A novel allergen-specific therapy for allergy using CD40-silenced dendritic cells

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Background: Induction of RNA interference with small interfering RNA (siRNA) has demonstrated therapeutic potential through the knockdown of target genes. We have previously reported that systemic administration of CD40 siRNA is capable of attenuating allergic symptoms but in an allergen-nonspecific fashion. However, siRNA-based allergen-specific therapy for allergy has not been developed.

Objective: We attempted to develop a new allergen-specific therapy for allergy using CD40-silenced and allergen-pulsed dendritic cells (DCs).

Methods: Bone marrow–derived DCs were silenced with CD40 siRNA and pulsed with ovalbumin (OVA). Mice had allergy after intraperitoneal sensitization with OVA and keyhole limpet hemocyanin, followed by intranasal challenge with the same allergens. The mice were treated with CD40-silenced and OVA-pulsed DCs (CD40-silenced OVA DCs) either before allergic sensitization or after establishing allergic rhinitis.

Results: Mice receiving CD40-silenced OVA DCs either before or after the establishment of allergic rhinitis showed remarkable reductions in allergic symptoms caused by OVA challenge, as well as anti-OVA IgE levels in sera. Additionally, CD40-silenced OVA DCs suppressed eosinophil infiltration at the nasal septum, OVA-specific T-cell responses, T-cell production of IL-4 and IL-5 after stimulation with OVA, and $CD4^+CD25^-$ effector T-cell responses. Furthermore, CD40-silenced OVA DCs facilitated the generation of $CD4^+CD25^+$ forkhead box protein 3–positive OVA-specific regulatory T cells, which inhibit allergic responses *in vivo*. However, CD40-silenced OVA DCs suppressed only OVA-specific allergy but did not inhibit keyhole limpet hemocyanin–induced allergy, suggesting that CD40-silenced OVA DCs induce allergen-specific tolerance.

Conclusions: This study is the first to demonstrate a novel allergen-specific therapy for allergy through DC-mediated immune modulation after gene silencing of CD40. (J Allergy Clin Immunol 2010;125:737-43.)

Key words: *Allergy, CD40, small interfering RN, dendritic cells, regulatory T cells*

IgE-mediated allergic diseases caused by excessive T_H2 responses are a growing international concern.[1] However, present clinical therapy is nonspecific and does not improve early events within the allergic cascade. Therefore a novel antigen-specific therapy that targets upstream causative events is highly desired. RNA interference with small interfering RNA (siRNA) is a potent, selective, and easy approach for blocking expression of specific genes.[2] Previous methods of gene inhibition, such as antisense oligonucleotides, have shown poor to mediocre clinical results.[3-5] Therapeutic use of siRNA is more promising because of its high specificity[6,7] and superior potency[8-10] compared with other approaches, such as antisense oligonucleotides and ribozymes. RNA interference has gained recognition, especially after Andrew Fire and Craig Mello won a 2006 Nobel Prize in Physiology or Medicine for its discovery.[11]

We previously reported that systemic inhibition of CD40 by directly administering CD40 short hairpin RNA (shRNA)–expressing vector (ie, CD40 shRNA, the CD40 siRNA–expressing vector) attenuated allergy not only before allergic sensitization[12] but also after allergic sensitization.[13] However, clinical translation of such a direct approach can be associated with several hurdles. Direct administration might induce hyper-IgM syndrome, which causes susceptibility to infection through inhibiting CD40 expression in B cells.[14] Indeed, CD40 shRNA inhibited CD40 expression in B cells both *in vitro*[12] and *in vivo*.[13] Direct administration might also induce other side effects because CD40 is expressed in various cells, such as dendritic cells (DCs), B cells, monocytes, macrophages, thymic epithelium cells, vascular endothelial cells, fibroblasts, and smooth muscle cells.[15] Also, the vector itself can cause side effects, although shRNA is more versatile and stable *in vivo* than siRNA because siRNA tends to be unstable *in vivo*.[16,17] Furthermore, direct administration of shRNA is not allergen-specific therapy.

DCs are the most influential allergen-presenting cells. The lack of 1 or more activation signals causes suppressive DCs (tolerogenic DCs) to inhibit T-cell responses through apoptosis, anergy, and generation of regulatory T (Treg) cells.[18-20] We previously showed a method of selectively delivering siRNA to DCs by using CD40 siRNA–bearing immunoliposomes decorated with DC-specific antibody.[21] Although administration of these immunoliposomes resulted in reduction of T-cell

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Abbreviations used
CD40L: CD40 ligand
DC: Dendritic cell
Foxp3: Forkhead box protein 3
KLH: Keyhole limpet hemocyanin
OVA: Ovalbumin
shRNA: Short hairpin RNA
siRNA: Small interfering RNA
Treg: Regulatory T

responses, mAbs used for this method might induce side effects *in vivo*. Furthermore, this method is not allergen-specific therapy, although allergen-specific therapy is expected for allergy.

In this study we sought to increase the safety, potency, and specificity of immune modulation in allergy by modifying DCs with allergen and siRNA. We were the first to discover that CD40-silenced and allergen-pulsed DCs are useful in allergen-specific therapy.

METHODS

The majority of the methods used in this study are described in the Methods section of this article's Online Repository at www.jacionline.org.

siRNA and gene silencing

siRNA specific to CD40 (CD40 siRNA, UUCUCAGCCCAGUGGAACA) was synthesized and annealed by the manufacturer (Dharmacon, Inc, Lafayette, Colo). Transfection of siRNA into DCs was conducted as described previously.[2] CD40 siRNA (2 μg) was incubated with 20 μL of GeneSilencer reagent (Gene Therapy Systems, San Diego, Calif) in 50 μL of serum-free RPMI 1640 medium at room temperature for 30 minutes. The mixture was then added to 400 μL of DCs cultured in 12-well plates. Control cells were treated with transfection reagent alone or siRNA specific to the Luciferase gene GL2 Duplex (Dharmacon, Inc), representing a sham silencing control. After 4 hours of incubation, an equal volume of RPMI 1640 supplemented with 20% FBS, 20 ng/mL recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ), and 20 ng/mL IL-4 (PeproTech) was added to the cell suspension.

Treatment and immunization

Bone marrow–derived DCs were transfected with CD40 siRNA or GL2 siRNA on day 6, as described above, and then pulsed with ovalbumin (OVA) on day 7. DCs were then activated with LPS, extensively washed, and used for subsequent transfer experiments.

