ vs group of PBS alone or control siRNA.

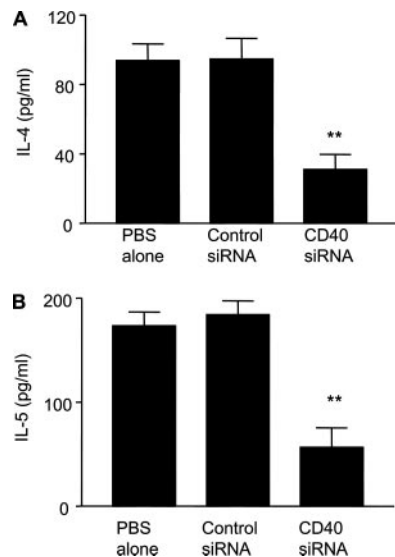


FIGURE 6. CD40 siRNA modulate cytokine production from splenocytes by stimulation of OVA. Mice were immunized and challenged with OVA after treatment of PBS alone, control siRNA, and CD40 siRNA. The levels of IL-4 and IL-5 production from splenocytes by stimulation of OVA were measured by ELISA. *A*, Level of IL-4 production is shown. **, $p < 0.01$ vs group of PBS alone or control siRNA. *B*, The level of IL-5 production is shown. **, $p < 0.01$ vs group of PBS alone or control siRNA.

