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IL-10 controls Th2-type cytokine production and eosinophil infiltration in a mouse model of allergic airway inflammation

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ABSTRACT

Interleukin-10 was originally described as a factor that inhibits cytokine production by murine Th1 clones. Recent studies have since shown that IL-10 can also downregulate Th2 clones and their production of IL-4 and IL-5. Because of its immuno-suppressive properties, IL-10 has been suggested as a potential therapy for allergic inflammation and asthma. However, the pathophysiological role of IL-10 *in vivo* has not been clearly elucidated. We investigated the effects of IL-10 administration on the production of IgE, cytokine and allergen-induced Th2 cytokine production as well as its effects on eosinophilic inflammation. We established GATA-3/TCR double transgenic (GATA-3/TCR-Tg) mice by crossing GATA-3 transgenic mice with ovalbumin (OVA)-specific TCR transgenic mice; these mice were then sensitized using an intraperitoneal injection of OVA adsorbed to alum and challenged with the intratracheal instillation of an allergen. When GATA-3/TCR-Tg mice sensitized with OVA and alum were injected with C57-IL-10 cells before OVA inhalation, the levels of IL-5, IL-13, and IL-4 decreased by 40–85% and number of eosinophils decreased by 70% ($P < 0.03$) in the murine bronchoalveolar lavage fluid (BALF). These results suggest that IL-10 plays an important role downstream of the inflammatory cascade in the Th2 response to antigens and in the development of BALF eosinophilia and cytokine production in a murine model of asthma. These immunosuppressive properties in animal models indicate that IL-10 could be a potential clinical therapy for the treatment of allergic inflammation.

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Introduction

Interleukin-10 is an important immunosuppressive cytokine. For example, IL-10 inhibits the production of proinflammatory cytokines such as IL-1 α , IL-1 β , IL-6, IL-8, IL-12, TNF- α , and GM-CSF by monocytes and cytokines such as IL-1 β , IL-6, IL-8, TNF- α , IFN- γ , and GM-CSF by Th1 cells (Moore et al. 2001; de Waal Malefyt et al. 1991; Fiorentino et al. 1991; Del Prete et al. 1993; D'Andrea et al. 1993). IL-10 downregulates the expression of MHC class II molecules and co-stimulatory molecules on monocytes/macrophages and dendritic cells (Moore et al. 2001; Buelemis et al. 1995; Ding et al. 1993). IL-10 suppresses the production of cytokines by T cells through direct (Taga et al. 1993; de Waal Malefyt et al. 1993; Schandene et al. 1994) and indirect mechanisms

(Fiorentino et al. 1991; Del Prete et al. 1993), although the direct effect on T cells has been confirmed only in humans. These *in vitro* studies raise the possibility that IL-10 could be beneficial for the treatment of various immune diseases *in vivo*. For example, both in human subjects and in animal models, treatment with IL-10 improved inflammatory bowel disease (Barbara et al. 2000; Steidler et al. 2000; van Deventer et al. 1997) and psoriasis (Asadullah et al. 1998, 2001), which are mainly mediated by Th1 immune responses. In individuals who have received bee venom-specific immunotherapy or who have recently received multiple bee stings, the upregulation of endogenous IL-10 may play an important role in preventing anaphylaxis (Akdis et al. 1998). However, thus far, the inhibitory effects of IL-10 have been analyzed almost always *in vitro*, and the mechanisms of inhibition *in vivo* have not been well examined. Therefore, the clinical application of IL-10 for the treatment of immune disorders remains difficult.

Recently, the role of IL-10 in the generation of regulatory T cells has been highlighted. The chronic activation of CD4⁺ cells in the presence of IL-10 *in vitro* reportedly induces the development of T-regulatory (Treg) 1 cells, which produce the majority

Abbreviations: OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; AHR, airway hyperreactivity; i.v., intravenously.

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of IL-10 (Grpux et al. 1997). Akbari et al. reported that the stimulation of CD4⁺ cells with IL-10-producing DCs, which were generated after exposure to inhaled antigen (Ag), induces IL-10-producing Treg cells. These reports suggest that IL-10-producing Treg cells develop in the presence of IL-10 (Akbari et al. 2001, 2002). However, whether *in vivo* IL-10 alone can induce the generation of Treg cells has not been fully clarified. Bronchial asthma is a chronic disorder characterized by eosinophilic airway inflammation, mucus hypersecretion, and an increase in airway hyperreactivity (AHR) (Bochner et al., 1994). The process of airway inflammation involves various types of cells, such as eosinophils, mast cells, T lymphocytes, and DCs (Humbert et al. 1999; Möller et al. 1996). Because of its immunosuppressive effects, IL-10 is expected to downregulate the airway immune response. In humans, the relative underproduction of IL-10 by alveolar macrophages and in the sputum of patients with asthma has been reported (Takanashi et al. 1999), suggesting an essential role for IL-10 in airway inflammation, and AHR is not well understood in either humans or animal models. Cumulative evidence suggests that Th2 cytokines play important roles in inducing the eosinophilic airway inflammation and bronchial hyperresponsiveness (BHR) that are characteristic of asthma (Virchow, 1996). The expression of Th2 cytokines is increased in allergic inflammation and in asthma (Virchow, 1996). Animals with low levels of interleukin-4 and interleukin-5 exhibit reduced eosinophilia and BHR (Egan et al. 1995; Foster et al. 1996; Mauser et al. 1995), and IL-5 overexpression enhances eosinophilia and BHR (Lee et al. 1997). In contrast, the Th1 cytokine interferon (IFN)- γ inhibits Th2 responses and attenuates allergen-induced eosinophilia, thereby inhibiting the onset of allergic asthma (Coyle et al. 1996).

The pathophysiological role of the Th2 cytokine IL-10 in asthma has not been clearly elucidated. Interleukin-10 was originally described as a factor that inhibits cytokine production from murine Th1 clones (Fiorentino et al. 1989). Subsequent studies showed that IL-10 also downregulates Th2 clones and their production of IL-4 and IL-5 (Borish 1999). In addition, IL-10 exerts a wide variety of effects on other immune cells, such as the stimulation of B cell differentiation and Ig secretion (Moore et al. 1993). The actual biological effects of IL-10 on immune responsiveness vary considerably (Lelievre et al. 1998). However, adult mice deficient in IL-10 develop CD4⁺ T cell-dependent, IFN- γ -mediated enterocolitis (Rennick et al. 1997).

