Video Article

Intra-tracheal Administration of *Haemophilus influenzae* in Mouse Models to Study Airway Inflammation

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Abstract

Here, we describe a detailed procedure to efficiently and directly deliver *Haemophilus influenzae* into the lower respiratory tracts of mice. We demonstrate the procedure for preparing *H. influenzae* inoculum, intra-tracheal instillation of *H. influenzae* into the lung, collection of broncho-alveolar lavage fluid (BALF), analysis of immune cells in the BALF, and RNA isolation for differential gene expression analysis. This procedure can be used to study the lung inflammatory response to any bacteria, virus or fungi. Direct tracheal instillation is mostly preferred over intranasal or aerosol inhalation procedures because it more efficiently delivers the bacterial inoculum into the lower respiratory tract with less ambiguity.

Video Link

The video component of this article can be found at https://www.jove.com/video/53964/

Introduction

Inflammation is a fundamental immune mechanism of defense against infectious agents. It promotes pathogen eradication and repair of damaged tissue. It also facilitates the recovery to a normal healthy state[1]. However, dysregulated inflammation often leads to chronic inflammatory diseases[2]. Airway inflammation is an initial trigger for different pulmonary diseases such as chronic obstructive pulmonary disease (COPD), asthma and pulmonary fibrosis[3].

The non-typeable (unencapsulated) *Haemophilus influenzae* (NTHi) is associated with chronic upper and lower lung inflammatory diseases[4,5]. It is the dominant species isolated from the lower airways of children and adults with chronic obstructive pulmonary disease[4,6,7]. The inflammatory response after NTHi infection is characterized by the upregulation of proinflammatory cytokines (such as TNF and IL-1β), and it is mediated by mitogen activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) through toll-like receptors (TLRs)[8].

Mouse models are very useful tools for analyzing the underlying pathology of lung inflammatory disease because of the availability of different gene-deficient lines. Several methods have been used to inoculate live/attenuated bacteria and bacterial products, including intranasal instillation and aerosolized inhalation[9,10]. Here, we demonstrate intra-tracheal instillation. Although used less frequently, this approach is more efficient and highly reproducible because of the direct delivery of the inoculum to the lower respiratory tract.

Protocol

All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Baylor Research Institute.

1. Culturing Non-typeable *Haemophilus influenzae* (NTHi) and Preparing the Inoculum

1. Plate NTHi on a chocolate agar plate and keep the plate upside down in a humidified CO_2 incubator overnight at 37 °C and 5% CO_2. The following day, culture the bacteria in brain heart infusion broth. Then, add 1 ml of the broth into a sterile 1.5 ml tube.
2. Centrifuge at 4,000 x g for 5 min at room temperature. Then, discard the supernatant and re-suspend the pellet in 1 ml of sterile 1x PBS. Repeat this step once.
3. Transfer 100 µl of re-suspended solution into 900 µl of 1x PBS and measure the absorbance of the diluted culture at 600 nm wavelength in a spectrophotometer.
4. Determine the viability and colony forming units (CFUs) of the inoculum by culturing 1:10 serial dilutions on chocolate agar plates and incubating overnight at 37 °C and 5% CO_2.
5. Based on the viability and CFUs determined from the previous step, adjust the inoculum to a final concentration of 20 x 10^6 CFU/ml.

2. Intra-tracheal (i.t) Instillation

1. Inject the mouse intraperitoneally (i.p) with 0.1 ml/10 g body weight of mouse with a 150 mg/ml ketamine + 20 mg/ml xylazine solution. The procedure should be done in the hood with a light source to keep the animal warm.
2. Pinch the interdigital space of the mouse to verify that the mouse is adequately anesthetized.
3. Place the mouse on its back. Shave the ventral area of the neck and disinfect with chlorhexidine and 70% ethyl alcohol.
4. Lift the skin of the upper ventral part of the neck using rat tooth forceps. Make a small incision (0.5-1.0 cm) above the thymus using a scalpel and carefully dissect the muscle to expose the trachea.
    1. Using a 1 ml syringe with a 27 G needle, slowly inject 0.05 ml air, followed by 0.05 ml NTHi obtained from Section 1 or saline as a control, followed by another 0.05 ml air. Keep animal vertical for around 1 min.
5. Close the incision with surgical glue. Keep the mouse laterally recumbent and warm for about 10 min.

