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Makoto Dohi

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High Expression of IL-22 Suppresses Antigen-Induced Immune Responses and Eosinophilic Airway Inflammation via an IL-10–Associated Mechanism

Kazuyuki Nakagome,^{*,†,1} Mitsuru Imamura,^{*} Kimito Kawahata,^{*} Hiroaki Harada,^{*} Katsuhide Okunishi,^{*} Taku Matsumoto,^{*} Oh Sasaki,^{*} Ryoichi Tanaka,^{*} Mitsunobu R. Kano,[‡] He Chang,[§] Haruo Hanawa,[§] Jun-ichi Miyazaki,[¶] Kazuhiko Yamamoto,^{*} and Makoto Dohi^{*}

Allergic inflammation in the airway is generally considered a Th2-type immune response. However, Th17-type immune responses also play important roles in this process, especially in the pathogenesis of severe asthma. IL-22 is a Th17-type cytokine and thus might play roles in the development of allergic airway inflammation. There is increasing evidence that IL-22 can act as a proinflammatory or anti-inflammatory cytokine depending on the inflammatory context. However, its role in Ag-induced immune responses is not well understood. This study examined whether IL-22 could suppress allergic airway inflammation and its mechanism of action. BALB/c mice were sensitized and challenged with OVA-Ag to induce airway inflammation. An IL-22–producing plasmid vector was delivered before the systemic sensitization or immediately before the airway challenge. Delivery of the IL-22 gene before sensitization, but not immediately before challenge, suppressed eosinophilic airway inflammation. IL-22 gene delivery suppressed Ag-induced proliferation and overall cytokine production in CD4⁺ T cells, indicating that it could suppress Ag-induced T cell priming. Antagonism of IL-22 by IL-22–binding protein abolished IL-22–induced immune suppression, suggesting that IL-22 protein itself played an essential role. IL-22 gene delivery neither increased regulatory T cells nor suppressed dendritic cell functions. The suppression by IL-22 was abolished by deletion of the IL-10 gene or neutralization of the IL-10 protein. Finally, IL-22 gene delivery increased IL-10 production in draining lymph nodes. These findings suggested that IL-22 could have an immunosuppressive effect during the early stage of an immune response. Furthermore, IL-10 plays an important role in the immune suppression by IL-22. *The Journal of Immunology*, 2011, 187: 000–000.

Allergic inflammation in the airway is generally considered a Th2-type response-mediated process (1). However, recent studies have demonstrated that Th1- and Th17-type immune responses also play some role (2–8). For example, the passive transfer of OVA-specific Th1 cells exacerbated OVA-induced airway eosinophilia (2, 3). Furthermore, the passive transfer of OVA-specific Th17 cells induced steroid-resistant airway inflammation and airway hyperresponsiveness (4). Among

Th17-type immune responses, IL-17 is thought to be important for the development of severe asthma as it induces neutrophilic inflammation (5), a hallmark of severe asthma (6). IL-17 concentration in the sputum correlates with the clinical severity of asthma (7). Expression of IL-17 in the airway increases only in severe asthmatics (8). Therefore, inhibition of Th17 cells or IL-17 could be a reasonable strategy for controlling severe asthma.

IL-22 is a member of the IL-10 cytokine family together with IL-10, IL-19, IL-20, IL-24, IL-26, IL-28A, IL-28B, and IL-29 (9–11). IL-10 is an immunosuppressive cytokine that signals through a heterodimer comprising IL-10R1 and IL-10R2 (12). We previously reported that IL-10 has a potent suppressive effect on allergic airway inflammation by downregulating APC functions and migration (13). In contrast, IL-22 transduces signals through a heterodimer comprising IL-22R1 and IL-10R2. Although IL-10R2 is expressed on various types of cells including immune cells, the expression of IL-22R1 is not found on immune cells and is restricted to certain tissues such as epithelial cells (9–11, 14). Therefore, IL-22 does not act directly on immune cells, in contrast to IL-10.

IL-22 is also made by Th17 cells (15). Therefore, IL-22 might play some role in the pathogenesis of bronchial asthma, especially in severe cases of asthma. However, increasing evidence suggests that IL-22 is regulated differently from IL-17 and other Th17-related cytokines (16–21). IL-22 expression can be induced solely by IL-6, whereas IL-17 expression was found to require both TGF- β and IL-6 (16). IL-22 expression is dependent on the transcription factor aryl hydrocarbon receptor (17), and this is in contrast to expression of other Th17-related cytokines that are highly dependent on the transcription factor retinoic acid orphan

^{*}Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [†]Department of Respiratory Medicine and Allergy Center, Saitama Medical University, Saitama, Japan; [‡]Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [§]Division of Cardiology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan; and [¶]Division of Stem Cell Regulation Research, Osaka University Graduate School of Medicine, Osaka, Japan

¹Current address: Department of Pediatrics, University of Wisconsin School of Medicine and Public Health, Madison, WI.

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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

Abbreviations used in this article: alum, aluminum hydroxide; BALF, bronchoalveolar lavage fluid; DC, dendritic cell; FR4, folate receptor 4; GPCR, glucocorticoid-induced TNF receptor; IL-22BP, IL-22–binding protein; PAS, periodic acid–Schiff; RagDof, Rag2^{−/−} DO11.10 scurfy; SA, physiologic saline; SP, signal peptide; Treg, regulatory T cell.

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receptor γ t (18). Furthermore, a distinct population of human CD4⁺ T cells that produce IL-22, without the cosynthesis of IL-17, was identified recently (19–21). Therefore, IL-22 might act on immune responses in a different way from IL-17.

Although patients with psoriasis, rheumatoid arthritis, and inflammatory bowel disease are known to have elevated levels of IL-22 (22–24), the role of IL-22 in these immune diseases is still controversial (16, 22, 24–37). For example, IL-22 plays a central role in the development of psoriasis-like skin inflammation (16, 22, 25–27). Deletion of IL-22 inhibited collagen-induced arthritis (28), suggesting a proinflammatory role for IL-22 in the pathogenesis of rheumatoid arthritis. In contrast, IL-22 ameliorated intestinal inflammation in a mouse model of inflammatory bowel disease (24, 29, 30). IL-22 plays a protective role in T cell-mediated murine hepatitis (31–33). Delivery of the IL-22 gene ameliorated experimental autoimmune myocarditis in rats (34). Furthermore, IL-22 is crucial for the control of pulmonary or gastrointestinal tract infections (35, 36). These findings suggest that IL-22 could function as an immunoprotective cytokine in some situations. However, whether IL-22 suppresses allergic inflammation in the airway has not been investigated.

IL-22-binding protein (IL-22BP) is considered a natural antagonist of IL-22 (38). It binds an epitope on IL-22 and neutralizes IL-22 activity such as STAT3 activation *in vitro* (38). Recently, attempts have been made to antagonize endogenous IL-22 using IL-22BP *in vivo* (29, 39). For example, neutralization of IL-22 by administration of IL-22BP-Fc before sepsis induction enhanced the accumulation of inflammatory cells and reduced bacterial load at the site of infection (39). Gene delivery of IL-22BP suppressed goblet cell restitution during the recovery phase of acute colitis, although gene delivery of IL-22 induced its restitution (29). However, the effect of IL-22BP on Ag-induced immune responses or allergic inflammation was not investigated.

The purpose of the current study is first to examine whether IL-22 suppresses Ag-induced immune responses in the airway, and then to elucidate the mechanism of its action. To examine its effect fully, IL-22 was delivered into mice using an IL-22-producing plasmid. The delivery of IL-22 gene prior to systemic sensitization suppressed Ag-induced T cell priming and eosinophilic airway inflammation, although it neither generated regulatory T cells (Tregs) nor suppressed the functions of dendritic cells (DCs). Administration of IL-22BP abolished the immune suppression by IL-22 gene delivery, suggesting that IL-22 protein itself played an essential role. The suppression by IL-22 was abrogated by deletion of the IL-10 gene or neutralization of the IL-10 protein. Furthermore, IL-22 gene delivery increased the IL-10 production in regional lymph nodes. These results indicate that *in vivo* overproduction of IL-22 suppresses Ag-induced immune responses, and IL-10 plays an important role in this process.

Materials and Methods

Mice

Male BALB/c and C57BL/6 mice were obtained from Charles River Japan (Kanagawa, Japan). OVA TCR-transgenic DO11.10 mice, C57BL/6-IL-10^{−/−} mice, and scurfy mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Rag2^{−/−} BALB/c mice were obtained from Taconic Farms (Germantown, NY). Rag2^{−/−} DO11.10 mice were obtained by crossing Rag2^{−/−} BALB/c mice with DO11.10 mice. Rag2^{−/−} DO11.10 scurfy (RagDOsf) mice were obtained by crossing Rag2^{−/−} DO11.10 mice with scurfy mice. All animal experiments were approved by the Animal Research Ethics Board of the Department of Allergy and Rheumatology, University of Tokyo.

Delivery of IL-22 into mice

IL-22 was delivered *in vivo* by a hydrodynamic-based method through the i.v. injection of plasmid DNA (13, 34, 40–44). The plasmid pCAGGS-rat

IL-22-Ig-GLU-tag (34) was amplified in *Escherichia coli* and purified with a Qiagen Endo Free Plasmid Giga kit (Qiagen GmbH, Hilden, Germany). The plasmid pCAGGS-signal peptide (SP)-Ig-GLU-tag (34) was used as a control. Plasmid DNA [100 μ g (40 μ g/ml); pCAGGS-rat IL-22-Ig-GLU-tag or pCAGGS-SP-Ig-GLU-tag] in lactated Ringer's solution (0.1 ml/g body weight) was injected intravenously via the tail, and the injection was completed within 5 s. Mouse IL-22 shares 92% amino acid sequence identity with rat IL-22, and thus mouse IL-22 could act on rat cells, and vice versa, as confirmed by the induction of STAT3 phosphorylation (45).

Immunization of mice and evaluation of allergic airway inflammation

Mice were immunized as reported previously (13, 40–42). Seven-week-old animals were sensitized with an i.p. injection of 2 μ g OVA (Sigma-Aldrich, St. Louis, MO) plus 2 mg aluminum hydroxide (alum; Serva, Heidelberg, Germany) on days 0 and 11. Control mice received injections of physiologic saline (SA) on days 0 and 11. Mice were challenged with an aerosolized solution of 3% OVA or PBS for 10 min from day 18 to day 20. Some mice received plasmid DNA (100 μ g; pCAGGS-rat IL-22-Ig-GLU-tag or pCAGGS-SP-Ig-GLU-tag) intravenously on day −3 (before systemic immunization). In another experiment, mice received plasmid DNA on day 17 (immediately before aerosol challenge; in the effector phase). On day 21, samples of serum and bronchoalveolar lavage fluid (BALF) were obtained. After perfusion with SA, the right lungs were cut out and fixed with 10% neutralized buffered formalin (Wako, Osaka, Japan). Three-micrometer-thick sections were prepared and subjected to H&E or periodic acid–Schiff (PAS) staining to evaluate mucus hypersecretion. The left lungs were homogenized in 1.0 ml PBS containing 0.5% Triton X-100 and complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany). The lung homogenates were cleared of debris and cells by centrifugation at 10,000 $\times$ g for 10 min and subjected to measurements of cytokine concentrations. Bronchial lymph nodes were also obtained.