Male C57BL/6 mice (Charles River Canada, Saint-Constant, Quebec, Canada) between 6 and 8 weeks of age were used. For treatment before allergic sensitization, CD40-silenced and OVA-pulsed DCs (CD40-silenced OVA DCs), scramble siRNA-treated and CD40-expressing DCs (control OVA DCs; 5×10^6 cells per mouse), or PBS were injected intraperitoneally on days 0 and 14. Mice were also injected intraperitoneally with 10 μg of OVA, 10 μg of keyhole limpet hemocyanin (KLH), and 4 mg of $Al(OH)_3$ on days 2 and 16, and each group consisted of 4 mice. The same mice were challenged intranasally on days 18 through 25 with OVA (600 μg) and KLH (30 μg), on day 26 with only KLH, and on day 27 with only OVA. Immediately after the nasal challenge on days 26 and 27, the number of sneezing and nasal rubbing movements were counted for 20 minutes according to the method previously described,[22] and samples from blood, the spleen, lymph nodes, and the nose were collected on day 28.

All mice were housed in the animal facility at the University of Western Ontario. The protocols were approved by the "Guidelines for care and use of animals" at the University of Western Ontario.

RESULTS

Silencing CD40 on DCs *in vitro* with siRNA

We performed standard 6-day DC cultures, followed by transfection with CD40 siRNA, GL2 siRNA (control siRNA), or transfection reagent alone (no siRNA), and then pulsed with OVA. Results of RT-PCR (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org) and real-time PCR (see Fig E1, *B*) showed that CD40 siRNA treatment resulted in potent inhibition of CD40 gene expression at the mRNA level compared with control siRNA or no siRNA. Transfection of DCs with siRNA did not alter their viability or their ability to respond to maturation stimuli of LPS. Altogether, these *in vitro* data demonstrate the feasibility and efficacy of CD40 siRNA to silence the expression of CD40 in DCs.

CD40-silenced OVA DCs specifically reduce OVA-induced allergy

Mice that had been primed with CD40-silenced OVA DCs, CD40-expressed and OVA-pulsed DCs (control siRNA-treated and OVA-pulsed DCs; ie, control OVA DCs), or PBS alone were injected intraperitoneally and challenged intranasally with OVA and KLH, as described in the Methods section. We investigated the effect of CD40-silenced OVA DCs on nasal allergic symptoms by quantifying sneezing and nasal rubbing. The frequency of sneezing and nasal rubbing in mice treated with CD40-silenced OVA DCs was significantly less than that of the mice that received control OVA DCs or PBS (Fig 1, *A* and *B*) when mice were challenged with OVA. However, the number of sneezing and nasal rubbing movements did not significantly differ among the 3 groups when mice were challenged with KLH (Fig 1, *C* and *D*).

Next, serum levels of OVA-specific antibodies and KLH-specific antibodies in mice were measured. Mice treated with CD40-silenced OVA DCs produced significantly less OVA-specific IgE (Fig 1, *E*), IgG1 (data not shown), and IgG2a (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org) than mice given control OVA DCs or PBS. However, there was no significant difference in KLH-specific IgE (Fig 1,F), IgG1 (data not shown), and IgG2a (see Fig E2, *B*) levels among the 3 groups. This suggests that administration of CD40-silenced OVA DCs suppresses only OVA-specific IgE, IgG1, and IgG2a.

T cells were stimulated with OVA or KLH to examine allergen-specific T-cell responses. The OVA-specific T-cell response in mice that received CD40-silenced OVA DCs was less than that of the mice receiving control OVA DCs or PBS (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org), suggesting that CD40-silenced OVA DCs reduced the OVA-specific response. However, there was no significant difference in KLH-specific T-cell responses among mice treated with CD40-silenced OVA DCs, control OVA DCs, or PBS (see Fig E3, *B*).

Furthermore, we measured cytokine production from T cells stimulated with OVA or KLH *in vitro*. T cells from mice injected with CD40-silenced OVA DCs released significantly less IL-4 and IL-5 than T cells from mice that had received control OVA DCs or PBS (see Fig E3, *C* and *D*). However, there was no significant difference between IL-4 and IL-5 production of T cells stimulated with KLH among the 3 groups (see Fig E3, *E* and *F*).

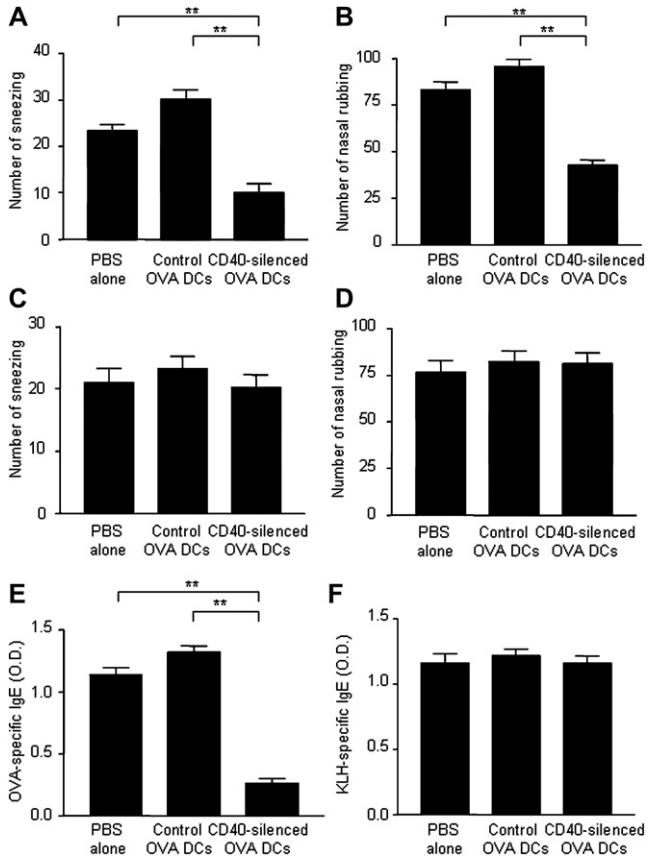

FIG 1. Allergic nasal symptoms and IgE levels in sera by administration of CD40-silenced OVA DCs before sensitization. **A** and **B**, The number of sneezing (Fig 1, *A*) and nasal rubbing (Fig 1, B) after nasal OVA challenge. **C** and **D**, The number of sneezing (Fig 1, *C*) and nasal rubbing (Fig 1, *D*) movements after nasal KLH challenge. **E** and **F**, OVA-specific IgE (Fig 1, *E*) and KLH-specific IgE (Fig 1, *F*) levels. n = 5 per group. $^{**}P < .01$.

