Chapter 14

An Inhalation Model of Allergic Fungal Asthma: *Aspergillus fumigatus*-Induced Inflammation and Remodeling in Allergic Airway Disease

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Abstract

The ability to accurately mimic normal processes for sensitization and allergen challenge in an experimental animal model are useful in that they allow researchers to critically manipulate the complex interactions of multiple cell types. In the context of the allergic lung, multiple cell types form complex cellular networks and function to regulate a variety of temporal and spatial changes. Mouse models of allergic airway disease have proven to be highly useful for dissecting these complex interactions, particularly in addressing remodeling of the allergic airway in chronic asthma. Until we can better represent the normal processes that initiate and perpetuate asthma, our understanding of the mechanisms of tissue injury leading to chronic remodeling of the airways and effective therapeutic strategies to treat this disease will remain limited. It was with this goal in mind that we set about devising an inhalational model of *Aspergillus fumigatus*-induced fungal asthma in a murine experimental system.

Key words Asthma, Allergy, Model, Aspergillus, Remodeling, Inhalation, Fungus

1 Introduction

The lung is a fabulously complex organ that employs over 50 cell types to carry out its primary function of gas exchange. From our first breath to our last, its delicate network of air spaces with walls comprising a single cell's thickness is constantly under mechanical stress as the alveoli are stretched and released. Its function and composition dictate that it must routinely rid itself of inhaled and cellular debris. The lung must withstand the regular assault of toxic exposures in the form of chemicals ranging from cigarette smoke to air fresheners. Often the assault is in the form of microorganisms that may be ignored, blocked, or attacked depending upon the level of threat. Not only does the immune response in the lung need to quickly block or eliminate and remove microbial pathogens from infecting the body through this highly vulnerable site of

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entry, but it must also retain function through the response and repair process. It is little wonder, then, that sometimes the pulmonary immune response is associated with a host-derived pathology. Whether the immunopathology of allergic asthma is a result of an aberrant response that incorrectly interprets an innocuous antigen as a pathogenic threat or a vestige of an appropriate immune response that has unintended consequences, the resulting response can lead to acute and chronic pulmonary dysfunction.

Asthma is a clinical condition affecting more than 300 million persons worldwide [1]. Its treatment is expensive both in personal expense, which can include medication costs, office and emergency center visits, and hospitalization, and reduced workforce productivity. In the USA alone, the economic burden associated with asthma is $56 billion annually [2] and continues to increase. As a disease that can develop in childhood and persist into senescence, the cost for an individual may be accrued for decades.

Asthma is characterized by acute exacerbations punctuating a persistent disease. The cumulative effects of these exacerbations may lead to permanent damage of the airways, particularly when the individual is sensitized to fungal allergens. Sensitization to fungi in the context of asthma presents a severe clinical scenario that is difficult to treat, accounting for a disproportionately large number of emergency center visits and hospitalizations [3, 4]. The inflammation and airway hyperresponsiveness that accompany an acute asthma attack are well-recognized factors that demand immediate medical intervention. However, while the chronic dysfunction that is associated with the remodeling of the airway wall may be less obvious, it is responsible for considerable morbidity associated with allergic asthma. This immunopathologically mediated transformation of the airway is typified by airway and blood vessel smooth muscle cell hyperplasia, increased mucus production, and peribronchial fibrosis. Airway obstruction in acute asthma is reversible; in contrast, the cumulative dysfunction caused by long-term airway remodeling is not.

The experimental model that is explained here was built upon the foundation of other intratracheal inoculation models of *A. fumigatus*-induced disease [5]. The nose-only inhalation of aerosolized *Aspergillus* conidia by a mouse that has been sensitized to *Aspergillus* antigens elicits an allergic phenotype with many of the immunological signs and physiological parameters that afflict human patients with asthma, including airway wall remodeling and exacerbation following rechallenge [6, 7].

