

Mouse Models Applied to the Research of Pharmacological Treatments in Asthma

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Abstract

Models developed for the study of asthma mechanisms can be used to investigate new compounds with pharmacological activity against this disease. The increasing number of compounds requires a preclinical evaluation before starting the application in humans. Preclinical evaluation in animal models reduces the number of clinical trials positively impacting in the cost and in safety. In this chapter, three protocols for the study of drugs are shown: a model to investigate corticoids as a classical treatment of asthma; a protocol to test the effects of retinoic acid (RA) on asthma; and a mouse model to test new therapies in asthma as monoclonal antibodies.

Key words Animal model, Antibody, Corticoids, Monoclonal, Mouse

1 Introduction

In predisposed individuals, initial exposure to the allergen leads to the activation of allergen-specific Th2 cells and IgE synthesis (allergic sensitization). Subsequent exposure to the allergen causes inflammatory cell recruitment, activation, and mediator release (histamine, leukotrienes, and cytokines). According to this, potential therapeutic interventions are proposed for the treatment of allergic diseases. Current treatments are primarily focused on pharmacological intervention to decrease the symptoms of the disease [1]. Among them the most used are corticosteroids, which are very effective drugs for disease control [2]. Although these treatments are highly effective for most individuals, many patients must take these drugs lifetime.

Mouse models can also be used to investigate the effect of other potential compounds. This is the case of all-trans-retinoic acid (ATRA). The ATRA derived from vitamin A in the diet [3] regulates cell growth, differentiation, and matrix formation in various cell types and plays a major role in the maintenance of a normal epithelial mucociliar phenotype [4].

Besides its modulatory role on epithelial cells, RA suppresses the differentiation of eosinophil and basophil from bone marrow precursors [5], most likely by controlling the IL-5 receptor expression on their surface [6], and by preventing mitogen-induced proliferation and IL-4-dependent IgE production [7]. It has been suggested a potential therapeutic effect of RA in asthma.

Corticosteroid treatment may not be effective in some patients or resistances to inhaled corticoids may appear [8]. The therapeutic use of monoclonal antibodies presents an opportunity for the treatment of these patients. Omalizumab was the first therapeutic antibody approved by FDA (U.S. Food and Drug Administration) for treatment of asthma and the first approved therapy designed to target immunoglobulin E (IgE) [9]. Today, several monoclonal antibodies for treating asthma are directed against different target [10].

Another strategy is the allergen-specific immunotherapy (SIT) that modifies the allergen-specific immune response and the course of disease [11]. Allergen-specific immunotherapy is based on repeated administration of disease-causing allergens in order to modify the allergen-specific immune response to reach tolerance [12]. In this chapter, we describe mouse models based on the experimental study of different treatments for asthma.

2 Materials

2.1 Animals

1. 6–8 Weeks old female BALB/c mice (Charles Rivers Lab).

2.2 Common Materials

1. 1× PBS (Phosphate-Buffered Saline): 1.37 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄. Adjust the pH to 7.4 with HCl.
2. Tuberculin syringes (BD Biosciences).
3. Needle 20G short (BD Biosciences).
4. Ketamine chlorhydrate (Imalgene 1000, Merial).
5. Medetomidine hydrochloride (Medetor, Virbac).
6. 100 µl micropipette and appropriate tips (Thermo-Fischer).

2.3 Corticoid Model

1. Dexamethasone (Sigma-Aldrich).
2. Ovalbumin (OVA) (Sigma-Aldrich).
3. Aluminum Hydroxide (Sigma-Aldrich).

2.3.1 House Dust Mite (HDM)-Induced Asthma Model

1. Budesonide (Sigma-Aldrich).
2. House Dust Mite (HDM) Extract (Allergopharma).

2.3.2 House Dust Mite (HDM)-Induced Severe Asthma Model

1. Budesonide (Sigma-Aldrich).
2. House Dust Mite (HDM) Extract (Allergopharma).
3. Freund's adjuvant: 1 ml contains 1 mg of *Mycobacterium tuberculosis* (H37Ra, ATCC 25177), heat killed and dried, 0.85 ml paraffin oil, and 0.15 ml mannide monooleate (Sigma-Aldrich).

