Chapter 19

A Mouse Model for Evaluating the Contribution of Fibrocytes and Myofibroblasts to Airway Remodeling in Allergic Asthma

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Abstract

Airway remodeling is a term used to collectively indicate bronchial structural changes that may lead to irreversible airflow obstruction and progressive decline in lung function in asthmatic patients. Bronchial myofibroblasts contribute to airway remodeling by producing collagenous proteins in the subepithelial zone and by increasing the density of contractile cells in the bronchial wall. A substantial proportion of bronchial myofibroblasts in asthma differentiate from circulating mesenchymal progenitor cells known as fibrocytes. Here, we describe a mouse model of allergic asthma for evaluating the functional role of fibrocytes and myofibroblasts in this disease and the inhibitory effects of novel therapeutic candidates.

Key words Airway remodeling, Asthma, Fibrocytes, Mice, Myofibroblasts

1 Introduction

1.1 Airway Remodeling in Asthma and in the Animal Model of Allergic Disease

Asthma is a common disorder of the airways characterized by recurrent episodes of airflow obstructions of variable duration, which may be triggered by various stimuli such as the exposure to environmental allergens or viral infections in genetically predisposed individuals [1]. The main histopathologic abnormalities include a chronic inflammatory infiltrate of the bronchial mucosa and a series of structural alterations collectively referred to with the term "airway remodeling" [2, 3] (Fig. 1). Structural alterations such as the thickening of the lamina reticularis and the increase in the smooth muscle mass are characteristic features of asthma [4]. These alterations have become major targets for the development of new therapeutic agents because they may cause fixed airway narrowing and contribute to a progressive loss of lung function in patients with severe disease and frequent clinical exacerbations [2, 3]. The thickening of the lamina reticularis is particularly evident in

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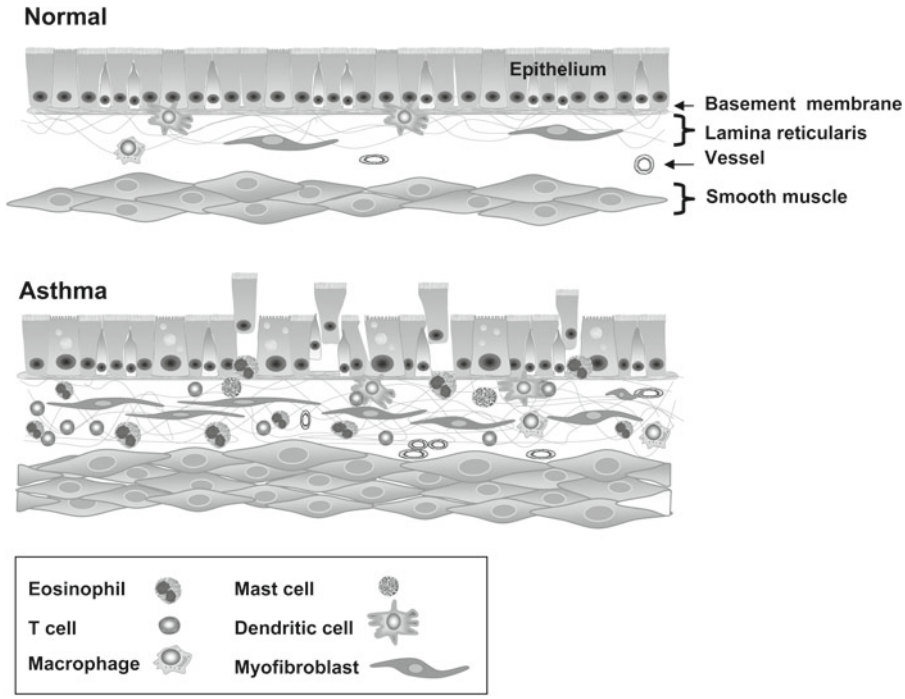

Fig. 1 Schematic illustration of the main inflammatory and structural changes that can be observed in the bronchial mucosa of patients with allergic asthma. Features of airway remodeling include hyperplasia and hypertrophia of the goblet cells, subepithelial fibrosis with thickening of the lamina reticularis, increased density of fibroblasts and myofibroblasts in the lamina propria, increased vascularity, and increased smooth muscle mass. Designed by using objects of the ScienceSlides 2005 software (VisiScience Corporation, Chapel Hill, NC, USA)

forms of asthma where the inflammatory infiltrate is predominantly composed of T helper type 2 (Th2) cells and eosinophils [5, 6] and reflects an increased deposition of collagen types I (COL1), III (COL3), and V (COL5), fibronectin, hyaluronan, various proteoglycans, and tenascin-C [7–11]. These changes in the composition of the lamina reticularis are collectively termed "subepithelial fibrosis" and are thought to be caused by the abnormal accumulation of fibroblasts and myofibroblasts in the lamina propria of the asthmatic bronchial mucosa [12, 13]. Like smooth muscle cells, bronchial myofibroblasts express the contractile protein α-smooth muscle actin (α-SMA) [12, 13]. Subepithelial fibrosis and the expansion of the population of contractile elements resulting from myofibroblast accumulation and increased smooth muscle mass in asthma may contribute to generate persistent airway narrowing by stiffening and contracting the bronchial wall [2, 3].

In allergic asthmatics, every exposure to the clinically relevant allergen can induce a further increase in the accumulation of fibroblasts and myofibroblasts in the lamina propria of their bronchial mucosa and further deposition of collagenous proteins in the