Measurement of cytokines and Ig

IL-5, IL-10, IL-13, IL-22, IFN- γ , TGF- β 1, IgE, and IgG concentrations were measured using ELISA kits (IL-5, IL-10, IFN- γ , and IgE from BD Pharmingen, San Diego, CA; IL-13, IL-22, and TGF- β 1 from R&D Systems, Minneapolis, MN; and IgG from Bethyl Laboratories, Montgomery, TX). The IL-17 concentration was measured by ELISA using anti-mouse IL-17 and biotinylated anti-mouse IL-17 Abs (BD Pharmingen). OVA-specific IgE or IgG was measured using an ELISA kit for IgE or IgG, although the plate was coated with OVA (1000 μ g/ml) at 4°C overnight instead of anti-mouse IgE or IgG Ab. The OVA-specific IgE or IgG standard was derived by pooling sera from five OVA-sensitized mice. Results are expressed as a percentage of the value for the standard.

Preparation of spleen cells and lymph node cells

Spleen cells were prepared as reported previously (13, 40–42). For the preparation of mesenteric or bronchial lymph node cells, lymph nodes were collected and minced, then incubated at 37°C for 30 min with 150 U collagenase (Sigma-Aldrich) in complete DMEM. Cell suspensions were obtained by pressing the tissues through a 70- μ m nylon filter. Splenic CD4⁺ T cells and splenic DCs were prepared using magnetic beads as described previously (13, 40–42). Briefly, spleen cells were incubated with anti-CD4 mAb-coated or anti-CD11c mAb-coated microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Bead-bound cells were then isolated using magnetic separation columns. The purities of the enriched CD4⁺ and CD11c⁺ cell populations were 95% and 85%, respectively (data not shown). More than 95% of the CD4⁺ cells were CD3⁺ T cells (data not shown).

Ag-induced immune response in bronchial lymph nodes

Lymph node cells (2.5×10^5 cells/well) were cultured with OVA (10 μ g/ml) in complete DMEM for 120 h, and then cytokine concentrations in the supernatants were measured by ELISA.

Effect of IL-22 treatment on Ag-induced immune responses in spleen

Mice were sensitized with OVA/alum or SA on day 0. Some mice received plasmid (100 μ g; pCAGGS-rat IL-22-Ig-GLU-tag or pCAGGS-SP-Ig-GLU-tag) on day −3. On day 11, spleen cells were obtained and cultured (2.5×10^5 cells/well) with OVA (20 μ g/ml) in complete DMEM. In some experiments, positively selected CD4⁺ T cells (2×10^5 cells/well) were cultured with freshly isolated mitomycin C (Sigma-Aldrich) treated splenocytes (2×10^5 cells/well) and OVA (20 μ g/ml). After 72 h, the

proliferation was assessed with a cell proliferation ELISA BrdU kit (Roche Applied Science). After 96 h, cytokine concentrations in the supernatant were measured by ELISA. To examine the direct effect of IL-22 on CD4⁺ T cell functions, OVA-sensitized CD4⁺ T cells were incubated with PMA (1 ng/ml; Sigma) and ionomycin (1 μ g/ml; Sigma) for 24 h or plate-bound anti-CD3 Ab (10 μ g/ml; BD Pharmingen) for 48 h in the presence of IL-22 (10 or 200 ng/ml). Then, the proliferation was assessed, and the IL-5 concentration in the supernatant was measured.

Effect of mouse IL-22BP on Ag-specific immune response

Mice were sensitized with OVA/alum or SA on day 0 and received plasmid (100 μ g; pCAGGS-rat IL-22-Ig-GLU-tag or pCAGGS-SP-Ig-GLU-tag) on day -3. Some pCAGGS-rat IL-22-Ig-GLU-tag-injected mice received i.p. injection of mouse IL-22BP-Fc (R&D Systems) on days -1 (25 μ g) and 0 (2 h before and 4 h after OVA sensitization; 25 μ g each). In some experiments, to elucidate the role of endogenous IL-22 in the Ag-specific immune response, mice were sensitized with OVA/alum or SA on day 0 (without plasmid injection), and some OVA-sensitized mice received IL-22BP-Fc i.p. on days -1 (25 μ g) and 0 (2 h before and 4 h after the OVA sensitization; 25 μ g each). On day 11, spleen cells were obtained, and cell proliferation was examined as described previously. To elucidate the role of endogenous IL-22 in the OVA-induced eosinophilic airway inflammation, mice were sensitized with OVA/alum (without plasmid injection) on day 0, and some OVA-sensitized mice received IL-22BP-Fc as described above. Then, the mice were challenged with OVA or PBS from day 18 to day 20, and airway inflammation was assessed on day 21.

Detection of Tregs and apoptosis

For detection of Foxp3⁺ CD4⁺, Foxp3⁺ Helios⁺ CD4⁺, or Foxp3⁺ Helios⁻ CD4⁺ T cells, spleen cells were obtained without collagenase to reduce the contamination of autofluorescent cells and were stained with PE-Cy5 anti-CD4 mAb (BioLegend, San Diego, CA) for 30 min not stimulating with OVA or PMA/ionomycin/brefeldin A. Then, the cells were incubated with the fixation/permeabilization solution (eBioscience, San Diego, CA) for 2 h at 4°C. After washing with permeabilization buffer, the cells were stained with PE anti-Foxp3 Ab (eBioscience) or PE-rat IgG2a (BioLegend) and FITC anti-Helios Ab (BioLegend) or FITC-Armenian hamster IgG (BioLegend) for 30 min. For detection of CD25⁺ CD4⁺, CD103⁺ CD4⁺, glucocorticoid-induced TNF receptor (GITR)⁺ CD4⁺, or folate receptor 4⁺ (FR4⁺) CD4⁺ T cells, spleen cells were stained with PE-Cy5 anti-CD4 Ab and PE anti-CD25 Ab (BD Pharmingen), FITC anti-CD103 Ab (BD Pharmingen), PE anti-GITR Ab (BioLegend), or FITC anti-FR4 Ab (BioLegend) for 30 min. For detection of intracellular CTLA-4⁺ CD4⁺ T cells, spleen cells were stimulated with lymphocyte activator mixture (PMA/ionomycin/brefeldin A; BD Pharmingen) for 5 h. The cells were stained with PE-Cy5 anti-CD4 Ab for 30 min and then incubated with intracellular fixation buffer (eBioscience) for 20 min. After washing with permeabilization buffer (eBioscience), the cells were stained with PE anti-CTLA-4 Ab (BioLegend) for 30 min. For detection of IL-10⁺ CD4⁺ T cells, spleen cells were stimulated with OVA (100 μ g/ml) for 4 d and then restimulated with lymphocyte activator mixture for 5 h. After staining with PE-Cy5 anti-CD4 Ab and incubation with intracellular fixation buffer, the cells were stained with PE anti-IL-10 Ab (BD Pharmingen) for 30 min. The stained cells were analyzed by flow cytometry (EPICS XL System II; Coulter). The cells were gated according to forward scatter/side scatter parameters.

Apoptosis was examined by annexin/propidium iodide assay as described previously (40). In another experiment, TUNEL staining of spleen sections was performed on the basis of labeling of DNA strand breaks using an in situ apoptosis detection kit (Takara Bio, Shiga, Japan).

Analyses of APC functions

In this study, we analyzed the functions of splenic DCs from OVA-sensitized mice or OVA-sensitized and plasmid-injected mice. To analyze allogeneic MLR, mitomycin C-treated BALB/c (H-2^d) CD11c⁺ cells (1×10^6 /well) from the spleen were cultured with positively selected C57BL/6 (H-2^b) CD4⁺ T cells (2.5×10^5 /well) in complete DMEM. For the measurement of OVA-Ag-presenting capacity, CD11c⁺ cells (1×10^5 /well) from the spleen were cultured with OVA-sensitized CD4⁺ T cells (2.5×10^5 /well) and OVA (20 μ g/ml). In some experiments, mitomycin C-treated CD11c⁺ cells (2.5×10^5 /well) were cultured with DO11.10 CD4⁺ T cells (2.5×10^5 /well). After 48 or 72 h, the proliferation was assessed with a cell proliferation ELISA BrdU kit. For measurement of the production of cytokines by CD11c⁺ cells, CD11c⁺ cells (1.25×10^5 /well) were incubated with LPS (1 μ g/ml; Sigma) for 24 h, and then cytokine concentrations in the supernatant were measured. To analyze the direct effect of IL-22 on DC functions in vitro, splenic CD11c⁺ cells from naive mice were

incubated with mouse IL-22 (10 or 200 ng/ml) and OVA (1000 μ g/ml) for 24 h. The cells were collected and washed three times, and then OVA-Ag-presenting capacity was examined. In some experiments, mitomycin C-treated whole spleen cells were used for analyses of overall APC functions.

Effect of anti-IL-10 mAb on Ag-induced immune response of CD4⁺ T cells

Mice were sensitized with OVA/alum or SA on day 0 and received plasmid (100 μ g; pCAGGS-rat IL-22-Ig-GLU-tag or pCAGGS-SP-Ig-GLU-tag) on day -3. Some pCAGGS-rat IL-22-Ig-GLU-tag-injected mice received anti-IL-10 Ab or control IgG i.p. on day 0 (2 h before and 4 h after the OVA sensitization; 250 μ g each). On day 11, spleen cells were obtained, and cell proliferation was examined as described previously.

Effect of IL-22 gene delivery on the expression of IL-10 in regional lymph nodes

Mice were sensitized with OVA/alum or SA on day 0 and received plasmid (100 μ g; pCAGGS-rat IL-22-Ig-GLU-tag or pCAGGS-SP-Ig-GLU-tag) on day -3. On day 2, mesenteric or bronchial lymph node cells (1×10^6 /well) were incubated with LPS (1 μ g/ml; Sigma) for 24 h, and the concentration of IL-10 in the supernatant was measured. RT-PCR for IL-10 in LPS-stimulated lymph node cells was performed using previously reported methods (13). The sense primer for IL-10 was 5'-TCA AAC AAA GGA CCA GCT GGA CAA CAT ACT GC-3', and the antisense primer was 5'-CTG TCT AGG TCC TGG AGT CCA GCA GAC TCA A-3'. The sense primer for β -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'. Densitometric analysis of mRNA expression was performed using Image software (National Institutes of Health, Bethesda, MD). Normalized values for IL-10 mRNA expression in each sample were calculated as the relative quantity of IL-10 divided by the relative quantity of β -actin.

Statistics

Values are expressed as means $\pm$ SEM. Statistical analyses were performed with a one-way ANOVA followed, when differences were significant, by appropriate post hoc tests using the Tukey-Kramer test. For analysis of the differences between two groups, we used Student *t* test. Values of *p* < 0.05 were considered statistically significant.

