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ORIGINAL PAPER

IL-12p40 is essential for the down-regulation of airway hyperresponsiveness in a mouse model of bronchial asthma with prolonged antigen exposure

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Summary

Background We previously reported a mouse model of bronchial asthma showing eosinophilic inflammation, but not airway hyperresponsiveness (AHR), after prolonged antigen exposure. This model showed an increase of IL-12 in the lung.

Objective The aim of this study was to investigate the role of IL-12p40 in a murine asthma model with prolonged antigen exposures.

Methods An ovalbumin (OVA)-induced asthma model was first established in wild-type (WT) and IL-12p40-deficient (IL-12p40$^{-/-}$) mice. Both strains of mice were further exposed to either OVA (prolonged exposure group) or phosphate-buffered saline (positive control group) 3 days per week for 3 weeks. During week 4, both groups of mice were given a final challenge with OVA.

Results Prolonged antigen exposures resulted in marked suppression of airway eosinophilia in both WT and IL-12p40$^{-/-}$ mice. However, AHR persisted in IL-12p40$^{-/-}$ but not in WT mice. There were no significant differences of IL-5, IL-13 or IFN-γ levels in bronchoalveolar lavage fluid between WT and IL-12p40$^{-/-}$ mice. The hydroxyproline content of the lung and peribronchial fibrosis were, however, significantly increased in IL-12p40$^{-/-}$ mice.

Conclusion The results suggest that endogenous IL-12p40 is essential for inhibition of AHR and peribronchial fibrosis, but not eosinophilic inflammation, in a murine asthma model with prolonged antigen exposures.

Keywords airway hyperresponsiveness, airway remodelling, bronchial asthma, IL-12
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Introduction

Bronchial asthma is characterized by the presence of a variable airflow limitation, chronic airway inflammation and airway hyperresponsiveness (AHR) [1]. The pathophysiology of AHR is complex, with many factors contributing to its development. Airway mucosal inflammation, which is the most important factor for the development of AHR, is characterized by an influx of activated eosinophils and T lymphocytes [2]. Numerous investigations have shown that Th2 cytokines, particularly IL-4, IL-5 and IL-13, play critical roles in orchestrating the allergic inflammatory response leading to AHR [3–5]. Furthermore, airway structural changes – remodelling – occur in response to persistent inflammation and contribute to AHR. Airway remodelling includes subepithelial fibrosis, hyperplasia of mucus glands, proliferation of myofibroblasts and smooth muscle cells and vascular changes [6]. However, a dissociation has sometimes been observed between AHR and airway inflammation [7–9].

We previously reported an example of the dissociation between eosinophilic airway inflammation and AHR in a mouse model with prolonged antigen exposure [10]. The prolonged ovalbumin (OVA) exposure almost completely abolished AHR in spite of the presence of attenuated, but significant, eosinophilic inflammation. Several groups have investigated the suppression of AHR and airway inflammation, but the mechanisms remain unresolved [11–13]. In this setting, we found that IL-12 levels in bronchoalveolar lavage fluid (BALF) were strikingly increased following prolonged OVA exposure, but were not increased in conventional short-term OVA-sensitized and -challenged mice.

IL-12 is a heterodimeric cytokine produced by activated macrophages, neutrophils and dendritic cells. The p40 subunit of IL-12 is shared by IL-23, a cytokine with

actions similar to but distinct from IL-12 [14]. Systemic administration of IL-12 has been reported to abolish AHR and pulmonary eosinophilia in a mouse model of allergic asthma [15–18]. IL-12p40-deficient (IL-12p40$^{-/-}$) mice have enhanced bone marrow and BAL eosinophilia after antigen challenge when compared with their wild-type (WT) controls [19]. A previous report suggested that differential effects of IL-12 blockade depended upon the timing of antigen dose administration in a mouse model of asthma [20]. During priming, blockade of IL-12 resulted in enhanced Th2 responses. In contrast, during airway challenges, blockade of IL-12 dampened AHR and Th2 inflammation in an IFN-γ-dependant manner. Thus, the various roles of IL-12 in allergic inflammation are complex.

Based on our previous observation that IL-12 levels in BALF were strikingly increased in a mouse model with prolonged antigen exposure, we hypothesized that such an increase of endogenous IL-12 may contribute to the attenuation of AHR. Using IL-12p40$^{-/-}$ mice, we attempted to elucidate the roles of IL-12p40 for AHR, eosinophilic inflammation and remodelling in the asthma model with prolonged antigen exposures. We also examined these phenotypes in an asthma model of IL-12p40$^{-/-}$ mice using conventional, short-term exposure to antigen. We found that endogenous IL-12p40 may selectively inhibit AHR and airway remodelling, but not eosinophilic inflammation, in the asthma model with prolonged antigen exposure.