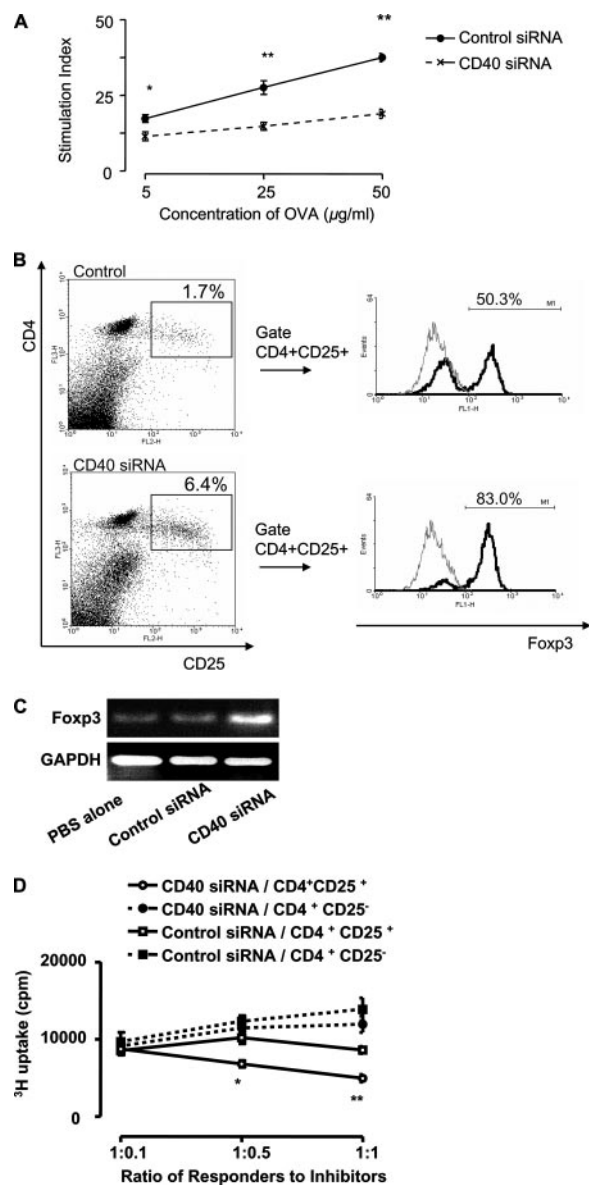


FIGURE 7. Immune modulation by CD40 siRNA treatment. Mice were treated with control siRNA, or CD40 siRNA before immunization. After immunization and nasal challenge with OVA, splenic lymphocytes were isolated from the spleen. **A**, Inhibition of OVA-specific T cell response. Lymphocytes were cultured in 96-well plates at a concentration of 4×10^5 cells/well. Ag-specific recall responses were performed in the presence of OVA, as described in *Materials and Methods*. An [^{3} H]thymidine incorporation assay was performed as described for MLR. Data were expressed as stimulation index. **, $p < 0.01$ vs counterpart group. **B**, Phenotypic analysis of in vivo-generated Treg cells. Splenic T cells were triple-stained with Cy5-labeled anti-CD4 mAb, PE-labeled anti-CD25 mAb, and FITC-labeled anti-Foxp3 mAb and analyzed by flow cytometry. **C**, Up-regulated expression of Foxp3 genes. Mice were treated with PBS alone, control siRNA, or CD40 siRNA before immunization. RNA from the splenic T cells was extracted by means of TRIzol, and RT-PCR was performed to assess the expression of Foxp3 using primers described in *Materials and Methods*. **D**, Inhibition of ongoing OVA-specific T cell response by Treg cells. Mice were treated with control siRNA, or CD40 siRNA before immunization. CD4⁺CD25⁺ T cells or CD4⁺CD25⁻ T cells used as inhibitors, isolated from mice injected with control siRNA or CD40 siRNA, were added to OVA-specific recall response, as described in *Materials and Methods*. *, $p < 0.05$; **, $p < 0.01$ vs group of control CD4⁺CD25⁺ cells.

Immune modulation by CD40 siRNA treatment

Allergy is associated with Ag-specific T cell response (1). To assess the T cell response in the CD40 siRNA-treated mice, we collected splenic T cells from the mice after immunization and challenge with OVA following treatment of CD40 siRNA or control siRNA. Splenic T cells were stimulated with OVA Ag to examine OVA Ag-specific T cell response. Splenic T cells showed strong OVA-specific T cell response in the mice that did not receive CD40 siRNA. OVA-specific T cell response in the mice that received CD40 siRNA was lower than OVA-specific T cell response in the mice that received control siRNA (Fig. 7A) or PBS alone (data not shown), suggesting that CD40 siRNA reduced OVA-specific response.

As previously mentioned, it has been reported that blockade of CD40-CD40L interaction actively generates Treg cells (21), and we have also shown this result in vitro by silencing CD40 in DCs (Fig. 1A). Therefore, we investigated whether treatment with CD40 siRNA increases the number of Treg cells in vivo. We treated mice with CD40 siRNA and examined Treg population in OVA-immunized mice. Increases in CD4⁺CD25⁺ Treg cells were observed in the mice treated with CD40 siRNA (Fig. 7B), as compared with mice treated with control siRNA (Fig. 7B) or PBS alone (data not shown) ($p < 0.01$). CD4⁺CD25⁺ T cells isolated from mice treated with CD40 siRNA expressed higher levels of the Foxp3 gene (Fig. 7B) compared with levels in mice treated with control siRNA (Fig. 7B) or PBS alone (data not shown) ($p < 0.01$). Results of RT-PCR of Foxp3 also showed that CD40 siRNA treatment increased Foxp3 gene expression compared with control siRNA or PBS alone (Fig. 7C). These data suggest that CD40 siRNA treatment increased the Treg population in vivo.

Next, we determined the function of CD4⁺CD25⁻ T cells and CD4⁺CD25⁺, which were isolated from the spleen in allergic mice that received CD40 siRNA or control siRNA. We isolated CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells from the spleen of allergic mice treated with CD40 siRNA or control siRNA and added these cells to OVA-specific T cells responding to OVA to evaluate suppression. CD4⁺CD25⁻ T cells isolated from mice treated with CD40 siRNA and control siRNA both enhanced OVA-specific response (Fig. 7D). CD4⁺CD25⁺ T cells isolated from mice treated with CD40 siRNA significantly inhibited OVA-specific T cell response, as compared with CD4⁺CD25⁺ T cells isolated from mice injected with control siRNA (Fig. 7D). These findings suggest that CD40 siRNA administered in the contexts of an OVA immunization event gives rise to the expansion of CD4⁺CD25⁺ Treg cell population in vivo.

