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經由口服給予塵蟎重組蛋白於小鼠氣喘模式降低呼吸道發炎之研究

The Effects of Oral Delivery of Recombinant House Dust Mite Allergen on Airway Inflammation in Murine Model of Asthma

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降低呼吸道發炎之研究

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本論文係簡芊卉君（R96450005）在國立臺灣大學口腔生物科學所完成之碩士學位論文，於民國 98 年 07 月 24 日承下列考試委員審查通過及口試及格，特此證明。

考試委員簽章	吳文超	李昆原	江伯倫						
備註									

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芊卉 于

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中文摘要

氣喘是兒童常見的過敏性疾病之一，根據統計全球約一成的人口有氣喘的困擾，歐洲塵蟎 (*Dermatophagoides pteronyssinus*) 是八成台灣過敏病患的過敏原之一。氣喘的臨床症狀包含：呼吸道過度反應、嗜酸性白血球浸潤於呼吸道、活化的過敏原特異性第二型輔助型 T 細胞增加、呼吸道黏液增加、甚至是呼吸道重塑等等。口服耐受性，是指透過口服抗原而引發抗原特異性的免疫反應下降的現象，目前被認為具潛力成為過敏性氣喘的治療方式。本篇研究希望透過口服餵食基因重組第二類與第一類歐洲塵蟎過敏原 (recombinant *D. pteronyssinus* group 2 allergen, rDp2; recombinant *D. pteronyssinus* group 1 allergen, rDp1)，藉此來降低由歐洲塵蟎粗萃蛋白所引發的呼吸道發炎現象。我們利用腹腔注射粗萃蛋白致敏 BALB/c 雌性小鼠，並給予氣管粗萃蛋白引發呼吸道發炎。結果顯示，腹腔注射高劑量 (50 μg) 的粗萃蛋白可有效誘發過敏性氣喘的臨床症狀，包含血清中塵蟎蛋白特異性的 IgE 含量增加、促進嗜酸性白血球浸潤於肺部、肺部沖洗液中介白素-5 (Interleukin-5, IL-5) 含量增加、小鼠脾臟細胞經粗萃蛋白刺激可產生大量 Th2 的細胞激素 (IL-5 與 IL-13) 並且大量增生。更進一步，我們利用口服餵食的方式，連續七天給予致敏小鼠 0.2 或 1.0 mg 劑量的 rDp2 或粗萃蛋白，探討是否能有效降低粗萃蛋白引發的氣喘症狀。結果發現，給予 1.0 mg 的 rDp2 可有效降低呼吸道過度反應，並減緩呼吸道發炎現象。因此，我們認為餵食較高劑量的基因重組蛋白或粗萃蛋白，有減緩過敏性氣喘症狀的傾向；未來將需要更多的實驗，以尋找合適的餵食計量與時間。

關鍵字：過敏性氣喘；歐洲塵蟎；第二類歐洲塵蟎過敏原；口服耐受性。

Abstract

Asthma is one of the most common allergic diseases in children; in addition, about 80% of asthmatic patients in Taiwan are sensitized by house dust mite -- *D. pteronyssinus*. The characteristics of asthma such as AHR, eosinophils infiltration, antigen-specific T helper 2 cells activation, increased mucus secretion and even airway remodeling. Antigens-specific immune tolerance by prior oral administration of antigens might be a therapeutic strategy for allergic asthma. Therefore, we aimed to apply oral administration of rDp2 to decrease the airway inflammation induced by *D. pteronyssinus*. The female BALB/c mice were used and given with crude mite extract of *D. pteronyssinus* as the allergic asthma. In present study, we sensitized mice with peritoneal injection, and then challenged with intratracheal injection of crude mite extract. The results showed that peritoneal injection with high-dose could induce the clinical features of asthma significantly, including elevated mite-specific IgE in serum, production of Th2 cytokines (IL-5, IL-13) of splenocytes, and crude mite-specific lymphoproliferation. Furthermore, oral delivery of rDp2 or crude mite extract 0.2 or 1.0 mg/day for consecutive 7 days at the beginning of sensitization showed some beneficial

effects on airway inflammation. Oral feeding 1.0 mg/day of rDp2 reduced AHR and slightly decreased the airway inflammation induced by crude mite extract. In conclusion, we suggest that oral delivery of high dose of single recombinant allergen seems to be more benefit on airway inflammation induced by the complex crude mite extract, and the feeding dose and feeding period need further investigation.

Keywords: allergic asthma; *Dermatophagoides pteronyssinus*; Dp2; oral tolerance.

Brief Contents

Full Contents

Contents of Figures

Abbreviation

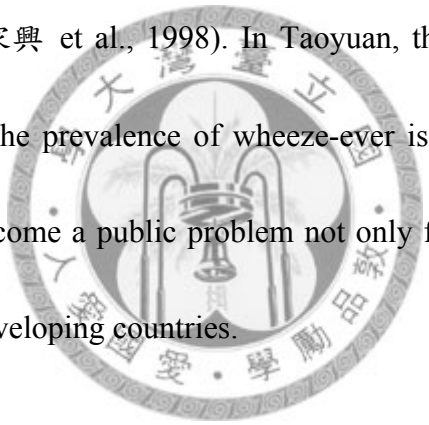
AHR	Airway hyperresponsiveness
BALF	Bronchoalveolar larvage fluid
CD	Cluster of differentiation
Con A	Concanavalin A
c.p.m.	Counts per minutes
DC	Dendritic cell
D. pteronyssinus	*Dermatophagoides pteronyssinus*
Dp1	Group 1 allergen derived from the *D. pteronyssinus*
Dp2	Group 2 allergen derived from the *D. pteronyssinus*
ELISA	Enzyme linked immunosorbent assay
E.U.	ELISA unit
HRP	Horseradish peroxidase
IFN-γ	Interferon-γ
IL	Interleukin
i.p.	Intraperitoneal
i.t.	Intratracheal
Methacholine	Acetyl-β-methylcholine chloride
OD	Optical density
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

Penh	Plethysmography and enhanced pause
rpm	Rotation per minute
PT	Pertussis toxin
SDS	Sodium dodecyl sulfate
PAGE	Polyacrlamide gel electrophoresis
Th1	Type 1 helper cell
Th2	Type 2 helper cell
TGF-β	Transforming growth factor β
TMB	3,3',5,5'-Tetramethylbenzidine

INTRODUCTION

Epidemiology of allergic asthma

Allergic asthma is the most common chronic respiratory disease of children, and its prevalence has been increased in resent decades. According to World Health Organization estimation, as many as 300 million people of all age suffer from asthma and asthma caused 255 thousand deaths in 2005. Based on WHO estimation, asthma affects about 10% of population in worldwide. In northern Taiwan, the prevalence of asthma of school-children has an increased trend, from 5.1% in 1985 to 10.2% in 1998 (吳家興 et al., 1998). In Taoyuan, the prevalence of diagnosed asthma is 12.2%, and the prevalence of wheeze-ever is 16.8% (Kao et al., 2005). Allergic asthma has become a public problem not only for developed countries but for all the regardless developing countries.



Risk factors for asthma

Childhood asthma has familial clustering which is due to a complex interaction between unknown genes and environmental factors. To study the genes involved in asthma, many genome screens have been carried out. Asthma shows some genetic linkages overlap with type 1 diabetes (also known as insulin-dependent diabetes), multiple sclerosis, rheumatoid arthritis, and ankylosing spondylitis (Cookson, 2002).

In addition, both polymorphisms of CD14 and Interleukin-13 (IL-13) have been reported that associated with the level of total IgE in serum (Baldini et al., 1999). The variation of *FCERIB* gene alters the activity of FcεRI of mast cells, and might affect the expression level as well (Donnadieu et al., 2003). Furthermore, ADAM33 expressed by bronchial smooth muscle (Su et al., 2008), DPP10 encodes a dipeptidyl peptidases (Allen et al., 2003; Blakey et al., 2009), both of which have been identified and the effects on asthma are still working.

Strachan proposed a hypothesis, which was called the hygiene hypothesis, to explain the increased the prevalence of asthma in children (Strachan, 1989). The hygiene hypothesis suggested that the increase in the prevalence of allergic diseases in the past decades seems to be resulted from the decrease in the frequency of childhood infection (Strachan, 1989), and also decreased regulatory cytokine levels (Weiss, 2002). Braun-fahrlander and coworkers even indicated that endotoxin levels in the dust were inversely related to the occurrence of atopic asthma (Braun-Fahrlander et al., 2002). Moreover, it has been proposed that endotoxin stimulation may play a role in normal immune system development (Weiss, 2002). Therefore, the westernized life style, urbanization (Lin et al., 2001), and compounds surround in environment, such as cell-wall component of fungi (Weiss, 2002) and air

pollution molecules (Ho et al., 2007) could be the factors for developing asthma.

Definition of asthma

According to the guideline provided by National Institutes of Health on 2007, asthma composes of airway inflammation, AHR, bronchoconstriction, and results in individual variant and recurring symptoms. Many cells are involved in airway inflammation, such as eosinophils, mast cells, and Type 2 helper cells (Th2 cells). The interactions among these cells result in airway inflammation, including AHR, and mucus hypersecretion. Consequently, asthmatic patients and susceptible individuals have clinical symptoms, including night coughing, wheezing, breathlessness, and bronchoconstriction quickly after a variety of stimulations.

Mechanism of asthma

The mechanism of asthma has been studied for a long time, and it involved various cell types and cytokines. Firstly, the innocuous antigens are uptaken by airway dendritic cells (DCs) throgh extending dendrites into airway lumen (Jahnsen et al., 2006) or the allergens digest the tight junction to penetrate the epithelial cells barrier (Wan et al., 1999). The allergen bearing DCs migrate to draining lymph

nodes to present allergen to naïve Th cells and induce the naïve Th cells differentiate into Th2 cells in the presence of interleukin 4 (IL-4) (Demeure et al., 1995). Consequently, these Th2 cells produce Th2 cytokines, including IL-4, IL-5, IL-9 and IL-13 to affect other immune cells. The IL-4 and IL-13 are essential for IgE class switching in B cell, and IL-5, IL-6, and IL-9 can also enhance the IgE production (Robinson, 2000). Both IL-5 and IL-9 promotes eosinophils development, survival and infiltration (Robinson, 2000; Takahashi et al., 1992). IL-9 overexpression increases number of mast cells in lung, accumulates mucus-like materials, and elevates AHR (Temann et al., 1998). IL-13 plays a role in mucus hypersecretion and AHR (Zhu et al., 1999). Consequently, while asthmatic individual re-encounter the allergens, both innate and adaptive immune responses are activated. The mast cells activated by cross-linking the FcεRI on the membrane (Novak et al., 2001), release histamine, leukotrienes (LTC_4), prostaglandins (PGD_2), cytokines, chemokines, and growth factors by degranulation. The activated eosinophils release pro-inflammatory mediators (Gelfand, 2004), such as cysteinyl leukotrienes, major basic protein, eosinophil peroxidase, and cytokines. Finally, these episodes lead to vascular permeability increase, smooth-muscle constriction, and mucus hypersecretion.

Animal model for allergic asthma

Animal models reproducing many features of human asthma were used for a hundred years for research. Clinical asthma symptoms include serum IgE elevation, airway inflammation, goblet cell hyperplasia, epithelial hypertrophy, AHR, and even airway remodeling (Vignola et al., 2000). Current knowledge of the pathology of asthma are resulted from animal studies such as sheep, dogs, guinea pigs, rats and mice (Zosky and Sly, 2007). The mouse is the commonly used laboratory animals, due to its easier to handle and more supports in transgenic technology. Ovalbumin (OVA) is often used as allergen to address issue of asthma, but other allergens, such as pollen, house dust mite, and cockroach, are also used to mimic clinical cases. The acute sensitization and challenge protocol usually contains several systemic administration of the allergen with an adjuvant and then challenged animals with allergen through airway for a period (Nials and Uddin, 2008). The mouse strains, the allergens, and the sensitization and challenge protocols affect the features of the animal model.

Drugs for asthma treatment

In addition to allergen avoidance, the most common treatment of asthma are

inhaling corticosteroids, β2-adrenoceptor agonists, mediator antagonists, and phosphodiesterase inhibitors (Holgate and Polosa, 2008). The corticosteroids suppress the Th2-cell-mediated inflammation by inhibiting the expression of cytokines and chemokines which are regulated by nuclear factor-κB (NF-κB) and activator protein 1 (AP1) (Barnes and Adcock, 1998). However, corticosteroids inhalation does not influence the disease essentially. The β2-adrenoceptor agonists suppress the bronchoconstriction (Usmani et al., 2005) by binding β2-adrenoceptor, activating protein kinase A, opening Ca^{2+}-dependent K^{+} channels. H1-antihistamines (Manjra et al., 2009), leukotriene inhibitors (Ishimura et al., 2009), and phosphodiesterase inhibitors (Kita et al., 2009) were also used to relax asthmatic symptoms.

Oral tolerance

The definition of oral tolerance is that the specific suppression of cellular and/or humoral immune responses to an antigen in induced by prior administration of the antigen through the oral route (Weiner, 2000). Two major mechanisms are involved in oral tolerance, including antigen-specific regulatory T cells induced by lower and repeating doses of oral antigen (Tsuji and Kosaka, 2008) and clonal deletion (Chen

et al., 1995) or anergy induced by higher single dose of oral antigen (Cobbold and Waldmann, 1998). Many different kinds of regulatory T cells (Treg) are involved in oral tolerance including natural Treg (nTreg), inducible Type 3 helper cells (Th3), and T regulatory type 1 (Tr1) cells. As Sakaguchi defined, nTreg are thymus-derived $CD4^+CD25^+Foxp3^+$ T cells and their suppression are major through cytotoxic T lymphocyte antigen 4 (CTLA-4) (Wing et al., 2008). Another report showed that the indoleamine 2,3-dioxygenase (IDO)-expressing DCs in Peyer's patches from orally tolerized mice are involved in the generation of $CD4^+CD25^+Foxp3^+$ T cells in a collagen-induced arthritis murine model (Park et al., 2008). Oral tolerance can be induced in absent of nTreg; however, oral tolerance dependents on transforming growth factor β (TGF-β) production (Mucida et al., 2005). Previous studies discovered a population of $CD4^+$ T cells with latency-associated peptide (bearing the membrane-bound form of TGF-β) and the ability to produce TGF-β, such as Th3, are considered as potent regulatory T cells involved in oral tolerance (Chen et al., 2008; Oida et al., 2003). T regulatory type 1 (Tr1) cells are defined as a subset cells, which are developed in the presence of IL-10 and have the ability to secret IL-10 (Martinez-Forero et al., 2008). Previous study reported that Peyer's patch cells produce IL-10 to suppress systemic inflammation after low-dose oral tolerance

induction (Tsuji et al., 2001).

