

# In Vivo IL-10 Gene Delivery Suppresses Airway Eosinophilia and Hyperreactivity by Down-Regulating APC Functions and Migration without Impairing the Antigen-Specific Systemic Immune Response in a Mouse Model of Allergic Airway Inflammation<sup>1</sup>

Kazuyuki Nakagome,\* Makoto Dohi,<sup>2\*</sup> Katsuhide Okunishi,\* Yoshinori Komagata,\* Katsuya Nagatani,\* Ryoichi Tanaka,\* Jun-ichi Miyazaki,<sup>†</sup> and Kazuhiko Yamamoto\*

IL-10 is an immunosuppressive cytokine. Although previous studies have reported that exogenous delivery of IL-10 reduced airway inflammation in experimental allergic airway inflammation, the mechanism of action has not been fully clarified. In this report, we elucidated a mechanism of action of IL-10 in vivo. BALB/c mice were immunized and aerosol challenged with OVA-Ag. We delivered the IL-10 gene to the mice before systemic sensitization or during aerosol Ag challenge by administering an IL-10-producing plasmid vector. Not only presensitization delivery of IL-10, as reported, but also delivery during inflammation strongly suppressed the development of airway eosinophilia and hyperreactivity. Presensitization delivery suppressed the Ag-specific Th2-type immune response in both the lung and spleen. In contrast, delivery in the effector phase suppressed the Th2 response only in the lung, whereas that in the spleen was not affected. IL-10 gene delivery did not induce the development of a regulatory phenotype of T cells or dendritic cells; rather, it suppressed the overall functions of CD11c<sup>+</sup> APCs of the lung such as Ag-presenting capacity, cytokine production, and transportation of OVA-Ag to lymph nodes, thus attenuating Th2-mediated allergic airway inflammation. Further, IL-10 revealed a distinct immunosuppressive effect in the presence of Ag and APCs. These results suggest that suppression of APC function in the lung, the site of immune response, played a critical role in the IL-10-mediated suppression of Ag-induced airway inflammation and hyperreactivity. Therefore, if delivered selectively, IL-10 could site specifically suppress the Ag-specific immune response without affecting systemic immune responses. *The Journal of Immunology*, 2005, 174: 6955–6966.

Interleukin-10 is an important immunosuppressive cytokine. For example, IL-10 inhibits the production of proinflammatory cytokines such as IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-12, TNF- $\alpha$ , and GM-CSF by monocytes and cytokines such as IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , IFN- $\gamma$ , and GM-CSF by Th1 cells (1–6). IL-10 down-regulates the expression of MHC class II molecules and costimulatory molecules on monocytes/macrophages and dendritic cells (DCs)<sup>3</sup> (1, 7–10). IL-10 suppresses the production of cytokines by T cells through direct (11–13) and indirect (APC-dependent) mechanisms (4, 5, 7), 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 var-

ious immune diseases in vivo. For example, both in human subjects and in animal models, treatment with IL-10 improved inflammatory bowel disease (14–17) and psoriasis (18–20), which are mainly mediated by Th1 immune response. In individuals who have received bee venom-specific immunotherapy or who recently have received multiple bee stings, up-regulation of endogenous IL-10 may play an important role in preventing anaphylaxis (21, 22). 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 to treat immune disorders is difficult.

Recently, the role of IL-10 in the generation of regulatory T (Treg) cells has been highlighted. Groux et al. reported that chronic activation of CD4<sup>+</sup> cells in the presence of IL-10 in vitro induces the development of T-regulatory 1 (Tr1) cells, which produce much IL-10 (23). Akbari et al. reported that stimulation of CD4<sup>+</sup> cells with IL-10-producing DCs, which were generated after exposure to inhaled Ag (24), induces IL-10-producing Treg cells (25). These reports suggest that IL-10-producing Treg cells develop in the presence of IL-10. However, whether in vivo IL-10 alone could 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) (26). The process of airway inflammation involves various types of cells, such as eosinophils, mast cells, T lymphocytes, and DCs (27, 28). Due to

\*Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; and <sup>†</sup>Division of Stem Cell Regulation Research, Osaka University Graduate School of Medicine, Osaka, Japan

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<sup>2</sup> Address correspondence and reprint requests to Dr. Makoto Dohi, Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail address: mdohi-ky@umin.ac.jp

<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; Treg, regulatory T; Tr1, T regulatory 1; AHR, airway hyperreactivity; SA, physiological saline; Mch, methacholine; Penh, enhanced pause; BALF, bronchoalveolar lavage fluid; MFI, mean fluorescence intensity.

its immunosuppressive effects, IL-10 is expected to down-regulate the airway immune response. In humans, relative underproduction of IL-10 by alveolar macrophages and in the sputum of patients with asthma has been reported (29, 30), which suggests an essential role for IL-10 in regulating airway inflammation. However, the role of IL-10 in airway inflammation and AHR is not well understood in either humans or animal models. For example, the effect of deleting endogenous IL-10 on Ag-induced eosinophilic inflammation in mouse models of allergic airway inflammation has been controversial. Some reports have indicated that IL-10-deficient mice exhibit exaggerated eosinophilic airway inflammation provoked by a systemic sensitization to Ag and local inhalation of Ag (31, 32), whereas another report indicated that IL-10-deficient mice show diminished eosinophilic airway inflammation (33). In contrast, several reports have indicated that administration of IL-10 to the lung suppresses eosinophilic airway inflammation. The instillation of recombinant murine IL-10 into the lung, administered concurrently with Ag, inhibits eosinophilic airway inflammation (34, 35). CD4<sup>+</sup> Th cells engineered to produce IL-10 prevent AHR and inflammation induced by the transfer of OVA-specific Th2 cells and OVA challenge, although their effect on eosinophilic inflammation was not confirmed in sensitized and challenged mice (36). Further, administration of IL-10 cDNA into the lung via an adenoviral vector, at least if done before sensitization, inhibits eosinophilic inflammation and AHR (37). Although these studies show that exogenous delivery of IL-10 would have some inhibitory effect on experimental allergic airway inflammation, the mechanism of action in vivo, especially in the lung, has not been clarified.

The purpose of the present study was to elucidate the mechanism of IL-10 gene delivery in vivo. We found that IL-10 gene delivery, even after systemic Ag sensitization has been completed, suppressed Ag-induced eosinophilic airway inflammation and AHR. We also found that IL-10 gene delivery down-regulated APC functions in the lung and migration of Ag-captured CD11c<sup>+</sup> cells to regional peribronchial lymph nodes, although it did not affect the systemic immune response to Ag. These results indicated that the immunosuppressive effect of IL-10 is site specific, occurring in tissue where APCs encounter the Ag and thus an Ag-specific immune response is driven.

## Materials and Methods

### Mice

Male BALB/c and C57BL/6 mice were obtained from Charles River Japan. Male OVA TCR-transgenic DO11.10 mice and C57BL/6-IL10<sup>-/-</sup> mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were approved by the Animal Research Ethics Board of the Department of Allergy and Rheumatology, University of Tokyo (Tokyo, Japan).

### Immunization of mice and evaluation of allergic airway inflammation

Mice were immunized as reported previously (38, 39). Seven-week-old animals were sensitized with an i.p. injection of 2  $\mu$ g of OVA (Sigma-Aldrich) plus 2 mg of aluminum hydroxide (alum) on days 0 and 11. Control mice received injections of physiological saline (SA) on days 0 and 11. Some groups of mice were sacrificed on day 18 for analyses of immune response in the spleen. In another experiment, for analyses of allergic airway inflammation, mice were challenged with an aerosolized solution of 3% OVA or PBS for 10 min from day 18 to day 20. On day 21, AHR to methacholine (Mch) was measured with the enhanced pause (Penh) system as described previously (38, 39). Results were expressed for each concentration of Mch as a percentage of the baseline Penh value after exposure to SA. Then, samples of serum and bronchoalveolar lavage fluid (BALF) were obtained. The lungs were cut out and fixed with 10% neutralized buffered formalin (Wako). Sections 3  $\mu$ m thick were prepared and subjected to H&E staining.

### Delivery of IL-10 into mice

In the present study, we delivered IL-10 in vivo using a hydrodynamics-based method through the i.v. injection of plasmid DNA with previously reported methods (40, 41). The plasmid pCAGGS-IL-10 (40) was amplified in *Escherichia coli* and purified with a Qiagen Endo Free plasmid Giga kit (Qiagen). The empty plasmid pCAGGS was used as a control. Plasmid DNA in lactated Ringer's solution (0.1 ml/g body weight) was injected i.v. into the tail. The injection was completed within 5 s. As reported originally, with this delivery system, the plasmid was trapped in the liver, and the cytokine was produced there and then moved into the bloodstream and to the perfused organs (40, 41). For study of the kinetics of IL-10 gene delivery, mice were sacrificed on days 1, 4, 7, 10, 13, 20, and 23 after the plasmid injection. Concentrations of IL-10 in serum and BALF were measured using an ELISA kit (BD Pharmingen) according to the manufacturer's instructions. Some mice received plasmid DNA (100  $\mu$ g; pCAGGS-IL-10 or control pCAGGS) i.v. on day -3 (before systemic immunization: pre) or on day 17 (during aerosol challenge; after systemic immunization: post).

### Measurement of cytokines and Ig

IL-4, IL-5, IL-10, IL-12 p70, IL-13, IFN- $\gamma$ , TGF- $\beta$ , and IgE concentrations were measured with ELISA kits (IL-4, IL-5, IL-10, IL-12 p70, IFN- $\gamma$ , and IgE, BD Pharmingen; IL-13 and TGF- $\beta$ , R & D Systems). OVA-specific IgE was measured using an ELISA kit for IgE except that the plate was coated with OVA (1000  $\mu$ g/ml) at 4°C overnight instead of anti-mouse IgE Ab. The OVA-specific IgE standard was derived by pooling sera from five OVA-sensitized mice. Results were expressed as a percentage of the value of the standard.

### Preparation of spleen and lung cells

For the preparation of spleen cells, a 2-ml solution of 450 U of collagenase (Sigma-Aldrich) in complete DMEM (Life Technologies) supplemented with 10% FCS, 10 mM HEPES (Life Technologies), 0.1 mM nonessential amino acids (Life Technologies), 1 mM sodium pyruvate, 2 mM sodium glutamate (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), 100  $\mu$ g/ml streptomycin (Sigma-Aldrich), and  $5.0 \times 10^{-5}$  M 2-ME (Sigma-Aldrich) were injected into the spleen, and the spleen was incubated at 37°C for 20 min. Cell suspensions were collected by pressing the tissues through a 70- $\mu$ m pore size nylon filter into HBSS (Sigma-Aldrich) and centrifuged. The cell pellets were resuspended in RBC lysis solution (Sigma-Aldrich) for 1 min and washed; then the cell suspensions were filtered again to remove clumps and aggregates. Viable cell numbers were determined by trypan blue dye exclusion. The preparation of lung cells was conducted essentially using previously reported methods with a slight modification (42, 43). Briefly, lungs were excised, minced, and digested for 90 min at room temperature with 150 U of collagenase in complete DMEM. Cell suspensions were obtained by pressing the tissues through a 70- $\mu$ m pore size nylon filter.