The model entails allergen sensitization through injections of soluble fungal extracts in adjuvant followed by an inhalation challenge with unmanipulated, airborne fungal spores. Directions for assembling a simple apparatus that allows the hydrophobic fungal spores to be blown into a nose-only inoculation chamber are included in the notes section (*see* **Note 1**). At prescribed time points after fungal inhalation, restrained plethysmography is

employed to assess airway responses before and after acetyl-β-methacholine injection. Blood, BAL fluid, and lung tissue may then be collected from each animal and stored or prepared for further analyses, which may include morphometric analysis of airway cells, histological visualization of inflammation and airway remodeling, protein and nucleic acid assessment, flow cytometry, and other measurements of the disease process.

2 Materials

2.1 Airborne Fungal Inhalation Apparatus (See Note 1)

1. Apparatus assembly: ¾-in. barbed female thread fitting; 1-in. × ¾-in. female threaded coupler; 1-in. coupler; 1-in. schedule 40 PVC; ¾-in. × 1-in. male threaded adaptor; ¾-in. male threaded to ½-in. barbed fitting; PVC cement; jigsaw with a PVC blade; drill with 5/16-in. drill bit or a drill press; ½-in. tubing; ¾-in. tubing; two 500-ml vacuum flasks; acidic sporicidal solution.

2.2 Fungal Sensitization and Challenge

1. Animals and husbandry: Specific pathogen-free C57BL/6 or BALB/c mice; Alpha-dri paper bedding.
2. Sensitizing fungal antigen and adjuvant for injections: 100 μg/ml of *Aspergillus fumigatus* antigen (Greer Laboratories, Lenoir, NC, USA) in normal saline (NS) that has been mixed immediately before injection with an equal volume of Imject Alum (Pierce, Rockford, IL, USA); 100 μl is required per injection.
3. Sensitizing fungal antigen for intranasal inoculation: 1 mg/ml of *Aspergillus fumigatus* antigen extract (Greer Laboratories) in NS delivered with a micropipette; 20 μl is required per inoculation.
4. Fungal culture for airborne challenge: *Aspergillus fumigatus*, *Fresenius* fungal culture stock (strain NIH 5233, American Type Culture Collection (ATCC), Manassas, VA, USA); 1× PBS; 0.4-ml Eppendorf tubes; 4 °C refrigerator; 25-cm^2 cell culture flasks coated on one large surface with 10–12 ml of Sabouraud Dextrose Agar (SDA).
5. Airborne delivery: Assembled apparatus (*see* **Note 1**), set up in a class II biological safety hood; anesthesia cocktail of 75 mg/kg of ketamine and 25 mg/kg of xylazine (*see* **Note 2**) delivered by injection with a tuberculin syringe with 26-gauge needle; warming blankets or heaters for post-anesthesia recovery.

2.3 Airway Plethysmography and Ventilation

1. Anesthesia: 0.01 mg of sodium pentobarbital/g body weight in a volume of <0.5 ml of sterile PBS per mouse (*see* **Note 2**).
2. Airway canulation: Small animal restraint board; 70 % EtOH; surgical scissors; forceps; 19-gauge beveled tracheal tube; surgical sutures.

3. Airway assessment: whole body, restrained plethysmograph for mouse (for example, Buxco, Troy, NY, USA, or flexiVent, SciReq, Montreal, Canada); small animal respirator (Harvard Apparatus, Holliston, MA, USA); 480 μg/kg of acetyl-β-methacholine in a 0.1-ml volume.

