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行政院國家科學委員會補助專題研究計畫

■期末進度報告

評估表現細胞激素的腺病毒載體對過敏氣喘造成的免疫調控影響

計畫類別：■個別型計畫　□ 整合型計畫
計畫編號：NSC 93－　2314－B－　038－　026－
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中華民國95年10月30日

中英文摘要：

在大家致力研發氣喘疾病的治療新方法中，有兩種策略是被認為最具有發展潛力的：

(1)發展出能誘發氣喘病人體內第一型 T 輔助細胞(Th1)免疫反應的疫苗，用來抑制過敏性第二型 T 輔助細胞(Th2)的免疫作用。而目前已知細胞激素 IL-12, IL-18, IL-27 以及 IFN-γ皆可用於引發 Th1 細胞的免疫反應。

(2)發展出能誘發氣喘病人體內調節性 T 細胞(Tr)免疫反應的疫苗，用來抑制過敏性第二型 T 輔助細胞(Th2)的免疫反應。而目前則認為細胞激素 IL-10 可做為佐劑，用來幫助病人體內調節性 T 細胞的生成，進而抑制 Th2 細胞的免疫作用。

多年前我們實驗室就已經開始將 IL-12 應用到過敏氣喘動物的治療，在對於將 IL-12 蛋白質以及 IL-12 基因治療氣管發炎的小鼠上皆獲得不錯的成績。目前，則更進一步將具有單一鏈的 IL-12 融合基因之腺病毒載體送到有氣喘症狀的小鼠肺部，來治療其氣管發炎的現象，而初步的研究成果令人滿意。現在為致力於開發更有效的基因治療方式，我們的實驗室未來將進一步使用各種能表現細胞激素基因的腺病毒載體，包括 IL-12 以及 IL-10；將這些腺病毒載體搭配送到小鼠肺部表現，在此氣管發炎的動物模式下，來了解是否能建立一個更有效的做為 DNA 疫苗佐劑或基因治療的新方法。

總合以上，本研究計畫的主要目的，是利用細胞激素基因來研發未來將細胞激素應用在預防過敏疾病或治療過敏疾病的可行性。

英文摘要:

Thus far, a broad range of therapeutic strategies is under development and two approaches seem to have the greatest potential.

Firstly, vaccines inducing type 1, T-helper cells (Th1) responses may prove successful in inhibiting allergic type2, T-helper cells (Th2) responses.

Hypothesis 1: Interleukin-12 (IL-12), IL-18, and IL-27 can induce Th1 responses that inhibit the development of Th2 cells.

Secondly, the induction of T regulatory (Tr) cells is able to suppress the development of allergic Th2 responses.

Hypothesis 2: It is potential for IL-10 in treatment of allergic asthma by using IL-10 as an adjuvant to induce Tr cells which can inhibit the development of Th2 responses.

In an effort to enhance the efficacy of gene therapy, our studies will be attempted to address the immunomodulatory role of different cytokine (IL-10,

IL-12) -expressing adenoviruses for asthma treatment. We believe that our project might shed light further understanding the regulatory mechanisms and designing immunotherapy for atopic diseases.

報告內容:

(一)前言以及研究目的

According to a worldwide survey by a Global Institute for Asthma in 2003 and 2004, it is estimated that as many as 300 million people suffer from asthma and 255,000 people died of asthma in 2005(Masoli et al., 2004). The rate of asthma is still increasing and it's estimated that there will be another 100 million people suffering from asthma by 2025. If there are no efficient preventions, asthma will become a major problem of public health in the next decade. Asthma is a chronic disease characterized by recurrent attacks of breathlessness and wheezing, which vary in severity and frequency from person to person. If patients receive treatment during asthma attack, asthma is a relatively non-fatal disease compared with other chronic diseases. The major treatment to asthma now is medicinal intervention and there are many new treatments under research, such as gene therapy.

