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利用口服過敏原及基因合併細胞激素基因來治療氣喘的動物模式(3/3)

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行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

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報告類型(依經費核定清單規定繳交)：□精簡報告　■完整報告

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中文摘要

本計畫的主要目的是要建立一個氣喘的動物模式，以評估我們建立的細胞激素基因載體是否能夠達到改善呼吸道發炎的反應。所以我們在今年的計畫中分別建立了幾個可以表現細胞激素基因的載體，分別包括如 IL-4、IL-10、sIL-12 和 TGF-b 等基因。我們在建立氣喘的動物模式後又進一步將這些細胞激素的基因載體由氣管內給予，再追蹤這些小鼠內的過敏原特異性的抗體，結果發現不同得組別中抗體的濃度並沒有特別的變化。我們又進一步分析這些細胞激素基因對呼吸道氣管阻力的影響，結果發現 IL-10 和 TGF-b 基因可以有效地抑制這些小鼠的呼吸道的阻力，而且其抑制效果甚至比我們以前做的研究結果 sIL-12 來得好。同時，更進一步的研究結果顯示 IL-10 和 TGF-b 基因可以有效地降低呼吸道沖洗液中的嗜酸性白血球，而嗜酸性白血球是在氣管發炎反應中扮演著一個相當重要的發炎細胞，也可以說是主要的發炎細胞。所以，如果能夠有效地抑制這些嗜酸性白血球在呼吸道沖洗液中的數目，也等於說是抑制了氣喘的發炎反應。我們進一步將這些小鼠的肺部取出後作病理組織分析，也發現在 IL-10、TGF-b 和 sIL-12 治療的小鼠中的確可以有效地改善肺部的發炎反應。而在進一步的分析中，也發現 eotaxin 和前列腺素的濃度在 IL-10 組也有降低的情形。由這些結果來看，我們發現這些 IL-10、TGF-b 和 sIL-12 的基因在氣喘動物模式的誘發前經由氣管給予，發現可以有效地改善呼吸道發炎反應。顯示利用細胞激素基因，尤其是一些抗發炎反應的細胞激素的確可以降低發炎反應。更何況，IL-10 和 TGF-b 其實與所謂的調節性 T 細胞有關，未來應該可以針對這方面來進行更詳細的研究。

關鍵詞:

Abstract

Summary

Background An increasing prevalence of allergic diseases, such as atopic dermatitis, allergic rhinitis and bronchial asthma, has been noted worldwide. Allergic asthma strongly correlates with airway inflammation caused by the unregulated production of cytokines secreted by allergen-specific type-2 T helper (Th2) cells.

Objective This study aims to explore the therapeutic effect of the intra-tracheal gene transfer of IL-12, IL-10 and TGF-β genes on airway inflammation in a mouse model of allergic asthma.

Methods BALB/c mice were sensitized to ovalbumin (OVA) by intraperitoneal injections with OVA and challenged by nebulized OVA. Different cytokine gene plasmids or non-coding vector plasmids were instilled daily into the trachea up to one day before the inhalatory OVA challenge phase.

Results Intratracheal administration of IL-10 or TGF-β gene plasmids can efficiently inhibit antigen-induced airway hyper-responsiveness and is able to significantly lower the number of eosinophils and neutrophils in bronchoalveolar lavage fluid of ovalbumin (OVA) sensitized mice during the effector phase. Furthermore, the effect of IL-10 plasmids is more remarkable than any other cytokine gene plasmid. On the other hand, local administration of IL-4 gene plasmids before antigen challenge can induce severe airway hyper-responsiveness and eosinophilia.

Conclusion Our data demonstrated that anti-inflammatory cytokines, particularly IL-10, have more therapeutic potential than the Th1-driving cytokine- IL-12 in the treatment of allergic diseases.

Keywords cytokine, gene therapy, asthma

一、 前言、研究目的、文獻探討

Asthma is an immunological disease that has increased dramatically in prevalence over the past two decades. It is characterized by airway hyperreactivity to a variety of specific and non-specific stimuli, severe chronic airway inflammation with pulmonary eosinophils, mucus hypersecretion, and increased serum IgE levels. Activation of Th2 cells in the respiratory tract is now believed to be responsible, in part, for the pathogenesis of this disease. Th2 cells secreting IL-4, IL-5, and IL-13 have been identified in the airways of asthmatics (1). Th2 cytokines produced in the respiratory tract, airway eosinophilia, high levels of serum IgE, and mast cell activation (2-3), are all believed to contribute to the pathological consequences inducing airway hyper-responsiveness (AHR), epithelial damage, and mucus hypersecretion.

Whereas the immunological mechanisms that induce asthma and allergies are relatively well characterized, the specific mechanisms that transpire *in vivo* to downmodulate Th2 cell-mediated allergic inflammatory responses are not yet clear. The Th1-relatived cytokines, such as IL-12 and IFN-γ, are the candidate cytokines for the treatment of allergic diseases as they downregulate Th2 responses (4). There is strong evidence regarding the therapeutic effect of Th1 cytokine administration. Using Th1-related cytokine proteins (5-7) and constructed plasmids expressing cytokine genes (8-10), airway inflammation could be decreased. According to our previous study (11), we also demonstrated that the local transfer of the IL-12 gene to the respiratory tract could modify allergic inflammation and airway hyper-responsiveness (AHR). However, recent studies have shown that not only Th1-related cytokines but also other anti-inflammatory cytokines, including TGF-β and IL-10, can downregulate Th2 responses and might also play an important role in regulating pulmonary inflammation and asthma (12, 13). IL-10 and TGF-β, which are pleiotropic cytokines with significant anti-inflammatory and immunosuppressive properties, are key regulators in the maintenance of immunological homeostasis. It has already been reported that allergic individuals express lower levels of IL-10 in locally affected sites (14, 15). In addition, IL-10 can inhibit MHC class II expression and macrophage activation and might induce T-cell anergy by enhancing the suppressive effects of TGF-β (16). TGF-β inhibits the production of proinflammatory cytokines from macrophages, B cells, and T cells and is a potent inhibitor of T cell-mediated immune responses, both *in vitro* (17,18) and *in vivo* (19, 20). Moreover, TGF-β has been postulated in the mechanism of oral tolerance, which is mediated by regulatory T cells that produce TGF-β preferentially induced at mucosal sites, possibly under the influence of IL-10 and/or IL-4 (21). Recently, Hansen et al. showed that not only TGF-β-producing T cells but also IL-10-producing T cells can abolish AHR and airway inflammation in a murine model of asthma (22, 23). However, the precise role of these anti-inflammatory cytokines in the lungs is not clear.