Oral tolerance has been regarded as a potential strategy for autoimmune such as collagen-induced arthritis (Khare et al., 1995), type I diabetes (Maron et al., 1996), and multiple sclerosis (Martinez-Forero et al., 2008). Merill Chase showed that oral administration of contact-sensitizing agent could prevent guinea pigs from allergic disease induced by the contact-sensitizing agent (Chase, 1946). Other research also showed that oral feeding with OVA prevent and improve the asthma symptoms (Chung et al., 2002).

House dust mite

Dermatophagoides pteronyssinus (*D. pteronyssinus*), the predominant house dust mite in Taiwan (Kuo et al., 1999), has been discovered that over 15 proteins of which can induce IgE production. Particularly, the group 1 and 2 allergens derived from house dust mites are considered major allergens based on the frequency of patients sensitized (Thomas et al., 2002).

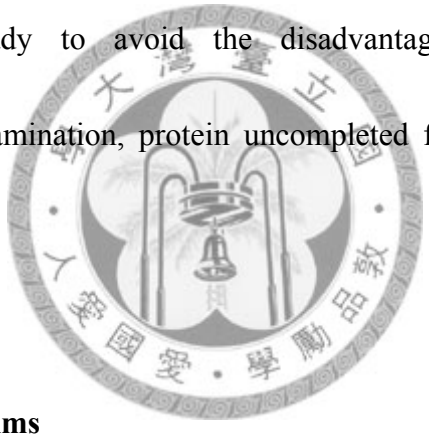
Group 1 allergen derived from the *D. pteronyssinus* (Dp1) in mite feces has been described as an aeroallergen. Dp1 is composed of major 222 amino acids with a molecular mass of 25 kD, 19-residues signal peptide, and 80-residues pro-domain

sequence (Zhang et al., 2009). Dp1 block the IgE producing feedback through cleaving CD23 (FcεRII) on B cells (Schulz et al., 1995), inhibit differentiation to Type 1 helper (Th1) linage though cleaving CD-25 (IL-2Rα) on T cells (Schulz et al., 1998), facilitate its own passage cross the barrier through digest the intercellular tight junctions of airway epithelial cells (Kalsheker et al., 1996; Wan et al., 1999), and induce mast cells and basophils degranulation to release proinflammatory cytokines (Mita et al., 1995). A clinical research showed that among 47 patients sensitized by *D. pteronyssinus* with allergic rhinitis with or without asthma, 31 patients (63.8%) had positive IgE to Dp1 (Taketomi et al., 2006).

Group 2 allergen derived from the *D. pteronyssinus* (Dp2) in male mite reproductive tract (Thomas and Chua, 1995) is composed of 129 amino acids. Dp2 has 35% identity to the human epididymal epithelial cell secreted protein (HE1), which is known to bind cholesterol with high affinity. Dp2 has been reported that it can promote toll like receptor 4 (TLR4) signaling to enhance the response of epithelial cells to LPS (Trompette et al., 2009). Additionally, 87.8% of *D. pteronyssinus* -sensitized asthmatic patients in Taiwan had a positive reaction in purified Dp2 skin-prick tests (Tsai et al., 2000).

Recombinant allergens

Due to the high cost of money to purify some rare or unstable allergen, protein expression systems seem to be an efficient strategy to gain allergen. Many hose cells are used to express recombinant proteins, such as *Escherichia coli* (*E. coli*), *Pichia pastoris*, drosophila cells, mammalian cells and so on. The *E. coli* and *Pichia pastoris* protein expression systems have been studied for a long time and utilized in lots of researches. *Pichia pastoris* systems were used to express recombinant allergens in this study to avoid the disadvantages in *E. coli* systems, lipopolysacchride contamination, protein uncompleted folding, and different gene codes usage.



Hypothesis and specific aims

In the previous data, we have known that the oral administration of recombinant Dp2 (rDp2) to rDp2 sensitized and challenged mice can decrease Dp2-specific IgE, total IgE, AHR, cell infiltration to lung, IL-4 levels in bronchoalveolar larvage fluid (BALF) and splenocyte cultured supernatant, and increase IL-10 level in BALF (李佩芸, 2004). Most asthmatic patients are not sensitized by Dp2 but the whole mite; thereby, the crude mite extract was used in this study rather than rDp2 to mimic the

symptoms of clinical asthma. We hypothesized that the airway inflammation induced by crude mite extract from *D. pteronyssinus* can be decreased by prior oral delivery with mite allergens, rDp2 or recombinant Dp1 (rDp1) or crude mite extract. Based on this hypothesis, the 3 specific aims were in the present study. Firstly, we would like to prepare large amount of rDp2 and rDp1 produced by *Pichia pastoris* for oral delivery. Secondary, we will select an appropriate intraperitoneal (i.p.) injection dose of crude mite extract to establish an asthmatic murine model. Finally, we would like to investigate the effect of oral delivery of rDp2 or crude mite extract on the airway inflammation induced by crude mite.

MATERIALS & METHODS

Preparation of crude mite extract

Crude mite extraction was prepared by following the protocol established previous in lab (何祥, 2002) with modifications. In breif, 1 gram of house dust whole culture mite (4960, Allergon AB, Sweden) was dissolved in 50 mL ether, mixed by a stirrer (Fisher Scientific, USA) at 4 ℃ for 48 hours, and the pellet was collected by centrifuging at 3000 rpm for 30 minutes. The pellet was suspended with 30 mL sterilized PBS, ground the pellet on ice, and mixed by a stirrer at 4 ℃ for 48 hours. The supernatant was collected by centrifuging at 10000 rpm for 30 minutes, dialyzed (Spectra/Por® Molecularporous membrane tubing MWCO: 6000-8000, Spectrum, USA) with PBS for 48 hours. Finally, the supernatant was filtered with 0.22μm filter (Acrodisc® Syringe Filter, PALL Life Sciences, USA), determined the concentration by BCA protein assay (Thermo Scientific, Rockford, USA), and stored at -80 ℃.

Preparation of rDp2

The *Pichia pastoris* GS115 transformed with *der p 2* gene in pPICZα-A (pPICZα-A-*der p 2*) was kindly provided by Dr. KY Chua, and the expression by a fermentor was kindly provided by Dr. KT Lee (Institute of Microbiology and Biochemistry, National Taiwan University). The yeast cultured supernatant from a

fermentor was collected, lyophilized by a freezer, and dissolved with ddH_2O. The cultured supernatant was depleted the alcohol oxidase by applying to Amicon® Ultra-15 PL-30 Ultrafiltration. Then protein in the fraction below 30 kD was dialyzed (Spectra/Por® Molecularporous membrane tubing MWCO: 6000-8000, Spectrum, CA, USA) with PBS at 4℃ for 48 hours. After that, the fraction containing rDp2 was concentrated and desalted by applying to Amicon® Ultra-15 PL-3 Ultrafiltration. Finally, the concentrated rDp2 protein was filtered with 0.22 μm filter (Acrodisc® Syringe Filter, PALL Life Sciences, NY, USA), determined the concentration by BCA protein assay (Thermo Scientific, Rockford, IL, USA), and stored at -80℃.

Expression of rDp1

The *E. coli* DH5α containing *der p 1* gene in pPICZα-A (pPICZα-A-*der p 1*) was kindly provided by Dr. KT Lee (Institute of Microbiology and Biochemistry, National Taiwan University). The pPICZα-A-*der p 1* was amplified and extracted by following the protocol provided by Plasmid DNA Extraction (Viogene, Taiwan). The concentration of pPICZα-A-*der p 1* was measured by a spectrophotometer (DU®800, Beckman Coulter, USA).

Six μg pPICZα-A-*der p 1* were digested into linear form by 20 Unit *Sac*I (New England BioLabs, USA) in a 37 ℃ water bath (YIHDERN) for 1 hour. The liner pPICZα-A-*der p 1* was transformed into the *Pichia pastoris* X33 by the protocol provided by the EasySelect^{TM} *Pichia* Expression Kit (Invitrogen, USA). Briefly, the 50 μL *Pichia pastoris* were added with 3 μg linear pPICZα-A-*der p 1* and 1 mL solution II. And then the *Pichia pastoris* were incubated in a 30℃ dry bath incubator (Violet BioScience, Taipei, Taiwan) for 1 hour, heat shocked in 42℃ water bath (YIHDERN) for 10 minutes, and added with 1mL fresh YPDZ and incubated at 30℃ with shacking 250 rpm for 1 hour. The yeast cells pellet were collected by centrifuging at 3000 rpm for 5 minutes, washed with 0.5 mL solution III, resuspended with 150 μL of solution III, and spread on YPDSZ plates and incubated in 30℃ incubator until colony formed (3 to 5 days). Colonies were streaked again on YPDSZ plates, and incubated at 30 ℃ until colony formed.

Colony polymerase chain reaction (PCR) was performed to confirm the successful transformation. Primers including *Eco*RI-*der p 1* and *der p 1-Xba*I (**Appendix. 3**) were used to confirm the insertion of *der p 1*. The colony PCR was performed by T3 thermocycler (Biometra, Germany) with the protocol that 95 ℃ for 5 minutes, 30 cycles (95 ℃ for 1 minute, 55 ℃ for 1 minute, and 72 ℃ for 1 minute),

and 72 ℃ for 5 minute. The PCR product was examined by agarose gel electrophoresis.

The pPICZα-A-*der p 1* inserted into *Pichia pastoris* genome may result in the destruction of AOX1 gene and poor metabolism of alcohol. MD and MM plates were used to select strains which can sustain in alcohol metabolites. Colonies from the YPDSZ plates were selected to streak on the MD and MM plates, and the colonies from the MM plates were selected to express protein in a small scale for further selection. The bigger single colonies were streaked into 5 mL BMG medium in sterilized 50 mL centrifuge tube, incubated in a shaking incubator (200 rpm) at 30 ℃ until OD_{600} reached 2-4 (about 16-18 hours). The *Pichia pastoris* were harvested by centrifuging at 2000 × g for 10 minutes at room temperature, and suspended to an OD_{600} of 1.0 in BMM medium. After overnight incubation at 30℃, the suspension was added with 100% methanol every 24 hours to reach the final concentration of 0.5% methanol. The supernatant was collected after induction for 96 hours by centrifuging at 9000 rpm for 15 minutes at 4 ℃, and for further Western blot confirmation.

The cultured supernatant from a fermentor was depleted the alcohol oxidase by applying to Amicon® Ultra-15 PL-50 Ultrafiltration. The fraction below 50 kD

dialyzed with 5 L of PBS in a dialysis bag (Spectra/Por® Molecularporous membrane tubing MWCO: 6000-8000, Spectrum, USA) at 4 ℃ for 24 hours. The fraction of rDp1 was concentrated and desalted by applying to Amicon® Ultra-15 PL-10 Ultrafiltration. Finally, the concentrated rDp1 protein was filtered with 0.22 μm filter (Acrodisc® Syringe Filter, PALL Life Sciences, USA), determined the concentration by BCA protein assay (Thermo Scientific, USA), and stored at -80 ℃.

Western blotting of rDp2 and rDp1

The sample proteins were mixed with 4 × loading dye and boiled at 95 ℃ for 10 minutes. Firstly, the samples and protein ladder (PageRuler™ Prestainged protein ladder, Fermentas, Canada) were run on SDS-polyacryamide gel (80V for 5% stacking gel, 100V for 15% running gel). Next, the gels were electro-blotted onto PVDF membrane (PALL Life Sciences, USA) at 150V for 90 minutes. After blocking with 1% BSA in TBS at room temperature for 1 hour, 5000-fold diluted mouse anti-Dp2 IgG, or 1000-fold diluted mouse anti-Dp1 IgG, or 10000-fold diluted mouse anti-His tag IgG were added and incubated at 4 ℃ overnight. The PVDF membranes were washed with TBST (120 rpm, 15 minutes) 3 times, and covered with 5000-fold or 10000-fold diluted goat anti-mouse IgG antibody at room

temperature for 30-60 minutes. The membranes were washed 6 times, added with ECL (PerkinElmer, USA), and the luminescence was detected by exposing to X-ray film (Super RX, Fujifilm, Japan).

Animals

Six to eight-week-old female BALB/c mice were purchased from the Animal Center of the College of Medicine at National Taiwan University for sensitization and challenge experiment, and from National Laboratory Animal Center for the oral feeding experiment. The animal room has a 12 hours light-dark cycle, a constant temperature (25±2 ℃) and humidity, and the mice are fed with chow diet (Lab Rodent Chow; Ralson Purina, USA). Each group in both experiments has 5 to 6 mice at beginning.

Sensitization and challenge of crude mite extract to establish allergic airway inflammation

The sensitization and challegne protocol was followed the protocol established previous in lab (Lee et al., 2001) with modifications. As summarized in **Fig.4**, each mouse was received 5 times i.p. injection with crude mite extract and 4 mg Imject®

Alum (Pierce, USA) plus with 200 ng pertussis toxin (PT, List Biological Lab, USA), and after the 5th i.p., mice were intratracheally (i.t.) challenged with crude mite extract. The mice in negative control group were sensitized and challenged with PBS.

