

Original Article

Low-dose exogenous interleukin (IL)-12 enhances antigen-induced interferon- γ production without affecting IL-10 production in asthmatics

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ABSTRACT

Background: Although interleukin (IL)-12, IL-10 and interferon (IFN)- γ are key cytokines that control the T helper (Th) 1/Th2 balance in human allergic disorders, details of their interactions in humans have not been clarified. Recently IL-10, one of the Th2 cytokines, has been shown to have an anti-inflammatory effect against allergic responses. To clarify the effect of IL-12 on the production of IFN- γ and IL-10, in the present study we examined responses of peripheral blood mononuclear cells (PBMC) from asthmatics to stimulation by *Dermatophagoides farinae* (Df) antigen in the presence of exogenous IL-12.

Methods: Peripheral blood mononuclear cells of Df-sensitized ($n = 7$) and non-sensitized ($n = 5$) asthmatics were stimulated by Df antigen after incubation with exogenous IL-12 (100 pg/mL). Interferon- γ and IL-10 produced in culture supernatants were measured by ELISA. The effect of IL-12 on lymphocyte proliferation was assayed by [³H]-thymidine incorporation in both groups.

Results: The production of IFN- γ by PBMC was significantly enhanced by incubation with IL-12 in both patient groups ($P < 0.05$), while IL-12 did not affect

the production of IL-10 in either group. Lymphocyte proliferation induced by Df antigen was significantly higher in the Df-sensitized group ($P < 0.01$). This lymphocyte proliferation was significantly enhanced by exogenous IL-12 only in the Df-sensitized group ($P < 0.05$).

Conclusions: These observations indicate that IL-12 enhances Th1-shifted immune responses without affecting IL-10 production and suggest that IL-12 may effectively inhibit the Th2 dominant state of bronchial asthma by regulating the Th1/Th2 balance.

Key words: bronchial asthma, *Dermatophagoides farinae*, interferon- γ , interleukin-10, interleukin-12, peripheral blood mononuclear cells.

INTRODUCTION

Allergic inflammation is mainly mediated by CD4⁺ T lymphocytes producing T helper (Th) 2-type cytokines, such as interleukin (IL)-4 and IL-5. Because both mRNA and protein levels of these cytokines are elevated in bronchial wall and bronchoalveolar lavage (BAL) cells in asthmatics,¹ bronchial asthma is thought to be caused by deviation to a Th2 dominant immune response. Interleukin-4 is an essential factor for IgE switching and enhances inappropriate IgE synthesis.² Interleukin-5 has selective biological effects on eosinophils and their precursors and promotes accumulation of eosinophils into the bronchial mucosa in asthmatics.³ In contrast, allergic inflammation is inhibited by interferon (IFN)- γ , which is one of the Th1 cytokines, and IFN- γ has been shown to

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Received 29 February 2000. Accepted for publication 27 November 2000.

suppress both IgE class switching and the proliferation of Th2 cells.^{4,5}

Recently, IL-12, which is a heterodimeric cytokine primarily produced by monocytes and macrophages,⁶ has been shown to be a key cytokine modulating differentiation of Th0 cells to Th1 cells.⁷ Because IL-12 is a potent inducer of IFN- γ ^{8,9} and a suppressor of IL-4, IL-5 and IgE,^{10–13} an immunomodulatory role of IL-12 has been investigated in allergic animal models. Interleukin-12 inhibited antigen-induced airway hyperresponsiveness and eosinophil recruitment into the airway mucosa in murine models.^{14–17} Therefore, IL-12 may have a beneficial role in the treatment of bronchial asthma by suppressing Th2 responses.

Interleukin-10 was initially characterized as a Th2 cytokine that could inhibit IFN- γ production and suppress Th1 cell development in murine systems,^{18,19} but recent studies have shown that IL-10 also has potent anti-inflammatory activities in immune responses.^{20–24} Moreover, BAL fluid (BALF) from asthmatics contained less IL-10 than BALF from normal subjects.²⁵ Thus, IL-10 has the potential for preventing allergic inflammation and suppression of IL-10 may cause exacerbation of allergic diseases. The effect of IL-12 on the production of IL-10 is still controversial.^{26,27} Accordingly, to elucidate the immunomodulatory effect of IL-12 on the Th1/Th2 balance in allergic disorders, we investigated antigen-induced lymphocyte proliferation and production of IFN- γ and IL-10 in the presence of IL-12 in asthmatics sensitized by *Dermatophagoides farinae* (Df). We found that IL-12 enhanced IFN- γ production in Df-sensitized and non-sensitized patients and that Df-antigen-induced lymphocyte proliferation was enhanced by exogenous IL-12 in Df-sensitized patients.