2.4 Tissue Collection, Processing, Storage

1. Bronchoalveolar lavage (BAL) cells and fluid: Tuberculin syringe with sterile NS fitted with a blunt 19-G needle; 1.5-ml Eppendorf tubes; ice bucket.
2. Blood collection: Forceps; sterile 1.5-ml Eppendorf tubes; micropipettors; microfuge; −20 °C freezer.
3. Lung tissue collection for protein or nucleic acid, histological, and flow cytometric analyses: Surgical scissors; forceps; 5-ml snap top tubes; liquid nitrogen in a dewar; −80 °C freezer; tuberculin syringes with 26-gauge needles; 10 % neutral buffered formalin; 50-ml tubes; 5-ml snap top tubes with cell culture medium; ice buckets.
4. Tissue preparation for nucleic acid or protein analysis: Tissue homogenizer (Tissue-Tearor, BioSpec Products, Bartlesville, OK); cold DMEM with a protease inhibitor cocktail (Roche Complete Mini or similar, Roche Applied Science, Indianapolis, IN); micropipettors, nucleic acid isolation kits (any); ELISA Abs and kits (R&D Systems, Minneapolis, MN, or others).

3 Methods

3.1 Murine Sensitization and Challenge with Live, Airborne Cultures of A. fumigatus

1. Obtain prior approval for these studies from the appropriate institutional office(s) for the use of animals in research and for the use of biological safety level (BSL) 2 biological organisms.
2. Reconstitute a single lyophilized *A. fumigatus* culture in PBS in a volume recommended by ATCC and store 60-μl aliquots of the suspension in 0.4-ml Eppendorf tubes at 4 °C until use.
3. Purchase animals from a reputable laboratory animal facility and maintain them in a specific pathogen-free facility for the duration of the study. Feed and water animals ad libitum throughout the study on a general mouse chow diet and house them on Alpha-dri paper bedding or a similar low-microbial bedding choice.
4. Divide mice into groups of 5–6 animals (*see* **Note 3**) for each time point. Sensitize mice with a subcutaneous (SC) and an intraperitoneal (IP) injection of 5 μg of soluble *A. fumigatus* antigen dissolved in 0.05 ml of PBS and 0.05 ml of Imject Alum totaling 10 μg between the two injections. Two weeks after the injections, inoculate the mice with a series of 3, weekly 20-μg intranasal (IN) inoculations consisting of soluble *A. fumigatus* antigen dissolved in 20 μl of NS (*see* **Note 4**).

5. One week after the final sensitizing inoculation, prepare the inhalation challenge chamber in a class II biological safety hood by fitting the neck of the culture flask to the input end (*see* **Note 5**). Deliver air through the culture flask at 2 psi to liberate the hydrophobic spores and allow their delivery through the inoculation port. For the initial run, place tape over each of the nose holes in the inoculation apparatus to allow the airborne spores to coat the inside of the apparatus. Turn off the air, remove the tape, and replace the culture flask with a new one for the first group of animals.
6. To expose the animals to live airborne conidia, anesthetize three mice with a ketamine/xylazine anesthesia cocktail and place their noses in one of the three inoculation ports. Adjust the airflow to 2 psi and allow the animals to breathe aerosolized conidia for 10 min. Return the animals to clean cages with heat support and monitor until they recover from anesthesia. Change the *Aspergillus* culture with each set of three mice. After the last group has been treated, decontaminate the apparatus (*see* **Note 6**).

3.2 AHR: Plethysmography and Ventilation by Cannulated Trachea

1. At the appropriate time point after allergen challenge, anesthetize the mice in a group one at a time with an SC injection of sodium pentobarbital. This will be the terminal procedure for each group of mice. Place the animal on a surgical restraint board, and tracheostomize. For tracheostomy, a length of surgical suture taped to the top of the restraint board should be used to catch the animal's front teeth to restrain the head for tracheal surgery. A drop of 70 % EtOH on the trachea helps to wet the fur, making surgery easier. Tracheostomize the mouse by opening the hide with a small snip along the trachea. Put the point of the surgical scissors in the cut and open the blades to extend the opening sagittally. Expose the trachea. Make a small snip in the membrane that covers the trachea. Insert the tip of the surgical scissors in the cut and open the blades to extend the opening sagittally. Using curved, sharp-nosed forceps, make a path behind the trachea and pull a 4-in. length of suture around the back of the trachea. Make a horizontal cut across the front of the trachea anterior to where the surgical suture is positioned, being careful not to cut through the back of the trachea. Insert the 19-gauge bevel tracheal tube and tie it into place securely with surgical suture. Connect the trachea tube to the ventilator. Measure and record the baseline compliance/resistance for airway response (per optimized settings for the plethysmography of choice). Inject 0.1 ml of methacholine (480 μg/kg) by tail vein injection (*see* **Note 7**), and record the postinjection peak airway resistance (Fig. 1).