The roles of IL-10 in allergic inflammation and airway hyperresponsiveness (AHR) are apparently contradictory. Some investigators have found that IL-10 mRNA expression is reduced both in peripheral blood mononuclear cells and in lymphocytes from bronchoalveolar lavage (BAL) (Borish 1999), whereas others have demonstrated elevated levels in asthmatic patients (Robinson et al. 1996; Tillie-Leblond et al. 1994; Magnan et al. 1998). Nevertheless, its evident immunosuppressive properties in animal models and *in vitro* suggest that IL-10 could function as a therapeutic strategy for treating allergic inflammation and asthma.

We examined the role of IL-10 in regulating the development of allergic inflammation and AHR in a mouse model of eosinophilic airway inflammation and allergen-driven alterations in airway function. We crossed GATA-3 transgenic (Tg) mice with OVA-specific TCR transgenic mice to produce double (GATA-3/TCR)-Tg mice, which were then sensitized with OVA plus alum. GATA-3/TCR-Tg double transgenic mice were used because these mice exhibited a significant response against OVA. When IL-10 was administered prior to OVA-restimulation in mice that had already been sensitized with OVA and alum, the production of IL-4 and IL-13 cytokine and the resulting eosinophil count in the BALF were inhibited remarkably. However, the OVA-specific and total IgE antibody titers were also not suppressed.

Our findings implied that IL-10 plays a major role in the regulation of airway function downstream of the inflammation cascade. IL-10 may be a useful inhibitory cytokine for the prevention of allergic disease.

Materials and methods

Retroviral vector and transduction of C57 cells

Murine IL-10 complementary DNA (cDNA) was kindly provided by Dr. Kawai (Toho University School of Medicine). The cDNA was inserted into the Bam HI-Hpa I sites of a retroviral vector (pLXN) using the Retro-X system (Clontech, Tokyo, Japan), and the resulting recombinant vector was designated pLXSN-IL-10. Empty virus (EV) vectors or pLXSN-IL-10 were then transfected into a packaging cell line (PT67) to generate the retroviruses LXSN-EV and LXSN-IL-10, respectively. The retroviral titers were estimated using NIH3T3 cells according to the manufacturer's instructions (Clontech) and were both found to be $\sim 1.0 \times 10^5$ colony forming units (CFU)/mL. Finally, C57 cells (5.0×10^4) were infected with either LXSN-IL-10 or LXSN-EV to generate the cell lines C57-IL-10 and C57-EV, respectively (Murakami et al. 2002). We maintained the C57L, C57-EV and C57-IL-10 cell lines in 10% FBS (ICN Biomedical, OH, USA) containing Dulbecco's modified Eagle's Medium (DMEM; Sigma) at 37 °C, 5% CO₂ at a cell density of 7×10^3 – 7×10^4 /cm². The primers used to determine the IL-10 gene expression levels of these cells extracts, to confirm the levels using RT-PCR, and to detect IL-10 were as follows:

IL-10-(forward: F): 5'-TGCTGCCTGCTCTTACTGACTG-3',
IL-10-(backward: B): 5'-CCTGCTCCACTGCCTTGCTCTTAT-3';
IL-10-neo^F-F: 5'-TGCTGCCTGCTCTTACTGACTG-3',
IL-10-neo^F-B: 5'-TCCCGCTCAGAAGAACTCGTCAAG-3';
neo^F-F: 5'-GGCGCCCGGTTCTTTTGTCA-3',
neo^F-B: 5'-TCCCGCTCAGAAGAACTCGTCAAG-3'; and
GAPDH-F: 5'-TTGGATCCGTCAGTGCCGGCCTCGTCTCATAG-3',
GAPDH-B: 5'-TTCTCGAGGACCCTTTTGGACCACCCTTCAG-3'.

After amplification using an IL-10-specific oligoprobe, the gene was detected using Southern hybridization with the following probes:

IL-10-probe, 5'-GTTGCCAAGCCTTATCGGAAATG-3';
neo^F-probe, 5'-CTACCTGCCCATTCGACCACCA-3'; and
GAPDH-probe, 5'-ACGGCAAGTCAATGGCACAG-3'.

Mice

Hemizygous GATA-3 transgenic (Tg) mice were crossed with the homozygous type of another transgenic mouse for the TCR-recognizing OVA_{323–339} peptide in the context of I-A^d restriction. These double-transgenic and non-GATA-3 TCR transgenic mice were designated as GATA-3/TCR-Tg and WT/TCR-Tg mice, respectively, in this study. We previously reported the characteristics of GATA-3/TCR-Tg and WT/TCR-Tg mouse (Tamauchi et al. 2004). C57BL/6 mice were obtained from Clea (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions, and the experiments were performed according to the Guidelines for Animal Experimentation published by the Japanese Association for Laboratory Animal Science (1987).

Bioactive measurement of IL-10 in the C57-IL-10 cell line

We cultured supernatant from the C57-IL-10 cell line with uric acid crystal and the macrophage cell line J774.1, derived from a

mouse, and measured the quantity of TNF- α production in the culture supernatant 24 h later using an ELISA kit (Biosource, California, USA).

Examination of the presence of the C57-IL-10 cell line in lung tissue

To measure the quantity of IL-10 in murine BALF after treatment with C57-IL-10 cells and the exogenous IL-10 gene expression level in lung tissue, we intravenously (i.v.) injected 5×10^5 C57-IL-10 cells into each mouse and measured IL-10 production over time.

The production of IL-10 was measured using an ELISA kit (Endogen Inc., MA, USA). Lung tissue was resected using a 27-gauge needle then frozen and crushed; the RNA was then extracted using ISOGEN (Japan Gene, Tokyo) according to the manufacturer's manual. We measured the mRNA expression using real-time RT-PCR with a 7700 ABI Prism (Applied Biosystems, CA, USA). The RT-PCR response was determined using a QuantiTect(R) SYBR(R) Green PCR Kit (Qiagen, CA, USA). RT-PCR for IL-10, IL-10-pLXSN and GAPDH consisted of 15 s of denaturation at 94 °C, 30 s of annealing at 57 °C, and 30 s of extension at 72 °C for 45 cycles.

