

# **Persisting *Cryptococcus* Yeast Species *Vishniacozyma victoriae* and *Cryptococcus neoformans* Elicit Unique Airway Inflammation in Mice Following Repeated Exposure**

## **Methods Collection**

### **Fungal Cultivation and Sample Preparation**

*Vishniacozyma victoriae* (ATCC MYA-305) was cultivated in Potato Dextrose Broth (BD Biosciences, San Jose, CA) at 15°C. *C. neoformans* (ATCC 32045) was grown in liquid cultures in Yeast Mold Broth (BD Biosciences, San Jose, CA) at 37°C. Cells were collected during the logarithmic growth phase (approximately 48 hours post-inoculation for *V. victoriae* and 24 hours post-inoculation for *C. neoformans*) washed and diluted in phosphate-buffered saline (PBS) at concentrations of  $2 \times 10^5$  cells/mL and  $2 \times 10^7$  cells/mL corresponding to  $10^4$  and  $10^6$  cells per 50  $\mu$ L aspiration, respectively. Cells were diluted from fresh cultures each exposure day immediately prior to administration.

### **Animal Exposures**

Randomly grouped 6- to 8-week-old female BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) were used for all experiments. Mice (n = 8-10 per group) were housed in the AAALAC International-accredited animal facility at the National Institute for Occupational Safety and Health (NIOSH). Mice received high-efficiency particulate absorbing (HEPA)-filtered air and were housed with 12-hour light/dark cycles and provided food and water *ad libitum*. All animal procedures were approved by the West Virginia University or CDC-Morgantown Animal Care and Use Committee.

Mice were exposed to yeast via oropharyngeal aspiration of a 50  $\mu$ L yeast suspension prepared in PBS. Mice were anesthetized, exposed via oropharyngeal aspiration every other day for a total of six exposures, and then humanely euthanized 1 day or 21 days after the final exposure. All mice were humanely euthanized with an intraperitoneal injection of pentobarbital at 100-300 mg/kg body weight (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). Lung tissue and

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bronchoalveolar lavage fluid (BALF) were collected following euthanasia for subsequent analyses.

### **Histopathological Analysis**

Right lung lobes (n= 3/group) from repeatedly exposed mice were inflated with 10% paraformaldehyde fixative, tied off, and collected. Lung tissue was then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E), Periodic-acid Schiff (PAS), or Grocott's methenamine silver (GMS) stains for histopathological evaluation by iHisto services (Salem, MA). H&E-stained sections were examined to evaluate lung architecture, inflammation severity and location, and inflammatory cell types. PAS-stained sections were analyzed to identify changes in airway epithelium, specifically the proliferation of goblet cells and mucous production. Lastly, GMS-stained sections were examined for yeast organisms and GMS<sup>+</sup> cytoplasmic granules. Scores were determined for various parameters based on a standard qualitative toxicologic scoring scale (0 – none, 1 – minimal, 2 – mild, 3 – moderate, 4 – marked, and 5 – severe). Slides were imaged and analyzed by a blinded histopathologist, and scores and findings were reported.

### **Flow Cytometric Analysis of Lung Tissue and Bronchoalveolar Lavage Fluid**

BALF cells were collected in 2 mL of PBS, washed, and immediately underwent flow cytometric staining. To analyze the remaining cells in the lung tissue, BALF-depleted lungs were then processed for flow cytometry. Following BALF collection, the lungs were washed with PBS, minced, and incubated in digestion buffer (5% fetal bovine serum, 1 mg/mL collagenase, 30 µg/mL DNase Type IV in Dulbecco's Modified Eagle Medium) for 30 minutes at 37°C on a shaker. Digested lung tissue was filtered (0.2 µm), washed, filtered, and centrifuged at 400 x g to isolate lung cells. Lung cells then underwent flow cytometric staining.

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Cell pellets from lung tissue were resuspended in 1 mL red blood cell lysis buffer for 5 min at room temperature, then washed in FACS buffer (4% fetal bovine serum + 2mM ethylenediaminetetraacetic acid in PBS) and centrifuged at 400 x g for 5 min. Next, lung and BALF cells were blocked in Fc Block (10% Rat Serum, 5% anti-mouse CD16/CD32 clone 2.4G2 antibody in PBS) for 5 min at room temperature. Cells were then incubated with a surface stain cocktail for 25 min in the dark at 4°C. Following a wash, the cells were then fixed with Cytofix for 10 min at room temperature, washed, and resuspended in 250 ul of FACS buffer. Data were acquired using an LSR II flow cytometer (BD Biosciences). Total cell counts per sample were calculated utilizing Spherotech AccuCount Beads per the manufacturer's instructions. Flow cytometric analysis was performed utilizing FlowJo version 10.6 (FlowJo, Becton, Dickinson and Company, NJ).

### **Cytokine Multiplex**

Frozen lung tissue was homogenized utilizing the TissueLyser II system (Qiagen, Hilden, Germany). Briefly, lung tissues were placed in 2 mL Eppendorf tubes containing Tissue Protein Extraction Reagent (T-PER, Thermo Fisher Scientific, Waltham, MA) with a 5 mm metal bead (OPS Diagnostics, Lebanon, NJ) and lysed for three 30-second cycles at 4.5 Hz. Homogenized samples were centrifuged at 1,400 rpm for 10 min and the supernatant was collected and stored at -80°C for later use. Cytokine levels of murine IL-4, IL-5, IL-13, IL-33, and Eotaxin were assessed using a custom ProcartaPlex Multiplex Immunoassay (Invitrogen, Waltham, MA). The plate was prepared per the manufacturer's instructions and run on a Luminex 200 system (Luminex, Austin, TX). In-software analysis was performed, and cytokine concentrations were extrapolated from averaged technical duplicates.