subepithelial zone [14–16]. These features of ongoing airway remodeling persist after the resolution of the acute inflammatory response elicited by allergen inhalation [15]. In 2003, we provided the first evidence that a substantial proportion of the fibroblasts and myofibroblasts emerging in asthmatic airways between 4 and 24 h following allergen exposure have the phenotypic characteristics of fibrocytes [17]. The fibrocytes are circulating $CD45^+CD34^+COL1^+$ mesenchymal progenitor cells that constitutively produce extracellular matrix components relevant to asthma, in addition to COL1, and differentiate into myofibroblast-like cells upon stimulation with various growth factors and cytokines [18–21]. These cells also constitutively express CD11b, CD13, the class II major histocompatibility complex HLA-DR, the co-stimulatory molecules CD80 and CD86, the C-C motif chemokine receptors (CCRs) for the C-C motif chemokine ligand (CCL)5 (CCR3 and CCR5), CCL11 (CCR3 and CCR5), CCL24 (CCR3), and the C-X-C motif chemokine receptor (CXCR) for the C-X-C motif chemokine ligand (CXCL)12 (CXCR4) [18, 20]. In normal individuals, the frequency of circulating fibrocytes [22–25] and the density of fibrocytes in the bronchial mucosa [22, 26] are very low, although circulating cells can be isolated in long-term cultures of peripheral blood mononuclear cells under conditions that selectively favor their survival and proliferation [18–21]. Further studies on the role of fibrocytes in asthma, following our initial observations [17], have demonstrated an increased frequency of circulating fibrocytes in patients with allergen-exacerbated asthma [22, 23] and in those with persistently severe, treatment-refractory disease [24, 25]. In persistent asthma, fibrocyte infiltration of the lamina propria correlates with disease severity [24] and extent of subepithelial fibrosis [26]. Fibrocyte infiltration also involves the bronchial smooth muscle bundle [24], and most of the $CD34^+COL1^+$ cells detected in the bronchial wall of the asthmatic individuals express α-SMA and have an elongated, fibroblast-like shape [17, 24, 26].

The asthmatic bronchial epithelium and the bronchial smooth muscle produce chemokines and growth factors capable of inducing the recruitment of circulating fibrocytes and their local proliferation or differentiation into myofibroblast-like cells [17, 22–24, 27, 28] (Fig. 2). Th2 lymphocytes and eosinophils also release cytokines and growth factors that are known to promote the contractility and profibrotic function of fibrocytes [17, 20, 22], such as interleukin (IL)-4, IL-13, and transforming growth factor-β (TGF-β) (Fig. 2).

In order to evaluate the contribution of fibrocytes and fibrocyte-derived myofibroblast-like cells to airway remodeling in allergic asthma, we developed a murine model of human disease where animals were systemically sensitized to the antigen ovalbumin (OVA) and then subjected to repeat inhalation challenges with controlled levels of aerosolized OVA every week for up to 8 weeks. Following

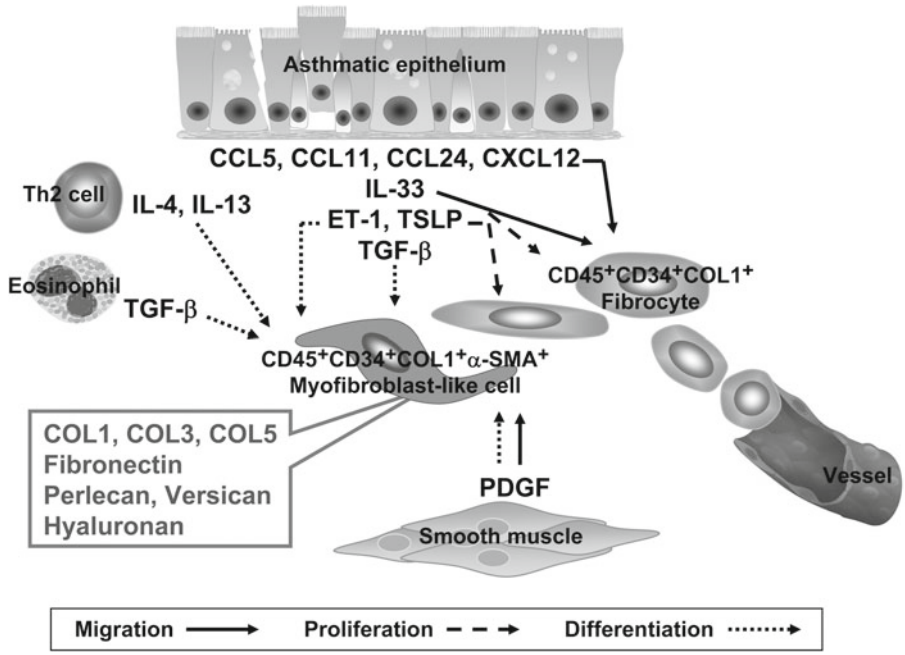

Fig. 2 Schematic illustrations of the factors that may induce the recruitment of circulating fibrocytes to the bronchial mucosa of asthmatic patients and promote their local proliferation and differentiation into myofibroblast-like cells. α-*SMA* α-smooth muscle actin, *CCL* C-C motif chemokine ligand, *COL1* type I collagen, *COL3* type III collagen, *COL5* type V collagen, *CXCL* C-X-C motif chemokine ligand, *ET-1* endothelin-1, *PDGF* platelet-derived growth factor, *TGF*-β transforming growth factor-β, *TSLP* thymic stromal lymphopoietin. Designed by using objects of the ScienceSlides 2005 software (VisiScience Corporation, Chapel Hill, NC, USA)

repeated exposures to the antigen for more than 4 weeks, the airway wall of these mice demonstrated many of the histopathologic abnormalities associated with the human condition. These abnormalities included sustained infiltration of the lamina propria and epithelium with eosinophils, the appearance of fibrocytes and myofibroblast-like cells below the epithelium, and a progressive thickening of the subepithelial zone, which reflected increased deposition of fibronectin and collagens [17] (Figs. 3 and 4). By tracking labeled circulating fibrocytes to the bronchial wall after an inhalation challenge with OVA at 6 weeks of repeated exposures, we provided direct evidence that these cells were recruited to areas of ongoing subepithelial fibrosis [17]. The recruited fibrocytes produced higher levels of intracellular COL1 than circulating fibrocytes and expressed α-SMA within 24 h following their migration at the tissue site [17]. We describe below the protocol for the specific evaluation of fibrocytes and myofibroblasts in this model of chronic allergic asthma. Various studies from other groups have recently confirmed the development and persistence of features of airway remodeling relevant to asthma in sensitized mice that are subjected to repeat allergen

