

Efficiency of Biolistic DNA Vaccination in Experimental Type I Allergy

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Abstract

Gene gun-mediated delivery of allergen-encoding plasmid DNA has been in focus for many years now as being a needle-free alternative to the protein-based desensitization regimen used in specific immunotherapy. Biolistic immunization with the Helios gene gun has proven to be potent in the induction of antigen-specific CD4⁺ and CD8⁺ T cells. Here we describe biolistic vaccination in experimental mouse models of IgE-mediated type I allergy as well as allergen-induced airway inflammation.

Key words: Allergy, DNA vaccination, Gene gun, IgE, Allergic airway inflammation

1. Introduction

The crucial event in the pathogenesis of type I allergy (atopic disease) is excessive production of allergen-specific IgE antibodies, promoted by the elicitation of a distinct Th2 immune response. Since Raz et al. (1) reported for the first time that intradermal injection of antigen-encoding DNA prior to sensitization efficiently suppressed specific IgE immune responses, genetic immunization has been in focus as an attractive alternative to specific immunotherapy (SIT) which until now represents the only causative treatment of allergy. Various routes of DNA administration have been tested to modulate allergic sensitization in animal models of type I allergies (reviewed in ref. (2)) including bombardment with DNA-coated gold particles using the helium-powered gene gun. In contrast to intramuscular or intradermal needle injection, biolistic

transfection of the skin allows for the direct introduction of plasmids into cutaneous antigen-presenting cells (epidermal Langerhans cells, dermal dendritic cells) and therefore requires 10–100 times lesser amounts of DNA to elicit an immune reaction (3, 4). We and others have shown that gene gun-mediated immunization using antigen-encoding plasmid DNA activates CD4⁺ T cells, which in addition to interleukin-(IL)-4 and IL-5 release high amounts of interferon-(IFN)- γ (1, 5–8). In addition, considerable numbers of antigen-specific IFN- γ -producing CD8⁺ effector T cells were demonstrated in gene gun-immunized mice (5, 6, 9). IFN- γ -producing CD4⁺ as well as CD8⁺ T cell populations are known to play important roles in the inhibition of IgE responses. Consequently, IgE production induced by sensitization with β -galactosidase (β Gal) protein as model antigen was significantly suppressed by preceding biolistic immunization with β Gal-encoding plasmid vectors (Fig. 1) (6, 10, 11). Moreover, eosinophilic infiltration into the lungs after intranasal challenge of β Gal-sensitized mice was strongly reduced by prophylactic application of β Gal-encoding plasmids in an experimental model of allergic airway inflammation (Fig. 2) (12). Although biolistic transfection improved Th2-associated pathology, antigen-induced airway hyperreactivity (AHR) was not alleviated by biolistic transfection (Fig. 3), probably due to immigration of neutrophils and/or IFN- γ producing T cells into the lung (12). In conclusion, gene gun-mediated biolistic transfection represents an attractive alternative therapeutic approach to genetic immunization by intramuscular or intradermal needle injection.

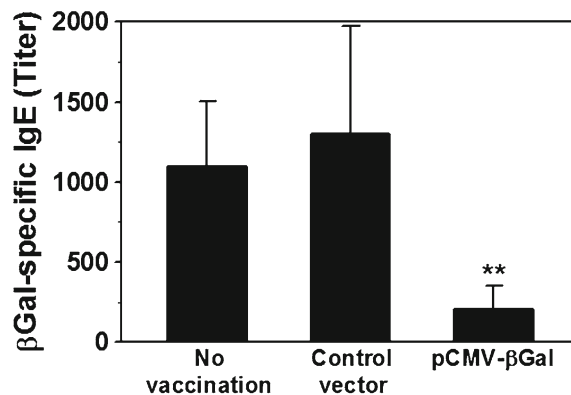


Fig. 1. Inhibition of IgE production by prophylactic gene gun-mediated vaccination. BALB/c mice ($n=4$) were vaccinated every week by a total of five biolistic transfections with pCMV- β Gal or a non-encoding control vector or were left unvaccinated. Subsequently mice were sensitized by six intraperitoneal injections of 1 μ g β Gal adsorbed to aluminum hydroxide as adjuvant at intervals of 2 weeks. Four days after the sixth application of β Gal, levels of β Gal-specific IgE in sera were determined by ELISA. Data represent mean β Gal-specific IgE titer $\pm$ SD. ** $P<0.01$.