Generation of OVA-specific Treg cells is facilitated by CD40-silenced OVA DCs

We examined whether CD40-silenced OVA DCs were capable of generating Treg cells *in vivo*. After treatment, mice were sensitized and challenged with OVA and KLH, as described in the Methods section. Lymph nodes were collected, and Treg cell populations were examined. An increase in the $CD25^+$ forkhead box protein 3 (Foxp3)–positive subpopulation of $CD4^+$ T cells was observed in mice treated with CD40-silenced OVA DCs compared with the population seen in mice treated with control OVA DCs or PBS (Fig 2, *A*). The results of real-time PCR also showed that CD40-silenced OVA DCs increased Foxp3 gene expression compared with that seen in the control groups (Fig 2, *B*). Thus CD40-silenced OVA DCs increased the Treg cell population *in vivo*.

We also examined allergen specificity of Treg cells generated by CD40-silenced OVA DCs. Mice that did not receive DCs were immunized with OVA, KLH, and alum. Splenocytes were collected, and the allergen-specific T-cell response was examined by means of OVA or KLH stimulation. Splenic T cells showed vigorous OVA-specific and KLH-specific T-cell responses (data not shown). $CD4^+CD25^+$ T cells were also isolated from spleens and lymph nodes in other mice that received only CD40-silenced OVA DCs. These $CD4^+CD25^+$ T cells significantly inhibited OVA-specific T-cell responses (Fig 2, *C*) but did not inhibit KLH-specific T-cell responses (Fig 2, *D*). These findings suggest that the administration of CD40-silenced OVA DCs gives rise to the expansion of an allergen-specific $CD4^+CD25^+$ Treg cell population *in vivo*.

Primary response after administration of CD40-silenced OVA DCs

It is important to study the safety of CD40-silenced OVA DCs. If administration of CD40-silenced OVA DCs before allergic sensitization causes allergic sensitization, it is difficult to develop CD40-silenced OVA DCs for clinical therapy. Mice were injected with CD40-silenced OVA DCs or control OVA DCs on days 0 and 14 (treatment with DCs on days 0 and 14 and sample collection on day 28) to investigate whether CD40-silenced OVA DCs themselves induce allergic sensitization. These mice were not injected intraperitoneally and challenged intranasally with OVA. Splenic T cells showed a strong OVA-specific T-cell response in mice that received control OVA DCs (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). However, T cells did not show an OVA-specific T-cell response in the mice that received CD40-silenced OVA DCs (see Fig E4, *A*). Also, T cells of mice injected with control OVA DCs produced IL-4 and IL-5 (see Fig E4, *B* and *C*). However, we could not detect IL-4 and IL-5 production from T cells of mice that received CD40-silenced OVA DCs (see Fig E4, *B* and *C*). Furthermore, OVA-specific IgE and IgG1 levels in sera were not detected in mice that received CD40-silenced OVA DCs, although there were high levels of OVA-specific IgE and IgG1 in sera of mice injected with control OVA DCs (see Fig E4, *D* and *E*). These suggest that CD40-silenced OVA DCs themselves do not induce detectable T-cell responses and allergic sensitization.

Therapeutic effects of CD40-silenced OVA DCs after establishing allergic rhinitis

Mice that had been sensitized intraperitoneally and challenged intranasally with OVA were treated with CD40-silenced OVA DCs, control OVA DCs, or PBS and then rechallenged intranasally with OVA (priming on days 0 and 14, challenge on days 18-24, treatment with DCs on days 26 and 30, rechallenge on days 28-34, and sample collection on day 35) to investigate the therapeutic effects of CD40-silenced OVA DCs on already established allergic rhinitis. The number of sneezing and nasal rubbing movements immediately after nasal challenge on day 24 (after 7 days of nasal challenge) significantly increased compared with those immediately after nasal challenge on day 18 (after the first challenge, data not shown), suggesting that allergic rhinitis is established. There were no significant differences of the numbers on day 24 among 3 treatments (data not shown). The number of sneezing and nasal rubbing movements immediately after the last nasal challenge on day 34 in mice treated with CD40-silenced OVA DCs were significantly lower than in mice having received control OVA DCs or PBS (Fig 3, *A* and *B*). CD40-silenced OVA DCs also significantly reduced the number of eosinophils infiltrating the nasal septum (Fig 3, *C*), OVA-specific IgE levels in sera (Fig 3, *D*), and production of IL-4 (Fig 3, *E*) and IL-5 (data not shown) from splenocytes stimulated with OVA. Also, real-time PCR results showed that CD40-silenced OVA DCs increased Foxp3 gene expression (Fig 3, *F*). These data suggest that

