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

Motohiko Suzuki,* Xiufen Zheng,* Xusheng Zhang,* Mu Li,* Costin Vladau,* Thomas E. Ichim,§ Hongtao Sun,* Lisa R. Min,* Bertha Garcia,* and Wei-Ping Min2*†‡

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) 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 provide 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...
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 \times 10^6 cells/ml) in 2 ml of complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 \mu g/ml streptomycin, 50 \mu g/ml 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 CO_2.

Construction of CD40 siRNA-expressing vector

CD40 siRNA-expressing vectors were generated by the Silencer Express kit (Ambion). Sense (ACACTACACAATGTCCACTGGGCT GAGAGCGGTGTTCCTCTTCTCCAAAG) and antisense (CGGC GAAGCCCTTTTTTTAAAATCTTCAAGCGGCTTGAACACTACA AATGTT) hairpin siRNA template oligonucleotide, specific to CD40 mRNA, were used.

Gene silencing

Transfection was conducted according to the method previously described (32). Briefly, 1 \mu g of vector that expresses no siRNA, CD40 siRNA, scramble siRNA (control siRNA), or transfection reagent alone was incubated with 5 \mu l of Geneporter (Gene Therapy Systems) for 15 min. This then was added to 400 \mu 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 Flow cytometry. T cells were isolated from spleens in allergic model mice with control CD40 siRNA, 50 \mu g of control siRNA, or PBS alone twice on days 0 and 14. Mice were also injected i.p. with 10 \mu 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 \mu 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 \times 10^5/well). Control siRNA or CD40 siRNA-treated DCs (1–10 \times 10^6/well from BALB/c) were used as stimulators. Cells were cultured for 3 days and pulsed with 1 \mu 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 \times 10^5/well) and irradiated (3000 rad) BALB/c spleen cells as stimulators (5 \times 10^6/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 sera 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 \times 10^6 cells/ml) were cultured for 72 h in complete medium and 100 \mu 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 supernatants were determined by ELISA. ELISA plates were coated with OVA. Nonspecific binding was blocked, and sera were added to the plates. The plates were washed, and biotin-labeled anti-mouse IgE (Zymed Laboratories) was added. The plates were incubated with avidin-peroxidase. After washing, the same substrate was applied, as described.

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 \times 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 \(5 \times 10^5\) cells/well with 400 \(\mu\)g/ml OVA Ag in the presence of CD4\(^+\)CD25\(^+\) T cells or CD4\(^+\)CD25\(^-\) T cells from the spleen of mice injected with control siRNA or CD40 siRNA for 72 h. An \([\text{H}]\)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 \(\mu\)g/ml) using 96-well plates. \(\text{[H]}\)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-\(\mu\)m 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 \(\times\) 40). The observer assessed eosinophilia blinded to the treatment of the mice.

Statistical analysis

Data are expressed as mean ± 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. 1B 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 \(\mu\)l of CD40 siRNA (50 \(\mu\)g per each mouse), control siRNA (50 \(\mu\)g 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.

Alternation 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 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, CD4\(^+\)CD25\(^-\)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 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 \(\mu\)g of CD40 siRNA, 50 \(\mu\)g 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.
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 lower 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 show 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 allogenic 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.

**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 x 10⁵/well) were incubated with siRNA-treated DCs at the indicated concentration for 72 h. Proliferation was 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 to 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 x 10⁵ cells/well) and C57BL/6 spleen cells (irradiated at 3000 rad) as stimulators (5 x 10⁶ 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 x 10⁵ 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.
Materials and Methods

in vitro. After total RNAs were 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 eosinophilia (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)
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.
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 naïve 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+ T 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+ T 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, eosinophil 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 thera
tropic potential for the patients without identified causative all
ergens 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.

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Disclosures

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References


