

Enhanced airway inflammation and decreased subepithelial fibrosis in interleukin 6-deficient mice following chronic exposure to aerosolized antigen

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Summary

Background Airway inflammation and remodelling are characteristic features of chronic asthma.

Objective To elucidate the role of interleukin (IL)-6 in airway responses to chronic antigen exposure.

Methods We compared airway inflammation, subepithelial collagen deposition, cytokine mRNA expression, and airway responsiveness between IL-6-deficient and wild-type (WT) mice following sensitization and repeated exposure to ovalbumin (OVA) three times a week for 8 weeks.

Results The repeated exposure to OVA induced infiltration of eosinophils, neutrophils, and lymphocytes into the airway, and caused thickening of the basement membrane and subepithelial fibrosis. IL-6-deficient mice exhibited more pronounced infiltration of these cells, a thinner basement membrane, and decreased subepithelial fibrosis, compared with WT mice. The repeated OVA exposure increased expression of IL-4, IL-13, eotaxin, monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor- β 1 mRNA in WT mice. Among these factors, expression of IL-13 and MCP-1 mRNA was further enhanced in IL-6-deficient mice, compared with WT mice. However, both WT and IL-6-deficient mice exhibited similar levels of airway responsiveness to increasing doses of methacholine, even after repeated exposure to OVA.

Conclusion These results suggest that IL-6 has dual roles in the chronic phase of asthma: down-regulation of inflammatory cell infiltration and enhancement of airway remodelling.

Keywords airway hyper-responsiveness, airway inflammation, airway remodelling, chemokines, chronic antigen exposure, cytokines, IL-6-deficient mice

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Introduction

Allergen-induced airway hyper-responsiveness (AHR) is associated with the influx of eosinophils into the airway in acute allergic asthma [1]. Chronic eosinophilic inflammation is invariably observed in the airway of patients with chronic asthma [2]. However, several lines of evidence have demonstrated dissociation between eosinophilic airway inflammation and AHR in patients with chronic asthma [3, 4]. Hence, other pathological changes such as airway remodelling may contribute to the AHR observed in asthmatic patients [5].

Recurrent injuries to the airway due to chronic exposure to allergens induce proliferation of subepithelial myofibroblasts [6], resulting in subepithelial fibrosis and an increase in types I–IV collagen, fibronectin, and tenascin within the lamina propria layer beneath the true epithelial basement membrane [7–9]. Resultant subepithelial fibrosis partially contributes to increased airway wall stiffness, poor reversibility of the airway, and poor responsiveness to inhaled corticosteroids even after a prolonged therapy with corticosteroids [10–12].

Cytokines and mediators produced and released by inflammatory cells, epithelial cells, and fibroblasts may be responsible for the myofibroblast proliferation and the subsequent subepithelial fibrosis [13]. However, the role of individual cytokines or mediators in this process remains to be elucidated.

IL-6 is a multi-functional cytokine that exerts important biological effects on inflammation, immunity, and stress [14, 15]. Accumulating evidence indicates that IL-6 levels increase in blood [16], bronchoalveolar lavage fluid (BALF) [17], and lung tissues [18] of asthmatic patients. Transgenic mice expressing the human IL-6 gene in their airways exhibit airway lymphocytic inflammation, airway thickening, and subepithelial fibrosis without an increase in airway resistance or responsiveness [19, 20]. These observations suggest the potential involvement of IL-6 in the airway remodelling process. However, because IL-6 has both pro- and anti-inflammatory properties [21–25], the precise roles of endogenous IL-6 in the airway remodelling process following chronic allergen challenge remain unclear.

Although *in vivo* animal models may provide useful information, most of the currently developed animal models of allergic airway inflammation are restricted to acute inflammatory changes following relatively short periods of allergen exposure, which do not simulate the clinical course of airway

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remodelling in chronic asthma. In addition, it is very difficult to separate the effects of IL-6 from the other cytokines in these situations. During chronic allergen exposure, it is also impossible to completely deplete and neutralize IL-6 by using monoclonal antibodies. We (unpublished data) previously observed that eosinophilic airway inflammation, IgE production, and Th2 polarization induced by acute exposure of ovalbumin (OVA) were reduced in OVA-sensitized IL-6-deficient mice compared with OVA-sensitized wild-type (WT) mice. This model may provide useful information on the mechanisms of acute asthmatic attack, but does not simulate the clinical course of airway remodelling in chronic asthma due to chronic exposure to allergens [26, 27]. Moreover, because IL-6 is multi-functional, it may exert distinct effects, depending on the phase of airway inflammation. Thus, in the present study, we evaluated the roles of endogenous IL-6 in chronic airway inflammation and remodelling induced by chronic allergen exposure in sensitized IL-6-deficient and WT mice.

Materials and methods

Reagents

OVA was obtained from Sigma Chemicals (St Louis, MO, USA), and aluminium hydroxide and methacholine were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Mice

Pathogen-free 8-week-old female BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan) and designated as WT mice in the following experiments. IL-6 gene-deficient mice were generated and backcrossed to BALB/c mice for six generations as previously described [28, 29], and were designated as IL-6-deficient (IL-6^{-/-}) mice. All animal procedures in this study complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals at Takaramachi Campus of Kanazawa University.

Experimental procedure

Sensitization of the mice to OVA was performed according to the method described by Temelkovski et al [30] with minor modifications. The mixed suspension of 50 µg OVA and 5 mg aluminium hydroxide in 0.1 mL physiological saline was administered intraperitoneally to mice twice at days 0 and 14. At day 21 and thereafter, mice were placed in a closed plastic inhalation chamber (length × width × height = 24 cm × 21 cm × 17 cm) and exposed to aerosol consisting of 3.0% w/v OVA suspended in physiological saline for 20 min, three times a week for 8 weeks. The aerosol, containing particle diameters between 1 and 10 µm, was generated and administered at a flow of 0.3 mL/min by using an ultrasonic nebulizer (Model C-13, Omron Co. Ltd, Tokyo, Japan). Control animals were treated similarly except that OVA and aluminium hydroxide were excluded from the solution.