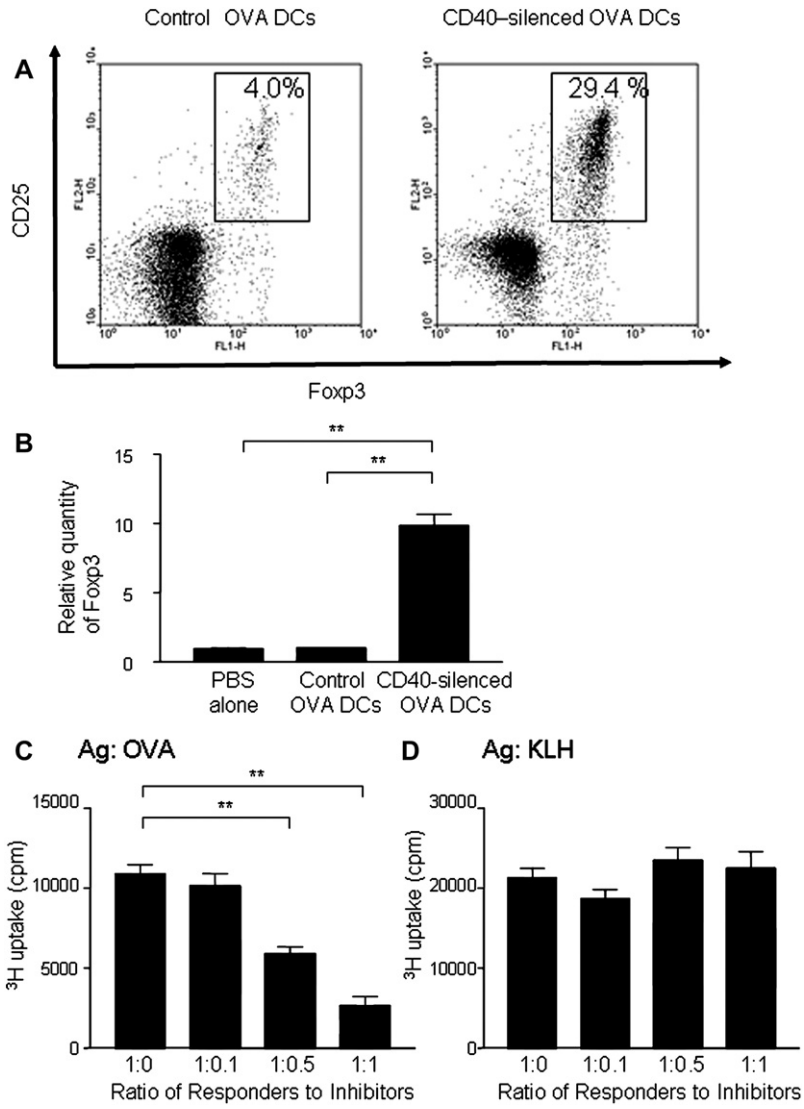

FIG 2. CD40-silenced OVA DCs facilitated OVA-specific Treg cells. **A**, $CD4^+$ T cells were analyzed by means of flow cytometry. **B**, Foxp3 expression was determined by means of real-time PCR. **C**, $CD4^+CD25^+$ T cells as inhibitors, isolated from mice injected with CD40-silenced OVA DCs, were added to OVA-specific T-cell response. **D**, $CD4^+CD25^+$ T cells were added to KLH-specific T-cell response. n = 5 per group. $^{**}P < .01$.

CD40-silenced OVA DCs are therapeutically useful, even when given after allergic rhinitis is established.

Therapeutic effects of CD40-silenced OVA DCs on $CD4^+CD25^-$ effector T cells

$CD4^+CD25^-$ effector T cells were isolated from mice treated with CD40-silenced OVA DCs, control OVA DCs, or PBS after establishment of allergic rhinitis (priming on days 0 and 14, challenge on days 18-24, treatment with DCs on days 26 and 30, rechallenge on days 28-34, and sample collection on day 35) to assess the effects of CD40-silenced OVA DCs on $CD4^+CD25^-$ effector T cells. OVA-specific T-cell responses were performed. Splenocytes isolated from allergic mice that were injected with OVA and alum (priming on days 0 and 14 and sample collection on day 28) were seeded in 96-well plates at a concentration of 5×10^4 cells per well and cocultured with OVA. Modulation of OVA-specific T-cell responses by $CD4^+CD25^-$ cells was also performed at the same time. Purified $CD4^+CD25^-$ T cells (5×10^4 cells per well) were added to the ongoing OVA-specific T-cell response. Although $CD4^+CD25^-$ T cells from mice that received control OVA DCs or PBS significantly enhanced the ongoing OVA-specific T-cell responses, $CD4^+CD25^-$ T cells from mice treated with CD40-silenced OVA DCs did not (Fig 4, *A*). We also measured levels of IL-4 and IL-5 produced by OVA-specific T-cell responses after addition of $CD4^+CD25^-$ effector T cells from mice treated with CD40-silenced OVA DCs, control OVA DCs, or PBS after establishment of allergic rhinitis. Although IL-4 and IL-5 production was not detected with adding of no $CD4^+CD25^-$ T cells or $CD4^+CD25^-$ T cells from mice that received CD40-silenced OVA DCs, IL-4 and IL-5 were produced with adding of $CD4^+CD25^-$ T cells from mice that received control OVA DCs or PBS (Fig 4, *B* and *C*). This suggested that unresponsiveness of $CD4^+CD25^-$ effector T cells is induced in mice treated with CD40-silenced OVA DCs.

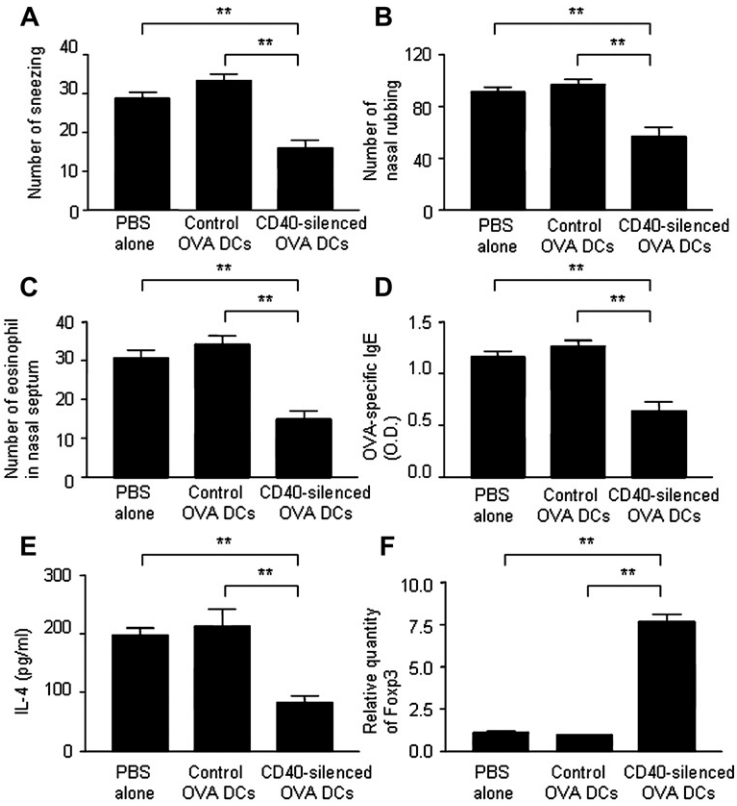

FIG 3. Reduction of allergic symptoms and responses by administration of CD40-silenced DCs in mice with established allergic rhinitis. **A-C**, The number of sneezing movements (Fig 3, *A*), nasal rubbing movements (Fig 3, *B*), and eosinophils in the nasal septum (Fig 3, *C*). **D**, OVA-specific IgE in sera. **E**, IL-4 production from splenocytes stimulated with OVA. **F**, Foxp3 expression determined by using real-time PCR. n = 5 per group. $^{**}P < .01$.

