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Keywords¡G asthma, gene therapy, interleukin-12, interleukin-23

The frequency of allergic diseases such as asthma and allergic rhinitis has increased rapidly during the past decade; however, the exact mechanisms have still not been established. Allergic asthma is characterized by airway hyperresponsiveness (AHR) to specific and nonspecific stimuli with elevated serum IgE levels and eosinophilic inflammation. Thus far, a broad range of therapeutic strategies is now under development. Although the topical glucocorticoids are now considered the cornerstone of therapy in the management of allergic diseases, many patients continue treatment with glucocorticoids despite the onset of serious adverse effects and poor clinical response. Immunotherapy, the intradermal injection of small but gradually increasing amounts of allergen, has been used as an effective treatment for allergic diseases. However, injection of allergen involves the risk of induction of mild and sometimes severe anaphylactic reactions. Allergic asthma is thought to be regulated by Th2 cells, and inhibiting this response is a promising mode of intervention.

IL-12 is a heterodimeric cytokine, which strongly promotes the differentiation of naïve $CD4^+$ T cells to the type-1 Th1 phenotype and suppresses the expression of Th2 cytokines. Preliminary data have demonstrated that IL-12 is a good candidate for the cytokine treatment of allergic diseases. In mice with Der p1-induced asthma, the local administration of IL-12 fusion gene into the lungs significantly prevented the development of AHR, abrogated airway eosinophilia, and inhibited type-2 cytokine production.

Recently, a novel cytokine, IL-23, is discovered and specifically acts on mouse memory T cells. Besides, human IL-23 stimulates IFN-γ production and proliferation in PHA blast T cells, as well as in memory T cells. In the future study, we will investigate the effect of coadministration of pscIL-12 and pIL-23 plasmids in OVA-induced animal model of airway inflammation. In summary, we will try to establish cytokine gene therapy for possible treatment of allergic diseases and might shed light on designing immunotherapy for atopic diseases.

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The incidence, morbidity, and mortality from asthma have been increasing worldwide over the last decade; however, the exact mechanisms have still not been established. Patients allergic to mite antigen have been demonstrated with elevated serum levels of allergic-specific IgE and local infiltration of inflammatory cells in which the presence of eosinophils is striking (Arm and Lee; 1992; Seminario et al., 1994; Nadel and Busse, 1998). It has been recognized that Th2 cells and their cytokines are responsible for the initiation and maintenance of allergic disorders (Azzawi et al., 1990). Allergen-specific T cells from atopic patients have a higher production of the type-2 cytokines IL-4 and IL-5 and a lower production of type-1 cytokine IFN-γ (Robinson et al., 1992; Brusselle et al., 1994; Foster et al., 1996). Thus, agents that decrease IgE levels or Th2 cytokine production or increase Th1 cytokine production may inhibit allergen-induced disorders. Hyposensitization with allergen has been documented to be effective for asthmatic children, however, the real mechanism are still not well defined.