Results

IL-22 expression in serum and in BALF after hydrodynamic-based gene delivery of plasmid DNA by i.v. injection

First, we examined the kinetics of IL-22 after i.v. injection of the plasmid DNA. Samples were collected at predetermined points in time after injection of the IL-22-expressing plasmid [100 μ g (40 μ g/ml); pCAGGS-rat IL-22-Ig-GLU-tag; p-IL-22] or control plasmid [100 μ g (40 μ g/ml); pCAGGS-SP-Ig-GLU-tag; p-Cont]. We confirmed the temporal expression of the IL-22 protein in serum (Fig. 1A) and in BALF (Fig. 1B). The level of IL-22 in both samples peaked 1 or 4 d after the injection and decreased thereafter. We also examined the kinetics of IL-22 after i.v. injection of the plasmid DNA in OVA-sensitized mice, and similar results were obtained (data not shown).

IL-22 gene delivery before systemic Ag sensitization suppresses Th2-mediated eosinophilic airway inflammation and mucus hypersecretion

We next elucidated the effect of delivering the IL-22 gene prior to sensitization on Th2-mediated allergic inflammation using an experimental model of allergic airway inflammation (Fig. 2). Mice were sensitized with either OVA/alum or SA and then challenged with nebulized OVA or PBS. First, we examined whether OVA sensitization and nebulization would increase endogenous IL-22. Although OVA sensitization and challenge slightly increased the concentrations of IL-22 in lung homogenates [13.2 ± 2.6 pg/ml (SA) versus 21.3 ± 3.2 pg/ml (OVA) measured on day 21, respectively], the differences were not significant. Then, we examined the effect of plasmid delivery. Injection of the plasmid (100

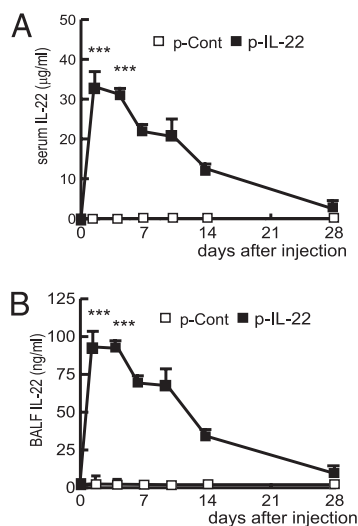


FIGURE 1. Hydrodynamic-based gene delivery of IL-22-expressing plasmid via i.v. injection increases the concentrations of IL-22 in serum and BALF. BALB/c mice received an i.v. injection of the plasmid [100 µg (40 µg/ml); pCAGGS-rat IL-22-Ig-GLU-tag (p-IL-22) or pCAGGS-SP-Ig-GLU-tag (p-Cont)] on day 0 without OVA/alum sensitization. Concentrations of IL-22 in serum (A) and BALF (B) were measured at the indicated times after the injection using ELISA. Values are presented as means $\pm$ SEM for six mice per group. The results presented are representative data from three independent experiments. *** $p < 0.001$ (compared with the value of p-Cont).

µg; p-IL-22 or p-Cont) was performed before the systemic sensitization to Ag. OVA sensitization and nebulization markedly increased the number of eosinophils in BALF (Fig. 2A). IL-22 gene delivery almost completely suppressed the infiltration by eosinophils (Fig. 2A). The OVA-sensitized and OVA-challenged mice showed marked infiltration of the peribronchial area by eosinophils and hypersecretion of mucus by bronchial epithelial cells (Fig. 2B). In the mice that received p-IL-22, these changes were much less extensive (Fig. 2B). BALF IL-13 and TGF- β 1 concentrations also decreased in the p-IL-22-treated mice (Fig. 2C). IL-22 treatment significantly suppressed OVA-specific IgE and IgG production (Fig. 2D). These results indicated that IL-22 gene delivery during the initial stage of sensitization suppressed Th2-mediated allergic airway inflammation *in vivo*.

We next examined whether the IL-22 gene could suppress the production of Th1- and Th17-type cytokines in the lung. As IFN- γ and IL-17 concentrations in BALF did not increase in OVA-sensitized and -challenged mice in our system (data not shown), we measured cytokine concentrations in the lung. Th2-type (IL-5 and IL-13), Th1-type (IFN- γ), and Th17-type (IL-17) cytokines all increased in the lung of OVA-sensitized and -challenged mice, and IL-22 treatment significantly suppressed their production (Fig. 2E). We also observed that IL-22 treatment suppressed the production of IL-5, IL-13, IFN- γ , and IL-17 by bronchial lymph node cells in response to OVA (data not shown). These results indicated that IL-22 gene delivery prior to sensitization suppressed the overall OVA-induced immune response in the lung. However, judging from cytokine productions, Th2 response was particularly suppressed, although Th1 and Th17 responses were also inhibited.

IL-22 gene delivery immediately before the challenge with Ag suppresses neither eosinophilic airway inflammation nor cytokine production in the lung

Next, we examined whether IL-22 suppresses allergic inflammation, when its gene is delivered before the challenge. The plasmid (p-IL-22 or p-Cont) was injected immediately before Ag challenge

(in the effector phase; on day 17). Delivery of the IL-22 gene before the challenge did not suppress the infiltration of eosinophils in BALF (Fig. 3A). Histologically, IL-22 treatment did not decrease the infiltration of inflammatory cells in the peribronchial area or hypersecretion of mucus (data not shown). It did not suppress IL-13 and TGF- β 1 concentrations in BALF (Fig. 3B) or OVA-specific IgE or IgG production (data not shown). These results indicated that delivery of the IL-22 gene immediately before the challenge did not suppress Th2-mediated allergic airway inflammation. Furthermore, IL-22 treatment did not suppress Th2-, Th17-, or Th1-type cytokine production in the lung (Fig. 3C). Moreover, it did not suppress Th2-, Th17-, or Th1-type cytokine production by bronchial lymph node cells in response to OVA (data not shown). Therefore, IL-22 gene delivery had no effect on the overall Ag-induced immune response in the lung when delivered in the effector phase.

In another experiment, we administered 1 µg IL-22 protein intratracheally to OVA-sensitized mice on day 18 (2 h before first OVA nebulization). This treatment exhibited a tendency to suppress eosinophilic airway inflammation (data not shown). Therefore, IL-22 administration in the effector phase could suppress eosinophilic airway inflammation, although our current delivery system using i.v. injection of IL-22-expressing plasmid did not suppress it.

*IL-22 gene delivery suppresses the Ag-induced immune response of CD4⁺ T cells *ex vivo**

As IL-22 gene delivery immediately before Ag challenge had no effect, we next focused on *ex vivo* analyses of the sensitization step. We first examined whether IL-22 gene delivery would suppress CD4⁺ T cell priming in Ag-induced immune responses. Mice received either plasmid (100 µg; p-IL-22 or p-Cont) on day -3 and were sensitized with OVA/alum on day 0. On day 11, whole spleen cells from treated mice were incubated with OVA for 72 or 96 h, and Ag-induced proliferation or cytokine production was examined. Spleen cells obtained from OVA-sensitized mice proliferated (Fig. 4A) and produced significantly higher amounts of IL-5, IFN- γ , and IL-17 (Fig. 4B) in response to OVA, compared with those of SA-treated mice. When assessed by BrdU cell proliferation assay, the proliferation of spleen cells from SA-treated mice was ~50% compared with that of OVA-sensitized mice, which was consistent with previous reports (13, 40, 41). We think that BrdU assay to detect the difference of proliferation is not so sensitive compared with [³H]thymidine incorporation assay. Spleen cells from OVA-sensitized and p-IL-22-injected mice failed to proliferate (Fig. 4A) or produce IFN- γ and IL-17 (Fig. 4B), or produced little IL-5 (Fig. 4B), in contrast to those from the OVA-sensitized mice or from the OVA-sensitized and p-Cont-injected mice. As the physiological ratio of CD4⁺ T cells in the spleen differed between p-IL-22 mice and p-Cont mice, we then confirmed the effect of IL-22 on the function of purified CD4⁺ T cells. On day 11, splenic CD4⁺ T cells from treated mice were incubated with APCs from naive mice and OVA for 72 or 96 h, and Ag-induced proliferation or cytokine production of CD4⁺ T cells was examined. IL-22 gene delivery suppressed the OVA-induced proliferation and overall cytokine production of splenic CD4⁺ T cells (Fig. 4C, 4D). These results indicated that delivery of the IL-22 gene before sensitization suppressed the expansion of Ag-stimulated CD4⁺ T cells. We next examined the direct effect of IL-22 on CD4⁺ T cell functions. CD4⁺ T cells from OVA-sensitized mice were incubated with PMA/ionomycin for 24 h or plate-bound anti-CD3 Ab for 48 h in the presence or absence of IL-22. Proliferation (Fig. 4E, 4F) or IL-5 production (data not shown)

