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

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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.

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llergic 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 eosinophilia (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 autoimmun eencephalomyelitis (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 eosinophilia (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) 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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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 EcoRI 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 mice 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 on day 17 (during aerosol challenge; in the effector phase). On day 21, spleen cells were obtained from the animals, 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.

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 × 105 cells/well) with OVA (20 μg/ml) in complete DMEM. In some experiments, positively selected CD4+ T cells (2 × 105 cells/well) were cultured with freshly isolated mitomycin C-treated (Sigma-Aldrich) splenocytes (2 × 105 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 acidicification.

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-2d) CD11c+ cells (1 × 105 or 1 × 104 cells/well) were cultured with positively selected C57BL/6 (H-2b) CD4+ T cells (2.5 × 105 cells/well) in complete DMEM. For the measurement of OVA Ag-presenting capacity, mitomycin C-treated CD11c+ cells (2.5 × 105 cells/well) or bronchial lymph node cells (2.5 × 105 cells/well) were cultured with DO11.10 CD4+ T cells (2.5 × 105 cells/well). In some experiments, CD11c+ cells (1 × 105 cells/well) from the spleen were cultured with OVA-sensitized CD4+ T cells (2.5 × 105 cells/well) and OVA (20 μg/ml). After 72 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 × 105 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, spleen 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 × 10⁵). 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 TRFK5) 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 hydrodynamics-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. 1A) and in BALF (Fig. 1B). 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.

**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. 2A). IFN-γ gene delivery almost completely diminished the infiltration of eosinophils (Fig. 2A). 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. 2B). In the mice that received pCAGGS IFN-γ, these findings strongly declined (Fig. 2B).

BALF IL-13 and TGF-β1 concentrations also decreased in the pCAGGS IFN-γ-treated mice (Fig. 2C). IFN-γ treatment significantly suppressed OVA-specific IgE and IgG production (Fig. 2D). The concentration of OVA-specific IgG1 in serum increased in the OVA-sensitized and -challenged mice, and IFN-γ treatment suppressed this increase (Fig. 2E). 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. 2E). 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 strongly suppressed the production of these cytokines (Fig. 2F). 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. 2G). 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. 3A) and produced IL-5, IFN-γ, and IL-17 (Fig. 3B) 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. 3A) or produce these cytokines (Fig. 3B), 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
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.
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. 4A) or TGF-β1 (data not shown) by whole spleen cells in response to OVA. CD4+ T cells from OVA-sensitized mice did not produce IL-10 (Fig. 4B) 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. 4C) 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 in vivo IFN-γ gene delivery did not induce the development of Treg cells.

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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. 6A) and OVA-presenting capacity (Fig. 6B). IFN-γ suppressed the production of IL-10 (Fig. 6C) and IFN-γ (Fig. 6D) by splenic DCs in response to LPS, whereas it did not affect the production of IL-12p70 (Fig. 6E) or IL-12p40 (Fig. 6F). 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. 6G). In contrast, IFN-γ treatment suppressed this migration (Fig. 6G). Moreover, IFN-γ strongly suppressed Ag-induced IL-5 production (Fig. 6H) or IL-5 secretion (Fig. 6I) 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. 6J), which was consistent with previous studies (42–44). Moreover, pre-treatment of DCs with IFN-γ attenuated eosinophilic inflammation induced by transfer of OVA-pulsed DCs and inhalation of OVA (Fig. 6K), 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.
infiltration of inflammatory cells in the peribronchial area and mucus hypersecretion (Fig. 7B). IFN-γ suppressed IL-13 and TGF-β1 concentrations in BALF (Fig. 7C), although it did not suppress OVA-specific IgE or IgG production (Fig. 7D). 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. 7E). 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. 7F). 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.
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. 8A) and OVA-presenting capacity of lung CD11c+ APCs (Fig. 8B). IFN-γ suppressed IL-10 production by lung CD11c+ APCs in response to LPS (Fig. 8C). It also suppressed the expression of MHC class II in lung CD11c+ APCs (Fig. 8D). In contrast, IL-12p70 or IL-12p40 production was not affected (Fig. 8E and F). Furthermore, IFN-γ suppressed the migration of lung APCs into the regional lymph nodes (Fig. 8G 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.
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 sensitization. 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. 2F). This result was confirmed with immunocytes obtained from bronchial lymph nodes ex vivo (Fig. 2G). In addition, IFN-γ significantly suppressed the production of all of the Th2-, Th17-, and Th1-type cytokines from whole spleen cells (Fig. 3B) or CD4+ T cells (Fig. 3D). 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. 4C). 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. 6F). 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. 6K). Moreover, in vivo IFN-γ gene delivery suppressed allogenic MLR (Fig. 6A) and OVA Ag-presenting capacity (Fig. 6B). 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. 6G) 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. 2E). 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 IgG2a 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. 1B). 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 per- fused 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 our study (57) vs 100–800 ng/ml in our study (Fig. 1A)). 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 airspace (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 thionovirus 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-, andTh17-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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The authors have no financial conflict of interest.

References
IFN-γ SUPPRESSES Ag-INDUCED OVERALL IMMUNE RESPONSE