Although the real cause of asthma is not well understood, the strong risk factors are inhaled asthma irritants. These include house dust mites, pet dander, pollens, moulds and chemical irritants in work place. Why asthma patients over-react to these substances is still unknown. However, the hygiene hypothesis may be part of the answer. This hypothesis states that inadequate exposure to environmental microorganism results in inappropriate or defective development of immunological regulatory control(Wills-Karp et al., 2001). The hypothesis also explains why prevalence of asthma is higher in developed countries than in developing countries.

Asthma is a chronic inflammatory disease. When susceptible patient first exposed to allergen, their B lymphocytes were activated and started to secret allergen-specific IgE antibodies. As allergen-specific IgE antibodies circulate in the body, they have chance to be caught by the FcεRI on mast cells. Subsequent exposure to the same allergen causes rapid activation of mast cells through allergen and IgE cross-linking. Activated mast cells release histamine and leukotrienes immediately to cause increases in vascular permeability, smooth-muscle constriction and mucus hyper-secretion. Late phase allergic responses are characterized by releasing cytokines (IL-4, IL-5, IL-9 and IL-13) and chemokines. IL-4, IL-5 and IL-13 have been implicated in major asthmatic diathesis, including IgE production, goblet cells hyperplasia and airway eosinophilia. IL-4 and IL-13 can facilitate Ig-isotype class switching in B cells and result in the synthesis of IgE. IL-5 not only helps the

proliferation of eosinophils but also recruits eosinophils into the lung tissues. IL-9 and IL-13 participate in the development airway hyperresponsiveness (AHR). By monitoring these factors, we can know that whether our treatment works or not.

Currently, replication-deficient adenovirus vector (Ad) is one of the most efficient gene transfer vehicles for human gene therapy. Importantly, Ad is not oncogenic in human; the genomes of common Ad are completely defined. The Ad genome can be easily modified, and recombinant Ad can be easily produced in large quantities and highly concentrated without modifying the ability of the virus to infect target cells(Ali et al., 1994). The aim of our studies is to investigate the immuno-modulatory roles of IL-10 and IL-12 expressing adenoviruses (Ad-IL-10 and Ad-IL-12) in a murine model of ovalbumin (OVA)-induced asthma. IL-10 is a potent anti-inflammatory cytokine, but its pathophysiological role in asthma is still unknown. IL-10 has been reported that it has the ability to inhibit the synthesis of numerous cytokines, including those implicated in the pathogenesis of asthma (van Scott et al., 2000). IL-12 is documented for its inhibitory effect at low dose on eosinophilia and production of cytokines which relate with asthma (Lee et al., 1999). In order to treat asthma, we will evaluate whether the different doses of the combinations of Ad-IL10 and Ad-IL-12 could get a synergistic effect and inhibit the development of airway inflammation symptoms in a mouse model of allergic asthma.

(二)研究方法

Animals and allergic sensitization

Six to eight week old female BALB/c mice were purchased from the animal center of the College of Medicine of National Taiwan University and maintained in the animal center of Taipei Medical University. The sensitization protocol followed the treatment regiment was shown in figure 1. All mice were sensitized by intraperitoneal injections of OVA on *day 0, 14, 28*. The injections consisted of 50 μg (*day 0*) or 30 μg (*day 14, 28*) of OVA and 4 mg of alum in 200 μl. We drew blood form the retro-orbital venous plexus *on day 0, 13, 27, 33* and the blood was centrifuged at 15000 rpm for 5min to obtain serum. The serum was stored at -20℃ for the follow-up OVA-specific antibody analysis. On *day 35*, mice were treated by intratracheal instillation of adenoviral vectors. On *day 37 and 38*, mice were exposed for 25 min daily to aerosolized OVA (5% wt/vol in 0.9% saline). On day 39, mice were evaluated airway hyperresponsiveness (AHR) and then sacrificed. Using a cannula, their lungs were immediately lavaged through the trachea for three times. Firstly we lavaged lung with 1ml HBSS then 2 times of 1ml HBSS with 2% FBS. The first collected BAL fluid was centrifuged at 3000 rpm for 7min and the supernatant was collected and stored at -20℃ for cytokines analysis. The cells pellet was mixed with 2nd and 3rd lavaged

fluids for further cell differential counts.