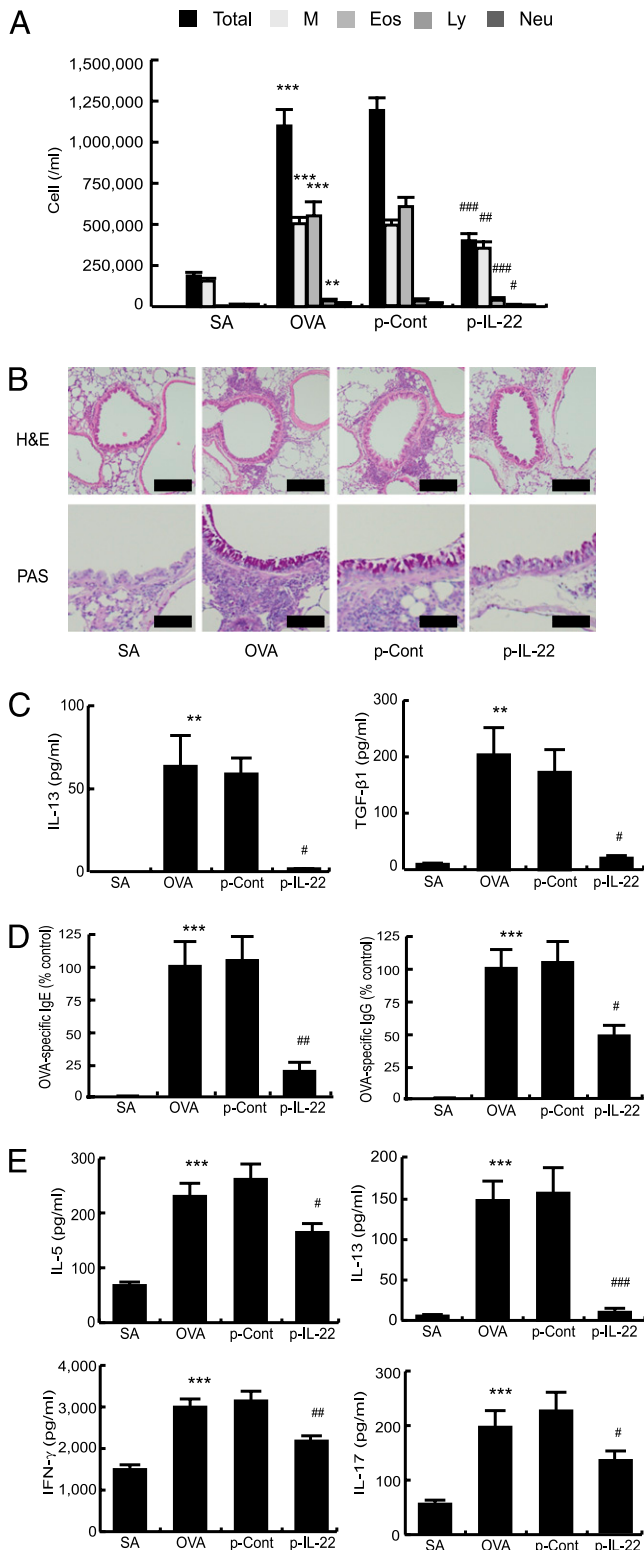


FIGURE 2. IL-22 gene delivery before systemic sensitization suppresses eosinophilic airway inflammation in vivo. BALB/c mice were sensitized with OVA/alum (OVA) or SA on days 0 and 11. Some OVA-sensitized mice received an i.v. injection of the plasmid (p-IL-22 or p-Cont) on day -3. The mice were nebulized with OVA or PBS from day 18 to day 20. On day 21, the mice were analyzed. **A**, Delivery of IL-22 prior to sensitization reduces the eosinophil count in BALF. BALF analyses were performed ($n = 8$). Leukocytes were identified by morphologic criteria. M, macrophages; Eos, eosinophils; Ly, lymphocytes; Neu, neutrophils. **B**, Histological findings. Lungs were excised and subjected to H&E and PAS staining. Scale bar, 200 μ m (H&E), 50 μ m (PAS). **C**, Cytokine concentrations in BALF. BALF supernatant was assayed for IL-13 and TGF- β 1 by ELISA ($n = 8$). **D**, OVA-specific IgE and IgG concentrations. Blood samples were obtained from treated mice. OVA-specific IgE and IgG concentrations were measured by ELISA ($n = 8$). Pooled sera from five OVA-sensitized mice were used as a control (OVA; 100%). **E**, Cytokine concentrations in the lung. Lung homogenate was assayed for cytokines by ELISA ($n = 8$). The results presented are representative data from five independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ (compared with the value for SA); $^{\#}p < 0.05$, $^{###}p < 0.001$ (compared with the value for p-Cont).

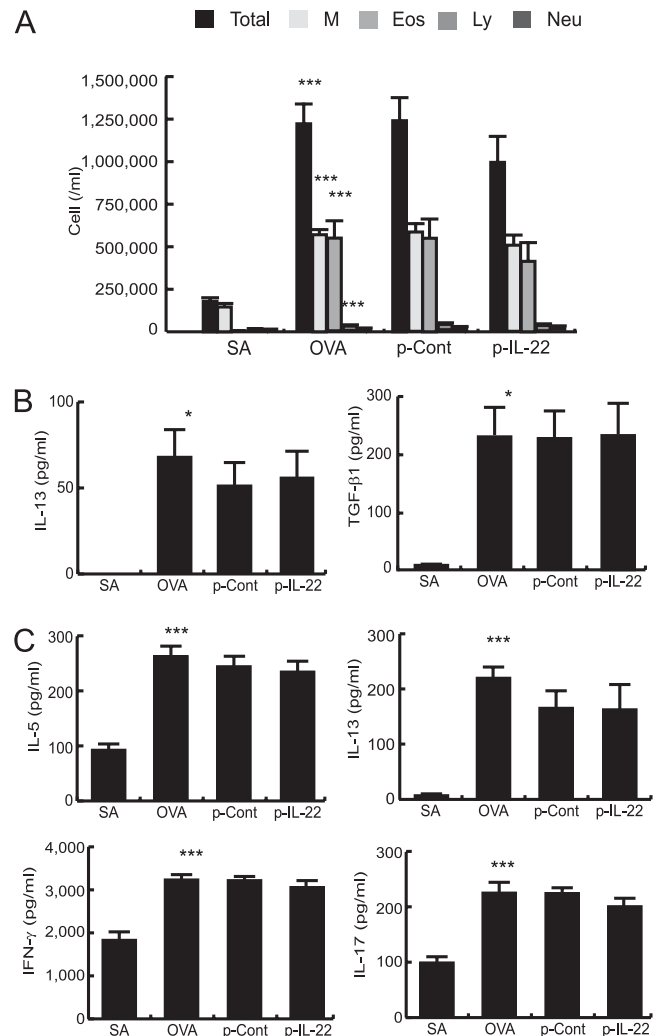


FIGURE 3. IL-22 gene delivery immediately before Ag challenge does not suppress eosinophilic airway inflammation. BALB/c mice were sensitized with OVA/alum (OVA) or SA on days 0 and 11. Some OVA-sensitized mice received an i.v. injection of p-IL-22 or p-Cont on day 17. The mice received inhalation of OVA or PBS from day 18 to day 20. On day 21, the mice were analyzed. **A**, IL-22 gene delivery immediately before Ag challenge does not suppress the eosinophil count in BALF. BALF analyses were performed ($n = 8$). Leukocytes were identified by morphologic criteria. M, macrophages; Eos, eosinophils; Ly, lymphocytes; Neu, neutrophils. **B**, Cytokine concentrations in BALF. BALF supernatant was assayed for IL-13 and TGF- β 1 by ELISA ($n = 8$). **C**, Cytokine concentrations in the lung. Lung homogenate was assayed for cytokines by ELISA ($n = 8$). The results presented are representative data from five independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ (compared with the value for SA).

of CD4 $^{+}$ T cells was not suppressed by IL-22 treatment (10 or 200 ng/ml), suggesting that IL-22 did not suppress CD4 $^{+}$ T cells directly.

trations in BALF. BALF supernatant was assayed for IL-13 and TGF- β 1 by ELISA ($n = 8$). **D**, OVA-specific IgE and IgG concentrations. Blood samples were obtained from treated mice. OVA-specific IgE and IgG concentrations were measured by ELISA ($n = 8$). Pooled sera from five OVA-sensitized mice were used as a control (OVA; 100%). **E**, Cytokine concentrations in the lung. Lung homogenate was assayed for cytokines by ELISA ($n = 8$). The results presented are representative data from five independent experiments. $^{*}p < 0.01$, $^{***}p < 0.001$ (compared with the value for SA); $^{\#}p < 0.05$, $^{###}p < 0.001$ (compared with the value for p-Cont).

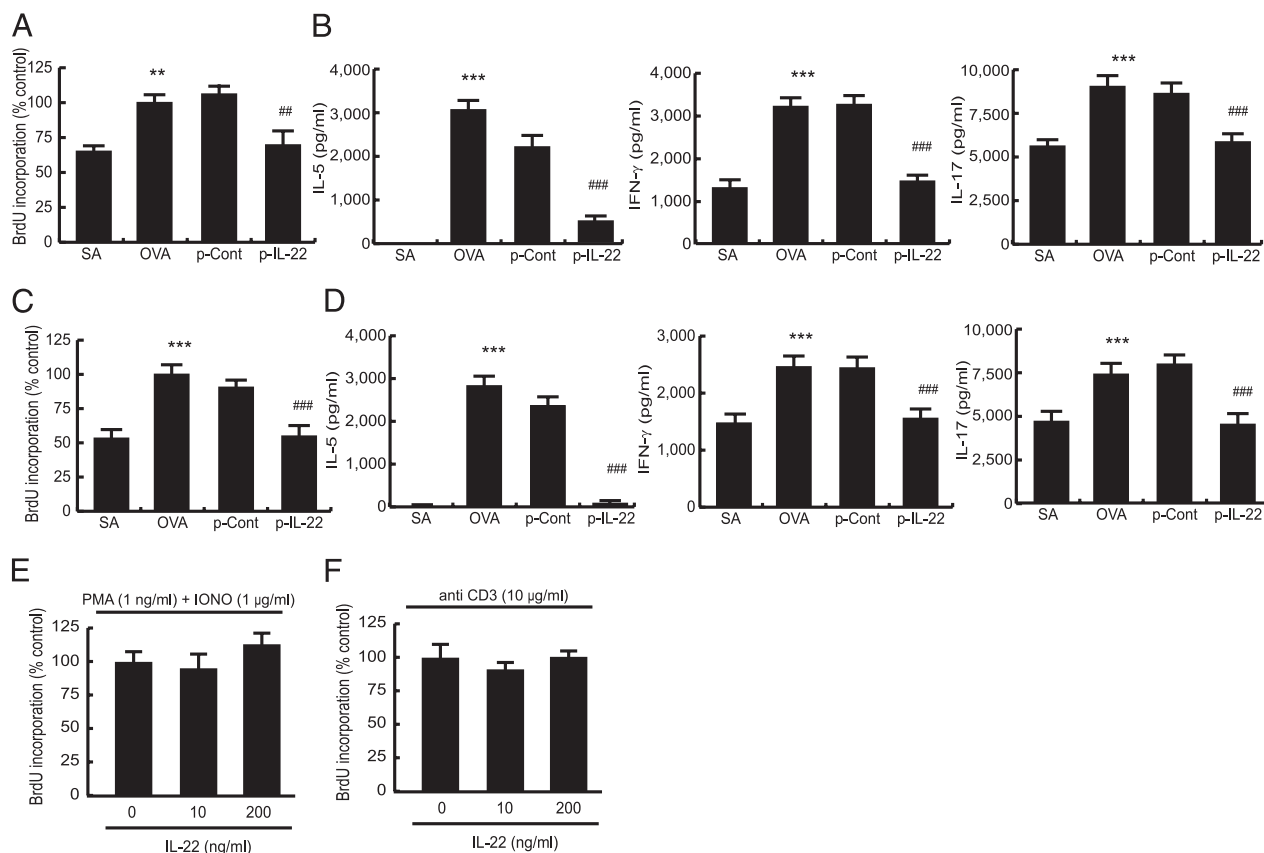


FIGURE 4. IL-22 gene delivery suppresses the overall Ag-induced immune responses. BALB/c mice were sensitized with OVA/alum (OVA) or SA on day 0. Some OVA-sensitized mice received plasmid (p-IL-22 or p-Cont) on day -3. On day 11, the mice were analyzed. **A** and **B**, Proliferation and cytokine production in whole spleen cells in response to OVA. Whole spleen cells (2.5×10^5 /well) from treated mice were incubated with OVA (20 μ g/ml). **A**, After 72 h, the proliferation was assessed using BrdU incorporation ($n = 6$). The maximum proliferation observed in response to OVA for spleen cells from OVA-sensitized mice was used as a control (OVA; 100%; OD at 450 nm = 1.10 ± 0.05). **B**, After 96 h, cytokine levels in the supernatant were measured ($n = 6$). **C** and **D**, Proliferation and cytokine production in CD4⁺ T cells in response to OVA. For the preparation of splenic CD4⁺ T cells, spleen cells were incubated with anti-CD4 mAb-coated microbeads, and bead-bound cells were isolated using magnetic separation columns. Splenic CD4⁺ T cells (2×10^5 /well) from treated mice were cultured with mitomycin C-treated splenocytes (APCs) (2×10^5 /well) from naive mice and OVA (20 μ g/ml). **C**, After 72 h, the proliferation was assessed ($n = 6$). The maximum proliferation observed in response to OVA for splenic CD4⁺ T cells from OVA-sensitized mice was used as a control (OVA; 100%; OD = 1.23 ± 0.06). **D**, After 96 h, cytokine levels in the supernatant were measured ($n = 6$). **E** and **F**, Direct effect of IL-22 on CD4⁺ T cells. OVA-sensitized splenic CD4⁺ T cells (1.25×10^5 /well) were incubated with PMA (1 ng/ml) and ionomycin (1 μ g/ml) for 24 h (**E**) or plate-bound anti-CD3 Ab (10 μ g/ml) for 48 h (**F**) in the presence or absence of IL-22. The proliferation was assessed using BrdU incorporation ($n = 6$). The maximum proliferation observed without IL-22 was used as a control [100%; OD = 1.10 ± 0.07 (**E**), 1.05 ± 0.09 (**F**)]. The results presented are representative data from five independent experiments. ** $p < 0.01$, *** $p < 0.001$ (compared with the value for SA); ## $p < 0.01$, ### $p < 0.001$ (compared with the value for p-Cont).

