IFN-γ Attenuates Antigen-Induced Overall Immune Response in the Airway As a Th1-Type Immune Regulatory Cytokine[1]

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Allergic inflammation in the airway is generally considered a Th2-type immune response. However, recent studies demonstrated that Th1- and Th17-type immune responses also play important roles in this process. IFN-γ is a Th1-type cytokine that generally counteracts the Th2 response. Although previous studies suggest that exogenous IFN-γ suppresses allergic airway inflammation, the mechanism of suppression has not been fully clarified. In this study, we elucidated whether IFN-γ suppresses Ag-induced immune responses including the production of Th1- and Th17-type cytokines in the lung, and examined its mechanism of action. BALB/c mice were sensitized and challenged with OVA-Ag to induce airway inflammation. An IFN-γ-producing plasmid vector was delivered before systemic Ag sensitization. IFN-γ suppressed indicators of Th2-type immune responses such as airway eosinophilia, IL-5 and IL-13 production in the lung, and bronchial mucus production. Moreover, IFN-γ also suppressed the production of IL-17 and IFN-γ itself. The suppression was not mediated by inducing regulatory T cells or by inducing apoptosis in immunocytes. Instead, IFN-γ suppressed the Ag-presenting capacity and cytokine production of splenic dendritic cells and thus subsequently suppressed OVA-induced activation of $CD4^+$ T cells. Furthermore, IFN-γ also attenuated allergic airway inflammation when delivered during the OVA challenge. Various functions of lung $CD11c^+$ APCs and their migration to regional lymph nodes were also suppressed. These results suggest that the Th1 cytokine IFN-γ has broad immune regulatory potential through suppressing APC functions. They also suggest that delivery of IFN-γ could be an effective strategy for regulating Ag-induced immune responses in the lung. ***The Journal of Immunology,*** **2009, 183: 209–220.**

Allergic inflammation in the airway is generally considered a Th2-type response-mediated process (1). However, the Th1-type response also plays an important role in this process. For example, passive transfer of OVA-specific Th1 cells exacerbated OVA-induced airway eosinophila (2, 3). IL-18-generated Th1 cells could produce a Th2-type cytokine, thus deteriorate allergic airway inflammation (4). Th1 cells promoted the recruitment of Th2 cells and eosinophils into the airway after viral infection in an Ag-independent manner (5). Furthermore, recently, the role of Th17 cells in allergic airway inflammation has been highlighted (6–8). IL-17 plays an important role in the development of neutrophilic inflammation (9), which is known to be a hallmark of severe asthma (10). An elevated sputa IL-17 concentration correlates with clinical severity of asthma (8), suggesting that the Th17-type response also plays an important role. Therefore, suppression of not only Th2 but also Th1- and Th17-mediated immune responses would be more effective for controlling asthma.

Some Th1 cells have regulatory roles. For example, deletion of T-bet, a transcription factor required for Th1 cell differentiation (11), worsened Th2- and Th17-type immune responses in allergic airway inflammation (12). These findings strongly indicate heterogeneity among Th1 cells in terms of conventional phenotype, inflammatory phenotype, and immune regulatory phenotype, as was observed in Th2 cells also (13). However, which cytokine plays an essential role in the immune regulatory Th1 cells has not been fully clarified.

IFN-γ is a Th1-type cytokine that enhances the development of naive T cells into Th1 cells and enhances Th1-type immune responses (14). It inhibits Th2 cell proliferation in vitro (15) and antagonizes *Leishmania*-induced Th2-type responses in vivo (16, 17). In addition to this conventional role, recent studies clarified that IFN-γ attenuates autoimmune encephalomyelitis (18, 19) and collagen-induced arthritis (20, 21) in mice, which have traditionally been considered Th1-mediated diseases, but are now considered Th17-mediated diseases (22). So, IFN-γ might work as an immune modulator for other types of immune responses such as Th1- and Th17-mediated responses in some cases. However, this possibility has not been studied in allergic inflammation.

Not many studies have examined the effect of IFN-γ on allergic airway inflammation. Intratracheal administration of an IFN-γ-producing plasmid attenuated airway eosinophila (23). IFN-γ suppressed the recruitment of eosinophils in the airway by inhibiting the infiltration of $CD4^+$ T cells (24). Recently, Stock et al. (25) reported that some Th1 cells that produce IL-10 and IFN-γ could function as a novel type of regulatory T (Treg)[3] cell and suppress airway hyperresponsiveness. Although these studies suggest that IFN-γ suppresses Th2-type responses in the airway, the mechanism of suppression has not been fully examined, and whether

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[3] Abbreviations used in this paper: Treg, regulatory T; alum, aluminum hydroxide; SA, physiologic saline; BALF, bronchoalveolar lavage fluid; DC, dendritic cell; PI, propidium iodide; IP-10, IFN-γ-inducing protein 10.

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IFN-γ could suppress the production of Th1- or Th17-type cytokine in the airway has not been studied.

The purpose of the present study is first to examine whether IFN-γ suppresses Ag-induced immune responses in the airway, and then to elucidate the mechanism of suppression. To fully examine the effect of IFN-γ, we delivered IFN-γ to mice using an IFN-γ-producing plasmid, and conducted analyses. IFN-γ significantly suppressed the production of all of the Th2 (IL-5 and IL-13), Th1 (IFN-γ), and Th17-type (IL-17) cytokines in the lung. Furthermore, IFN-γ suppressed $CD11c^+$ APC functions in the lung as well as the activation of Ag-induced immune responses of $CD4^+$ T cells. These results indicate a novel role for IFN-γ as a broad immune suppressor in the lung.

Materials and Methods

Mice

Male BALB/c and C57BL/6 mice were obtained from Charles River Breeding Laboratories Japan. OVA TCR-transgenic DO11.10 mice were obtained from The Jackson Laboratory. All animal experiments were approved by the Animal Research Ethics Board of the Department of Allergy and Rheumatology, University of Tokyo.

Delivery of IFN-γ into mice

IFN-γ was delivered in vivo by a hydrodynamic-based method through the i.v. injection of plasmid DNA (26–30). The plasmid pCAGGS IFN-γ was constructed by inserting mouse IFN-γ cDNA into the unique *Eco*RI site between the CAG (cytomegalovirus immediate-early enhancer-chicken β-actin hybrid) promoter and the 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector. The IFN-γ expression capacity of the resulting pCAGGS IFN-γ plasmid DNA was confirmed by transient transfection into cultured cells, followed by the measurement of IFN-γ in the culture supernatant by ELISA (data not shown). The plasmid was amplified in *Escherichia coli*, and purified with a Qiagen Endo Free plasmid Giga kit. The empty plasmid pCAGGS was used as a control. Plasmid DNA (10 μg; pCAGGS IFN-γ or control pCAGGS) in lactated Ringer's solution (0.1 ml/g body weight) was injected i.v. via the tail, and the injection was completed within 5 s.

Immunization of mice and evaluation of allergic airway inflammation

Mice were immunized as reported previously (26–28). Seven-week-old animals were sensitized with an i.p. injection of 2 μg of OVA (Sigma-Aldrich) plus 2 mg of aluminum hydroxide (alum) 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 (pCAGGS IFN-γ or control pCAGGS) i.v. on days −3 and 8 (before systemic immunization). In another experiment, mice received plasmid DNA on day 17 (during 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 Pure Chemical). The 3-μm thick sections were prepared and subjected to H&E or periodic acid-Schiff staining to evaluate mucus hypersecretion. The left lungs were homogenized in 1.0 ml of PBS containing 0.5% Triton X-100 and complete protease inhibitor cocktail (Roche Applied Science). The lung homogenates were cleared of debris and cells by centrifugation at 10,000 × *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-12p40, IL-12p70, IL-13, IFN-γ, TGF-β1, IgE, and IgG concentrations were measured using ELISA kits (IL-5, IL-10, IL-12p40, IL-12p70, IFN-γ, and IgE from BD Pharmingen; IL-13 and TGF-β1 from R&D Systems; IgG from Bethyl Laboratories). 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 μg/ml) at 4°C overnight instead of anti-mouse IgE or IgG Ab. OVA-specific IgG1 or IgG2a was measured by ELISA using OVA for capture and HRP-conjugated anti-mouse IgG1 or anti-mouse IgG2a Ab (BD Pharmingen) for detection. The OVA-specific IgE, IgG, or IgG1 standard was derived by pooling sera from five OVA-sensitized mice. As OVA/alum sensitization alone did not produce OVA-specific IgG2a (data not shown), the OVA-specific IgG2a standard was derived by pooling sera from five pCAGGS IL-12-injected and OVA-sensitized mice. Results are expressed as a percentage of the value for the standard.

Preparation of spleen cells, lung cells, and lymph node cells

Spleen cells and lung cells were prepared as reported previously (26). For the preparation of bronchial lymph node cells, bronchial lymph nodes were collected and minced, then incubated at 37°C for 30 min with 150 U of collagenase (Sigma-Aldrich) in complete DMEM. Cell suspensions were obtained by pressing the tissues through a 70-μm nylon filter. Splenic $CD4^+$ T cells, splenic dendritic cells (DCs), and lung $CD11c^+$ APCs were prepared using magnetic beads as reported previously (26, 28).

