

Eosinophils and the Ovalbumin Mouse Model of Asthma

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Abstract

Mouse models of asthma are essential to understand asthma pathogenesis and eosinophil recruitment in the airways, and to develop new therapeutic strategies. Animal models try to mimic features of the human disease including airway hyperresponsiveness (AHR), eosinophilic inflammation, and remodeling, which are the typical asthma-related characteristics. The mouse is now the species of choice for asthma research due to the availability of transgenic animals and a wide array of specific reagents and techniques available. Cellular responses may be studied with innovative imaging and flow cytometry methods while lung mechanics may be precisely measured by the forced oscillation technique, and airway responsiveness approached by barometric plethysmography in conscious and unconstrained animals. Here, we describe procedures to generate acute models of hypereosinophilic asthma in mice, with ovalbumin (OVA) as the allergen. The presented allergic asthma models offer a large and reproducible eosinophil recruitment, measured in the bronchoalveolar lavage (BAL), accompanied with AHR, inflammation, and remodeling, and are particularly suited to assess the activity of drug candidates. We here present the classical 21-day allergic asthma model to OVA, and adjustments for a rapid 8-day model of airway allergic hypereosinophilia, and a more chronic 57-day model suitable for C57BL/6 mice to develop AHR together with airway eosinophilic inflammation and remodeling.

Key words Allergic asthma, Mouse model, Ovalbumin, Airway eosinophilia

1 Introduction

Human allergic asthma is defined as a chronic eosinophilic inflammatory disorder of the airways and is characterized clinically by airway hyperresponsiveness (AHR) and intermittent, reversible airway obstruction [1]. Histopathological features of asthma show the presence of a large eosinophilic infiltrate in the bronchi, and structural changes grouped as “airway remodeling” including sub-epithelial fibrosis, goblet cell hyperplasia, airway smooth muscle thickening, and increased vascularity. It is accepted that AHR and remodeling result from repeated exposure to allergen, that lead to chronic inflammation of the airways [2]. Studies in laboratory animals have produced a large amount of knowledge on the mechanisms responsible for allergic asthma. Among animal models, the

mouse is now the most widely used species. Mice are not naturally prone to asthma, but asthma-like artificial reactions may be induced in the respiratory tract to develop inflammation and symptoms that are reflecting those of the human disease. Some differences persist however in the pathophysiology of asthma between mouse and human. AHR in mice follows allergen challenge and is transient, while it is present at any time in human even in the absence of symptoms [3]. Chronic models appear difficult to develop in mice, probably due to the absence of environmental cofactors such as pollution, lung infection, and also probably due to the employed allergen. These observations suggest that allergic asthma models in mice do not exactly replicate the human disease, but the observations made in these mouse models support most existing paradigms and are useful to develop new antiasthma strategies [4].

The most commonly used strain for allergen challenge models and eosinophil recruitment is the Th2-type BALB/c mice, but the C57BL/6 or A/J strains have also been used successfully for allergen challenge studies and development of asthma features [5]. A/J mice seem more sensitive to allergen and are suitable for development of chronic models [6]. Allergens that may have clinical relevance, such as house dust mite or cockroach extracts [7, 8], have been recently successfully developed for asthma models in the mouse. However, ovalbumin (OVA) derived from chicken egg is until now a more frequently used allergen since it induces a robust and reproducible allergic bronchial inflammation, particularly in acute allergic responses [9]. Various sensitization and challenge procedures have been developed to induce acute asthma symptoms to OVA. For priming, allergen sensitization requires multiple systemic administrations of OVA in the presence of an adjuvant via intraperitoneal route. Aluminum hydroxide ($\text{Al}(\text{OH})_3$) is a commonly used adjuvant to promote the development of a Th2 immune response to OVA [10]. After the sensitization period allowing IgE production (usually from 5 to 21 days), all protocols require local challenges with the allergen. OVA may be applied to the airways by nebulization, or by intratracheal (i.t.) or intranasal (i.n.) instillation [11, 12]. Following these procedures, mice develop key features of clinical asthma, including increased levels of allergen-specific IgE in serum, eosinophil and Th2 cell infiltration into the airways referred to as airway inflammation, mucus hyperproduction and collagen deposition as airway remodeling, and AHR to methacholine.

