

Intranasal, Liposome-Adjuvanted Cockroach Allergy Vaccines Made of Refined Major Allergen and Whole-Body Extract of *Periplaneta americana*

Panissara Meechan^a Anchalee Tungtrongchitr^b Urai Chaisri^d
Khuanwalai Maklon^b Nitaya Indrawattana^e Wanpen Chaicumpa^b
Nitit Sookrung^c

^aGraduate Program in Immunology, Department of Immunology, ^bDepartment of Parasitology,

^cDepartment of Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University,

^dDepartment of Tropical Pathology, ^eDepartment of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Key Words

Arginine kinase · Cockroach allergy vaccines · Cytokines ·
Per a 9 · *Periplaneta americana*

Abstract

Background: Cockroach (CR) allergens frequently cause severe asthma in CR-sensitized subjects. Allergen-specific immunotherapy causes a shift of allergic Th2 responses towards Th1 and/or regulatory T cell (Treg) responses which reduce airway inflammation and prevent disease progression. Data are relatively limited on immunotherapy via CR allergy vaccine. **Methods:** The therapeutic efficacy of an intranasal liposome-adjuvant vaccine made of a refined *Periplaneta americana* arginine kinase (AK) was compared to the liposome-entrapped *P. americana* crude extract (CRE) vaccine. Adult BALB/c mice were rendered allergic to CRE. Three allergic mouse groups were immunized intranasally on alternate days with 8 doses of liposome-entrapped CRE (L-CRE), liposome-entrapped AK and placebo, respectively. One week later, all mice received a nebulized CRE provocation. Evaluation of vaccine efficacy was performed 1 day after provocation. **Results:** Liposome-entrapped native AK atten-

uated airway inflammation after the CRE provocation and caused a shift of allergic Th2 to Th1 and Treg responses. The L-CRE also induced a shift from the Th2 to the Th1 response but did not induce a Treg response and could not attenuate the airway inflammation upon allergen reexposure. **Conclusions:** Intranasal liposome-adjuvant CR allergy vaccine containing native AK (Per a 9) is better than L-CRE in attenuating allergic airway inflammation. The findings of this study not only document a more comprehensive and beneficial immune response induced by the refined allergen vaccine but also raise the point that the shift from the Th2 to the Th1 response alone might not correlate with improved airway histopathology, clinical outcome and quality of life.

Copyright © 2013 S. Karger AG, Basel

Introduction

Cockroach (CR) proteins are important indoor allergens worldwide, especially among inhabitants of crowded inner cities [1, 2]. Exposure to CR allergens leads to sensitization, particularly in atopic subjects. The sensitized subjects develop predominantly allergen-specific Th0/

Th2 responses, including antigen-specific Th2 proliferation, production of Th2 cytokines, antigen-specific IgE, IgE-sensitized mast cells and basophils, mast cell maturation and inflammatory cell infiltration into the respiratory tissue [3, 4]. Allergic subjects also have a reduced Th1 response manifested by a decrease in interferon (IFN)- γ production [5]. Varying degrees of allergic morbidity, particularly allergic rhinitis and asthma, occur in sensitized subjects upon reexposure to the allergens, which accentuate airway inflammation by cross-linking the specific IgE on the cell surface [6] and also by proteases contained in the allergenic mixture [7]. Asthmatic subjects usually have airway hyperresponsiveness (AHR) caused by chronic airway inflammation which is characterized by inflammatory cell and T lymphocyte infiltration into the respiratory tissues and tissue remodeling, i.e. epithelial cell hyperplasia, hypersecretion of mucus by goblet cells, fibrosis of subepithelial basement membrane and smooth muscle degeneration and thickening [8]. CR allergen-mediated atopic asthma is usually more prolonged and severe than that produced by other indoor allergens such as house dust mites and pets [1, 9]. Asthmatic attacks have a serious impact on quality of life and may even be fatal due to respiratory insufficiency, suffocation and brain anoxia if not treated promptly and properly, especially in young children.

Currently, antigen-specific immunotherapy is the only measure that can attenuate the allergen-induced pathogenic Th2 response, thereby ameliorating the symptom severity, inhibiting disease progression, reducing drug use and increasing the patients' quality of life. An allergen to which the patient is sensitive is given in increasing quantities either parenterally (subcutaneously) or mucosally (sublingually or intranasally) over an extended period of time [10, 11]. The mechanisms and outcomes of individual therapeutic allergy vaccines vary depending on the allergens used and the route of vaccination. Results from previous studies indicate that allergy vaccines act as an immune modifier to induce a deviation from the pathogenic Th0/Th2 response of the patient towards the nonpathogenic Th0/Th1 response or to cause T cell anergy via a regulatory T cell (Treg) response, or both [12, 13]. The allergen-specific Th1 immune response protects against the development of allergic manifestations by inducing the production of interleukin (IL)-12/IL-18, which alleviate the allergic lung inflammation through induction of IFN- γ upregulation (as well as pulmonary IL-10), thus inhibiting the development of Th2 cells [14, 15]. The Treg cytokines IL-10 and transforming growth factor (TGF)- β suppress IgE production

and induce isotype switching of the allergen-specific B cells to favor IgG4 and IgA antibody production, respectively [16]. Moreover, IL-10 and TGF- β directly or indirectly suppress effector cells which initiate allergic inflammation (e.g. mast cells, eosinophils and neutrophils) [17], thereby preventing the release of cellular mediators and late-phase inflammation. Immunotherapy has been shown to be effective for allergies caused by *Hymenoptera* [18, 19], some pollens [20], grasses [21], trees [22] and house dust mites [23]. However, data in the literature are relatively scarce on vaccine efficacy for allergic morbidity, especially asthma, caused by the American CR (ACR; *Periplaneta americana*), which is the predominant CR species in Thailand and many other tropical countries.

In this study, intranasal CR allergy vaccine made from refined ACR allergen, i.e. native Per a 9 (arginine kinase, AK), entrapped in liposome was tested for its therapeutic efficacy in comparison with the liposome-entrapped whole-body crude extract of the ACR (CRE) vaccine and liposome-entrapped PBS (placebo). The intranasal route was chosen, not only because this route is painless, but also because evidence indicates that immune responses induced in the nasal-associated lymphoid tissue commonly act at the lower respiratory effector sites including the larynx-associated lymphoid tissue and the bronchus-associated lymphoid tissue. The vaccine components applied intranasally may reach the macrophages in the trachea and lung; the antigen-activated macrophages mature during their drainage to lymph nodes and stimulate the local immune response [24, 25]. For CR allergy, the most serious clinical manifestation is asthma due to lower airway inflammation and functional abnormality. Thus, the local Th1 cell-mediated effector responses in the lower respiratory lymphoid tissues should reach the target site and exert the expected therapeutic activity, i.e. attenuation of the Th2-mediated airway inflammation. Per a 9 (AK) was chosen as a vaccine component because this CR protein is a major allergen that reacted to serum IgE of 100% CR-allergic Thai patients tested [26]. Multilamellar liposome was used as the vaccine delivery vehicle in this study because, not only is it easy to prepare, but it has also been shown over more than two decades to be a safe and promising immunological adjuvant in provoking humoral and/or cell-mediated immune responses to a variety of entrapped antigens [27–29]. To our knowledge, this is the first report on the efficacy of a mucosally delivered, liposome-entrapped, refined *P. americana* allergen vaccine for treatment of CR allergy.

Materials and Methods

Vaccine Components

Whole-body extracts of adult ACR (CRE) were prepared as described previously [30]. The protein content of the CRE was quantified using Bradford reagents. Native Per a 9 (AK) was prepared from the *P. americana* extract (CRE) as described previously by using Per a 9 specific monoclonal antibody (Mab38G6)-based affinity resin [26, 30]. The average content of AK in CRE preparations was 0.28%. The concentrations of endotoxin in the CRE and native AK preparations were measured using a test kit (Biolasco, Taiwan).

CRE and Sham Mice Sensitization and Specimen Collection

The animal experiments were approved by the Animal Ethics Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (FTM-ACUC 012/2009). Male BALB/c mice, 6–8 weeks old, were sourced from the National Laboratory Animal Center, Mahidol University. The mice were sensitized by intraperitoneal injection with 3 doses of 150 µg of CRE (containing 0.42 µg of AK) in normal saline solution (NSS) mixed 2:1 v/v with alum (Thermo Scientific, USA) on days 0, 7 and 14. On day 21, each mouse was challenged intranasally with 100 µg of CRE in 10 µl of NSS (5 µl into each nostril). On days 23, 25 and 27, all mice were nebulized with 10 ml of aerosolized PBS containing 10 mg of CRE. Sham mice were injected intraperitoneally with NSS mixed with alum, challenged intranasally with 10 µl (5 µl per nostril) of PBS and nebulized with 10 ml of PBS using the same timeline as for the allergenized mice. One day after nebulization (day 28), all allergenized and sham mice were bled, and their sera were collected individually for determination of CRE-specific serum IgE and IgG1 levels by indirect ELISA. After bleeding, 7 allergenized, 4 sham and 4 normal mice were sacrificed. The right bronchus of each mouse was ligated, and bronchoalveolar lavage fluid (BALF) was harvested from the left side of the respiratory tract using a needle connected to a syringe containing 500 µl of sterile PBS. The BALF of each mouse (200 µl) was cytospun and Giemsa stained for inflammatory cell enumeration. The diaphragmatic lobe of the right lung was preserved with 5% paraformaldehyde and 4% sucrose in PBS for 24 h. The fixed lung tissue was embedded in paraffin, and 5-µm sections were prepared. Each section was stained with hematoxylin and eosin dye and examined under a light microscope for the histological features of the bronchioles and lung tissue. The apical, azygous and cardiac lobes of the lungs were preserved in an RNA stabilization reagent (RNA later™, Qiagen GmbH, Germany) at –80°C, and the total RNA was isolated to be used as a template in the cytokine study by quantitative real-time PCR (qPCR). The experimental timeline for mouse sensitization, challenge and allergy model analysis is shown in online supplementary figure S1a (for all online suppl. material, see www.karger.com/doi/10.1159/000348314).

