

Reduced Serum Antibody Production and Acute Airway Inflammation in Interleukin 6-deficient Mice Challenged with Ovalbumin

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ABSTRACT

Background: IL-6 is a unique cytokine that has pro- and anti-inflammatory property. The purpose of this study was to clarify the role of IL-6 in the allergic airway inflammation.

Methods: We challenged wild-type and IL-6-deficient mice with ovalbumin (OVA) inhalation after sensitization to OVA.

Results: OVA challenge induced an intrapulmonary eosinophil infiltration and airway hyperresponsiveness in wild-type mice with increased serum IgE levels and enhanced intrapulmonary mRNA expression of T helper type 2 (Th2) cytokines, IL-5 and IL-13. IL-6-deficient mice developed a similar level of airway hyperresponsiveness despite an attenuated eosinophil infiltration and reduced elevation in serum IgE levels after OVA challenge. After OVA challenge, intrapulmonary IL-5 and IL-13 mRNA expression was less evident in IL-6-deficient mice than wild-type mice, while intrapulmonary IFN- γ mRNA expression was more enhanced. Intravenous administration of anti-IL-6 antibodies during sensitization period in wild-type mice caused similar effects as observed in IL-6 deficient mice.

Conclusions: Endogenous IL-6 may have important roles in Th2 polarization in sensitization period accompanied by eosinophil infiltration after acute exposure to aerosolized antigen.

KEY WORDS

animal, asthma, cytokine, eosinophil, IL-6

INTRODUCTION

Asthma is a chronic inflammatory disorder characterized by airway eosinophilia, high levels of serum IgE, airway hyper reactivity and mucus hypersecretion.^{1,2} Th2 cell activation in the respiratory tract has a crucial role for these pathological changes in asthma. Accumulating evidence suggests that IL-5 and IL-13 have a major role in eosinophil infiltration and Th2 polarization, respectively, in sensitized animal model for asthma.^{3,4} IL-4 promotes mucus secretion⁵ while IFN- γ inhibits mucus production in cultured cell lines.⁶ These observations suggest that Th2 polariza-

tion may also regulate mucus secretion as well.

IL-6 is a multifunctional cytokine that plays an important role in immune and inflammatory responses, and elevated levels of IL-6 are seen in serum and bronchoalveolar lavage (BAL) fluid in asthmatic patients.⁷⁻⁹ However, the role of IL-6 in asthma is not clear because it can exert multiple effects on inflammatory reactions. IL-6 can induce terminal differentiation and proliferation of B cells¹⁰ as well as proliferation and activation of T lymphocytes.¹¹ Moreover, IL-6 may be able to promote IL-4 production by T helper precursor cells,¹² thus inducing Th2 differentiation. Furthermore, IL-6 could suppress IFN- γ and TNF- α

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Received 31 August 2004. Accepted for publication 10 December 2004.

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expression by inhibiting the activation of NF- κ B, an essential transcription factor for these genes.^{13,14} These observations suggest an important role of IL-6 in Th2 polarization.

Simultaneously, IL-6 also can induce the expression of multiple factors with anti-inflammatory properties, including IL-1 receptor antagonist,¹⁵ soluble TNF receptor,¹⁵ IL-10,¹⁶ acute phase reactive proteins, glucocorticoids^{17,18} and protease inhibitors such as tissue inhibitor of metalloproteinase-1.¹⁹ Moreover, IL-6 can attenuate tissue injury in inflammatory lung diseases^{20,21} although IL-6 has been presumed to be a major pro-inflammatory cytokine. These results suggest that the balance between these pro- and anti-inflammatory actions determine the actual roles of IL-6 in a particular inflammatory process.

Progression of asthma consists of the sensitization phase, allergic inflammation at tissue sites and chronic phase with airway remodeling.²² IL-6 may play different actions at different phases of airway allergy. Wang and his colleagues reported that endogenous and exogenous IL-6 inhibited OVA-induced Th2-mediated inflammation in lung when mice were sensitized for a total of 12 days.²³ However, the role of IL-6 in acute antigen-induced airway inflammation has not been fully understood especially whether antibody production may be influenced by IL-6 or whether responses to antigen may be influenced by the level of antibody.

To answer these questions we studied the antigen-induced airway inflammation and antibody production induced by exogenous OVA challenge in wild-type and IL-6 deficient mice.