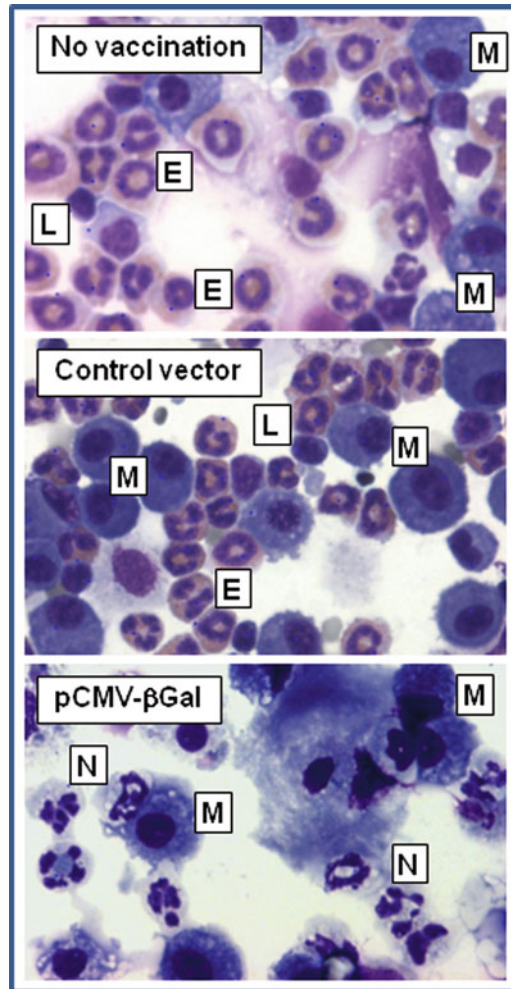


Fig. 2. Reduction of pulmonary eosinophilic infiltration by prophylactic gene gun-mediated vaccination. BALB/c mice ($n=4$) were vaccinated every week by a total of three biolistic transfections with pCMV- β Gal or a non-encoding control vector. Subsequently vaccinated mice as well as untreated control mice were sensitized at a 10 day interval by two intraperitoneal injections of 1 μ g β Gal adsorbed to aluminum hydroxide as adjuvant. One week later all mice were challenged with antigen on three consecutive days by intranasal application of 50 μ g β Gal. Twenty-four hour after the last challenge BAL was performed and BAL cells were differentiated using Diff-Quick staining. *M* monocyte/macrophage; *L* lymphocyte; *E* eosinophil; *N* neutrophil.

2. Materials

Unless otherwise indicated use ultrapure water for preparation of the solutions (Milli-Q-Integral System, Millipore, Schwalbach, Germany) and reagents of analytical grade.

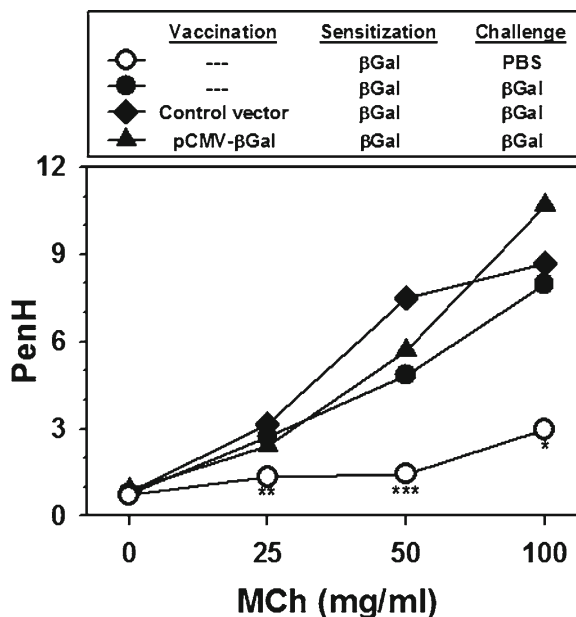


Fig. 3. Measurement of airway reactivity after prophylactic gene gun-mediated vaccination. BALB/c mice ($n=4$) were vaccinated every week by a total of three biolistic transfections with pCMV- β Gal or a non-encoding control vector. Subsequently vaccinated mice as well as untreated control mice were sensitized at a 10 day interval by two intraperitoneal injections of 1 μ g β Gal adsorbed to aluminum hydroxide as adjuvant. One week later (all) mice were challenged with antigen or saline on three consecutive days by intranasal application of 50 μ g β Gal, a separate control group of sensitized mice was treated with PBS. Twenty-four hours after the last challenge airway reactivity was measured by non-invasive plethysmography in response to increasing doses of methacholine (MCh) as enhanced pause (PenH). Data represent means. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