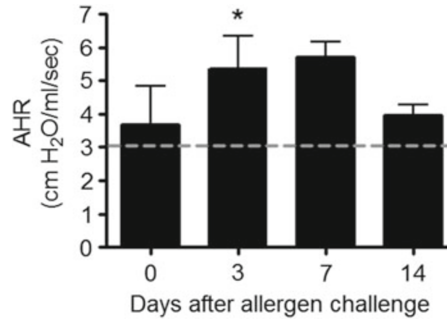

Fig. 1 Airway hyperresponsiveness at days 0, 3, 7, 14, 21, and 35 after conidia challenge in *A. fumigatus*-sensitized BALB/c mice challenged with airborne conidia. The baseline airway resistance in all groups was similar prior to the methacholine provocation (1.52 ± 0.061 cm H_2O/ml/s). Peak increases in airway resistance were stimulated by using an intravenous methacholine injection dose of 480 μg/kg. Naïve values after methacholine are represented with a *dashed line*. Values are expressed as the mean ± SEM; $n = 5$ mice/group (Modified from data originally published in [8] and reproduced with permission from Informa Healthcare)

3.3 Blood Collection per Orbital Bleed Exsanguination

1. Remove the mouse from the ventilator and, under anesthesia, exsanguinate the animal by removing one or both eyeballs. Collect the blood in a 1.5-ml Eppendorf tube. Approximately 500 μl of blood can be collected efficiently by this method. Centrifuge the blood at 15,000 × *g* for 10 min and transfer the serum to a new tube. Store the serum at −20 °C until use. Sera can be used for various protein analyses by standard ELISA methods (Fig. 2a, c, d).

3.4 BAL Fluid Collection via Trachea Tube Cannula, Cell Differential, and Fluid Collection

1. Open the chest cavity, exposing the lungs. Connect a 19-gauge blunt needle fitted to a tuberculin syringe loaded with 1.0 ml of sterile PBS to the tracheal tube and lavage the bronchoalveolar space. Place the lavage fluid in a 1.5-ml Eppendorf tube on ice. After all samples are collected, centrifuge to pellet cells. Remove the supernatant, transfer the BALF to a clean tube, and freeze at −20 °C until use for protein analysis (Fig. 2b). Resuspend the cells in PBS (*see* **Note 8**) and cytospin onto coded glass microscope slides. Dry the slides and perform a standard quick dip differential stain. Differential counts on lymphocytes (B and T cells), monocyte/macrophages, neutrophils, and eosinophils can be recorded by counting at least 300 cells per slide from 1,000× random fields (Fig. 3).

3.5 Lung Dissection for Histology, Nucleic Acid Assessment, or Protein Analysis

1. Dissect whole left lungs from each mouse. Inflate the lung ex vivo by injecting 1 ml of 10 % neutral buffered formalin (NBF) through a single injection into the peripheral lung tissue until the entire left lung is inflated. Place the left lungs from one group in a 50-ml tube containing 10 % NBF and fix overnight for histological processing and staining (Fig. 4, *see* **Note 9**).