Determination the levels of crude mite specific antibodies in serum

Mice were bled by retro-orbital venous plexus on day 0, 37, 44, 51. After incubating at room temperature for 2 hours, the sera were collected by centrifuging at 5500 rpm for 10 minutes at 4 ℃. The levels of mite-specific IgE, IgG1, and IgG2a in sera were determined by enzyme-linked immunosorbent assay (ELISA). 96-well plates (Nunc, Denmark) were coated with 100 μL per well of 10 μg/mL crude mite extract in coating buffer (151 mM Na_2CO_3 (Merck, Germany), 35 mM $NaHCO_3$ (Wako, Japan) , 3 mM NaN_3 (Ferak, Germany), and pH 6.0 in ddH_2O) and incubated at 4 ℃ overnight. The plates were washed with PBST (0.5 % Tween-20 (Riedel-de-Haën, Germany) in PBS), blocked with 200 μL per well of block buffer (3% BSA (Sigma, USA) in PBS) at 4 ℃ overnight. The sera were used at 1:50 dilution for IgE, 1:500 dilution for IgG2a, and 1:10000 dilution for IgG1 determinations. After washing, the plates were added 100 μL per well of the sera

diluted with block buffer and incubated at 4 ℃ overnight. Next, the plates were washed and loaded with with 100 μL per well of the 2 μg/mL detection antibody (biotinylated rat anti-mouse IgE (553419, BD Bioscience PharMingen, USA), IgG1 (553441, BD Bioscience PharMingen, USA) and IgG2a (553388, BD Bioscience PharMingen, USA) antibody) in block buffer and incubated at room temperature for 1 hour. After washing, the plates were loaded with with 100 μL per well of streptavidin-HRP (R&D, USA) used at 1:5000 in block buffer, avoid from direct light, incubated at room temperature for 20 minutes. The plates were washed, 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB, KPL, USA) per well was loaded and incubated at room temperature for 10 to 20 minutes. Finally, the reaction was stopped by 50 μL per well of the 2N H_2SO_4 and the optical density (OD was measured at 450 nm and 540 nm as a reference filter) was measured with a VERSAmax microplate reader (Molecular Devices, USA). The results were presented in ELISA unit (E.U. =(ODsample - ODblank)/ (ODpositive control -ODblank)).

Determination the levels of total IgE in serum

The levels of total IgE in sera were determined by ELISA. Briefly, 96-well

plates (Nunc, Denmark) were coated with 100 μL per well of 2 μg/mL purified rat anti-mouse IgE (553413, BD Bioscience PharMingen, USA) in PBS and incubated at 4 ℃ overnight. The plates were washed with PBST (0.5 % Tween-20 (Riedel-de-Haën, Germany) in PBS), blocked with 200 μL per well of block buffer (3% BSA (Sigma, USA) in PBS) and incubated at 4 ℃ overnight. The sera were used at 1:500 dilutions for total IgE determinations, and the standards (557079, BD Bioscience PharMingen, USA) were used in a serial two-fold dilution with block buffer. After washing, the plates were loaded with 100 μL per well of the sera diluted with block buffer and incubated at 4 ℃ overnight. The plates were washed and loaded with 100 μL per well of the 2 μg/ mL detection antibody (biotinylated rat anti-mouse IgE (553419, BD Bioscience PharMingen, USA)) in block buffer and incubated at room temperature for 45 minutes. After washing, the plates were loaded with 100 μL per well of streptavidin-HRP (R&D, USA) used at 1:5000 in block buffer, avoid from direct light, and incubated at room temperature for 30 minutes. The plates were washed and loaded with 100 μL per well of TMB, and incubated at room temperature for 10 to 20 minutes. Finally, the reaction was stopped by 50 μL per well of the 2N H_2SO_4 and the optical density (OD was measured at 450 nm and 540 nm as a reference filter) was measured with a VERSAmax microplate reader

(Molecular Devices, USA).

Measurement of AHR

Pulmonary function of mice was measured on the day after the 2nd i.t. challenge. Mice were placed in a barometric whole-body plethysmographic chamber (Buxco Electronics, Sharon, USA) and given different doses of acetyl-β-methylcholine chloride (use at 0, 3.125, 6.25, 12.5, 25 and 50 mg/ml in PBS, methacholine, Sigma, USA). After inhalation of nebulized methacholine for 3 minutes, the respiratory pressure curves were recorded for the following 3 minutes. The averages of methacholine-induced airway obstruction were presented as plethysmography and enhanced pause (Penh= pause× (peak expiratory box flow/peak inspiratory box flow) values.

Analysis of cellular composition of BALF

After mouse was sacrificed, the trachea was immediately lavaged via a trachea cannula (Angiocatch®, BD, USA) with 1 mL HBSS (Hank's balanced salts (Sigma, USA) in 1L ddH_2O, pH to 7.2- 7.4 by using filtered 7.5% $NaHCO_3$) for the 1st time and 1 mL HBSS with 2% FBS for twice. The lavages were centrifuged 1500 rpm, at

4 ℃ for 10 minutes, and then collect the supernatant of 1st lavage were collected for cytokine analysis. In addition, the cell pellet was collected and resuspended by HBSS with 2% FBS for cell composition analysis. The total cell number in the BALF was counted with standard hemocytometer (Sigma, USA), and then 1×10^5 cells were cytocentrifuged (Cytospin, Shandon, UK) onto glass slide at 500 rpm for 4 minutes. The BALF cells were immediately stained with Liu's stain. A minimum of 300 cells were counted per slide and differentiated as eosinophils, neutrophils, macrophages/monocytes, and lymphocytes by standard morphological criteria of leukocytes.

Determination of cytokines secretion of splenocytes by ELISA

Spleens were harvested from all mice by sterile scissors and forceps, and were grinded into single-cell suspensions in RPMI-1640 medium (1% L-glutamine (Biological Industries, USA), 1% PSA (Biological Industries, USA), 1% HEPES (Gibco Brl, USA), and 5% Fetal Bovine Serum (FBS, Hyclone, USA) in RPMI-1640 (Hyclone, USA)). To remove the large connective tissue, the cell suspensions stood at room temperature for 10 minutes and the supernatants were transferred to new tubes. After removal of erythrocytes by ACK lysis buffer (150 mM NH_4Cl (Ferak,

Germany), 10 mM $KHCO_3$ (Ferak, Germany), and 0.1 mM Na_2EDTA (Sigma, USA) in ddH_2O), splenocytes were washed with HBSS buffer for twice. Splenocytes were seeded in 48-well plates with 5×10^6 cells per well for cytokine profile analysis and in 96-well plates with 2×10^5 cells per well for proliferation assay in 5% FBS RPMI-1640 medium and incubated in a 37 ℃ and 5% CO_2 incubator. In addition, splenocytes were cultured with different concentrations of crude mite extract (0, 2.5, 5, 10, 20 μg/mL) or the nonspecific stimulation of mitogen Concanavalin A (Con A, Sigma, USA , 5 μg/mL).

After 48 hours incubation, the splenocytes in 48-well plates were centrifuged to collect the supernatants. The levels of IL-5, IL-10, IL-13, eotaxin, TGF-β and INF-γ in splenocytes culture supernatant were determined by sandwich-ELISA (DuoSet ELISA Development kit, R&D, USA). The 96-well plates (Nunc, Denmark) were coated with 100 μL per well of capture antibody in PBS and incubated at room temperature overnight. Then plates were washed with PBST (0.5 % Tween-20 (Riedel-de-Haën, Germany) in PBS), blocked with 200 μL per well of block buffer (1% BSA (Sigma, USA) in PBS) and incubated at room temperature for 1 hour. After washing, the plates were loaded with 100 μL per well of the supernatant and standard, serial two-fold diluted with reagent diluents (1% BSA (Sigma, USA) in

PBS for IL-5, IL-10, IL-13, and eotaxin determination; 1.4% BSA (Sigma, USA) and 0.05% Tween 20 (Riedel-de-Haën, Germany) in PBS for TGF-β determination; 0.1% BSA and 0.05% Tween 20 (Riedel-de-Haën, Germany) in TBS for INF-γ determination), and incubated at 4 ℃ overnight. The plates were washed and loaded with 100 μL per well of biotinylated detection antibody in reagent diluents and incubated at room temperature for 2 hour. After washing, the plates were loaded with 100 μL per well of streptavidin-HRP used at 1:200 in reagent diluents, avoid from direct light, and incubated at room temperature for 20 minutes. The plates were washed and loaded with 100 μL per well of TMB and incubated at room temperature for 10 to 20 minutes. Finally, the reaction was stopped with 50 μL per well of the 2N H_2SO_4, and the optical density (OD was measured at 450 nm and 540 nm as a reference filter) was measured with a VERSAmax microplate reader (Molecular Devices, USA).

Determination the lymphoproliferation of splenocytes

After 48 hours incubation, the splenocytes in 96-well plates were pulsed with 1 μCi per well of [^{3}H]-thymidine (Ameresco, USA) for 16 to 18 hours. Then the cells were harvested onto glass fiber paper (Packard, Meriden, USA) by a semi-automated

harvester (Filtermate 196, Packard, Meriden, USA), and the value of counts per minute (c.p.m.) were read by a β-counter instrument (Direct Beta Counter, Packard, Meriden, USA). The results were presented in c.p.m. (cpm_{sample}-$cpm_{medium\ alone}$).

Oral feeding protocol

The time line was summarized in **Fig. 12**. Crude mite extract-sensitized mice were divided into five groups (fed with 0.2 or 1.0 mg/day of rDp2 or crude mite extract, and a positive control group fed with PBS) and a PBS-sensitized group (as negative control), and each group consisted of 3 to 5 animals. Mice were tube-fed with 0.5 mL of rDp2 or crude mite extract dissolved in PBS for consecutive 7 days started from day 1.

Statistical Analysis

All the results were expressed as means with standard deviation. Statistical analysis was determined by Students' *t*-test, and a *p* value less than 0.05 was considered to be significant.

RESULTS

Successful expression of rDp2 protein from a fermentor produced by *Pichia pastoris*

To get large amount of rDp2, the fermentor were used for yeast culture. The *Pichia pastoris* was used as the recombinant protein expression system for its endotoxin-free, larger scale of protein expression, and the ability to secrete the recombinant protein into cultured medium. The rDp2 was collected from the cultured supernatant of *Pichia pastoris* from a fermentor and concentrated by Amicon tubes. The concentrated rDp2 was confirmed by SDS-PAGE (**Fig. 2a**) and Western blot with anti-Dp2 antibody (**Fig. 2b**). Since rDp2 contains 6 histidines at its C terminus (**Appendix. 1**), the rDp2 was confirmed by Western blot with anti-His tag antibody (**Fig. 2b**). As **Fig. 2a** shown, the rDp2 is the major protein after concentration and the size of rDp2 can be predicted similar with Dp2 (14kD) purified from house dust mite.

Expression of rDp1 protein produced by *Pichia pastoris*

The *E. coli* DH5α containing pPICZα-A- *der p 1* gene was kindly provided by Dr. KT Lee (Institute of Microbiology and Biochemistry, National Taiwan University). The *der p 1* gene was inserted into pPICZα-A, a commercial plasmid for

yeast protein expression system, and the sequencing result was shown in **Appendix. 2**. The *der p 1* gene was inserted by *Eco*RI and *Xba*I between α-factor and c-myc epitope in the pPICZα-A. The 153th- 155th, the sequence start from the restriction enzyme cutting site (*Eco*RI), had a variation that TAC was replaced by CAC, and the 375th- 377th had variation that GTA was replaced by GCA (Chua et al., 1993). The 328th-330th showed a base wobbles, GCA was replaced by GCG, and it did not affect the counterpart amino acid. The sequencing result demonstrated that the pPICZα-A-*der p 1* contained the correct sequence of *der p 1* gene.

The pPICZα-A-*der p 1* was amplified and collected from DH5α, and then transformed into *Pichia pastoris* as a linear form by the EasySelect^{TM} *Pichia* Expression Kit (Invitrogen, USA). The 8 clones (clone 8, 14, 15, 16, 17, 21, 27, and 30) growing on the YPDSZ plate were pricked to confirm the insertion of *der p 1* by directly colony PCR (**Fig. 3a**). Six clones (clone 8, 14, 15, 16, 17, and 21) in the 8 clones confirmed by colony PCR had inserted by pPICZα-A-*der p 1*. Among them 4 clones (clone 8, 16, 17, and 21) were selected to express rDp1 in a small scale. After induction for 96 house, the supernatant were collected, confirmed by Western blot (**Fig. 3b**), and the size of rDp1 produced by the 4 selected clones were similar with Dp1 purified from house dust mite. These data suggested that the 4 clones selected

(clone 8, 16, 17, and 21) can produce rDp1 successfully.

To get large amount of rDp1, the fermentor were used for the large scale yeast culture. The rDp1 was collected from cultured supernatant of *Pichia pastoris* and concentrated. The concentrated rDp1 was confirmed by SDS-PAGE (**Fig. 3c**) and Western blot (**Fig. 3d**). The data suggested that the size of rDp1 can be predicted similar with Dp1 (25kD) purified from house dust mite and slightly higher than 25kD.

Sensitization with crude mite extract elevated specific immunoglobulin

To assess the effectiveness of the sensitization and challenge protocol, mice were bled on day 0 (pre-i.p.), 37 (i.p. 4), 44 (i.p. 5) and 51 (Sacrifice) to analyze the antibody level in serum. The experimental design was summarized in **Fig. 4** and the flow chart of the sacrifice was shown in **Fig. 5**. Increased IgE production is one of the most common symptoms of asthma, and an apparent parameter for the confirmation of the effectiveness of the sensitization and challenge protocol.

As summarized in **Fig. 6a** and **Fig. 6b**, the total IgE levels and mite-specific IgE levels in serum of 3 experimental groups (Low, Mid, and High; crude mite extract sensitized) were significantly higher than that of the negative control group (NC;

PBS sensitized), except the serum before i.p. (pre-i.p.). Mite-specific IgE, IgG1, IgG2a (**Fig. 6c**) levels in sera of mice at the sacrificed timepoint (day 51) were also significantly higher than that of NC. The data suggested that the IgE level was significantly elevated by the sensitization and challenge protocol.

Sensitization and challenge with crude mite extract increased lymphoproliferation of splenocytes

To measure the lymphoproliferative ability of splenocytes after sensitization and challenge, ^{3}H-incorporation proliferation assay was used to determine the lymphoproliferation. Data summarized in **Fig. 7**, crude mite extract sensitized and challenged groups had apparently proliferation under crude mite extract stimulation. The proliferative response of splenocytes under 5 μg/mL of Con A stimulation showed no difference among each group indicated that the proliferative ability of splenocytes was not affected by the sensitization and challenge protocol. In brief, the splenocytes of crude mite extract sensitized and chllanged groups had significantly higher lymphoproliferative abilities under crude mite extract stimulation than that of NC.

Sensitization and challenge with crude mite extract increased inflammatory cytokine secretions of splenocytes

To assess the systemic immune response after crude mite extract sensitization and challenge, the cytokines profiles in the supernatant of splenocytes cultured with different stimulations were analyzed. Since allergic asthma is a disease major driven by the Th2 cells, and IL-4 and IL-5 are the major cytokines produced by Th2 cells. The levels of IL-4 and IL-5 were determined by ELISA.

