

IL-21 Administration into the Nostril Alleviates Murine Allergic Rhinitis¹

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Type I allergic diseases such as allergic rhinitis are caused by IgE-mediated humoral immune responses, while eosinophils also fulfill important roles in the etiology of IgE-mediated allergy. IL-21 regulates growth, differentiation, and function of T, B, and NK cells, while the production of IgE is also influenced by IL-21. In this study we examined whether IL-21 is capable of controlling IgE-mediated allergic reactions *in vivo* by using the allergic rhinitis mouse model that was established by repetitive sensitization and intranasal challenge with OVA. Intranasal administration with recombinant mouse IL-21 (rmIL-21) significantly reduced the number of sneezes, as well as the serum concentration of OVA-specific IgE, in comparison with that of untreated allergic mice. The rmIL-21 treatment also suppressed germline *Cε* transcription in the nasal-associated lymphoid tissues, which may have, at least partly, resulted from the up-regulation of Bcl-6 mRNA caused by IL-21. Local expression of IL-4, IL-5, and IL-13 was also inhibited by the intranasal cytokine therapy whereas, in contrast, the expression of endogenous IL-21 mRNA was induced by exogenous rmIL-21. Moreover, IL-21 acted on nasal fibroblasts to inhibit production of eotaxin. This novel function of IL-21 may be associated with the attenuation of eosinophil infiltration into nasal mucosa that was revealed by histopathological observation. These results indicated that IL-21 nasal administration effectively ameliorated allergic rhinitis through pleiotropic activities, *i.e.*, the prevention of IgE production by B cells and eotaxin production by fibroblasts. *The Journal of Immunology*, 2007, 179: 7157–7165.

The incidence of allergic rhinitis is dramatically increasing in industrial countries, causing important medical and social problems. Although antiallergic and antihistamine agents are commonly used for the clinical treatment, the effect is transient and they can suppress only terminal branches of allergic responses. A novel therapeutic strategy for allergic rhinitis that strikes at the root of its pathogenesis is desired.

In allergic states, the allergen triggers a Th 2-predominant immune response, leading to the generation of memory B cells that produce Ag-specific IgE. Subsequent exposure to the allergen provokes IgE-binding mast cells to release chemical mediators that cause allergic reactions. In this context, IgE production is the crucial step in the immunopathological cascade of allergic disorders and abrogation of this process should be quite important for the artificial control of allergy, although therapeutic means that achieve IgE suppression have not been clinically available to date.

In the meantime, allergic diseases are also characterized by the propagation and infiltration of eosinophils. Suppression of eosinophil infiltration may also provide a feasible method to treat allergy, although this concept still remains controversial (1–4).

IL-21 is a member of the type I cytokine family with significant sequence homology to IL-2, IL-4, and IL-15 (5). The IL-21 receptor complex is composed of two subunits; IL-21R has sequence

homology with the β component of the IL-2 receptor complex while the common γ -chain is shared by the cell surface receptor complexes for IL-2, IL-4, IL-7, IL-9, and IL-15 as an essential signal transducing subunit (5). IL-21 or IL-21R null mutant mice develop normally and are fertile; they do not develop any apparent disease (6, 7).

Activated CD4⁺ T cells produce IL-21, although conflicting results have been reported as to whether this cytokine is produced by Th1 or Th2 cells (8, 9). IL-21 exerts pleiotropic activities on a variety of target cells, alone or in combination with other cytokines (10, 11). Several studies have indicated that IL-21 regulates the differentiation, growth, and activation of Th1 (9, 11–13), Th2 (14), and CTL (15–17) as well as NK cells (11), whereas myeloid cells, including dendritic cells (18, 19) and macrophages (14, 20), are also stimulated by this cytokine (21).

With regard to B cells, IL-21 may provide both positive and negative regulatory control, depending on the conditions of stimulation. IL-21 potently facilitates the proliferation of B cells after engaging CD40 (22–24) or BCR (23–25), whereas the B cell proliferation promoted by LPS or CpG DNA is inhibited by IL-21 (23, 24). IL-21 also elicits the differentiation of naive or memory B cells into postswitch Ag-secreting plasma cells (26, 27) in combination with BCR and CD40 stimuli (27), while IL-21 induces class switch recombination (CSR)³ to IgG1 and IgG2a in human B cells that are activated with anti-CD40 (28). IL-21 transgenic mice exhibited elevated serum IgM and IgG1 levels (26), while IL-21 and IL-21R gene-deficient mice showed low IgG1 and high IgE production after immunization (6, 7). Other reports indicated that

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Received for publication January 30, 2007. Accepted for publication August 27, 2007.

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¹ This study was supported by a grant-in-aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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³ Abbreviations used in this paper: CSR, class switch recombination; EPO, eosinophil peroxidase; *i.n.*, intranasal; *m*, mouse (prefix); NALT, nasal-associated lymphoid tissue; *rm*, recombinant mouse (prefix).

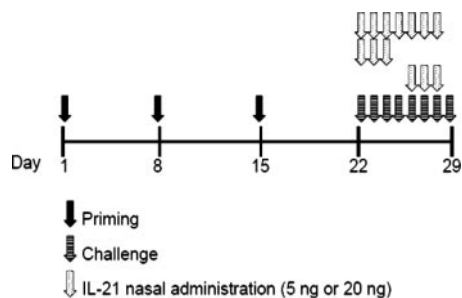


FIGURE 1. Protocols for sensitization, challenge, and rmIL-21 administration. Allergic mice were prepared by i.p. immunization with OVA plus alum on days 1, 8, and 15 (priming) followed by daily i.n. challenge with OVA solution on days 22–29. Some groups of mice were treated with daily i.n. rmIL-21 administration on days 22–29, 22–24, or 26–28. Normal (non-allergic) mice were given only the last i.n. challenge on day 29.

IL-21 induces the apoptosis of B cells under certain circumstances (24, 29).