Vaccine Delivery Vehicle (Liposome)

Multilamellar liposomes were prepared from Lipoid-S-100 phosphatidylcholine derived from fat-free soybean lecithin (a kind gift from Lipoid AG, Switzerland) and cholesterol (Sigma-Aldrich, Germany) using didecyldioctadecylammonium bromide (DDAB; Fluka, Germany) as a cationic surfactant. To prepare a lipid stock solution, DDAB (153 mg), phosphatidylcholine (148 mg) and cholesterol (72.5 mg) were mixed (molar ratio 2:

1:1) [31] using 25 ml of dichloromethane as a solvent. One milliliter of the lipid stock (30 µM) was rotated in a round-bottom flask until a thin film of dried lipids was obtained. The internal surface of the flask was purged with nitrogen gas to remove residual moisture.

Formulation of Vaccines and Placebos

Two vaccine formulations were prepared, i.e. liposome-entrapped CRE (L-CRE) and liposome-entrapped AK (L-AK). Liposome-entrapped PBS (L-P) served as a placebo. For the L-CRE vaccine, 3.75 mg of CRE in 500 µl of PBS were added to the lipid gel film prepared from 1 ml of the lipid stock and mixed manually until the gel film collapsed and a homogeneous suspension was obtained. For the L-AK vaccine, 125 µg of purified AK in 500 µl of PBS were added to a gel film prepared from 1 ml of lipid stock. For the L-P placebo, 500 µl of PBS were mixed with the lipid gel. The zeta potentials, diameters and size distributions of liposome preparations were analyzed by dynamic light scattering using a Zetasizer (Nano-ZS, Malvern Instruments, UK) at 25°C. For the measurements, the samples were diluted in the appropriate volume of double-distilled water. Data analysis was performed in automatic mode. The particle size distribution (polydispersity index) of each liposome preparation was reported as the mean ± standard deviation (SD). Triplicate measurements were performed for each parameter of each preparation. The percentage of antigen entrapment in the liposome was determined. For this, each liposome formulation was centrifuged at 12,000 g, 4°C, for 20 min, and the protein in the supernatant (nonentrapped protein) was determined (Bradford reagent). The percentage of antigen entrapment was calculated from the original antigen added to the lipid gel.

Immunization and Provocation of the CRE-Allergic Mice

Two weeks after the CRE nebulization (day 41), the remaining CR-allergic mice were divided into 3 groups of 8–10 mice each. The mice in groups 1 and 2 were vaccinated with L-CRE and L-AK, respectively; the mice in group 3 were given placebo vaccinations. The immunization procedure described previously [32] for local nasal immunotherapy of house dust mite allergy was followed. The allergic mice of groups 1 and 2 were vaccinated with 20 µl of 150 µg of L-CRE and 5 µg of L-AK (10 µl to each nostril), respectively, while the allergic mice in group 3 received L-P intranasally. All primed mice were given 7 booster doses of the respective vaccines/placebo (same dose and route) on alternate days. Sham mice were given 8 doses of PBS intranasally instead of the vaccines/placebo. One week after the last vaccine/placebo booster (day 62), the mice in groups 1–3 were provoked by aerosol challenge with 10 mg of CRE in 10 ml of PBS. The sham mice were nebulized with 10 ml of PBS.

Vaccine Efficacy Evaluation

Evaluation of vaccine efficacy was performed on day 63 or 1 day postprovocation (PP). All mice were bled, and their sera were collected for quantification of CRE-specific IgE and IgG1. Thereafter, mice from each group were sacrificed. Their BALF samples were collected individually for inflammatory cell enumeration. Their lung tissues were processed for histopathological and cytokine gene expression studies. The timeline for mouse immunization, provocation and vaccine efficacy evaluation is shown in online supplementary figure S1b.

Indirect ELISA

For determination of CRE-specific IgE and IgG1 in sera of normal, sham and allergic mice (before and after vaccination and provocation), CRE (20 µg/ml carbonate-bicarbonate buffer, pH 9.6) was added (100 µl) to each well of an ELISA plate, and the plate was incubated at 37°C until dried. All wells were washed with PBS containing 0.05% Tween-20 (PBST), blocked with 200 µl of 1% BSA in PBS (blocking solution) for 1 h and washed again. Triplicate CRE-coated wells were filled with individual mouse sera (diluted 1:4 for IgE and 1:1,000 for IgG1), and the plate was kept at 37°C for 1 h and at 4°C overnight. Wells filled with only serum diluent (0.2% BSA and 0.2% gelatin in PBS) served as blanks. All wells were washed, and 100 µl of rat antimouse IgE-biotin (diluted 1:3,000 in PBST) were added for specific IgE determination. For IgG1 determination, 100 µl of goat antimouse IgG1-biotin (diluted 1:10,000) were added. The plate was incubated at 37°C for 1 h, then washed with PBST, and each well was filled with 100 µl of streptavidin-horseradish peroxidase conjugate (Southern Biotech; diluted 1:8,000 in serum diluent). After further incubation at 37°C for 40 min, the wells were washed, freshly prepared 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) substrate (KPL, USA) was added and the plate was kept in the dark at 25°C for 20 min for IgE and 5 min for IgG1. The color reaction was stopped by adding 50 µl of 1% SDS. The optical density at 405 nm ($OD_{405\text{ nm}}$) of the content of each well was determined against the blanks. Data are presented as means $\pm$ SD of $OD_{405\text{ nm}}$ of sera of mice of the same treatment group.

Lung Histopathology

For each mouse, 6–9 hematoxylin and eosin-stained lung sections covering 20–77 microscopic fields were studied under a light microscope (magnification $\times 400$) by a pathologist who was blinded to the mouse treatment groups. The degree of histopathological change in the bronchi and lungs was evaluated using a scoring system [33, 34] with modifications. The number of inflammatory cells which infiltrated into peribronchiolar areas and the morphology of the bronchiolar epithelial and smooth muscle cells were graded arbitrarily using a scale from 0 to 4 (online suppl. table S1).

Cytokine Study

The cytokine gene expression in the lung tissue of each mouse was studied by qPCR [4]. After treating the total RNA with RNase-free DNase 1 (Invitrogen), cDNA was synthesized using RevertAid H minus (Fermentas, Lithuania). The cDNA was used as a template for the quantification of cytokine mRNA which included IL-4, IL-5, IL-10, IL-13, tumor necrosis factor (TNF)- α , IFN- γ , IL-12 and IL-18. The nucleotide primers for amplification of individual genes are listed in online supplementary table S2 [35–37]. The qPCR was performed on 1 µl of cDNA and 300 nM of each PCR primer in SYBR Green PCR Master Mix (Applied Biosystems, USA). Reactions were run using a StepOne™ Real-Time PCR System for 40 cycles. StepOne™ software version 2.1 was used for data analysis. The expression of each target gene was normalized to the housekeeping β -actin gene.

Statistical Methods

SPSS 11.5 was used as statistical software. The data were analyzed using one-way ANOVA followed by post hoc comparison using the least significant difference and the independent t test for BALF, histopathological and cytokine data. The paired t test and

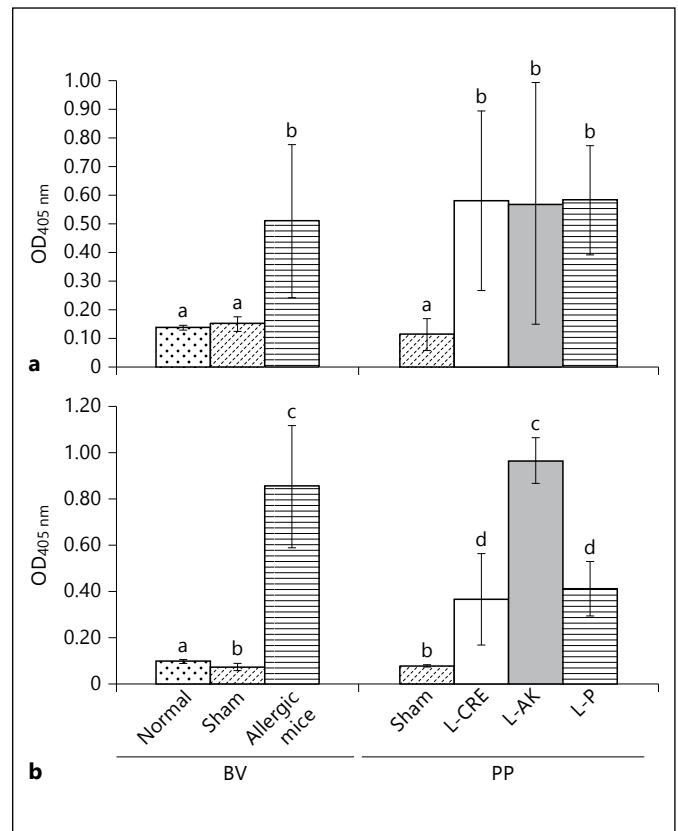


Fig. 1. Mean indirect ELISA $OD_{405\text{ nm}}$ values of serum CRE-specific IgE (a) and IgG1 (b) of mice. Bars with different letters are statistically different.

the Mann-Whitney U test were used for analysis of the IgE and IgG1 levels. Data are expressed as means $\pm$ SD. $p < 0.05$ was considered statistically significant.

Results

Serum Specific IgE and IgG1

The means $\pm$ SD of the indirect ELISA $OD_{405\text{ nm}}$ values of CRE-specific IgE in the sera of normal and sham mice were 0.139 ± 0.008 and 0.152 ± 0.026 , respectively. The allergenized mice had significant rises in serum specific IgE; the mean $\pm$ SD was 0.509 ± 0.266 , significantly higher than those of the sham and normal mice [$p < 0.001$, before receiving vaccines/placebo (BV); fig. 1a]. Based on the CRE-specific IgE data, all allergenized mice were allergic to CRE.