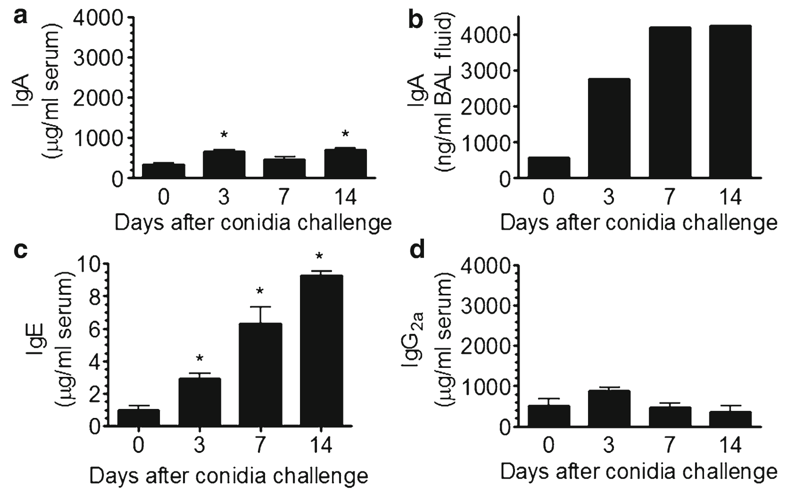

Fig. 2 Antibody levels from serum and BAL fluid after allergen challenge with aerosolized *A. fumigatus* conidia in BALB/c mice. Ab isotypes were quantified by specific ELISA in serum and BAL fluid at days 3, 7, and 14 and compared to sensitized mice that were not challenged with inhaled fungal conidia (day 0). Serum levels were analyzed using an unpaired, student's two-tailed t test with Welch's correction. All values are expressed as the mean ± SEM. $n = 4$–5 mice/group, $^*p < 0.05$ was considered statistically significant. BAL samples were pooled, and no statistical analysis was run on them

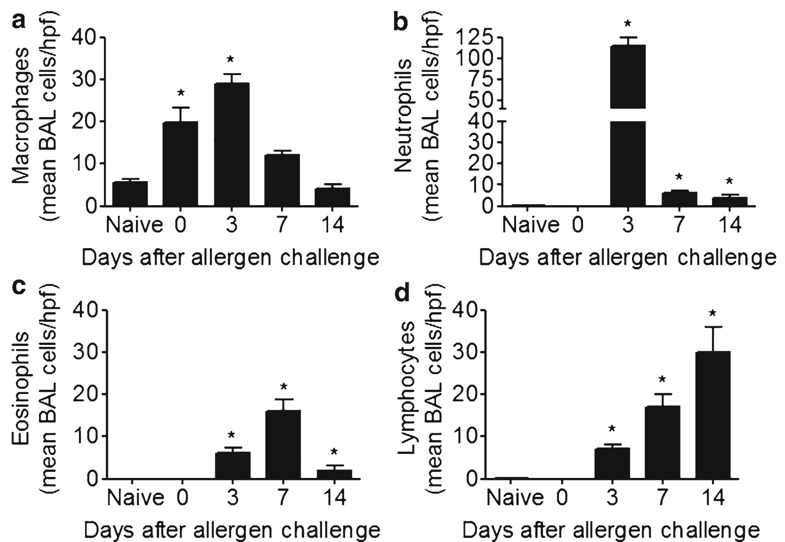

Fig. 3 BAL leukocyte counts in *A. fumigatus*-sensitized BALB/c mice at days 0, 3, 7, 14, and 21 after airborne conidia challenge. Cells washed from the airways at various times after allergen challenge were cytospun onto coded microscope slides and assessed by morphometric characteristics. Data are expressed as the mean number of cells per HPF (1,000×) ± SEM; $n = 5$ mice/group