METHODS

REAGENTS AND CELLS

The following chemicals were used; ovalbumin (OVA; Sigma, St. Louis, MO, USA), aluminum hydroxide (Al[OH]₃), and methacholine (Wako Pure Chemical Ind., Osaka, Japan). Polyclonal antibodies to mouse IL-6 were prepared as previously described.²⁴

MICE

Specific pathogen-free, 8-week-old female BALB/c mice were obtained from Clea Japan, Inc. (Tokyo, Japan) and designated as wild-type mice in the following experiments. We prepared IL-6-deficient mice on a BALB/c genetic background as described previously.²⁵ All animal procedures complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University.

OVA SENSITIZATION AND CHALLENGE

OVA sensitization and challenge were performed according to the previously described protocols with some modifications.²⁶ Briefly, 8-week old wild-type

and IL-6-deficient mice were given 200 μ g of OVA mixed with Al[OH]₃ intraperitoneally twice at a 3-week interval. Three weeks after the second injection, animals received an aerosol challenge with OVA (10 mg/ml) in PBS or PBS alone for 20 min. The aerosol was generated via an Omron NE-C13 jet nebulizer (Omron Healthcare, Tokyo, Japan). In another series of experiments, wild-type BALB/c mice received an intraperitoneal injection of the rat anti-murine IL-6 monoclonal IgG antibody (clone 6B4, a generous gift of Dr. Van Snick, 200 μ g/body) or rat IgG (200 μ g/body, CosmoBio Ltd., Osaka, Japan) three times a week for 6 weeks between the first intraperitoneal injection of OVA and the aerosol inhalation of OVA.

EVALUATION OF AIRWAY RESPONSIVENESS

Airway responsiveness was measured 24 h after the inhaled antigen challenge. Mice were anesthetized by an intraperitoneal injection of 75 μ g/g of sodium pentobarbital and were placed in a supine position. After the trachea was cannulated with a polyethylene tube, the animals were ventilated artificially 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, the lateral pressure of the tracheal tube (pressure at the airway opening; Pao) (cmH₂O), was measured using a pressure transducer (Model TP-603T, Nihon Koden Kogyo Co., Ltd., Tokyo, Japan),^{27,28} and used as an overall index of bronchial responsiveness to methacholine as previously described.²⁹ The mice were exposed to increasing doses of methacholine (25, 50, 100 and 200 μ g/ml) at five-minute intervals under continuous ventilation. The aerosol was generated during a 20-second period by an ultrasonic nebulizer developed for small animals at our institution.

ANALYSIS OF BRONCHOALVEOLAR LAVAGE (BAL) FLUID CELLS

Measurements of BAL and histopathologic analysis were performed 24 h after the inhaled antigen challenge. After anesthesia was administered and the trachea was cannulated, the airways were lavaged via the tracheal cannula with 1 ml sterile saline prewarmed at 37°C. The fluid was recovered by gentle aspiration with a disposable syringe. The supernatants were pooled, and the cells were counted manually in a Turk Chamber. Cytoцентрифугed preparations were stained with May-Giemsa for a differential cell count of 300 cells.

HISTOPATHOLOGIC ANALYSIS

Mice were anesthetized, the trachea was cannulated as described above, and the lungs were then inflated to a 25-cm pressure with 10% formalin in PBS (pH 7.40), removed from the chest, postfixed in 10% for-

Table 1 Sequences of primers used for RT-PCR

	Sense Primer	Anti-sense Primer
IL-6	GCTCCCTACTTCACAAGTCC	GCAGGTTTGCCGAGRAGATC
IFN- γ	ACTGGCAAAAGGATGGTGAC	TGAGCTCATTGAATGCTTGG
IL-5	CCCACAAAAGTGTGCAAATG	GGTATGTGATCCTCCTGCGT
IL-13	CAGCTCCCTGGTTCTCTCAC	TGTGTCCACAGCTGAGATGC
RANTES	ATCTTGCAAGTCGTGTTTGTAC	GAAATGCTGATTCTTGGGTTT
Eotaxin	CTCCACAGCGCTTCT ATTCC	TTTGGAGTTTTTGGTCCAGG
β -actin	TGTGATGGTGGGAATGGGTGAC	TTTGATGTCACGCACGATTTC

malin in PBS for 24 h, placed in paraffin, and sectioned. Hematoxylin and eosin stains were performed for histopathological analysis.

