

Mutant Mice and Animal Models of Airway Allergic Disease

Marie-Renée Blanchet, Matthew Gold, and Kelly M. McNagny

Abstract

Eosinophilia is a hallmark of allergic airway inflammation, and eosinophils represent an integral effector leukocyte through their release of various granule-stored cytokines and proteins. Numerous mouse models have been developed to mimic clinical disease and they have been instrumental in furthering our understanding of the role of eosinophils in disease. Most of these models consist of intranasal (i.n.) administration of antigenic proteases including papain and house dust mite (HDM) or the neo-antigen ovalbumin, with a resulting Th2-biased immune response and airway eosinophilia. These models have been particularly informative when combined with the numerous transgenic mice available that modulate eosinophil frequency or the mechanisms involved in their migration. Here, we describe the current models of allergic airway inflammation and outline some of the transgenic mice available to study eosinophils in disease.

Key words Asthma, Inflammation, Eosinophil, Mouse

1 Introduction

Allergic airway disease such as asthma is characterized by airway inflammation, airway hyperresponsiveness, and tissue remodelling [1]. In recent years, the vast majority of the research on asthma has focused on furthering our understanding of the development of the pulmonary allergic reaction using animal models and assessing their response to a variety of naturally occurring antigens, such as house dust mite (HDM) extract [2], papain [3] and model antigens such as ovalbumin (OVA) [4]. Inhalation of these antigens leads to a pulmonary allergic reaction, involving recruitment of granulocytes and a strong inflammatory response. Eosinophils are known to play a major role in the development of Th2-driven allergic airway inflammation in response to exposure to airborne allergens. Coincident with eosinophil and mast cell infiltration and degranulation, airway hyperresponsiveness develops and, after repeated attacks and chronic inflammation, airway remodelling ensues. These three hallmarks of asthma (eosinophil infiltration, airway hyperresponsiveness and tissue remodelling) are tightly linked and mimic the symptoms occurring in asthmatic patients.

As stated, a major contribution to the understanding of the role of eosinophils in the pathophysiology of this disease has been the development of animal models which mimic either one or all of the characteristics of human asthma [5]. This has been further enhanced through the use of genetically-modified mice including those completely lacking eosinophils [6, 7], and has led to several breakthrough findings on the role of this cell population in airway inflammation and other disease. Here we briefly describe several genetically-modified mouse strains and their relative utility in assessing eosinophil function (*PHIL*, *dbl-GATA*, *eoCRE*, *Cd34^{-/-}*, *Rora^{tg/tg}*) and provide in-depth protocols for three different mouse models of allergic airway inflammation (the OVA model of asthma, the HDM model of asthma, and the papain model of eosinophilia) which are widely used to study eosinophil function. Finally, we also present a non-eosinophilic model of airway inflammation, hypersensitivity pneumonitis (HP), which is a useful and important negative control for distinguishing between Th2 and Th1/17-driven disease and eosinophil function.

1.1 Mouse Strains to Study the Role of Eosinophils in Allergic Responses

The PHIL Mouse

The *PHIL* mouse [6] was one of the first genetically-modified mouse strains developed and used to study the importance of eosinophils in disease. In these mice, transgenic expression of diphtheria toxin is driven by the eosinophil peroxidase promoter, leading to ablation of all eosinophils shortly after the commitment of precursors to the eosinophil lineage. These mice therefore do not possess mature eosinophils and have been widely used to study the role of eosinophils in disease and various phenotypes [6, 8].

The dblGATA Mouse

Another eosinophil-deficient mouse which is available to study the role of the presence of eosinophils in disease is the *dblGATA* mouse [8, 9]. These mice carry a site-directed mutation of tandem GATA-1 binding sites in the eosinophil specific GATA-1 promoter. This very subtle mutation selectively ablates expression of GATA-1 in the eosinophil lineage and ablates development of eosinophils during early stages of precursor commitment while all other GATA-1-dependent lineages (erythroid and megakaryocytic) are spared. Thus, like the *PHIL* mice, the *dblGATA* mice are extremely useful for delineating general eosinophil-dependent aspects of normal development and disease.