The influx of inflammatory cells in the airways, in particular eosinophil infiltration, is studied in the bronchoalveolar lavage (BAL) [20]. BAL is performed after euthanasia and differential cells are counted by optical microscopy as eosinophils, neutrophils, macrophages, and lymphocytes [15]. For more specific assessments, flow cytometric methods can be applied, e.g., to identify T cell phenotypes in the cell composition of BAL fluid in asthma models [21].

We here describe the optimized procedures to sensitize and challenge mice to OVA, in order to develop reproducible features of airway inflammation, e.g., eosinophil infiltration assessed in BAL fluid, remodeling, and hyperresponsiveness. To do so, we present the classical acute 21-day asthma model in Balb/c mice and special adjustments for a rapid 8-day model of airway allergic hypereosinophilia and a more chronic 57-day model suitable for C57BL/6 mice to develop AHR associated with inflammation and remodeling. In a following step, we describe an easy-to-perform, inexpensive, and reproducible manner to obtain BAL to measure inflammatory cell recruitment in mouse airways.

2 Materials

Prepare all solutions using sterile and pyrogen-free commercial saline (NaCl 0.9 %, B. Braun, Boulogne, France), and all reagents on ice. OVA solutions are prepared, aliquoted, and frozen ready-to-use to increase reproducibility of the experiment.

2.1 Ovalbumin Solutions and Aluminum Hydroxide Powder

1. Prepare a sterile 2 mg/ml solution of OVA as follows: weigh 80 mg OVA (Sigma-Aldrich, A5503) and dissolve it in 40 ml cold sterile saline in a 50 ml Falcon tube with a vortex (at maximal speed, *see Note 1*)
2. For sensitization procedures in the 8-day and 21-day models, take 25 ml of this solution and distribute as 1.1-ml aliquots into 1.5-ml microtubes. Immediately freeze and store aliquots at -80°C .
3. For challenge procedures in the 8-day and 21-day models, take 8 ml of the remaining 2 mg/ml solution and dilute it 1/5th by adding 32 ml cold sterile saline in a 50-ml Falcon tube with a vortex (at maximal speed, *see Note 1*). Distribute as 1-ml aliquots in 1.5-ml microtubes. Immediately store aliquots at -80°C .
4. For sensitization and challenge procedures in the 57-day model, take 8 ml of the 2 mg/ml solution and dilute it 1/2.5th by adding 12 ml cold sterile saline in a 50-ml Falcon tube with a vortex (at maximal speed, *see Note 1*). Distribute as 1-ml aliquots in 1.5-ml microtubes. Immediately store aliquots at -80°C .
5. In addition, prepare aliquots of ready-to-use sterile saline for control experiments. Transfer 1 ml sterile saline into 1.5-ml microtubes and 4 ml saline into sterile culture tubes (BD Falcon, 352003). Immediately store the aliquots at -80°C .
6. As soon as the aluminum hydroxide powder [$\text{Al}(\text{OH})_3$, alum, Sigma-Aldrich, cat. no. 239186] (*see Note 2*) arrives at the lab, weigh aliquots of 80 mg in 5-ml sterile culture tubes. Be careful to store alum aliquots protected from light at room temperature.

2.2 Ovalbumin and Alum Conjugates

Conjugation of OVA to alum is a critical step to guarantee the reproducibility of the sensitization.

1. Before use, bring an aliquot of 2 mg/ml OVA solution and an aliquot of 4-ml sterile saline to room temperature.
2. Take an aliquot of alum (80 mg), add 1 ml OVA solution (2 mg/ml) and 3 ml sterile saline. This suspension should be prepared fresh every day.
3. Gently homogenize the OVA–alum suspension for 4 h at 4 °C on a rotator mixer to allow adsorption of OVA on alum. (Alum is not entirely soluble and this will be presented as a suspension.)
4. Before administration to animals, bring the suspension to room temperature on a rotator mixer for 10 min (18–23 °C).

2.3 Anesthetic Solution for in Administration

1. In a 15 ml Falcon tube, add 1.5 ml of a 100 g/l commercial solution of ketamine, Imalgene[®]), 0.5 ml of a 20 g/l commercial solution of xylazine (Bayer, Rompun[®] 2 %) and 10 ml of sterile saline. The prepared solution contains 12.5 mg/ml ketamine base and 0.83 mg/ml xylazine base from hydrochloride. This preparation may be stored at 4 °C for 10 days.
2. Before use in animals, bring the solution to room temperature. Inject 100 µl of the anesthetic solution per mouse (25 g), i.e., 4 ml/kg. The administered dose is 50 mg/kg ketamine and 3.3 mg/kg xylazine.