In this study we investigated the effect of four different cytokine genes including IL-12, IL-10, TGF-β, and IL-4 on the effector phase of allergen-induced AHR and asthma. In addition, we also investigated whether these cytokine genes could affect the expression of inflammatory mediators.

二、 研究方法

Animals

Female BALB/c mice were obtained from and maintained at the Animal Center of the College of Medicine of National Taiwan University. Animals were used between six and 10 weeks of age and were age-matched within each experiment. Animal care and handling conformed to the *NIH Guide for the Care and Use of Laboratory Animals*.

Plasmids and preparation of lipid-plasmid DNA complexes

For the construction of plasmid DNA encoding murine IL-4, IL-10 or TGF-β, the cDNA for murine IL-4, IL-10, or TGF-β was cloned by reverse transcription- polymerase chain reaction (RT-PCR) from normal mouse spleen cells, using primers based on the published cytokine

sequence. The cDNA was sequenced and *in vitro* expression was confirmed by enzyme-linked immunosorbent assay (ELISA) and bioassay (data not shown). The cytokine gene expression vector utilized the human cytomegalovirus (CMV) immediate-early promoter and the simian virus 40 (SV40) polyadenylation sequence. The vector without a gene insert (empty vector) served as a control for *in vivo* gene delivery studies.

The construction of pscIL-12 vectors were described previously (24). Briefly, the p40 and p35 subunits of the murine IL-12 gene were connected by a linker of 54-bp in length in the pscIL-12 plasmids. The p40 and p35 subunits were obtained by polymerase chain reaction (PCR) from the BLpSV35 and BLpSV40 plasmids. Recombinant PCR, using the p40 and P35 PCR products as the DNA templates and the *Sal* I-sontaining and the *Bam* HI-containing primers as such primers, generated the single-chain IL-12 genes. The resulting recombinant PCR fragments were cloned at the *Sal* I and *Bam* HI sites of the pCMV vector. Plasmid DNA were subsequently introduced into the *Escherichia coli* DH5α by transformation. The plasmid were purified using EndoFree plasmid kits (QIAGEN, Valencia, CA) and suitable for gene therapy. No CpG motif sequence was noted in the IL-4, IL-10, IL-12 and TGF-β genes.

For intra-tracheal delivery, lipid-DNA complexes were prepared by combining 20 μL lipofectAMINE (Life Technologies, Gaithersburg, MD), per 10 μg of plasmid DNA at a final volume of 30 μl in water. The expression of cytokine plasmid in pulmonary tissues was determined by the cytokine ELISA of BALF collected 48 hr post-injection (24).

Administration of DNA-lipid complexes

Intra-tracheal administration was accomplished by the use of a No. 23 steel gavage tube and a 1.0-ml syringe. Animals were anesthetized (pentobarbital sodium salt, Tokyo Chemical Industry, Tokyo, Japan, 10 mg/ml solution, 0.005 ml/g body weight) prior to intra-tracheal injection and placed in dorsal recumbency on an inclined board. The gavage tube was directed into the proximal trachea, and then the lipid-DNA solution was injected slowly. Proper positioning of the tube was assured by visualization of movement of the fluid meniscus and by palpation of the gavage tube moving across the tracheal rings. A volume of 30 μl lipid-DNA mixture was injected intratracheally, such that each mouse received 10 μg of plasmid DNA. This technique works well without involving any surgical procedure and allows the aspirated material to spread over the whole lung.

Administration of cytokine plasmid into allergen-sensitized mice

BALB/c mice were sensitized by an intraperitoneal injection with OVA (Sigma, St. Louis, MO, 10 μg) complexed with aluminum potassium sulfate (Imject Alum, Pierce Biotechnology Inc., Rockford, IL, 2 mg) on day 0. On day 14, the mice were boosted with OVA (30 μg) adsorbed to alum. As the negative control group, the mice were injected with PBS only. To examine the therapeutic effects of different cytokine plasmids, each group of mice received intra-tracheal delivery of 10 μg pCDNA vector only or a single chain IL-12 DNA plasmid or TGF-β plasmid or IL-10 plasmid or IL-4 plasmid liposome complexes, respectively, two days before the inhalation challenge on day 26, 27, and 28. On day 28, 29, and 30, mice were exposed to aerosolized 0.2% (W/V) OVA over a 20-minute period, by placing them in a chamber which could contain up to six to eight mice concurrently. One day after the last challenge (day 31), AHR was measured. On day 32, mice were bled and then terminated. (Fig.1)

Measurement of airway hyper-responsiveness

Airway responsiveness was measured as a change in function after challenge with aerosolized mechacholine (Mch) in conscious, spontaneously breathing animals by barometric plethysmography (Buxco, Troy, NY) as described in the literature (25). Pressure differences were measured between the main chamber of the plethysmograph, containing the animal and a reference chamber (box pressure signal). Mice were challenged with aerosolized saline (for the

baseline measurement) or Mch (3.13 to 50 mg/ml) for three minutes and readings were taken and averaged for three minutes after nebulization. The Penh value for each minute was recorded and after the third recorded value, the average Penh value was divided by the Penh of normal saline and was presented as a relative percentage increase of Penh.

