

Chapter 11

Subcutaneous and Sublingual Immunotherapy in a Mouse Model of Allergic Asthma

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Abstract

Allergic asthma, caused by inhaled allergens such as house dust mite or grass pollen, is characterized by reversible airway obstruction, associated with an eosinophilic inflammation of the airways, as well as airway hyper responsiveness and remodeling. The inhaled allergens trigger a type-2 inflammatory response with involvement of innate lymphoid cells (ILC2) and Th2 cells, resulting in high production of immunoglobulin E (IgE) antibodies. Consequently, renewed allergen exposure results in a classic allergic response with a distinct early and late phase, 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 and both subcutaneous AIT (SCIT) as well as sublingual AIT (SLIT) have proven clinical efficacy in long term suppression of the allergic response. Although these treatments are very successful for rhinitis, application of AIT in asthma is hampered by variable efficacy, long duration of treatment, and the risk of severe side-effects. A more profound understanding of the mechanisms by which AIT achieves tolerance to allergens in sensitized individuals is needed to improve its efficacy. Mouse models have been very valuable as a preclinical model to characterize the mechanisms of desensitization in AIT and to evaluate novel approaches for improved 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 absorbed to aluminum hydroxide to induce allergic sensitization, followed by subcutaneous injections (SCIT) or sublingual administrations (SLIT) of the allergen as immunotherapy treatment. Finally, mice are challenged by three intranasal allergen administrations. We will describe the protocols as well as the most important read-out parameters including measurement of invasive lung function measurements, serum immunoglobulin levels, isolation of broncho-alveolar lavage fluid (BALF), and preparation of cytopins. Moreover, we describe how to restimulate lung single cell suspensions, perform flow cytometry measurements to identify populations of relevant immune cells, and perform ELISAs and Luminex assays to measure the cytokine concentrations in BALF and lung tissue.

Key words Allergic asthma, Subcutaneous immunotherapy (SCIT), Sublingual immunotherapy (SLIT), Mouse model, Grass pollen (GP), House dust mite (HDM), FlexiVent, Flow cytometry, Bronchoalveolar lavage, Eosinophilia

1 Introduction

Asthma is the result of a complex interaction between genetic susceptibility and environmental factors that lead to the inception of the disease only in the susceptible individual. 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 an eosinophilic inflammation, hyper responsiveness and remodeling of the airways. The worldwide prevalence of allergic asthma has dramatically increased over the last 25 years, currently affecting over 330 million people.

The inflammatory response in allergic asthma is 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 [3]. These cytokines contribute to the pathological changes of the airways observed in allergic asthma such as the influx of eosinophils, mucus hypersecretion, airway hyper responsiveness and airway wall remodeling. In addition, the Th2 dominated adaptive response to the inhaled allergens result in the presence of allergen-specific IgE. Allergen-induced cross-linking of IgE bound to the cell surface of mast cells and basophils through the high affinity IgE receptor will induce degranulation of these cells resulting in the acute allergic response, leading to bronchoconstriction and vasodilation. The subsequent influx of inflammatory cells including Th2 cells will result in activation of these cells upon local antigen presentation of the causative allergen, inducing the late-phase response. Upon recurrent exposure to the allergen, a chronic and poorly resolving inflammation around the small airways results in permanent changes to the airway wall.

Currently available asthma therapies are focused on controlling the ongoing inflammatory process, mainly with inhaled corticosteroids (ICS), in combination with long acting beta agonists (LABA) or leukotriene receptor antagonists (LTRA) [4]. Notwithstanding the clinical success in achieving asthma control, current asthma treatment regimens fail to cure the disease, as evidenced by ongoing airway wall remodeling even in well-controlled asthma patients and an unaltered risk for asthma attacks either upon daily ICS use compared to intermittent ICS use or upon cessation of ICS treatment [5, 6]. Moreover, a subset of patients with severe asthma exists that does not respond to steroid treatment [7, 8]. These shortcomings of current mainstream asthma therapy indicate the failure of this therapeutic approach to address the underlying, causative immunological mechanisms, resulting in 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) [9]. Unlike standard asthma treatment, AIT has been shown to provide a long-term protection against asthma attacks that is maintained upon cessation of therapy and to reduce medication use in allergic asthma. AIT involves the administration of gradually increasing amounts of allergen extracts

for a period of 3–5 years with the aim of achieving a state of immunological tolerance and subsequently a reduction of clinical manifestations in the allergic patients [10]. A Cochrane systematic review on the application of subcutaneous AIT (SCIT) in asthma reported an overall efficacy in suppression of symptom score, medication use and both allergen-specific and nonspecific bronchial hyperresponsiveness [11]. Sublingual AIT (SLIT) treatment for house dust mite allergy reduced the use of ICS (relative mean reduction of 50%) while maintaining asthma control [12]. In addition to suppressing asthma symptoms and achieving reduced medication use, grass pollen AIT has also been found to prevent progression to asthma in children with seasonal allergic rhinoconjunctivitis [10]. Also AIT treatment has been shown to prevent progression to asthma in a subset of children [13].

Although the mechanism behind successful immunotherapy remains, at least in part, unknown, the beneficial effects of AIT are ascribed to a shift from the Th2 profile seen in allergic asthma towards a protective T regulatory (Treg) profile that suppresses allergen specific responses [14]. Clinical improvement after AIT is associated with increased levels of neutralizing antibodies, production of IL-10 and TGF- β , and increased CD4⁺FoxP3⁺ Treg numbers and resolution of Th2 inflammation [15–18]. For instance, GP SCIT treatment has been shown to induce regulatory T cell numbers in nasal tissue, associated with reduced tissue Th2 and eosinophil cell numbers [17]. Moreover, PBMCs from AIT treated patients cultured in the presence of allergens produce increased levels of IL-10 and TGF- β , reflecting an increased presence or activity of regulatory T cells [18]. In addition, a recent study employing tetramer technology to track individual Th cell clones in GP allergic patients elegantly showed that successful GP SCIT was associated with a selective loss of those allergen-specific Th2 clones that were found to be increased in number in GP allergic patients compared to non-allergic control subjects [16]. Furthermore, blocking IgGs has been suggested to contribute to successful AIT, since increased levels of IgGs in serum of AIT-treated patients has been associated with the alleviation of allergic symptoms [19–21]. The blocking IgG hypothesis states that blocking antibodies produced by B cells upon tolerance induction contribute to successful AIT in two ways. First, by binding to the allergen directly and thereby competing with specific IgE preventing cross-linking and subsequent mast cell and basophil degranulation as well as preventing IgE facilitated antigen presentation. Second, the specific IgG antibodies can bind to Fc γ RIIB expressed by B cells, mast cells, and basophils, which can, upon allergen-mediated IgG cross-linking, provide a negative intracellular signal and prevent activation, even upon co-aggregation with Fc ϵ RI.

While SCIT and SLIT have been widely accepted as effective therapeutic alternatives for allergic rhinoconjunctivitis, application

in asthma is hampered by the long duration of treatment required to achieve long-lasting relieve of symptoms, the variable efficacy in allergic asthma and concerns regarding the safety of treatment. SCIT injections of allergen extracts have a potential risk of anaphylactic reactions, with an incidence of severe anaphylactic responses reported to be at around one in a million injections [22]. Therefore, outpatient clinic visits are required for the administration and monitoring of the therapy [23]. In developing more convenient alternatives, SLIT has been developed as a less invasive alternative with proven clinical effects for patients suffering from allergic rhinitis [24]. Herein, uptake of the allergen takes place in the oral mucosa, where mucosal Langerhans cells in humans and oral macrophage-like cells in mice have been implicated [25]. Although the mechanism of action remains to be elucidated, advantages include the absence of the involvement of the clinic, ease of use (droplets or fast dissolving tablets), and relative costs. Understanding the mechanisms by which AIT suppresses the allergen-induced asthma symptoms is needed to redesign AIT for better efficacy and optimal safety, in particular in asthmatic patients.