Discussion

DCs, as professional APCs, can potentially initiate naive T cells to differentiate into effector T cells in immune responses. Control of immunity by DCs is based on the ability of this cell, once mature, to provide three signals to T cells during activation: signal 1, the antigenic signal that is communicated via the high expression of MHC molecules found on DCs; signal 2, the "costimulatory" signal, comprising membrane-bound molecules such as CD40, CD80/86, and OX-40 ligand, which are essential for T cell expansion and escape from anergy; and signal 3, soluble cytokines that induce T cell differentiation into Th1 phenotype (IFN- γ) or Th2 phenotype (IL-4). Mature DCs provide strong costimulation for T cell activation, whereas immature DCs express low levels of costimulatory molecules, and thus, are not able to activate T cells. Among the known costimulatory molecules, CD40 plays critical roles in T cell

activation and differentiation (13–15, 41). Blocking the CD40-CD40L interaction has demonstrated to effectively induce tolerance (16–21, 36). In this study, we showed that CD40 siRNA knocked down CD40 signals in DCs at the protein and RNA levels. We also showed that CD40-silenced DCs failed to stimulate an allogeneic T cell response but reduced IL-4 production from T cell. Furthermore, CD40-silenced DCs increased Treg cell population. These findings suggest that CD40 siRNA generates CD40-silenced DCs, which function as immunoregulatory or tolerogenic cells.

It was reported that CD40-deficient mice have a profoundly reduced nasal eosinophilia, reduced IgE and IgG1 levels, and lower levels of IL-4 and IL-5 produced from nasal cells when compared with wild-type mice of the same strain (19, 20). These previous studies imply that CD40 is an ideal targeting molecule for gene silencing in terms of allergy treatment. There is also a dose-dependent effect about blockade of CD40-CD40L interaction by anti-CD40 Ab (42), suggesting that near complete blockade is necessary for optimal biological efficacy. However, the efficacy of gene silencing in this study was relatively low. Despite low gene silencing, CD40 siRNA inhibited allergic responses and symptoms. The first possibility is that partial blocking of CD40 can attenuate allergy. The second is that Treg cells induced by CD40 siRNA may have inhibited allergic responses and symptoms. In fact, CD40 siRNA increased CD4⁺CD25⁺Foxp3⁺ Treg cells over five times in vitro by FACS and real-time PCR results (Fig. 2, B and D). CD40 siRNA also increased CD4⁺CD25⁺Foxp3⁺ Treg cells over six times in vivo (Fig. 7B). The third is that CD40 siRNA attenuate allergy through reduction of B cell function.

It is known that T cells stimulate IgE production and induce sensitization of allergy through IL-4 (3). T cells also activate eosinophils and induce allergic symptoms through IL-5 (4, 38–40). We found that CD40 siRNA suppressed OVA Ag-specific T cell response, consequently resulting in the inhibition of IL-4 and IL-5 production, reduction in levels of IgE and IgG1 Abs, and suppression of eosinophilia. This result suggests that CD40 siRNA is useful for control of allergic responses. We further demonstrated that CD40 siRNA significantly reduced the sneezing and nasal rubbing, suggesting that CD40 siRNA is effective in controlling the allergic symptoms of mice.

Blockade of CD40-CD40L interaction generates Treg cells (21). It has been reported that Treg cells inhibits T cell response against allergic Ags and production of Th2 cytokines (43–46). Because silencing of CD40 was also associated with acquisition of a tolerogenic or Treg-generating DC subset, we sought to determine whether allergic responses might benefit from such an approach. Indeed, the present study showed that augmentation of CD4⁺CD25⁺Foxp3⁺ T cells was observed in mice treated with CD40 siRNA. CD4⁺CD25⁺ T cells isolated from mice that received CD40 siRNA significantly inhibited OVA-specific T cell responses. This finding suggests that immune suppression and therapeutic effects of CD40 siRNA in allergy may be associated with Treg generation.

