行政院國家科學委員會專題研究計畫成果報告

計畫名稱:過敏原及淋巴介質基因在呼吸道發炎動物模式的調整機制探討

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† 八十六年度及以前的一般國科會專題計畫(不含產學合作研究計畫)亦可選擇適用，惟較特殊的計畫如國科會規劃案等，請先洽得國科會各學術處同意。

一、中文摘要

介白質-12 是促進第一型 T 輔助細胞及抑制第二型 T 輔助細胞活性的重要細胞激素。我們之前的研究結果已經證明在利用過敏原誘發的免疫反應中，加上 IL-12 的治療可以避免免疫蛋白 E 的產生，第二型 T 輔助細胞激素的製造及氣管支氣管沖洗液內發炎細胞的嗜伊紅性白血球的數目。但是進一步的研究結果，在之前利用 IL-12 治療過的老鼠如果反覆地再利用過敏原刺激，體內 IgE 的濃度還是會逐漸增加。但是在已經建立第二型 T 輔助細胞反應的老鼠體內，再給予 IL-12 的治療，o 現能夠有效地抑制肺部的發炎反應及肺沖洗液內嗜伊紅性白血球的數目。由上述的研究結果，我們發現 IL-12 蛋白質應用在過敏疾病的治療上，似乎治療的效果要比預防的效果來得更好。

關鍵詞：介白質-12，氣喘

Interleukin-12 (IL-12) is a key cytokine, which promotes Th1-type cell-mediated immunity and inhibits Th2-type responses. It has been previously shown here that IL-12 administration during active immunization following a single allergen exposure can prevent antigen-induced increases in IgE formation, Th2 cytokine production and bronchoalveolar lavage (BAL) eosinophils in a murine model of allergic airway inflammation. Thus, these studies have now been extended and two IL-12 treatment protocols on this murine model were evaluated. Administration of IL-12 during the active immunization strikingly increased Der p 1-specific serum IgG2a and transiently decreased the levels of IgG1 and IgE antibodies following multiple allergen challenges. Such early treatment of IL-12 down-regulated IL-5 production and modestly up-regulated IFN-γ production but did not effect BAL eosinophilia. These results suggest that repeated exposure to antigen and IL-12 is necessary to maintain a persistent Th1-recall response. Further, administration of IL-12 to actively immunized mice, in which Th2-associated responses were established, had a significant effect on IgG2a synthesis and a modest effect on IgE levels, also down-regulation of IL-5 production and markedly increased IFN-γ production and abolished eosinophils recruitment. Therefore, these data indicate that IL-12 can inhibit antigen-induced eosinophils infiltration into airways, despite that Th2-associated response existed. Taken together, these studies suggest that IL-12 may be useful as an immunotherapeutic agent in treatment of such pulmonary allergic disorders as bronchial asthma.

Key words: Interleukin-12, asthma

本年度計畫已發表的論文：

Lee, Y.-L., Fu, C-L. Ye, Y.-L. and Chiang, B.-L. Administration of IL-12 prevents mite Der p1 allergen-IgE antibody production and eosinophils infiltration in animal model of airway inflammation. Scand J Immunol 1999; 49:229-236.*

Lee, Y.-L., Fu, C-L. and Chiang, B.-L.

Administration of IL-12 exerts therapeutic instead of longterm preventive effect on Der p1 allergen-linduced animal model of airway inflammation. Immunology 1999; 97: 232-240.*

Administration of Interleukin-12 Prevents Mite Der p 1 Allergen-IgE Antibody Production and Airway Eosinophil Infiltration in an Animal Model of Airway Inflammation

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The aim of the present study was to examine the *in vivo* effect of interleukin (IL)-12 on a murine model of asthma induced by *Dermatophagoides pteronyssinus*-derived Der p 1 allergen. C57BL/6 mice immunized with Der p 1 allergen adsorbed to alum/pertussis toxin developed a T-helper type 2 (Th2)-dominant immune response characterized by the presence of IgE antibody, airway eosinophil infiltration and increased production of Th2 cytokine. Intraperitoneal injection of IL-12 (1 or 0.1 μg per day) for 5 days (day -1 to $+3$) simultaneously with each immunization, inhibited the production of IgE and IgG_1 antigen-specific antibodies, whereas production of IgG_{2a} was strongly enhanced. In addition, mice receiving both doses of IL-12 showed a strong inhibition of IL-5 but up-regulation of IFN-γ production by spleen cells stimulated with antigen. Administration of IL-12 also prevented antigen-induced eosinophil infiltration into the bronchoalveolar area in a dose-dependent manner and the primary inflammatory mediator serotonin in bronchoalveolar lavage (BAL) fluids was also reduced significantly. Taken together, the data indicate that IL-12 has a potent immunomodulatory effect on house-dust-mite-induced allergic disorders and may be used as an efficient agent for immunotherapy.

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INTRODUCTION

The house dust mite is one of the most important inhalant allergens in respiratory disorders such as bronchial asthma and allergic rhinitis [1]. Among many species of mites in house dust, *Dermatophagoides pteronyssinus* (Dp) is reported to be dominant, as many basic and clinical studies have indicated. However, mite extracts are heterogeneous and contain various proteins of different molecular weights, although most of the IgE antibody to Dp is directed against Der p 1 in the sera of mite-allergic subjects. Der p 1, a 25-kDa glycoprotein found in mite faeces, has been purified and shown to be the predominant antigen [2]. Patients allergic to mite antigen have been demonstrated to have elevated serum levels of allergen-specific IgE and local infiltration of inflammatory cells in which the presence of eosinophils is striking. It has been recognized that T-helper type 2 (Th2) cells and their cytokines are responsible for the initiation and maintenance of allergic disorders [3]. Allergen-specific T cells isolated from atopic patients produce higher levels of the type-2 cytokines interleukin (IL)-4 and IL-5 and a lower level of type-1 cytokine interferon (IFN)-γ [4]. Furthermore, Th1 and Th2 cells have been found to show reciprocal regulation. Thus, agents that decrease IgE levels or Th2 cytokine production, or increase Th1 cytokine production may inhibit allergen-induced disorders.

It has been well documented that activation of naive T cells in the presence of IL-12 promotes differentiation to Th1 cells [5, 6]; conversely, IL-4 promotes Th2 development [7, 8]. IL-12 is a characterized heterodimeric cytokine that plays a primary role in the induction of cell-mediated immune functions. Previous studies have shown that treatment with IL-12 inhibits Th2 cytokines and related antibody production both *in vitro* and *in vivo* [9, 10]. Taken together, the apparent ability of exogenous IL-12, given as a biological adjuvant co-incident with antigen exposure, to promote a Th1-dominant response suggests a

potentially valuable therapeutic strategy that might reduce morbidity and mortality in asthmatic patients.

It has been demonstrated that CBA and C57BL/6 strains of mice are high IgE responders to Der p 1 antigen [11]. Therefore, to determine the potential role of IL-12 in the pathogenesis of allergic diseases, a murine model of airway inflammation induced by Der p 1 allergen has been developed. This model resembles human asthma in that it is characterized by an increase in serum level of antigen-specific IgE, the presence of peribronchial inflammatory infiltrates and an increased level of Th2 cytokine. In the present study, IL-12 treatment during allergic sensitization not only promotes Th1 immune responses but also inhibits airway inflammation. These results indicated that IL-12 might be useful as a therapeutic agent to reduce the duration of immunotherapy or even to prevent the attack of severe allergic diseases.

MATERIALS AND METHODS

Animals. Female, BALB/c and C57BL/6 mice between 6 and 8 weeks of age were obtained from and maintained in the Animal Center of the College of Medicine, National Taiwan University.

Preparation of antigens. The allergen Der p 1 was isolated by affinity column from spent mite media, kindly provided by Dr KY Chua (The National University of Singapore). Firstly, 5 g of the spent mite media was mixed with 100 ml of 0.1 M Tris–HCl (pH 7.6) and stirred overnight at 4°C. The mite extract was collected after centrifugation at 19000 ***g*** for 30 min at 4°C and passed through the anti-Der p 1 affinity column. The column was washed with phosphate-buffered saline (PBSA) and then Der p 1 protein was eluted with NH_4OH (pH 11) at 4°C. Immediately, 0.1 M Tris–HCl (pH 6.8) was added to neutralize the eluted fractions. The pooled fractions were dialysed against PBSA and further concentrated. Finally, the concentrated product was monitored at OD 562 nm and stored at −20°C before use.

The lyophilized house dust mite, Dp, was purchased from Allergon (Angelholm, Sweden). The allergen was prepared as described previously [12]. Briefly, 1 g of lyophilized mite body was defatted with 100 ml ether, homogenized and stirred continuously in 25 ml PBSA for 48 h at 4°C. After centrifugation (12000 ***g***., 30 min), the crude extract was dialysed with PBSA and the mite extract dissolved in PBSA and stored at −20°C.

Immunizations and inhalation exposure of mice. To establish an animal model of asthma, BALB/c and C57BL/6 mice were immunized by an intraperitoneal injection of Der p 1 at doses of 1, 5 and 10 μg with either alum alone or alum plus 400 ng pertussis toxin (List Biological Lab. Inc., Campbell, CA, USA) as the adjuvant. Every 2 weeks the mice were boosted with the same dose of Der p 1 and adjuvant. On days 0, 14, 28 and 42, mice were bled from the retro-orbital venous plexus. Sera were collected and tested for the presence of Der p 1-specific antibodies using isotype-specific enzyme-linked immunosorbent assay (ELISA).

To further examine the *in vivo* effects of mouse rIL-12 (R&D, Minneapolis, MN, USA) on this murine model of asthma induced by Der p 1, C57BL/6 mice were immunized by an intraperitoneal injection of 10 μg Der p 1 with 2 mg alum plus 400 ng pertussis toxin as the adjuvant. On days 14 and 28, the mice were boosted with the same dose of Der p 1 and adjuvant. In addition, two groups of mice were treated with different doses IL-12 for 5 days (day −1 to +3) simultaneously with each immunization. IL-12 was administered at dose of 1 μg/mouse/day or 0.1 μg/mouse/day. Control-sensitized mice received PBSA instead of IL-12. Each group is composed of 8–12 mice. On days 0, 14 and 28, the mice were bled from the retro-orbital venous plexus. Sera were collected and stored at −20°C until analysis. On day 42, all groups of mice were exposed to aerosolized crude mite extract (*Derma ophagoides pteronyssinus*) over a 20-min period, by placing them in a chamber which could hold 6–8 mice concurrently. The aerosols were generated in the chamber using an ultrasonic nebulizer (DeVilbiss, Somerset, PA). The output of the nebulizer was 0.3 ml/min and the produced particles had a size range of 0.5–5 μm. The concentration of crude mite extract in the nebulizer was 0.1% (wt/vol).