Previously, animal models have proven to be valuable as a pre-clinical model to improve AIT and to unravel the mechanisms of desensitization involved. The development of a predictive and reproducible AIT protocol was initially based on a mouse model of allergic asthma using the standard model antigen ovalbumin (OVA) [26]. Herein, mice were sensitized with OVA without the use of an adjuvant in seven intraperitoneal injections, followed after 2 weeks by SCIT treatment using three injections of OVA (1 mg), and after another 2 weeks challenged by OVA (2 mg/ml) inhalation once a day (5 min) for 8 consecutive days. The outcome parameters of SCIT treatment were OVA-specific immunoglobulin (Ig) levels: sp-IgE, -IgG1 and IgG2a, airway eosinophilia, airway hyperresponsiveness (AHR), and proinflammatory cytokine production by antigen specific T cells (Th2 mediated response), such as interleukin 4 (IL-4), IL-5, and IL-13. In the initial studies, no adverse events were recorded in the BALB/c strain of mice, while the experimental SCIT treatment effectively reduced airway inflammation and AHR and induced spIgG1 and spIgG2a serum levels. In addition, spIgE levels were also increased, in accordance with the initial rise of spIgE in human subjects treated with SCIT. Importantly however, SCIT treatment in the OVA mouse model prevented the increase of spIgE levels after allergen challenge, which is readily observed in control treated mice [27]. After several improvements, the protocol was reduced to two intraperitoneal injections containing OVA accompanied by a sensitizing adjuvant, Alum (mixture of aluminum hydroxide and magnesium hydroxide) and three intranasal challenges containing high dose aerosolized OVA. In addition, OVA SCIT treatment was also found to be effective in sensitized mice that were challenged by

OVA inhalation prior to SCIT treatment, indicating the ability of the experimental treatment to suppress an established allergic airway inflammatory response [28]. Throughout the years, this mouse model for SCIT has been used to improve our understanding of the mechanisms of desensitization [26, 29–31]. Examples include the requirement on neutralizing antibody responses [28], the crucial role of IL-10 in the induction of tolerance, the essential contribution of CD4⁺FoxP3⁺ T regulatory cells (Treg). Aside from studies on the involvement of B and T lymphocytes, the crucial role of the antigen presenting cells, mostly DCs, have been investigated extensively. DCs play a key role in the generation of adaptive T cell subsets and, depending on their maturation state, DCs can respond either immunogenic, in a sensitizing fashion, or tolerogenic [32]. Tolerogenic DCs have a semi-mature or immature phenotype, characterized by increased expression of major histocompatibility complex class II (MHC-II) and B7-2, low expression of CD40, and no production of proinflammatory cytokines (IL-6 and TNF α). Studies have shown that incubation of immature DCs with CD4⁺T cells induce antigen specific Tregs [33, 34], postulating that immature DCs play a critical role in Treg cell generation and peripheral tolerance. Based on these findings, AIT can be improved when allergen administration is accompanied by inhibition of DC maturation or prevention of DC dependent costimulation. One approach explored to this end is the use of 1,25(OH)₂vitamin D₃, the active metabolite of vitamin D, since it inhibits NF- κ B signaling via the RelB protein, which is essential for DC differentiation and maturation. Indeed, using the OVA-SCIT mouse model, co-administration of 1,25(OH)₂VitD₃ has been shown to potentiate the suppressive effects of AIT [29]. In addition, CTLA4-Ig (Abatacept) was found to enhance efficacy of SCIT in the OVA mouse model [35].

Although using this mouse model of immunotherapy has provided a much more detailed insight in the immunological mechanisms, it should not be forgotten that it is merely an experimental preclinical model using a purified protein (OVA) that lacks the properties of natural allergens, and which in fact is able to induce tolerance when inhaled by naïve mice [36]. Therefore, the classical model allergen OVA was replaced with a natural allergen extract that is also used in human SCIT, such as GP and HDM, and the GP and HDM SCIT protocols for allergic asthma have been optimized regarding administered allergen doses (*unpublished data*). Second, other administrative routes were optimized, based on a SLIT mouse model of allergic rhinitis [37]. Both administrative routes have been validated and standardized allowing a head-to-head comparison. This model 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 use of alternative formulations and adjuvantia.

In this protocol, we explain how subcutaneous and sublingual routes of allergen-specific immunotherapy can be applied in a mouse model of allergic asthma using natural allergen extracts. We provide an overview of 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 measurement of lung tissue and airway inflammation. Moreover, we describe how to restimulate lung single cell suspensions, perform flow cytometry measurements to identify populations of relevant immune cells, and perform ELISAs and Luminex assays to measure the cytokine concentrations in BALF and lung tissue.

2 Materials

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

1. 7–9-week-old BALB/cByJ mice (females) housed in individually ventilated cages (IVC).
2. Syringes (1 ml) and 25 G needles.
3. 20 µl pipet and tips.
4. 15 ml tubes.
5. Sterile phosphate buffered saline (PBS).
6. Rough extract grass pollen (GP, *Phleum pratense*; *Phl p*) or crude extract house dust mite (HDM, *Dermatophagoides pteronyssinus*; *Der p*), resuspended according to **Note 1**.
7. Sterile aluminum hydroxide (Al(OH₃)), 20 % (Imject® Alum).

2.2 Ear Swelling Test and 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-trifluoroethane, 4.5 %, halocarbon, isoflurane USP, liquid for inhalation).
3. Oxygen (1.5 ml/min O₂).
4. Sterile phosphate buffered saline (PBS).
5. Rough extract grass pollen (GP, *Phleum pratense*; *Phl p*) or crude extract house dust mite (HDM, *Dermatophagoides pteronyssinus*; *Der p*).
6. 30 G Insulin syringes (0.3 ml 0.30 mm × 8 mm).
7. 1 ml MiniCollect® serum tubes (Greiner Bio-One).
8. Glass microcapillary tubes: micro hematocrit tubes (Na-Heparinized 80 IU/ml).
9. 1.5 ml tubes and a centrifuge.
10. Digimatic pressure-limited micrometer.
11. Regular hand tissues, hand gloves, and sterile tissues.

2.3 Lung Function Measurement and Section

1. Ligatures (6/0 and 3/0), silicon tubing (0.28 mm and OD: 0.61 mm), syringes (1 and 5 ml), and 25 G needles.
2. 20 G intravenous cannula for tracheal cannulation and calibration (pink, 20GA 1.16IN 1.1 × 30 mm BD Insyte-W™).
3. 1.5 ml tubes, 15 ml tubes, 24-well cell culture plates, MiniCollect® serum tubes, and 2 ml cryogen vials.
4. Sterile PBS, PBS supplemented with bovine serum albumin (BSA, 5%, heat shock fraction, protease free, low endotoxin, suitable for cell culture, pH 7, ≥98%) and a complete protease inhibitor cocktail tablet (cOmplete Mini), and sterile RPMI1640.
5. Ketamine (100 mg/ml), Domitor (1 mg/kg), Rocuronium bromide (10 µg/ml), sterile methacholine.
6. Chirurgical microscope (40×) and basic micro-chirurgical instrument set.
7. FlexiVent (SCIREQ), computers, and FlexiWare Software.
8. Weighing scale (precision > 0.1 g).
9. Manometer with syringe and closing valves.
10. Micro pulse-oxygenometer for small animals.
11. Heating mats for small animals.

2.4 Analysis of the Infiltration of Inflammatory Cells in BAL Fluid

1. Microscope slides (76 × 26 mm), Shandon filter cards and 70 % ethanol.
2. Cytospin cuvette and metal cytospin slide holder and its driver.
3. Pipet-man® with associated pipettes and tips.
4. Cyto-centrifuge (Shandon, cytospin 3), regular centrifuge and aspirator.
5. Cell counter machine, 10 ml Coulter counter cups, Isoton II, and Lyzerglobin.
6. Sterile PBS, bovine serum albumin (BSA, 5%, heat shock fraction, protease free, low endotoxin, suitable for cell culture, pH 7, ≥98%).
7. Sterile lysis buffer containing 155 mmol/l NH₄Cl, 10 mmol/l KHCO₃, and 0.1 mmol/l EDTA.
8. Medion Diagnostics Diff-Quick staining set.
9. A light microscope with 20×, 40×, and 100× magnification lenses, immersion oil, and a differential cell counter.

2.5 Preparation of Single Cell Suspensions of Lung Tissue, Spleen, and Draining Lymph Nodes

1. Standard laboratory equipment including ice, 24-well plates, petri dishes, sterile scalpels, pipets, Pipet-man with associated pipets, 50 ml tubes, 70 µm nylon cell strainers and the plunger of a 5 ml syringe.
2. Safety cabinet (down-flow cabinet) with a closed suction system, and a standard centrifuge.

3. Sterile RPMI1640, and sterile 500 ml RPMI1640 supplemented with: 10 % (50 ml) fetal calf serum (FCS, complement inactivated), L-glutamine (200 mM in 0.85 % NaCl solution),
 5 ml of 10,000 U/ml penicillin with 10,000 µg/ml streptomycin (PenStrep), (*optional*) 3 % bovine serum albumin (BSA, heat shock fraction, protease free, low endotoxin, suitable for cell culture, pH 7, $\geq 98\%$), (*optional*) 5 ml MEM vitamin solution (100×).
4. 4 mg/ml collagenase A and 0.1 mg/ml DNase I.
5. Cell counter machine, 10 ml Coulter counter cups, Isoton II, and Lyzerglobin.
6. Cryogen vials, a Stratagene box, $-80\text{ }^{\circ}\text{C}$ freezer, and liquid nitrogen storage.
7. Storage medium for cells in liquid nitrogen: 50 % HBSS, 40 % FCS, and 10 % DMSO.