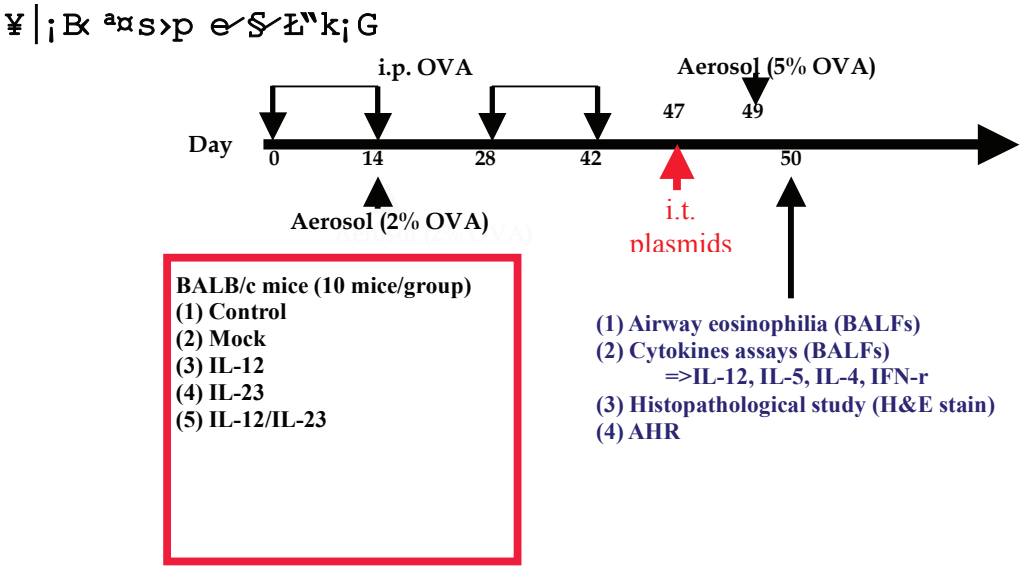
It has been well documented that activation of naive T cells in the presence of Interleukin-12 promotes differentiation to Th1; conversely, IL-4 promote Th2 development. IL-12 is a heterodimeric cytokine composed of a 35-kDa subunit (p35) and a 40-kDa subunit (p40), linked by disulfide bonds (Schoenhaut et al., 1992). In general, IL-12 upregulates the interferon (IFN)-γ production of T-cells and natural killer (NK) cells, promotes the development of type- 1 T helper (Th1) cells, and suppresses the expression of Th2 cytokines (Hsieh et al., 1993; Manetti et al., 1993; Wolf et al., 1994). These immunoregulatory properties of IL-12 have led to its therapeutic use in a variety of applications. Indeed, several studies have demonstrated that systemic administration of the IL-12 protein can decrease the eosinophil infiltration and airway inflammation in murine models of asthma (Gavett et al., 1995; Iwamoto et al., 1996; Kips et al., 1996; Sur et al., 1996; Lee et al., 1999a, b; 2001). However, this approach is costly, and prolonged exposure to IL-12 has been shown to have deleterious effects (Gately et al., 1994; Sarmiento et al., 1994). Recent studies in murine models of airway inflammation have shown promise, with marked suppression of allergic responses after the *in vivo* administration of IL-12 systemically. Several groups have shown that IL-12 administration at the time of antigen sensitization prevents the development of antigen-induced airway hyperresponsiveness (AHR), the recruitment of eosinophils, and the increases in antigen-specific IgE levels (Gavett et al., 1995; Iwamoto et al., 1996 Kips et al., 1996). Collectively, these studies demonstrated the ability of IL-12 given early during the immune response to redirect a Th2-mediated immune response to inhaled antigens. However, toxicities, including mild temperature elevation, anemia, lymphopenia, neutropenia, splenomegaly, and skeletal muscle degeneration have been observed in animals dosed daily with 1 mg of IL-12 for 7 days (Gately et al., 1994; Sarmiento et al., 1994; Eng et al., 1995). Therefore, further clinical trials with IL-12 should be illustrative in determining the optimal dosage regimens for these indications. Whether IL-12 is effective in redirecting immune responses to inhaled antigens when administered after initial antigenic sensitization has been controversial. The discrepancies in these studies are probably due to differences in the frequency of IL-12 administration in the various studies. A recent murine study has highlighted the potential utility of using IL-12 as an adjuvant in immune therapeutic approaches to the treatment of allergic airway responses (Kim et al., 1997). Kim et al. have demonstrated that, in a setting of pre-existing or ongoing immunity characterized by high IL-4 and IgE synthesis, the ovalbumin-IL-12 fusion protein can significantly reduce IgE level,

increase IgG2a, and greatly enhance Th1 cytokine.