Mite-allergen-specific antibody assay. Sera anti-Der p 1 IgE, IgG_1 and IgG_{2a} antibody titres were determined by ELISA. Briefly, 96-well microtitre plates were coated with 4 μg/ml Der p 1 diluted in $NaHCO_3$ buffer, pH 8.2. After overnight incubation at 4°C, plates were washed and blocked with 3% bovine serum albumin (BSA) in PBSA for 2 h at 37°C. Serum samples were diluted and added to each well overnight at 4°C. Plates were then washed. Either biotin-conjugated antimouse IgE (0.5 μg/ml, Serotec, Raleigh, NC, USA) or IgG_1 (1 : 500, Pharmingen, San Diego, CA, USA) or IgG_{2a} (1 : 500, Pharmingen), diluted in 3% BSA–PBSA buffer, was added for 1 h at 37°C. Streptavidin-conjugated alkaline phosphatase (1 : 2000, Sigma, St Louis, MO, USA) was then added for an additional 2 h at room temperature. Finally, the reaction was developed by phosphatase substrate pNPP and the absorbance determined at 405 nm in a microplate reader. The levels of antibody were compared with IgG_1, IgE and IgG_{2a} standards with predetermined concentrations (immunoglobulin concentrations: $IgG_1 = 28.2$ μg/ml, IgE = 1.1 μg/ml, $IgG_{2a} = 16.7$ μg/ml). The concentration of standard serum was arbitrarily assigned 1 ELISA unit (1 EU).

Antigen-specific proliferative assay. To measure the Der p 1-specific T-cell proliferative response, Der p 1-immunized mice were sacrificed and 3×10^5/well spleen cells were cultured with Der p 1 (10 μg/ml) or phytohaemagglutinin (PHA, 10 μg/ml) or medium only in RPMI-1640 medium containing 2% TCM (mouse serum replacement, Celox Corp., Hopkins, MN) plus 2% fetal calf serum (FCS). Cells were cultured in round bottom 96-well microculture plates at 37°C in 5% CO_2. After 2 days in culture, the cells were pulsed with 1 μCi/well of [^{3}H]TdR for 15–17 h. Specific incorporation of TdR was analysed using a β-counter (Packard Instrument Co., Meriden, CT) and results were expressed as stimulation index (SI).

Cytokines assay. To measure the levels of cytokines, splenocytes (1×10^7/well) of immunized mice treated with or without IL-12 after inhalation were cultured in 0.5 ml AIM-V medium supplemented with 2% TCM in the presence of 10 μg/ml Der p 1 or PHA (10 μg/ml) in 48-well microtitre plate at 37°C for 48 h. The culture supernatants were collected and centrifuged at 400 ***g*** at 4°C. The cell-free supernatants were stored at −20°C until they were used for the cytokine assay. The levels of IL-5 and IFN-γ in the culture supernatants were evaluated by sandwich-ELISA. Briefly, ELISA plates were coated with 2 μg/ml of monoclonal rat antimouse IL-5 (Pharmingen) or rat antimouse IFN-γ (Pharmingen) in $NaHCO_3$ buffer, pH 9.6 at 4°C overnight and blocked with 2% BSA–PBSA at room temperature for 2 h. The diluted supernatants were added and incubated for 1 h at 37°C. Biotinylated antimouse IL-5 (1 μg/ml, Pharmingen) or IFN-γ (1 μg/ml, Pharmingen) was applied to the plates and incubated at 37°C for 1 h. Avidin–horseradish peroxidase (1 : 1000, Immunopure, Pierce, Rockford, IL, USA) was then added for 30 min at 37°C. After the wells were washed, colour was generated with the addition of substrate 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid). The absorbance at 420 nm was measured on an ELISA reader. The concentration of cytokines was measured by

converting the OD values of the samples to pg from the standard curve. The levels of sensitivity for the IL-5 and IFN-γ assay were 60 pg/ml and 150 pg/ml, respectively.

Bronchoalveolar lavage and histopathological study. Twenty-four hours after the aerosol exposure, all groups of mice were bled from the retro-orbital venous plexus and then sacrificed. In addition, the naive mice exposed to aerosolized allergen were used as the negative controls. The lung was immediately lavaged via the trachea cannula with 3 × 1 ml of Hank's balanced salt solution (HBSS), free of ionized calcium and magnesium. The lavage fluid was centrifuged at 400 *g* for 10 min at 4°C. After washing, the cells were resuspended in 1 ml HBSS and total-cell counts were determined by counting in a haemocytometer. Cytocentrifuged preparations were stained with Liu's stain for differential cell counts. A minimum of 200 cells was counted and classified as macrophages, lymphocytes, neutrophils and eosinophils, based on standard morphologic criteria.

To evaluate the effects of IL-12 on allergen-induced lung inflammation, each group of animals was sacrificed for histopathological examination. After the lavage, the lungs were immediately removed and fixed in sublimate formalin solution. The tissues were subsequently embedded in paraffin and cut into 5-μm-thick sections. These frozen sections were stained with haematoxylin–eosin and examined for histological changes using light-microscopy changes.

The levels of serotonin in bronchoalveolar lavage fluids. Bronchoalveolar lavage (BAL) fluids were assayed 24 h after the antigen challenge. Animals were sacrificed, and BAL fluids were collected and centrifuged at 400 *g* for 10 min at 4°C. The supernatants were collected and stored at −20°C until assay. Concentrations of serotonin in lavage fluids were measured by serotonin ELISA kit (IBL, Hamburg, Germany) according to the manufacturer's recommendations. Absorbance values, read at 405 nm were converted to concentration in BAL fluids by comparison with a standard curve. The level of sensitivity for serotonin assay was 0.03 ng/ml. Because the sample preparation leads to a 207.25-fold dilution, the values read from the standard curve have to be corrected by multiplying by 207.25.

Statistical analysis. Unpaired Student's *t*-test was used to analyse the data throughout the study. A *P*-value of <0.05 was considered to be significant.

RESULTS

Antibody responses of different mouse strains against Der p 1

To establish an animal model of airway inflammation, many factors involved in the regulation of Th1 and Th2 responses have been taken into consideration, including the route of antigen entry, the dose of antigen, the type of adjuvant and the genetics of mouse strains. Table 1 illustrates the immune responses to the allergen Der p 1 in BALB/c and C57BL/6 mouse strains. The IgG_1 antibody response against Der p 1 allergen was observed in all groups. In general, 10 μg of antigen without pertussis toxin PT immunization was enough to induce persistent anti-Der p 1 IgG_1 antibody response in both mouse strains. The anti-Der p 1 IgG_1 antibody response was further enhanced significantly in BALB/c mice receiving Der p 1 immunization plus PT. In contrast, the anti-Der p 1 IgG_{2a} responses were poor and there was no difference among groups. Notably, the anti-Der p 1 IgE antibody titre was low except C57BL/6 strain mice in which IgE anti-Der p 1 antibody was significantly enhanced after immunization in the presence of PT. In general, C57BL/6 tended towards a relatively higher and more persistent IgE response to Der p 1 allergen than did BALB/c. Consequently, C57BL/6 strain mice were used in the following experiments.

IL-12 suppresses the synthesis of IgG_1 and IgE and enhances the production of IgG_{2a}

To examine the influence of IL-12 on Der p 1-specific immune responses *in vivo*, two different doses of IL-12 treatment on the murine model of airway inflammation were evaluated. C57BL/6 mice immunized with 10 μg of the allergen Der p 1 adsorbed to 2 mg alum plus 400 ng PT induced a strong IgG_1 response (Fig. 1A). Furthermore, substantial amounts of IgE

Table 1. The titers of anti-Der p 1 antibodies from mice immunized with different doses of antigen

	Immunization		IgE		IgG_1		IgG_{2a}	
Strain	Der p 1 (μg)	PT	Day 0	Day 42	Day 0	Day 42	Day 0	Day 42
BALB/c	1	—	0.02 ± 0.04	0.08 ± 0.09	0	0.35 ± 0.12	0.01 ± 0.01	0.03 ± 0.02
	5	—	0	0.09 ± 0.05	0	1.27 ± 0.29	0.01 ± 0.00	0.12 ± 0.05
	10	—	0	0.04 ± 0.03	0	1.20 ± 0.15	0	0.14 ± 0.08
	10	+	0.01 ± 0.01	0.11 ± 0.06	0.01 ± 0.01	1.88 ± 0.32**	0.08 ± 0.02	0.20 ± 0.05
C57BL/6	1	—	0	0.02 ± 0.02	0	0.64 ± 0.42	0	0.09 ± 0.06
	5	—	0	0.02 ± 0.01	0	0.70 ± 0.32	0	0.05 ± 0.07
	10	—	0	0.02 ± 0.02	0	1.13 ± 0.23	0	0
	10	+	0.01 ± 0.02	0.40 ± 0.28*	0	1.38 ± 0.44	0	0.03 ± 0.03

Eight groups of mice were immunized by intraperitoneal injections of Der p 1 at doses of 1, 5 and 10 μg adsorbed to either 2 mg alum alone or plus 400 ng pertussis toxin (PT) as the adjuvant. Mice of each group were boosted biweekly with the same doses as priming doses. The mice were bled at days 0, 14, 28 and 42 and serum Ab levels were determined by ELISA. The values represent the mean ± SD of 5–6 mice per group. *$P<0.05$ and **$P<0.005$ compared with that of 10 μg Der p 1 + alum immunized mice.