OVA-specific antibodies analysis and grouping

The titers of sera anti-OVA IgE, IgG1 and IgG2a were measured by ELISA. In brief, 96-well plates were coated with 10 μg/ml OVA diluted in $NaHCO_3$ buffer, pH8.2. After overnight incubation at 4℃, plates were washed and blocked with 3% BSA in PBS for 2 hr at 37℃. Serum samples were diluted and added to each well for an overnight period at 4℃. Plates were then washed. The biotin-conjugated anti-mouse IgE (1:1000) or IgG1 (1:500) or IgG2a (1:500), diluted in 3%BSA-PBS buffer, was added for an additional 1 hr at 37℃. The avidin-conjugated HRP (1:20000) was added for an additional 2 hr at room temperature. Finally, the reaction was developed with substrate ABTS. Plates were read in a microplate reader at 405 nm. The levels of antibody were compared with IgE, IgG1 and IgG2a standard. We chose the previous sample with OD_{405nm} over 1.5 as our standard. The concentration of standard serum was assigned as 1 ELISA unit (E.U.).

We assigned all sensitized mice into the following six groups. Each group contained 6-8 mice and was treated with different kind of adenovirus vectors.

1st group: positive control
2nd group: 1 x 10^8 pfu Ad-Mock/mouse
3rd group: 1 x 10^8 pfu Ad-IL-10/mouse
4th group: 1 x 10^8 pfu Ad-IL-12/mouse
5th group: 1 x 10^8 pfu Ad-IL-10/mouse + 1 x 10^8 pfu Ad-IL-12/mouse
6th group: 2 x 10^8 pfu Ad-IL-10/mouse + 2 x 10^8 pfu Ad-IL-12/mouse
7th group: naïve mouse

pfu : plaque forming unit
Ad-Mock: vectors contain no cytokine gene
Ad-IL-10: vectors contain IL-10 gene
Ad-IL-12: vectors contain IL-12 gene

Measurement of Airway Hyperresponsiveness

In order to investigate the effect of adenoviral vectors on airway hyperresponsiveness, the Buxco system was used to evaluate the extent of airway constriction. This system is a non-invasive way to measure airway hyperresponsiveness. The instrument collects data from the transducer and uses it to calculate the Penh values. Penh = Pause x PIF/PEF; Pause = (Te-Tr)/Tr (PIF: peak inspiratory flow; PEF: peak expiratory flow; Te: expiratory time; Tr: relaxation time). On *day 39*, mice were put

into the chamber and given aerosolized normal saline, 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, 50 mg/ml and 100 mg/ml methacholine serially. Each methacholine concentration was given for 3min and Penh value was recorded for 3 min after inhalation. We use the relative increase ratio to represent the severity of airway constriction.

Relative increase ratio = $Penh_{MCh}$ / $Penh_{NS.}$(MCh: methacholine; NS.: normal saline)

Cytokine analysis of bronchoalveolar lavage fluid

TNF-α, IFN-γ, IL-4, IL-5 and eotaxin were measured with ELISA kits. All procedure followed manufacturers' directions.

Cell differentiation counts

The cell numbers in BALF were determined by using a hemocytometer. The mixed lavages were centrifuged at 3000 rpm for 7 min at 4℃. After centrifugation, we discarded the supernatant and the cells pellet was resuspended in 1xHBSS to final concentration 1.25 x 10^5 cell/ml. 800μl cell suspension was loaded to the cytocentrifuge and cytospun at 800rpm for 5 min at 4℃. Cytocentrifuged slides were stained with Liu's stain for cell differentiation counts. Two hundred cells were counted and classified as macrophage, neutrophil, eosinophil or lymphocyte.

Data Analysis

Data are expressed as means ± standard error of the mean (SEM). Statistical analyses were performed using unpaired student's T test. $p < 0.05$ was considered significant.