For the preparation of splenic CD4<sup>+</sup> T cells or DCs, spleen cells were incubated with anti-CD4 mAb-coated or anti-CD11c mAb-coated microbeads (Miltenyi Biotec). Bead-bound cells were then isolated using magnetic separation columns. The purities of the enriched CD4<sup>+</sup> and CD11c<sup>+</sup> cell populations were 95 and 85%, respectively (data not shown). More than 95% of the CD4<sup>+</sup> cells were CD3<sup>+</sup> T cells (data not shown). For the preparation of lung CD11c<sup>+</sup> cells, cells were purified using Nycoprep (AXIS-SHIELD) before incubation with anti-CD11c mAb-coated microbeads, and bead-bound cells were then isolated. The purity of the enriched CD11c<sup>+</sup> cells was 80% (data not shown). This purity was similar to that of previous studies that isolated CD11c<sup>+</sup> cells from the lung (42, 43).

### In vitro proliferation and cytokine assays

Spleen or lung cells were cultured ( $5 \times 10^5$  cells/well) with or without OVA in complete DMEM. In some experiments, positively selected CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well) were cultured with freshly isolated mitomycin C (Sigma-Aldrich)-treated splenocytes ( $2.5 \times 10^5$  cells/well) in the presence or absence of OVA. After 72 or 96 h, the proliferation was assessed with a cell proliferation ELISA BrdU kit (Roche Applied Science). After 120 h, cytokine concentrations in the supernatants were measured using ELISA kits. To examine the direct effect of IL-10 on CD4<sup>+</sup> T cell functions in vitro, OVA-sensitized CD4<sup>+</sup> T cells were incubated with plate-bound anti CD3 Ab (10  $\mu$ g/ml; BD Pharmingen) for 48 h or PMA (1 ng/ml; Sigma-Aldrich) and ionomycin (1  $\mu$ g/ml; Sigma-Aldrich) for 24 h in the presence or absence of IL-10. Then, the proliferation was assessed, and the IL-4 concentration in the supernatants was measured. To examine the effect of coinubation with IL-10 on the Ag-specific immune response

of CD4<sup>+</sup> T cells, OVA-sensitized CD4<sup>+</sup> T cells were incubated with mitomycin C-treated splenocytes from naive mice and OVA (20  $\mu$ g/ml) for 72 h in the presence or absence of IL-10. In another experiment, to examine the effect of preincubation with IL-10, OVA-sensitized spleen cells ( $5 \times 10^6$  cells/ml) were preincubated with or without IL-10 (50 ng/ml) for 24 h. After IL-10 was removed from the culture medium, CD4<sup>+</sup> T cells were positively selected from the spleen cell population and incubated with mitomycin C-treated splenocytes from naive mice and OVA for 72 h. Then, the proliferation was assessed and the IL-4 concentration was measured. For measurement of the production of cytokines by CD11c<sup>+</sup> cells, CD11c<sup>+</sup> cells ( $1.25 \times 10^5$  cells/well) were incubated with LPS (1  $\mu$ g/ml) or TNF- $\alpha$  (50 ng/ml) for 24 h, and then cytokine concentrations in the supernatants were measured using ELISA kits.

### RT-PCR

RNA was extracted from CD4<sup>+</sup> T cells by the acid-guanidium phenol chloroform method using Isogen (Nippon Gene). RT-PCR for Foxp3 or  $\beta$ -actin was performed using previously reported methods (38, 44). PCR for Foxp3 consisted of 40 s of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C for 26 cycles. PCR for  $\beta$ -actin consisted of 1 min of denaturation at 94°C, 1 min of annealing at 61°C, and 1 min of extension at 72°C for 21 cycles. The sense primer for Foxp3 was 5'-GGC GAA AGT GGC AGA GAG GTA T-3', and the antisense primer was 5'-AAG ACC CCA GTG GCA GCA GAA-3'. The sense primer for  $\beta$ -actin was 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', and the antisense primer was 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. PCR products were electrophoresed in a 3% agarose gel, and the results were visualized by ethidium bromide staining. Densitometric analysis of mRNA expression was performed using NIH Image software (National Institutes of Health, Bethesda, MD). Normalized values for Foxp3 mRNA expression in each sample were calculated as the relative quantity of Foxp3 divided by the relative quantity of  $\beta$ -actin.

### Allogenic MLR and Ag-presenting capacity of CD11c<sup>+</sup> cells

For analyses of allogeneic MLR, mitomycin C-treated BALB/c (H-2<sup>d</sup>) CD11c<sup>+</sup> cells ( $1 \times 10^4$  or  $2.5 \times 10^4$  cells/well) were cultured with positively selected C57BL/6 (H-2<sup>b</sup>) CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well) in complete DMEM. In some experiments, C57BL/6 CD11c<sup>+</sup> cells ( $2.5 \times 10^4$  cells/well) were cultured with BALB/c CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well). For the measurement of OVA-Ag-presenting capacity, mitomycin C-treated CD11c<sup>+</sup> cells ( $2.5 \times 10^4$  cells/well) were cultured with DO11.10 CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well). In some experiments, CD11c<sup>+</sup> cells ( $1.25 \times 10^5$ ) from the spleen were cultured with OVA-sensitized CD4<sup>+</sup> T cells ( $1.25 \times 10^5$  cells/well) and OVA (30  $\mu$ g/ml). After 48–96 h, the proliferation was assessed with a cell proliferation ELISA BrdU kit. For measurements of the capacity of regional lymph node cells to activate OVA-specific TCR-transgenic CD4<sup>+</sup> T cells, mitomycin C-treated lymph nodes cells ( $2.5 \times 10^5$  cells/well) were cultured with DO11.10 CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well). After 72 h, the proliferation was assessed.

### Flow cytometry

Spleen or lung cells ( $1 \times 10^6$ ) were incubated with PE anti-CD11c mAb (HL3; BD Pharmingen) and biotin anti-mouse MHC class II mAb (anti I-A/I-E mAb; 2G9; BD Pharmingen), anti-CD40 mAb (3/23; BD Pharmingen), anti-CD80 mAb (16-10A1; BD Pharmingen), or anti-CD86 mAb (GL1; BD Pharmingen) in the presence of anti-CD16/32 mAb (2.4G2; BD Pharmingen) for 30 min and washed twice. Then, cells were incubated with FITC-streptavidin (BD Pharmingen) for 30 min and washed twice. Dead cells were excluded by propidium iodide staining. Briefly, cells were incubated with propidium iodide (2  $\mu$ g/ml; Sigma-Aldrich) for 15 min at room temperature. Then stained cells were analyzed by flow cytometry (EPICS XL System II).

### Fluorescence study

Fluorescence microscopy was performed using previously reported methods with some modification (45, 46). Mice were sensitized with OVA on days 0 and 11. Some mice received pCAGGS-IL-10 or control pCAGGS (100  $\mu$ g) i.v. on day 17. Mice were given FITC-labeled OVA (Molecular Probes), FITC-dextran (Sigma-Aldrich), or FITC alone intratracheally on day 20 (500  $\mu$ g each). On day 21, the bronchial lymph nodes were excised, embedded in cryomolds containing Tissue-Tek OCT compound (Sakura Finetek), and frozen in liquid nitrogen. Sections were cut 5  $\mu$ m thick by cryostat. Some sections were fixed in acetone and treated with PE anti-CD11c mAb. The sections were subsequently observed under a fluorescence microscope (BX51).

### Preparation of OVA-pulsed splenic DCs and transfer into naive mice

Splenic CD11c<sup>+</sup> cells were positively selected by magnetic cell sorting from naive mice and incubated with OVA (1000  $\mu$ g/ml) in the presence or absence of GM-CSF (10 ng/ml). IL-10 was added to some culture media (50 ng/ml). After 20 h, cells were collected and washed three times. The cells were examined for Ag (OVA)-presenting capacity and cell surface marker expression. In some experiments, the cells were transferred into naive mice i.v. ( $5 \times 10^5$ ). The mice were challenged with nebulized 3% OVA in PBS for 1 h twice daily on days 7, 8, and 15 and sacrificed on day 16 for BALF analysis.

### Statistics

Values are expressed as means  $\pm$  SEM. Statistical analyses were performed using a one- or two-way ANOVA followed by Fisher's least significant difference test or Scheffé's procedure.  $p < 0.05$  was considered significant.

## Results

### IL-10 expression in serum and in BALF after hydrodynamics-based gene delivery by i.v. injection of plasmid DNA

First, we examined the kinetics of IL-10 gene delivery after i.v. injection of the plasmid DNA. Samples were collected at the indicated times after the injection of pCAGGS-IL-10 or control pCAGGS. Endogenous IL-10 concentrations in serum and in BALF from mice before gene delivery were  $8.5 \pm 2.4$  and  $97.5 \pm 28.1$  pg/ml, respectively. The temporal pattern of IL-10 protein expression in serum (Fig. 1A) and in BALF (Fig. 1B) was confirmed, as described previously (40). The level of IL-10 peaked 1 day after the injection and gradually decreased thereafter. On day 20, IL-10 delivery did not differ between mice that received pCAGGS-IL-10 and mice that received control pCAGGS.

### In vivo IL-10 gene delivery suppresses Ag-induced immune response of CD4<sup>+</sup> T cells and DC functions ex vivo

Next, we confirmed the effect of in vivo IL-10 gene delivery on the Ag-induced immune response by conducting ex vivo analyses.