Determination of OVA-specific antibody levels

Total IgE concentrations and OVA-specific IgE, IgG1, and IgG2a antibody concentrations in serum were measured by ELISA. Briefly, microtiter plates were coated with 1.0 mg OVA protein in 100 ml carbonate coating buffer per well and incubated at 4℃ overnight. The plates were then washed twice with 1× PBS containing 0.05% Tween 20. Then, the plates were blocked with blocking solution (1%BSA-0.05% Tween 20 in 1× PBS) and washed three times after reaction. Serum sample were diluted with blocking solution (3%BSA in 1× PBS), and 100μl per well was added. After two hours of incubation, biotinylated anti-IgE antibody (02122D; BD PharMingen, San Diego, CA) was used for detecting antibodies, and the reaction was amplified with avidin-horseradish peroxidase. To detect IgG1 and IgG2a, biotinylated anti- IgG1 and biotinylated anti- IgG2a antibodies (02172D and 02012D; BD PharMingen) were used. The OVA-specific antibody titers of samples were related to an internal pooled standard, which was arbitrarily designated in ELISA units (EU).

Analysis of bronchoalveolar lavage (BAL) fluid and lung histology

At 48 hours after the last aerosol exposure, all groups of mice were bled from the retro-orbital venous plexus and terminated. The lungs were immediately lavaged via the tracheal cannula with 3 × 1 ml of HBSS, free of ionized calcium and magnesium. The lavage fluid was centrifuged at 400 ×g for 10 minutes at 4℃. After washing, the cells were resuspended in 1 ml HBSS, and total cells counts were determined by counting in a hemocytometer. Cytocentrifuged preparations were stained with Liu's stain for different cell counts. A minimum of 200 cells were counted and classified as macrophages, lymphocytes, neutrophils, and eosinophils, based on standard morphological criteria.

After the lavage, the lungs were immediately removed and fixed in 10% neutral-buffered formalin, routinely processed, and embedded in paraffin wax. Five-micrometer sections were prepared and stained with hematoxylin and eosin (H&E).

Measurement of Eotaxin and LTB4 levels in bronchoalveolar lavaged fluid

The concentration of eotaxin was assayed with an ELISA kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. Briefly, the bronchoalveolar lavage of each condition was added to wells precoated over-night at 4℃ with anti-eotaxin Ab. After two hours of incubation, the plates were washed and biotin-conjugated Ab was added. After two more hours at room temperature, HRP-avidin was then added, and the OD (at 450 nm) values were converted to concentrations of chemokine in the BALF. The sensitivity of this assay was 1.9 pg/ml for eotaxin.

LTB4 levels in the BALF were determined using the LTB4 enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's instructions. The detection limits for LTB4 is 47 pg/ml.

Statistical analysis

Data are expressed as the mean±SEM for each group. The statistical significance of the differences between various treatment groups was assessed with the Mann-Whitney U test for non-parametric data.

三、 結果與討論

The levels of OVA-specific serum antibodies in OVA-sensitized mice after treatment with different cytokine gene plasmids

Ovalbumin-sensitized mice had increased total serum IgE concentrations and produced OVA-specific IgE and IgG1 antibodies after airway challenge with OVA (Figure 2). Low levels of OVA-specific IgG2a were also detected. Intra-tracheal administration of mock vector DNA did not change OVA-specific antibody levels. Intra-tracheal treatment with the IL-4 gene resulted in increased concentrations of OVA-specific IgG1 ($p= 0.019$). The OVA-specific IgE concentrations were also increased, but the increase was not significant. Furthermore, administration of scIL-12, IL-10, or TGF-β plasmid DNA did not significantly change OVA-specific IgE, IgG1, or IgG2a levels.

The effect of different cytokine genes on methacholine-induced increase in AHR

In order to examine the effect of different cytokine genes, lipid- plasmid DNA complexes were administered intratracheally 48 hours prior to OVA challenge in OVA-sensitized mice. Our previous studies have demonstrated the expression of genes and proteins of delivered genes 24-48 hours after the administration (32). One day after the last allergen challenge, each group of mice was measured for airway responsiveness to aerosolized methacholine (Figure 3). We measured the extent of airway constriction of mice using the Buxco system. The Penh (pause of enhance) increased as the concentration of mechacholine increased. The mice sensitized with OVA but only admistered mock vector-only developed marked increased airway responsiveness to methacholine challenge compared with mice challenged without prior sensitization. There was no statically significant difference between mice given IL-4 gene plasmids and the vector-only group in airway hyperresponsiveness. Similar to our previous study (11), local administration of single-chain IL-12 gene plasmids exerted the therapeutic effect in OVA-induced asthma model as in the Der p 1-induced asthma model. Further, administration of TGF-β gene plasmids and IL-10 gene plasmids has also been found to inhibit the increase in airway responsiveness to methacholine after aerosol challenge in OVA-sensitized mice when compared with that of the mock vector-only group.

The effect of different cytokine gene plasmids on airway eosinophilia

Further, we analyzed the cellular composition in the BAL fluid of sensitized mice 48 hours after the last challenge to determine whether the local transfer of cytokine gene plasmids could alleviate airway inflammation.. In vector-only treated sensitized mice, exposure to aerosolized OVA often induced a marked increase in the number of neutrophils and eosinophils in BALF (Figure 4). In contrast, a few cells were noted in non-sensitized mice. However, administration of scIL-12-encoding vector inhibited the recruitment of eosinophils ($p=0.12$). Administration of IL-10 gene plasmids ($p=0.009$) had a more significant decrease in the level of eosinophilia than those given TGF-β ($p=0.04$) and IL-12 ($p=0.12$) encoding vector. Furthermore, the recruitment of neutrophils was almost completely inhibited by the treatment of IL-10 encoding vector ($p=0.019$). On the other hand, through the administration of IL-4 gene plasmids in sensitized mice, a sharp increase in neutrophils, eosinophils, and lymphocytes was noted ($p=0.026$, compared to negative control).

Histopathologically (Figure 5), many cells infiltrated around the bronchial and lung alveoli in both the control and vector treated group; in the contrast, the damage and infiltrative cells were less severe in the IL-10 plasmid (Figure 5D) or TGF-β plasmid (Figure 5E) or scIL-12 plasmid treated group(Figure 5C). However, pulmonary tissues from IL-4 plasmid treated mice (Figure 5F) showed severe airway inflammation. These results demonstrated that intratracheal delivery with scIL-12 plasmid, TGF-β plasmid or IL-10 plasmid could efficiently inhibit the infiltration of the cells and reduce the pathological damage within the lung in this mouse model.

