



Chapter 20

Methods for Experimental Allergen Immunotherapy: Subcutaneous and Sublingual Desensitization in Mouse Models of Allergic Asthma

Laura Hesse, Arjen H. Petersen, and Martijn C. Nawijn

Abstract

Allergic asthma is characterized by airway hyperresponsiveness, remodeling, and reversible airway obstruction. This is associated with an eosinophilic inflammation of the airways, caused by inhaled allergens such as house dust mite or grass pollen. The inhaled allergens trigger a type-2 inflammatory response with the involvement of innate lymphoid cells (ILC2) and Th2 cells, resulting in high immunoglobulin E (IgE) antibody production by B cells and mucus production by airway epithelial cells. As a consequence of the IgE production, subsequent allergen reexposure results in a classic allergic response with distinct early and late phases, both resulting in bronchoconstriction and shortness of breath. Allergen-specific immunotherapy (AIT) is the only treatment that is capable of modifying the immunological process underlying allergic responses including allergic asthma. Both subcutaneous AIT (SCIT) as well as sublingual AIT (SLIT) have shown clinical efficacy in long-term suppression of the allergic response. Although AIT treatments are very successful for rhinitis, application in asthma is hampered by variable efficacy, long duration of treatment, and risk of severe side effects. A more profound understanding of the mechanisms by which AIT induces tolerance to allergens in sensitized individuals is needed to be able to improve its efficacy. Mouse models have been very valuable in preclinical research for characterizing the mechanisms of desensitization in AIT and evaluating novel approaches to improve its efficacy. Here, we present a rapid and reproducible mouse model for allergen-specific immunotherapy. In this model, mice are sensitized with two injections of allergen adsorbed to aluminum hydroxide, followed by subcutaneous injections (SCIT) or sublingual administrations (SLIT) of allergen extracts as an immunotherapy treatment. Finally, mice are challenged by intranasal allergen administrations. We will also describe the protocols as well as the most important readout parameters for the measurements of invasive lung function, serum immunoglobulin levels, isolation of bronchoalveolar lavage fluid (BALF), and preparation of cytospin slides. Moreover, we describe how to perform ex vivo restimulation of lung single-cell suspensions with allergens, flow cytometry for identification of relevant immune cell populations, and ELISAs and Luminex assays for assessment of the cytokine concentrations in BALF and lung tissue.

Key words Allergic asthma, Subcutaneous immunotherapy (SCIT), Sublingual immunotherapy (SLIT), BALB/cByJ and C57BL/6 mouse models, Grass pollen (GP), House dust mite (HDM), FlexiVent, Flow cytometry, Bronchoalveolar lavage, Eosinophilia

Electronic Supplementary Material: The online version of this chapter (https://doi.org/10.1007/978-1-0716-1001-5_20) contains supplementary material, which is available to authorized users.

Kumi Nagamoto-Combs (ed.), *Animal Models of Allergic Disease: Methods and Protocols*, Methods in Molecular Biology, vol. 2223, https://doi.org/10.1007/978-1-0716-1001-5_20, © Springer Science+Business Media, LLC, part of Springer Nature 2021

1 Introduction

Asthma is the result of a complex interaction between genetic susceptibility and environmental factors. The most common asthma phenotype is allergic asthma, which is caused by inhaled allergens such as grass pollen (GP), ragweed, cat and dog allergens, and house dust mites (HDM) [1, 2]. Patients suffering from allergic asthma have reversible airway obstruction associated with eosinophilic inflammation as well as airway hyperresponsiveness (AHR) and remodeling. The worldwide prevalence of allergic asthma has dramatically increased over the last 25 years, currently affecting over 300 million people [3].

The inflammatory responses in allergic asthma are characterized by the presence of high levels of cytokines such as IL-4, IL-5, and IL-13, produced by both innate lymphoid cells (ILCs) and T helper 2 (Th2) cells [4]. These cytokines contribute to the pathological changes of the airways observed in allergic asthma, including influx of eosinophils, mucus hypersecretion, airway hyperresponsiveness, and airway wall remodeling. In addition, the Th2-dominated adaptive immune response to inhaled allergens results in the presence of allergen-specific IgE. Allergen-induced crosslinking of IgE that is bound to the cell surface of mast cells and basophils through the high affinity IgE receptor triggers degranulation of these cells resulting in acute allergic responses, leading to bronchoconstriction and vasodilation. The subsequent influx of inflammatory cells, including Th2 cells, into the tissue will result in activation of these cells and late-phase responses. Upon recurrent exposures to the allergen, chronic and poorly resolving inflammation around the small airways is induced, resulting in permanent structural changes to the airway wall.

Currently available asthma therapies are focused on controlling the chronic inflammatory process, mainly using inhaled corticosteroids in combination with long-acting beta agonists or leukotriene receptor antagonists [5]. Notwithstanding the clinical success in achieving asthma control, current asthma treatment regimens fail to cure the disease. This lack of a cure is evidenced by ongoing airway wall remodeling even in well-controlled asthma patients [6, 7]. Moreover, a subset of patients with severe asthma does not respond to steroid treatment [8, 9]. These shortcomings of current mainstream asthma therapy indicate that this therapeutic approach fails to address the underlying, causative immune mechanisms, achieving merely a transient suppression of symptoms of asthma in most patients.

The only treatment known to date that is capable of modifying the immunological process underlying allergic asthma is allergen-specific immunotherapy (AIT) [10]. AIT provides long-term protection against asthma attacks, which is even maintained upon

cessation of therapy and reduces medication use in allergic asthma. AIT involves the administration of gradually increasing amounts of allergen for a period of 3–5 years, aiming to achieve a state of immunological tolerance and a subsequent reduction of clinical manifestations of the disease [11]. Although the immune mechanisms behind successful immunotherapy remain unknown, the beneficial effects of AIT associated with a shift from Th2 activity toward a T-regulatory (Treg) profile that suppresses allergen-specific responses are known [12]. Successful AIT is characterized by increased levels of neutralizing antibodies, production of IL-10 and TGF- β , and increased CD4⁺FoxP3⁺ Treg numbers [13–16].

While subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) have been widely accepted as effective therapeutic alternatives for allergic rhinoconjunctivitis, application in asthma is hampered by the long duration of treatment, the variable efficacy in allergic asthma, and concerns regarding the safety of treatment. SCIT injections of allergen extracts have a risk of inducing anaphylactic reactions, with an incidence of severe anaphylactic responses at around one in a million injections [13]. Therefore, outpatient clinic visits are required for the administration and monitoring of the therapy [14]. In developing more convenient alternatives, SLIT has been developed as a less invasive alternative with proven clinical effects for patients suffering from allergic rhinitis [15]. Herein, uptake of the allergen involves the oral mucosa, where mucosal Langerhans cells in humans and oral macrophage-like cells in mice have been implicated to be important [16]. Although the exact mechanism of action remains to be elucidated, advantages of SLIT include the ease of use (droplets or fast-dissolving tablets), the application in a home setting, and relative costs. A better understanding of the mechanisms by which AIT suppresses allergen-induced asthma phenotypes is needed to improve the efficacy and safety of AIT, in particular in asthmatic patients.

Previously, animal models have proven to be valuable as a preclinical model to improve AIT by unraveling the immune mechanisms of allergen desensitization. The development of a predictive and reproducible AIT protocol was based on the classic OVA-driven mouse model of allergic asthma [17]. In this study, mice were sensitized to OVA in seven intraperitoneal injections. Two weeks later, SCIT treatment was performed using three injections of OVA (1 mg), followed by allergen challenges after another 2 weeks by OVA (2 mg/mL) inhalation once a day (5 min) for 8 consecutive days. In these initial studies, no adverse events of SCIT treatment were recorded in the BALB/c strain of mice, while the experimental SCIT treatment effectively suppressed airway inflammation and AHR and induced serum levels of antigen-specific immunoglobulin (spIg)G1 and spIgG2a. In addition, spIgE levels were increased, which matches the initial rise of

spIgE in human subjects treated with SCIT. Importantly, SCIT treatment in the OVA mouse model prevented the increase of spIgE levels after allergen challenges, which readily occurs in control-treated mice [18]. After several improvements, the protocol for sensitization was reduced to two intraperitoneal injections of OVA using a sensitizing adjuvant, Alum (mixture of aluminum hydroxide and magnesium hydroxide), and three intranasal challenges containing a high dose of aerosolized OVA. Importantly, OVA SCIT treatment was shown to be also effective in sensitized mice that were challenged by OVA inhalation prior to SCIT treatment, indicating the ability of SCIT treatment to suppress an established allergic airway inflammatory response [19]. Throughout the years, this mouse model for SCIT has been used to characterize the mechanisms of desensitization [17, 20–22], including the relevance of the neutralizing antibody responses [19], the role of IL-10 in the induction of tolerance, and the contribution of CD4⁺FoxP3⁺ T-regulatory cells (Treg). In addition, the role of dendritic cells (DCs) and their phenotypic modulation has been investigated extensively. DCs play a key role in the generation of adaptive T-cell subsets and can respond in either immunogenic or in a tolerogenic fashion [23]. Tolerogenic DCs have a semimature or immature phenotype, characterized by high expression of major histocompatibility complex class II (MHC-II) and B7-2, low expression of CD40, and lack of proinflammatory cytokine expression (IL-6 and TNF α). Studies have shown that incubation of immature DCs with CD4⁺ T cells induces antigen-specific Tregs [24, 25], indicating that immature DCs can play a critical role in Treg cell generation and peripheral tolerance. Based on these findings, it has been studied whether AIT can be improved when allergen administration is accompanied by inhibition of DC maturation or prevention of DC-dependent costimulation. One approach is the use of 1,25(OH)₂Vitamin D₃ (VitD₃), the active metabolite of vitamin D, which suppresses DC differentiation and maturation. Indeed, using the OVA-SCIT mouse model, administration of 1,25(OH)₂VitD₃ has been shown to potentiate AIT [20]. In addition, CTLA4-Ig (Abatacept) was found to enhance the efficacy of SCIT in the OVA model, most likely by affecting the DC function [26].

Although using this mouse model of immunotherapy has provided insight into the immunological mechanisms of AIT, its value as an experimental preclinical model is limited by the use of a purified protein (OVA) that lacks the properties of natural allergens and induces tolerance when inhaled by naïve mice [27]. Therefore, the classical model allergen OVA was more recently replaced with a natural allergen extract that is also used in human SCIT, such as GP and HDM. The GP and HDM SCIT protocols for allergic asthma have been optimized first with regard to the allergen dosage needed to achieve suppression of phenotypes of allergic asthma [28–

30]. Second, other administrative routes were optimized, based on a SLIT mouse model of allergic rhinitis [28, 31]. SCIT and SLIT have been validated and standardized allowing a head-to-head comparison [28]. This model therefore allows in-depth characterization of the mechanisms of SCIT and SLIT treatment for allergic asthma as well as their optimization using novel approaches including peptide SCIT treatment or the use of alternative formulations and adjuvantia. Herein, we found that, while SLIT suppresses mainly AHR, GP SCIT suppresses Th2 profile and induces neutralizing antibodies. Furthermore, we showed that using purified allergens derived from crude extracts of HDM, like *Dermatophagoides pteronyssinus* (*Der p*) Der p1 and Der p2, allows suppression of AHR and inflammation, but also has superior activity toward suppression of type 2 cytokines [29].

The use of crude allergen extracts with IgE-crosslinking capacity has safety concerns, and although occurring in very low frequency, there is a risk of anaphylaxis [32, 33]. When studying mechanisms of allergen-induced tolerance induction in murine models, the same risk should be taken into consideration [34–36]. BALB/c mice have traditionally been considered an appropriate strain for developing allergy mouse models [35, 37]. The allergic phenotype of these mice has led this strain to be widely used for characterizing classic (IgE-FcεRI-mast cell-histamine) and alternative dependent pathways (IgG-FcγRIII-macrophage-platelet-activating factor) and for establishing the immunoregulatory mechanism underlying tolerance, which suppresses both Th 1 and Th 2 responses. Smit et al. demonstrate that in three different mouse strains (BALB/c, C3H/HeOuJ, and C57BL/6), components of the classic and alternative anaphylactic cascade are differently expressed, leading to different outcomes in parameters of allergic disease and food-induced systemic anaphylaxis. To overcome strain-dependent differences in optimizing allergen immunotherapy for allergic asthma, we performed our GP SCIT protocol in C57BL/6J mice and found that these mice are more prone toward anaphylaxis than BALB/cByJ mice (*unpublished data*).

In the protocol provided here, we explain how subcutaneous and sublingual routes of allergen-specific immunotherapy can be applied in both BALB/cByJ and C57BL/6 mouse models of allergic asthma using natural allergen extracts. We provide detailed methods to obtain the most important outcome parameters for translational studies, including invasive lung function measurements for AHR, specific IgE and IgG levels in serum, ear swelling tests for the early phase response, and inflammation of lung tissue and airways. Moreover, we describe how to restimulate lung cells with allergen extracts, perform flow cytometric measurements to identify populations of relevant immune cells, and perform ELISAs and Luminex assays to measure the cytokine concentrations in bronchoalveolar lavage fluid (BALF) and lung tissue. In C57BL/6 mice, we

included an adapted SCIT treatment protocol, including monitoring of immediate responses such as severity of shock and body temperature after the first injections, to avoid anaphylaxis in this mouse strain.