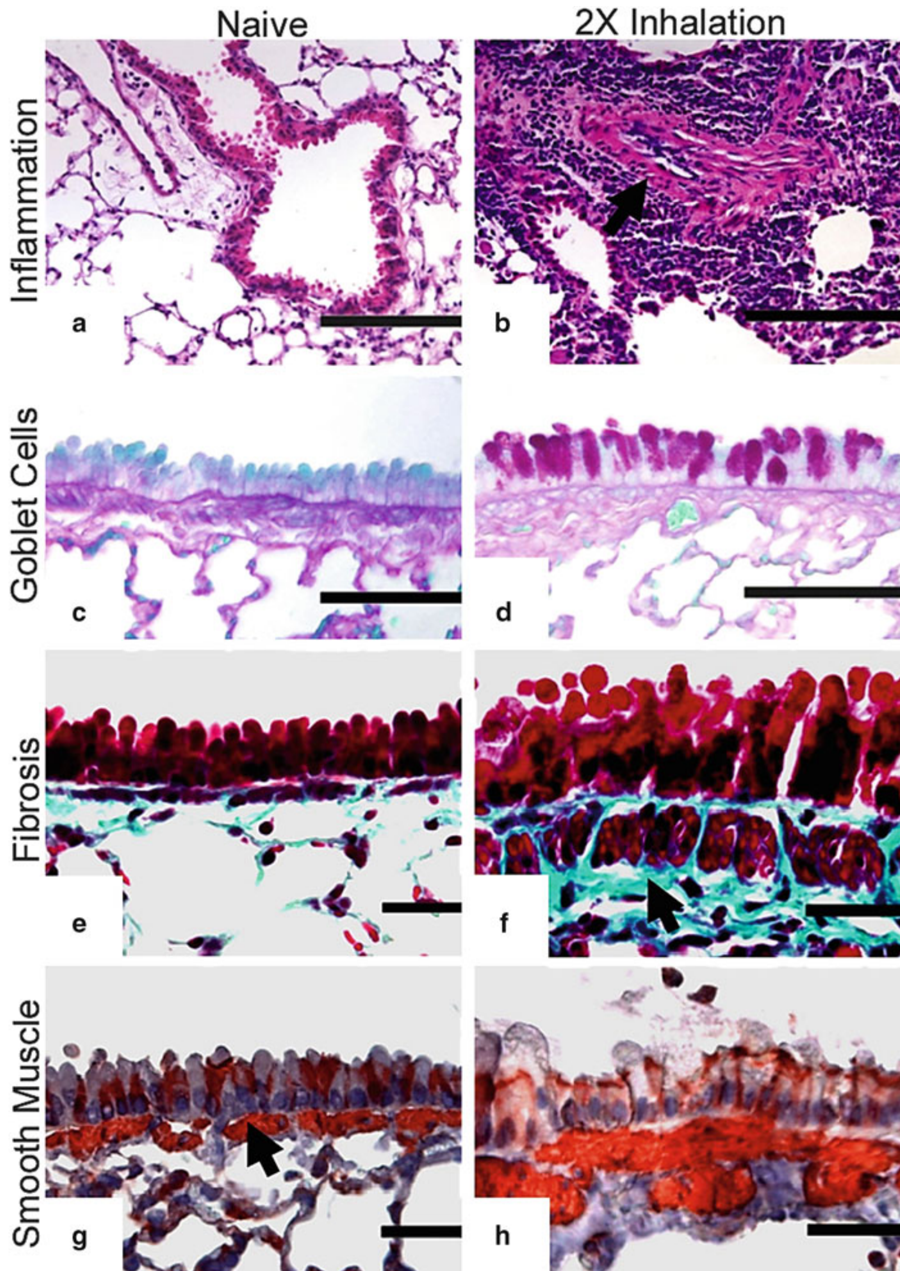

Fig. 4 Representative photomicrographs showing inflammation, goblet cell metaplasia, subepithelial fibrosis, and peribronchovascular smooth muscle cell changes in naïve controls or at day 7 after two inhalational challenges with *A. fumigatus*. H&E-stained histological sections from naive (*left*) and fungus-challenged (*right*) lungs were assessed for inflammation by H&E stain (**a** and **b**, *arrows* indicate perivascular smooth muscle cell increases), goblet cell metaplasia by periodic acid Schiff's stain (**c** and **d**, *magenta* stain), fibrosis by Gomori's trichrome stain (**e** and **f**, *blue* stain), and peribronchial smooth muscle by IHC for α-smooth muscle actin (**g** and **h**, *red* stain). Scale bars for **a**, **b** = 200 μm; for **c**, **d** = 100 μm; for **g**, **h** = 50 μm (Modification of original reproduced from [6] with permission from Elsevier)

Alternatively, each lung can be processed separately for paired analysis with AHR or other measurements.