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

1. Safety cabinet (down-flow cabinet) with a closed suction system, standard centrifuge, and a CO₂ incubator.
2. Petri dishes, sterile scalpels, pipets, and Pipet-man® with associated pipets.
3. Supplemented RPMI1640 and a single cell suspension resulted from Subheading 2.5.
4. A sterile U-bottom 96-well cell culture plate.
5. Rough extract grass pollen (GP, *Phleum pratense*; *Phl p*) or crude extract house dust mite (HDM, *Dermatophagoides pteronyssinus*, *Der p*).

2.7 Homogenization of Lung Tissue for Total Protein and Cytokine Analysis

1. Homogenizer, 1.5 ml tubes, 96-well flat bottom ELISA plates, cryogen vials, and ELISA plate reader.
2. BCA protein assay kit.
3. Demi water, Tween 20, and 70 % ethanol.
4. Luminex buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.002 % Tween, pH 7.5, and (*optional*) one tablet complete protease inhibitor cocktail tablet/100 ml buffer (cOmplete Mini), and (*optional*) one tablet PhosSTOP phosphatase inhibitor cocktail tablet/100 ml buffer. Luminex buffer can be aliquoted and stored at $-20\text{ }^{\circ}\text{C}$.

**2.8 Analysis
of Immunoglobulin
Levels in Serum
and Cytokine Levels
in BAL Fluid,
Supernatant
of Restimulated Single
Cell Suspensions,
and Lung Tissue
Homogenates**

1. ELISAs for immunoglobulins: total IgE, -IgA, -IgG1, and -IgG2a, as well as specific (sp)IgE, spIgG1, and spIgG2a.
2. ELISAs for cytokines of interest like, IL-4, IL-5, IL-10, IL-13, and IFN γ .
3. Luminex assay for mouse cytokines, choose your cytokines of interest and combine them in a single Mouse Magnetic Luminex Screening Assay [LXSAMSM].
4. For an overview of all antibodies included in the immunoglobulin-ELISAs, we would like to refer to Table 1. For the allergen specific IgE ELISA, we use a “home-made” biotinylated allergen as a second antibody (*see* Note 13). To include a reference line in all specific Ig-ELISAs we use a collection of pooled sera from asthmatic mice (positive controls). As an example, we include here the total Immunoglobulin E levels in serum:
 - (a) NUNC Maxisorp 96-well flat bottom ELISA plates, multichannel pipet and associated tips, ELISA plate-washer, -shaker, -and reader.
 - (b) ELISA buffer containing: 6.06 g/l Tris (50 mM), 8 g/l NaCl (136.9 mM), 0.05 % Tween 20, 0.744 g/l EDTA (2 mM), pH 7.2, and freshly add 5 g/l BSA.
 - (c) Wash buffer containing PBS and 0.05 % Tween 20.
 - (d) Coat: purified rat anti-mouse IgE, clone R35-72 (*see* Table 1).
 - (e) Standard: purified mouse IgE κ isotype control, clone C38-2.
 - (f) Samples: mouse serum collected on different time points.
 - (g) Second antibody: biotin rat anti-mouse IgE, clone R35-118.
 - (h) Avidin horseradish peroxidase (HRP), OPD peroxidase substrate, and 4 M H $_2$ SO $_4$.

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

1. FACS tubes (polystyrene) for samples and singles during staining, 30 μ m filter top FACS tubes, standard centrifuge, three laser flow cytometer (FacsVerse).
2. FACS buffer containing: 1 % BSA in PBS.
3. Block buffer containing: 2 % normal rat serum (NRS) and 5 % FcBlock (purified and unlabeled CD16/32 antibody) in FACS buffer. For intracellular blocking, use 2 % NRS and 5 % FcBlock in PERM buffer (*see* Subheading 2.9, item 6).
4. Staining antibodies diluted in FACS buffer according to manufacturers' protocol (*see* Tables 2 and 3). In some cases, the dilution of every antibody can be adjusted depending on the intensity of the fluorescent signal.

Table 1
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	Mice Sera 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	Mice Sera 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	Mice Sera samples (Pre- and Post-sera)	Pure	1:50	Animal Centre
	Standard	Purified Mouse IgG2a κ Isotype Control (GL15-178)	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 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	Mice Sera 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

spIgE	Capture Block	Purified Rat Anti-Mouse IgE (R35-72) ELISA buffer containing 3% powdered milk (ELK)	0.5 mg/ml Pure	1:500 300 µl	BD Bioscience Campina
	Sample Standard Detection	Mice Sera samples (Pre- and Post-sera) Pooled reference serum positive Biotinylated allergen (<i>see</i> Note 13)	Pure Pure 3.9 mg/ml	Pre 1:30, Post 1:60 Start 1:2, twofold dilution steps 1:150	Animal Centre Animal Centre Lab EXPIRE
	Capture	Crude or rough extract Allergen (<i>see</i> Note 1)	1 mg/ml	1:100	ALK Abello/ Citeq Biologics
	Block Sample Standard Detection	ELISA buffer containing 1% BSA Mice Sera samples (Pre- and Post-sera) Pooled reference serum positive Biotin Rat Anti-Mouse IgG1 (A85-1)	Pure Pure Pure 0.5 mg/ml	300 µl 1:300,000 Start 1:50,000, twofold dilution steps 1:500	Lab EXPIRE Animal Centre Animal Centre BD Bioscience
spIgG2a	Capture	Crude or rough extract Allergen (<i>see</i> Note 1)	1 mg/ml	1:100	ALK Abello/ Citeq Biologics
	Block Sample Standard Detection	ELISA buffer containing 1% BSA Mice Sera samples (Pre- and Post-sera) Pooled reference serum positive Biotin Rat Anti-Mouse IgG2a (R19-15)	Pure Pure Pure 0.5 mg/ml	300 µl 1:50 Start 1:25, twofold dilution steps 1:500	Lab EXPIRE Animal Centre Animal Centre BD Bioscience

Table 2
Antibodies used for FACS analysis of innate lymphocytes

Specificity	Clone	Isotype	Staining	Supplier
Brilliant Violet 605™ anti-mouse Ly-6A/E (Sca-1) Antibody	D7	Rat IgG2a, κ	Extracellular	BioLegend
PerCP/Cy5.5 anti-mouse/human KLRG1 (MAFA) Antibody	2F1/KLRG1	Syrian hamster IgG	Extracellular	BioLegend
Anti-Mouse CD3e PE	145-2C11	Armenian Hamster IgG	Extracellular	eBioscience
Anti-Mouse CD5 PE	53-7.3	Rat IgG2a, κ	Extracellular	eBioscience
Anti-Mouse CD19 PE	eBio1D3 (1D3)	Rat IgG2a, κ	Extracellular	eBioscience
Anti-Mouse NK1.1 PE	PK136	Mouse IgG2a, κ	Extracellular	eBioscience
Anti-Mouse Fc epsilon Receptor I alpha (FceR1) PE	MAR-1	Armenian Hamster IgG	Extracellular	eBioscience
Anti-Mouse CD11b PE	M1/70	Rat IgG2b, κ	Extracellular	eBioscience
Anti-Mouse CD11c PE	N418	Armenian Hamster IgG	Extracellular	eBioscience
Anti-Mouse Ly-6G (Gr-1) PE	RB6-8C5	Rat IgG2b, κ	Extracellular	eBioscience
Anti-Mouse TER-119 PE	TER-199	Rat IgG2b, κ	Extracellular	eBioscience
T1/ST2 (IL-33R) Monoclonal Antibody, FITC	DJ8	IgG1	Extra- and Intracellular	mdbiosciences
Anti-Mouse CD45 APC	30-F11	Rat IgG2b, κ	Extracellular	eBioscience
Anti-Mouse CD127 APC-eFluor® 780	A7R34	Rat IgG2a, κ	Extracellular	eBioscience
Anti-Human/Mouse Gata-3 PE-Cyanine7	TWJ	Rat IgG2b, κ	Intracellular	eBioscience

Table 3
Antibodies used for FACS analysis of T cells and DCs

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

5. Fixable live/dead (L/D) cell stain: 1/1000 dilution in PBS (V450 dye).
6. Foxp3/transcription factor staining buffer set containing: fixation/permeabilization concentrate (00-5123), fixation/permeabilization diluent (00-5223), and permeabilization buffer (10×, 00-8333, eBioscience). Dilute the concentrate (1 part) with the diluent (3 parts) prior to use (is called FIX). Dilute the buffer 10× in demi water (PERM buffer).