2 Materials

2.1 Subcutaneous and Sublingual Immunotherapy in a Mouse Model of Allergic Asthma

1. BALB/cByJ mice or C57BL/6 mice: 7- to 9-week-old females housed in individually ventilated cages (IVC).
2. 1-mL Syringes and 25G needles.
3. P20 pipette and tips.
4. 15-mL tubes.
5. Sterile phosphate buffered saline (PBS): 1 mM potassium phosphate monobasic (KH_2PO_4), 154 mM sodium chloride (NaCl), 3 mM sodium phosphate dibasic ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), pH 7.4. Dissolve 144 mg of KH_2PO_4 , 9.0 g of NaCl , and 795 mg of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ in approximately 800 mL of ultrapure water. Adjust the pH and make the final volume up to 1 L. Use a 0.25- μm filter or autoclave to sterilize.
6. Rough extract of grass pollen (GP, *Phleum pratense*; *Phl p*): Dissolve 204 mg of dry matter of *Phleum pratense*, 225 (MP225PHLpra, 1006674 or 1031225) in 2.125 mL of sterile PBS to obtain a solution containing 60 kSQ/ μL ($\sim 96 \mu\text{g}/\mu\text{L}$). Aliquot this stock in 100- μL portions and store at -20°C .
7. Crude extract of house dust mite (HDM, *Dermatophagoides pteronyssinus*; *Der p*): Dissolve 25.0 mg of DP extract FD 12C27 in 500 μL of sterile PBS to get a solution containing 50 $\mu\text{g}/\mu\text{L}$ HDM and aliquot this stock in 25- μL portions and store at -20°C .
8. Imject[®] Alum or equivalent aluminum hydroxide adjuvant: 20% $\text{Al}(\text{OH})_3$.
9. Isoflurane: used for anesthesia at 4.5% with 1 mL/min O_2 .
10. Heating mats with temperature control.

2.2 Blood Withdrawal via Orbital Puncture

1. Small animal anesthesia device compatible with isoflurane and a connected induction chamber.
2. Isoflurane: 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane. Used for inhalation anesthesia at 4.5% with 1 mL/min O_2 .
3. Sterile phosphate buffered saline (PBS): See Subheading 2.1, item 5.
4. 30G Insulin syringes: 0.3 mL, needle 0.3 mm $\times$ 8 mm (e.g., BD Micro-Fine[™] 0.3 mL).

5. 1-mL MiniCollect[®] serum tubes (Greiner Bio-One) or equivalent.
6. Glass microcapillary tubes: micro hematocrit tubes (Na-Heparinized 80 IU/mL).
7. 1.5-mL Microfuge tubes.
8. Centrifuge.

2.3 Ear Swelling Test

1. Positive allergen test solutions: Prepare either 1 μ g of *Phl p* or 0.5 μ g of *Der p* in 10 μ L of sterile PBS per mouse as positive allergen test solutions for GP or HDM sensitivity, respectively.
2. Negative allergen test solution: sterile PBS, pH 7.4.
3. Mitutoyo Digimatic Micrometer or equivalent: 0.5 ± 0.15 N. Used for measuring ear thickness.
4. Regular hand tissues, hand gloves, and sterile tissues.

2.4 Lung Function Measurement

1. SCIREQ[®] FlexiVent (SCIREQ Scientific Respiratory Equipment Inc.).
2. A computer installed with FlexiWare software (SCIREQ Scientific Respiratory Equipment Inc.).
3. Silicon tubing: 0.28 mm and OD. 0.61 mm.
4. Syringes: 1 mL and 5 mL.
5. Anesthesia: Combine 100 mg/mL ketamine and 1 mg/kg Domitor as shown in Table 1.
6. Manometer with syringe and closing valves.
7. Weighing scale (precision >0.1 g).
8. Surgical microscope (40 $\times$).
9. Dissection instrument set.
10. Ligatures: 6/0 and 3/0.
11. 25G needles.
12. 20G intravenous cannula: for tracheal cannulation and calibration (pink, 20GA 1.16IN 1.1 $\times$ 30 mm BD Insyte-W[™] or equivalent).
13. Bulldog clamp.
14. Sterile PBS.
15. Rocuronium bromide: Prepare a working solution of 0.125 mg/mL from a 10- μ g/mL stock in sterile PBS.
16. Sterile methacholine solutions: For concentrations and dosage, see Tables 2 and 3.
17. Micro pulse-oximeter for small animals.
18. Heating mats for small animals.

Table 1
Dilution scheme for anesthesia

	Solution	Administration	Dose
Ketamine	100 mg/mL	75 mg/mL	0.75 μ L/g mouse
Domitor	0.5 mg/mL	1 mg/mL	1 μ L/g mouse
Dilution scheme (100 mg/mL)			
	1 mL	5 mL	10 mL
Domitor	200 μ L	1000 μ L	2000 μ L
Ketamine	75 μ L	375 μ L	750 μ L
Saline	725 μ L	3625 μ L	7250 μ L

Table 2
The dosages of methacholine and the concentrations of methacholine solutions used for intravenous injections during the lung function test

Dose (μg/kg)	Dose mg/mL
0	0
50	0.01562
100	0.03125
200	0.0625
400	0.125
800	0.25

19. Supplemented PBS: 3% (w/v) bovine serum albumin (BSA, heat shock fraction, protease free, low endotoxin, suitable for cell culture, $\geq 98\%$) with protease inhibitor prepared in PBS, pH 7.4. Dissolve 0.3 g of BSA and 1 tablet of commercially available protease inhibitor cocktail tablet in 10 mL of sterile PBS.
20. Sterile RPMI 1640: supplemented with 10% fetal calf serum (FCS), L-glutamine (200 mM in 0.85% NaCl stock solution), 100 U/mL penicillin, 100 μ g/mL streptomycin. To make 500 mL, add 50 mL of FCS (complement inactivated) and 0.5 mL of 2 mM L-glutamine in 0.85% NaCl working solution (freshly diluted from stock), and 5 mL of PenStrep (10,000 U/mL penicillin and 10,000 μ g/mL streptomycin). (*see Note 1*).
21. 1.5-mL Microfuge tubes.
22. 15-mL Tubes.
23. 24-Well cell culture plates.

Table 3
Weights of mice and corresponding volumes of the methacholine solutions to be injected

Weight (g)	Injection volume (μL)
20	64
21	67
22	70
23	74
24	77
25	80
26	83
27	86
28	90
29	93
30	96
31	99
32	102
33	106
34	109
35	112
36	115
37	118
38	122
39	129
40	128

24. 1-mL MiniCollect[®] serum gel tubes (Greiner Bio-One) or equivalent.
25. 2-mL cryogenic vials.

**2.5 Analysis
of the Infiltration
of Inflammatory Cells
in BALF**

1. Microscope slides: 76×26 mm.
2. Shandon filter cards.
3. Cytospin cuvette.
4. Cytospin metal slide holder and its driver.
5. Cytocentrifuge (e.g., Shandon Cytospin 3).
6. Temperature-controlled centrifuge.
7. Aspirator.

8. 70% Ethanol: To make 100 mL, mix 70 mL of absolute ethanol with 30 mL of ultrapure water.
9. Micropipettes with associated pipette tips.
10. Automated cell counter or hemocytometer (e.g., Coulter Counter Z1, single-threshold model, Beckman Coulter).
11. Red blood cell (RBC) lysis buffer: commercially available (e.g., Lyzerglobin, Avantor B.V. Deventer).
12. Sterile PBS.
13. 5% bovine serum albumin (BSA): Dissolve in PBS, pH 7. Use heat shock fraction, protease free, low endotoxin, suitable for cell culture, $\geq 98\%$.
14. Sterile lysis buffer: 155 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM EDTA.
15. Diff-Quick staining set: commercially available.
16. Light microscope with 20 $\times$, 40 $\times$, and 100 $\times$ objective lenses.
17. Immersion oil.
18. Differential cell counter.

**2.6 Preparation
of Single-Cell
Suspensions of Lung
Tissue, Spleen,
and Draining Lymph
Nodes (DLNs)**

1. Ice in a bucket.
2. 24-Well culture plates.
3. Petri dishes.
4. Sterile scalpels and scalpel blades.
5. Micropipettes with associated pipette tips.
6. 50-mL Conical tubes.
7. 70- μm Nylon cell strainers.
8. 5-mL Syringe.
9. Biosafety cabinet: down-flow cabinet with a closed suction system.
10. Temperature-controlled centrifuge.
11. Sterile RPMI 1640: supplemented with 10% fetal calf serum (FCS), L-glutamine (200 mM in 0.85% NaCl stock solution), 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin. To make 500 mL, add 50 mL of FCS (complement inactivated) and 0.5 mL of 2 mM L-glutamine in 0.85% NaCl working solution (freshly diluted from stock), and 5 mL of PenStrep (10,000 U/mL penicillin and 10,000 $\mu\text{g}/\text{mL}$ streptomycin). *see Note 1.*
12. Collagenase A: 4 mg/mL.
13. DNase I: 0.1 mg/mL.
14. Cell counter.
15. 10-mL Coulter counter cups.

16. Flow cytometry diluent reagent: ISOTON[®] II Diluent (Beckman Coulter) or equivalent.
17. Red blood cell (RBC) lysis buffer: commercially available (e.g., Lyzerglobin, Avantor B.V. Deventer).
18. Cryogenic vials.
19. Benchtop cooler or ice bucket.
20. -80°C Freezer.
21. Liquid nitrogen storage.
22. Hanks' Balanced Salt solution (HBSS): 140 mM NaCl, 5.0 mM KCl, 1.0 mM CaCl_2 , 0.4 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.4 mM KH_2PO_4 , 6.0 mM D-glucose, and 4.0 mM NaHCO_3 , pH 7.4. To make 1000 mL, dissolve 8.0 g of NaCl, 0.4 g of KCl, 140 mg of CaCl_2 , 100 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mg of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 60 mg of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 60 mg of KH_2PO_4 , 1 g of D-glucose, and 350 mg of NaHCO_3 in about 800 mL of ultrapure water. Adjust pH to 7.4 and bring the volume up to 1000 mL. Sterilize with a $0.25\text{-}\mu\text{m}$ filter. Store at 4°C .
23. Storage medium for cells in liquid nitrogen: 40% FCS and 10% DMSO in HBSS.

2.7 Restimulation of Lung Cells and Draining Lymph Node (DLN) Cells

1. Biosafety cabinet: down-flow cabinet with a closed suction system.
2. Single-cell suspension resulted from Subheading 3.10.
3. Temperature-controlled centrifuge.
4. CO_2 incubator.
5. Petri dishes.
6. Sterile scalpels and scalpel blades.
7. Micropipettes with associated pipette tips.
8. Supplemented RPMI 1640: See Subheading 2.6, item 11.
9. Sterile U-bottom 96-well cell culture plate.
10. GP rough extract or HDM crude extract: See Subheading 2.1, items 6 (GP) and 7 (HDM).

2.8 Quantification of Lung Single-Cell Suspensions Using Flow Cytometry

1. FACS tubes (polystyrene) for samples and singles during staining.
2. $30\text{-}\mu\text{m}$ Filter top FACS tubes.
3. Temperature-controlled centrifuge.
4. Three-laser flow cytometer (e.g., FACSVerse).
5. FACS buffer: 1% BSA in PBS.

6. Block buffer for extracellular blocking: 2% normal rat serum (NRS) and 5% FcBlock (purified and unlabeled CD16/32 antibody) in FACS buffer.
7. Block buffer for intracellular blocking: 2% NRS and 5% FcBlock in PERM buffer (*see item 10* in this section).
8. Extracellular staining antibodies: diluted in FACS buffer according to suppliers' recommendations (*see* Tables 2 and 3). In some cases, the dilution of every antibody can be adjusted depending on the intensity of the fluorescence signal.
9. Fixable LIVE/DEAD™ (L/D) V450 cell stain: Predilute to 1:1000 in PBS prior to use.
10. Foxp3/transcription factor staining buffer set: a commercial kit containing the following components:
 - (a) Fixation/permeabilization concentrate.
 - (b) Fixation/permeabilization diluent (FIX).
 - (c) Permeabilization buffer (PERM buffer).

2.9 Homogenization of Lung Tissue for Total Protein and Cytokine Analysis

1. Homogenizer (e.g. IKA Werke T10 basic Ultra-Turrax, Germany).
2. 1.5-mL Microfuge tubes.
3. 96-Well flat-bottom ELISA plates.
4. Cryogenic vials.
5. ELISA plate reader.
6. BCA protein assay kit: commercially available.
7. Demineralized water.
8. Tween 20.
9. 70% Ethanol.
10. Luminex buffer: 50 mM Tris-HCl, 150 mM NaCl, 0.002% Tween 20, pH 7.5. To make 100 mL, add 0.6 g of Tris-HCl, 0.9 g of NaCl, and 2.0 μ L of Tween 20. Optionally, add 1 tablet of complete protease inhibitor cocktail (e.g., cComplete Mini) per 100 mL of buffer and 1 tablet of PhosSTOP phosphatase inhibitor cocktail per 100 mL of buffer. Luminex buffer can be aliquoted and stored at -20°C .

2.10 Biotinylation of Allergens for splgE ELISA

We use a “home-made” biotinylated allergen for the detection of allergen-specific IgE using ELISA. Biotinylation of GP and HDM is performed using a commercially available biotinylation reagent.

1. Biotinylation reagent: EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) or equivalent.
2. PBS: pH 7.4. Used as reaction buffer.
3. Desalting columns or dialysis units: Slide-A-Lyzer Dialysis Cassettes 0.1–0.5 mL or equivalent with a molecular-weight cutoff of 3500 kDa. Use for buffer exchange.

2.11 Analysis of Immunoglobulin Levels in Serum with ELISA

For total IgE, IgA, IgG1, IgG2a, and spIgE, unconjugated rat monoclonal antibodies against respective mouse immunoglobulins are used as the capture antibodies. For spIgG1 and spIgG2a ELISA, the allergen extracts are used to coat ELISA plates. For spIgE ELISA, biotinylated antigens are used for detection (*see* Subheadings 2.10 and 3.13, **step 1**). *See* Table 6 for an overview of all antibodies, reagents, and sample types used for these ELISAs.