Ag-induced immune response in bronchial lymph nodes

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

Effect of in vivo treatment with IFN-γ on Ag-induced immune response in spleen

Mice were sensitized with OVA/alum or SA on day 0. Some mice received plasmid (pCAGGS IFN-γ or control pCAGGS) on day −3. In another experiment, mice were sensitized with OVA (2 μg)/CFA on day 0. On day 11, spleen cells were obtained, and cultured (2.5×10^5 cells/well) with OVA (20 μg/ml) in complete DMEM. In some experiments, positively selected $CD4^+$ T cells (2×10^5 cells/well) were cultured with freshly isolated mitomycin C-treated (Sigma-Aldrich) splenocytes (2×10^5 cells/well) and OVA (20 μg/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 supernatants were measured by ELISA. For the measurement of TGF-β1 concentrations, we used serum-free medium X-vivo 15 (Cambrex Bioscience) instead of complete DMEM. We measured total TGF-β1 concentrations after complete activation by acidification.

Detection of $Foxp3^+$ $CD4^+$ T cells

$Foxp3^+$ $CD4^+$ T cells were detected using anti-mouse Foxp3 staining set (eBioscience). Briefly, spleen cells or bronchial lymph node cells were stained with FITC anti-CD4 mAb (BD Pharmingen) for 30 min. Then, the cells were incubated with the fixation/permeabilization solution for 18 h at 4°C. After washing with permeabilization buffer, the cells were stained with PE anti-Foxp3 Ab for 30 min. Then stained cells were analyzed by flow cytometry (EPICS XL System II; Coulter).

Detection of apoptosis

Apoptosis was examined by Annexin/propidium iodide (PI) assay (31, 32). Spleen cells were stained with FITC-Annexin V and PI (5 μg/ml) for 15 min at room temperature. Then stained cells were analyzed by flow cytometry. Annexin V-positive and PI-negative staining indicated early apoptotic cells. Annexin V-positive and PI-positive staining indicated late apoptotic or necrotic cells. In another experiment, TUNEL staining of spleen sections was performed to detect apoptosis based on the labeling of DNA strand breaks using an in situ apoptosis detection kit (Takara Bio).

Analyses of APC functions

For analyses of allogenic MLR, mitomycin C-treated BALB/c ($H\text{-}2^d$) $CD11c^+$ cells (1×10^4 or 1×10^5 cells/well) were cultured with positively selected C57BL/6 ($H\text{-}2^b$) $CD4^+$ T cells (2.5×10^5 cells/well) in complete DMEM. For the measurement of OVA Ag-presenting capacity, mitomycin C-treated $CD11c^+$ cells (2.5×10^4 cells/well) or bronchial lymph node cells (2.5×10^5 cells/well) were cultured with DO11.10 $CD4^+$ T cells (2.5×10^5 cells/well). In some experiments, $CD11c^+$ cells (1×10^5 cells/well) from the spleen were cultured with OVA-sensitized $CD4^+$ T cells (2.5×10^5 cells/well) and OVA (20 μg/ml). After 60–96 h, the proliferation was assessed with a cell proliferation ELISA BrdU kit. For the measurement of the production of cytokines by $CD11c^+$ cells, $CD11c^+$ cells (1.25×10^5 cells/well) were incubated with LPS (1 μg/ml) for 24 h, and then cytokine concentrations in the supernatants were measured. The expression of MHC class II, CD40, CD80, or CD86 was measured by flow cytometry as reported previously (26). For analyses of the direct effect of IFN-γ on DC functions in vitro, splenic DCs from naive mice were incubated with IFN-γ (200 ng/ml) for 24 h, and then LPS (1 μg/ml) was added to the culture. After 24 h, the cells were collected and washed three times,

and then examined for allogenic MLR. In cell transfer study, splenic DCs from naive mice were incubated with IFN-γ (200 ng/ml) for 24 h, and OVA (1000 μg/ml) was added to the culture. After 24 h, the cells were collected, washed, and transferred into naive mice (5×10^5). The mice were challenged with nebulized 3% OVA in PBS for 1 h twice daily on days 7, 8, 14 and 15 and sacrificed on day 16 for BALF analyses. The migration of lung APCs to regional lymph nodes was examined as reported previously (26).

ELISPOT assay

IL-5-secreting cells in bronchial lymph nodes were detected using ELISPOT assay kit (Mabtech). Briefly, polyvinylidene fluoride-based membrane plates were coated with anti IL-5 mAb (clone TRFK5) in PBS at 4°C overnight. After blocking with complete DMEM, cells were incubated with or without OVA on coated membrane for 48 h at 37°C. After washing with PBS, the membrane was incubated biotinylated anti-IL-5 mAb (clone TRFK4) for 2 h. The IL-5 spots were developed by streptavidin-alkaline phosphatase and substrate solution. The number of spots was counted with a dissecting microscope and expressed per million cells.

Statistics

Values are expressed as mean ± SEM. Statistical analyses were performed with a one-way ANOVA followed, when differences were significant, by appropriate post hoc tests using the Turkey-Kramer test. For analysis of the differences between two groups, we used Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

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

First, we examined the kinetics of IFN-γ after i.v. injection of the plasmid DNA. Samples were collected at predetermined points in time following injection of the IFN-γ-expressing plasmid (pCAGGS IFN-γ) or control plasmid (control pCAGGS). We confirmed the temporal expression of the IFN-γ protein in serum (Fig. 1*A*) and in BALF (Fig. 1*B*). We also confirmed the increased expression of IFN-γ-inducing protein 10 (IP-10) mRNA in lung or BALF cells (data not shown), indicating that IFN-γ did actually work on lung or BALF cells. The level of IFN-γ in both samples peaked 1 day after the injection, and rapidly decreased thereafter.

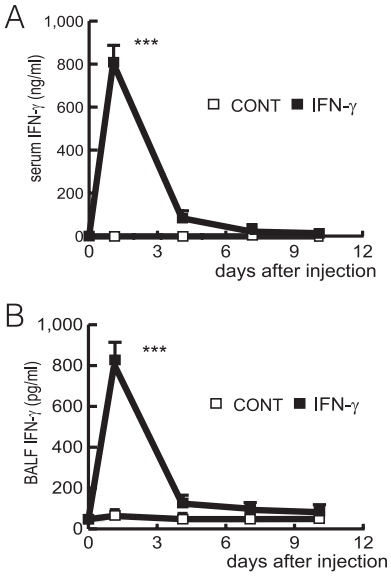

FIGURE 1. IFN-γ concentrations in serum and in BALF after a hydrodynamics-based gene delivery of plasmid DNA via i.v. injection. Mice received an i.v. injection of pCAGGS IFN-γ (IFN-γ) or control pCAGGS (CONT) plasmid on day 0. Concentrations of IFN-γ in serum (*A*) and in BALF (*B*) were measured at the indicated times after the injection using ELISA. Results are presented as mean ± SEM for $n = 6$ mice per group. ***, $p < 0.001$ compared with the value of control pCAGGS.

In vivo IFN-γ gene delivery before systemic Ag sensitization suppresses Th2-mediated eosinophilic airway inflammation and mucus hypersecretion

We next elucidated the effect of IFN-γ gene delivery before 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. Injection of the plasmid (pCAGGS IFN-γ or control pCAGGS) was performed before the first or second systemic Ag sensitization (on days −3 and 8). OVA sensitization and nebulization markedly increased the number of eosinophils in BALF (Fig. 2*A*). IFN-γ gene delivery almost completely diminished the infiltration of eosinophils (Fig. 2*A*). The histology of OVA-sensitized and OVA-challenged mice showed a prominent infiltration of eosinophils into the peribronchial area and mucus hypersecretion by bronchial epithelial cells (Fig. 2*B*). In the mice that received pCAGGS IFN-γ, these findings strongly declined (Fig. 2*B*). BALF IL-13 and TGF-β1 concentrations also decreased in the pCAGGS IFN-γ-treated mice (Fig. 2*C*). IFN-γ treatment significantly suppressed OVA-specific IgE and IgG production (Fig. 2*D*). The concentration of OVA-specific IgG1 in serum increased in the OVA-sensitized and -challenged mice, and IFN-γ treatment suppressed this increase (Fig. 2*E*). The concentration of OVA-specific IgG2a in serum did not increase in the OVA-sensitized and -challenged mice or in the pCAGGS IFN-γ-treated mice (Fig. 2*E*). These results indicated that in vivo IFN-γ gene delivery during the initial stage of sensitization suppressed Th2-mediated allergic airway inflammation.

In vivo IFN-γ gene delivery before systemic Ag sensitization also suppresses the production of IFN-γ and IL-17 in the lung

We next examined whether IFN-γ gene delivery could suppress the production of Th1- and Th17-type cytokines in the lung. Because IFN-γ and IL-17 concentrations in BALF did not increase in OVA-sensitized and -challenged mice in our system (data not shown), first, 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 IFN-γ treatment suppressed the production of these cytokines (Fig. 2*F*). Next, we measured cytokine production by bronchial lymph node cells. In the OVA-sensitized and -challenged mice, lymph node cells produced IL-5, IL-13, IFN-γ, and IL-17 in response to OVA, and IFN-γ treatment strongly suppressed the production of these cytokines (Fig. 2*G*). These results indicated that IFN-γ gene delivery before sensitization could suppress Ag-induced overall immune responses in the lung.

