



Investigating MIF in Mouse Models of Severe Corticosteroid-Resistant Neutrophilic Asthma

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Abstract

Experimental mouse models of asthma are widely used to investigate the underlying mechanisms of this complex and heterogeneous disease. Using mouse models of ovalbumin-induced asthma, previous investigators have established a crucial role for MIF in the development of type 2-mediated eosinophilic asthma. Surprisingly, however, the role of MIF in other phenotypes of asthma has received little attention. MIF is an important mediator of neutrophilic inflammation, and also acts to antagonize the actions of corticosteroids. Thus, MIF may play a role in the development of severe forms of asthma in which airway neutrophilia and corticosteroid insensitivity are major features. In this chapter, we provide an experimental protocol that may be used to investigate the role of MIF in a mouse model of severe corticosteroid-resistant neutrophilic asthma.

Key words Severe asthma, Corticosteroid resistance, Neutrophil, Mouse model, House dust mite

1 Introduction

Macrophage migration inhibitory factor (MIF) was first identified as a mediator of asthma about 20 years ago. Studies published in the late 1990s reported increased levels of MIF protein in the bronchoalveolar lavage fluid (BALF), induced sputum, and serum of asthmatic subjects [1, 2]. In the subsequent decade, several investigators using *Mif* gene-deficient mice, neutralizing MIF antibodies, or the small-molecule MIF antagonist ISO-1 clearly demonstrated a role for MIF in mouse and rat models of ovalbumin (OVA)-induced asthma, which mostly recapitulate eosinophilic sub-phenotypes of asthma. In these studies, MIF inhibition protected against type 2-mediated eosinophilic inflammation, structural remodeling of the airway wall, and airway hyperreactivity, all of which are classic features of the allergic asthmatic response [3–8].

Over the past decade or so, it has become increasingly apparent, however, that asthma is a heterogeneous disease. Indeed, asthma

incorporates a spectrum of respiratory conditions which appear to be mediated by distinct and overlapping molecular mechanisms. This spectrum includes a group of patients who do not achieve or maintain control of their symptoms, despite adequate and, in many cases, high-dose treatment with anti-inflammatory corticosteroids. Defined as having severe asthma, this group constitutes about 5–10% of the asthmatic population, but accounts for nearly 50% of the health care costs of asthma, thus contributing a significant health and social burden [9].

MIF is a critical regulator of the immune response, and has the capacity to antagonize the anti-inflammatory effects of corticosteroids [10]. It is also a potent mediator of neutrophilic inflammation [11, 12]. MIF facilitates neutrophil recruitment to sites of inflammation, including the lung [13, 14], and prolongs neutrophil survival [15]. The increased recruitment of neutrophils to the airways in asthmatic subjects is one of the most important determinants of disease severity, and the lack of therapeutic response to corticosteroids [16, 17]. Thus, given that MIF regulates neutrophil trafficking and survival, and inhibits corticosteroid activity, anti-MIF therapies may offer a unique advantage in severe asthma, where neutrophilic inflammation and the lack of corticosteroid responsiveness present major therapeutic issues. In this chapter, we provide a detailed experimental protocol that may be used to investigate the role of MIF in a mouse model of severe corticosteroid-resistant neutrophilic asthma.

2 Materials

2.1 Induction of Severe Corticosteroid-Resistant Neutrophilic Asthma in Mice

1. C57BL6 female mice 8–12 weeks of age (*see Note 1*).
2. Isoflurane anesthetic system.
3. Mortar and pestle.
4. 1 mL Sterile syringe.
5. 25-gauge Needle.
6. 20 μ L Pipette and tips.
7. 0.1% House dust mite (*Dermatophagoides pteronyssinus*) extract.
8. 1 $\times$ Dulbecco's phosphate-buffered saline (PBS) (Life Technologies).
9. Complete Freund's adjuvant (CFA) (Sigma Aldrich).
10. 4,5-Dihydro-3-(4-hydroxyphenyl)-5-isoxazoleacetic acid methyl ester (ISO-1) (Tocris Bioscience).
11. Isoflurane (Veterinary Companies of Australia Pty Ltd).