One of mechanisms of DC-induced tolerance is through generation of Treg cells (47). In this study, CD40-silenced DCs increased CD4⁺CD25⁺Foxp3⁺ T cells in vitro. CD4⁺CD25⁺ T cells incubated with CD40 silenced DCs inhibited allogeneic T cell response, although CD4⁺CD25⁺ T cells incubated with control DCs did not silence the response. Considering these outcomes, CD40 siRNA may be thought to increase Treg cell population and inhibit allergic responses in vivo through knocking down CD40 signals of DCs.

Allergic response is not only dependent on IgE but also associated with IgG1 in the mouse (37). The present study showed that CD40 siRNA reduced IgE and IgG1 in sera, suggesting that CD40

siRNA can reduce allergic response through reduction of IgE and IgG1. Also, CD40 siRNA inhibited IL-4 production from splenocytes. IL-4 plays an important role in the production of allergen-specific IgE and IgG1 (3). Therefore, CD40 siRNA may have decreased IgE and IgG1 through the reduction of IL-4.

Mice immunized and challenged with OVA showed eosinophil accumulation in the nasal mucosa. The eosinophil has been known as a primary effector in terms of inducing airway damage by releasing detrimental mediators such as major basic protein, eosinophilic peroxidase, and eosinophil cationic protein (4, 48, 49). CD40 siRNA reduced OVA Ag-induced eosinophil infiltration in the nasal mucosa and the number of eosinophil in the bone marrow, indicating that CD40 siRNA inhibited nasal allergic symptoms through inhibition of eosinophilia. IL-5 plays a central role not only in eosinophil differentiation and proliferation but also in eosinophil accumulation (4, 40). Administration of IL-5 results in eosinophilic infiltration and activation (4, 38, 50). Conversely, IL-5 deficiency results in abrogation of eosinophilic accumulation in mice with allergy (4, 39). In this study, CD40 siRNA reduced IL-5 production from splenocytes, which may contribute to the inhibition of eosinophil infiltration.

The Ag-dependent therapy is not applicable for many patients when the causative allergen is unknown. Additionally, there is increasing incidence of patients with allergies suffering from sensitization to multiple allergens (2), making the Ag-specific tolerance induction hard to achieve. This study showed that administration of CD40 siRNA alone can effectively inhibit allergic response and attenuate symptoms of allergy. Thus, CD40 siRNA-based treatment may be an alternative Ag-independent therapy with therapeutic potential for the patients without identified causative allergen or with multiple causative allergens.

In summary, we report in this study a novel immune therapy for allergy through gene silencing. Silencing CD40 using siRNA results in generation of immunoregulatory DCs that suppress allergic responses through Treg cells. Furthermore, treatment with CD40 siRNA significantly attenuates allergic symptoms and pathological changes, suggesting potential clinical uses of CD40 siRNA.