2.4 Anesthetic Solution for Bronchoalveolar Lavage Procedure

1. In a 15 ml Falcon tube, add 4.5 ml of a 100 g/l commercial solution of ketamine, 1.5 ml of a 20 g/l commercial solution of xylazine, and 6 ml of sterile saline. The prepared solution contains 37.5 mg/ml ketamine base and 24.9 mg/ml xylazine base from hydrochloride. This preparation may be stored at 4 °C for 10 days.
2. Before use in animals, bring the solution to room temperature. Inject 100 µl of the anesthetic solution per mouse (25 g), i.e., 4 ml/kg. The administered dose is 150 mg/kg ketamine and 10 mg/kg xylazine.

2.5 Solutions for Bronchoalveolar Lavage Procedure

1. Saline–EDTA 2.6 mM: in a sterile 1-l DURAN[®] glass bottle, add 970 mg of EDTA (ethylenediaminetetraacetic acid disodium salt dihydrate), 9 g of sodium chloride, and 1-l of ultrapure water. Add a PTFE coated stir bar in the bottle and place it on a magnetic stirrer for 30 min until dissolved. Adjust pH to 7.3 with a 2 N sodium hydroxide solution. This preparation may be stored at 4 °C for 1 week.
2. Potassium chloride 0.6 M: in a sterile 500-ml DURAN[®] glass bottle, add 22.36 g of potassium chloride and 500 ml of ultrapure water.

3 Methods

This procedure describes how mice sensitized and challenged to OVA will develop reproducible and robust allergic asthma features, in particular eosinophil infiltration to be recovered in the BAL. Mice should be sensitized intraperitoneally, then challenged by intranasal administration at the indicated times (Fig. 1). Repeated exposure to OVA is required to induce cytokine production and increase eosinophil recruitment (Fig. 2). Eosinophils recovered in the BAL fluid are presented in Fig 3. Control mice are sensitized with the OVA–alum suspension for the 8-day and 21-day models or with OVA in the absence of adjuvant for the 57-day model. They are challenged with solvent (saline) alone.

Asthma features will be assessed 24 h after the last OVA challenge: AHR may be measured by whole body plethysmography as described [13] or the forced oscillation technique [14] and inflammatory cell recruitment in particular eosinophils assessed in the collected BAL [15] described below and presented on Fig. 3.