2. Dissect a small piece of the right lung for nucleic acid analysis and a large piece for protein analysis. Snap freeze in liquid N_2 and store at −80 °C until use. Process the tissues for real-time RT-PCR by isolating total RNA by standard methods. Process the tissues for protein analysis by grinding with a tissue homogenizer in cold DMEM with a protease inhibitor cocktail and analyzing by standard ELISA methods (*see* **Note 10**).

4 Notes

1. Using a jigsaw with a PVC blade, cut the schedule 40 PVC into a 10¾-in. length. Using a 5⁄16-in. drill bit, make nose holes for exposure by first drilling one hole into the middle of the pipe, and then drilling one hole to the left and one to the right of the central hole with approximately 2.5-in. spacing. It is important that the holes are exactly in line. We suggest drilling the holes prior to assembling the apparatus as it may take multiple attempts to achieve this without a drill press.

   Place the ¾-in. × 1-in. male threaded adaptor on the right end of the PVC pipe and the 1-in. PVC coupler on the left end. Insert the 1 × ¾-in. female adaptor into the 1-in. coupler on the left end. Fasten all of these together using PVC cement. Once the cement has dried, place the ¾-in. female adaptor on the right end and the ¾-in. male thread × ½-in. barbed fitting on the opposite end. Attach a short length of ¾-in. tubing to the right side and approximately 2.5-ft of ½-in. tubing to the left side.

   Assemble the inoculation chamber in a class II biological safety hood (Fig. 5). Prepare the 25 cm^2 cell culture flask containing an 8-day-old culture of *A. fumigatus* by boring a hole into the rear top and back end of the flask (distal from the neck) with a ½-in. cork hole borer heated over a Bunsen burner. The air input tubing is placed into these holes and adjusted to 2 psi. to liberate the spores when the animals are in place. Attach the culture flask neck to the ¾-in. tubing on the right side of the inoculation chamber. The ½-in. tubing on the left end is used to connect the apparatus to two vacuum flasks containing a sporicidal agent. Connect the vacuum flasks in a series so that the incoming air flows into the top of the first flask down through a plastic pipette fitted through a rubber stopper and into the liquid. The air and spores should bubble into the liquid of flask 1 and the exhaust air and any residual spores from that flask will continue out the side port, through another length of ½-in. tubing into the top of a second

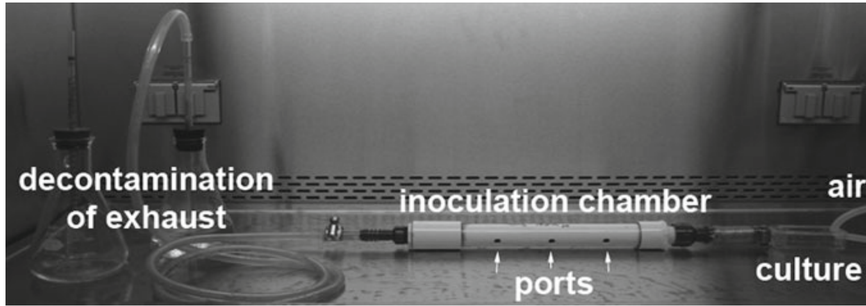

Fig. 5 Airborne inoculation apparatus. From *right* to *left*, the *Aspergillus* inoculation apparatus consists of two air inputs that blow air over a mature, sporulating culture that has been grown on a solid SDA medium in a 25-cm^2 culture flask. Two holes are bored into the plastic immediately before use to provide access for the air hoses. The culture is connected to the inoculation chamber by a short piece of flexible tubing. The inoculation chamber has three, nose-only ports where the anesthetized animals are placed for inoculation. The exhaust air is decontaminated by bubbling through two flasks of sporicidal liquid. The entire apparatus is contained in a class II biological safety hood

flask and down into another volume of sporicidal agent. Finally, the air is allowed to escape from that flask through the side port.