Acknowledgments

We thank Weihau Liu for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

- Del Prete, G. 1992. Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. *Allergy* 47: 450–455.
- Arbes, S. J., Jr., P. J. Gergen, L. Elliott, and D. C. Zeldin. 2005. Prevalences of positive skin test responses to 10 common allergens in the US population: results from the third National Health and Nutrition Examination Survey. *J. Allergy Clin. Immunol.* 116: 377–383.
- Snapper, C. M., F. D. Finkelman, and W. E. Paul. 1988. Regulation of IgG1 and IgE production by IL 4. *Immunol. Rev.* 102: 51–75.
- Hamelmann, E., and E. W. Gelfand. 2001. IL-5-induced airway eosinophilia: the key to asthma? *Immunol. Rev.* 179: 182–191.
- Waterhouse, P. M., M. B. Wang, and T. Lough. 2001. Gene silencing as an adaptive defense against viruses. *Nature* 411: 834–842.
- Hill, J. A., T. E. Ichim, K. P. Kusznierek, M. Li, X. Huang, X. Yan, R. Zhong, E. Cairns, D. A. Bell, and W. P. Min. 2003. Immune modulation by silencing IL-12 production in dendritic cells using small interfering RNA. *J. Immunol.* 171: 691–696.
- Tong, A. W., Y. A. Zhang, and J. Nemunaitis. 2005. Small interfering RNA for experimental cancer therapy. *Curr. Opin. Mol. Ther.* 7: 114–124.
- Bertrand, J. R., M. Pottier, A. Vekris, P. Opolon, A. Maksimenko, and C. Malvy. 2002. Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. *Biochem. Biophys. Res. Commun.* 296: 1000–1004.
- Thompson, J. D. 2002. Applications of antisense and siRNAs during preclinical drug development. *Drug Discov. Today* 7: 912–917.
- de Fougerolles, A., H. P. Vornlocher, J. Maraganore, and J. Lieberman. 2007. Interfering with disease: a progress report on siRNA-based therapeutics. *Nat. Rev. Drug Discov.* 6: 443–453.