The IL-22 protein itself plays a critical role in the suppression of the immune response of spleen cells induced by the IL-22 gene

Next, we examined whether the effect of the IL-22 gene is indeed achieved by the IL-22 protein. Mice received either plasmid (0.1, 1, 10, or 100 μ g; p-IL-22 or p-Cont) on day -3 and were sensitized with OVA/alum on day 0. Delivery of the IL-22 gene in vivo suppressed the Ag-specific proliferation of spleen cells in a dose-dependent manner of the plasmid (Fig. 5A). We then examined the effect of antagonism of IL-22 by IL-22BP on the suppression of immune responses by IL-22. Mice received either plasmid (100 μ g; p-IL-22 or p-Cont) on day -3 and were sensitized with OVA/alum on day 0. Some p-IL-22-injected mice received IL-22BP-Fc i.p. on days -1 (25 μ g) and 0 (2 h before and 4 h after the OVA sensitization; 25 μ g each). On day 11, the proliferation of spleen cells in response to OVA was examined. The administration of mouse IL-22BP-Fc abolished the suppression of the Ag-specific proliferation of spleen cells induced by the p-IL-22 injection (Fig. 5B). These results confirmed that the IL-22 protein itself played an

essential role in triggering the hyporesponsiveness of spleen cells induced by delivery of the IL-22 gene in vivo.

We next examined the role of endogenous IL-22 in Ag-induced immune responses by neutralizing endogenous IL-22 using IL-22BP. Mice were sensitized with OVA/alum on day 0, and some OVA-sensitized mice received mouse IL-22BP-Fc i.p. as described earlier. However, administration of IL-22BP-Fc did not modify OVA-induced proliferation or cytokine production in spleen cells (Fig. 5C and data not shown). We then examined the effect of IL-22BP on eosinophilic airway inflammation in the absence of IL-22 overexpression. Mice were sensitized with OVA/alum and received IL-22BP-Fc as described earlier. The mice were challenged with OVA from day 18 to day 20, and airway inflammation was assessed on day 21. Administration of IL-22BP-Fc did not modify the infiltration of eosinophils into the airway (Fig. 5D and data not shown). Furthermore, administration of IL-22BP-Fc affected neither the concentration of IL-13 or of TGF- β 1 in BALF (Fig. 5E) nor the production of OVA-specific IgE or IgG in serum (Fig. 5F). These results suggested that neutralization of endogenous IL-22 by IL-22BP did not affect Ag-induced immune re-

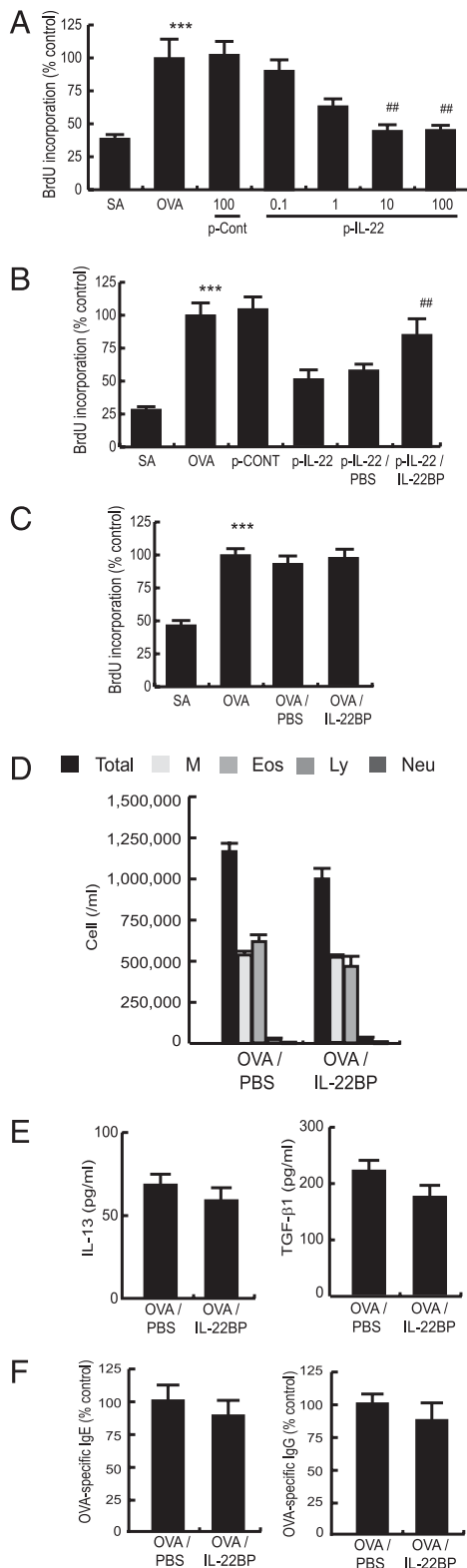


FIGURE 5. The IL-22 protein itself plays a critical role in the suppression of spleen cells induced by IL-22 gene delivery. **A**, Effect of the dose of IL-22 plasmid on the proliferation of spleen cells. BALB/c mice received plasmid (p-IL-22 or p-Cont; 0.1, 1, 10, or 100 μ g) on day -3 and were sensitized with OVA/alum (OVA) or SA on day 0. On day 11, the proliferation of spleen cells was examined using BrdU incorporation ($n = 6$). The maximum proliferation observed in response to OVA for spleen cells from OVA-sensitized mice was used as a control (OVA; 100%; OD = 1.14 ± 0.21). The results presented are representative data from five independent experiments. *** $p < 0.001$ (compared with the value for SA);

sponses or Ag-induced eosinophilic airway inflammation in our experimental system.

IL-22 gene delivery does not induce the development of Tregs or apoptosis

We examined the possibility that this suppression was mediated through the production of Tregs in vivo. First, we evaluated the production of immune-suppressive cytokines. For the measurement of cytokine production by CD4⁺ T cells, splenocytes obtained from naive mice were used as APCs. IL-22 gene delivery, however, did not increase the production of IL-10 (Fig. 6A) and TGF- β 1 (data not shown) by whole spleen cells in response to OVA. Furthermore, CD4⁺ T cells from OVA-sensitized and p-IL-22-injected mice did not produce IL-10 (Fig. 6B) or TGF- β 1 (data not shown). These findings suggested that IL-22 gene delivery in vivo would not induce the production of immune-suppressive cytokines by spleen cells. We next examined whether IL-22 gene delivery increased the expression of Foxp3, a transcription factor specifically expressed in Tregs (46–48), in CD4⁺ T cells. On day 11, spleen cells from OVA-sensitized and plasmid-injected mice were obtained without collagenase to reduce the contamination of autofluorescent cells and were stained with PE-Cy5 anti-CD4 mAb and PE anti-Foxp3 Ab or its isotype Ab (PE-rat IgG2a) without stimulation. IL-22 gene delivery in vivo did not increase the ratio of Foxp3⁺ cells in CD4⁺ T cells in spleen (Fig. 6C, 6D). We confirmed the validity of Foxp3 staining using RagD^{osf} mice, which do not develop the scurfy disease despite the absence of Foxp3⁺ Tregs (naturally occurring or peripherally induced Foxp3⁺ Tregs) (49) (Fig. 6E). Similar results were obtained when we examined the expression of Foxp3 in CD4⁺ T cells after 4-d OVA stimulation (data not shown). It is reported that Helios is a good indicator to distinguish naturally occurring from peripherally induced Foxp3⁺ Tregs (50), and thus we examined the expression of Helios in Foxp3⁺ CD4⁺ T cells. IL-22 gene delivery slightly

$p < 0.01$ (compared with the value for p-Cont). **B**, Effect of mouse IL-22BP on Ag-induced proliferation of spleen cells of IL-22-treated mice. BALB/c mice received plasmid (100 μ g; p-IL-22 or p-Cont) on day -3 and were sensitized with OVA/alum (OVA) or SA on day 0. Some p-IL-22-injected mice received mouse IL-22BP or PBS i.p. on days -1 (25 μ g) and 0 (2 h before and 4 h after the OVA sensitization; 25 μ g each). On day 11, the proliferation of spleen cells was examined using BrdU incorporation ($n = 6$). The maximum proliferation observed in response to OVA for spleen cells from OVA-sensitized mice was used as a control (OVA; 100%; OD = 1.06 ± 0.14). The results presented are representative data from four independent experiments. *** $p < 0.001$ (compared with the value for SA); ## $p < 0.01$ (compared with the value for p-IL-22/PBS). **C**, Effect of antagonism of endogenous IL-22 by IL-22BP on Ag-induced proliferation of spleen cells. BALB/c mice were sensitized with OVA/alum (OVA) or SA on day 0. Some OVA-sensitized mice received mouse IL-22BP or PBS i.p. as described for **B**. On day 11, the proliferation of spleen cells was examined using BrdU incorporation ($n = 6$). The maximum proliferation observed in response to OVA for spleen cells from OVA-sensitized mice was used as a control (OVA; 100%; OD = 1.52 ± 0.05). The results presented are representative data from four independent experiments. *** $p < 0.001$ (compared with the value for SA). **D–F**, Effect of antagonism of endogenous IL-22 by IL-22BP on Ag-induced eosinophilic airway inflammation. BALB/c mice were sensitized with OVA/alum (OVA) on days 0 and 11. Some OVA-sensitized mice received mouse IL-22BP or PBS i.p. as described for **B**. The mice were nebulized with OVA from day 18 to day 20. On day 21, the mice were analyzed. **D**, BALF analyses ($n = 8$). M, macrophages; Eos, eosinophils; Ly, lymphocytes; Neu, neutrophils. **E**, Cytokine concentrations in BALF ($n = 8$). **F**, OVA-specific IgE and IgG concentration ($n = 8$). The results presented are representative data from three independent experiments.