DETERMINATION OF SERUM IgG SUBCLASSES AND IgE CONCENTRATIONS

Blood samples were taken before sensitization and 24 h after the inhaled antigen challenge.

Relative serum levels of IgG subclasses were determined with an immunoglobulin isotyping kit (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Serum IgE levels were measured using the combination of rat anti-mouse IgE monoclonal antibody (clone R35-72, Pharmingen) and biotinylated anti-mouse IgE monoclonal antibody (clone R35-92) according to the manufacturer's instructions. IgE concentrations were calculated using purified mouse IgE (Pharmingen) as standards.

EXTRACTION OF TOTAL RNA FROM LUNG AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNAs were extracted from a portion of the lung tissues by means of RNeasyTM (Biotex Laboratory Inc., Houston, TX, USA) according to the manufacturer's instructions. One μ g of total RNA was reverse-transcribed at 42°C for 1 h in 20 μ L of reaction mixture containing mouse Moloney murine leukemia virus reverse transcriptase (Toyobo, Osaka, Japan) and hexanucleotide random primers (Pharmacia-Biotech, Uppsala, Sweden). Thereafter, 0.5 μ L of cDNA product was amplified for 30 cycles, consisting of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, together with Taq polymerase (Takara Shuzo, Kyoto, Japan) and the sets of the primers that specifically amplify mouse IL-6, IL-5, IL-13, IFN- γ , RANTES, eotaxin, or β -actin cDNA (Table 1). Under these conditions, the quantity of amplified products did not reach a saturable level. The amplified PCR products were fractionated on a 1% agarose gel and visualized by ethidium bromide. The intensity of the bands was measured with the aid of NIH Image Analysis software (version 1.55, National Institutes of Health, Bethesda, MD, USA) and the ratio to β -actin was determined.

STATISTICAL ANALYSIS

Mean and S.E.M. were calculated on all parameters. Statistical analysis were performed by two-way ANOVA followed by Fisher's protected least significant difference (PLSD). A *p* value of 0.05 or less was considered to indicate a statistically significant difference.

RESULTS

OVA CHALLENGE-INDUCED AIRWAY INFLAMMATION IN WILD-TYPE MICE

Wild-type mice exhibited a significant and marked increase in total leukocytes in BAL fluid after antigen challenge ($1.37 \pm 0.31 \times 10^5$ BAL leukocytes after PBS challenge *vs.* $2.83 \pm 0.87 \times 10^5$ BAL leukocytes after antigen challenge) ($p < 0.0001$, $n = 6$). Of note is that among various types of leukocytes, antigen challenge caused a remarkable increase in BAL eosinophils (from $0.3 \pm 0.2\%$ to $15.8 \pm 4.3\%$), neutrophils (from $3.5 \pm 0.6\%$ to $17.3 \pm 2.9\%$), and lymphocytes (from $7.0 \pm 1.4\%$ to $12.5 \pm 1.7\%$) but not macrophages (Fig. 1, upper panel). Histopathological analyses demonstrated that a large number of eosinophils and lymphocytes were accumulated in subepithelial areas in wild-type mice challenged with OVA (Fig. 1, panel A; higher magnification and C; lower magnification). We next examined whether OVA challenge induced airway hyperresponsiveness in wild-type mice. OVA challenge increased the bronchoconstrictor responses to methacholine in wild-type mice (Fig. 2). These results indicate that OVA challenge induced airway inflammation characterized by eosinophil and lymphocyte infiltration and airway hypersensitivity in wild-type mice.