2.4 Asthma Model for Acid All-Trans-Retinoic (ATRA) Treatment

1. All-Trans-Retinoic Acid (ATRA) (Sigma-Aldrich).
2. DPLC (dilauroylphosphatidylcholine) (Sigma-Aldrich).
3. Butanol.
4. Isopropanol.

2.5 Asthma Model for Treatment with Monoclonal Antibodies

1. Ovalbumin (OVA) (Sigma-Aldrich).
2. Aluminum Hydroxide (Sigma-Aldrich).
3. Anti-mouse-IL-5 monoclonal.
4. Isotype control monoclonal antibody.

3 Methods

3.1 Corticoid Models

In this chapter, we will review a protocol of acute asthma in mouse to be treated with dexamethasone.

3.1.1 Acute Model of Asthma for Corticoid Treatment

1. Perform the experiments with 6–8 weeks old female BALB/c mice.
2. Animals must be kept confined and handled to meet the current ethics rules of handling animals for experimentation.
3. It starts with 6–8 weeks old female BALB/c mice, for example eight mice. Divide the animals into two groups (Fig. 1):
 - Group 1: Four mice that will not be injected with OVA Intraperitoneal/Intranasal (in place 1× PBS will be given).

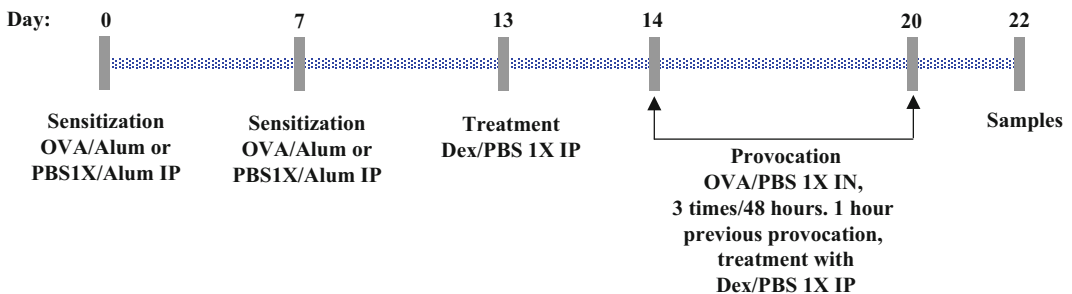


Fig. 1 Corticoids: acute mouse model. *IP* intraperitoneal, *IN* intranasal, *Dex* dexamethasone, *PBS* phosphate-buffered saline, *OVA* ovalbumin, *Alum* aluminum hydroxide

- Group 2: Four mice in which OVA Intraperitoneal/Intranasal will be administered.
4. On day 0 (start of experiment), inject the group 2 by intraperitoneal route, with 20 μ g of OVA emulsified in 0.2 ml 1 $\times$ PBS containing 2 mg of aluminum hydroxide (these doses are administered to each mouse).
 5. In the controls, group 1, inject with 0.2 ml of 1 $\times$ PBS containing 2 mg of aluminum hydroxide.
 6. Keep the animals stabled for 1 week (7 days) for the OVA hyper-sensitization to occur.
 7. On day 7, administer the second OVA dose to reinforce the hyper-sensitization. For this, the OVA intraperitoneal injection is repeated as in **step 4**.
 8. Keep the mice housed another 7 days.
 9. On day 13 of the experiment, split each of the mice populations in two, as described below (*see* **Note 1**):
 - Group 1: Without OVA
 - (a) Two mice with 1 $\times$ PBS (control).
 - (b) Two mice with dexamethasone (control treated with dexamethasone).
 - Group 2: With OVA.
 - (a) Two mice with 1 $\times$ PBS (asthma without treatment).
 - (b) Two mice with dexamethasone (asthma with treatment).
 10. That same day, carry out the first intraperitoneal injection of corticosteroid (dexamethasone) or 1 $\times$ PBS, based on the designed groups of mice.
 11. Use dexamethasone at a concentration of 3 mg/kg. Inject each mouse with 60 μ g of dexamethasone dissolved into 0.2 ml of 1 $\times$ PBS (*see* **Note 2**).
In summary, we have the following treatment groups:
 - Intraperitoneal/Intranasal 1 $\times$ PBS + 1 $\times$ PBS intraperitoneal.
 - Intraperitoneal/Intranasal 1 $\times$ PBS + dexamethasone intraperitoneal.
 - Intraperitoneal/Intranasal OVA + 1 $\times$ PBS intraperitoneal.
 - Intraperitoneal/Intranasal OVA + dexamethasone intraperitoneal.
 12. The next day, day 14, perform other injection with intraperitoneal dexamethasone or 1 $\times$ PBS, 1 h before intranasal provocation.

