

Signals through CD40 play a critical role in the pathophysiology of *Schistosoma mansoni* egg antigen-induced allergic rhinitis in mice

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ABSTRACT

Background: Interaction between CD40 and CD40L is thought to regulate immune responses in several allergic diseases. However, little is known about its *in vivo* role in the pathophysiology of allergic rhinitis. We sought to determine whether the lack of signals through CD40 affects the pathophysiology of allergic rhinitis using a murine model.

Methods: Wild type (WT) and CD40-deficient BALB/c (CD40^{-/-}) mice were sensitized intranasally to *Schistosoma mansoni* egg antigen (SEA). After repeated sensitization, histamine responsiveness, serum antibody titer including immunoglobulin E (IgE), nasal eosinophilia, and cytokine production by nasal mononuclear cells were determined in each group.

Results: Intranasal sensitization with SEA in WT mice elicited a strong Th2 response including SEA-specific IgE production, nasal eosinophilia, and interleukin (IL)-4, and IL-5 production by nasal mononuclear cells after antigen challenge. Production of SEA-specific IgE and IgG1 was abolished in SEA-sensitized CD40^{-/-} mice. These mice showed impaired nasal eosinophilia and displayed markedly reduced histamine-induced nasal hyperresponsiveness as compared with WT mice. Furthermore, reduced production of IL-4 and IL-5 by nasal mononuclear cells was seen in CD40^{-/-} mice.

Conclusion: These results show that signals through CD40 play a critical role in not only IgE production but also pathophysiology of allergic rhinitis such as nasal hyperresponsiveness and nasal eosinophilia. (Am J Rhinol 20:165–169, 2006)

A member of the tumor necrosis factor receptor superfamily, CD40 is expressed on thymic epithelial cells, B cells, and a number of professional antigen-presenting cells. Many studies have indicated that the interaction of CD40 with its ligand CD40L, which is expressed on activated T cells, plays a critical role in induction of T-cell-dependent B-cell responses. Thus, CD40/CD40L interaction regulates B-cell proliferation, immunoglobulin (Ig) production, isotype switching, and induction of B-cell memory.^{1,2} Studies using both CD40^{-/-} and CD40L^{-/-} mice as well as blocking antibody (Ab) have shown that CD40/CD40L interaction also modulates inflammatory responses by up-regulating production of proinflammatory cytokines and nitric oxide from monocytes.^{3,4}

Allergic rhinitis is associated with the expansion of Th2-type immune responses characterized by the production of allergen-specific IgE, nasal eosinophilia, and nasal hyperresponsiveness.^{5,6} Several studies using CD40- or CD40L-deficient mice have shown a role for CD40/CD40L interaction in the pathophysiology of asthma, one of the most typical allergic diseases in the lower airway.^{7–10} However, it remains unclear whether CD40/CD40L interaction also is involved in pathogenesis of

allergic rhinitis, and to date, no reports have established the *in vivo* role of CD40 or CD40L in the initiation of allergic rhinitis. We have developed a murine model of allergic rhinitis, which received repeated intranasal administration of *Schistosoma mansoni* egg antigen (SEA) in the absence of an adjuvant and consequently showed Th2 response and nasal mucosa inflammation with eosinophil infiltration.¹¹ To determine whether CD40/CD40L interaction plays a role in pathogenesis of allergic rhinitis *in vivo*, we compared nasal hyperresponsiveness, Ab production, nasal eosinophilia, and nasal mononuclear cell cytokine production in SEA-sensitized CD40^{-/-} mice with that in similarly sensitized wild-type (WT) mice. We believe that this is the first study showing the *in vivo* role of CD40 in nasal pathophysiology such as nasal eosinophilia and nasal hyperresponsiveness in a murine model of rhinitis.

MATERIALS AND METHODS

Animals

Homozygous CD40-deficient BALB/c (CD40^{-/-}) mice and WT BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Six- to 7-week-old female mice were used in all of the experiments. These mice were maintained in the specific pathogen-free condition in accordance with instituted guidelines.