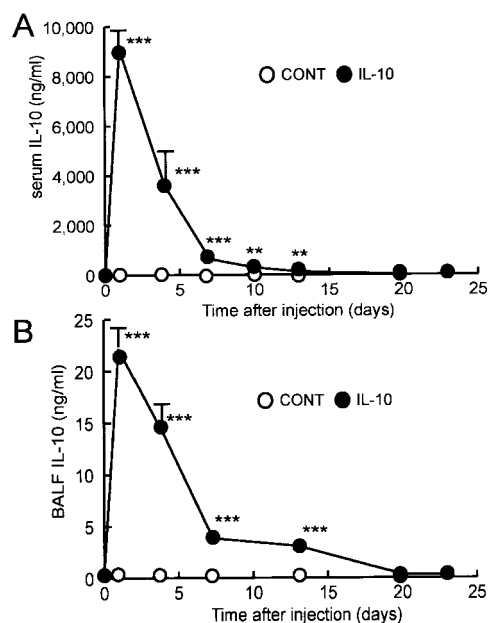


**FIGURE 1.** IL-10 concentrations in serum and in BALF after hydrodynamics-based gene delivery by i.v. injection of plasmid DNA. Mice received an i.v. injection of pCAGGS-IL-10 (IL-10) or control pCAGGS plasmid (CONT) on day 0. Concentrations of IL-10 in serum (A) and in BALF (B) were measured at the indicated times after the injection using ELISA. Values are presented as means  $\pm$  SEM for 6–10 mice per group. \*\*,  $p < 0.01$  and \*\*\*,  $p < 0.001$  compared with the value of CONT.

Splenic CD4<sup>+</sup> T cells obtained from OVA-sensitized mice strongly proliferated (Fig. 2A) and produced Th1- and Th2-type cytokines (Fig. 2B), compared with those of SA-treated mice. Splenic CD4<sup>+</sup> T cells from OVA-sensitized and pCAGGS-IL-10-injected mice failed to proliferate (Fig. 2A) or produce Th1- and Th2-type cytokines (Fig. 2B) in response to OVA, in contrast to those from the OVA-sensitized and control pCAGGS-injected mice. Similar results of cell proliferation and cytokine production were obtained when we used whole spleen cells instead of CD4<sup>+</sup> T cells (data not shown). Then, we examined the possibility that this suppression was mediated through production of Treg cells in vivo. CD4<sup>+</sup> T cells from OVA-sensitized and pCAGGS-IL-10-injected mice did not produce IL-10 (Fig. 2B) or TGF- $\beta$  (data not shown) in response to OVA, which suggested that this treatment did not induce the development of Tr1 (23)-like cells or Th3 (47)-like cells. In vivo IL-10 gene delivery also did not increase the mRNA expression of Foxp3, a transcription factor that was thought to be specifically expressed in naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 2C). In addition, IL-10 did not alter the ratio of CD4<sup>+</sup>CD25<sup>+</sup> cells to CD4<sup>+</sup>CD25<sup>-</sup> cells either (data not shown). These results suggested that in vivo IL-10 gene delivery would not induce the development of Treg cells. Next, we examined the effect of IL-10 treatment on DC functions after Ag sensitization. IL-10 treatment decreased the MHC alloreactivity (Fig. 2D), OVA-presenting capacity (Fig. 2E), and MHC class II expression of splenic DCs (Fig. 2F). IL-10 treatment also suppressed CD40, CD80, and CD86 expression in splenic DCs, although there was no significant difference in CD80 or CD86 expression (mean fluorescence intensity (MFI): CONT/OVA; CD40 71.1  $\pm$  5.6, CD80 56.0  $\pm$  4.4, CD86 51.0  $\pm$  3.6; vs IL-10/OVA; CD40 38.0  $\pm$  3.3, CD80 33.0  $\pm$  4.0, CD86 29.3  $\pm$  4.1, respectively). Further, IL-10 treatment also suppressed the production of IL-10 in response to LPS (Fig. 2G) or TNF- $\alpha$  (Fig. 2H). These results suggested that IL-10 gene delivery induced an overall suppression of DC activities, not a switch to a suppressive phenotype. They also indicated that our system of in vivo IL-10 gene delivery effectively suppressed the Ag-induced immune response.

*IL-10 has a suppressive effect on CD4<sup>+</sup> T cells only in the presence of Ag and APCs in vitro*

As described above, IL-10 gene delivery suppressed DC function and CD4<sup>+</sup> T cell priming in vivo. Then, we examined whether IL-10 could directly suppress CD4<sup>+</sup> T cell functions in vitro. When OVA-sensitized splenic CD4<sup>+</sup> T cells were stimulated with plate-bound anti-CD3 Ab or PMA-ionomycin, IL-10 did not suppress the proliferation or IL-4 production of CD4<sup>+</sup> T cells (Fig. 3, A and B). So IL-10 did not suppress CD4<sup>+</sup> T cells directly. Coincubation of IL-10 with OVA and APCs significantly suppressed the proliferation of CD4<sup>+</sup> T cells (Fig. 3, C and D). In contrast, pretreatment of OVA-sensitized spleen cells with IL-10, but without OVA, had no effect on the proliferation of CD4<sup>+</sup> T cells upon further OVA stimulation (Fig. 3, E and F). These results suggested that IL-10 has a suppressive effect on CD4<sup>+</sup> T cells only in the presence of Ag and APCs.

*In vivo IL-10 gene delivery, even after systemic Ag sensitization has been completed, suppresses Ag-induced eosinophilic airway inflammation and AHR*

We next elucidated the effect of IL-10 gene delivery on Th2-mediated allergic inflammation using an experimental model of allergic airway inflammation. Mice were sensitized with OVA or SA and then challenged with nebulized OVA or PBS. Injection of plasmid (pCAGGS-IL-10 or control pCAGGS) was performed before the systemic Ag sensitization (pre, on day -3) or during the

aerosol challenge (after systemic Ag sensitization; post, on day 17). In vivo IL-10 delivery in both cases almost completely diminished the eosinophil number in BALF (Fig. 4A). Histologically, a slight infiltration of inflammatory cells into the peribronchial area was detected in both groups of pCAGGS-IL-10-injected mice (Fig. 4B), but prominent infiltration of eosinophils into the peribronchial interstitial area and goblet cell formation of bronchial epithelial cells, which were provoked in the OVA-sensitized and OVA-challenged mice (Fig. 4B), were not observed. In the mice that received control pCAGGS before the sensitization or during the aerosol challenge, prominent infiltration of eosinophils and goblet cell formation were observed, respectively (Fig. 4B). BALF IL-13 and TGF- $\beta$  concentrations were also decreased in pCAGGS-IL-10-injected mice (Fig. 4C). Moreover, AHR to Mch decreased in pCAGGS-IL-10-injected mice (Fig. 4D). These results indicated that in vivo IL-10 gene delivery, not only before systemic sensitization but also during ongoing airway immune responses, suppressed eosinophilic airway inflammation and AHR.

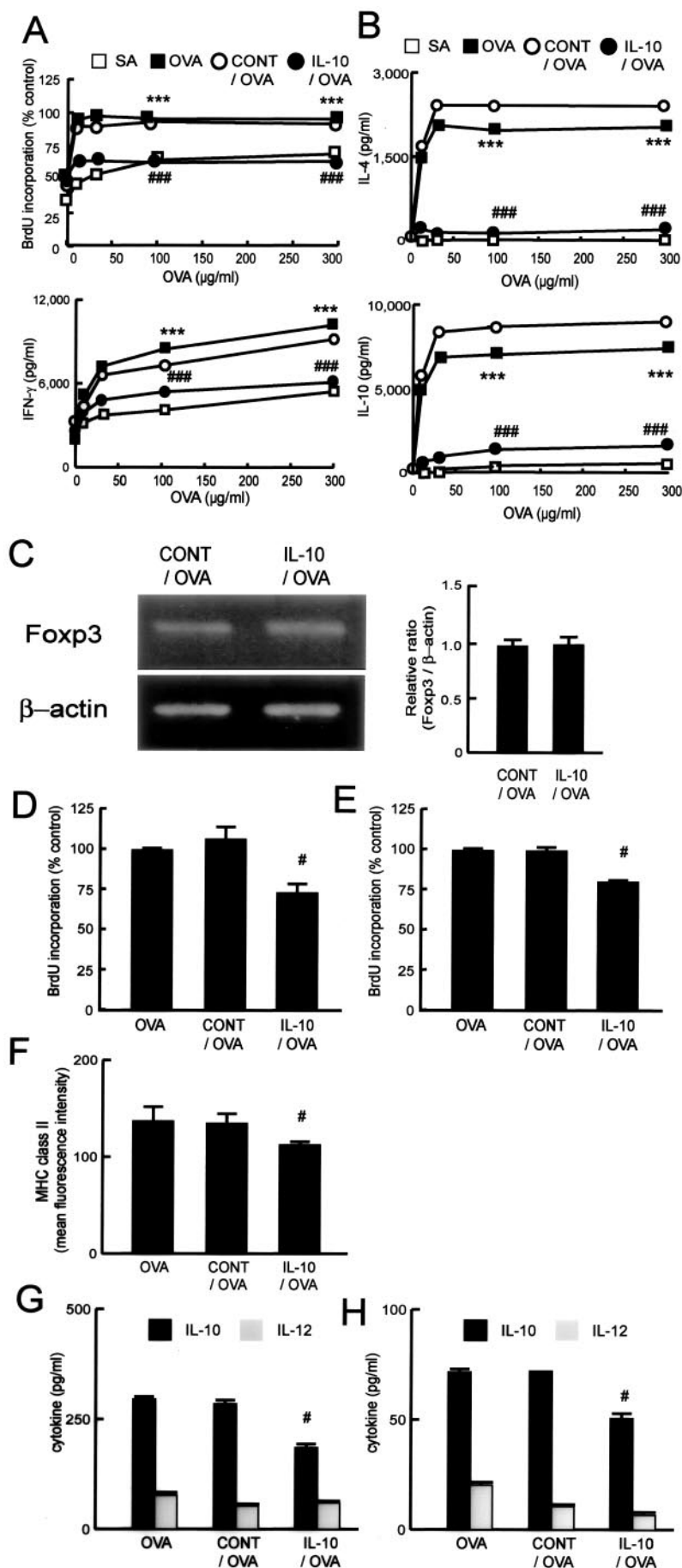
*In vivo IL-10 gene delivery during aerosol challenge suppresses production of Th2 cytokines by lung cells, but not spleen cells*

Next we examined the effect of IL-10 gene delivery on the immune response in the lung and compared it with that in the spleen. Control pCAGGS injection before sensitization or during aerosol challenge did not significantly suppress Th2 cytokine production in the lung (Fig. 5A). IL-10 delivery before sensitization and that during aerosol challenge suppressed the Th2 cytokine production by lung cells, although the suppression after IL-10 gene delivery during aerosol challenge was moderate compared with that after delivery before sensitization (Fig. 5A). In contrast, in vivo IL-10 delivery during aerosol challenge did not suppress the cell proliferation or cytokine production of spleen cells, whereas IL-10 delivery before sensitization suppressed them (Fig. 5, B and C). Further, in vivo IL-10 delivery during aerosol challenge did not suppress total or OVA-specific IgE production, whereas IL-10 delivery before sensitization suppressed both (Fig. 5D).