The effect of different cytokine gene plasmids on eotaxin and leukotriene B_4 (LTB4) levels in BAL fluid

In order to investigate the effects and underlying mechanism(s) of the action of different cytokine gene plasmids on eosinophils recruitment, the inflammatory mediators implicated in regulating eosinophils accumulation was also determined. Allergen challenge via the airway in sensitized mice resulted in a sharp increase in eotaxin levels in BALF (P=0.005, compared with the negative control). In our previous *in vitro* study, Ye et al. (26) have demonstrated that IL-4 could stimulate lung cells to secret eotaxin, but IL-12 could suppress eotaxin secretion from IL-13 or IL-4 stimulated primary lung cell culture. In present study, *in vivo* experiment also supports this result. Administration of IL-4 gene plasmid could increased the level of eotaxin in the BALF, but the level of eotaxin was decreased in the mice treated with scIL-12 plasmid. Furthermore, the eotaxin levels in BAL fluid significantly decreased through the delivery of IL-10 (P=0.019) and TGF-β encoding vector (P=0.007) (Figure 6A). The data shows that the eotaxin levels correlate with the reduction in eosinophils in BALF.

LTB4 is a potent eicosanoid lipid mediator that is involved in numerous homeostatic biological functions and inflammation (27). The interaction between eicosanoid may represent means to regulate the release of inflammatory mediators, and may be important for the regulation of cell functions and inflammatory disorders, such as allergic asthma. As shown in Figure 6B, the levels of LTB4 in the BAL fluid were also determined after administration of different cytokine gene plasmid. The level of LTB4 in BAL fluid did not show a significant difference among groups treated with different cytokine gene plasmids. However, LTB4 concentrations in the BAL fluid of the IL-10 gene-treated group was obviously lower than that of vector-only treated group (p=0.085). This result was proven that IL-10 gene plasmid could decrease the production of LTB4 as previous study.

DISCUSSION

The prevalence of asthma worldwide has been increasing significantly in the past two decades, such that asthma now affects as many as 10% of individuals in industrialized countries (28, 29). Allergic diseases are characterized by the presence of Th2 cells and related cytokines, such as interleukin-4 (IL-4), IL-5, IL-9, and IL-13 with the subsequent development of eosinophils infiltration and chronic inflammation. Although the immunologic mechanisms that induce asthma and allergic diseases are relatively well characterized, the specific mechanisms that transpire *in vivo* to downmodulate Th2-mediated allergic inflammatory responses are yet to be clarified. However, blocking the release or effects of proinflammatory cytokines in allergic asthma has provided the basis for the development of novel treatments (12). In this study, we employed a liposome-mediated genetic transfer approach to examine the therapeutic efficacy of the local pulmonary delivery of various cytokine gene plasmids in an established murine model of asthma in OVA-sensitized mice.

Although the control of allergic inflammation and asthma is complex, involving several different mechanisms and several different cell types and cytokines. Interleukin-12 is the endogenous regulator of Th1 cell development and determines the balance between Th1 and Th2 cells (30). Several studies have demonstrated that IL-12 protein can decrease allergen-specific IgE and eosinophils infiltration in a mouse model of airway inflammation (5, 6, 9, 31). Previous studies have shown that intravenous injection of single chain IL-12 DNA plasmids mixed with liposome achieved the highest protein expression in the lungs and can alleviate airway hyperresponsiveness in an animal model of asthma. (32). Furthermore, the local IL-12 gene transfer to the lung before the tracheal allergen challenge resulted in a remarked decrease in IL-5 levels and a similarly marked increase in IFN-γ, this being consistent with a shift from a Th2 to a Th1 profile (11). The results of our present study also support the finding that intra-tracheal delivery of IL-12 encoding DNA plasmids can decrease eosinophils infiltration in a murine model of airway inflammation.

IL-10 is a pleiotropic cytokine with significant anti-inflammatory and immuno- modulatory properties. IL-10 can down-regulate cytokine production not only from Th1 cells (33) but also from Th2 cells (34). Borish et al. (14) reported that asthmatic had a comparatively decreased ability to produce IL-10 by BALF and mononuclear cells. In a subsequent report, Hobbs and associates (35) documented that polymorphisms were putatively associated with defective transcription in the promoter region of IL-10 genes in patients with allergies and asthma. These associations led to the speculation that constitutive expression of IL-10 in the airway might contribute to maintain the normal state of allergen nonresponsiveness. However, the role of IL-10 in regulating Th2-mediated diseases such as asthma is controversial. IL-10 has been considered to be an essential Th2 cytokine (36), particularly because IL-10 inhibits Th1cytokine production by inhibiting IL-12 synthesis (37) and B7 expression (38). In addition, IL-10 plays an important role in inhibiting Th1-mediated autoimmune diseases such as experimental allergic encephalomyelopathy (39), suggesting that IL-10 might contribute to the pathogenesis of allergic diseases. Indeed, IL-10 is required for the development of AHR and administration of IL-10 enhanced AHR, though it reduces eosinophilia (40). The overall physiologic effect of IL-10 is to decrease inflammation. It accomplishes this role primarily by down-regulating synthesis of a number of cytokines, both Th1- and Th2-associated. In the present study we investigated whether the expression of IL-10 in the airway environment could modulate OVA-specific immune-inflammatory responses in OVA-sensitized and challenged mice. As shown in Figures 2-4, expression of IL-10 in the airway can inhibit the inflammatory response elicited by repeated OVA challenge. The inhibitory effect was not restricted to decrease airway hyper-reactivity but also affected the severity of eosinophilia and neutrophilia. Stampfli et al. showed that concurrent expression of IL-10 at the time of mucosal sensitization altered the immune response to OVA (41). Here, we showed IL-10 expression at the time of mucosal challenge phase also can inhibit OVA-induced airway inflammation. These results support the concept that IL-10 plays an inhibitory regulatory role in allergic asthma. In recent years, it is generally accepted that IL-10-producing regulatory T cells mediates antigen-specific tolerance that protects against allergic diseases and airway inflammation. For example, regulatory T cells producing IL-10 are thought to develop after successful bee venom- specific immunotherapy (42) and during T-cell tolerance induced with respiratory exposure to antigen (43, 44). The inhibitory effects of regulatory T cells were dependent on the production of IL-10, because neutralization of the IL-10 with an anti-IL-10 monoclonal antibody reversed the inhibitory effect of the regulatory T cells (23). Therefore, we speculate that IL-10 deviate the Th2- polarized immune response at the time of mucosal sensitization but directly inhibit the Th2 inflammatory response at the challenged phase.