The means $\pm$ SD of the indirect ELISA $OD_{405\text{ nm}}$ values of CRE-specific IgG1 in the sera of normal and sham mice were 0.100 ± 0.008 and 0.071 ± 0.014 , respectively. The

allergenized mice had significant rises in serum specific IgG1; the mean $\pm$ SD of OD_{405 nm} was 0.853 ± 0.263 , significantly higher than those of the sham and normal mice ($p < 0.001$, BV; fig. 1b). The ratio of mean IgE to mean IgG1 of the allergic mice was 0.60.

Inflammatory Cells in BALF of Allergic Mice

Examples of cells in the BALF of normal, sham and allergic mice are shown in online supplementary figure S2. There were only sloughed off epithelial cells in the BALF of normal mice and few inflammatory cells in the BALF of sham mice. On the other hand, the BALF of allergic mice showed pronounced inflammatory cell infiltration. They were predominantly neutrophils (100 ± 38 cells/microscopic field), followed by eosinophils (19 ± 7 cells/microscopic field).

Lung Histopathology of Allergic Mice

Histopathologic features of the mouse lungs of the arbitrary grades 0–4 are shown in online supplementary figure S3. The means $\pm$ SD of the arbitrary grades of lung histopathology in normal, sham and allergic mice are shown in figure 2 (BV). Normal mice had negligible histopathological lesions (the arbitrary grade was 0.147 ± 0.038), while sham mice had some inflammatory and histopathological features in their lungs with an average grade of 0.745 ± 0.146 , which was significantly higher than the normal mice. The average histopathological grade of CRE-allergic mice (2.199 ± 1.012) was significantly higher than those of the sham and normal mice ($p < 0.01$). Inflammatory cells, including neutrophils, eosinophils and lymphocytes, were abundant in the peribronchiolar areas of the allergic mice.

Cytokines in the Lungs of Allergic Mice

The results of qPCR for cytokine gene expression in the lungs of normal, allergic and sham mice are shown in figure 3 (BV). Allergic mice showed a significant upregulation of IL-4, IL-5, IL-10, IL-13 and TNF- α , in comparison with the respective gene expressions in normal and sham mice. The levels of IFN- γ and IL-12 mRNAs in the allergic and sham mice were not different but were higher than in the normal mice. IL-18 in the sham and allergic mice was much lower than in normal mice.

Characteristics of Liposome and Liposome-Entrapped Vaccines/Placebo

The sizes, zeta potentials, polydispersity and percentage immunogen entrapment of the two vaccine formulations and the placebo are shown in table 1. The liposome

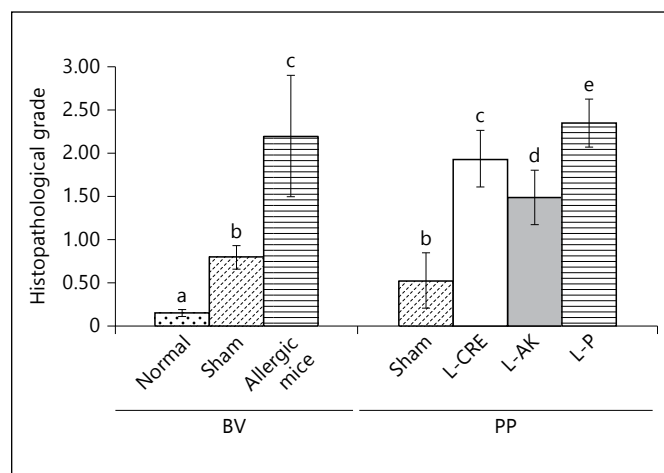


Fig. 2. Mean $\pm$ SD histopathological grades of lung tissues of mice. Bars with different letters are statistically different.

Table 1. Characteristics of liposome-entrapped CR allergy vaccines and placebo

Parameter	Vaccine		Placebo
	L-CRE	L-AK	L-P
Average size, nm	3,060.33 $\pm$ 52.27	1,413.33 $\pm$ 17.04	921.80 $\pm$ 5.01
Polydispersity	0.085 $\pm$ 0.039	0.202 $\pm$ 0.053	0.121 $\pm$ 0.067
Zeta potential	21.77 $\pm$ 0.74	39.07 $\pm$ 0.12	23.36 $\pm$ 1.12
Immunogen entrapment, %	88.54	93.28	ND

Values represent means $\pm$ SD, except where indicated otherwise. ND = No data.

vesicles with CRE, AK and PBS were cathodic, with sizes of $3,060.33 \pm 52.27$, $1,413.33 \pm 17.04$ and 921.81 ± 5.01 nm, respectively, which were statistically different ($p < 0.001$). The polydispersity values of L-CRE, L-AK and L-P were 0.085 ± 0.039 , 0.202 ± 0.053 and 0.121 ± 0.067 , respectively. The polydispersity values of L-CRE and L-AK were different ($p = 0.036$), but there were no differences between L-CRE and L-P ($p = 0.455$) and L-AK and L-P ($p = 0.075$). The immunogen entrapment percentages of L-CRE and L-AK were 88.54 and 98.38%, respectively. Each dose of L-CRE, L-AK and L-P contained 23, 7 and 3 EU of endotoxin, respectively.

Serum Specific IgE and IgG1 after Provocation

The indirect ELISA OD_{405 nm} values of CRE-specific IgE in sera of the sham and allergic mice that received vaccines/placebo and provocation with aerosol CRE/PBS are

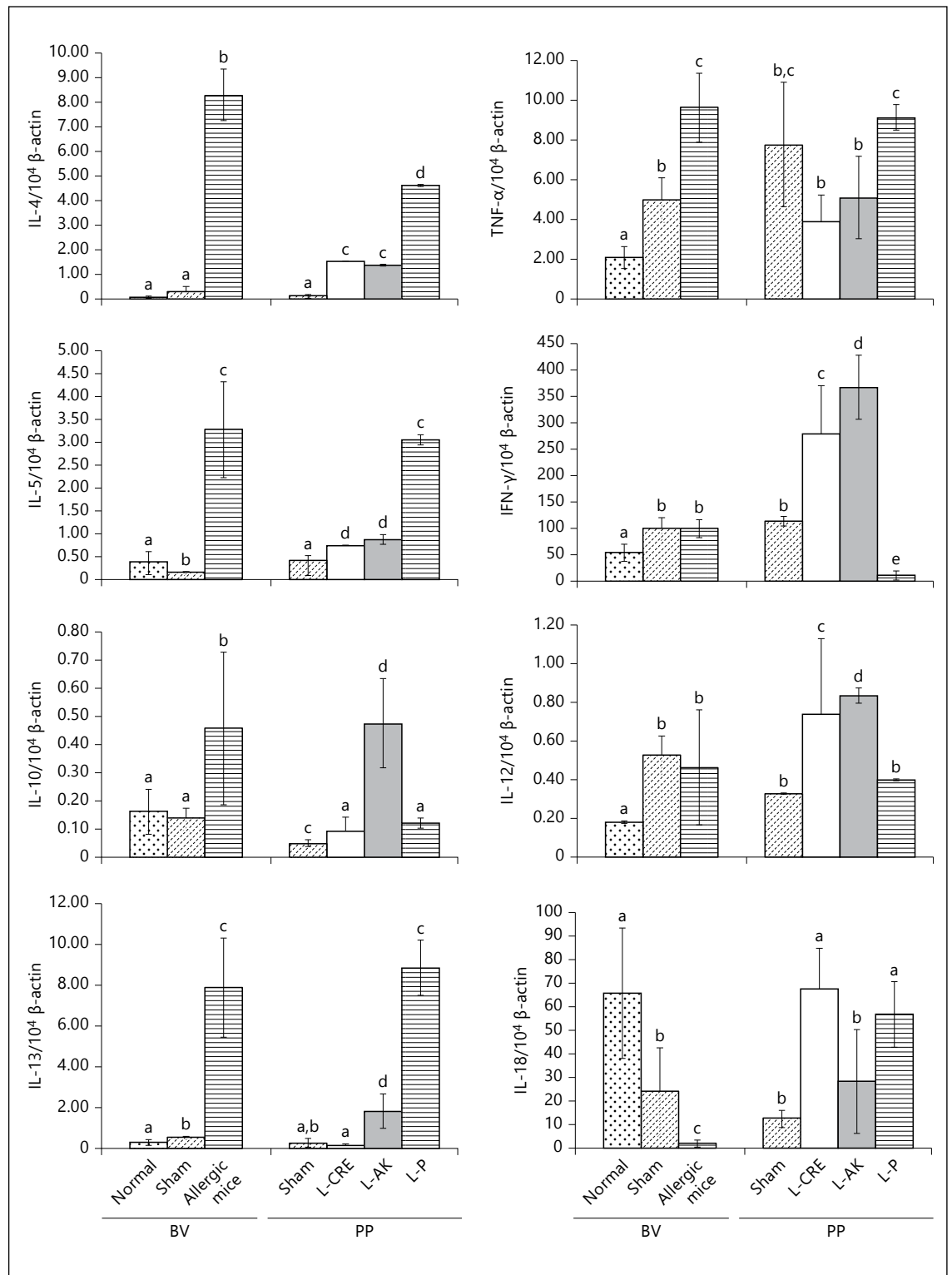


Fig. 3. Arbitrary values of cytokine gene expressions in lung tissues of mice. Bars with different letters are statistically different.