Sensitization of Mice

We sensitized WT and CD40^{-/-} mice as previously described.¹¹ In brief, 5 µg of SEA was instilled into the anterior nose once a week for 3 weeks (on days 1, 8, and 15). One week later, mice were challenged intranasally with 1 µg of SEA for 7 consecutive days (on days 22–28; nasal challenge). Mice were bled by tail snipping on day 6 after the third sensitiza-

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tion and 18 hours after the final challenge. After 18 hours following the final challenge, the mice were killed and nasal mononuclear cells were obtained for further analysis as described before.¹¹

Measurement of Nasal Hyperreactivity

To assess nasal hyperreactivity in SEA-sensitized mice, responsiveness to intranasally administered histamine was measured by determining the concentration of histamine that causes nasal rubbing and sneezing. Briefly, 10 μ L of histamine (Sigma, St. Louis, MO) in different doses was administered into the nostrils of SEA-sensitized CD40^{-/-} and WT mice. Nasal symptoms (nasal rubbing and sneezing) were evaluated by counting the number of nasal rubbings and sneezing that were caused for 5 minutes after nasal administration of histamine. The concentration of histamine at which mice rubbed >40 times and also sneezed >5 times was identified as the threshold concentration. This point was expressed to the limiting concentration of histamine (log₁₀ M). The nasal responsiveness was measured 1 day before both the first sensitization and the death (day 0 and day 29, respectively). The nose symptom assay was examined blindly.

Ab Enzyme-Linked Immunosorbent Assay

The levels of SEA-specific IgG₁ and IgG_{2a} in the serum were measured by enzyme-linked immunosorbent assay (ELISA) as previously described.¹¹ The serum total IgE and SEA-specific IgE was determined also by ELISA as previously described.¹¹

Histological Examination

The heads were removed, fixed, and decalcified. Coronal nasal sections were stained with Luna solution and the number of eosinophils in the posterior part of the nasal septum was counted microscopically under a high-power field (10 $\times$ 40).¹² This count was done blindly.

In vitro Culture of Nasal Mononuclear Cells and Cytokine Determination

Nasal mononuclear cells from WT and CD40^{-/-} mice were isolated by enzyme extraction, as previously described, with slight modification.¹² Cells were cultured in 96-well flat-bottom plates in 5% CO₂ at 37°C with and without plate-bound purified hamster anti-mouse CD3e monoclonal Ab (1 μ g/mL; Biosciences Pharmingen, San Jose, CA). After 72 hours of culture, supernatants were harvested. Interleukin (IL)-4, IL-5, and interferon (IFN) γ production in culture supernatant were measured by captured ELISA as previously described.¹³ The detection limit for IL-4, IL-5, and IFN- γ in this system was 10, 200, and 100 pg/mL, respectively.

Statistics

Nonparametric Mann-Whitney *U* test was used to evaluate the differences in the values obtained. A value of *p* < 0.05 was considered significant. Values were given as mean $\pm$ SEM.

RESULTS

Impaired Histamine-Induced Nasal Responsiveness in SEA-Sensitized CD40^{-/-} Mice

Nasal allergic symptoms such as sneezing and nasal rubbing were determined before the sensitization (day 0) and

after the final nasal challenge (day 28). Before sensitization, histamine responsiveness was not different between WT and CD40^{-/-} mice. In WT mice, histamine responsiveness was remarkably enhanced after the final challenge (*p* < 0.01). However, histamine responsiveness was not changed in CD40^{-/-} mice after the final challenge, and the responsiveness was significantly lower as compared with that of control mice (Fig. 1).

SEA-Sensitized CD40^{-/-} Mice Show Reduced Recruitment of Eosinophils into Nasal Mucosa

After intranasal sensitization with SEA, WT mice displayed significant eosinophil infiltration into nasal mucosa (Fig. 2A). In contrast, only a few eosinophils were present in nasal mucosa from SEA-sensitized CD40^{-/-} mice (*p* = 0.003, Fig. 2B). The number of eosinophils infiltrating into nasal septum per field (10 $\times$ 40) was 26.5 $\pm$ 6.0 and 8.0 $\pm$ 5.5 (median value with range) in WT (*n* = 6) and CD40^{-/-} mice (*n* = 8), respectively. There were no significant differences in the numbers of infiltrating lymphocytes between the groups.