The eos-CRE Mouse

The CRE recombinase system is widely used to target mutations to specific cell lineages. These mice provide an invaluable method for inactivating genes that would otherwise be embryonic lethal selectively in one lineage or tissue via transgenic expression of CRE. This strategy is extremely useful in determining the cell autonomous function of a particular gene in the absence of possible bystander effects in other lineages. Recently, an eosinophil lineage-specific CRE mouse was developed by expressing CRE-recombinase

under the control of the eosinophil peroxidase promoter [10]. This now paves the way for evaluating the functional evaluation of a number of critical genes selectively in the eosinophil lineage. Such mice will likely quickly become widely used to study the importance of gene expression in eosinophils in disease such as allergic airway inflammation.

The Cd34^{-/-} Mouse

Although several eosinophil-deficient mouse strains exist and can be used to evaluate the absolute requirement for eosinophils in disease, they do not permit the analysis of more subtle aspects of eosinophil function. Recently, we showed that eosinophils express the hematopoietic stem cell marker CD34 and that, although these mice produce normal numbers of eosinophils in the bone marrow and peripheral blood, they fail to traffic efficiently to the sites of Th2 driven inflammation [4]. Thus, CD34-deficient mice offer an interesting opportunity for distinguishing the functional significance of inflammatory eosinophil recruitment from steady state eosinophilopoiesis. However, it is also important to note that CD34 is expressed by a number of other mature hematopoietic cells in mice including mast cells and dendritic cells [4, 11, 12], and this needs to be taken into consideration when using this mouse to study cell trafficking in disease.

The Rora^{sg/sg} Mouse

Group 2 innate lymphoid cells (ILC2s) are a recently described subset of the innate immune compartment identified by their unique expression of surface antigens (CD45⁺Lineage⁻CD127⁺CD25⁺CD90⁺ST2⁺Sca1⁺) [13–15]. They reside in multiple tissue sites but predominantly at mucosal surfaces and other potential portals of pathogen entry and they are potent producers of a variety of Th2 polarizing cytokines, predominantly IL-5 and IL-13. As such, these cells play a critical innate role in the initiation of Th2-driven disease and the recruitment and frequency of eosinophils [16–21]. While ILC2 development has been shown to be dependent on the transcription factors *Id2* [13], *Gata3* [17, 18], and *Tcf7* [20], two groups independently identified the transcription factor ROR α to be highly expressed in ILC2 development and mice deficient in ROR α activity have a specific loss of ILC2 cells without abnormalities in most other hematopoietic compartments [22, 23]. Therefore, these mice too can be used to investigate the significance of eosinophil recruitment to specific sites of Th2-driven inflammation as well as the upstream processes that initiate Th2 inflammation.

1.2 Mouse Models to Study the Role of Eosinophils in Disease

Ovalbumin Model of Asthma

The OVA-based model of asthma has, historically, been the most widely used model and has been studied in depth. It involves a systemic priming step via intraperitoneal OVA injection, with or without aluminum hydroxide (Al(OH)₃), as a Th2 polarizing adjuvant. Typically, following sensitization, mice are “rested” for a minimum of 7 days to permit time for the production of

OVA-specific antibodies (predominantly IgG₁ and IgE), before mice are subsequently challenged either via intranasal instillation (i.n.) or aerosol exposure to OVA. Several different temporal regimens for sensitization and challenges have been published in the literature but these seem to create similar responses. Here, we present the exact timing we have used for over 10 years and with which we obtain reproducible eosinophil-driven responses [5, 24–26]. We find that when combined with intraperitoneal adjuvant priming, OVA induces a potent and robust eosinophil recruitment to the lung and thus this is a useful measure of their function in an allergic disease.