Materials and methods

Mice

Homozygous IL-12p40$^{-/-}$ (B6.129S1-Il12b^{tm1Jm}) mouse strains on a C57BL/6 background, and age- and sex-matched control WT mice were purchased from Charles River Laboratories (Kanagawa, Japan). Animals were maintained in our facilities according to the guidelines for the ethical use of animals in research of Hiroshima University, Japan.

Protocols for ovalbumin-induced allergic airway inflammation

Figure 1 summarizes the protocols and schedules used for antigen or phosphate-buffered saline (PBS) administration to mice. All mice were sensitized on days 0 and 14 by intraperitoneal injection of 10 μg OVA (grade V; Sigma-Aldrich, St Louis, MO, USA) and 20 mg $Al(OH)_3$ in 0.2 mL of PBS (pH 7.4). After sensitization, animals were exposed to either aerosolized 1% OVA/PBS or PBS-only for 20 min once a day for 4 consecutive days (days 27, 28, 29 and 30). On day 31, mice were analysed for AHR, cell number and differentials in BALF.

Five days after the final challenge, a second set of mice was divided into two groups. For prolonged exposure, we used aerosolized OVA of a fixed concentration (1%). One group (prolonged exposure group) was exposed to 1% OVA aerosols (20 min) on 3 consecutive days per week for 3 weeks. The other group (positive control group) was exposed only to aerosolized PBS by the same schedule for 3 weeks. Beginning on day 56, both groups were challenged with 1% OVA aerosols for 3 consecutive days. All mice were killed on day 59.

Measurement of airway hyperresponsiveness and analysis of bronchoalveolar lavage fluid

AHR was determined 24 h after the final aerosol challenge. AHR was a measure of the total respiratory system

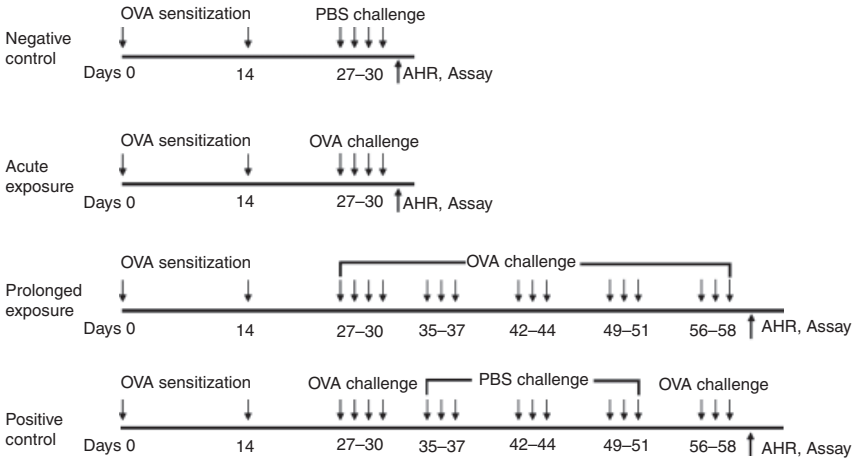

Fig. 1. Experimental protocols. All mice were sensitized with ovalbumin (OVA) plus alum on days 0 and 14, and then challenged with aerosolized OVA (1%) for 4 consecutive days. Mice from negative control and acute exposure groups were examined on day 31. Following acute exposure procedures, mice were divided into two treatment groups. Prolonged exposure group mice were repeatedly exposed to 1% OVA aerosol on 3 consecutive days per week for the next 3 weeks. Positive control group mice were exposed to phosphate-buffered saline (PBS) instead of OVA. Mice of these groups were finally challenged with 1% OVA aerosol for 3 days.

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resistance response. Mice were connected to a ventilator (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) at a rate of 160 breaths/min with a stroke volume of 175 μL. A plethysmograph (Buxco Electronics Inc., Troy, NY, USA) was sealed and readings were monitored by a computer. A pressure transducer measured alterations in tracheal pressure as a function of airway calibre. Once stabilized, mice were challenged with PBS, followed by increasing concentrations of methacholine (Mch; 0.5, 1, 2, 4, 8, 16 mg/mL). Aerosols were generated with the Aeroneb nebulizer system for mice (Buxco) and delivered to the inspiratory line. Each aerosol was delivered for a period of 3 min, during which pressure and flow data were continuously recorded, and a computer program (BioSystemXA; Buxco) was used to calculate lung resistance (R_L). Peak values were taken as the mean response to the concentration of Mch being tested. Analyses were based upon mean values of R_L vs. Mch concentration.

BALF was obtained as previously described [21]. Briefly, the trachea of each mouse was cannulated and lavaged three times with 0.5 mL PBS (total 1.5 mL). Cells in BALF were counted with a haemocytometer. The differential cell count was obtained by Diff-Quick using cytospin preparations (Shandon, Lipshaw, PA, USA).