TGF-β, which is also an anti-inflammatory cytokine, is a key immunoregulatory factor in the development of unresponsiveness to antigens in the gastrointestinal tract, an anatomic site that is closely related developmentally to the respiratory tract. TGF-β inhibits the production of proinflammatory cytokines from macrophages, B cells, and T cells, and is a potent inhibitor of T cell-mediator of T cell-mediated immune responses both *in vitro* (17-18) and *in vivo* (19-20). Hansen and his colleagues (22) have demonstrated that CD4+ T helper cells engineered to produce TGF-β1 in the respiratory mucosa can indeed reverse allergen-induced airway hyperreactivity and inflammation. TGF-β- secreting T cells, called Th3 cells, have been shown to play a regulatory role at mucosal sites- e.g., in the induction of oral tolerance (45). In addition, TGF-β- secreting T cells might also play a significant role in modulating allergic inflammation (22). However, TGF-β is also a potent inducer of myofibroblasts and collagen synthesis. It has been reported that eosinophils might produces TGF-β to prevent allergen-induced AHR at the late phase (46). In our study, the result has shown that administration of TGF-β plasmid in OVA-sensitized and challenged mice can decrease airway hyper-reactivity, eosinophilia and neutrophilia. Thus, TGF-β plays a role in immunoregulation, wound healing, and to shorten the inflammatory response when it is applied in the treatment of allergen-induced asthma.

IL-4 is a critical factor for Th2 differentiation and the generation of the IgE response. IL-4 also plays a role in eosinophils recruitment, although the main instigator of eosinophil is IL-5 (47). In this study, our results also showed that local IL-4 gene transfer to the lung before the allergen challenge could cause severe eosinophilia and lymphocyte infiltration when compared with the vector-only group. However, the serum titer of OVA-specific IgE was not increased after

local IL-4 gene transfer, since local injection of IL-4 plasmid is probably not stronger enough to affect the systemic production of serum antibody.

As suggested by the effect of IL-10 and TGF-β on airway eosinophilia, IL-10 and TGF-β appears to play a role in regulating eosinophils recruitment. Amongst eosinophil-active chemoattractants, eotaxin specifically attracts and activates eosinophils as demonstrated *in vitro* for both mouse and human eotaxin using assays for chemotaxis and calcium release (48, 49). Eotaxin has also been demonstrated to selectively induce eosinophil recruitment to the airways and to the skin *in vivo* (50). As shown in Figure 6A, we found that eotaxin levels in BAL fluid were significantly decreased by treatment with IL-10 or TGF-β encoding vector. It is possible that IL-10 and TGF-β can suppress OVA-induced airway eosinophils recruitment directly by down-regulating the production of eotaxin.

Moreover, we also detected the level of inflammatory mediators in the BALF to determine the effect of these cytokine-encoding vectors. Both leukotriene B4 (LTB4) and prostaglandin E2 (PGE) is a potent pro- inflammatory mediator and is involved in several inflammatory diseases (27). The result of our study indicates that IL-10 can significantly decrease the level of LTB4 in the BALF (Figure 6B), but other cytokine gene plasmids may not show a similar effect. In contrast, all cytokine plasmids do not have any effect on the level of PGE2 in the BALF (data not shown). LTB4 is a potent neutrophil chemoattractant that enhances neutrophil-endothelial interactions and stimulates neutrophil activation (51). LTB4 may contribute to airway narrowing by producing local edema and increasing mucus secretion. The overproduction of LTB4 plays an important role in the pathogenesis of asthma and acute lung injury (52). In recent study, Harizi et al. (53) demonstrated that endogenous IL-10 could inhibit the production of LTB4 from dendritic cells. Our experiment data also support that expression of IL-10 in the airway could affect the production of LTB4 to alleviate the symptom of airway inflammation.

Collectively, these data suggests that immunosuppressive cytokines, such as TGF-β and IL-10, as well as Th1-related IL-12, can alleviate the symptom of airway inflammation in a murine model of asthma. However, therapeutic mechanisms of these cytokine gene plasmids are different and affect different inflammatory mediators. It still needs further investigation to analyze the further detailed mechanisms.

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Legends

Figure 1 **Treatment regimen.** Time line representation of the OVA protocol used and the intratracheal injection of cytokine plasmid. i.p., intraperitoneal; i.t., intra-tracheal.