2.1. Mice

1. Female BALB/c mice (see Note 1).
2. BALB/c mice were vaccinated three to five times in weekly intervals with a plasmid encoding β Gal as model antigen under control of the ubiquitously active CMV promoter (pCMV- β Gal) (9) (see also Chapter 17 in this volume).

2.2. Mouse Model of IgE-Mediated Type I Allergy

1. Recombinant β Gal from *Escherichia coli* (Sigma-Aldrich, Deisenhofen, Germany) as antigen. Prepare a 1 mg/mL β Gal stock solution in phosphate buffered saline (PBS).
2. Aluminum hydroxide (e.g., Imject® Alum, Thermo Fisher Scientific, Rockford, IL, USA) as adjuvant.
3. 15 mL polypropylene tubes.
4. 1 mL syringe with 0.7 mm cannula.
5. 96 well ELISA microtiter plates (see Note 2) and ELISA plate seals.
6. Precision microplate reader.
7. Microplate washer.
8. PBS: 137.5 mM NaCl, 10 mM NaH_2PO_4 , pH 7.2.

9. Coating Buffer: 0.1 M NaHCO_3 , pH 8.2.
10. Detection antibody: monoclonal rat anti-mouse-IgE (clone EM-95.3 (13)).
11. Secondary antibody: biotin-conjugated mouse anti-rat-IgG (e.g., Dianova, Hamburg, Germany).
12. Streptavidin-conjugated horseradish peroxidase (e.g., ExtrAvidin-Peroxidase, Sigma-Aldrich).
13. Dilution buffer: PBS with 1% bovine serum albumin.
14. Washing buffer: PBS with 0.05% Tween 20.
15. Substrate buffer: 0.2 M NaH_2PO_4 , 0.1 M sodium citrate, pH 5.0.
16. Ortho-phenylenediamine (OPD) (Sigma-Aldrich).
17. 30% H_2O_2 .
18. Stop solution: 1 M H_2SO_4 .

2.3. Mouse Model of Allergen-Induced Airway Inflammation

2.3.1. Intranasal Challenge with βGal

1. Recombinant βGal (Sigma-Aldrich). Prepare a 1 mg/mL βGal stock solution in PBS.
2. 1.5 mL Eppendorf tubes.
3. Forene® (Abbott GmbH & Co. KG, Wiesbaden, Germany) as narcotic agent (active substance: isoflurane).
4. Exsiccator.

2.3.2. Bronchoalveolar Lavage

1. 1 mL syringe with 0.7 mm cannula.
2. Intravenous catheter Vasofix Safety® (Braun, Melsungen, Germany).
3. 1.5 mL LoBind tubes (Eppendorf, Hamburg, Germany).
4. PBS: 137.5 mM NaCl, 10 mM NaH_2PO_4 , pH 7.2.
5. Bronchoalveolar lavage (BAL) solution: PBS with 10% fetal calf serum.
6. Gey's lysis buffer: 10 mM KHCO_3 , 155 mM NH_4Cl , 100 μM EDTA, pH 7.4; sterile by filtration (0.2 μm).
7. Microscope glass slides with frosted ends and 16 mm cover glasses.
8. Cytospin centrifuge with cell funnels and filters.
9. 50 mL polypropylene tubes.
10. Diff-Quick staining kit (Siemens Healthcare Diagnostics, Eschborn, Germany).
11. Entellan® rapid embedding agent for microscopy (Merck, Darmstadt, Germany).
12. Hemocytometer.
13. Stereo microscope with digital camera unit.
14. Trypan blue.
15. Foamed polystyrene plate.

16. Canullas 0.7 mm.
17. Tweezers.
18. Anatomical scissors.

**2.3.3. Noninvasive
Measurement of Lung
Function**

1. Four-chamber whole-body plethysmography system (Buxco Research Systems, Wilmington, NC, USA).
2. Methacholine (Acetyl- β -methylcholine chloride) (Sigma-Aldrich).
3. PBS: 137.5 mM NaCl, 10 mM NaH_2PO_4 , pH 7.2.
4. Nebulizer.
5. BioSystem XA Software (Buxco Research Systems).