Table 2. Inflammatory cells in BALF of allergic mice at BV and PP compared to sham mice

Group	BV			PP		
	neutrophils	others	total	neutrophils	others	total
L-CRE	100.2±38.1	19.41±7.23 (Eo)	119.78±37.3 ^a	124.07±75.31	17.97±4.45 (Eo), 2.05±1.89 (L)	144.10±80.73 ^c
L-AK	100.2±38.1	19.41±7.23	119.78±37.3 ^a	42.43±24.58	0	42.43±24.58 ^d
L-P	100.2±38.1	19.41±7.23	119.78±37.3 ^a	124.22±31.18	8.25±9.80 (L)	137.22±35.95 ^c
Sham	1.37±0.11	0	1.37±0.11 ^b	0	0	0 ^e

Values represent means ± SD of cell numbers from 15–20 microscopic fields (×400). Numbers of neutrophils/other cells/total inflammatory cells at BV were the same in all vaccinated/placebo groups as these numbers are average numbers of the allergic mice. ‘Others’ includes eosinophils (Eo), lymphocytes (L) and macrophages. Values with different superscript letters are significantly different ($p < 0.05$).

shown in figure 1a. The background OD_{405 nm} of the IgE of the sham mice at PP (0.108 ± 0.051) was not different from BV. Likewise, the OD_{405 nm} values of the CRE-specific IgE of allergic mice that received vaccines/placebo and provocation (0.553 ± 0.298 , 0.543 ± 0.401 and 0.555 ± 0.182 for L-CRE, L-AK and L-P, respectively) were not different from the average BV level (0.509 ± 0.266 ; $p > 0.05$).

There was a significant increase in the mean ± SD of the indirect ELISA OD_{405 nm} of serum CRE-specific IgG1 in L-AK-treated mice at PP (1.072 ± 0.109) compared to the average BV OD_{405 nm} of allergic mice (0.853 ± 0.263 ; $p < 0.05$). In contrast, at PP the specific IgG1 in sera of L-CRE-treated mice (0.407 ± 0.219) and placebo mice (0.457 ± 0.131) was significantly reduced from the average OD_{405 nm} at BV. The ratios of mean IgE to mean IgG1 of the L-CRE, L-AK and L-P mice at PP were 1.359, 0.51 and 1.214, respectively, indicating that only allergic mice treated with L-AK had an improved IgE to IgG1 ratio after the vaccination and provocation.

Inflammatory Cells in BALF after Provocation

The numbers of inflammatory cells in the BALF of sham mice and allergic mice that received vaccines/placebo at BV and PP are shown in table 2. Sham mice had negligible numbers of inflammatory cells in their BALF at all times. After provocation, the number of inflammatory cells in the BALF of the L-AK-treated mice (42.43 ± 24.58 cells/microscopic field) was found to be significantly lower than the average number in allergic mice at BV (119.78 ± 37.30 cells/microscopic field) and the L-P group at PP. The numbers of inflammatory cells (neutrophils/eosinophils and some lymphocytes) at PP in the BALF of the L-CRE (144.10 ± 80.73 cells/microscopic field) and L-P mice (137.22 ± 35.95 cells/microscopic field) were significantly higher than the average number in allergic mice at BV.

Lung Histopathology of Vaccinated/Placebo Mice after Provocation

The lung histopathology grades of sham mice at BV and PP were not statistically different (fig. 2). The mean ± SD histopathological grade for the L-AK-vaccinated mice at PP (1.676 ± 0.868) was significantly reduced from the BV grade (2.199 ± 1.012 ; $p < 0.001$). The lung histopathological grade of the L-CRE-vaccinated group at PP (2.124 ± 0.885) was not different from the BV grade ($p = 0.412$), while the features in the placebo mice were worsened at PP (2.643 ± 0.836) compared to BV ($p < 0.001$). Sham mice had an unchanged histopathology grade (0.798 ± 0.139 at BV vs. 0.594 ± 0.360 at PP).

Expression of Cytokine Genes in Lung Tissues

The arbitrary values of various cytokine gene expressions (mRNA levels) in the lungs of vaccinated, placebo and sham mice are shown in figure 3. At PP, the expression levels of IL-4, IL-5, IL-13 and TNF-α in the L-CRE and L-AK mice were markedly reduced compared to the respective average levels in allergic mice at BV and were significantly lower than the levels in the placebo mice. Nevertheless, at PP the placebo mice had a significant reduction in the expression of the IL-4 gene (fig. 3, bar with superscript b vs. bar with superscript d), indicating a placebo effect of liposome on IL-4 expression. Nevertheless, the IL-4 expression level of the placebo mice was still significantly higher than that of the vaccinated mice ($p < 0.05$). The IL-5, IL-13 and TNF-α expression of the allergic mice that received placebo remained the same as that of allergic mice at BV (fig. 3). Overall results indicated that allergic mice treated with both L-CRE and L-AK vaccines had significantly reduced Th2 cytokine gene expressions after the allergen reexposure.

The IFN- γ expression (fig. 3) in allergic mice that received L-CRE and L-AK was markedly increased at 1 day PP compared to BV, while the allergic mice that received placebo (L-P) and provocation had a much reduced IFN- γ expression compared to BV and the vaccinated mice at PP. The sham mice had the same levels of IFN- γ expression at BV and PP.

At PP, the arbitrary values of the IL-12 mRNA levels of the allergic mice that received L-CRE and L-AK treatment were higher than at BV. The IL-12 expression of the placebo mice and sham mice was not different at BV and PP (fig. 3).

At BV, the allergic and sham mice had significantly lower IL-18 mRNA compared to normal mice. A significantly higher expression of the cytokine gene was found in the L-CRE- and L-AK-vaccinated mice at PP compared to BV ($p < 0.05$). Placebo mice also had a significant rise in IL-18 mRNA at PP. The IL-18 mRNA levels of sham mice at BV and PP were not different (fig. 3).

At PP, the IL-10 mRNA expression in the L-AK-treated group increased markedly, while the cytokine expression in the L-CRE and L-P groups was lower than at BV. The low IL-10 expression of the sham mice at BV and PP did not change (fig. 3).

Discussion

Pharmacologic drugs for allergy temporarily ameliorate or abrogate the clinical symptoms but cannot prevent disease progression and sometimes produce more unwanted side effects than beneficial ones. Thus, an effective immunotherapy in the form of a CR allergy vaccine is needed. In this study, vaccines and placebo were administered intranasally for the reasons stated earlier. Multilamellar liposome was chosen as the immunogen delivery vehicle, not only because it is safe, inexpensive and easy to prepare [38], but also because liposome reduces the toxicity/adverse activity of the entrapped components [39]. The lipid micelles also protect and deliver the cargo immunogens directly to the antigen-presenting cells [40] and serve as immunological adjuvants for Th1- and cell-mediated immune responses [27, 41]. Thus, liposome is well suited as a delivery vehicle (and adjuvant) for allergy vaccines that aim to counterbalance the dominant Th2 response of allergic subjects [42]. However, the surface charges, sizes and lipid composition of the liposome determine the biodistribution, immunogenicity and innocuousness of the vaccine [43]. In this study, Lipoid-S-100 phosphatidylcholine, which

is a saturated and neutral lipid, and cholesterol were used to prepare multilamellar liposomes. Both lipids are normal constituents of the host and therefore biocompatible and safe. The two vaccine formulations (L-CRE and L-AK) and placebo (L-P) did not cause any detectable untoward reactions in the immunized mice. Liposome consisting of phosphatidylcholine has an enhanced binding capacity to human and animal macrophages [44], thus increasing the antigenic peptide presentation. Liposome surface charges influence the interaction of lipid micelles with antigen-presenting cells, particularly dendritic cells (DC). Anionic liposomes interact with a limited fraction of the DC population, while cationic liposomes react with a much larger percentage of DC, and larger amounts of the cargo antigen could be detected intracellularly [45]. In this study, DDAB was successfully used as a cationic surfactant in creating positive surface charges for immunogen/PBS-containing liposome vesicles. Cationic liposomes may deliver the entrapped vaccine component directly into the cytoplasm of the coalescent host cells for cytosolic pathway antigen processing, hence stimulating the cell-mediated immune response. Alternatively, the immunogen-containing lipid vesicles may be phagocytosed, whereupon the antigen is processed in the endosome and the peptides are presented by the MHC class II pathway to T helper cells. The resulting outcome may be either Th1 and/or Th2 responses, depending upon the costimulations and the cytokines present in the microenvironment. As mentioned earlier, liposome is an adjuvant for the Th1 and cell-mediated response; thus, the cationic liposomal CR vaccines in this study should induce and enhance the development of Th1 and cell-mediated immune responses, suppressing the pathological Th2 disorder. Existing data indicate that liposomal vesicles larger than 200 nm are usually directed to early endosomes and induce a Th1 response to the entrapped antigen following parenteral or mucosal administration [46, 47]. Although the liposome vaccines containing crude CR extract (L-CRE) and refined AK (L-AK) and placebo (L-P) had statistically different sizes due to different viscosity of the preparations (L-CRE had the highest viscosity and the largest size, followed by L-AK and L-P, respectively), all of them were larger than 200 nm with low polydispersity; thus they should be processed, largely if not solely, in early endosomes and induce the required Th1 response in a similar manner.

The CRE-allergized mice had the characteristic features of chronic allergic airway inflammation including

inflammatory cell infiltration into BALF and peribronchiolar areas, high levels of serum specific IgE, different degrees of airway tissue remodeling and typical upregulation of Th2 cytokine gene expressions, i.e. IL-4, IL-5, IL-10 and IL-13 as well as TNF- α . IL-10 has pleiotropic activities and may be regarded as either a Th2 or Treg cytokine. Under most circumstances, IL-10 upregulates IL-12 and IFN- γ , which prevent allergy establishment and suppress allergic inflammation [48]. However, IL-10 may enhance allergic inflammatory responses which worsen the allergic responsiveness by inhibiting the production of IFN- γ and IL-12 [49, 50]. In this study, allergic mice had higher IL-10 expression and significantly lower IFN- γ and IL-12 mRNA than sham mice, implying that in these circumstances IL-10 was a Th2 cytokine which promoted the allergic manifestations in the CRE-primed and challenged mice.