3 Methods

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

1. Sensitization

Mice are injected intraperitoneally on days 1 and 14, with GP or HDM allergen extract absorbed to alum dissolved in PBS (total volume 100 μ l, *see* Fig. 1 and **Note 2**). For GP sensitization, a rough extract of grass pollen (GP, *Phleum pratense*; *Phl p*) containing 8 μ g *Phl p*5a total protein dissolved in 80 μ l PBS (*see* **Note 1**, for GP stock solutions) is added to 20 μ l Inject alum for each mouse that needs to be sensitized. For sensitization against HDM, (*Dermatophagoides pteronyssinus*; *Der p*) 5 μ g HDM extract in 80 μ l PBS is added to 20 μ l Inject alum for each mouse (*see* **Note 1** for HDM stock solutions).

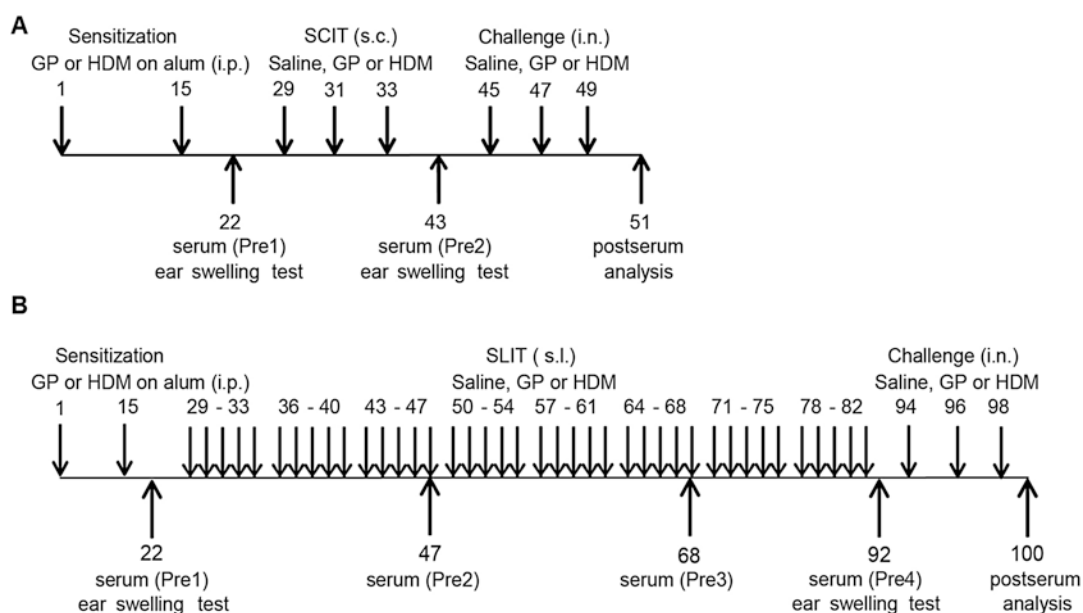


Fig. 1 Overview of AIT-treatment protocols in BALBcByJ mice. **(a)** Outline of the SCIT protocol. **(b)** Outline of the SLIT protocol. Serum is taken before SCIT (Pre1), before challenge (Pre2 in case of SCIT and Pre4 in case of SLIT), and after challenges (Post). In case of SLIT, we include two extra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

2. SCIT treatment

Mice are SCIT treated on days 29, 31, and 33, by subcutaneous injections (*see Note 3*) of the allergen dissolved in PBS (total volume of 100 μ l, Fig. 1a). For GP-SCIT (*Phleum pratense*; *Phl p*), dissolve 500 μ g *Phl p* protein in 100 μ l PBS per injection per mouse. For HDM-SCIT (*Dermatophagoides pteronyssinus*; *Der p*) use 250 μ g HDM in 100 μ l PBS per injection per mouse.

3. SLIT treatment

Mice are SLIT treated for 5 days a week for 8 consecutive weeks (from day 29 until day 82), by sublingual administration (*see Note 4*) of the allergen dissolved in PBS (total volume of 5 μ l, Fig. 1b). For GP-SLIT (*Phleum pratense*; *Phl p*), use 500 μ g *Phl p* in 5 μ l PBS per administration per mouse. For HDM-SLIT (*Dermatophagoides pteronyssinus*; *Der p*), use 250 μ g HDM protein in 5 μ l PBS per administration per mouse.

4. Allergen Challenges

SCIT treated mice are challenged on days 45, 47, 49, while SLIT treated mice are challenged on days 94, 96, 98, by intranasal administration (*see Note 5*) of the allergen dissolved in PBS (total volume of 25 μ l, Fig. 1). For GP challenges, use 40 μ g *Phl p* protein in 25 μ l PBS per administration per mouse. For HDM challenges, use 25 μ g HDM in 25 μ l PBS per administration per mouse.

3.2 Ear Swelling Test and Blood Withdrawal via Orbital Puncture

After sensitization, during SLIT and after SCIT (*see Fig. 1*), all mice receive a retro-orbital puncture to collect blood (*see Note 6*, PreI-serum; max. 8 ml/kg in 14 days), while under anesthesia (4.5 %, 1.5 ml isoflurane/min O₂). Per sample ten small drops are collected in miniCollect tubes and centrifuged in the lab for 11 min at 18,000 $\times g$, after which serum is collected in 100 μ l aliquots in 1.5 ml Eppendorf tubes and stored at -80°C for analysis of immunoglobulins and cytokines. In combination with these procedures, the ear swelling tests (EST) are performed (*see Figs. 1 and 2*), before and after SCIT and SLIT.

The early phase response to allergen provocation is measured by performing an ear swelling test (EST) before and after SCIT and SLIT treatment. Mice are anesthetized using 4.5 %, 1.5 ml isoflurane/min O₂ and 10 μ l of PBS containing a low amount of allergen (either 1 μ g GP or 0.5 μ g HDM) is injected intradermal in the right ear of the mouse (*see Fig. 2*). As a control, 10 μ l of PBS is injected intradermally in the mouse's left. After 1 or 2 h, ear thickness of both ears is measured using a digimatic pressure limited force-micrometer at 0.5 N (± 0.15 N, *see Note 7*). The net thickness (Δ , in mm) can be calculated by subtracting the thickness of the left ear from the right ear (*see Fig. 3a*).



Fig. 2 Photos representing 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 digimatic force-micrometer. It is important to keep the micrometer in a horizontal position

3.3 Lung Function Measurement and Section

Here, the methods and protocols described are based on the FlexiVent version 5.3 (Factory assigned IP address: 192.168.1.200). While we routinely use intravenous administration of methacholine and flexible cannulas for tracheal intubation, alternative approaches including methacholine inhalation and the use of rigid cannulas for tracheal intubation have been described as well [38]. In case immunohistochemical analyses of lung tissue, airway wall remodeling or inflammation is required, this is best performed on a separate group of mice as the forced oscillation technique of invasive airway resistance measurements can be disruptive of local tissue architecture.

1. **Calibration of FlexiVent** should be performed according to the manufacturer's instructions. Briefly, both airway and cylinder pressure need to be calibrated per mouse (weight adjusted) using a 1 ml syringe, a manometer, and a closed and open cannula. For the airway pressure, the first pressure point is zero, the second is the pressure after putting in 1 ml of air. For the cylinder pressure, a dynamic tubing calibration is required using the open and closed cannula according to the manufacturer's instructions.
2. Two days after the final challenge, mice were **weighed and anesthetized** with a mixture of 75 mg/kg ketamine (100 mg/ml) and 1 mg/kg domitor (0.5 mg/ml, *see Note 8*) by intraperitoneal injection (10 μ l mix per gram bodyweight). Repeat this injection with 25% of the initial dose every 20 min. Be aware that during the entire procedure the mice should be kept warm. Furthermore, heart rate, blood pressure and O₂ saturation should be monitored using a pulse-oximeter and recorded.
3. All anesthetized mice were cannulated with a 20 G **trachea cannula** and a jugular vein cannula, then placed on a computer-controlled small-animal ventilator (FlexiVent, SCIREQ).