In vivo IFN-γ gene delivery suppresses the Ag-induced Th2-, Th1-, and Th17-type cytokine production of $CD4^+$ T cells ex vivo

Next, we conducted ex vivo analyses. Mice received either pCAGGS IFN-γ or control pCAGGS 3 days before being sensitized to OVA/alum. On day 11, whole spleen cells or $CD4^+$ cells were subjected to analyses. Spleen cells obtained from OVA-sensitized mice strongly proliferated (Fig. 3*A*) and produced IL-5, IFN-γ, and IL-17 (Fig. 3*B*) in response to OVA, compared with those of SA-treated mice. Spleen cells from OVA-sensitized and pCAGGS IFN-γ-injected mice failed to proliferate (Fig. 3*A*) or produce these cytokines (Fig. 3*B*), in contrast to those from the OVA-sensitized mice or from the OVA-sensitized and control pCAGGS-injected mice. As the physiological ratio of $CD4^+$ T cells in the spleen had

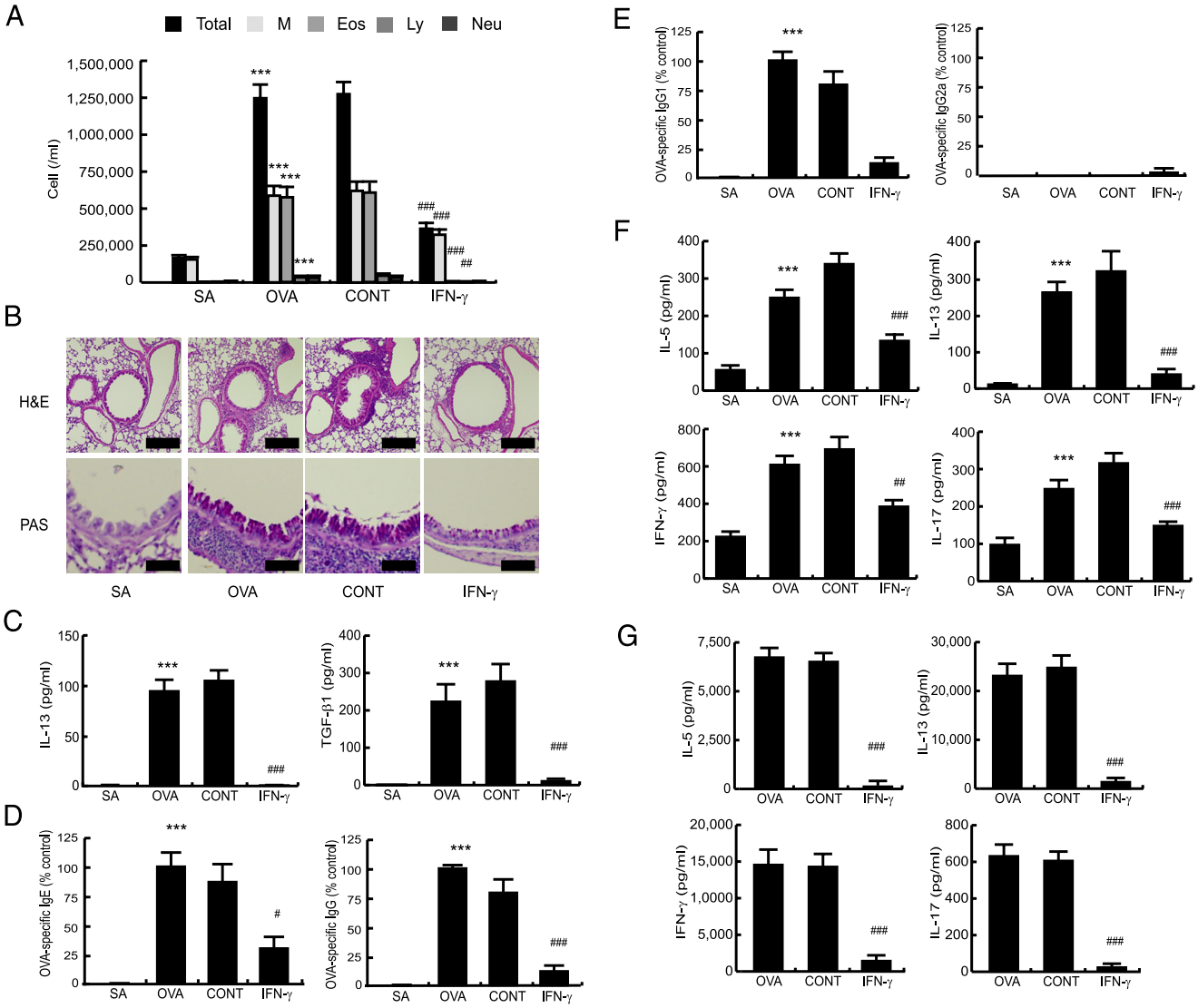

FIGURE 2. In vivo IFN-γ gene delivery before systemic sensitization suppresses the production of not only Th2-type but also Th1- and Th17-type cytokines in the lung. Mice were sensitized with OVA/alum (OVA) or SA on days 0 and 11. Some mice received an i.v. injection of pCAGGS-IFN-γ (IFN-γ) or control pCAGGS (CONT) on days −3 and 8. The mice were nebulized with OVA or PBS from day 18 to day 20. On day 21, the mice were analyzed. *A*, In vivo IFN-γ delivery before sensitization reduces the eosinophil count in BALF. BALF analyses were performed ($n = 8$). Leukocytes were identified by morphologic criteria. *B*, Histological findings. Lungs were excised and subjected to H&E and periodic acid-Schiff staining. Scale bar represents 200 μm (H&E) and 50 μm (periodic acid-Schiff). *C*, Cytokine concentrations in BALF. BALF supernatant was assayed for IL-13 and TGF-β1 concentrations by ELISA ($n = 8$). *D*, OVA-specific IgE and IgG concentrations. Blood samples were obtained from the mice. OVA-specific IgE and IgG concentrations were measured by ELISA ($n = 8$). Pooled sera from five OVA-sensitized mice were set as a control (OVA; 100%). *E*, OVA-specific IgG1 and IgG2a concentrations. OVA-specific IgG1 and IgG2a concentrations were measured by ELISA ($n = 8$). Pooled sera from five OVA-sensitized mice were set as a control for OVA-specific IgG1 (OVA; 100%). Pooled sera from five pCAGGS IL-12-injected and OVA-sensitized mice were set as a control for OVA-specific IgG2a (100%). *F*, Cytokine concentrations in the lung. Lung homogenate was assayed for cytokine concentrations by ELISA ($n = 8$). *G*, Cytokine production of bronchial lymph node cells in response to OVA. Lymph node cells (2.5×10^5 cells/well) were incubated with OVA (10 μg/ml). After 120 h, cytokine levels of the supernatants were measured ($n = 6$). ***, $p < 0.001$ compared with the value of SA-sensitized mice. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ compared with the value of control pCAGGS.

differed between pCAGGS IFN-γ mice and control pCAGGS mice, we then examined the effect of IFN-γ on purified $CD4^+$ T cells, and obtained similar results (Fig. 3, *C* and *D*). These results indicated that in vivo IFN-γ gene delivery before sensitization suppressed the Ag-induced overall immune response of $CD4^+$ T cells.

In vivo IFN-γ gene delivery also suppresses Th1 response of $CD4^+$ T cells ex vivo

Next, we examined whether in vivo IFN-γ gene delivery could suppress Th1 response. Mice received either pCAGGS IFN-γ or control pCAGGS on day −3 and were sensitized to OVA/CFA on day 0. On day 11, whole spleen cells or $CD4^+$ T cells were analyzed. Spleen cells obtained from OVA/CFA-sensitized mice produced IFN-γ in response to OVA (Fig. 3*E*), but did not produce IL-5 (data not shown), indicating that a Th1 response was induced. IFN-γ strongly suppressed the production of IFN-γ itself by spleen cells (Fig. 3*E*). Similar results were obtained when we used $CD4^+$ T cells instead of whole spleen cells (Fig. 3*F*). These results confirmed that IFN-γ gene delivery could suppress Th1 response as well.