Measurement of airway responsiveness

Twenty to 24 h after the last inhalation of antigen, mice were anaesthetized by an intraperitoneal injection of 250 µg

pentobarbital sodium and were placed in a supine position. After the trachea was cannulated with a 20-gauge nylon needle (inner diameter, 0.80 mm; length, 32 mm), the animals were artificially ventilated using a small-animal respirator (Model 1680, Harvard Apparatus Co. Inc., South Natick, MA, USA) adjusted to deliver a tidal volume of 0.05 mL/g at a rate of 80 strokes/min. The change in lung resistance to inflation, represented by the lateral pressure in the tracheal tube (pressure at the airway opening; P_{ao} , cmH₂O), was determined using a pressure transducer (Model TP-603T, Nihon Koden Kogyo Co. Ltd, Tokyo, Japan), and used as an overall index of airway responsiveness to methacholine as previously described [31]. Five minutes after the initiation of artificial ventilation, when the P_{ao} was stabilized, the mice were exposed to increasing doses of aerosolized methacholine (100, 200, 400, and 800 µg/mL) at 5-min intervals. The aerosol was produced over a 20-s period by an ultrasonic nebulizer (Model NE-U06, Omron Co. Ltd). The amount of aerosol was 15.2 µL/min. The P_{ao} was continuously measured as described above and expressed as a percentage of the baseline value.

Collection of BALF and differential cell count

After anaesthesia was administered and cannulation was performed as described above, animals were killed with an overdose of pentobarbital. BALF was then collected by an injection of 1 mL sterile saline into the lungs and withdrawal by gentle suction through an intratracheal needle. After the total cell number was determined using a Turk chamber (Erma, Tokyo, Japan), differential cell counts were determined using May-Grunwald-Giemsa-stained cytocentrifuged preparations by counting at least 400 cells according to standard morphologic criteria.

Histological analysis

Immediately after collection of BALF, the right lung was removed and stored in liquid nitrogen for total RNA isolation. The left lung was then inflated with 1 mL of 10% neutral buffered formalin and excised. After fixation overnight in formalin, the tissue was embedded in paraffin. The sections were cut in the longitudinal plane of bronchus and lung, stained in one batch with either haematoxylin-eosin (H-E) or elastin-van Gieson (EVG) solution. Then the sections were coded for morphometry. Histological analysis was performed by an observer without prior knowledge of the experimental procedures.

The thickness of the epithelial cell layer and basement membrane was measured on H-E-stained sections. For each of five animals per group, a section and an initial field of examination at one end of the section were randomly chosen. The analyses were made along the either side of longitudinally cut airway wall. The measurements were made only in trachea, bronchi, and bronchioles more than 150 µm in diameter and at locations where the epithelium appeared intact and well-orientated to minimize the influence of tangential sectioning, using an oil immersion objective ($\times 1000$) with the help of an eyepiece graticule as previously described [30, 32]. The data were recorded to the nearest half division on the reticle (0.5 µm). After the measurements were completed on

one field, the microscope stage was advanced at a linear distance of two microscopic fields and the same measurements were repeated. For an individual animal, the thickness of the epithelial cell layer and basement membrane was determined in an average of 20 fields and at least 75 points (mean 81 ± 3) at 20 μm intervals. The areas included equated to 1.6 mm of basement membrane length. Then, the mean thickness of the epithelial cell layer and basement membrane was calculated.

The density of eosinophils in the epithelial cell layer and the lamina propria was also measured in H-E-stained sections. The lamina propria was defined as the zone between the epithelial basement membrane and the luminal border of the tracheal cartilage plates and smooth muscle layer in bronchi or bronchioles. Eosinophils were readily identified on the basis of their specifically staining granules in cytoplasm. When eosinophils were counted in a field, the length of basement membrane directly underneath the epithelium was also measured. For each animal at least 15 fields, equivalent to 2.3 mm of epithelial basement membrane length, was analysed, and the number of cells per 100 μm basement membrane calculated. Measurements of the epithelial and basement thickness were undertaken in the same fields as for cell counting, with at least 23 (mean 28 ± 2) measurements made for each animal.

The thickness of subepithelial collagen deposition areas was analysed on EVG-stained sections in the same way as described above in the determination of thickness of epithelium basement membrane. On average, the positive zones beneath the epithelium were measured in 20 fields and 80 points on each section. The data were taken along 1.5–1.8 mm of basement membrane from which the mean thickness of subepithelial collagen layer was calculated for every mouse.

Extraction of total RNA and RT-PCR

Total RNAs were extracted from the right lung tissue by using RNeasyTM (Biotex Laboratory Inc., Houston, TX, USA) according to the manufacturer's instructions. Two micrograms of total RNA was reverse-transcribed for 10 min at 30 °C and subsequently for 1 h at 42 °C in a 20 μL reaction mixture containing murine Moloney leukaemia virus reverse transcriptase (ReverTra Ace-a[®], Toyobo, Osaka, Japan) and hexanucleotide random primers (Amersham Pharmacia Biotech Japan, Tokyo, Japan). Thereafter, 0.5 μL of cDNA product was amplified with Taq polymerase (Takara Shuzo, Tokyo, Japan) and sets of primers that specifically amplify mouse IL-4, IL-5, IL-13, IL-6, IFN- γ , monocyte chemoattractant protein-1 (MCP-1), eotaxin, TGF- β 1, platelet-derived growth factor A chain (PDGF-A), basic fibroblast growth factor (bFGF), and β -actin (Table 1) were used. A cycle consisted of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The amplification used 30 cycles except for the β -actin gene, which required only 25 cycles. The amplified products were electrophoresed through a 1.5% agarose gel and then stained with ethidium bromide. The intensity of each band was measured with the aid of NIH Image Analysis Software (version 1.62, National Institutes of Health, Bethesda, MD, USA) and the ratio to β -actin was determined. The relative intensity was calculated on the assumption that the ratio of the control WT mice was 1.00.