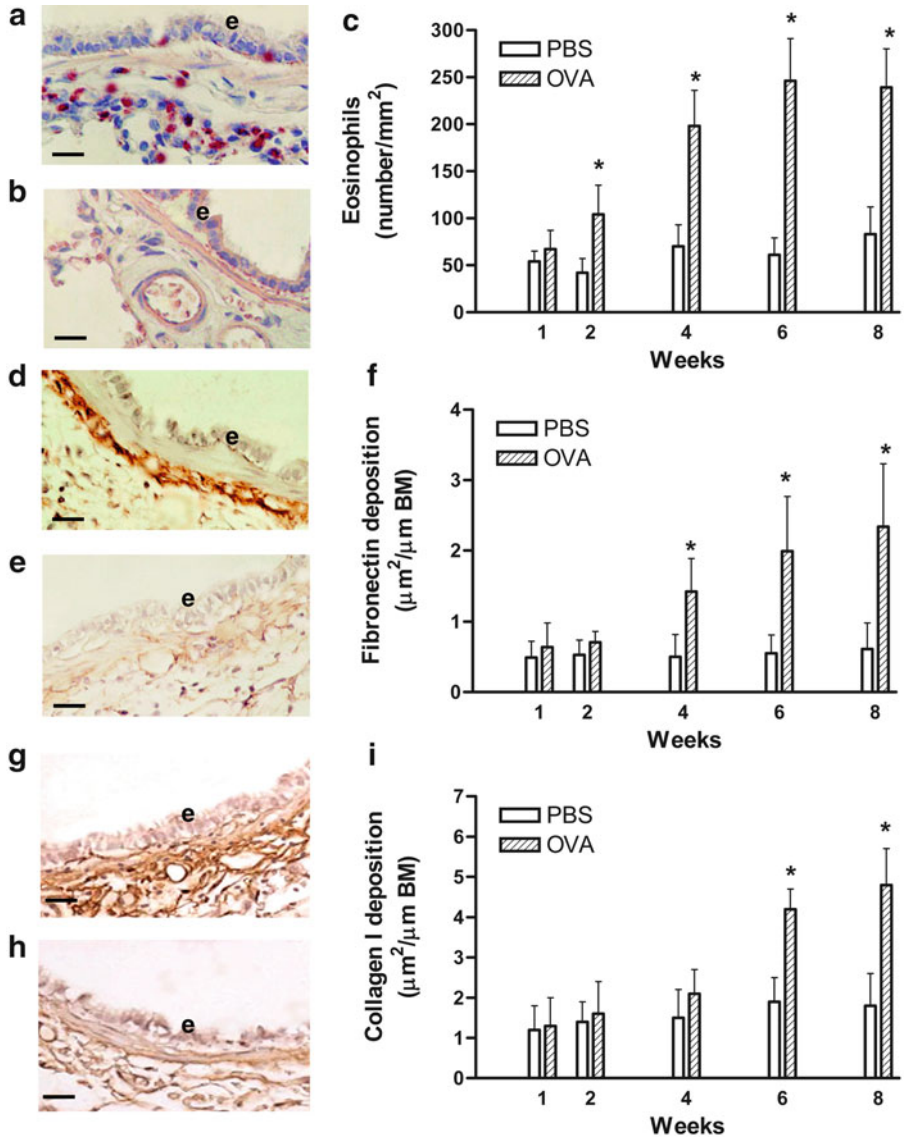

Fig. 3 Airway eosinophilia (**a**–**c**), and progressive increase in the deposition of fibronectin (**d**–**f**) and collagen type I (**g**–**i**) in the bronchial wall of BALB/c mice systemically sensitized to ovalbumin (OVA) and subjected to repeated challenges with aerosolized 2.5 % OVA in phosphate-buffered saline (PBS) three times a week over a period of 8 weeks, using a whole-body exposure system. Control mice systemically sensitized to OVA were subjected to repeated challenges with PBS alone. The response was assessed at the time-points indicated in (**c**), (**f**), and (**i**), 24 h after the last challenge. The microphotographs in (**a**) and (**b**), respectively, show the infiltration with eosinophils (*red* cells) of the lamina propria and epithelium of an OVA-exposed mouse at 2 weeks and the absence of a similar infiltration in the bronchial wall of a control mouse. The microphotographs in (**d**) and (**g**), respectively, show thickening of the subepithelial zone with increased deposition of fibronectin and type I collagen (*brown* stain) in the bronchial wall of OVA-exposed mice at 6 weeks. Less marked changes were observed in the bronchial wall of OVA-exposed mice at 2 (**e**) or 4 (**h**) weeks. Quantitative results are reported in (**c**), (**f**), and (**i**) and the data are expressed as the means and standard error. Significant differences between groups of mice are indicated by an *asterisk*. *BM* basement membrane, *e* epithelium. Scale bar = 50 μm. Reproduced with permission from ref. 17. Copyright 2003. The American Association of Immunologists, Inc