(三)研究結果

The levels of OVA-specific IgE, IgG1 and IgG2a during sensitization

The sensitized mice bled from retro-orbital venous plexus on day 0, 13, 27 and 33. Serum was assayed for OVA-specific IgE, IgG1 and IgG2a (Fig.2). After challenging, the levels of OVA-specific IgE and IgG1 levels were increased. In contrast, the level of OVA-specific IgG2a was low. This meant that OVA-immunized mice showed Th2 type immune response. From the high levels of serum IgE and IgG1 on day 33, we verified the success of sensitization.

Intra-tracheal delivery of Ad-IL-10/Ad-IL-12 only or combined Ad-IL-10 and Ad-IL-12 alleviated the severity of AHR and antigen-induced eosinophil infiltration

To determine the therapeutic effect of adenovirus-expressing IL-10 and IL-12, Ad-IL-10 and Ad-IL-12 were administered intra-tracheally 3 days prior to the last OVA challenge. One day after the last OVA challenge, mice were measured for airway

responsiveness (Fig.3). The positive control group was OVA-sensitized but without treating any adenovirus vectors. The relative increase ratio (RIR) of positive control group increased markedly as giving more and more methacholine treatment. The Ad-Mock group also showed a significant RIR. In Ad-IL-10 or Ad-IL-12 group, administration of adenovirus could ease airway hyperresponse when challenging concentrations of methacholine were 25, 50 and 100 mg/ml. The decrease of RIR reached a statistical significance compared with positive control when giving 100 mg/ml methacholine. The decrease pattern of RIR could also be observed in 1:1 group. This group seemed to have a greater therapeutic effect because its decrease of RIR reached a statistical significance when giving lower concentration of methacholine (50 mg/ml). The 2:2 group had no therapeutic effect in AHR.

Analysis of the cellular composition of the BALF showed eosinophil infiltration in positive control and Ad-Mock group (Fig.4). Administration of adenovirus could lower the numbers of eosinophils in lung and the treatments of Ad-IL-10 and 1:1 seemed to be better choices for therapy.

Cytokines in BALF after intra-tracheal delivery of adenovirus-expressing IL-10 and IL-12 gene

After AHR test, mice were sacrificed and we used 1ml 1xHBSS to lavage lung for 3 times. We kept the first 1 ml of lavage fluid to assay cytokine levels. Compared with the positive control group, administration of Ad-IL-12 induced large amount of TNF-α production (Fig.5A). However, giving adenovirus seemed to have no effect in IFN-γ expression (Fig.5B). IL-4 was suppressed by giving either Ad-IL-10 or Ad-IL-12 and the 1:1 group reached statistical significance (Fig.5C). The IL-5 result also revealed the therapeutic effect of Ad-IL-10 and Ad-IL-12(Fig.5D). Except for cytokines, we also assayed the BALF for eotaxin, an eosinophil chemotactic factor (Fig.6). The 1:1 group showed less expression of eotaxin in lung.

(四)討論

Although there are many researches about how IL-10 participates in immune-regulation in asthmatic patients, the precise role of IL-10 remains controversial. Makela et al. pointed IL-10 is required for the development of AHR and administration of IL-10 enhanced AHR, though it reduces eosinophilia (Makela et al., 2000). However, some reports indicate that IL-10 can prevent AHR (Jae-Won et al., 2002). Untill now, the definite opinion about IL-10 is its potential to decrease inflammation (Zuany-Amorim et al., 1995). Our studies demonstrated that the immuno-modulatory effects of intra-tracheal administration of Ad-IL-10 in a murine model of OVA-induced asthma. The subsequent pulmonary expression of IL-10 in

lung alleviates the severity of AHR (Fig.3) and reduces the infiltration of eosinophils (Fig.4).

