行政院國家科學委員會專題研究計畫　成果報告

利用細胞激素基因來治療氣管發炎的動物模式

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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

利用細胞激素基因來治療氣管發炎的動物模式

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成果報告類型(依經費核定清單規定繳交)：■精簡報告□完整報告
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執行單位：北醫微免科

中華民國九十三年九月二十九日

一、計畫中文摘要：請於五百字內就本計畫要點作一概述，並依本計畫性質自訂關鍵詞。關鍵詞：關鍵字: 氣喘, 基因治療, 細胞激素

從 1970 年代以來，許多報告都指出，全世界氣喘病的罹患率持續在增加，病情轉趨嚴重，住院率和死亡率也隨著增高，台灣地區也不例外。在氣喘病人身上可觀察到幾項特徵：包括血液中的 IgE 增加，體內第二型 T 輔助細胞 (Th2) 數目增加，氣管聚集大量的嗜伊紅性白血球細胞 (eosinophilia) 以及發炎媒介物的產生。過去對氣喘疾病的治療大部分偏重於抑制氣管的發炎現象和舒緩氣管的收縮程度，而吸入性類固醇的使用為目前對於氣喘症狀控制和肺功能的改善最有效的方法；但是類固醇的使用只能抑制發炎現象，對氣喘仍無法根治，而且對於人體仍有潛在的副作用，所以病人除了儘量避免接觸過敏原外，要徹底治療氣喘疾病，目前治療方法為減敏療法。減敏治療必須持續幾年的治療，此種耗時並且需要大量純化過敏原的治療方式並不是最理想的治療方法。基於此，其病因的探討和新治療的方法刻不容緩。

目前已經知道細胞激素 IL-12 會促進 T 細胞分化為 Th1 輔助細胞，使其產生大量的 Th1 型細胞激素 IFN-γ以及 IL-2, 並抑制 Th2 型細胞激素 IL-4 以及 IL-5 的分泌。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，其構築出具有單一鏈的 IL-12 融合基因於質體上，並將此質體配合 liposome 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。

近年來新的細胞激素 IL-23 被發現，其和 IL-12 有相似的構造和生物活性。IL-23 和 IL-12 都是異雙合體，且皆可促進 T 細胞分泌 IFN-γ。IL-23 的這些特性使我們看好其在基因治療上的可行性，也相信 IL-12 在 IL-23 的搭配之下更能找出更有效的治療方法。

貳、計畫英文摘要：請於五百字內就本計畫要點作一概述，並依本計畫性質自訂關鍵詞。

Keywords：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.

三、研究計畫之背景及目的：

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,

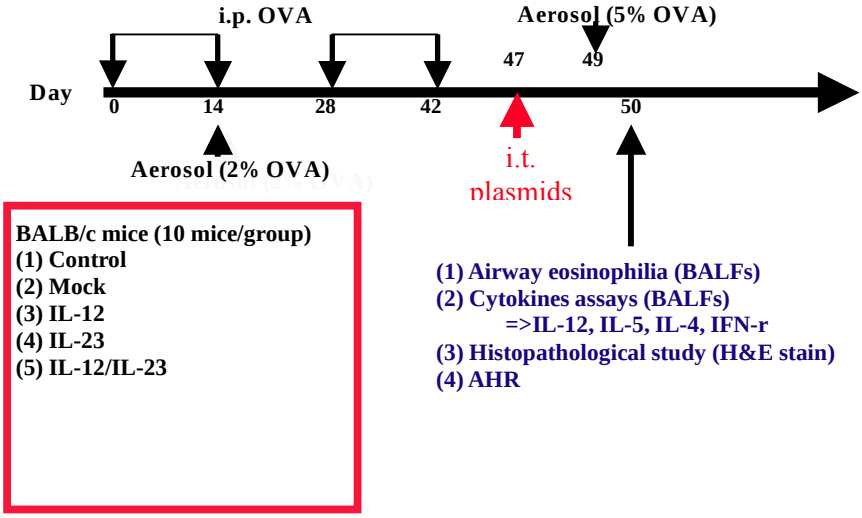
increase IgG2a, and greatly enhance Th1 cytokine.

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.