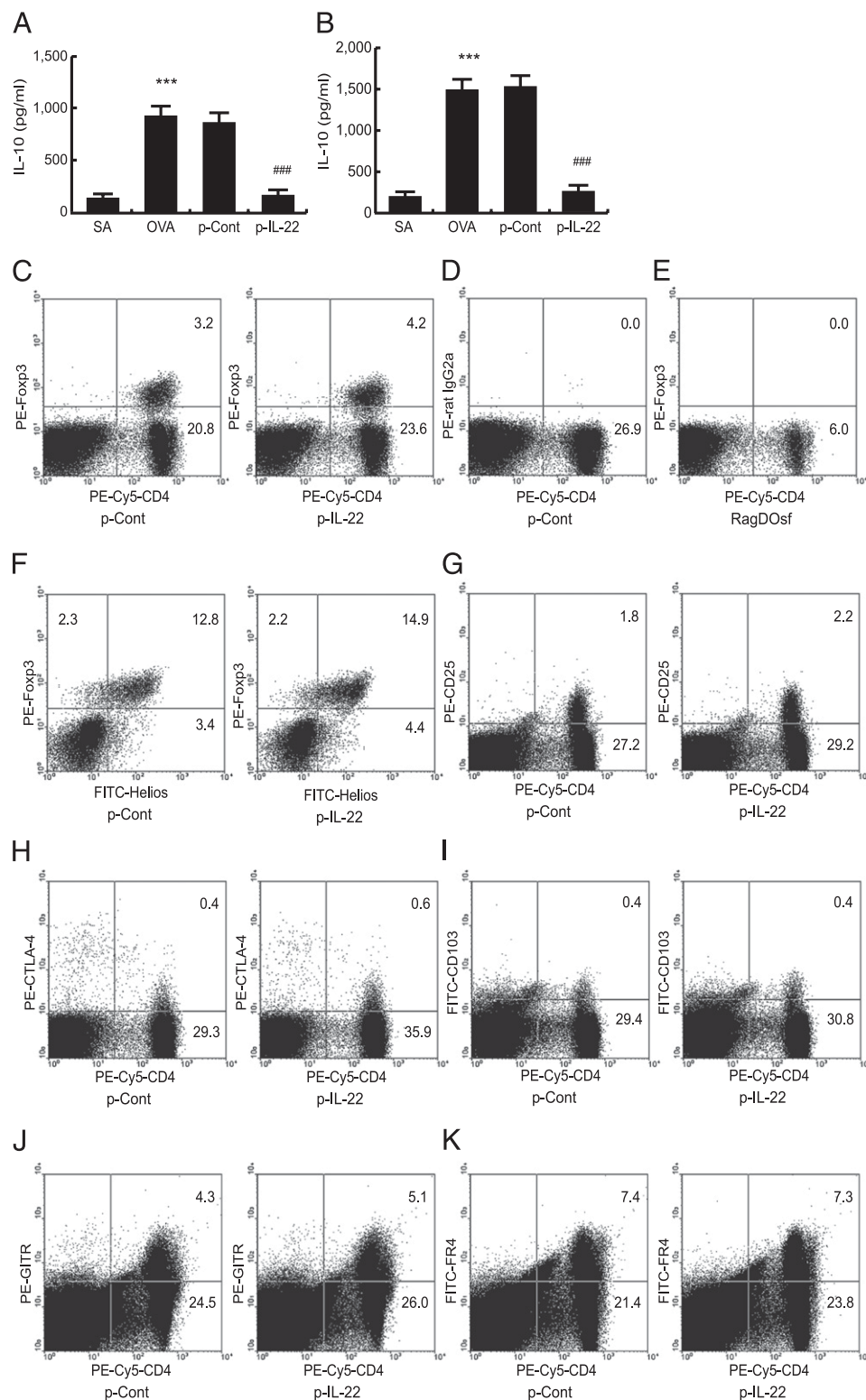


FIGURE 6. IL-22 gene delivery does not induce the development of Tregs. BALB/c mice were treated as described in Fig. 4. **A**, IL-10 production by spleen cells in response to OVA. On day 11, spleen cells (2.5×10^5 /well) from treated mice were incubated with OVA (20 μ g/ml). After 96 h, the concentration of IL-10 in the supernatant was assayed ($n = 6$). **B**, IL-10 production by CD4⁺ T cells in response to OVA. For the preparation of splenic CD4⁺ T cells, spleen cells were incubated with anti-CD4 mAb-coated microbeads, and bead-bound cells were isolated using magnetic separation columns. On day 11, splenic CD4⁺ T cells (2×10^5 /well) from treated mice were cultured with mitomycin C-treated splenocytes (APCs) (2×10^5 /well) from naive mice and OVA (20 μ g/ml). After 96 h, the concentration of IL-10 was assayed ($n = 6$). The results presented are representative data from five independent experiments. *** $p < 0.001$ (compared with the value for SA); ### $p < 0.001$ (compared with the value for p-Cont). **C** and **D**, IL-22 gene delivery does not increase the expression of Foxp3 in CD4⁺ T cells of spleen. On day 11, spleen cells were stained with PE-Cy5 anti-CD4 Ab and PE anti-Foxp3 Ab (**C**) or its isotype Ab (PE-rat IgG2a) (**D**) not stimulating with OVA or PMA/ionomycin/brefeldin A. Foxp3⁺ CD4⁺ T cells were detected by flow cytometry. **E**, Foxp3⁺ CD4⁺ T cells were not detected in RagDOsf mice. Spleen cells from RagDOsf mice were stained with PE-Cy5 anti-CD4 Ab and PE anti-Foxp3 Ab. RagDOsf mice do not develop the scurfy disease despite the absence of Foxp3⁺ Tregs. **F**, Expression of Helios in Foxp3⁺ CD4⁺ (Figure legend continues)

increased the proportion of Foxp3⁺ Helios⁺ cells in CD4⁺ T cells in spleen; however, the difference was not significant (Fig. 6F). IL-22 gene delivery did not increase the number of Foxp3⁺ Helios⁺ CD4⁺ T cells (Fig. 6F), suggesting that it neither increased thymus-derived Tregs nor induced Tregs. Furthermore, expressions of other Treg-related surface markers such as CD25 (48, 51), intracellular CTLA-4 (48, 52–54), CD103 (48, 55), GITR (48, 56, 57), and FR4 (48, 58) were not upregulated in CD4⁺ T cells from mice subjected to IL-22 gene delivery (Fig. 6G–K). For detection of IL-10–producing CD4⁺ T cells, we used CD4⁺ T cells after OVA restimulation *ex vivo* from OVA-sensitized mice, as we could not detect intracellular IL-10 in CD4⁺ T cells without OVA restimulation (data not shown). However, IL-22 gene delivery did not increase IL-10–producing CD4⁺ T cells (data not shown). These results suggested that the suppression of immune responses by IL-22 would not be mediated by the generation of Tregs in our experimental system.

We then examined the possibility that the suppressive effect of IL-22 was mediated by apoptosis of immunocompetent cells. IL-22 gene delivery did not increase apoptosis, as assessed by the annexin/propidium iodide assay of spleen cells or TUNEL of the splenic tissue (data not shown), suggesting that the suppression by IL-22 would not be mediated by apoptosis.

IL-22 gene delivery does not directly affect DC function

The initiation of Ag-induced immune response is under the control of DCs, the most potent of all APCs (59). Furthermore, DCs play an essential role not only in the development but also in the maintenance of Ag-induced immune response in the airway (60, 61). Therefore, we next examined the effect of IL-22 gene delivery on splenic DC functions. Because DCs have a capacity to stimulate allogeneic CD4⁺ T cells, to present Ag to CD4⁺ T cells, and to produce cytokines such as IL-10, we evaluated DC functions by allogeneic MLR (by incubation with CD4⁺ T cells from C57BL/6 mice), OVA-presenting capacity (by incubation with CD4⁺ T cells from OVA-sensitized BALB/c mice and OVA), and IL-10 production (by stimulation with LPS). IL-22 tended to suppress allogeneic MLR (Fig. 7A) and OVA-presenting capacity (Fig. 7B); however, the differences were not significant. IL-22 slightly, but not significantly, suppressed the production of IL-10 by splenic DCs in response to LPS (Fig. 7C). These findings suggested that IL-22 gene delivery *in vivo* did not have a suppressive effect on DC functions. We then examined the direct effect of IL-22 on splenic DC functions. Splenic DCs from naive mice were incubated with mouse IL-22 and OVA for 24 h, and then OVA–Ag–presenting capacity was examined. Treatment of splenic DCs with IL-22 *in vitro* did not suppress OVA-presenting capacity (Fig. 7D). These results suggested that IL-22 had no direct effect on DC functions and that IL-22R was not expressed in DCs.

Because a secondary *ex vivo* immune response of spleen should not require professional APCs (DCs) but could use monocytes, macrophages, and B cells for Ag presentation to class II MHC, we examined overall APC functions of whole spleen cells. We also