METHODS

Subjects

We studied 12 adult patients with mild or moderate asthma, who met the criteria of the Japanese Society of Allergology and the American Thoracic Society,²⁸ and also five healthy subjects (median age 42 years; age range 38–50 years). None of the patients required regular oral steroid medications. The patient group included seven Df-sensitized asthmatics (median age 41 years; age range 25–56 years) and five who were Df-non-sensitized asthmatics (median age 50 years; age range 39–64 years). The Df-sensitized asthmatics were characterized by positive immediate skin reactions

(positive response ≥ 10 mm mean diameter) to dust mite antigen (Torii Pharmaceuticals, Tokyo, Japan) and positive scores for the radioallergosorbent test (RAST) to Df antigen. A RAST score > 1 was considered to be positive on a scale of 0–4. The Df-non-sensitized asthmatics were defined as those negative for immediate skin reactions and RAST. Peripheral blood was collected when there were no symptoms of asthma. Informed consent was obtained from all patients prior to their participation in this study.

Cell preparation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation on Histopaque (Sigma Chemical Co., St Louis, MO, USA). Cell viability was $> 98\%$, as determined by Trypan blue dye exclusion. Peripheral blood mononuclear cells were resuspended in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS), glutamine (2 mmol/L), penicillin (100 units/mL) and streptomycin (100 μ g/mL).

Antigen

Crude Df extract was provided by Torii Pharmaceuticals. The Df antigen was used at a concentration of 10 μ g/mL, which was found to be an optimal concentration for the induction of PBMC proliferation and cytokine production in preliminary experiments (data not shown) and is also based on a concentration used in a previous report.²⁹

Proliferation assay

Peripheral blood mononuclear cells (1×10^5 /well) were cultured in triplicate in 96-well flat-bottomed plates (Corning; Costar, Corning, NY, USA) in medium alone or with Df antigen in the presence or absence of IL-12 (100 pg/mL; Genetics Institute, Cambridge, MA, USA) at 37°C in a humidified atmosphere with 5% CO₂ in air. After 3 days culture, PBMC were pulsed with 37 kBq [³H]-thymidine (Amersham Japan, Tokyo, Japan) to each well. Eighteen hours later, cells were harvested and their filter-associated radioactivity was determined with a liquid scintillation counter. The incubation time used in the present study was based on that in a previous report.²⁷ Proliferative responses were evaluated with a stimulation index (SI), which was calculated as follows:

$$SI = (\text{mean c.p.m. of the test culture} / \text{mean c.p.m. of the control culture})$$

Cytokine production

To study the effect of exogenous IL-12 on Df antigen-induced production of IFN- γ and IL-10, PBMC were incubated with or without Df antigen (10 μ g/mL) in 24-well culture plates in the presence or absence of recombinant human IL-12. Fifteen minutes before the addition of Df antigen, IL-12 was added to cultures at a final concentration of 100 pg/mL. To rule out the effect of endogenous IL-12, a neutralizing test was also performed with an anti-IL-12 monoclonal antibody (mAb; R&D Systems, Minneapolis, MN, USA). According to the application note, the Neutralization Dose₅₀ (ND₅₀) for this anti-IL-12 mAb has been determined to be approximately 0.3–0.9 μ g/mL, using the human phytohemagglutinin-activated PBMC proliferation assay. An anti-IL-12 antibody was added, with the final concentration ranging from 0.1 to 10 μ g/mL. Supernatants were collected after 48 h incubation and were kept at -70°C until measurement of cytokines. The production of IFN- γ and IL-10 in the supernatants gradually increased for up to 48 h, and then plateaued. Concentrations of IFN- γ and IL-10 were determined by ELISA assays (Endogen, Boston, MA, USA). Detection limits for IL-10 and IFN- γ were 2 and 3 pg/mL, respectively.

Statistical analysis

All results are presented as the mean $\pm$ SEM. Data were analyzed statistically by using two-tailed Student's *t*-test or

paired *t*-test. Significant differences were indicated when $P < 0.05$.

RESULTS

Production of IFN- γ and IL-10 from Df antigen-stimulated PBMC

Because IL-12 could be detected in the supernatant of Df antigen-stimulated PBMC of Df-sensitized asthmatics in preliminary experiments at concentrations less than 20 pg/mL (data not shown) and high concentrations of IL-12 were shown to cause serious toxicity in a previous report,³⁰ IL-12 was added at concentrations ranging from 0.1 to 100 pg/mL. The results showed that IFN- γ production from Df antigen-stimulated PBMC was affected by the addition of IL-12 in a dose-dependent manner (Fig. 1). However, IL-10 production was not affected by exogenous IL-12. From these observations, the concentration of IL-12 used was set at 100 pg/mL in the following experiments.