TNF- α is a proinflammatory cytokine that is involved in both Th1- and Th2-mediated inflammation [48]. TNF- α contributes to clinical manifestations of allergic rhinitis and atopic asthma. This cytokine is released from mast cells/macrophages by a specific IgE-mediated mechanism and causes inflammatory cell extravasation (by upregulating cellular adhesion molecules on endothelial cells, e.g. E-selectin, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) and migration into the bronchiolar wall which subsequently activates the profibrotic mechanism [48, 49]. Thus, TNF- α is an initiator of both airway inflammation and AHR in atopic asthmatic subjects. In this study, allergenized mice (with high serum IgE) had significant TNF- α mRNA upregulation in comparison to sham and normal mice, likely mediated by the specific IgE. Sham mice also had significant expression of TNF- α but negligible CRE-specific serum IgE, indicating that the TNF- α involvement was mediated by Th1 and caused by the sham manipulation and not by the IgE. The overall features of the CRE-allergenized mice indicated that they were allergic to the ACR allergens and thus suitable for testing the therapeutic efficacies of the allergy vaccines.

Crude extract contains a mixture of allergenic and nonallergenic proteins and other unidentified components. The allergen amounts are variable depending on the sources of the raw material. Several studies have indicated that specific allergens are more clinically relevant than crude preparations in identifying allergic patients and their allergic status [50, 51]. Moreover, purified allergen seems to be better than the crude extract in reducing allergic immune responses [52, 53]. Among

several allergenic proteins derived from *P. americana*, Per a 9 (native AK) has the highest allergenicity as this protein reacted with serum IgE of all CR-allergic patients tested [26]. Thus, it was logical to test the immunotherapeutic efficacy of a vaccine made of AK in comparison to a CRE-containing vaccine for ACR allergy. Previous studies by others have demonstrated that vaccines made of crude extracts at 100–1,000 μ g per dose and purified native allergens at 5–100 μ g per dose could induce therapeutic efficacy [23, 32, 54, 55]. In this study, CRE and AK at 150 and 5 μ g per dose, respectively, were used as 'low-dose vaccines' [55]. Each dose of L-CRE, L-AK and L-P contained 23, 7 and 3 EU of endotoxin, respectively, which are equivalent to 5.4, 1.4 and 0.6 ng. Although the presence of endotoxin in products for injection can result in a pyrogenic response, and the US Food and Drug Administration has set limits on the concentration of endotoxin at 5.0 EU/kg/h in parenteral drugs and 0.2 EU/kg/h for intrathecal drugs, there is no endotoxin limit for the mucosally administered preparation. Normally, respiratory and digestive mucosae are unresponsive to a plethora of inhaled/ingested foreign substances. Indeed, studies on vaccine adjuvants in mice have shown that addition of 10–100 μ g of endotoxin (lipopolysaccharide) to a protein antigen potentiates the production of Th1 differentiation to the protein and production of IFN- γ [56]. Thus, the low levels of endotoxin impurities in the intranasal CR allergy vaccines may foster the immune response towards the required therapeutic efficacy [57].

The levels of allergen-specific IgE in the sera of allergenized mice were higher than in normal and sham mice. The IgE levels of the allergic mice that received vaccination/placebo did not rise any further after provocation, indicating that the level at BV was already maximal and sustained until PP. The L-CRE result of this study, however, was similar to the data reported previously which showed that CRE-allergic mice treated with subcutaneous CRE vaccine did not have a significant reduction of IgE, even 6 weeks after the last vaccine dose [54]. Unfortunately, a longer time period was not studied.

The role of IgG in immediate-type allergy is conflicting. IgG may have anaphylactic properties; however, IgG antibodies may also act as allergen-blocking antibodies and induce an immunotherapeutic effect [58]. In this study, only allergic mice treated with L-AK vaccine had a rise in serum specific IgG1 at PP (fig. 1b); hence, there was a significant reduction of the specific IgE to IgG1 ratio from 0.60 in allergic mice at BV to 0.51 in the L-AK-treated mice at PP. In contrast, allergic mice treated with

L-CRE and L-P had marked reductions of serum specific IgG1 and hence significant increases in the IgE to IgG1 ratio (from 0.60 to 1.359 and 1.214, respectively). Together with the other findings, this indicates that the IgG1 induced by the L-AK vaccination should exert a beneficial rather than a harmful effect on the treated mice.

Allergic mice that received L-AK vaccine had reduced lower airway inflammation (fewer inflammatory cells in BALF and lower lung histopathology grades) after allergen provocation compared to allergic mice that were treated with L-CRE vaccine and placebo. This finding indicates that the vaccine made of the single well-defined major allergen (native AK) was more effective in mitigating/alleviating the airway inflammation than crude extract vaccine. The reduced inflammation mediated by the L-AK should correlate directly with an improved clinical outcome.

Many previous studies have shown that IFN- γ inhibits Th2 cytokine production and suppresses airway inflammation [59]. Allergic mice that received L-CRE and L-AK treatment had a shift from the major Th2 cytokine response towards major Th1 cytokine expression, i.e. increased IFN- γ mRNA, which was consistent with previous findings and confirmed the anti-inflammatory role of the vaccine-induced IFN- γ [60]. Usually, IL-12 and/or IL-18 drive CD4⁺ T cells towards Th1 differentiation and production of IFN- γ . IL-12 has been shown to mitigate airway inflammation, lung histopathology and AHR of allergic mice by inhibiting IL-4 and IL-5 production and eosinophilia through IFN- γ production [61, 62]. Also, IL-12 can mediate suppression of allergic lung inflammation by an IFN- γ -independent mechanism [63]. IL-18 is a pleiotropic cytokine. In the presence of IL-12 it has antiallergic activity [64]. However, IL-18 increased production of IL-4, IL-13 and histamine by basophils [65]. In this study, the IL-12 and IL-18 mRNA levels of allergic mice that received L-CRE and L-AK at PP correlated with the increased IFN- γ mRNA, implying that both cytokines were associated with IFN- γ upregulation. At PP, the placebo mice had a significant upregulation of IL-18 expression but they had decreased IFN- γ expression, which tended to indicate that the IL-18 in the placebo mice was not antiallergic. Besides, the placebo mice had the same IL-12 expression level as the sham mice. The data indicate that the placebo mice did not have a shift from the Th2 to the Th1 response.

One of the expected outcomes of allergen-specific immunotherapy is the generation of adaptive Treg (inducible Treg, iTreg) that produce immunosuppressive cyto-

kines including IL-10 and TGF- β . IL-10 has been shown to reduce proinflammatory cytokine release from Th2 and mast cells, downregulate eosinophil activities and suppress IL-5 production [66]. After provocation, only the allergic mice treated with L-AK had a significant rise in IL-10 mRNA, indicating that this refined allergen vaccine was more effective in inducing the iTreg response than the vaccine made of crude ACR extract. Currently, the mechanisms underlying the induction of iTreg are only partially known [67]. Factors involved in the conversion of conventional CD4⁺CD25^{low} T cells to become antigen-specific CD4⁺CD25^{high} iTreg include the cytokine milieu, the density of T cell receptor, T cell receptor affinity to the antigen presented by antigen-presenting cells, the strength of costimulatory signals, IL-2 receptor, programmed cell death-1 ligand 1, TGF- β receptor and notch [67, 68]. Experiments are needed to elaborate the mechanism and signaling pathway of the iTreg induction by L-AK.

In conclusion, allergic mice that were treated with liposome-entrapped native Per a 9 (AK) via the mucosal route had significantly reduced respiratory tract inflammation (less inflammatory cell infiltration into the BALF and lower lung histopathology grade) after provocation. The L-AK-treated allergic mice also had an immune deviation from the allergic Th2 response to the nonpathogenic Th1 and Treg responses as indicated by the lung cytokine profiles. Allergic mice that received vaccine made of crude extract (L-CRE) also showed a shift from the Th2 to the Th1 response; nevertheless, the vaccine did not induce a significant Treg response and could not attenuate the airway inflammation upon allergen reexposure. Although the mouse ACR allergy model may not be directly transferable to human ACR allergy, the findings in this study raise the point that the shift from the Th2 to the Th1 response might not correlate with improved airway histopathology, clinical outcome and quality of life.

Acknowledgements

We thank Lipoid AG, Switzerland, for the supply of Lipoid phosphatidylcholine. The work was supported by the Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand; the National Research University Project of The Office of Higher Education Commission, the Ministry of Education, Thailand, and the DPG5380001 grant of the Thailand Research Fund. Nitat Sookrung received the 'Chalermprakiat' grant of the Faculty of Medicine, Siriraj Hospital, Mahidol University. Nitat Sookrung and Nitaya Indrawattana are Thailand Research Fund new scholars.