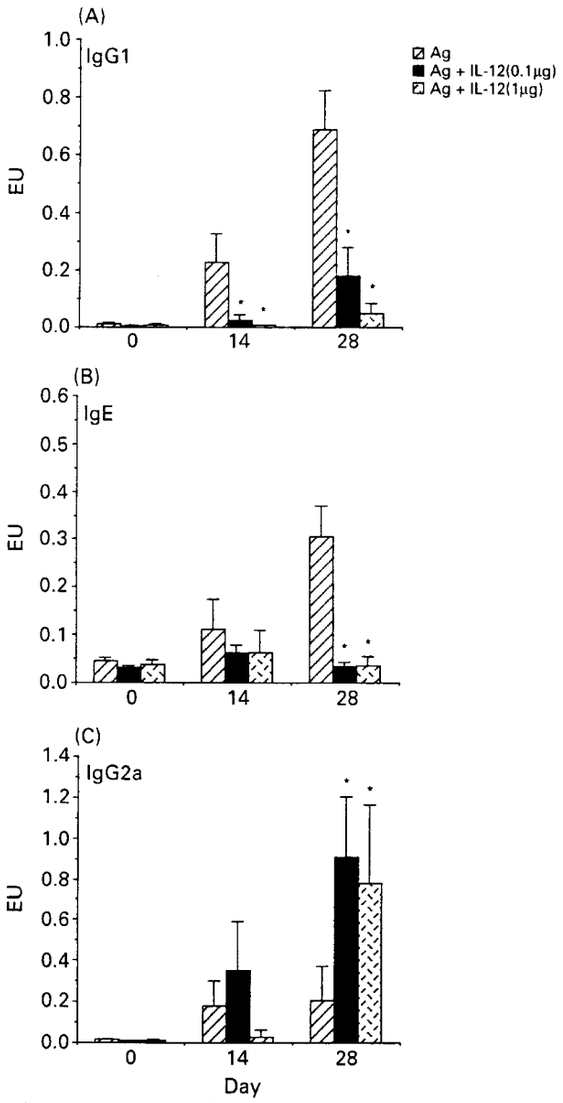

Fig. 1. Antigen-specific antibody isotype pattern in the sera of mice immunized with Der p 1 adsorbed to alum/PT with or without IL-12 treatment. C57BL/6 mice were immunized intraperitoneally three times with 10 μg/mouse Der p 1 adsorbed to alum plus PT every 2 weeks. IL-12 was administered to mice intraperitoneally at 0.1 μg/day or 1 μg/day for 5 days (day −1 to +3) simultaneously with each immunization. Blood was collected on days 0, 14 and 28, and sera levels of anti-Der p 1 antibodies IgG_1 (A), IgE (B) and IgG_{2a} (C) were assayed by using ELISA. Data are shown as mean ± SD for 10 mice per group. Significant differences ($^*P < 0.05$) from the immunized IL-12-untreated group are indicated.

were produced (Fig. 1B), whereas synthesis of IgG_{2a} was low (Fig. 1C). The data suggested that the Der p 1-specific IgG_1 response on day 28 was suppressed 14-fold or 3.8-fold by a daily dose of 1 μg or 0.1 μg of IL-12. However, the most striking effect of both doses of IL-12 on humoral immune responses is a significant inhibition (8–9-fold) of IgE level on day 28 (Fig. 1B).

In contrast, either dose of IL-12 administration during immunization up-regulated synthesis of IgG_{2a} about four-fold on day 28. Thus, IL-12 clearly promotes the development of characteristic Th1-associated pattern of antibody response in Der p 1-sensitized mice that normally develops in a Th2 manner.

IL-12 inhibits antigen-induced Th2-like cytokine production and increases Th1-like cytokine expression in sensitized mice

Twenty-four hours after the inhalation, splenocytes isolated from immunized mice treated with or without IL-12 were examined for Th-cell proliferative responses by stimulation with Der p 1 *in vitro*. Spleen cells from mite-exposed control mice were used as negative controls. The results are shown in Fig. 2. A dose response was observed when cells from Der p 1-sensitized mice were incubated with increasing amounts (0, 5, 10 and 20 μg/ml) of Der p 1 *in vitro* (data not shown). Der p 1-specific proliferative responses were reduced modestly by IL-12 treatment. In some experiments, McKinght *et al.* [6] observed a 20–30% reduction of T-cell proliferative response after IL-12 administration. However, the reason for this phenomenon is yet to be defined.

To determine whether the *in vivo* administration of rIL-12 affects antigen-specific T-cell function, the *in vitro* production of IFN-γ and IL-5 of spleen cells in IL-12-treated mice was examined. Both doses of IL-12 suppressed IL-5 production

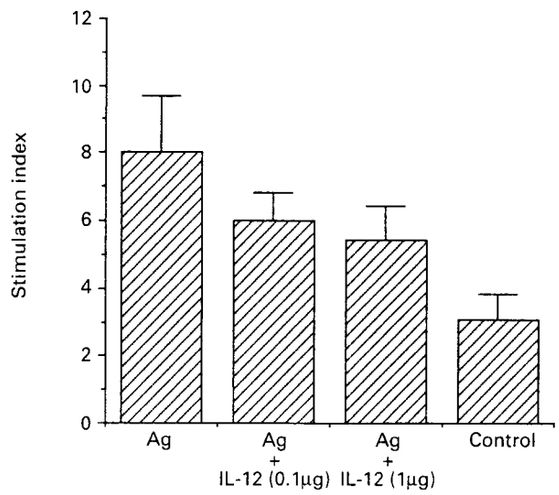

Fig. 2. The effect of IL-12 on Der p 1-antigen-specific T-cell proliferative response. C57BL/6 mice were treated as described in Fig. 1. On day 42, each group of sensitized mice was challenged with inhaled *D pteronyssinus* and nonsensitized mice were also treated with the same protocol as the control group. Spleen cells were taken from these allergen-challenged mice after 24 h and restimulated with 10 μg/ml Der p 1 *in vitro*. Proliferation was measured by [^{3}H]thymidine incorporation on day 3. The background values of spleen cells without Der p 1 stimulation were between 734 and 895 cpm in this assay. The results are expressed as SI and shown as mean ± SD for 8–10 mice per group.

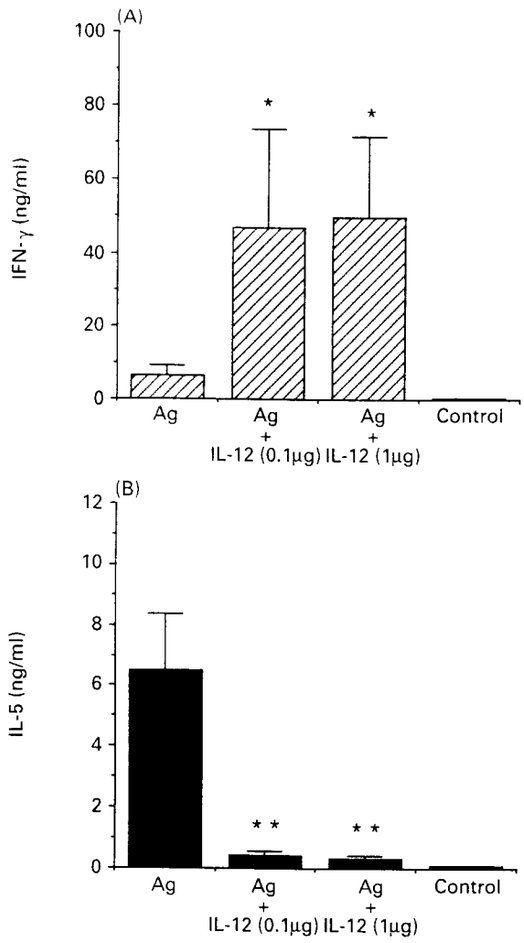

Fig. 3. The effect of IL-12 on IFN-γ, IL-5 production by splenocytes of mice after a single antigen challenge. Splenocytes were stimulated with 10 μg/ml Der p 1 *in vitro* and culture supernatants were collected after 48 h, and the levels of cytokine production IFN-γ (A) and IL-5 (B) were measured by ELISA. Values shown are mean ± SD of 8–12 mice per group. Significant differences (*$P < 0.05$, **$P < 0.01$) compared with those of mice without IL-12 treatment.

almost completely and enhanced IFN-γ secretion dramatically in allergen-challenged mice to a similar degree (Fig. 3A, B). These recall responses cannot be explained by residual IL-12 due to the *in vivo* IL-12 treatment because the spleens were removed 12 days later after the last injection of IL-12 and the serum half-life of IL-12 is only 2–5 h in rodents [13]. These results indicated that *in vivo* IL-12 treatment enhanced the differentiation of Der p 1-specific Th1 cells and suppressed the development of Th2 cells in response to immunization.

IL-12 inhibits airway inflammation induced by a single antigen challenge

To examine whether IL-12 could alleviate allergen-induced airway inflammation, both the cellular composition and inflammatory mediators in BAL fluids were analysed after aerosol exposure. In actively sensitized mice, a single exposure to aerosolized Dp induced a marked increase in the percentage of neutrophils, lymphocytes and eosinophils in BAL fluids, compared with those of the nonsensitized control mice exposed to aerosolized allergen (Table 2). The ratio of neutrophils and lymphocytes was increased slightly in mite-exposed control mice following inhalation. The mice receiving 1 μg/day of IL-12 treatment showed a significant decrease in neutrophils and lymphocytes, whereas the percentage of these cells was not significantly affected by treatment with 0.1 μg/day of IL-12. It seemed that IL-12 exerted a dose-dependent inhibition of antigen-induced airway inflammation. Both low and high doses of IL-12 significantly inhibited the eosinophil recruitment.

Histopathological examination of lung taken from sensitized mice after inhalation demonstrated inflammatory infiltrates in peribronchial and peribronchiolar regions (Fig. 4A). The infiltrates consisted of admixtures of predominantly eosinophils, neutrophils and lymphocytes. In contrast, the IL-12-treated animals were not noted to show significant airway inflammation (Fig. 4B). The serotonin levels in BAL fluids from allergen-challenged mice were measured and are shown in Fig. 5. The level of serotonin was increased markedly in sensitized mice

Table 2. Change in cellular composition in BAL of sensitized mice exposed to aerosolized allergen

		BAL			
Group	Treatment	Monocytes (%)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)
1	Der p 1	28.8 ± 5.9	20.0 ± 3.4	16.6 ± 4.6	34.6 ± 8.1
2	Der p 1/IL-12(0.1 μg)	68.0 ± 14.1	15.1 ± 9.6	14.9 ± 6.3	2.0 ± 1.6*
3	Der p 1/IL-12 (1 μg)	94.4 ± 2.2*	3.1 ± 1.4*	2.0 ± 1.3*	0.5 ± 0.6*
4	None	85.6 ± 4.1	5.0 ± 2.9	8.9 ± 3.1	0.5 ± 0.5

BAL was performed 24 h after *Dermatophagoides pteronyssinus* inhalation in control and IL-12-treated mice. The differential counts of BAL were determinated and data are mean ± SEM for 8–12 mice in each group.

* $P < 0.001$ for difference from the mean value of the corresponding response in Der p 1 sensitized mice.