IL-12 is a good candidate for asthmatic treatment. IL-12 not only inhibits the expression of IL-4 and IL-5 in lung but also decreases eosinophilic infiltration. According to our previous studies, administration of IL-12 could reduce eosinophilic infiltration, decrease expression of IL-5 and increase expression of IFN-γ(Lee et al., 1999; Lee et al., 2001). IFN-γ is a potent anti-inflammatory cytokine, but the role of IFN-γ in human asthma is still unclear. From our study, giving Ad-IL-12 can inhibit expression of IL-5(Fig.5D) and infiltration of eosinophil (Fig.4) in lung, but we can't observe high expression level of IFN-γ (Fig.5B) like our previous study. In another experiment, we found that giving higher dose of Ad-IL-12 could induce expression of IFN-γ. It seems that the dose of Ad-IL-12 used in this study is too low to induce IFN-γ expression. Although IFN-γ expression level in Ad-IL-12 group has no significant difference from the positive control group (Fig.5B), it still shows that administration of Ad-IL-12 has therapeutic effect for asthma. This result implicates that the therapeutic effect of low dose Ad-IL-12 is independent of IFN-γ production. There might be some routes to inhibit asthma when the dose of Ad-IL-12 is low. In Fig.5A, Ad-IL-12 induces mass expression of TNF-α. TNF-α is a pro-inflammatory cytokine. Numerous reports have proposed a role for TNF-a in the development of allergic airway inflammation and AHR in human asthmatics(Kips et al., 1993; Shah et al., 1995). In addition, administration of TNF-α causes AHR in allergen-sensitized mice. It has been demonstrated that intra-nasal administration of a monoclonal antibody to TNF-α before the last challenge significantly reduces the number of eosinophil, neutrophil and lymphocyte(Yu et al., 1997). These reports confirmed that TNF-α causes lung inflammation, AHR and eosinophilic infiltration.

IL-10 and IL-12 participate in asthma is still controversial. Using IL-10 or IL-12 to treat asthma has their pros and cons respectively. The disadvantage of using IL-10 is that we don't know the role in the development of AHR. Some reports indicate IL-10 can enhance AHR, but there are also some adverse reports. The major disadvantage of using IL-12 to treat asthma is that IL-12 induces large amount of TNF-α. TNF-α makes the inflammation of lung worse and attenuates the therapeutic effect. Since the use of Ad-IL-10 or Ad-IL-12 only has some therapeutic effect, we further want to find out whether combination of Ad-IL-10 and Ad-IL-12 may have better therapeutic effect. Truly, the 1:1 group markedly inhibits the development of AHR when the concentration of methacholine is greater than 50 mg/ml (Fig.3). However, the suppression of AHR in Ad-IL-10 and Ad-IL-12 are significant only

when concentration of methacholine is 100 mg/ml.

The result represents that the combined use of two adenoviruses is better than single use in AHR test. The cell number of eosinophil in BALF still has significant decrease (Fig.4), although the degree of decrease is not as obvious as Ad-IL-10 group. This difference might come from the effect of Ad-IL-12. Furthermore, in 1:1 group, IL-4 and IL-5 are reduced to a lower level than Ad-IL-10 or Ad-IL-12 group (Fig.5C, 5D). Besides, we also see that the production of eotaxin is decreased in 1:1 group (Fig.6). The combination treatment of 2:2 is not an ideal choice. Giving too much adenovirus reveals the side effects of IL-10 and IL-12. Compared with 1:1 group, the 2:2 group has less inhibitory effect on AHR and the production of IL-4 and IL-5.

Summing up the results of this summer, we conclude that the combination of low dose of Ad-IL-12 and Ad-IL-10 (1:1) might have best therapeutic effect to inhibit airway inflammation, reduce airway eosinophilia and suppress the expression of IL-4 and IL-5.