*In vivo IL-10 gene delivery suppresses resident lung APC functions*

To elucidate the difference in the mechanism of suppression between lung and spleen, we examined lung APC functions. Lung CD11c<sup>+</sup> cells obtained from OVA-sensitized and OVA-nebulized mice enhanced the MHC alloreactivity (Fig. 6A), OVA-presenting capacity (Fig. 6B), cytokine production (Fig. 6C), and expression of MHC class II (Fig. 6, D and E), compared with those of SA-treated, PBS-nebulized mice. IL-10 delivery before sensitization and that during aerosol challenge suppressed these resident lung APC functions of CD11c<sup>+</sup> cells (Fig. 6, A-E). IL-10 treatment also suppressed the expression of other costimulatory molecules such as CD40, CD80, and CD86 on lung CD11c<sup>+</sup> cells, although there were no significant differences (MFI: CONT (pre), CD40 38.4  $\pm$  3.4, CD80 52.9  $\pm$  4.4, CD86 38.0  $\pm$  2.6; IL-10 (pre), CD40 27.7  $\pm$  3.6, CD80 43.2  $\pm$  3.7, CD86 28.1  $\pm$  3.1; CONT (post), CD40 37.6  $\pm$  4.0, CD80 58.1  $\pm$  6.4, CD86 37.2  $\pm$  3.2; IL-10 (post), CD40 29.1  $\pm$  2.8, CD80 40.1  $\pm$  7.4, CD86 28.2  $\pm$  2.9). This is probably because OVA sensitization and challenge itself did not induce high levels of expression of these molecules. In the spleen, IL-10 delivery before sensitization suppressed the OVA-presenting capacity of DCs, whereas postsensitization delivery of IL-10 did not suppress it (Fig. 6F).

**FIGURE 2.** In vivo IL-10 gene delivery suppresses Ag-induced immune response of CD4<sup>+</sup> T cells and DC functions ex vivo. **A** and **B**, Proliferation and cytokine production of CD4<sup>+</sup> T cells in response to OVA. Mice were sensitized with OVA or SA on days 0 and 11. Some mice received an i.v. injection of pCAGGS-IL-10 or control pCAGGS on day -3. On day 18, splenic CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well) were positively selected by magnetic cell sorting, and they were cultured with freshly isolated mitomycin C-treated splenocytes ( $2.5 \times 10^5$  cells/well) in the presence or absence of OVA. **A**, After 96 h, the proliferation was assessed based on BrdU incorporation ( $n = 6$  per group). The maximum proliferation observed in response to OVA for splenic CD4<sup>+</sup> T cells from OVA-sensitized mice was set as a control (OVA; 100%). **B**, After 120 h, the cytokine levels of the supernatants were assayed ( $n = 6$  per group). \*\*\*,  $p < 0.001$  compared with the value of CONT/OVA. ###,  $p < 0.001$  compared with the value of CONT/OVA. **C**, In vivo IL-10 gene delivery does not up-regulate the expression of Foxp3 mRNA. On day 18, RNA was extracted from splenic CD4<sup>+</sup> T cells by the acid-guanidium phenol chloroform method. PCR products were electrophoresed in a 3% agarose gel, and the results were visualized by ethidium bromide staining. Normalized values for Foxp3 mRNA expression in each sample were calculated as the relative quantity of Foxp3 divided by the relative quantity of  $\beta$ -actin. The values presented are a Foxp3: $\beta$ -actin ratio from CD4<sup>+</sup> T cells of OVA-sensitized and pCAGGS-IL-10-injected mice (IL-10/OVA) to Foxp3: $\beta$ -actin from CD4<sup>+</sup> T cells of OVA-sensitized and control pCAGGS-injected mice (CONT/OVA) ( $n = 4$  per group). **D–H**, In vivo IL-10 treatment suppresses DC functions. Mice were sensitized with OVA on day 0. Some mice received an i.v. injection of pCAGGS-IL-10 or control pCAGGS on day -3. On day 11, splenic CD11c<sup>+</sup> cells were positively selected and subjected to analyses. **D**, Allogenic MLR. Splenic CD11c<sup>+</sup> cells ( $1 \times 10^4$  cells/well) from BALB/c mice (H-2<sup>d</sup>) were cultured with CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well) from C57BL/6 mice (H-2<sup>b</sup>). After 96 h, the proliferation was assessed based on BrdU incorporation ( $n = 6–10$  per group). The proliferation of CD4<sup>+</sup> T cells from C57BL/6 mice in response to CD11c<sup>+</sup> cells from OVA-sensitized BALB/c mice was set as a control (OVA). **E**, Ag-presenting capacity. Splenic CD11c<sup>+</sup> cells ( $1.25 \times 10^5$  cells/well) were cultured with CD4<sup>+</sup> T cells ( $1.25 \times 10^5$  cells/well) from OVA-sensitized mice and OVA (30  $\mu$ g/ml). After 96 h, the proliferation was assessed based on BrdU incorporation ( $n = 6–10$  per group). The proliferation of CD4<sup>+</sup> T cells from OVA-sensitized mice in response to CD11c<sup>+</sup> cells from OVA-sensitized mice with OVA was set as a control (OVA). **F**, MHC class II expression of splenic CD11c<sup>+</sup> cells was measured by flow cytometry ( $n = 6–10$  per group). **G** and **H**, Cytokine production. **G**, Splenic CD11c<sup>+</sup> cells ( $1.25 \times 10^5$  cells/well) were cultured with LPS (1  $\mu$ g/ml). After 24 h, the cytokine levels of the supernatants were assayed ( $n = 6–10$  per group). **H**, Splenic CD11c<sup>+</sup> cells ( $1.25 \times 10^5$  cells/well) were cultured with TNF- $\alpha$  (50 ng/ml). After 24 h, the cytokine levels of the supernatants were assayed ( $n = 6–10$  per group). #,  $p < 0.05$  compared with the value of CONT/OVA.



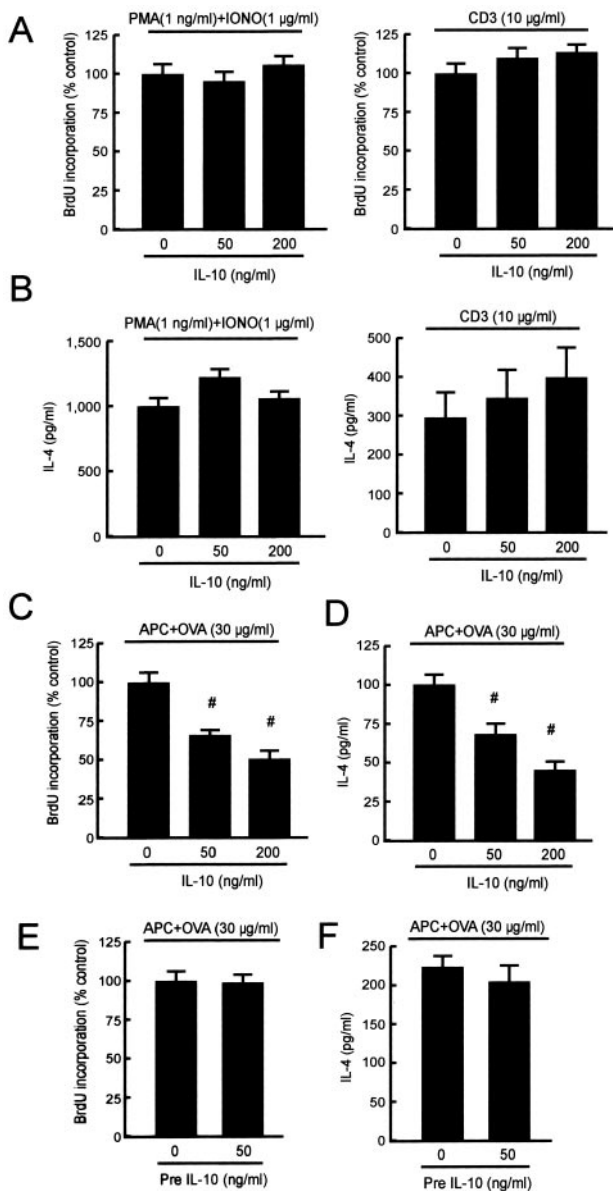


**FIGURE 3.** IL-10 has a suppressive effect on CD4<sup>+</sup> T cells only in the presence of Ag and APCs. *A* and *B*, Direct effect of IL-10 on CD4<sup>+</sup> T cells. Mice were sensitized with OVA on days 0 and 11. On day 18, splenic CD4<sup>+</sup> T cells ( $1.25 \times 10^5$  cells/well) were incubated with PMA (1 ng/ml) and ionomycin (IONO, 1 μg/ml) for 24 h or plate-bound anti-CD3 Ab (10 μg/ml) for 48 h in the presence or absence of IL-10. *A*, The proliferation was assessed based on BrdU incorporation ( $n = 6$  per group). The proliferation of CD4<sup>+</sup> T cells in response to PMA-ionomycin or plate-bound anti-CD3 Ab in the absence of IL-10 was set as a control (100%). *B*, IL-4 concentration in the supernatants was measured by ELISA ( $n = 6$  per group). *C* and *D*, IL-10 has a suppressive effect on CD4<sup>+</sup> T cells in the presence of Ag and APCs. Mice were sensitized with OVA on days 0 and 11. On day 18, splenic CD4<sup>+</sup> T cells ( $1.25 \times 10^5$  cells/well) were incubated with mitomycin C-treated splenocytes from naive mice ( $1.25 \times 10^5$  cells/well) and OVA (20 μg/ml) for 72 h in the presence or absence of IL-10. *C*, The proliferation was assessed based on BrdU incorporation ( $n = 6$  per group). The proliferation of CD4<sup>+</sup> T cells in response to OVA in the absence of IL-10 was set as a control. *D*, IL-4 concentration in the supernatants was measured by ELISA ( $n = 6$  per group). #,  $p < 0.05$  compared with the value in the absence of IL-10. *E* and *F*, IL-10 does not suppress CD4<sup>+</sup> T cell proliferation in the absence of Ag. Mice were sensitized with OVA on days 0 and 11. On day 18, spleen cells were preincubated with or without IL-10 for 24 h; then CD4<sup>+</sup> T cells ( $1.25 \times 10^5$  cells/well) were positively selected from the spleen cells and incubated with mitomycin C-treated splenocytes from naive mice ( $1.25 \times 10^5$  cells/well) and OVA

### *In vivo IL-10 gene delivery suppresses migration of CD11c<sup>+</sup> cells into draining lymph nodes*

Next, we examined the effect of IL-10 gene delivery on the migration of lung CD11c<sup>+</sup> cells into draining lymph nodes. First, we examined the capacity of regional lymph node cells (APCs) to activate OVA-specific TCR-transgenic CD4<sup>+</sup> T cells after inhalation of OVA. Lymph node cells from mice that received OVA sensitization, control pCAGGS injection, and OVA challenge induced a strong proliferation of DO11.10 CD4<sup>+</sup> T cells, which suggested that OVA-captured APCs actually migrated into lymph nodes (Fig. 7A). *In vivo* IL-10 gene delivery suppressed the proliferation of DO11.10 CD4<sup>+</sup> T cells (Fig. 7A), which suggested that IL-10 inhibited the migration of OVA-captured APCs into lymph nodes.