13. An hour later, perform the first intranasal provocation with OVA or 1× PBS.
14. First, anesthetize the animal. Administer an intraperitoneal dose of Ketamine (75 mg/kg)+Medetomidine (1 mg/kg). Wait for a complete sedation of the animal, before starting to handle (*see* **Note 3**).
15. In sedated animal, standing upright with his nose up, give 50 µl of 1× PBS to the control group or 50 µl of OVA (0.1 % in 1× PBS) to the experimental group. Administer the mixture drop wise on the snout, favoring inoculation during the inspiratory process of the animal.
16. Keep the animal in that position for 1–2 min. Return the animal to the cage, and place it horizontally on its side for favor ventilation.
17. Repeat **steps 12–17**, three times with a frequency of 2 days (48 h) between a provocation and the next. In this protocol, it corresponds to day 16 (second provocation), 18 (third provocation), and 20 (fourth provocation).
18. On day 22, proceed to the collection of samples, 2 days after the last challenge (48 h).
19. Anesthetize the animal with the same mixture described in **step 14**.
20. With the anesthetized animal, proceed to the necropsy. The mouse is placed in *decubitus supino*, fixing the animal to a surface by the legs.
21. For obtaining samples:
 - Blood is obtained by cardiac puncture at the ventricular level. This puncture serves as blood gathering and euthanasia. Cellular and soluble factors can be analyzed.
 - BALF (Bronchoalveolar Lavage) can be obtaining by cannulating the trachea, injecting 1 ml of 1× PBS, two times, and collect. BALF is used for cellular and soluble factor studies.
 - Collect the organs of interest for molecular biology as well as histological studies.
 - Collection of hematopoietic organs can be performed for in vitro cell culture.

3.1.2 Chronic Model of Asthma for Corticoid Treatment

1. Perform the experiments with 6–8 weeks old female BALB/c mice (Fig. 2).
2. Animals must be kept confined and handled to meet the current ethics rules of handling animals for experimentation.
3. It starts with a population of 6–8 weeks old female BALB/c mice. Divide the animals into two groups:

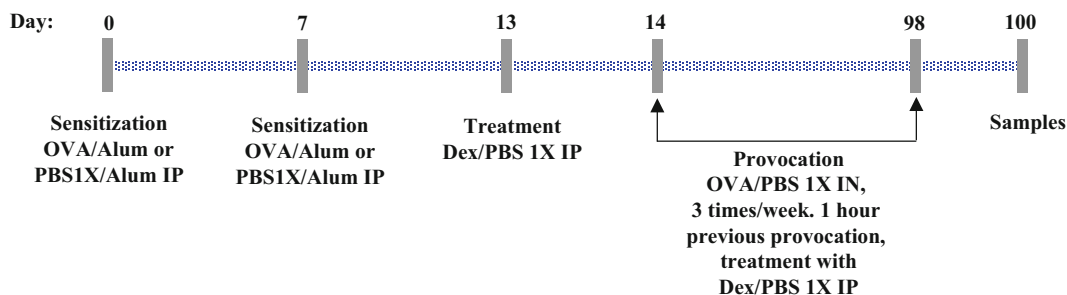


Fig. 2 Corticoids: chronic mouse model. *IP* intraperitoneal, *IN* intranasal, *Dex* dexamethasone, *PBS* phosphate-buffered saline, *OVA* ovalbumin, *Alum* aluminum hydroxide