The following primers were used:

IL-10-(real-time: r) F: 5'-TGAATTCCTGGGTGAGAAG-3',
IL-10-rB: 5'-TGGCCTTGTAGACACCTTGG-3';
IL-10-pLXSN-rF: 5'-AGCTCCAAGACCAAGGTGTC-3',
IL-10-pLXSN-rB: 5'-GGGGACTTTCCACACCTAA-3'; and
GAPDH-rF: 5'-TGCACTGGCAAAGTGGAGATT-3',
GAPDH-rB: 5'-ATTTGGCGTGAGTGGAGTCAT-3'.

The expression level was calculated using the $\Delta\Delta C_t$ (the comparative threshold cycle) method relative to the production of murine GAPDH.

Immunization and challenge protocol

We immunized GATA-3/TCR-Tg and WT/TCR-Tg mice intraperitoneally (i.p.) with OVA (50 μ g) plus alum (1 mg) on days 0 and 7. The immunized mice were challenged by exposure to aerosolized 1% OVA in saline for 20 min to determine the cytokine production and the infiltration of eosinophils in the bronchoalveolar lavage fluid (BALF) *in vivo*. At 24 h after the last inhalation challenge, BALF (1 mL of PBS) was collected (Fig. 1, protocol a). In the second group, C57-IL-10 was administered i.v. to GATA-3 mice at the time of sensitization using OVA and alum (Fig. 1, protocol b). In the third group, GATA-3 mice were injected (i.v.) with C57-IL-10 on the 12th day and were subjected to OVA inhalation using a nebulizer on sensitization day 14 after having been sensitized with OVA and alum (Fig. 1, protocol c). BALF was collected as described above. The BALF was stored at -80°C before the cytokine assays.

Estimation of cytokines and eosinophils in BALF

To determine the cytokine concentrations in BALF, contaminating cells were removed by centrifugation at 1200 rpm for 5 min. The cytokines in the BALF were then assayed using sandwich ELISA kits. The ELISAs for IL-4, IL-5, and IFN- γ were performed using the respective ELISA kits (Endogen Inc., MA, USA). The IL-13 assay was conducted using the mouse IL-13 Quantikine ELISA kit (R&D Systems, MN, USA) in accordance with the manufacturer's instructions.

The cell numbers in 100- μ L aliquots of BALF were counted under a microscope. BALF cells were prepared by cytopspin and were stained with Diff-Quik (International Reagents Co., Kobe, Japan) to distinguish the eosinophils based on their morphology and staining characteristics. The cells were counted under a microscope.

Measurement of OVA-specific Ig in sera

Ovalbumin-specific antibodies in the sera of the immunized mice were measured using a sandwich ELISA as described previously (Tamauchi et al. 2004). Serum samples and serial dilutions of the controls were incubated in 96-well microtiter plates coated with OVA (100 μ g/mL; Sigma Chemical, St. Louis, MO) for 1.5 h at room temperature. Biotin-labeled rat mAbs against mouse IgM, IgG1, IgG2a, IgE and IgA (Zymed, South San Francisco, CA) were added for a second incubation for 1.5 h at room temperature. The plates were then washed and incubated with streptavidin-horseradish peroxidase (Pharmingen). Finally, the color that resulted from a peroxidase reaction with the TMB substrate was determined at 450 nm using an ELISA plate reader. Standard curves for anti-OVA Ab were generated using hyperimmune serum from a BALB/c mouse (Tamauchi et al. 2004).

Statistical analysis

Data are expressed as the mean $\pm$ S.E.M. Statistical significance was tested using the Student *t*-test. The differences were considered significant if $P < 0.05$.

Results

Establishment of the C57-IL-10 cell line

A steady supply of IL-10 is important for clinical and other applications, but its estimated biological half-life is 20 min. We generated C57, C57-EV and C57-IL-10 cell lines harboring a retroviral gene and examined the endogenous and exogenous IL-10 DNA gene expression using PCR and real time-PCR (Fig. 2a), and protein expression using an ELISA. The C57-IL-10 cells expressed about 15 ng/mL of IL-10 protein (data not shown). When this culture supernatant was added to J774.1 and then stimulated with 150 μ g of MSU (uric acid crystals), the TNF- α production was regulated, compared with in the group without the addition of IL-10 (Fig. 2b). The culture supernatant of the C57-IL-10 cells or the culture supernatant of the C57-EV cells was added to J774.1 cells and cultured, and the quantities of TNF- α production by the J774.1 cells were compared.

As a result, the C57-IL-10 cell culture supernatant induced the production of TNF- α at a level 10–12 times higher than that induced by the C57-EV cell culture supernatant.

Kinetics of IL-10 production and IL-10 mRNA expression in vivo

To measure the activity of IL-10 *in vivo*, 5×10^5 C57-IL-10 cells were injected i.v. into C57BL/6 mice and the production of IL-10 and IL-10 gene expression in the BALF were measured over time. The cells produced IL-10 for 14 days after injection, followed by a decrease in production (Fig. 2c). The levels of IL-10 in the pulmonary tissues were measured using real-time RT-PCR to determine the therapeutic effect of C57-IL-10 (Exogenous; pLXSN-IL-10) on the lungs. The total amount of IL-10, including the extent IL-10, increased by about 7-times compared with the amount immediately after the injection of the cells, then gradually decreased 2–3 days later (Fig. 2c). The level of C57-IL-10-specific pLXSN-IL-10 mRNA reached a maximum on day 1 and persisted for 14 days (Fig. 2c). These results showed that our mouse model using retroviral vectors can produce IL-10 over a long time period. The expression level of the IL-10 gene derived from endogenous sources was about $1/10^5$ of the expression level of the IL-10 gene of exogenous origin. This value was similar to the expression level in lung tissue that was not injected with cells.