11. Maneechotesuwan, K., Y. Xin, K. Ito, E. Jazrawi, K. Y. Lee, O. S. Usmani, P. J. Barnes, and I. M. Adcock. 2007. Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3. *J. Immunol.* 178: 2491–2498.
12. Nigo, Y. I., M. Yamashita, K. Hirahara, R. Shinnakasu, M. Inami, M. Kimura, A. Hasegawa, Y. Kohno, and T. Nakayama. 2006. Regulation of allergic airway inflammation through TLR 4-mediated modification of mast cell function. *Proc. Natl. Acad. Sci. USA* 103: 2286–2291.
13. Grewal, I. S., and R. A. Flavell. 1996. The role of CD40L in costimulation and T cell activation. *Immunol. Rev.* 153: 85–106.
14. Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med.* 180: 1263–1272.
15. Peng, X., A. Kasran, P. A. Warmerdam, M. de Boer, and J. L. Ceuppens. 1996. Accessory signaling by CD40 for T cell activation: induction of Th1 and Th2 cytokines and synergy with IL-12 for IFN- γ production. *Eur. J. Immunol.* 26: 1621–1627.
16. Kirk, A. D., P. J. Blair, D. K. Tadaki, H. Xu, and D. M. Harlan. 2001. The role of CD154 in organ transplant rejection and acceptance. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 356: 691–702.
17. Blazar, B. R., P. A. Taylor, A. Panoskaltsis-Mortari, J. Buhlman, J. Xu, R. A. Flavell, R. Korngold, R. Noelle, and D. A. Valleria. 1997. Blockade of CD40L-CD40 interaction impairs CD4⁺ T cell-mediated alloreactivity by inhibiting mature donor T cell expansion and function after bone marrow transplantation. *J. Immunol.* 158: 29–39.
18. Tang, A., T. A. Judge, B. J. Nickoloff, and L. A. Turka. 1996. Suppression of murine allergic contact dermatitis by CTLA4Ig: tolerance induction of Th2 responses requires additional blockade of CD40-ligand. *J. Immunol.* 157: 117–125.
19. Hattori, H., M. Okano, S. Kariya, K. Nishizaki, and A. R. Satoskar. 2006. Signals through CD40 play a critical role in the pathophysiology of *Schistosoma mansoni* egg Ag-induced allergic rhinitis in mice. *Am. J. Rhinol.* 20: 165–169.
20. Pochanke, V., S. Hatak, H. Hengartner, R. M. Zinkernagel, and K. D. McCoy. 2006. Induction of IgE and allergic-type responses in fur mite-infested mice. *Eur. J. Immunol.* 36: 2434–2445.
21. Taylor, P. A., T. M. Friedman, R. Korngold, R. J. Noelle, and B. R. Blazar. 2002. Tolerance induction of alloreactive T cells via ex vivo blockade of the CD40: CD40L costimulatory pathway results in the generation of a potent immune regulatory cell. *Blood* 99: 4601–4609.
22. Suri-Payer, E., and B. Fritzsche. 2006. Regulatory T cells in experimental autoimmune disease. *Springer Semin. Immunopathol.* 28: 3–16.
23. Taylor, J. J., M. Mohr, and E. J. Pearce. 2006. Regulatory T cell responses develop in parallel to Th responses and control the magnitude and phenotype of the Th effector population. *J. Immunol.* 176: 5839–5847.
24. Xu, G., Z. Mou, H. Jiang, L. Cheng, J. Shi, R. Xu, Y. Oh, and H. Li. 2007. A possible role of CD4⁺CD25⁺ T cells as well as transcription factor Foxp3 in the dysregulation of allergic rhinitis. *Laryngoscope* 117: 876–880.
25. Poulsen, L. K., and L. Hummelshøj. 2007. Triggers of IgE class switching and allergy development. *Ann. Med.* 39: 440–456.
26. Bacharier, L. B., and R. S. Geha. 2000. Molecular mechanisms of IgE regulation. *J. Allergy Clin. Immunol.* 105: S547–S558.
27. Jelinek, D. F. 2000. Regulation of B lymphocyte differentiation. *Ann. Allergy Asthma Immunol.* 84: 375–387.
28. Linhart, B., S. Bigenzahn, A. Hartl, C. Lupinek, J. Thalhammer, R. Valenta, and T. Wekerle. 2007. Costimulation blockade inhibits allergic sensitization but does not affect established allergy in a murine model of grass pollen allergy. *J. Immunol.* 178: 3924–3931.
29. Knechtle, S. J. 2000. Knowledge about transplantation tolerance gained in primates. *Curr. Opin. Immunol.* 12: 552–556.
30. Kirk, A. D., L. C. Burkly, D. S. Batty, R. E. Baumgartner, J. D. Berning, K. Buchanan, J. H. Fechner, Jr., R. L. Germond, R. L. Kampen, N. B. Patterson, et al. 1999. Treatment with humanized mAb against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat. Med.* 5: 686–693.