Some previous studies reported contrary results as to whether IL-21 enhances or suppresses IgE production by B cells. The mice deficient for the IL-21R (6) or the IL-21 (7) gene exhibited enhanced IgE production in response to Ag immunization. IL-21 decreased serum IgE levels when injected into immunized mice, while the IgE production by LPS plus IL-4-stimulated murine splenic B cells was suppressed by IL-21 in culture through the inhibition of germline C ϵ transcription (30). In contrast, Wood et al. (31) reported that IL-21 augmented IgE production by unfractionated PBMC or B cells driven by anti-

CD40 plus either IL-4 or IL-13, whereas IgE secretion by PBMC was blocked by IL-21 when PHA plus IL-4 were used as the stimuli. Caven et al. (32) showed that IL-21 elevated IgE production and the proliferation of IgE-producing B cells when the cytokine was added to PBMC or purified tonsillar B cells provoked by anti-CD40 and IL-4 and that this effect was synergized with IL-10, whereas IgE production was either enhanced or diminished by IL-21 depending on the cell density in case murine B cells were cultured with anti-CD40 and IL-4 with or without IL-21. Pene et al. (33) reported that IL-21 did not inhibit IL-4-induced germline C ϵ transcription in purified human peripheral blood and spleen B cells but down-regulated IgE production through the induction of IFN- γ in an IL-21R genotype-dependent manner.

In this context, it is intriguing to ask whether the administration in vivo of IL-21 suppresses IgE production and allergic symptoms in allergic model mice. In this report we used allergic rhinitis mice and administered them with IL-21 in an attempt to analyze the in vivo effects of IL-21 on allergic disease. We further analyzed part of the molecular basis of the antiallergic activity by focusing on germline C ϵ transcription in nasal-associated lymphoid tissue (NALT) as well as eotaxin expression in nasal fibroblasts.

Materials and Methods

Mice

Female BALB/c mice (6- to 8-wk-old) were purchased from Shimizu Laboratory Suppliers. The mice received humane care in accordance with the institutional guidelines of the Kyoto Prefectural University of Medicine

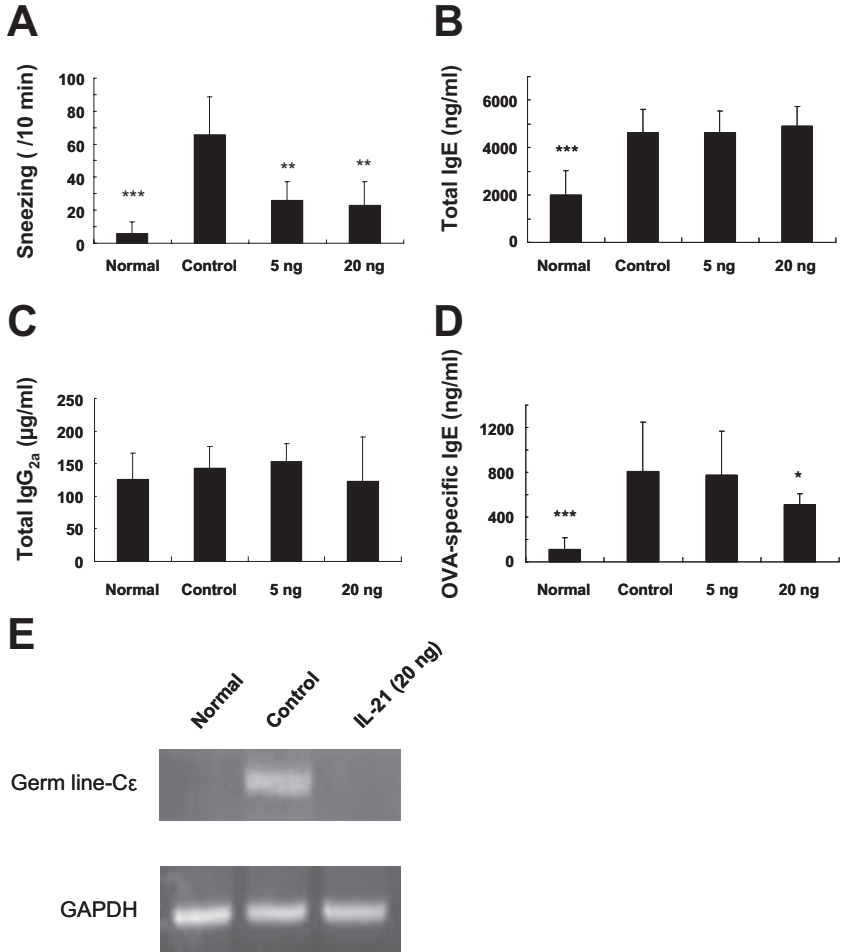
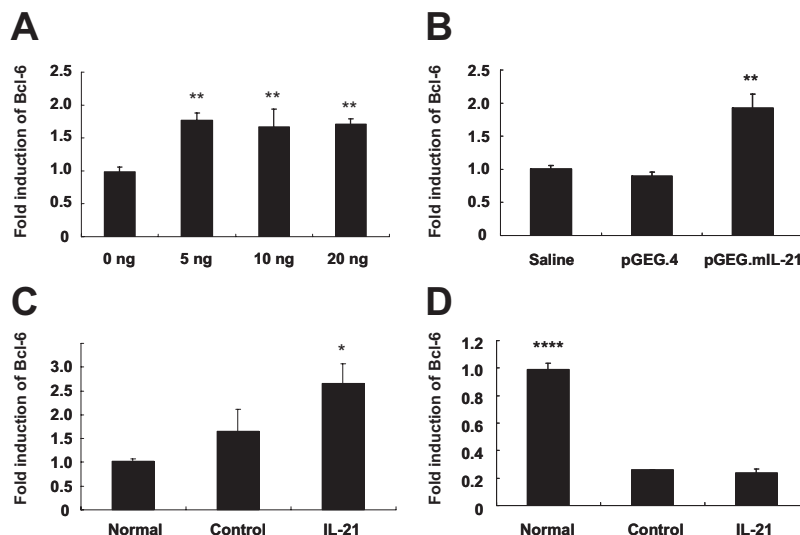


FIGURE 2. Effects of rmIL-21 on OVA-induced allergic reaction and Ig concentrations. Mice were prepared as in Fig. 1, where rmIL-21 treatment was performed at doses of 5 or 20 ng on days 22–29. **A**, Sneezes were counted for 10 min after the last challenge. **B–D**, Mice were killed 2 h after the last challenge, and the concentrations of total IgE (**B**), total Ig G_{2a} (**C**), and OVA-specific IgE (**D**) in the sera were evaluated by ELISA. Each value represents the mean $\pm$ SD of six mice in each group. **E**, Total RNA was extracted from the NALT cells of mice 2 h after the last challenge, and RT-PCR was performed using the indicated primers. *, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.0005$ vs control group.