Table 1. Primer sequences of cytokine and growth factor gene products

Gene	Primer sequences	Product size (bp)
β -actin	Sense 5'-TGTGATGGTGGGAATGGGTCAG-3'	514
	Antisense 5'-TTTGATGTACGCACGATTTC-3'	
IL-6	Sense 5'-GCTCCCTACTTCACAAGTCC-3'	561
	Antisense 5'-GCAGGTTTGCCGAGTAGATC-3'	
IL-4	Sense 5'-CCTCAGCAACGAAGAACA-3'	393
	Antisense 5'-TTCAAGCATGGAGTTTCCC-3'	
IL-5	Sense 5'-CCCACAAAAGTGTGCAATG-3'	455
	Antisense 5'-GGTATGTGATCCTCCTGCGT-3'	
IL-13	Sense 5'-CAGCTCCCTGGTCTCTCAC-3'	353
	Antisense 5'-TGAGTCCACAGCTGAGATGC-3'	
IFN- γ	Sense 5'-ACTGGCAAAAGGATGGTGAC-3'	273
	Antisense 5'-TGAGCTCATTGAGATGCTTGG-3'	
TGF- β 1	Sense 5'-TGAGTGGCTGTCTTTGACG-3'	293
	Antisense 5'-TCTCTGTGGAGCTGAAGCAA-3'	
bFGF	Sense 5'-TTCAGTGGCTGAAGCACATC-5'	367
	Antisense 5'-TGGAAGTCGCTCTTCTTGGT-3'	
PDGF-A	Sense 5'-CAAGACCAGGACGGTCATTT-3'	315
	Antisense 5'-CCGGCTCTATCTCACCTCAC-3'	
MCP-1	Sense 5'-AAGCCAGCTCTCTCTTCTC-3'	247
	Antisense 5'-CCTCTCTCTTGAGCTTGGT-3'	
Eotaxin	Sense 5'-CTCCACAGCGTCTATTCC-5'	272
	Antisense 5'-TTTGGAGTTTTGGTCCACG-3'	

bFGF, basic fibroblast growth factor; PDGF-A, platelet-derived growth factor A chain; MCP-1, monocyte chemoattractant protein-1.

Statistical analysis

The mean and standard deviation (SD) were calculated for all parameters determined in this study. Statistical analyses were performed by using two-way ANOVA followed by Fisher's protected least significant difference. $P < 0.05$ was accepted as statistically significant.

Results

Analyses of BALF and lung tissues after chronic antigen exposure

We first evaluated the changes in BALF cells after chronic exposure to OVA. No significant differences were observed between non-sensitized WT and IL-6-deficient mice exposed to saline, in terms of total cell number and differential cell count (Fig. 1). Moreover, no apparent morphological differences were observed in the airways between the two animal groups (Figs 2a and b). Chronic exposure of OVA-sensitized WT mice to OVA resulted in significant increases in the total number of cells, neutrophils, eosinophils, and lymphocytes in BALF (Fig. 1). Histological analysis further demonstrated peribronchial and perivascular infiltration of inflammatory cells, predominantly consisting of eosinophils, and basement membrane thickening (Fig. 2c). Sensitized IL-6-deficient mice exposed to OVA exhibited augmented increases in the total number of cells, neutrophils, eosinophils, and lymphocytes, compared with WT mice (Fig. 1). Moreover, after chronic exposure to OVA, peribronchial, perivascular, and intra-epithelial eosinophil and neutrophil infiltrations were more conspicuous in IL-6-deficient mice than in WT mice (Fig. 2d).

Quantitative analyses verified that there were more eosinophils in the epithelium and lamina propria of the airway of IL-6-deficient mice than of WT mice after chronic OVA

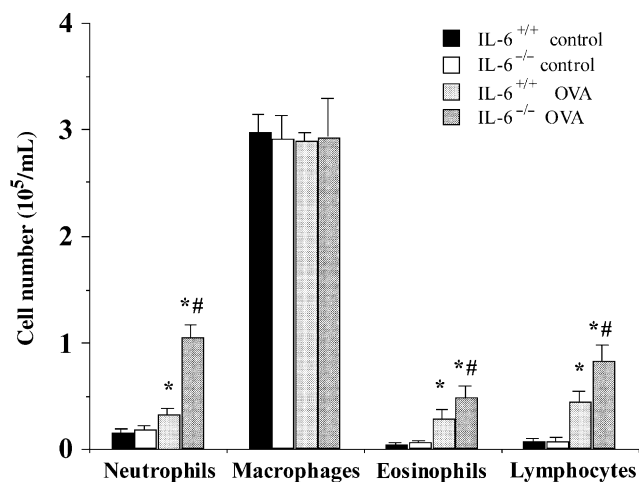


Fig. 1. Bronchoalveolar lavage cell differentials in wild-type (WT) and IL-6-deficient mice with or without sensitization and chronic exposure to ovalbumin (OVA) ($n = 6$). * $P < 0.05$ compared with WT and IL-6-deficient saline groups; # $P < 0.05$ compared with WT mice exposed to OVA.

exposure (epithelium: WT, 0.68 ± 0.14 ; IL-6-deficient mice, 0.85 ± 0.19 ; lamina propria: WT, 0.39 ± 0.07 ; IL-6-deficient mice, 0.53 ± 0.11 cells/100 μm basement membrane, $P < 0.05$), whereas basement membrane thickness was less evident in IL-6-deficient mice than in WT mice (Fig. 2d and Table 2).

We next evaluated changes in subepithelial collagen and epithelial thickness. Consistent with the morphological analyses of H-E-stained tissue samples, we observed no significant differences in subepithelial collagen content and epithelial thickness between IL-6-deficient and WT mice exposed to saline (Fig. 3 and Table 2). Chronic OVA exposure increased both subepithelial collagen and epithelial thickness in WT mice. IL-6-deficient mice exhibited an increase in subepithelial collagen thickness albeit to a lesser degree than in WT mice (Fig. 3 and Table 2). In contrast, chronic exposure of IL-6-deficient mice to OVA induced a small but significant enhancement in epithelial thickness, as compared with WT mice (Table 2).

mRNA expression of cytokines, chemokines, and growth factors

IFN- γ and IL-5 mRNAs were expressed constitutively in the lungs of both WT and IL-6-deficient mice, and their

