

Intramuscular delivery of mIL-12 gene reduces the expression of CD44/CD49d on pulmonary leucocytes and inhibits ovalbumin-induced airway hyperreactivity

H. Li¹, Q. Xie², H. Wang³, C. Shao¹, W. Chen¹

¹ Institute of Immunology, Zhejiang University, 388 Yuhangtang Road, Hangzhou, 310058, P. R. China, Fax: (++86-571) 8820 8285, e-mail: chenwei566@zju.edu.cn

² Zhejiang Respiratory Drugs Research Laboratory of State Drugs Administration of China, Medical School of Zhejiang University, Hangzhou, 310058, P. R. China

³ Yinzhou Pepole's Hospital, Ningbo, 315040, P. R. China

Received 10 March 2007; returned for revision 6 April 2007; returned for final revision 24 June 2007; accepted by A. Falus 18 July 2007

Abstract. *Objective:* To investigate the effects of murine IL-12 (mIL-12) gene on the expression of CD44 and CD49d on pulmonary leukocytes.

Material and methods: BALB/c mice (n = 24) were sensitized to ovalbumin (OVA) by intraperitoneal injection and challenged with OVA aerosol for 7 consecutive days. mIL-12 plasmid was then intramuscularly administered in eight BALB/c mice every second day for three times during OVA challenge.

Results: Administration of mIL-12 plasmid significantly reduced airway hyperreactivity, the expression of CD44 and CD49d on pulmonary leukocytes, pulmonary infiltration of inflammatory cells, Th2 cytokines production and goblet cell hyperplasia ($P < 0.05$). Importantly, the percentages of CD44+/CD49d+ leukocytes showed positive correlations with numbers of inflammatory cells, goblet cells hyperplasia and levels of IL-4 and IL-5 ($P < 0.01$).

Conclusions: Our study suggests that CD44 and CD49d might contribute to the preventive effects of mIL-12 gene in asthma.

Key words: Asthma – CD44 – Gene transfer – Interleukin-12 – Very late antigen-4

Introduction

Allergic asthma is a chronic inflammation disease, characterized by airway inflammation including infiltration of inflammatory cells, goblet cell hyperplasia and airway hyperreactivity (AHR) [1]. Animal experiments have demonstrated that airway inflammation in asthma arises from the Th1/Th2 imbalance with an increase of Th2 cytokines [2, 3]. Clinical investigations also showed that the levels of Th2 cytokines, such as IL-4 and IL-5, correlated with the severity of the disease [4, 5]. IL-12 is a heterodimeric cytokine

which promotes the differentiation of naive CD4+ T cells to Th1 phenotype and inhibits the expression of Th2 cytokines. Several studies have demonstrated the ability of IL-12 to inhibit the airway inflammatory reactions in murine models of asthma. Administration of recombinant murine IL-12 (mIL-12) plasmid before or during the period of allergen challenge was found to be able to diminish AHR, airway eosinophilia and the production of antigen-specific IgE [6, 7]. Moreover, mucosal IL-12 gene delivery could prevent the development of allergic disease and suppress the established allergic airway inflammation [8]. However, the molecular and cellular mechanisms accounting for the effects of IL-12 in allergic asthma are still not fully investigated.

Recruitment of leukocytes including lymphocyte and eosinophil into the allergic lung is of paramount importance for the pathogenesis of allergic asthma. Adhesion molecule CD44 is an important receptor of hyaluronic acid (HA). CD44-HA interactions are contributory to the rolling of leukocytes over endothelial cells and the migration of leukocytes to inflammatory tissue [9, 10]. The $\alpha 4$ integrin subunit CD49d is a component of the very late antigen (VLA)-4. The interaction of VLA-4 with vascular cell adhesion molecule-1 is involved in leukocyte adherence to pulmonary endothelium and transendothelial migration [11]. Previous studies have demonstrated the critical role of CD44 and CD49d in the development of allergic asthma, suggesting the potentially tempting targets of CD44 and CD49d for asthma prevention and therapy [10–12].

In this study, we determined whether CD44 and CD49d play a potential role in the preventive effect of mIL-12 gene on allergic asthma. To this end, mIL-12 plasmid was intramuscularly administered in a murine asthma model and the indices including airway hyperreactivity, the expression of CD44 and CD49d on leukocytes in bronchoalveolar lavage fluid (BALF), pulmonary infiltration of leukocytes, local Th2 cytokines production and goblet cell hyperplasia were examined. The relationships between the percentages of CD44/CD49d positive leukocytes in BALF and other parameters were also analyzed.

Methods

Animals

Male BALB/c mice, 6 to 8 weeks old (weigh 20–22 g) and free of specific pathogens, were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). Experiments were performed according to protocols approved by the Animal Studies Committee of China.

Vector construction and delivery

The construction of mIL-12 expression plasmid and the bioassay of expressed mIL-12 were described in our previous report [13]. In particular, the mIL-12 plasmid was transfected into COS-7 cells and the expression of mIL-12 p70 protein in the supernatant was detected by ELISA 48 h after transfection [13]. Biological activity of expressed mIL-12 was demonstrated *in vitro* based on its ability to stimulate NK activity in naive splenocytes. Splenocytes were incubated with various supernatant for 12 h and cytotoxic activity of splenocytes against YAC-1 cells was determined by MTT assay [13]. The NK activity of splenocytes incubated with mIL-12-expression supernatant was (42.9 ± 3.78) %, whereas the NK cytotoxicity of splenocytes incubated with supernatant from COS-7 cells transfected with pcDNA3.1 vector was (21.4 ± 3.89) % [13].