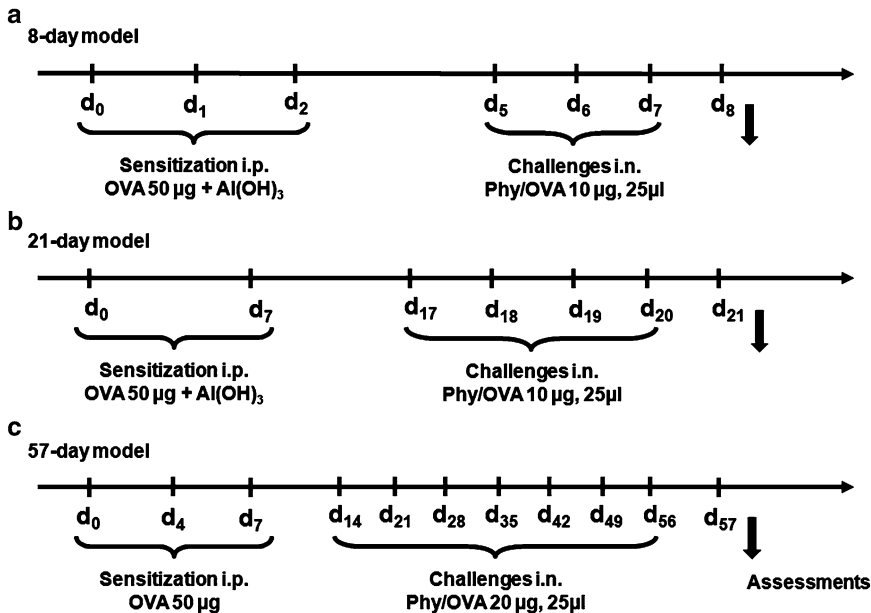


Fig. 1 Allergic asthma models to ovalbumin (OVA) in mice. (a) The 8-day rapid model of hyper eosinophilia is composed of a sensitization step with intraperitoneal (i.p.) administrations of OVA–alum suspension on days 0, 1, and 2, followed by intranasal (i.n.) challenges with OVA or saline for control mice on days 5, 6, and 7. (b) The 21-day acute model of asthma is composed of a sensitization step with i.p. administrations of OVA–alum suspension on days 0 and 7, followed by i.n. challenges with OVA or saline for control mice on days 17, 18, 19, and 20. (c) The 57-day chronic model of asthma is composed of a sensitization step with i.p. administrations of OVA alone (no adjuvant) on days 0, 4, and 7, followed by i.n. challenges with OVA or saline for control mice weekly for 7 weeks. Assessments of AHR, OVA-specific IgE production, airway inflammatory cell recruitment, and airway remodeling are performed 24 h after the last OVA challenge

**3.1 Perform
Ovalbumin
Sensitization
with Adjuvant
in the 8-day
and 21-day Models**

1. Take the OVA–alum suspension (*see* Subheading 2.2).
2. Hold the mouse (*see* Note 3) in your hand by the dorsal skin so that its head is up and its rear legs are down. Maintain its tail in a fixed position with your fingers.
3. Use 1-ml syringes and 25-G needles to inject the OVA–alum suspension and administer 4 ml/kg, i.e., 100 μ l per mouse (of 25 g) by intraperitoneal injection. Gently homogenize the