Various studies using genetically modified mice have demonstrated the importance of eosinophils in this Th2 airway inflammatory response. Interestingly, initial analyses of eosinophil-deficient *PHIL* mice [6] and *dblGATA* mice using this model, came to different conclusions on the importance of eosinophils in allergic airways disease [6, 8, 9]. With *PHIL* mice, the presence of eosinophils was fully necessary to induce airway inflammation and airway hyperresponsiveness, two major hallmarks of the disease. In stark contrast, the *dblGATA* mice failed to show a significant defect in airway hyperresponsiveness and in mucus secretion and only exhibited protection from airway remodelling and collagen deposition. Thus, these authors argued for a relatively minor role for eosinophils in the full asthma phenotype. This discrepancy was later resolved to be an effect of the genetic backgrounds of the mice used (B6 for *PHIL* and Balb/c for *dblGATA*). These two background strains are well-known to have different predispositions to Th2 driven immune responses. Indeed, when both strains were evaluated in a B6 background, it was found that eosinophils are essential for the development of airway allergic responses [8].

The conclusions on the significance of eosinophils in OVA-induced asthma are further supported by studies in the *Cd34*^{-/-}, eosinophil migration-deficient mouse. Studies utilizing these mice revealed that efficient eosinophil migration to the lung is crucial for development of airway inflammation and AHR, even in the presence of normal levels of Th2 cytokines and bone marrow blood eosinophil precursors [4], thus highlighting the importance of local recruitment. Therefore, there is currently strong evidence for a role for eosinophils in the development of the allergic airway inflammation and AHR in this model, making it one of the best models to study eosinophil function and recruitment to the lung in allergy.

House Dust Mite (HDM)
Model of Asthma

While the use of the OVA model of asthma helped increase our understanding of eosinophil function in this disease, it quickly became evident that a mouse model based on naturally-occurring antigens, using the natural intranasal route of sensitization and which did not require the use of synthetic adjuvants such as

Al(OH)₃ was necessary. The HDM extract-based model of asthma was first described in 1996 [27] and it fulfills these criteria. Briefly, mice are sensitized i.n. for 3 consecutive days with HDM extract. The fact that HDM extract leads to a local sensitization to this antigen is likely linked to the intrinsic protease activity of several proteins in this antigen extract [28] which directly stimulate the airway epithelium to cause sensitization. Following sensitization, several i.n. challenges are used to induce allergic airway disease characterized in part by the recruitment of eosinophils to the alveolar space and lung tissue.

Although it is quickly increasing in popularity amongst the asthma basic research community, the characterization of this model and of the role of eosinophils in this model remains in its infancy. For example, no studies using eosinophil-deficient mice have been used in this model. However, as exposure to HDM causes very similar inflammation to OVA-based models (a Th2 cytokine response with high levels of IL-5 and IL-13, recruitment of eosinophils in the alveolar space and bronchi, AHR and antigen-specific IgE), this model is very likely to be dependent on the presence and recruitment of eosinophils to the lung as has previously shown with the OVA model. Likewise the dearth of antigen-specific transgenic TCR mice for HDM antigens has, until recently, impaired the ability to study downstream T cell driven responses. This has recently been remedied [29], and therefore, this model that uses a common human allergen is likely to be important for most future studies on eosinophil function in allergy.

Intriguingly, *Cd34*^{-/-} and *Rora*^{tg/tg} mice have been used in this model (Gold et al., unpublished and submitted data) and confirm an important role for eosinophils migration (*Cd34*^{-/-} mice) and recruitment (*Rora*^{tg/tg}) in disease induced by HDM. Studies using *Rora*^{tg/tg} mice in particular show that recruitment of eosinophils in response to a local antigen route of priming with HDM is solely dependent on the presence of innate lymphoid ILC2 cells, and that without ILC2 cells (and consequently, eosinophils), mice did not develop airway inflammation in response to HDM (unpublished, submitted data).

Papain Model of Eosinophilia

Although mouse models of asthma are useful tools to study eosinophil biology, they are complex and require the highly coordinated interaction of several hematopoietic cell populations. For some purposes, it would be more desirable to have a simple and acute model which consists of a relatively pure eosinophil-recruitment reaction. The papain-induced eosinophilia model is almost entirely eosinophilic, and fulfills these criteria [3].