For intramuscular delivery, BALB/c mice were anesthetized intraperitoneally with pentobarbital sodium (50 mg/kg) and the leg hair was shaven. Fifty microliter of 25 % sucrose in PBS was injected into the left quadriceps muscles to facilitate the diffusion of plasmid DNA. Fifteen minutes later, 100 µg of mIL-12 plasmid or 100 µg of pcDNA 3.1 vector in 50 µl of PBS was administered at the same site. The muscle injections were performed by using a 1-ml syringe with a 27-gauge needle, fitted with a plastic collar to limit needle penetration to 3 mm. The time of injection was over 30 s and the needle was held *in situ* for another 5 s before withdraw.

Antigen challenge and experimental protocol

A murine model of allergic asthma was established in our previous work [14]. Particularly, mice were immunized to ovalbumin (OVA) (Sigma Chemical, St Louis, MO, USA) with intraperitoneal injection of 100 µg OVA absorbed in 2 mg alum on day 0 and 9. From day 14 to day 20, mice were exposed daily to an aerosol of OVA (10 mg/ml) in PBS using an ultrasonic nebulizer for 30 min. Control mice (Control) were injected intraperitoneally with 0.2 ml PBS and then exposed to aerosolized PBS using similar equipments and schedules. All OVA-sensitized and challenged mice ($n = 24$) were separated in three groups: mIL-12 plasmid group (100 µg mIL-12 plasmid administered 1 h after OVA challenge on day 14, 16 and 18), vector group (100 µg pcDNA3.1 vector 1 h after OVA challenge on day 14, 16 and 18) and OVA group (100 µl PBS 1 h after OVA challenge on day 14, 16 and 18). On day 21, the airway responsiveness to methacholine (Sigma Chemical) was assessed and animals were killed.

Airway responsiveness measurements

Airway reactivity was measured *in vivo* 24 h after the last aerosol exposure using a method as described previously [15]. Mice were anesthetized with intraperitoneal injection of urethane (1,300 mg/kg) and placed in a whole-body plethysmography chamber. The lung resistance (R_L) and dynamic compliance (C_{dyn}) were measured at baseline. The methacholine of 0.125, 0.25, 0.5, 1.0 and 2.0 µg/kg were then administered intravenously at 4 min intervals and dose-response curves were obtained.

Analysis of BALF

After AHR measurements the right lung was lavaged six times with 1 ml D-Hang's solution. Total BALF cells were counted from a 0.05-ml al-

iquot. The remaining fluid was centrifuged (1,000 g for 10 min at 4 °C). The supernatant was collected for cytokine analysis and the cell pellet was resuspended with D-Hang's solution. Differential cell count was prepared using a cytospin and stained with Wright-Giemsa.

Flow cytometry

Cells derived from BALF were resuspended and stained with FITC-conjugated anti-CD44, PE-conjugated anti-CD49d, APC-conjugated anti-CD45 and the respective isotype controls according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). The fluorescent antibodies were added (1 µg/100 µl) and incubated for 30 min at 4 °C. Flow cytometry was carried out with FACS LSRII and data were analyzed with FACSDiva software (Becton Dickinson).

Cytokine ELISAs

The concentration of cytokines in BALF and serum were measured using ELISA Kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Lung histology and quantitation of epithelial cells

After mice were sacrificed, noninflated lung tissue was removed from left lung, then fixed with 10 % formalin and embedded in paraffin. Sections (5 µm) were stained with hematoxylin-eosin (HE) for general morphology and Alcian blue/periodic acid Schiff (AB/PAS) for detection of goblet cells. Colorized digital images of lung sections were captured using an inverted microscope and a Photometric CCD camera (Olympus, Tokyo, Japan). Quantification of AB/PAS-stained and epithelial areas in bronchial epithelium was performed as described previously [16].

Statistical analysis

All data were expressed as mean $\pm$ SEM. Differences between groups were examined for statistical significance by one-way analysis of variance using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). A *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results

Intramuscular administration of mIL-12 plasmid inhibits OVA-induced AHR

To demonstrate the production of mIL-12 protein *in vivo* after the administration of mIL-12 plasmid, the serum was collected after mice were sacrificed on day 21 and analyzed with ELISA. Figure 1 shows the decrease on the concentration of mIL-12 in mice sensitized and challenged with OVA compared with that in controls, suggesting Th1/Th2 imbalance in the murine model of asthma. Intramuscular administration of mIL-12 plasmid during OVA challenge resulted in a significant increase of mIL-12 production in serum compared with OVA-challenged mice ($P < 0.05$), whereas administration of control vector had no significant effect.

To test the ability of mIL-12 plasmid to inhibit AHR in the asthmatic model, R_L and C_{dyn} responding to methacholine were employed. The OVA-challenged mice developed significant AHR compared with the control mice, as evidenced by an increase in R_L and a decrease in C_{dyn} (Fig. 2A and B). Administration of mIL-12 plasmid during OVA challenge

decreased R_L and increased C_{dyn} in OVA-challenged mice, suggesting the inhibition of AHR ($P < 0.01$). Meanwhile, administration of control vector during OVA challenge did not show a measurable effect on AHR. These data suggested that administration of mIL-12 plasmid could prevent the development of AHR.