**2.3.4. Invasive
Measurement of Lung
Function**

1. Four-chamber resistance and compliance (RC) system (Buxco Research Systems).
2. Methacholine (Acetyl- β -methylcholine chloride) (Sigma-Aldrich).
3. PBS: 137.5 mM NaCl, 10 mM NaH_2PO_4 , pH 7.2.
4. Narcoren® (Merial GmbH, Halbergmos, Germany) as narcotic agent (active substance: pentobarbital). Prepare 1:5 Narcoren® stock solution in PBS.
5. 2 mL Eppendorf tubes.
6. Tweezers.
7. Anatomical scissors.
8. Scalpel.
9. Spatula.
10. Medical yarn.
11. 18 gauche tracheal tube.
12. Esophagus tube.
13. Ventilation pump.
14. BioSystem XA Software (Buxco Research Systems).

3. Methods

3.1. Mouse Model of IgE-Mediated Type I Allergy

3.1.1. Treatment Protocol

1. *Sensitization:* Dilute β Gal stock solution to 10 $\mu\text{g}/\text{mL}$ in PBS. Thoroughly mix β Gal working solution with an equal volume of aluminum hydroxide. Immunize gene gun-vaccinated as well as naïve control mice up to five times in intervals of 2 weeks by intraperitoneal injection of 200 μL of β Gal/adjuvant mixture (see Note 3) into the lower right quadrant of the abdomen ([10](#), [11](#)) (see Note 4).

2. Collect blood various times during the vaccination and sensitization period by puncture of the retro-orbital plexus or by tail vein bleeding (see Note 5). Remove clot of blood after 60 min at room temperature (RT) and centrifuge the cell suspension for 5 min at $10,000 \times g$. Store the supernatant as serum in safe lock tubes at -20°C until thawing for determination of βGal -specific IgE titers (see Note 6).

*3.1.2. Detection of
 βGal -Specific IgE in Sera
by ELISA*

1. Dilute βGal stock solution to $5 \mu\text{g}/\text{mL}$ in coating buffer. Add $100 \mu\text{L}$ of antigen solution to the wells of a 96-well ELISA microtiter plate. Keep plate at 4°C overnight.
2. Wash plate with washing buffer using the ELISA washer (see Note 7).
3. Add $200 \mu\text{L}$ of dilution buffer to each well for 1 h at RT to block residual binding sites.
4. Wash plate with washing buffer.
5. Add $100 \mu\text{L}$ of dilution buffer to each well. Prepare serial dilutions (1:2) of the serum: pipet $100 \mu\text{L}$ of serum into the first well and after careful mixing transfer $100 \mu\text{L}$ of solution to the next well. Repeat seven times. To evaluate background reaction by the reagents, add diluent buffer only to separate antigen-coated wells as blank control. After sealing the plate, incubate 2 h at RT or overnight at 4°C .
6. Wash plate twice with washing buffer.
7. Add $100 \mu\text{L}$ of detection rat anti-mouse IgE antibody (1:1,000 in dilution buffer) to the wells and incubate plate for 45 min at RT.
8. Wash plate twice with washing buffer.
9. Add $100 \mu\text{L}$ of secondary biotin-conjugated mouse anti-rat-IgG antibody ($1 \mu\text{g}/\text{mL}$ in dilution buffer) to each well and incubate plate for 45 min at RT.
10. Wash plate twice with washing buffer.
11. Add $100 \mu\text{L}$ of streptavidin-conjugated horseradish peroxidase (1:2,000 in dilution buffer) as enzyme (see Note 8) to each well for 30 min at RT.
12. Wash plate four times with washing buffer.
13. Prepare OPD solution ($1 \text{ mg}/\text{mL}$) in substrate buffer. Immediately before use add $1 \mu\text{L}$ $30\% \text{H}_2\text{O}_2/\text{mL}$ OPD solution. Apply $100 \mu\text{L}$ of the mixture to the wells until color changes into yellow.
14. Stop the enzymatic reaction by adding $100 \mu\text{L}$ of $1 \text{ M H}_2\text{SO}_4$ to each well (see Note 9).
15. Measure optical density (OD) of the samples at a wave length of 490 nm using the Microplate reader.

