

## **Interferon $\gamma$ Regulates Antigen-induced Eosinophil Recruitment into the Mouse Airways by Inhibiting the Infiltration of CD4<sup>+</sup> T Cells**

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### **Summary**

We have previously shown that antigen-induced eosinophil recruitment into the tissue of sensitized mice is mediated by CD4<sup>+</sup> T cells and interleukin 5. To determine whether interferon  $\gamma$  (IFN- $\gamma$ ) regulates antigen-induced eosinophil recruitment into the tissue, we studied the effect of recombinant (r) murine IFN- $\gamma$  and of anti-IFN- $\gamma$  monoclonal antibody (mAb) on the eosinophil infiltration of the trachea induced by antigen inhalation in mice. The intraperitoneal administration of rIFN- $\gamma$  prevented antigen-induced eosinophil infiltration in the trachea of sensitized mice. The administration of rIFN- $\gamma$  also decreased antigen-induced CD4<sup>+</sup> T cell but not CD8<sup>+</sup> T cell infiltration in the trachea. On the other hand, pretreatment with anti-IFN- $\gamma$  mAb enhanced antigen-induced eosinophil and CD4<sup>+</sup> T cell infiltration in the trachea. These results indicate that IFN- $\gamma$  regulates antigen-induced eosinophil recruitment into the tissue by inhibiting CD4<sup>+</sup> T cell infiltration.

Eosinophil infiltrate is a characteristic feature of allergic inflammation such as asthma. In a murine model of airway late-phase reaction, we have previously shown that antigen-induced eosinophil recruitment into the tissue of sensitized mice is mediated by CD4<sup>+</sup> T cells and IL-5 (1). However, the mechanism that regulates antigen-induced eosinophil recruitment into the tissue is unknown. It has been suggested that CD4<sup>+</sup> Th cells can be divided into two subsets (Th1 and Th2 cells) on the basis of their different patterns of cytokine secretion (2); Th1 cells produce IL-2, IFN- $\gamma$ , and lymphotoxin, and Th2 cells produce IL-4, IL-5, and IL-6. Furthermore, IFN- $\gamma$  has been shown to be a major regulatory cytokine that inhibits the proliferation of Th2 cells in vitro (3, 4) and antagonizes the in vivo Th2-type responses such as IL-4-dependent IgE antibody production (5). Therefore, to determine whether IFN- $\gamma$  regulates antigen-induced eosinophil recruitment into the tissue, we studied the effect of recombinant murine IFN- $\gamma$  and of anti-IFN- $\gamma$  mAb on the eosinophil infiltration of the trachea induced by antigen inhalation in mice. Our results indicate that IFN- $\gamma$  regulates antigen-induced eosinophil recruitment into the tissue by inhibiting CD4<sup>+</sup> T cell infiltration.

### **Materials and Methods**

**Mice and Immunization.** 8-wk-old BALB/c mice (Charles River Laboratories, Shizuoka, Japan) were immunized intraperitoneally twice with 1  $\mu$ g of OVA (Sigma Chemical Co., St. Louis, MO) in 4 mg of aluminum hydroxide at a 2-wk interval. 10–14 d after

the second immunization, the sensitized mice were challenged with aerosolized OVA as described below.

**Antigen-induced Eosinophil Infiltration in Mouse Trachea.** The eosinophil infiltration into the trachea was induced by the inhalation of antigen in sensitized mice, and the number of eosinophils infiltrating into the submucosal tissue of trachea was evaluated as described previously (1). Briefly, the sensitized mice inhaled aerosolized OVA (50 mg/ml) dissolved in 0.9% saline by a nebulizer (646; DeVilbiss Corp., Somerset, PA) for 20 min. As a control, 0.9% saline alone was administered by the nebulizer. At various intervals after the inhalation, the mice were killed by cervical dislocation and the tracheas were excised. After the tracheas were fixed in 10% formalin, the specimens were embedded in paraffin, sectioned 3  $\mu$ m thick, and stained with Luna solution and hematoxylin-eosin solution. The number of eosinophils in the submucosal tissue of trachea was counted in Luna-stained sections and expressed as the number of eosinophils per the length of the basement membrane of trachea, which was measured with a digital curvimeter.