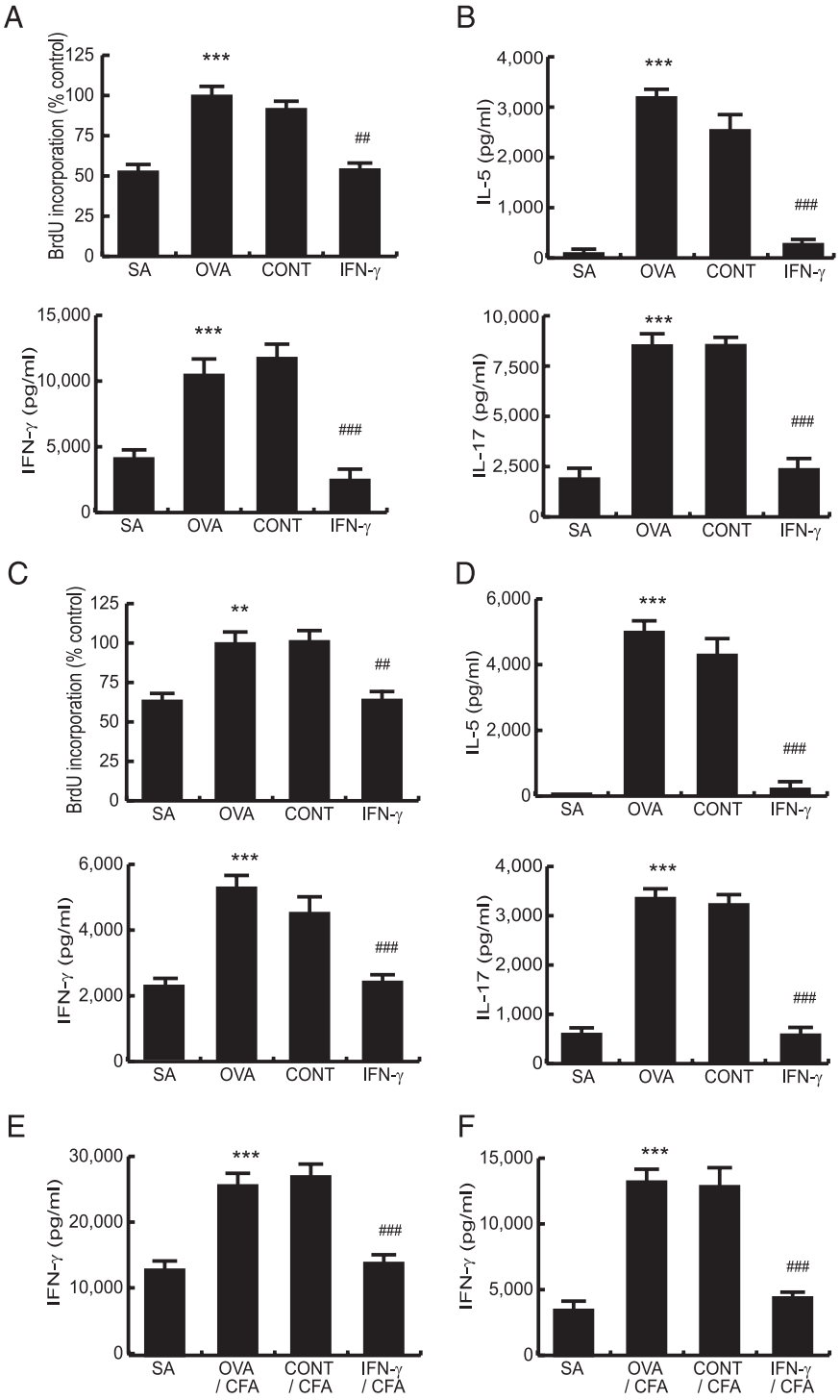

FIGURE 3. In vivo IFN-γ gene delivery suppresses the Ag-induced not only Th2-type but also Th1- and Th17-type cytokine production of $CD4^+$ T cells. *A–D*, Effect of IFN-γ gene delivery on Ag-induced immune responses. Mice were sensitized with OVA/alum or SA on day 0. Some mice received plasmid (pCAGGS-IFN-γ or control pCAGGS) on day −3. On day 11, the mice were analyzed. *A* and *B*, Proliferation and cytokine production of whole spleen cells in response to OVA. Whole spleen cells (2.5×10^5 cells/well) were incubated with OVA (20 μg/ml). *A*, After 72 h, the proliferation was assessed based on BrdU incorporation ($n = 6$). The maximum proliferation observed in response to OVA for spleen cells from OVA-sensitized mice was set as a control (OVA; 100%). *B*, After 96 h, cytokine levels of the supernatants were measured ($n = 6$). *C* and *D*, Proliferation and cytokine production of $CD4^+$ T cells in response to OVA. Splenic $CD4^+$ T cells (2×10^5 cells/well) were positively selected by magnetic cell sorting and cultured with freshly isolated mitomycin C-treated splenocytes (2×10^5 cells/well) and OVA (20 μg/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 set as a control (OVA; 100%). *D*, After 96 h, cytokine levels of the supernatants were measured ($n = 6$). *E* and *F*, Effect of IFN-γ gene delivery on Th1 response. Mice were sensitized with OVA/CFA or SA on day 0. Some mice received plasmid (pCAGGS IFN-γ or control pCAGGS) on day −3. Analyses were conducted in the same manner as the experiments with OVA/alum sensitization, described for *A* and *B*. IFN-γ production by whole spleen cells (*E*) and $CD4^+$ T cells (*F*) was measured ($n = 6$ each). **, $p < 0.01$ and ***, $p < 0.001$ compared with the value of SA. ##, $p < 0.01$ and ###, $p < 0.001$ compared with the value of control pCAGGS.

In vivo IFN-γ gene delivery does not induce the development of Treg cells

We examined the possibility that this suppression was mediated through the production of Treg cells in vivo. IFN-γ gene delivery, however, did not increase the production of immune suppressive cytokines such as IL-10 (Fig. 4*A*) or TGF-β1 (data not shown) by whole spleen cells in response to OVA. $CD4^+$ T cells from OVA-sensitized and pCAGGS IFN-γ-injected mice did not produce IL-10 (Fig. 4*B*) or TGF-β1 (data not shown). Furthermore, IFN-γ did not increase the expression of Foxp3, transcription factor that was specifically expressed in Treg cells (33, 34), in $CD4^+$ T cells in spleen (Fig. 4*C*) or in bronchial lymph nodes (data not shown). In addition, IFN-γ did not alter the ratio of $CD4^+CD25^+$ cells to $CD4^+CD25^-$ cells, either (data not shown). These results suggested that in vivo IFN-γ gene delivery would not induce the development of Treg cells. So, the suppression of immune responses by IFN-γ would not be mediated by the induction of Treg cells in our experimental system.

In vivo IFN-γ gene delivery does not induce the apoptosis of spleen cells

IFN-γ plays an essential role in the induction of apoptosis (18, 31, 32, 35–37). Therefore, we examined the possibility that the suppressive effect of IFN-γ was mediated by apoptosis. Mice received

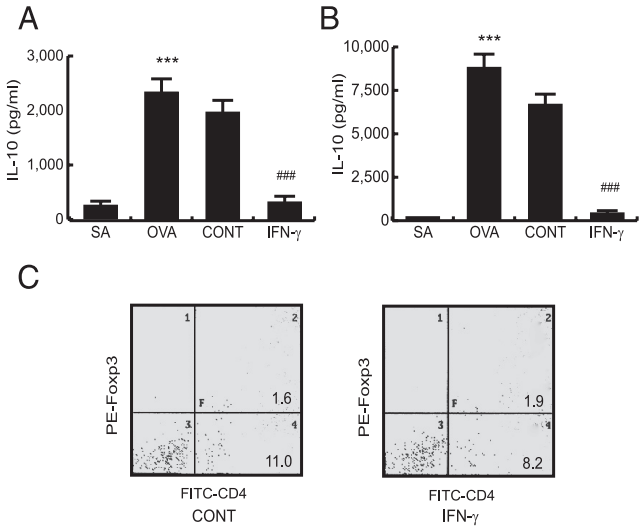

FIGURE 4. In vivo IFN-γ gene delivery does not induce the development of Treg cells. Mice were sensitized with OVA/alum or SA on day 0. Some mice received plasmid (pCAGGS-IFN-γ or control pCAGGS) on day −3. On day 11, the mice were analyzed. *A*, IL-10 production by whole spleen cells in response to OVA. On day 11, whole spleen cells (2.5×10^5 cells/well) were incubated with OVA (20 μg/ml). After 96 h, the concentration of IL-10 in the supernatants was assayed ($n = 6$). *B*, IL-10 production by $CD4^+$ T cells in response to OVA. On day 11, splenic $CD4^+$ T cells (2×10^5 cells/well) were positively selected by magnetic cell sorting and cultured with freshly isolated mitomycin C-treated splenocytes (2×10^5 cells/well) and OVA (20 μg/ml). After 96 h, the concentration of IL-10 was assayed ($n = 6$). *C*, IFN-γ gene delivery does not increase $Foxp3^+$ $CD4^+$ T cells in spleen. On day 11, whole spleen cells were obtained and $Foxp3^+$ $CD4^+$ T was detected by flow cytometry. Representative images were shown. ***, $p < 0.001$ compared with the value of SA. ###, $p < 0.001$ compared with the value of control pCAGGS.

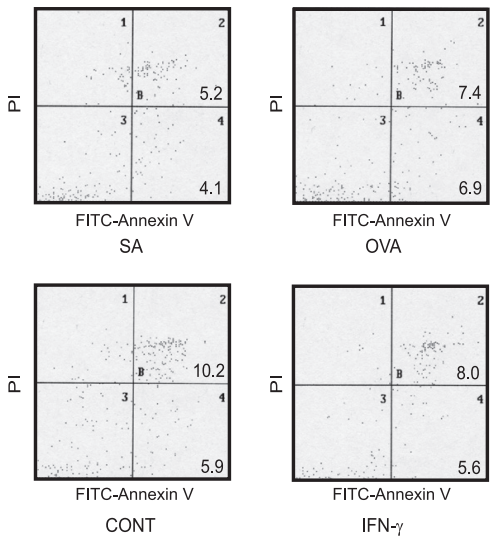

FIGURE 5. In vivo IFN-γ gene delivery does not induce the apoptosis of spleen cells. Mice were sensitized with OVA/alum or SA on day 0. Some mice received plasmid (pCAGGS-IFN-γ or control pCAGGS) on day −3. On day 11, whole spleen cells were obtained and apoptosis was assessed by Annexin/PI staining. Annexin V-positive and PI-negative cells (lower right quadrant; field no. 4) were early apoptotic cells. Annexin V-positive and PI-positive cells (upper right quadrant; field no. 2) were late apoptotic or necrotic cells. Representative images of Annexin/PI staining were shown.

either pCAGGS IFN-γ or control pCAGGS on day −3 and were sensitized with OVA/alum or SA on day 0. On day 11, whole spleen cells were subjected to analyses. IFN-γ gene delivery did not increase apoptosis, as assessed by the Annexin/PI assay (Fig. 5) or TUNEL staining of the splenic tissues (data not shown). In some experiments, we used spleen cells that were not treated with collagenase or RBC lysis solution to reduce treatment-induced cell death in the Annexin/PI assay, and obtained similar results (data not shown). When spleen was obtained 1 or 3 days after plasmid injection (on day −2 or day 0), the results were similar (data not shown). These results suggested that the suppression of immune responses by IFN-γ in this system would not be mediated by apoptosis.