To confirm this, we examined the presence of OVA-captured APCs by fluorescence microscopy (45, 46). In mice that received the OVA sensitization and control pCAGGS injection, after instillation of FITC-OVA, FITC signal was detected into paracortical regions of the lymph nodes (Fig. 7B), whereas little signal was detected after instillation of FITC alone (data not shown). Next, we confirmed the coexistence of OVA and CD11c<sup>+</sup> cells by double staining. Some areas in the lymph nodes were double-positive for FITC-OVA and CD11c, indicating the presence of the OVA-captured CD11c<sup>+</sup> APCs (Fig. 7C). *In vivo* IL-10 gene delivery suppressed the migration of the OVA-captured APCs (Fig. 7D). In another experiment, FITC signal was also detected in the lymph nodes after instillation of FITC-dextran, instead of OVA, in mice that received OVA sensitization and control pCAGGS injection (data not shown). IL-10 treatment also suppressed this migration (Fig. 7E). Thus, IL-10 suppressed the migration of Ag-captured APCs irrespective of the kind of Ag.

### *IL-10-deficient mice exhibit up-regulated lung APC functions and exaggerated eosinophilic airway inflammation*

To further confirm the significant role of IL-10 in the lung immune response, we elucidated the effect of deleting endogenous IL-10 and the effect of IL-10 gene delivery during aerosol challenge into IL-10-deficient mice. First, we examined lung APC functions and the migration of CD11c<sup>+</sup> cells into regional lymph nodes in IL-10-deficient mice. Lung CD11c<sup>+</sup> cells from IL-10-deficient mice exhibited increased MHC alloreactivity (Fig. 8A) and MHC class II expression (Fig. 8B) compared with those from wild-type mice. In addition, the migration of CD11c<sup>+</sup> cells of IL-10-deficient mice into regional lymph nodes dramatically increased (Fig. 8C). IL-10 gene delivery into IL-10-deficient mice during aerosol challenge suppressed APC functions of lung CD11c<sup>+</sup> cells (Fig. 8, A and B) and the migration of CD11c<sup>+</sup> cells into regional lymph nodes (data not shown). IL-10-deficient mice exhibited exaggerated neutrophilic as well as eosinophilic airway inflammation provoked by Ag systemic sensitization and local Ag inhalation (Fig. 8D), which was consistent with previous reports (31, 32). IL-10 gene delivery into IL-10-deficient mice during aerosol challenge suppressed the airway inflammation (Fig. 8D). These results also supported the finding that IL-10 played a critical role in down-regulating resident lung APC functions and migration into regional lymph nodes.

(20 μg/ml) for 72 h. *E*, The proliferation was assessed based on BrdU incorporation ( $n = 6$  per group). The proliferation of CD4<sup>+</sup> T cells in response to OVA in the absence of IL-10 was set as a control. *F*, IL-4 concentration in the supernatants was measured by ELISA ( $n = 6$  per group).

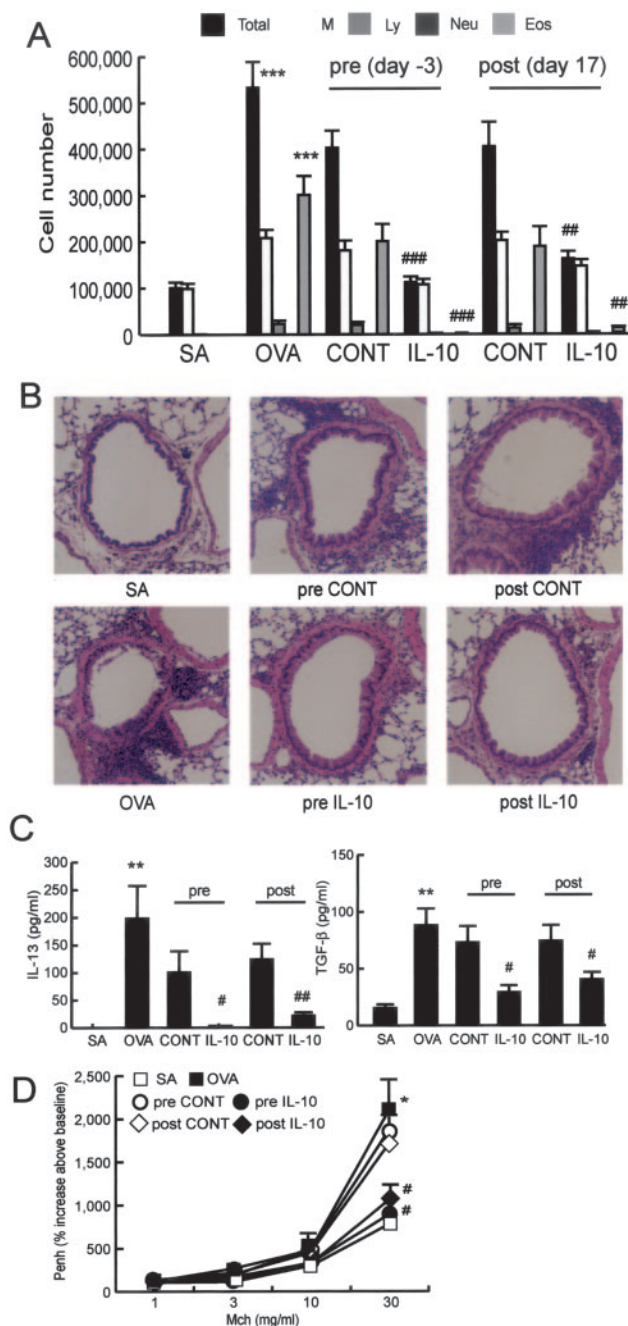


**FIGURE 4.** In vivo IL-10 gene delivery before systemic sensitization (pre) and that during aerosol challenge (post) suppress eosinophilic (Eos) airway inflammation and AHR. Mice were sensitized with OVA or SA on days 0 and 11. Some mice received an i.v. injection of pCAGGS-IL-10 (IL-10) or control pCAGGS (CONT) on day -3 (pre) or on day 17 (post). Then, mice were challenged with 3% OVA or PBS for 10 min from day 18 to day 20. On day 21, mice were analyzed. **A**, In vivo IL-10 delivery suppresses eosinophil numbers in BALF. Bronchoalveolar lavage was performed ( $n = 11$ – $12$  per group). Leukocytes were identified by morphological criteria. \*\*\*,  $p < 0.001$  compared with the value of SA. ##,  $p < 0.01$  and ###,  $p < 0.001$  compared with the value of CONT. **B**, Histological findings. Lungs were excised and subjected to H&E staining. Magnification,  $\times 40$  each. **C**, BALF cytokine concentrations. Supernatant of BALF was assayed for IL-13 and TGF- $\beta$  concentrations by ELISA ( $n = 11$ – $12$  per group). \*\*,  $p < 0.01$  compared with the value of SA. #,  $p < 0.05$  and ##,  $p < 0.01$  compared with the value of CONT. **D**, In vivo IL-10 gene delivery suppresses AHR. AHR to Mch was measured as described in *Materials and Methods* ( $n = 11$ – $12$  per group). Results were expressed for each Mch concentration as the percentage of the baseline Penh value after SA exposure. \*,  $p < 0.05$  compared with the value of SA. #,  $p < 0.05$  compared with the value of CONT.