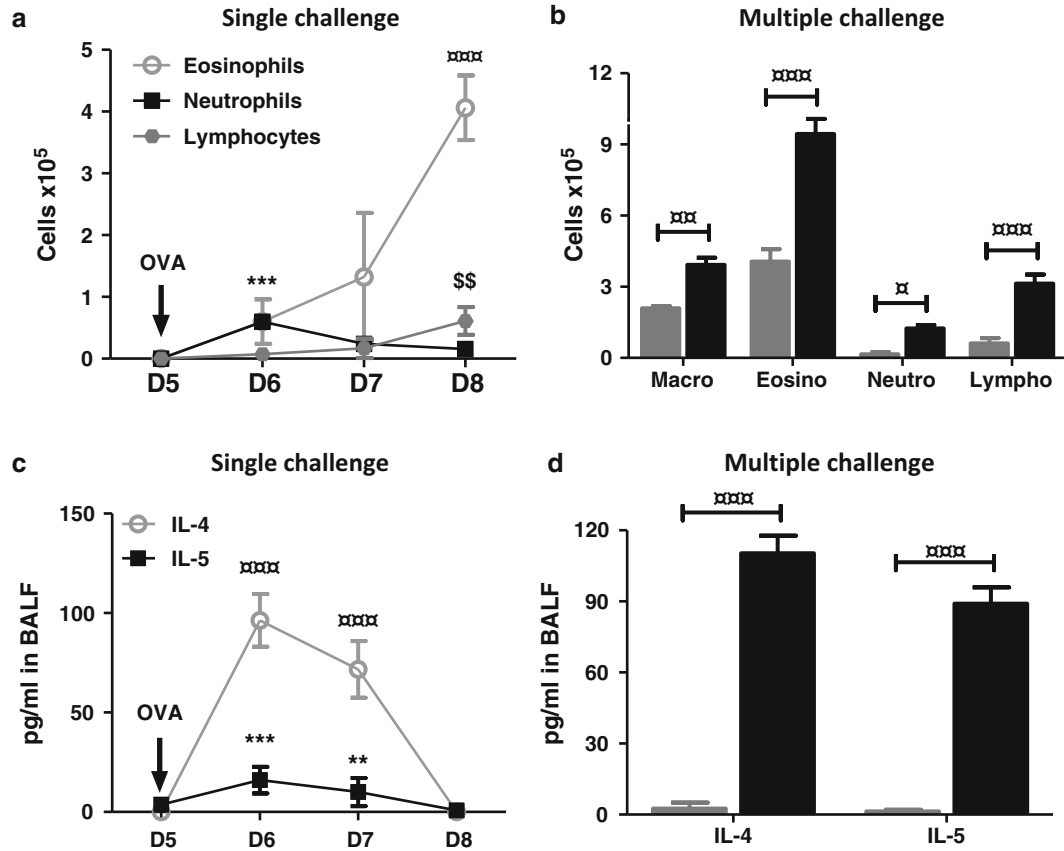


Fig. 2 Repeated exposure to OVA is required to induce both eosinophil recruitment and cytokine production. Example of the 8-day model. **(a)** Evolution of absolute numbers of eosinophils, neutrophils, and lymphocytes in the bronchoalveolar lavage fluid (BALF) 24 h (D6), 48 h (D7), and 72 h (D8) after a single challenge to OVA (performed on D5 and represented by the arrow) in mice sensitized to OVA. Dots are means and bars are SEM values ($n=3-6$ mice per group). **** $P \leq 0.001$ versus D5 for eosinophils, *** $P \leq 0.001$ versus D5 for neutrophils, and \$\$\$ $P \leq 0.01$ versus D5 for lymphocytes. **(b)** Absolute numbers of macrophages (Macro), eosinophils (Eosino), neutrophils (Neutro), and lymphocytes (Lympho) in BALF on D8 after a single challenge to OVA on D5 (grey blocks) or 3 challenges to OVA on 3 consecutive days (D5, D6, and D7) (black blocks). Blocks are means and bars are SEM values ($n=6$ mice per group). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. **(c)** Evolution of IL-4 and IL-5 cytokine levels recovered in BALF 24, 48, and 72 h after a single challenge to OVA on D5 (represented by the arrow). Dots are means and bars are SEM values ($n=3-6$ mice per group). **** $P \leq 0.001$ versus D5 for IL-5 and ** $P \leq 0.01$, *** $P \leq 0.001$ versus D5 for IL-4. **(d)** Levels of IL-4 and IL-5 recovered in BALF on D8 after a single challenge to OVA on D5 (grey blocks) or 3 challenges to OVA on 3 consecutive days (D5, D6, and D7) (black blocks). Blocks are means and bars are SEM values ($n=6$ mice per group). **** $P \leq 0.001$.

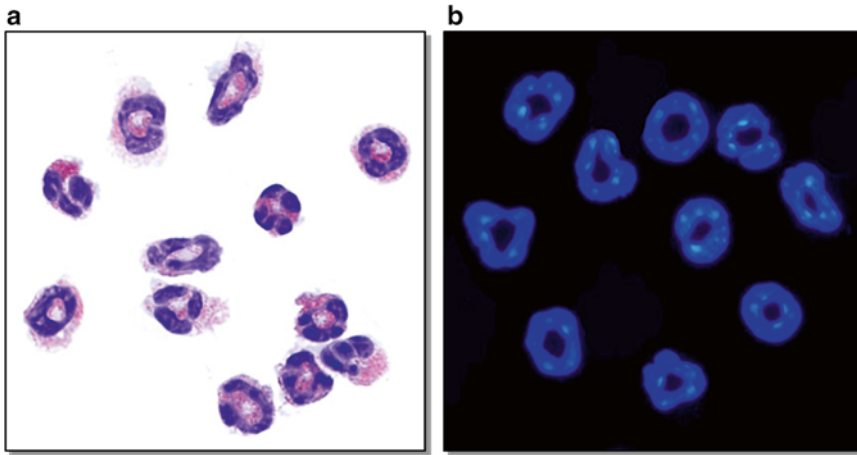


Fig. 3 Photograph of eosinophils recovered in BALF in the 21-day acute model of asthma to OVA in mice. (a) Bright field photograph of cytopun eosinophils stained with Diff-Quick. (b) Fluorescent photograph of eosinophils stained with DAPI to highlight the typical circular nucleus of murine eosinophils

suspension between each injection (*see Note 4*). Each mouse receives 50 μ g OVA adsorbed on 2 mg alum in saline.

3.2 Perform Ovalbumin Sensitization in the 57-day Model

1. Bring an aliquot of 0.8 mg/ml OVA solution to room temperature and vortex for 5 s at maximal speed (*see Note 1*).
2. Hold the mouse (*see Note 3*) in your hand by the dorsal skin so that its head is up and its rear legs are down. Maintain its tail in a fixed position with your fingers.
3. Use 1-ml syringes and 25-G needles to inject OVA and administer 5 ml/kg, i.e., 125 μ l per mouse (of 25 g) by intraperitoneal injection. Each mouse receives 50 μ g OVA in saline.