The administration of mIL-12 plasmid reduces the expression of CD44 and CD49d on pulmonary leukocytes

To test the effect of mIL-12 treatment on the expression of CD44 and CD49d on leukocytes, the leukocytes (CD45 positive) derived from BALF were analyzed by flow cytometry. After exposure to OVA, larger numbers of CD44-positive and CD49d-positive leukocytes were observed when compared with the controls (Fig. 3). The mean fluorescence intensity (MFI) of CD44 and CD49d on pulmonary leukocytes was also increased in OVA-challenge mice compared with control mice. Treatment of mIL-12 plasmid could reduce the percentages of CD44/CD49d positive leukocytes and the MFI of these two molecules markedly ($P < 0.05$). In mice treated with control vector, no such effect was observed. These data suggested that OVA sensitization and challenge could up-regulate the expression of CD44 and CD49d on leukocytes, whereas treatment of mIL-12 plasmid significantly attenuated this change.

mIL-12 plasmid suppresses the pulmonary infiltration of leukocytes

Our previous report showed that mIL-12 plasmid reduced the number of eosinophils in BALF [14]. In this study, we fur-

ther investigated the numbers of total inflammatory cells and four cell populations in BAL fluid on day 21. After sensitized and challenged with OVA, the numbers of total inflammatory cells, macrophages, lymphocytes, eosinophils and neutrophils increased significantly in BALF (Fig. 4). Administration of mIL-12 plasmid during OVA challenge decreased the number of eosinophils, lymphocytes and neutrophils ($P < 0.01$), whereas the macrophages were not significantly affected. Treatment with control vector had no effect on the number of inflammatory cells in OVA-challenge mice. Furthermore, the number of inflammatory cells was found to significantly correlate with the percentage of CD44-positive leukocytes in BALF ($r = 0.988$, $P < 0.01$) and the percentage of CD49d-positive leukocytes ($r = 0.986$, $P < 0.01$), suggesting the potential role of CD44 and CD49d in the infiltration of leukocytes in lung.

We also examined the lung histology and inflammatory cells infiltration. Lung sections from OVA-challenged mice showed a dense peribronchial inflammation consisting of eosinophils (Fig. 5). Meanwhile, mice received mIL-12 plasmid treatment during OVA challenge exhibited less

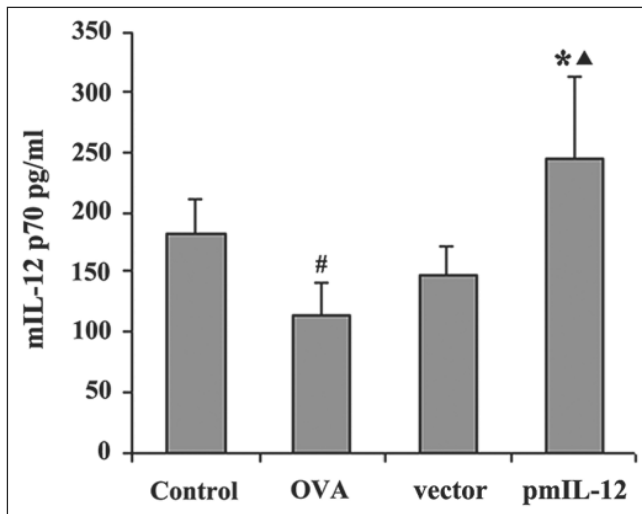


Fig. 1. mIL-12 production in serum after intramuscular administration of mIL-12 plasmid. Mice were divided into four groups: Control, OVA sensitized and challenged (OVA), pcDNA3.1 vector administered during OVA challenge (vector), mIL-12 plasmid administered during OVA challenge (pmIL-12). The concentration of mIL-12 p70 in serum was assessed by ELISA on day 21. Data denote the mean $\pm$ SEM for each group ($n = 8$). [#]Significant difference ($P < 0.05$) versus Control group; ^{*} $P < 0.05$ versus OVA group; [▲] $P < 0.05$ versus vector group.

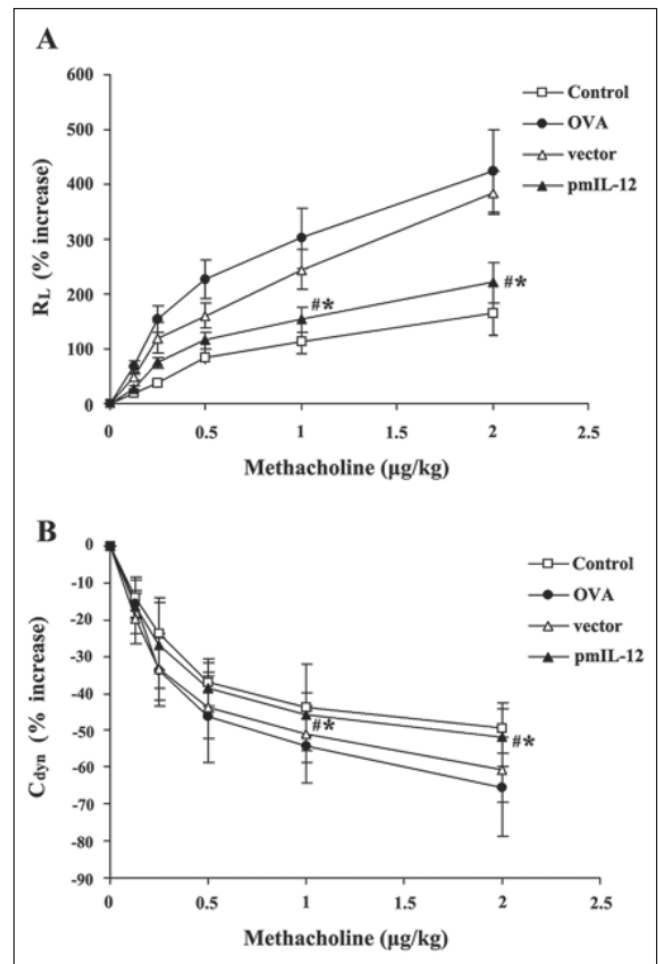


Fig. 2. mIL-12 plasmid inhibits AHR. Airway responsiveness to increased concentration of methacholine was examined 24 h after the final OVA challenge in each group. R_L (A) and C_{dyn} (B) for each group were calculated as described in Methods (means $\pm$ SEM, $n = 8$). [#] $P < 0.01$ versus OVA group; ^{*} $P < 0.01$ versus vector group.