Proliferative responses induced by Df antigen

The Df-sensitized asthmatics showed higher proliferative responses to Df antigen than non-sensitized asthmatics when responses were compared by SI (1.80 ± 0.50 vs 0.94 ± 0.52 , respectively; $P < 0.01$; Fig. 2). The SI value for healthy subjects was 1.03 ± 0.21 ($n = 5$; data not shown).

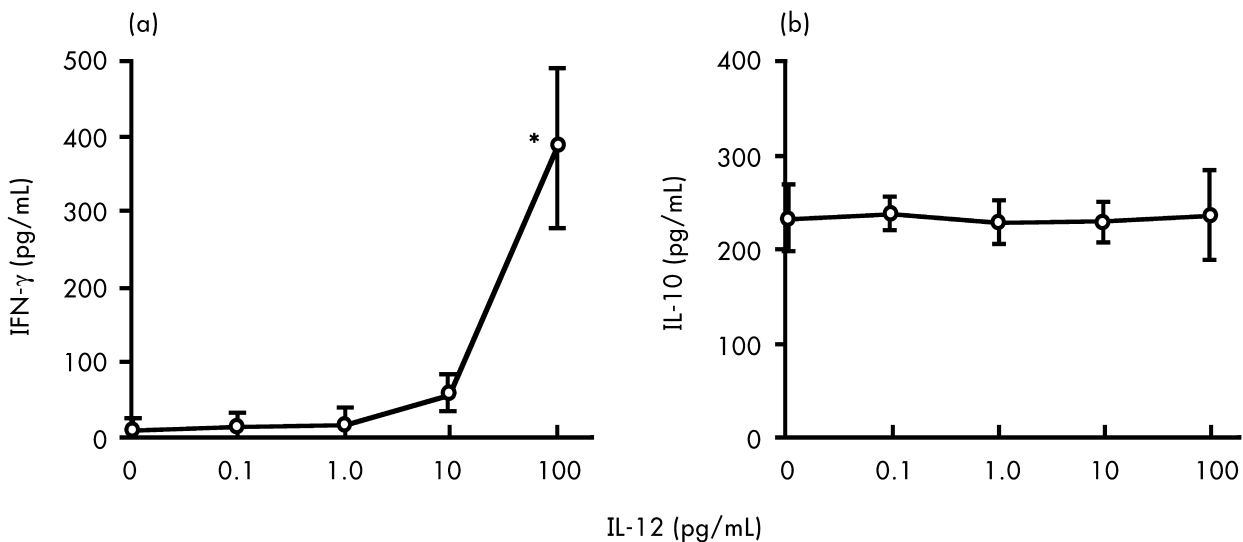


Fig. 1 (a) Interferon (IFN)- γ production and (b) interleukin (IL)-10 production from peripheral blood mononuclear cells of asthmatics incubated with 10 μ g/mL *Dermatophagoides farinae* (Df) antigen for 48 h. Data are the mean $\pm$ SEM of three experiments. * $P < 0.05$ compared with control.

Effect of IL-12 on the production of IFN- γ and IL-10

The concentration of IFN- γ produced by Df-stimulated PBMC from Df-sensitized asthmatics in the presence or absence of IL-12 was 755.16 ± 261.70 and 88.86

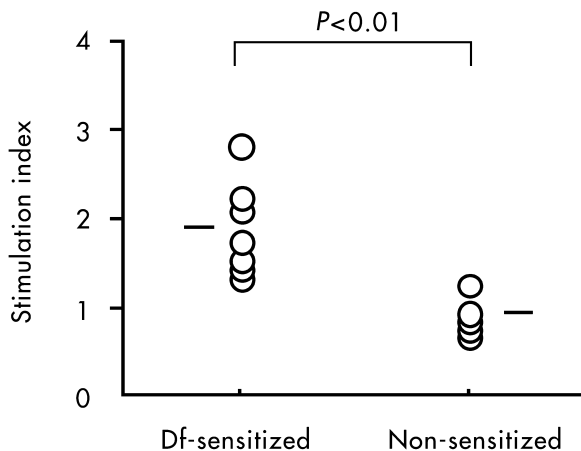


Fig. 2 Proliferative response of peripheral blood mononuclear cells (PBMC) from *Dermatophagoides farinae* (Df)-sensitized and non-sensitized asthmatics. The PBMC were incubated for 3 days with $10 \mu\text{g/mL}$ Df antigen and were then pulsed with [^3H]-thymidine and harvested after 18 h of additional culture. Data are expressed as stimulation index (SI). Each line represents the change in individual donors. The mean of the data in each category is indicated by the horizontal line.