Analysis of Ab Response in SEA-Sensitized CD40^{-/-} and WT Mice

After the nasal sensitization, WT mice produced significant amounts of Th2-associated total IgE and SEA-specific IgE and IgG₁ but no Th1-associated IgG_{2a} (Figs. 3 and 4). On the other hand, similarly sensitized CD40^{-/-} mice abolished the production of both total IgE and SEA-specific IgE during the

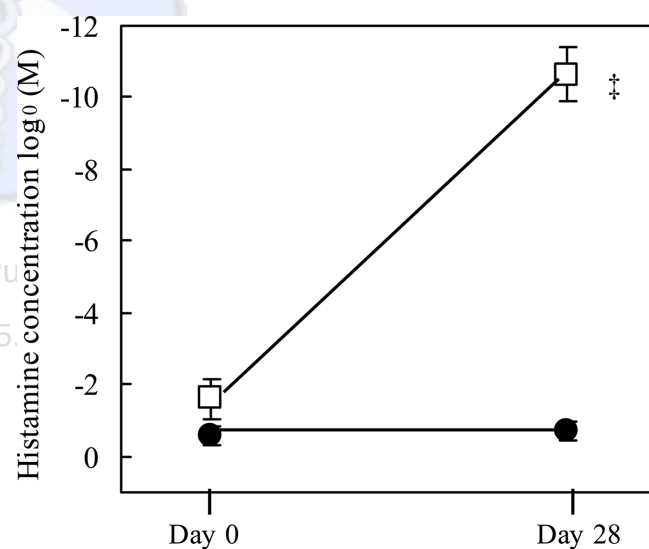
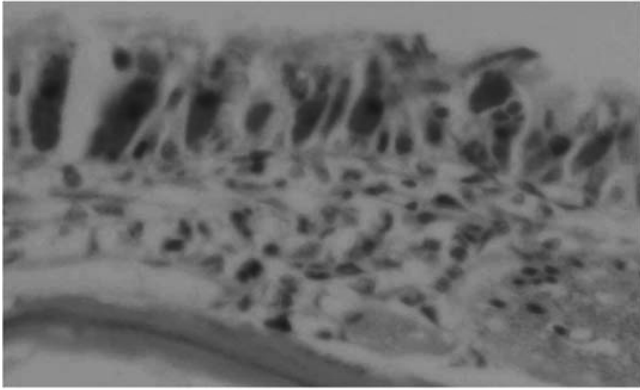


Figure 1. Histamine-induced nasal responsiveness. WT mice and CD40^{-/-} mice were intranasally sensitized with SEA once a week for 3 weeks. Then, 1 week later, all mice were challenged with SEA for 7 consecutive days. The nasal hyperresponsiveness for histamine was determined before sensitization (day 0) and after nasal challenge (day 28). Histamine responsiveness of WT (open square) and CD40^{-/-} mice (closed circle) was expressed by the strongest histamine concentration that caused mice both >5 times sneezing and >40 times nasal rubbing. Data shown are mean $\pm$ SEM of five mice per group. Data are representative of two separate experiments ($\ddagger p$ < 0.01 compared with the WT group).

A



B

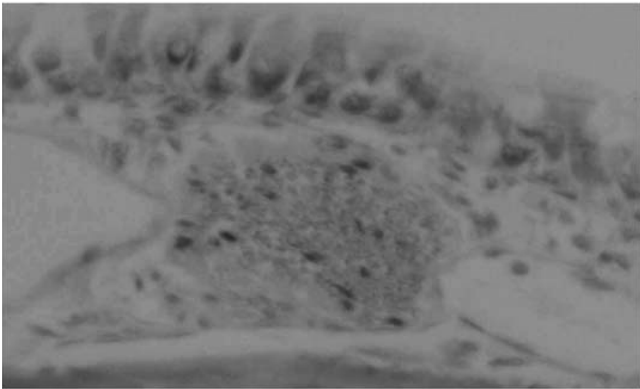


Figure 2. Nasal eosinophilia of mice intranasally sensitized with SEA. WT and CD40^{-/-} mice were intranasally sensitized with SEA. After nasal challenge, mice were killed, and coronal nasal sections were fixed, decalcified, and stained with Luna solution. Typical nasal sections are shown: (A) WT mouse and (B) CD40^{-/-} mouse.

period observed (Fig. 3). CD40^{-/-} mice also failed to produce significant levels of SEA-specific IgG₁ and IgG_{2a} (Fig. 4).