AIRWAY HYPERRESPONSIVENESS WITH REDUCED INFLAMMATORY CELL INFILTRATION IN IL-6-DEFICIENT MICE CHALLENGED WITH OVA

In order to delineate the roles of IL-6 in OVA-induced airway inflammation and bronchial hyperresponsiveness, we compared IL-6-deficient mice with wild-type mice. After OVA challenge, IL-6-deficient mice exhibited a significant increase in BAL total leukocyte number (from $1.37 \pm 0.39 \times 10^5$ to $1.85 \pm 0.24 \times 10^5$ BAL leukocytes) but to a lesser degree compared

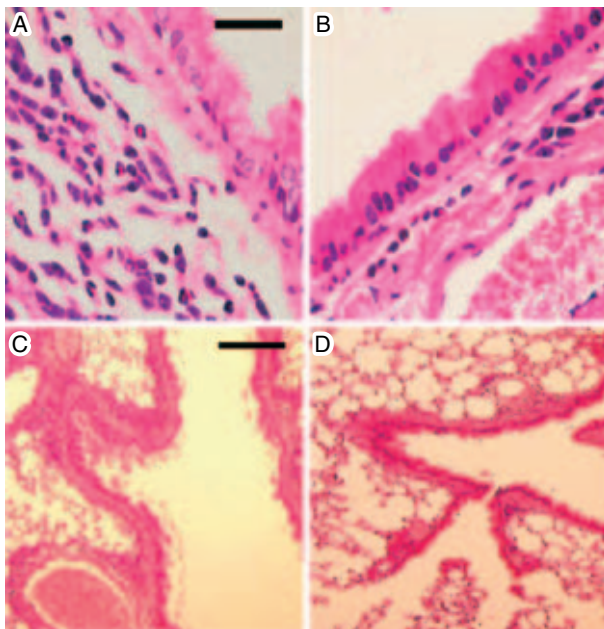
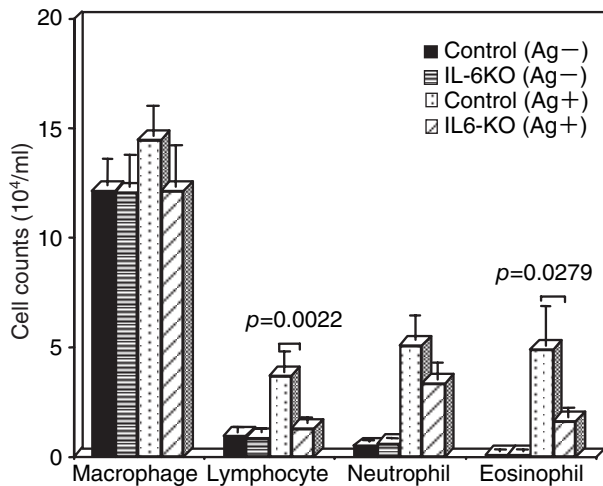


Fig. 1 Differential cell counts of BAL cells obtained from wild-type and IL-6-deficient mice (upper panel) and histological analyses on the lungs of OVA-challenged wild-type (**A** and **C**) and IL-6-deficient mice (**B** and **D**). Wild-type and IL-6-deficient mice were sensitized and exposed to inhaled OVA (Ag+) in PBS or PBS alone (Ag-). Twenty-four hrs after OVA challenge, total cell numbers and differential counts were performed on BALs. Each group consists of at least 5 mice. Bars indicate 20 μ m length in panel **A** and 80 μ m in panel **C**.

with wild-type mice ($p = 0.038$). Of note, BAL eosinophil and lymphocyte numbers in OVA-challenged IL-6 deficient mice were less than one third of those in OVA-challenged wild-type mice (eosinophils; IL-6 deficient mice, $1.6 \pm 0.4 \times 10^4/\text{ml}$; wild-type mice, $4.9 \pm 1.8 \times 10^4/\text{ml}$; $p = 0.0279$; lymphocytes; IL-6 deficient mice, $1.3 \pm 0.3 \times 10^4/\text{ml}$; wild-type mice, $3.7 \pm$

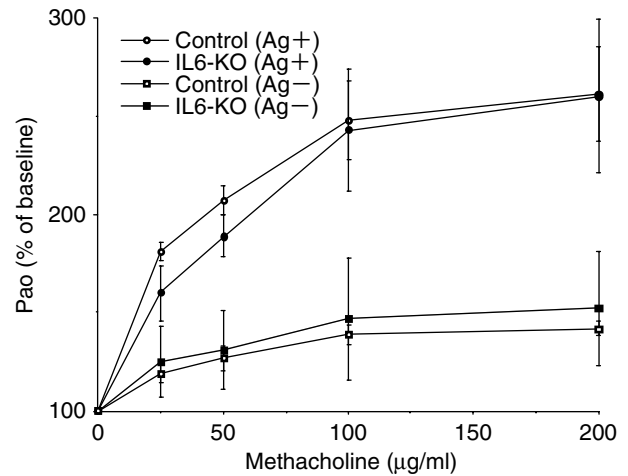


Fig. 2 Airway responsiveness in OVA-challenged wild-type and IL-6-deficient mice. Wild-type and IL-6-deficient mice were sensitized with OVA and challenged with inhaled OVA, and responsiveness to inhaled methacholine was measured as a percent increase of pressure of airway opening (Pao). Each group consists of at least 5 animals. Mean and SE are shown here.