1. NUNC Maxisorp 96-well flat-bottom ELISA plates or equivalent.
2. Multichannel pipettes and associated tips.
3. ELISA plate washer (optional).
4. Plate shaker.
5. Spectrophotometric microplate reader.
6. ELISA buffer: 50 mM Tris-HCl, 136.9 mM NaCl, 0.05% Tween 20, 2 mM EDTA, 1% BSA, pH 7.2. To make 1000 mL, dissolve 6.06 g of Tris, 8 g of NaCl, 0.744 g of EDTA, and 10 g of BSA in ultrapure water. Adjust pH.
7. Wash buffer: 0.05% Tween 20 in PBS.
8. Capture antibody: rat antimouse IgE (R35-72), rat anti-mouse IgG1 (A85-3), rat antimouse IgG2a (R11-89), or rat anti-mouse IgA antibody (RMA-1).
9. Coating antigen: GP rough extract or HDM crude extract. *See* **items 6** and **7** in Subheading 2.1.
10. Immunoglobulin standards: mouse IgE κ isotype control (C38-2), mouse IgG1 κ isotype control (MOPC-31C), mouse IgG2a κ isotype control (G155-178), mouse IgA, or pooled positive reference serum.
11. Samples: mouse sera collected at different time points (*see* Fig. 1).
12. Detection antibody/antigen: biotinylated rat antimouse IgE, (R35-118), biotinylated rat antimouse IgG1 (A85-1), biotinylated rat antimouse IgG2a (R19-15), biotinylated rat anti-mouse IgA (C10-1), biotinylated GP rough extract, or HDM crude extract (*see* Subheadings 2.10 and 3.13, **step 1**).
13. Avidin horseradish peroxidase (Avidin-HRP).
14. Peroxidase substrate: *o*-phenylenediamine dihydrochloride (OPD).
15. Stop solution: 4 M H₂SO₄.

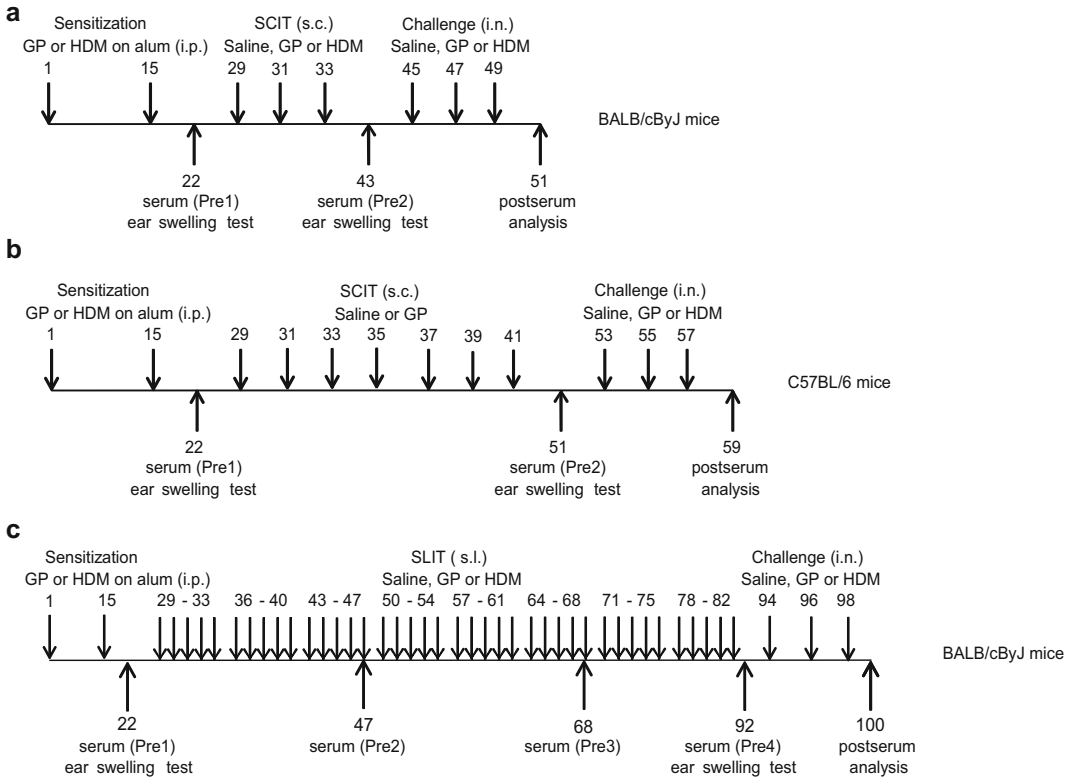


Fig. 1 Overview of AIT-treatment protocols in BALB/cByJ and C57BL/6 mice. **(a, b)** Outline of the SCIT protocols in both mouse strains. **(c)** Outline of the SLIT protocol. Serum is taken before SCIT (Pre1), before challenge (Pre2 in case of SCIT and Pre4 in case of SLIT), and after challenges (Post). In case of SLIT, we include two extra serum time points (Pre2 and Pre3) during SLIT. Ear swelling tests (ESTs) are performed before AIT (to confirm sensitization) as well as after AIT (to confirm the effect of AIT).

2.12 Analysis of Cytokine Levels in BALF, Supernatant of Restimulated Single-Cell Suspensions and Lung Tissue Homogenates

1. Commercially available ELISA kits for cytokines of interest, such as IL-4, IL-5, IL-10, IL-13, and IFN γ .
2. Mouse cytokine multiplex assay: Mouse Magnetic Luminex Screening Assay (R&D Systems) or equivalent multiplex assay. Choose your cytokines of interest and combine them in a single assay system.
3. Multichannel pipettes and associated tips.
4. ELISA plate washer (optional).
5. Plate shaker.
6. Spectrophotometric microplate reader.

3 Methods

3.1 Sensitization

For allergen sensitization, both BALB/cByJ and C57BL/6 mice are given either GP or HDM allergen by intraperitoneal injections on Days 1 and 14, using alum-adsorbed allergen extracts.

1. Before preparing the sensitization solution, thaw the aliquoted stocks of grass pollen (GP, *Phleum pratense*; *Phl p*) or house dust mite (HDM, *Dermatophagoides pteronyssinus*; *Der p*) and mix Inject Alum (or an equivalent aluminum hydroxide adjuvant) well by shaking or vortexing until thoroughly emulsified.
2. Under a sterile condition, add 20 μL of the alum adjuvant to 80 μL of each allergen stock. The final concentration of allergens will be 8 μg of *Phl p*5a for GP sensitization and 5 μg of *Der p* for HDM sensitization, in a total of 100 μL of allergen-alum solution per mouse. Prepare fresh.
3. Randomly assign mice to GP or HDM sensitization groups.
4. Using 1-mL syringes with 25G needles, intraperitoneally inject 100 μL of the appropriate sensitization solution to each mouse assigned for the allergen (*see Note 2*).
5. Repeat **steps 1–4** again on Day 14.

3.2 SCIT Treatments

3.2.1 SCIT Treatment of BALB/cByJ Mice

For SCIT treatment of BALB/cByJ mice, subcutaneous allergen injections are given on Days 29, 31, and 33. *See* Fig. 1a for the timeline of the treatment schedule.

1. Randomly assign the allergen-sensitized mice to SCIT or control groups to start the treatments on Day 29.
2. On Day 29, thaw the aliquoted stocks of grass pollen (GP) or house dust mite (HDM) to prepare allergen solutions for SCIT injections. Dilute each allergen stock in sterile PBS to achieve the final concentrations of SCIT solutions below (100 μL /mouse).
 - (a) For GP: Dilute 5.2 μL of the *Phl p* stock (96 $\mu\text{g}/\mu\text{L}$) in 94.8 μL of sterile PBS to the final concentration of 500 μg *Phl p* in 100 μL of PBS.
 - (b) For HDM: Dilute 5 μL of the *Der p* stock (50 $\mu\text{g}/\mu\text{L}$) in 95 μL of sterile PBS to the final concentration of 250 μg of *Der p* in 100 μL of PBS.
3. Subcutaneously inject 100 μL of the appropriate SCIT solution to each mouse using 1-mL syringes with 25G needles (*see Note 3*). Use sterile PBS to inject SCIT control mice.
4. Repeat **steps 1–3** again on Days 31 and 33.

3.2.2 SCIT Treatment of C57BL/6 Mice

For SCIT treatment of C57BL/6 mice, subcutaneous allergen injections are given on Days 29, 31, 33, 35, 37, 39, and 41. *See* Fig. 1b for the timeline of the treatment schedule. Given the sensitivity of C57BL/6 for anaphylactic responses, we use an incremental up dosing scheme for SCIT treatments (*see step 2* below). In addition, the mice should be monitored for potential adverse responses during the first injections. For this reason, we routinely

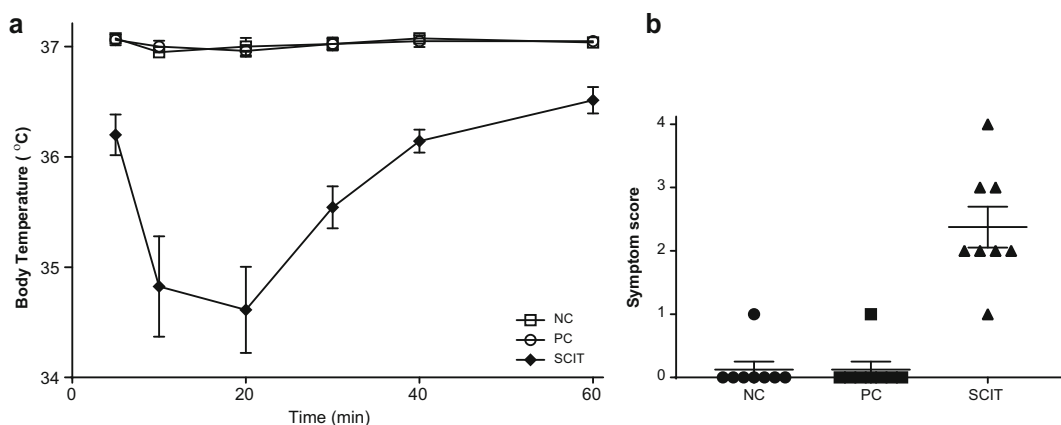


Fig. 2 Monitoring symptoms of anaphylaxis in sensitized mice following the first subcutaneous GP injections. **(a)** Changes in rectal temperature following SCIT at the indicated time points. **(b)** Peak anaphylactic symptom score of each individual mouse within 40 min after the first SCIT. Absolute values are expressed as mean $\pm$ SEM ($n = 8$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ compared to the positive control. NC negative control, PBS challenged, PC positive control, GP challenged; SCIT-treated mice (50 μ g of GP), GP challenged

measure body temperature and score shock symptoms after each injection (*see* **Note 4**, Fig. 2).

1. Randomly assign the allergen-sensitized mice to SCIT or control groups to start the treatments on Day 29.
2. Thaw the aliquoted stocks of GP or HDM to prepare allergen solutions for SCIT injections (*see* **Note 4**). Dilute each allergen stock in sterile PBS to achieve the final concentrations of SCIT solutions below (100 μ L/mouse).
 - (a) For GP: Dilute 0.5 μ L of the GP stock (96 μ g/ μ L) in 99.5 μ L of sterile PBS to the final concentration of 50 μ g of *Phl p* in 100 μ L of PBS.
 - (b) For HDM: Dilute 5.0 μ L of the HDM stock (50 μ g/ μ L) in 95.0 μ L of sterile PBS to the final concentration of 250 μ g of *Der p* in 100 μ L of PBS.
3. Subcutaneously inject 100 μ L of the appropriate SCIT solution to each mouse using 1-mL syringes with 25G needles (*see* **Note 3**). Use sterile PBS to inject SCIT control mice.
4. Measure body temperature at 20, 40, and 60 min after the SCIT injection using a rectal thermometer (*see* Fig. 2). Observe mice and record shock symptom scores according to the scoring criteria listed in **Note 4**.

5. Repeat **steps 1–4** again on Days 31, 33, 35, 37, 39, and 41, *except* change the concentrations of GP SCIT solution by appropriately diluting the GP stock as follows (100 μ L/mouse):
 - (a) Day 31: Dilute 1.0 μ L of the GP stock in 99.0 μ L of sterile PBS to the final concentration of 100 μ g of *Phl p* in 100 μ L of PBS.
 - (b) Day 33: Dilute 2.1 μ L of the GP stock in 97.9 μ L of sterile PBS to the final concentration of 200 μ g of *Phl p* in 100 μ L of PBS.
 - (c) Day 35: Dilute 4.2 μ L of the GP stock in 95.8 μ L of sterile PBS to the final concentration of 400 μ g of *Phl p* in 100 μ L of PBS.
 - (d) Days 37, 39, and 41: Dilute 5.2 μ L of the GP stock in 94.8 μ L of sterile PBS to the final concentration of 500 μ g of *Phl p* in 100 μ L of PBS.

For HDM SCIT in C57BL/6 mice, simply recalculate a similar up dosing scheme as described above.

3.3 SLIT Treatments

SLIT treatment is applied 5 days a week for a total of 8 consecutive weeks from day 29 through day 82 by sublingual administration (*see Note 5*) of the allergen in PBS. *See Fig. 1c* for the timeline of the treatment schedule.

1. Randomly assign the allergen-sensitized mice to SLIT or control groups to start the treatments on Day 29.
2. Thaw the aliquoted stocks GP) or HDM to prepare allergen solutions for SLIT applications. Dilute each allergen stock in sterile PBS to achieve the final concentrations of SLIT solutions below 5 μ L/mouse.
 - (a) For GP: Dilute 5.2 μ L of the GP stock in 94.8 μ L of sterile PBS to the final concentration of 500 μ g of *Phl p* in 100 μ L of PBS.
 - (b) For HDM: Dilute 5 μ L of the HDM stock in 95 μ L of sterile PBS to the final concentration of 250 μ g of *Der p* in 100 μ L of PBS.
3. Sublingually apply 5 μ L of the appropriate SLIT solution to each mouse using a P20 pipette (*see Note 5*). Use sterile PBS for SLIT control mice.
4. Repeat **steps 1–3** again 5 days a week for 8 consecutive weeks until day 82 (*see Fig. 1c*).