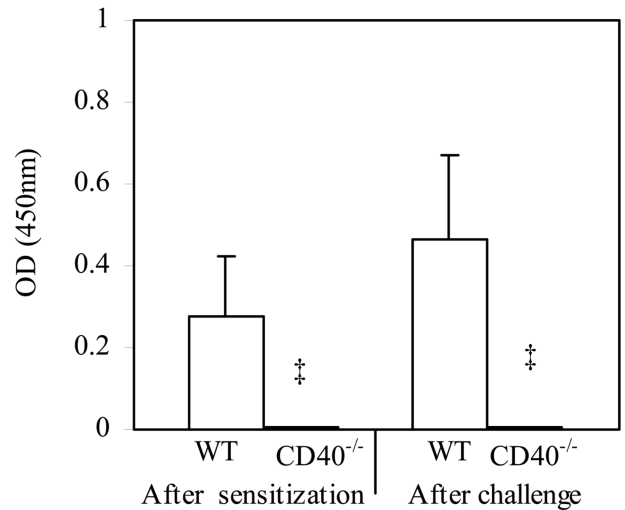
Analysis of Cytokine Production by Nasal Mononuclear Cells in SEA-Sensitized CD40^{-/-} and WT Mice

After *in vitro* stimulation with anti-CD3, nasal mononuclear cells from WT mice produced significant levels of IL-4 and IL-5. In contrast, nasal lymphocytes from CD40^{-/-} mice produced only basal levels of IL-4 or IL-5 (Fig. 5, A and B). As regards IFN- γ , there were no significant differences between WT and CD40^{-/-} mice (Fig. 5C).

DISCUSSION

In this study, we showed that CD 40^{-/-} mice sensitized intranasally with SEA mount poor Th2 response, produce

A



B

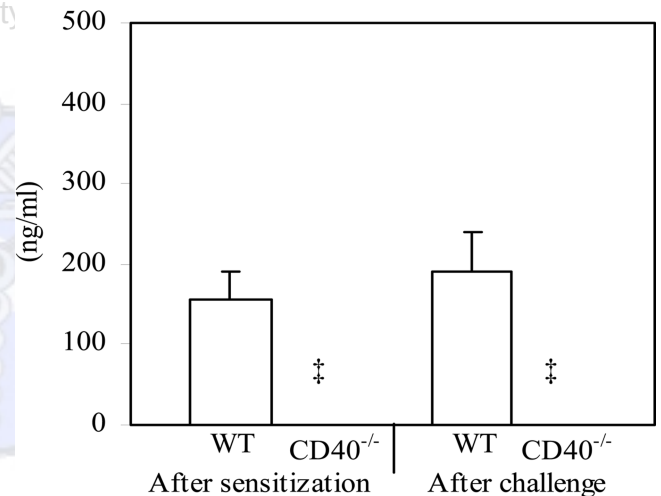


Figure 3. (A) Titers of SEA-specific IgE and (B) amount of total IgE. WT mice and CD40^{-/-} mice were intranasally sensitized with SEA once a week for 3 weeks. Then, 1 week later, all mice were challenged with SEA for 7 consecutive days. WT (open column) and CD40^{-/-} mice (closed column) were bled by the tail snipping on day 6 after the third sensitization and 18 hours after the final challenge. SEA-specific IgE and serum total IgE were detected with sandwich ELISA. Results show (A) the mean optical density at 450 nm $\pm$ SEM at 1:4 dilution and (B) the mean nanograms per milliliter $\pm$ SEM of five serum samples from each group. Data are representative of two separate experiments (* p < 0.01 compared with the WT group).

little or no IL-4 and IL-5 as well as SEA-specific IgG₁ and IgE. Furthermore, CD40^{-/-} mice display markedly reduced nasal eosinophilia and nasal hyperresponsiveness in response to histamine. These findings indicate that CD40 plays a critical role in the pathophysiology of SEA-induced murine allergic rhinitis.