Long-term effect of CD40-silenced OVA DCs on established allergic rhinitis

It is not clear how long CD40-silenced OVA DCs attenuate allergy. Mice that had been sensitized intraperitoneally and challenged intranasally with OVA were treated with CD40-silenced OVA DCs or PBS and then rechallenged intranasally with OVA (priming on days 0 and 14, challenge on days 18-24, treatment with DCs on days 26 and 30, rechallenge on days 28-86, and sample collection on day 86) to investigate the long-lasting effect of CD40-silenced OVA DCs. CD40-silenced OVA DCs significantly decreased the number of sneezing and nasal rubbing movements on days 44, 51, 58, 65, 72, 79 (data not shown), and 86 (see Fig E5, *A* and *B*, in this article's Online Repository at www.jacionline.org). CD40-silenced OVA DCs also reduced the number of eosinophils and the level of OVA-specific IgE in sera 8 weeks after treatment (see Fig E5, *C* and *D*). Also, real-time PCR results showed that CD40-silenced OVA DCs significantly increased Foxp3 gene expression in the spleen 8 weeks after treatment (see Fig E5, *E*).

Therapeutic effects of Treg cells induced by CD40-silenced OVA DCs on established allergic rhinitis *in vivo*

$CD4^+CD25^+$ Treg cells were isolated from mice that received CD40-silenced OVA DCs (treatment with DCs on days 0 and 7 and sample collection on day 21) to study the effects of Treg cells on allergy *in vivo*. Mice with established allergic rhinitis were treated with Treg cells or PBS intravenously and then rechallenged intranasally with OVA (priming on days 0 and 14, challenge on days 18-24, treatment with 7×10^5 Treg cells on day 26, rechallenge on days 27-32, and sample collection on day 33). Treg cells derived from mice treated with CD40-silenced OVA DCs significantly reduced the number of sneezing and nasal rubbing movements immediately after the last nasal challenge, the number of eosinophils in nasal mucosa, and OVA-specific IgE levels in sera (Fig 5).

DISCUSSION

Our experiments suggested that (1) CD40 siRNA effectively knocked down CD40 gene expression in DCs; (2) CD40-silenced OVA DCs did not induce allergic responses and sensitization *in vivo*, although CD40-expressing and OVA-pulsed DCs (control OVA DCs) did; (3) CD40-silenced OVA DCs inhibited allergic nasal symptoms and allergic responses not only before sensitization but also after establishing allergic rhinitis; (4) treatment with CD40-silenced OVA DCs is an allergen-specific therapy; (5) CD40-silenced OVA DCs prevented the function of effector T cells; and (6) CD40-silenced OVA DCs facilitated the generation of allergen-specific Treg cells, which reduced allergic responses *in vivo*.

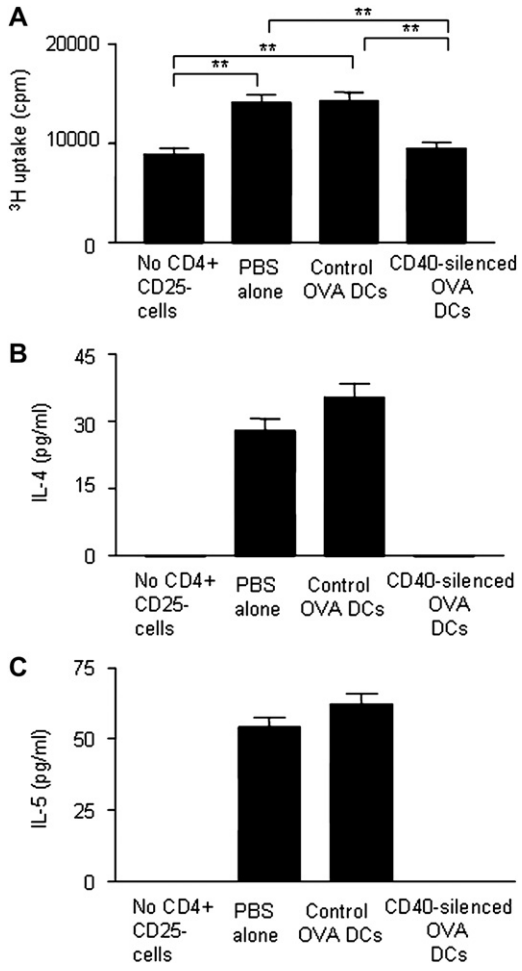

FIG 4. Effect of CD40-silenced OVA DCs on effector T cells. OVA-specific T-cell responses were performed. Splenocytes isolated from allergic mice that were injected with OVA and alum were seeded in 96-well plates and cocultured with OVA. $CD4^+CD25^-$ effector T cells were isolated from mice with allergic rhinitis treated with PBS, control OVA DCs, or CD40-silenced OVA DCs. **A**, Modulation of OVA-specific T-cell responses by $CD4^+CD25^-$ effector T cells. Purified $CD4^+CD25^-$ effector T cells were added to ongoing OVA-specific T-cell response. **B** and **C**, The levels of IL-4 (Fig 4, *B*) and IL-5 (Fig 4, *C*) produced by splenocytes cocultured with OVA and purified $CD4^+CD25^-$ effector T cells. n = 5 per group. $^{**}P < .01$.

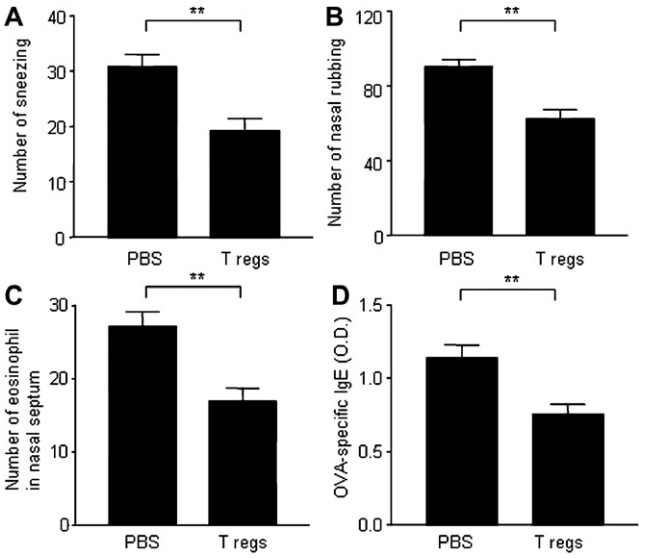

FIG 5. The effect of $CD4^+CD25^+$ Treg cells on allergic responses. Five mice with established allergic rhinitis were treated with PBS alone or Treg cells from mice that received CD40-silenced OVA DCs. The number of sneezing **(A)** and nasal rubbing **(B)** movements were counted after the last nasal challenge. **C**, The number of eosinophils in nasal septum. **D**, Level of OVA-specific IgE in sera. $^{**}P < .01$.