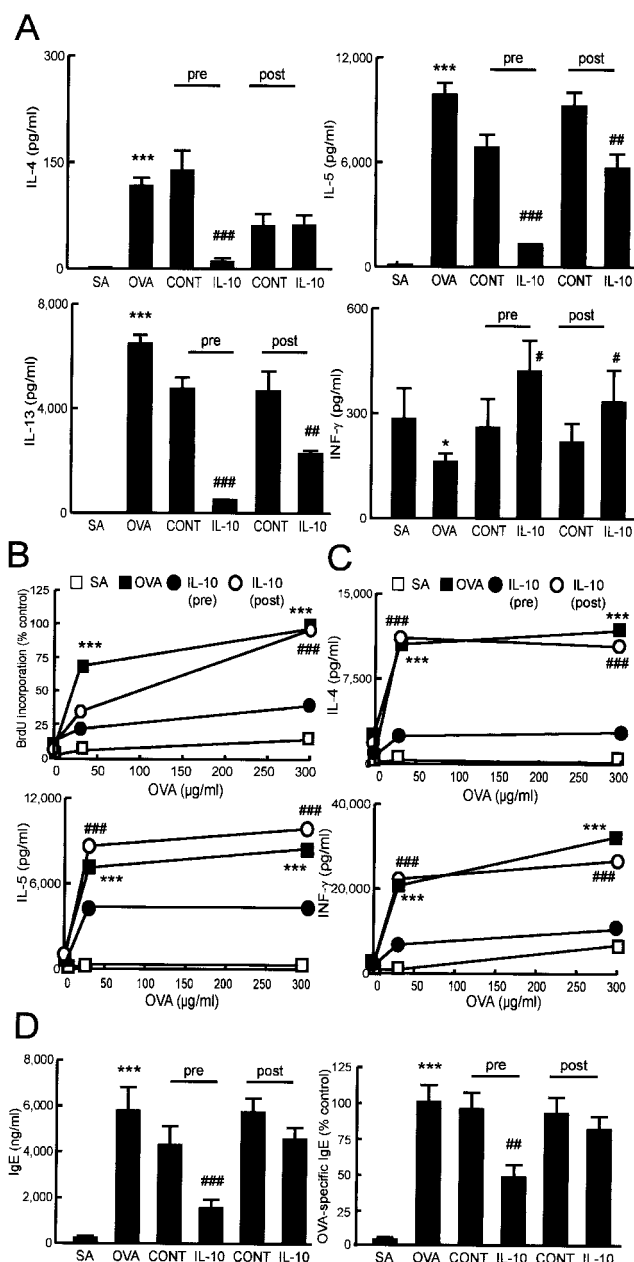


**FIGURE 5.** In vivo IL-10 gene delivery during aerosol challenge suppresses Th2 cytokine production by lung cells, but not by spleen cells. Mice were treated as described in Fig. 4. On day 21, mice were analyzed. **A**, Cytokine production by lung cells. Lung cells were incubated ( $5 \times 10^5$  cells/well) with OVA (200  $\mu$ g/ml). After 120 h, the cytokine levels of the supernatants were assayed ( $n = 6$  per group). \*,  $p < 0.05$  and \*\*\*,  $p < 0.001$  compared with the value of SA. #,  $p < 0.05$ , ##,  $p < 0.01$ , and ###,  $p < 0.001$  compared with the value of CONT. **B** and **C**, Proliferation and cytokine production of spleen cells. Spleen cells were incubated ( $5 \times 10^5$  cells/well) with OVA. **B**, After 72 h, the proliferation was assessed based on BrdU incorporation ( $n = 6$  per group). The control was the maximum proliferation observed in response to OVA for spleen cells from OVA-sensitized mice (OVA; 100%). **C**, After 120 h, the cytokine levels of the supernatants were assayed ( $n = 6$  per group). \*\*\*,  $p < 0.001$  compared with the value of SA. ###,  $p < 0.001$  compared with the value of IL-10 (pre). **D**, Serum IgE concentrations. On day 21, blood samples were obtained from the mice presented in Fig. 4, and total and OVA-specific IgE concentrations were measured by ELISA ( $n = 11$ – $12$  per group). For measurement of OVA-specific IgE, sera from five OVA-sensitized mice was set as a control. \*\*\*,  $p < 0.001$  compared with the value of SA. ##,  $p < 0.01$  and ###,  $p < 0.001$  compared with the value of CONT.

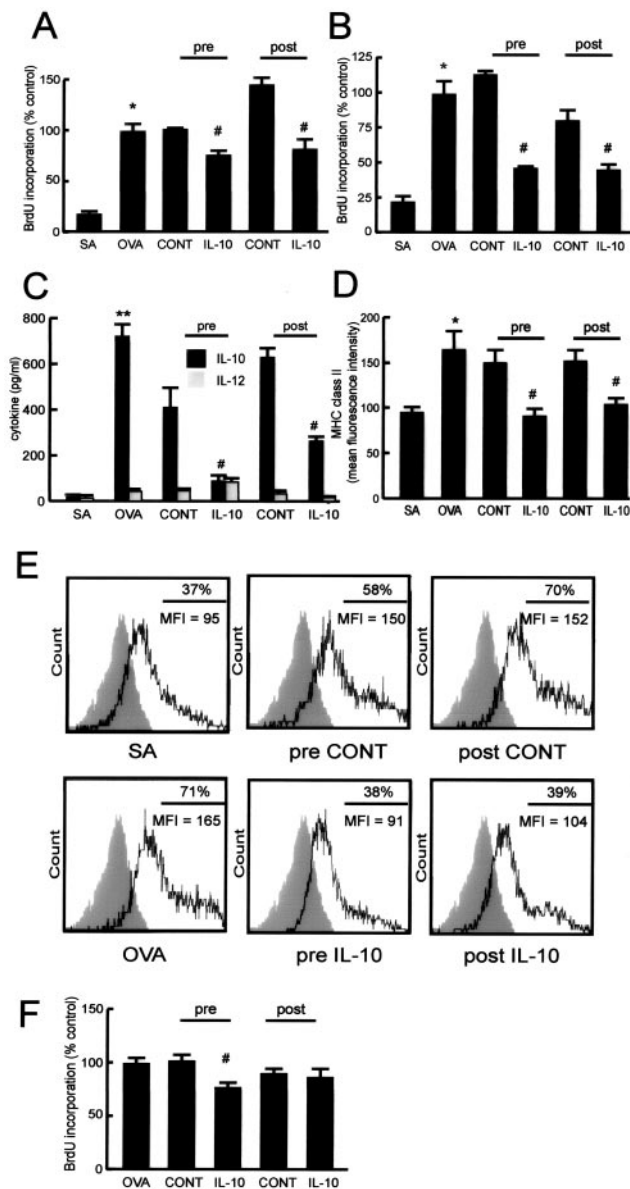


**FIGURE 6.** In vivo IL-10 gene delivery suppresses resident lung APC functions. Mice were treated as described in Fig. 4 and were evaluated on day 21. **A**, Allogenic MLR. Lung CD11c<sup>+</sup> cells ( $2.5 \times 10^4$  cells/well) were cultured with CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well) from C57BL/6 mice. After 60 h, the proliferation was assessed based on BrdU incorporation ( $n = 6-10$  per group). The control was the proliferation of CD4<sup>+</sup> T cells from C57BL/6 mice responding to lung CD11c<sup>+</sup> cells from OVA-sensitized and -nebulized BALB/c mice (OVA; 100%). \*,  $p < 0.05$  compared with the value of SA. #,  $p < 0.05$  compared with the value of CONT. **B**, OVA-presenting capacity of lung CD11c<sup>+</sup> cells. Lung CD11c<sup>+</sup> cells ( $2.5 \times 10^4$  cells/well) were cultured with CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well) from DO11.10 mice. After 60 h, the proliferation was assessed based on BrdU incorporation ( $n = 6-10$  per group). The control was the proliferation of CD4<sup>+</sup> T cells from DO11.10 mice in response to lung CD11c<sup>+</sup> cells from OVA-sensitized and -nebulized BALB/c mice (OVA). \*,  $p < 0.05$  compared with the value of SA. #,  $p < 0.05$  compared with the value of CONT. **C**, Cytokine production. Lung CD11c<sup>+</sup> cells ( $1.25 \times 10^5$  cells/well) were cultured with LPS (1  $\mu$ g/ml). After 24 h, the cytokine levels of the supernatants were assayed ( $n = 6-10$  per group). \*\*,  $p < 0.01$  compared with the value of SA. #,  $p < 0.05$  compared with the value of CONT. **D**, MHC class II expression of lung CD11c<sup>+</sup> cells was measured by flow cytometry ( $n = 6-10$  per group). \*,  $p < 0.05$  compared with the value of SA. #,  $p < 0.05$  compared with the value of CONT. **E**, Representative figures of MHC class II expression. **F**, OVA-presenting capacity of splenic DCs. Splenic CD11c<sup>+</sup> cells ( $1.25 \times 10^5$  cells/well) were cultured with

### Treatment of OVA-pulsed DCs with IL-10 in vitro directly prevents the development of eosinophilic airway inflammation

Suppression of splenic DC functions (Fig. 2, D–H) or lung APC functions (Fig. 6, A–E) by IL-10 gene delivery seems to be mild. Therefore, to confirm the significance of the suppression of APC functions by IL-10 to inhibit subsequent airway inflammation, we finally performed a transfer experiment. Stimulation of DCs with OVA activated DCs, and GM-CSF treatment further up-regulated these functions such as OVA-Ag presenting capacity (Fig. 9A), MHC class II expression (Fig. 9B), and expression of cell surface markers such as CD40, CD80, and CD86 (data not shown). Treatment with IL-10 in vitro suppressed these functions by almost 25% in both GM-CSF-treated and untreated DCs (Fig. 9, A and B, and data not shown). The degree of suppression was equivalent to the suppression of splenic DC functions (Fig. 2, D–H) or lung APC functions (Fig. 6, A–E) obtained by in vivo IL-10 gene delivery. In an in vivo experiment, transfer of OVA-pulsed DCs into naive mice and subsequent OVA inhalation induced eosinophilic airway inflammation, and GM-CSF-treatment of DCs increased the intensity of the eosinophilic airway inflammation (Fig. 9C). The transfer of DCs treated with IL-10 in vitro attenuated the eosinophilic airway inflammation (Fig. 9C). These results confirmed that the suppression of APC functions by IL-10 could sufficiently inhibit the development of subsequent eosinophilic inflammation in vivo.

### Discussion

In the current study, we demonstrated that in vivo IL-10 gene delivery suppresses Ag-induced eosinophilic airway inflammation and AHR even during airway Ag challenge, an effector phase of the immune response. In vivo IL-10 gene delivery during airway challenge suppressed local ongoing immune responses of the lung without impairing the Ag-specific systemic immune response, and this suppression was mediated by down-regulating the functions of resident lung APCs as well as the migration of CD11c<sup>+</sup> cells into regional lymph nodes. The present study confirmed a significant role for IL-10 as an Ag-specific, site-specific immune regulator.

First, we confirmed the effect of IL-10 on the Ag-induced immune response ex vivo (Fig. 2). IL-10 suppressed the Ag-induced immune response of CD4<sup>+</sup> T cells (Fig. 2, A and B) and DC functions (Fig. 2, D–H). Generally, IL-10 down-regulates the expression of MHC class II and costimulatory molecules on monocytes/macrophages and DCs (1, 7–10, 48), thus suppressing the Ag-induced immune response. Our data from spleen cells are consistent with these previous studies.

Chronic activation of CD4<sup>+</sup> T cells with Ag in the presence of IL-10 induces the development of Tr1 cells, which produce IL-10, but not IL-2 and IL-4 (23). Some reports have indicated that TGF- $\beta$  converts CD4<sup>+</sup>CD25<sup>+</sup> T cells to Treg cells in vitro though the induction of Foxp3 (44, 49). A recent report showed that CD4<sup>+</sup> T cells expressing membrane-bound TGF- $\beta$  and Foxp3 are involved in the tolerance induced by Ag inhalation (50). In the current study, IL-10 treatment did not up-regulate IL-10 production (Fig. 2B) or TGF- $\beta$  production (data not shown) by CD4<sup>+</sup> T cells. In addition, in vivo IL-10 gene delivery did not enhance TGF- $\beta$  production in the lung (Fig. 4C). Further, IL-10 did not increase

CD4<sup>+</sup> T cells ( $1.25 \times 10^5$  cells/well) from OVA-sensitized mice and OVA (30  $\mu$ g/ml). After 96 h, the proliferation was assessed based on BrdU incorporation ( $n = 6-10$  per group). The control was the proliferation of CD4<sup>+</sup> T cells from OVA-sensitized mice in response to splenic CD11c<sup>+</sup> cells from OVA-sensitized and -nebulized BALB/c mice and OVA (OVA). #,  $p < 0.05$  compared with the value of CONT.