31. Pluvinet, R., J. Petriz, J. Torras, I. Herrero-Fresneda, J. M. Cruzado, J. M. Grinyo, and J. M. Aran. 2004. RNAi-mediated silencing of CD40 prevents leukocyte adhesion on CD154-activated endothelial cells. *Blood* 104: 3642–3646.
32. Li, M., X. Zhang, X. Zheng, D. Lian, Z. X. Zhang, W. Ge, J. Yang, C. Vladau, M. Suzuki, D. Chen, et al. 2007. Immune modulation and tolerance induction by ReIB-silenced dendritic cells through RNA interference. *J. Immunol.* 178: 5480–5487.
33. Suzuki, M., N. Ohta, W. P. Min, T. Matsumoto, R. Min, X. Zhang, K. Toida, and S. Murakami. 2007. Immunotherapy with CpG DNA conjugated with T cell epitope peptide of an allergenic Cry j2 protein is useful for control of allergic conditions in mice. *Int. Immunopharmacol.* 7: 46–54.
34. Min, W. P., D. Zhou, T. E. Ichim, G. H. Strejan, X. Xia, J. Yang, X. Huang, B. Garcia, D. White, P. Dutartre, et al. 2003. Inhibitory feedback loop between tolerogenic dendritic cells and regulatory T cells in transplant tolerance. *J. Immunol.* 170: 1304–1312.
35. Lechler, R., W. F. Ng, and R. M. Steinman. 2001. Dendritic cells in transplantation—friend or foe? *Immunity* 14: 357–368.
36. Brown, D. L., D. K. Bishop, S. Y. Wood, and P. S. Cederna. 2006. Short-term anti-CD40L costimulatory blockade induces tolerance to peripheral nerve allografts, resulting in improved skeletal muscle function. *Plast. Reconstr. Surg.* 117: 2250–2258.
37. Miyajima, I., D. Dombrowicz, T. R. Martin, J. V. Ravetch, J. P. Kinet, and S. J. Galli. 1997. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc γ RIII: assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *J. Clin. Invest.* 99: 901–914.
38. Shi, H. Z., C. Q. Xiao, D. Zhong, S. M. Qin, Y. Liu, G. R. Liang, H. Xu, Y. Q. Chen, X. M. Long, and Z. F. Xie. 1998. Effect of inhaled IL-5 on airway hyperreactivity and eosinophilia in asthmatics. *Am. J. Respir. Crit. Care Med.* 157: 204–209.
39. Foster, P. S., S. P. Hogan, A. J. Ramsay, K. I. Matthaei, and I. G. Young. 1996. IL 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J. Exp. Med.* 183: 195–201.
40. Yamaguchi, Y., T. Suda, J. Suda, M. Eguchi, Y. Miura, N. Harada, A. Tominaga, and K. Takatsu. 1988. Purified IL 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J. Exp. Med.* 167: 43–56.
41. Mackey, M. F., J. R. Gunn, C. Maliszewsky, H. Kikutani, R. J. Noelle, and R. J. Barth, Jr. 1998. Dendritic cells require maturation via CD40 to generate protective antitumor immunity. *J. Immunol.* 161: 2094–2098.
42. Quesenberry, P. J., S. Zhong, H. Wang, and M. Stewart. 2001. Allogeneic chimerism with low-dose irradiation, Ag presensitization, and costimulator blockade in H-2 mismatched mice. *Blood* 97: 557–564.
43. Nagato, T., H. Kobayashi, M. Yanai, K. Sato, N. Aoki, K. Oikawa, S. Kimura, Y. Abe, E. Celis, Y. Harabuchi, and M. Tateono. 2007. Functional analysis of birch pollen allergen Bet v 1-specific regulatory T cells. *J. Immunol.* 178: 1189–1198.
44. Li, J., M. Bracht, X. Shang, J. Radewonuk, E. Emmell, D. E. Griswold, and L. Li. 2006. Ex vivo activated OVA specific and non-specific CD4⁺CD25⁺ regulatory T cells exhibit comparable suppression to OVA mediated T cell responses. *Cell Immunol.* 241: 75–84.
45. Prescott, S. L., and J. A. Dunstan. 2005. Immune dysregulation in allergic respiratory disease: the role of T regulatory cells. *Pulm. Pharmacol. Ther.* 18: 217–228.
46. Hadeiba, H., and R. M. Locksley. 2003. Lung CD25 CD4 regulatory T cells suppress type 2 immune responses but not bronchial hyperreactivity. *J. Immunol.* 170: 5502–5510.
47. Bacchetta, R., S. Gregori, and M. G. Roncarolo. 2005. CD4⁺ regulatory T cells: mechanisms of induction and effector function. *Autoimmun. Rev.* 4: 491–496.
48. Corrigan, C. J., and A. B. Kay. 1992. T cells and eosinophils in the pathogenesis of asthma. *Immunol. Today* 13: 501–507.
49. Nishioka, K., C. Saito, T. Nagano, M. Okano, Y. Masuda, and T. Kuriyama. 1993. Eosinophil cationic protein in the nasal secretions of patients with mite allergic rhinitis. *Laryngoscope* 103: 189–192.
50. Shi, H., S. Qin, G. Huang, Y. Chen, C. Xiao, H. Xu, G. Liang, Z. Xie, X. Qin, J. Wu, et al. 1997. Infiltration of eosinophils into the asthmatic airways caused by IL 5. *Am. J. Respir. Cell Mol. Biol.* 16: 220–224.