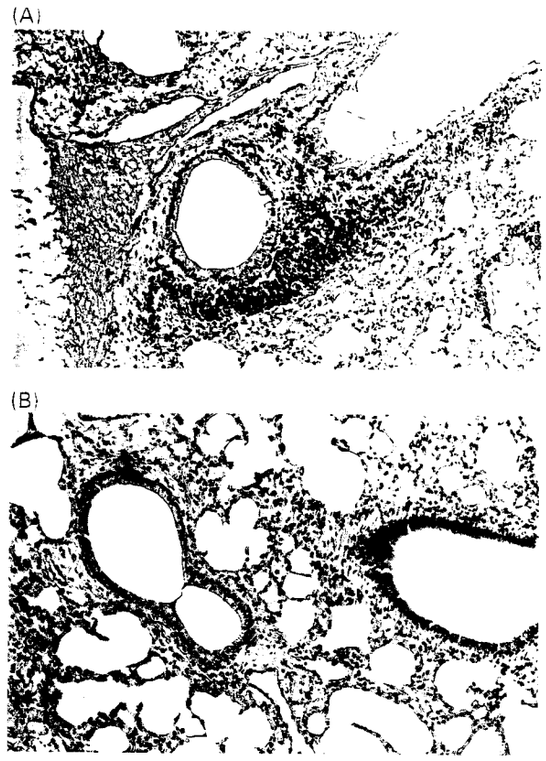

Fig. 4. Histological study of the lungs of immunized mice with or without IL-12 treatment (haematoxylin–eosin stain). Lung tissue from mice without IL-12 treatment (A) demonstrates extensive cellular infiltration of the periairway region (×200). In contrast, lung tissue from IL-12-treated mice (B) demonstrates a complete absence of histologically significant inflammation (×200).

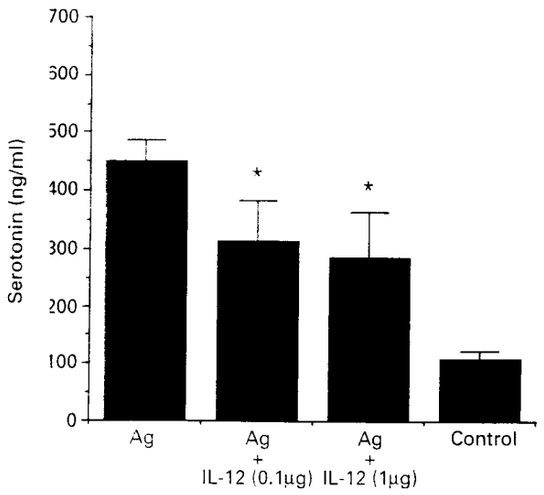

Fig. 5. The effect of IL-12 on serotonin production in BAL from mice after a single antigen challenge. C57BL/6 mice were treated as described in Fig. 1. On day 42, mice were challenged with inhaled *D. pteronyssinus* and BAL samples were taken from the mice with allergen challenged after 24 h. BAL fluids from nonsensitized mice exposed to aerosolized allergen were measured by using a specific ELISA. Data represent the mean ± SEM of 8–12 samples per group. $*P<0.05$ compared with the level of immunized mice without IL-12 treatment.

without IL-12 treatment and was reduced significantly with either dose of IL-12 treatment.

DISCUSSION

Dermatophagoides pteronyssinus is dominant among the many species of mite found in house dust, and many studies have been devoted to the characterization of the Der p 1 involved in the pathogenesis of allergic diseases [14–16]. However, only a few studies have described an animal model of airway inflammation induced by Der p 1 allergen. Attention has therefore been directed at setting up a Der p 1-sensitized murine model of airway inflammation and investigating the application of IL-12 to modulate such an allergic inflammatory response. In studies designed to optimize the production of anti-Der p 1 IgE in mice, a range of doses of both antigen and pertussis toxin as the IgE adjuvant was investigated. The data showed that a significant anti-Der p 1 IgE antibody response can be detected in C57BL/6 mice immunized with 10 μg of Der p 1 plus 2 mg of alum and 400 ng of PT intraperitoneally. It was recognized that different kinds of adjuvants were able to selectively activate one of the two $CD4^+$ T-cell subpopulations, Th1 or Th2. For example, in conjunction with the same antigen, alum activated Th2 cells whereas Freund's complete adjuvant (FCA) activated Th1 cells [17, 18]. PT, a protein toxin of *Bordetella pertussis*, was also used as the IgE adjuvant and the stimulation of IgE production by PT in response to protein antigens has been well documented [19–21]. In our studies, C57BL/6 mouse strain immunized with Der p 1 allergen + alum + PT may be used as a murine model of airway inflammation and as a prelude to further studies designed to examine and manipulate the immune response to the Der p 1 allergen at the molecular level, which may allow exploration of possible agents for immunotherapy.

IL-12 is a heterodimeric cytokine that is produced primarily by antigen-presenting cells, and plays an important role in the regulation of both innate and adaptive immune responses. One of the most striking effects of IL-12 is its ability to promote the development of Th1-type immune responses. Exogenous rIL-12, added to cultured bulk lymphocytes from atopic donors during *in vitro* stimulation with Der p 1, favoured the differentiation of antigen-specific T-cell lines and clones producing high concentrations of IFN-γ and low concentrations of IL-4 [5]. Following *in vivo* challenge with various intracellular pathogens, IL-12 has been shown to promote Th1 responses while inhibiting Th2 responses. In a murine leishmaniasis model, it has been demonstrated that IL-12 had to be given within the first week after infection to be effective in providing immunity, before the establishment of a Th2 response [22, 23]. In addition, Nabors *et al.* [24] have found that IL-12 plus the leishmanicidal drug given 3 weeks after infection cured 70% of the mice. Recently, a

IL-12 gene transfer approach for nucleic acid vaccination or for gene therapy has been used in various models [25, 26]. Therefore, the potential use of IL-12 plasmid in future as an application to the treatment of Der p 1-induced airway inflammation should be investigated further.

Consistent with the role of IL-12 in promoting Th1- and suppressing Th2-type responses, IL-12 also exerts regulatory effects on humoral immune responses. In CBA/J mice immunized with protein antigens adsorbed to alum, administration of IL-12 induced strong increases in antigen-specific IgG_{2a}, IgG_{2b} and IgG_3 production [27]. Kiniwa *et al.* [28] demonstrated that IL-12 dramatically inhibited IL-4-induced IgE synthesis by peripheral blood mononuclear cells (PBMC) *in vitro*. Since the distinguishing feature between an atopic and a nonatopic individual is their ability to produce a sustained high-level IgE response to allergens, IgE antibody has been defined to be the marker for sensitization. Recent studies have disclosed a correlation between serum IgE and airway responsiveness in asthmatic patients as compared with that of normal controls [29]. In the murine model of Der p 1-induced asthma, it has been demonstrated that administration of IL-12 at 1 μg/day inhibited the Der p 1-specific IgE response by 88% and the Der p 1-specific IgG_1 response by 93%. In contrast, the production of Der p 1-specific IgG_{2a} was enhanced strongly by either dose of IL-12. Likewise, high dose of 1 μg IL-12/day treatment given to allergen phospholipase A_2 (PLA_2)-sensitized CBA/J mice could reduce the IgE response. Nevertheless, the suppression phenomenon was not permanent, because IgE synthesis was up-regulated in mice after repeated antigen challenge without IL-12 [30].

Airway inflammation is the major pathological change of bronchial asthma [31]. It has been proposed that mast cells and T cells are the key effector cells involved in triggering inflammatory responses in allergic diseases. Mast cells present in the airway mucosa, stimulated by inhaled allergens, and releasing mediators such as histamine and serotonin are responsible for the acute phase reactions. Eosinophils are prominent inflammatory cells in the airways of asthmatic subjects and have been proposed to mediate tissue injury and airway hyper-responsiveness [32]. Recently, it has been observed that IL-12 has a profound effect on antigen-induced airway inflammation. IL-12 was able to abolish antigen-induced airway hyper-responsiveness and pulmonary eosinophilia in sensitized mice after an intratracheal (IT) challenge with sheep red blood cell antigen [33]. Similarly, the administration of IL-12 during active immunization decreased ragweed-specific serum IgE and BAL eosinophilia following IT allergen challenges [34]. In the animal model of ovalbulmin-induced lung inflammation, the treatment of mice with IL-12 either before or during the aerosolized antigen challenge abrogated airway eosinophilia, whereas administration of IL-12 during the aerosolized antigen challenge did not decrease the level of specific IgE [35, 36]. In our study, it has been demonstrated that administration of IL-12 during active immunization strikingly decreased airway inflammatory cells and the levels of serotonin in BAL fluids after an inhaled challenge. Collectively, these studies demonstrated the ability of IL-12 given early during the immune response to redirect a Th2-mediated immune response to inhaled antigens.

There was a discrepancy between our findings regarding the levels of IFN-γ secretion and those regarding serotonin production in IL-12-treated mice. The data demonstrated that IL-12 treatment strongly augmented the production of IFN-γ and markedly reduced IL-5 secretion by antigen-stimulated spleen cells. However, IL-12 treatment only modestly decreased serotonin production in BAL fluids. Firstly, it is presumed that exogenous IL-12 might potently enhance IFN-γ production not only by steering Der p 1-specific $CD4^+$ T cells to Th1-associated activation patterns but also by transient activation of natural killer (NK) cells. Secondly, the lack of a dramatic effect of IL-12 on serotonin production may be due to the long interval between the last dose of IL-12 and the assay of BAL in IL-12-treated mice. Indeed, it has been reported that up-regulating effects of IL-12 on BAL cell IFN-γ expression last for up to 2 days but faded out by 8 days [34]. Furthermore, the endogenous release of IFN-γ was not enough to completely inhibit aspects of mast cell function such as the release of serotonin.

In this study, the effects of IL-12-treatment protocols on a murine model of Der p 1-induced asthma have been evaluated. It has been demonstrated that the intraperitoneal administration of IL-12 during active immunization prevented the production of Der p 1-specific serum IgE levels, airway eosinophilia provoked by aerosol challenge and Th2-type cytokines production. In addition, allergen-induced eosinophils recruitment was also strikingly inhibited in the group treated with a dose of 1 μg/day of IL-12. Thus, IL-12 may be useful as a single immunotherapeutic agent, in combination with chemotherapeutic agents, or as a vaccine adjuvant for treatment of atopic diseases.