12. 9 α -Fluoro-16 α -methyl-11 β ,17 α ,21-trihydroxy-1,4-pregna-diene-3,20-dione (Dexamethasone) (Sigma Aldrich).
13. 80% Ethanol.

2.2 Measurement of Airway Reactivity

1. flexiVent System (SCIREQ) (*see Note 2*),
2. 18-gauge Blunted cannula.
3. Spring scissors.
4. Fine scissors, sharply angled.
5. Two sets of small curved forceps.
6. Non-sterile silk thread.
7. Dissection board.
8. Absorbent paper towels.
9. Anesthetic agent (e.g., 1 mL of ketamine (100 mg/mL):0.1 mL xylazine (100 mg/mL):8.9 mL sterile water cock-tail solution).
10. 10% Acetyl- β -methyl choline chloride (methacholine) (Sigma Aldrich).
11. 1x Dulbecco's phosphate-buffered saline (PBS) (Life Technologies).
12. 80% Ethanol.

2.3 Euthanasia and Collection of Biological Specimens

1. 23-gauge Needle.
2. Spring scissors.
3. Fine scissors.
4. Forceps.
5. Dissection board.
6. Absorbent paper towels.
7. 1.5 mL Eppendorf tubes.
8. Portable liquid nitrogen container.
9. Ice bucket.
10. Liquid nitrogen.
11. Pentobarbitone sodium (Lethobarb) (Virbac Animal Health).
12. 1 $\times$ Hanks' balanced salt solution (HBSS).
13. RNA*later*[®] (Life Technologies).
14. 10% Neutral buffered formalin.

2.3.1 Bronchoalveolar Lavage Fluid (BALF) Processing

1. Refrigerated benchtop centrifuge (with rotor to accommodate 2 mL tubes).
2. 1.5 mL Eppendorf tubes.
3. 1 mL Pipette and tips.

4. $1 \times$ HBSS.
5. Red blood cell lysis buffer.

2.3.2 Blood Processing

1. Refrigerated benchtop centrifuge (with rotor to accommodate 2 mL tubes).
2. 1.5 mL Eppendorf tubes.
3. 1 mL Pipette and tips.

2.3.3 Cell Slide Preparation

1. Microscope ($10\times$, $20\times$, $100\times$ objectives).
2. Hemocytometer.
3. Coverslips.
4. Cytospin.
5. Shandon™ Single Cytofunnel™ with White Filter Cards.
6. Glass slides.
7. 1.5 mL Eppendorf tubes.
8. 1 mL Pipette and tips.
9. 20 μ L pipette and tips.
10. Trypan blue.
11. Differential Quik Stain Kit (Modified Giemsa) (Polysciences, Inc.)

3 Methods

3.1 Induction of Severe Corticosteroid-Resistant Neutrophilic Asthma in Mice

To induce severe corticosteroid-resistant neutrophilic asthma, C57BL/6 mice are sensitized with the clinically relevant allergen house dust mite (HDM) in the presence of complete Freund's adjuvant (CFA), and then 14 days later challenged with a single dose of house dust mite. This protocol elicits profound airway neutrophilia, mucus hypersecretion, and airway hyperresponsiveness [18].

3.1.1 Preparation of House Dust Mite Extract and CFA Emulsion for Mouse Sensitization

1. Dissolve lyophilized house dust mite extract in PBS to achieve a concentration of 1 μ g/1 μ L (0.1%), and then transfer to a mortar which is to be kept on ice.
2. Slowly add an equal amount of CFA to the mortar.
3. Pulverize in a clockwise direction using the pestle (*see Note 3*).
4. After continuous pulverizing, an emulsion of house dust mite extract in CFA will form; this is used to sensitize mice as described below.