Papain is a protease antigen that is a causative agent of occupational asthma [30]. While intradermal administration has been used to model basophil recruitment and Th2 development [31],

inhalation of papain induces a robust and relatively selective recruitment of airway eosinophils. This response is independent of T and B cells as papain-induced eosinophil recruitment is not affected in *Rag2*^{-/-} mice [31]. Papain induces release of IL-33 [32], a potent activator of group 2 innate lymphoid cells (ILC2s) that release large amounts of IL-5 and IL-13 and are responsible for papain-induced airway eosinophilia [15]. This model has become invaluable in determining early innate mediators of airway eosinophilia and is especially useful in highlighting the importance of the newly defined ILC2 subset of the innate immune cell repertoire.

*Hypersensitivity
Pneumonitis as a Negative
Control to Study Eosinophil
Function*

One of the challenges of working with genetically modified mice and models to study eosinophil function is the lack of a robust Th1/Th17 allergic airway model to serve as a good negative control, i.e., in which it has been clearly shown that eosinophils do not play a role. In terms of a negative control for the eosinophilic response, the mouse model of hypersensitivity pneumonitis (HP) is the perfect candidate. HP is a Th1/Th17 allergic airway inflammatory disease characterized by an important alveolar infiltration of lymphocytes and lack of AHR. Eosinophils are not characteristic of the inflammation observed in HP, nor are any of the common eosinophil-related cytokines including IL-3, IL-5, or IL-13. Rather, HP relies on the release of Th1 and Th17 cytokines including IFN- γ and IL-17 [33, 34]. Therefore, the mouse model of HP remains one of the few non-Th2 allergic airway inflammatory models, and it can be used to rule out the role of eosinophils in genetically modified mice phenotypes.

In mouse, HP is induced by i.n. administration of purified antigen from the bacteria *Saccharopolyspora rectivirgula* (SR), which grows in humid environments such as damp hay. The specific SR-dependent form of HP is also less formally known as “Farmer’s lung” [35]. Typically, the SR antigen is obtained by bead-crushing live purified SR bacteria. The highly purified antigen preparation is complex to prepare, as contamination with other bacterial strains during preparation can easily occur and specific equipment is needed to produce efficient antigen. Indeed, in our hands, antigen produced by techniques such as sonication does not demonstrate the appropriate antigenic properties (unpublished observation). It is therefore highly recommended to get in touch with specialists in the field to obtain highly purified antigen.

The SR-induced Th-1/Th17 inflammatory response is characterized by an alveolar lymphocytosis and SR-specific IgG. In response to SR, neither humans nor mice develop AHR, which further separates this model from the mouse models of eosinophil-driven asthma previously described. It is therefore an appropriate and very useful negative control for the study of eosinophils or the significance of molecules on the molecules elaborated by eosinophils in disease [11].

2 Materials

2.1 Ovalbumin Model of Asthma

1. Mice (minimum of 6 weeks old at start of experiment and age and sex matched). All animal experiments must be conducted according to your institutions guidelines.
2. Albumin from chicken egg white, Grade V.
3. Injectable Alum.
4. Phosphate Buffered Saline.
5. 1 mL syringes.
6. 26–28 G needles.
7. Isoflurane.

2.2 House Dust Mite Model of Asthma

1. HDM lyophilized extract, *Dermatophagoides pteronyssinus* (Greer Labs, cat #: XPB82D3A), resuspended to 2.5 mg/mL in sterile PBS.

2.3 Papain Model of Eosinophilia

1. Papain (from *Carica papaya*, Sigma-Aldrich, supplied as a 25 mg/mL solution).

2.4 Hypersensitivity Pneumonitis: Negative Control for Eosinophilic Responses

1. SR antigen (home-grown or contact specialists in the field to obtain efficient antigen).

2.5 Assessment of Airway Inflammation

1. Ketamine/xylazine (20/1 mg/mL; 100 µl/10 g IP).
2. Surgical scissors and forceps.
3. 70 % Ethanol.
4. Catheters 22 g.
5. Suture thread.
6. 1 mL Tuberculin syringe.
7. PBS.
8. 15 mL conical tubes.
9. 10 % neutral buffered formalin.
10. Red Cell Lysis Buffer (Invitrogen, cat #: A1049201).
11. Hemacytometer.
12. Trypan Blue solution, 0.4 %.
13. Microscope slides.
14. Shandon Filter Cards.
15. Cytospin 4 Cytocentrifuge.