FIGURE 3. Induction of Bcl-6 by rmIL-21. *A*, Splenic B cells were cultured with rmIL-21 at the indicated doses for 6 h. Total RNA was prepared from B cells and subjected to real-time RT-PCR using a pair of primers corresponding to the Bcl-6 sequence. *B*, Mice were i.v. injected with saline or 25 μ g of either pGEG.4 or pGEG.mIL-21 as indicated. Twelve hours later, total RNA was prepared from enriched splenic B cells and subjected to real-time RT-PCR as in *A*. *C* and *D*, Normal, allergic, and rmIL-21-treated allergic mice were prepared as in Fig. 1, and rmIL-21 treatment was given at a dose of 20 ng on days 22–29. Two hours after the last challenge, total RNA was prepared from the NALT (*C*) and spleen (*D*), and subjected to real-time RT-PCR to detect Bcl-6 mRNA as in *A*. Shown are means $\pm$ S.D. of the fold increase of Bcl-6 mRNA levels. Each value represents the mean $\pm$ SD of six mice in each group. *, $p < 0.05$; **, $p < 0.005$; and ****, $p < 0.00005$ vs control group.



(Kyoto, Japan). These mice were maintained in horizontal laminar flow cabinets and provided sterile food and water in a specific pathogen-free facility.

Nasal allergic model and recombinant mouse (rm) IL-21 administration

According to published procedures (34), mice were administered i.p. 0.5 mg/ml OVA (fraction V; Sigma-Aldrich) and 20 mg/ml Al(OH)₃ (Wako Pure Chemical Industries) in saline at a dosage of 0.2 ml/mouse. The sensitization was repeated three times at weekly intervals (days 0, 7, and 14) followed by daily injections of OVA solution (40 mg/ml in saline) into the nostrils (0.02 ml/mouse) on days 21–29 (challenge) (Fig. 1). Some groups of mice were administered 5 or 20 ng of rmIL-21 (R&D Systems) into the nostrils daily on days 22–24, 26–28, or 22–28 (rmIL-21-treated allergic mice), whereas another group of mice were not treated with the cytokine (control mice). As a negative control, a group of mice received neither

sensitization nor challenge treatment, except for the last challenge on day 29 (normal mice). Nasal symptoms were evaluated by counting the number of sneezes during 10 min immediately after the last OVA intranasal (i.n.) provocation on day 29.

Plasmid vectors and gene transfer

The plasmids pGEG.4 and pGEG.mIL-21 were described previously (10, 15). They were purified using Qiagen Maxi Prep EndoFree kits (Qiagen). The in vivo gene transfer was performed as described (17). Briefly, BALB/c mice were i.v. injected via the tail vein with 25 μ g of plasmid dissolved in 1.6 ml of saline. The injection was completed within 5 s.

Splenic B cell preparation

Spleens from female BALB/c mice were homogenized in RPMI 1640 medium. Spleen cells were separated into enriched populations of B cells

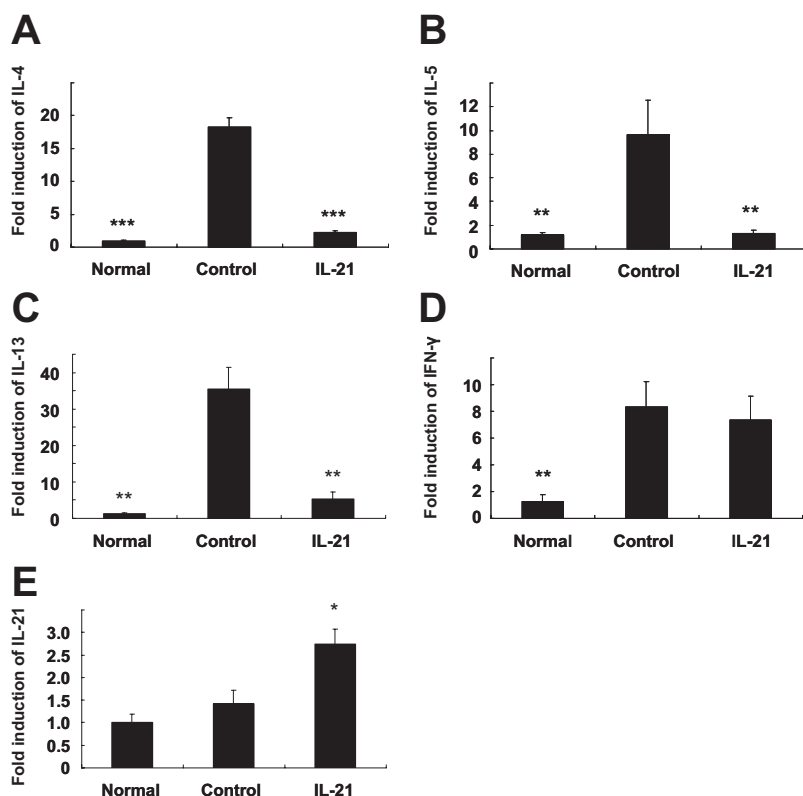


FIGURE 4. Cytokine expression profiles in the nasal tissue. Normal, allergic, and rmIL-21-treated allergic mice were prepared as in Fig. 1, and rmIL-21 treatment was given at a dose of 20 ng on days 22–29. Total RNA was extracted from the NALT, and real-time RT-PCR was performed to detect mRNA of the indicated cytokines. Each value represents the mean $\pm$ SD of seven mice in each group. *, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.00005$ vs control group.

using the MACS magnetic separation system (Miltenyi Biotec) according to the manufacturer's protocol.