3.4 Allergen Challenges

Allergen challenges are performed by intranasal administration (*see Note 6*; Fig. 1) 2 weeks after the cessation of SCIT or SLIT treatments. For SCIT-treated BALB/cByJ mice, the challenge

days are days 45, 47, and 49, while SCIT-treated C57BL/6 mice are challenged on days 53, 55, and 57. For SLIT treatment, BALB/cByJ mice are challenged on days 94, 96, and 98.

1. Thaw the aliquoted stocks of GP or HDM to prepare allergen solutions for intranasal challenge. Dilute each allergen stock in sterile PBS to achieve the final concentrations of allergen solutions below 25 μL /mouse.
 - (a) For GP: Dilute 1 μL of the GP stock in 59 μL of sterile PBS to the final concentration of 40 μg of *Phl p* in 25 μL of PBS.
 - (b) For HDM: Dilute 1 μL of the HDM stock in 50 μL of sterile PBS to the final concentration of 25 μg of *Der p* in 25 μL of PBS.
2. Anesthetize a mouse using 4.5% isoflurane in combination with 1 mL/min oxygen until its breathing starts to slow down. Remove the mouse from anesthesia.
3. Restrain a mouse by gently gripping the nape with one hand and anchoring the tail between the small finger and the palm. Hold the mouse in a supine position with the head elevated.
4. Right before the mouse wakes up (approximately within 1 min), position the end of a micropipette at or in the external nares. Administer 25 μL of the appropriate allergen solution to each mouse, by placing it as a droplet on the nose. Watch as the mouse strongly inhales the droplet, usually splitting into both nasal cavities (*see* **Note 6**).
5. Repeat **steps 1–4** until all the mice are challenged.

3.5 Blood Withdrawal via Retro-Orbital Puncture

To monitor the response to SLIT or SCIT treatments, blood is collected by retro-orbital puncture after allergen challenge. The timing of blood collections for each experimental paradigm is indicated as “Pre1 and Pre 2” for SCIT and “Pre1–4” in the “SLIT protocol” in Fig. 1a–c.

1. Anesthetize a mouse using 4.5% isoflurane in combination with 1 mL/min oxygen. Confirm deep anesthesia with the absence of a pedal reflex.
2. Place the anesthetized mouse on a flat surface. Gently press the body to force its blood from the thorax to the head.
3. Using the forefinger of the same hand holding the mouse down, pull the dorsal eyelid back to produce slight exophthalmos (bulging of the eye).
4. Penetrate the orbital conjunctiva at the medial or lateral canthus of the eye with a glass microcapillary tube. As soon as blood accumulates in the capillary, lift up the mouse and hold it above the MiniCollect tube to collect 10 drops of blood (*see* **Note 7**).

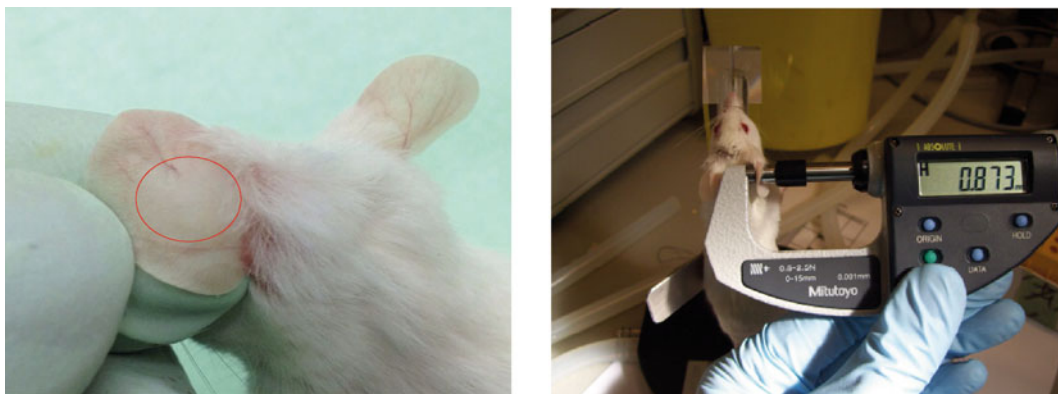


Fig. 3 The ear swelling test. *Left*: Anesthetized mice are intradermally injected with 10 μ L of PBS in the left ear as a control using a small insulin syringe. A small swelling will be visible just below the skin. *Right*: after 2 h, ear thickness of both ears is measured using a micrometer. It is important to keep the micrometer in a horizontal position

5. Process further to collect serum. Ensure that the MiniCollect[®] Cross-Cut Cap is properly placed back on the tubes and the tubes are centrifuged at $3000 \times g$ for 10 min at room temperature. Gel separation tubes should be centrifuged no later than 2 h after collection.

3.6 Ear Swelling Test

To monitor modulation of the early phase response to allergen provocation by SCIT or SLIT, the ear swelling test (EST) is performed before and after the treatments. To minimize discomfort to the experimental animals due to repeated anesthesia, the EST is carried out at the same time as the blood draws (Fig. 1).

1. Prepare test solutions to inject in the ear to determine local responses. For a positive control, use either 1 μ g of *Phl p* or 0.5 μ g of *Der p* in 10 μ L of sterile PBS. For a negative control, use sterile PBS only.
2. Anesthetize a mouse using 4.5% isoflurane/min O_2 as described in Subheading 3.5, step 1.
3. Inject the mouse with the selected allergen test solution intradermally in the right ear (Fig. 3).
4. Inject sterile PBS intradermally in the mouse's left ear as a negative control reference.
5. After 1–2 h, anesthetize the mice again using 4.5% isoflurane/min O_2 and measure the ear thicknesses in micrometers (*see Note 8*).
6. Calculate the allergen-induced net increase in ear thickness (Δ , in μ m) by subtracting the left ear thickness from that of the right ear (Fig. 4a).

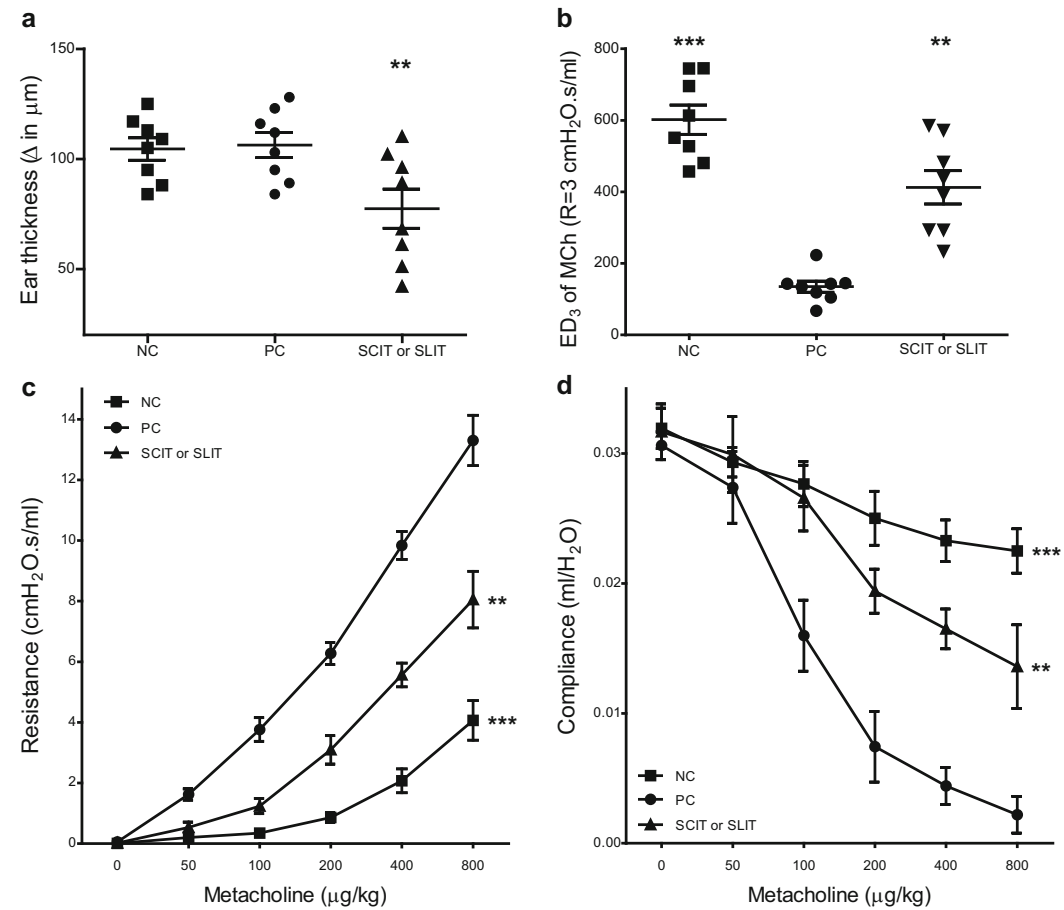


Fig. 4 Clinical manifestations after AIT. (a) IgE-dependent allergic response performed after AIT. Ear thickness (μm) in the right ear was measured 2 h after GP injection (1 kSQ) and differences in the thickness were compared to the left ear, which received PBS as a control. Placebo-treated mice (NC and PC) showed a similar swelling since both had not been challenged yet. (b) Effective Dose (ED_3) of methacholine, when the airway resistance reaches $3 \text{ cmH}_2\text{O.s/mL}$. Airway hyperactivity (AHR) was measured by FlexiVent and plotted as (c) airway resistance (R in $\text{cmH}_2\text{O.s/mL}$) and as (d) airway compliance (c in $\text{mL/cmH}_2\text{O}$). Absolute values are expressed as mean $\pm$ SEM ($n = 8$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ compared to positive control. NC negative control, PBS challenged, PC positive control, GP challenged, SCIT- or SLIT-treated mice (300 kSQ), GP challenged

3.7 Lung Function Measurement

Lung functions of experimental mice are tested 2 days after the final allergen challenge. Here, we describe the method to assess lung functions using the FlexiVent version 5.3. In this setup, we routinely use intravenous methacholine administration in combination with the use of flexible cannulas for tracheal intubation, although the use of inhaled methacholine and rigid tracheal intubation has also been described by others [38]. In our experience, invasive measurement of airway resistance affects immunohistochemical analyses of lung tissue, including airway wall remodeling and

inflammation. Therefore, we recommend that histological analyses be performed in a separate group of mice to make sure that lung tissue architecture is not disrupted by the prior FlexiVent analysis.

3.7.1 Preparation

1. Prior to the measurement, calibrate the computer-controlled small-animal ventilator (e.g., FlexiVent) according to the manufacturer's instructions (*see Note 9*). Perform weight-adjusted calibration of both airway and cylinder pressure for each animal using a 1-mL syringe, a manometer, and a closed and an open cannula (*see Note 9*).
2. Two days after the final allergen challenge, weigh and anesthetize mice with 75 mg/kg ketamine (100 mg/mL) and 1 mg/kg domitor (0.5 mg/mL) in sterile PBS by intraperitoneal injection (10 μ L of mix per gram bodyweight). *See* the dilution scheme shown in Table 1.
3. Upon confirmation of deep anesthesia with the absence of a pedal reflex, place the mouse in a supine position on an operating table for tracheal and jugular cannulations (*see Note 10*).
4. For tracheal cannulation, first, make an incision in the middle of the neck and carefully remove the underlying tissue with two sharp pairs of forceps to reveal the muscle bundles that cover the trachea. Do not touch the glands around the trachea, as this will trigger enhanced mucus production.
5. Move the muscle bundles aside to reveal the trachea, which is then freed from the underlying tissue.
6. Place two ligatures under the trachea, one more proximal to the oral cavity but still leaving room for making the incision to insert the cannula, and the other more distally, close to the bifurcation leading to the primary bronchi.
7. Open the trachea by making a small cut in between the tracheal cartilage rings, leaving the two ligatures below the incision site. Place the cannula in the trachea and fix the tracheal cannula in an airtight fashion by carefully closing both ligatures to prevent the cannula from sliding up or down within the trachea.
8. Attach the tracheal cannula to the FlexiVent and ventilate using the standard breathing program with the script running on basic breathing (*see Note 11*).

3.7.2 Methacholine Challenge

1. Prepare methacholine solutions with different concentrations for intravenous injections according to Tables 2 and 3.
2. Methacholine will be introduced via the jugular vein. To position the jugular vein, draw an imaginary line from the right ear and its left armpit, and between the chin and its right armpit. The crossing point of these two lines identifies the location of

incision (Suppl. Fig 1). Make a 1.5-cm vertical incision downward.

3. Carefully remove the underlying tissue with two sharp pairs of forceps until the jugular vein is revealed, clearing any fat or surrounding connective tissue (*see Note 12*).
4. Place a ligature (6/0) around the upper part of the jugular vein and close it lightly (Suppl. Fig 1). Secure the ligature to the operating table with a small piece of tape, in order to put some tension on it.
5. After the tension is sufficient to stretch the jugular vein slightly, place a second ligature (6/0) approximately 0.5 cm below the first and close it lightly but not completely, still allowing sufficient space for the cannula to be placed into the jugular vein.
6. Place a bulldog clamp on the lower ligature to increase the tension on the jugular vein and make a small cut in the vein between the two ligatures. Carefully place the cannula into the jugular vein through this opening, and tightly close the bottom ligature (*see Note 13*).
7. Once the proper placement of the cannula is confirmed, fix it in position by tightly closing both upper and lower ligatures. Now, the jugular vein cannula can be used for intravenous methacholine delivery when prompted by the FlexiVent's protocol (*see Note 11*).
8. Prior to the FlexiVent measurements, administer an intraperitoneal injection of rocuronium bromide (1 $\mu\text{L/g}$ mouse body weight).
9. Set the Positive end-expiratory pressure (PEEP) at 20 mmH_2O .
10. Administer the appropriate volume of saline (0 $\mu\text{g/kg}$ methacholine) intravenously through the jugular cannula as a blank for the subsequent methacholine injections. *See Table 3* for injection volumes.
11. Measure airway responsiveness by obtaining airway resistance (R in $\text{cmH}_2\text{O.s/mL}$), the Newtonian resistance (the resistance of the central or conducting airways, Rn in $\text{cmH}_2\text{O.s/mL}$), and lung compliance (C in $\text{mL/H}_2\text{O}$).
12. Inject the next dose of methacholine (50 $\mu\text{g/kg}$ body weight) listed in Table 3. After the injection of methacholine solution, immediately flush the tubing of the jugular vein cannula with 30 μL of saline to make sure that all the methacholine enters the body in a single bolus (*see Note 14*). Measure airway responsiveness as described in **step 11**.