An engagement of CD40 with CD40L provides an essential

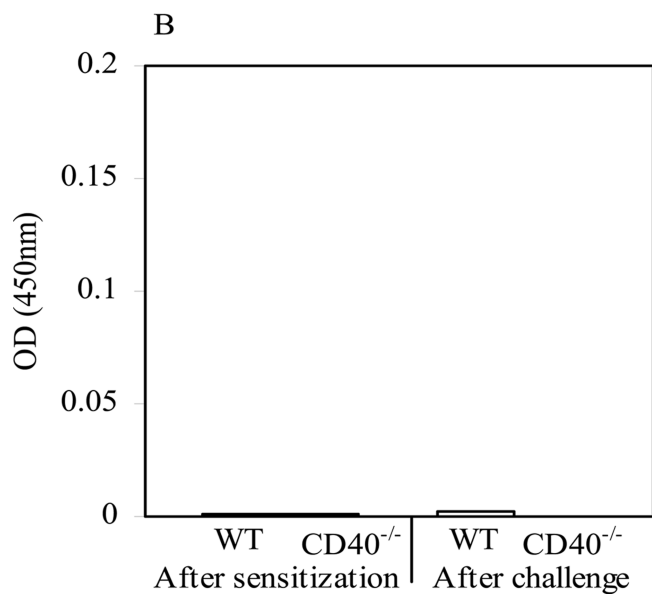
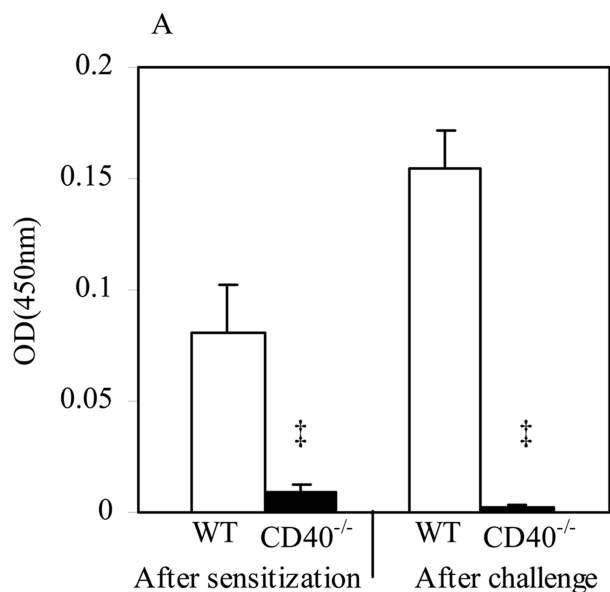


Figure 4. SEA-specific (A) IgG₁ and (B) IgG_{2a} isotypes. After boosting sensitization with SEA, WT mice and CD40^{-/-} mice were intranasally challenged with SEA for 7 consecutive days. Sera were taken from WT (open column) and CD40^{-/-} mice (closed column) 6 days after last boosting sensitization and 18 hours after last nasal challenge. They were assayed for SEA-specific IgG₁ and IgG_{2a} in indirect ELISA. Results show the mean optical density at 450 nm $\pm$ SEM of five serum samples from each group at 1:100 dilutions. Data are representative of two separate experiments ($\dagger p < 0.01$ compared with the WT group).

costimulatory signal to the B cell that is required for T-cell-dependent class switching for all Ig's except IgM.¹ A previous study has shown that CD40/CD40L interaction is required for initiation of the immune response to antigens that inherently induce a very strong Th2 response and production of IL-4, which facilitates isotype switching to IgE and IgG₁.^{7,14} On the other hand, Ferlin *et al.* found that CD40 signaling causes