2. Ketamine is a USDEA Schedule III drug, and pentobarbital is a USDEA Schedule II drug. They are controlled substances for which appropriate drug licensure is required for purchase. The University's Attending Veterinarian in charge of animal care may be an appropriate point of contact to procure regulated drugs and to ensure the proper use, storage, documentation, and disposal of the same.
3. Male and/or female mice can be used but should be age and sex matched for each study. In our experience, 5–6 animals per time point provide a reliable assessment of the inflammatory and remodeling aspects of the model. However, a larger sample size may be required for other types of assessments, for example cell sorting for ex vivo experiments. By convention, the day 0 time point represents sensitized animals that have not received the allergen inhalation challenge. These and/or naïve mice are used as controls. In groups that are to receive two or more aerosol challenges, we have found that a 1–2-week interval between inoculations provides robust responses, but does not result in observable physical difficulties for the animals. Likewise, they do not succumb to fungal outgrowth.
4. For intranasal inoculation, pick up the mouse with a hold that immobilizes the head. Draw up the entire 20-μl volume with a micropipettor and deliver half of the volume to each nare allowing the animal to sniff in the inoculum before returning it to the cage.

5. To bore holes through the top and back end of the fungal culture flask, heat a ½-in. metal cork borer over a Bunsen burner and bore holes through the plastic.
6. The interior space of the safety cabinet should be considered contaminated with spores throughout the experiment and until it is thoroughly wetted and wiped down with sporicidal solution. Typically, UV irradiation is insufficient to kill *Aspergillus fumigatus* spores. For decontamination of the apparatus, submerge it in a sporicidal bath in the hood after each use.
7. Warming lights or oil of wintergreen help to vasodilate the tail vein for methacholine injections. The 480 μg/kg dose for tail vein injection has been shown to double the baseline AHR in a naïve mouse, which is then used as the definition of "airway hyperresponsiveness" for the study. Alternatively, a range of increasing injected doses may be used sequentially or inhaled methacholine can be introduced through a nebulization port. If nebulized methacholine is used, care must be taken to prevent the inadvertent exposure of methacholine in the apparatus for the next baseline measurement.
8. To ensure countable BAL cell differentials, we have found that reconstitution in 200 μl of PBS is appropriate for day-0, -7, and -14 samples from a single challenge. Reconstitution in 1 ml of PBS is needed for day-3 samples from a single or a double challenge and for day-7 samples from a double challenge.
9. Columnar epithelial thickness and peribronchial fibrosis can be quantified by measuring the thickness of the cell layer (for epithelial cells) or stained collagen (fibrosis) perpendicularly to the basement membrane. We find that the second (L2) and third (L3) lateral branch of the large airway is an appropriate location to measure continuous lengths of airway to get a representative sampling. Although a skilled technician is still required, L2 and L3 are landmarks that are most easily reproduced when samples are sectioned. At least 50 discrete points for epithelium and at least 100 discrete points for collagen should be measured at intervals of 50 μm, taking care not to include those points that are directly adjacent to a blood vessel as this would artificially increase the measurement.
10. Care should be exercised when interpreting data from ELISAs run on whole-lung homogenates. While serum or BAL fluid results in data that is linear with dilution and reproducible across different manufacturers' platforms, this is not necessarily the case with whole-lung homogenates. Our assessment is that as the protein content of the lung changes dramatically over the course of the model, it may adversely impact the signal-to-noise ratio of antibody-based ELISAs.

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