Table 4
Antibodies used for FACS analysis of innate lymphocytes

Specificity	Clone	Isotype	Staining
Brilliant Violet 605™ anti-mouse Ly-6A/E (Sca-1) antibody	D7	Rat IgG2a, κ	Extracellular
PerCP/Cy5.5 anti-mouse/human KLRG1 (MAFA) antibody	2F1/ KLRG1	Syrian hamster IgG	Extracellular
Anti-Mouse CD3e PE	145-2C11	Armenian Hamster IgG	Extracellular
Anti-Mouse CD5 PE	53-7.3	Rat IgG2a, κ	Extracellular
Anti-Mouse CD19 PE	eBio1D3 (1D3)	Rat IgG2a, κ	Extracellular
Anti-Mouse NK1.1 PE	PK136	Mouse IgG2a, κ	Extracellular
Anti-Mouse Fc epsilon Receptor I alpha (FcεR1) PE	MAR-1	Armenian Hamster IgG	Extracellular
Anti-Mouse CD11b PE	M1/70	Rat IgG2b, κ	Extracellular
Anti-Mouse CD11c PE	N418	Armenian Hamster IgG	Extracellular
Anti-Mouse Ly-6G (Gr-1) PE	RB6-8C5	Rat IgG2b, κ	Extracellular
Anti-Mouse TER-119 PE	TER-199	Rat IgG2b, κ	Extracellular
T1/ST2 (IL-33R) Monoclonal Antibody, FITC	DJ8	IgG1	Extra- and Intracellular
Anti-Mouse CD45 APC	30-F11	Rat IgG2b, κ	Extracellular
Anti-Mouse CD127 APC-eFluor® 780	A7R34	Rat IgG2a, κ	Extracellular
Anti-Human/Mouse Gata-3 PE-Cyanine7	TWJ	Rat IgG2b, κ	Intracellular

13. Continue with 100, 200, 400, and 800 µg/kg methacholine doses (*see* Tables 3 and 4), with an additional administration of rocuronium bromide after the methacholine dosage of 100 µg/kg. Repeat the measurements as described in **step 11** after each methacholine administration.
14. For the analysis of the lung function test, export all FlexiVent data as a comma separated value (CSV) format and store the data files in an appropriate location such as a backed-up network drive for analysis (*see* **Note 15**).

3.8 Collection of Blood, Bronchoalveolar Lavage, and Lung Tissue

1. Immediately after the completion of FlexiVent measurements, sacrifice the mouse by collecting a large volume of blood through the vena cava (post-serum). Under continued anesthesia, open the abdomen and reposition the bowels to reveal the vena cava. After removing fat, puncture the vena cava using

a 25G needle on a 1-mL syringe. Up to 1 mL of blood should be collected.

2. For the collection of the bronchoalveolar lavage fluid (BAL), open the diaphragm to allow the lungs to collapse. It is important to make sure not to damage (puncture) the lungs when opening up the thoracic cavity.
3. Using the tracheal cannula and a 1-mL syringe, lavage the lungs with 1 mL of the supplemented PBS at room temperature. During the first drawback of this BALF, a small volume may remain behind in the lungs. Store this first 1 mL of BALF separately in a 1.5 mL tube and keep on ice until the cells and fluid are separated by centrifugation in **step 5** below.
4. Repeat the lung lavage four more times using regular nonsupplemented PBS at room temperature. Pool the BALF from the four lavages in a 15-mL tube and keep on ice.
5. Spin down the initial BALF collected in **step 3** at $590 \times g$ at 4°C for 5 min. Transfer the supernatant in clean microcentrifuge tubes in 100 μL aliquots and store at -80°C until used for cytokine ELISA in Subheading 3.14.
6. Resuspend the resulting cell pellets from **step 5** in 1 mL of PBS and combine with the rest of the BALF sample collected in a 15-mL tube in **step 4**. Keep this BAL cell suspension on ice until used for cytopsin slide preparation or flow cytometry in Subheading 3.9.1 or Subheading 3.12, respectively.
7. Finally, collect individual lung lobes and any other necessary organs in 1 mL of ice-cold RPMI 1640 medium for preparation of a single-cell suspension in Subheading 3.10.
8. Alternatively, collect individual lung lobes in cryogenic vials, snap-freeze immediately by submerging the tubes in liquid nitrogen, and store at -80°C until processed for ELISA in Subheadings 3.13 and 3.14 (*see Note 16*).

3.9 Analysis of the Infiltration of Inflammatory Cells in BALF

Cell compositions of BALF or lung tissue can be analyzed either by cytopsin preparations or by flow cytometry. The cytopsin preparations require minimal time investment on the section day of the experiment but need to be differentially counted thereafter, which requires expertise and additional time. In contrast, flow cytometric measurements have a greater capacity to phenotype cells based on their granularity and allow immediate analysis.

3.9.1 Cytological Analysis with Cytopsin Preparations

1. Label cytopsin slides for the identification of the samples.
2. To coat the slides with BSA, assemble each cytopsin holder by inserting a labeled slide and a Shandon filter card and attaching a cuvette in the holder above the filter card.

- Place the assembled holder into the cytocentrifuge. Add 20 μL of PBS containing 1% BSA in the cuvette and spin at $550 \times g$ for 1 min.
- Centrifuge the BALF cells from **step 6** in Subheading 3.8 at $590 \times g$ for 5 min at 4°C . Discard the supernatant.
- Resuspend the cell pellets in 500 μL of RBC lysis buffer and incubate for 1 min at room temperature.
- Centrifuge the cells $590 \times g$ for 5 min at 4°C , discard the supernatant, and resuspend the pellet in 200 μL of PBS containing 1% BSA.
- Count the cell number in the BALF using a cell counter or hemocytometer for total BALF cell count (Fig. 5a). Adjust the

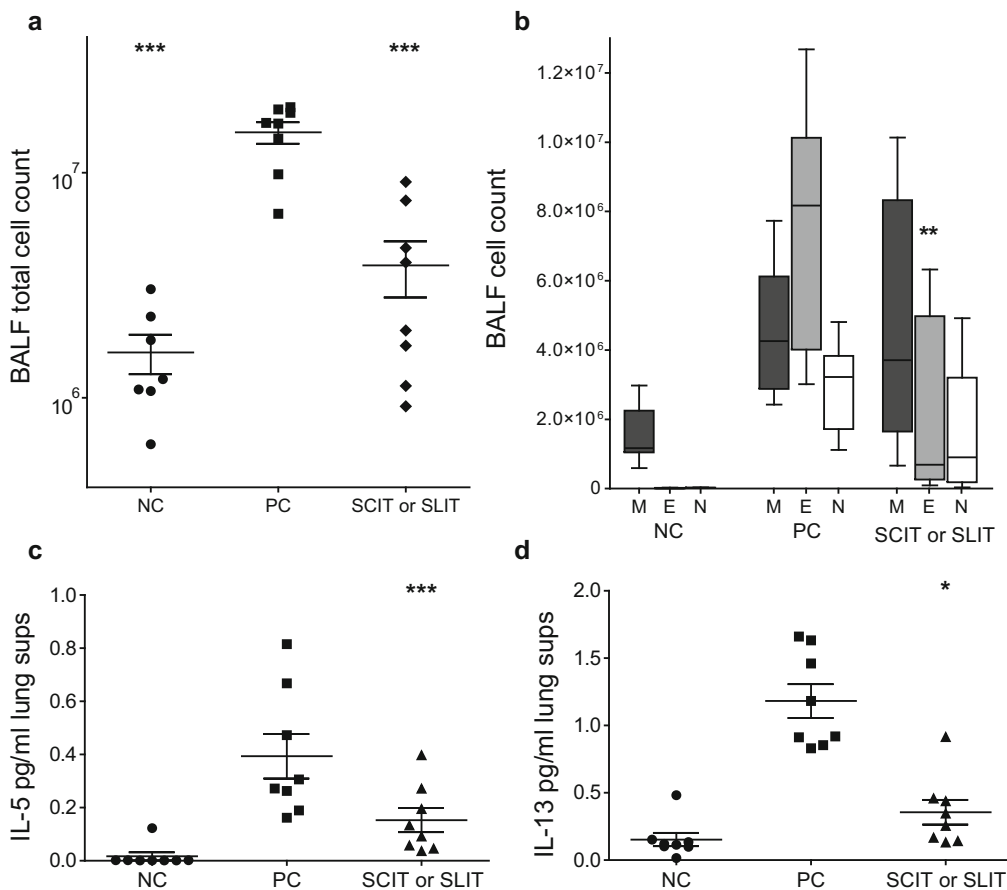


Fig. 5 Eosinophilic and proinflammatory cytokine responses after AIT. **(a)** Total cell counts in bronchoalveolar lavage fluid (BALF). **(b)** Differential cytospin cell counts in BALF. *M* mononuclear cells, *E* eosinophils, *N* neutrophils. Absolute numbers are plotted as median and 10–90 interquartile. **(c, d)** Concentrations of IL-5 and IL-13 measured in restimulated single-cell suspensions of lung cells. Concentrations were calculated as the concentration after 5 day restimulation minus unstimulated control (PBS) and expressed as mean $\pm$ SEM ($n = 8$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ compared to positive control

volume of the cell suspension with PBS with 1% BSA to achieve a BALF cell density of 1×10^6 cells/mL.

8. Prepare cytopspins by spinning down 100 μ L of the cell suspension (1×10^5 cells) onto the coated slides at $550 \times g$ for 5 min at room temperature. Carefully release the slides from the holder and let the slides air-dry at room temperature for 10 min. Clean the cuvettes using demineralized water and 70% ethanol solution.
9. Stain the cytospin slides using a Diff-Quick staining set according to the manufacturer's protocol. Dry thoroughly and cover-slip the slides.
10. Perform differential counts of 300 cells per cytospin by identifying mononuclear cells (M), neutrophils (N), and eosinophils (E) by standard morphology using a light microscope at $100\times$ magnification (*see* **Note 17**). The results may be graphed as shown in Fig. 5b.
11. Alternatively, the BAL cell suspension from **step 7** may be used for flow cytometric analysis. *See* Subheading 3.11 to proceed with flow-cytometry-based BALF cell analyses.

3.10 Preparation of Single-Cell Suspensions of Lung Tissue, DLNs, and the Spleen

3.10.1 Single-Cell Suspensions from Lung Tissue and DLNs

1. After dissecting the lung tissue from the mouse in **step 7**, Subheading 3.8, transfer the largest left lung lobe to a Petri dish in a biosafety cabinet (*see* **Note 18**).
2. Using a scalpel, cut the lobe into a homogenous paste and resuspended in 2 mL of RPMI 1640 medium with 4 mg/mL Collagenase A, 0.1 mg/mL DNase I, and 1% BSA. Incubated the cells for 1.5 h at 37 °C.
3. To remove tissue fragments, run the lung cell suspension through a 70- μ m cell strainer into a 50-mL tube. Wash the cell strainer with 2–5 mL of RPMI 1640 at room temperature in order to flush out the remaining cells.
4. Centrifuge the cell suspension at $350 \times g$ for 5 min at 4 °C and discard the supernatant. Resuspend the cell pellet in 1 mL of RBC lysis buffer and incubated for 3 min at room temperature.
5. Centrifuge the cell suspension again at $350 \times g$ for 5 min at 4 °C and discard the supernatant. Count the cells using an automated cell counter or hemocytometer.

3.10.2 Single-Cell Suspensions from the Spleen

Single-cell suspensions of spleen cells are obtained in a similar fashion as from the lung tissue, although enzymatic digestion is only required for analysis of DC subsets.

1. Remove the spleen from the mouse in **step 7**, Subheading 3.8. Mince the tissue with a scalpel and strain through a 70- μ m cell strainer in a biosafety cabinet as described in **steps 2** and **3** of

Subheading 3.10.1. Rinse the strainer with 5 mL of RPMI 1640.

2. Centrifuge the spleen cell suspension at $550 \times g$ for 5 min at 4 °C and discard the supernatant. Resuspend the cell pellet in 1 mL of RBC lysis buffer and incubate for 10 min at room temperature.
3. Centrifuge again at $550 \times g$ for 5 min at 4 °C and resuspend in 1 mL of RPMI 1640.
4. Repeat **step 3** one more time, and the cells are ready for further use (*see Note 19*).

3.11 Restimulation of Lung Cells and DLN Cells

To evaluate the T-cell responses to allergen recall, lung and DLN cells prepared in Subheading 3.10 are restimulated with allergens in culture.

1. In U-bottom 96-well plates, seed 200,000 cells/well in 250 μ L of RPMI 1640 supplemented with 10% FCS, pen/strep; 50 μ M beta-mercaptoethanol in the presence of either 0 μ g or 30 μ g of GP or HDM extract in triplo (*see Note 20*).
2. Culture the cells for 5 days at 37 °C and 5% CO₂.
3. Collect the culture media from all wells and store at –80 °C for analysis of cytokines (*see Subheading 3.14*).

3.12 Quantification of DCs, T-Cell Populations, and Innate Lymphoid Cells in Lung Single-Cell Suspensions Using Flow Cytometry

3.12.1 Staining of Extracellular and Intracellular Targets

1. Divide all lung single-cell suspensions in FACS tubes, using one tube per mouse for each staining. In addition, pipette approximately 5 μ L out of every mouse sample and prepare a pooled sample containing a small number of cells from each mouse for the single stains.
2. Wash the cells using 300 μ L of the FACS buffer and centrifuge the cells at $590 \times g$ for 5 min at 4 °C to remove the supernatant.
3. Resuspend the cells in 100 μ L of the FACS buffer. Add 100 μ L of the block buffer (to a total of 200 μ L) for extracellular blocking and incubate for 10 min at room temperature.
4. Centrifuge at $590 \times g$ for 5 min at 4 °C to remove the supernatant. Resuspend the cells in 100 μ L of FACS buffer.
5. Add 100 μ L of an antibody cocktail containing appropriately diluted primary antibodies for extracellular staining to a total of 200 μ L (*see Tables 4 and 5*). Incubate for 30 min at room temperature in the dark.
6. Centrifuge at $590 \times g$ for 5 min at 4 °C and wash in 1 mL of FACS buffer. Repeat the centrifugation and wash again in 1 mL of PBS. Centrifuge again to remove the supernatant.