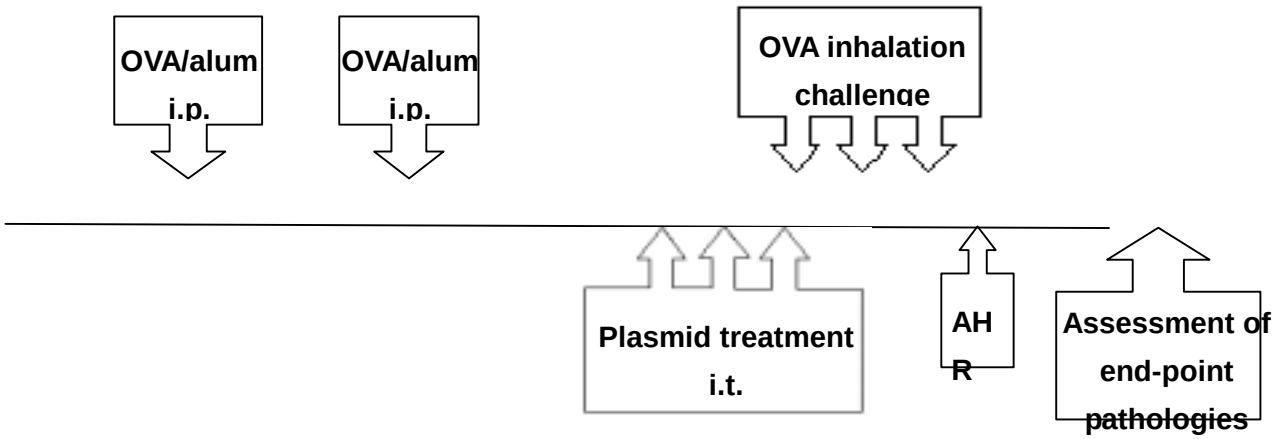


Figure 2 **Effect of intra-tracheal cytokine gene plasmids on serum OVA-specific immunoglobulin.** BALB/c mice were immunized i.p. with OVA in alum twice on Day 0 and 14. OVA sensitized mice were treated with different cytokine gene plasmid as described in *Material and Methods*. Mice were bled 48 hours after the last aerosol challenge from OVA sensitized mice with or without treatment of different cytokine gene plasmids. Serum OVA - specific IgE, IgG1, and IgG2a were tested by ELISA. Data are representative of three separate experiments with similar results. The columns and error bars represent mean±SEM of 4 to 8 mice per group.
* $P < 0.05$, **, $P < 0.01$ vs. vector-only treated control group

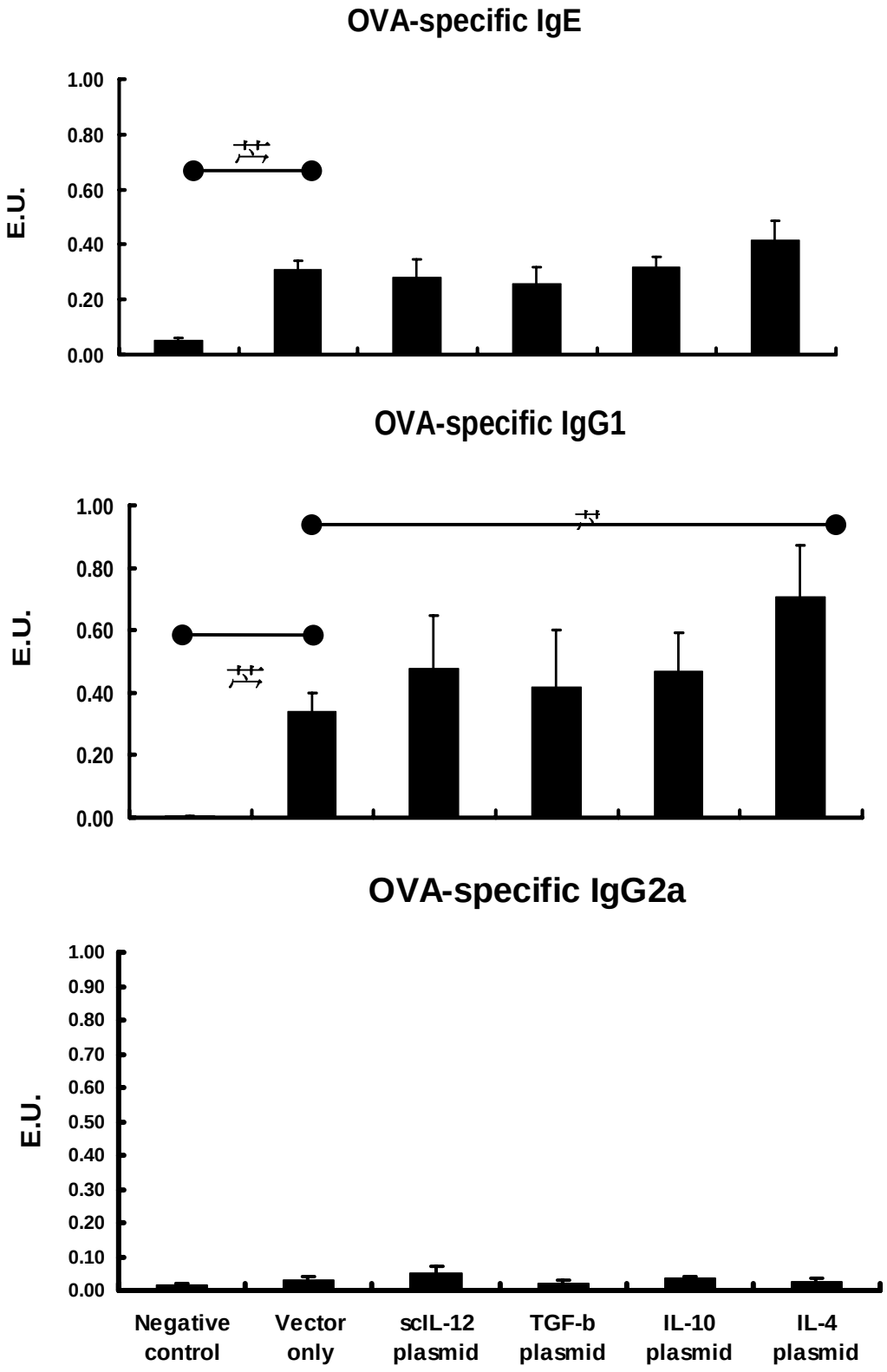

OVA-specific IgE
E.U.
1.00
0.80
0.60
0.40
0.20
0.00
OVA-specific IgG1
E.U.
1.00
0.80
0.60
0.40
0.20
0.00
OVA-specific IgG2a
E.U.
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Negative control
Vector only
scIL-12 plasmid
TGF-b plasmid
IL-10 plasmid
IL-4 plasmid

Figure 3 **The effect of different cytokine genes on methacholine- induced increases in AHR.** Female BALB/c mice were sensitized with OVA and treated with different cytokine gene plasmid as described in *Material and Methods*. Mice were nebulized with titrated doses of methacholine and tested for AHR by whole-body plethysmograph 24 hr. after last aerosol challenge. The columns and error bars represent mean Penh ± SEM of 4 to 8 mice per group. Data are representative of three separate experiments with similar results. The columns and error bars represent mean Penh ± SEM of 4 to 8 mice per group.§ P<0.1, * P < 0.05, # P <0.01 vs. vector-only treated control group