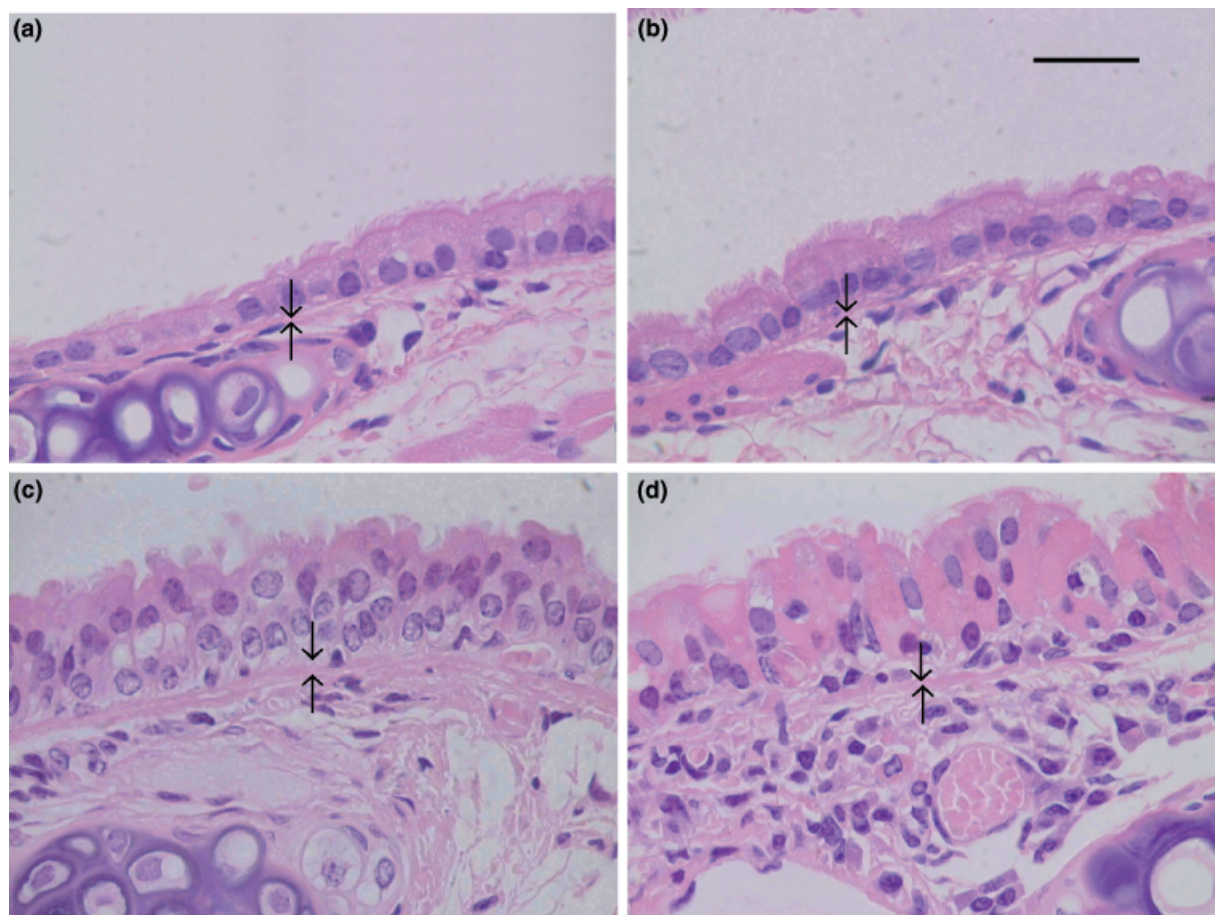


Fig. 2. Thickness of epithelial basement membrane and number of eosinophils in epithelium and lamina propria layer of airway in wild-type (WT) and IL-6-deficient mice with or without sensitization and chronic exposure to ovalbumin (OVA). Lung tissues were obtained from WT (a, c) and IL-6-deficient mice (b, d) exposed chronically to saline (a, b) and OVA (c, d), and were stained with haematoxylin–eosin solution. Representative results from five animals in each group are shown here. Original magnification, $\times 1000$, bar = 20 μm .

expression was not enhanced after chronic exposure to OVA. In contrast, IL-4 and IL-13 mRNA expression was enhanced in both WT and IL-6-deficient mice after chronic exposure to OVA (Fig. 4). Moreover, the increase in IL-13 but not IL-4 mRNA was more pronounced in IL-6-deficient than in WT mice, suggesting that chronic allergen exposure induces a more pronounced Th2 polarization in the absence of IL-6

Table 2. The thickness of basement membrane, subepithelial collagen layer, and epithelium of airway in IL-6^{+/+} and IL-6^{-/-} mice with or without sensitization and chronic exposure to ovalbumin (OVA) (mean $\pm$ SD, $n = 5$)

Group	Thickness of basement membrane (μm)	Thickness of subepithelial collagen layer (μm)	Epithelial thickness (μm)
IL-6 ^{+/+} Control	1.7 $\pm$ 0.2	3.0 $\pm$ 0.1	15.3 $\pm$ 1.0
IL-6 ^{-/-} Control	1.6 $\pm$ 0.2	3.0 $\pm$ 0.3	14.5 $\pm$ 0.9
IL-6 ^{+/+} OVA	2.5 $\pm$ 0.3*	4.1 $\pm$ 0.2*	19.4 $\pm$ 1.7*
IL-6 ^{-/-} OVA	2.1 $\pm$ 0.1*,**	3.7 $\pm$ 0.1*,**	21.7 $\pm$ 1.7*,**

* $P < 0.05$ compared with both control groups; ** $P < 0.05$ compared with IL-6^{+/+} OVA group.

gene. Among chemokines that we examined, MCP-1 mRNA expression was enhanced after chronic exposure to OVA and the enhancement was more evident in IL-6-deficient than in WT mice (Fig. 4).

Because increased collagen deposition was observed after chronic exposure to OVA, we evaluated mRNA expression of growth factors that exhibit fibrogenic activities, including TGF- β 1, PDGF-A, and bFGF. Among these growth factors, only TGF- β 1 mRNA expression was enhanced after chronic exposure to OVA (Fig. 4). However, no significant difference was observed in TGF- β 1 mRNA levels between WT and IL-6-deficient mice repeatedly exposed to OVA.