Table 5
Antibodies used for FACS analysis of T cells and DCs

Specificity	Clone	Isotype	Staining
Brilliant Violet 605™ anti-mouse CD4 Antibody	GK1.5	Rat IgG2b, κ	Extracellular
Anti-Mouse CD103 (Integrin alpha E) FITC	2E7	Armenian Hamster IgG	Extracellular
Anti-Mouse CD11b APC	M1/70	Rat IgG2b, κ	Extracellular
Anti-Mouse CD11c APC-eFluor® 780	N418	Armenian Hamster IgG	Extracellular
Anti-Mouse F4/80 Antigen PE-Cyanine7	BM8	Rat IgG2a, κ	Extracellular
Anti-Human/Mouse Gata-3 PerCP-eFluor® 710	TW4J	Rat IgG2b, κ	Intracellular
Anti-Mouse/Rat Foxp3 PE	FJK-16 s	Rat IgG2a, κ	Intracellular

7. Resuspend the cells in 100 µL of PBS and add 75 µL of fixable L/D V450 cell stain (1:1000 prediluted in PBS). Incubate for 15 min at room temperature in the dark.
8. Wash once in 1 mL of PBS as described in **step 6** and resuspend the cells in 1 mL of FIX. Incubate for 30 min at room temperature in the dark.
9. Without washing, add 1 mL of PERM buffer. After one minute, centrifuge at $590 \times g$ at 4 °C and remove the supernatant. Resuspend the cells in 100 µL of intracellular block buffer and incubate for 10 min at room temperature.
10. Centrifuge at $590 \times g$ at 4 °C, remove the supernatant, and resuspend in 100 µL of PERM buffer.
11. Add 100 µL of the antibody cocktail containing primary antibodies appropriately diluted in PERM buffer for intracellular staining (*see* Tables 4 and 5). Incubate for 30 min at room temperature in the dark.
12. Without washing, add 1 mL of PERM buffer, centrifuge $590 \times g$ at 4 °C, and remove the supernatant. Wash once in 1 mL of FACS buffer and remove the supernatant.
13. Resuspend the cell pellet in 200 µL of FACS buffer and transfer the cells to a FACS tube with a 35-µm cell strainer cap to remove any clumps. The samples are now ready for flow cytometry.

3.12.2 Flow Cytometry

Use different gating strategies depending on the cell types of interest. Here, the gating strategy for ILC2s is described as an example.

1. Set compensation using single stain controls for the antibody panel used.

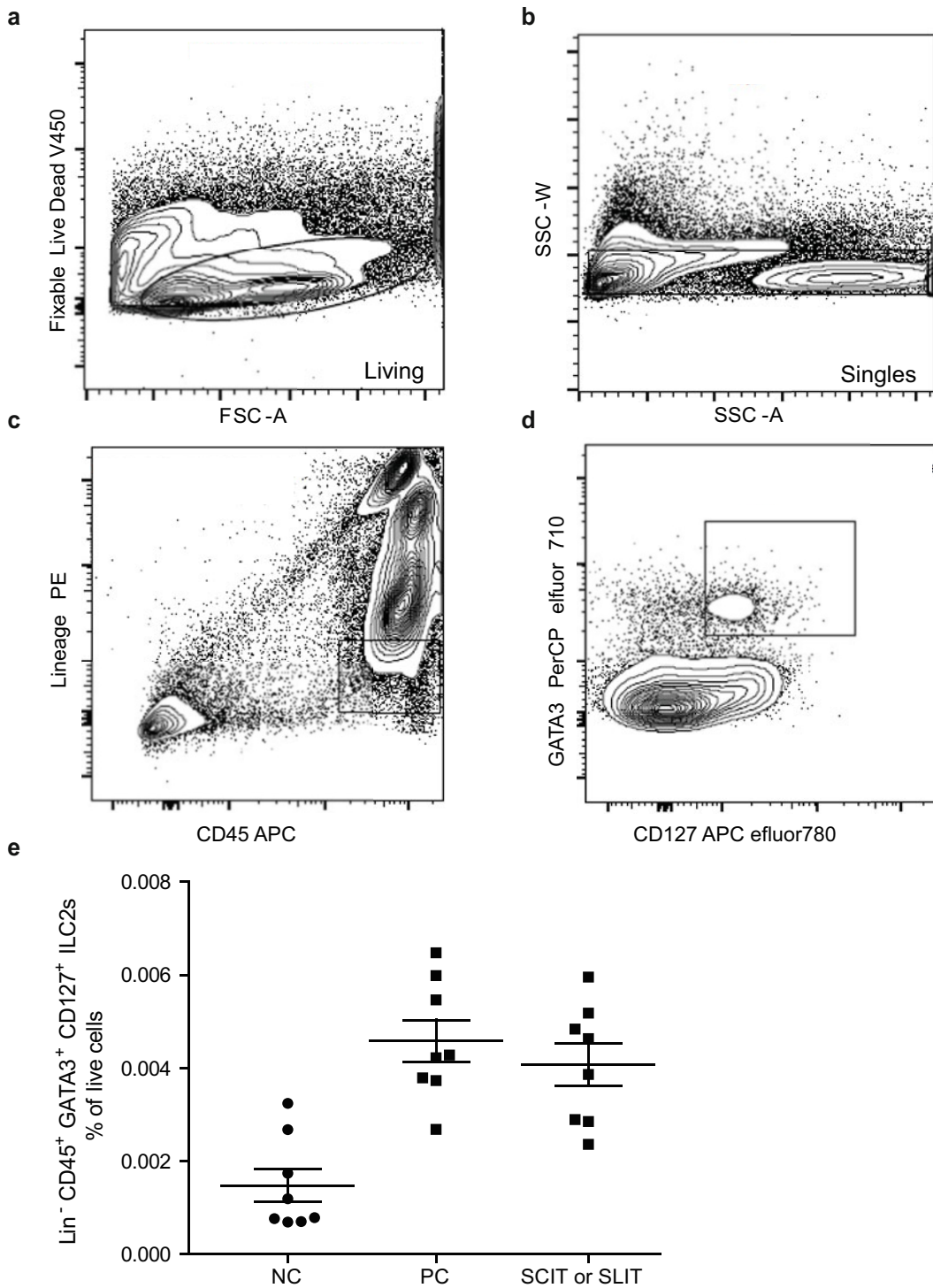


Fig. 6 Gating strategy for the identification of innate lymphoid cells type 2 (ILC2s) in the lung. **(a)** Gating of live cells identified by L/D V450 stain. **(b)** Doublet cell exclusion. **(c)** Gating out the lineage negative cells and including the CD45 positive cells. **(d)** Gating for only the GATA3 and CD127 double-positive cells. **(e)** Lineage $^-$ CD45 $^+$ GATA3 $^+$ CD127 $^+$ ILC2s plotted as % of live cells

2. Exclude all dead cells by plotting the area of the forward scatter (FSC-A) against the fixable L/D marker (Fig. 6a).
3. Exclude all doublets using a small selection gate in the plot of the area of the side scatter (SSC-A) against the width of the side scatter (SSC-W, Fig. 6b).
4. ILC2s may be plotted as Lineage negative cells, CD45-positive cells (*see* Table 4, Fig. 6c), followed by gating on the GATA3- and CD127-positive cells, markers that are both expressed by ILC2s (Fig. 6d).
5. For the identification of other cell types, use CD4 and GATA3 for Th2 cells, CD4 and FoxP3 for Treg cells, and CD11b and CD103 for subpopulations of conventional DCs (*see* Table 5).

3.13 Homogenization of Lung Tissue for Total Protein and Cytokine Analysis

The levels of specific cytokines, chemokines, or other mediators can be measured from either freshly dissected lung lobes or from snap-frozen lung lobes stored at -80°C . Use the identical lung lobe for all mice in the experimental and control groups. Keep the lung tissue on ice at all times.

1. Weigh the cryogenic vials containing the lung lobe and correct for the empty cryogenic vial weight to obtain the net lung lobe weight (milligram of lung tissue).
2. Add Luminex buffer at a ratio of 1:5 (weight: volume). For example, to 1 mg of lung tissue, add 4 μL of the buffer.
3. Homogenize the lung with a homogenizer on ice for at least 1 min, until no large pieces are visible anymore. Clean the homogenizer with tap water and ethanol between samples.
4. Centrifuge the lung homogenate samples at $12,000 \times g$ for 20 min at 4°C .
5. Collect the supernatants in clean microcentrifuge tubes in 100- μL aliquots (*see* Note 21). Store the samples at -80°C until used for ELISA in Subheading 3.14. Discard the pellets.

3.14 Analysis of Immune Responses by ELISA

Immunoglobulin levels in serum and cytokine levels in BALF, culture media of restimulated single-cell suspensions, and lung tissue homogenates can be determined using ELISA (*see* Note 22).

3.14.1 Biotinylation of Allergens for splgE ELISA

Biotinylation of *Phl p* and *Der p* is performed using a commercial biotinylation reagent such as Thermo Scientific EZ-Link Sulfo-NHS-LC-Biotin. The biotinylated allergens are specifically used to detect spIgE by ELISA (*see* Subheading 3.12.2, step 7). According to the manufacturers' protocol, we can adjust the molar ratio of Sulfo-NHS-LC-Biotin to a protein to obtain the level of incorporation desired (~4–6 biotin groups per allergen particle). Since the rough extracts contain a mixture of proteins with various molecular weights, we make an estimate based on SDS-PAGE results. For the rough extract of GP, we use an average molecular weight of 10,000 Dalton.

1. Calculate millimoles of biotin reagent to add to the reaction for a 20-fold molar excess: $\text{mmol biotin} = \text{mL protein} \times (\text{mg protein}/\text{mL protein}) \times (\text{mmol protein}/\text{mg protein}) \times (20 \text{ mmol biotin}/\text{mmol protein})$.
An example: $1000 \text{ } \mu\text{L GP} \times (3.9 \text{ mg of GP}/1 \text{ mL GP}) \times (1 \text{ mmol GP}/10,000 \text{ mg of GP}) \times (5 \text{ mmol biotin}/1 \text{ mmol GP}) = 1.95 \times 10^{-3} \text{ mmol biotin}$.
2. Calculate microliters of 10 mM biotin reagent solution (stock) to add to the reaction: $\mu\text{L biotin} = \text{mmol biotin} \times (1,000,000 \text{ } \mu\text{L}/\text{L}) \times (1/10 \text{ mmol})$.
An example: $1.95 \times 10^{-3} \text{ mmol biotin} \times (1 \times 10^6 \text{ } \mu\text{L}/\text{L}) \times (1/10 \text{ mmol}) = 195 \text{ } \mu\text{L of biotin}$.
3. For the biotinylation reaction, take the following steps: add 180 μL of ultrapure water to the 1 mg microtube to prepare a 10 mM solution of the biotin reagent (stock) and take out the calculated volume of biotin to add to your protein solution.
4. Incubate for 2 h on ice or for 30 min at room temperature.
5. Hydrate the membrane of a Slide-A-Lyzer™ G2 Dialysis Cassette in PBS for 2 min in order to purify the labeled proteins for optimal performance.
6. Using a 1-mL syringe with an 18G needle, slowly fill the cassette with your biotin–allergen mixture and withdraw any remaining air.
7. Dialyze in 2 L of PBS for 2 h at room temperature.
8. Refresh the 2 L PBS and dialyze for another 2 h at room temperature.
9. Change the buffer again and dialyze overnight at 4 °C.
10. The next day, collect the purified sample using a 1-mL syringe with an 18G needle. Store in aliquots at –20 °C.

3.14.2 Detection of Analytes in Serum, BALF, Culture Media, and Tissue Homogenates by ELISA

For the detection of immunoglobulins, ELISA is carried out using antibodies and reagents listed in Table 6. For cytokine measurements in BALF, the supernatant of restimulated single-cell suspensions and lung tissue homogenates, ELISA and multiplex are carried out using kits available from a wide range of commercial suppliers according to the manufacturers' instructions.

1. Coat a high-affinity binding 96-well flat-bottom ELISA plate with 100 μL /well of an appropriate capture antibody diluted in PBS overnight at 4 °C (*see* Table 6).
2. Wash the plate five times using 300 μL of the wash buffer either using an ELISA plate washer or by hand.
3. Block the ELISA plate using 300 μL of ELISA buffer with 1% BSA at room temperature for 1 h.