**Effect of Murine rIFN- $\gamma$ .** To determine whether IFN- $\gamma$  has a regulatory role in antigen-induced eosinophil recruitment into the tissue, we examined the effect of murine rIFN- $\gamma$  on antigen-induced eosinophil infiltration in the trachea of sensitized mice. OVA-sensitized mice were injected intraperitoneally with murine rIFN- $\gamma$  (300–30,000 U; Genentech, Inc., South San Francisco, CA) every 12 h from 1 d before the inhaled OVA challenge until 24 h after the challenge. As a control, OVA-sensitized mice were injected intraperitoneally with saline. The eosinophil infiltration into the trachea was evaluated at 9, 24, and 48 h after OVA inhalation.

**Effect of Anti-Murine IFN- $\gamma$  mAb** To determine whether endogenous IFN- $\gamma$  is involved in regulating antigen-induced eosinophil recruitment into the tissue, we examined the effect of anti-mu-

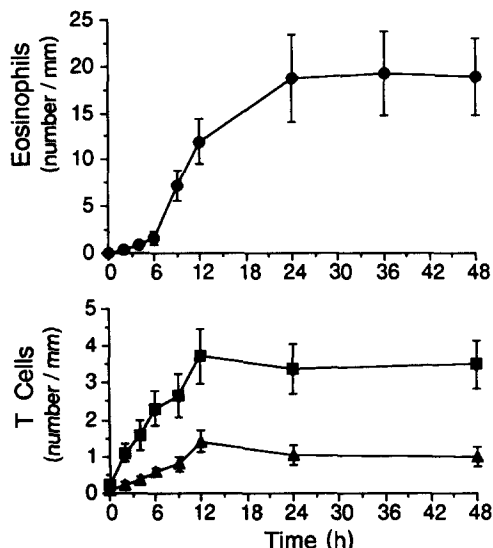
rine IFN- $\gamma$  mAb on antigen-induced eosinophil infiltration in the trachea of sensitized mice. OVA-sensitized mice were injected intraperitoneally once with 1 mg of rat anti-murine IFN- $\gamma$  mAb (R4-6A2) (6) 24 h before the inhaled OVA challenge. As a control, OVA-sensitized mice were injected intraperitoneally with purified rat IgG (1 mg) 24 h before the inhaled OVA challenge. The eosinophil infiltration into the trachea was evaluated at 9 and 24 h after OVA inhalation.

**Immunocytochemistry.** T cell infiltration into the trachea was assessed by direct staining with streptavidin-biotinylated antibody technique (7). Briefly, the trachea was removed and frozen with OCT compound (Miles Laboratories, Naperville, IL) in a liquid nitrogen bath. After acetone-fixed cryostat sections (3  $\mu$ m thick) were treated with normal rabbit serum, the sections were incubated with biotinylated anti-Thy-1.2, anti-L3T4, or anti-Lyt-2 mAbs (Becton Dickinson & Co., Mountain View, CA) at room temperature for 2 h. As a negative control, biotinylated normal rat IgG was used. Sections were then incubated with the streptavidin conjugated with horseradish peroxidase (Vector Laboratories, Burlingame, CA) at room temperature for 30 min, followed by the reaction with 3', 3'-diaminobenzidine in Tris-HCl buffer containing H<sub>2</sub>O<sub>2</sub> for 5 min. The immunostained cells were counted and expressed as described above.

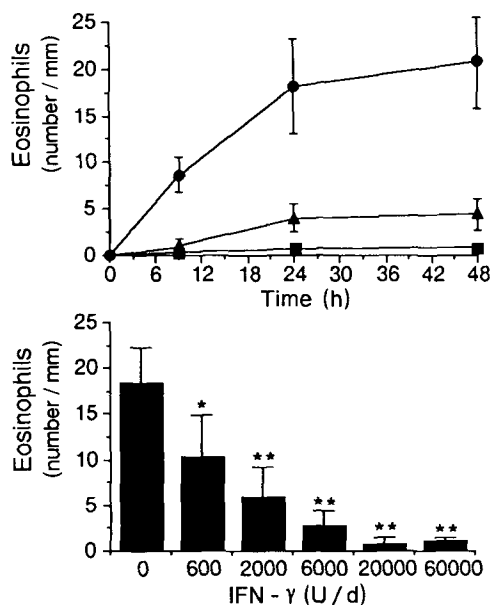
**Data Analysis.** Data are summarized as mean  $\pm$  SD. The statistical analysis of the results was performed by the analysis of variance using Fisher's least significant difference test for multiple comparisons. Values of  $p < 0.05$  were considered significant.