In vivo IFN-γ gene delivery suppresses overall DC functions ex vivo

DCs play an essential role in the initiation and development of Ag-induced immune responses (38–40). So, we next examined the effect of IFN-γ gene delivery on splenic DC functions. IFN-γ significantly decreased the MHC alloreactivity (Fig. 6*A*) and OVA-presenting capacity (Fig. 6*B*). IFN-γ suppressed the production of IL-10 (Fig. 6*C*) and IFN-γ (Fig. 6*D*) by splenic DCs in response to LPS, whereas it did not affect the production of IL-12p70 (Fig. 6*E*) or IL-12p40 (Fig. 6*F*). IFN-γ slightly suppressed MHC class II expression, however, it did not suppress costimulatory molecules such as CD40, CD80, and CD86 (data not shown). Furthermore, IFN-γ did not increase the apoptosis of splenic DCs, as assessed by Annexin/PI staining (data not shown). Recently, Kool et al. (41) reported that after i.p. injection of OVA/alum, OVA-captured inflammatory monocytes migrated to the bronchial lymph nodes via abdominal lymphatics, and then acquired a DC phenotype that induced persistent Th2 response. So, we examined whether IFN-γ could suppress this process. Bronchial lymph node cells from OVA-sensitized and control pCAGGS-injected mice induced the proliferation of DO11.10 $CD4^+$ cells, indicating that OVA-captured APCs actually migrated into bronchial lymph node (Fig. 6*G*). In contrast, IFN-γ treatment suppressed this migration (Fig. 6*G*). Moreover, IFN-γ strongly suppressed Ag-induced IL-5 production (Fig. 6*H*) or IL-5 secretion (Fig. 6*I*) in bronchial lymph node cells, suggesting that IFN-γ suppressed Th2-cell priming in the airway. In another experiment, pretreatment of splenic DCs from naive mice with IFN-γ in vitro directly suppressed the Ag-presenting capacity of DCs (Fig. 6*J*), which was consistent with previous studies (42–44). Moreover, pretreatment of DCs with IFN-γ attenuated eosinophilic inflammation induced by transfer of OVA-pulsed DCs and inhalation of OVA (Fig. 6*K*), although this suppressive effect was mild. These results suggested that IFN-γ gene delivery did not merely conduct a shift from a Th2 to Th1-type immune response, but induced an overall suppression of Ag presentation by DCs.

In vivo IFN-γ gene delivery during Ag airway challenge suppresses the production of all of the Th2-, Th1-, and Th17-type cytokines in the lung

We next examined whether IFN-γ gene delivery suppresses allergic inflammation, even when the gene is delivered in the effector phase. First, we confirmed the effect of delivery during the Ag challenge on Th2-mediated allergic inflammation. The injection of plasmid (pCAGGS IFN-γ or control pCAGGS) was performed during a local Ag challenge (on day 17). IFN-γ delivery during the challenge strongly suppressed the infiltration of eosinophils (Fig. 7*A*). Histologically, IFN-γ decreased the