16. Wright-Giemsa Stain.
 - (a). Fixative (Fisher Scientific, cat #: 122-929), or use methanol (Fisher Scientific, cat #: A412).
 - (b). Solution I: PROTOCOL HEMA-3 (Fisher Scientific, cat #: 122-937).
 - (c). Solution II: PROTOCOL HEMA-3 (Fisher Scientific, cat #: 122-952).
17. Permount Mounting Medium.

2.6 Lung Inflation and Fixation for Histology

1. 30 mL Luer-Lok syringe.
2. 18 G needle.
3. 500 mL volume Erlenmeyer Filter Flask.
4. 10 mL polystyrene serological pipette.

3 Methods

(Note that disease timelines are presented in Fig. 1 for all four models described below (Subheadings 3.1–3.4)).

3.1 Ovalbumin Model Disease Induction [4]

1. Dilute OVA in PBS to make a 4 mg/mL solution.
2. Shake the aluminum hydroxide solution well before use, then dilute the 4 mg/mL OVA solution 1:1 with the alum, for a final OVA concentration of 2 mg/mL.
3. Mix the OVA/Alum mixture for 30 min at room temperature in order for the alum to effectively adsorb the OVA antigen.
4. Immunize mice intraperitoneally on days 0 and 7 with 100 μ L of the OVA/alum mixture using a 1 mL syringe and 28 G needle.
5. For intranasal challenges on days 21, 22, 23, 25, and 27, dilute OVA in PBS to make a 10 mg/mL solution.
6. Anesthetize mice until breathing rate reduces to approximately one per second (oxygen 1.5 L/min with 3.5 % isoflurane).
7. Once mice are appropriately sedated, intranasally administer 50 μ L of 10 mg/mL OVA solution, adding dropwise through the nares.
8. Monitor the mice until they recover from anesthesia (approximately 5 min).

3.2 HDM-Extract Model Disease Induction [2]

1. Reconstitute lyophilized HDM extract to a stock concentration of 2.5 mg/mL in PBS.
2. Anesthetise mice with isoflurane as described in **step 6** of Subheading 3.2.