$\pm 47.26 \text{ pg/mL}$, respectively. Similarly, in non-sensitized asthmatics, the concentration of IFN- γ produced was higher in the presence than the absence of IL-12 (555.50 ± 258.03 vs $17.06 \pm 8.64 \text{ pg/mL}$, respectively). In healthy subjects, the concentration of IFN- γ produced in the presence or absence of IL-12 was 532.02 ± 89.75 and $11.93 \pm 3.98 \text{ pg/mL}$, respectively (Fig. 3a). Although values fluctuated considerably from case to case in all groups, the differences in cytokine production were significantly different between the presence and absence of IL-12 ($P < 0.05$). The production of IL-10 by Df-stimulated PBMC was not significantly affected by 100 pg/mL IL-12 for all Df-sensitized asthmatics (131.15 ± 21.5 vs $126.79 \pm 26.17 \text{ pg/mL}$ for the absence vs presence of IL-12, respectively), non-sensitized asthmatics (96.03 ± 19.00 vs $109.21 \pm 27.44 \text{ pg/mL}$ for the absence vs presence of IL-12, respectively) and healthy subjects (176.95 ± 20.87 vs $178.98 \pm 33.34 \text{ pg/mL}$ for the absence vs presence of IL-12, respectively; Fig. 3b). The production of IFN- γ and IL-10 under in the presence of IL-12 without Df antigen stimulation showed lower concentrations of cytokines produced compared with production following stimulation of PBMC with Df antigen. The production of IFN- γ in Df-sensitized asthmatics, non-sensitized asthmatics and healthy subjects was 10.73 ± 3.14 , 5.13 ± 1.70 and $2.71 \pm 1.18 \text{ pg/mL}$, respectively, while the production of IL-10 in

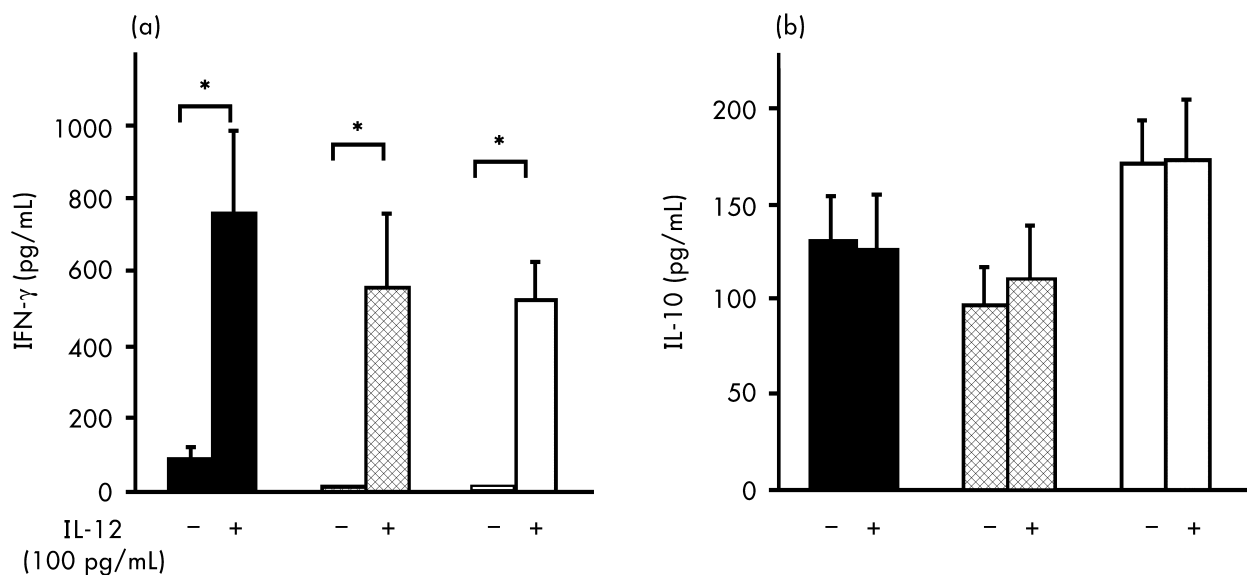


Fig. 3 (a) Interferon (IFN)- γ production and (b) interleukin (IL)-10 production from peripheral blood mononuclear cells of *Dermatophagoides farinae* (Df)-sensitized (■; $n = 7$) and non-sensitized (▨; $n = 5$) asthmatics stimulated with $10 \mu\text{g/mL}$ Df antigen in the presence or absence of 100 pg/mL IL-12. (□), healthy subjects. Data are the mean $\pm$ SEM. * $P < 0.05$.

these same groups was 9.99 ± 2.63 , 6.70 ± 1.55 and 9.40 ± 2.37 pg/mL, respectively.