ACKNOWLEDGMENT

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Administration of interleukin-12 exerts a therapeutic instead of a long-term preventive effect on mite *Der p* I allergen-induced animal model of airway inflammation

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SUMMARY

Interleukin-12 (IL-12) is a key cytokine, which promotes T helper type 1 (Th1) cell-mediated immunity and inhibits Th2-type responses. It has been previously shown that IL-12 administration during active immunization following a single allergen exposure can prevent antigen-induced increases in immunoglobulin E (IgE) formation, Th2 cytokine production and bronchoalveolar lavage (BAL) eosinophils in a murine model of allergic airway inflammation. Thus, these studies have now been extended and two IL-12 treatment protocols on this murine model were evaluated. Administration of IL-12 during the active immunization strikingly increased *Der p* I-specific serum IgG2a and transiently decreased the levels of IgG1 and IgE antibodies following multiple allergen challenges. Such early treatment of IL-12 down-regulated IL-5 production and modestly up-regulated interferon-γ production but did not effect BAL eosinophilia. These results suggest that repeated exposure to antigen and IL-12 is necessary to maintain a persistent Th1-recall response. Furthermore, administration of IL-12 to actively immunized mice, in which Th2-associated responses were established, had a significant effect on IgG2a synthesis and a modest effect on IgE levels, also down-regulation of IL-5 production, and markedly increased interferon-γ production and abolished recruitment of eosinophils. Therefore, these data indicate that IL-12 can inhibit antigen-induced eosinophil infiltration into airways, despite the existence of a Th2-associated response. Taken together, these studies suggest that IL-12 may be useful as an immunotherapeutic agent in the treatment of such pulmonary allergic disorders as bronchial asthma.

INTRODUCTION

The house dust mite is one of the most important inhalant allergens in respiratory disorders, such as bronchial asthma and allergic rhinitis.[1] Among many species of mite in house dust, *Dermatophagoides pteronyssinus* (Dp) is dominant, as many basic and clinical studies have indicated. *Der p* I, a 25 000 MW glycoprotein found in mite faeces, has been purified and shown to be the predominant antigen.[2] However, no study has described an animal model of airway inflammation induced by *Der p* I allergen. Allergic asthma is characterized mainly by elevated specific immunoglobulin E (IgE) antibody production and eosinophilic inflammation.[3,4] It has been proposed that eosinophils mediate tissue injury and airway hyperresponsiveness.[5] Since IgE production and eosinophil differentiation and recruitment are positively controlled by the type 2 cytokines interleukin-4 (IL-4) and IL-5, respectively.[6–9] It has been recognized that T helper type 2 (Th2) cells and their cytokines are responsible for the initiation and maintenance of allergic disorders.[10] Thus, agents which decrease IgE levels or Th2 cytokine production or increase Th1 cytokine production may inhibit allergen-induced disorders.

Interleukin-12 (IL-12) is a key cytokine produced by macrophages to promote Th1-type cell-mediated immune functions.[11,12] Previous studies have shown that treatment with IL-12 inhibits Th2 cytokines and related antibody production *in vitro* and *in vivo*.[13–16] These biological activities form the basis for many studies examining the therapeutic potential of IL-12. Recent studies in several murine models have shown that IL-12 has tremendous potential as a vaccine-adjuvant in promoting a Th1 response.[17–20] However, such enthusiasm for IL-12 as a biological adjuvant, founded primarily on striking data obtained in these short-term experimental systems, makes it impossible to determine whether IL-12 has lasting impact on Th1-recall responses following repeated antigen exposure in the absence of IL-12. In addition, several studies have demonstrated that IL-12 needs to be administered early in the sensitization process to induce a Th1-mediated immune response to inhaled antigen.[14,21]

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Abbreviations: BAL, bronchoalveolar lavage; Dp, *Dermatophagoides pteronyssinus*; IFN-γ, interferon-γ; IL-12, interleukin-12.

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Collectively, a key to the development of IL-12 as a vaccine adjuvant or a clinical therapeutic agent for allergic asthma will be better understanding of modes for using IL-12 to establish long-lived immune memory of Th1 responses, to reverse or inhibit existing Th2 responses. Therefore, to address these issues, a well-defined mouse model of airway inflammation induced by *Der p* I allergen was used and two protocols of IL-12 treatment were tested.

MATERIALS AND METHODS

Animals

Female, 7-week-old C57BL/6 mice were obtained from and maintained in the Animal Center of the College of Medicine, National Taiwan University.

Preparation of antigens

The allergen *Der p* I was isolated by affinity column from spent mite media, which was kindly provided by Dr K-Y. Chua (The National University of Singapore). Firstly, 5 g of spent mite media were mixed with 100 ml of 0·1 M Tris–HCl (pH 7·6) and then stirred overnight at 4°. The mite extract was collected after centrifugation at 19 000 ***g*** for 30 min at 4° and passed through the anti-*Der p* I affinity column. The column was washed with phosphate-buffered saline (PBS) and then *Der p* I protein was eluted with NH_4OH (pH 11) at 4°. Immediately, 0·1 M Tris–HCl (pH 6·8) was added to neutralize the eluted fractions. The pooled fractions were dialysed against PBS and further concentrated. Finally, the concentrated product was monitored at optical density (OD) 562 nm and stored at −20° before use.

The lyophilized house dust mite, Dp, was purchased from Allergon (Angelholm, Sweden). The allergen was prepared as described.[22] Briefly, 1 g of lyophilized mite body was defatted with 100 ml ether, then homogenized and stirred continuously in 25 ml PBS for 48 hr at 4°. After centrifugation (12 000 ***g***, 30 min), the crude extract was dialysed with PBS, then the mite extract was dissolved in PBS and stored at −20°.

Immunization and inhalation exposure of mice

For systemic immunization, 10 μg *Der p* I was mixed with 2 mg alum plus 400 ng pertussis toxin (List Biological Lab. Inc., Campbell, CA) as the adjuvant and injected intraperitoneally in a volume of 100 μl.

To examine the effects of recombinant mouse IL-12 (rIL-12; R & D, Minneapolis, MN), two groups of mice were in addition treated with IL-12 for 5 days (day −1 to +3) simultaneously with immunization indicated. IL-12 was administered intraperitoneally at 1 μg/mouse/day. Control mice received PBS instead of IL-12.

Aerosol immunization was performed with crude mite extract (*Dermatophagoides pteronyssinus*) solution. The aerosols were generated into the chamber using an ultrasonic nebulizer (DeVilbiss, Somerset, PA). The output of the nebulizer was 0·3 ml/min, and the produced particles had a size range of 0·5–5 μm. The concentration of crude mite extract in the nebulizer was 0·1% (w/v). The mice were exposed to 8 ml suspension over a 20-min period, by placing them in a chamber, which could contain six to eight mice concurrently.

Experimental design

The experimental design is summarized in Fig. 1. Group 1 ($n=8$) was immunized three times intraperitoneally with *Der p* I in $Al(OH)_3$ plus pertussis toxin at days 0, 14 and 28. On day 42, day 56 and day 70, the mice were aerosolized with crude mite extract. As a control, one group was given PBS on days −1 to 3, days 13 to 17 and days 27 to 31.

Group 2 ($n=8$) was immunized intraperitoneally with *Der p* I in $Al(OH)_3$ plus pertussis toxin at days 0, 14 and 28 and subsequently aerosolized with the allergen as described above. In addition, rIL-12 was administered for 5 days each time (days −1 to 3, days 13 to 17, days 27 to 31).

Group 3 ($n=8$) was immunized intraperitoneally with *Der p* I in $Al(OH)_3$ plus pertussis toxin at days 0, 14, 28 and 42 and thereafter aerosolized with crude mite extract on day 56. As control group, PBS was given for 5 days (days 27–31) and after 2 weeks for another 5 days (days 41–45).

Group 4 ($n=8$) was immunized intraperitoneally with *Der p* I in $Al(OH)_3$ plus pertussis toxin at days 0, 14, 28 and 42 and subsequently aerosolized with the allergen on day 56. These mice were treated with rIL-12, administered from days

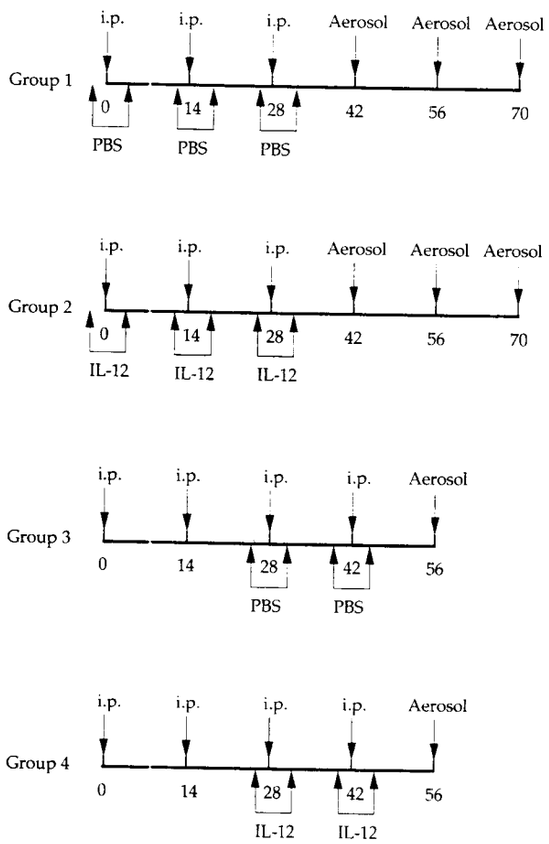

Figure 1. Immunization protocol of the four experimental groups of C57BL/6 mice ($n=8$). Intraperitoneal (i.p.) *Der p* I injections consisted of 10 μg of *Der p* I, 2 mg $Al(OH)_3$ and 400 ng pertussis toxin dissolved in 100 μl of PBS/dose. Dp inhalation was performed with 8 mg of Dp dissolved in 8 ml of PBS/time. Intraperitoneal IL-12 consisted of 1 μg of IL-12 dissolved in 50 μl of PBS/dose and treated mice for 5 days (day −1 to +3) simultaneously with immunization indicated; i.p. PBS consisted of 50 μl of PBS/dose.

27 to 31 and from days 41 to 45. Our preliminary study has found that administration of IL-12 caused more severe adverse effects in Group 4 mice than in mice of Group 2. Furthermore, a short course of IL-12 delivery was designed for the purpose of treatment. Therefore, there were only two occasions of IL-12 treatment in Group 4 mice to explore the therapeutic effects of IL-12 on airway inflammation.