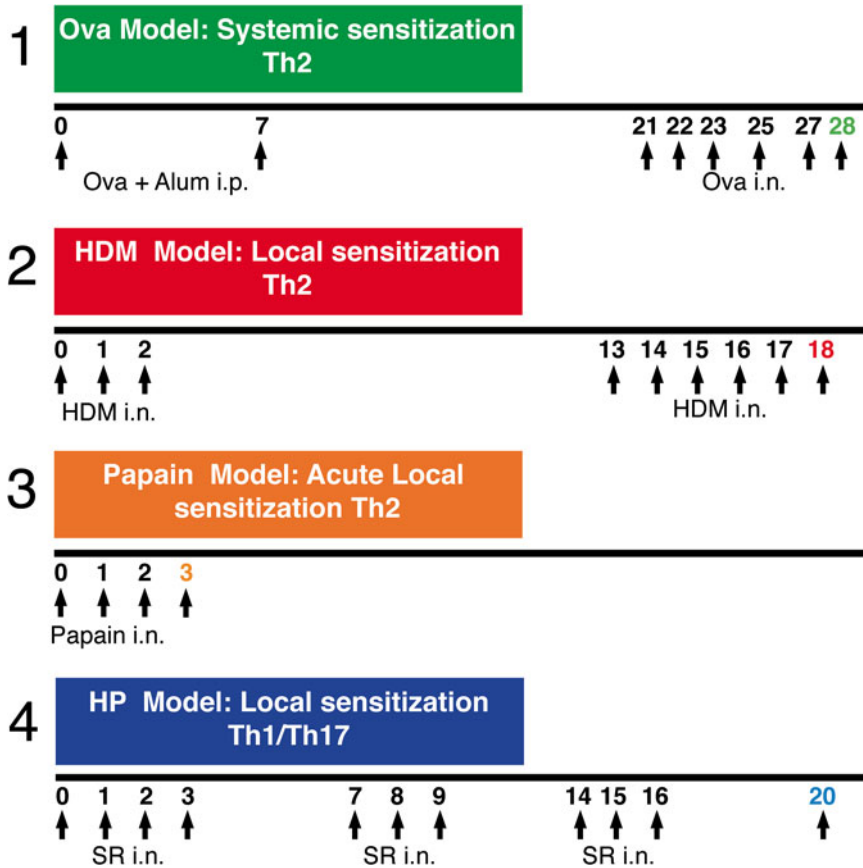


Fig. 1 Timelines for each model presented in the chapter

3. For HDM challenges on days 0, 1, and 2, dilute stock HDM solution to 2 mg/mL in PBS, intranasally administer 50 μ L dropwise through the nares.
4. For HDM challenges on days 13–17, dilute stock HDM solution to 500 μ g/mL in PBS and intranasally administer 50 μ L dropwise through the nares.
5. Monitor the mice until they recover from anesthesia (approximately 5 min).

3.3 Papain Model Disease Induction [22]

1. Papain is supplied as a 25 mg/mL solution, dilute to 250 μ g/mL with sterile PBS.
2. Anesthetise mice with isoflurane as described in **step 6** of Subheading 3.2.
3. Administer 40 μ L of 250 μ g/mL papain solution dropwise through the nares. Administer PBS alone or heat-inactivated papain as a negative control (*see Note 6*) on days 0, 1, and 2.
4. Monitor the mice until they recover from anesthesia (approximately 5 min).

3.4 HP Model Disease Induction [10]

1. Reconstitute lyophilized SR antigen to a stock concentration of 4 mg/mL in PBS.
2. Anesthetise mice with isoflurane as described in **step 6** of Subheading 3.2.
3. For challenges on all challenge days, administer 50 μ L dropwise through the nares.
4. Monitor the mice until they recover from anesthesia (approximately 5 min).

3.5 Analysis of Disease Severity: Broncho-alveolar Lavage (BAL)

1. Anesthetise mice with a ketamine/xylazine overdose (20/1 mg/mL; 100 μ L/10 g IP).
2. Place the mice ventral side up and pin down forepaws and hind-paws.
3. Using scissors and forceps, remove skin above the neck area. Carefully pull the parotid and submaxillary gland laterally.
4. Upon visualization of the trachea, remove the muscle layer surrounding it, then make a pinhole partial cut of the trachea just inferior of the larynx and thyroid. Carefully insert the flexible 22 G catheter into the trachea, using the suture thread to secure the catheter.
5. Prepare three 1 mL slip-tip syringes filled with 1 mL of PBS. Lavage the lungs three times with 1 mL of PBS, slowly advancing and recovering the PBS lavage liquid. Pool the collected lavages into a 15 mL conical tube and store on ice until further use.
6. After collecting the BAL, dissect open the thoracic cavity, exposing the left lung and the four lobes of the right lung (superior, middle, inferior, and post-caval).
7. Dissect out some lung specimens for histology, and place in ten volumes worth of 10 % buffered formalin. Store specimens at 4 °C for 24 h and then replace the formalin with 70 % ethanol for long term storage at 4 °C before paraffin embedding and histology.

3.6 Analysis of BAL Fluid: Enumeration and Differential Counts of BAL Leucocytes

1. Record collected BAL volume and then centrifuge at $1,200 \times g$ for 5 min.
2. Resuspend cell pellet in 1 mL of red-cell lysis buffer and incubate for 3–5 min, then dilute with PBS and centrifuge at $1,200 \times g$ for 5 min.
3. Resuspend cell pellet in 1 mL of PBS or media. Collect aliquot of cells for dilution with trypan blue and enumeration on hemocytometer.
4. Count total number of BAL cells and report it on the BAL volume (total number of BAL cells/mL BAL fluid).