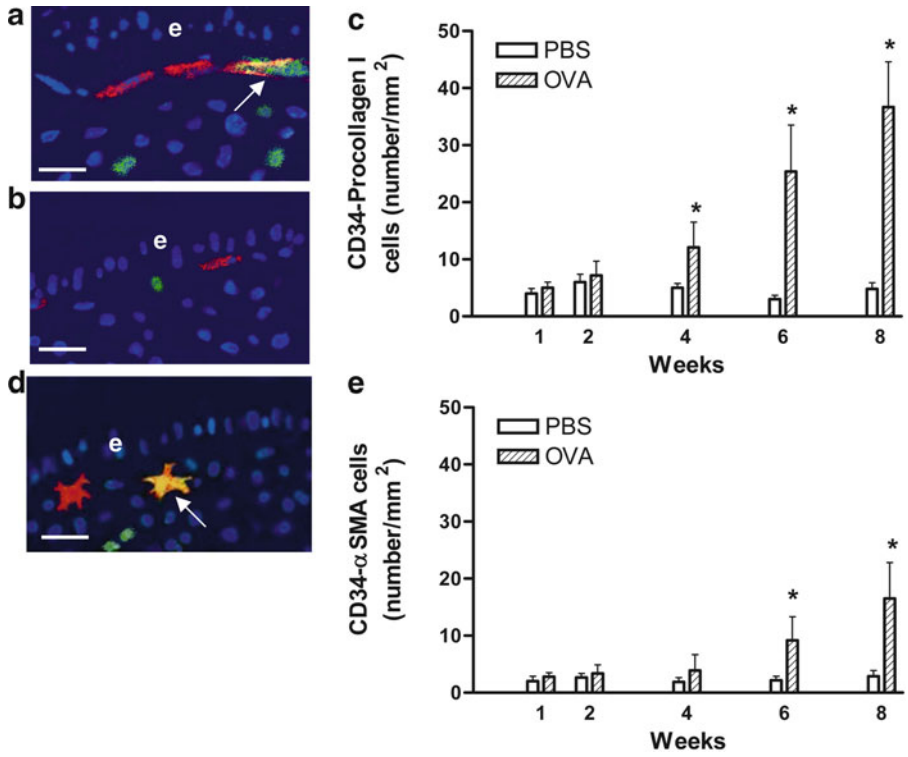

Fig. 4 Detection of fibrocytes in the airway wall of BALB/c mice systemically sensitized to ovalbumin (OVA) and subjected to repeated challenges with aerosolized 2.5 % OVA in phosphate-buffered saline (PBS) three times a week over a period of 8 weeks, using a whole-body exposure system. Control mice systemically sensitized to OVA were subjected to repeated challenges with PBS alone. The response was assessed at the time-points indicated in (**c**), and (**e**), 24 h after the last challenge. The microphotograph in (**a**) shows a representative section of the bronchial wall of an OVA-exposed mouse at 6 weeks after staining with fluorochrome-labeled antibodies against CD34 (*green*) and the intracellular precursor of type I collagen (procollagen I) (*red*). The *arrow* points to a fibrocyte that can be easily identified as a double-labeled cell (*yellow*). The microphotograph in (**b**) shows the absence of a similar double-stained cell in the bronchial wall of a control mouse. The microphotograph in (**d**) shows a representative section of the bronchial wall of an OVA-exposed mouse at 6 weeks after staining with fluorochrome-labeled antibodies against CD34 (*green*) and the smooth muscle/myofibroblast marker α-SMA (*red*). The *arrow* points to a double-labeled cell (*yellow*), representing a fibrocyte-derived myofibroblast. In all sections, nuclei were counterstained blue with DAPI. Quantitative results are reported in (**c**) and (**e**) and the data are expressed as the means and standard error. Significant differences between groups of mice are indicated with an *asterisk*. *e* epithelium. Scale bar = 50 μm. Reproduced with permission from ref. 17. Copyright 2003. The American Association of Immunologists, Inc

challenges every week for at least 5 weeks, irrespective of the method used for delivering the allergen to the airways [29–33]. Two of these studies [29, 30] evaluated the presence of myofibroblasts and myofibroblast-like cells in the bronchial wall and demonstrated an allergen-induced accumulation of $COL1^{+}\alpha\text{-}SMA^{+}$ myofibroblasts

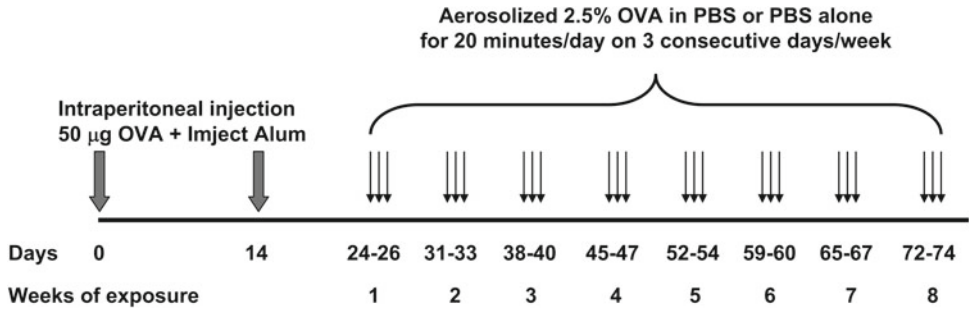

Fig. 5 Schematic illustration of the experimental procedures and timelines for systemic sensitization to ovalbumin (OVA) and repeated challenges with aerosolized 2.5 % OVA in phosphate-buffered saline (PBS) or PBS alone as control

and α-SMA$^+$ myofibrocytes in concomitance with the development of subepithelial fibrotic changes. Interestingly, the administration of a low-molecular-weight antagonist of CCR3, a receptor expressed by eosinophils [34] and fibrocytes [20, 28], not only reduced the eosinophilic inflammation but also prevented the increased accumulation of myofibrocytes beneath the epithelium and the excessive subepithelial deposition of collagens that were elicited by repeated challenge with aerosolized OVA for at least 8 weeks [30].

1.2 Overview of the Experimental Procedures and Timelines

Pathogen-free, 6–8-week-old female or male BALB/c mice should be employed in all experiments because these mice develop a good Th2-biased immunological response and produce higher levels of allergen-specific IgE antibodies than other strains upon repeated exposure to OVA following systemic sensitization to this antigen [35–37]. Sensitization to OVA is achieved by repeat intraperitoneal administrations of the antigen absorbed to an adjuvant, such as alum, which boosts the immune response and promotes the production of Th2-derived cytokines. Two intraperitoneal injections of 50 μg OVA with sterile alum solution are performed at a distance of 14 days (Fig. 5). Starting on day 24, sensitized mice are then challenged with an aerosolized solution of 2.5 % OVA in phosphate-buffered saline (PBS) in a whole-body exposure chamber for 20 min/day on three consecutive days of each week over a period of 8 weeks (Fig. 5). Control mice are challenged with an aerosolized solution of PBS alone in the same way and for the same period of time (Fig. 5). Assessment of the response to repeat inhalation challenges with aerosolized OVA or PBS is conducted every week for the first 2 weeks and every 2 weeks thereafter, 24 h following the end of the last OVA or PBS challenge. Different subgroups of mice subjected to the same experimental procedure are sacrificed at each time point (e.g., on days 27, 34, 48, 61, and 75).

2 Materials

2.1 Reagents for Systemic Sensitization

1. Grade V, ≥98 % pure chicken egg OVA (Sigma-Aldrich, St. Louis, MO, USA).
2. 10× stock solution of PBS, 1 l, pH 7.4 ± 0.05: Add 80 g of NaCl, 2 g of KCl, 11.5 g of $Na_2HPO_4 \cdot 7H_2O$, 2 g of KH_2PO_4 to a graduated cylinder. Add distilled water to the 1-l mark. Dilute the stock solution in distilled water.
3. Imject Alum (Thermo Fisher Scientific/Pierce Biotechnology, Rockford, IL, USA).
4. 15-ml conical tubes.
5. 0.45 μm filters.
6. Sterile 100-ml graduated flask.
7. Sterile stir bar.
8. Stir plate.
9. Sterile gloves.
10. 1-ml syringe.