3. BALF Collection

1. Sacrifice the mouse by cervical dislocation 24 hr after infection.
2. Open the abdominal cavity of the mouse with anatomical scissors and cut the ventral aorta to bleed. Expose the ventral part of the lung.
3. Expose the trachea and intubate it with a 20 gauge catheter. Instill 0.8 ml of BALF buffer (1x PBS, 0.1% dextrose, 10 U/ml heparin) and collect the lavage by gentle aspiration. Repeat this procedure three times.
4. Count the total number of cells in 100 µl of BALF suspension using a hemocytometer under 10X magnification with Trypan Blue staining.
5. Prepare slides for modified Giemsa staining. Aliquot 100 µl of BALF into the appropriate wells of the cytospin and centrifuge at 500 x g for 5 min. Dry the slides for 10 min and stain the cells using modified Giemsa stain according to the manufacturer's protocols.
6. Use the remaining cells for subsequent analysis, such as florescence activated cell sorting (FACS), confocal microscopy, gene expression and western blot[11-13].

4. Histopathology Preparation

1. For histopathology analysis, infect mice with NTHi as described in step 2.4. After 24 hr of infection, sacrifice the animals by cervical dislocation. Cut open the thoracic cavity with anatomical scissors to expose the lungs and the trachea as mentioned before. Then, insert the 20 gauge catheter into the trachea.
2. Fill the lung with 2% warm agarose solution prepared in 4% formalin. Once the lungs are fully inflated, place ice on top of the lungs to solidify the agarose solution.
3. Then, carefully remove the thoracic plug and put it in a 50 ml solution of 4% formalin in 1x PBS until it is processed for hematoxylin and eosin (H&E) sections.

5. FACS Staining Cells in the BALF

1. Put 1 x 10^5 cells from the BALF fluid in a 96-well V-bottom micro titer plate.
2. Wash cells with 300 µl of cold 1x PBS and spin at 4,000 x g for 3 min at 4 °C.
3. Stain the cells with cell stain solution in 100 µl 1x PBS (1:1,000 dilution) and keep at room temperature for 15 min.
4. Wash cells with 300 µl of cold 1x PBS at 4,000 x g for 3 min at 4 °C.
5. Keep cells in 100 µl of FACS wash buffer [Wash buffer (1x PBS, 2% FBS, 1 mM EDTA, 0.1% sodium azide] containing 1:100 diluted Fc-Block for 15 min at 4 °C.
6. Centrifuge cells at 4,000 x g for 3 min and discard supernatant.
7. Stain cells with 100 µl surface staining cocktail (1:100 dilution) in FACS wash buffer for 30 min at 4°C.
8. Wash cells thrice with 300 µl of FACS wash buffer at 4,000 x g for 3 min at 4 °C.
9. Fix cells in 100 µl of 4% paraformaldehyde for 15 min at room temperature.
10. Wash cells twice with 300 µl 1x PBS at 400 x g for 3 min at 4 °C and re-suspend in 300 µl of FACS wash buffer.
11. Make single-color staining controls using flow cytometry beads.
    1. Add one drop of beads into the 96-well V-bottom microliter plate and wash with 200 µl 1x PBS.
    2. Add 1 µl of staining antibody to 100 µl 1x PBS and beads and incubate at room temp for 5 min.
    3. Wash beads twice with 300 µl 1x PBS.
12. Analyze single-stained controls and cells by flow cytometry[14].

6. Homogenization of Lung Tissue to Isolate RNA

1. After infection, collect a small piece of lung (20-40 mg) and keep it in RNA stabilizing reagent at 4 °C overnight. Then, store it at -80 °C until the next step.
2. Wash the lung tissues in cold 1x PBS to get rid of the RNA stabilization reagent.
3. Place the tissue into a 1.5 ml centrifuge tube containing lysis buffer (1 ml lysis buffer + 10 µl β-mercaptoethanol).
4. Chop the tissue into small pieces using pointed scissors and homogenize using a cordless motor pellet pestle for 20-40 sec.
5. Keep the lysate on ice for 5 min and proceed with step 7.1.