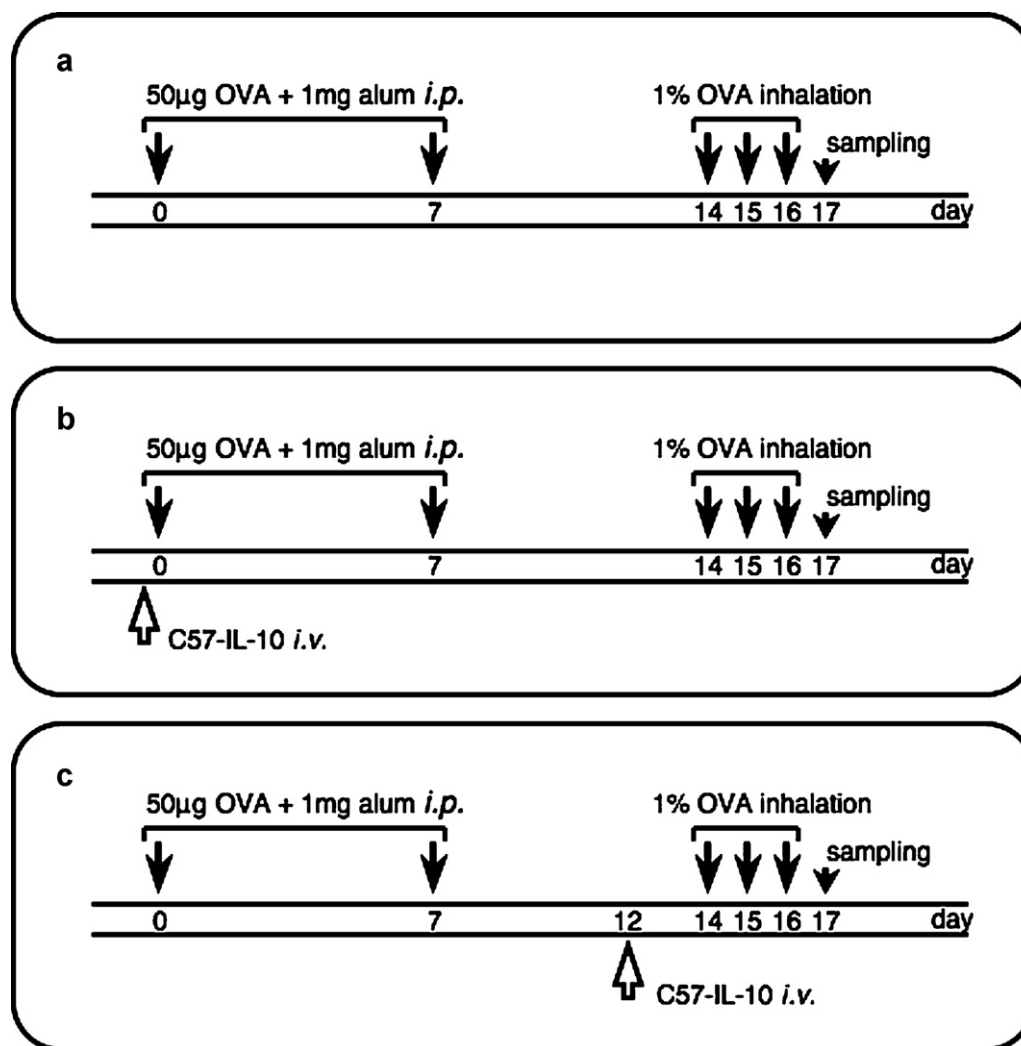


Fig. 1. Experimental protocols. Protocol a: OVA and alum were administered i.p. to GATA-3/TCR-Tg and WT/TCR-Tg mice on days 0 and 7. Protocol b: GATA-3/TCR-Tg and WT/TCR mice were inoculated with C57-IL-10 cells on day 0 in addition to protocol a. Protocol c: GATA-3/TCR-Tg and WT/TCR mice were inoculated with C57-IL-10 cells on day 12 in addition to protocol a. Aerosolized OVA was administered on days 14, 15 and 16 after immunization with OVA plus alum. Mice were anesthetized on day 17, and the samples were collected.

Effect of IL-10 produced by C57-IL-10 cells on Th2-type cytokine production and influence on eosinophil differentiation in BALF

When C57-IL-10 cells were injected into OVA-sensitized GATA-3/TCR-Tg mice prior to the inhalation of the OVA antigen (Fig. 1c), the production of IL-5 and the IL-13 levels in the BALF were suppressed and the ratio of eosinophils in the BALF decreased, compared with the C57-EV cell control group (Figs. 3b and 4b). Approximately 30% of the IL-5 production and 60% of the IL-13 production was inhibited, compared with the situation after C57-EV administration in the C57-IL-10 group. When the C57-IL-10 cells were challenged at the same time as sensitization with OVA plus alum and injected, the production of IL-5 and IL-13 in the BALF increased. In the C57-IL-10 cell-treated group, IL-5 and IL-13 production increased by approximately 50%. However, no significant change in IL-4 and IFN- γ production was observed, regardless of the time of C57-IL-10 administration. Also, as for the number of eosinophils in the BALF, the number was inhibited by approximately 40% compared with that in the CD57-EV treated group. The number of eosinophils in the BALF increased in proportion to the amounts of IL-5 and IL-13 produced (Figs. 3 and 4). These findings indicate that IL-10 caused an increase in cytokine production and in the ratio of eosinophils, depending upon the time of adminis-

tration. Therefore, whether the C57-IL-10 was administered at the same time of sensitization or after sensitization with OVA and alum, the production of Th2-type cytokines, particularly IL-5 and IL-13 was affected.

Influence of C57-IL-10 on serum IgE production

Because the IgE antibody is associated with Th2-type cytokine production and action, we examined serum IgE production after the injection of C57-IL-10 cells. Simultaneous C57-IL-10 administration and OVA antigen sensitization caused an increase in the amount of total IgE and OVA-specific IgE production in the blood (Fig. 5a and b). On the other hand, C57-IL-10 inoculation before OVA inhalation did not significantly alter the levels of total and OVA-specific IgE (Fig. 5c and d). As for the quantity of OVA-specific IgE, an increase by approximately 1.5 times was observed simultaneously with an increase in the total IgE level by approximately 200 times, compared with the C57-EV control, when the C57L-IL-10 cells were injected with OVA plus alum. However, the quantity of OVA-specific IgE and total IgE did not change when the C57-IL-10 cells were injected before OVA inhalation.

Although the data are not shown, the production of total IgE and OVA-specific IgE increased similarly in the blood of mice that

were sensitized with OVA plus alum when the C57-IL-10 cells were injected at the same time.