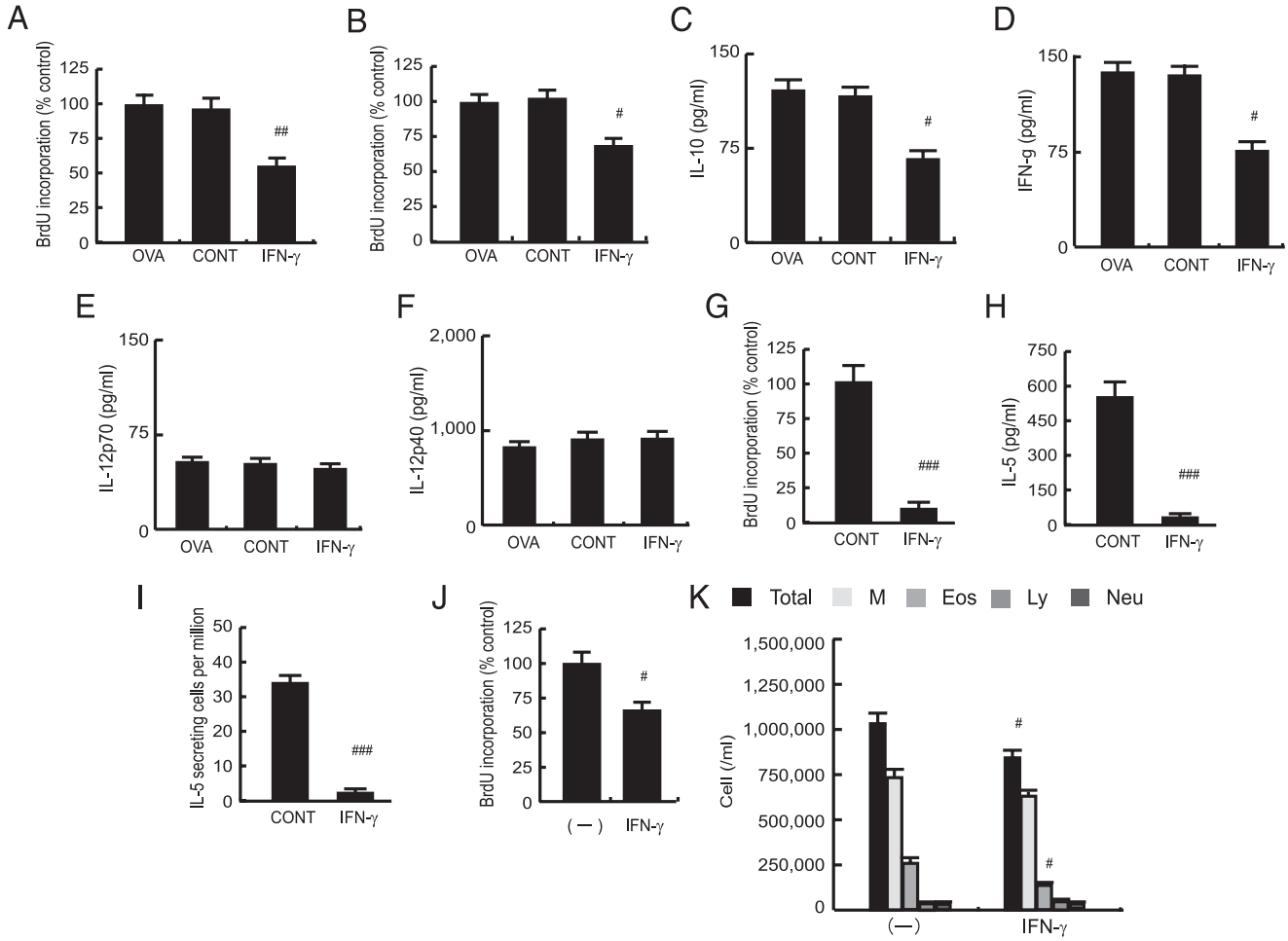

FIGURE 6. In vivo IFN-γ gene delivery suppresses DC functions. *A–F*, Effect of IFN-γ gene delivery on splenic DC functions. Mice were sensitized with OVA/alum on day 0. Some mice received an i.v. injection of pCAGGS IFN-γ or control pCAGGS on day −3. On day 11, splenic DCs were positively selected and subjected to analyses. *A*, Allogenic MLR. Splenic DCs (1×10^4 cells/well) from BALB/c mice (H-2^d) were cultured with $CD4^+$ T cells (2.5×10^5 cells/well) from C57BL/6 mice (H-2^b). After 72 h, the proliferation was assessed based on BrdU incorporation ($n = 6$). The proliferation of $CD4^+$ T cells from C57BL/6 mice in response to DCs from OVA-sensitized BALB/c mice was set as a control (OVA; 100%). *B*, Ag-presenting capacity. Splenic DCs (1×10^5 cells/well) were cultured with $CD4^+$ T cells (2.5×10^5 cells/well) from OVA-sensitized mice and OVA (20 μg/ml). After 72 h, the proliferation was assessed based on BrdU incorporation ($n = 6$). The proliferation of $CD4^+$ T cells from OVA-sensitized mice in response to DCs from OVA-sensitized mice with OVA was set as a control (OVA; 100%). *C–F*, Cytokine production. Splenic DCs (1.25×10^5 cells/well) were cultured with LPS (1 μg/ml). After 24 h, concentrations of IL-10 (*C*), IFN-γ (*D*), IL-12p70 (*E*), and IL-12p40 (*F*) of the supernatants were assayed ($n = 6$). *G*, Effect of IFN-γ gene delivery on migration of OVA-captured APCs to bronchial lymph nodes via abdominal lymphatics. Mice were sensitized and received plasmid as described. On day 1, after the OVA/alum sensitization, bronchial lymph nodes were excised. Mitomycin C-treated lymph node cells (2.5×10^5 cells/well) were cultured with DO11.10 $CD4^+$ T cells (2.5×10^5 cells/well). After 96 h, the proliferation was assessed based on BrdU incorporation ($n = 6$). The proliferation of $CD4^+$ T cells from DO11.10 mice in response to lymph node cells from OVA-sensitized and control pCAGGS-injected mice was set as a control (CONT; 100%). *H* and *I*, Effect of IFN-γ gene delivery on Th2-type cell priming in bronchial lymph nodes. Mice were sensitized and received plasmid as described. On day 11, bronchial lymph nodes were excised. *H*, IL-5 production. Lymph nodes cells (2.5×10^5 cells/well) were cultured with OVA (10 μg/ml). After 120 h, IL-5 concentrations of the supernatants were assayed ($n = 6$). *I*, IL-5 secretion. Lymph node cells were incubated with OVA (10 μg/ml) on polyvinylidene fluoride-based membrane for 48 h at 37°C. IL-5-secreting cells were detected using ELISPOT assay ($n = 6$). The number of spots was counted with a dissecting microscope and expressed per million cells. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ compared with the value of control pCAGGS. *J* and *K*, Direct effect of IFN-γ on DC functions. *J*, Allogenic MLR. Splenic DCs from naive mice were incubated with IFN-γ (200 ng/ml) for 24 h, and then LPS (1 μg/ml) was added in the culture. After 24 h, the cells were collected and washed three times, and then examined for allogenic MLR ($n = 6$). *K*, Effect of IFN-γ-pretreated and OVA-pulsed DCs on eosinophilic airway inflammation. IFN-γ-pretreated (200 ng/ml) DCs were incubated with OVA (1000 μg/ml) for 24 h. Syngenic recipients received OVA-pulsed DCs or IFN-γ-pretreated and OVA-pulsed DCs on day 0 (5×10^5). The mice were challenged with nebulized 3% OVA for 1 h twice daily on days 7, 8, 14, and 15, and sacrificed on day 16 for BALF analysis ($n = 8$). #, $p < 0.05$ compared with the value without IFN-γ.

infiltration of inflammatory cells in the peribronchial area and mucus hypersecretion (Fig. 7*B*). IFN-γ suppressed IL-13 and TGF-β1 concentrations in BALF (Fig. 7*C*), although it did not suppress OVA-specific IgE or IgG production (Fig. 7*D*). These results indicated that in vivo IFN-γ gene delivery during the effector phase also suppressed Th2-mediated allergic airway inflammation. Furthermore, IFN-γ treatment suppressed the production of not only IL-5 and IL-13 but also IFN-γ and IL-17 in the lung (Fig. 7*E*). Moreover, IFN-γ strongly suppressed the production of IL-5, IL-13, IFN-γ, and IL-17 by bronchial lymph node cells in response to OVA (Fig. 7*F*). These results suggested that IFN-γ could also suppress the Ag-induced overall immune response in the lung, even when delivered in the effector phase.

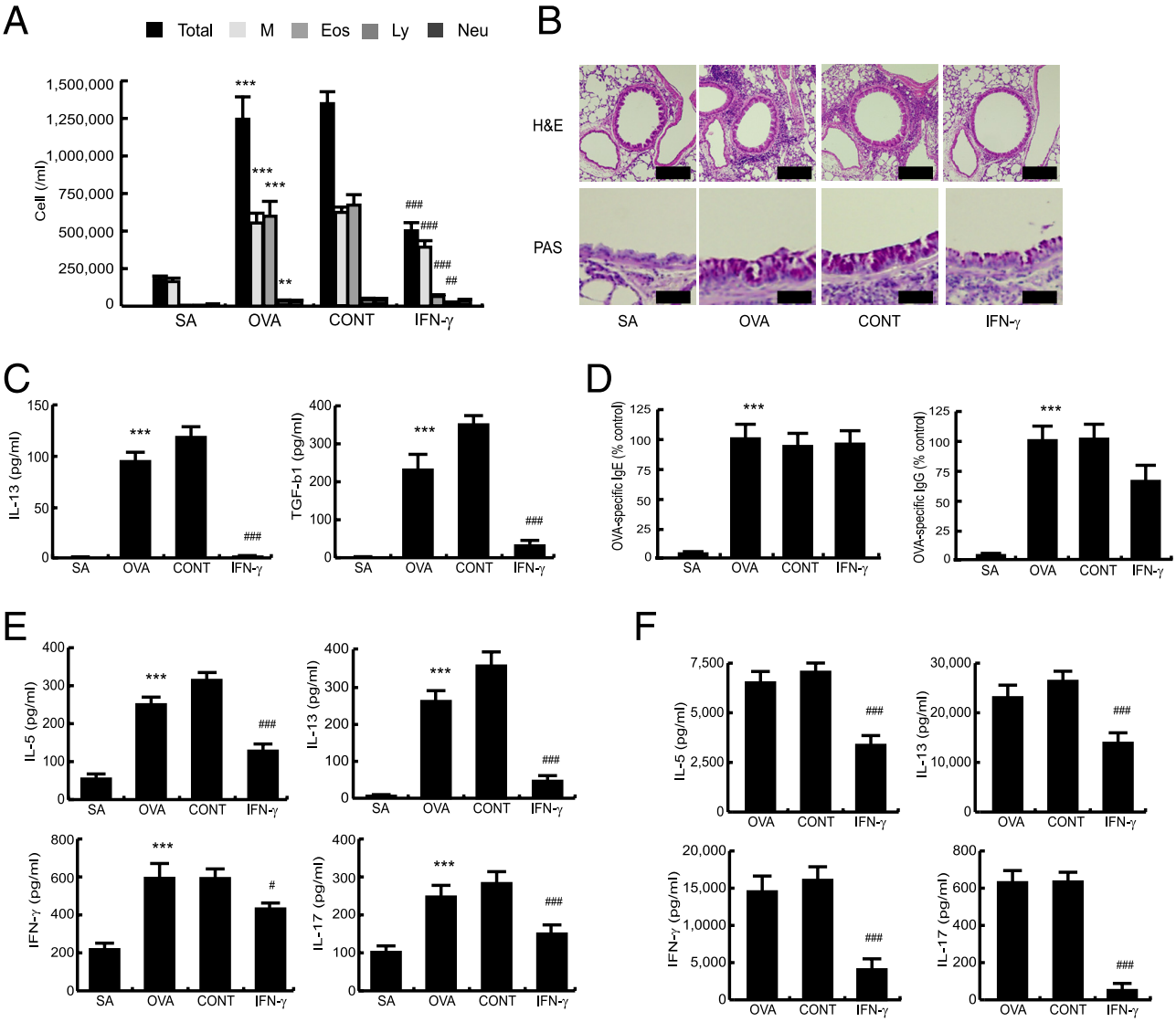

FIGURE 7. In vivo IFN-γ gene delivery during Ag challenge also suppresses the production of not only Th2-type but also Th1- and Th17-type cytokines in the lung. Mice were sensitized with OVA/alum (OVA) or SA on days 0 and 11. Some mice received an i.v. injection of pCAGGS-IFN-γ (IFN-γ) or control pCAGGS (CONT) on day 17. The mice received by inhalation, OVA or PBS from day 18 to day 20. On day 21, the mice were analyzed. *A*, In vivo IFN-γ delivery during Ag challenge suppresses the eosinophil count in BALF. BALF analyses were performed ($n = 8$). Leukocytes were identified by morphologic criteria. *B*, Histological findings. Lungs were excised and subjected to H&E and periodic acid-Schiff staining. Scale bar represents 200 μm (H&E) and 50 μm (periodic acid-Schiff). *C*, Cytokine concentrations in BALF. Supernatant of BALF was assayed for IL-13 and TGF-β1 concentrations by ELISA ($n = 8$). *D*, OVA-specific IgE and IgG concentrations. OVA-specific IgE and IgG concentrations in serum were measured by ELISA ($n = 8$). Pooled sera from five OVA-sensitized mice were set as a control (OVA; 100%). *E*, Cytokine concentrations in the lung. Lung homogenate was assayed for cytokine concentrations by ELISA ($n = 8$). *F*, Cytokine production of bronchial lymph node cells in response to OVA. Lymph node cells (2.5×10^5 cells/well) were incubated with OVA (10 μg/ml). After 120 h, cytokine levels of the supernatants were measured ($n = 6$). **, $p < 0.01$ and ***, $p < 0.001$ compared with the value in SA-sensitized samples. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ compared with the value in control pCAGGS-injected samples.

In vivo IFN-γ gene delivery during Ag challenge suppresses lung CD11c$^+$ APC functions

Finally, we examined the effect of IFN-γ gene delivery during the Ag challenge phase on lung CD11c$^+$ APC functions. IFN-γ treatment significantly decreased the MHC alloreactivity (Fig. 8*A*) and OVA-presenting capacity of lung CD11c$^+$ APCs (Fig. 8*B*). IFN-γ suppressed IL-10 production by lung CD11c$^+$ APCs in response to LPS (Fig. 8*C*). It also suppressed the expression of MHC class II in lung CD11c$^+$ APCs (Fig. 8*D*). In contrast, IL-12p70 or IL-12p40 production was not affected (Fig. 8, *E* and *F*). Furthermore, IFN-γ suppressed the migration of lung APCs into the regional lymph nodes (Fig. 8, *G* and *H*). These results demonstrated that IFN-γ gene delivery during the effector phase suppressed lung CD11c$^+$ APC functions and migration.