The IL-4 levels of 3 experimental groups treated with crude mite extract were significantly higher than that of NC (**Fig. 8a**). Splenocytes of 3 experimental groups produced higher amount of IL-5 (**Fig. 8b**) than that of NC with crude mite extract stimulation. Furthermore, the IL-5 levels of low-dose and high-dose groups treated with 5 μg/mL of Con A (non-specific stimulation) were higher than that of NC (**Fig. 8b**). The data suggested that the splenocytes of crude mite extract sensitized groups produced more Th2 cytokines under crude mite extraction stimulations.

Sensitization and challenge with crude mite extract induced AHR

To assess the effects of sensitization and challenge protocol on lung function, the AHR was measured after i.t. challenges. AHR is another important parameter of

clinical symptoms of asthma. Acetylcholine stimulation induces bronchoconstriction, and methacholine was used to mimic the stimulation induced by the short-half life of acetylcholine. Aerosolized methacholine induces the bronchoconstriction, and with increasing concentration of methacholine accompanied by the marked bronchoconstriction.

The ratio of Penh value had an increasing trend with the increased concentration of methacholine (**Fig. 9**). The high-dose group had higher Penh ratio significantly compared with that of NC. Therefore, the data demonstrated that 50 μg crude mite extract as the i.p. dose and i.t. dose can induce AHR significantly.

Sensitization and challenge with crude mite extract facilitated eosinophils infiltration in BALF

To assess the lung inflammation, the levels of cytokines and compositions of infiltrated cells in BALF were analyzed. Eosinophils infiltration into lung is a parameter for asthma and airway inflammation. Eotaxin is a chemokine for eosinophils recruitment; additionally, IL-5 is a major cytokine produced by Th2 cells and has been reported to be involved in eosinophils development.

To assay the compositions of infiltrated cells, both the percentage and number

of cell populations in BALF were summarized in **Fig. 6a** and **Fig. 6b**, respectively. The eosinophils of mid-dose and high-dose groups were signigicantly increased in percentage (**Fig. 10a**), but not the number (**Fig. 10b**).The eosinophils of low-dose group were significantly higher than that of NC in number (**Fig. 10b**) but not the percentage (**Fig. 10a**). The number of lymphocytes of high-dose group was significantly higher than that of NC (**Fig. 10b**). Moreover, the high-dose group showed higher neutrophils and lower monocytes both in number (**Fig. 10b**) and percentage (**Fig. 10a**).

The BALF were collected, and both eotaxin and IL-5 levels were determined by ELISA (**Fig. 11**). The eotaxin levels of mid-dose and high-dose groups were slightly higher than that of NC (**Fig. 11a**). The IL-5 level of high-dose groups was higher than that of NC without significance (**Fig. 11b**). To sum up, the high-dose group showed more severe airway inflammation, including higher percentage of eosinophils in BALF, and higher eotaxin and IL-5.

Oral feeding showed no effects on the levels of immunoglobulin in serum

The experimental design was summarized in **Fig. 12** and the flow chart of the sacrifice was shown in **Fig. 5**. To evaluate the effect of oral feeding on the levels of

mite-specific immunoglobulin in serum, the sera were collected at the sacrificed timepoint (day 43) for the measurement of the antibody levels. As summarized in **Fig. 13**, the total IgE, mite-specific IgE, and mite-specific IgG1 levels in serum of crude mite sensitized groups (PC, rD2 0.2, rD2 1.0, Mite 0.2, and Mite 1.0 group) were significantly higher than that of PBS sensitized group (NC). These data suggested that the level of total IgE, mite-specific IgE and mite-specific IgG1 were not affected by oral feeding.

The lymphoproliferation were not affected by oral feeding with mite allergens

To assess whether the lymphoproliferative ability of splenocytes was affected by the oral feeding with mite allergens or not, ^{3}H-incorporation proliferation assay was used to determine the lymphoproliferation. As summarized in **Fig. 14**, the crude mite sensitized and challenged groups had higher proliferation compared with that of NC. The data suggested that the proliferation of splenocytes under crude mite extract stimulation was not affected oral feeding with mite allergens.

IL-4 productions of splenocytes decreased after oral feeding

To assess the systemic immune response after oral feeding, the cytokines

profiles in splenocytes cultured supernatant were analyzed. The Mite 0.2 group decreased IL-4 production under 5 and 10 μg/mL of crude mite extract treatment (**Fig. 15a**). The rD2 0.2 group had higher IL-5 production than that of PC which were treated with crude mite extract, and the rD2 1.0 group had higher IL-5 production than that of PC which were treated with 2.5 μg/mL of crude mite extract (**Fig. 15b**). Summarily, oral feeding with crude mite extract had slightly effects on decreasing Th2 cytokines production of splenocytes.

Oral feeding with mite allergens slightly elevated IL-10 levels of splenocytes

To evaluate whether oral feeding with mite allergen increases the regulatory cytokines or not, the IL-10 and TGF-β levels in splenocytes cultured supernatants were measured. The IL-10 productions under specific or non-specific stimulation showed no statistical difference and the rD2 0.2 group had slightly higher IL-10 level than that of PC (**Fig. 16a**). Furthermore, the TGF-β showed more variable levels among each group, and the Mite 1.0 group under 5 μg/mL of crude mite extract treatment showed higher TGF-β levels than that of PC without statistic difference (**Fig. 16b**). To sum up, the IL-10 levels were slightly elevated by oral feeding with mite allergens.

Oral feeding with higher dose of mite allergens reduced the AHR

To assess the effect of oral feeding with mite allergens on lung function, mice were measured the AHR at day 42. As summarized in **Fig. 17**, the ratio of Penh value of rD2 1.0 group was lower than PC under 3.125, 6.25, 12.5, and 25 mg/mL of methacholine stimulation. Under 25 mg/mL of methacholine stimulation, Mite 1.0 group showed lower ratio of Penh valure than that of PC. Therefore, these data demonstrated that oral feeding with 1.0 mg/day of rDp2 or crude mite eactract for constitutive 7 days reduced the AHR.

The airway inflammation were slightly decreased after oral feeding

To evaluate the effect of oral feeding mite allergens on the lung inflammation, the levels of cytokines and compositions of infiltrated cells in BALF were analyzed. The percentage and number of cell populations in BALF were summarized in **Fig. 18b** and **Fig. 18a**, respectively. The groups sensitized and challenged with crude mite extract had higher eosinophils (**Fig. 18**). The rD2 0.2, rD2 1.0, and Mite 1.0 groups had lower neutrophil than that of PC in percentage (**Fig. 18a**). The rD2 0.2, Mite 0.2, and Mite 1.0 groups had lower total cell numbers than that of PC (**Fig. 18b**).

The BALF were collected for eotaxin, IL-5, IL-10, and IL-13 measurement by ELISA (**Fig. 19**). The rD2 1.0 and Mite 1.0 groups had lower eotaxin (**Fig. 19a**) and IL-10 levels (**Fig. 19c**) than that of PC. The rD2 1.0, Mite 0.2, and Mite 1.0 groups had lower levels of IL-5 (**Fig. 19b**) and IL-13 (**Fig. 19d**). To sum up, the infiltrated neutrophils percentage in BALF were slightly reduced, and the inflammatory cytokines were slightly decreased after oral feeding.

DISCUSSION & CONCLUSION

Allergic asthma model induction

To figure out the suitable i.p. dose for sensitization, the modified protocol had 5 times i.p. injections with different doses of crude mite extract in the presence of adjuvants and twice i.t. challenge. BALB/c female mice were used in this study based on its Th2 biased immunological response (Boyce and Austen, 2005; Melgert et al., 2005), such as higher IgE response and severe airway hypersensitiveness (Leong and Huston, 2001). Crude mite extract from house dust mite *D. pteronyssinus* were utilized as the allergen here rather than OVA for its greater clinical relevance; moreover, crude mite extract had been used as the allergen used in sensitization (Johnson et al., 2004) and challenge phase (Tournoy et al., 2000) in other reports. The parameters used in this study to evaluate the protocol are the specific IgE elevation, AHR induction, and the airway inflammation.

As the data shown (**Fig. 6**), the levels of mite-specific antibodies IgE and IgG in crude mite immunized mice were elevated significantly after 5 times i.p. injection. IgG1 and IgE production *in vivo* have been linked to Th2 cytokines, such as IL-4 (Finkelman et al., 1988) and IL-13; likewise, IgG2a has been linked to Th1 cytokines, such as interferon-γ (IFN-γ) (Snapper et al., 1988). Pertussis toxin increases IFN-γ, IL-2 (Ryan et al., 1998), and IL-4 production of naïve T cells (Mu and Sewell, 1993).

It has been suggested that pertussis toxin activates antigen presenting cells to promote clonal expansion and differentiation of Th1 and Th2 cells and enhances both Th1 and Th2 cytokines production (Shive et al., 2000). So that pertussis toxin has widely used as an adjuvant in animal models of Th1-mediated autoimmune disease (Munoz et al., 1984) and delayed-type hypersensitivity (Sewell et al., 1987). Additionally, the crude extract of *D. pteronyssinus* plus with alum salt sensitized mice elevated mite specific IgE, IgG1, IgG2a, and IgG2b (Sato et al., 2002). Based on the adjuvant effects and the property of crude extract of *D. pteronyssinus*, mite specific IgE, IgG1, and IgG2a significantly increased in the crude mite extract sensitized mice after sensitization. The splenocytes derived from crude mite immunized mice secreted higher Th2 cytokines and exhibited higher proliferative abilities than negative control group under crude mite stimulation but there were no difference among 3 experimental groups. These data suggest that no differences in systemic responses among different sensitization dose might be caused by the potent enhancement of adjuvants.

The symptoms induced by crude mite extract were similar with that induced by rDp2, including elevated allergen specific IgE and IgG, increased AHR, eosinophil infiltration in the lung, airway inflammation, and a splenic Th2 response (何祥, 2002;

李佩芸, 2004). These are also similar with the symptoms induced by natural Dp1, such as high titer of allergen specific IgG1, elevated AHR, eosinophil infiltration into lung, increased IL-5 level in BALF (Lee et al., 1999; Lee et al., 2001). However, crude mite extract induced significantly mite specific IgE levels after 5 times of i.p. injection, which differ from rDp2 sensitization, which induced rDp2-IgE levels after 4 times of i.p. injection. That might be because the allergens are only a small part of crude mite extract; thereby, the crude mite extract needs more boost to elevate specific IgE levels.

To sum up, the present study showed that the systemic responses were similar among 3 different doses of i.p. injection, and the local airway inflammation and AHR response were more significantly induced by the high dose of i.p. injection (50 μg of crude mite extract).

The effects of oral delivery with rDp2 on airway inflammation

Oral tolerance has been studied for a long time as a potential strategy to modulate autoimmune and allergic disease (Mayer and Shao, 2004). Some evidences suggested that the regulatory T cells induced at the gut might migrate to periphery to exert their suppressive function by direct or indirect mechanism (Tsuji and Kosaka,

2008). Our previous studies showed that oral delivery of the single allergen decreases the allergen-specific IgE and airway inflammation (李佩芸, 2004; 徐凡琪, 2004; 游詩怡, 2007). Thus, we aimed to evaluate the effects of an oral tolerance response induced by low doses to rDp2 on crude mite induced allergic airway inflammation.

As the results shown (**Fig. 13**), the serum antibody levels, including total IgE and mite-specific antibodies, did not be affected by the oral feeding strategy. These data were different from our previous studies in which oral feeding 0.5 mg/day of OVA for 5 days reduced OVA-specific IgE (游詩怡, 2007), and oral feeding 0.2 mg/day of rDp2 for 7 days reduced Dp2-specific IgE and total IgE (李佩芸, 2004). Oral delivery of crude extract of *D. pteronyssinus* to *D. pteronyssinus* sensitized A/Sn mice decreased the IgE response (Carvalho et al., 2004). In a serial studies, oral feeding crude extract of *D. pteronyssinus* decreased the allergen specific IgE and IgG in crude mite sensitized A/Sn mice which might be through the production of autoantibody IgG anti-IgE (Sato et al., 1998; Sato et al., 2002). However, another report demonstrated that oral delivery of recombinant Der f 2 (rDf2) could not decrease the level of Der f 2-specific IgE in *Dermatophagoides farinae* plus with rDf2 sensitized A/J mice (Yasue et al., 1997). It has been reported that there are about 0.1 μg Dp2 in 1 mg fecal extract and there are about 1 μg Dp2 in 1 mg body

extract of D. *pteronyssinus* (Heymann et al., 1989). Therefore, we speculated that the oral feeding induced Dp2-specific regulatory T cells could decrease the Dp2 specific systemic response by direct suppression but might be not enough to decrease the crude mite systemic immune response through indirect mechanism. However, this needs to be confirmed further.

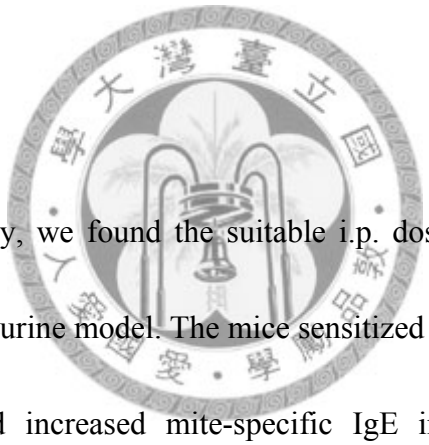
In the mice fed with 1.0 mg/day of rDp2, IL-4 secretion of splenocytes were slightly decreased, TGF-β secretion of splenocytes were slightly elevated, AHR were significantly reduced, eotaxin and IL-13 levels in BALF were slightly retarded, and the number of neutrophil were slightly decreased. Our previous studies showed that oral delivery of 0.2 mg/day of rDp2 could decrease the IL-4 production of splenocytes, and oral feeding with 1.0 mg/day of rDp2 decreased AHR, the eosinophils number in BALF, and the IL-4 level in BALF (李佩芸, 2004; 徐凡琪, 2004). The differences between the present study and the previous studies might be due to the antigens used for feeding and sensitization. It has been well demonstrated that oral delivery with an antigen could decrease the same antigen induced allergic response in our and other groups' previous studies. Additionally, oral feeding with rDf2 slightly decreased the neutrophil infiltration in BALF in *Dermatophagoides farinae* plus with rDf2 sensitized A/J mice (Yasue et al., 1997). We had showed that

oral delivery with 1.0 mg/day of rDp2 significantly decreased AHR and slightly reduced IL-13, AHR and airway inflammation. Therefore, we suggested that oral delivery with single allergen, even its natural content is small, might alleviate the complex crude mite induced inflammation.