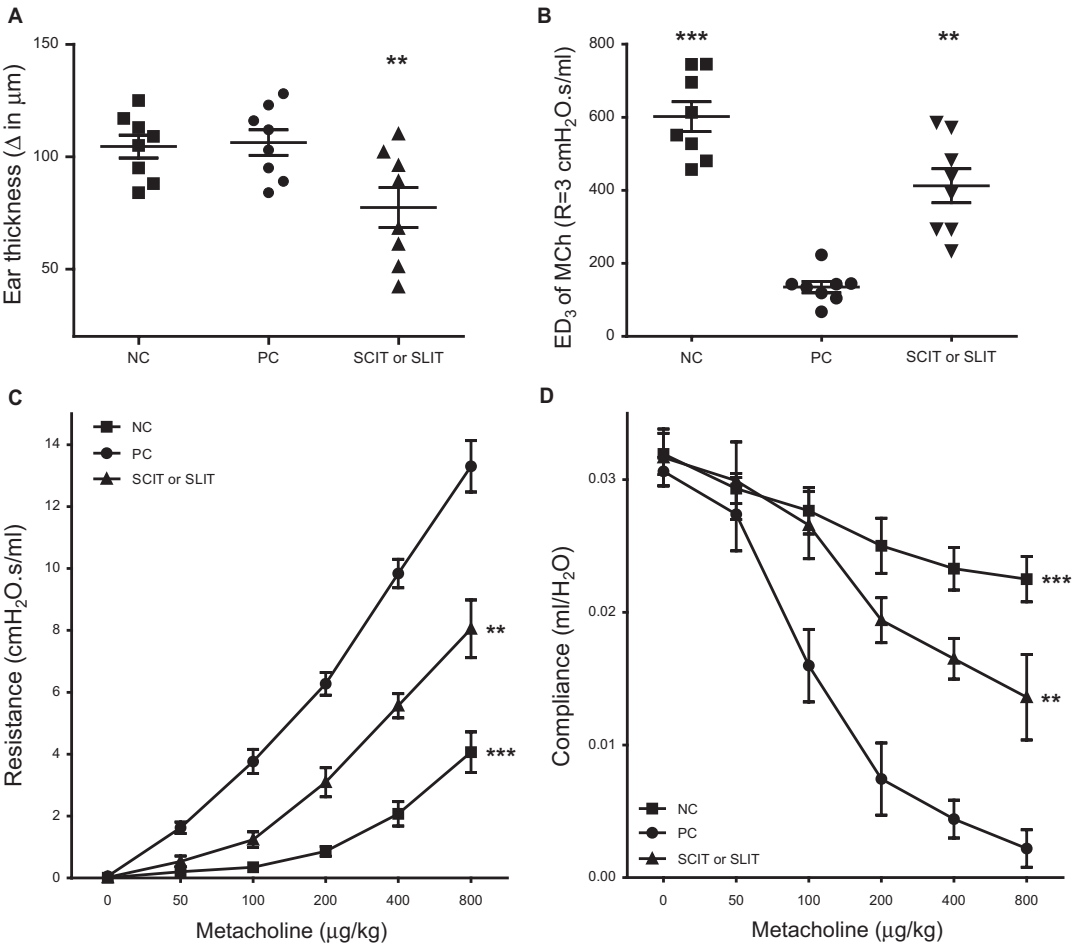


Fig. 3 Clinical manifestations after AIT. (a) IgE dependent allergic response plotted as net ear thickness (mm) 2 h after GP injection (1 kSQ) in the right ear and PBS in the left ear as a control, performed after AIT. Placebo-treated mice (NC and PC) provide similar swelling since both have been placebo treated and are not challenged yet. (b) Effective Dose (ED) of methacholine, when the airway resistance reaches 3 cmH₂O s/ml. (c) Airway hyperactivity (AHR) was measured by FlexiVent and plotted as airway resistance (R in cmH₂O s/ml) and as (d) Airway compliance (C in ml/cmH₂O). 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

Tracheal cannulation: First, make an incision in the middle of the neck and tear the underlying tissue carefully with two sharp pair of tweezers till you see the muscle where the trachea is lying in. Be careful not to touch the gland around the trachea, to prevent increased mucus production. Then, tear the muscle open carefully, completely free the trachea from underlying tissue and place two ligatures, one at the top of the trachea and one at the bottom. Third, cut a hole in the trachea between the trachea rings and place the cannula in the

trachea and close both ligatures. Attach the upper ligature to the cannula with a tight knot. Finally, attach the mice to the FlexiVent machine and set the program to standard breathing (script running on basic breathing, *see* **Note 9**).

Jugular vein cannulation: Make an imaginary line from the right armpit to the left foreleg and from the right armpit to the middle of the jaw, then make a 1.5 cm vertical incision (downwards) on the crossing. Similar as with the trachea cannula, tear the underlying tissue carefully with two sharp pairs of tweezers until you can see the jugular vein and clear it of any fat or surrounding connective tissue, allowing a curved surgical tweezer to maneuver underneath the jugular vein. Then, place a ligature (6/0) around the upper part of the jugular vein, close it and attach this ligature with some tape to the operating table to put some tension on it. Now, place a second ligature (6/0) 0.5 cm down, but do not close the knot completely. Place a bulldog clamp on the lower ligature to increase the tension and cut a hole in the vein between the two ligatures. Place the cannula through the hole in the jugular vein and close the bottom ligature. Test whether the cannula is in place by carefully pulling the plunger of the syringe. A properly cannulated jugular vein will allow blood to be present in the cannula. Fix the cannula with both ligatures. Now, several dosages of methacholine can be given through this cannula according to the FlexiVent's protocol (*see* **Notes 9** and **10**).

4. Before starting the **FlexiVent measurements**, all mice receive a muscle relaxant, 1 $\mu\text{l/g}$ mouse rocuronium bromide (diluted to 0.125 mg/ml), which is repeated after the methacholine dosage of 100 $\mu\text{g/kg}$. When starting the protocol, make sure the positive end-expiratory pressure (PEEP) is set at 20 mm H_2O . According to the weight table (*see* **Note 11**), methacholine is administered intravenously in doses 0, 50, 100, 200, 400, and 800 $\mu\text{g/kg}$, and airway responsiveness is measured by obtaining airway resistance (R in $\text{cmH}_2\text{O s/ml}$), the Newtonian resistance (the resistance of the central or conducting airways, R_n in $\text{cmH}_2\text{O s/ml}$), and compliance (C in $\text{ml/H}_2\text{O}$). Immediately after each methacholine dose flush the tubing with 30 μl saline to make sure that all methacholine enters the body.
5. Immediately after this procedure, all mice were **sacrificed** under continued anesthesia by collecting blood through a vena cava puncture (post-serum) in a miniCollect tube. To start, the abdomen of the mouse is opened and the bowels are repositioned so that the vena cava is clearly visible. After fat removal, the vena cava can be punctured using a 25 G needle on a 1 ml syringe and take out approximately 1 ml of total blood volume.

6. Prepare the body for **Broncho-alveolar lavage** by opening the diaphragm so the lungs can fully collapse, but make sure not to damage the lungs by puncturing. Using a syringe containing 1 ml supplemented PBS (10 ml PBS+1 protease cocktail inhibitor tablet and 3% BSA on room temperature) on the trachea cannula, the lungs can be flushed gently. During draw-back of this first ml of supplemented PBS, it is normal that a small amount remains in the lungs. Store the first ml in a 1.5 ml tube. Repeat this lavage for four times using regular PBS (non-supplemented, on room temperature) and collect this in a 15 ml tube. Store the first ml lavage on ice as BALF and the remaining 4 ml on ice to collect extra cells.
7. Hereafter, individual lung lobes and all other necessary organs can be collected and placed in RPMI1640 medium (~1 ml at 4 °C) and kept on ice. Also, individual lung lobes can be stored separately in cryogen-vials, and snap-frozen immediately after isolation in liquid nitrogen and stored at -80 °C for future analysis. Make sure that the empty cryovials have been weighed prior to dissection to allow measurement of the net weight of the lung lobe retrieved during section.
8. Finally, export all FlexiVent data to Excel files and store them according at the appropriate locations (backed-up network drive), for analysis of resistance and compliance values (*see Note 12*). After analysis of the data, resistance and compliance values can be plotted in a graph against the doses of MCh (*see Fig. 3c, d*). Furthermore, in analyzing the resistance data, one could calculate the effective dose (ED) of MCh necessary to increase AHR to an R of 3 cmH₂O s/ml (ED3) (*see Fig. 3b*). Appropriate statistical evaluation includes ANOVA, or in case of missing values a GEE analysis [39].

3.4 Analysis of the Infiltration of Inflammatory Cells in BAL Fluid

Inflammatory cell composition of BAL and lung tissue can be analyzed by manual counting of cytopsin preparations or by flow cytometric analysis. While the latter allows for a far more detailed analysis, the former does require minimal time investment on the section day of the experiment.