Effect of IL-12 on proliferative responses

Proliferative responses of PBMC stimulated by Df antigen in Df-sensitized asthmatics in the absence or presence of IL-12 were 1.80 ± 0.50 and 2.22 ± 0.21 SI, respectively. Exogenous IL-12 significantly enhanced proliferation of PBMC in Df-sensitized asthmatics ($P < 0.05$; Fig. 4a). However, in non-sensitized asthmatics, proliferative responses of PBMC in the absence or presence of IL-12 were 0.94 ± 0.52 and 1.32 ± 0.16 SI, respectively, and there was no significant difference between these two groups (Fig. 4b).

Neutralizing test of IL-12

To rule out the effect of endogenous IL-12, we performed a neutralizing test by adding an anti-IL-12 antibody to cultures examining the production of IFN- γ or IL-10 by Df-stimulated PBMC. The production of IFN- γ by PBMC stimulated with Df antigen in the presence of IL-12 was inhibited by the addition of an anti-IL-12 antibody in a dose-dependent manner (Fig. 5). At a concentration of $10 \mu\text{g/mL}$, the anti-IL-12 antibody inhibited IFN- γ production almost completely ($> 95\%$ inhibition). The production of IFN- γ by Df stimulated PBMC in the

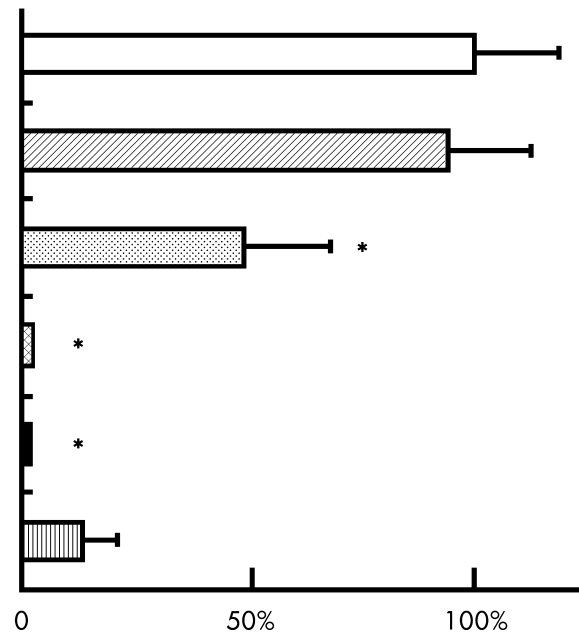


Fig. 5 Neutralizing test for interleukin (IL)-12 using anti-IL-12 antibody for interferon (IFN)- γ production. Peripheral blood mononuclear cells from *Dermatophagoides farinae* (Df)-sensitized asthmatics were incubated with Df antigen and/or 100 pg/mL of IL-12 and/or three different concentrations of anti-IL-12 antibody (Ab) for 48 h. Data are the mean $\pm$ SEM of three experiments. * $P < 0.05$ compared with the group incubated with Df and IL-12 ($\square$). ($\square$), Df + IL-12 + $0.1 \mu\text{g/mL}$ $\alpha\text{IL-12Ab}$; ($\square$), Df + IL-12 + $1.0 \mu\text{g/mL}$ $\alpha\text{IL-12Ab}$; ($\square$), Df + IL-12 + $10 \mu\text{g/mL}$ $\alpha\text{IL-12Ab}$; ($\blacksquare$), Df + $10 \mu\text{g/mL}$ $\alpha\text{IL-12Ab}$; ($\square$), Df.