inflammatory cells infiltration in peribronchial region than OVA-challenged mice. No significant difference was found between mice treated with control vector and OVA-challenged mice.

mIL-12 plasmid decreases Th2 cytokines production in BALF

The concentrations of IFN- γ and Th2-phenotype cytokines in BALF supernatants were measured using ELISA. In line with the data in our previous report [14], a significant in-

crease in IL-4 and IL-5 production and a decrease in IFN- γ production were observed in mice sensitized and challenged with OVA (Fig. 6). Administration of mIL-12 plasmid during OVA challenge significantly increased the production of IFN- γ and suppressed the OVA-induced IL-4 and IL-5 production ($P < 0.01$), suggesting a shift from Th2-like phenotype to Th1-like phenotype. No significant changes of IFN- γ , IL-4 and IL-5 production were observed in mice treated with control vector when compared with the OVA-challenge mice. Moreover, the concentrations of IL-4 showed significant correlations with the percentage of CD44-positive leukocytes in BALF ($r = 0.974$, $P < 0.01$) and the percentage of CD49d-positive leukocytes ($r = 0.948$, $P < 0.01$). Same applied to the correlations between IL-5 and the percentages of CD44-positive leukocytes ($r = 0.989$, $P < 0.01$) and CD49d-positive leukocytes ($r = 0.963$, $P < 0.01$).

mIL-12 plasmid alleviates goblet cells hyperplasia

Few AB/PAS positive staining cells were found in bronchial epithelial tissue of control mice (Fig. 7). However, OVA-challenged mice showed a strong AB/PAS positive staining in bronchial epithelial tissue. The abundant positive staining cells were elongated in shape, indicating goblet cell hyperplasia. Administration of mIL-12 plasmid during antigen challenge could significantly alleviate goblet cell hyperplasia, whereas control vector had no obvious effect. The percentage of AB/PAS positive staining area of epithelium significantly correlated with the percentages of CD44-positive leukocytes ($r = 0.983$, $P < 0.01$) and CD49d-positive leukocytes ($r = 0.992$, $P < 0.01$).

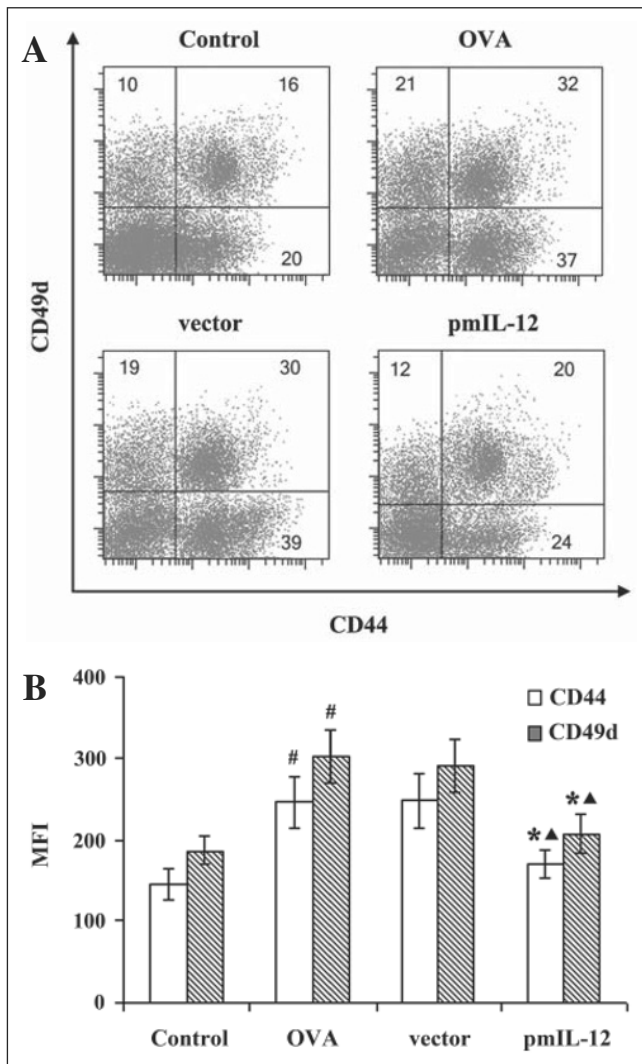


Fig. 3. Effects of mIL-12 plasmid on the expression of CD44 and CD49d on leukocytes derived from BALF. Single-cell suspensions prepared from BALF were treble-stained with FITC-conjugated anti-CD44, PE-conjugate anti-CD49d, and APC-conjugated anti-CD45. CD45 positive leukocytes were gated in plots and expression of CD44 and CD49d were analyzed by flow cytometry. (A) Percentage of CD44- and CD49d-positive cells. The numbers are the averaged values of three independent experiments. (B) Mean fluorescence intensity (MFI) of CD44 and CD49d. Results show the mean of MFI $\pm$ SEM for each group ($n = 8$). [#] $P < 0.05$ versus Control group; ^{*} $P < 0.05$ versus OVA group; [△] $P < 0.05$ versus vector group.