$0.9 \times 10^4/\text{ml}$; $p = 0.0022$) with no significant differences in the number of BAL neutrophil and macrophage numbers between wild-type and IL-6 deficient mice (Fig. 1, upper panel). Consistent with the changes in BAL fluid cell number, eosinophil and lymphocyte infiltration in peribronchial and perivascular areas was markedly reduced in OVA-challenged IL-6-deficient mice, compared with wild-type mice (Fig. 1, panel B; higher magnification, and D; lower magnification). However, OVA-challenged IL-6-deficient mice exhibited similar bronchoconstrictor responses to methacholine as wild-type mice (Fig. 2).

REDUCED TH2-MEDIATED CHANGES IN SERUM Ig LEVELS IN IL-6-DEFICIENT MICE

We measured Th2-mediated changes in serum immunoglobulin levels, characterized by increases in serum IgE and IgG1 levels in mice (Fig. 3). Serum IgE levels in wild-type mice increased about 10-fold 6 wk after OVA sensitization ($0.778 \pm 0.209 \mu\text{g/ml}$ before sensitization vs. $19.875 \pm 4.092 \mu\text{g/ml}$ after sensitization). Serum IgE levels in IL-6-deficient mice were significantly less than in wild-type mice both before and after OVA sensitization ($0.137 \pm 0.035 \mu\text{g/ml}$ before sensitization vs. $9.836 \pm 0.725 \mu\text{g/ml}$ after sensitization). IL-6-deficient mice also exhibited less elevation in serum IgG1 levels after the sensitization than in wild-type mice. In contrast, antigen sensitization did not induce significant increases in serum IgG2a, IgG2b and IgG3 levels in both wild-type and IL-6 defi-

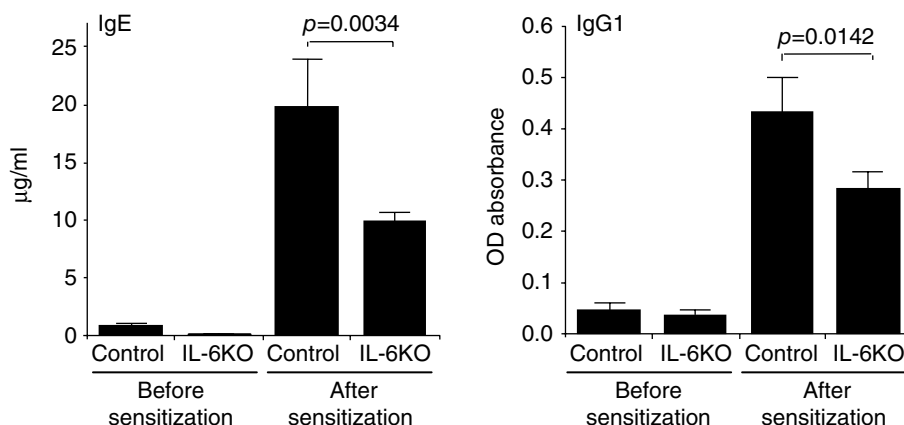


Fig. 3 Serum levels of IgE and IgG1 in wild-type (Control) and IL-6-deficient mice (IL-6KO) before and after OVA sensitization. Relative serum levels of IgG1 were determined on sera diluted at 1 : 10000. Each group consists of at least 5 mice. Means and SE are shown here.

cient mice (data not shown). These observations indicate that Th2-mediated changes in serum immunoglobulin levels might depend on endogenous IL-6.