16. Subtract OD of blank wells from OD of sample wells. Use linear regression analysis to calculate the antibody titer of the serum as the reciprocal serum dilution yielding an absorbance value of OD = 0.2.

3.2. Mouse Model of Allergen-Induced Airway Inflammation

3.2.1. Treatment Protocol

1. *Sensitization*: Immunize gene gun-vaccinated as well as naïve control mice three times in weekly intervals by intraperitoneal injection of β Gal as described in Subheading 3.1.1, step 1.
2. *Challenge*: Anesthetize mice 7 days after the last sensitization by sedation with Forene® in an exsiccator. Sprinkle paper towel on the bottom of the exsiccator with the narcotic and place a mouse in the tank until it is quiescent (see Notes 10 and 11). Challenge the mice by intranasal instillation of 50 μ L β Gal stock solution (see Notes 12 and 13). Control mice receive PBS alone intranasally (see Note 14). Repeat the procedure on the two subsequent days.

3.2.2. Differential Staining of BAL Cells

Perform a BAL to analyze the cells infiltrating the lung after intranasal challenge, and differentiate the cells recovered according to their morphology and staining behavior.

1. Kill the mouse by intraperitoneal injection of a lethal dose of 200 μ L of Narcoren® working solution (1:5 in PBS).
2. Place the mouse in a supine position on a foamed polystyrene plate and fix the extremities with cannulas to obtain a slight tension on the thorax.
3. Prepare the thorax by opening the skin of the mouse with an Y-cut, starting ventral of the body and ending at the forelimbs. Fix the skin with cannulas on the plate.
4. Open the thorax by cutting the dermal layer directly below the sternum and further along the costal arch.
5. Carefully retain the liver from the diaphragm. Perforate the diaphragm without injuring the lobes of the lungs by cutting a hole in the diaphragm where the heart is visual through the diaphragm (marked by a darker spot).
6. Carefully prepare the trachea by removing the adjacent tissue and dermal layer (see Note 15).
7. Carefully perforate the trachea in the upper quarter using a 0.7 mm cannula.
8. Place an intravenous catheter (without needle) into the perforated hole and steadily inject 1 mL of BAL solution into the lung.
9. Aspirate the BAL solution in a continuous procedure using the syringe.
10. Determine the volume of the BAL solution recovered from the lung (see Note 16).

11. Centrifuge the BAL solution (10 min, $300\times g$, 4°C). Collect the supernatant as BAL fluid (BALF) in 1.5 mL LoBind tubes and immediately freeze at -20°C (see Note 17).
12. If lysis of erythrocytes is necessary (see Note 18), resuspend the pellet in 0.5 mL Gey's lysis buffer and incubate 45 s. Subsequently, add 10 mL PBS, centrifuge cells as outlined in step 11 and discard the supernatant. Wash the BAL cells two additional times as described.
13. Resuspend the cell pellet in 0.5 mL PBS. Mix an aliquot of the cell suspension 1:10 with trypan blue and determine the total living cell number under a stereo microscope using a hemocytometer by trypan blue exclusion (see Note 19).
14. Add 2 mL of PBS and centrifuge the cell suspension (10 min, $300\times g$, 4°C). Discard the supernatant and adjust the cell number with PBS to 1×10^7 cells/mL.
15. Cytospin (5 min, 500 Upm) 5×10^5 cells in a volume of 50 μL onto a microscope glass slide using the cytopspin centrifuge according to the manufacturer's instructions (see Note 20).
16. Fill the three Diff-Quick staining solutions into separate 50 mL polypropylene tubes and slowly dip the microslide into each solution five times as follows:
 - (a) Diff-Quick Fixation.
 - (b) Diff-Quick I.
 - (c) Diff-Quick II.
 - (d) Rinse the slide with water.
17. Air dry smear of cells at RT.
18. Add one drop of Entellan[®] rapid embedding agent onto the smear and apply a suitable cover glass on this position.
19. Differentiate at least 300 BAL cells per slide based on conventional criteria like morphology and staining behavior (see Note 21) using a microscope with digital camera unit.
20. Calculate the relative frequencies of the various cell populations in the BAL (monocytes/macrophages, lymphocytes, eosinophils, neutrophils). To determine the absolute cell number (in cells/ml BAL), multiply the percentage of the respective cell population by the total cell number (extrapolated to 1 mL) as determined before.