Discussion

Several reports have shown that IL-10 regulates immune reactions through Th1-type cytokines (Verma and Weitzman 2005; Miller and Miller 1994; Miller 1996). However, other studies have indicated that IL-10 also regulates Th2-type immune reaction *in vivo*, although considerable uncertainty remains (van Oosterhout and Bloksma 2005; Sasaki et al. 2005; van Scott et al., 2000). Since IL-10 is involved in immune reactions *in vivo* (Connors et al., 1994), we have induced allergic reactions in GATA-3 Tg mice using IL-10 to examine the possibility of gene therapy against Th2-type immunity. We induced OVA-specific inflammatory reactions in GATA-3 mice that enhanced the Th2-type immune response (Tamauchi et al. 2004).

A previous report showed that the instillation of IL-10 protein into the lung also suppresses eosinophilic airway inflammation (Nakagome et al. 2005). However, the half-lives of recombinant cytokines are generally very short. In fact, the circulating half-life of a systemically delivered rIL-10 protein is only 20 min (Chernoff et al. 1995). Therefore, we examined the effect of IL-10 on immune disorders using an experimental system to

maintain the level of IL-10 using an IL-10 gene introduction transfectant (C57-IL-10) to functionalize IL-10 *in vivo*. The C57-IL-10 cell made it possible to maintain IL-10 production for long time periods *in vivo*, and the presence of IL-10 in BALF was observed until administration day 14, as shown in Fig. 2a.

We examined the effect of IL-10 treatment. Mice that were already sensitized were exposed to a same antigen. Fig. 1b shows the schedule that was used to examine the inhibition of allergic airway inflammation using IL-10. We examined whether this model could prevent symptom provocation in asthmatic patients. Fig. 1c shows the prevention of sensitization using IL-10 in animals that had been newly sensitized to a new antigen. This concept is known as an allergy march (Jonathan 2010a,b) and is known to exist in humans with allergic disease. Therefore, we designed this experiment to examine the influence of IL-10 on the induction of allergic disease, since patients with allergies often become newly sensitized to different antigens.

We regulated the levels of IL-5 and IL-13 in the alveolar space using IL-10-producing C57-IL-10 cells line in GATA-3/TCR-Tg mice with allergic airway inflammation induced by OVA (Fig. 2). When IL-10-producing C57-IL-10 cells were administered prior to OVA inhalation in OVA-sensitized GATA-3 Tg mice, the IL-5 and IL-13 productions in the BALF were remarkably inhibited and the number of eosinophils in the BALF decreased (Figs. 3b and 4b).

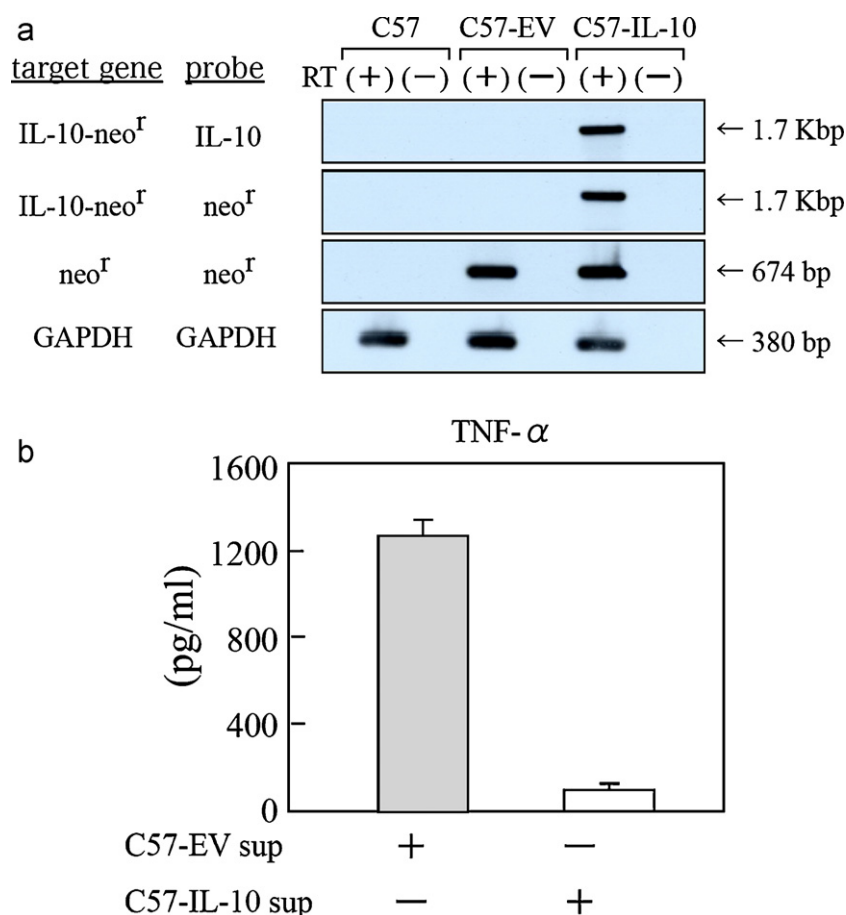


Fig. 2. Presence of C57-IL-10 cells used to introduce the IL-10 gene and measurement of their biological activity. (a) Presence of C57-IL-10 cells. We amplified RNA extracted from C57, C57-EV and C57-IL-10 cells using RT-PCR and then analyzed the fragments using Southern blotting. RT (+) and (-), presence or absence of reverse transcriptase, respectively. Representative reverse transcription RT-PCR results showing IL-10 (1.7 kbp), neo^I (674 bp) and GAPDH (380 bp). (b) Production of IL-10 in C57-IL-10 cells. Culture supernatant from C57-IL-10 or C57-EV cells was added to J774.1 cells, which were then stimulated with MSU. The amount of TNF-α production in the culture supernatants after 24 h was measured using an ELISA. A total of 5×10^5 C57-IL-10 cells were injected into C57BL/6 mouse *i.v.*, and the production of IL-10 in BALF was examined using an ELISA. (c) Endogenous IL-10 protein and (d) endogenous and (e) exogenous IL-10 gene expression levels in lung tissue as determined using RT-PCR. Data are shown as the mean $\pm$ SD.