## Results

**Kinetics of Antigen-induced Eosinophil and T Cell Infiltration into the Mouse Trachea.** The inhalation of aerosolized OVA caused the infiltration of eosinophils into the trachea of OVA-sensitized mice. The eosinophil infiltration into the tracheal submucosa of OVA-sensitized BALB/c mice began at 6 h after



**Figure 1.** Time course of antigen-induced eosinophil and T cell infiltration into the mouse trachea. OVA-sensitized BALB/c mice were challenged with the inhalation of OVA, and the number of eosinophils and T cells (CD4<sup>+</sup> [■] and CD8<sup>+</sup> [▲]) infiltrating into the submucosal tissue of trachea was counted at various intervals after the inhalation. Data are means  $\pm$  SD for 10 mice at each time point.

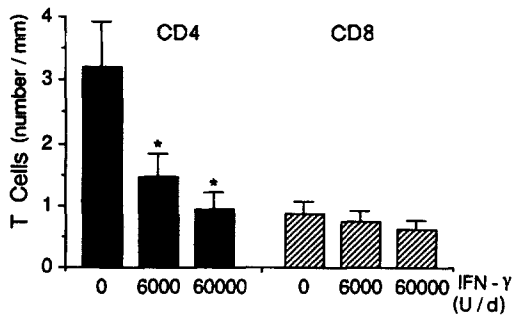


**Figure 2.** Effect of murine rIFN- $\gamma$  on antigen-induced eosinophil infiltration in mouse trachea. (Top) OVA-sensitized mice were injected intraperitoneally with murine rIFN- $\gamma$  or saline from 1 d before the inhaled OVA challenge until 24 h after the challenge (saline [●], 6,000 U/d [▲], and 60,000 U/d [■];  $n = 5-6$  mice in each group). The mean values in the treated group are significantly different from the mean value of the corresponding control response (saline),  $p < 0.001$ . (Bottom) The dose-dependent effect of rIFN- $\gamma$  (600–60,000 U/d) on the eosinophil infiltration was examined at 24 h ( $n = 5$  mice in each group). (\* and \*\*) Significantly different from the mean value of the control response (saline): \* $p < 0.01$ ; \*\* $p < 0.001$ .

OVA inhalation, increased at 9–24 h, and then reached a plateau ( $n = 10$  mice at each time point) (Fig. 1). The antigen-induced eosinophil recruitment into the mouse trachea was shown to be mediated by CD4<sup>+</sup> T cells and IL-5 in our previous study (1).

CD4<sup>+</sup> and CD8<sup>+</sup> T cell infiltration into the trachea also occurred 2 h after OVA inhalation and increased to reach a peak at 12 h ( $n = 10$  mice at each time point) (Fig. 1). The majority of T cell infiltrates (70–75%) were CD4<sup>+</sup> T cells (Fig. 1).

**Effect of IFN- $\gamma$  on Antigen-induced Eosinophil Infiltration into the Trachea.** IFN- $\gamma$  prevented antigen-induced eosinophil recruitment into the mouse trachea. The intraperitoneal preinjection with rIFN- $\gamma$  (6,000 U/d) from 1 d before the inhaled OVA challenge significantly decreased OVA-induced eosinophil infiltration into the trachea of OVA-sensitized mice at 9–48 h by 78–87% (control [18.2  $\pm$  4.9] vs. rIFN- $\gamma$  [4.0  $\pm$  1.6] eosinophils/mm at 24 h, mean  $\pm$  SD;  $n = 5$ ,  $p < 0.001$ ) (Fig. 2). The preinjection with rIFN- $\gamma$  (60,000 U/d) also decreased the OVA-induced eosinophil infiltration at 9–48 h by 96–97% (0.6  $\pm$  0.4 eosinophils/mm at 24 h;  $n = 6$ ,  $p < 0.001$ ) (Fig. 2). In addition, a single intraperitoneal administration of rIFN- $\gamma$  (30,000 U) 2 h before OVA inhalation also significantly decreased the OVA-induced eosinophil infiltration at 24 h by 68% (control [17.0  $\pm$  3.4] vs. rIFN- $\gamma$  [5.5  $\pm$  2.9] eosinophils/mm;  $n = 10$ ,  $p < 0.001$ ).