1. First, all **cytopsin slides** need to be numbered and coated. Open the slide holder by its driver, insert a clean slide with appropriate label in the slide holder, place a clean Shandon filter card on the slide in the holder, insert a clean cuvette in the holder above the filter card, and put the holder containing slide—filter card—cuvette in the cyto-centrifuge. Add 20 µl PBS containing 1% BSA in the whole of cuvette and run the centrifuge at 550×*g* for 1 min.
2. Centrifuge the first ml of the **BAL** cell suspension at 590×*g*, 4 °C for 5 min and store the supernatant in 100 µl aliquots at

–80 °C. Do the same for the 15 ml tube containing the additional BAL cells, only discard the supernatant. Now, resuspend the pellet of the 15 ml tube in 500 µl lysis buffer and add this to the pellet of the first ml and incubate for 1 min at room temperature. Then, centrifuge the cells at $590\times g$, 4 °C for 5 min, discard the supernatant and resuspend the pellet in 200 µl PBS containing 1% BSA.

3. To **count** the cells, use an appropriately calibrated method for cell counting (resulting in a BALF **total cell count** (*T*)) and adjust the number of cells to 1×10^6 cells/ml (*see* Fig. 4a).
4. Prepare **cytospins** by adding 100 µl of the cell suspension (1×10^5 cells) to the cuvette of the coated slides and centrifuge at $550\times g$ at room temperature for 5 min. Carefully release the slides from the holder using the driver and let the slides air dry for 10 min at room temperature. Clean the cuvettes using demi-water and 70% ethanol solution overnight.
5. Cytospin preparations are stained with a Diff-Quick staining set (manufacturer's protocol) and 300 cells per cytospin were evaluated using the differential cell counter and differentiated by an observer blinded to the experimental groups into **mononuclear cells** (M), **neutrophils** (N), and **eosinophils** (E) by standard morphology using a light microscope with 100 $\times$ magnification in immersion oil (*see* Fig. 4b).

3.5 Preparation of Single Cell Suspensions of Lung Tissue, Spleen, and Draining Lymph Nodes

1. In preparing a **single cell suspension** from **mouse lung**, the left lung lobe (largest) of every mouse is transferred into a petri dish in the flow cabinet. Using a scalpel, the lobe is cut into a homogenous paste, dissolved in 2 ml RPMI1640 medium including 4 mg/ml collagenase A, 0.1 mg/ml DNase I, and 1% BSA, and incubated for 1.5 h at 37 °C. A single cell suspension is generated by flushing the lung digest through a 70 µm cell strainer into a 50 ml tube. Cell strainers are washed with 2–5 ml of RPMI (at room temperature) in order to flush out remaining cells. The collection tubes are centrifuged at $350\times g$ for 5 min at 4 °C and the supernatant can be discarded. Pellets are resuspended in 1 ml lysis buffer and incubated for 3 min at room temperature. Cells are then centrifuged again at $350\times g$ for 5 min at 4 °C and supernatant is discarded. The cells are counted using a Coulter counter and used for cytospin (*see* Subheading 2.4 BAL Fluid) and restimulation (*see* Subheading 2.6).
2. **Single cell suspensions of spleen cells** are obtained in a similar fashion, although enzymatic digestion is only required in case of DC subset analyses. Single cell suspensions are generated by flushing the spleen tissue suspension through a 70 µm cell strainer and rinsing the strainer with 5 ml sterile RPMI1640,

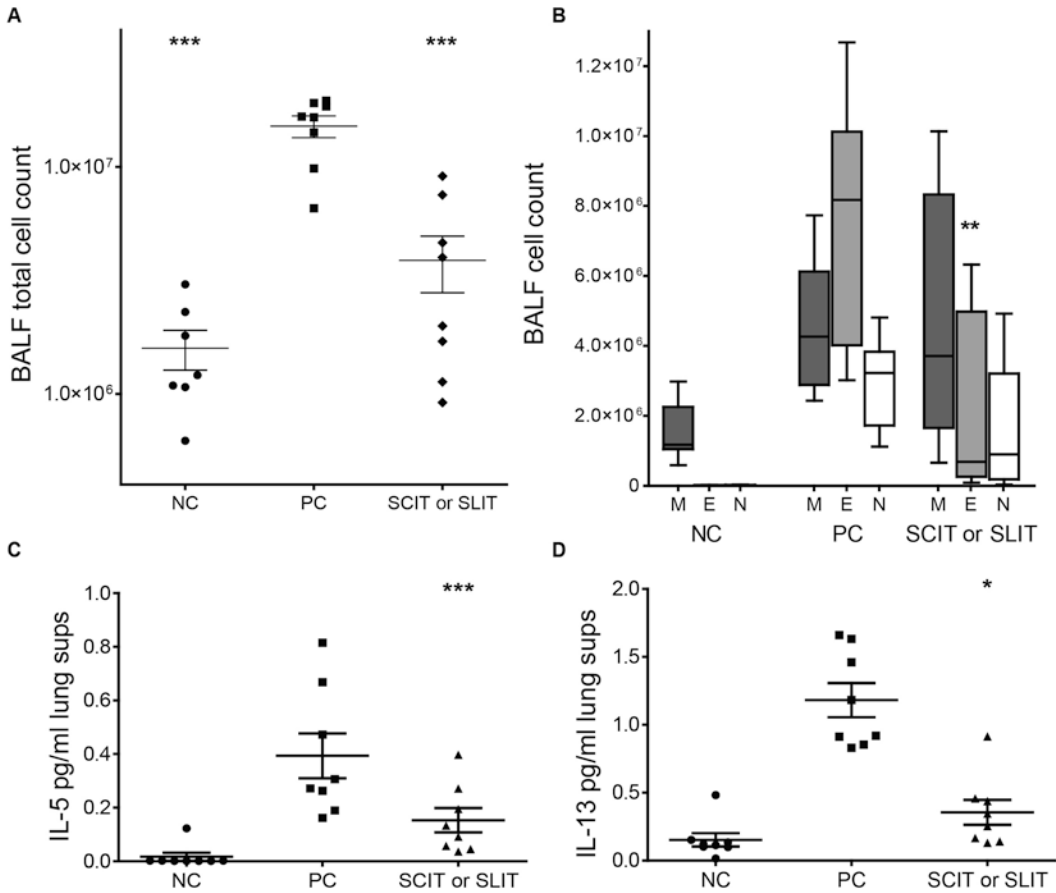


Fig. 4 The eosinophilic and proinflammatory cytokine response after AIT. **(a)** Total cell counts in bronchoalveolar lavage fluid (BALF). **(b)** Differential cytopsin cell counts in BALF. *M* mononuclear cells, *E* eosinophils, *N* neutrophils. Absolute numbers are plotted as median and 10–90 interquartile. **(c and 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

before centrifuging the spleen cells at $550 \times g$ for 5 min. Discard the supernatant, add 1 ml lysis buffer and incubate for 10 min at room temperature. Wash the cells with 1 ml RMPI1640 twice and cells are ready for further use. For storage of the cells in liquid nitrogen, resuspend the cell pellet in 50% HBSS, 40% FCS, and add slowly 10% DMSO, then divide the suspension over both cryogen vials, and place them in a Stratagene® box in the -80°C overnight. The day after, transfer the vials into the liquid nitrogen.

- Cells originating from the (lung) **draining lymph nodes** (DLN) can be processed in a similar fashion as the lung tissue cells.

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

1. To evaluate the T cell responses in **lung tissue single cell suspensions**, 200,000 cells/well are restimulated in 250 μ l RPMI1640 (supplemented with 10% FCS, pen/strep; 50 μ M beta-ME) with either 0 or 30 μ g of GP or HDM extract *in triplo* in 96-well plates (U-bottom). Lung cell cultures are incubated for 5 days at 37 °C and 5% CO₂. The supernatant of all wells are pooled per mouse, aliquoted *in triplo* and stored in –80 °C, for analysis of cytokines.
2. Cell suspensions from draining **lymph nodes** can be restimulated in a similar way as described above. Using a round-bottom 96-well plate, 200,000 cells/well can be restimulated with GP or HDM extracts (30 μ g/well). Since lymph node-cell suspensions usually do not contain as much cells as the lung suspensions, here measurements can be done *in duplo*.

3.7 Homogenization of Lung Tissue for Total Protein and Cytokine Analysis

To measure levels of specific cytokines, chemokines or other mediators in lung tissue, individual lung lobes are used either freshly at the day of section, or at a later stage using lung lobes snap-frozen on the day of section and retrieved from –80 °C for analysis. Use the identical lung lobe for all mice in the experimental and control groups. Keep the lung lobes on ice at all times.