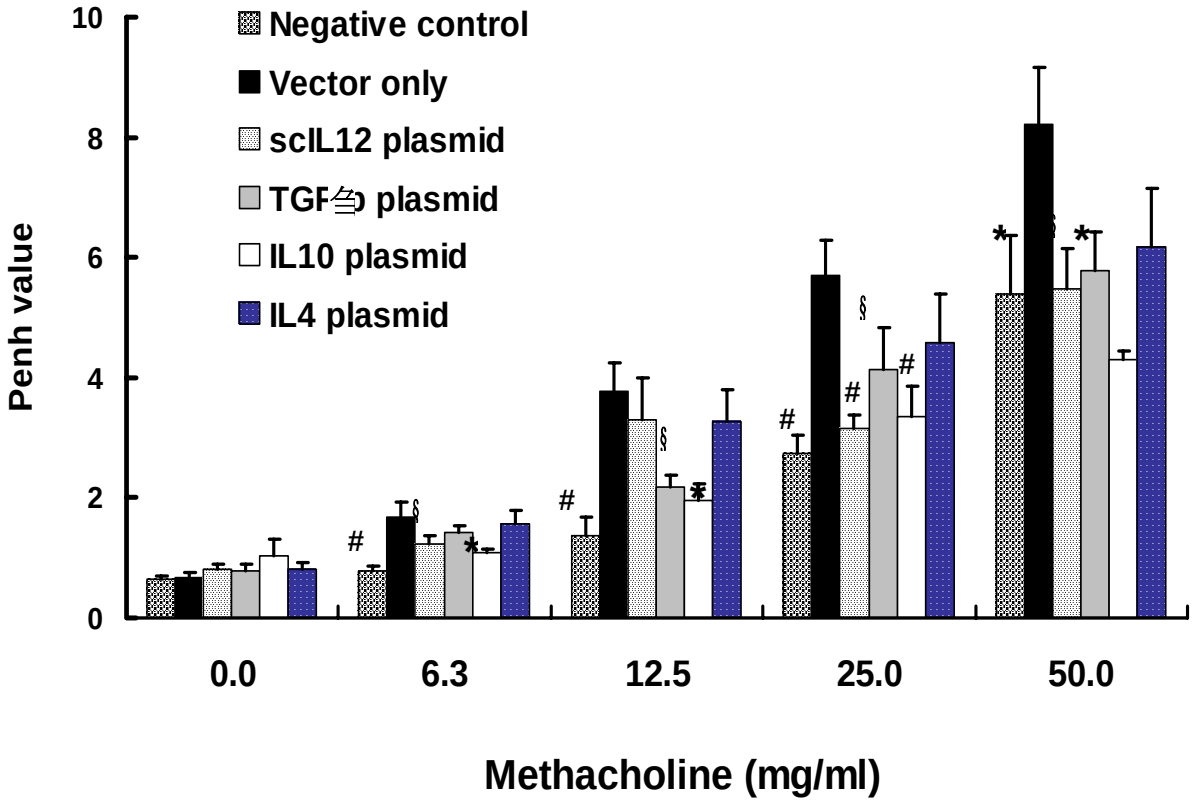


Figure 4 **Different cell counts of BALF in mice treated with different cytokine gene plasmids after aerosol challenge.** Female BALB/c mice were sensitized with OVA and treated with different cytokine gene plasmid as described in *Material and Methods*. Mice were killed 48 hr. after last aerosol challenge and the differential of the BAL cells are shown. Bars represent, from left to right, untreated mic(negative control), mice exposed OVA and treated with vector plasmid (vector only), single-chain IL-12 plasmid (scIL-12 plasmid), TGF-β plasmid, IL-10 plasmid and IL-4 plasmid. Data are representative of three separate experiments with similar results. The columns and error bars represent mean ± SEM of 4 to 8 mice per group.