四、研究計畫之方法：



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

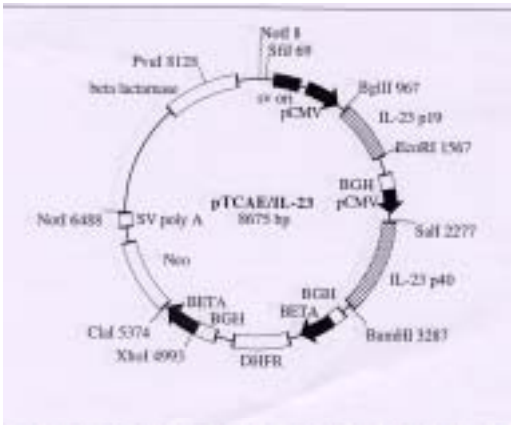
The quantities of IL-12, IFN-γ IL-4 and IL-5 in the BAL fluid supernatants are evaluated using an appropriate ELISA kit. These assays have a threshold of detection of 2.5 pg/ml, 2 pg/ml, 2pg/ml and 7pg/ml, respectively. The cytokine levels are calculated by linear- regression analysis based on the values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

五、研究計畫之結果與評估：

我們將已把 IL-23 的 p19 以及 p40 單元體構築好的 pIL-23 質體(**Figure 1**)挑選兩個各自送入小鼠的肺部細胞表現。在送入之後的 48hrs 和 72hrs，收肺部細胞抽取其 RNA 將其做 RT-PCR，來檢測 pIL-23 質體在肺部細胞表現的狀況；同時抽取小鼠的肺部沖洗液，以 p40ELISA 來測定其表現的情形。由 **Figure 2** 圖示知：IL-23 蛋白質在小鼠體內 48hrs 為表現高峰，而在 72hrs 之後表現趨弱(data 未 show)。在確定 pIL-23 質體可在小鼠體內表現後，我們進一步的將 pIL-23 質體配合 pscIL-12 質體，以及對照組的 pCMV 質體，應用在氣喘動物的模式上來評估基因治療的效果。基因治療的程序和檢測的項目請參照方法中的流程圖所示。首先我們抽取小鼠的肺部沖洗液測其細胞的變化，由 **Table 1** 所示：pIL-23 質體單獨使用時其治療成效不明顯，但在配合 pscIL-12 質體共同使用下則有加成的效果。若進一步的將收集的肺部沖洗液測各種細胞激素的表現量(**Figure 3**)，則可觀察到：在有 pscIL-12 質體存在下，可偵測到 Th1 型 IFN-r 的表現增高，而配合 pIL-23 質體使用並不能增加 IFN-r 的表現量。至於在 Th2 型 IL-5 的表現方面則知：pIL-23 質體單獨使用並不能抑制 IL-5 的表現量，而 pIL-23 質體配合 pscIL-12 質體則可抑制 IL-5 的表現程度。最後根據測量小鼠呼吸道收縮反應(airway hyperresponsiveness)結果(Figure 4)推測：IL-23 蛋白質的表現並不能減緩小鼠呼吸道收縮反應。

整體而言：將 pIL-23 質體放在基因治療的目的上成效並不大。由於 IL-23 的角色是在幫助已形成的記憶性 Th1 細胞增殖，可惜在氣喘動物身上現存的記憶性 Th1 細胞數量太少，以致 IL-23 的存在無法抑制 Th2 細胞所產生的影響。因此若要使 pIL-23 質體發揮較大的作用，那麼在策略上可能是在以過敏原致敏小鼠初期，即要同時加入 pIL-23 質體以及 pscIL-12 質體，使對過敏原有專一性的 Th1 細胞數目能增加，如此在後期再加入 pIL-23 質體配合 pscIL-12 質體做基因治療時，才可能觀察到 IL-23 在此氣喘動物模式上呈現較明顯的治療效果。

<Figure 1.>



<Figure 2.>

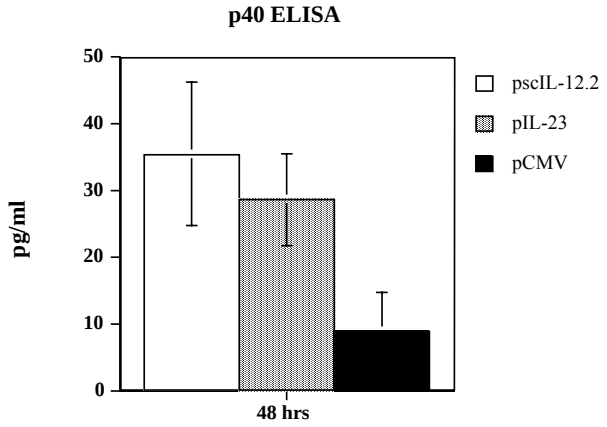


Table 1. Effect of local IL-12/IL-23 genes delivery on allergen-induced cells infiltration into the mouse airway