Discussion

In our previous work, we reported that administration of mIL-12 plasmid before or during OVA challenge reduced the number of eosinophils in BALF and the production of Th2 cytokines in a murine model of asthma, suggesting the

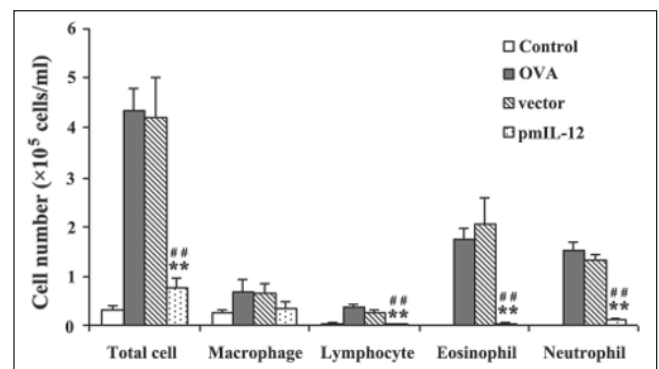


Fig. 4. Effects of mIL-12 plasmid on numbers of BAL inflammatory cells. The number of total cells, macrophages, lymphocytes, eosinophils and neutrophils were determined one day after the final challenge with OVA. Values were shown as mean $\pm$ SEM of eight mice per group. [#] $P < 0.05$ and ^{###} $P < 0.01$ versus OVA group; ^{*} $P < 0.05$ and ^{**} $P < 0.01$ versus vector group.

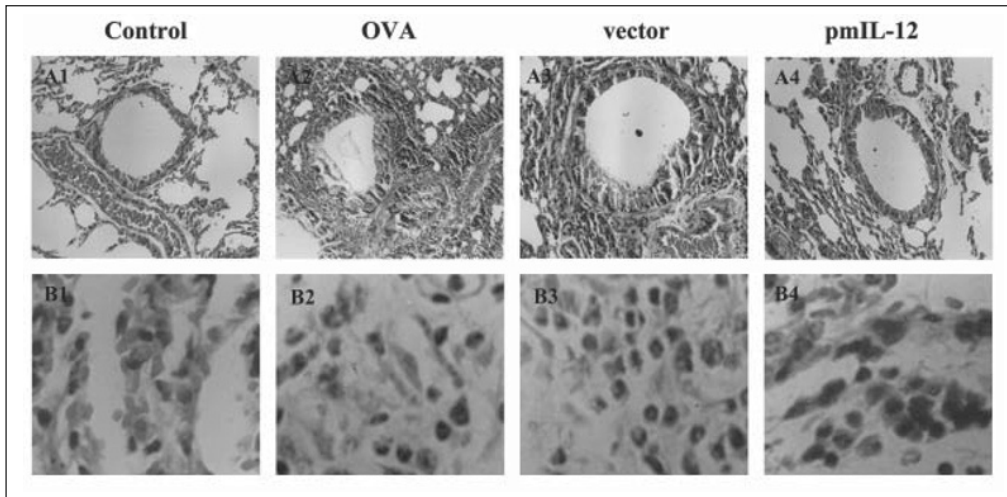


Fig. 5. Lung histology of BALB/c mice treated with mIL-12 plasmid. Lung tissues were obtained one day after the final challenge from Control mice, OVA sensitized and challenged mice, mice treated with vector, mice treated with pmIL-12. Sections were stained with HE: A1-4, magnification $\times 100$; B1-4 magnification $\times 800$.

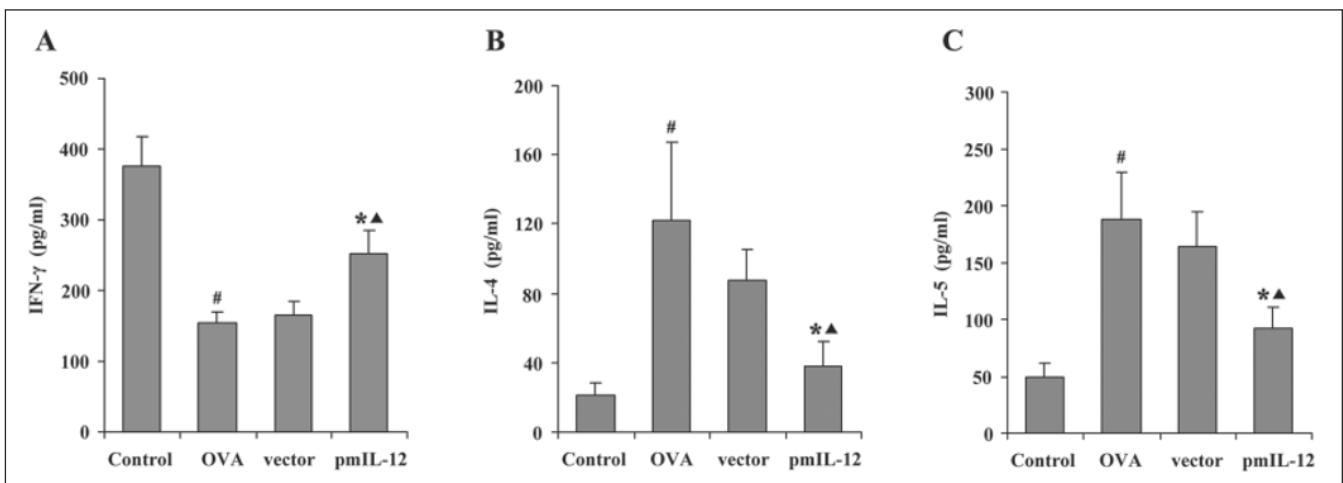


Fig. 6. Effects of mIL-12 plasmid on cytokines production in BALF. One day after the last OVA challenge, concentrations of IFN- γ (A), IL-4 (B) and IL-5 (C) in the supernatants of BALF were assessed by ELISA. Data are presented as means $\pm$ SEM of eight mice per group. [#]Significant difference ($P < 0.01$) versus Control group; $^*P < 0.01$ versus OVA group; $^{\blacktriangle}P < 0.01$ versus vector group. One representative of three independent experiments is shown.