NALT cell preparation

After exsanguinations of the mice, the fore teeth were cut off. The lower jaw and cheek muscles were removed, and NALT was exposed by carefully peeling away the plate. NALT localized on the posterior part of the plate was teased out with syringe needles in ice-cold RPMI 1640 culture medium supplemented with 10% FBS, and then the cells were passed through a 70- μ m Falcon cell strainer. RBC were lysed in Tris-buffered ammonium chloride solution (0.83% NH_4Cl and 20 mM Tris/Cl).

Fibroblast culture

Embryonic fibroblasts were prepared from BALB/c mouse embryos at the gestational age of day 16. Briefly, the heads and internal viscera were removed and the carcasses were finely minced with scissors. The cells were plated onto tissue culture dishes. After 3 to 4 days, when the growth of fibroblasts was established, embryo fragments were removed. Embryonic fibroblasts were maintained as a monolayer culture, and cells of the second or later passages were used. Primary cell lines were also established from the extirpated nasal mucosa and cartilage of BALB/c mice as described above. Fibroblasts were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in 5% CO_2 and 95% humidified air (standard conditions). Fibroblasts were plated at a density of 4×10^5 cell in 6-well tissue culture plates and cultivated for 24 h in the presence or absence of 10 ng/ml rmIL-4 (35, 36) and/or 20 ng/ml rmIL-21 before the culture supernatant was collected.

Flow cytometric analysis

Primary nasal fibroblasts were detached from culture dishes using a non-enzymatic cell dissociation solution and then incubated for 15 min at 4°C in the presence of anti-mouse IL-4R (purified rat monoclonal IgG2b clone 129801; R & D Systems) or anti-mouse (m) IL-21R (purified rat monoclonal IgG1 clone 155502 (R & D Systems)). After washing the samples were incubated with FITC-conjugated goat anti-rat IgG (Serotec). Following further washing, the cells were analyzed by FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences).

ELISA

Sera were collected from mice 2 h after the last challenge. Concentrations of total IgE, total IgG2a, and OVA-specific IgE were evaluated using corresponding ELISA kits purchased from the Morinaga Institute of Biological Science, Bethyl Laboratories, and Dainippon Sumitomo Pharma, respectively. The levels of eotaxin-1 in the culture supernatants were also measured by ELISA with eotaxin-1-specific ELISA kits (R&D Systems). The sensitivity of the eotaxin-1 detection was 3 pg/ml.

Histology

The head of each mouse was excised and immersed in 10% neutral buffered formalin. After fixation, the heads were decalcified in 8.8% formic acid for 6 days. The specimens were embedded in paraffin, coronally sectioned at a thickness of 3 μ m, and the resultant coronal nasal sections were stained with H&E. Luna staining was also performed to detect eosinophils in the nasal mucosa.

RT-PCR

NALT was obtained as described above and RNA was prepared using Isogen solution (Nippon Gene). RT-PCR was performed to detect germline C ϵ transcripts using a pair of forward (5'-GCACAGGGGGCAGAAGAT-3') and reverse (5'-CGTTGAATGATGGAGGAT-3') primers as described elsewhere (37). RT-PCR for GAPDH mRNA was also performed to control the sample-to-sample variation in RNA isolation and integrity by using a pair of forward (5'-CGTCCGTAGACAAAATGGT-3') and reverse (5'-CCCTGTTGCTGTAGCCGTAT-3') primers. The PCR conditions were as follows. For amplification of the germline C ϵ transcript sequence, predenaturation at 95°C for 1 min was followed by 32 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 1 min. For amplification of the GAPDH cDNA sequence, predenaturation at 95°C for 1 min was followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 1 min. PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Real-time RT-PCR

RNA was prepared from the NALT or cultured fibroblasts as described above and reverse transcribed using an Omniscript kit (Qiagen) accord-