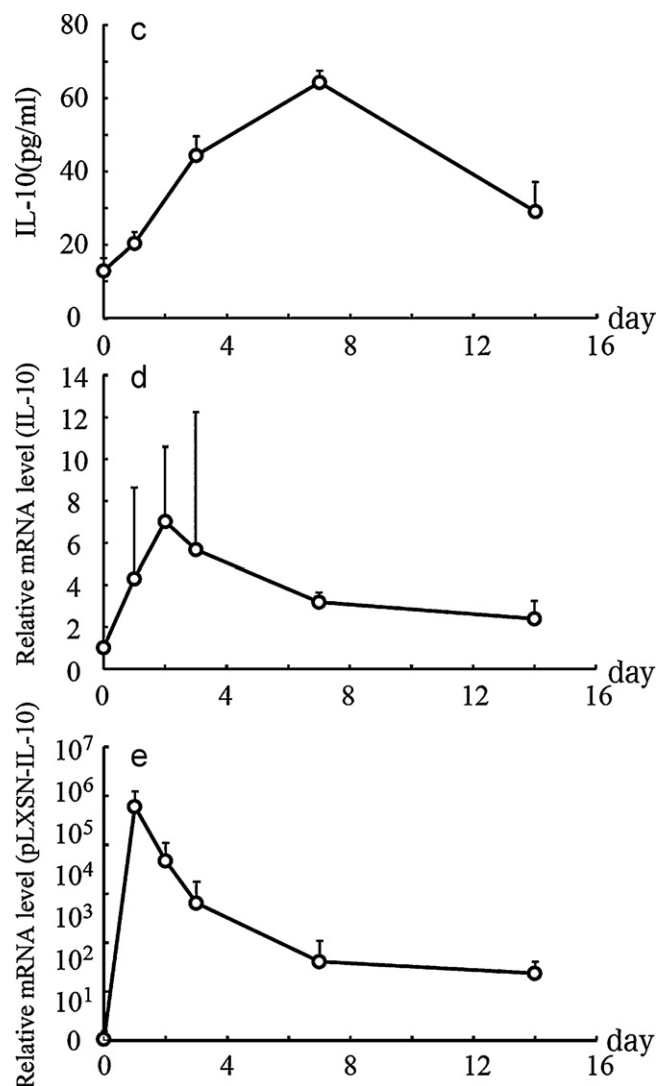


Fig. 2. Continued.

On the other hand, when the IL-10-producing C57-IL-10 cells were injected at the same time as the sensitization of GATA-3/TCR-Tg mice with OVA and alum, the IL-5 and IL-13 productions increased by 2–4 times the levels seen in the control group; furthermore, the number of eosinophils in the BALF doubled (Figs. 3a and 4a). Eosinophil infiltration was regulated by the IL-5 and IL-13 levels in the BALF (Figs. 3 and 4), which in turn were controlled by the injection of C57-IL-10 cells. These findings suggested that continuous stimulation with IL-10 is necessary to regulate eosinophil infiltration *in vivo*. Eosinophil infiltration in the BALF is known to be suppressed by the administration of IL-10 intranasally (van Scott et al., 2000). However, no change in IL-4 or IFN- γ production was observed in either system shown in Fig. 1b or c. We speculated that this conflicting response originated as a result of the use of the GATA-3 Tg mouse model, in which a small amount of OVA antigen is applied at the time of IL-10 administration. Moreover, our results might have differed from those for Th2-type immune reactions, which were regulated by the injection of C57-IL-10 cells *in vivo*. The possibility of IL-10 gene therapy for the treatment of Th2-type immune diseases seems evident. IL-10 reportedly suppresses IL-5 production in BALF obtained from IL-10 knockout mice (Yang et al. 2000). Our results agree with these previous findings. Together, this evidence indicates that IL-10 can both suppress and evoke the Th2 immune response.

Here, we examined the activities of IL-10 *in situ* and in mice *in vivo*. The effect of IL-10 is thought to be exerted through the participation of the IgE antibody (Yang et al. 2000), although the IgE antibody has been assumed to perform a key role in models of allergic diseases. The function of IL-10 *in vivo* was clarified by the finding that IgE antibody could be induced in GATA-3/TCR-Tg mice independently of extant bronchial asthma and that the amount of IgE antibody produced in serum could be an index of generalized Th2-type immunity. Therefore, we examined the effect of IL-10 on total IgE and OVA-specific IgE antibody production. As shown in Fig. 1c, IL-10 inhibited the OVA-specific and total IgE antibody production after an OVA antigen challenge in mice that had already been sensitized with OVA antigen but enhanced OVA-specific and total IgE antibody production when administered at the same time as sensitization with the OVA antigen (Fig. 1b).

Therefore, the relationship between IgE antibody production and IL-10 *in vitro* has been investigated. CD40, which induces non-specific IgE antibody but not transcription, is stimulated when IL-4 and CD40 coexist on human B cells (Bacharier and Geha 2000).

Previous reports have indicated that IL-10 enhances IgE antibody production. The stimulation of IL-10 reportedly enhanced IgE antibody production in the presence of IL-4 and CD40 mAb using PBMC from donors with allergic disease (Jeannin et al., 1997). In another report, highly purified human peripheral blood B cells were