7. RNA Isolation from the Lung

1. Centrifuge the lysate at 18,000 x g for 3 min. Remove the supernatant by pipetting and carefully transfer it to a new 1.5 ml tube.
2. Add 1 volume of 70% ethanol to the lysate. Mix immediately by pipetting and wait for 2-4 min.
3. Transfer up to 700 µl of lysate to a spin column placed in a 2 ml collection tube.
4. Centrifuge at 9,600 x g for 15 sec and discard the flow-through.
5. Add 350 µl stringent wash buffer and centrifuge at 9,600 x g for 15 sec. Discard the flow-through.
6. Add 80 µl DNase I directly to the spin column membrane and keep it at room temperature for 15 min.
7. Add 350 µl stringent wash buffer to the spin column and centrifuge at 9,600 x g for 15 sec. Discard the flow-through.
8. Add 500 µl RNA wash buffer and centrifuge at 9,600 x g for 15 sec. Discard the flow-through.
9. Add 500 µl RNA wash buffer and spin at 9,600 x g for 2 min. Discard the flow-through.
10. Place the spin column in a new 2 ml collection tube and centrifuge at 9,600 x g for 1 min. Discard the flow-through.
11. Add 30-50 µl RNase-free water directly to the spin column and centrifuge at 9,600 x g for 1 min. Store RNA at -80 °C.
12. Perform RNAseq analysis[14].

Representative Results

Intra-tracheal instillation resulted in a markedly increased number of leukocytes in the BALF (**Figure 1A**, left panel) than installation with saline. The differential count analysis of the leukocytes clearly showed increased neutrophil infiltration (**Figure 1**, right panel). The FACS analysis of the cells in the BALF further confirmed the increased number of neutrophils (**Figure 1B**). Histological analysis of H&E-stained sections of the lung tissue showed increased airway inflammation (**Figure 1C**). These data collectively suggest that intra-tracheal instillation induces airway inflammation in mice. To support the use of this approach for studying signaling pathways that regulate airway pathogenesis, we used mice that are deficient for the E3 ubiquitin ligase Itch. Itch is known to be involved in regulating inflammatory signaling pathways[15]. We inoculated age- and sex-matched wild type C57BL/6 control and Itch-/- mice with NTHi. We isolated RNA from the lung tissues of control WT and Itch-/- mice and performed whole transcriptome sequencing to identify the inflammatory genes that are differentially expressed. As shown in **Figure 2**, Itch deficiency resulted in differential expression of several genes. This suggests that intra-tracheal instillation can be used to investigate lung inflammation, gene expression profiles and signaling pathways.

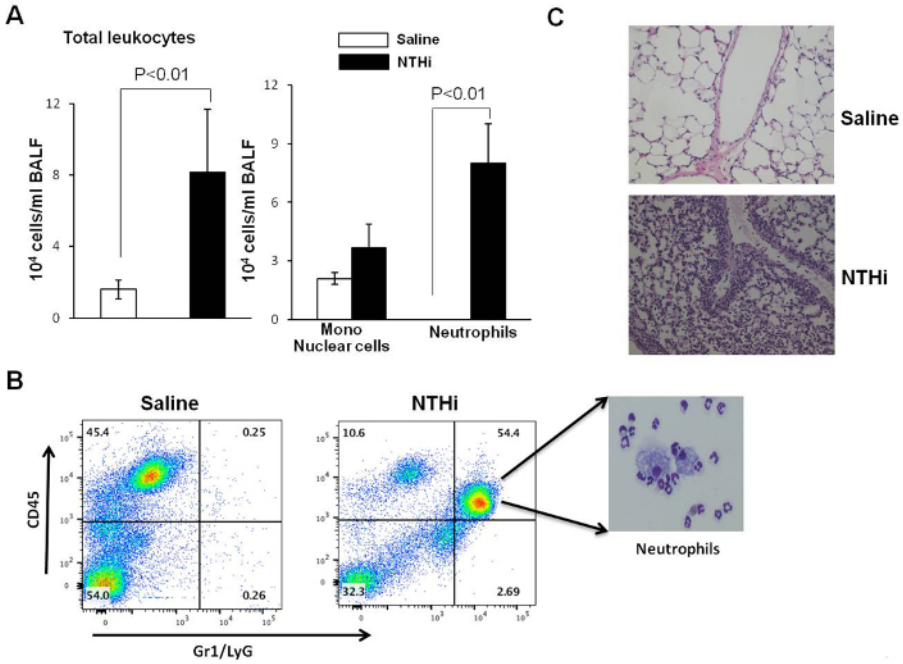

Figure 1: Intra-tracheal inoculation of NTHi induces lung inflammation in mice. Mice were inoculated with NTHi (10^6 CFU/mouse) or a saline control. 24 hr after the inoculation, mice were sacrificed (**A**) BALF was collected. The total leukocyte, monocyte and neutrophil numbers were determined. Data are presented as means ± SEM. n = 4 mice/group. * Indicates p <0.05 compared to saline-treated mice. (**B**) The cells in the BALF were stained with anti-Gr1 antibody and analyzed by FACS. (**C**) H&E-stained lung sections showing leukocyte infiltration and inflammation. Please click here to view a larger version of this figure.