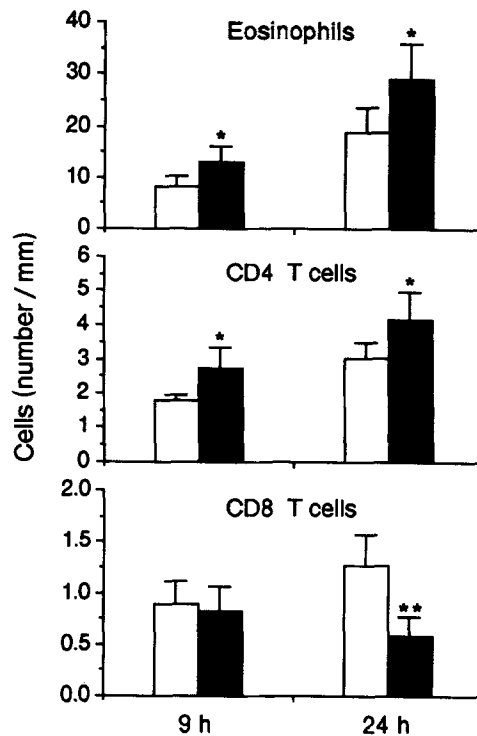
**Figure 3.** Effect of murine rIFN- $\gamma$  on antigen-induced T cell infiltration in mouse trachea. CD4<sup>+</sup> and CD8<sup>+</sup> T cell infiltration in the trachea was examined at 24 h ( $n = 10$  mice in each group). (\*) Significantly different from the mean value of the control response (saline); \* $p < 0.001$ .

The dose-dependent effect of rIFN- $\gamma$  on antigen-induced eosinophil infiltration of the trachea was then examined. The intraperitoneal preinjections with rIFN- $\gamma$  (600–60,000 U/d) from 1 d before OVA inhalation decreased the OVA-induced eosinophil infiltration at 24 h in a dose-dependent manner with a significant minimum effect at 600 U/d ( $n = 5$ ,  $p < 0.01$ ) and an almost complete inhibition at  $>20,000$  U/d ( $n = 5$ ,  $p < 0.001$ ) (Fig. 2). However, the preinjections with rIFN- $\gamma$  (600–60,000 U/d) did not significantly affect blood eosinophil counts at 24 h compared with those of control mice (data not shown).

**Effect of IFN- $\gamma$  on Antigen-induced T Cell Infiltration into the Trachea.** IFN- $\gamma$  decreased antigen-induced CD4<sup>+</sup> T cell infiltration into the mouse trachea. The intraperitoneal preinjection with rIFN- $\gamma$  (6,000 and 60,000 U/d) from 1 d before OVA inhalation significantly decreased OVA-induced CD4<sup>+</sup> T cell infiltration in the trachea of OVA-sensitized mice at 24 h by 54 and 70%, respectively ( $n = 10$ ,  $p < 0.001$ ) (Fig. 3). In contrast, the preinjection with rIFN- $\gamma$  did not significantly affect CD8<sup>+</sup> T cell infiltration into the trachea ( $n = 10$ ) (Fig. 3). In addition, there were no significant changes in CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets of spleen cells by FACS<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA) analysis after the intraperitoneal administration of rIFN- $\gamma$  (60,000 U/d) (data not shown).

**Effect of Anti-IFN- $\gamma$  mAb** Pretreatment with anti-IFN- $\gamma$  mAb (increased antigen-induced eosinophil recruitment into the mouse trachea. The intraperitoneal preinjection with anti-murine IFN- $\gamma$  mAb (R4-6A2; 1 mg) 24 h before the inhaled OVA challenge significantly increased OVA-induced eosinophil infiltration into the mouse trachea at 9 h by 60% (control rat IgG [ $8.1 \pm 2.1$ ] vs. anti-IFN- $\gamma$  mAb [ $12.9 \pm 3.3$ ] eosinophils/mm;  $n = 10$ ,  $p < 0.05$ ) (Fig. 4). The preinjection with anti-IFN- $\gamma$  mAb also increased the OVA-induced eosinophil infiltration at 24 h by 54% (control rat IgG [ $18.9 \pm 4.7$ ] vs. anti-IFN- $\gamma$  mAb [ $29.0 \pm 6.9$ ] eosinophils/mm;  $n = 10$ ,  $p < 0.05$ ) (Fig. 4).