Airway responsiveness to methacholine

We lastly evaluated airway responsiveness to methacholine. Chronic exposure to OVA-induced AHR in both IL-6-deficient and WT mice, as compared with animals exposed to saline (Fig. 5). However, OVA-exposed IL-6-deficient mice exhibited similar levels of airway responsiveness as OVA-exposed WT mice.

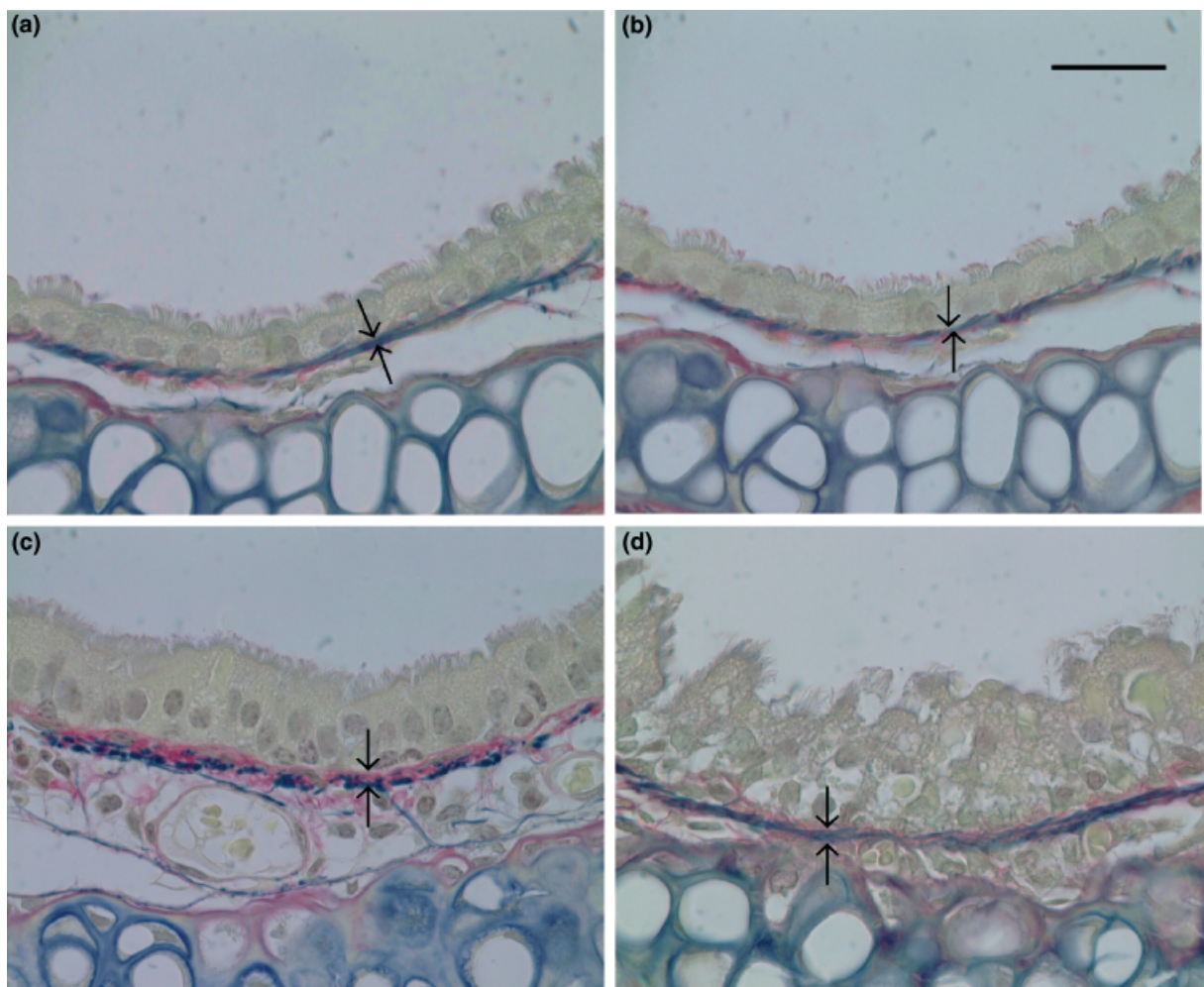


Fig. 3. Collagen deposition in subepithelial layer of airway in wild-type (WT) and IL-6-deficient mice with or without sensitization and chronic exposure to ovalbumin (OVA). Lung tissues were obtained from WT (a, c) and IL-6-deficient mice (b, d) exposed chronically to saline (a, b) and OVA (c, d), and were stained with elastin-van Gieson solution. Representative results from five mice in each group are shown here. Original magnification, $\times 1000$, bar = 20 μm .