- Group 1: Four mice that will not be treated with OVA Intraperitoneal/Intranasal (in place 1× PBS will be given).
 - Group 2: Four mice that will be treated with OVA Intraperitoneal/Intranasal.
4. On day 0 (start of experiment), inject the animals of the group 2, by intraperitoneal route, with 20 µg of OVA emulsified in 0.2 ml 1× PBS containing 2 mg of aluminum hydroxide (these doses are administered to each mouse).
Inject the animals of the control group, group 1, with 0.2 ml of 1× PBS containing 2 mg of aluminum hydroxide.
 5. Keep the animals stabled for 1 week (7 days) for the reaction of hyper-sensitization to occur with OVA.
 6. On day 7, administer the second dose of OVA to reinforce the hyper-sensitization. For this, the intraperitoneal injection is repeated as in **step 4**.
 7. Keep the mice housed for another 7 days.
 8. On day 13 of the experiment, divide each population of mice into two groups, as described below:
 - Group 1: Without OVA.
 - (a) Two mice with 1× PBS (control).
 - (b) Two mice with dexamethasone (control treated with dexamethasone).
 - Group 2: With OVA.
 - (a) Two mice with 1× PBS (asthma without treatment).
 - (b) Two mice with dexamethasone (asthma with treatment).
 9. That same day carry out the first intraperitoneal injection of corticosteroid (dexamethasone) or 1× PBS, based on the groups of mice performed.

10. Use dexamethasone at a concentration of 3 mg/kg, thus injecting mice with 5 µg of dexamethasone into 0.2 ml of 1× PBS.

In summary, we have the following treatment groups:

- Intraperitoneal/Intranasal 1× PBS + 1× PBS intraperitoneal.
 - Intraperitoneal/Intranasal 1× PBS + dexamethasone intraperitoneal.
 - Intraperitoneal/Intranasal OVA + 1× PBS intraperitoneal.
 - Intraperitoneal/Intranasal OVA 1× + dexamethasone intraperitoneal.
11. The next day, day 14, perform other intraperitoneal injection of 1× PBS or dexamethasone 1 h before the intranasal provocation.
 12. An hour later, perform the first intranasal provocation with OVA or 1× PBS.
 13. First anesthetize the animal. Administer an intraperitoneal dose of Ketamine (75 mg/kg) + Medetomidine (1 mg/kg). Wait for a complete sedation of the animal before starting.
 14. Stand upright the sedated animal, with his nose up and give 50 µl of 1× PBS to the control group or 50 µl of OVA (0.1 % in 1× PBS) to the experimental group. Administer drop wise to the snout, favoring inoculation during the inspiratory process of the animal.
 15. Keep the animal in that position for 1–2 min.
 16. Return the animal to the cage, putting horizontally on its side, for favoring his ventilation.
 17. In this chronic model, it is interesting to monitor the clinical course of the mice. Use a classification or Clinical Score that assigns a numerical value increasing with the severity of the response according to the clinical signs developed.
 18. Repeat the procedure described in **steps 12–17**, three times per week for 12 weeks (3 months).
 19. Observe and record the signs in mice during this period.
 20. After that time, proceed to the collection of samples.
 21. Perform the necropsy of the animals, 2 days (48 h) after the last provocation.
 22. Anesthetize the animal with the same mixture described in paragraph 14.
 23. With the anesthetized animal, proceed to the necropsy. The mouse is placed in *decubitus supino* fixing it by the legs to a surface.

24. For obtaining samples:

- Blood is obtained by cardiac puncture at the ventricular level. This puncture serves as blood gathering and euthanasia. Cellular and soluble factors can be analyzed.
- BALF (Bronchoalveolar Lavage) can be obtained by cannulating the trachea, injecting 1 ml of 1× PBS, two times, and collect. BALF is used for cellular and soluble factors studies.
- Collect the organs of interest for molecular biology as well as histological studies.
- Collection of hematopoietic organs can be performed for in vitro cell culture.

3.1.3 House Dust Mite (HDM)-Induced Asthma Model for Corticoid Treatment

All allergen-specific models, as well as those described in Chapter 16 of this book, may be used for corticosteroid treatment studies.

