Online Data Supplement

Inhalation of *Stachybotrys chartarum* fragments induces pulmonary arterial remodeling

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SUPPLEMENTAL MATERIALS

Supplemental Materials E1. Fungal cultures

Conidia from each strain was inoculated on malt extract agar plates for 10-14 days at 26°C and harvested. Autoclaved Mahatma white rice (10 grams) (Riviana Foods Inc., Houston, TX) was inoculated with 5 mL of $2.5x10^5$ conidia/mL and incubated 21-28 days before desiccation. Heat-inactivated conidia were prepared by placing the *S. chartarum* conidia laden rice culture flasks in an 80°C bead bath (Lab Armor, LLC, Cornelius, OR) for 2 hours. Viable and heat-inactivated conidia were desiccated 7 days prior to inhalation exposures. Fungal test articles were aerosolized using a computer-controlled acoustical generator system (AGS) as previously documented (24-26). Particle size distributions and counts were recorded using an aerodynamic particle sizer (APS; TSI Inc., Shoreview, MN), and the raw counts obtained following exposure were normalized to 1000 particle counts for comparison between each strain and exposure group.

Supplemental Materials E2. Histopathology

Left lung lobes (n=3) were harvested, tied off and perfused with formalin, then paraffin embedded and sectioned at 5 microns as previously described (26). Slides were stained with hematoxylin and eosin (H&E) for routine histopathology evaluation. Images were captured using an Olympus BX53 microscope equipped with a DP73 camera and cellSens Standard software (Olympus Corporation, Shinjuku, Japan).

Supplemental Materials E3. Flow cytometry and cell differential analysis

BAL was performed *in situ* 3 times with Phosphate Buffered Saline (pH 7.2). Cells derived from BALF (n=7/group) were enumerated using a Cellometer Vision (Nexcelom Bioscience, Lawrence, MA) and prepared for flow cytometric analysis as previously described (26). To minimize non-

specific and Fc receptor binding, cells were incubated with Fc Block (CD16/CD32) (BD Biosciences, San Jose, CA, USA) and rat serum (Sigma-Aldrich, St Louis, MO, USA) prior to staining. Cells were subsequently stained using fluorochrome-conjugated antibodies (Table 7). Data were collected via BD LSR II (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo (BD Biosciences).

Supplemental Materials E4. Proteomic and RNA analysis

Frozen right lung lobes (n=3/group) were sent to MS Bioworks (Ann Arbor, MI) for quantitative proteomic analysis as previously described (26). Briefly, the lung tissues were lysed in modified RIPA buffer (50mM Tris HCl, pH 8.0, 150mM NaCl, 2.0% SDS, 0.1% TX100, 1X Roche Complete Protease Inhibitor). Samples were heated, and the supernatants were collected and quantified by Qubit Fluorometry (Life Technologies). Extracted proteins were separated using a 10% bis tris SDS-PAGE gel followed by in-gel digestion of proteins with trypsin. Digested peptides were pooled and analyzed by nano-liquid chromatography tandem mass spectrometry with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive Orbitrap. Data was processed through the MaxQuant software which recalibrated the mass spectrometric data, filtered at the 1% protein and peptide false discovery rate (FDR) and calculated the peak areas for detected peptides and proteins. Data was searched against the *Mus musculus* genome database (Swissprot).

The mRNA analyses were conducted as previously described (25). First, RT^2 First Strand Kit (Qiagen, CA) converted the extracted total RNA to cDNA. The cDNA was mixed in prefabricated PCR plates that contained primers of genes involved in the Th1-Th2 response and Th17 response pathways (RT^2 Profiler™ PCR Array Mouse Th1 & Th2 Responses and RT^2 Profiler™ PCR Array Mouse Th17 Response), and then subjected to real-time PCR (ABI 7900HT,

Applied Biosystems, Thermo Fisher Scientific, CA) according to manufacturer's instructions (Qiagen, CA). RT² SYBR Green ROX qPCR Mastermix was used for cDNA quantification (Qiagen, CA). Th1, Th2 and Th17 gene expression data were normalized to beta-glucuronidase (*gusb*). Secondly, the expression of additional genes (Table 8) were measured by converting the extracted total RNA to cDNA by the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, MA). Additional murine gene assay identifiers (Life Technologies, MA) are reported in Table 8. Equal amounts of cDNA were then subjected to real-time PCR. TaqMan® Fast Universal PCR Master Mix (2x), no AmpErase® UNG (Life Technologies, MA) was used for quantification of cDNA. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). Heat maps were generated by the online analysis tool, CIMminer (https://discover.nci.nih.gov/cimminer/).

Fold changes and false discovery rates of the differentially expressed miRNAs, genes, and proteins were analyzed by Ingenuity Pathway Analysis (IPA) software (Qiagen) for core, comparative, and miRNA target filter analysis (25).