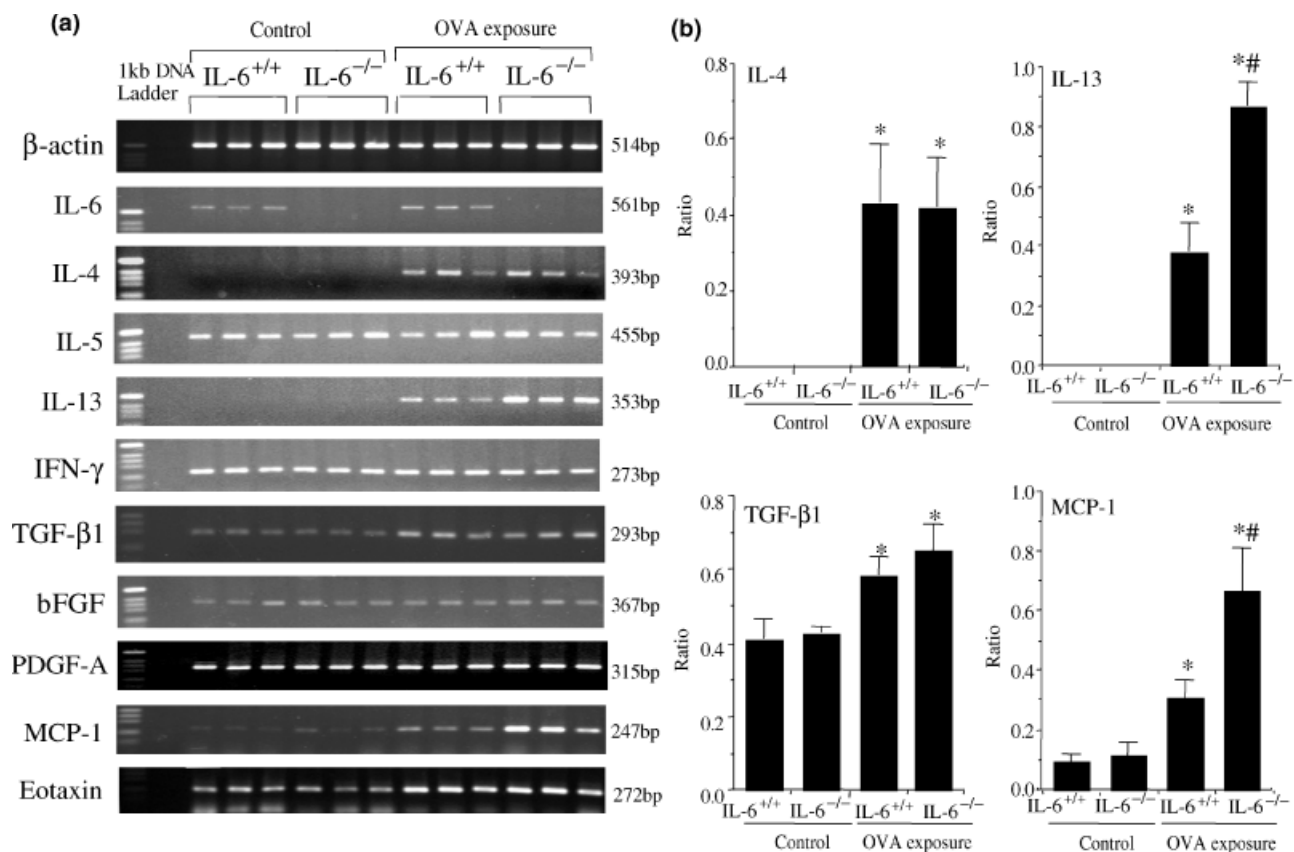


Fig. 4. RT-PCR analysis of mRNA expression of Th cytokines, growth factors, and chemokines in lung tissues from wild-type (WT) and IL-6-deficient mice with or without sensitization and chronic exposure to ovalbumin (OVA) ($n = 6$). * $P < 0.05$ compared with both saline groups; # $P < 0.05$ compared with WT mice exposed to OVA.

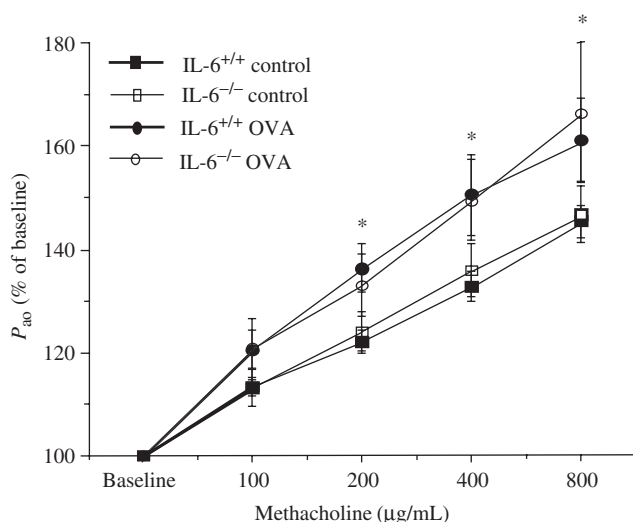


Fig. 5. Airway responsiveness to methacholine in wild-type (WT) and IL-6-deficient mice with or without sensitization and chronic exposure to ovalbumin (OVA) ($n = 6$). * $P < 0.05$ compared with WT and IL-6-deficient mice exposed to saline.

Discussion

To assess the role of endogenous IL-6 in chronic airway inflammation and airway remodelling in asthma, we examined the cellular components of BALF and subepithelial collagen

deposition in the airway of WT and IL-6-deficient mice following OVA sensitization and a 2-month long exposure to OVA. The results demonstrated that IL-6-deficient mice had less subepithelial fibrosis and decreased collagen deposition despite more conspicuous inflammatory cell infiltration into the airway compared with WT mice. However, the chronic exposure to OVA increased airway responsiveness to methacholine to the same degree in WT and IL-6-deficient mice.

Eosinophil infiltration into the airway is presumed to be regulated by Th2 cytokines and chemokines having chemotactic effects on eosinophils, and the infiltration contributes to the pathogenesis of asthma [33, 34]. Among Th2 cytokines, IL-5 plays an important role in eosinophil infiltration in concert with chemokines such as eotaxin and RANTES [34]. In the present study, chronic exposure to OVA augmented mRNA expression of eotaxin, but not IL-5 or RANTES, in WT and IL-6-deficient mice. We previously observed that a single OVA challenge enhanced IL-5 mRNA expression but not eotaxin or RANTES in both WT and IL-6-deficient mice sensitized by the same procedure (unpublished data). These observations suggest that the profile of cytokines regulating eosinophil infiltration may be different between acute and chronic asthma.

Accumulating evidence suggests a crucial role for Th2 cytokines, particularly IL-4 and IL-13 in allergen-induced asthma [35, 36]. Wang et al. [25] observed that IL-6 inhibited Th2-mediated airway inflammation but did not influence Th1 polarization in an acute allergen exposure model. However, in

our previous study, IL-6-deficient mice exhibited decreased eosinophil infiltration, reduced IL-5 and IL-13, but increased IFN- γ mRNA expression 24 h after a single allergen challenge, compared with WT mice (unpublished data). In contrast, in the present chronic allergen exposure model, IL-6-deficient mice exhibited more prominent eosinophil infiltration and enhanced IL-13 mRNA expression, compared with WT mice. It is possible that IL-6 may exert contradictory effects on Th2 polarization, depending on the phase of allergen exposure.