protective effect of mIL-12 plasmid on airway inflammation [14]. However, administration of mIL-12 plasmid during challenge might be more relevant to the clinical application of mIL-12 plasmid in asthma and was thus further investigated in the present study. The data in this study showed that intramuscular administration of mIL-12 plasmid during OVA challenge significantly prevented airway hyperreactivity, decreased Th2 cytokines production, reduced numbers of four different inflammatory cells in BALF and alleviated goblet cell hyperplasia.

Importantly, this study demonstrated that OVA sensitization and challenge could up-regulate the numbers of CD44/CD49d-positive pulmonary leukocytes and enhance the MFI of CD44 and CD49d, whereas delivery of mIL-12 plasmid significantly inhibited this change. Moreover, the percentages of CD44/CD49d-positive leukocytes showed significant correlations with several important indices of asthma, including numbers of lung inflammatory cells, levels of IL-4 and IL-5 in BALF as well as hyperplasia of airway goblet cells. These findings suggested that CD44/CD49d-positive leukocytes might play a crucial role in the preventive effects of IL-12 in allergic asthma.

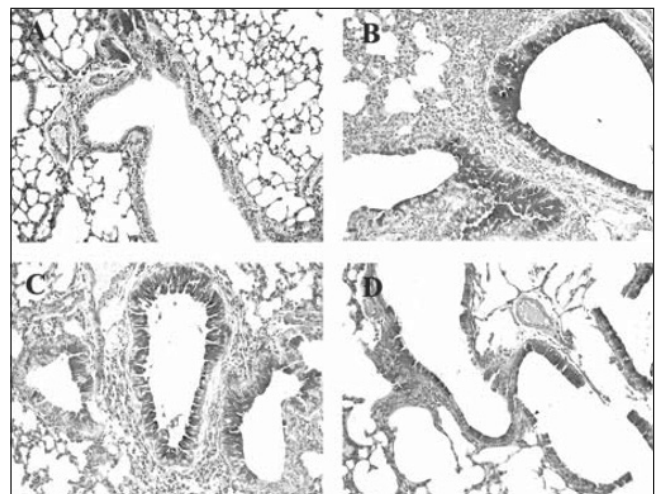


Fig. 7. Effects of mIL-12 plasmid on goblet cells hyperplasia. Lung sections from OVA-treated mice were stained with AB/PAS (see details in Methods) (magnification $\times 100$). (A) Control mice, (B) OVA sensitized and challenged mice, (C) mice treated with vector and (D) mice treated with pmIL-12.

CD44 and CD49d were believed to be essential for the recruitment of leukocytes into lung in asthma. Pulmonary lymphocytes and eosinophils expressed higher level of CD44 in BALF than in peripheral blood with allergic airway diseases [17, 18]. Proinflammatory stimuli enhanced the HA-binding ability of CD44 on leukocytes [10, 19]. Moreover, administration of anti-CD44 mAb prevented the accumulation of leukocytes in lung and subsequently blocked the AHR [10]. The expression of VLA-4 (CD49d/CD29) was also increased on lymphocytes and eosinophils in lung compared with cells in peripheral blood, while anti-CD49d mAb reduced the infiltration of eosinophils and lymphocytes in inflammatory sites in animal asthma models [20, 21]. Our results showed a positive correlation between the percentages of CD44/CD49d-positive leukocytes and the numbers of inflammatory cells in BALF. Therefore, it could be speculated that the mIL-12 gene transfer might down-regulate the expression of CD44 and CD49d on the leukocytes and hence reduce the trafficking of leukocytes into airway.

However, the mechanism of the reduction of CD44 and CD49d expression on leukocytes induced by mIL-12 is still not well understood. Stimulation with IL-5 could up-regulate the expression of CD44 on eosinophils *in vitro* [22]. Clinical study also showed the significant correlation between concentrations of IL-5 and amounts of soluble CD44 in BALF in eosinophilic pneumonia [18]. IL-5 transgenic mice exhibited similar characteristics of asthma including AHR and administration of anti-CD49d antibody resulted in immediate inhibition of AHR [23]. This study showed a significant correlation between the concentration of IL-5 and percentages of CD44/CD49d-positive cells in BALF. Based on these findings, we speculated that mIL-12 might down-regulate the expression of CD44 and CD49d on leukocytes by reducing the concentration of IL-5.