3.1.2 Allergen Sensitization via Subcutaneous Injection

1. Fill a 1 mL syringe with the HDM/CFA emulsion, and then fit the syringe with a 25-gauge needle.
2. Inject 100 μ L of the HDM/CFA emulsion subcutaneously. Note, control mice are injected with PBS only.
3. Monitor mice for the development of lesions every second day (*see* **Note 4**).

3.1.3 Allergen Challenge via the Intranasal Route

1. 14 days after sensitization, place mice in the induction chamber of an isoflurane anesthetic system.
2. Adjust the oxygen flow rate to 1–2 L/min and the isoflurane vaporizer to 3% (*see* **Note 5**).
3. Mice will become immobilized and lightly sedated after approximately 2–3 min; this indicates that a suitable plane of anesthesia has been reached.
4. Once anesthesia is achieved, manually restrain the mouse in the supine position, with the head elevated.
5. Administer 100 μ g HDM in a volume of 20 μ L PBS into the nares using a 20 μ L pipette. Note, control mice receive PBS only.
6. Once allergen administration is completed, mice can be immediately returned to their respective cages.

3.1.4 Administration of ISO-1 and Dexamethasone

1. To confirm corticosteroid-resistant airway neutrophilia, administer the corticosteroid dexamethasone (1 mg/kg) via oral gavage using a 20-gauge stainless steel curved feeding needle 1 h prior to allergen challenge (*see* **Note 6**).
2. To inhibit airway neutrophilia, mucus hypersecretion, and AHR, administer the MIF inhibitor ISO-1 (35 mg/kg) via intraperitoneal injection using a 25-gauge needle 1 h prior and 6 h post intranasal HDM administration. Control mice are injected with vehicle only.

3.2 Measurement of Airway Reactivity Using the flexiVent System

Prior to measuring airway reactivity, prepare all reagents and calibrate the flexiVent system. Following this, anesthetize the mice, cannulate the trachea, and measure airway reactivity as described below. Detailed instructions on how to measure airway reactivity using the flexiVent system have been previously published [19, 20].

3.2.1 Preparation of Reagents

1. Prepare the anesthetic agent by mixing 1 mL ketamine (100 mg/mL), 0.1 mL of xylazine (100 mg/mL), and 8.9 mL sterile water for injection in a 15 mL conical tube (*see* **Note 7**).
2. Prepare a stock solution of 10% methacholine by dissolving 100 mg of methacholine in 1 mL PBS. Perform serial dilutions

in PBS to generate concentrations of 50, 25, 10, 5, 2.5, 1.25 mg/mL or less, if needed (*see* **Note 8**).

3.2.2 Calibration of the flexiVent System

1. Weigh the mouse.
2. Enter the weight in the flexiVent operating software.
3. Proceed with the calibration of the system using the appropriate endotracheal cannula, according to the manufacturer's instructions (*see* **Note 9**).

3.2.3 Preparation of Mice

1. Anesthetize mice by injecting ketamine:xylazine cocktail 100 μ L/10 g via the intraperitoneal route.
2. Return mice to their respective cages until they are sedated; this takes approximately 10–15 min.
3. Evaluate the animal's toe pinch reflex. A lack of response to the toe pinch indicates that the mouse has reached the surgical plane of anesthesia.
4. Place adsorbent paper towels on the dissection board, and then place the anesthetized mouse in a supine position on the dissection board, under a heat lamp to maintain body temperature.
5. Wipe down the fur on the neck area with 80% ethanol.
6. Make a vertical incision, starting between the forelimbs near the lower jaw, such that the submaxillary glands become visible.
7. To expose the trachea, separate the lobes of the submaxillary glands, and then, using fine scissors, dissect the sheath of muscular tissue that surrounds the trachea.
8. Place a 7–10 cm piece of thread underneath the trachea by holding the thread with forceps and dragging it below the trachea through to the other side. Once the thread is properly positioned, using spring scissors, make a small incision between cartilaginous rings on the trachea.
9. Insert an 18-gauge blunted cannula into the incision. Slide the cannula past the incision to make sure there is an adequate length of cannula inside the trachea. Tie the thread around the trachea in order to seal the cannula very tightly (*see* **Note 10**).