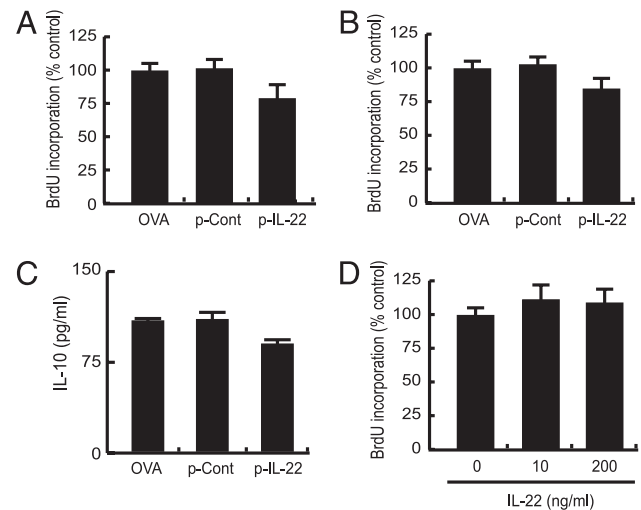


FIGURE 7. IL-22 gene delivery does not directly affect DC functions. **A–C,** Effect of IL-22 gene delivery on splenic DC functions. BALB/c mice were treated as described in Fig. 4. On day 11, splenic DCs were subjected to analyses. For preparation of splenic DCs, spleen cells were incubated with anti-CD11c mAb-coated microbeads, and bead-bound cells were then isolated using magnetic separation columns. **A,** Allogeneic MLR. Splenic DCs (1×10^4 /well) from treated BALB/c mice (H-2^d) were cultured with CD4⁺ T cells (2.5×10^5 /well) from naive C57BL/6 mice (H-2^b). After 72 h, the proliferation was assessed using BrdU incorporation ($n = 6$). The proliferation of CD4⁺ T cells from naive C57BL/6 mice in response to DCs from OVA-sensitized BALB/c mice was used as a control (OVA; 100%; OD = 0.76 ± 0.03). **B,** Ag-presenting capacity. Splenic DCs (1×10^5 /well) from treated BALB/c mice were cultured with CD4⁺ T cells (2.5×10^5 /well) from OVA-sensitized BALB/c mice and OVA (20 μ g/ml). After 72 h, the proliferation was assessed using BrdU incorporation ($n = 6$). The proliferation of CD4⁺ T cells from OVA-sensitized BALB/c mice in response to DCs from OVA-sensitized BALB/c mice with OVA was used as a control (OVA; 100%; OD = 0.73 ± 0.04). **C,** IL-10 production. Splenic DCs (1.25×10^5 /well) from treated mice were cultured with LPS (1 μ g/ml). After 24 h, the IL-10 concentration in the supernatant was assayed ($n = 6$). **D,** Direct effect of IL-22 on DC functions. Splenic DCs were positively selected by magnetic cell sorting from naive BALB/c mice and incubated with OVA (1000 μ g/ml). IL-22 was added to some cultures (10 or 200 ng/ml). After 24 h, OVA-pulsed CD11c⁺ cells were collected (2.5×10^4 /well) and cultured with CD4⁺ T cells from DO11.10 BALB/c mice (2.5×10^5 /well). After 48 h, the proliferation was assessed using BrdU incorporation ($n = 6$). The control was the proliferation of CD4⁺ T cells from DO11.10 mice in response to OVA-pulsed CD11c⁺ cells in the absence of IL-22 (100%; OD = 1.11 ± 0.04). The results presented are representative data from five independent experiments.

confirmed that IL-22 gene delivery did not significantly affect overall APC function of whole spleen cells such as allogeneic MLR and OVA-presenting capacity (data not shown).

IL-10 plays an important role in the suppression of Ag-induced immune responses by IL-22 gene delivery

As IL-22 produced IL-10 in epithelial cells (62), we examined the role of IL-10 in IL-22-induced Ag-specific immune suppression.

T cells. On day 11, spleen cells were stained with PE–Cy5 anti-CD4 Ab, PE anti-Foxp3 Ab, and FITC anti-Helios Ab. Foxp3⁺ Helios⁺ or Foxp3⁺ Helios⁺ CD4⁺ T cells were detected by flow cytometry. The cells were gated according to PE–Cy5–CD4 parameters. **G,** Expression of CD25 in CD4⁺ T cells. On day 11, spleen cells were stained with PE–Cy5 anti-CD4 Ab and PE anti-CD25 Ab. **H,** Expression of intracellular CTLA-4 in CD4⁺ T cells. On day 11, spleen cells were stimulated with PMA/ionomycin/brefeldin A for 5 h and then stained with PE–Cy5 anti-CD4 Ab. After incubation with intracellular fixation buffer, the cells were stained with PE anti-CTLA-4 Ab. **I,** Expression of CD103 in CD4⁺ T cells. On day 11, spleen cells were stained with PE–Cy5 anti-CD4 Ab and FITC anti-CD103 Ab. **J,** Expression of GITR in CD4⁺ T cells. On day 11, spleen cells were stained with PE–Cy5 anti-CD4 Ab and PE anti-GITR Ab. **K,** Expression of FR4 in CD4⁺ T cells. On day 11, spleen cells were stained with PE–Cy5 anti-CD4 Ab and FITC anti-FR4 Ab. Representative results are shown. The results presented are representative data from two to four independent experiments.

We first measured IL-10 concentrations in serum; however, we could not detect any increase in IL-10 after delivery of the IL-22 gene (data not shown). We then elucidated the effect of IL-22 with IL-10-deficient mice. CD4⁺ T cells from OVA-sensitized and plasmid-injected IL-10-deficient mice were incubated with APCs from naive mice and OVA, and then proliferation of CD4⁺ T cells was examined. As we would like to exclude the influence of the deletion of IL-10 gene in APCs, we obtained APCs from naive mice. In IL-10-deficient mice, delivery of the IL-22 gene in vivo prior to sensitization could not suppress the expansion of Ag-stimulated CD4⁺ T cells, although delivery of the IL-10 gene suppressed it (Fig. 8A), indicating that IL-10 plays an essential role in Ag-induced immune suppression by IL-22 in this system. We also confirmed the crucial role of IL-10 in the suppression of Ag-induced immune responses by IL-22 treatment using a neutralizing Ab for IL-10 (Fig. 8B). Furthermore, delivery of the IL-22 gene into IL-10-deficient mice before their systemic sensitization did not suppress eosinophilic infiltration in BALF (Fig. 8C) or in the lungs (data not shown) induced by sensitization to and inhalation of OVA. The IL-22 gene did not suppress IL-13 and TGF- β 1 concentrations in BALF (Fig. 8D) or OVA-specific IgE or IgG production in serum (Fig. 8E).

We finally examined whether IL-22 gene delivery could increase the production of IL-10 in draining lymph node after OVA sensitization. Mice received plasmid on day -3 and were sensitized with OVA/alum on day 0. Mesenteric lymph node cells were obtained on day 2. The cells were incubated with LPS (1 μ g/ml) for 24 h, and then the production of IL-10 and expression of IL-10 mRNA were examined. IL-22 gene delivery increased the production of IL-10 and expression of IL-10 mRNA of mesenteric lymph node cells in response to LPS (Fig. 8F, 8G). Furthermore, we examined the effect of IL-22 on IL-10 production by bronchial lymph node cells, as OVA-captured APCs actually migrated into bronchial lymph node 2 d after OVA sensitization (40, 63). IL-22 gene delivery also increased the production of IL-10 by bronchial lymph node cells in response to LPS (data not shown). These findings suggested that IL-22 could increase IL-10 production and IL-10 mRNA expression in draining lymph node after OVA sensitization. Therefore, IL-10 plays an important role in the suppression of Ag-induced immune responses or eosinophilic airway inflammation by IL-22 gene delivery.

Discussion

The results of the current study clearly demonstrated that IL-22 acts as an immune-modulating cytokine and attenuates Ag-induced immune responses. Delivery of the IL-22 gene prior to systemic sensitization, but not immediately before challenge, reduced Ag-induced eosinophilic airway inflammation. It also suppressed the Ag-induced proliferation and overall cytokine production in splenic CD4⁺ T cells. Administration of IL-22BP abolished the immune suppression by IL-22 gene delivery, suggesting that IL-22 protein itself played an essential role in the suppression. IL-22 gene delivery in vivo neither generated Tregs nor directly suppressed the function of DCs. The suppression was abrogated by deletion of the IL-10 gene or neutralization of the IL-10 protein. Finally, IL-22 gene delivery increased IL-10 production in draining lymph nodes, suggesting that IL-10 plays an important role in the immune suppression by IL-22 gene delivery. These findings suggested the novel role of IL-22 as a suppressive cytokine in Ag-induced immune responses.

IL-22 is also made by Th17 cells (15). Although its expression is known to be increased in patients with psoriasis, rheumatoid arthritis, and inflammatory bowel disease (22–24), its role in these immune diseases is not well understood. Some reports suggested

that IL-22 exacerbates psoriasis (16, 22, 25–27) and arthritis (28), indicating its proinflammatory role. In contrast, many reports suggest an immune-suppressive property of IL-22 (24, 29–37), although the mechanisms of this suppression have not been fully elucidated. As IL-22R is absent on immune cells, IL-22 is not considered to act directly on them (9–11, 14). For example, IL-22 had an ameliorating effect in a mouse model of ulcerative colitis by enhancing the production of mucus and restitution of mucus-producing goblet cells (29). IL-22 also protected against T cell-mediated murine hepatitis by inducing the expression of antiapoptotic and mitogenic proteins (31). IL-22 suppressed experimental autoimmune myocarditis by inhibiting the gene expression of PG synthetases, IL-6, and chemokines in non-cardiomyocytic non-inflammatory cells (34). These findings indicate that IL-22 suppresses the development of these immune diseases without acting directly on immune cells.

The role of IL-22 in the development of lung disease is not well understood (64, 65). The expression of IL-22 as well as IL-17 is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients (64), suggesting that IL-22 plays some role in the airway inflammation. In another study, IL-22 suppressed IL-8 production by alveolar epithelial cells, suggesting an anti-inflammatory role (65). Whether IL-22 can suppress allergic airway inflammation in the lung remains to be investigated.

Our study demonstrated that delivery of the IL-22 gene prior to systemic sensitization suppressed the expansion of Ag-stimulated CD4⁺ T cells and eosinophilic airway inflammation (Figs. 2, 4). However, the results might be due to secondary effects caused by the high level of IL-22 expression. We confirmed in this study that gene delivery of 0.1 μ g IL-22 plasmid before OVA sensitization did not suppress Ag-induced immune responses (Fig. 5A), although it induced moderate expression of IL-22 [800–1200 ng/ml (serum), 1000–1500 pg/ml (BALF), and 4000–5000 pg/ml (lung)]. Furthermore, when 1 μ g IL-22 protein was administered by i.p. injection to mice on day 0 (2 h before OVA sensitization), OVA-induced proliferation or cytokine production in spleen cells was not inhibited (data not shown). These findings suggested that, if delivered systemically, high expression on IL-22 would be required to suppress the CD4⁺ T cell priming in the Ag-induced immune responses.

In contrast, IL-22 gene delivery immediately before OVA challenge did not suppress eosinophilic airway inflammation (Fig. 3). The reason why delivery of the IL-22 gene in the effector phase did not suppress eosinophilic airway inflammation is unclear. We found in another experiment that intratracheal IL-22 administration in the effector phase exhibited a tendency to suppress eosinophilic airway inflammation (data not shown). Therefore, the description that IL-22 had no effect in the effector phase is incorrect. As IL-22 expression in the airway by hydrodynamic-based gene delivery is not so high (50–100 ng/ml) compared with that in the serum (20–40 μ g/ml), and the suppressive effect of IL-22 on Ag-induced immune responses is weaker than that of IL-10 (13) or IFN- γ (40), we think that IL-22 gene delivery during the effector phase could not suppress eosinophilic airway inflammation. Amounts of IL-22 in the lung might affect the results of eosinophilic airway inflammation if delivered just before OVA nebulization.