EFFECTS OF THE ANTI-IL-6 MONOCLONAL ANTIBODY ON OVA-INDUCED AIRWAY INFLAMMATION

To exclude the possibility that the phenotypes of IL-6-deficient mice may be secondary to congenital defect of IL-6 gene, we administered the anti-IL-6 antibody to wild-type mice during the whole course of OVA sensitization. As shown in Figure 4, IL-6 antibody treatment reduced the elevation in serum IgE level and eosinophil infiltration into the lung, observed after OVA challenge. These results imply that the phenotypes of OVA-challenged IL-6-deficient mice can be ascribed directly to IL-6 gene deficiency.

CHANGES IN CYTOKINE GENE EXPRESSION IN LUNG

Next, we examined intrapulmonary cytokine mRNA expression in wild-type and IL-6-deficient mice (Fig. 5). OVA challenge caused a 2-fold increase in IL-6 mRNA in wild-type mice, whereas no IL-6 mRNA was detected in IL-6-deficient mice. Moreover, mRNA expression of two representative Th2 cytokines, IL-5 and IL-13, was enhanced in wild-type mice challenged with OVA, compared with untreated ones. After OVA challenge, IL-6-deficient mice also exhibited enhanced expression of IL-5 and IL-13, but to a lesser degree than wild-type mice. In contrast, IFN- γ mRNA expression was enhanced in IL-6-deficient mice challenged with OVA to a greater degree than wild-type mice. The mRNA expression of two chemokines with potent eosinophil chemotactic activities, RANTES and eotaxin, did not change before and after OVA challenge in both groups. Collectively, in the absence

of IL-6, Th2-dominated response in OVA-challenged wild-type mice may be reduced and shifted to a Th1-type response.

DISCUSSION

In this study we observed that OVA-challenged IL-6-deficient mice exhibited reduced serum IgE and IgG1 levels, IL-5 and IL-13 gene expression, and tissue eosinophilia, but elevated IFN- γ gene expression compared with those of OVA-challenged wild type mice. Collectively, endogenous IL-6 can enhance sensitization and Th2 polarization but attenuate Th1 polarization in OVA-induced acute model of airway hyperresponsiveness.

Inhaled OVA-challenge caused marked increase of eosinophils in BAL suggesting that this experimental model has a characteristic feature of acute allergic airway inflammation induced by aerosolized antigen. The results of tissue cytokine mRNA analysis also suggest that this eosinophilia is caused by IL-5, not by eotaxin or RANTES.

Evidence is accumulating to indicate that IL-6 can promote Th2 polarization.¹³⁻¹⁵ La Flamme *et al.* showed that IL-6 up-regulates IL-10 production which, together with IL-6, negatively regulates IL-12 and IFN- γ production.¹⁷ They suggested that IL-6 can negatively regulate Th1 polarization directly, or indirectly by IL-10 induction. In the acute model of allergic airway inflammation we considered the antigen-antibody reaction to be essential for the trigger of reaction. To study the effect of IL-6 on antibody development we sensitized wild-type and IL-6-deficient mice for 42 days and observed IgE and IgG antibody production. In accordance with these previous reports, IL-6-deficient mice showed reduced serum IgE and IgG1 levels. We also observed reduced intrapulmonary gene expression of Th2 cytokines such as IL-