We previously reported that direct administration of CD40 shRNA attenuated allergy.[12] However, this direct administration has several potential problems: (1) hyper-IgM syndrome through inhibition of immunoglobulin class switching of B cells; (2) knocking down of CD40 expression not only in target cells but also in other cells; (3) the side effect of vector; and (4) allergen-independent therapy. The cellular therapy with CD40-silenced OVA DCs can solve these problems because (1) siRNA is transfected only *in vitro* but not *in vivo* and therefore CD40 expression in B cells is not affected, avoiding hyper-IgM syndrome; (2) DCs are transfected with siRNA *in vitro*, suggesting that only target DCs are affected by siRNA; (3) use of a vector is not necessary for the *in vitro* transfection with siRNA; and (4) in this study CD40-silenced OVA DCs suppressed only OVA-specific allergic response but did not inhibit KLH-specific response, suggesting that use of CD40-silenced OVA DCs is an allergen-specific therapy.

Our data showed that CD40-silenced OVA DCs themselves did not induce OVA-specific T-cell responses or production of IL-4, IL-5, IgG1, and IgE *in vivo*, indicating that CD40-silenced OVA DCs do not cause allergic responses and sensitization; in this respect CD40-silenced OVA DCs appear safe and promising.

In this study CD40-silenced OVA DCs inhibited allergic responses. There are 2 possibilities to explain this mechanism: unresponsiveness of effector T cells and generation of Treg cells. Tolerance of effector T cells can be induced by DCs.[23] CD40 plays an important role in DC maturation,[23] and immature or semimature DCs by lack of CD40–CD40 ligand (CD40L) signal inhibit T-cell responses through anergy.[19] CD40-CD40L ligation is also necessary for prolongation of T-cell activation and immunity.[24] Indeed, $CD4^+CD25^-$ effector cells in mice that received CD40-silenced OVA DCs did not enhance the OVA-specific T-cell response or production of IL-4 and IL-5 in this study, although $CD4^+CD25^-$ effector cells in mice that received control OVA DCs or PBS did. Immature or semimature DCs also induce allergen-specific Treg cells, which can regulate allergen-dependent effector T cells.[25] The present study showed that augmentation of $CD25^+Foxp3^+$ T cells was observed in mice treated with CD40-silenced OVA DCs. This suggests that the therapeutic effects of CD40-silenced OVA DCs in allergic patients are associated with Treg cell generation. The Treg cell population produced by CD40-silenced OVA DCs inhibited OVA-specific T-cell responses. However, these Treg cells did not inhibit KLH-specific T-cell responses, suggesting allergen specificity. Even nonspecific immune modulation, if it occurs during an ongoing response, can induce antigen-specific immune suppression by Treg cells. Treatment with anti-CD3 mAb generated antigen-specific Treg cells in the case of type I diabetes.[26] Anti-CD40L antibody also induced antigen-specific Treg cells and antigen-specific transplantation tolerance.[27] Encounter of preactivated cells with CD40-silenced OVA DCs might generate specific

Treg cells because CD40-silenced OVA DCs present OVA peptide to preactivated cells.

In summary, we report a novel, cellular allergen-specific therapy through immune modulation by CD40-silenced OVA DCs. Treatment with allergen-pulsed CD40-silenced OVA DCs significantly attenuates allergen-specific allergic symptoms and pathologic changes, suggesting potential clinical uses as an allergen-specific immunotherapy.

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Clinical implications: The ability of CD40-silenced OVA DCs to inhibit only OVA-specific allergic responses suggests that use of CD40-silenced OVA DCs is a useful strategy for allergen-specific therapy.

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Correction

With regard to the December 2009 article "Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome" (J Allergy Clin Immunol 2009;124:1289-1302), the name of co-author Necil Kutukculer was misspelled in the list of authors.

METHODS

Generation of bone marrow–derived DCs

DCs were generated from bone marrow progenitor cells, as described previously.[E1] Briefly, bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice (Charles River Canada) and cultured in complete medium supplemented with recombinant GM-CSF and recombinant IL-4.

RT-PCR and real-time PCR

RNA was isolated by using Trizol (Invitrogen Life Technologies, Carlsbad, Calif). The SuperScript Preamplification System (Invitrogen Life Technologies) was used to generate the first-strand cDNA. The CD40, Foxp3, and glyceraldehyde-3-phosphate dehydrogenase primers described before[E1] were used in this study. Real-time PCRs were performed by using a method described previously.[E1]

Flow cytometry

A phenotypic analysis of T cells was performed on a FACScan, as previously described.[E1] T cells were stained with anti-mouse CD4 and CD25 mAb. Foxp3 expression was also assessed by means of intracellular staining with a cell permeabilization kit.

Isolation of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from splenocytes and lymph nodes of mice by using a method described previously.[E1]

Antigen-specific T-cell response

By using a centrifugation gradient over Ficoll-Paque (Amersham Pharmacia Biotech, Little Chalfont, Bucks, United Kingdom), T cells were isolated from spleens. All cells were cultured in 96-well plates at a concentration of 4×10^5 cells per well for 72 hours in the presence or absence of OVA or KLH.

Splenocytes were isolated from allergic mice that were injected twice with OVA (10 μg per mouse), KLH (10 μg per mouse), and alum (4 mg per mouse) to assess the effects of Treg cells. These splenocytes were seeded in 96-well plates at a concentration of 5×10^4 cells per well and cocultured with OVA or KLH. Purified $CD4^+CD25^+$ cells (5×10^4 cells per well) were added to the ongoing splenocyte-allergen reaction for 72 hours.