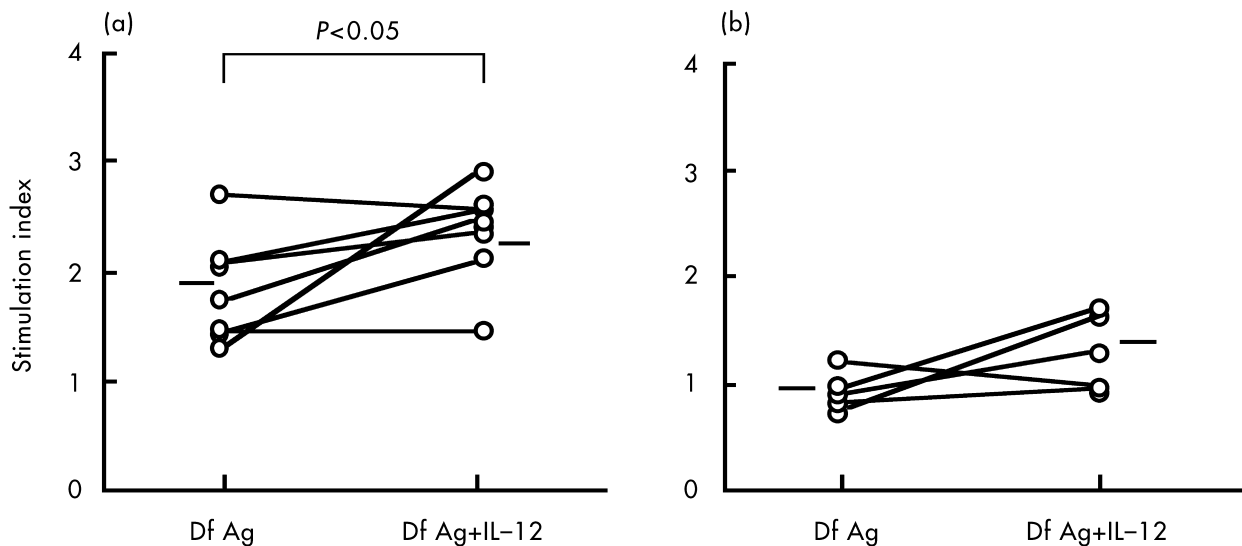


Fig. 4 Proliferative response of peripheral blood mononuclear cells (PBMC) from (a) *Dermatophagoides farinae* (Df)-sensitized ($n = 7$) and (b) non-sensitized ($n = 5$) asthmatics. The PBMC were incubated for 3 days with $10 \mu\text{g/mL}$ Df antigen (Ag) in the presence or absence of 100 pg/mL interleukin (IL)-12. Cells were pulsed with [^3H]-thymidine and harvested after 18 h of additional culture. Results are expressed as stimulation index (SI).