Many neutrophils and eosinophils are present in the airways of patients with moderate-to-severe asthma [37, 38]. Moreover, several lines of evidence suggest that production of lipid mediators, reactive oxygen intermediates, and proteases by neutrophils may contribute to airway obstruction, epithelial cell damage, and remodelling [39]. In the present study, after repeated exposure to OVA, the increase in neutrophils as well as eosinophils was more prominent in IL-6-deficient than in WT mice. Concomitantly, MCP-1 mRNA expression was enhanced in IL-6-deficient mice repeatedly exposed to OVA. Johnston et al. [40] observed that chronic immunization with adjuvant increased the expression of CCR2, a specific receptor for MCP-1, on neutrophils and that MCP-1 elicited an enhanced transendothelial migration of neutrophils, but not monocytes/macrophages, in chronic adjuvant-immunized animals. Thus, repeated exposure to OVA is believed to induce CCR2 expression on neutrophils and MCP-1 production in lungs, and the absence of endogenous IL-6 can further enhance MCP-1 expression, thereby resulting in augmented neutrophil accumulation in the airway.

IL-6-deficient and WT mice exhibited similar levels of AHR, despite different magnitudes of inflammatory cell infiltration and airway remodelling. This may be explained by the assumption that inflammatory cell infiltration and airway remodelling have profound and complicated effects on airway responsiveness. Infiltrating inflammatory cells release chemical mediators, cytokines, and chemokines in the local microenvironment, thereby directly affecting airway responsiveness. Concomitantly, these mediators can induce tissue injuries and subsequent tissue repair and remodelling consisting of an incremental increase in airway wall thickness and subepithelial fibrosis [13]. Increased airway wall thickness can enhance the effect of a given smooth-muscle contraction on airway resistance as explained by Poiseuille's law for laminar flow, and can contribute to AHR [41]. Subepithelial fibrosis in asthmatic airways may enhance airway narrowing by reducing the number of mucosal folds and changing the mucosal buckling pattern as calculated by Wiggs et al. [42]. In contrast to these assumptions, the intensity of airway inflammation is not always correlated with that of AHR [4]. Moreover, increased stiffness of the inner airway due to subepithelial fibrosis might reduce AHR and attenuate excessive airway narrowing [43]. Furthermore, an animal model of chronic allergen exposure revealed that subepithelial fibrosis could enhance or reduce AHR, depending upon the time of allergen exposure and the intensity or distribution of remodelling [44].

After chronic exposure to OVA, IL-6-deficient mice exhibited more pronounced inflammatory cell infiltration and reduced airway remodelling as represented by subepithelial fibrosis, compared with WT mice. These observations suggest

that fibrotic changes can occur independent of inflammatory cell infiltration. Accumulating evidence indicates that myofibroblasts are responsible for extracellular matrix protein accumulation and subsequent fibrotic changes in organs and that myofibroblast proliferation and activation are the first step leading to the fibrotic changes [6, 45]. Thus, inflammatory cell infiltration may not be directly associated with myofibroblast proliferation and activation.

Among the fibrogenic factors that we examined, only TGF- β 1 mRNA expression was modestly enhanced after prolonged exposure to OVA in both WT and IL-6-deficient mice. No apparent difference was observed in PDGF-A or bFGF mRNA expression levels. Although we cannot exclude the possibility that a minor but significant change in fibrogenic factor mRNA expression occurred below the detection limit of our RT-PCR because it was performed on whole lung tissue in spite of airway fragments, these results suggest that IL-6 exerts a direct effect on fibrotic changes. The effects of IL-6 on fibroblast proliferation and collagen production are still unresolved [46–49]. However, we previously observed that prolonged injection of carbon tetrachloride caused collagen accumulation in the liver of WT, but not IL-6-deficient, mice [29]. Overexpression of human IL-6 in the airway of transgenic mice has been shown to result in subepithelial fibrosis and airway thickening [20]. In addition, IL-6 has been shown to induce hepatic myofibroblast proliferation [48]. Thus, we favour the notion that the absence of IL-6 directly attenuated fibrotic changes in the present animal model. If so, IL-6 may be a novel target for preventing airway remodelling of chronic asthma.

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References

- 1 Rossi GA, Crimi E, Lantero S et al. Late-phase asthmatic reaction to inhaled allergen is associated with early recruitment of eosinophils in the airway. *Am Rev Respir Dis* 1991; 144:379–83.
- 2 Bousquet J, Chanez P, Lacoste J-Y et al. Eosinophilic inflammation in asthma. *J Clin Invest* 1990; 323:1033–9.
- 3 Ollerenshaw SL, Woolcock AJ. Characteristics of the inflammation in biopsies from large airways of subjects with asthma and subjects with chronic airflow limitation. *Am Rev Respir Dis* 1992; 145:922–7.
- 4 Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 1998; 157:4–9.
- 5 Wiggs BR, Bosken C, Pare PD, James A, Hogg JC. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1992; 145:1251–8.
- 6 Brewster CEP, Howarth PH, Djukanovic R, Wilson J, Holgate ST, Roche WR. Myofibroblast and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1990; 3:507–11.
- 7 Roche WR, Beasley R, Williams JH, Holgate ST. Sub-epithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989; 1:520–4.