1. For the acute model, dissolve HDM in 1× PBS at a concentration of 10 µg of extract (plus 0.5 mg aluminum hydroxide) in 50 µl of 1× PBS per mouse in the experimental group. In control mice, give 50 µl of 1× PBS with 0.5 mg of aluminum hydroxide.
2. Perform the experiments with 6–8 weeks old female BALB/c mice.
3. Start for example from a population of 8 mice. Divide the animals into two groups:
 - Group 1: Four mice without Intraperitoneal HDM (in place will be given 1× PBS).
 - Group 2: Four mice with HDM Intraperitoneal
4. The first day of the experiment (day 0), perform the first administration of HDM extract or 1× PBS (plus adjuvant for both), depending on the group (sensitization phase). In this case, it is administered by intraperitoneal (Fig. 3).

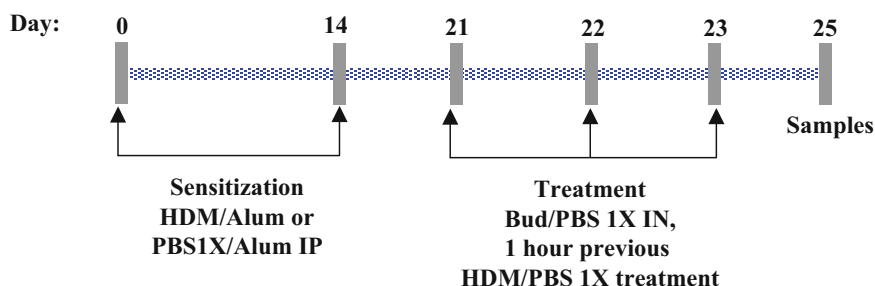


Fig. 3 Corticoids: house dust mite (HDM)-induced asthma model. *IP* intraperitoneal, *IN* intranasal, *Bud* budesonide, *PBS* phosphate-buffered saline, *HDM* house dust mite, *Alum* aluminum hydroxide

5. Keep the animals housed for 14 days.
6. On day 14, repeat **step 3**.
7. Maintain mice housed until day 21 of the experiment.
8. Prepare HDM at a concentration of 10 μg of extract in 50 μl of 1 $\times$ PBS without aluminum hydroxide.
9. On day 21, perform the first administration of corticosteroids.
10. Use budesonide (0.3 mg/kg in 1 $\times$ PBS), administer intranasal.
11. Furthermore, perform the first challenge by administering the allergen prepared in paragraph eight for intranasal route.
12. Administer an intraperitoneal dose of ketamine mixture (75 mg/kg)+ Medetomidine (1 mg/kg), achieving a smooth sedation.
13. Administer corticosteroid 1 h prior to sensitization to HDM.
14. Perform allergen deposition intranasal.
15. Repeat **steps 10–13**, days 22 and 23 of the experiment.
16. On day 25 (48 h after the last allergen exposure and the last dose of corticosteroid), proceed to the samples collection.
17. Anesthetize the animal with the same mixture described in **step 12**.
18. With the anesthetized animal, proceed to the necropsy, setting the animal in *decubitus supino* position.
19. Obtain samples (blood, BALF, tissues, and/or hematopoietic organs).

3.1.4 House Dust Mite (HDM)-Induced Severe Asthma Model for Corticoid Treatment

1. Dissolve HDM in 1 $\times$ PBS at a concentration of 100 μg extract more complete Freund's adjuvant in 50 μl of 1 $\times$ PBS per mouse in the experimental group. In control mice, give 50 μl of 1 $\times$ PBS plus adjuvant (Fig. 4).
2. Perform the experiments with 6–8 weeks old female BALB/c mice.