3.2.4 Measurement of Airway Reactivity

1. Start the flexiVent ventilator by selecting a predefined ventilation profile.
2. Connect the cannula to the ventilator system via the Y-tubing (*see* **Note 11**).
3. Run a deep inflation perturbation by double-clicking on the perturbation name to verify there is no leakage between the cannula insertion and Y-tubing (*see* **Note 12**).

4. Initiate a predefined script for measurement of airway reactivity by double-clicking on the script title in the software to run the automated measurement sequences.
5. Perform a baseline measure of airway reactivity in the absence of methacholine or PBS to ensure there are no aberrations in the trachea cannulation.
6. Load the nebulizer with PBS or methacholine, then activate the nebulizer to generate an aerosol for inhalation.
7. Measure airway reactivity to PBS and increasing concentrations of methacholine.

3.3 Euthanasia, Collection, and Processing of Blood, Bronchoalveolar Lavage Fluid, and Lung Tissue

1. Once measurements of airway reactivity are complete, stop the ventilator and detach the mouse from the flexiVent system.
2. Euthanize the mouse with pentobarbital (100 mg/kg) via the intraperitoneal route.
3. Blood samples, if required, must be collected immediately after euthanasia.
4. To obtain blood samples via cardiac puncture, place the mouse in a supine position on the dissection board covered with absorbent paper towels. Use a pair of fine scissors to open the chest cavity, being careful not to damage the lungs. Then carefully remove the collar bones, being careful not to damage the trachea. Following this, insert a 21- or 23-gauge needle into the heart and collect the blood. It is possible to collect approximately 600–700 μ L of blood using this method.
5. Allow the blood to coagulate at room temperature for at least 30 min, then spin in a refrigerated centrifuge at $18,800 \times g$ for 5 min. Then carefully remove tubes from the centrifuge and collect the serum (which separates out as a distinct top layer) using a 1 mL pipette, and transfer to a new microcentrifuge tube. Store serum samples at -80°C for later analysis.
6. If bronchoalveolar lavage fluid (BALF) is required, attach the hub of a syringe to the cannula that is attached to the trachea and slowly inject 1 mL of HBSS into the lungs in a uniform motion. Then immediately withdraw the HBSS from the lungs in a slow and continuous motion. The collected fluid can be stored in a 1.5 mL Eppendorf tube on ice for later processing, as described in **step 7**.
7. To pellet cells, spin the BALF in a refrigerated centrifuge at $900 \times g$ for 5 min. Carefully remove tubes from the centrifuge, being careful not to disturb the cell pellet, and transfer the supernatant to a clean 1.5 mL Eppendorf tube. Store BALF samples at -80°C for later analysis. Then add 500 μ L of red blood cell lysis buffer to the remaining cell pellet, and incubate on ice for 10 min. Spin cells in a refrigerated centrifuge at