Recently, direct genetic transfer (a form of gene therapy) has been used in various models to modify the course of the immune response (Hogan et al., 1998). Thus, the ability to transfer the IL-12 gene into the lungs may localize cytokine expression and potentially reduce any associated systemic toxicity in the treatment of pulmonary allergic diseases. To apply genetic therapy to the IL-12 gene, we have constructed murine IL-12 vectors, pscIL-12.2, expressing the bioactive heterodimeric component and the single-chain IL-12 protein. In mice with Der p 1-induced asthma, the local administration of this IL-12 fusion gene into the lungs significantly prevented the development of AHR, abrogated airway eosinophilia, and inhibited type-2 cytokine production (Lee et al., 2001).

Recently, a new heterodimeric cytokine termed IL-23 that consists of p40 and p19 subunits (Oppmann et al., 2000). The IL-23 p19 component is first identified and characterized during sequence database searches for members of the IL-6 cytokine family. At the sequence level, IL-23 p19 is most closely related to the IL-12 p35 subunit. Similar to IL-12, IL-23 is produced mainly by activated murine and human dendritic cells. Whereas the biological activities of IL-12 are mediated by the high-affinity IL-12R, consisting of a β1 and a β2 chain (Presky et al., 1996), IL-23 binds to IL-12Rβ1 but fails to engage IL-12Rβ2. Now we know that IL-23 binds the IL-23R complex, composed of IL-23R and IL-12Rβ1. Structurally, human IL-23R shares many features with human IL-12Rβ2. IL-23R complex are expressed in $CD4^{+}CD45RB^{low}$ memory T cells and this contrasts with IL-12Rβ2 expression, which is munch higher in the $CD4^{+}CD45RB^{high}$ population. IL-23 uses many of the same signal-transduction components as IL-12, including Janus kinase 2, Tyk2, signal transducer and activator of transcription (Stat)1, Stat3, Stat4, and Stat5 (Lankford and Frucht, 2003). IL-23 induces strong proliferation of mouse memory T cells, a unique activity of IL-23 as IL-12 has no effect on this cell population. Like IL-12, IL-23 enhances proliferation and production of IFN-γ by activated human PHA blast T cells. The maximum levels of IFN-γ production induces by IL-23 are always lower than those induced by IL-12, even at saturating levels of the added cytokines. This characteristic may make IL-23 a more suitable entity to stimulate the cell-mediated immune response, as IL-12 administration leads to severe side effect associated with extremely high IFN-γ production. To this point, we will try to evaluate whether the combination of low doses of IL-12 and IL-23 could get a synergistic effect and inhibit the development of airway symptoms in a mouse model of allergic asthma.

In this study, using an established murine model of OVA-induced asthma, we will try to intratracheally deliver the IL-12 plasmid and IL-23 plasmid to cationic liposomes as a therapeutic reagent to the lungs. The treated mice are then evaluated for changes in their airway hyperresponsiveness, airway eosinophilia, histology of lung tissues, and cytokines production. In summary, our project might shed light on further understanding the regulatory mechanisms and designing immunotherapy for atopic diseases.



Plasmid preparation

The p40 and p19 subunits of the murine IL-23 gene are constructed in the pTCAE plasmid. The pCMV vector and the construction of the pscIL-12.2 vector were described previously (Lee et al., 1998). These clones are subsequently introduced into the *Escherichia coli* DH5 by transformation. The plasmids are purified using EndoFree plasmid kits and suitable for gene therapy.

Animals

Female Balb/c mice aged 6-8 weeks are obtained from and maintained in the Animal Center of the Taipei Medical University.