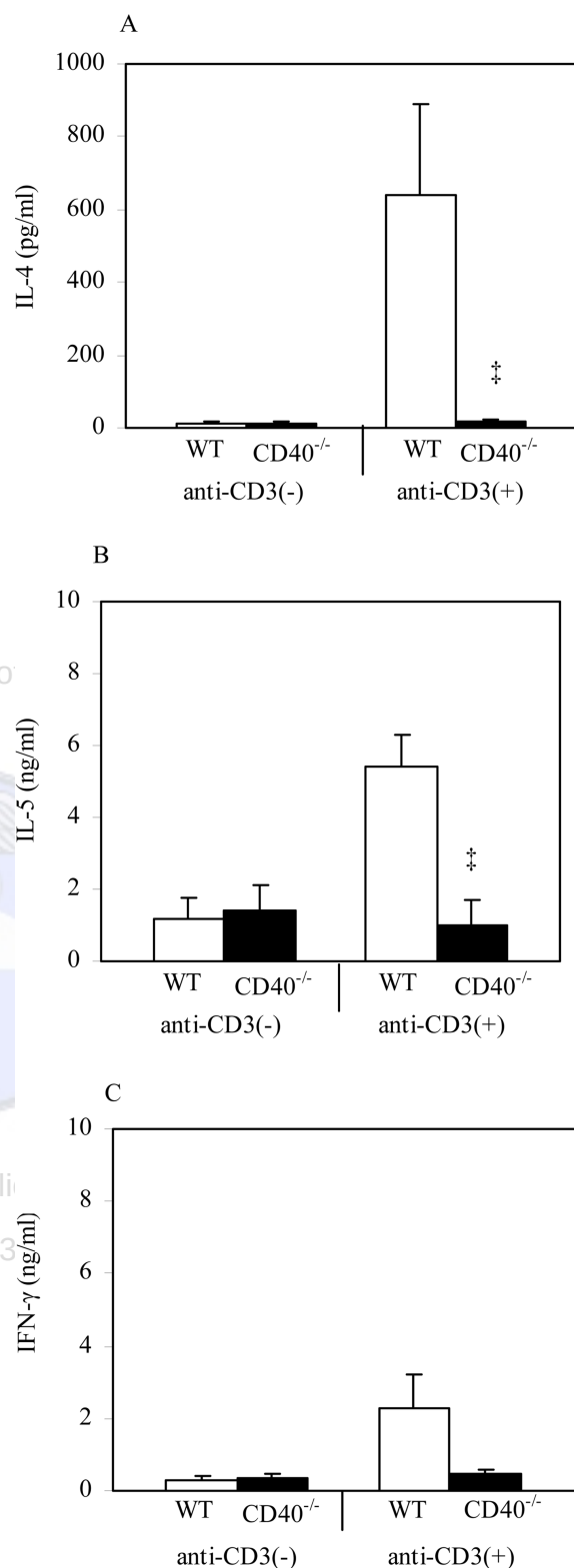


Figure 5. Production of (A) IL-4, (B) IL-5, and (C) IFN- γ by nasal mononuclear cells from WT (open column) and CD40^{-/-} (closed column) mice sensitized intranasally with SEA. Nasal mononuclear cells were isolated and cultured *in vitro* for 72 hours without or with plate-bound antimouse CD3e monoclonal Ab. Cytokines were measured by ELISA. Results were expressed the mean $\pm$ SEM. Data are representative of two separate experiments ($\dagger p < 0.05$, $\ddagger p < 0.01$).

IL-4-independent IgE switching *in vivo*.¹⁵ We have previously reported that SEA-sensitized IL-4-deficient mice fail to produce significant levels of IgE, suggesting that IL-4 is critical for IgE production in SEA-induced rhinitis.¹⁶ Therefore, it is perhaps not surprising that SEA-sensitized CD40^{-/-} mice, which produced significantly less IL-4, displayed low titers of SEA-specific IgE, IgG₁, and total IgE. The lack of SEA-specific IgE and IgG₁ production in CD40^{-/-} mice observed in this study is also consistent with the similar observation reported by others using these mice.^{1,2,7,9}

IL-5 is a Th2-derived cytokine that and plays a central role in the differentiation, proliferation, accumulation, and migration of eosinophils to the site of inflammation.¹⁷ In this study, reduced production of IL-5 by nasal mononuclear cells was seen in CD40^{-/-} mice that could contribute to reduced levels of eosinophilia. The impaired infiltration of eosinophils in this study is not simply caused by the lack of IL-4 or IgE production because we have previously shown that IL-4-deficient mice, which are unable to produce SEA-specific IgE, show significant numbers of eosinophils in their nasal mucosa after intranasal sensitization with SEA.¹⁶ The production of other cytokines, such as granulocyte-macrophage colony-stimulating factor and IL-13 or chemokines, such as eotaxin, may influence the inflammatory process.¹⁸ The present results together with our previous findings suggest that CD40-dependent but IL-4/IgE-independent mechanism regulates eosinophil trafficking into nasal mucosa after SEA sensitization.

In this study, we found that histamine-induced nasal hyperresponsiveness was markedly reduced in SEA-sensitized CD40^{-/-} mice. Although there are several reports investigating the role of CD40 in bronchial hyperresponsiveness,^{7,9,10} this is the first report regarding the role of CD40 in the hyperresponsiveness in the upper airway. In fact, our result is not consistent with the previous reports focusing on bronchial hypersensitivity.^{7,9,10} Takahashi *et al.* showed that enhanced bronchial hyperresponsiveness accompanied the increase of lung eosinophilia in CD40^{-/-} mice.¹⁰ On the other hand, Mehlhop *et al.* have suggested that CD40L but not CD40 is central to bronchial hyperresponsiveness, although both CD40^{-/-} mice and CD40L^{-/-} mice sensitized with *Aspergillus* showed the same level of lung eosinophilia as WT mice and produced no serum IgE.⁹ These conflicting observations may be caused by several factors such as the type of antigen (SEA, ovalbumin, or *Aspergillus*), the route for sensitization (intranasal versus intraperitoneal), phase examined (induction or effector phase), or usage of adjuvants (*e.g.*, alum).

In conclusion, CD40^{-/-} BALB/c mice fail to display reduced nasal hyperresponsiveness after repeated intranasal challenge with SEA. These findings show that CD40 plays a central role in pathophysiology allergic rhinitis. These results have implications for future investigations on understanding the underlying mechanism of allergic rhinitis and may have therapeutical implication for this disease.

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