(五)圖表

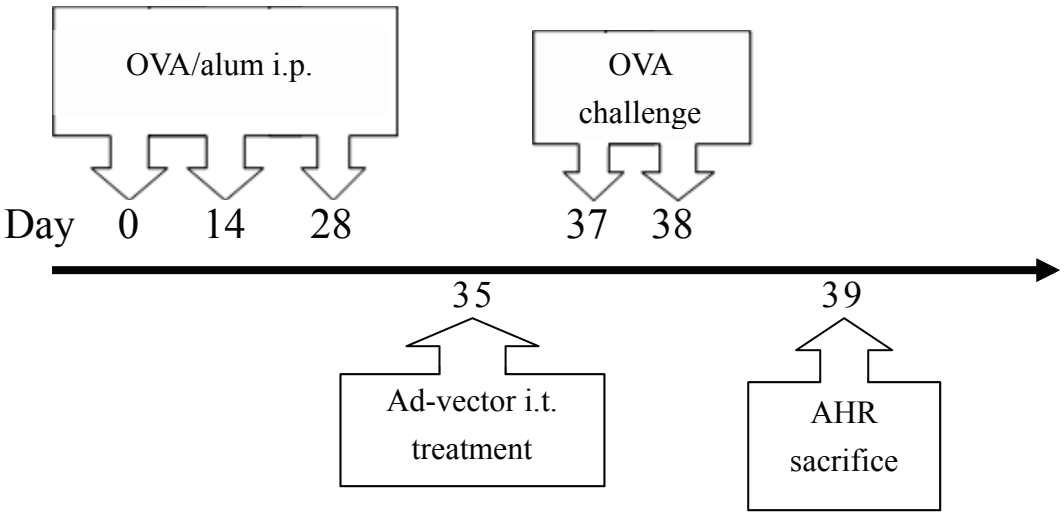

Fig.1 The protocol of treatment regimen

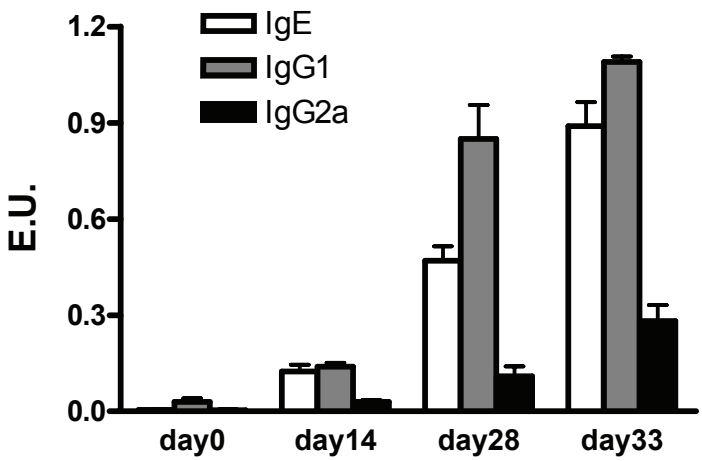

Fig.2 The expression levels of serum IgE, IgG1 and IgG2a.