Preparation and administration of DNA-lipid complexes

Small cationic liposomes are prepared in a 5% (w/v) dextrose solution in the following fashion. Dimethyldioctadecylammonium bromide (DDAB) in chloroform is mixed with cholesterol in a 1:1 molar ratio, and the solvent is removed slowly under reduced pressure at 55°C on a rotary evaporator. The dry lipid film is hydrated with 5% dextrose solution pre-warmed to 55°C and the container is sealed under argon. The hydrated lipid suspension is sonicated in a bath sonicator for 5-10 min. at 55°C. The final concentration of liposomes is 5mM cationic lipid and the size of the liposome is measured by a dynamic light scattering to be 100 ± 30 nm. The liposomes are stored until use under argon at 4°C. For intratracheal administration, cationic lipid-DNA complexes are formed by mixing 9 nmol of lipid per 1 g of plasmid DNA in 10% glucose in water. Groups of mice are anesthetized and instilled intratracheally with cationic lipids alone, or cationic lipid-DNA complexes. Each mouse receives a volume of 30 µl of lipid-DNA complexes that contain 5 µg of plasmid DNA.

Immunization and inhalation-exposure of mice

For the systemic immunization, mice are sensitized by intraperitoneal injections of 10 µg OVA mixed with 4 mg alum on day 0, day 14 and day 28. On day 35, they are challenged with a nebulized OVA for 30 min. The aerosols are generated in a chamber using an ultrasonic nebulizer and the concentration of the OVA in the nebulizer was 5% (w/v). Forty-eight hours prior to the OVA challenge, the cationic lipids or lipid-plasmid DNA complexes are administered intratracheally as a single dose. For the positive control group, the sensitized mice are not treated with lipid or DNA plasmid intratracheally but receive only an antigen challenge.

Measurement of airway function

On day 36, the airway responsiveness to aerosolized methacholine (MCh) is measured in unrestrained, conscious mice. The mice are placed in the main chamber of a whole-body plethysmograph and challenged with aerosolized 0.9% normal saline, and then with increasing doses of MCh (12.5-100 mg/ml). Each nebulization lasts 3 minutes. After each nebulization, recordings are taken and averaged for the 3 min. The Penh (enhanced pause = pause x [peak expiratory box flow/peak inspiratory box flow]) values are determined and data expressed as Penh values.

Bronchoalveolar lavage fluid (BALF) study

To measure airway inflammation, we study the accumulation of the inflammatory cells in the BAL fluid. After the measurement of the pulmonary function parameters, the mouse is sacrificed and the trachea is immediately lavaged three times via a trachea cannula with 1 ml of HBSS, free of ionized calcium and magnesium. The lavage fluid is cooled on ice and centrifuged (400 xg) at 4°C for 10 min. After washing, the cell pellets are re-suspended in 1 ml HBSS and the total number of the cells in the BALF are counted with a standard hemocytometer. A differential count is made on a smear prepared with a cytocentrifuge and stained with Liu's stain. A minimum of 200 cells are counted and classified as macrophages, lymphocytes, neutrophils and eosinophils, based on standard morphological criteria.

Histopathological study

To evaluate the effects of the IL-12/IL-23 treatment on the allergen-induced pulmonary inflammation, each group of animals is sacrificed for histopathological examination. After the lavage, the lungs are immediately removed and fixed in 10% buffered formalin. The pulmonary tissues are subsequently sliced and embedded in paraffin, and cut into 5-µm thick sections. These frozen sections are stained with hematoxylin-eosin (H&E) and examined by light microscopy for histological changes.

BALF cytokine measurement

The quantities of IL-12, IFN-γ IL-4 and IL-5 in the BAL fluid supernatants are evaluated using an appropriate ELISA kit. These assays have a threshold of detection of 2.5 pg/ml, 2 pg/ml, 2pg/ml and 7pg/ml, respectively. The cytokine levels are calculated by linear- regression analysis based on the values obtained from a standard curve

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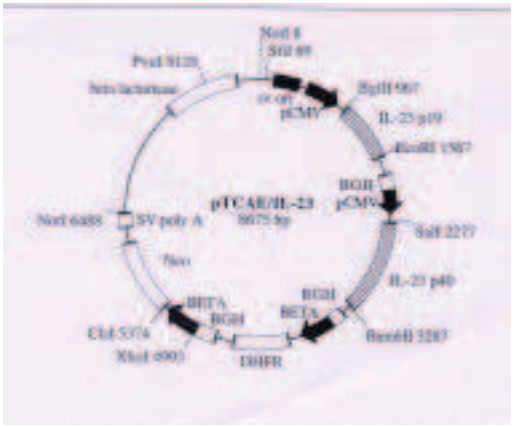