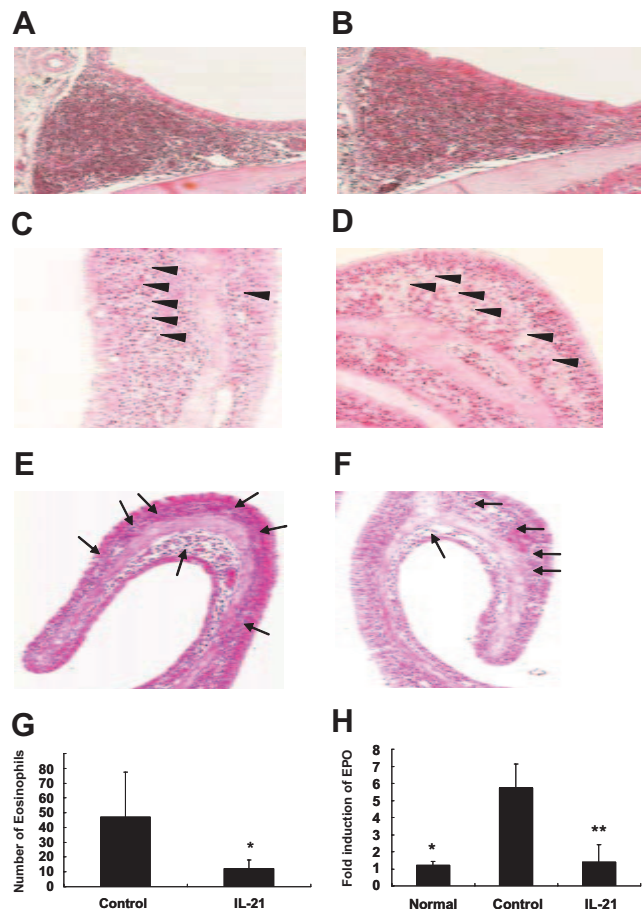


FIGURE 5. Histological examination of NALT and nasal mucosa treated with rmIL-21. Mice were sensitized and challenged with the allergen as in Fig. 1. *A–F*, A group of mice were given daily administrations with 5 ng of rmIL-21 on days 22–28 (*B*, *D*, and *F*), whereas the other group of mice was not treated with the cytokine (*A*, *C*, and *E*). Two hours after the last challenge, NALT (*A* and *B*) and nasal mucosa (*C–F*) tissues were dissected and fixed with formalin. After decalcification, the specimens were embedded in paraffin and sliced into sections that were subsequently stained with HE (*A*, *B*, *E*, and *F*) or Luna (*C* and *D*). The arrowheads in *C* and *D* indicate the infiltration of eosinophils. The arrowheads in *E* and *F* represent the multilayered epithelium while the arrows in *E* and *F* indicate the infiltration of inflammatory cells. Original magnification was $\times 400$. *G*, The number of eosinophils infiltrating the nasal mucosa was counted based on the Luna-stained sections as in *E* (mean $\pm$ SD, $n = 5$ fields at a magnification of $\times 400$). *H*, Total RNA was prepared from the nasal tissues of mice from the normal, allergic, and IL-21 (20 ng)-treated groups, and subjected to real-time RT-PCR using the primers/probes specific for EPO. Each value represents the mean $\pm$ SD of seven mice in each group. *, $p < 0.05$; **, $p < 0.005$ vs control group.

ing to the manufacturer's directions. The resultant cDNA was subjected to the real-time PCR using TaqMan Assay-on Demand gene expression primer/probe sets (Applied Biosystems) for β -actin (assay identifier Mm00607939_s1), Bcl-6 (assay identifier Mm 00477633_A1), eotaxin-1 (assay identifier Mm00441238_m1), eotaxin-2 (assay identifier Mm00444701_m1), and eosinophil peroxidase (EPO) (assay identifier Mm00514768_m1) cDNA sequences. Cytokine gene expression was also examined by real-time RT-PCR using primers for mIL-4 (assay identifier Mm00445259_m1), mIL-5 (assay identifier Mm00439646_m1), mIL-13 (assay identifier Mm00434204_m1), mIL-21 (assay identifier Mm00517640_m1), and mIFN- γ (assay identifier Mm00801778_m1).

Statistical evaluation

Student's *t* test was used for ELISA and comparison of the number of sneezes between groups. Data are presented as mean values $\pm$ SD. $p < 0.05$ was considered statistically significant.

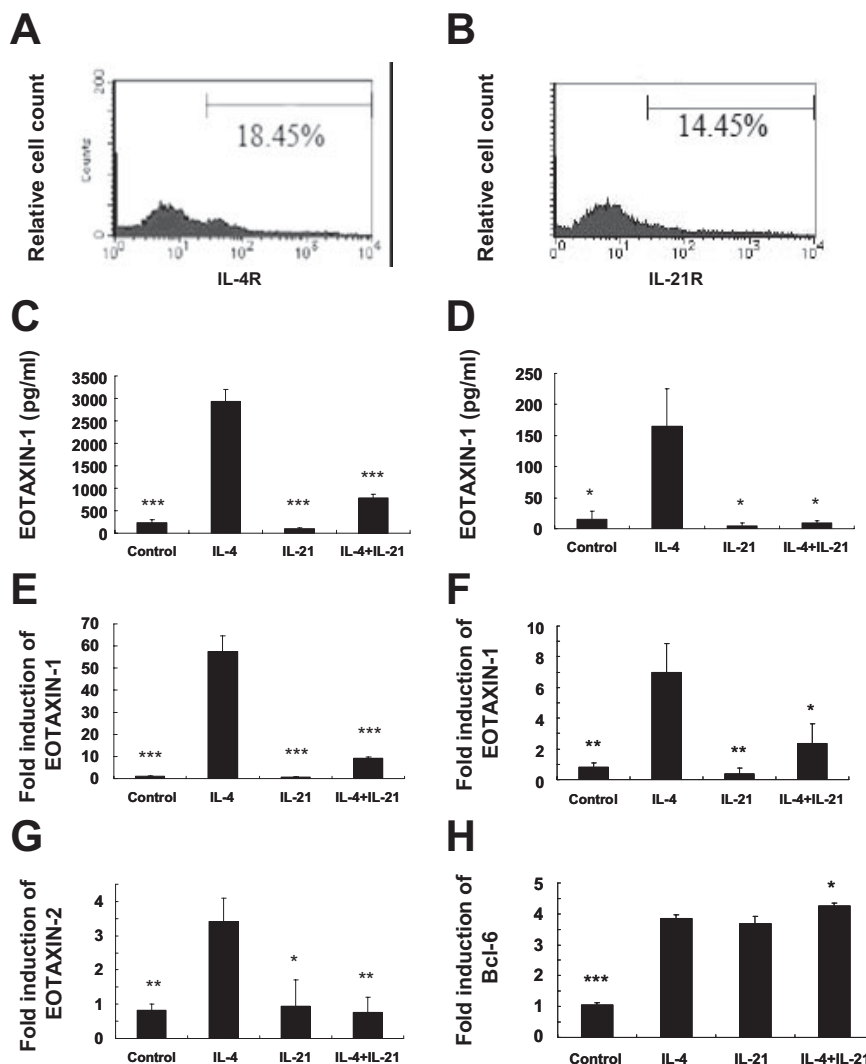


FIGURE 6. IL-21 down-regulated eotaxins that were otherwise induced by IL-4 in fibroblasts. Primary fibroblast cultures were established from nasal mucosa of adult mice (A, B, D, F, and G) or from mouse embryos at the gestational age of day 16 (C and E). A and B, Cells were stained with the indicated Abs and analyzed by flow cytometry. The numbers represent proportions of positive cells in each histogram. C and D, cells were cultured with rmIL-4 and/or rmIL-21 for 24 h and the culture supernatant was subjected to ELISA to measure eotaxin-1 concentrations. E–H, total RNA was isolated and served for real-time RT-PCR using the primers/probes specific for eotaxin-1 (E and F), eotaxin-2 (G), or Bcl-6 (H). Each value represents the mean $\pm$ SD ($n = 3$ in each group). *, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.0005$ vs IL-4 group.