1. Weigh the cryogen vials containing the lung lobe and correct for the empty cryogen vial weight to obtain the net lung lobe weight (mg lung tissue).
2. Add Luminex buffer in a ratio of 1:5 weight/volume of each lung (1 mg lung + 4 μ l buffer).
3. Homogenize the lung with the homogenizer (on ice!) for at least 1 min, until no more large pieces are visible. Then, clean the homogenizer (dismantle the crusher) with tap water (once) and ethanol (once) in between samples. Clean and dry the homogenizer.
4. Centrifuge the crushed lung samples at 12,000 $\times g$ for 20 min at 4 °C.
5. Collect supernatants, store them in 100 μ l aliquots at –80 °C until further analysis, and discard the pellets.
6. When evaluating cytokine responses between experimental groups, it is important to measure cytokine-levels (ELISA) and plot them relative to protein content instead of per ml lung tissue homogenate. Protein concentrations retrieved from homogenization of lung lobes vary between individual mice, so this needs to be corrected for. Therefore, a BCA analysis can be performed to quantitate protein content of the lung tissue homogenates according to the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA).

**3.8 Analysis
of Immunoglobulin
Levels in Serum
and Cytokine Levels
in BAL Fluid,
Supernatant
of Restimulated Single
Cell Suspensions
and Lung Tissue
Homogenates**

1. ELISA measurements can be performed to determine the **immunoglobulin levels in serum** of total as well as GP- or HDM-spIgG1, GP- or HDM-spIgG2a, GP- or HDM-spIgE in serum taken at three different time points (pre1-, pre2-, and post-serum, stored at -80°C).
2. Cytokine measurements in BAL fluid, supernatant of restimulated single cell suspensions and lung tissue homogenates can be performed by ELISA or multiplex analysis (a wide range of commercial suppliers available). For accurate quantification of cytokine levels by ELISA, samples should be stored at -80°C until measurement, and, as a rule of thumb, samples should be processed within 3 months from isolation.
3. Protocol for Ig-ELISA measurements in sera: coat a high affinity binding 96-well flat bottom ELISA plate with 100 μl purified rat anti-mouse IgE/G1/G2a (*see* Table 1) in PBS overnight at 4°C . Wash the plate five times using 300 μl wash buffer (PBS containing 0.05 % Tween 20) either with a ELISA plate washer or by hand. Block the plate using 300 μl ELISA buffer with 1 % BSA at room temperature for 1 h. Empty the plate, wash once with wash buffer, make sure the wells are emptied and add the samples and standard (both *in duplo*) in a total volume of 100 μl and incubate for 2 h at room temperature on the shaker 300 rpm. For concentration of the reference curve and the appropriate dilution of the serum samples please refer to Table 1. Usually, pre-sera samples will be diluted 1:30 and post-sera samples 1:60 in ELISA buffer; however several dilutions can be tested for optimal results. Wash the plate five times using 300 μl wash buffer. Add detection Ab diluted in ELISA buffer (*see* Table 1 and Note 13) to the plate in 100 μl and incubate for 1.5 h at room temperature on the shaker at 300 rpm. Wash the plate five times using wash buffer. Add 100 μl horseradish peroxidase (HRP) diluted 1:200 in ELISA buffer and incubate for 1 h at room temperature on the shaker 300 rpm. Wash the plate again and add 100 μl OPD peroxidase substrate (according to manufacturers' instructions) and incubate for 10 min in the dark until the coloring reaction is complete. Stop the reaction by adding 75 μl 4 M H_2SO_4 to each well. Read the optical density at 490 nm on the ELISA plate reader. Calculate the concentrations using of the sera samples based on the reference curve a four parameter model. The fit of the standard curve should be at least 0.95 (r^2). When performing multiple 96-well plates in one measurement, each plate should contain a standard curve as well as a few reference samples that are included on all plates for plate-plate comparison.

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

Divide all lung single cell suspensions in the FACS tubes per mouse per mixture and use a small amount of cells for the single staining (two donors per single). Start with a wash using 300 μ l FACS Buffer, centrifuge the cells at $590\times g$ for 5 min and remove supernatant. Then, resuspend in 100 μ l FACS buffer, add an extracellular block using 100 μ l Block buffer (2% NRS, 5% FcBlock in FACS buffer) and incubate for 10 min on room temperature. Centrifuge, remove supernatant and resuspend in 100 μ l FACS buffer, add 100 μ l extra-cellular staining, and incubate for 30 min on room temperature in the dark (*see* Tables 2 and 3 for the mixtures). Centrifuge and wash in 1000 μ l FACS buffer then wash again in 1000 μ l regular PBS. Resuspend in 100 μ l PBS, add 75 μ l fixable live dead V450 (1:1000 diluted in PBS) incubate for 15 min on room temperature in the dark. Wash once in 1000 μ l regular PBS and resuspend in 1000 μ l FIX, incubate for 30 min on room temperature in the dark. Without washing add 1000 μ l PERM buffer. Centrifuge and remove supernatant and then resuspend in 100 μ l PERM buffer containing 2% NRS and 5% FcBlock for intracellular blocking. After 10 min incubation, centrifuge, remove supernatant, and resuspend in 100 μ l PERM buffer. Then add 100 μ l intracellular staining (antibodies diluted in PERM buffer), and incubate for 30 min on room temperature in the dark (*see* Tables 2 and 3 for the mixtures). Without washing add 1000 μ l PERM buffer, centrifuge and remove supernatant. Wash once more in 1000 μ l FACS buffer and remove supernatant. Finally, resuspend 200 μ l FACS buffer and transfer the cells to the 35 μ m cell strainer FACS tube. Measure all singles and samples using a three laser flow cytometer (eight fluorochromes).

In analyzing the FCS files derived from the measurements, always start by setting the compensation settings using the single stained cells, depending on the program used. Once, these are corrected throughout the entire experiment, start the analysis by excluding all dead cells as well as doublets. The first can be done by plotting the area of the forward scatter (FSC-A) against the fixable live dead marker and exclude the dead cells (*see* Fig. 5a). Second, all doublets can be excluded using a small selection gate when plotting the area of the side scatter (SSC-A) against the width of the side scatter (SSC-W, *see* Fig. 5b). Then, depending on the mixing strategies can be adjusted depending on which cell type needs to be identified and or quantified. Here, as an example, the gating strategy for ILC2s can be plotted as Lineage negative cells versus CD45 positive cells (*see* Table 2 and Fig. 5c). Subclassification can be done using GATA3 and CD127, markers that are both expressed by ILC2s (Fig. 5d).

For the identification of other cell types, CD4 and GATA3 can be used to identify Th2 cells, CD4 and FoxP3 can be used to identify