Table 6
Overview of immunoglobulin ELISA antibodies

ELISA	Layer	Antibody	Stock	Dilution	Supplier
Total IgE	Capture	Purified rat anti-mouse IgE (R35-72)	0.5 mg/mL	1:500	BD Bioscience
	Block	ELISA buffer containing 1% BSA	Pure	300 µL	Lab EXPIRE
	Sample	Mouse serum samples (pre- and post-sera)	Pure	Pre 1:30, post 1:60	Animal Centre
	Standard	Purified mouse IgE κ isotype control (C38-2)	0.5 mg/mL	Start 2500 ng/mL, twofold dilution steps	BD Bioscience
	Detection	Biotin rat anti-mouse IgE (R35-118)	0.5 mg/mL	1:500	BD Bioscience
Total IgG1	Capture	Purified rat anti-mouse IgG1 (A85-3)	0.5 mg/mL	1:200	BD Bioscience
	Block	ELISA buffer containing 1% BSA	Pure	300 µL	Lab EXPIRE
	Sample	Mouse serum samples (pre- and post-sera)	Pure	1:300,000	Animal Centre
	Standard	Purified mouse IgG1, κ isotype control (MOPC-31C)	0.5 mg/mL	Start 750 ng/mL, threefold dilution steps	BD Bioscience
	Detection	Biotin rat anti-mouse IgG1 (A85-1)	0.5 mg/mL	1:500	BD Bioscience
Total IgG2a	Capture	Purified rat anti-mouse IgG2a (R11-89)	0.5 mg/mL	1:200	BD Bioscience
	Block	ELISA buffer containing 1% BSA	Pure	300 µL	Lab EXPIRE
	Sample	Mouse serum samples (pre- and post-sera)	Pure	1:50	Animal Centre
	Standard	Purified mouse IgG2a κ isotype control (G155-178)	0.5 mg/mL	Start 500 ng/mL, twofold dilution steps	BD Bioscience
	Detection	Biotin rat anti-mouse IgG2a (R19-15)	0.5 mg/mL	1:500	BD Bioscience
Total IgA	Capture	Purified rat anti-mouse IgA Antibody (RMA-1)	0.5 mg/mL	1:100	BioLegend
	Block	ELISA buffer containing 1% BSA	Pure	300 µL	Lab EXPIRE
	Sample	Mouse serum samples (post-sera)	Pure	Post 1:50	Animal Centre
	Standard	Purified mouse IgA	1000 ng	Start 1000 ng/mL, threefold dilution steps	Bethyl Laboratories
	Detection	Biotin rat anti-mouse IgA (C10-1)	0.5 mg/mL	1:250	BD Bioscience

(continued)

Table 6
(continued)

ELISA	Layer	Antibody	Stock	Dilution	Supplier
spIgE	Capture	Purified rat anti-mouse IgE (R35-72)	0.5 mg/mL	1:500	BD Bioscience
	Block	ELISA buffer containing 3% powdered milk (ELK)	Pure	300 μ L	Campina
	Sample	Mouse serum samples (pre- and post-sera)	Pure	Pre 1:30, Post 1:60	Animal Centre
	Standard	Pooled positive reference serum	Pure	Start 1:2, twofold dilution steps	Animal Centre
	Detection	Biotinylated allergen	3.9 mg/mL	1:150	Lab EXPIRE
spIgG1	Capture	Crude or rough extract allergen	1 mg/mL	1:100	ALK Abello/ Citeq Biologics Lab EXPIRE
	Block	ELISA buffer containing 1% BSA	Pure	300 μ L	Lab EXPIRE
	Sample	Mouse serum samples (pre- and post-sera)	Pure	1:300,000	Animal Centre
	Standard	Pooled positive reference serum (<i>see Note 14</i>)	Pure	Start 1:50,000, twofold dilution steps	Animal Centre
	Detection	Biotin rat anti-mouse IgG1 (A85-1)	0.5 mg/mL	1:500	BD Bioscience
spIgG2a	Capture	Crude or rough extract allergen	1 mg/mL	1:100	ALK Abello/ Citeq Biologics Lab EXPIRE
	Block	ELISA buffer containing 1% BSA	Pure	300 μ L	Lab EXPIRE
	Sample	Mouse serum samples (pre- and post-sera)	Pure	1:50	Animal Centre
	Standard	Pooled positive reference serum (<i>see Note 14</i>)	Pure	Start 1:25, twofold dilution steps	Animal Centre
	Detection	Biotin rat anti-mouse IgG2a (R19-15)	0.5 mg/mL	1:500	BD Bioscience

4. Empty the plate by flicking and wash once with the wash buffer, making sure that the wells are empty.
5. Add the samples and standards, both in duplo, appropriately diluted in the ELISA buffer in a total volume of 100 μ L (*see Note 22* and Table 6 for appropriate dilutions for serum samples and standards). Incubate for 2 h at room temperature on a bench-top plate shaker at 300 rpm.
6. Wash the plate five times with 300 μ L of the wash buffer each time. In each well, add 100 μ L of a respective detection antibody, or biotinylated allergen for spIgE (*see Subheading 3.13, step 1*), appropriately diluted in ELISA buffer (*see Note 22* and

Table 6) and incubate for 1.5 h at room temperature on the shaker at 300 rpm.

7. Wash the plate again five times with 300 μ L of wash buffer each time. Add 100 μ L of Avidin-HRP diluted 1:200 in ELISA buffer and incubate for 1 h at room temperature on the shaker at 300 rpm.
8. Wash the plate again three times with 300 μ L of the wash buffer each time. Add 100 μ L of the OPD peroxidase substrate to each well and incubate for 10 min in the dark until the coloring reaction is complete, visible by the color development in the standard wells.
9. Stop the reaction by adding 75 μ L of 4 M H_2SO_4 to each well. Read the optical density at 490 nm on an ELISA plate reader.
10. Calculate the concentrations of analytes in the serum samples based on the standard curve, using a four-parameter model. The fit of the standard curve should be at least $r^2 = 0.95$. When performing with multiple 96-well plates, each plate should contain a standard curve as well as a few reference samples that are included on all plates for plate–plate comparisons.

4 Notes

1. The medium may be optionally supplemented with 3% BSA (w/v, cell culture grade) and 5 mL of 100 $\times$ MEM Vitamin Solution, which is commercially available as a growth supplement.
2. Sensitization with intraperitoneal injections is performed on nonanesthetized animals. Restrain a mouse by gently gripping the nape with one hand and anchoring the tail between the small finger and the palm to secure the lower body. Tilt the mouse head down at a 35–40° angle so that the intestines fall away from the injection site. The needle is inserted into the lower right quadrant of the abdomen slightly off the midline anterior to the bladder. Slight negative pressure is applied to the syringe. To avoid the likelihood of puncturing or lacerating abdominal organs, a 1/2 in., 27G needle or insulin syringe is recommended. This route of administration allows for a volume of up to 1 mL to be given safely to a mouse.
3. For subcutaneous injections, a nonanesthetized mouse is held by the nape using one hand. A syringe with a 25G needle is inserted with the other hand at the base of a skin “tent” created by the thumb and forefinger. A slight negative pressure is applied to the syringe to ensure that the needle placement is subcutaneous and did not accidentally damage a local vein. A total of 100 μ L is injected.

4. During the first SCIT injections in C57BL/6 mice, it is important to monitor immediate responses like severity of shock and drop in body temperature. The severity of shock is scored as follows: (1) mild shock (itching, ruffling of fur, dyspnea, self-isolation, and decrease in spontaneous activity); (2) moderate shock (prostration, sluggish gait, no response to whisker stimuli, puffiness around eyes and or mouth, and slight activity after prodding); (3) severe shock (complete paresis and no activity following prodding with or without convulsions); (4) death within 30 min. At the same time, body core temperature must be registered every 20 min using a rectal thermometer after the first SCIT injection.
5. For sublingual administration, restrain a nonanesthetized mouse by gently gripping the nape with one hand and tuck its tail between the fingers to secure the lower body. Hold the mouse up at a fully vertical position. Tighten the grip of the fur between the thumb and index finger until the mouse sticks out its tongue slightly. Using a P20 pipette, place 5 μ L of the allergen solution under the tongue, while ensuring that it stays there for 30 s minimum. It is of paramount importance that the mouse does not swallow the allergen during the administration. After the administration, maintain the restrained animal for 30 s vertically to prevent swallowing before returning the mouse to the individual ventilated cage (IVC).
6. The intranasal challenges are performed with the mice that have been lightly anesthetized. This method facilitates the inhalation of the allergen into the lungs as the mouse wakes up. Check visually whether the allergen solution has been inhaled by the mouse.
7. Retro-orbital puncture targets the venous sinus located behind the eye. When correctly performed on these long-anesthetized mice using 4.5% isoflurane in combination with 1 mL/min O₂, the eyes and health of the animal remain unaffected. The rate of blood flow through the tube may vary significantly from mouse to mouse. When punctured properly, act quickly. The maximum amount of blood that should be collected from a mouse is 8 mL/kg body weight in 14 days.
8. When measuring ear thickness using a micrometer, it is important to keep the device perfectly horizontal. Therefore, all mice should be maintained in the same horizontal position during the measurement. Mice receiving a light anesthesia using 4.5% isoflurane in combination with 1 mL/min O₂ can be placed in a stand, similar to that for intratracheal administrations, to make sure the ears are in the same horizontal position every time (*see* Fig. 3 right panel).

9. For airway pressure, the first measurement is set to zero, while the second point is the pressure measurement after administration of 1 mL of air. For the cylinder pressure, a dynamic tubing calibration is required, using the open and closed cannula as per the manufacturer's instructions.
10. Maintain the anesthesia by repeating the injections every 20 min with 25% of the initial dose. Keep the anesthetized mice warm and record the heart rate, blood pressure, and O₂ saturation during the entire procedure.
11. A standard script is available within the FlexiWare software, which can be adjusted to the following format:

```

FlexiVent script
=====
Script: MCh IV DRC
Author: LH
Date: 15-02-2012
Changes: LH: 8-6-2016
Adapted to new software and doses
Adapted to new doses and comments of Scireq crew
1. Added snapshot after TLC (for quality control)
2. Quick prime 3 instead of quick prime 2 (+ reduction of
number of perturbations)
3. In template adaptation: addition of constant phase model for
the quick prime perturbations
(c) SCIREQ Inc. 2001-04
// Start of command section
// -----
Start Script, Format = 3.0;
Title = MCh DRC with TLC;
//Start DEFAULT ventilation (Depending on Template)
0:00 Ventilation DEFAULT;
// Perform TLC maneuver immediately after attaching the animal
0:02 Prompt MESSAGE=Attach the animal, then click OK to perform
two TLC maneuvers.;
0:00 Marker TEXT=Performing two TLC maneuvers at the start of
the experiment.;
0:00 Ventilation MODE=CFlow; F=10 br/min; Vt=40 mL/kg; IER=1;
Pmax=30 cmH2O;
0:05 Ventilation DEFAULT;
0:15 Ventilation MODE=CFlow; F=10 br/min; Vt=40 mL/kg; IER=1;
Pmax=30 cmH2O;
0:05 Ventilation DEFAULT;
//Do a snapshot for quality control (check for leakage)
0:05 Perturbation NAME=Snapshot-240;
// Log the substance

```

```
0:05 Marker TEXT=Start of MCh Dose-response measurement.;
// Start loop for Baseline
0:02 Loop Begin
// Log next dose
0:00 Marker TEXT=Next dose: Baseline ;
// Prepare to inject the mice
0:00 Prompt BEEP ;
0:00 Prompt MESSAGE=Press OK to start baseline measurement.
0:02 Marker TEXT=Mouse is being injected: Baseline;
// Take 6 measurements directly after each other
0:00 Loop Begin
0:00 Perturbation NAME=QPrime3;
0:00 Loop Return MAXCOUNT=6;
// Take 4 measurements for 4 times
0:00 Loop Begin
0:00 Perturbation NAME=QPrime3;
0:40 Loop Return MAXCOUNT=4
// Prompt user for next dose
0:20 Query MESSAGE=Continue with next dose? (No to restart
previous dose);
0:00 Loop Return REPLY=NO ;
// Start loop for 0 µg/kg
0:02 Loop Begin
// Log next dose
0:00 Marker TEXT=Next dose: 0 ;
// Prepare to inject the mice
0:00 Prompt BEEP ;
0:00 Prompt MESSAGE=Press OK when ready to inject mouse (0 µg/
kg), and inject immediately thereafter. ;
0:02 Marker TEXT=Mouse is being injected:0 µg/kg;
// Take 6 measurements directly after each other
0:00 Loop Begin
0:00 Perturbation NAME=QPrime3;
0:00 Loop Return MAXCOUNT=6;
// Take 4 measurement for 4 times
0:00 Loop Begin
0:00 Perturbation NAME=QPrime3;
0:40 Loop Return MAXCOUNT=4;
// Prompt user for next dose
0:20 Query MESSAGE=Continue with next dose? (No to restart
previous dose);
0:00 Loop Return REPLY=NO;
// Start loop for 50 µg/kg → repeat for the dosages 100, 200,
400, and 800 µg/kg
// End of script
```

12. Any fat or surrounding connective tissue needs to be cleared from the jugular vein, allowing sufficient space for a curved surgical tweezer to maneuver underneath the jugular vein.
13. To test whether the cannula is correctly in place, slightly pull the plunger of the syringe connected to the cannula. A properly cannulated jugular vein will allow blood to flow into the cannula upon the application of negative pressure.
14. Before putting a new syringe on the IV-line, make sure there is no air remaining in the syringe and cannula (use a 5-mL syringe with saline to fill the 1-mL syringe).
15. Analysis of resistance and compliance data can be performed with the exported files using any spreadsheet or statistical program and plotted against the methacholine doses (Fig. 4c, d). In short, highlight the values of interest (resistance and compliance), the COD, and the PEEP. Every dose of methacholine gives a peak value in resistance ($\text{cmH}_2\text{O.s/mL}$) and a minimum value in compliance ($\text{mL/H}_2\text{O}$). Select these values for every dose of methacholine and for every mouse and plot them.

To ensure high-quality data, use the coefficient of determination (COD)-value with a cutoff of 8.0. This is a quality control parameter measuring the quality of the single-compartment model fit. Next, check whether the PEEP was registered as $\sim 20 \text{ mmH}_2\text{O}$ or $\sim 2 \text{ cmH}_2\text{O}$. Alternatively, all values that are measured with sufficient technical quality (use the COD value as a cutoff) can be plotted to calculate an area under the curve for each individual methacholine dose. Another relevant variable is the effective dose (ED) of methacholine (MCh) necessary to increase AHR to an R of $3 \text{ cmH}_2\text{O.s/mL}$ (ED3) (*see* Fig. 4b). As the data are not normally distributed, but represent linked measurements in a dose-range of methacholine challenges, an appropriate statistical evaluation is ANOVA, or in case of missing values, a GEE analysis [39].