With the extensive application of IL-12 in experimental asthma models, it is generally believed that IL-12 is protective against excessive Th2 sensitization and is considered as a potential therapy for asthma [6, 8, 24, 25]. Nevertheless, Meyts et al. reported a dual role of IL-12 in allergic airway inflammation in a mouse model [26]. They found that blockage of IL-12 during the challenge phase attenuated AHR and reduced CD4⁺ T cells and Th2 cytokine production in BALF, suggesting that IL-12 contributes to allergic airway inflammation in the effector phase of asthma. Their work also showed that the pro-inflammatory effect of IL-12 was IFN- γ dependent [26]. However, numerous studies demonstrated that administration of IL-12 at the effector stage of lung inflammation significantly attenuated the typical features of allergic asthma, such as AHR, Th2 cytokine production and antigen-specific IgE [6, 8, 27, 28]. Moreover, Flaishon et al. reported that administration of low dose of IFN- γ during allergen challenge dramatically inhibited airway inflammation in an asthma model [29]. Low level IFN- γ down-regulated integrin-mediated adhesion and migration of Th2 cells to site of inflammation and hence implemented the anti-inflammatory effect [29]. Our study also showed that treatment of IL-12 plasmid down-regulated the expression of CD44 and CD49d on leukocytes, reduced the infiltration of lymphocytes and eosinophils in lung and subsequently took anti-inflammatory effects.

Goblet cell hyperplasia has been described as an important histological change of airway allergic diseases. Our data showed that repeated OVA challenge induced extensive hyperplasia of goblet cells in sensitized mice while mIL-12 plasmid alleviated this airway change. It appears that both IL-4 and the recruitment of eosinophils and neutrophils contribute to allergen-induced goblet cell hyperplasia [30–32]. Recently, Kanehiro and colleagues reported that CD49d might also be essential for triggering the goblet cell hyperplasia as goblet cell hyperplasia could be blocked by the administration of anti-CD49d mAb [33]. The results in our study suggested the significant correlation of the percentage of CD49d positive leukocytes with the number of goblet cells and the percentage of AB/PAS-positive stained area in lung tissue. Our data also support the potential role of CD49d in goblet cell hyperplasia.

In summary, our study at this stage suggested that mIL-12 plasmid treatment decreased the asthmatic responses associated with the decreased number of CD44/CD49d-positive leukocytes. Treatment with mIL-12 plasmid might down-regulate the number of CD44/CD49d-positive leukocytes by altering the cytokine network, and, therefore, reduce the infiltration of leukocytes in lung and decrease the asthmatic responses. Further study is needed to elucidate the role of CD44 and CD49d in the preventive effects of IL-12 for asthma.

Acknowledgements. We thank Haiya Zheng for expert technical assistance and Dr Dajing Xia for critical reading of the manuscript. This work was supported by National Natural Science Foundation Grant (30500446), and Department of Science and Technology Grant, Zhejiang Province (2003C33004).

Nonstandard abbreviations used: AB/PAS, Alcian blue/periodic acid Schiff; AHR, airway hyperreactivity; BALF, Bronchoalveolar lavage fluid; C_{dyn}, dynamic compliance; HA, hyaluronic acid; HE, hematoxylin-eosin; R_L, lung resistance; mIL-12, murine IL-12; OVA, ovalbumin; VLA, very late antigen.

References

- [1] Oh SW, Pae CI, Lee DK, Jones F, Chiang GK, Kim HO et al. Trypsin inhibition blocks airway inflammation in a mouse asthma model. *J Immunol* 2002; 168: 1992–2000.
- [2] Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; 326: 298–304.
- [3] Lack G, Bradley KL, Hamelmann E, Renz H, Loader J, Leung DY et al. Nebulized IFN-gamma inhibits the development of secondary allergic responses in mice. *J Immunol* 1996; 157: 1432–9.
- [4] Lee YC, Lee KH, Lee HB, Rhee YK. Serum levels of interleukins (IL)-4, IL-5, IL-13, and interferon-gamma in acute asthma. *J Asthma* 2001; 38: 665–71.
- [5] Azzawi M, Bradley B, Jeffery PK, Frew AJ, Wardlaw AJ, Knowles G et al. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am Rev Respir Dis* 1990; 142: 1407–13.
- [6] Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med* 1995; 182: 1527–36.
- [7] Kips JC, Brusselle GJ, Joos GF, Peleman RA, Tavernier JH, Devos RR et al. Interleukin-12 inhibits antigen-induced airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* 1996; 153: 535–9.