Splenocytes were isolated from allergic mice that were injected twice with OVA (10 μg per mouse) and alum (4 mg per mouse) to study the effects of CD40-silenced OVA DCs on effector cells. These splenocytes were seeded in 96-well plates at a concentration of 5×10^4 cells per well and cocultured with OVA. Purified $CD4^+CD25^-$ T cells (5×10^4 cells per well) were added to the ongoing splenocyte-allergen reaction for 72 hours. A tritiated thymidine incorporation assay was performed.

Measurements of allergen-specific antibodies and cytokines

OVA-specific IgE, IgG1, IgG2a, IL-4, and IL-5 levels were measured by means of ELISA, as described previously.[E1,E2] KLH-specific IgE levels were measured by means of ELISA. Briefly, plates were coated with anti-mouse IgE mAb. After nonspecific binding was blocked, sera were added. After adding biotinylated KLH, KLH-specific IgE levels were measured by using the same method as that for OVA-specific IgE levels. KLH-specific IgG1 and IgG2a levels were also measured by means of ELISA. After plates were coated with KLH, levels of KLH-specific IgG1 or IgG2a were assessed by using the same method used for OVA-specific IgG1 or IgG2a levels, respectively.

Pathology

Nasal tissues were stained with Luna staining. The number of eosinophils at the nasal septum was counted microscopically in a high-power field (10×40). The counting of eosinophils by the observer was completed in a blind fashion.

Statistical analysis

Data are expressed as means ± SEMs. Statistical comparisons between groups were performed by using the Student *t* test, paired *t* test, or one-way ANOVA, followed by the Newman-Keuls test. Differences with *P* values of less than .05 were considered significant.

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E1. Suzuki M, Zheng X, Zhang X, Li M, Vladau C, Ichim TE, et al. Novel vaccination for allergy through gene silencing of CD40 using small interfering RNA. J Immunol 2008;180:8461-9.

E2. Suzuki M, Zheng X, Zhang X, Ichim TE, Sun H, Kubo N, et al. Inhibition of allergic responses by CD40 gene silencing. Allergy 2009;64:387-97.

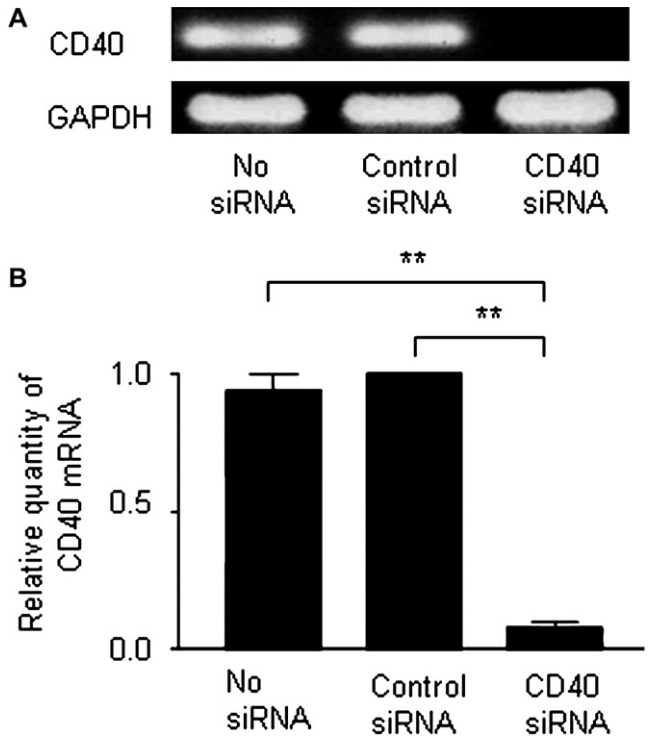

FIG E1. CD40-silencing DCs with CD40 siRNA. Bone marrow–derived DCs were transfected with no siRNA, control siRNA, or CD40 siRNA. Total RNAs were extracted from DCs. **A**, CD40 expression determined by means of RT-PCR *in vitro*. RT-PCR was performed by using primers specific to CD40 and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*. **B**, CD40 expression was determined by means of real-time PCR. $^{**}P < .01$.

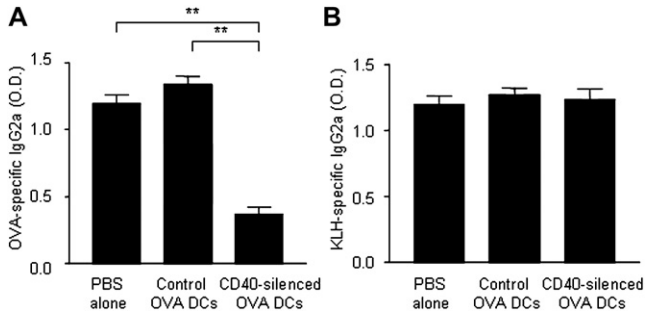

FIG E2. OVA -specific and KLH-specific IgG2a levels in sera by administration of CD40-silenced OVA DCs before immunization. Five mice were sensitized and challenged with OVA and KLH after treatment with PBS, control OVA DCs, or CD40-silenced OVA DCs. **A**, OVA-specific IgG2a in sera. **B**, KLH-specific IgG2a in sera. $^{**}P < .01$.