Cytokine levels were determined with commercial ELISA kits according to the manufacturers' instructions. ELISA kits for IL-5, IL-12 (p70) and IFN-γ were from Endogen (Boston, MA, USA), IL-13, TGF-β1, monocyte chemoattractant protein-1 (MCP-1) and vascular endothelial growth factor (VEGF) were from R&D Systems (Minneapolis, MN, USA) and plasminogen activator inhibitor-1 (PAI-1) was from Innovation Research (Southfield, MI, USA). The detection limits were 5 pg/mL for IL-5, 12 pg/mL for IL-12, 1.5 pg/mL for IL-13, 10 pg/mL for IFN-γ, 4.61 pg/mL for TGF-β1, 2 pg/mL for MCP-1, 3.0 pg/mL for VEGF and 0.02 ng/mL for PAI-1.

Hydroxyproline assay

To evaluate lung fibrosis, hydroxyproline content of whole mouse lungs was measured in each group 24 h after final exposure as previously described [22].

Purification of RNA and quantitative reverse transcriptase-polymerase chain reaction

Murine left lungs were harvested 24 h after final OVA exposure and stored at −80 °C. Total RNA was extracted from lungs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further purified with RNeasy Mini Kit (Qiagen, Hilden, Germany). After extraction, total RNA was reverse-transcribed into cDNA, quantitative RT-PCR was performed using sequence-specific TaqMan primers and probes for the ABI 7500Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Murine IL-12 and ribosomal 18S protein (18S) were analysed. As previously described [23, 24], the measured mRNA levels were expressed as relative to 18S mRNA levels used as an endogenous reference.

Histology

Following bronchoalveolar lavage, the lungs were perfused with 10 mL PBS by puncture of the right ventricle. Lungs were removed and fixed with 4% paraformaldehyde, embedded in paraffin and cut into 5-μm sections. The sections were stained with haematoxylin and eosin, elastica-Masson's trichrome or Alcian blue at pH 2.5/periodic acid-Schiff (AB/PAS). The number of eosinophils, goblet cells (AB/PAS-positive cells) and total epithelial cells (total number of nuclei in the epithelium) were counted using an image analysis system. The slides stained with haematoxylin and eosin (for eosinophils) or AB/PAS (for goblet and total epithelial cells) were examined with a light microscope (AX80, Olympus, Tokyo, Japan) and image capture software (Lumina Vision, Ver2.20, Mitani Corporation, Tokyo, Japan). Eosinophils in the subepithelial area were counted (within the limits of 0.1 mm in depth under subepithelial basement membrane). Data are expressed as the number of cells per millimetre of subepithelial basement membrane.

The morphometric analysis of airway fibrosis used a method described previously with some modification [25]. Briefly, using recorded images of the intrapulmonary airway stained by elastica-Masson's trichrome, we measured a basement membrane perimeter (P_{bm}) and the external perimeter (P_{ex}) of the blue stained area. Assuming that both P_{bm} and P_{ex} are circular, the area of collagen deposition (A_{cd}) under basement membrane is given by:

$$A_{cd} = (P_{ex})^2/4\pi - (P_{bm})^2/4\pi.$$

Statistical analysis

Results are expressed as mean±SEM unless otherwise stated. Repeated measures ANOVA was used for AHR results. A Mann–Whitney *U*-test was used for comparisons between groups. A *P*-value of <0.05 was considered statistically significant. Analyses used SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Effects of prolonged antigen exposure in the asthma model for wild-type mice

Throughout this section, implied reference is made to Fig. 1 that summarizes the treatment schedules for the

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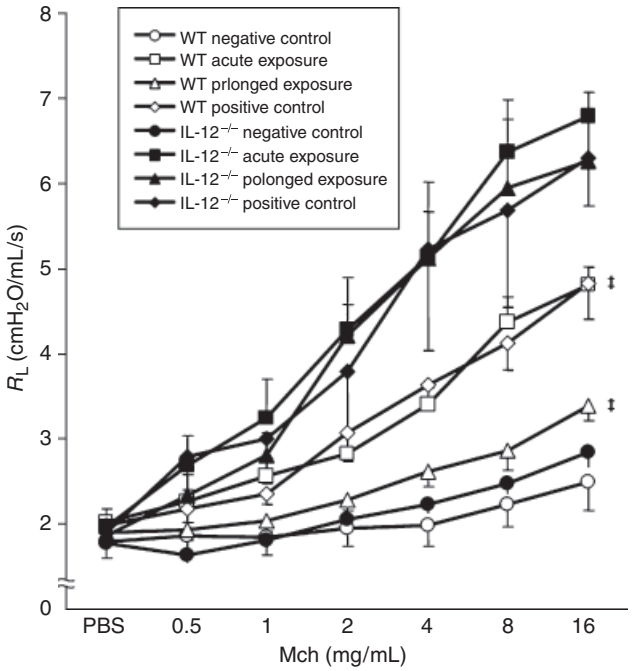