The effects of oral delivery with crude mite extract

Oral feeding with 0.2 mg/day of crude mite extract significantly decreased IL-4 and TGF-β levels, increased IL-5 production of splenocytes, and slightly reduced AHR. Oral feeding with 1.0 mg/day of crude mite extract showed reduced AHR, lower eotaxin level in BALF, reduced neutrophil percentage and total infiltrated cell number in BALF, and mild lymphoproliferation of splenocytes. OVA sensitized BALB/c and BP2 mice, which were oral administered with OVA in drinking water, showed reduced AHR, mucus hypersecretion, the levels of Th2 type cytokines in BALF, and the levels of OVA-specific IgE (Russo et al., 2001). Oral delivery of crude extract of *D. pteronyssinus* to *D. pteronyssinus* sensitized A/Sn mice decreased neutrophils and lymphocytes infiltration in BALF (Carvalho et al., 2004), significantly decreased IL-4 and IL-5 and increased TGF-β production of splenocytes (Sato et al., 2001). The difference might be caused by the different oral feeding doses

and days, and the different animal model. Another reason for the unclear trend was that the number of mice in oral feeding experiment was 3 to 5 per group, which was too few to represent a clear trend. In addition, the mice used in 2 animal experiments were from different origins, which might result in some difference between these 2 experiments. Therefore, we only suggest that feeding with a higher dose (≧1 mg) of single recombinant allergen or with a longer period (≧ 7 days) might have much more benefit on complex-crude-mite-induced airway inflammation.



Conclusion

In the present study, we found the suitable i.p. dose of crude mite extract to establish an asthmatic murine model. The mice sensitized and challenged by 50 μg of crude mite extract had increased mite-specific IgE in serum, elevated airway hypersensitiveness, and a splenic Th2 immune response to crude mite stimulation. The data suggested that the sensitization and challenge protocol induces an asthmatic murine model to mimic clinical asthma symptoms for further study. We also showed that oral delivery of 1.0 mg/day of rDp2 for consecutive 7 days reduced AHR, slightly decreased the eotaxin level in BLAF, and elevated TGF-β production of splenocytes in an asthmatic model established by crude mite extract. We suggested

that oral delivery of high dose (1 mg/day) of single recombinant allergen seems to be more beneficial on airway inflammation induced by the complex crude mite extract, but the proper feeding dose and feeding period need further investigation. In the future, the oral feeding experiment is needed to be repeated to conclude a significant effect of oral delivery with a recombinant major allergen on crude-mite-induced airway inflammation and then we would like to further investigate its mechanism.

FIGURES

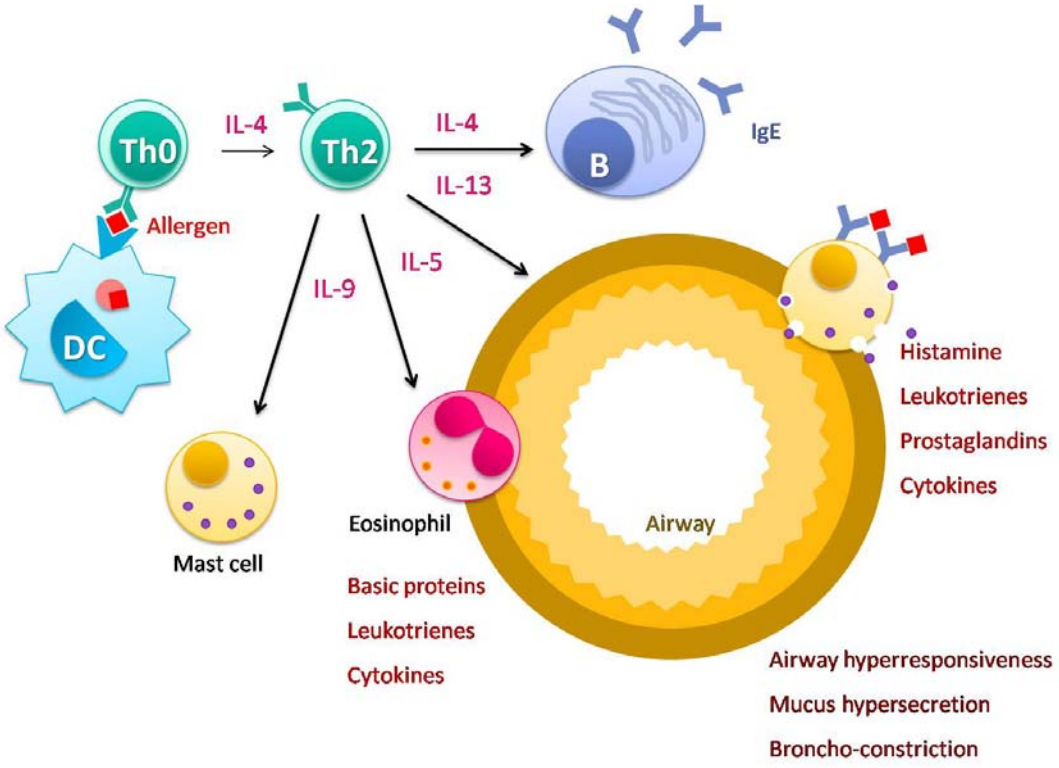


Figure 1. The scheme of allergic asthma

In the sensitization phase, the airway DCs present the processed allergen to naïve Th cells, which develop into Th2 cells in the presence of early IL-4. The Th2 cells secrete IL-4, IL-13, IL-5, and IL-9 to promote IgE production by B cells, AHR, mucus hypersecretion, and support eosinophils and mast cells development. In the challenge phase, the allergen bearing IgE binding to mast cells induces degranulation of mast cells, and releases of histamine, leukotrienes, prostaglandins, and cytokines. Finally, all the events result in increasing mucus production, broncho-constriction, and wheezing. The infiltrated eosinophils release basic proteins, leukotrienes, and pro-inflammatory cytokines to enhance the airway inflammation.

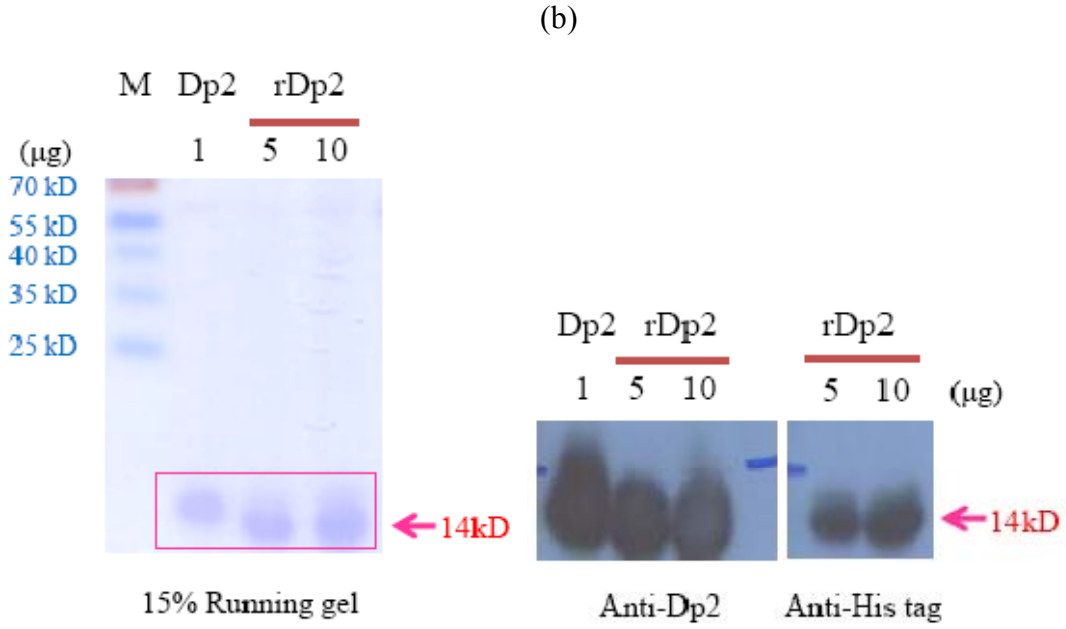


Figure 2. Successful expression of rDp2 protein from a fermentor produced by *Pichia pastoris*

The rDp2 protein was concentrated from *Pichia pastoris* cultured supernatant collected from a fermentor. The concentrated rDp2 was confirmed the size with SDS-PAGE (a) and western blot by anti-Dp2 and anti-His tag antibody (b). (Dp2, natural Dp2 purified from mite was as a control for rDp2 size confirmation; M, marker)

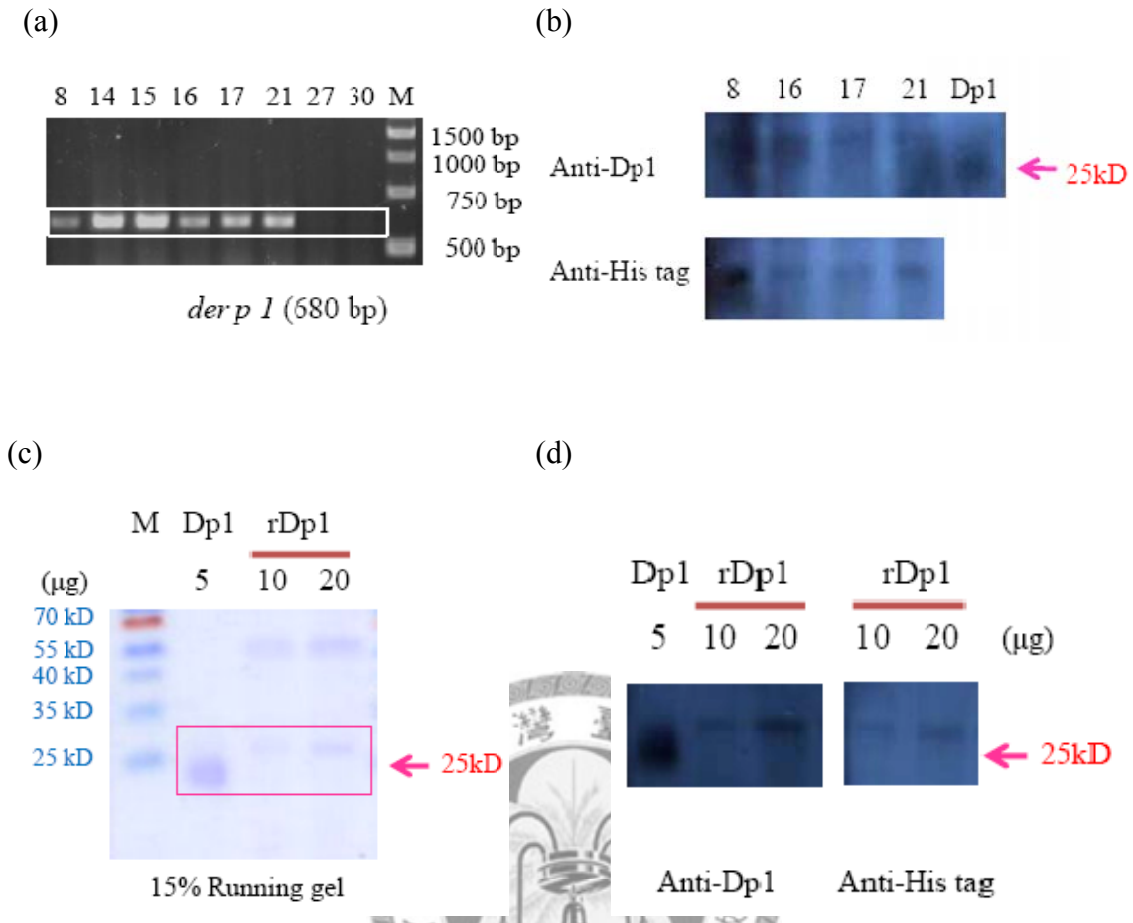
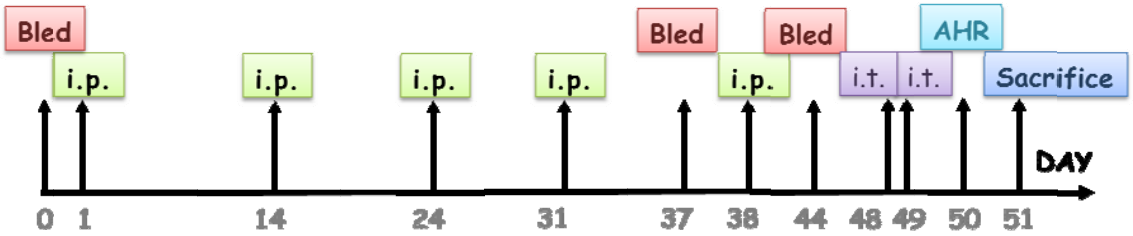


Figure 3. Expression of rDp1 protein produced by *Pichia pastoris*

Eight clones (8, 14, 15, 16, 17, 21, 27, and 30) were selected to confirm whether the *der p 1* gene insert into *Pichia pastoris* by colony polymerase chain reaction (PCR) (a). Four clones (8, 16, 17, and 21) were induced by methanol to express recombinant Dp1 protein (rDp1) in a small scale, and the concentrated cultured supernatant were confirmed by Western blot by anti-Dp1 and anti-His tag antibody (b). The rDp1 was concentrated from a large scale expression by a fermentor, the concentrated rDp1 was confirmed the size with SDS-PAGE (c) and Western blot (d). (Dp1, natural Dp1 purified from mite was used as a control for rDp1 size confirm; M, marker)

(a) Time line of sensitization and challenge experiment



(b) The groups design of sensitization and challenge experiment

Groups	i.p.	i.t.
NC	PBS	PBS
Low	10 μg Mite + 4 mg Alum + 200 ng PT	50μg Mite
Mid	30 μg Mite + 4 mg Alum + 200 ng PT	
High	50 μg Mite + 4 mg Alum + 200 ng PT	

Figure 4. The experimental design of sensitization and challenge experiment

As summarized in (a), mice were i.p. injected 5 times, on day 1, 14, 24, 31, and 38. After the 5th i.p. injection, mice were i.t. challenged on day 48 and 49, measured the AHR on day 50, and sacrificed on day 51. As shown in (b), mice were divided into four groups, negative control (NC), low-dose (Low), middle-dose (Mid), high-dose group (High), and each group consisted of 4- 6 animals. (Mite, crude mite extract.)