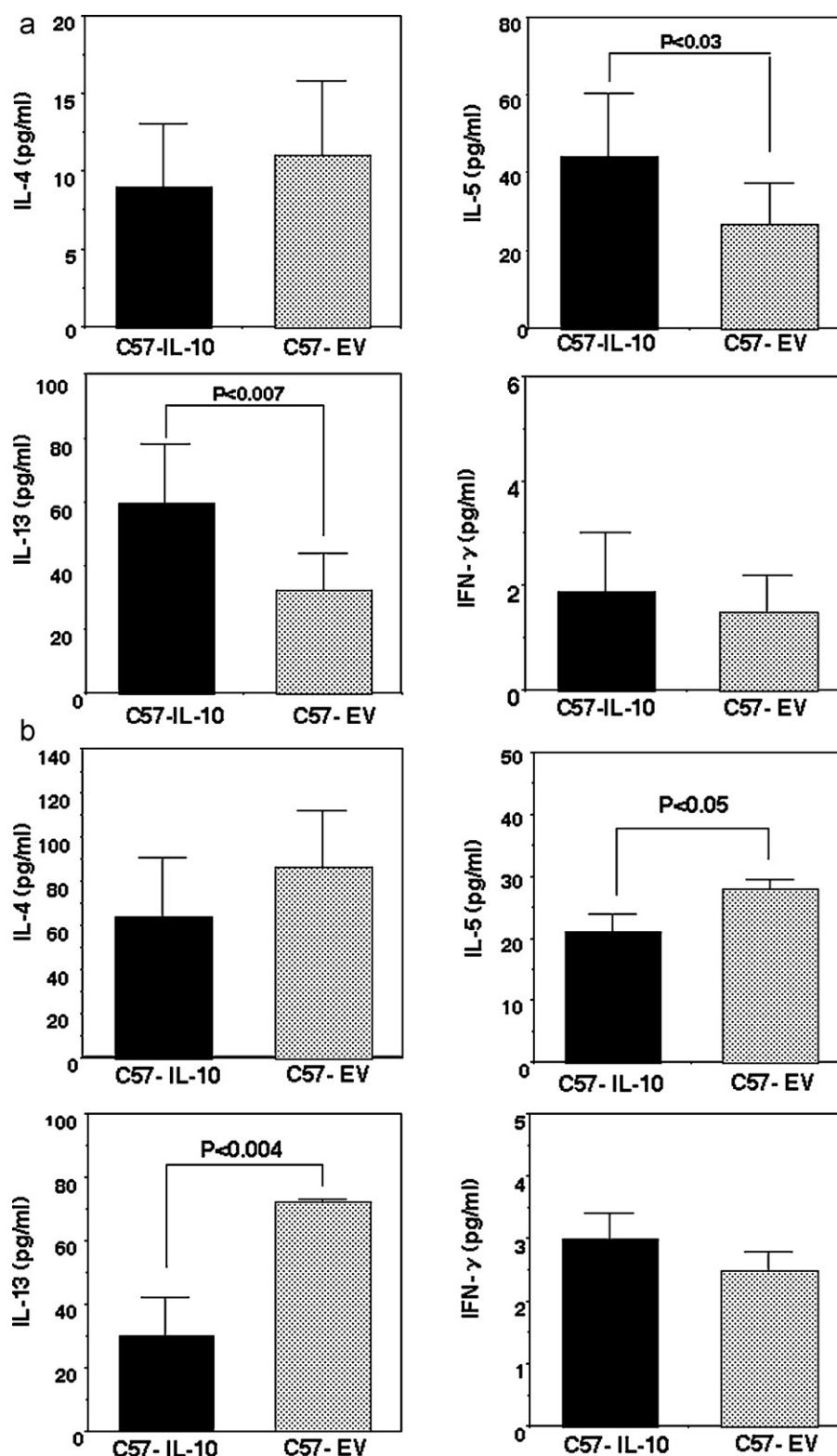


Fig. 3. Influence of time of C57-IL-10 administration on cytokine production in BALF. GATA-3/TCR-Tg mice were immunized with OVA plus alum according to protocol a. On day 7 after the first immunization, OVA plus alum was re-inoculated to antigen-sensitized GATA-3/TCR-Tg mice and OVA was inhaled using a nebulizer on days 14, 15 and 16. The BALF samples from the sensitized and inhaled GATA-3/TCR-Tg mice were recovered on day 17, and the cytokine levels (IL-4, IL-5, IL-13 and IFN-γ) were measured using an ELISA (protocol a). (a) C57-IL-10 cells were injected into GATA-3/TCR-Tg mice at the same time as the OVA and alum sensitization according to protocol b. (b) Before the second immunization according to protocol c, the C57-IL-10 cells were injected into OVA plus alum-sensitized GATA-3/TCR-Tg mice and BALF was recovered and the cytokine levels were measured using an ELISA. C57 cells harboring an empty vector were used as controls. Data are shown as the mean $\pm$ SD.

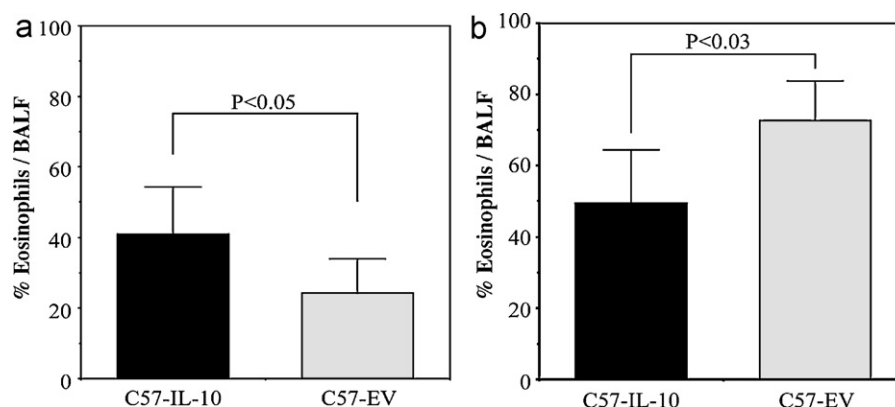


Fig. 4. Influence of time of C57-IL-10 cell administration on eosinophil infiltration in BALF of GATA-3/TCR-Tg mice. GATA-3/TCR-Tg mice were injected with C57-IL-10 cells at the time of sensitization with OVA plus alum according to protocol b. Seven days later, the antigen-sensitized GATA-3/TCR-Tg mice were re-immunized with OVA plus alum and were subsequently treated according to protocol b. (b) C57-IL-10 cells were administered before OVA inhalation (on day 12) according to protocol c. BALF was recovered on day 17, and the number of eosinophils in the BALF was measured using Diff-Quik. C57 cells harboring empty vectors were used as a control group. The ratio of eosinophils stained with Diff-Quik among all the BALF cells was calculated. Data are shown as the mean $\pm$ SD.

cultured with cross-linked CD40 mAb and IL-4, and the B cells differentiated into antibody-forming cells; when IL-10 was added, a larger quantity of IgE antibodies was produced (Kobayashi et al. 2002).