References

- ▶ 1 Rosenstreich DL, Eggleston P, Kattan M, Baker D, Slavin RG, Gergen P, Mitchell H, McNiff-Mortimer K, Lynn H, Ownby D, Malveaux F: The role of cockroach allergy and exposure to cockroach allergens in causing morbidity among inner-city children with asthma. *N Engl J Med* 1997;336:1356–1363.
- ▶ 2 Togias A, Fenton MJ, Gergen PJ, Rotrosen D, Fauci AS: Asthma in the inner city: the perspective of the National Institute of Allergy and Infectious Diseases. *J Allergy Clin Immunol* 2010;125:540–544.
- 3 Kindt TJ, Goldsby RA, Osborne BA: *Kuby Immunology*, ed 6. New York, Freeman and Company, 2007, pp 371–388.
- ▶ 4 Sookrung N, Indrawattana N, Tungtrongchitr A, Karuhassuwan C, Chaisri U, Chaicumpa W: A murine model of allergy caused by American cockroach (CR), *Periplaneta americana*. *Asian Pac J Allergy Immunol* 2008;26:143–149.
- ▶ 5 Poon AH, Eidelman DH, Martin JG, Laprise C, Hamid Q: Pathogenesis of severe asthma. *Clin Exp Allergy* 2012;42:625–637.
- ▶ 6 Tantilipikorn P, Auewarakul P: Airway allergy and viral infection. *Asian Pac J Allergy Immunol* 2011;29:113–119.
- ▶ 7 Sokol CL, Barton GM, Farr AG, Medzhitov R: A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol* 2008;9:310–318.
- ▶ 8 Sumi Y, Hamid Q: Airway remodeling in asthma. *Allergol Int* 2007;56:341–348.
- ▶ 9 Olmedo O, Goldstein IF, Acosta L, Divjan A, Rundle AG, Chew GL, Mellins RB, Hoepner L, Andrews H, Lopez-Pintado S, Quinn JW, Perera FP, Miller RL, Jacobson JS, Perzanowski MS: Neighborhood differences in exposure and sensitization to cockroach, mouse, dust mite, cat, and dog allergens in New York City. *J Allergy Clin Immunol* 2011;128:284–292.
- ▶ 10 Abramson MJ, Puy RM, Weiner JM: Injection allergen immunotherapy for asthma. *Cochrane Database Syst Rev* 2010;8:CD001186.
- ▶ 11 Cox LS, Linnemann DL, Nolte H, Welden D, Finegold I, Nelson HS: Sublingual immunotherapy: a comprehensive review. *J Allergy Clin Immunol* 2006;117:1021–1035.
- ▶ 12 Umetsu DT, DeKruyff RH: The regulation of allergy and asthma. *Immunol Rev* 2006;212:238–255.
- ▶ 13 Robinson DS: Regulatory T cells and asthma. *Clin Exp Allergy* 2009;39:1314–1323.
- ▶ 14 Manetti R, Parronchi P, Giudizi MG, Piccinini MP, Maggi E, Trinchieri G, Romagnani S: Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* 1993;177:1199–1204.
- ▶ 15 Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H: Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol* 2001;19:423–474.
- ▶ 16 Meiler F, Klunker S, Zimmermann M, Akdis CA, Akdis M: Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors. *Allergy* 2008;63:1455–1463.
- ▶ 17 Akdis M, Akdis CA: Therapeutic manipulation of immune tolerance in allergic disease. *Nat Rev Drug Discov* 2009;8:645–660.
- ▶ 18 Kussebi F, Karamloo F, Rhyner C, Schmid-Grendelmeier P, Salagianni M, Mannhart C, Akdis M, Soldatova L, Markovic-Housley Z, Von Beust BR, Kündig T, Kemeny DM, Blaser K, Cramer R, Akdis CA: A major allergen gene-fusion protein for potential usage in allergen-specific immunotherapy. *J Allergy Clin Immunol* 2005;115:323–329.
- ▶ 19 Karamloo F, Schmid-Grendelmeier P, Kussebi F, Akdis M, Salagianni M, von Beust BR, Reimers A, Zumkehr J, Soldatova L, Housley-Markovic Z, Müller U, Kündig T, Kemeny DM, Spangfort MD, Blaser K, Akdis CA: Prevention of allergy by a recombinant multi-allergen vaccine with reduced IgE binding and preserved T cell epitopes. *Eur J Immunol* 2005;35:3268–3276.
- ▶ 20 Höiby AS, Strand V, Robinson DS, Sager A, Rak S: Efficacy, safety, and immunological effects of a 2-year immunotherapy with Depigoid birch pollen extract: a randomized, double-blind, placebo-controlled study. *Clin Exp Allergy* 2010;40:1062–1070.
- ▶ 21 Pfaar O, Urry Z, Robinson DS, Sager A, Richards D, Hawrylowicz CM, Bräutigam M, Klimek L: A randomized placebo-controlled trial of rush preseasonal depigmented polymerized grass pollen immunotherapy. *Allergy* 2012;67:272–279.
- ▶ 22 Kulis M, Li Y, Lane H, Pons L, Burks W: Single-tree nut immunotherapy attenuates allergic reactions in mice with hypersensitivity to multiple tree nuts. *J Allergy Clin Immunol* 2011;127:81–88.
- ▶ 23 Tourdot S, Airouche S, Berjont N, Da Silveira A, Mascarell L, Jacquet A, Caplier L, Langelot M, Baron-Bodo V, Moingeon P: Evaluation of therapeutic sublingual vaccines in a murine model of chronic house dust mite allergic airway inflammation. *Clin Exp Allergy* 2011;41:1784–1792.
- ▶ 24 Holt PG, Schon-Hegrad MA, Oliver J, Holt BJ, McMenamin PG: A contiguous network of dendritic antigen-presenting cells within the respiratory epithelium. *Int Arch Allergy Appl Immunol* 1990;91:155–159.
- ▶ 25 Xia W, Pinto CE, Kradin RL: The antigen-presenting cell activities of Ia+ dendritic cells shift dynamically from lung to lymph node after an airway challenge with soluble antigen. *J Exp Med* 1995;181:1275–1283.
- ▶ 26 Sookrung N, Chaicumpa W, Tungtrongchitr A, Vichyanond P, Bunnag C, Ramasoota P, Tongtawe P, Sakolvaree Y, Tapchaisri P: *Periplaneta americana* arginine kinase as a major cockroach allergen among Thai patients with major cockroach allergies. *Environ Health Perspect* 2006;114:875–880.
- ▶ 27 Gregoriadis G: Immunological adjuvants: a role for liposomes. *Immunol Today* 1990;11:89–97.
- ▶ 28 Alving CR: Liposomes as carriers of antigens and adjuvants. *J Immunol Methods* 1991;140:1–13.
- ▶ 29 Gregoriadis G, Wang Z, Barenholz Y, Francois MJ: Liposome-entrapped T-cell peptides help for a co-entrapped B-cell peptide to overcome genetic restriction in mice and induce immunological memory. *Immunology* 1993;80:535–540.
- ▶ 30 Sookrung N, Diraphat P, Chaicumpa W, Tongtawe P, Sakolvaree Y, Tapchaisri P, Mahakittikun V, Tungtrongchitr A, Vichyanond P, Bunnag C: Cockroach allergen detection and cockroach allergens of allergic Thai patients. *Asian Pac J Allergy Immunol* 2003;21:1–9.
- ▶ 31 Badiiee A, Jaafari MR, Khamesipour A, Samiei A, Soroush D, Kheiri MT, Barkhordari F, McMaster WR, Mahboudi F: The role of liposome charge on immune response generated in BALB/c mice immunized with recombinant major surface glycoprotein of *Leishmania* (rgp63). *Exp Parasitol* 2009;121:362–369.
- ▶ 32 Liu Z, Guo H, Wu Y, Yang H, Li J: Local nasal immunotherapy: efficacy of *Dermatophagoides farinae*-chitosan vaccine in murine asthma. *Int Arch Allergy Immunol* 2009;150:221–228.
- ▶ 33 Underwood S, Foster M, Raeburn D, Bottoms S, Karlsson JA: Time-course of antigen-induced airway inflammation in the guinea-pigs and its relationship to airway hyperresponsiveness. *Eur Respir J* 1995;8:2104–2113.
- ▶ 34 Lindner I, Meier C, Url A, Unger H, Grassauer A, Prieschi-Grassauer E, Doerfler P: Beta-escin has potent anti-allergic efficacy and reduces allergic airway inflammation. *BMC Immunol* 2010;11:24.
- ▶ 35 Abebe F, Mustafa T, Nerland AH, Bjune GA: Cytokine profile during latent and slowly progressive primary tuberculosis: a possible role for interleukin-15 in mediating clinical disease. *Clin Exp Immunol* 2005;143:180–192.
- ▶ 36 Tsuzaka K, Nozaki K, Kumazawa C, Shiraishi K, Setoyama Y, Oshimoto K, Suzuki K, Abe T, Takeuchi T: DNA microarray gene expression profile of T cells with the splice variants of TCR mRNA observed in systemic lupus erythematosus. *J Immunol* 2006;176:949–956.
- ▶ 37 Overbergh L, Valckx D, Waer M, Mathieu C: Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine* 1999;11:305–312.
- ▶ 38 Thueng-in K, Maneewatch S, Srimanote P, Songserm T, Tapchaisri P, Sookrung N, Tongtawe P, Channarong S, Chaicumpa W: Heterosubtypic immunity to influenza mediated by liposome adjuvanted H5N1 recombinant protein vaccines. *Vaccine* 2010;28:6765–6777.