Fig. 2. Airway hyperresponsiveness (AHR) to methacholine challenge (Mch). Airway responsiveness was assessed by the lung resistance (R_L; see 'Materials and methods') for four treatment groups of mice (see Fig. 1). Open symbols are results for wild-type (WT) mice; filled symbols are results for IL-12p40$^{-/-}$ mice. Results are mean values±SEM of six to nine mice. There are significant differences between acute exposure, positive control and prolonged exposure groups of WT mice and the corresponding groups of IL-12p40$^{-/-}$ mice, as determined by repeated measures ANOVA: $^{\ddagger}P<0.05$.

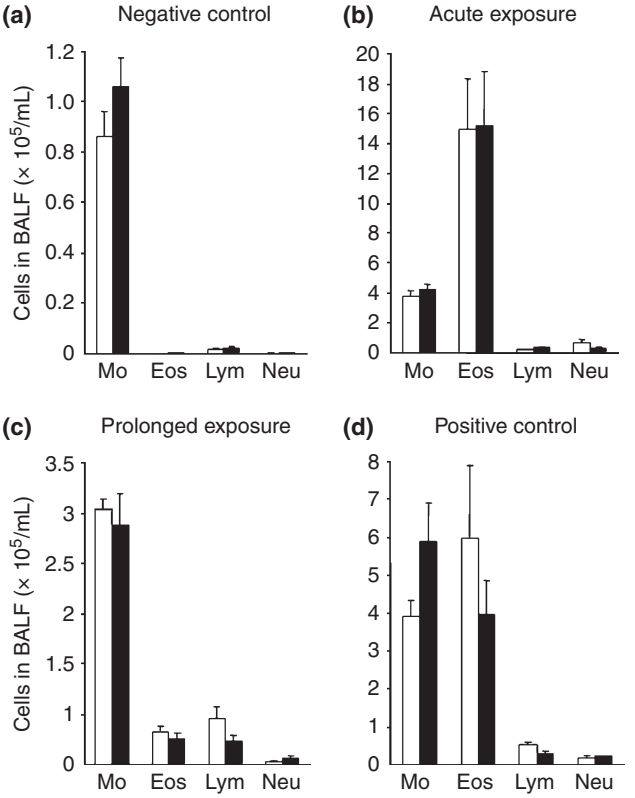

Fig. 3. Analysis of cells in bronchoalveolar lavage fluid (BALF). Open columns (□) are results for wild-type (WT) mice; filled columns (■) are results for IL-12p40$^{-/-}$ mice. (a) Negative control mice, (b) acute exposure mice, (c) prolonged exposure mice and (d) positive control mice. Results are mean values±SEM of six to nine mice.

experimental groups of mice. As shown in Fig. 2, using a conventional exposure schedule, the OVA-primed and challenged (acute exposure) WT mice developed a marked AHR to Mch challenge. Further exposure to antigen (prolonged exposure) resulted in significantly attenuated AHR, which is in accordance with previous findings [26, 27]. The PBS-treated WT mice (positive control) showed the same degree of AHR as the acute exposure mice.

As shown in Fig. 3, in the mice from the acute exposure group, severe BALF eosinophilia was observed [$(15.0\pm 3.3)\times 10^5\,mL^{-1}$]. Four weeks later, the number of eosinophils was decreased in the positive control group [$(6.0\pm 1.9)\times 10^5\,mL^{-1}$]. However, WT mice treated with aerosolized antigen (prolonged exposure group) showed a much more prominent decrease of eosinophils in BALF [$(0.3\pm 0.1)\times 10^5\,mL^{-1}$] when compared with the positive control group. Prolonged exposure to antigen resulted in a specific reduction of eosinophils without any significant effects on the other inflammatory cells in BALF (Fig. 3).

The level of IL-12 in BALF and expression of IL-12 mRNA in lung tissues in the prolonged exposure group were increased significantly when compared with the positive control and other groups (data not shown).

Effects of prolonged antigen exposure in the asthma model for interleukin-12-deficient mice

Because an association between an increase of IL-12 and prolonged antigen exposure was suggested in the WT mice, we explored the roles of IL-12 in phenotypic changes observed in prolonged exposure mice by using IL-12p40$^{-/-}$ mice. As shown in Fig. 2 for IL-12p40$^{-/-}$ mice, prolonged exposure to antigen did not result in attenuated airway responsiveness, as the same degree of AHR was observed comparable with that for the positive control and acute exposure groups. The AHR results of the acute exposure and positive control groups in IL-12p40$^{-/-}$ mice were significantly higher than those in WT mice, possibly due to contra-suppression of AHR by a lack of IL-12. There are significant differences between the acute exposure, positive control or prolonged exposure groups of WT and the corresponding groups of IL-12p40$^{-/-}$ mice.