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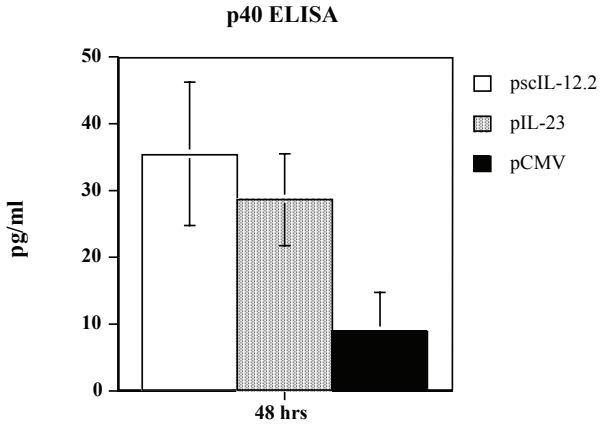


Table 1. Effect of local IL-12/IL-23 genes delivery on allergen-induced cells infiltration into the mouse airway

Groups	total cells $1x10^5$/ml	BALFs Macrophages (%)	Lymphocytes (%)	Neutrophils (%)
control	5.8 ¡ 1.1	44.9 ¡ 12.8	12.0 ¡ 2.8	18.0 ¡ 5.2	25.0 ¡ 10.5
Mock	5.0 ¡ 1.2	52.8 ¡ 12.7	6.3 ¡ 2.5	24.6 ¡ 6.7	16.3 ¡ 9.8
pIL-23	5.8 ¡ 1.9	49.8 ¡ 5.1	9.3 ¡ 3.5	28.2 ¡ 4.7	12.7 ¡ 3.1
pscIL-12	3.0 ¡ 0.7	81.8 ¡ 7.1	4.0 ¡ 2.1	8.9 ¡ 4.1	5.2 ¡ 2.8
pscIL-12 pIL-23	3.3 ¡ 0.7	76.8 ¡ 8.2	7.2 ¡ 3.6	14.0 ¡ 5.0	2.0 ¡ 0.9