3.2.3. Noninvasive Measurement of Lung Function

To quantify the degree of airway reactivity after intranasal challenge with the antigen, noninvasive whole-body plethysmography is a quick and easy-to-handle method to measure the lung function in mice. The normally breathing mice are not anesthetized and the system provides the chance for repetitive and longitudinal measurements like kinetic experiments. No tracheal surgery is necessary. Four mice can be analyzed at the same time.

1. Calibrate the noninvasive whole-body plethysmography system according to the manufacturer's guidelines (to calibrate the system has the outcome to validate all measurements and to reach a proper and consistent dynamic range).
2. Prepare a 50 mg/mL stock solution of methacholine in PBS and serially dilute (1:2) the stock solution (25, 12.5, 6.25 mg/mL).
3. Place individual mice into each of the four chambers of the plethysmography system and start the measurement protocol.
4. Start the nebulizer to deliver an aerosol of PBS to each chamber for 30 s.
5. Record the baseline airway reactivity as enhanced pause (Penh) response for 5 min (see Note 22).
6. Start the nebulizer to deliver an aerosol of the lowest concentration of methacholine (6.25 mg/mL) to each chamber for 30 s.
7. Record the change of lung function by provocation to aerosolized methacholine as enhanced pause (Penh) response for 5 min (see Note 22).
8. Recovery time (3 min).
9. Repeat steps 6–8 with increasing doses of methacholine (12.5–50 mg/mL).
10. End of measurement.
11. Analyze the raw data with the BioSystem XA Software. Calculate the fold increase of Penh measured for each concentration of methacholine in relation to baseline Penh (see Note 23).

3.2.4. Invasive Measurement of Lung Function

The invasive measurement of the lung function is a very sensitive and effective method, characterized by controlled ventilation, bypassing the upper airways and a local delivery of the aerosolized bronchoconstrictor via a tracheal tube (see Note 24). It is a technically demanding method that requires anesthetization and tracheal surgery of the mice. Therefore no repetitive measurements are possible.

1. Calibrate each of the four chambers of the RC system according to the manufacturer's guidelines.
2. Prepare a 50 mg/mL stock solution of methacholine in PBS and serially dilute (1:2) the stock solution (25, 12.5, 6.25, 3.125 mg/mL).
3. Prepare Narcoren® working solution for anesthetization of mice by thoroughly mixing Narcoren® stock solution and PBS (1:5) in a 2 mL Eppendorf tube on a shaker.
4. Depending on the body weight of the mouse inject an appropriate volume of Narcoren® working solution intraperitoneally.

The recommended dose of Narcoren® for anesthetization of mice is 75 mg/kg body weight.

5. Pinch the foot of the mouse with tweezers to check the surgical tolerance of the mouse.
6. Lift the coat in the upper part of the cervix with the tweezers and cut off a piece of coat with the anatomical scissors, until a coat-free circle of skin can be seen.
7. Perform a vertical cut of the skin with the scalpel above the thymus and use two tweezers to carefully pull the tissue apart until the esophagus and the trachea are visible.
8. Remove the skin and muscle tissue covering the trachea using surgical scissors, two tweezers and a scalpel.
9. Carefully pull up the trachea with the tweezers and put the flat part of the spatula underneath the trachea but on top of the esophagus (see Note 25). Insert a medical yarn under the trachea, which will subsequently be used to tie the trachea to the tracheal tube.
10. Perform a tiny horizontal cut between two tracheal rings
11. Place the 18 gauge tracheal tube into the cut and carefully push it in the airways until you feel a resistance. Then pull it a few millimeters back out (see Note 26).
12. Pull up both ends of the medical yarn with the tweezers to bind the tracheal tube tight to the trachea with a surgical knot.
13. Place the mouse into a chamber of the RC system. Depending on the measurement method an esophagus tube, connected to a transducer, is placed well.
14. Connect the implanted tracheal tube to the free adjustable ventilation pump and close the chamber to avoid environmental influences.
15. Start the following study protocol:
 - (a) Record the baseline resistance and compliance for 60 s.
 - (b) Deliver aerosolized methacholine at the lowest concentration (3.125 mg/mL) for 30 s.
 - (c) Record the change of resistance and compliance for 5 min.
 - (d) Repeat step 16b, c with increasing doses of methacholine (6.25, 12.5, 25, 50 mg/mL).
16. Analyze the raw data with the BioSystem XA Software. Calculate the fold increase of resistance and compliance measured for each concentration of methacholine in relation to baseline resistance and compliance (see Note 23).