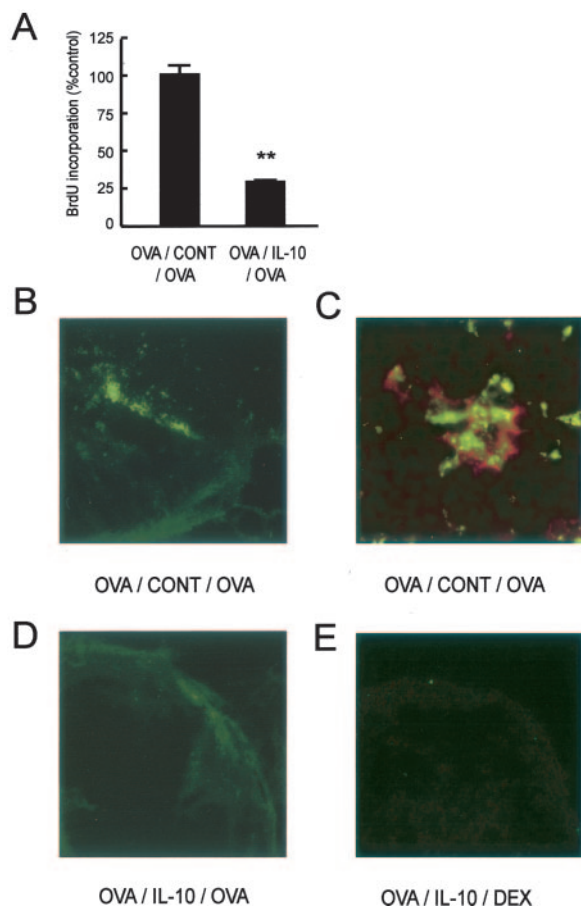


**FIGURE 7.** In vivo IL-10 gene delivery suppresses the migration of CD11c<sup>+</sup> cells into draining lymph nodes. **A**, Capacity of regional lymph node cells to activate OVA-specific TCR-transgenic CD4<sup>+</sup> T cells. Mice were sensitized with OVA on days 0 and 11. Mice received pCAGGS-IL-10 or control pCAGGS i.v. on day 17 and then were challenged with OVA from days 18 to 20. On day 21, regional lymph nodes were excised. Mitomycin C-treated lymph node cells ( $2.5 \times 10^5$  cells/well) were cultured with DO11.10 CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well). After 72 h, the proliferation was assessed based on BrdU incorporation ( $n = 6$  per group). \*\*,  $p < 0.01$  compared with the value for mice that received OVA sensitization, control pCAGGS injection, and OVA challenge (OVA/CONT/OVA). **B**, Fluorescence micrograph of a lymph node. Mice were sensitized with OVA on days 0 and 11. Mice received control pCAGGS on day 17. On day 20, mice were given FITC-labeled OVA (500  $\mu$ g) intratracheally. On day 21, bronchial lymph nodes were excised and subjected to fluorescence microscopy. FITC-positive cells (green) were clearly detected in the regional lymph nodes of mice (OVA/CONT/OVA). Magnification,  $\times 40$ . **C**, Double staining. The section was fixed in acetone and treated with PE anti-CD11c mAb. Some FITC-positive cells from mice that received OVA sensitization and control pCAGGS injection (OVA/CONT/OVA) were positive for CD11c (red). Magnification,  $\times 200$ . **D**, In vivo IL-10 gene delivery suppresses the migration of the OVA-captured APCs. Mice were sensitized with OVA on days 0 and 11. Mice received pCAGGS-IL-10 on day 17. On day 20, mice were given FITC-labeled OVA (500  $\mu$ g) intratracheally. On day 21, bronchial lymph nodes were excised (OVA/IL-10/OVA). Magnification,  $\times 40$ . **E**, IL-10 suppresses the migration of Ag-captured APCs irrespective of the kind of Ag. Mice were sensitized with OVA on days 0 and 11. Mice received pCAGGS-IL-10 on day 17. On day 20, mice were given FITC-labeled dextran (500  $\mu$ g). On day 21, bronchial lymph nodes were excised (OVA/IL-10/DEX). Magnification,  $\times 40$ .

Foxp3 mRNA expression in CD4<sup>+</sup> T cells either (Fig. 2C). Therefore, IL-10 gene delivery did not induce the development of Treg cells in our study. In a preliminary study, Foxp3 mRNA expression

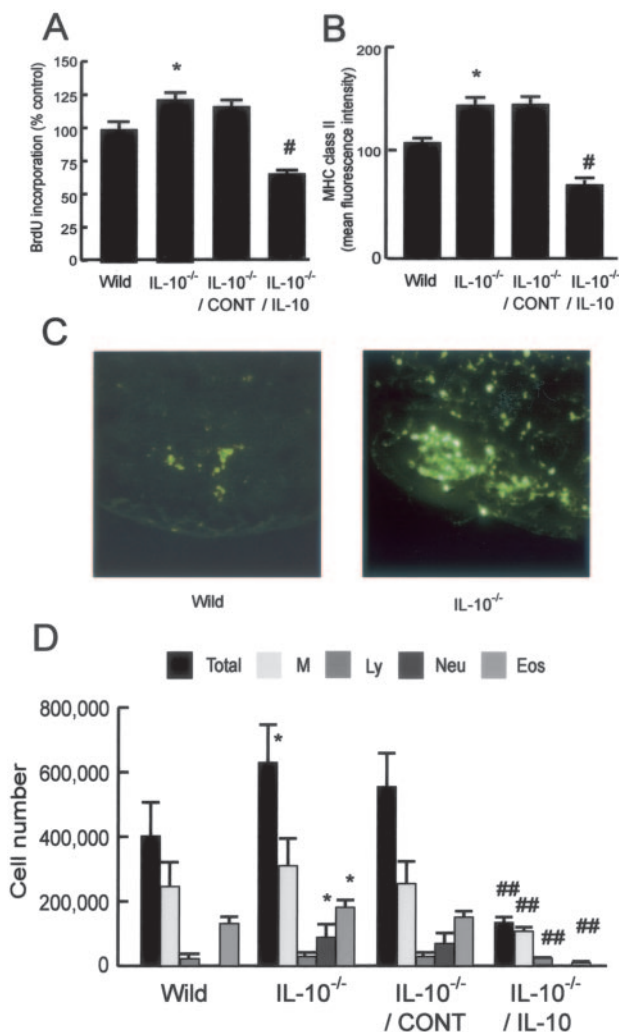


**FIGURE 8.** IL-10-deficient mice exhibit exaggerated eosinophilic airway inflammation accompanied by up-regulation of lung APC functions. Wild-type C57BL/6 mice and IL-10-deficient mice were sensitized with OVA on days 0 and 11. Some IL-10-deficient mice received pCAGGS-IL-10 or control pCAGGS i.v. on day 17. Then, mice were challenged with OVA from days 18 to 20. On day 21, the lungs were subjected to analyses. **A**, Allogeneic MLR. Lung CD11c<sup>+</sup> cells ( $2.5 \times 10^4$  cells/well) were cultured with CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  cells/well) from BALB/c mice. After 60 h, the proliferation was assessed based on BrdU incorporation ( $n = 6$  per group). The control was the proliferation of CD4<sup>+</sup> T cells from BALB/c mice in response to lung CD11c<sup>+</sup> cells from OVA-sensitized and -nebulized wild-type C57BL/6 mice (Wild; 100%). **B**, MHC class II expression of lung CD11c<sup>+</sup> cells was measured by flow cytometry ( $n = 6$  per group). **C**, Presence of FITC-labeled OVA in lymph nodes. Mice were sensitized with OVA on days 0 and 11. On day 20, mice were given FITC-labeled OVA intratracheally. On day 21, bronchial lymph nodes were excised. The sections were cut by cryostat and observed. Magnification,  $\times 40$  each. **D**, BALF cell findings. Leukocytes were identified by morphological criteria ( $n = 8$ –10 per group). M, Macrophages; Ly, lymphocytes; Neu, neutrophils; Eos, eosinophils. \*,  $p < 0.05$  compared with the value for wild-type mice. #,  $p < 0.05$  and ##,  $p < 0.01$  compared with the value for IL-10-deficient mice that received control pCAGGS (IL-10<sup>-/-</sup>/CONT).

in CD4<sup>+</sup> T cells or the ratio of CD4<sup>+</sup>CD25<sup>+</sup> cells to CD4<sup>+</sup>CD25<sup>-</sup> cells were not altered in the IL-10-deficient mice (data not shown). Moreover, in vivo IL-10 gene delivery into IL-10-deficient mice did not affect them either (data not shown). These results also support the speculation that Treg cells would not be induced by IL-10 gene delivery. Thus, IL-10 revealed its effect on the immune

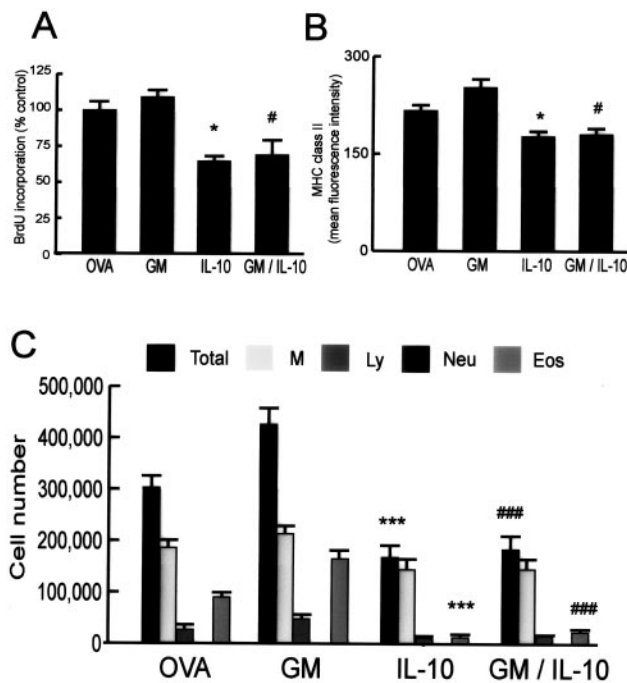


**FIGURE 9.** OVA-pulsed, IL-10-treated DCs prevent the development of eosinophilic airway inflammation provoked by a combination of DC transfer and subsequent OVA inhalation. **A**, IL-10-treated DCs impair OVA-presenting capacity. Splenic CD11c<sup>+</sup> cells were positively selected by magnetic cell sorting from naive mice and incubated with OVA (1000  $\mu$ g/ml) in the presence or absence of GM-CSF (10 ng/ml). IL-10 was added to some culture media (50 ng/ml). After 20 h, OVA-pulsed CD11c<sup>+</sup> cells were collected ( $2.5 \times 10^4$ ) and cultured with CD4<sup>+</sup> T cells from DO11.10 mice ( $2.5 \times 10^5$ ). After 48 h, the proliferation was assessed based on BrdU incorporation ( $n = 6$  per group). The control was the proliferation of CD4<sup>+</sup> T cells from DO11.10 mice in response to OVA-pulsed CD11c<sup>+</sup> cells (OVA; 100%). **B**, MHC class II expression of OVA-pulsed CD11c<sup>+</sup> cells was measured by flow cytometry ( $n = 6$  per group). **C**, OVA-pulsed and IL-10-treated DCs attenuate transfer-mediated eosinophilic airway inflammation. Syngeneic recipients received OVA-pulsed CD11c<sup>+</sup> cells (OVA), OVA-pulsed GM-CSF-treated CD11c<sup>+</sup> cells (GM), OVA-pulsed IL-10-treated CD11c<sup>+</sup> cells (IL-10), or OVA-pulsed GM-CSF- and IL-10-treated CD11c<sup>+</sup> cells (GM/IL-10) on day 0 ( $5 \times 10^5$ ). The mice were challenged with nebulized 3% OVA for 1 h twice daily on days 7, 8, and 15 and sacrificed on day 16 for BALF analysis ( $n = 6-8$  per group). M, Macrophages; Ly, lymphocytes; Neu, neutrophils; Eos, eosinophils. \*,  $p < 0.05$  and \*\*\*,  $p < 0.001$  compared with the value of OVA. #,  $p < 0.05$  and ###,  $p < 0.001$  compared with the value of GM.

response by some mechanism other than inducing production of Treg cells in our experimental system.