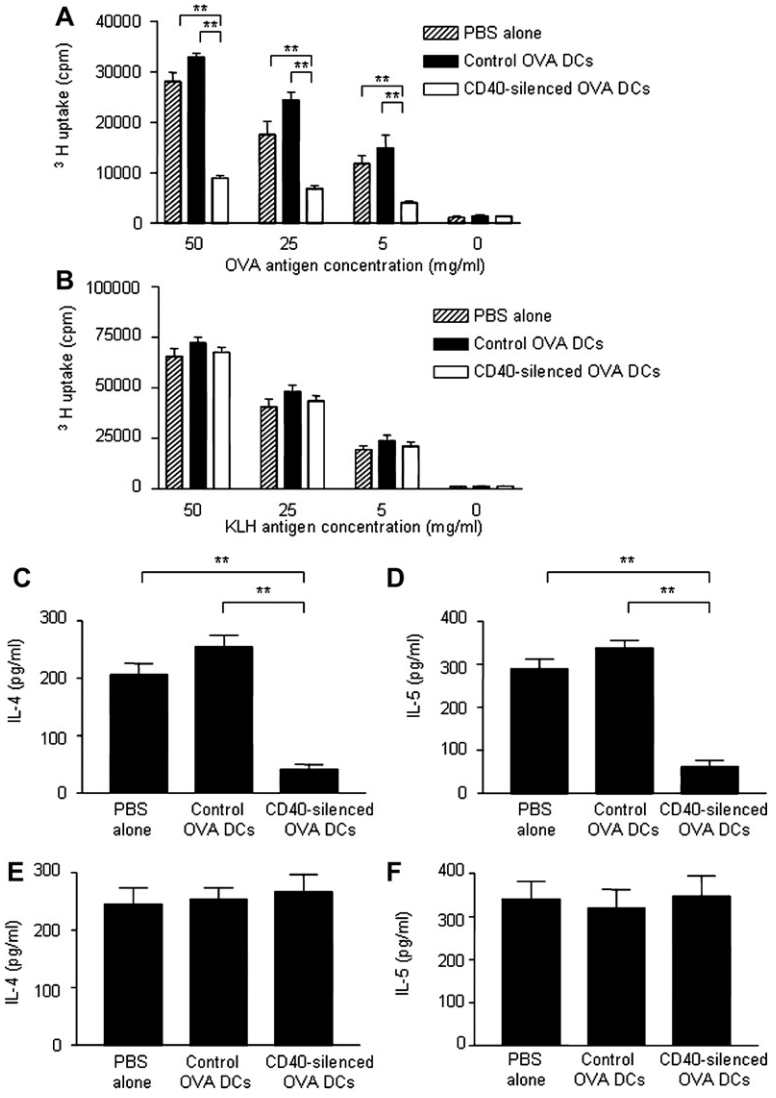

FIG E3. Administration of CD40-silenced OVA DCs before sensitization modulates the OVA-specific T-cell response. **A**, OVA-specific recall responses. **B**, KLH-specific recall responses. **C** and **D**, The levels of IL-4 (Fig E3, *C*) and IL-5 (Fig E3, *D*) by splenocytes stimulated with OVA. **E** and **F**, The levels of IL-4 (Fig E3, *E*) and IL-5 (Fig E3, *F*) by splenocytes stimulated with KLH. n = 5 per group. $^{**}P < .01$.

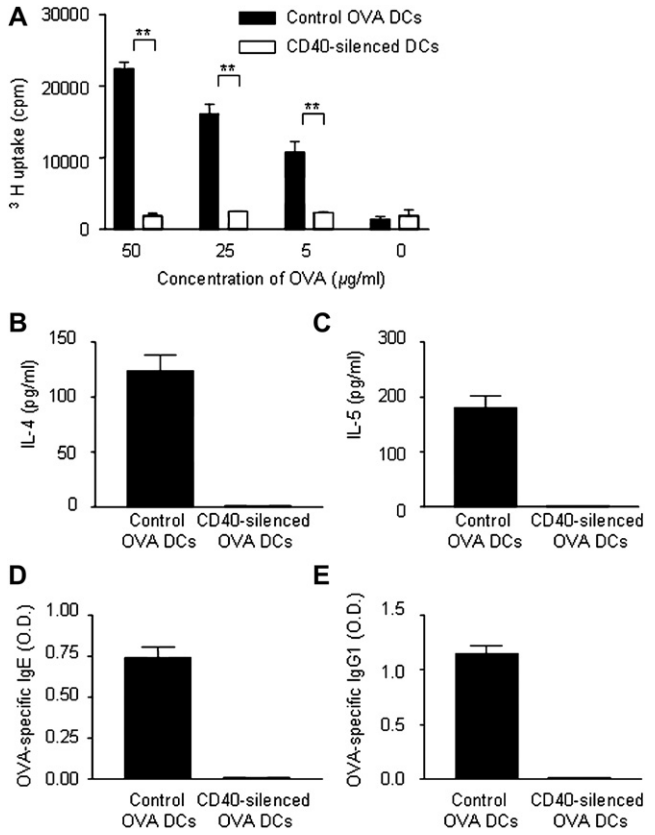

FIG E4. Prime responses by CD40-silenced OVA DCs. Five mice were injected with control OVA DCs or CD40-silenced OVA DCs. **A**, OVA-specific T-cell response of splenic cells. **B** and **C**, The level of IL-4 (Fig E4, *B*) and IL-5 (Fig E4, *C*) productions from splenocytes stimulated by OVA. **D** and **E**, The level of OVA-specific IgE (Fig E4, *D*) and IgG1 (Fig E4, *E*) in sera. $^{**}P < .01$.

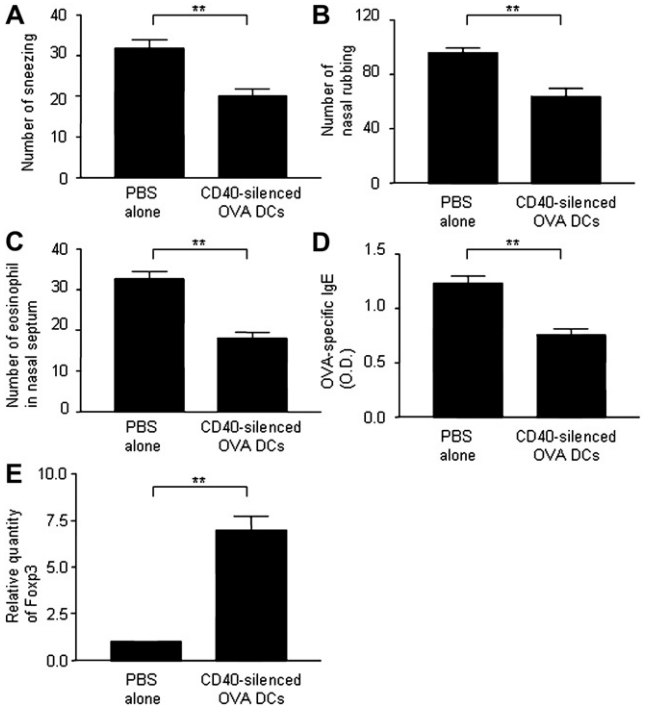

FIG E5. Long -term effect of CD40-silenced DCs. Five mice were treated with PBS alone or CD40-silenced OVA DCs after establishment of allergic rhinitis. The number of sneezing **(A)** and nasal rubbing **(B)** was counted after nasal challenge 8 weeks after treatment. **C**, The number of eosinophil in nasal mucosa 8 weeks after treatment. **D**, Level of OVA-specific IgE in sera 8 weeks after treatment. **E**, Foxp3 gene expression in 8 weeks after treatment was determined by means of real-time PCR. $^{**}P < .01$.