2.2 Equipment for Whole-Body Exposure

1. A whole-body exposure system for mice, equipped with components (**items 2–14**; Fig. 6) from ToxoRes Technologies (Dublin, Ireland) if not otherwise specified (*see* **Notes 1** and **2**).

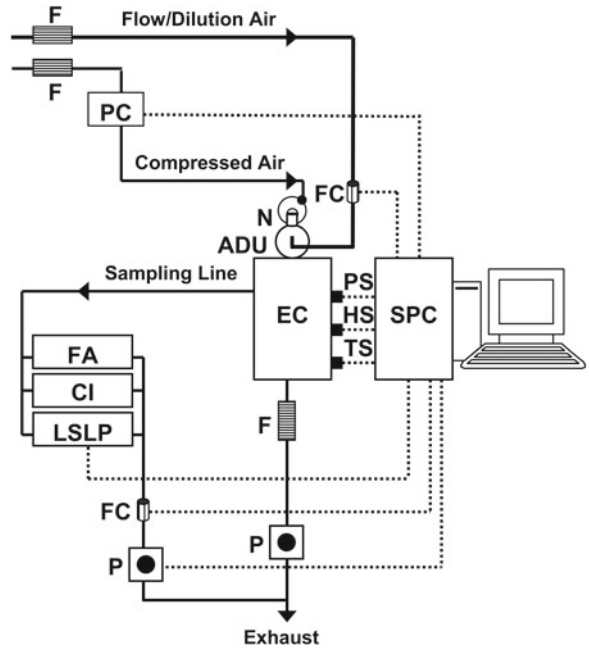

Fig. 6 Overview of the whole-body exposure system. *ADU* aerosol dilution unit, *CI* cascade impactor, *EC* exposure chamber, *F* filter, *FA* filter analyzer, *FC* flow control, *HS* humidity sensor, *LSLP* light-scattering laser photometer, *N* nebulizer, *P* pump, *PC* pressure control, *PS* pressure sensor, *SPC* signal-processing cabinet, *TS* temperature sensor

2. Stainless steel 0.3 m^3 whole-body exposure chamber with front safety glass for animal observation, a front air inlet, a side outlet for aerosol sampling, and back exhaust.
3. Wire flow-through cage with stainless steel cage support and body restraint.
4. Two lines for air supply to the exposure chamber and to the nebulizer with in-line flow controllers.
5. Line for exhaust air.
6. High-efficiency particulate air (HEPA) filters.
7. Air compressor with compressor control.
8. 3-jet Collison nebulizer (BGI, Waltham, MA, USA).
9. Aerosol diluting unit.
10. Sensors for recording the temperature and humidity in the exposure chamber.
11. Two high-volume pumps (Emerson, St. Louis, MO, USA).
12. Line for aerosol sampling.
13. Filter analyzer, multi-jet cascade impactor, and light-scattering laser photometer (TSE Systems, Bad Homburg, Germany) with the associated software for real-time and off-line measurement of aerosol mass concentration, aerosol particle size, and particle size distribution.
14. Exposure control cabinet for signal processing with real-time analysis of airflow, back-pressure, and airflow supply to the nebulizer; pressure, humidity, and temperature in the exposure chamber; aerosol mass concentration; and particle size distribution.

2.3 Reagents for Whole-Body Exposure

1. Grade V, ≥98 % pure chicken egg OVA (Sigma-Aldrich).
2. 1× PBS.
3. Sterile 50-ml conical tubes and flasks.
4. 0.45 μm filters.

2.4 Materials for Lung Tissue Processing

1. Anesthetic for intraperitoneal injection (e.g., 60 mg/kg of sodium pentobarbital or a combination of 10 mg/ml of ketamine and 1 mg/ml of xylazine).
2. 1-ml syringe.
3. Gloves, goggles, and protective clothing.
4. Blunt-ended needles.
5. Infusion apparatus.
6. 4 % paraformaldehyde solution: Add 8 g of reagent-grade, crystalline paraformaldehyde (Sigma-Aldrich) to 100 ml of tap water. Heat to 60 °C in a fume hood. Add a few drops of 1 N NaOH to help dissolve. When the solid has completely dissolved, let the solution cool to room temperature. Add 100 ml of 2× PBS

and adjust the pH to 7.4 with 1 N NaOH. This solution should be prepared fresh on the same day as the fixation.

7. 30 % sucrose in PBS for cryoprotection: Add 30 g of sucrose to a 500-ml graduated cylinder. Add 1× PBS to the 100 ml mark. Stir vigorously with a stir bar and store the solutions in a refrigerator.
8. Tissue-Tek optimal-cut-temperature (OCT) embedding compound (Miles Laboratories, Naperville, IL, USA).
9. Beakers.
10. Insulated thermos.
11. Long forceps.
12. Liquid nitrogen, in an appropriate storage tank (−196 °C).
13. Mounting blocks.
14. Freezer (−70 °C).
15. Zip-lock bags.
16. Isopentane.

2.5 Materials for Preparation of Frozen Tissue Sections

1. Gloves, goggles, and protective clothing.
2. Cryostat.
3. Clean glass slides.
4. 0.1 % poly-L-lysine solution (Sigma-Aldrich).
5. Staining racks and dishes.
6. Oven.
7. Freezer (−70 °C).

2.6 Materials for Immunohistochemical Analysis

1. Gloves, goggles, and protective clothing.
2. Staining racks and dishes.
3. Coplin jars.
4. Pipettes.
5. Humid chamber for incubations.
6. PBS.
7. Normal goat serum (Dako, Carpinteria, CA, USA) diluted 1:10 and 1:100 in PBS.
8. BLOXALL Blocking Solution (Vector Laboratories, Burlingame, CA, USA).
9. Fc Receptor Blocker (Innovex Biosciences, Richmond, CA, USA).
10. Rabbit antibody (IgG) against mouse COL1 (Merck Millipore, Darmstadt, Germany).
11. Rabbit antibody (IgG) against mouse fibronectin (Merck Millipore).

12. Rabbit antibody (IgG) against mouse collagen III (Abcam, Cambridge, United Kingdom).
13. VECTASTAIN Elite ABC Kit for rabbit IgG (Vector Laboratories).
14. Absolute alcohol, 95 % ethanol, and xylene.
15. VectaMount (Vector Laboratories).
16. Irrelevant antibodies raised in the same species as the primary antibodies listed above.
17. Glass coverslips.
18. Light microscope and image analysis system.