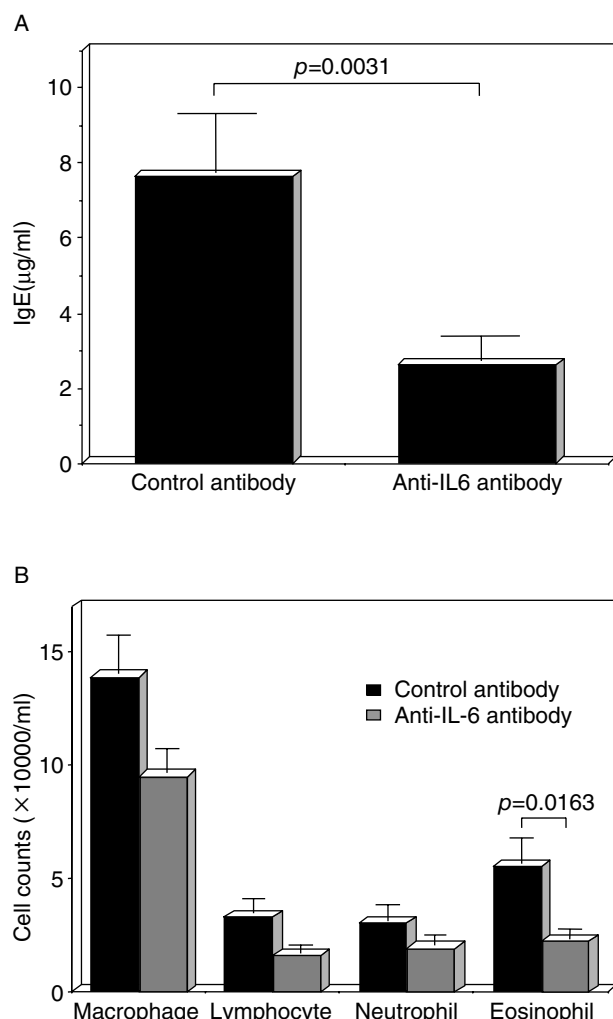


Fig. 4 The effects of anti-IL-6 antibody treatment on antigen-induced responses. Wild-type mice were sensitized with OVA for 6 wks with concomitant injection with an anti-IL-6 antibody or a control antibody three times a week, and challenged with inhaled OVA. Twenty-four hr later, serum IgE levels and cell composition in BAL fluid were determined as described in Methods. Each group consists of at least 5 mice.

5 and IL-13, and increased IFN- γ gene expression in IL-6-deficient mice after inhaled antigen challenge. Moreover, reduced serum IgE levels and intrapulmonary eosinophilia were observed in wild-type mice treated with anti-IL-6 antibody during the sensitization. Thus, reduced serum IgE levels with reduced intrapulmonary eosinophilia was based directly on the absence of endogenous IL-6 gene, but not due to secondary defects from congenital IL-6 deficiency.

In contrast to these reports, several groups reported the possibility that IL-6 can inhibit Th2 type response.³⁰ A previous study reported that IL-6-deficient mice from C57BL/6 genetic background ex-

hibited exaggerated OVA-induced Th2 inflammation.²³ However, in their studies, they sensitized mice for 12 days. Thus, under their experimental conditions, specific antibody generation may not be complete. Moreover, in their experimental conditions, OVA-challenged wild-type mice exhibited more than a ten-fold increase in the total BAL cell number and nearly three quarters of the total cells were eosinophils. In contrast, we observed only a two-fold increase in total BAL cell number and eosinophils were less than 20 percent of the total cells. In this sense, the conditions of our experiments may be more physiological than those of their experiments as a model of bronchial asthma.

However, we cannot exclude the possibility that the role of IL-6 may be different in different animal backgrounds. Th1 and Th2 polarization can predominate in C57BL/6 and BALB/c mice, respectively, when they were exposed to various conditions such as *Leishmania* infection. Moreover, these two background mice exhibited discrete responses in terms of fibrotic changes, even if the same IL-6 gene is deleted. IL-6-deficient mice on a C57BL/6 background exhibited exaggerated hepatocyte necrosis and increased fibrosis compared with wild-type mice, when they were treated with carbon tetrachloride.³¹ In contrast, we observed that IL-6-deficient mice on a BALB/c genetic background exhibited attenuated liver fibrosis compared with wild-type mice, when they were treated with carbon tetrachloride.²⁵ Thus, the phenotypic differences may be explained by the differences in genetic backgrounds.

Although eosinophil and lymphocyte infiltration is less severe in IL-6 deficient mice, similar levels of bronchial hyperresponsiveness (BHR) were observed in both groups after OVA challenge. One limitation is that measurements of Pao are not so sensitive because of the small size of the mice lung. Therefore, the difference of Pao between wild type and IL-6 deficient mice might be under the detection limit. However, recent clinical trials demonstrated that late asthmatic response or BHR persisted despite reduced eosinophil infiltration or airway inflammation by the administration with a neutralizing IL-5 monoclonal antibody,^{32,33} suggesting that BHR could develop independently of airway inflammation. Another possibility is that IL-6 may play a pivotal role in a yet unknown manner in mediator release and maintenance of epithelial integrity. Since IL-6 is reported to have a growth inhibitory effect on airway epithelial cells,³⁴ IL-6 might have a direct effect on epithelial damage.