Omori et al. reported that IL-10 acts only on B cells that receive an antigenic stimulus. However, their report describes the production of non-specific, rather than antigen-specific IgE antibodies (Ohmori et al. 1995). Furthermore, the augmentation effect of IL-

10 on IgE antibody production was examined using transfectants that introduced the co-stimulatory molecules of CD40/CD32 or CD27/CD70 into cells (Rousset et al. 1992, 1995; Kobayashi et al. 2002). In particular, in an experiment using a CD27/CD70 transfectant, the production of IgE antibody was enhanced when IL-10 was administered with an antigen in an *in vitro* system. B cell proliferation was augmented by the addition of the CD27 ligand (CD70) to IL-10. The addition of IL-2 further augmented immunoglobulin

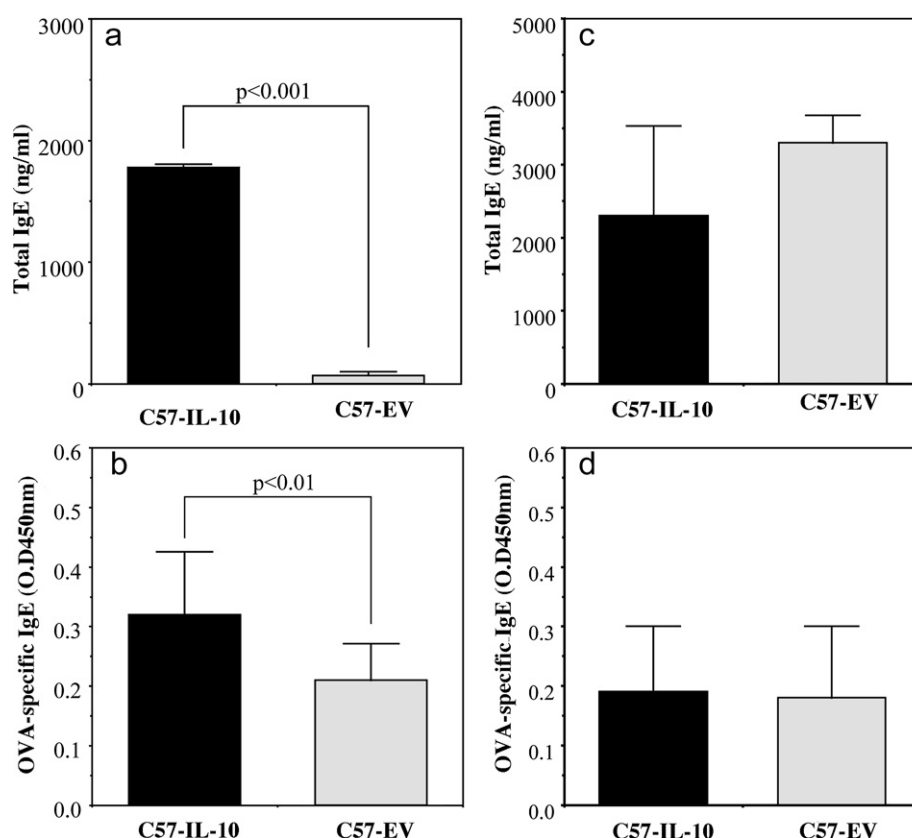


Fig. 5. Influence of time of C57-IL-10 cell administration on serum total and OVA-specific IgE antibody titers in GATA-3/TCR-Tg mice. (a) GATA-3/TCR-Tg mice were injected with C57-IL-10 cells at the time of sensitization with OVA plus alum according to protocol b. Seven days later, the antigen-sensitized GATA-3/TCR-Tg mice were re-inoculated with OVA plus alum. After the antigen-sensitized GATA-3/TCR-Tg mice had inhaled OVA 3 times, blood was collected on day 17 and the serum sample was subsequently divided and used to measure the IgE antibody titers. (b) C57-IL-10 cells were administered according to protocol c. After the final sensitization, C57-IL-10 was administered into antigen-sensitized GATA-3/TCR-Tg mice that had inhaled OVA on 3 different days. The following day, the blood was recovered and the serum sample was divided. The IgE titer was measured using an ELISA. C57 cells harboring empty vectors were used as a control group. The amounts of IgE antibody and OVA-specific IgE antibody in the serum were measured using an ELISA. Data are shown as the mean $\pm$ SD.

production, which was synergistically enhanced by IL-10 and CD27 triggering. Together, these results indicated that the cooperative response to IL-10 and CD27/CD70 interactions appeared to regulate B-cell immunoglobulin production (Agematsu et al., 1998; Nagumo and Agematsu, 1998; Agematsu et al., 1999). IL-10 is known to augment antibody production in activated B cells in some *in vivo* systems (Stein et al. 1997; Herminajeng et al. 2001; Finnegan et al. 2003) as well as in some *in vitro* systems (Kindler and Zubler, 1997; Choe and Choi, 1998; Xu et al. 2004).

In contrast, IL-10 has been shown to decrease IgE production in purified peripheral blood B cells in the presence of IL-4 and soluble CD154 (Akdis et al. 1998). Also Kobayashi et al. showed that CD27 signaling reduces IgE production in the presence of IL-10, IL-4 and CD40 mAb/CD32T (Kobayashi et al. 2002). Furthermore, the contribution of CD28 molecules and IL-10 has been suggested by the activated mechanism of T cells, but remains uncertain (Rulifson et al. 1987; Webb and Feldman 1995). These facts suggest that costimulatory molecules may be involved in the augmentation and suppression of the antibody production induced by IL-10, but further detailed examinations are needed. The present study showed that IL-10 affects not only the Th1-type, but also the Th2-type immune reaction *in vitro* and *in vivo* (Fiorentino et al. 1991; Del Prete et al. 1993).

In mice, IL-10 administration before allergen treatment induced antigen-specific T-cell tolerance and demonstrated the pivotal role of IL-10 in the establishment of peripheral T-cell anergy.

We showed here that IL-10 produced *in vitro* possesses biological activity similar to that of rIL-10 and that it can be stably supplied by C57-IL-10 cells (data not shown). These findings suggest that a retroviral vector can be used to treat Th2-type immune diseases and that immunological diseases can be treated using gene therapy.

We carried out a similar experiment using rIL-10. However, as the half-life of IL-10 is relatively short, we administered the IL-10 frequently (data not shown).

In summary, *in vitro* studies on the suppression of IL-10 and its augmentation effect have been reported, with results that were similar to those of the present *in vivo* study. Our study showed not only the effective functions of IL-10, but also some drawbacks.

These findings should be helpful for therapeutic studies of allergic disease. Nevertheless, further studies are needed to elucidate the details of whether IL-10 inhibits the function of immunocompetent cells enhanced in allergic disease.

As for IL-10, the inhibition-related activities of this cytokine may be useful for the prevention and possible treatment of airway inflammation of humans.

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