2.7 Materials for Immunofluorescence Analysis

1. Gloves, goggles, and protective clothing.
2. Staining racks and dishes.
3. Coplin jars.
4. Pipettes.
5. Humid chamber for incubations.
6. Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium.
7. Fc Receptor Blocker (Innovex Biosciences, Richmond, CA, USA).
8. Normal donkey serum diluted 1:10 and 1:100 in PBS.
9. 0.1 % saponin (Sigma-Aldrich).
10. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD34 monoclonal antibody (Cederlane, Burlington, ON, Canada).
11. Cyanine(Cy)3-conjugated antibody against α-SMA, clone 1A4 (Sigma-Aldrich).
12. Goat antibody (IgG) against mouse intracellular COL1 precursor, recognizing the N-terminus of procollagen I, α2 chain (Santa Cruz Biotechnology, Santa Cruz, CA, USA).
13. Rhodamine Red-X-AffiniPure or Cy3-AffiniPure and FITC-AffiniPure F(ab′)2 fragment donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, PA, USA).
14. Irrelevant antibodies raised in the same species as the primary antibodies listed above.
15. ProLong Gold Antifade Reagent with DAPI (Life Technologies/Invitrogen, Paisley, United Kingdom).
16. Glass coverslips.
17. Wide-field fluorescence microscope connected to a laser scanning confocal system with appropriate filters.

2.8 Materials for Determination of Serum OVA-Specific IgE and IgG Levels

1. Serum-separator tubes.
2. Centrifuge.
3. Adjustable pipettes and repeat pipettor.
4. Distilled or deionized water.
5. Mouse total IgE assay kit (Chondrex, Redmond, WA, USA).
6. Mouse serum anti-OVA IgE antibody assay kit (Chondrex).
7. Anti-Ovalbumin IgG_1 (mouse) EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA).
8. Plate reader capable of measuring absorbance at 450 nm, possibly using a dual beam at 450/630 nm.

3 Methods

3.1 Animal Housing and Care

1. Mice should be housed in conventional autoclaved cages, in a pathogen-free and air-conditioned room at 21–23 °C, with relative humidity of 55–65 %, and subjected to a 12-h light/dark cycle.
2. Provide free access to irradiated OVA-free food and acidified water, except when the mice are in the exposure chamber.
3. Permit acclimatization to the exposure chamber for at least 1 h/day during the week preceding the actual exposure (*see* **Note 3**).

3.2 Systemic Sensitization

1. Pour 2.5 mg of OVA into a 15-ml conical tube.
2. Add 5 ml of 1× PBS. The OVA concentration will be 0.5 μg/μl.
3. Vortex the tube three to four times.
4. Pass the solution through a 0.45 μm filter into a new 15-ml conical tube (*see* **Note 4**).
5. Place the 100-ml flask on the stir plate and start the stir bar.
6. Shake the capped bottle of Imject Alum well.
7. Pour the OVA solution into the flask, take 5 ml of Imject Alum out of the bottle, and add the solution dropwise to mix the OVA and Alum in a 1:1 ratio (vol:vol).
8. Continue stirring for at least 30 min after the addition of the entire 5 ml of Imject Alum.
9. Take the mixture into a 1-ml syringe and inject 0.2 ml containing 50 μg of OVA plus Imject Alum into the mouse peritoneal cavity (*see* **Notes 5** and **6**).

3.3 Whole-Body Exposure

1. Prepare a PBS solution containing 2.5 % OVA (*see* **Note 7**).
2. Pass 100 ml of this solution and 100 ml of PBS alone through a 0.45 μm filter into separate sterile flasks.

3. Depending on whether the experiment involves mouse exposure to OVA or PBS alone, pour the appropriate solution into the nebulizer (*see* **Note 8**).
4. Place the selected mouse in the exposure cage and insert the cage into the chamber. The nose of the mouse must be oriented toward the front air/aerosol inlet of the chamber and the animal must be fixed in that position with the body restraint.
5. Draw filtered aerosol diluting air through the chamber at a flow rate of 180 l/min with the mouse inside the chamber (*see* **Notes 9**).
6. Turn on the nebulizer by delivering compressed air at a pressure of 179 kPa and a flow rate of 10 l/min and allow the aerosol to equilibrate in the chamber for 5 min.
7. Check the aerosol concentration and reduce or increase the air supply to the nebulizer via the pressure control to obtain and maintain an aerosol concentration of 10–20 mg/m^3 (*see* **Note 10**), taking into account that the 3-jet Collison nebulizer requires a minimum airflow of 6 l/min.
8. Keep the temperature and relative humidity inside the exposure chamber at 21–23 °C and 40–50 %, respectively (*see* **Note 11**).
9. Expose the mice to controlled aerosol concentrations for 20 min.
10. Turn off the nebulizer and let fresh diluting air run into the chamber to flush the system for 5 min.
11. Stop the air supply and remove the animals from the chamber.

3.4 Lung Tissue Processing

1. Deeply anesthetize the animal via intraperitoneal injection of the anesthetic.
2. Arrange the animal on the dissection tray and secure the limbs.
3. Wash fur with 70 % alcohol.
4. Make a midline incision through the skin to expose the abdomen, thorax, and neck.
5. Expose the posterior abdominal aorta, and kill the animal by exsanguinations.
6. Expose the trachea and remove the ventral thoracic wall.
7. Place two ligatures around the trachea, below the larynx.
8. Make a straight narrow incision in the inter-cartilaginous space between the larynx and the first cartilaginous tracheal ring.
9. Cannulate the trachea with a small blunt-ended needle connected to the tube of the infusion apparatus and secure with the bottom ligature.
10. Infuse the lung with 4 % paraformaldehyde solution for 1 min at 25 cm fluid height. Infusion height is measured as the distance

between the lungs and the meniscus of the fixative in the reservoir. The volume of fixative used to fill the adult mouse lung will be 1–1.4 cm^3.