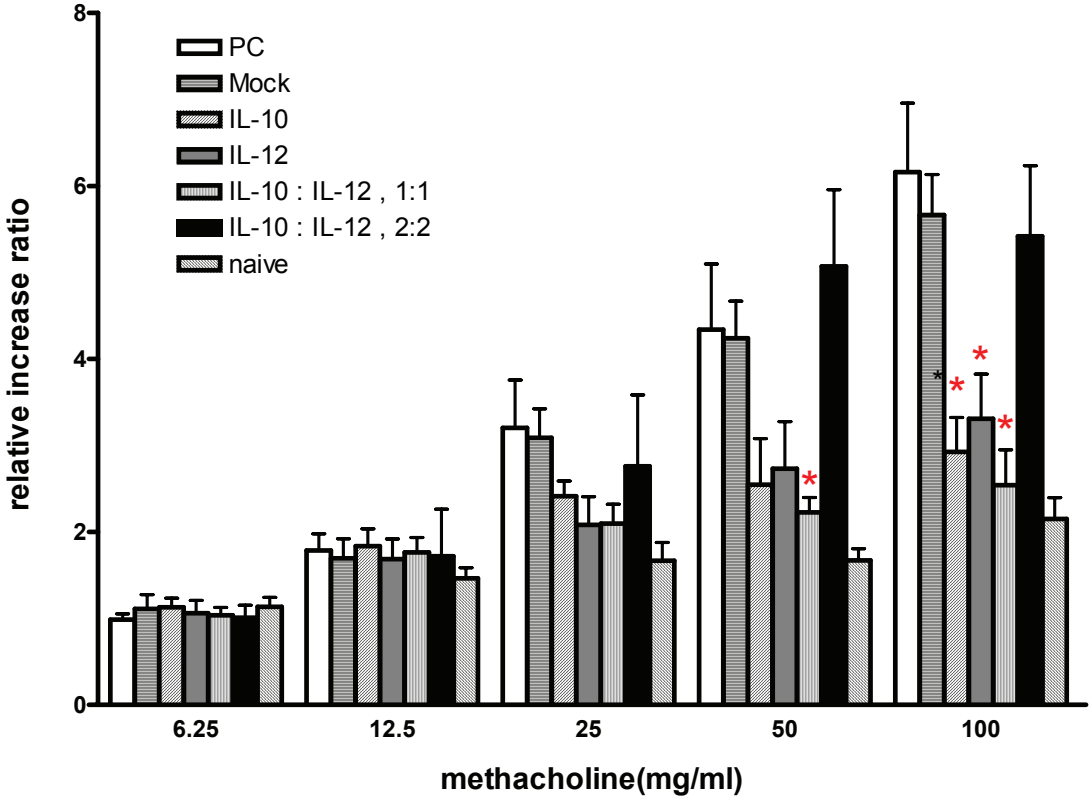

Fig.3 Effect of Ad-IL-10 and Ad-IL-12 on AHR when OVA-sensitized mice were challenged with aerosolized methacholine. Values presented as relative increase ratio($Penh_{methacholine}$ / $Penh_{normal\ saline}$). Significant difference from positive control group, *$p < 0.05$.

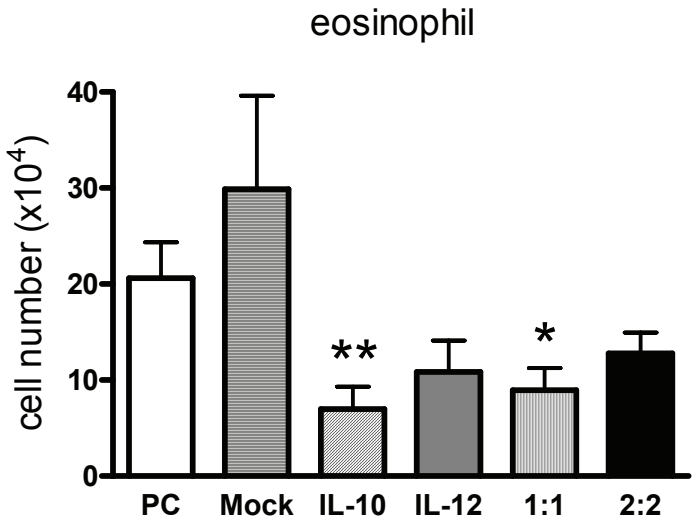

Fig.4 Effect of Ad-IL-10 and Ad-IL-12 on airway eosinophil infiltration. Data was presented as Mean±SEM. Significant difference from positive control group, *$p <$ 0.05, **$p < 0.01$.