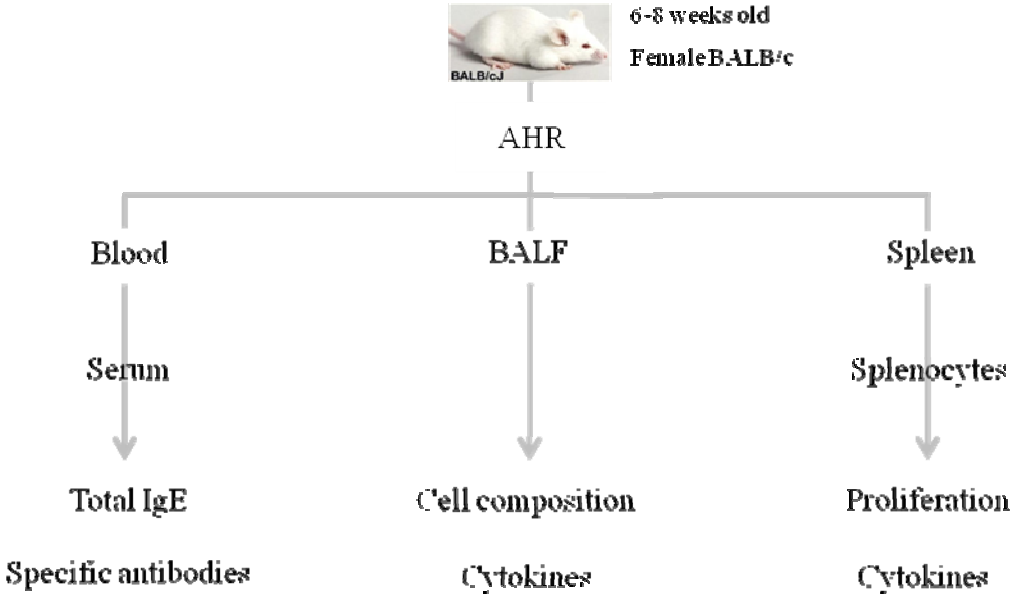
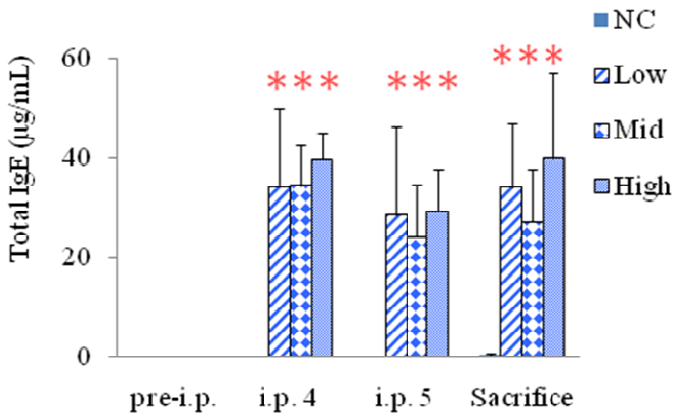


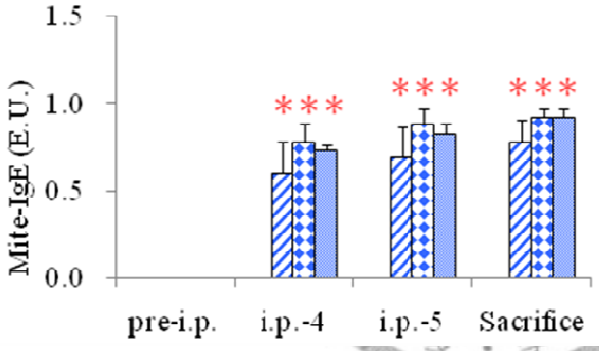
Figure 5. The flow chart of the sacrifice

After sensitization and challenge or oral feeding, mice were measured AHR and sacrificed to collect blood, BALF, and spleens. The serum were separated from blood for total IgE and specific antibodies determination. The cell composition and cytokine levels in BALF were analyzed. Splenocytes were isolated from spleen for proliferation assay and cytokine levels determination.

(a) Total IgE



(b) Mite-specific IgE



(c) Mite-specific IgE, IgG1, IgG2a

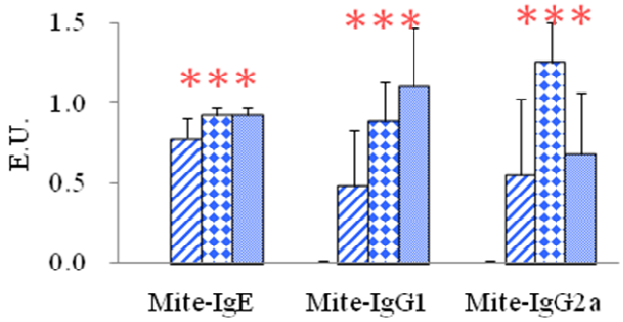


Figure 6. Sensitization with crude mite extract elevated specific immunoglobulin

The levels of total IgE in sera before i.p. (pre-i.p.), after 4th i.p. (i.p.-4), after 5th i.p. (i.p.-5), and the sacrificed timepoint (Sacrifice) were shown in (a), and the level of mite-specific IgE were shown in (b). The levels of mite-specific IgE, IgG1, and IgG2a of the sacrificed day were shown in (c). (NC, negative control group; Low, low-dose group; Mid, middle group; High, high-dose group) Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means and * presents $P < 0.05$ compared to the negative control group with statistical difference.

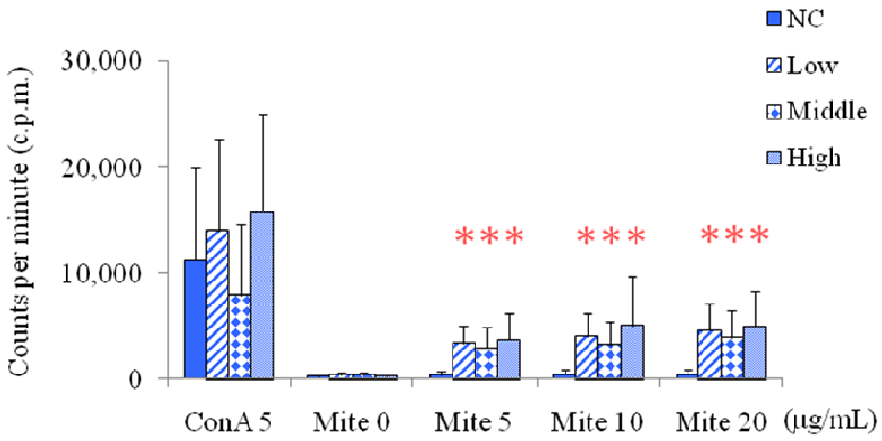
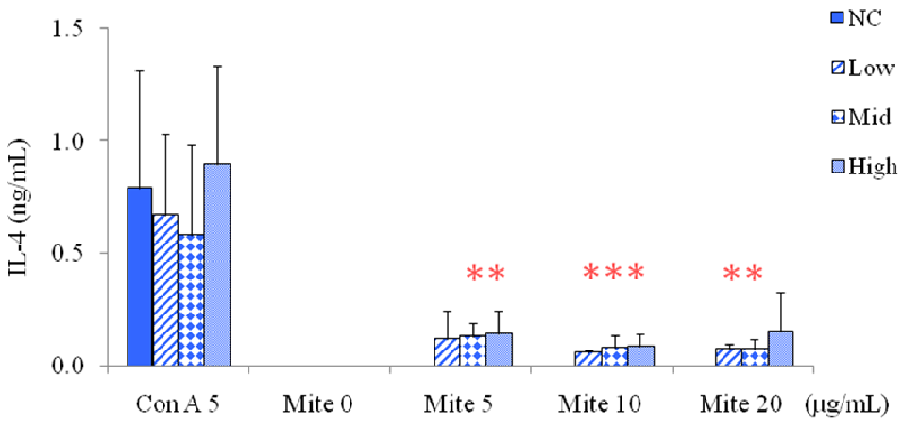


Figure 7. Sensitization and challenge with crude mite extract increased lymphoproliferation of splenocytes

Splenocytes were cultured with Con A (5 μg/mL) or crude mite extract (0, 5, 10, 20 μg/mL) for 48 hour. The lymphoproliferation was determined by ^{3}H incorporation assay. The results were presented in counts per minute (c.p.m. = cpm_{sample}-$cpm_{medium\ alone}$). Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means and * presents $P < 0.05$ compared to the negative control group with statistical difference.

(a) IL-4 levels under stimulation



(b) IL-5 levels under stimulation

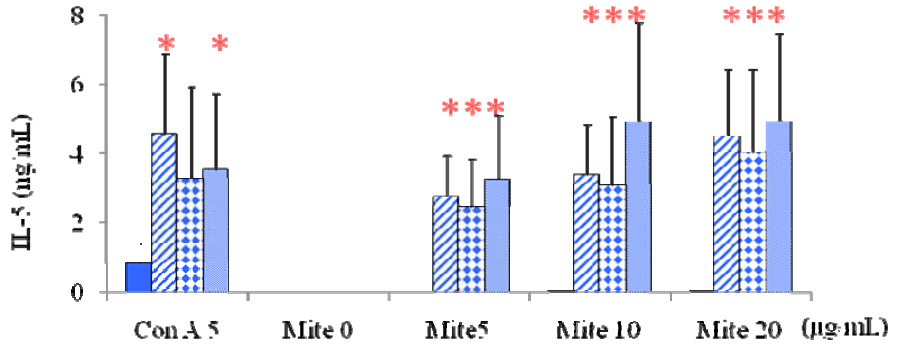


Figure 8. Sensitization and challenge with crude mite extract increased inflammatory cytokine secretions of splenocytes

Splenocytes were cultured with Con A (5 μg/mL) or crude mite extract (0, 5, 10, 20 μg/mL) for 48 hours. The levels of IL-4 (a) and IL-5 (b) in the supernatant were determined by ELISA. Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means and * presents $P < 0.05$ compared to the negative control group with statistical difference.

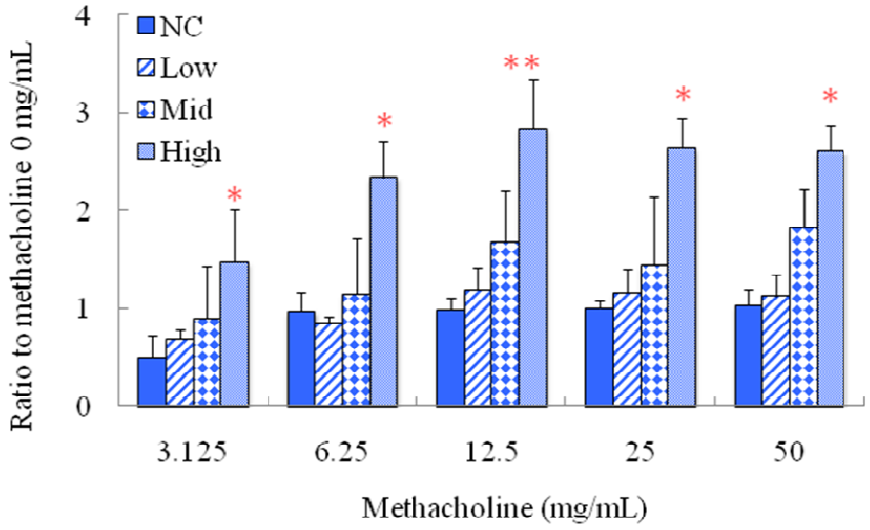
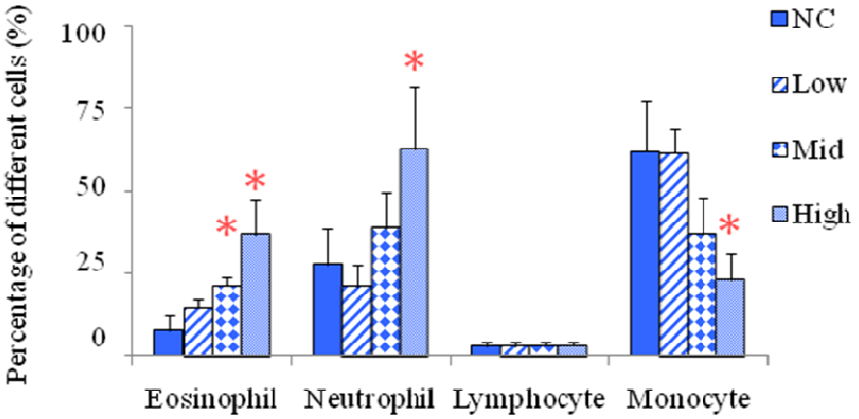


Figure 9. Sensitization and challenge with crude mite extract induced AHR

The pulmonary functions of mice were measured on the day 50. The bars represent averages of the ratio of the Penh value under respective concentrations of methacholine to that under methacholine 0 mg/mL treatment. Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means and * presents $P < 0.05$ compared to the negative control group with statistical difference.

(a) Percentage of infiltrated cells in BALF



(b) Number of infiltrated cells in BALF

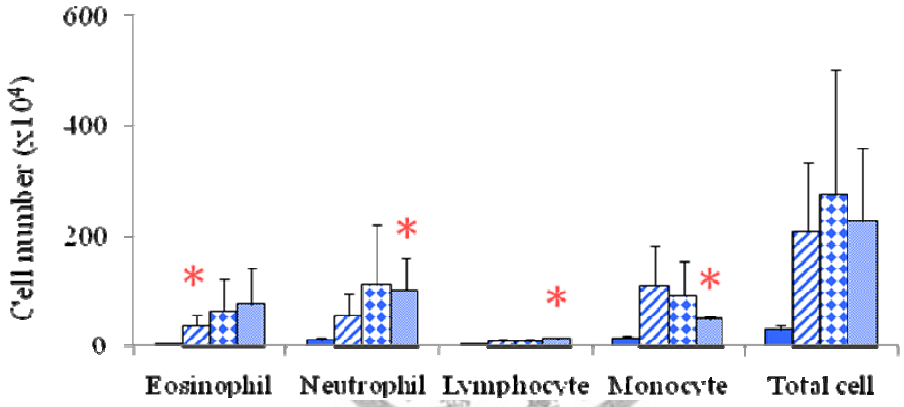
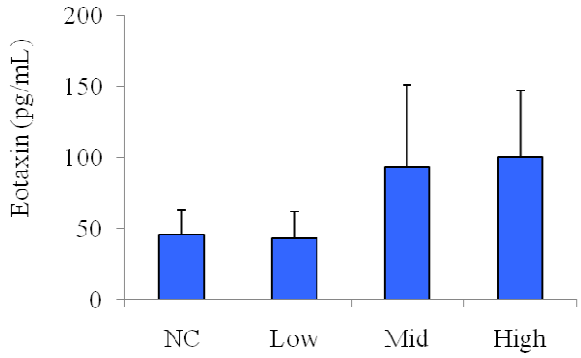


Figure 10. Sensitization and challenge with crude mite extract facilitated eosinophils infiltration in BALF

The cell composition analysis was counted a minimum of 300 cells per slide and the cell populations were shown in number (a) and in percentages (b). Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means and * presents $P < 0.05$ compared to the negative control group with statistical difference.