Mite allergen-specific antibody and total antibody assays

Der p I-specific IgE, IgG1 and IgG2a sera antibody titres were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtitre plates were coated with 4 μg/ml *Der p* I diluted in $NaHCO_3$ buffer, pH 8·2. After overnight incubation at 4°, plates were washed twice and blocked with 3% bovine serum albumin (BSA) in PBS for 2 hr at 37°. Serial dilution of sera were added to each well for an overnight period at 4°. Plates were washed and incubated with biotin-conjugated anti-mouse IgE (0·4 μg/ml, Serotec, Raleigh, NC) or IgG1 (1:500, PharMingen, San Diego, CA) or IgG2a (1:500, PharMingen) diluted in 3%BSA–PBS buffer for 1 hr at 37°. After further washes, streptavidin-conjugated alkaline phosphatase (1:2000, Sigma, St Louis, MO) was added for an additional 2 hr at room temperature. After extensive washing, wells were developed by phosphatase substrate p-nitrophenyl phosphate (pNPP) and absorbance at 405 nm was determined using a microplate reader. The levels of antibody were compared with IgG1, IgE and IgG2a standards with predetermined concentrations. (immunoglobulin concentrations: IgG1 = 28·2 μg/ml, IgE = 1·1 μg/ml, IgG2a = 16·7 μg/ml). For determination of serum total IgE level, microtitre plates were coated with 2 μg/ml of anti-mouse IgE (PharMingen) and blocked as described above. Serial dilutions of the sera and the IgE standard were added for 1 hr at 37° and then incubated with a biotin-conjugated anti-mouse IgE (1:500, PharMingen), followed by 1:2000 dilution of alkaline phosphatase-conjugated avidin and the substrate pNPP in reaction buffer. The levels of total IgG2a were measured by radial immunodiffusion (RID, The Binding Site, Birmingham, UK) method. The concentration was determined by measuring the ring diameter of the tested samples and reading off a RID reference table.

Antigen-specific proliferative assay

To measure the *Der p* I-specific T-cell proliferative response, the spleens were removed aseptically from rIL-12-treated or control (PBS-treated) mice 24 hr after the last allergen inhalation. The cells were plated into 96-well round-bottomed plates at a concentration of 3×10^5/well and were stimulated with 10 μg/ml of *Der p* I. In addition, phytohaemagglutinin (PHA; 10 μg/ml) was used as a positive mitogenic control and ovalbumin (4 μg/ml) was used as a negative control antigen. Control wells contained cells only. After 2 days in culture, the cells were pulsed with 1 μCi/well of [^{3}H]TdR for 15–17 hr. Specific incorporation of TdR was determined by β-counter (Packard Instrument Co., Meriden, CT) and results were expressed as c.p.m.

Cytokines assay

To measure cytokine secretion, splenocytes (1×10^7/well) of immunized mice treated with or without IL-12 after the last allergen inhalation were cultured in 0·5 ml AIM-V medium (serum-free lymphocyte medium, Gibco BRL, Grand Island, NY) supplemented with 2% TCM (mouse serum replacement, Celox Lab., Hopkins, MN) in the presence of 10 μg/ml *Der p* I or PHA (10 μg/ml) in a 48-well microtitre plate at 37° for 48 hr. After the culture, the culture supernatants were collected and centrifuged at 400 *g* at 4°. The cell-free supernatants were stored at −20° until they were used for the cytokine assay. The quantities of IL-5 and interferon-γ (IFN-γ) in the culture supernatants of spleen cells were evaluated by sandwich-ELISA (PharMingen). The levels of sensitivity for the IL-5 and IFN-γ assay were 60 pg/ml and 150 pg/ml, respectively.

Bronchoalveolar lavage (BAL) and histopathological study

At 24 hr after the last aerosol exposure, all groups of mice were bled from the retro-orbital venous plexus and killed. In addition, the naive mice exposed to aerosolized allergen were used as negative controls. The lung was immediately lavaged via the trachea cannula with 3 × 1 ml of Hanks' balanced salt solution (HBSS), free of ionized calcium and magnesium. The lavage fluid was centrifuged at 400 *g* for 10 min at 4°. After washing, the cells were resuspended in 1 ml HBSS and total cells were determined by counting in a haemocytometer. Cytocentrifuged preparations were stained with Liu's stain for differential cell counts. A minimum of 200 cells was counted and classified as macrophages, lymphocytes, neutrophils and eosinophils, based on standard morphological criteria.

To evaluate the effects of IL-12 treatment on allergen-induced lung inflammation, each group of animals was killed for histopathological examination. After the lavage, the lungs were immediately removed and fixed in 10% neutral-buffered formalin. The tissues were subsequently embedded in paraffin and cut into 5 μm thick sections. These frozen sections were stained with haematoxylin and eosin and examined using light microscopy for histological changes.

The levels of serotonin in BAL fluids

The levels of serotonin in lavage fluids were measured by serotonin ELISA kit (IBL, Hamburg, Germany) according to the manufacturer's recommendations. Absorbance values, read at 405 nm were converted to concentration in BAL fluids by comparison with a standard curve. The level of sensitivity for serotonin assay was 0·03 ng/ml. Because the sample preparation leads to a 207·25-fold dilution, the values read from the standard curve have to be corrected by multiplying by 207·25.

Statistical analysis

Individual experimental values were compared by the paired two-tailed Student's *t*-test. Differences between two groups were considered significant at $P < 0·05$.

RESULTS

Effects of IL-12 treatment on long-term antibody responses to *Der p* I allergen

To evaluate the *in vivo* impact of rIL-12 as vaccine adjuvant on the maintenance of Th1 activity and the capacity of rIL-12 to enhance Th1-associated responses under Th2-dominated conditions, administration of IL-12 was timed to interfere with either initial allergen sensitization (Group 2) or boosting allergen exposure (Group 4). In Groups 1 and 3, antibodies

of the IgG1 were produced in large amounts (Fig. 2a, Fig. 3a). Furthermore, substantial amounts of IgE were produced, whereas the synthesis of IgG2a was low (Fig. 2b, c; Fig. 3b, c). The data indicated allergic sensitization of these mice that normally develops in a Th2 manner. In Group 2, IL-12 treatment transiently suppressed *Der p* I-specific IgG1 (Fig. 2a, $P<0{\cdot}001$) and IgE (Fig. 2b, $P<0{\cdot}005$) responses but failed to show a difference from Group 3 since day 42. However, the IgG2a levels were strongly augmented and consistently maintained in Group 2 even without IL-12 treatment for a long period (Fig. 2c, $P<0{\cdot}001$). In Group 4, IL-12 treatment did not significantly down-regulate IgG1 production (Fig. 3a) and the serum IgE levels were not decreased until day 56 (Fig. 3b, $P<0{\cdot}05$). Whereas the synthesis of IgG2a was significantly up-regulated after two cycles of IL-12 treatment in Group 4 (Fig. 3c, $P<0{\cdot}001$).

The total IgE and IgG2a serum levels were summarized in Table 1. The results suggested that there was no significant difference in the level of total IgE among the four groups. It further indicated that the reduced levels of *Der p* I-specific IgE in mice of Group 2 and Group 4 were regulated by an antigen-specific process. In contrast, the level of total IgG2a was significantly enhanced as early as at day 28 in mice of Group 2 and such results were consistent with the level of *Der p* I-specific IgG2a antibodies. Nevertheless, the levels of total IgG2a showed no significant difference between the mice of Group 3 and Group 4.

Effects of IL-12 treatment on T-cell response to *Der p* I allergen

The results of *Der p* I-specific T-cell proliferative responses are summarized in Fig. 4. In Groups 2 and 4, proliferative responses were reduced 45% ($P\leq0{\cdot}05$) and 24% ($P\leq0{\cdot}001$), respectively. In some experiments, McKinght *et al.* have observed a 20–30% reduction of T-cell proliferation after IL-12 administration.[17] However, the reason for this phenomenon is yet to be defined.

To determine whether the *in vivo* administration of rIL-12 affects antigen-specific T-cell function and thereby inhibits antigen-induced eosinophil recruitment, the *in vitro* production of IFN-γ and IL-5 of spleen cells in IL-12-treated mice was examined. In Group 2, IL-12 treatment modestly enhanced IFN-γ secretion about fourfold (Fig. 5a, $P<0{\cdot}05$ versus Group 1) and markedly suppressed IL-5 production by 94% as compared with that of Group 1 control mice (Fig. 5b, $P<0{\cdot}001$). In Group 4, IL-12 treatment significantly elicited IFN-γ secretion about 12-fold (Fig. 5a, $P<0{\cdot}001$ versus Group 3) and inhibited IL-5 production by 85% (Fig. 5b, $P<0{\cdot}05$ versus Group 3).

Effects of IL-12 treatment on allergen-induced airway inflammation

The results of cellular composition and inflammatory mediators in BAL fluids were shown in Table 2. Consistent with our previous findings, the numbers of macrophages, neutrophils, lymphocytes and eosinophils in the mice of Groups 1 and 3 were significantly higher than in those of Groups 2 and 4, indicating *Der p* I-induced cell recruitment in these sensitized mice. In Group 4, IL-12 treatment decreased recruitment of allergen-induced eosinophils by 98% compared

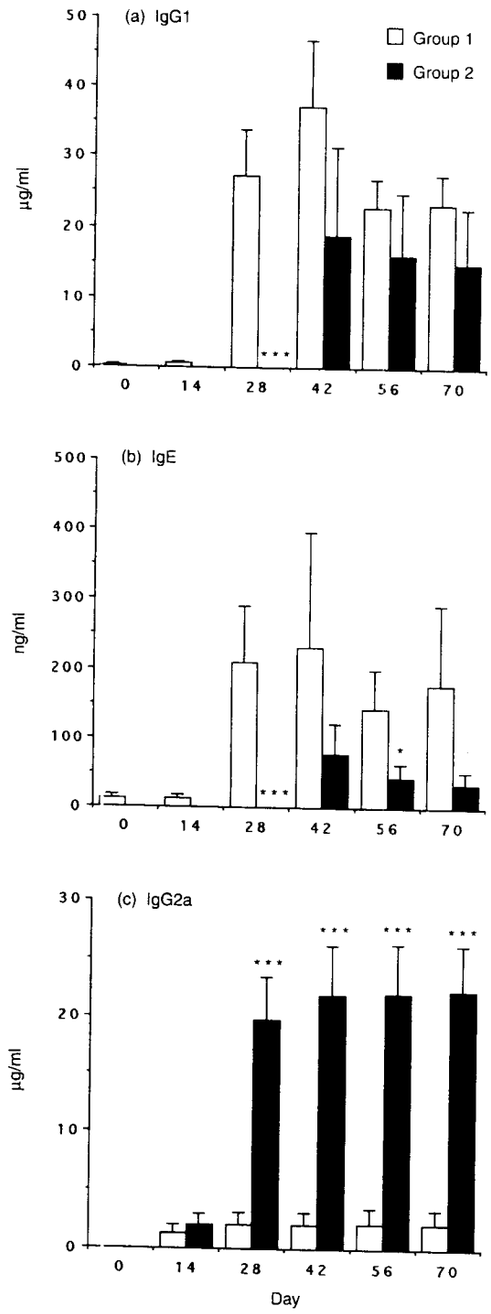

Figure 2. Serum IgG1, IgE and IgG2a antibody responses to *Der p* I. Groups 1 and 2 are described in the Materials and Methods. C57BL/6 mice were immunized i.p. three times with 10 μg/mouse *Der p* I adsorbed to alum plus pertussis toxin at biweekly intervals. In Group 2, IL-12 was administered i.p. at 1 μg/day for 5 days (day −1 to +3) to mice simultaneously with each systemic immunization. After 2 weeks, sensitized mice were aerosolized three times with Dp extract on days 42, 56 and 70. Blood was collected on the days indicated and sera levels of anti-*Der p* I antibodies IgG1 (a), IgE (b) and IgG2a (c) were assayed by using ELISA. Data are shown as mean ± SEM for eight mice per group. Significant differences (*$P<0{\cdot}05$; ***$P<0{\cdot}001$) from the immunized IL-12-untreated Group 1 are indicated.