- ▶ 39 Gondal JA, Preuss HG, Swartz R, Rahman A: Comparative pharmacological, toxicological and antitumoral evaluation of free and liposome-encapsulated cisplatin in rodents. *Eur J Cancer* 1993;29A:1536–1542.
- ▶ 40 Nair S, Zhou F, Reddy R, Huang L, Rouse BT: Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro. *J Exp Med* 1992;175:609–612.
- ▶ 41 Ludewig B, Barchiesi F, Pericin M, Zinkernagel RM, Hengartner H, Schwendener RA: In vivo antigen loading and activation of dendritic cells via liposomal peptide vaccine mediates protective antiviral and anti-tumor immunity. *Vaccine* 2001;19:23–32.
- ▶ 42 Moingeon P: Adjuvants for allergy vaccines. *Hum Vaccin Immunother* 2012, E-pub ahead of print.
- ▶ 43 Oussoren C, Zuidema J, Crommelin DJ, Storm G: Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. Influence of liposomal size, lipid composition and lipid dose. *Biochim Biophys Acta* 1997;1328:261–272.
- ▶ 44 Filder IJ: Targeting of immunomodulators to mononuclear phagocytes for therapy of cancer. *Adv Drug Deliv Rev* 1988;2:69–106.
- ▶ 45 Foged C, Ariqita C, Sundblad A, Jiskoot W, Storm G, Frokjaer S: Interaction of dendritic cells with antigen-containing liposome: effect of bilayer composition. *Vaccine* 2004;22:1903–1913.
- ▶ 46 Alving CR, Wassef NM: Cytotoxic T lymphocytes induced by liposomal antigens: mechanisms of immunological presentation. *AIDS Res Hum Retroviruses* 1994;10:S91–S94.
- ▶ 47 Rao M, Wassef NM, Alving CR, Krzych U: Intracellular processing of liposome-encapsulated antigens by macrophages depends upon the antigen. *Infect Immun* 1995;63:2396–2402.
- ▶ 48 Neurath MF, Fuss I, Pasparakis M, Alexopoulou L, Haralambous S, Meyer zum Büschenfelde KH, Strober W, Kollias G: Predominant pathogenic role of tumor necrosis factor in experimental colitis in mice. *Eur J Immunol* 1997;27:1743–1750.
- ▶ 49 Rollins BJ, Yoshimura T, Leonard EJ, Pober JS: Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE. *Am J Pathol* 1990;136:1229–1233.
- ▶ 50 Matsuo H, Dahlström J, Tanaka A, Kohno K, Takahashi H, Furumura M, Morita E: Sensitivity and specificity of recombinant omega-5 gliadin-specific IgE measurement for the diagnosis of wheat-dependent exercise-induced anaphylaxis. *Allergy* 2008;63:233–236.
- ▶ 51 Nicolaou N, Poorafshar M, Murray C, Simpson A, Winell H, Kerry G, Härlin A, Woodcock A, Ahlstedt S, Custovic A: Allergy or tolerance in children sensitized to peanut: prevalence and differentiation using component-resolved diagnostics. *J Allergy Clin Immunol* 2010;125:191–197.
- ▶ 52 Ball T, Linhart B, Sonneck K, Blatt K, Herrmann H, Valent P, Stoecklinger A, Lupinek C, Thalhamer J, Fedorov AA, Almo SC, Valenta R: Reducing allergenicity by altering allergen fold: a mosaic protein of Phl p 1 for allergy vaccination. *Allergy* 2009;64:569–580.
- ▶ 53 Valenta R: The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol* 2002;2:446–453.
- ▶ 54 Srivastava D, Mehta AK, Arora N, Gaur SN, Singh BP: Proteolytically inactive Per a 10 allergen of *Periplaneta americana* modulates Th2 response and enhances IL-10 in mouse model. *J Clin Immunol* 2010;30:426–434.
- ▶ 55 Yu HQ, Li XH, Guo H, Liu ZG, Ran PX, Ji KM, Wang J: Sublingual immunotherapy efficacy of *Dermatophagoides farinae* vaccine in a murine asthma model. *Int Arch Allergy Immunol* 2010;152:41–48.
- ▶ 56 Schenk J, Hargie M, Brown M, Eberi D, Yoo AH: The enhancement of antibody formation by *E. coli* lipopolysaccharide and detoxified derivatives. *J Immunol* 1969;114:770–775.
- ▶ 57 Verthelyi D, Wang V: Trace levels of innate immune response modulating impurities (IIRMI)s synergize to break tolerance to therapeutic proteins. *PLoS One* 2010;5:e15252.
- ▶ 58 Van der Zee JS, Aalberse RC: The role of IgG in immediate-type hypersensitivity. *Eur Respir J Suppl* 1991;13:91s–96s.
- ▶ 59 Maggi E, Parronchi P, Manetti R, Simonelli C, Piccinni MP, Rugiu FS, De Carli M, Ricci M, Romagnani S: Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones. *J Immunol* 1992;148:2142–2147.
- ▶ 60 McHugh SM, Deighton J, Stewart AG, Lachmann PJ, Ewan PW: Bee venom immunotherapy induces a shift in cytokine responses from a TH-2 to a TH-1 dominant pattern; comparison of rush and conventional therapy. *Clin Exp Allergy* 1995;25:828–838.
- ▶ 61 Kips JC, Brusselle GJ, Joos GF, Peleman RA, Tavernier JH, Devos RR, Pauwels RA: Interleukin-12 inhibits antigen-induced airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* 1996;153:535–539.
- ▶ 62 Iwamoto I, Kumano K, Kasai M, Kurasawa K, Nakao A: Interleukin-12 prevents antigen-induced eosinophil recruitment into mouse airways. *Am J Respir Crit Care Med* 1996;154:1257–1260.
- ▶ 63 Durrant DM, Metzger DW: IL-12 can alleviate Th17-mediated allergic lung inflammation through induction of pulmonary IL-10 expression. *Mucosal Immunol* 2010;3:301–311.
- ▶ 64 Sugaya M, Nakamura N, Tamaki K: Interleukin-18 and 12 synergistically upregulated interferon- γ production by murine dendritic epidermal T cells. *J Invest Dermatol* 1999;113:50–54.
- ▶ 65 Yoshimoto T, Tsutsui H, Tominaga K, Hoshino K, Okamura H, Akira S, Paul WE, Nakanishi K: IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. *Proc Natl Acad Sci USA* 1999;96:13962–13966.
- ▶ 66 Hawrylowicz CM, O'Garra A: Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol* 2005;5:271–283.
- ▶ 67 Adalid-Peralta L, Fragoso G, Fleury A, Sciutto E: Mechanisms underlying the induction of regulatory T cells and its relevance in the adaptive immune response in parasitic infections. *Int J Biol Sci* 2011;7:1412–1426.
- ▶ 68 Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK: Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006;441:235–238.

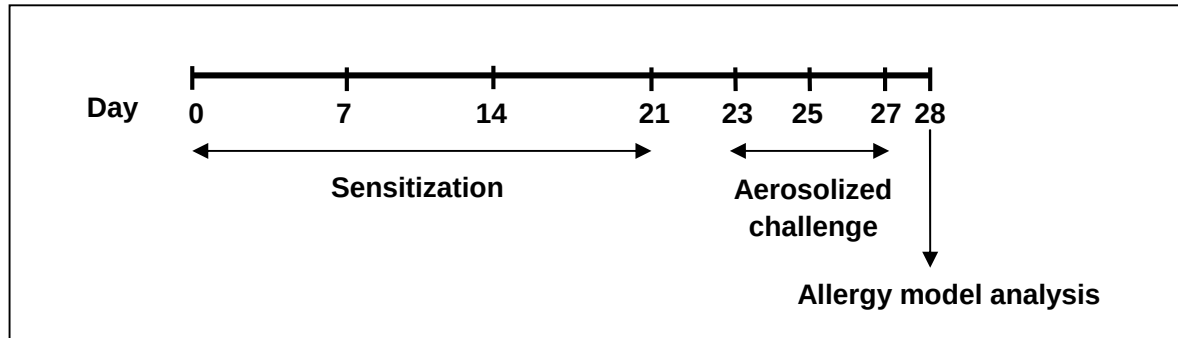
ONLINE SUPPLEMENTARY MATERIAL

Meechan T., et al.

Intranasal, Liposome-adjuvanted Cockroach Allergy Vaccines Made of Refined Major Allergen and Whole Body Extract of *Periplaneta americana*

Int Arch Allergy Immunol 2013, DOI: 10.1159/000348314

(a)



(b)

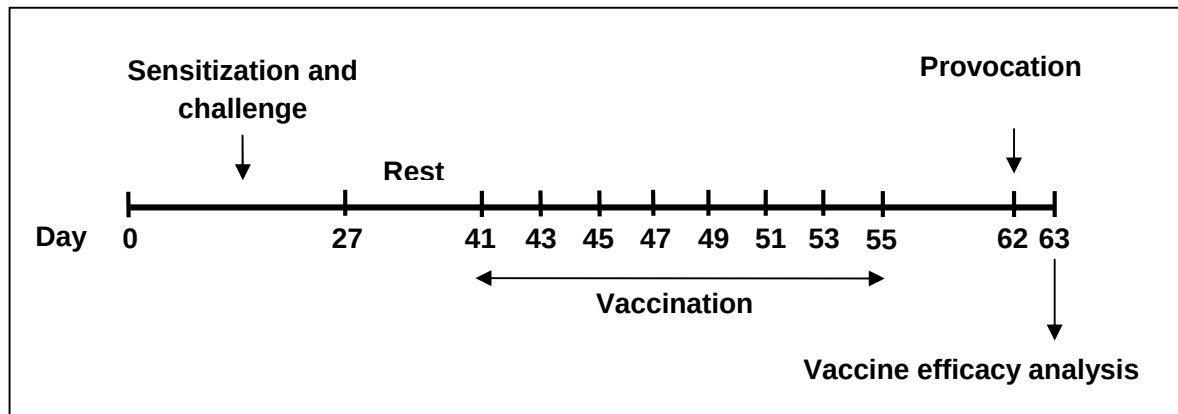


Fig. S1. Timelines of animal experiments. Timeline for preparing allergic mice (a); timeline for vaccination and provocation of the allergic mice and vaccine efficacy analysis (b). Sham mice were treated with PBS instead of CRE using the same timelines as the allergic mice.