- 2500 $\times g$ for 5 min. Carefully remove tubes from the centrifuge, being careful not to disturb the pellet. Discard the supernatant, and resuspend cells in 1 mL HBSS.
8. To determine total cell counts, dilute 20 μ L of the cell suspension in a 1:1 ratio with trypan blue. Transfer 20 μ L of the cell suspension to a hemocytometer and count cells under a microscope with 10 $\times$ magnification.
 9. To perform differential cell counts, vortex the remaining 1 mL cell suspension at low speed. Load 150 μ L of cell suspension onto labeled double-frosted microscope slides that have been secured into Shandon™ Single Cytofunnel™ with white filter cards, and cytospin at 600 rpm for 5 min. Allow slides to air-dry overnight, and then stain using Differential Quik Stain Kit (Modified Giemsa) according to the manufacturer's instructions. Allow stained slides to air-dry overnight. Perform differential cell counts using a microscope equipped with a 100 $\times$ oil-immersed objective lens (*see* **Notes 13** and **14**).
 10. The left lung lobe may be collected for histological analysis, while the right lung may be collected for measurement of gene and protein expression, if required, as described in **step 11** below.
 11. To begin this process, ligate the right lung lobe using a suture thread. Then, with a second suture thread, support the trachea by placing the thread directly under it, below the end of the cannula, and loop the thread in a half-tightened knot. Once this is done, inflate the left lung lobe with 600 μ L of 10% neutral buffered formalin. Be careful not to overinflate the lung, as this will distort tissue histology. Once the left lobe is fully inflated, tighten the half-tied suture thread, and remove the syringe and cannula from the trachea. Then, dissect out the right lung lobe, place it in a 1.5 mL Eppendorf tube, and immediately snap-freeze in liquid nitrogen. If the right lung lobe is required for gene expression studies, add an appropriate volume of RNAlater® to the 1.5 mL Eppendorf tube. Then, to collect the left lung lobe, grasp the excess suture thread with the forceps and gently lift the trachea. Carefully excise the left lung lobe, with the heart still attached, and place the inflated left lobe in a 50 mL conical tube containing 50 mL of 10% neutral buffered formalin

4 Notes

1. All procedures are performed in living animals; thus, investigators must obtain appropriate approvals for working with animals at their institution, prior to conducting experiments.

2. Other alternatives are available for measurement of airway reactivity.
3. Ensure the emulsion is pulverized in one direction, preferably clockwise. Pulverization should be performed quickly in an uninterrupted manner until the emulsion forms. If not pulverized correctly, the emulsion will break and lose its physical stability.
4. Subcutaneous injection of the allergen/CFA emulsion may result in local inflammation and cause granulomatous lesions, skin ulceration, and abscesses at the site of injection. It is therefore important to monitor mice regularly, as serious lesions may require attention.
5. Exposure to isoflurane may occur during filling of the isoflurane anesthetic system, or during anesthesia, if the isoflurane escapes to the atmosphere. Exposure to isoflurane may cause severe respiratory distress and medullary paralysis; thus, proper use of the isoflurane anesthetic system and the waste scavenging system is essential.
6. To perform the oral gavage, restrain the mouse tightly by holding the scruff of its neck so that its head and body are in a straight vertical line. This allows easy passage of the feeding needle into the esophagus.
7. Measurement of airway reactivity in each mouse will take approximately 40–50 min. To maintain anesthesia for the duration of this period, administer additional anesthetic (one-third the original ketamine/xylazine dose) after 30 min have elapsed.
8. Methacholine is highly hygroscopic; fresh solutions must be prepared on the day of use.
9. The flexiVent system need only be calibrated at the start of each day. However, it is necessary to perform tube calibration prior to measuring airway reactivity in each individual mouse on any given day.
10. If necessary, adjust the position of the cannula to avoid aberrant breathing patterns during ventilation.
11. Position the animal as straight as possible on the flexiVent stand to ensure direct flow of air from the ventilator to the lungs. The mouse may be held in position by restraining the limbs with masking tape. When ready to proceed with mechanical ventilation, start the ventilator before attaching the cannula to avoid any interruption of airflow to the lungs.
12. To confirm there is no leakage around the cannula, deep inflation perturbation should be performed prior to commencing measurements of airway reactivity. This also allows standardization of lung volumes across different mice.

13. To ensure optimal results, slides for differential cell counts should be prepared the same day cells are retrieved from the mouse. It is also important that slides are completely dry prior to staining with Differential Quik Stain Kit (Modified Giemsa).
14. Eosinophils, neutrophils, and macrophages can be distinguished on the basis of several features; eosinophils have a lobed blue nucleus and cytoplasmic granules that stain reddish/orange, neutrophils have a lobed dark blue nucleus and unstained cytoplasmic granules, macrophages have a large round violet-stained nucleus and a sky blue cytoplasm.

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