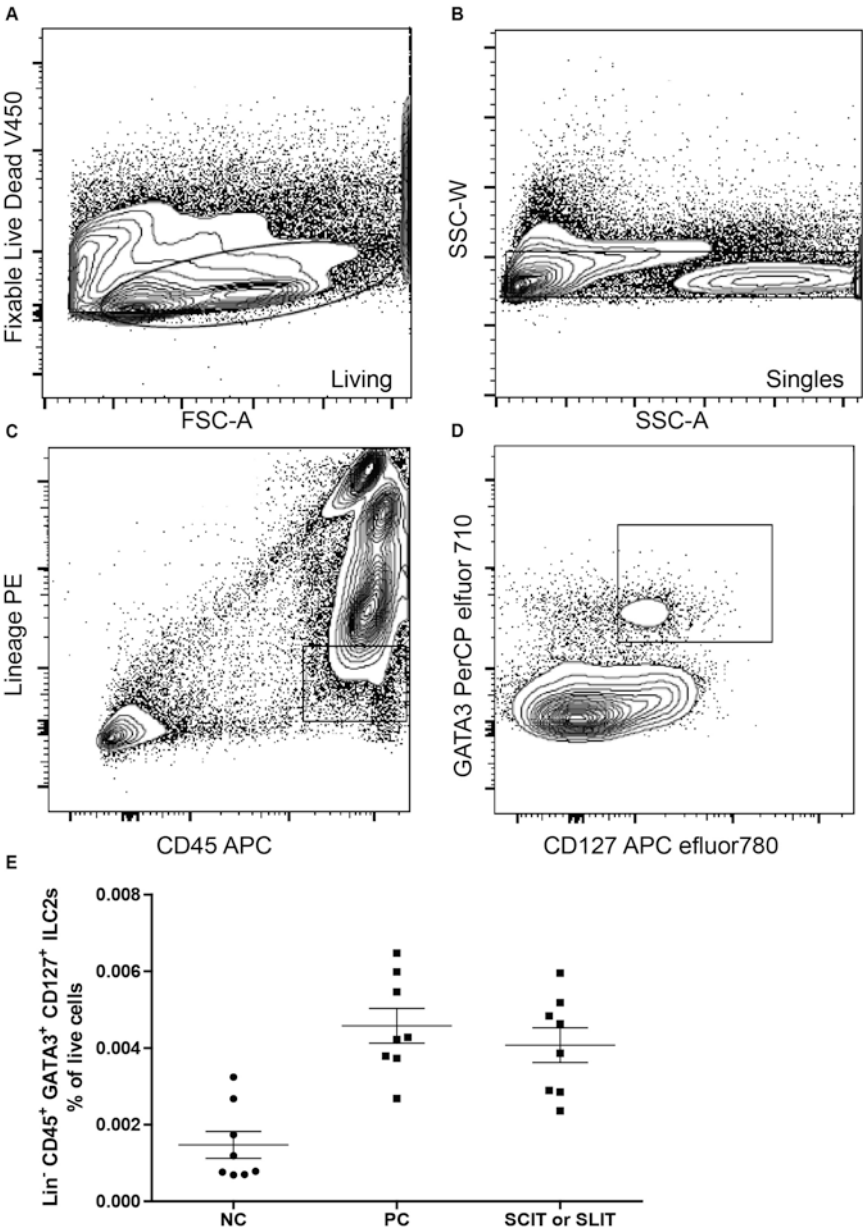


Fig. 5 Gating strategy for the identification of innate lymphoid cells type 2 (ILC2s) in the lung. (a) Gating of live cells. (b) Doublet cell exclusion. (c) Gating out the lineage negative cells and including the CD45 positive cells. (d) Gating for only the GATA3 and CD127 double positive cells. (e) ILC2s plotted as Lineage⁻CD45⁺GATA3⁺ and CD127⁺ % of live cells

Treg cells, and CD11b and CD103 can be used to identify subpopulations of different types of conventional DCs (see Table 3). The gating strategy starts similar as mentioned before, with the exclusion of dead cells and doublets.

4 Notes

1. **Stocks of GP:** dissolve the 204 mg dry matter of *Phleum pratense*, 225 (MP225PHLpra, 1006674 or 1031225) in 2.125 ml sterile PBS to get a solution containing 60 kSQ/ μ l ($\sim 96 \mu\text{g}/\mu\text{l}$). Aliquot this stock in small 100 μ l portions and store at -20°C . **Stocks of HDM:** dissolve 25.0 mg DP extract FD 12C27 in 500 μ l sterile PBS to get a solution containing 50 $\mu\text{g}/\mu\text{l}$ HDM and aliquot this stock in 25 μ l portions and store at -20°C .
2. Performing **intraperitoneal injections:** the non-anesthetized animal is restrained manually (one-handed) and tilted head down at $35\text{--}40^\circ$ 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 $\frac{1}{2}$ inch, 27 G needle or insulin syringe is recommended. This route of administration allows for a volume of up to 1 ml be given safely to a mouse.
3. **Subcutaneous injections** can be used for administration of the allergen extracts. The non-anesthetized animal is held using a standard single handed restraint technique and a 25 G needle is inserted at the base of a skin tent created by the thumb and forefinger. A slight negative pressure is applied to the syringe to ensure proper placement is subcutaneous and not having accidentally damaged a local vein. A total of 100 μ l is injected.
4. For **sublingual administration**, the non-anesthetized animal is restrained manually (one-handed) and the head is tilted up at full vertical position. Tightly fix the fir between thumb and index finger, until the mice slightly stick out their tongue. Using a P20 pipet, 5 μ l of allergen solution can be placed under the tongue, while ensuring that it stays there for 30 s minimum. It is of paramount importance that the mice do not swallow the allergen during the administration. After administration, maintain the restrained animal for 30 s up-straight to prevent swallowing until the mouse can be placed back in the individual ventilated cage (IVC).
5. The **intranasal challenges** are performed with the mouse lightly and quickly anesthetized using 4.5 % isoflurane in combination with 1 ml/min O_2 . Mice are taken out of the anesthesia as soon as their breathing starts to slow down a bit. The mouse is manually restrained and the tail anchored between the small finger and the palm. The mouse is held in a supine position with the head elevated. Right before the mouse wakes

up (should be within 1 min), the end of a micropipette is placed at or in the external nares and then the solution is poured in. Pipet 25 µl of the allergen extract dissolved in PBS, quickly on each of the nares as the mouse wakes up and inhales into the lungs. Check visually whether the allergen solution has been inhaled by the mouse.

- 6. **Retro-orbital bleeding** 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. Hold the anesthetized mouse on a flat surface and gently press it against the flat surface, to force blood from the thorax to the head. The forefinger of the same hand is used to pull the dorsal eyelid back producing slight exophthalmos (bulging of the eye). Then, use a glass microcapillary tube to penetrate the orbital conjunctiva at the medial or lateral canthus of the eye. As soon as blood accumulates in the capillary lift up the mouse and hold it above the MiniCollect tube and let ten drops of blood fall in. Be aware, the speed of which the blood drops in the tube can vary significantly! When punctured just right, you have to act quickly.
- 7. In using the **digimatic pressure-limited micrometer**, it is really important to keep it perfectly horizontal when measuring ear thickness. Therefore, all mice need to remain 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 on a small bridge/cup to make sure the ears are in the same horizontal position every time.
- 8. Mice can be anesthetized for invasive lung function measurement using the following dilution schedule:

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

- 9. 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 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 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 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 meas-
urement 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 → re-
peat for the dosages 100, 200, 400 and 800
µg/ kg // End of script

```

10. Before putting a new syringe on the IV-line, make sure there is no air remaining in the syringe and cannula (use 5 ml syringe with saline to fill the 1 ml syringe).
11. IV injection of several doses of methacholine: see dilution table and weights:

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

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

Weight (g)	Injection volume (μl)
34	109
35	112
36	115
37	118
38	122
39	129
40	128

12. Analysis of resistance and compliance data can be performed using the exported files. Herein, a clear stepwise report is made for every subject. When interested in the resistance and compliance values, simply highlight those, together with the COD values, the PEEP (and optional the Newtonian resistance R_N). Every dose of MCh gives a peak value in resistance (cmH₂O s/ml) and a bottom value in compliance (ml/H₂O). Highlight those for every dose of MCh and for every subject, and plot them. To ensure high quality data use the coefficient of determination (COD)-value with a cutoff of 8. This is a quality control parameter measuring the quality of the single compartment model fit. Alternatively, all values that are measured with sufficient technical quality (use COD value as a cutoff) can be plotted to calculate an area under the curve for each individual methacholine dose. Check whether the PEEP was registered as ~20 mm H₂O or ~2 cm H₂O.
13. Biotinylation of grass pollen and house dust mites is performed using the “Thermo Scientific EZ-Link Sulfo-NHS-LC-Biotin,” an intermediate-length, water-soluble biotinylation reagent for labeling proteins (Thermo Scientific, 21327). According to the manufacturers’ protocol, we can adjust the molar ratio of Sulfo-NHS-LC-Biotin to protein to obtain the level of incorporation desired (~4–6 biotin groups per allergen-particle). Calculate millimoles of biotin reagent to add to the reaction for a 20-fold molar excess: **mmol biotin** = ml protein × (mg protein/ml protein) × (mmol protein/mg protein) × (20 mmol biotin/mmol protein). Then, calculate microliters of 10 mM biotin reagent solution (stock) to add to the reaction: **μl biotin** = mmol biotin × (1,000,000 μl/l) × (l/10 mmol). Since, the rough extracts contain a mixture of proteins with a lot of different molecular weights, we make an estimate based on SDS-PAGE results. For the rough extract of GP, we calculated using an average molecular weight of 10,000 Da. An example: 1000 μl GP × (3.9 mg GP/1 ml GP) × (1 mmol GP/10,000 mg GP) × (5 mmol biotin/1 mmol GP) = 1.95×10^{-3} mmol biotin. Second, 1.95×10^{-3} mmol biotin × (1 × 10⁶ μl/l) × (l/10 mmol) = 195 μl

biotin. 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. Incubate for 2 h on ice or for 30 min on room temperature. For optimal performance and stability, we purify the labeled proteins using a Slide-A-Lyzer™ G2 Dialysis Cassettes, 3.5K MWCO, 0.5 ml according to the manufacturers' protocol (Thermo Scientific, 87722). In short, after hydrating the membrane in PBS for 2 min, using a 1 ml syringe with 18 G needle, slowly fill the cassette with your biotin–allergen mixture and withdraw any remaining air. Next, perform a typical dialysis procedure: 2 h at room temperature in 2 l PBS; then change the PBS and dialyze for another 2 h; change the buffer again and dialyze overnight at 4 °C. The next day, you can remove the purified sample and store aliquoted in the –20 °C freezer.

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