- [8] Hogan SP, Foster PS, Tan X, Ramsay AJ. Mucosal IL-12 gene delivery inhibits allergic airways disease and restores local antiviral immunity. *Eur J Immunol* 1998; 28: 413–23.
- [9] DeGrendele HC, Estess P, Siegelman MH. Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science* 1997; 278: 672–5.
- [10] Katoh S, Matsumoto N, Kawakita K, Tominaga A, Kincade PW, Matsukura S. A role for CD44 in an antigen-induced murine model of pulmonary eosinophilia. *J Clin Invest* 2003; 111: 1563–70.
- [11] Henderson WR Jr, Chi EY, Albert RK, Chu SJ, Lamm WJ, Rochon Y et al. Blockade of CD49d (alpha4 integrin) on intrapulmonary but not circulating leukocytes inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma. *J Clin Invest* 1997; 100: 3083–92.
- [12] Singh J, Adams S, Carter MB, Cuervo H, Lee WC, Lobb RR et al. Rational design of potent and selective VLA-4 inhibitors and their utility in the treatment of asthma. *Curr Top Med Chem* 2004; 4: 1497–507.
- [13] Chen W, Shao CS, Shen JG, Bao JF, Pan JP, Han W et al. Construction of bi-cistronic co-expression plasmid of mIL-12 and the expression in vitro or in vivo. *J Zhejiang university (medical sciences)* 2002; 31: 15–8.
- [14] Li HQ, Shao CS, Xie QM. The effect of a mouse IL-12 plasmid on airway inflammation and cytokine production in a mouse asthmatic model. *Chin J Tuberc Respir Dis* 2003; 26: 354–7.
- [15] Xie QM, Zeng LH, Zheng YX, Lu YB, Yang QH. Bronchodilating effects of bambuterol on bronchoconstriction in guinea pigs. *Acta Pharmacol Sin* 1999; 20: 651–4.
- [16] Wu CA, Puddington L, Whiteley HE, Yiamouyiannis CA, Schramm CM, Mohamadu F et al. Murine cytomegalovirus infection alters Th1/Th2 cytokine expression, decreases airway eosinophilia, and enhances mucus production in allergic airway disease. *J Immunol* 2001; 167: 2798–807.
- [17] Kennedy JD, Hatfield CA, Fidler SF, Winterrowd GE, Haas JV, Chin JE et al. Phenotypic characterization of T lymphocytes emigrating into lung tissue and the airway lumen after antigen inhalation in sensitized mice. *Am J Respir Cell Mol Biol* 1995; 12: 613–23.
- [18] Katoh S, Taniguchi H, Matsubara Y, Matsumoto N, Fukushima K, Kadota J et al. Overexpression of CD44 on alveolar eosinophils with high concentrations of soluble CD44 in bronchoalveolar lavage fluid in patients with eosinophilic pneumonia. *Allergy* 1999; 54: 1286–92.
- [19] Mohamadzadeh M, DeGrendele H, Arizpe H, Estess P, Siegelman M. Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. *J Clin Invest* 1998; 101: 97–108.
- [20] Gascoigne MH, Holland K, Page CP, Shock A, Robinson M, Foulkes R et al. The effect of anti-integrin monoclonal antibodies on antigen-induced pulmonary inflammation in allergic rabbits. *Pulm Pharmacol Ther* 2003; 16: 279–85.
- [21] Richards IM, Kolbasa KP, Hatfield CA, Winterrowd GE, Vonderfecht SL, Fidler SF et al. Role of very late activation antigen-4 in the antigen-induced accumulation of eosinophils and lymphocytes in the lungs and airway lumen of sensitized brown Norway rats. *Am J Respir Cell Mol Biol* 1996; 15: 172–83.
- [22] Matsumoto K, Appiah-Pippim J, Schleimer RP, Bickel CA, Beck LA, Bochner BS. CD44 and CD69 represent different types of cell-surface activation markers for human eosinophils. *Am J Respir Cell Mol Biol* 1998; 18: 860–6.
- [23] Borchers MT, Crosby J, Justice P, Farmer S, Hines E, Lee JJ et al. Intrinsic AHR in IL-5 transgenic mice is dependent on CD4(+) cells and CD49d-mediated signaling. *Am. J. Physiol. Lung Cell Mol Physiol* 2001; 281: L653–9.
- [24] Leonard P, Sur S. Interleukin-12: potential role in asthma therapy. *BioDrugs* 2003; 17(1): 1–7.
- [25] Kuribayashi K, Kodama T, Okamura H, Sugita M, Matsuyama T. Effects of post-inhalation treatment with interleukin-12 on airway hyper-reactivity, eosinophilia and interleukin-18 receptor expression in a mouse model of asthma. *Clin Exp Allergy* 2002 32(4): 641–9.
- [26] Meyts I, Hellings PW, Hens G, Vanaudenaerde BM, Verbinen B, Heremans H et al. IL-12 contributes to allergen-induced airway inflammation in experimental asthma. *J Immunol* 2006; 177(9): 6460–70.
- [27] Rais M, Wild JS, Choudhury BK, Alam R, Stafford S, Dharajiya N et al. Interleukin-12 inhibits eosinophil differentiation from bone marrow stem cells in an interferon-gamma-dependent manner in a mouse model of asthma. *Clin Exp Allergy* 2002; 32(4): 627–32.
- [28] Matsuse H, Kong X, Hu J, Wolf SF, Lockey RF, Mohapatra SS. Intranasal IL-12 produces discrete pulmonary and systemic effects on allergic inflammation and airway reactivity. *Int Immunopharmacol* 2003; 3(4): 457–68.
- [29] Flaishon L, Topilski I, Shoseyov D, HersHKoviz R, Fireman E, Levo Y et al. Cutting edge: anti-inflammatory properties of low levels of IFN-gamma. *J Immunol* 2002; 168(8): 3707–11.
- [30] Temann UA, Prasad B, Gallup MW, Basbaum C, Ho SB, Flavell RA et al. A novel role for murine IL-4 in vivo: induction of MU-C5AC gene expression and mucin hypersecretion. *Am J Respir Cell Mol Biol* 1997; 16: 471–8.
- [31] Agusti C, Takeyama K, Cardell LO, Ueki I, Lausier J, Lou YP et al. Goblet cell degranulation after antigen challenge in sensitized guinea pigs. *Am J Respir Crit Care Med* 1998; 158: 1253–8.
- [32] Henderson WR Jr, Lewis DB, Albert RK, Zhang Y, Lamm WJ, Chiang GK et al. The importance of leukotrienes in airway inflammation in a mouse model of asthma. *J Exp Med* 1996; 184: 1483–94.
- [33] Kanehiro A, Takeda K, Joetham A, Tomkinson A, Ikemura T, Irvin CG et al. Timing of Administration of Anti-VLA-4 Differentiates Airway Hyperresponsiveness in the Central and Peripheral Airways in Mice. *Am J Respir Crit Care Med* 2000; 162: 1132–9.