Severe BALF eosinophilia was observed [$(15.2\pm 3.6)\times 10^5\,mL^{-1}$] for acute exposure IL-12p40$^{-/-}$ mice, which was comparable with the eosinophilia observed in WT mice (Fig. 3). Four weeks later, decreased eosinophilia

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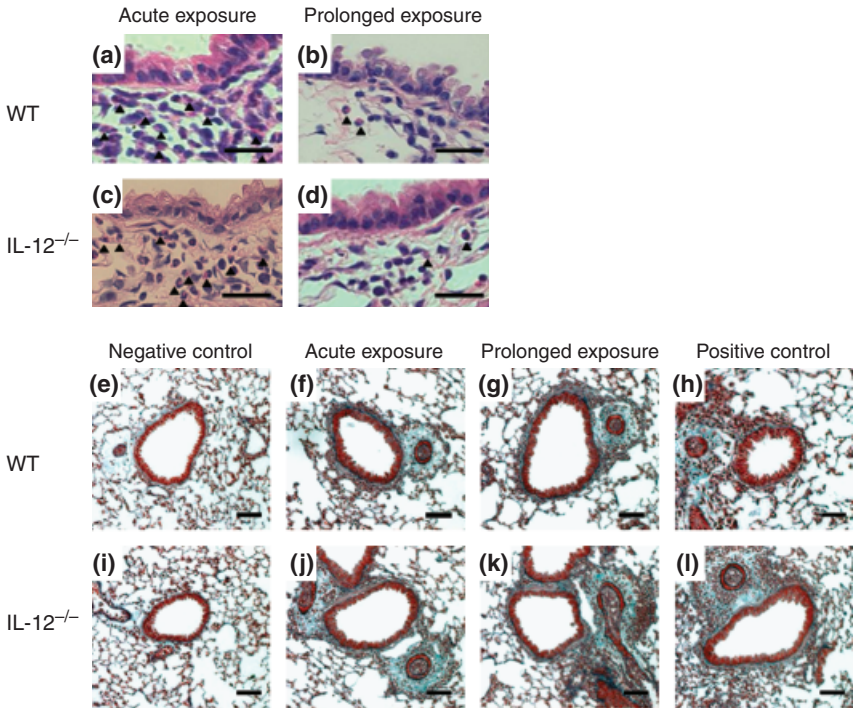

Fig. 4. Histological analyses of lung sections. Lung sections were stained with haematoxylin and eosin (a–d: bars = 50 μm; arrowheads show eosinophils) or elastica-Masson's trichrome (e–l: bars = 100 μm). Representative photomicrographs of the lungs from experimental groups in Fig. 1 are as follows: (1) negative control in wild-type (WT) mice (e) or in IL-12p40$^{-/-}$ mice (i), (2) acute exposure of WT mice (a, f) or IL-12p40$^{-/-}$ mice (c, j), (3) prolonged exposure in WT mice (b, g) or in IL-12p40$^{-/-}$ mice (d, k) and (4) positive control in WT mice (h) or in IL-12p40$^{-/-}$ mice (l).

was observed in the positive control group [$(4.0\pm 0.9)\times 10^5\,mL^{-1}$]. In the prolonged exposure group, the decreased numbers of eosinophils in BALF [$(0.3\pm1.1)\times 10^5\,mL^{-1}$] were observed. There were no significant differences in the numbers of inflammatory cells in BALF comparing WT and IL-12p40$^{-/-}$ mice (Fig. 3).

Comparisons of wild-type and IL-12p40$^{-/-}$ lung histological analyses

The panels in Fig. 4 show representative lung sections from WT and IL-12p40$^{-/-}$ mice. As described in the 'Materials and methods', three different staining techniques were used to reveal different histological characteristics of these sections (see legend to Fig. 4). Using haematoxylin and eosin (panels a–d), there were no significant differences of tissue eosinophilia between WT and IL-12p40$^{-/-}$ mice. Both WT and IL-12p40$^{-/-}$ mice in the acute exposure stage showed a marked subepithelial eosinophil-dominant cell infiltration (panels a and c) and less in the prolonged exposure groups (panels b and d).

Elastica-Masson's trichrome (panels e–l) was used to reveal peribronchial fibres. In WT mice, there were no significant differences in peribronchial collagen fibres among the acute exposure, prolonged exposure or positive control groups (panels f, g and h, respectively). However, in IL-12p40$^{-/-}$ mice, the collagen fibres around the airways were significantly accumulated in the prolonged exposure and positive control groups (panels k and l) compared with the acute exposure group (panel j).