3.3 Ovalbumin Challenge

1. Bring an aliquot of 0.4 mg/ml (8-day and 21-day models) or 0.8 mg/ml (57-day model) OVA solution to room temperature and vortex for 5 s at maximal speed (*see Note 1*).
2. Mice should be anesthetized for intranasal administration. Anesthetize mice as follows: Hold the mouse in your hand by the dorsal skin so that its head is up and its rear legs are down. Maintain its tail in a fixed position with your fingers and use 1-ml syringes and 25-G needles to inject 4 ml/kg of the anesthetic solution (*see Note 5*) by intraperitoneal injection.
3. Place the mouse in the cage and wait until vibrissae do not move any more.
4. Hold the mouse in your hand in a vertical position with its head up and its rear legs down. Administer drop by drop 12.5 μ l OVA solution, or saline alone for controls, in each nostril using sterile tips and a 20- μ l precision pipette (*see Note 6*). Each mouse receives 10 μ g (8-day and 21-day models) or 20 μ g (57-day model) of OVA.

5. Keep the mouse in your hand in a vertical position for at least 1 min so that the solution can be distributed in the airways, and check that the mouse breathes normally (*see Note 7*).
6. Next place the mouse in a horizontal decubitus on a heating blanket until the mouse is completely conscious.

3.4 Bronchoalveolar Lavage Procedure

1. Mice should be anesthetized for BAL procedure as follows: Hold the mouse in your hand by the dorsal skin so that its head is up and its rear legs are down. Maintain its tail with fingers and use 1-ml syringes and 25-G needles to inject 4 ml/kg of the anesthetic solution (*see Subheading 2.4*) by intraperitoneal injection.
2. Place the animal in a dorsal decubitus position until the mouse is deeply anesthetized.
3. Using scissors, make a small incision in the neck skin. Separate salivary glands, incise the sternohyoid muscle to expose the trachea, and place a cotton thread (black haberdashery cotton thread no. 40) under the trachea.
4. Make a small semi-incision of the trachea to allow a 21-G lavage tube to pass into the trachea (*see Note 8*). Stabilize the tube and needle by attaching them with a cotton thread.
5. Load a 1-ml syringe with 0.5 ml sterile saline–EDTA, place it in the 21-G lavage tube and inject 0.5 ml saline–EDTA into the lung. Massage the chest for 10 s, re-aspirate saline of the first lavage, and keep the recovered lavage fluid into a 5-ml tube placed on ice.
6. Repeat the procedure 10 times per animal (*see Note 9*).
7. Pool lavages recovered and centrifuge them for 5 min at $300 \times g$ at 4 °C to pellet the cells. Discard the supernatant and add 1500 μ l ultrapure water to the cell pellet for erythrocyte hemolysis. Wait for 10 s, add 500 μ l KCl (0.6 M), and homogenize by inverting.
8. Centrifuge the BAL fluid for 5 min at $300 \times g$ at 4 °C. Discard the supernatant, add 500 μ l saline–EDTA to the cell pellet, and homogenize by inverting.

3.5 Inflammatory Cell Counting

1. For total cell count: place 5 μ l of the cell suspension on a hemocytometer, count the cells, and calculate the total cell number in BALF.
2. For differential cell count: homogenize by inverting and dilute with saline–EDTA to obtain a final concentration of 250,000 cells/ml. In a cytofunnel place 200 μ l of the cell suspension. Cytocentrifuge for 10 min at $200 \times g$, air-dry the slide, and proceed to cell staining.

3. Cell staining with Diff-Quick staining kit: immerse the slide for 15 s in Diff-Quick fixative reagent, for 30 s in Diff-Quick solution I then for 15 s in Diff-Quick solution II and rinse the slide for 5 s in tap water. Let the slide air-dry (*see Note 10*).
4. Count and identify 400 cells under light microscopy on each slide. Magnification 1,000 $\times$; oil immersion. Manual counting allows to identify eosinophils, neutrophils, macrophages, and lymphocytes. The percentage of each cell population is reported as the total number of cells, and the absolute number of each cell population is then calculated in BALF.