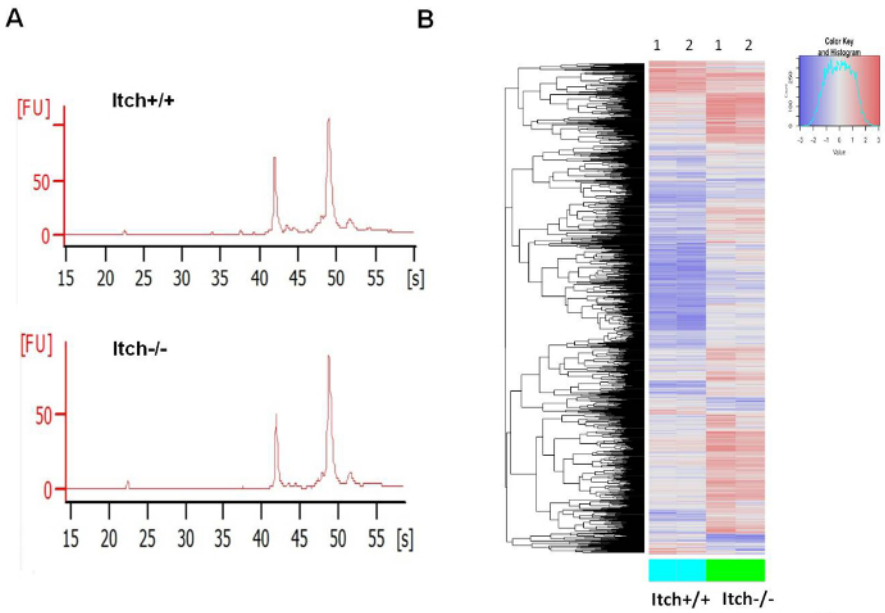

Figure 2: Differential gene expression in the lungs of WT and Itch-/- mice following intra-tracheal NTHi inoculation. 24 hr after NTHi inoculation, RNA was isolated from the lungs of two WT (1, 2) and two Itch-/-(1, 2) mice. (**A**) RNA quality was analyzed using a bioanalyzer. (**B**) Heat map showing Z-scores (interpreted as a measure of s.d. away from the mean) for the $\log_2$ count per million ($\log_2$ CPM) of 172 differentially expressed genes identified using edgeR in the Itch -/- compared to WT mice. Please click here to view a larger version of this figure.

Discussion

Herein, we describe a unique and minimally invasive procedure to inoculate the lungs of mice with a bacterial lung pathogen. We demonstrate that this procedure can be used to study the function of different genes using mice that are deficient in genes of inflammatory signaling pathways. This procedure can also be used to study the inflammatory responses to viral and fungal lung infections. The advantages of this procedure over other methods such as intranasal or aerosol inhalation are (1) in this procedure, the pathogenic inoculum is directly instilled to the lower respiratory tract; (2) the ability to control the inoculum size; and (3) reduced risk of bacterial exposure to the handler.

Exposure of the trachea surgically for instilling bacteria is a crucial step where care must be taken to avoid causing damage to surrounding tissues, especially the blood vessels.

Instillation of bacteria into the trachea should be done carefully to avoid the possibility of death of the mice due to asphyxiation. It is suggested that the inoculum (around 50 µl) be released slowly and air be injected before and after the inoculum. Keeping the mouse vertical following the instillation for a while and keeping them laterally recumbent facilitates faster recovery. The major disadvantage of this procedure is that multiple exposures within a short period of time are not possible due to the requirement of the surgery. A potential issue is that if the catheter is placed too deep in the bronchus, the BALF cell count will be lower. This problem can be solved by pulling the tube slightly upwards.

Since the incidence of airway inflammatory diseases are increasing worldwide[3,16], understanding the pathophysiology of these diseases is of prime importance. The technologies described in this study could help to identify key regulatory pathways and could facilitate the development of new treatment modalities.

Disclosures

The authors have nothing to disclose.

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