Our results indicated that IL-10 plays an important role in immune suppression by IL-22 gene delivery (Fig. 8). Suppression of the Ag-induced proliferation of CD4⁺ T cells by IL-22 was abrogated by deletion of the IL-10 gene or neutralization of the IL-10 protein (Fig. 8A, 8B). Suppression of eosinophilic airway inflammation or other indicators of a Th2-type response by IL-22 gene delivery was abolished by deletion of the IL-10 gene (Fig. 8C–E). Furthermore, IL-22 gene delivery in vivo increased the

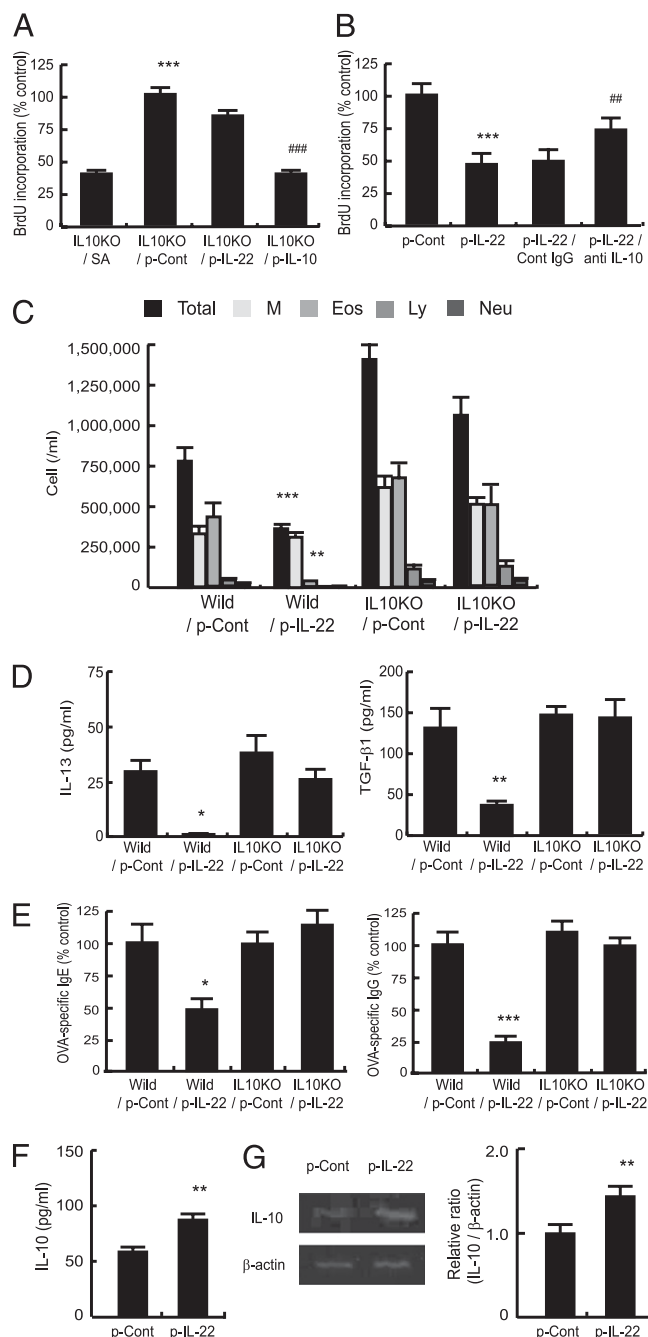


FIGURE 8. IL-10 plays an important role in Ag-induced immune suppression by IL-22. **A**, Effect of deletion of the IL-10 gene on the IL-22-induced immune suppression of CD4⁺ T cells. IL-10-deficient C57BL/6 mice were sensitized with OVA/alum or SA on day 0. Some OVA-sensitized IL-10-deficient mice received p-IL-22, p-Cont, or pCAGGS-IL-10 (p-IL-10) intravenously on day -3. On day 11, splenic CD4⁺ T cells (2×10^5 /well) from treated IL-10-deficient C57BL/6 mice were cultured with mitomycin C-treated splenocytes (APCs) (2×10^5 /well) from naive C57BL/6 mice and OVA (20 μ g/ml). After 72 h, the proliferation was assessed ($n = 6$). The maximum proliferation observed in response to OVA for spleen CD4⁺ T cells from OVA-sensitized, p-Cont-injected IL-10-deficient C57BL/6 mice was used as a control (IL10KO/p-Cont; 100%; OD = 1.76 ± 0.11). The results presented are representative data from four independent experiments. *** $p < 0.001$ (compared with the value for IL10KO/SA); ### $p < 0.001$ (compared with the value for IL10KO/p-Cont). **B**, Effect of neutralization of the IL-10 protein on the IL-22-induced immune suppression of spleen cells. BALB/c mice received plasmid on day -3 and were sensitized with OVA/alum on day 0. Some p-IL-22-injected mice received anti-IL-10 Ab or control IgG i.p. on day 0 (2 h

production of IL-10 and expression of IL-10 mRNA of mesenteric lymph node cells in response to LPS (Fig. 8F, 8G). Moreover, it also increased the production of IL-10 by bronchial lymph node cells in response to LPS (data not shown), suggesting that IL-22 could induce IL-10 production in draining lymph node after OVA sensitization. IL-10 is generally considered an immunosuppressive cytokine (12). Previously, we showed that delivery of IL-10 suppressed OVA-induced allergic airway inflammation by suppressing the functions of DCs as well as T cells (13). Therefore, in the current study, IL-10 expression induced by IL-22 could have suppressed airway inflammation via similar mechanisms, although we could not confirm significant suppression of DC functions, probably due to limitations of the system used (Fig. 7). In a preliminary study, i.p. injection of the IL-10-expressing plasmid did not increase the concentration of IL-10 in serum, but it suppressed OVA-induced proliferation or cytokine production in CD4⁺ T cells (K. Nakagome and M. Dohi, unpublished observations). Therefore, an undetectable or low level of IL-10 could have suppressed the OVA-induced immune response.

However, as the increase in IL-10 production in draining lymph nodes was not so strong (Fig. 8F), other mechanisms might play some role in the IL-22-induced immune suppression or IL-22-induced IL-10 production. For example, another cellular source of IL-10 might have existed in mice that received IL-22. Nagalakshmi et al. (62) reported that IL-22 induced production of IL-10 by colon epithelial cells. Therefore, other epithelial cells such as airway or alveolar epithelial cells could be a cellular source of IL-10. We tried to detect IL-10 on these epithelial cells using immunohistochemistry, but could not (data not shown). Furthermore, IL-22 treatment did not increase the expression of IL-10 mRNA or protein in the lung from OVA-sensitized and -challenged mice (data not shown).

There is evidence that non-immune structural cells such as epithelial cells regulate DC functions and thus Ag-induced immune responses (66, 67). For example, during house dust mite-driven

before and 4 h after the OVA sensitization; 250 μ g each). On day 11, the proliferation of spleen cells was examined using BrdU incorporation ($n = 6$). The maximum proliferation observed in response to OVA for spleen cells from OVA-sensitized p-Cont-injected BALB/c mice was used as a control (p-Cont; 100%; OD = 1.12 ± 0.12). The results presented are representative data from four independent experiments. *** $p < 0.001$ (compared with the value for p-Cont); ## $p < 0.01$ (compared with the value for p-IL-22/Cont IgG). **C–E**, Effect of deletion of the IL-10 gene on IL-22-induced suppression of eosinophilic airway inflammation. Wild-type C57BL/6 mice or IL-10-deficient C57BL/6 mice were sensitized with OVA/alum on days 0 and 11. Mice received p-IL-22 or p-Cont intravenously on day -3. Then, mice were challenged with OVA from days 18 to 20. On day 21, the lungs were subjected to analyses. **C**, BALF analyses ($n = 8$). M, macrophages; Eos, eosinophils; Ly, lymphocytes; Neu, neutrophils. **D**, Cytokine concentrations in BALF ($n = 8$). **E**, OVA-specific IgE and IgG concentration ($n = 8$). The results presented are representative data from four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared with the value for Wild/p-Cont). **F** and **G**, IL-22 gene delivery increases the production of IL-10 and the expression of IL-10 mRNA in draining lymph node. BALB/c mice received plasmid on day -3 and were sensitized with OVA/alum on day 0. On day 2, mesenteric lymph node cells were obtained and incubated with LPS (1 μ g/ml). **F**, IL-10 production. After 24 h, the IL-10 concentration in the supernatant was assayed ($n = 6$). **G**, RT-PCR. Normalized values for IL-10 mRNA expression in each sample were calculated as the relative quantity of IL-10 divided by the relative quantity of β -actin ($n = 4$). The results presented are representative data from four independent experiments. ** $p < 0.01$ (compared with the value for p-Cont).

allergic airway inflammation, TLR4 acting on structural cells causes the production of innate proallergic cytokines such as thymic stromal lymphopoietin and GM-CSF, and the mucosal DCs activated by these cytokines initiate Ag-induced immune responses in the airway (66). Exposure of the airway epithelium to house dust mite results in the specific and rapid secretion of CCL20, a chemokine attractant for immature DCs, through β -glucan-dependent pathways (67). These findings indicate that, although IL-22R is not expressed in immune cells such as DCs, IL-22 could modify the functions of DCs or other immune cells through the cytokines produced by non-immune structural cells, such as epithelial cells, as discussed earlier. As such, this possibility should be investigated further.

Another possible mechanism of IL-22-induced immune suppression is direct action on immune cells through another yet-to-be-defined receptor. Long-term exposure of T cells to IL-22 in vitro (total of 6 to 7 wk) downregulated IFN- γ production but upregulated IL-4 and IL-13 production in T cells (68), suggesting that IL-22 could act directly on T cells if they are exposed for longer. This finding suggests the existence of another receptor for IL-22. In the current study, the concentration of IL-22 protein (10–30 μ g/ml in serum; Fig. 1) was much higher than that used in most previous studies in vitro (1–100 ng/ml) (16, 22–26, 28, 29, 31, 32, 34–36, 62, 65). This high concentration, along with the longer exposure (14–28 d; Fig. 1), might have affected the ability of immune cells to induce suppression. As we could not completely exclude a direct effect of IL-22 on immune cells in this study, this possibility should be investigated further.

The role of endogenous IL-22 in Ag-induced immune responses has not been fully clarified. In a preliminary study, we found that OVA sensitization and nebulization did not increase the endogenous expression of IL-22. Furthermore, antagonism of endogenous IL-22 by administration of IL-22BP-Fc did not modify OVA-induced proliferation in spleen cells or eosinophilic airway inflammation (Fig. 5C–F and data not shown). Therefore, we think that endogenous IL-22 plays a limited role in Ag-induced immune responses. In contrast, although we tried to clarify the endogenous role of IL-22, it remains unclear. First, we did not examine the effect of IL-22 gene deletion using IL-22 knockout mice. Second, antagonism by IL-22BP-Fc in OVA-sensitized mice might be insufficient, although we confirmed the effect by administration of IL-22BP-Fc in mice that received IL-22 gene (Fig. 5B). Furthermore, interaction between endogenous IL-22 and exogenous factors should be considered. For example, overexpression of IL-22 might mimic an endogenous synergy that does not exist in this particular model system.

In conclusion, the current study demonstrated a novel role for IL-22 as a broad immune-suppressive cytokine suppressing Ag-induced CD4⁺ T cell priming. It also indicated that IL-22 could suppress immune responses in the airway. Therefore, IL-22 gene delivery could be an effective strategy for regulating Ag-specific immune responses in the lung.

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Disclosures

The authors have no financial conflicts of interest.

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