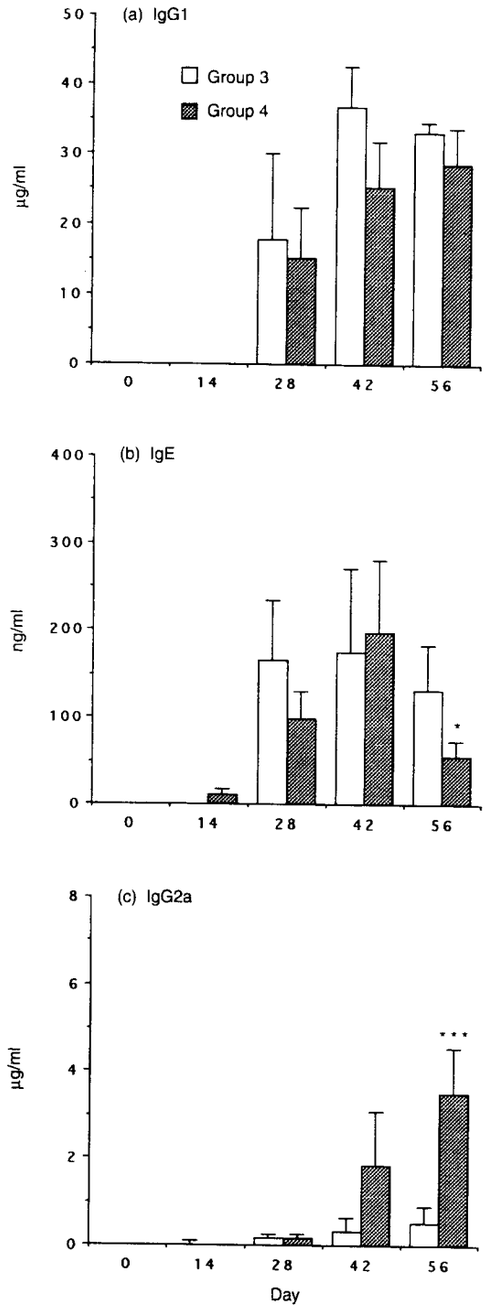

Figure 3. Serum IgG1, IgE and IgG2a antibody responses to *Der p* I. Groups 3 and 4 are described in the Materials and Methods. C57BL/6 mice were immunized i.p. four times with 10 μg/mouse *Der p* I adsorbed to alum plus pertussis toxin at biweekly intervals. In Group 4, IL-12 was administered i.p. at 1 μg/day for 5 days (day −1 to +3) to mice simultaneously with the third and fourth systemic immunizations. After 2 weeks, each group of sensitized mice was challenged with aerosolized Dp allergen. Blood was collected on the days indicated and sera levels of anti-*Der p* I antibodies IgG1 (a), IgE (b) and IgG2a (c) were assayed by using ELISA. Data are shown as mean ± *SEM* for eight mice per group. Significant differences (*$P<0{\cdot}05$; ***$P<0{\cdot}001$) from the immunized IL-12-untreated Group 3 are indicated.

to that of Group 3 ($P<0{\cdot}001$). Surprisingly, IL-12 treatment in Group 2 did not significantly affect the mean percentage of the eosinophils compared with that of Group 1. Collectively, it seemed that IL-12 treatment was more effective in inhibiting eosinophil recruitment in Group 4 than in Group 2. The percentage of neutrophils was decreased ($P<0{\cdot}05$ versus Group 1) following IL-12 treatment in Group 2, but was unaffected in Group 4 compared with that of Group 3. Compared with the control mice, the percentage of macrophages in both IL-12-treated groups were significantly increased ($P<0{\cdot}001$), whereas the percentage of lymphocytes did not change markedly.

Histopathological examination of lung taken from Groups 1 and 3 control mice after inhalation demonstrated that lung parenchyma was infiltrated with inflammatory cells (Fig. 6a, c). The infiltrates consisted of admixtures of predominantly eosinophils, neutrophils and lymphocytes. In contrast, the IL-12-treated animals were not noted to have histologically significant pulmonary inflammation (Fig. 6b, d).

In Fig. 7, the levels of serotonin were markedly increased in PBS-treated mice without IL-12 treatment. In Group 2, IL-12 administration led to a 36% reduction in serotonin levels compared with Group 1. Further, the levels of serotonin were strikingly reduced about 78% in Group 4 compared with that of Group 3.

DISCUSSION

The ability of endogenous IL-12 production to shape developing Th1 cells and their cytokine responses has stimulated much enthusiasm for the potential therapeutic use of IL-12. In several models of infectious diseases, exogenous administration of IL-12 exerts striking effects when given at the time of initial antigen exposure.[15,23–26] Similarly, prior studies in the murine model of allergic asthma have shown promise, with marked suppression of allergic responses after the *in vivo* administration of IL-12.[14,21,27] These studies have shown that IL-12 administration at the time of initial antigen sensitization inhibited airway eosinophilia, IL-4 and IL-5 expression, IgE production, and airway hyperresponsiveness in allergen-sensitized mice. However, the vast majority of studies have been short-term systems in which responses are evaluated soon after IL-12 treatment or following a single antigen, parasite, or viral challenge. In addition, the dramatic effects of IL-12 in these diseases are contingent on its administration in a narrow therapeutic window restricted by the time at which it must be administered to exert an effect. However, optimal treatment of allergic diseases requires that the cytokine profile of allergen-specific cells be redirected, with the conversion of Th2 profiles into Th1 cytokine profiles. Thus, this is thought to be the result of the difficulty of reversing a Th2 immune response once it has been established. In the murine model of airway inflammation, we have observed the effects of IL-12 administration to induce immune responses after multiple allergen challenges and to redirect Th1/Th2 balance after established Th2-dominated conditions.

To measure the persistence of the Th1 response in mice immunized with IL-12 as adjuvant, the antibody titre and isotype to *Der p* I have been determined following multiple inhaled allergen exposure in IL-12-treated mice. Despite the lower antigen-specific IFN-γ response from mice of early IL-12

Table 1. Total serum IgE and IgG2a levels in *Der p* I-immunized mice

Groups	Total IgE (μg/ml)			Total IgG2a (mg/ml)		
	day 0	day 28	day 56	day 0	day 28	day 56
1	0·1 ± 0·1	4·3 ± 0·5	4·7 ± 0·7	0·2 ± 0·1	1·1 ± 0·2	1·5 ± 0·1
2	0·1 ± 0·1	2·9 ± 0·9	5·9 ± 1·2	0·3 ± 0·1	7·1 ± 2·1*	14·1 ± 0·6*
3	0·2 ± 0·1	4·4 ± 0·6	4·9 ± 0·8	0·4 ± 0·1	2·6 ± 0·3	2·5 ± 1·1
4	0·1 ± 0·1	3·6 ± 0·4	5·1 ± 0·2	0·5 ± 0·1	1·8 ± 0·2	2·2 ± 1·6

Groups of mice were treated with IL-12 or PBS as in Fig. 1. Serum IgE and IgG2a levels were measured in samples collected on days 0, 28 and 56. Values are means ± SEM for eight mice in each group. *$P < 0.005$ compared to that of PBS-treated control mice.

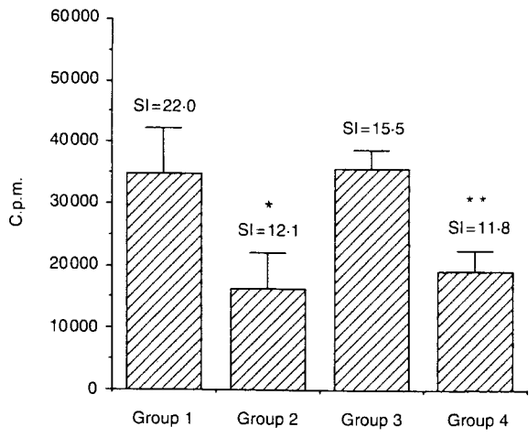

Figure 4. The effect of IL-12 on *Der p* I antigen-specific T-cell proliferative response. C57BL/6 mice were treated as described in Fig. 1. Following the last inhalation, spleen cells were taken from these sensitized mice after 24 hr and restimulated with 10 μg/ml *Der p* I *in vitro*. Proliferation was measured by [^{3}H]thymidine incorporation on day 3. The results are expressed as c.p.m. and shown as mean ± SEM for seven or eight mice per group. The background values of controls were between 1450 and 2534 c.p.m. Ovalbumin (4 μg/ml) was used as a negative control antigen and the responses were between 1045 and 3138 c.p.m. in this assay. The stimulation index (SI) was calculated as the mean c.p.m. of the stimulated wells divided by the mean c.p.m. of the control wells. Significant differences (*$P \leq 0.05$; **$P \leq 0.001$) from the immunized untreated controls are indicated.