In summary, we have shown that IL-6 has pivotal role in the sensitization phase of allergic airway inflammation. We also showed that in this acute exposure model, airway inflammation could be reduced in IL-6-deficient mice. Although the effects of IL-6 on allergic airway inflammation might be complicated by exerting pro- and anti-inflammatory activities simulta-

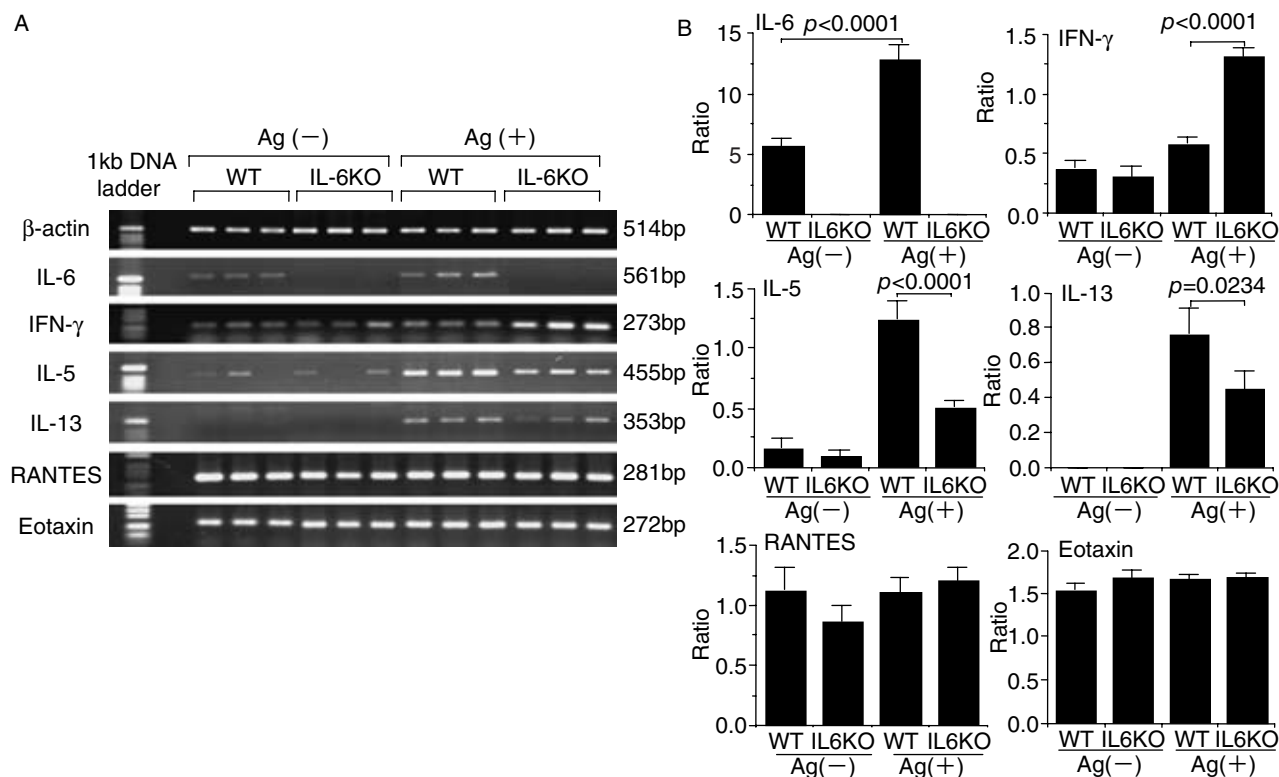


Fig. 5 Expression of IL-6, IFN- γ , IL-5, IL-13, RANTES and eotaxin in the lung of wild-type and IL-6-deficient mice. Total RNAs were extracted from either wild-type or IL-6-deficient mice before or 24 h after inhaled challenge with OVA. Cytokine transcripts were amplified as described in Methods. RT-PCR was performed on 6 individual animals, and the ratios of IL-6, IFN- γ , IL-5, and IL-13 to β -actin are shown here. Means $\pm$ SE are shown.

neously, the results of this study may help to further understand the role of IL-6 in atopy and antigen-induced airway reaction.

ACKNOWLEDGMENT

Z.Q. is a Sasagawa Research Fellow from the affiliated hospital of Jiangxi College of Traditional Chinese Medicine, PR China.

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