4. Notes

1. BALB/c mice are known to be high responders in terms of IgE production and are therefore recommended for type I allergy models. Female mice tend to produce higher amounts of immunoglobulins after sensitization. Follow the principles of laboratory animal care (NIH publication no. 85–23, revised 1985). Elicit approval of the experiments by the local ethics commission.
2. ELISA microtiter plates with high binding polystyrene surface (e.g., Microlon ELISA plates, Greiner bio-one, Frickenhausen, Germany) are recommended.
3. The quantity of β Gal injected per application should account for 1 μ g.
4. Alternatively, mice can be sensitized without adjuvant by repeated subcutaneous application of 10 μ g β Gal dissolved in 200 μ L PBS in weekly intervals (14). Subcutaneous injection is performed at the scruff of the neck and is verified by the observation of a fluid bubble forming under the skin during injection.
5. Minimum volume of blood sample should be 0.5 mL. Puncture of the retro-orbital plexus usually yields more blood than tail vein bleeding.
6. Usually substantial titers of specific IgE in unvaccinated control mice can be determined at the earliest after the third application of β Gal. IgE production drastically increases in mice with progressing time and with number of immunizations.
7. Remove the residuary washing buffer after each wash step by firmly blotting the plate on paper towel to avoid background substrate metabolism.
8. To avoid unspecific metabolism of the substrate, pipet the enzyme solution without touching the walls of the well.
9. Reaction should be stopped when the substrate solution in the blank control starts to become yellow. After addition of the stop solution color changes into orange/brown.
10. Because isoflurane is volatile and potentially harmful, always perform sedation under the laboratory hood, otherwise the indoor air may be contaminated.
11. Keep an eye on the mouse during the whole sedation procedure, which usually lasts 30–45 s. Be aware that the mouse is vital before intranasal application of antigen.
12. The quantity of β Gal applicated by intranasal installation should account for 50 μ g.

13. For intranasal treatment, position the mouse supine and fix the head between two fingers. Be aware that the mouse breathes normally. Continuously pipet 50 μ L of the antigen solution drop by drop into one nose hole. Stop applying the solution when the mouse is breathing out to prevent the formation of bubbles and thus to avoid incomplete inoculation of the antigen.
14. We recommend the use of PBS-treated control mice to assess antigen-independent side effects of the intranasal application itself.
15. Take care that arteries are not disrupted by preparation of the trachea, because massive bleeding might hamper the differentiation of BAL cells.
16. Usually 0.8–0.9 mL of BAL solution can be recovered from the lung.
17. We routinely determine cytokine levels (IL-5, IL-13, IFN- γ) in the BALF using ELISA (12).
18. Massive bleeding during preparation of the trachea often leads to an excess of erythrocytes in the BAL. If the pellet has a red color, erythrocytes have to be lysed.
19. Living cells, which exclude trypan blue, appear colorless under the microscope, while dead cells, which incorporate trypan blue, appear blue.
20. 5×10^5 cells is the maximum number of BAL cells that should be cytopspun onto the glass slide. If the cells are too densely located on the glass slide, try different cell numbers for centrifugation to ensure optimal identification of cells.
21. The various cell populations in the BAL can be differentiated as follows by conventional criteria such as morphology and staining behavior (see Fig. 2): monocytes/macrophages appear as large cells with violet, sometimes indented nucleus and light blue-gray cytoplasm, lymphocytes appear as small cells with a round, dense and dark-violet nucleus and a thin rim of peripheral light-blue cytoplasm, the polymorphonuclear granulocytes (with lobulated purple nucleus) are either eosinophils (with cytoplasm containing granules that are stained brightly pinkish-red) or neutrophils (with unstained cytoplasm).
22. Data are reported as the mean value of 30 values of Penh recorded every 10 s for 5 min.
23. To determine AHR, calculate provocative concentration value PC_{100} for every individual mouse by interpolation of the dose–response curve as the methacholine dose causing 100% increase above baseline reactivity.

24. The invasive measurement of airway resistance and compliance is the recommended standard for the determination of airway reactivity to be published in scientific journals.
25. It is important for the measurement to separate the trachea from the esophagus.
26. It is essential that this step is performed correctly to ensure delivery of the methacholine aerosol into the lower airways.

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