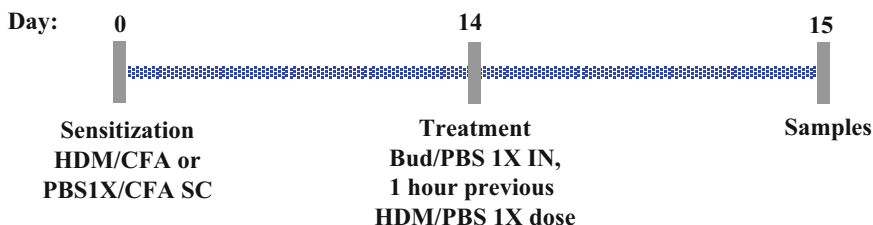


Fig. 4 Corticoids: house dust mite (HDM)-induced severe asthma model. SC subcutaneous, IN intranasal, Bud budesonide, PBS phosphate-buffered saline, HDM house dust mite, CFA complete Freund's adjuvant

3. On first day of the experiment (day 0), perform the first administration of HDM extract or 1× PBS (plus adjuvant for both), depending on the group (HDM or control) (sensitization phase). In this case, administer via subcutaneous.
4. Keep the mice housed for 14 days.
5. Prepare the HDM at a concentration of 100 µg of extract in 50 µl of 1× PBS, without adjuvant.
6. On day 14 day, perform corticosteroid administration in single dose.
7. Use budesonide as corticosteroid (0.3 mg/kg in 1× PBS), intranasal.
8. Furthermore, the provocation model is performed by administering the allergen prepared in **step 5**, via intranasal.
9. Administer an intraperitoneal dose of ketamine mixture (75 mg/kg)+ Medetomidine (1 mg/kg), achieving a mild sedation.
10. Deposit the corticosteroid 1 h before challenge.
11. The allergen deposition is performed intranasal.
12. On day 15 (24 h after challenge), proceed to the sample collection.
13. Anesthetize the animal with the same mixture described in **step 9**.
14. With the anesthetized animal, proceed to the necropsy, setting the animal in *decubitus supino* position.
15. Obtain the samples as needed (blood, BALF, tissues, and/or hematopoietic organs).

3.2 Asthma Model for All-Trans-Retinoic Acid (ATRA) Treatment

In this section, we will focus on a protocol design to study the effects of a pretreatment with ATRA over an acute model of asthma (*see Note 4*).

1. Initially, prepare a solution of 20 µg of OVA, emulsified in 0.2 ml of 1× PBS containing 2 mg of aluminum hydroxide (this dose is administered per mouse).
2. Secondly, prepare ATRA. ATRA is a fat-soluble molecule to be encapsulated in a vehicle to promote their distribution in the animal.
3. Prepare the liposome particle DPLC. Dissolve 100 mg DPLC in 1 ml of butanol. This volume will be split in two tubes, one will be supplemented by ATRA, the other not.
4. Moreover, dissolve ATRA in isopropanol at a concentration of 100 mg/kg. 0.2 ml 1× PBS of ATRA (10%, wt:wt) per animal and day of dosing (*see Note 5*).
5. Mix the solution of DPLC-ATRA with a ratio of 4 parts ATRA to 1 part DPLC. Prepare aliquots depending on the number of mice and days of inoculation.

6. Prepare all solutions with butanol or isopropanol, leave in a hood until complete evaporation of the alcohol. Once the dried pellet is obtained, store at -80°C until use.

7. Perform the experiments with 6–8 weeks old female BALB/c mice (*see* **Note 6**).

The mice will be divided into two experimental groups (Fig. 5):

- Control group without ATRA (for example, 4 mice).
 - Group treated with ATRA (for example, 4 mice).
8. One day before the sensitization of mice (day-1), administer the first dose of liposome-ATRA to the treated group, and the first dose of empty liposome to the control group.
9. Dissolve each aliquot (both liposome-ATRA as empty liposome) in 0.3 ml of $1\times$ PBS.
10. Inoculate intraperitoneal.
11. One day later (day 0), repeat **steps 8** and **9** an hour before the sensitization.
12. Next, divide the groups as follows:
- Control without ATRA:
 - (a) Two mice with $1\times$ PBS (control).
 - (b) Two mice with ovalbumin (OVA).
 - Group with ATRA:
 - (a) Two mice with $1\times$ PBS.
 - (b) Two mice with ovalbumin (OVA).