The preinjection with anti-IFN- $\gamma$  mAb increased OVA-induced CD4<sup>+</sup> T cell infiltration in the trachea at 9 and 24 h by 53 and 38%, respectively ( $n = 6$ ,  $p < 0.05$ ) (Fig. 4). In contrast, the preinjection with anti-IFN- $\gamma$  mAb de-



**Figure 4.** Effect of anti-murine IFN- $\gamma$  mAb on antigen-induced eosinophil and T cell infiltration in mouse trachea. OVA-sensitized mice were injected intraperitoneally with anti-murine IFN- $\gamma$  mAb R4-6A2 (■) or rat IgG (□) 24 h before OVA inhalation, and the eosinophil and T cell infiltration in the trachea was examined at 9 and 24 h ( $n = 6$ –10 mice in each group). (\* and \*\*) Significantly different from the mean value of the corresponding control response (rat IgG); \* $p < 0.05$ ; \*\* $p < 0.005$ .

creased the OVA-induced CD8<sup>+</sup> T cell infiltration at 24 h by 54% ( $n = 6$ ,  $p < 0.005$ ) (Fig. 4).

## Discussion

We have previously shown that antigen-induced eosinophil recruitment into the airways of sensitized mice is mediated by CD4<sup>+</sup> T cells and their cytokine IL-5 (1). In this study, we show that IFN- $\gamma$  regulates antigen-induced eosinophil recruitment into the tissue by inhibiting CD4<sup>+</sup> T cell infiltration. We found that the administration of murine rIFN- $\gamma$  efficiently prevented the eosinophil infiltration induced by antigen inhalation in the trachea of sensitized mice (Fig. 2), and that the inhibition of the eosinophil infiltration was associated with the decrement of CD4<sup>+</sup> T cell but not CD8<sup>+</sup> T cell infiltration (Fig. 3). However, there were no significant changes in the number of blood eosinophils nor of splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the treatment with rIFN- $\gamma$ . On the other hand, we also found that the administration of anti-murine IFN- $\gamma$  mAb enhanced the antigen-induced eosinophil and CD4<sup>+</sup> T cell infiltration in the trachea (Fig. 4). Taken together with the results of a previous study (1), these results suggest that IFN- $\gamma$  prevents antigen-induced CD4<sup>+</sup> T cell infiltration into the tissue and thereby decreases the following CD4<sup>+</sup> T cell and IL-5-dependent eosinophil recruitment into the tissue.

IFN- $\gamma$  has been shown to inhibit Th2 cell proliferation in vitro (3, 4) and antagonize Th2-type responses in vivo (5, 8, 9). It has also been suggested that the inhibitory effect of IFN- $\gamma$  on the in vivo Th2-type responses occurs at the time of initial CD4<sup>+</sup> T cell activation during the antigen presentation (9, 10). Therefore, it is possible that the inhibitory effect of IFN- $\gamma$  (both exogenous rIFN- $\gamma$  and endogenous IFN- $\gamma$ ) on antigen-induced CD4<sup>+</sup> T cell infiltration into the trachea of sensitized mice might be due to the interference of initial Th2 cell activation by the presence of IFN- $\gamma$  at the time of antigen inhalation.

IFN- $\gamma$  has also been shown to activate vascular endothelial cells to increase the expression of adhesion molecules (11) through which the adhesion of eosinophils to endothelial cells is increased (12). Therefore, it seems unlikely that the direct effect of IFN- $\gamma$  on vascular endothelial cells is involved in the inhibition of antigen-induced eosinophil infiltration into

the tissue. It is also unlikely that IFN- $\gamma$  directly acts on eosinophils and thereby inhibits the antigen-induced IL-5-dependent eosinophil infiltration, because it has been demonstrated that IFN- $\gamma$  is an activator for eosinophils to prolong the survival and enhance the cytotoxicity (13).

Our finding that rIFN- $\gamma$  effectively prevents antigen-induced eosinophil recruitment into the tissue suggests that rIFN- $\gamma$  would be useful for the treatment of atopic diseases such as asthma and atopic dermatitis. Because asthma is characterized by allergic eosinophil infiltration in the airways, treatment with rIFN- $\gamma$  would reduce airway eosinophil infiltration in asthma. Indeed, it has been reported that treatment with rIFN- $\gamma$  decreases cutaneous lesions of atopic dermatitis (14). In summary, we have shown that IFN- $\gamma$  regulates antigen-induced eosinophil recruitment into the tissue, indicating that IFN- $\gamma$  is a potent modulator of allergic late-phase reaction.

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