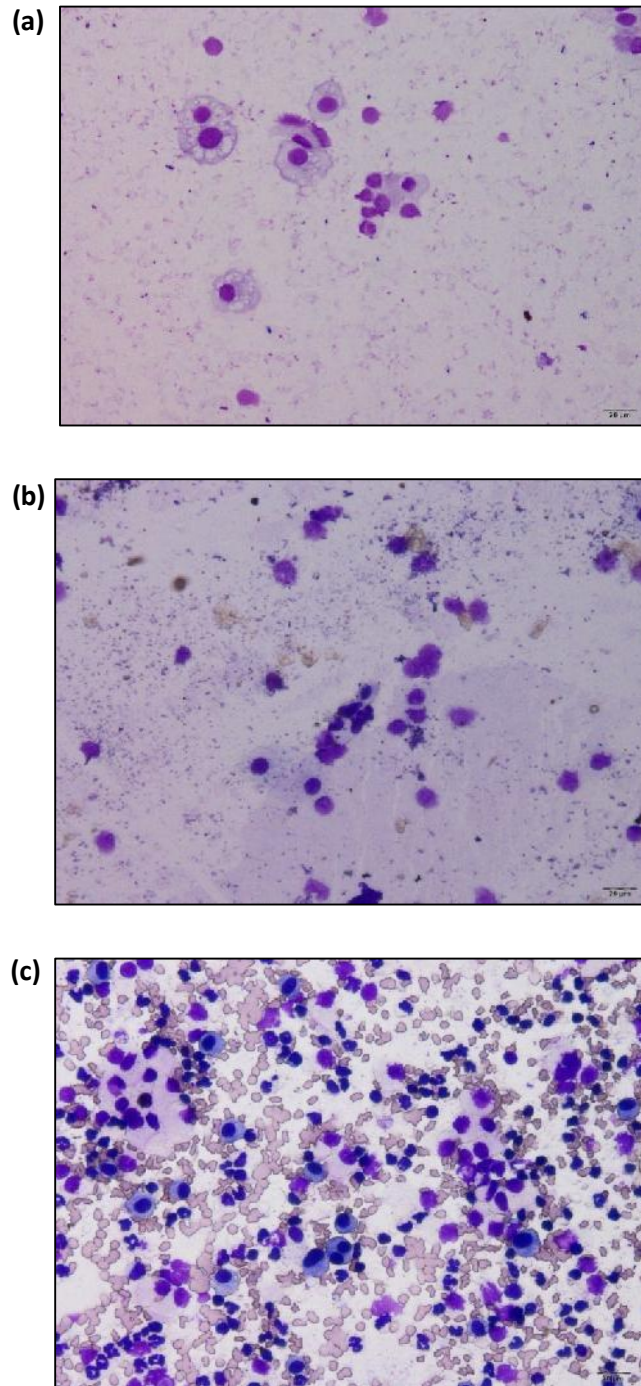


Fig. S2. Giemsa stained cells in the BALF of mice. BALF of normal (a), sham (b) and allergenized mice (c) (original magnification of 400 x). There were only a few sloughed-off epithelial cells in the BALF of normal mice, while the BALF of allergenized mice had intense inflammatory cell infiltration, predominantly neutrophils and to the less extent eosinophils. The BALF of sham mice contained sloughed off epithelial cells and few inflammatory cells.

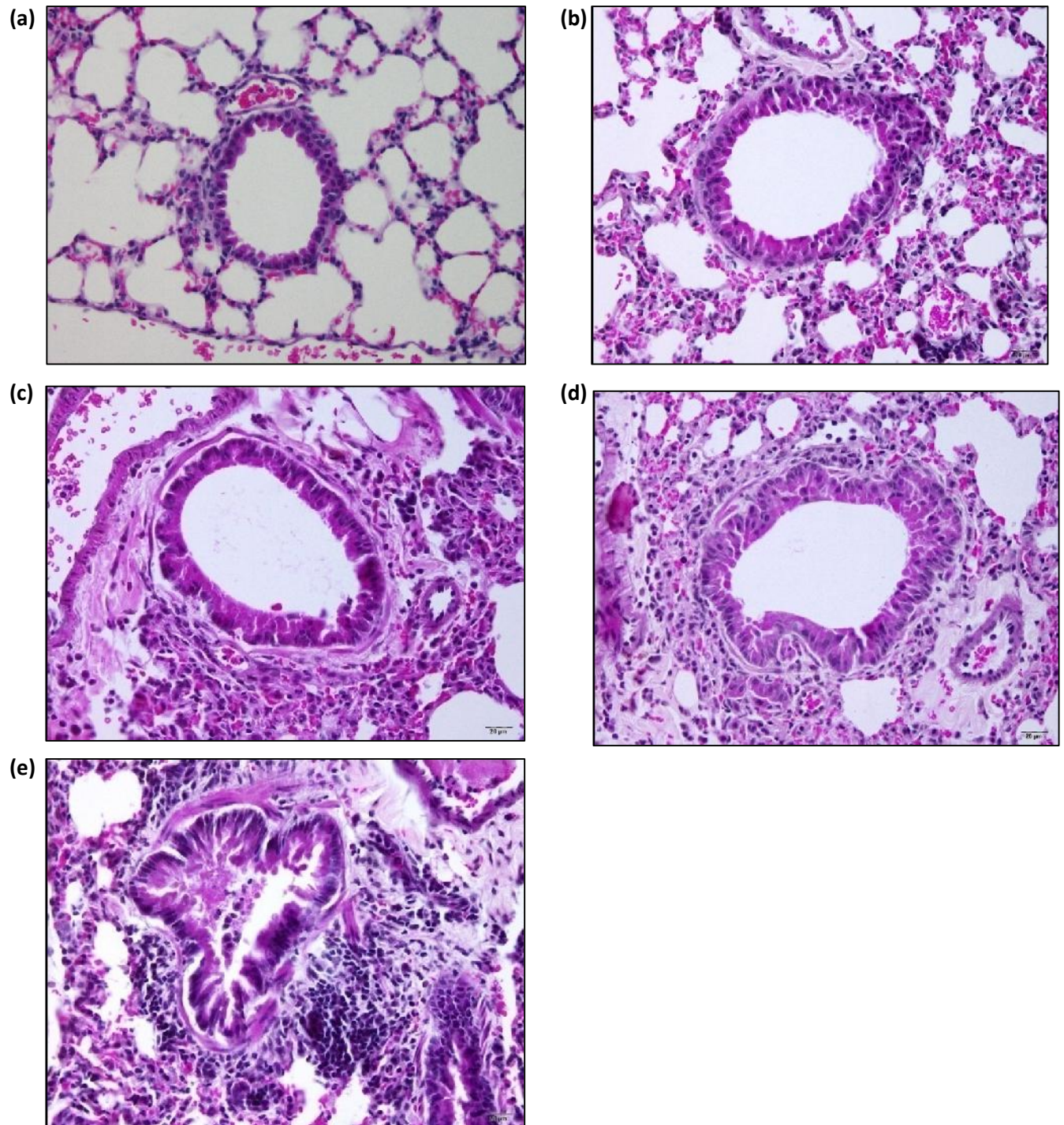


Fig. S3. Histopathological features of the arbitrary grades 0-4 of mouse lungs in this study. Grade 0 (normal): bronchiole with normal columnar epithelial cells, without inflammatory cell infiltration into peribronchiolar tissue with single layer of smooth muscle cells in the submucosa (a); Grade 1: thickening of ciliated pseudostratified columnar epithelium of bronchioles, few inflammatory cells in the interstitial area of neighboring alveoli and slight degeneration of smooth muscle cells (b); Grade 2: hyperplasia of bronchiolar epithelial cells, much more inflammatory cell infiltration into the peribronchiolar area than grade 1 and marked degeneration of bronchiolar smooth muscle cells (c); Grade 3: bronchiolar epithelial hyperplasia with two-three layers of inflammatory cells around bronchioles and degeneration and detachment of smooth muscle (d); and Grade 4: epithelial hyperplasia and sloughing which obstructed the bronchiolar lumen, intense inflammatory cell infiltration into the peribronchiolar area (more than four layers of inflammatory cells around bronchioles) and necrosis of smooth muscle cells (e).

Table S1. Designated histopathological features for arbitrary grades of mouse bronchioles and lung tissues

Grade of histopathology	Histological features
0 (normal)	Bronchiole with normal columnar epithelial cells, without inflammatory cell infiltration into peribronchiolar tissue and with single layer of smooth muscle cells at submucosa
1	Thickening of ciliated pseudo-stratified columnar epithelium of bronchiole, few inflammatory cells in the interstitial area of neighboring alveoli and slight degeneration of smooth muscle cell
2	Hyperplasia of bronchiolar epithelial cells, much more inflammatory cell infiltration into peribronchiolar area than grade 1 and marked degeneration of bronchiolar smooth muscle cells
3	Bronchiolar epithelial hyperplasia with 2-3 layers of inflammatory cells around bronchiole and degeneration and detachment of smooth muscle
4	Epithelial hyperplasia and sloughing which obstructed the bronchiolar lumen, intense inflammatory cell infiltration (Neutrophils, lymphocytes and eosinophils) into peribronchiolar area (more than 4 layers of inflammatory cells around bronchiole) and necrosis of smooth muscle cells

Table S2. Oligonucleotide primers used in quantitative polymerase chain reaction for determination of cytokine gene expression in mouse lung tissues

Gene encoding	Primer	Size of PCR product (bp)
Th2 cytokine		
<i>IL-4</i>	F: 5'-acaggagaagggacgccat-3' R: 5'-gaagccctacagacgagctca-3'	94
<i>IL-5</i>	F: 5'-agcacagtgggtgaaagagacctt-3' R: 5'-tccaatgcatagctgggtgattt-3'	116
<i>IL-10</i>	F: 5'-ggttgcaagccttatcgga-3' R: 5'-acctgctccactgccttgct-3'	191
<i>IL-13</i>	F: 5'-agaccagactcccctgtgca-3' R: 5'-tgggtcctgtagatggcattg-3'	123
Pro-inflammatory		
<i>TNF-α</i>	F: 5'-catcttctcaaaattcgagtgacaa-3' R: 5'-tgaggagtagacaaggtacaaccc-3'	174
Innate / Th1 cytokine		
<i>IFNγ</i>	F: 5'-tcaagtggcatagatgtggaagaa-3' R: 5'-tggctctgcaggattttcatg-3'	91
<i>IL-12</i>	F: 5'-ggaagcacggcagcagaata-3' R: 5'-aacttgagggagaagtaggaatgg-3'	179
<i>IL-18</i>	F: 5'-agacaactttggccgacttca-3' R: 5'-ttcacagagggtcacagcc-3'	110
House keeping		
<i>Jactin</i>	F: 5'-ggccaaccgtgaaaagatga-3' R: 5'-cacgctcggtcaggatcttc-3'	250

PCR = polymerase chain reaction; bp = base pairs; IL = interleukin; IFN = interferon; TNF = tumor necrosis factor; F = forward primer; R = reverse primer.