Supplemental Materials E5. Statistics

Statistical power tests were conducted to determine the appropriate number of samples needed for each endpoint measurement. The flow cytometry datasets (means±SEM) were analyzed using the unpaired t-test (two-tailed) in Prism 7.0 (Graphpad Software Inc, La Jolla, CA). Statistical analyses of the mRNA results were performed using Qiagen's GeneGlobe Data Analysis Center using Student's t-tests for replicate values of each gene. MiRNA analyses were performed by Exiqon Inc. using R/Bioconductor software (Limma package) and moderated t-tests with false discovery rates. Proteomic data was compared by independent sample t-test for each protein (26) and

statistical analysis performed using Perseus. Fold changes ≥ 2 or ≤ -2 were considered altered. *P*-values ≤ 0.05 were considered statistically significant (25, 26).

Supplemental Figure Legend

Figure E1. Charted weights for study mice. Weights were recorded weekly for each exposure group. Weights (grams) were reported following a (A) 4 week and 13 week (B) Strain A exposure, as well as a (C) 4 week and (D) 13 week Strain B exposure.

Figure E2. Lung tissue following heat-inactivated conidia exposure. Representative photomicrographs of H&E stained sections of lung from mice exposed to either Strain A HIC or Strain B HIC, and sacrificed at 24 hours after 13 weeks of exposure. Lung from an air exposed mouse is shown for reference. Pulmonary arteries (PA, arrows) near the terminal bronchiole (TB).

Table E1. Flow cytometry antibodies

Fluorochrome - Antibody	Fluorochrome	Clone
Ly-6G	FITC	1A8
Siglec-F	PE	E50-2440
CD11c	APC	N418

FITC – fluorescein isothiocyanate, PE – phycoerythrin, APC – allophycocyanin, , AF – Alexa Fluor®.

Table E2. Additional genes*

Target	Full name	Assay ID
Arg1	Arginase 1	Mm00475988_m1
Ccr7	Chemokine (C-C motif) receptor 7	Mm01301785_m1
Cd163	Scavenger receptor type 1	Mm00474091_m1
Chil3	Chitinase like 3	Mm00657889_mH
Clec4n	C-type lectin domain family 4, member N	Mm00490934_m1
Gapdh	Glyceraldehyde 3 phosphate dehydrogenase	Mm99999915_g1
Il33	Interleukin 33	Mm00505403_m1
Mrc1	Mannose receptor c type I	Mm00485148_m1
Retnla	Resistin-like molecule alpha	Mm00445109_m1
Tslp	Thymic stromal lymphopoietin	Mm00498739_m1

*Life Technologies, MA

Table E3. Proteomic profile following exposure

Please see attached excel file

Table E4. MicroRNA expression profile following exposure

Please see attached excel file

Figure E1

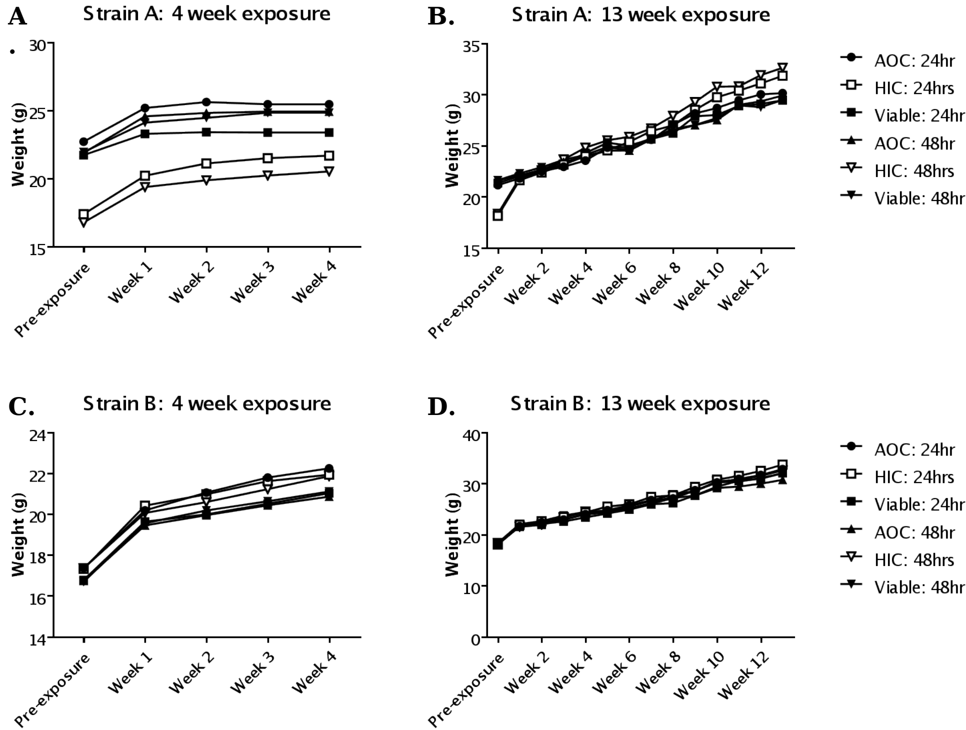

Figure E2