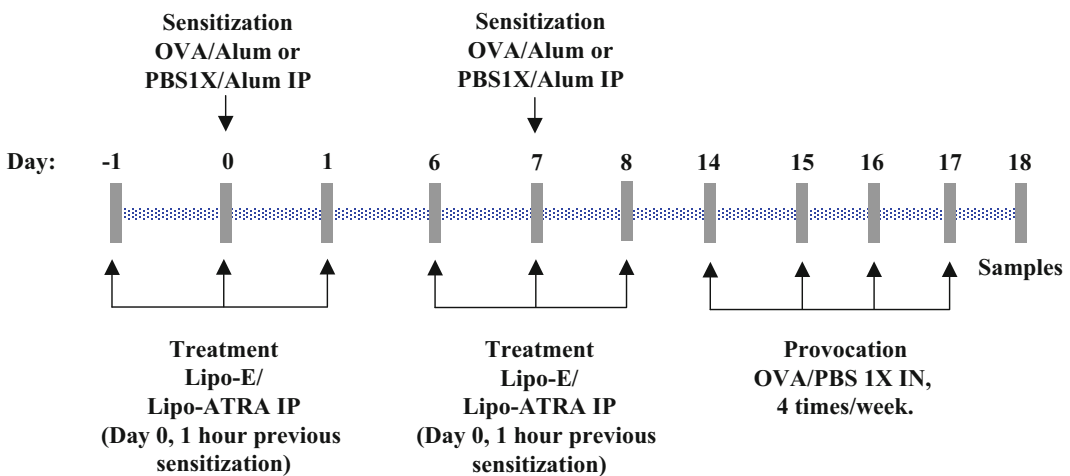


Fig. 5 All-trans-retinoic acid model (ATRA). *IP* intraperitoneal, *IN* intranasal, *PBS* phosphate-buffered saline, *OVA* ovalbumin, *Alum* aluminum hydroxide, *Lipo-ATRA* liposome-all-trans-retinoic acid, *Lipo-E* liposome-empty

13. One hour after injection of ATRA, carry out the sensitization. Inoculate intraperitoneal 0.2 ml of the OVA solution prepared in **step 3**. Inoculate 1× PBS to the controls.
14. The next day (day 1), administer another dose of liposome-ATRA (**steps 8–9**).
15. On day 6 (1 day before the second sensitization), administer the fourth dose of liposome-ATRA by repeating the **steps 8–9**.
16. On day 7, administer the fifth dose of liposome-ATRA 1 h before the sensitization repeating the **steps 8–9**.
17. One hour after ATRA treatment, repeat **step 13**.
18. On day 8, repeat the **steps 8–9** for the administration of the sixth dose of liposome-ATRA.
19. On day 14, begin to make provocations.

Perform the first intranasal provocation with OVA or 1× PBS according to the group.

20. Anesthetize the animal. To obtain a mild sedation, administer an intraperitoneal dose of Ketamine (75 mg/kg) + Medetomidine (1 mg/kg). Wait for a complete sedation of the animal before you start.
21. Stand upright the sedated animal, with his nose up, and administer 50 µl of 1× PBS to the control group or 50 µl of OVA (0.1% in 1× PBS) to the experimental group. Administer the mixture drop wise to the snout, favoring the inoculation during the inspiratory process of the animal.
22. Keep the animal in that position for 1–2 min.
23. Return the animal to the cage, placing flat on its side to favor ventilation.
24. Repeat the challenge, the days 15, 16, and 17 of the experiment.
25. On day 18 (24 h after the last challenge), proceed to the sample collection.
26. Anesthetize the animal with the same mixture described in the **step 20**.
27. With the anesthetized animal, proceed to the necropsy, setting the animal in *decubitus supino* position.
28. Proceed to obtain the samples (blood, BALF, tissues, and/or hematopoietic organs) (*see Note 7*).

3.3 Monoclonal Antibodies

This section relies on chronic corticosteroid treatment model described in this chapter, making some modifications.

1. On day 0 (start of experiment), inject the animals (4 mice), by intraperitoneal route, with 50 µg of OVA, emulsified in 0.2 ml 1× PBS containing 2 mg of aluminum hydroxide (these doses

are administered to each mouse). Inject the controls (4 mice) with 0.2 ml of 1× PBS containing 2 mg of aluminum hydroxide (Fig. 6).