Repetitive exposure of the respiratory tract to Ag could induce some kind of DCs that produce IL-10 to induce the development of Treg cells (24, 25) and thus may work as tolerogenic DCs. In the present study, IL-10 gene delivery did not up-regulate, but rather suppressed, the production of IL-10 by purified splenic DCs stimulated with LPS or TNF- $\alpha$  (Fig. 2, *G* and *H*). Thus, a single injection of the IL-10 expression plasmid did not suppress the OVA-induced immune response by generating IL-10-producing tolerogenic DCs.

In the current study, we delivered IL-10 via an expression plasmid. Instillation of IL-10 protein into the lung also suppresses eosinophilic airway inflammation (34, 35). However, generally, the half-lives of recombinant cytokines are very short. In fact, the circulating half-life of a systemically delivered rIL-10 protein is only 20 min (51). Further, the degree of suppression of eosino-

philic inflammation by IL-10 protein was weak in previous reports (1/4–1/5 as compared with positive control) (34, 35). Instillation of IL-10 protein even increases AHR unexpectedly (32, 35). In contrast, in this study, after a single injection of IL-10-expressing plasmid, both serum and BALF IL-10 concentrations remained high for 5–7 days (Fig. 1). IL-10 gene delivery almost completely suppressed eosinophilic inflammation (1/20–1/100 of control) (Fig. 4A) and it also suppressed AHR (Fig. 4D). Thus, IL-10 gene delivery was highly efficient and was superior to the instillation of IL-10 protein for controlling airway inflammation and AHR. In addition, we demonstrated that, even after systemic sensitization had been completed, a high level of IL-10 strongly suppressed Ag-induced eosinophilic inflammation (Fig. 4). In a previous report on the gene delivery system, IL-10 was effective only when administered before systemic sensitization (37). We, for the first time, clearly demonstrated the effect of IL-10 gene delivery after systemic sensitization had been completed, and the airway immune response was ongoing, which could lead to the clinical application of gene delivery for patients with asthma.

In vivo IL-10 gene delivery during inhalation suppressed the production of Th2 cytokines by lung cells in response to OVA (Fig. 5A). However, it did not suppress the proliferation or cytokine production of spleen cells (Fig. 5, *B* and *C*). It did not suppress OVA-specific IgE production either (Fig. 5D). In contrast, as expected, in vivo IL-10 gene delivery before sensitization suppressed the production of cytokines in both the lung and spleen (Fig. 5, *A–C*). OVA-specific IgE production was also significantly suppressed (Fig. 5D). In the case of presensitization delivery of the IL-10 plasmid, IL-10 suppressed the OVA-induced immune response from the initial stage of sensitization, so the overall response was suppressed both in the lung and in the spleen. Therefore, further immune responses induced by OVA inhalation were accordingly suppressed. In contrast, when IL-10 was delivered during OVA inhalation, systemic sensitization had already developed in the spleen, and IgE synthesis had been accomplished. Therefore, presensitization delivery exhibited stronger suppression than postsensitization delivery (Figs. 4 and 5).

In addition, as we have demonstrated in studies in vitro, IL-10 had a strong immunosuppressive effect where Ag and APCs coexisted (Fig. 3, *C* and *D*). Thus, when IL-10 was delivered during OVA inhalation into the airway, it suppressed the immune response more strongly in the lung, although the delivery of IL-10 was systemic. In other words, the immunosuppressive effect of IL-10 is essentially the same between lung and spleen, however, the presence of Ag determined the intensity of suppression. Zuan-Yan et al. (34) reported that the instillation of recombinant murine IL-10 with Ag to the lung reduces Ag inhalation-induced eosinophilic inflammation, whereas IL-10 alone does not show a suppressive effect. In another system, IL-10 delivered into the ankle attenuated arthritis that was initiated by direct injection of OVA-Ag into the ankle in presensitized mice without affecting the immune response in the spleen (52). These studies support our findings. In other words, IL-10 acts as an immunosuppressive cytokine especially at the site of the immune response where both Ag and APCs exist concurrently. If we could deliver the IL-10 gene selectively into the lung, the lung immune response would be specifically suppressed, which should be further investigated in future.

To further clarify the mechanism of action of IL-10 in the lung immune response, we next analyzed the effect of IL-10 on lung APC function. Generally, lung APCs capture inhaled Ag in situ, mature, and migrate into draining lymph nodes to activate naive T cells (45). Furthermore, recent reports have demonstrated that some resident DCs can mature and present Ag to T cells in the lung

before migration (53–55). In our study, IL-10 gene delivery suppressed resident lung APC functions such as Ag-presenting capacity and cytokine production (Fig. 6, A–C). The expression of MHC class II molecule on APCs was significantly down-regulated (Fig. 6, D and E). The expression of other costimulatory molecules such as CD40, CD80, and CD86 was also down-regulated, although a statistically significant difference was not detected, probably due to a low baseline expression with OVA treatment. Moreover, in vivo IL-10 gene delivery also suppressed the migration of OVA-captured CD11c<sup>+</sup> cells (DCs) into regional lymph nodes (Fig. 7). Taken together, at least in our experimental system, the mechanism of action of IL-10 was not to induce the regulatory function, but rather to suppress the overall function of APCs in the initial stage of Ag presentation and recognition. In contrast, the effect of postsensitization delivery of IL-10 on splenic DCs was slight and not significant (Fig. 6F).

Next, we confirmed whether inhibition of APC function by IL-10 directly leads to inhibition of eosinophilic airway inflammation. In IL-10-deficient mice treated with OVA sensitization and inhalation, lung APC function was up-regulated and stronger eosinophilic airway inflammation was provoked compared with that in wild-type mice (Fig. 8). Compensation by IL-10 gene delivery again down-regulated the APC function and thus attenuated eosinophilic airway inflammation. Further, in vitro treatment of splenic DCs with OVA activated the DCs, and the transfer of these cells into naive mice followed by subsequent OVA inhalation provoked eosinophilic inflammation (Fig. 9). IL-10 treatment in vitro down-regulated the Ag-presenting capacity and MHC class II expression of DCs by 25–50% (Fig. 9, A and B). Then, the intensity of this transfer-induced eosinophilic airway inflammation was strongly attenuated by this IL-10 treatment (Fig. 9C). This supports the reliability of data obtained from in vivo IL-10 gene delivery into sensitized and challenged mice, in which the APC function of lung cells was suppressed by 25–50% (Fig. 6, A–E), which resulted in a strong suppression of airway inflammation and AHR (Fig. 4). Taken together, these results indicated that suppression of APC function by IL-10 sufficiently led to inhibition of eosinophilic airway inflammation and that this was one definite mechanism by which IL-10 inhibits eosinophilic inflammation.

As IL-10 is a multipotent immunosuppressive cytokine, it could exhibit various effects on many resident cells and inflammatory cells, such as endothelial cells, monocytes/macrophages, lymphocytes, and mast cells (1, 56). In our experimental system, mechanisms other than the suppression of APC function could be attributed to the suppression of Ag-induced airway responses. For example, the possibility that IL-10 affected T cells directly cannot be completely ruled out, because in humans, the direct effect of IL-10 on T cell proliferation has been confirmed (11–13). In the current study, however, IL-10 suppressed DC functions in vitro (50 ng/ml; Fig. 9, A and B), whereas this concentration of IL-10 did not directly suppress CD4<sup>+</sup> T cell proliferation and cytokine production (Fig. 3, A and B). Thus, this possibility seems unlikely. IL-10 could also have modulated the expression of VCAM-1 on endothelial cells, thus reducing the recruitment of eosinophils into the lung. These possibilities should also be considered. In addition, at present there is a limitation to the purification of CD11c<sup>+</sup> cells from the lung. In the present study, the purity was 80–85%, which was similar to that in previous reports (42, 43). We confirmed that there was no difference in the purity of CD11c<sup>+</sup> cells among experimental groups in the same experiment (data not shown). Therefore, we consider that our results on APC function are, not completely but to a certain extent, reliable. However, the effect of contaminating cells should also be considered.

Strong Ag stimulation may produce qualitatively and quantitatively different Ag responses from a modest stimulation. In a preliminary study, we examined various concentrations of OVA-Ag and found that the protocol used in the current study induced a maximal response. Given that the purpose of this study was to examine the therapeutic effect of IL-10, we applied this protocol throughout the study to induce a specific intensity of immune response. However, differences in the intensity of Ag stimulation could affect how IL-10 would suppress the Ag-induced immune response.

In summary, IL-10 gene delivery achieved a high concentration of protein in the lung and suppressed OVA-Ag-induced eosinophilic airway inflammation and AHR, even after sensitization had been completed, by down-regulating lung APC functions and the subsequent Th2 response. IL-10 exhibited its suppressive effect in a site-specific manner in the tissue where DCs encountered Ag and thus an Ag-specific immune response was driven. Therefore, if done selectively, IL-10 gene delivery could be a very effective strategy for regulating Ag-specific immune responses in a local environment, such as the lung, without affecting the systemic response.

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## Disclosures

The authors have no financial conflict of interest.

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