Results

rmIL-21 administration improved symptoms of allergic rhinitis in mice

To examine whether i.n. administration with rmIL-21 has protective effects on allergic rhinitis, allergic mice were left untreated or treated with rmIL-21 at doses of 5 or 20 ng and the number of sneezes was counted after the last Ag challenge with OVA. The nontreated allergic mice showed a drastic increase in number of sneezes (65.6 ± 23.2 counts/10 min) compared with that in normal (nonsensitized) mice that had received only the last challenge immediately before the counting (5.8 ± 7.0 counts/10 min). The sneeze counts for rmIL-21-treated allergic mouse groups were 26.0 ± 11.1 counts/10 min (mice receiving 5 ng of rmIL-21) and 22.8 ± 14.4 counts/10 min (mice receiving 20 ng of rmIL-21), which were significantly smaller than that of the control (nontreated allergic) mice ($p < 0.005$, 5 ng vs control; $p < 0.005$, 20 ng vs control) (Fig. 2A). The results clearly indicate that i.n. rmIL-21 treatment significantly ameliorated allergic symptoms in this model system.

Serum concentration of OVA-specific IgE was reduced by rmIL-21 treatment

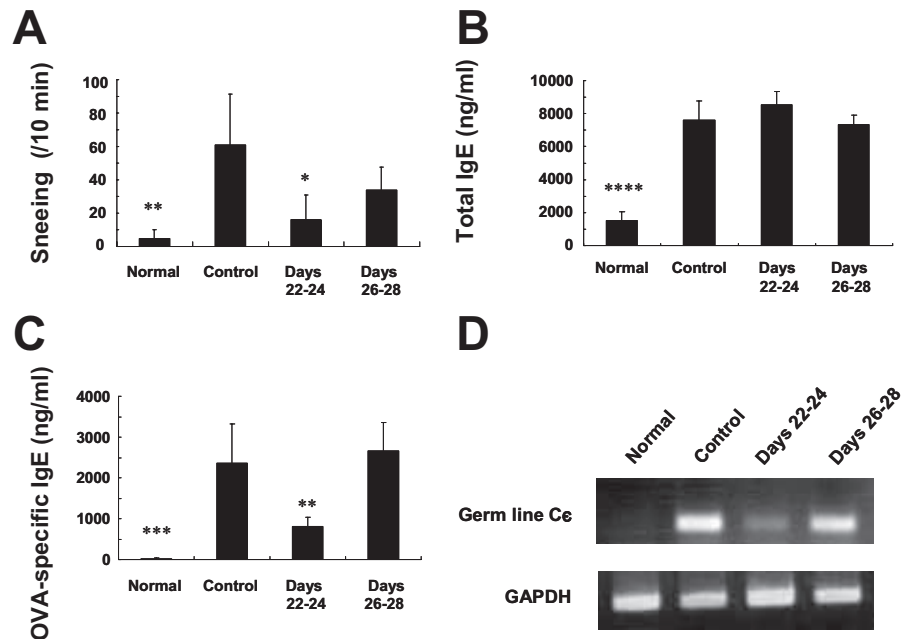
Next, we examined total IgE, total IgG2a, and OVA-specific IgE levels in the sera of mice. The immunization with OVA and alum increased serum levels of total ($p < 0.001$) and OVA-

specific ($p < 0.005$) IgE compared with those in normal mice (Fig. 2, B and D). The total IgE levels in the sera were not significantly affected by the treatment with rmIL-21, regardless of the doses of the cytokine (Fig. 2B). In contrast, rmIL-21 administration at a dose of 20 ng significantly reduced the serum concentration of OVA-specific IgE compared with that in nontreated allergic mice ($p < 0.05$), whereas a significant decrease in OVA-specific IgE level was not demonstrated after the treatment with 5 ng of rmIL-21 (Fig. 2D). The concentrations of serum IgG2a were comparable among all groups tested (Fig. 2C).

rmIL-21 hampered the induction of germline C ϵ transcript in NALT

To investigate the mechanism(s) of the IL-21-induced reduction in OVA-specific IgE, germline C ϵ transcript in the NALT was estimated by RT-PCR. As shown in Fig. 2E, nontreated allergic mice profoundly expressed the germline C ϵ transcript, a transcript that was not demonstrated in normal mice. In contrast, the administration of rmIL-21 almost completely abrogated the transcription of germline C ϵ . The results strongly suggest that B cells in NALT undergo a C ϵ class switch in the nasal allergic state, while IL-21 inhibits germline C ϵ transcription leading to a decrease in IgE production by NALT B cells (Fig. 2E).

FIGURE 7. Effective window for IL-21 treatment to suppress nasal allergy. Mice were prepared as in Fig. 1, where rmIL-21 treatment was performed on days 22–24 or 26–28. **A**, Sneezes were counted for 10 min after the last challenge. **B** and **C**, Mice were killed 2 h after the last challenge, and the concentrations of total (**B**) and OVA-specific (**C**) IgE in the sera were evaluated by ELISA. Each value represents the mean $\pm$ SD of six mice in each group. **D**, NALT was collected from the mice 2 h after the last challenge. RT-PCR analysis was performed to detect germline C ϵ transcript and GAPDH mRNA. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; and ****, $p < 0.00005$ vs control group.



Bcl-6 is transcriptionally up-regulated in the NALT after rmIL-21 stimulation

It has been shown that the Bcl-6 transcriptional factor is involved in germline C ϵ transcription (38, 39). Ozaki et al. (26) reported that Bcl-6 was up-regulated by IL-21 in cultured splenic B cells. Therefore, we examined whether Bcl-6 expression was augmented in vivo in the spleen and nasal lymphatic system in an attempt to examine the mechanism of IL-21-mediated suppression of the germline C ϵ transcript. Real-time RT-PCR analysis demonstrated that Bcl-6 mRNA was elevated ~2-fold in splenic B cells stimulated with IL-21 either in vitro or in vivo (Fig. 3, A and B). It was also demonstrated that Bcl-6 mRNA expression was significantly elevated in the NALT of allergic mice that received intranasal rmIL-21 administration (Fig. 3C), whereas Bcl-6 expression in the spleen was not influenced by the local rmIL-21 treatment (Fig. 3D).

rmIL-21 stimulation down-regulated Th2 cytokines

IL-4, IL-5, and IL-13 are important Th2 cytokines involved in allergic reaction, whereas IFN- γ is a typical Th1 cytokine. Real-time RT-PCR analyses were performed to investigate whether the expression of these cytokines in the nasal tissue is affected by the administration of IL-21. As shown in Fig. 4, A–D, IL-4, IL-5, and IL-13 mRNA declined in the nasal tissue of IL-21-administered mice, whereas the expression of IFN- γ was not influenced by IL-21.