- P < 0.05, ** P <0.01 vs. vector-only treated control group

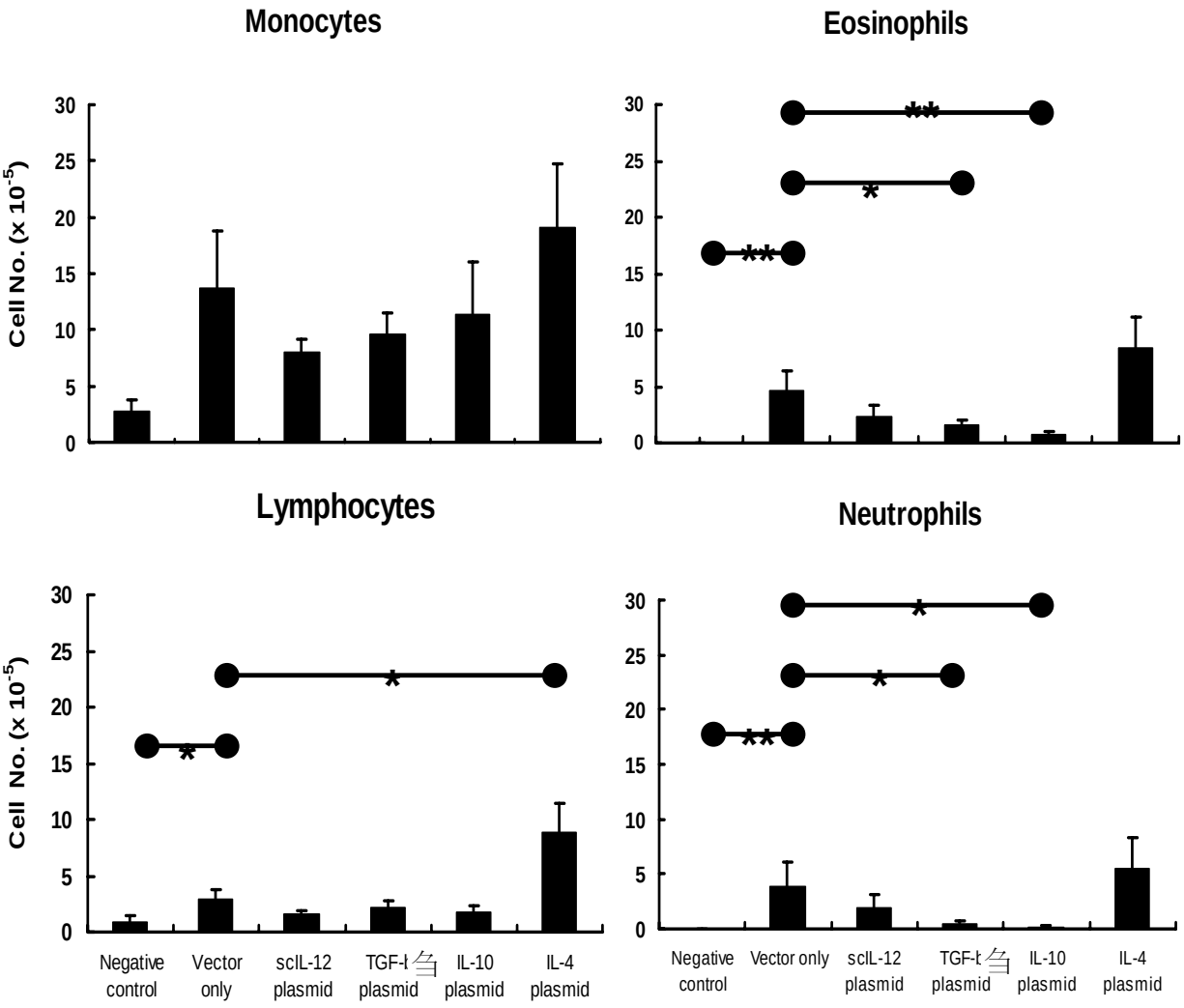
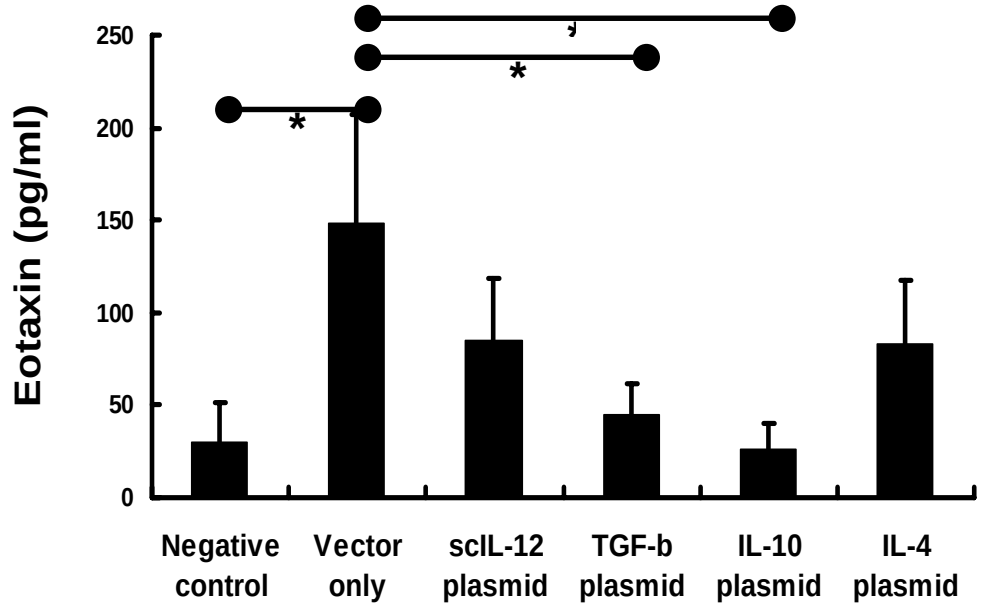


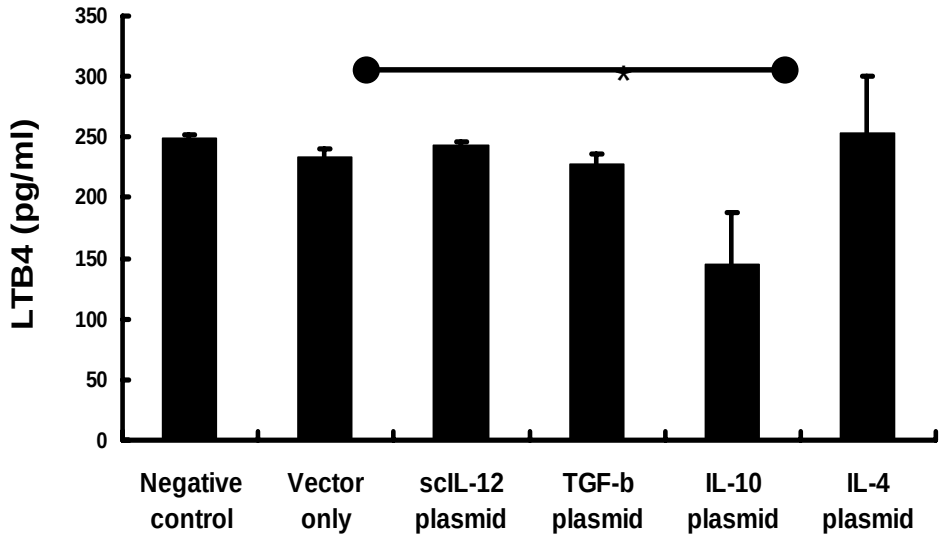
Figure 5 Effect of different cytokine gene plasmids on eotaxin and leukotriene B4 (LTB4) levels in BAL fluid from mice after aerosol challenge. Female BALB/c mice were sensitized with OVA and treated with different cytokine gene plasmid as described in *Material and Methods*. Mice were sacrificed 48 hr. after last aerosol challenge, and the BAL fluid was collected to determine the levels of eotaxin and LTB4 by ELISA. Data are representative of three separate experiments with similar results. The columns and error bars represent mean ± SEM of 4 to 8 mice per group.* $P < 0.05$, ** $P < 0.01$ vs. vector-only treated control group

(A)


Eotaxin (pg/ml)
250
200
150
100
50
0
*
*
Negative control
Vector only
scIL-12 plasmid
TGF-b plasmid
IL-10 plasmid
IL-4 plasmid

六.

(B)


LTB4 (pg/ml)
350
300
250
200
150
100
50
0
*
Negative control
Vector only
scIL-12 plasmid
TGF-b plasmid
IL-10 plasmid
IL-4 plasmid

五.