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

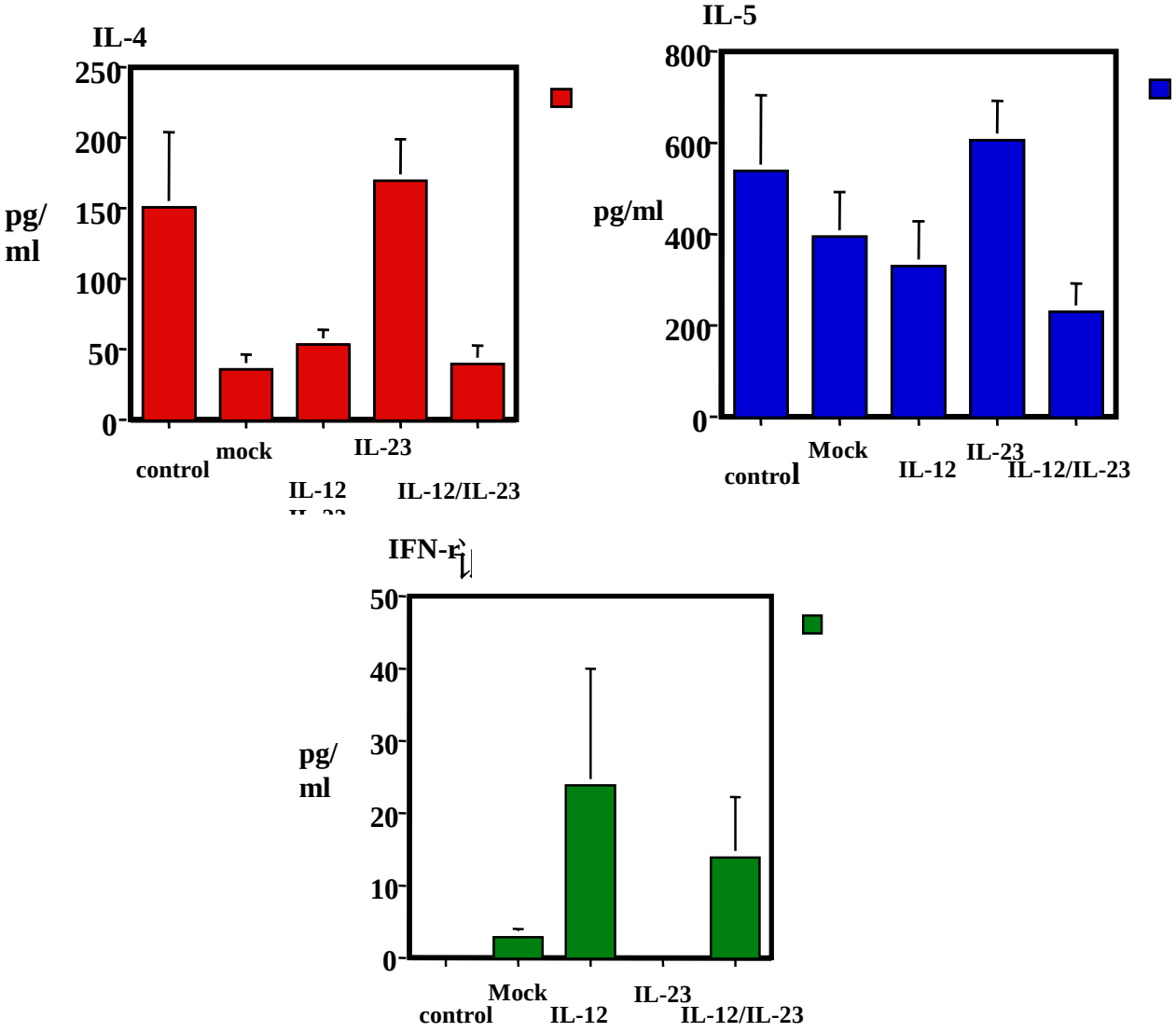
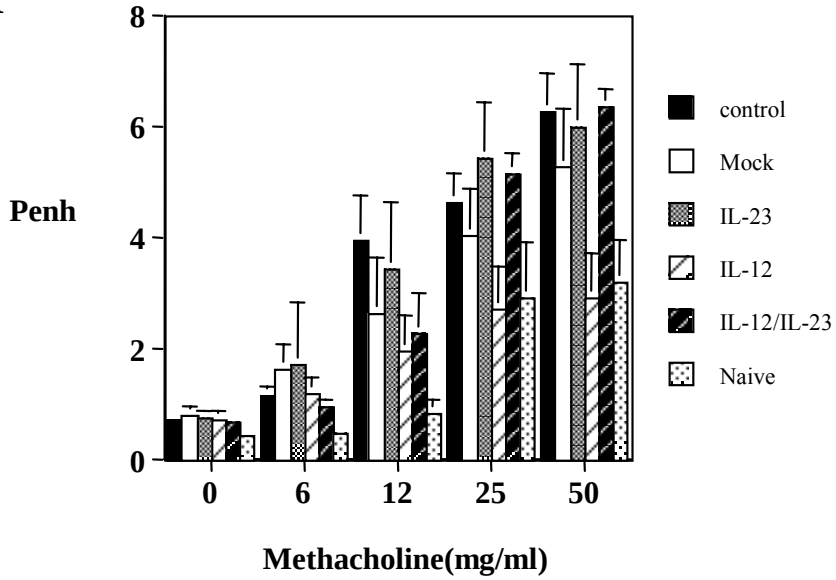


Figure 4 AHR



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