- 8 Wilson JW, Li X. The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway. *Clin Exp Allergy* 1997; 27:363–71.
- 9 Laitinen A, Altraja A, Kampe M, Linden M, Virtanen I, Laitinen LA. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med* 1997; 156:951–8.
- 10 Lange P, Parner J, Vestbo J, Schnohr P, Jensen GA. A 15-year follow-up study of ventilatory function in adults with asthma. *N Engl J Med* 1998; 339:1194–200.
- 11 Chetta A, Foresi A, Del Donno M, Bertorelli G, Pesci A, Olivieri D. Airway remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest* 1997; 111:852–7.
- 12 Selroos O, Pietinalho A, Lofroos A, Riska H. Effect of early vs late intervention with inhaled corticosteroids in asthma. *Chest* 1998; 108:1228–34.
- 13 Kips JC, Pauwels RA. Airway remodeling: does it occur and what does it mean? *Clin Exp Allergy* 1999; 29:1457–66.
- 14 Kishimoto T, Akira S, Narazaki M, Taga T. Interleukin-6 family of cytokines and gp 130. *Blood* 1995; 86:1243–54.
- 15 Papanicolaou DA, Wilder RL, Manolagas SC, Chrousos GP. The pathophysiologic role of interleukin-6 in human disease. *Ann Intern Med* 1998; 128:127–37.
- 16 Yokoyama A, Kohno N, Fujino S et al. Circulation interleukin-6 in patients with bronchial asthma. *Am J Crit Care Med* 1995; 151:1354–8.
- 17 Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* 1992; 89:958–67.
- 18 Marini M, Vittori E, Hollemborg J, Mattoli S. Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J Allergy Clin Immunol* 1992; 89:1001–9.
- 19 DiCosmo BF, Geba GP, Picarella D et al. Airway epithelial cell expression of interleukin-6 in transgenic mice. Uncoupling of airway inflammation and bronchial hyperreactivity. *J Clin Invest* 1994; 94:2028–35.
- 20 Kuhn C, Home RJ, Zhu Z et al. Airway hyperresponsiveness and airway obstruction in transgenic mice. Morphologic correlates in mice overexpressing interleukin (IL)-11 and IL-6 in the lung. *Am J Respir Cell Mol Biol* 2000; 22:289–95.
- 21 Vercelli D, Jabara H, Arai K, Yokota T, Geha RS. Endogenous IL-6 plays an obligatory role in IL-4 dependent IgE synthesis. *Eur J Immunol* 1989; 19:1419–24.
- 22 Rincon M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4 producing CD4⁺ T cells. *J Exp Med* 1997; 185:461–9.
- 23 Denis M. Interleukin-6 in mouse hypersensitivity pneumonitis; changes in lung free cells following depletion of endogenous IL-6 or direct administration of IL-6. *J Leukoc Biol* 1992; 52:197–201.
- 24 Xing Z, Gauldie J, Cox G et al. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* 1998; 101:311–20.
- 25 Wang J, Homer RJ, Chen Q, Elias JA. Endogenous and exogenous IL-6 inhibit aeroallergen-induced Th2 inflammation. *J Immunol* 2000; 165:4051–61.
- 26 Panettieri Jr RA, Murray PK, Eszterhas AJ, Bilgen G, Martin JG. Repeated allergen inhalations induce DNA synthesis in airway smooth muscle and epithelial cells *in vivo*. *Am J Physiol* 1998; 274:L417–24.
- 27 Trifilieff A, El-Hashim A, Bertrand C. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. *Am J Physiol* 2000; 279:L1120–8.
- 28 Kopf M, Baumann H, Freer G et al. Impaired immune and acute phase response in interleukin-6-deficient mice. *Nature* 1994; 368:339–42.
- 29 Natsume M, Tsuji H, Harada A et al. Attenuated liver fibrosis and depressed serum albumin levels in carbon tetrachloride-treated IL-6-deficient mice. *J Leukoc Biol* 1999; 66:601–8.
- 30 Temelkovski J, Hogan SP, Shepherd DP, Foster PS, Kumar PK. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax* 1998; 53:849–56.
- 31 Sogur N, Fujimura M, Mizuhashi K, Saito M, Xiu QY, Matsuda T. Involvement of thromboxane A2 in propranolol-induced bronchoconstriction after allergic bronchoconstriction in guinea pigs. *Am J Respir Crit Care Med* 1994; 149:1488–93.
- 32 Sullivan P, Stephens D, Ansari T, Costello J, Jeffery P. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. *Eur Respir J* 1998; 12:811–5.
- 33 Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airway hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996; 183:195–201.
- 34 Sahabuddin S, Ponath P, Schleimer RP. Migration of eosinophils across endoepithelial cell monolayers: interactions among IL-5, endothelial-activating cytokines, and C-C chemokines. *J Immunol* 2000; 164:3847–54.
- 35 Gavett SH, O'Hearn DJ, Karp CL et al. Interleukin-4 receptor blockade prevents airway responsiveness induced by antigen challenge in mice. *Am J Pathol* 1997; 272:L253–61.
- 36 Wills-Karp M, Luyimbazi J, Xu X et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998; 282:2258–60.
- 37 Sur S, Crotty TB, Kephart GM et al. Sudden-onset fatal asthma. A distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa? *Am Rev Respir Dis* 1993; 148:713–9.
- 38 Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 1995; 95:843–52.
- 39 Sampson AP. The role of eosinophils and neutrophils in inflammation. *Clin Exp Allergy* 2000; 30 (Suppl. 1):22–7.
- 40 Johnston B, Burns AR, Suematsu M, Issekutz TB, Woodman RC, Kubes P. Chronic inflammation upregulates chemokine receptors and induces neutrophils migration to monocyte chemoattractant protein-1. *J Clin Invest* 1999; 103:1269–76.
- 41 Moreno RH, Hogg JC, Pare PD. Mechanics in airway narrowing. *Am Rev Respir Dis* 1986; 133:1171–80.
- 42 Wiggs BR, Hrousis CA, Drazen JM, Kamm RD. On the mechanism of mucosal folding in normal and asthmatic airways. *J Appl Physiol* 1997; 83:1814–21.
- 43 Lambert RK, Codd SL, Alley MR, Pack RJ. Physical determinants of bronchial mucosal folding. *J Appl Physiol* 1994; 77:1206–16.
- 44 Palman E, Kips JC, Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. *Am J Respir Crit Care Med* 2000; 161:627–35.
- 45 Kuhn C, McDonald JA. The roles of myofibroblast in idiopathic pulmonary fibrosis: ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *Am J Pathol* 1991; 138:1257–65.
- 46 Shahar I, Fireman E, Topilsky M et al. Effect of IL-6 on alveolar fibroblast proliferation in interstitial lung diseases. *Clin Immunol Immunopathol* 1996; 79:244–51.
- 47 Jikko A, Wakisaki T, Iwamoto M et al. Effects of interleukin-6 on proliferation and proteoglycan metabolism in articular chondrocyte cultures. *Cell Biol Int* 1998; 22:615–21.
- 48 Choi I, Kang HS, Yang Y, Pyun KH. IL-6 induces hepatic inflammation and collagen synthesis *in vivo*. *Clin Exp Immunol* 1994; 95:530–5.
- 49 Gallucci RM, Simeonova PP, Matheson JM et al. Impaired cutaneous wound healing in interleukin-6-deficient and immunosuppressed mice. *FASEB J* 2000; 14:2525–31.