Comparisons of wild-type and IL-12p40$^{-/-}$ lung morphometric analyses

Using morphometric analysis, there were no significant differences in subepithial eosinophil infiltration and the number of goblet cells between WT and IL-12p40$^{-/-}$ mice (Fig. 5a and b). In both strains, the prolonged exposure groups had a significantly reduced number of subepithelial eosinophils when compared with the positive controls, although the numbers in these groups were far less than the numbers in the acute exposure group (Fig. 5a). Peribronchial collagen fibres in the prolonged exposure and positive control groups in IL-12p40$^{-/-}$ mice were significantly higher than in WT mice (Fig. 5c). In WT mice, there were no significant differences in the total lung hydroxyproline contents among the acute exposure, prolonged exposure and positive control groups. However, in IL-12p40$^{-/-}$ mice, lung hydroxyproline contents of the prolonged exposure or positive control groups were significantly higher than the acute exposure group. In addition, for the prolonged exposure and positive control groups, hydroxyproline contents of IL-12p40$^{-/-}$ mice were significantly higher than in WT mice (Fig. 5d).

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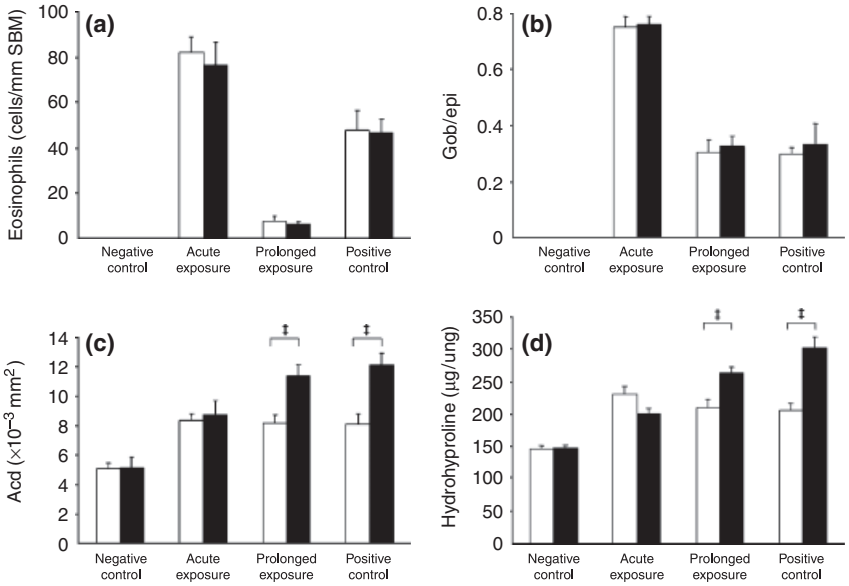

Fig. 5. Morphometric analysis of bronchial inflammation and airway remodelling. Open columns (□) are results for wild-type (WT) mice; filled columns (■) are results for IL-12p40$^{-/-}$ mice. (a) Subepithelial eosinophils expressed as the number of cells per millimetre subepithelial basement membrane (SBM), (b) goblet cell hyperplasia expressed as the goblet cells/epithelial cells ratio (Gob/epi), (c) area of collagen deposition (A_{cd}) of airway wall and (d) content of hydroxyproline in the lung. Results are mean values±SEM for $n = 6$–9 mice per group. Statistical comparisons were made between WT mice and IL-12p40$^{-/-}$ mice by Mann–Whitney U-test: $^{\ddagger}P < 0.05$.

Cytokines in bronchoalveolar lavage fluid from wild-type and IL-12p40$^{-/-}$ mice

Cytokine levels in BALF for WT and IL-12p40$^{-/-}$ mice were analysed, with the results shown in Fig. 6. Broadly, the cytokines studied were inflammation-associated (IFN-γ, IL-5 and IL-13; Fig. 6a–c) and pro-fibrotic (TGF-β1, VEGF, MCP-1 and PAI-1; Fig. 6d–g). Comparisons were made for the cytokine levels of different experimental groups between strains (WT vs. IL-12p40$^{-/-}$ mice).

From comparisons for the inflammation-associated cytokines, there were no significant differences in BALF concentrations of IFN-γ, IL-5 and IL-13 between WT and IL-12p40$^{-/-}$ mice (Fig. 6a–c). Also, the 'patterns' of inflammation-associated cytokines in different experimental groups were similar in both the WT and IL-12p40$^{-/-}$ mice. For the growth-promoting cytokines, TGF-β1 levels of the positive control group in IL-12p40$^{-/-}$ mice were significantly lower than in WT mice (Fig. 6d). In contrast, VEGF levels in BALF of these groups were significantly higher than WT mice (Fig. 6e). There were no significant differences of MCP-1 and PAI-1 levels between WT and IL-12p40$^{-/-}$ mice (Fig. 6f and g).