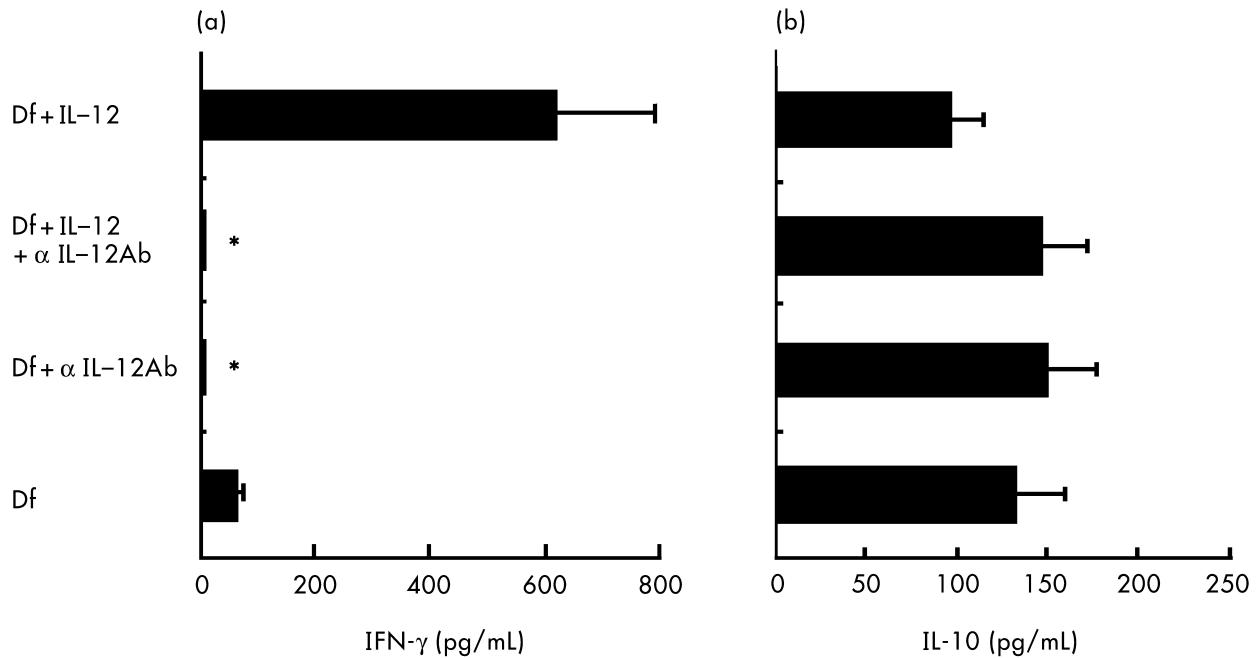


Fig. 6 Neutralizing test for interleukin (IL)-12 on the production of (a) interferon (IFN)- γ and (b) IL-10. Peripheral blood mononuclear cells from *Dermatophagoides farinae* (Df)-sensitized asthmatics were incubated with Df antigen and/or 100 pg/mL of IL-12 and/or 10 μ g/mL anti-IL-12 antibody (Ab) for 48 h. Data are the mean $\pm$ SEM of five experiments. * $P < 0.05$ compared with the group incubated with Df and IL-12.

absence or presence of anti-IL-12 antibody was 62.9 ± 28.0 and 14.0 ± 3.1 pg/mL, respectively. Anti-IL-12 antibody could inhibit IFN- γ production to the same degree (13.4 ± 4.7 pg/mL) in the presence of IL-12. Because 10 μ g/mL anti-IL-12 antibody neutralized exogenous IL-12 and completely inhibited IFN- γ production, IFN- γ production was largely induced by exogenous IL-12 in this system (Fig. 6a). Although IL-10 production from Df antigen-stimulated PBMC showed a tendency to decrease in the presence of IL-12 and to be increased in the presence of an anti-IL-12 antibody, the difference between these two conditions was not significant. The production of IL-10 by Df antigen-stimulated PBMC was not significantly affected by neutralizing both endogenous and exogenous IL-12 (Fig. 6b).

DISCUSSION

In the present study, we examined the effect of IL-12 on PBMC stimulated by Df antigen in both Df-sensitized and non-sensitized asthmatics. We demonstrated that Df-sensitized asthmatics showed greater lymphocyte proliferation in response to Df antigen compared with non-sensitized asthmatics. Interleukin-12 significantly enhanced not only lymphocyte proliferation, but also

IFN- γ production when PBMC were stimulated with Df antigen in Df-sensitized asthmatics. However, in non-sensitized asthmatics, IL-12 enhanced only IFN- γ production and failed to enhance lymphocyte proliferation. These results suggest that IL-12 can induce both resting and activated T cells to produce IFN- γ , while enhanced lymphocyte proliferation was observed only in activated T cells in sensitized asthmatics. Previous reports have shown that IL-12 enhances proliferation of PBMC or lymphocytes when they are activated by antigen.^{31–33} Recent evidence indicates that only activated T cells express receptors for IL-12³⁴ and antigen stimulation induces upregulation of IL-12 receptors on T cells, which allows an increased proliferative response to IL-12.³⁵ These data could be the reason why, in the present study, lymphocytes of Df-sensitized asthmatics stimulated by Df antigen showed a higher proliferation response in the presence of IL-12 compared with non-sensitized asthmatics. Furthermore, IL-12 did not increase lymphocyte proliferation in non-sensitized asthmatics, whereas it did increase IFN- γ production from PBMC. This dissociation between lymphocyte proliferation and IFN- γ production could not be explained by the regulation of the IL-12 receptor. However, because we showed that IFN- γ production under conditions in which the PBMC were not

stimulated by Df antigen in the presence of IL-12 were apparently lower than those when PBMC were stimulated by Df antigen, antigen stimulation was indispensable for the production of IFN- γ . This result indicates that the main source of IFN- γ may be activated T cells. Taken together, our data suggest that IL-12 may induce a potent Th1-type immune reaction in both Df-sensitized and non-sensitized asthmatics and, after that, it may regulate Df antigen-induced Th2-type immune responses.