4 Notes

1. OVA is a protein that may be difficult to dissolve in saline. In our experience, it is better to use a high speed vortex, with maximal speed $\geq 3,000$ rpm to dissolve OVA rapidly and prevent the formation of a turbid suspension composed of hydrated OVA particles.
2. Be very careful while choosing the aluminum hydroxide reagent. The quality of aluminum hydroxide influences the number of eosinophils recovered in BAL in the 8-day and 21-day models. We recommend to use aluminum hydroxide from Sigma-Aldrich (cat. no. 239186).
3. These protocols are developed to be used with 9-week-old male or female Balb/c mice to study AHR, OVA-specific IgE production, airway inflammatory cell recruitment, and airway remodeling [16–19]. C57BL6 mice may be used to study eosinophilic airway inflammation in the 8-day and 21-day models, but AHR cannot be measured (Fig. 4). To measure AHR together with eosinophil assessment in C57BL6 mice, we recommend the 57-day allergic asthma model. In addition, the described steps may be used for various other asthma models. Most of asthma models are dependent on the number of sensitizations and challenges, the dose of OVA and alum used, and the time between sensitizations and/or challenges.
4. Gently homogenize the suspension by 3–4 repeated reversal of the tube/syringe between each use so that the suspension does not drop at the bottom of the tube/syringe.
5. Anesthesia is key to successful intranasal administration. Too light anesthesia reduces reproducibility of inflammatory cells recruitment, and too deep anesthesia enhances respiratory failure during and after i.n. administration. It may be necessary to adapt the dose of anesthetic to warrant the best quality of anesthesia. It is often necessary to increase the dose over time.

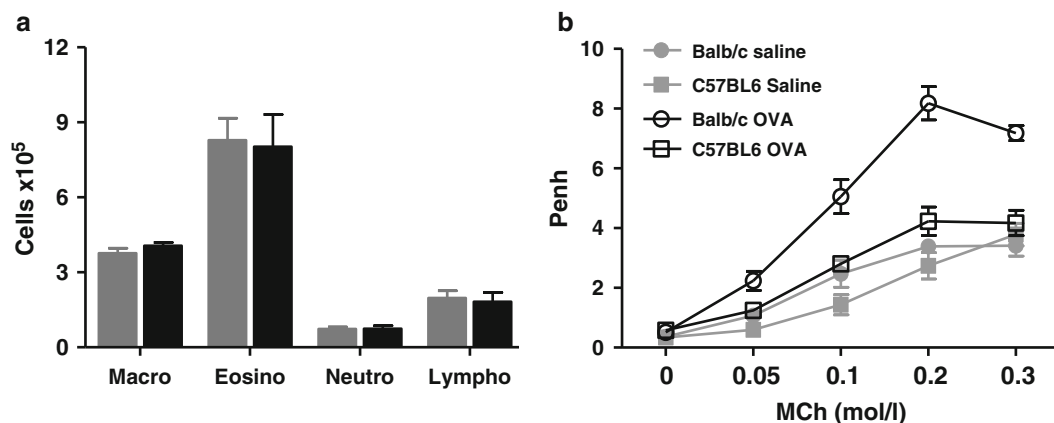


Fig. 4 Comparison of inflammatory cell recruitment and airway hyperresponsiveness between Balb/c and C57BL6 in the 21-day allergic asthma model. **(a)** Inflammatory cells in BALF on day 21 in Balb/c mice (grey blocks) or in C57BL6 mice (black blocks). Blocks are means and bars are SEM values. **(b)** Airway hyperresponsiveness to methacholine measured by plethysmography on vigil and unrestrained mice (EMKA Technologies, Paris, France) on day 21. Dots are means and bars are SEM values

6. The solution has to be administered drop by drop, slowly and very carefully, to maximize the distribution of OVA into the airways. Intranasal challenge is a simple and easy-to-perform technique, but it requires expertise to obtain reproducible responses.
7. If the mouse does not breathe normally, perform a thorax massage by pressing the rib cage several times, quickly but carefully. Please take care to identify each mouse who stops breathing, because this may affect AHR and eosinophilic inflammation of the airways.
8. While semi-incising the trachea, take care not to cut through the trachea. For tracheotomy, use a 21-G lavage tubing, carefully placed over a 21-G needle or a single-use Terumo surshield® IV catheter (Terumo, ISR*SFA2025A).
9. Supernatant from the first two lavages should be pooled and separated from the eight following lavages for cytokine or mucus analysis, since a higher concentration is present in the 2 first lavages. In this case, centrifuge the initial two first lavages for 5 min at 300×g at 4 °C, then freeze the supernatant for further analysis, and add the cell pellet to the eight following lavages.
10. We describe the staining of cytopun cells with the commercial Diff-Quick staining : this staining requires to be performed in less than an hour of cytopinning. Other commercial staining may be useful such as RAL 555 (RAL-diagnostics) or could be advantageously replaced by a homemade staining described in [15].

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