Figure 3 Cytokines assays

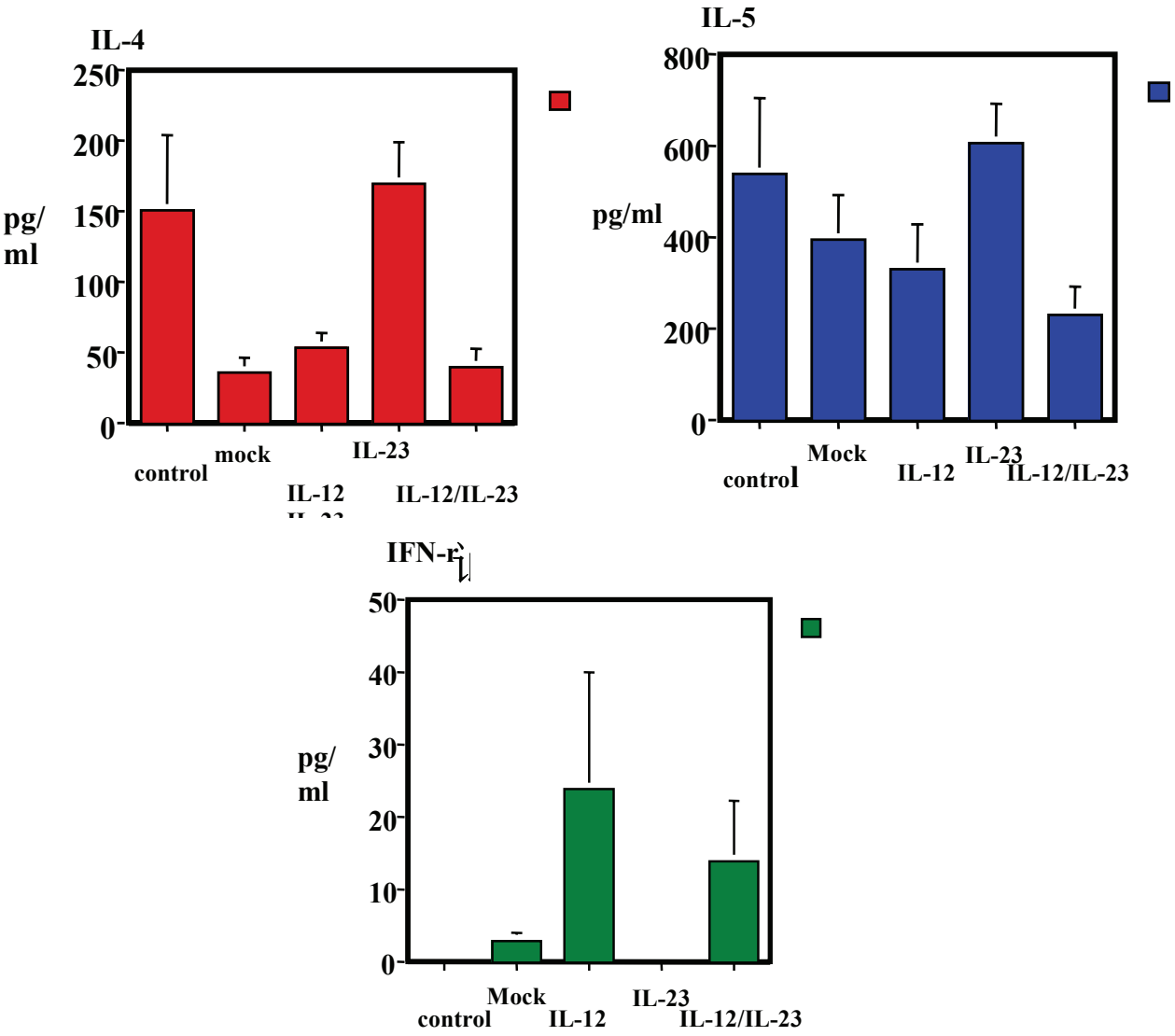
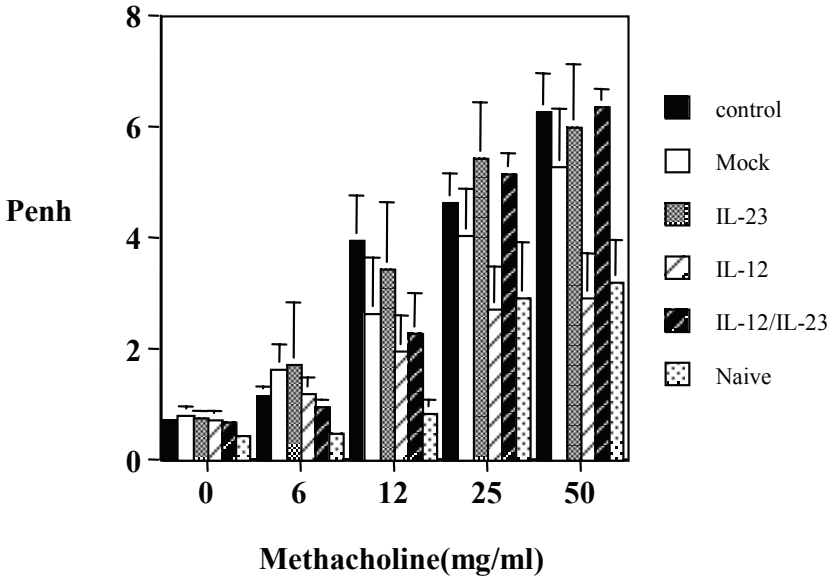


Figure 4 AHR



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