2. Perform a sensitization on day 14 of the experiment, as described in **step 1**.
3. On day 21 of the experiment, divide mice in two groups (for example, starting 4 per group):
 - Group 1: Without OVA.
 - (a) Two mice with isotype control monoclonal antibody (0.5 mg per injection).
 - (b) Two mice with anti-mouse-IL-5 monoclonal (0.5 mg per injection).
 - Group 2: With OVA.
 - (a) Two mice with isotype control monoclonal antibody (0.5 mg per injection).
 - (b) Two mice with anti-mouse-IL-5 monoclonal (0.5 mg per injection).
4. Challenge the animals with intranasal OVA 3 days/week for 6 weeks.
5. During the last 2 weeks of treatment with OVA, administer monoclonal antibody doses by intraperitoneal route, days 50, 55, 59, and 63.
6. On day 65, 48 h after the last challenge proceed to sample collection.

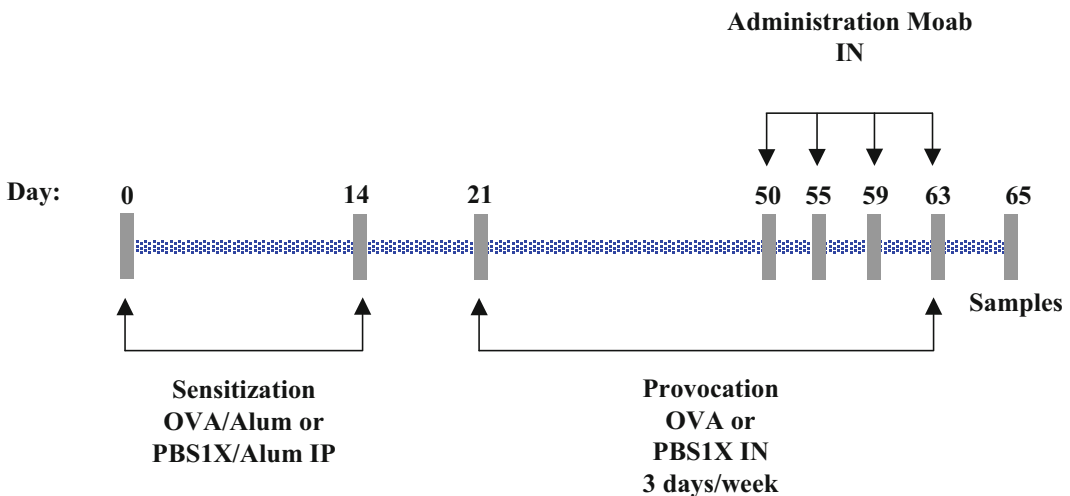


Fig. 6 Monoclonal antibodies. *IP* intraperitoneal, *IN* intranasal, *PBS* phosphate-buffered saline, *OVA* ovalbumin, *Alum* aluminum hydroxide, *Moab* monoclonal antibody

4 Notes

1. Six mice can be stored per cage. Keep mice in a cage for group 1 and group 2 in another. It is necessary to differentiate in each cage mice treated with dexamethasone from treated with 1× PBS. Optionally, mark the animals with an incision in the ear with an ear tag. Calculate the number of animals according to the requirements of the experiment.
2. This dose of dexamethasone is calculated for an average weight of 20 g per mice.
3. It is advisable to inject the anesthetic in one side of the animal body and dexamethasone in the other, avoiding overloading an area with an excessive number of inoculations.
4. The same protocols described in the treatment sections with OVA (chronic and acute) can be applied to the studies of the effect of ATRA in asthma, replacing the intraperitoneal injection of corticosteroid for the injection of ATRA.
5. The ATRA is a photosensitive molecule, which must be protected from light when it is lyophilized and when it is in solution. Store at -20°C .
6. It is necessary to know the amount of vitamin A that the animal is consuming in the diet.
7. To assess levels of ATRA, the HPLC methodology can be used.

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