We also evaluated endogenous IL-21 production in nasal tissue of normal, allergic, and rmIL-21-treated allergic mice. Real-time RT-PCR analyses revealed that IL-21 was not massively induced in the allergic state (Fig. 4E) but was remarkably up-regulated in the rmIL-21-treated group (Fig. 4D), suggesting that exogenous IL-21 induced the expression of endogenous IL-21 in the nasal tissue of allergic animals.

Pathological changes in nasal mucosa and NALT

Next, we analyzed the significance of morphological changes in rmIL-21-treated nasal and NALT tissues. After the last challenge with OVA, the tissues of allergic mouse displayed prominent changes including the conspicuous infiltration of eosinophils and

lymphocytes as well as submucosal fibrosis. The epithelium became multilayered. Acidophilic changes and individual cell necrosis were also found to be significantly increased in the submucosa (Fig. 5, C and E). In the NALT of allergic mice, small lymphocytes increased in number and accumulated to a high density (Fig. 5A). In contrast, the administration of rmIL-21 drastically prevented the infiltration of inflammatory cells and eosinophils in the submucosa, in which the development of fibrosis, acidophilic change, and necrosis were also normalized by the cytokine (Fig. 5, B, D, and F). The rmIL-21-mediated amelioration of eosinophilic infiltration was also confirmed by a significant decrease in the number of eosinophils (Fig. 5G) as well as by the reduction of EPO activity (Fig. 5H) in the submucosal tissue of the mice.

IL-21 also prohibited the accumulation of small lymphocytes in the NALT. These findings are consistent with the reduced allergic reaction after the cytokine treatment.

IL-21 prevented nasal fibroblasts from producing eotaxins

We investigated whether IL-21-mediated suppression of eosinophil infiltration into the nasal mucosa can be ascribed to the modulation of eotaxin production in nasal fibroblasts by the cytokine. Primary fibroblast cultures were established from nasal mucous as well as mouse embryos, and IL-21R expression on the cells was confirmed by flow cytometric analysis (Fig. 6B). The fibroblasts were then cultured in the presence or absence of rmIL-4 and rmIL-21, and the concentrations of eotaxin-1 in the culture supernatants were measured by ELISA. As shown in Fig. 6, C and D, rmIL-4 significantly induced the secretion of eotaxin-1 from the fibroblasts of both origins, which was clearly canceled by the addition of rmIL-21. Real-time PCR analysis also demonstrated that rmIL-21 drastically suppressed the eotaxin-1 mRNA that was otherwise induced by rmIL-4 (Fig. 6, E and F). Similarly, rmIL-4 prompted nasal mucosal fibroblasts to express eotaxin-2 mRNA, while the induction of the chemokine was almost completely impaired by the presence of rmIL-21 (Fig. 6G). These results strongly suggest that IL-21 counteracts the IL-4 effect on the nasal fibroblast in terms of production of eotaxins, which may partly explain the attenuation of eosinophil infiltration into allergic nasal mucosa by IL-21.

Intriguingly, IL-21 provoked nasal fibroblast to express Bcl-6 to a comparable extent as IL-4 (Fig. 6H).

Effect of IL-21 on allergic reactions was dependent on the timing of the administration

Next, we investigated whether the effect of IL-21 was dependent on the period and timing of administration. When two allergic mouse groups were administered with rmIL-12 on days 22–24 and 26–28 that corresponded to the earlier and later halves of the OVA challenge period, respectively (Fig. 1), the number of sneezes as well as OVA-specific IgE levels in the sera were significantly reduced in the former group whereas these parameters in the latter group were virtually comparable with those of the untreated allergic mice (Fig. 7, A and C). Any significant difference was demonstrated among the total IgE concentrations in the sera of these three groups (Fig. 7B).

We also examined whether the amount of germline C ϵ transcripts in the NALT also correlated with the timing of rmIL-21 treatment. The germline C ϵ transcription in the NALT of allergic mice was blocked by administration of rmIL-21 on days 22–24, whereas rmIL-21 failed to significantly affect this transcription when administered on days 26–28 (Fig. 7D).

Discussion

In the present study, we examined the effect of IL-21 treatment on allergic rhinitis in mice. Intranasal administration of rmIL-21 effectively ameliorated allergic symptoms and suppressed the production of allergen-specific IgE. As mechanisms of the antiallergic activity of IL-21 we found that the cytokine enhanced the expression of Bcl-6 in the NALT while inhibiting germline C ϵ transcription that was otherwise induced in the allergic state. The suppression of eotaxin expression may also contribute to the antiallergy outcome of the IL-21 treatment.

Although CSR was considered to take place in restricted areas, i.e., the germinal center, it was recently reported that B cells are capable of undergoing CSR to IgE in nasal mucosa when exposed to allergen (40–43). The spleen and the draining lymphoid tissue may differentially function with regard to IgE production in the murine Th2-biased responses induced by OVA/alum immunization. Ag-specific IgE is predominantly produced in the draining lymphoid tissue but not in the spleen, whereas the spleen serves as a major source for Ag-unidentified IgE (44). Therefore it should be important to control the production of Ag-specific IgE in the local area. In this respect, i.n. administration may be quite suitable for IL-21-based control of nasal allergy, providing an effective means to regulate specific IgE reactions in the nasal mucosa and its draining lymphatic tissue. Indeed, our results showed that OVA-specific IgE was significantly suppressed by the i.n. inoculation of 20 ng of rmIL-21 that probably affected draining lymphoid tissue while total IgE was virtually not affected by this treatment, suggesting that the local IL-21 treatment did not influence the production of non-specific IgE in the spleen (Fig. 2, B and D).