In a murine system, intraperitoneal administration of IFN- γ has been shown to decrease antigen-induced CD4⁺ T cells and to prevent eosinophil infiltration into tracheal mucosa.³⁶ This observation indicates that IFN- γ inhibits antigen-induced eosinophil recruitment into tissue by inhibiting Th2 cell infiltration. Other murine studies have shown that intraperitoneal administration of IL-12 suppresses airway eosinophilia¹⁴ and hyper-responsiveness^{15,37} without affecting IgE production.¹⁶ Furthermore, intranasal IL-12 administration has been reported to suppress eosinophil infiltration into the lung and airway hyperresponsiveness.¹⁷ Another *in vivo* study has shown that successful immunotherapy increases IL-12 mRNA-positive cells at allergen-challenged sites.³⁸ Moreover, reduced production of IL-12 or lack of IL-12 signaling in allergen-specific Th2 cells has been observed in allergic patients.^{39,40} Because the inhibitory effect of IFN- γ on Th2 cells is enhanced by IL-12, it may be useful in suppressing allergic inflammation.^{4,5} Thus, exogenous IL-12 could play a therapeutic role in the treatment of bronchial asthma.

Previously, IL-10 was recognized as a Th2 cytokine^{18,19} because it inhibited Th1-induced delayed-type hypersensitivity⁴¹ and exacerbated Th2-type allergic inflammation.⁴² Recently, an anti-inflammatory function of IL-10 has been demonstrated in immune responses.^{20–23} Interleukin-10 inhibited cytokine generation from mast cells⁴³ and both survival and cytokine production of eosinophils.⁴⁴ It was also shown that IL-10 induced an antigen-specific anergic state in CD4⁺ T cells.²³ Successful antigen-specific immunotherapy upregulated IL-10 production in bee venom allergy and caused antigen-specific anergy in peripheral T cells.⁴⁵ Moreover, BALF from asthmatics showed that constitutive production of IL-10 was diminished in asthmatics compared with normal controls;²⁵ inhaled corticosteroids increased IL-10 production by alveolar macrophages in asthmatics.⁴⁶ Because impaired IL-10 production permits the release of proinflammatory cytokines and leads to the development of bronchial inflammation in asthmatics, IL-10 may retain

the potential to reduce allergic bronchial inflammation.⁴⁷ We have shown that IL-12, at a dose of 100 pg/mL, did not affect IL-10 production in either Df-sensitized or non-sensitized asthmatics. This concentration of IL-12 was 10–100-fold lower than those used in previous studies. In the present study, we used preferentially lower doses of IL-12 because high doses of IL-12 have been reported to exert severe toxicities in an *in vivo* study.³⁰ The results of an IL-12 neutralizing test indicated that endogenous IL-12 was negligible and 100 pg/mL exogenous IL-12 was enough to regulate the production of IFN- γ . In addition, in the present study, the concentrations of IL-10 in asthmatic groups were lower than those in healthy subjects. This result indicates the possibility that lower production of IL-10 may induce prolonged allergic inflammation in asthmatic groups.

Regulation of IL-10 by IL-12 has been examined by other investigators and their results were different from ours because of different methods used or study conditions. Several studies have shown that IL-12 inhibits the production of IL-10,^{27,48} while others have found that IL-12 induces IL-10 production by T cells.^{26,49,50} These studies were performed with T cell lines or T cell clones, which aimed to clarify the immunologic functions of T cells in the presence of IL-12. To evaluate the whole immunologic reaction of immunocomponent cells, including T cells and antigen-presenting cells, we examined lymphocyte proliferation and the production of cytokines in a bulk culture system. Because the production of IL-10 is influenced by the culture conditions, regulatory mechanisms of IL-10 remain to be studied further.

Glucocorticoids (GC) are most important agents for the treatment of allergic inflammation. Physiologic concentrations of exogenous GC can inhibit IL-12 production, but do not affect IL-10 production *in vitro*.⁵¹ It has been shown that GC affect the function of antigen-presenting cells to develop Th2 lymphocytes.⁵² From these reports, GC are considered to possibly supporting Th2 development. Our data imply that immunomodulators, such as IL-12, could be used as complementary agents to GC to avoid shifting to a Th2 dominant immune reaction. Because exogenous systemic IL-12 administration has been shown to induce severe adverse effects in a clinical trial,³⁰ inhaled administration of IL-12 may be better than systemic administration because this method of administration will be able to provide a sufficient local IL-12 concentration without any adverse systemic effects. According to our results, because

low-dose IL-12 still has a preferable effect on allergic inflammation, inhalational administration of IL-12 may be an effective method of treatment.

In summary, our results suggest that low-dose IL-12 enhances IFN- γ production but does not inhibit IL-10 production when PBMC are stimulated by Df antigen. Because IFN- γ can suppress Th2 dominant allergic immune reactions and IL-10 can inhibit hyperactivation of sensitized lymphocytes, low-dose IL-12 could be a useful candidate therapeutic agent for the treatment of bronchial asthma.

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