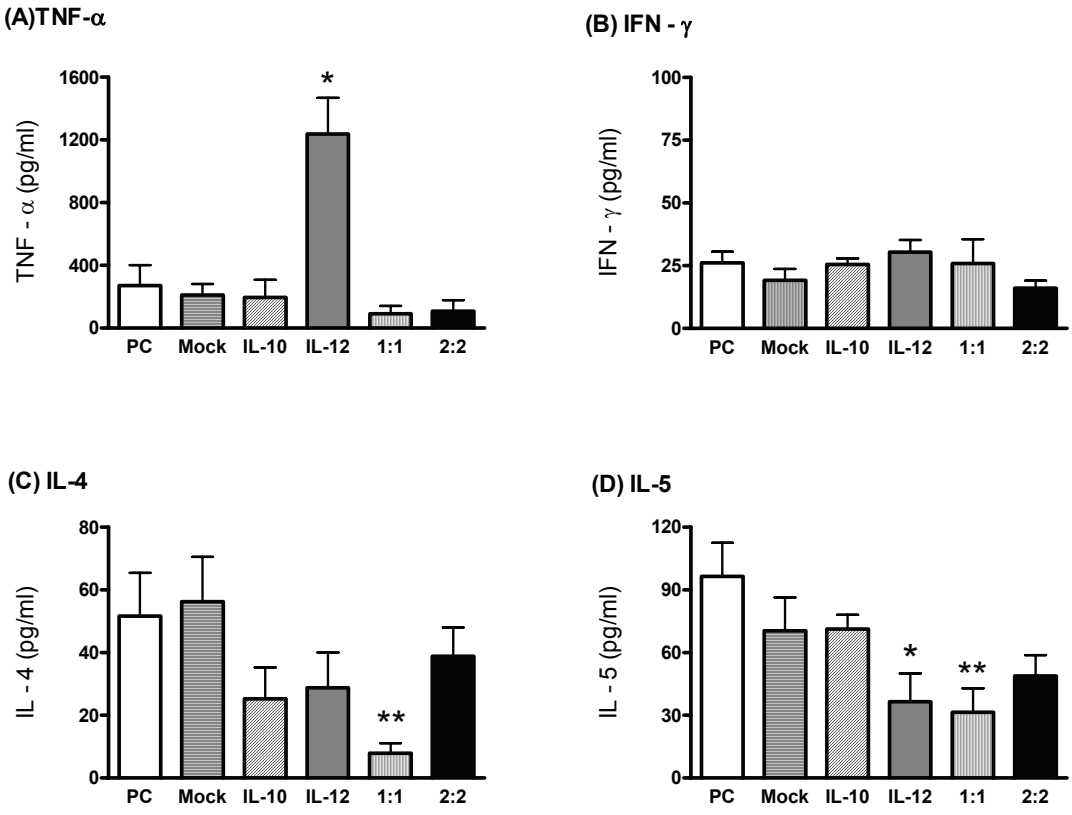

Fig.5 Cytokines expression levels in BALF after intra-tracheal administration of adenovirus vectors. Data was presented as Mean ± SEM, significant difference from positive control group, $*p < 0.05$、 $**p < 0.01$

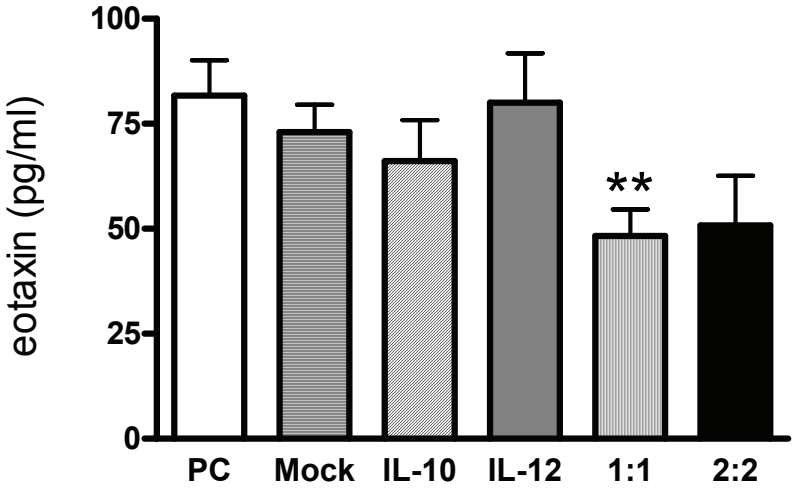

Fig.6 The levels of eotaxin in BALF. Data was presented as Mean ± SEM. Significant difference from positive control group, $^{**}p < 0.01$