Discussion

In this study, we demonstrated that prolonged exposure to antigen resulted in diminished lung eosinophilia and AHR, but not remodelling, in a mouse model of asthma. Although these observed phenotypic changes in a prolonged antigen exposure group of mice were accompanied with a mild increase of IFN-γ and a decrease of IL-5 and IL-13, we were particularly interested in the marked increases of IL-12 protein and mRNA in the lungs of these mice. Using IL-12p40$^{-/-}$ mice, we demonstrated that IL-12p40 was essential for down-regulation of AHR, but not the eosinophilia, observed in the prolonged antigen-exposure mice. Furthermore, with regard to remodelling of the airways, peribronchial fibrosis was increased in IL-12p40$^{-/-}$ mice of the positive control and prolonged antigen exposure groups but not the acute exposure group, suggesting an anti-fibrotic role of IL-12p40 in the late phase of airway fibrosis. In summary, IL-12p40 may selectively inhibit AHR and peribronchial fibrosis, but not eosinophilic inflammation or goblet cell hyperplasia, in the prolonged stage for this asthma model.

It should be noted that we could not definitively conclude that IL-12 was responsible for the regulatory processes described in this paper, as we could not exclude a possible contribution of IL-23. The heterodimeric cytokine IL-23 consists of IL-12p40 and IL-23p19. Thus, IL-23, as well as IL-12, is deficient in IL-12p40$^{-/-}$ mice. Although the differential triggering and regulation of IL-12 vs. IL-23 production have been described [14], the roles for IL-23 in the pathophysiology of asthma are unclear. Meyts et al. demonstrated that the effects of an anti-IL-12p40-blocking antibody on the phenotype of the experimental asthma model mice were nearly the same as those of an anti-IL-12p35 antibody [28]. Because the anti-IL12p40 antibody also neutralized IL-23, this

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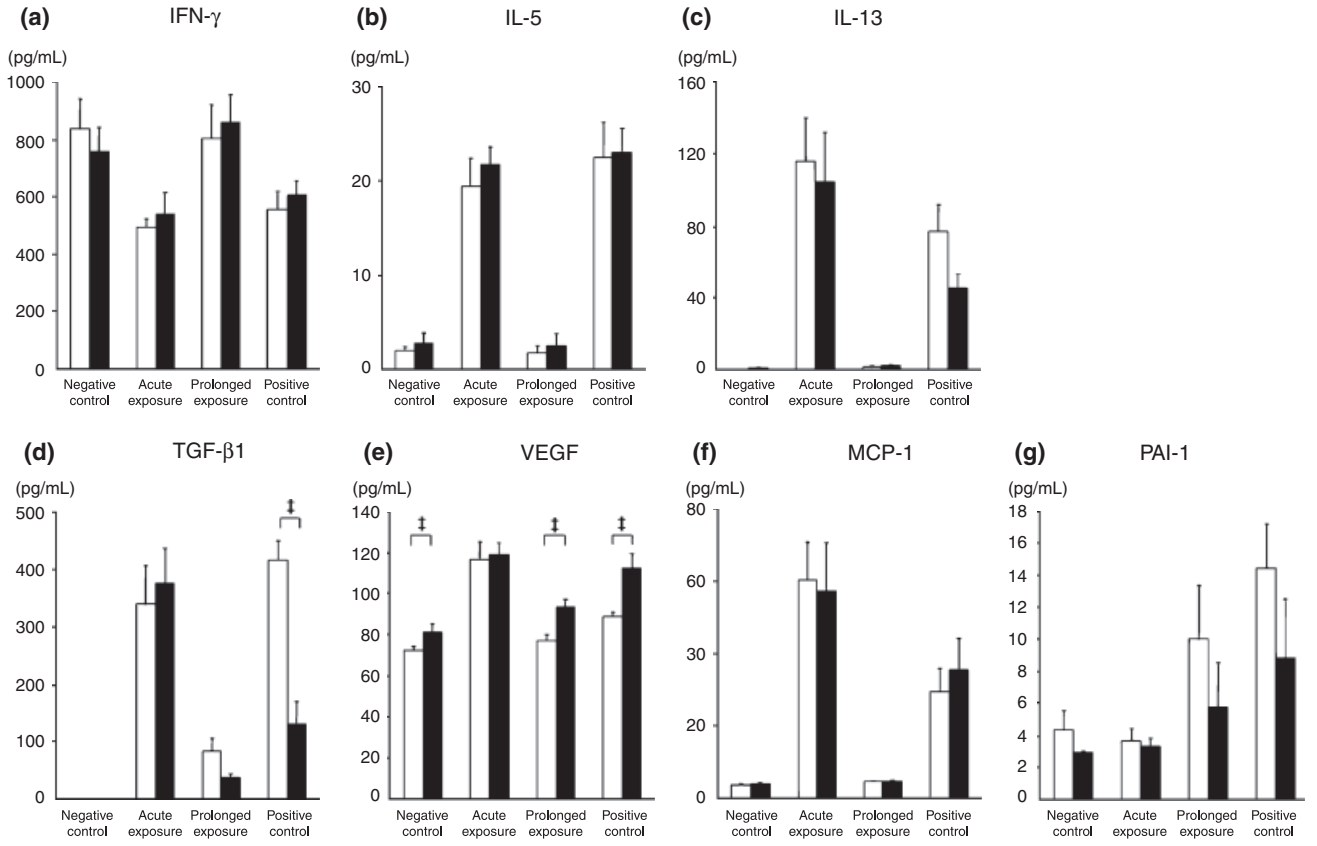