11. Withdraw the cannula and tie off the trachea with the upper ligature. Cut the trachea below the upper ligature.
12. Dissect the lungs out of the thorax and immerse the trachea and the lungs in cold fixative (20× the volume of the tissue) for 24 h.
13. Isolate the left lung and perform a traverse section (*see* **Note 12**).
14. Rinse the trachea and lower half of the sectioned left lung in three changes of cold PBS (20× the volume of the tissue) for 10 min each.
15. Transfer to a cold solution of 30 % sucrose in PBS (20× the volume of the tissue) for 24–72 h.
16. Transfer to a 2:1 mixture of 30 % sucrose solution and OCT embedding compound for an additional 24 h.
17. Wear goggles and gloves.
18. Blot off excess liquid and quickly immerse the tissue in OCT embedding compound in mounting blocks using long forceps.
19. Insert a beaker into an insulated thermos. Place melting isopentane in the beaker and carefully fill the space surrounding the beaker with liquid nitrogen, always wearing goggles and gloves. The temperature of the isopentane will drop to –140 °C in about 2 min.
20. Plunge the mounting block with the tissue specimens in isopentane for 10 s to freeze the tissue, using the long forceps (*see* **Note 13**).
21. Mark specimen orientation (longitudinal orientation for the trachea) on each block.
22. Store in zip-lock bags at –70 °C.

3.5 Preparation of Frozen Tissue Sections

1. Place clean glass slides in a staining rack.
2. Immerse the slides for 30 min in a large staining dish containing 1:10 dilution of 0.1 % poly-L-lysine solution in deionized water.
3. Remove the slides and oven dry for 1 h at 60 °C.
4. Fasten the mounting block to the block holder in the cryostat.
5. Align the knife to touch the surface of the tissue.
6. Set the section thickness to 4 μm.
7. Start cutting slowly until the section clings to the knife.
8. Bind the section to one of the poly-L-lysine-coated slides.
9. Allow the section to air-dry for 2–3 h at room temperature.

10. Repeat the steps above to prepare each additional section slide from the same frozen tissue sample and other samples.
11. Store the slides at –70 °C in slide boxes.

3.6 Immunohistochemical Analysis

1. Transfer the slides to a Coplin jar containing PBS and wash, dipping in and out of the solution, ten times.
2. Transfer the slides to another Coplin jar containing PBS for 5 min.
3. Quench endogenous peroxidase activity by incubating the slides in BLOXALL Blocking Solution for 10 min.
4. Wash slides in PBS for 5 min.
5. Cover sections with 3–6 drops of Fc Receptor Blocker to block the nonspecific binding of each primary antibody (rabbit anti-mouse IgG) to the Fc receptor present on the surface of several types of inflammatory cells and tissue macrophages. Incubate for 30 min at room temperature in the humidified chamber.
6. Rinse in PBS.
7. Cover sections with a solution containing 10 % normal goat serum in PBS and incubate for 15 min at room temperature in the humidified chamber.
8. Blot off excess serum from sections by placing one margin of the slightly angled slide on an absorbent paper towel.
9. Incubate replicate sections with each primary antibody or with the negative control antibody, all rabbit anti-mouse IgGs diluted 1:100–1:800 in PBS containing 1 % normal goat serum, for 30 min at room temperature (or overnight at 4 °C) in the humidified chamber (*see* **Note 14**).
10. Wash the slides two times in PBS containing 1 % normal goat serum for 5 min.
11. Use the VECTASTAIN Elite ABC Kit for rabbit IgG to apply the secondary anti-rabbit antibody and reveal antibody-binding sites by the immunoperoxidase procedure according to the manufacturer's instructions. Extracellular deposits of fibronectin, COL1, and COL3 beneath the bronchial epithelium and in the peribronchial zone will stain brown (Fig. 19.3d, e, g, h).
12. Counterstain sections in hematoxylin, if desired.
13. Dehydrate the slides in three changes of 95 % alcohol and two changes of absolute alcohol, and clear in two changes of xylene.
14. Mount with VectaMount and apply a coverslip.
15. Analyze each slide under a light microscope, using an image analysis system according to the manufacturer's instructions (*see* **Note 15**).

3.7 Immunofluorescence Analysis

1. Transfer the slides to a Coplin jar containing DPBS and wash, dipping in and out of the solution, ten times.
2. Transfer slides to another Coplin jar containing DPBS for 5 min.
3. Cover the sections with 3–6 drops of Fc Receptor Blocker. Incubate for 30 min at room temperature in the humidified chamber.
4. Rinse in DPBS.
5. Cover the sections with a solution containing 10 % normal donkey serum in DPBS and incubate for 15 min at room temperature in the humidified chamber.
6. Blot off excess serum from sections placing one margin of the slightly angled slide on an absorbent paper towel.
7. Incubate replicate sections with the FITC-conjugated antibody against α-SMA or the FITC-conjugated isotype-matched control, all diluted 1:100–1:800 in DPBS containing 1 % normal donkey serum, for 30 min at room temperature (or overnight at 4 °C) in the dark, in the humidified chamber (*see* **Note 14**).
8. Wash five times with DPBS containing 1 % normal donkey serum.
9. Cover the slides with a DPBS solution containing 1 % normal donkey serum and 0.1 % saponin for 10 min at room temperature in the dark. Saponin is used to permeabilize the cells and allow the binding of the primary antibodies against procollagen I and α-SMA to their intracellular target antigens.
10. Blot off excess solution.
11. Incubate replicate slides with the unlabeled goat antibody against procollagen I, Cy3-conjuaged antibody against α-SMA, or the corresponding control antibodies for 30 min at room temperature in the dark. All antibodies should be diluted in DPBS solution containing 1 % normal donkey serum and 0.1 % saponin.
12. Wash five times in DPBS solution containing 1 % normal donkey serum and 0.1 % saponin in the dark.
13. Incubate the slides previously incubated with either the unlabeled antibody against intracellular procollagen I or the unlabeled control antibody with the Rhodamine Red-X-AffiniPure F(ab′)2 fragment donkey anti-goat IgG for 30 min at room temperature in the dark.
14. Wash five times in DPBS.
15. Use ProLong Gold Antifade Reagent with DAPI to counterstain the cell nuclei and mount the slides.
16. Apply the glass coverslips.

17. Analyze the sections using a wide-field fluorescence microscope connected to a laser scanning confocal system with appropriate filters for the employed fluorochromes (*see* **Notes 15** and **16**).