(a) Eotaxin levels in BALF



(b) IL-5 levels in BALF

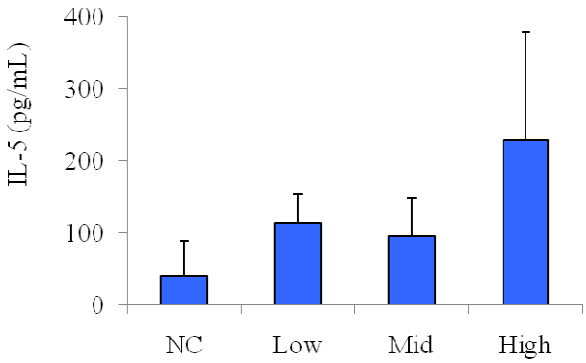
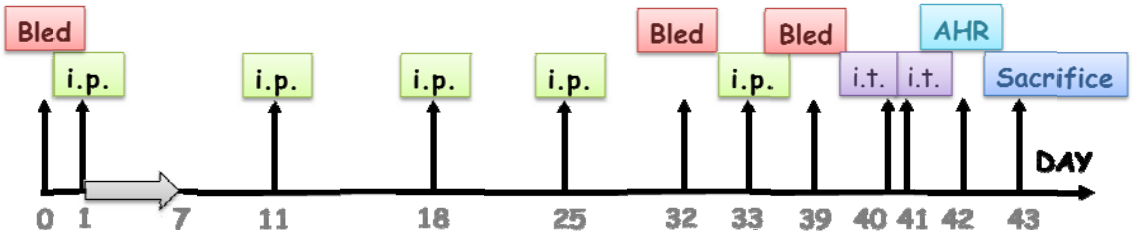


Figure 11. Sensitization and challenge with crude mite extract increased inflammatory cytokine levels in BALF

The levels of eotaxin (a) and IL-5 (b) were determined by ELISA. Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means.

(a) Time line of oral feeding experiment



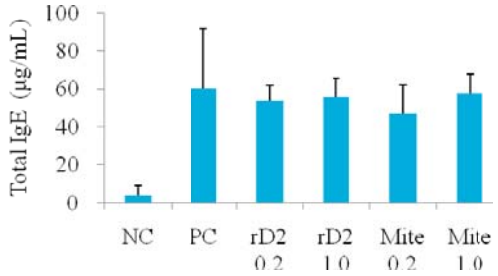
(b) The groups design of oral feeding experiment

Group	i.p.	Feeding	i.t.
NC	PBS	PBS	PBS
PC	50 μg Mite + 4 mg Alum + 200 ng PT	PBS	50 μg Mite
rD2 0.2		rDp2 0.2 mg	
rD2 1.0		rDp2 1.0 mg	
Mite 0.2		Mite 0.2 mg	
Mite 1.0		Mite 1.0 mg	

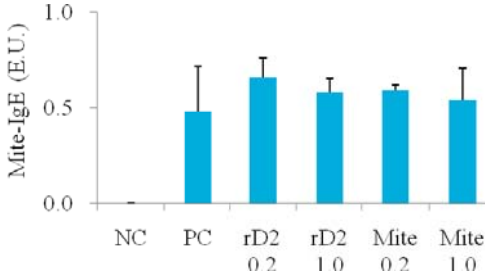
Figure 12. The experimental design of oral feeding experiment

As summarized in (a), mice were i.p. injected 5 times, i.t. challenged twice, measured the AHR, and sacrificed. Mice were fed with or without rDp2 or crude mite extract dissolved in PBS from day 1 to 7. As shown in (b), mice were divided into six groups, negative control (NC), positive control (PC), oral fed with 0.2 mg of rDp2 (rD2 0.2), oral fed with 1.0 mg of rDp2 (rD2 1.0), oral fed with 0.2 mg of crude mite extract (Mite 0.2), and oral fed with 1.0 mg of crude mite extract (Mite 1.0 group). Each group consisted of 3- 5 animals.

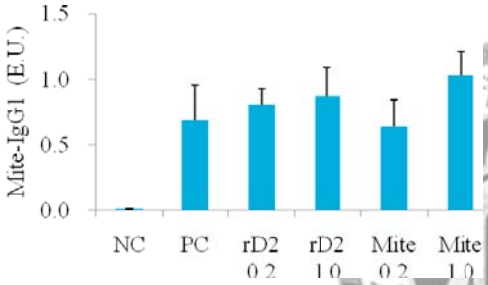
(a) Total IgE



(b) Mite-specific IgE



(c) Mite-specific IgG1



(d) Mite-specific IgG2a

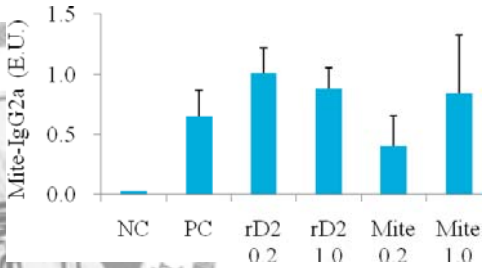


Figure 13. Oral feeding showed no effects on the levels of immunoglobulin in serum

The levels of total IgE (a), mite-specific IgE (b), mite-specific IgG1 (c), and mite-specific IgG2a (d) in serum were detected by ELISA. Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means. (NC, negative control group; PC, positive control group)

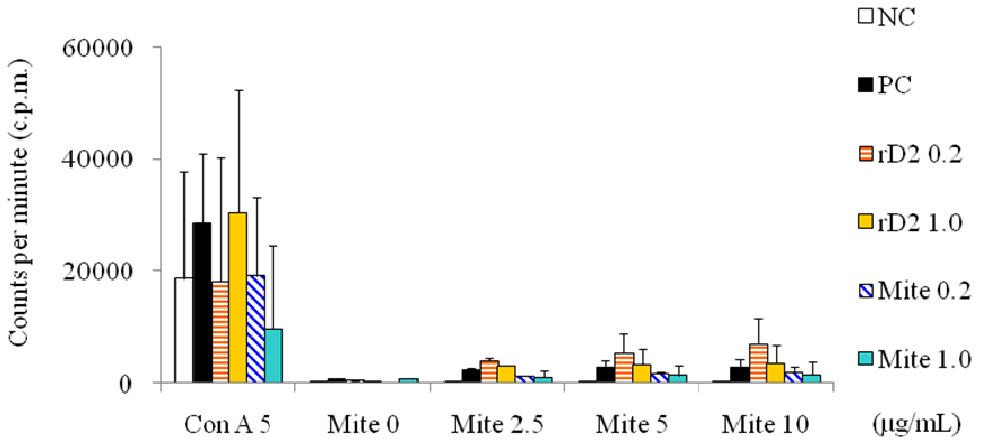
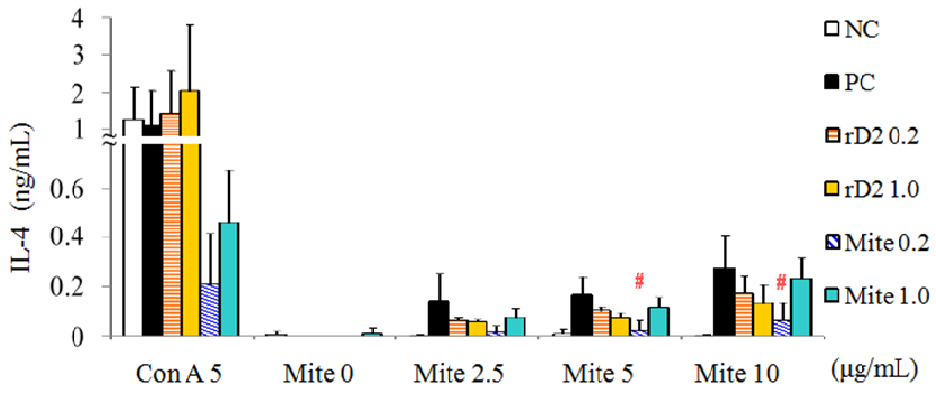


Figure 14. The lymphoproliferation were no affected by oral feeding with mite allergens

Splenocytes were cultured with Con A (5 μg/mL) or crude mite extract (0, 2.5, 5, 10 μg/mL) for 48 hour. The lymphoproliferation was determined by ^{3}H incorporation assay. The results were presented in counts per minute (c.p.m. = cpm_{sample}-$cpm_{medium\ alone}$). Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means and # presents $P < 0.05$ when compared to the positive control group with statistical difference.

(a) IL-4 levels under stimulation



(b) IL-5 levels under stimulation

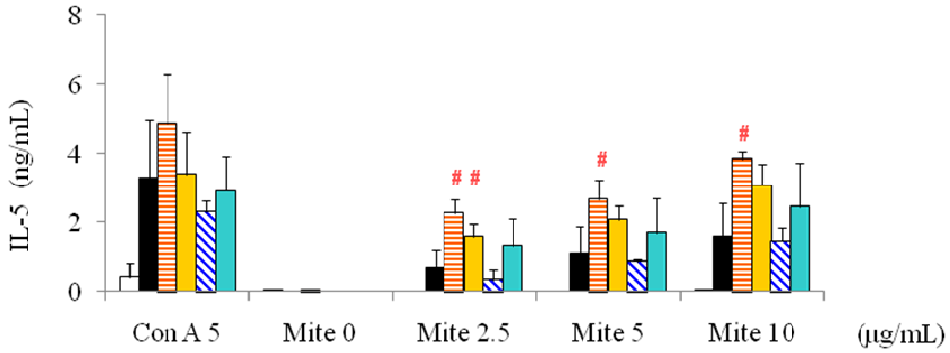
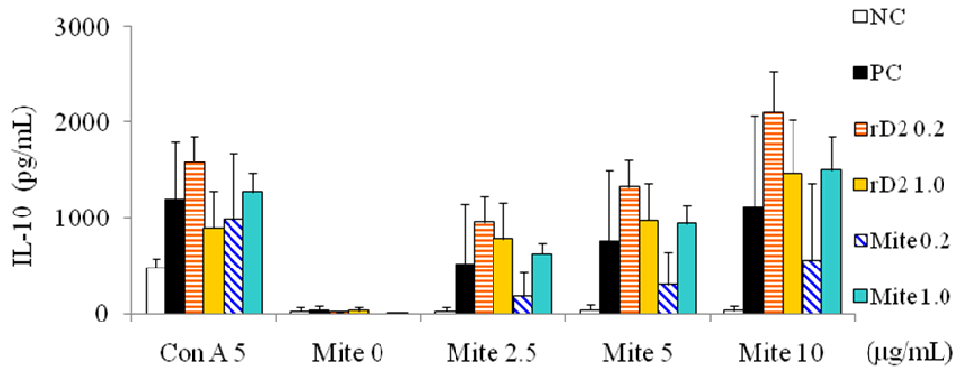


Figure 15. IL-4 productions of splenocytes decreased after oral feeding

Splenocytes were cultured with Con A (5 μg/mL) or crude mite extract (0, 2.5, 5, 10 μg/mL) for 48 hours. The levels of IL-4 (a) and IL-5 (b) in the supernatant were determined by ELISA. Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means.

(a) IL-10 levels under stimulation



(b) TGF-β levels under stimulation

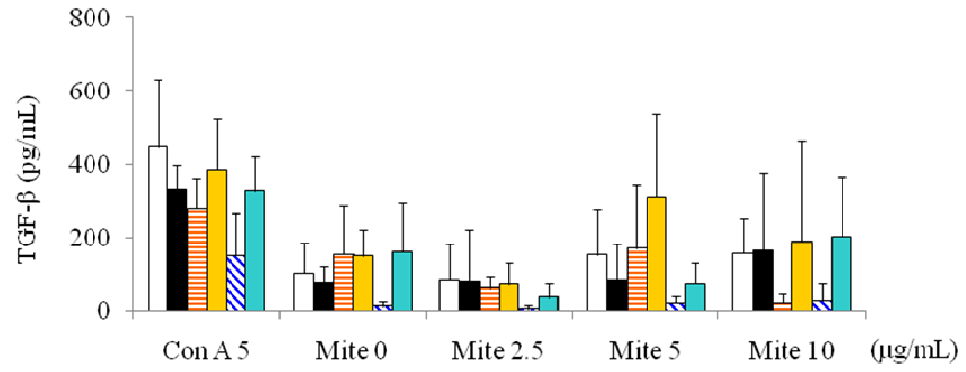


Figure 16. Oral feeding with mite allergens slightly elevated IL-10 levels of splenocytes

The levels of IL-10 (a) and TGF-β (b) in the splenocytes cultured supernatant were determined by ELISA. Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means.

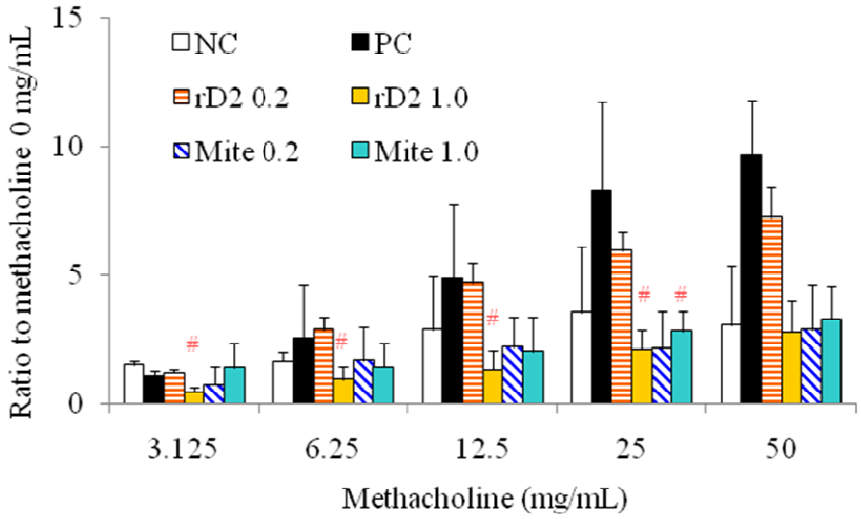
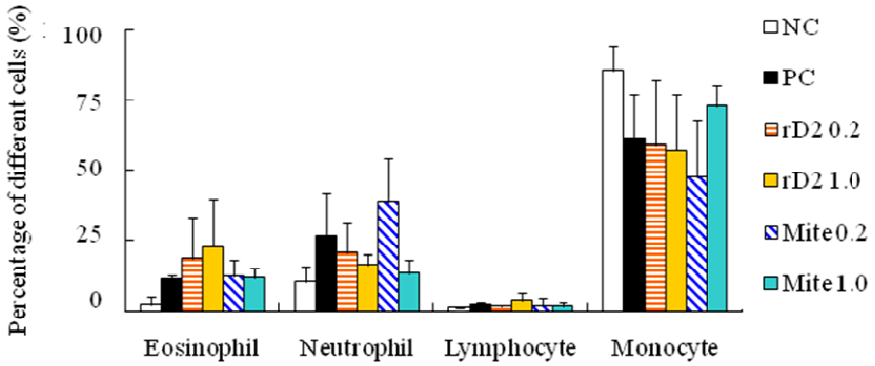


Figure 17. Oral feeding with higher dose of mite allergens reduced the AHR

The pulmonary functions of mice were measured on the day 42. The bars represent averages of the ratio of the Penh value under respective concentrations of methacholine to that under methacholine 0 mg/mL treatment. Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means and # presents $P < 0.05$ when compared to the positive control group with statistical difference.