Discussion

The results of the present study clearly demonstrated that IFN-γ acts as an immune-modulating cytokine and attenuates Ag-induced immune responses in the lung by suppressing the functions of CD11c$^+$ APCs and CD4$^+$ T cells. IFN-γ attenuated the indicators of a Th2-type response such as eosinophilic inflammation, IL-5 and IL-13 production in the lung, and IgE production. Moreover, IFN-γ also suppressed the production of IL-17 and IFN-γ itself.

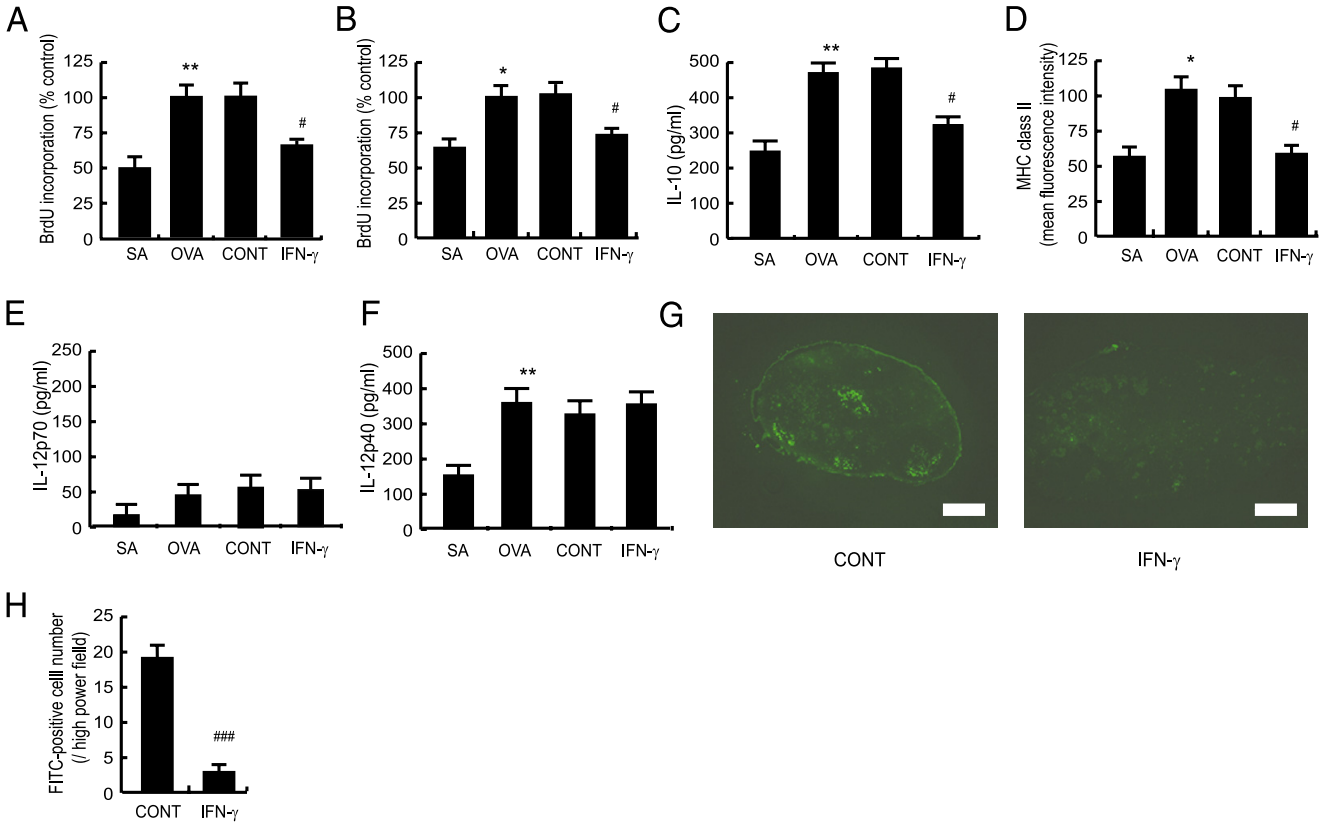

FIGURE 8. In vivo IFN-γ gene delivery during Ag challenge suppresses lung CD11c$^+$ APC functions and migration. *A–F*, APC functions in the lung. Mice were sensitized with OVA/alum (OVA) or SA on days 0 and 11. Some mice received an i.v. injection of pCAGGS-IFN-γ (IFN-γ) or control pCAGGS (CONT) on day 17. The mice received by inhalation, OVA or PBS from day 18 to day 20. On day 21, the mice were analyzed. *A*, Allogenic MLR. Lung CD11c$^+$ cells (1×10^5 cells/well) from BALB/c mice were cultured with CD4$^+$ T cells (2.5×10^5 cells/well) from C57BL/6 mice. After 60 h, the proliferation was assessed based on BrdU incorporation ($n = 6$). The proliferation of CD4$^+$ T cells from C57BL/6 mice in response to CD11c$^+$ cells from OVA-sensitized BALB/c mice was set as a control (OVA; 100%). *B*, Ag-presenting capacity. Lung CD11c$^+$ cells (2.5×10^4 cells/well) were cultured with CD4$^+$ T cells (2.5×10^5 cells/well) from DO11.10 mice. After 60 h, the proliferation was assessed based on BrdU incorporation ($n = 6$). The proliferation of CD4$^+$ T cells from DO11.10 mice in response to CD11c$^+$ cells from OVA-sensitized mice was set as a control (OVA; 100%). *C*, IL-10 production. Lung CD11c$^+$ cells (1.25×10^5 cells/well) were cultured with LPS (1 μg/ml). After 24 h, concentrations of IL-10 in the supernatants were assayed ($n = 6$). *D*, MHC class II expression of lung CD11c$^+$ cells was measured by flow cytometry ($n = 6$). *E* and *F*, IL-12 production. Lung CD11c$^+$ cells (1.25×10^5 cells/well) were cultured with LPS (1 μg/ml). After 24 h, concentrations of IL-12p70 (*E*) and IL-12p40 (*F*) in the supernatants were assayed ($n = 6$). *G* and *H*, Migration of lung APCs into regional lymph node. Mice were sensitized and received plasmid as described. On day 19, mice were administered FITC-labeled OVA (500 μg) intratracheally. On day 20, bronchial lymph nodes were excised, and subjected to fluorescence microscopy. *G*, Representative images. FITC-positive cells (green) were clearly detected in the regional lymph nodes of mice i.v. injected with control pCAGGS. Scale bar represents 300 μm. *H*, FITC-positive cell (migrated OVA-captured APC) numbers in regional lymph node. FITC-positive cells were enumerated directly in six random high-power fields (40X objective). *, $p < 0.05$ and **, $p < 0.01$ compared with the value of SA-sensitized samples. #, $p < 0.05$ and ###, $p < 0.001$ compared with the value of control pCAGGS samples.

IFN-γ reduced the Ag-presenting capacity and cytokine production of DCs, thus attenuating the proliferation and cytokine production of CD4$^+$ T cells. Finally, IFN-γ suppressed allergic airway inflammation not only when administered before systemic sensitization but also when delivered during the effector phase of an allergic immune response. To our knowledge, this report is the first to demonstrate a possible, novel role of IFN-γ as a broad immune-modulating cytokine in the lung.

IFN-γ is a Th1-type cytokine. It inhibits Th2 cell proliferation in vitro (15) and antagonizes *Leishmania*-induced Th2 responses in vivo (16, 17). So it is expected to antagonize or suppress Th2-mediated allergic inflammation. However, the effect of IFN-γ on allergic airway inflammation has been examined only in a few reports. Deletion of IFN-γ suppressed eosinophilic airway inflammation, indicating that endogenous IFN-γ plays some role in the development of allergic airway inflammation (45). In contrast, several studies demonstrated that the exogenous delivery of IFN-γ suppresses allergic airway inflammation (23, 24, 45). Iwamoto et al. (24) reported that the administration of a recombinant IFN-γ inhibited recruitment of eosinophils into the airway with inhibition of CD4$^+$ T cells but not CD8$^+$ T cells. They speculated that the mechanism of suppression was interference with the initial activation of Th2 cells and an effect on vascular endothelial cells. Li et al. (23) delivered IFN-γ into the lung by administering an IFN-γ-producing plasmid in the trachea, and found that IFN-γ treatment attenuated the conalbumin-induced recruitment of eosinophils in the airway as well as IL-4 and IL-5 concentrations in BALF. Although these early studies indicate that exogenous IFN-γ can modulate Th2-mediated immune responses in the airway, the precise mechanism of action has not been fully examined. Its effect on immunocytes other than T cells has not been examined either. Furthermore, these early studies did not analyze the effect of IFN-γ on the production of Th1- and Th17-type cytokines. To clarify these issues, in the current study we delivered IFN-γ in an experimental system of OVA-induced allergic airway immune responses, and conducted analyses.

IFN-γ gene delivery before systemic sensitization attenuated indicators of a Th2-type response (Fig. 2, *A–E*). Furthermore, IFN-γ also attenuated the concentrations of IL-17 and IFN-γ itself in the lung (Fig. 2*F*). This result was confirmed with immunocytes obtained from bronchial lymph nodes ex vivo (Fig. 2*G*). In addition, IFN-γ significantly suppressed the production of all of the Th2-, Th17-, and Th1-type cytokines from whole spleen cells (Fig. 3*B*) or $CD4^+$ T cells (Fig. 3*D*). Moreover, it suppressed the production of IFN-γ in the Th1-biased OVA/CFA system (Fig. 3, *E* and *F*). Taken together, the mechanism of suppression of IFN-γ was not merely antagonizing the Th2 response. Rather, IFN-γ suppressed the Ag-induced immune responses as a whole, including the production of IFN-γ and IL-17.