3.8 Determination of Serum OVA-Specific IgE and IgG Levels

1. Collect samples of the blood in serum separator tubes at the time the mice are sacrificed.
2. Allow samples to clot for 30 min at room temperature.
3. Centrifuge for 10 min at 1,000 × *g*.
4. Decant the serum and store samples at −70 °C.
5. Measure the serum concentrations of total IgE and OVA-specific IgE and IgG_1 using the commercially available kits according to the manufacturers' instructions (*see* **Note 17**).

4 Notes

1. Similar customized and preassembled whole-body exposure systems are currently provided by TSE Systems (European/Asian Headquarters: Bad Homburg, Germany; North America Headquarters: Chesterfield, MO, USA). The preassembled 12-port Small Animal Exposure System from InTox (Moriarty, NM, USA) allows the concomitant exposure to aerosols of at least six mice, placed into distinct whole-body mouse tubes. This system is similarly equipped with a jet nebulizer that can generate aerosol particles in the 1–3 μm range at an appropriate airflow rate and back-pressure. It may represent a valid alternative if the sampling line is connected to instruments for the real-time monitoring of aerosol concentrations (not currently provided by the manufacturer), such as the OptoPan or the SpectroPan from TSE and the DustTrack DRX from TSI (Shoreview, MN, USA). The latter instrument must be calibrated for aerosols made of liquid droplets rather than dust.
2. All instruments should be calibrated, operated, and maintained according to the manufacturers' or suppliers' instructions.
3. Number mice by toe clip and make sure that the toe clip number and the litter number are correct before starting any experimental procedure.
4. The OVA solution should be freshly prepared the day of the injection.
5. Insert the needle into the lower part of abdomen (e.g., near the groin) to avoid injecting the OVA–Alum mixture into the intestines.
6. Stop insertion as soon as you feel a sudden reduction in the resistance, indicating that the needle has penetrated through the abdominal wall.

7. The OVA solution should be freshly prepared the day of each whole-body exposure.
8. The liquid reservoir of the nebulizer can be refilled with fresh solutions of OVA or PBS alone while the experiment is running.
9. The parameters for delivering diluting air to the exposure chamber and for aerosol generation can vary depending on the whole-body exposure system used for the experiments. A working protocol should be established and validated in preliminary experiments.
10. Repeated exposures to aerosol concentrations lower than 10 mg/m^3 may cause sustained but slight airway remodeling changes, which almost exclusively consist of subepithelial fibrosis and epithelium hypertrophy and are predominantly localized to the trachea [38]. In our hands, repeated exposures to aerosol concentrations up to 100 mg/m^3 do not cause extensive and nonspecific inflammatory lesions of the parenchyma surrounding the conducting airways. However, measurements of the mass concentration of a given aerosol with instruments obtained from different manufacturers, or with different models of the same instrument, do not provide comparable results. The differences in the recorded values may vary from two- to five-fold. Thus, it is recommended that the initial experiments for the establishment of the working protocol for repeat allergen challenges be started with aerosol concentrations between 50 and 70 mg/m^3. The instrument for real-time monitoring of the aerosol concentrations should be calibrated for use with aerosols made of liquid droplets rather than dust.
11. Under these conditions, the particle sizes of the aerosol generated by the 3-jet Collison nebulizer within the breathing zone of mice will range from 0.5 to 7 μm, which is appropriate for preferential deposition in the bronchi, rather than in the upper airways (nose and trachea) [39–41].
12. Orientate the left lung to obtain a traverse section of the first-generation bronchus and the accompanying artery and vein.
13. The density of fibrocytes and other CD34$^+$ progenitor cells can be greatly underestimated in tissue sections from formalin-fixed and paraffin-embedded tissue because of the difficulties in retrieving the CD34 antigen on the cell surface. The protocol reported here for tissue fixation, cryoprotection, and freezing usually permits an excellent conservation of the tissue architecture.
14. In any immunohistochemical and immunofluorescence analysis, positive control sections should be used to test serial dilutions of each primary and secondary antibody for optimal staining.

Skin tissue and a traverse section of the thoracic aorta can be obtained, in addition to the lungs and trachea, at the time the mice are sacrificed. All tissue samples should be processed in an identical way. Sections of the skin tissue should be used as positive controls for the detection of extracellular matrix components (fibronectin, COL1, and COL3) and cells producing COL1 (fibroblasts and myofibroblasts will stain positively with antibodies against the intracellular COL1 precursor procollagen I). Sections of the thoracic aorta should be used as positive controls for tissue labeling with antibodies against α-SMA because the smooth muscle layer of the vessel will be stained positively.

15. Sections of the airway wall that are associated to adjacent vessels through connective tissue attachments should be excluded. On each section, the region of interest is a 20-μm subepithelial band that includes the extracellular matrix components and contractile elements (myofibroblasts and smooth muscle cells) of the bronchial wall. An appropriate image analysis system can estimate the area covered by the brown stain in different fields along the epithelial basement membrane. The mean area is calculated and expressed as square micrometers per length of basement membrane in micrometer (Fig. 3f, i).
16. $CD34^+$ cells will stain green. Cells producing the intracellular precursor of COL1 (procollagen I) will stain red. The cell nuclei will stain blue. Cells expressing α-SMA will also stain red. Cells double stained for CD34 and procollagen I or CD34 and α-SMA are fibrocytes and fibrocyte-derived myofibroblast-like cells, respectively, and will stain yellow (Fig. 4a, b, d). The density of fibrocytes and fibrocyte-derived myofibroblast-like cells present in the subepithelial zone along the epithelial basement membrane can be expressed as the number of cells per square millimeters of subepithelial bronchial mucosa (Fig. 4d, g). By double staining replicate sections with Cy3-conjugated antibody against α-SMA and unlabeled goat antibody against procollagen I, using the FITC-AffiniPure F(ab′)2 fragment donkey anti-goat IgG as secondary antibody, it is also possible to identify all myofibroblasts and myofibroblast-like cells [29].
17. To confirm the effectiveness of the systemic sensitization to OVA, anti-OVA antibody levels are usually measured in the serum or in the plasma. Both OVA-specific IgE and IgG_1 can now be assayed with commercially available kits. In general, elevated levels of anti-OVA IgE are indicative of successful sensitization to the antigen. The measurement of anti-OVA IgG_1 is commonly used for assessing the magnitude of the Th2-biased immune response [42, 43].

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