5. Prepare aliquots of cells for cytopins. Dilute aliquots to $5.0\text{--}7.5 \times 10^5$ cells/mL, and cytospin 100 μL of cell dilutions to each slide for 3 min at $200\times g$ (approximately 50,000–75,000 cells/slide).
6. Allow slides to dry for 2 h to overnight, before staining.
7. Insert the slides for 30 s each into methanol, solution I and solution II of the PROTOCOL HEMA-3 staining set. Allow excess methanol/stain to drip down in-between solutions.
8. Allow the slides to air-dry and then, if desired, mount with coverslip using Permount.
9. Perform differential cell counts from a minimum of 400 cells/slide using standard morphological criteria of macrophages, lymphocytes, neutrophils, and eosinophils.

3.7 Histology and Tissue Inflammation Analysis

1. Paraffin embed lung specimens and section 3–6 μm thick, then stain with H and E.
2. Under microscopic examination, score disease severity based on the following criteria in 4–5 airway sections per mouse and average the results:
 - (a) Perivascular infiltration, score of 0–4.
 - 0, no cells.
 - 1, a few cells.
 - 2, entire encirclement of vessel with infiltrate 1–2 cells deep.
 - 3, entire encirclement of vessel with infiltrate 3–5 cells deep.
 - 4, entire encirclement of vessel with infiltrate >5 cells deep.
 - (b) Peribronchiolar infiltration, score of 0–4.
 - 0, no cells.
 - 1, a few cells.
 - 2, entire encirclement of bronchiole with infiltrate 1–2 cells deep, slight loss of uniformity of airway epithelium.
 - 3, entire encirclement of bronchiole with infiltrate 3–5 cells deep, with a more pronounced hyperplasia and metaplasia of the airway epithelium.
 - 4, entire encirclement of vessel with infiltrate >5 cells deep, complete loss of airway epithelial structure including tissue damage leading to rupture of the bronchiole.

(c) Parenchymal infiltration, score of 0–4.

- 0, no cells.
- 1, a few scattered cells throughout the lung.
- 2, one quadrant of the lung with a high degree of infiltration.
- 3, two quadrants of the lung with a high degree of infiltration.
- 4, more than two quadrants of the lung with a high degree of infiltration.

4 Notes

1. Most methods mentioned above are tailored for mice on the C57Bl/6 J background. Due to the increased Th2 bias of the immune response in other inbred strains (Balb/cJ or 129S1/SvImJ for example), it would be advisable to titrate down the antigen concentration for both the OVA and HDM asthma models if these Th2-skewed strains were to be used.
2. It is important to use only age and sex matched mice for all asthma experiments due to the reported sex differences in asthma susceptibility in mice [36, 37].
3. It is important to insure that the levels of LPS are as low as possible (and similar between lots) for the OVA and HDM stocks. Increased levels of LPS will lead to a neutrophilic response rather than an eosinophilic response using these antigens.
4. The protease activity in the HDM stock should be verified and kept similar between lots to insure reproducibility.
5. We also notice that the aluminum hydroxide tends to lose its potency after 4–6 months. Be sure to shake the bottle carefully before using as it tends to precipitate.
6. As a negative control, treat mice i.n. with heat-inactivated papain. This can be easily achieved by boiling the papain mixture for 10 min at 100 °C.
7. To avoid the entrance of blood in the BAL, avoid performing cardiac punctures until after the collection of the BAL. The blood obtained can then be later used for serum antibody and cytokine quantifications.
8. For intranasal administration, it is imperative to treat the mice at the optimal sedation point to insure the antigen is aspirated into the lungs and not expelled (mouse not sedated enough) or drained directly into the throat and swallowed (mouse too sedated). One good indication of the right level of sedation is to count the number of breaths per second: antigen should be administered when the mouse breathes at the rate of one breath per second.

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