Treatment of mice with 20 ng of rmIL-21 almost completely inhibited germline C ϵ transcription while only partially decreasing OVA-specific IgE levels in serum (Fig. 2, D and E). This may be because the suppression of IgE CSR obstructs the generation of IgE-producing cells from progenitor IgM B cells but fails to affect the survival of and the IgE production by preexisting IgE B cells. In the meantime, treatment of mice with a low dose (5 ng) of rmIL-21 markedly suppressed sneezing while leaving OVA-specific IgE levels unchanged. This may suggest that this pleiotropic cytokine suppresses allergic reaction not only by inhibiting IgE CSR in B cells but also by acting on other target cells. We are currently investigating this issue in more detail.

It is noteworthy that rmIL-21 administration for only 3 days was as effective as the 7-day-regimen for the inhibition of allergic rhinitis, provided that the administration was initiated concurrently with the Ag challenge (Figs. 2 and 7), whereas the cytokine treatment alone at a later phase of Ag challenge failed to alleviate the allergic manifestation (Fig. 7). Therefore, the period of the initiation of IL-21 administration may be more important than the length of treatment for the cytokine control of allergy. These results may also indicate that the production of allergen-specific IgE was promptly and irreversibly directed by Ag exposure during the early term of the challenge so that the IL-21 medication starting on the 5th day of the challenge no longer canceled the proceeding of the reaction. Alternatively, it may take several days for the IL-21-mediated suppression of IgE CSR to result in the reduction of the Ag-specific IgE level to an extent insufficient to trigger the allergic reaction. Although this point should be clarified in future studies, the present results demonstrated that the rmIL-21 treatment effectively suppressed allergic rhinitis after sensitization of mice with the Ag (Figs. 1, 2, 5, and 7). This is an extremely important finding to consider regarding the i.n. clinical application of IL-21 medication for the treatment of allergic rhinitis patients in the future.

Another intriguing finding is that i.n. treatment of allergic mice with exogenous IL-21 resulted in a significant induction of endogenous IL-21 in nasal tissue (Fig. 4E). This may be consistent with recent reports showing that production of IL-21 can be regulated in an autocrine manner (45). The positive feedback regulation of IL-21 may have an important implication for the clinical application of this cytokine to patients with allergic diseases, because a relatively small dose of IL-21 may lead to stronger antiallergic action through expansion of the cytokine signal.

The Bcl-6 gene has been recognized as the proto-oncogene that is most frequently mutated in non-Hodgkin's lymphomas (46, 47). In Bcl-6 deficient mice, germinal center formation accompanying T cell-dependent immune responses is impaired, strongly suggesting that this transcriptional factor plays a prerequisite role in the development of germinal center B cells (48–50), while CD4⁺ Th cells preferentially differentiate into Th2, which induces fatal inflammatory diseases in Bcl-6 knockout mice (51, 52). In B cells, Bcl-6 binds to the Stat6 site in the I ϵ promoter in the Ig H chain locus and represses the IL-4-dependent induction of germline C ϵ transcripts, negatively modulating S μ to S ϵ CSR and IgE expression (38, 39). Consistent with the previous report showing that IL-21 up-regulated Blimp-1 and Bcl-6 in B cells in vitro (26), the present study demonstrated Bcl-6 induction in vivo in splenic (Fig. 3B) as well as in NALT B cells (Fig. 3C) in IL-21-treated animals, which may explain, at least partly, the mechanisms of the suppressive effect of IL-21 on allergen-specific IgE production.

Recent reports documented that IL-21 induced apoptosis in B cells that had undergone CSR to IgE (53). Although we demonstrated that IL-21 suppressed IgE CSR in NALT B cells of the diseased mice (Figs. 2E and 7D), apoptosis induction in ϵ B cells could partially be involved in the IL-21-mediated suppression of IgE.

In the meantime, IL-21 has been shown to down-regulate CD23, the low affinity Fc ϵ R (23), which may lead to the inhibition of IgE-dependent cytotoxicity and the phagocytosis of macrophages and eosinophils. This action may also participate in IL-21-mediated interference with allergic reactions.

The eotaxin family members are CC chemokines that exert a chemoattractive activity for eosinophils, while eotaxins also facilitate the differentiation of eosinophils as well as the rapid mobilization of eosinophils and their progenitors from the bone marrow (54, 55). Eotaxins have been shown to play pivotal roles in recruiting eosinophils to the nasal mucosa in allergic rhinitis, where

IL-4 provokes nasal fibroblasts to secrete eotaxins (36, 56–58), although there have been some contradictory reports on the roles of eotaxins in airway inflammation and allergy (1, 2, 59, 60). The IL-21R has been demonstrated on intestinal fibroblasts (61) as well as on macrophages and fibroblasts of the articular synovium of rheumatoid arthritis patients (62). The present study demonstrates for the first time that rhinal fibroblasts express IL-21R (Fig. 5B), and IL-21 drastically inhibited the production of eotaxin-1 and 2 in IL-4-stimulated nasal fibroblasts (Fig. 5D, F, and G). The inhibition of eotaxins may also contribute to IL-21-mediated attenuation of allergic reactions by preventing accumulation of eosinophils in the nasal mucosa (Fig. 4, D and F).

In conclusion, the present study has clarified the influence of exogenous IL-21 on the local allergic state in vivo. The results are consistent with our recent observation that systemic administration with rmIL-21 successfully alleviated peanut anaphylactic reactions in mice (T. Kishida, Y. Hiromura, M. Shin-Ya, H. Asada, H. Kuriyama, M. Sugai, A. Shimizu, Y. Yokota, T. Hama, J. Imanishi, Y. Hisa, and O. Mazda, unpublished data). Finally, it should be emphasized that i.n. treatment with IL-21 may offer novel and powerful prophylactic and/or therapeutic moieties to treat allergic rhinitis by directly interfering with allergen-specific IgE production and local eotaxin production, which are key events in the immunopathological cascade of the allergic reaction, rather than by nonspecifically suppressing immune responses or antagonizing chemical mediators as did conventional antiallergic therapeutic intervention.

Acknowledgment

We thank Dr. Wilfred T. V. Germeraad (University of Maastricht, Maastricht, The Netherlands) for critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

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