Fig. 6. Cytokine levels in bronchoalveolar lavage fluid (BALF). Open columns (□) are results for wild-type (WT) mice; filled columns (■) are results for IL-12p40$^{-/-}$ mice. (a) IFN-γ, (b) IL-5, (c) IL-13, (d) TGF-β1, (e) VEGF, (f) MCP-1 and (g) PAI-1 levels in BALF were measured by ELISA. Results are mean values±SEM for six to nine mice. Statistical comparisons were made between WT mice and IL-12p40$^{-/-}$ mice by Mann–Whitney *U*-test: $^{‡}P<0.05$.

study suggested that the blockade of IL-23 did not make significant differences for the phenotype of the mouse asthma model over that of IL-12 blockade alone [28]. However, the role of IL-23, if any, in the prolonged exposure model mice should be clarified in the future.

The mechanism for inhalational tolerance is unclear at present. Several candidates, such as anti-inflammatory cytokines, regulatory T cells, maturation disturbances of dendritic cells and diminished CD28 and ICOS on T cells, have been reported. But, these remain controversial [11–13]. Our results suggest that the down-regulation of AHR after prolonged antigen exposures was due to IL-12p40. The down-regulation of AHR may be due to increased levels of IFN-γ and decreased levels of IL-5 and IL-13 (Fig. 6). These cytokine level changes could be caused by increased IL-12 upstream effects in the lung, as IL-12 is known to induce Th1 cells and diminish pathological Th2 responses [29]. However, this would be unlikely. Only IL-12p40$^{-/-}$ mice of the prolonged antigen exposure group showed marked AHR in spite of similar amounts of BALF cytokines in both WT and IL-12p40$^{-/-}$ mice. Therefore, IL-12p40 *per se* would be important for down-regulation of AHR in our experimental model.

There were no significant differences of eosinophilic airway inflammation between the 'conventional' asthma model (acute exposure groups) of WT and IL-12p40$^{-/-}$ mice. Endogenous IL-12 has been reported to be needed for the full expression of eosinophilic inflammation [30]. OVA-primed and challenged IL-12p40$^{-/-}$ mice have significantly lower airway eosinophil counts than WT mice, and the decrease of eosinophils appeared to be due to decreased VCAM-1 expression in the lung of IL-12p40$^{-/-}$ mice [30]. The protocol used for this reported mouse model included twice the OVA sensitization and once the intranasal challenge and, therefore, the numbers of eosinophils in BALF were much smaller than observed in our present study. However, this may explain why the numbers of eosinophils we observed in positive control IL-12p40$^{-/-}$ mice tended to be less than that in positive control WT mice.

In our study, the airway remodelling in IL-12p40$^{-/-}$ mice for the positive control and the prolonged exposure groups was significantly more intense than WT mice. Previously reported regulators of airway fibrosis, including TGF-β1, MCP-1, VEGF and PAI-1, were investigated in the present study [31–34]. TGF-β1 levels of the positive control group in IL-12p40$^{-/-}$ mice were significantly

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lower than WT mice. The levels of VEGF in IL-12p40$^{-/-}$ BALF were significantly higher than WT mice. Furthermore, the levels of MCP-1 and PAI-1 were the same between WT and IL-12p40$^{-/-}$ mice. These results highlight that the relationship between cytokines and airway remodelling is, indeed, complex. A previous study demonstrated that pulmonary fibrosis and hydroxyproline content were increased in IL-12p40$^{-/-}$ compared with WT mice in bleomycin-induced lung fibrosis [35]. However, IL-12p40$^{-/-}$ mice exhibit decreased fibrosis in response to silica particles [36]. Although the role of IL-12 in pulmonary fibrosis is controversial, IL-12 may act directly upon fibrogenesis and inhibit airway fibrosis in asthma model mice.

In conclusion, IL-12p40 is essential for the down-regulation of AHR in a mouse model of asthma with prolonged antigen exposure. Prolonged antigen exposure in asthma model mice induces down-regulation of not only AHR but eosinophilia as well. However, IL-12p40 may be indispensable for down-regulation of airway eosinophilia. Our present study also disclosed an anti-fibrotic property of IL-12p40 for the airway fibrosis in asthma.

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