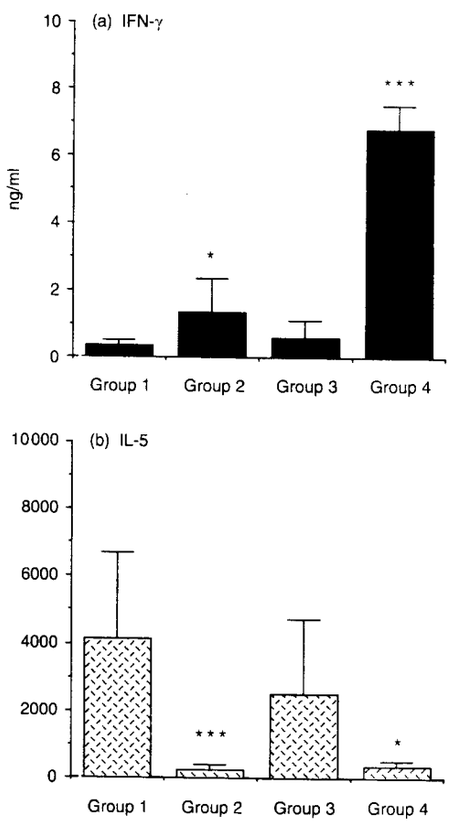

Figure 5. The effect of IL-12 on IFN-γ, IL-5 production by splenocytes of mice after the last allergen challenge. Splenocytes from IL-12 and control groups were stimulated with 10 μg/ml *Der p* I *in vitro* and culture supernatants were collected after 48 hr, and the levels of cytokine production IFN-γ (a) and IL-5 (b) were measured by ELISA. Values shown are mean ± SD of eight mice per group. Significant differences (*$P < 0.05$, ***$P < 0.001$) compared with paired control mice, respectively.

treatment (Group 2), IgG2a antibody persisted for as long as 40 days postimmunization with antigen in the absence of IL-12. The previous data demonstrated the IFN-γ level was much higher in early IL-12 treated mice without further repeated inhaled allergen challenge (data not shown). These allergen-specific Th1 or Th0 cells might be down-regulated by repeated challenge of inhaled allergen. However, allergen-specific IgG2a-secreting memory B cells are less susceptible to this modulation. In fact, Bliss *et al.* have examined the effects of IL-12 on recall responses to keyhole limpet haemocyanin and showed that antigen-specific IgG2a antibody promoted by IL-12 can last for 6 months.[28] However, in Group 2, the impact of IL-12 administration on antibody responses, such as *Der p* I-specific serum IgG1 and IgE production was transiently suppressed. This finding is consistent with that of German *et al.* in that they have demonstrated the suppression of IgE by IL-12 to be unstable and have even enhanced the synthesis of IgE antibodies when sensitized mice subsequently received repeated antigen challenge.[16]

Notably, there is discrepancy between findings regarding the BAL fluid and those regarding cytokine patterns in Group 2 mice. Because the synthesis of IL-5 in the splenocytes recall response to *Der p* I allergen is inhibited in the same group, the pathophysiological significance of increased eosinophils is

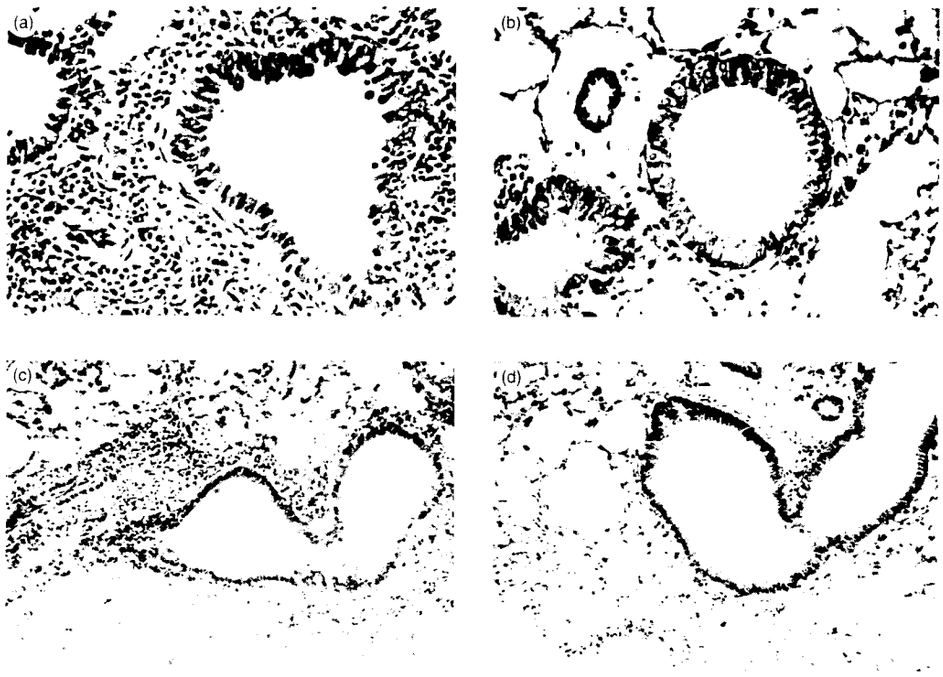

Figure 6. Representative light microscopic findings of PBS-treated mice (a, c) and IL-12-treated mice (b, d) (hematoxylin and eosin stain). Lung tissue of mice without IL-12 treatment (a, ×400, and c, ×200) demonstrates extensive cellular infiltration of the periairway regions. In contrast, lung tissue of IL-12-treated mice (b, ×400, and d, ×200) demonstrates complete absence of histologically significant inflammation.

questionable. It is possible that the lack of an effect of IL-12 on eosinophilia may have been caused by the long interval between the last dose of IL-12 and BAL in these IL-12-treated mice. IFN-γ is known to inhibit the eosinophilia and to inhibit aspects of mast cell functions, including the release of mediators such as serotonin.[29–31] Further, it has been reported that the up-regulating effects of IL-12 on IFN-γ expression last for up to 2 days but are lost by 8 days.[32] Therefore, such levels of IFN-γ in these mice were not enough completely to suppress eosinophil recruitment if the inhibitory effects of IL-12 on the antigen-induced eosinophilia and serotonin secretion were mediated by enhanced IFN-γ production. However, Brusselle *et al.* using IFN-γ receptor-deficient mice have demonstrated that inhibition of the allergen-induced airway eosinophilia by IL-12 is IFN-γ independent during the secondary allergen exposure.[33] Thus, it is possible that IL-12 may either directly inhibit eosinophil influx or stimulate the production of mediators other than IFN-γ that have such effects.

To examine the effects of IL-12 on allergen-induced changes when administered after the initial antigen presentation, once the T-cell development has been committed to a Th2 phenotype, mice were sensitized twice to *Der p* I on day 0 and 14, but were administered IL-12 only during subsequent antigen boosts from day 28. This study showed that IL-12 administration did not suppress the synthesis of *Der p* I IgG1 antibody, but modestly inhibited IgE response and enhanced the production of IgG2a after the second round of delayed IL-12 treatment in Group 4. Interestingly, the levels of total IgE and IgG2a antibodies were not significantly different between Group 3 and Group 4 during the follow-up. Because more long-term investigation of this immune response is clearly needed, it should not be concluded that IL-12 is unable to reverse the Th2 responses once Th2 effector cells are

Table 2. Change in total cell numbers and cellular composition in BAL of sensitized mice exposed to aerosolized allergen

		BAL				
		Total cells	Macrophages	Neutrophils	Lymphocytes	Eosinophils
Groups	Treatment i.p.	(1×10^5 cells/ml)	(%)	(%)	(%)	(%)
1	*Der p* I PBS (early)	10·9 ± 6·2	19·0 ± 4·1	36·0 ± 5·1	22·0 ± 4·1	23·0 ± 5·5
2	*Der p* I IL-12 (early)	1·7 ± 1·0	44·8 ± 8·5**	20·0 ± 10·1†	18·6 ± 6·1	16·6 ± 10·1
3	*Der p* I PBS (late)	8·3 ± 2·1	28·9 ± 5·4	32·2 ± 5·5	17·6 ± 5·0	21·3 ± 11·3
4	*Der p* I IL-12 (late)	1·5 ± 0·5	66·6 ± 10·4**	20·5 ± 10·4	12·5 ± 4·5	0·4 ± 0·2**

BAL was performed 24 hr after Dp inhalation in PBS- and IL-12-treated mice. The differential counts of BAL were determined and data are mean ± SEM for eight mice in each group. †$P < 0·05$; **$P < 0·001$ for difference from the mean value of corresponding response in PBS-treated control mice.

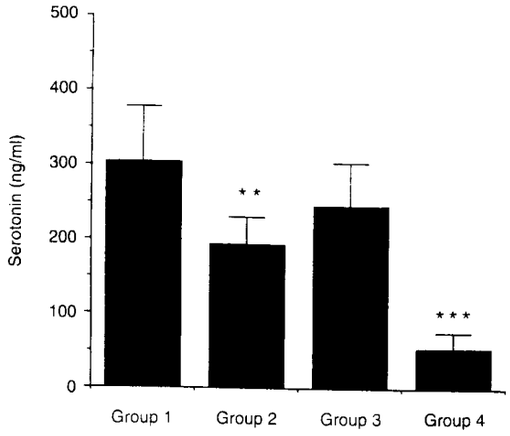

Figure 7. The effect of IL-12 on serotonin production in BAL of mice after the last antigen challenge. C57BL/6 mice were treated as described in Fig. 1. Mice were challenged with inhaled *Dermatophagoides pteronyssinus* and BAL samples were taken from the mice with allergen challenged after 24 hr. BAL fluids from each group of mice were measured by using a specific ELISA. Data represent the mean ± SD of eight samples per group. $^{**}P<0{\cdot}05$ compared with Group 1; $^{***}P<0{\cdot}001$ compared with Group 3.

dominated. However, it was found that administration of IL-12 resulted in up-regulation of IFN-γ production and down-regulation of IL-5 production in the IL-12-treated mice. Notably, delayed IL-12 treatment was effective in inhibiting antigen-induced eosinophilic inflammation after mite inhalation. Whether IL-12 is effective in redirecting immune responses to inhaled antigen when administered after initial antigen sensitization has been controversial. In contrast to these data, Sur *et al.* observed that IL-12 was not effective when given after the initiation of aerosolized antigen challenge.[32] On the other hand, Kips *et al.* demonstrated that, while IL-12 was given until the time of inhaled antigen challenge, it was effective in inhibiting eosinophil recruitment.[21] However, these studies covered only a few weeks of treatment (about 18–21 days) or were examined shortly after cytokine administration. Thus, compared to our results, the discrepancies in these studies are yet to be conclusive.

In conclusion, studies suggest that IL-12, as an adjuvant, promotes Th1 cells recall responses but did not suppress the development of Th2 cells after multiple antigen challenges in the absence of IL-12. Thus, the Th1-recall response may be suppressed by a Th2 response after repeated antigen challenges, so that repeated exposure to antigen and IL-12 is necessary to maintain a stable and dominated Th1 response. In addition, the present study indicates that IL-12 can inhibit IL-5 production, increase IFN-γ secretion and suppress antigen-induced airway inflammation in established Th2-type responses of *Der p* I-sensitized mice, despite the presence of circulating IgE. This supports the idea that IL-12 may be more useful as an immunotherapeutic agent than as a vaccine adjuvant in the treatment of such as atopic asthma.

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