(a) Percentage of infiltrated cells in BALF



(b) Number of infiltrated cells in BALF

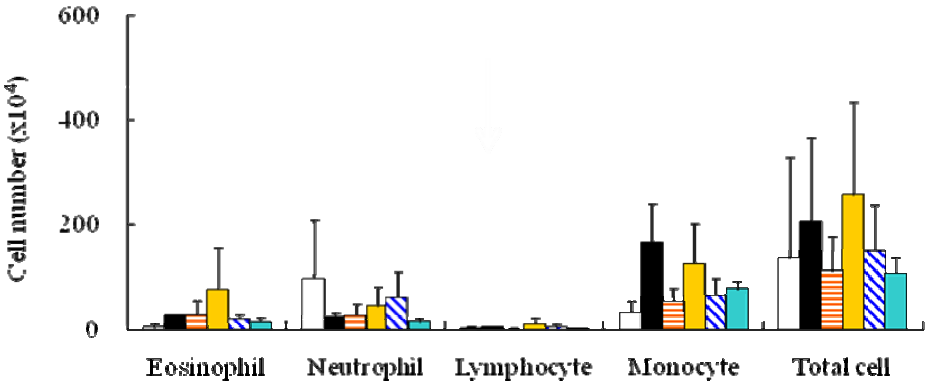
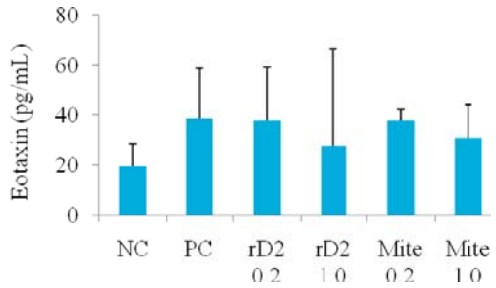


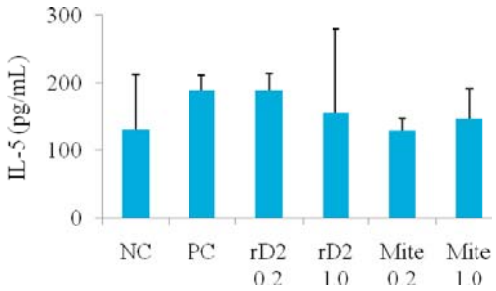
Figure 18. The infiltrated neutrophils percentage in BALF were slightly reduced after oral feeding

The infiltrated cells were classified and shown in percentages (a) and in number (b). Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means.

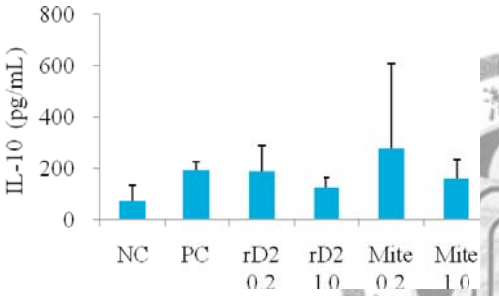
(a) Eotaxin levels in BALF



(b) IL-5 levels in BALF



(c) IL-10 levels in BLAF



(d) IL-13 levels in BLAF

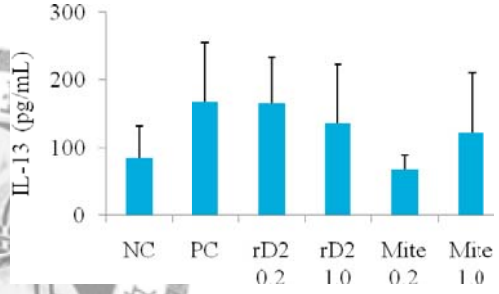


Figure 19. The inflammatory cytokine levels in BALF slightly decreased after oral feeding

The levels of eotaxin (a), IL-5 (b), IL-10 (c), and IL-13 (d) in BALF were determined by ELISA. Statistical analysis was determined by Students' *t*-test. Error bars represent the standard deviation of the means.

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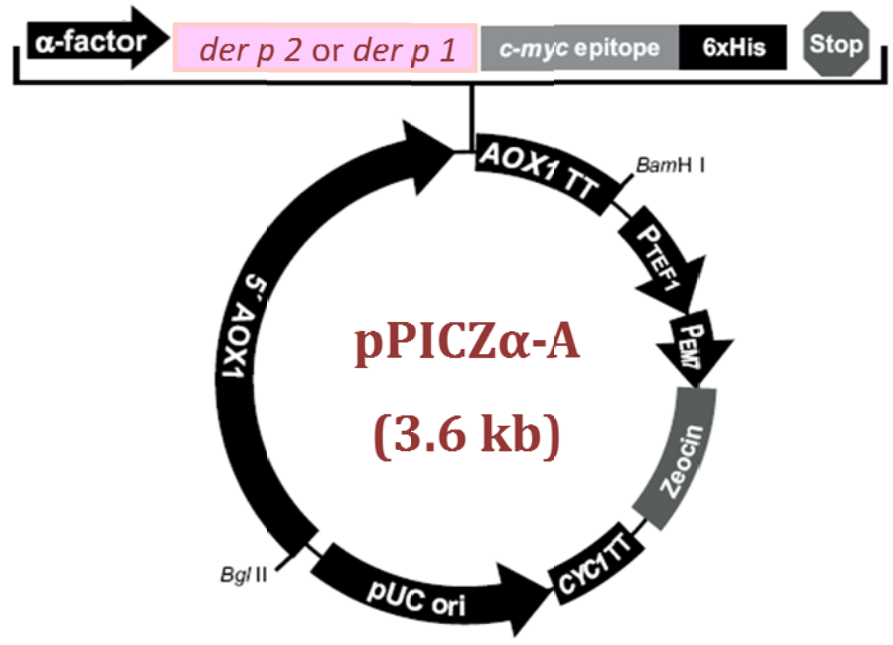
游詩怡 (2007). 探討過敏性氣喘小鼠引發口服耐受性之相關基因.

APPENDIX

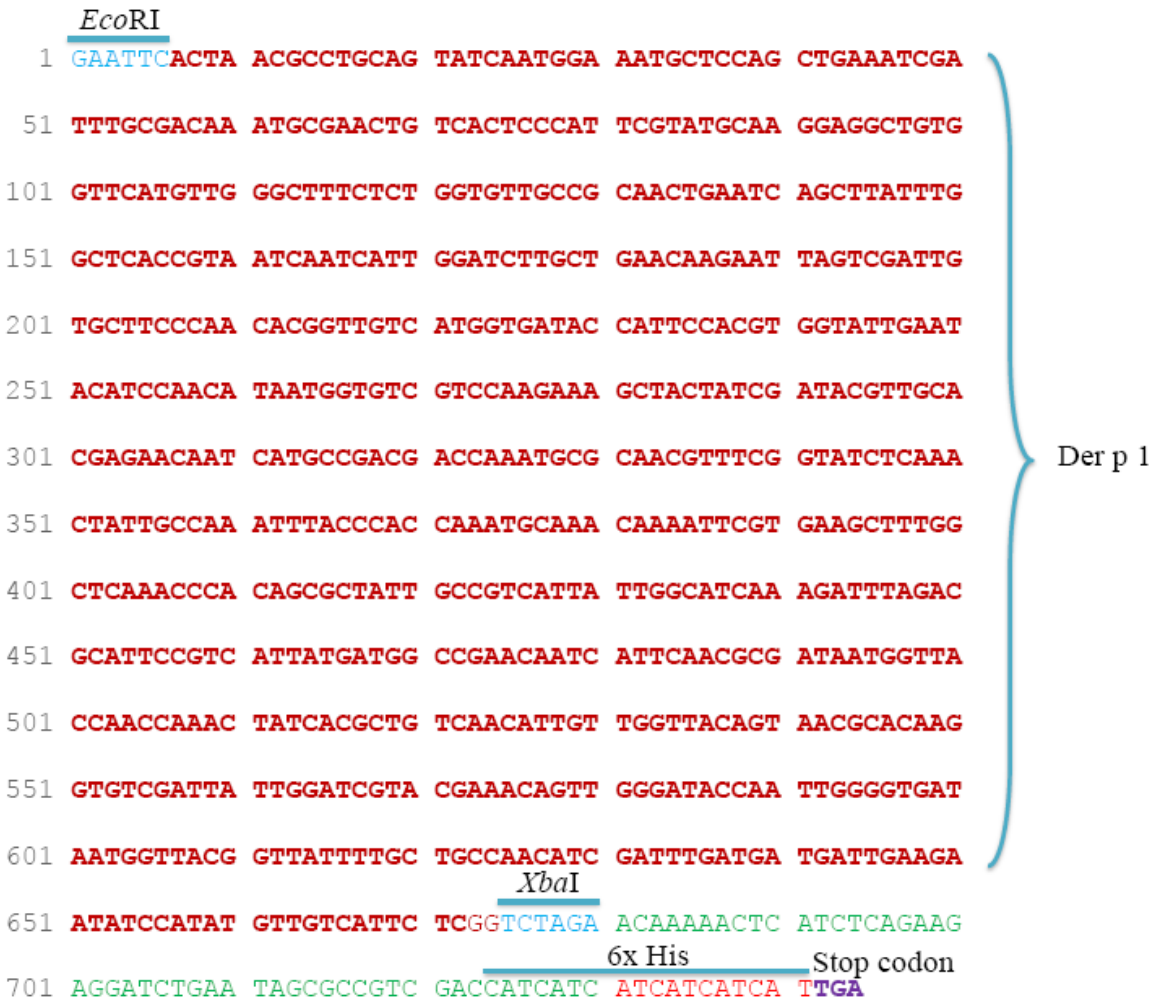
Appendix 1. Schematic representation of vector maps of rDp1 or rDp2

This is the figure and the complete sequence of pPICZα-A is available for downloading from Invitrogen company's World Wide Web site (www.invitrogen.com).



Appendix 2. Sequence of pPICZα-A-*der p 1*

The sequencing result of pPICZα-A-*der p 1*, which was kindly provided by Dr. KT Lee (Institute of Microbiology and Biochemistry, National Taiwan University).



Appendix 3. Reagents

Phosphate Buffer Solution, PBS

20.2 mM Na_2HPO_4 (Bionavas, USA), 137 mM NaCl (Ameresco, USA), 1.5 mM KH_2PO_4 (Wako, Japan), and 2.7 mM KCl (Merck, Germany) in ddH_2O

Tris-Buffered Saline, TBS

50 mM Trizma® base (Sigma, USA) and 154 mM NaCl (Ameresco, USA) in ddH_2O

Yeast extract peptone dextrose medium, YPD

1% Yeast extract (Merck, Germany), 2% Peptone-A (Bio Basic, Canada) 2% D(+)-Glucose (dextrose, Riedel-de-Haën, Germany), and 100 μg/mL Zeocin in ddH_2O

Yeast extract peptone dextrose plate, YPDSZ plate

1% Yeast extract (Merck, Germany), 2% Peptone-A (Bio Basic, Canada), 2% D(+)-Glucose (dextrose, Riedel-de-Haën, Germany), 2% Agar-agar (Merck, Germany), 1M Sorbitol (Bionavas), and 100 μg/mL Zeocin in ddH_2O

Minimal dextrose plates, MD plates

1.34% Yeast nitrogen base (Invitrogen, USA), 4× 10^{-5}% Biotin (Sigma, USA), 2% D(+)-Glucose (dextrose, Riedel-de-Haën, Germany), 1.5% Agar-agar (Merck, Germany), and 100 μg/mL Zeocin in ddH_2O

Minimal methanol plates, MM plates

1.34% Yeast nitrogen base (Invitrogen, USA), 4× 10^{-5}% Biotin (Sigma, USA), 0.5% Methanol (Mallinckrodt, USA), 1.5% Agar-agar (Merck, Germany), and 100 μg/mL Zeocin in ddH_2O

Buffered minimal glycerol, BMG

100 mM Potassium phosphate, pH 6.0 (Wako, Japan), 1.34% yeast nitrogen base (Invitrogen, USA), 4× 10^{-5}% Biotin (Sigma, USA), and 1% Glycerol (Nihon Shiyaku Reagent, Japan) in ddH_2O

Buffered minimal methanol, BMM

100 mM Potassium phosphate, pH 6.0 (Wako, Japan), 1.34% Yeast nitrogen base (Invitrogen, USA), 4× 10^{-5}% Biotin (Sigma, USA), and 0.5% Methanol

(Mallinckrodt, USA) in ddH_2O

Primers for colony PCR

*Eco*RI-*der p 1*, 5'-GAA TTC ACT AAC GCC TGC AGT ATC -3'; *der p 1-Xba*I, 5' –TCT AGA CCG AGA ATG ACA ACA TAT GGA-3'.

SDS-acryamide gel for Western blot

(mL)	15% running gel	5% stacking gel
ddH_2O	4.745	6.150
1.5 M Tris, pH 8.8	3.250	
0.5 M Tris, pH 6.8		2.500
10% SDS	0.130	0.100
40% Acryamide	4.875	1.250
10% APS*	0.975	0.075
TEMED*	0.013	0.010

* Added just before the gel loading