16. For quantification of net weight of lung tissue collected, it is imperative to weigh the empty cryogenic vials prior to dissection and again after collecting the lung lobes.
17. Differential counting of BALF cells should be performed by an observer who is blinded to the experimental groups.
18. Cells originating from the lung DLN can be processed in an identical fashion as the lung tissue cells.
19. For long-term storage of the cells in liquid nitrogen, resuspend the cell pellet in 50% HBSS, 40% FCS, and dropwise add 10% DMSO, then divide the cell suspension over cryogenic vials, and place the vials in a Stratagene[®] box at -80°C

overnight. The day thereafter, transfer the vials to liquid nitrogen.

20. Since DLNs do not usually yield as many cells as the lung, DLN cell cultures may only be performed in duplo.
21. Protein concentrations retrieved from homogenization of lung lobes vary considerably between individual mice. Therefore, a BCA protein assay should be performed to quantify the protein content of the lung tissue homogenates. The protein concentration for each sample is used to normalize cytokine levels detected by ELISA when evaluating cytokine responses between experimental groups.
22. The serum samples taken at the three (in SCIT) or five (in SLIT) different time points (pre1–4 and post-serum; *see* Fig. 1a–c) will be used to measure the total as well as allergen-specific IgG1, IgG2a, and IgE. Usually, pre-sera samples will be diluted 1:30 and post-sera samples 1:60 in ELISA buffer, although several dilutions can be tested to determine the optimal dilution. For accurate quantification of immunoglobulin or cytokine levels by ELISA, samples should be stored at -80°C until used. Samples should be processed within 3 months from isolation.

Acknowledgements

Laura Hesse is supported by the Dutch Lung Foundation (NAF10.060).

References

1. Kim HY, DeKruyff RH, Umetsu DT (2010) The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nat Immunol* 11:577–584. <https://doi.org/10.1038/ni.1892>
2. Weiss ST, Litonjua AA, Lange C, Lazarus R, Liggett SB, Bleecker ER, Tantisira KG (2006) Overview of the pharmacogenetics of asthma treatment. *Pharmacogenomics J* 6:311–326. <https://doi.org/10.1038/sj.tpj.6500387>
3. Backman H, Räisänen P, Hedman L, Stridsman C, Andersson M, Lindberg A, Lundbäck B, Rönmark E (2017) Increased prevalence of allergic asthma from 1996 to 2006 and further to 2016—results from three population surveys. *Clin Exp Allergy* 47:1426–1435. <https://doi.org/10.1111/cea.12963>
4. Lambrecht BN, Hammad H (2014) The immunology of asthma. *Nat Immunol* 16:45–56. <https://doi.org/10.1038/ni.3049>
5. Holgate ST, Polosa R (2008) Treatment strategies for allergy and asthma. *Nat Rev Immunol* 8:218–230. <https://doi.org/10.1038/nri2262>
6. Matte-martyn A, Sc B, Diaz-granados N, Sc B, Al-saidi F, Cooper AB, Guest CB, Mazer CD, Mehta S, Stewart TE, Barr A, Ph D, Cook D, Slutsky AS, Critical C, Trials C (2011) Long-term inhaled corticosteroids in preschool children at high risk for asthma. *New Engl J Med* 354:683–693. <https://doi.org/10.1056/NEJMoa1207363>
7. Guilbert TW, Martinez FD, Lemanske RF, Strunk RC, Covar R, Szefer SJ, Boehmer S, Jackson DJ, Sorkness CA, Pharm D, Gern JE, Kelly HW, Pharm D, Friedman NJ, Mellon MH, Schatz M, Morgan WJ, Chinchilli VM, Ph D, Raissy HH, Pharm D, Bade E, Malkarais J, Beigelman A, Taussig LM (2011) Daily or intermittent budesonide in preschool children with recurrent wheezing. *N Engl J Med*

- 365(21):1990–2001. <https://doi.org/10.1056/NEJMoa1104647>
8. Hancox RJ, Cowan JO, Flannery EM, Herbison GP, McLachlan CR, Taylor DR (2000) Bronchodilator tolerance and rebound bronchoconstriction during regular inhaled beta-agonist treatment. *Respir Med* 94:767–771. <https://doi.org/10.1053/rmed.2000.0820>
9. Yim RP, Koumbourlis AC (2013) Tolerance & resistance to β 2-agonist bronchodilators. *Paediatr Respir Rev* 14:195–198. <https://doi.org/10.1016/j.prrv.2012.11.002>
10. Jutel M (2014) Allergen-specific immunotherapy in asthma. *Curr Treat Options Allergy* 1:213–219. <https://doi.org/10.1007/s40521-014-0013-1>
11. Jacobsen L, Niggemann B, Dreborg S, Ferdousi HA, Halken S, Høst A, Koivikko A, Norberg LA, Valovirta E, Wahn U, Möller C (2007) Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10-year follow-up on the PAT study. *Allergy* 62:943–948. <https://doi.org/10.1111/j.1398-9995.2007.01451.x>
12. Passalacqua G (2014) Specific immunotherapy in asthma: a comprehensive review. *J Asthma* 51:29–33. <https://doi.org/10.3109/02770903.2013.853082>
13. Epstein TG, Liss GM, Murphy-Berendts K, Bernstein DI (2014) AAAAI/ACAAI surveillance study of subcutaneous immunotherapy, years 2008–2012: an update on fatal and non-fatal systemic allergic reactions. *J Allergy Clin Immunol Pract* 2:161–167.e3. <https://doi.org/10.1016/j.jaip.2014.01.004>
14. Jacobsen L, Wahn U, Bilo MB (2012) Allergen-specific immunotherapy provides immediate, long-term and preventive clinical effects in children and adults: the effects of immunotherapy can be categorised by level of benefit -the centenary of allergen specific subcutaneous immunotherapy. *Clin Transl Allergy* 2:8. <https://doi.org/10.1186/2045-7022-2-8>
15. Durham SR, Emminger W, Kapp A, de Monchy JGR, Rak S, Scadding GK, Wurtzen PA, Andersen JS, Tholstrup B, Riis B, Dahl R (2012) SQ-standardized sublingual grass immunotherapy: confirmation of disease modification 2 years after 3 years of treatment in a randomized trial. *J Allergy Clin Immunol* 129:717–725.e5. <https://doi.org/10.1016/j.jaci.2011.12.973>
16. Didier A, Worm M, Horak F, Sussman G, de Beaumont O, Le Gall M, Melac M, Malling H-J (2011) Sustained 3-year efficacy of pre- and coseasonal 5-grass-pollen sublingual immunotherapy tablets in patients with grass pollen-induced rhinoconjunctivitis. *J Allergy Clin Immunol* 128:559–566. <https://doi.org/10.1016/j.jaci.2011.06.022>
17. Janssen EM, van Oosterhout AJ, Nijkamp FP, van Eden W, Wauben MH (2000) The efficacy of immunotherapy in an experimental murine model of allergic asthma is related to the strength and site of T cell activation during immunotherapy. *J Immunol* 165:7207–7214. <https://doi.org/10.4049/jimmunol.165.12.7207>
18. Janssen EM, Wauben MH, Jonker EH, Hofman G, Van Eden W, Nijkamp FP, Van Oosterhout AJ (1999) Opposite effects of immunotherapy with ovalbumin and the immunodominant T-cell epitope on airway eosinophilia and hyperresponsiveness in a murine model of allergic asthma. *Am J Respir Cell Mol Biol* 21(1):21–29. <https://doi.org/10.1165/ajrcmb.21.1.3519>
19. Shirinbak S, Taher YA, Maazi H, Gras R, van Esch BCAM, Henricks PAJ, Samsom JN, Verbeek JS, Lambrecht BN, van Oosterhout AJM, Nawijn MC (2010) Suppression of Th2-driven airway inflammation by allergen immunotherapy is independent of B cell and Ig responses in mice. *J Immunol* 185:3857–3865. <https://doi.org/10.4049/jimmunol.0903909>
20. Taher YA, van Esch BCAM, Hofman GA, Henricks PAJ, van Oosterhout AJM (2008) 1,25-dihydroxyvitamin d3 potentiates the beneficial effects of allergen immunotherapy in a mouse model of allergic asthma: role for IL-10 and TGF- β . *J Immunol* 180:5211–5221. <https://doi.org/10.4049/jimmunol.180.8.5211>
21. Maazi H, Shirinbak S, Willart M, Hammad HM, Cabanski M, Boon L, Ganesh V, Baru AM, Hansen G, Lambrecht BN, Sparwasser T, Nawijn MC, van Oosterhout AJM (2012) Contribution of regulatory T cells to alleviation of experimental allergic asthma after specific immunotherapy. *Clin Exp Allergy* 42:1519–1528. <https://doi.org/10.1111/j.1365-2222.2012.04064.x>
22. Vissers JLM, van Esch BCAM, Hofman GA, van Oosterhout AJM (2005) Macrophages induce an allergen-specific and long-term suppression in a mouse asthma model. *Eur Respir J* 26:1040–1046. <https://doi.org/10.1183/09031936.05.00089304>
23. Kapsenberg ML (2003) Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol* 3:984–993. <https://doi.org/10.1038/nri1246>
24. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH (2000) Induction of interleukin 10-producing, nonproliferating CD4(+) T

- cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 192 (9):1213–1222. <https://doi.org/10.1084/jem.192.9.1213>
25. Weiner HL (2001) The mucosal milieu creates tolerogenic dendritic cells and T(R)1 and T(H)3 regulatory cells. *Nat Immunol* 2 (8):671–672. <https://doi.org/10.1038/90604>
 26. Maazi H, Shirinbak S, den Boef LE, Fallarino F, Volpi C, Nawijn MC, van Oosterhout AJM (2013) Cytotoxic T lymphocyte antigen 4-immunoglobulin G is a potent adjuvant for experimental allergen immunotherapy. *Clin Exp Immunol* 172:113–120. <https://doi.org/10.1111/cei.12041>
 27. Tan AM, Chen H-C, Pochard P, Eisenbarth SC, Herrick CA, Bottomly HK (2010) TLR4 signaling in stromal cells is critical for the initiation of allergic Th2 responses to inhaled antigen. *J Immunol* 184:3535–3544. <https://doi.org/10.4049/jimmunol.0900340>
 28. Hesse L, Brouwer U, Petersen AH, Gras R, Bosman L, Brimnes J, Oude Elberink JNG, van Oosterhout AJM, Nawijn MC (2018) Subcutaneous immunotherapy suppresses Th2 inflammation and induces neutralizing antibodies, but sublingual immunotherapy suppresses airway hyperresponsiveness in grass pollen mouse models for allergic asthma. *Clin Exp Allergy* 48:1035–1049. <https://doi.org/10.1111/cea.13169>
 29. Hesse L, van Ieperen N, Habraken C, Petersen AH, Korn S, Smilda T, Goedewaagen B, Ruiters MH, van der Graaf AC, Nawijn MC (2018) Subcutaneous immunotherapy with purified Der p1 and 2 suppresses type 2 immunity in a murine asthma model. *Allergy* 73:1–13. <https://doi.org/10.1111/all.13382>
 30. Hesse L, Feenstra R, Ambrosini M, de Jager WA, Petersen A, Victor H, Unger WWJ, van Kooyk Y, Nawijn MC (2019) Subcutaneous immunotherapy using modified Phl p5a-derived peptides efficiently alleviates allergic asthma in mice. *Allergy* 74 (12):2495–2498. <https://doi.org/10.1111/all.13918>
 31. Brimnes J, Kildsgaard J, Jacobi H, Lund K (2007) Sublingual immunotherapy reduces allergic symptoms in a mouse model of rhinitis. *Clin Exp Allergy* 37:488–497. <https://doi.org/10.1111/j.1365-2222.2006.02624.x>
 32. Hoffmann HJ, Valovirta E, Pfaar O, Moingeon P, Schmid JM, Skaarup SH, Cardell LO, Simonsen K, Larché M, Durham SR, Sørensen P (2017) Novel approaches and perspectives in allergen immunotherapy. *Allergy* 72 (7):1022–1034. <https://doi.org/10.1111/all.13135>
 33. Elliott J, Kelly SE, Johnston A, Skidmore B, Gomes T, Wells GA (2017) Allergen immunotherapy for the treatment of allergic rhinitis and/or asthma: an umbrella review. *CMAJ Open* 5(2):E373–E385. <https://doi.org/10.9778/cmajo.20160066>
 34. Jönsson F, Mancardi DA, Kita Y, Karasuyama H, Iannascoli B, Van Rooijen N, Shimizu T, Daëron M, Bruhns P (2011) Mouse and human neutrophils induce anaphylaxis. *J Clin Invest* 121(4):1484–1496. <https://doi.org/10.1172/JCI45232>
 35. Marco-Martín G, La Rotta HA, Vázquez De La Torre M, Higaki Y, Zubeldia JM, Baeza ML (2017) Differences in the anaphylactic response between C3H/HeOuJ and BALB/c mice. *Int Arch Allergy Immunol* 173:204–212. <https://doi.org/10.1159/000478983>
 36. Sun J, Arias K, Alvarez D, Fattouh R, Walker T, Goncharova S, Kim B, Wasserman S, Reed J, Coyle AJ, Jordana M (2007) Impact of CD40 ligand, B cells, and mast cells in peanut-induced anaphylactic responses. *J Immunol* 179:6696–6703. <https://doi.org/10.4049/jimmunol.179.10.6696>
 37. Finkelman FD (2007) Anaphylaxis: lessons from mouse models. *J Allergy Clin Immunol* 120:506–515. <https://doi.org/10.1016/j.jaci.2007.07.033>
 38. Robichaud A, Fereydoonzaad L, Urovitch IB, Brunet J-D (2015) Comparative study of three flexiVent system configurations using mechanical test loads. *Exp Lung Res* 41:84–92. <https://doi.org/10.3109/01902148.2014.971921>
 39. Twisk JW (2004) Longitudinal data analysis. A comparison between generalized estimating equations and random coefficient analysis. *Eur J Epidemiol* 19(8):769–776. <https://doi.org/10.1023/b:cejep.0000036572.00663.f2>