To elucidate the mechanism of the suppression, we analyzed whether IFN-γ induced the regulatory phenotype of T cells (Fig. 4). IFN-γ did not up-regulate IL-10 and TGF-β1 production by whole spleen cells or $CD4^+$ T cells (Fig. 4, *A* and *B*, and data not shown). In addition, IFN-γ did not alter the ratio of $CD4^+CD25^+$ cells to $CD4^+CD25^-$ cells, either (data not shown). Recently, Wang et al. (46) reported that IFN-γ converts $CD4^+CD25^-$ T cells to Treg cells in vitro though the induction of Foxp3 expression. So, we next examined the effect of in vivo IFN-γ gene delivery on the expression of Foxp3 in $CD4^+$ T cells. However, expression of Foxp3 did not increase after IFN-γ treatment (Fig. 4*C*). This indicated that IFN-γ does not generate $Foxp3^+$ Treg cells in our system.

IFN-γ plays an essential role in the induction of apoptosis in immunocytes (18, 31, 32, 35–37). Furthermore, recent reports suggest that it plays an important role in the apoptosis of Th1 cells (47, 48). Deletion of IFN-γ impaired the apoptosis of T cells while addition of IFN-γ to IFN-γ-deficient mice restored this function (18, 31, 35). In contrast, the effect of IFN-γ on apoptosis in wild-type mice remains controversial. A report suggests that IFN-γ induces apoptosis (37), whereas other studies suggest that addition of IFN-γ to T cells from wild-type mice does not affect apoptosis (18, 31, 35). In the present study, in vivo IFN-γ gene delivery did not increase apoptosis of splenocytes (Fig. 5) and lung cells (data not shown). The delivery of IFN-γ did not increase apoptosis of splenic DCs and lung $CD11c^+$ APCs, either (data not shown). Although a possibility that we could not detect early or mild apoptosis still remains, apoptosis of immunocytes would not be a major mechanism for the immune suppression by IFN-γ in our system.

We next examined the effect of IFN-γ on splenic DCs. The effect of IFN-γ on DC functions has been controversial (42–44, 49, 50). For example, bone marrow-derived DCs obtained from IFN-γ-deficient mice had impaired functions, which suggests that IFN-γ is an autocrine mediator for DC maturation (49). Akbar et al. (50) reported that DCs from IFN-γ transgenic mice had enhanced functions as compared with those from wild-type mice in a model of cancer. In contrast, IFN-γ-pretreated splenic DCs suppressed experimental autoimmune myasthenia gravis (42), autoimmune diabetes (43), and allergic encephalomyelitis (44), suggesting that IFN-γ suppresses DC functions in some situations. In this study, we confirmed the direct suppressive effect on splenic DC functions by in vitro IFN-γ pretreatment (Fig. 6*J*). We also confirmed that pretreatment of DCs with IFN-γ in vitro directly attenuated the transfer-induced eosinophilic inflammation, although the effect was mild (Fig. 6*K*). Moreover, in vivo IFN-γ gene delivery suppressed allogenic MLR (Fig. 6*A*) and OVA Ag-presenting capacity (Fig. 6*B*). It also suppressed IL-10 and IFN-γ production by splenic DCs with no effect on IL-12p70 or IL-12p40 production (Fig. 6, *C–F*). Furthermore, IFN-γ suppressed the migration of OVA-captured APCs into bronchial lymph nodes (Fig. 6*G*) and T cell priming in the airway (Fig. 6, *H* and *I*). These results suggest that IFN-γ exhibited its suppressive effect on DCs (APCs) by suppressing Ag presentation. Recently, Sun and Metzger (51) reported that IFN-γ suppresses the expression of MARCO, the class A scavenger receptor, on lung $CD11c^+$ cells and the phagocytosis, thus inhibited antibacterial immunity. Although Arredouani et al. (52) reported that deletion of MARCO enhances lung DC migration and Ag-induced eosinophilic inflammation, the role of MARCO in the Ag-induced immune response of wild-type mice has not been fully clarified. So, suppression of MARCO might also be involved in the IFN-γ-mediated suppression of T cell priming in this study, which should be elucidated in future.

Conversely, as direct suppressive effect on DCs by in vitro IFN-γ pretreatment was not so strong and IFN-γ was known to act on several cell types, another mechanism than direct suppression of DC functions also might play some role. The reason for the discrepancy in results between the study of Akbar et al. (50) and our study remains unclear. In their report, Ag-specific IgG production and Ag-specific T cell responses as well as DC functions increased in IFN-γ transgenic mice (50), which were completely different from the results we obtained by in vivo IFN-γ gene delivery. The difference in the expression of IFN-γ protein between their system (transgenic mice) and ours (plasmid gene delivery) might explain this discrepancy. In addition, the difference in the experimental system (cancer and allergic inflammation) should also be considered.

Finally, we confirmed that IFN-γ was effective even when delivered during airway inflammation (Figs. 7 and 8). IFN-γ suppressed the production of all of the Th2-, Th1-, and Th17-type cytokines (Fig. 7). IFN-γ suppressed $CD11c^+$ APC functions in the lung such as Ag presentation and cytokine production, and the migration of APCs to regional lymph nodes (Fig. 8). Taken altogether, these results indicate a potential role for IFN-γ as an immune suppressive cytokine that affects on APC functions such as Ag presentation, cytokine production, and migration in the lung.

IFN-γ is known to enhance the production of IgG2a (53). Although IL-12 is essential for IgG2a production, IFN-γ is not essential (53). In this study, IFN-γ treatment did not increase IgG2a production (Fig. 2*E*). Most of the previous findings that IFN-γ enhances IgG2a production were obtained from in vitro study (54, 55). In contrast, there were few in vivo studies on the effect of IFN-γ on IgG2a production. Finkelman et al. (56) examined in vivo effect of IFN-γ on IgG2a production. They sensitized mice with goat Ab-specific for mouse IgD, and this system produced IgG2a. Administration of low dose of IFN-γ protein enhanced IgG2a production. In contrast, high dose of IFN-γ protein did not increase, rather suppressed, IgG2a production. This finding is consistent with our result by OVA/alum sensitization. Therefore, the effect of IFN-γ on IgG2 production seems to depend on the dose of IFN-γ, type of Ag, and so on. Because Ag presentation by DCs to B cells as well as T cells initiates Ag-induced immune response, we speculated that IFN-γ treatment would suppress overall B cells functions by inhibiting DC functions.

Jaffe et al. (57) reported that systemic administration of IFN-γ protein did not induce the expression of IP-10 mRNA in alveolar macrophage, suggesting that IFN-γ is compartmentalized. In contrast, in our study, systemic delivery of IFN-γ gene increased IFN-γ concentrations in BALF (Fig. 1*B*). We also confirmed that the increased expression of IP-10 mRNA in lung or BALF cells (data not shown), indicating that IFN-γ did actually work on lung or BALF cells. As reported originally, with our gene delivery system, the plasmid was trapped in the liver and the cytokine was

produced there, then moved into the bloodstream and to the perfused organs (30). Therefore, our system did not directly transduce IFN-γ gene into lung cells. A reason for the discrepancy in the results would be due to a difference in the expression of IFN-γ protein in serum (0.1–0.8 ng/ml in their study (57) vs 100–800 ng/ml in our study (Fig. 1*A*)). We consider that IFN-γ protein was detected even in the BALF due to high expression of IFN-γ in serum by our system.

IL-10 was originally identified as a Th2-type cytokine (58). However, it is now recognized to have a broad range of immune regulatory effects on various kinds of cells (58, 59). Many kinds of Treg cells produce IL-10 to exhibit an immune suppressive effect (59). We previously reported that IL-10 has a potent suppressive effect on the immune response in the airway (26). The current study suggests that a Th1-type cytokine, IFN-γ, possesses similar potential as a broad immune modulator as a counterpart of the Th2-type immune suppressive cytokine IL-10. This potential might work in some regulatory Th1 cells in various immune responses.

Recently, a defensive role of IFNs against viral infection has been highlighted in patients with bronchial asthma (60, 61). IFN-β (60) or IFN-λ (61) plays a critical role in the exclusion of virus out of bronchial epithelial cells. IFN-γ is also considered to play some role in host defense against rhinovirus infection in asthmatic patients (62–64). In these reports, it is speculated that decrease in the production of IFN-γ by PBMCs or by lung cells may result in the exacerbation of asthma. These findings suggest that delivery of IFN-γ may also be beneficial in the treatment of asthma by inhibiting virus-induced exacerbation.

In conclusion, the current study demonstrated a novel role for IFN-γ as a broad immune suppressive cytokine to suppress the production of all Th2-, Th1-, and Th17-type cytokines in the lung. IFN-γ induced an overall suppression of DC functions, which subsequently led to the suppression of Ag-induced immune responses of $CD4^+$ T cells. Therefore, delivery of IFN-γ to the lung could be a very effective strategy for regulating Ag-specific immune responses in the lung.

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Disclosures

The authors have no financial conflict of interest.

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