Journal of Visualized Experiments

Cigarette Smoke Exposure in Mice using a Whole-Body Inhalation System

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61793R2
Full Title:	Cigarette Smoke Exposure in Mice using a Whole-Body Inhalation System
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Additional Information:	
Question	**Response**
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TITLE:

Cigarette Smoke Exposure in Mice using a Whole-Body Inhalation System

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KEYWORDS:

cigarette smoke, hematopoiesis, emphysema, hematopoietic stem and progenitor cells, bone marrow, COPD

SUMMARY:

This protocol demonstrates the study of the pathophysiologic effects of cigarette smoke (CS) with a whole-body inhalation (WBI) exposure system (WBIS) built in-house. This system can expose

animals to CS under controlled repeatable conditions for research of CS-mediated effects on lung emphysema and hematopoiesis.

ABSTRACT:

Close to 14% of adults in the United States were reported to smoke cigarettes in 2018. The effects of cigarette smoke (CS) on lungs and cardiovascular diseases have been widely studied, however, the impact of CS in other tissues and organs such as blood and bone marrow remain incompletely defined. Finding the appropriate system to study the effects of CS in rodents can be prohibitively expensive and require the purchase of commercially available systems. Thus, we set out to build an affordable, reliable, and versatile system to study the pathologic effects of CS in mice. This whole-body inhalation exposure system (WBIS) set-up mimics the breathing and puffing of cigarettes by alternating exposure to CS and clean air. Here we show that this do-it-yourself (DIY) system induces airway inflammation and lung emphysema in mice after 4-months of cigarette smoke exposure. The effects of whole-body inhalation (WBI) of CS on hematopoietic stem and progenitor cells (HSPCs) in the bone marrow using this apparatus are also shown.

INTRODUCTION:

Cigarette smoking remains one of the major causes of preventable diseases in the US despite the steady decline in the number of cigarette-smoking adults in the past 50–60 years[1]. It is widely known that smoking is linked to multiple diseases of the lungs and blood including chronic obstructive pulmonary disease (COPD), a group of diseases that includes emphysema and chronic bronchitis[2–4]. According to the Center for Disease Control (CDC), in 2014, COPD was the third leading cause of death in the United States with over 15 million Americans suffering from COPD[5].

CS has also recently been associated with a higher risk of developing clonal hematopoiesis (CH)[6,7], a condition in which a single hematopoietic stem cell disproportionately produces a large percentage of a person's peripheral blood. This finding indicates a potential connection between smoking and bone marrow function. Given the widespread and highly significant health implications of CS and given that murine models of diseases are a cornerstone of progress in biomedical research, it is useful to develop efficient and affordable systems to model CS in mice.

Here, we provide a step-by-step guide for building an affordable system for treating and studying the in vivo effects of CS on lung emphysema and bone marrow homeostasis. The assembly of this equipment does not require the user to have specialized knowledge and thus allows for DIY assembly.

PROTOCOL:

All the animals involved in the experiments and the development of this technique have been under our animal use protocol approved by the Institutional Animal Care and Use Committee (IACUC) and under Baylor College of Medicine and MD Anderson institutions that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

1. Building the apparatus

1.1. Assembling the air compressor with the valve system.

1.1.1. Connect the flowmeters (two 15 L/min with Y bar and 2 power takeoffs) to the miniature pressure regulator using a 1/8 inch threaded male adapter nipple fitting. Make sure to use the thread seal tape in all threaded ends.

1.1.2. Connect the assembled pressure regulator with the flowmeter to the medical air compressor instrument using the following: a 1/8 inch hex nipple on the compressor air outlet, a 1/8 inch threaded coupling fitting, and a 1/8 inch threaded male adapter nipple fitting that connects to the pressure regulator.

1.1.3. Install the oxygen swivel barbed connector on each (4) flowmeter.

1.1.4. Install a male adaptor on the air compressor's top air outlet (part included with the medical air compressor instrument).

1.2. Assembling exposure chambers (make 4 units)

1.2.1. Cut a 3/4 inch chlorinated polyvinyl chloride (CPVC) pipe into eight 4 inch segments.

1.2.2. Insert each segment to one 3/4 inch 90˚ elbow CPVC fitting and attach the fitting side of the elbow to a 3/4 inch diameter CPVC male adapter. There should be eight CPVC segments, each attached to one CPVC elbow fitting and one CPVC male adapter.

1.2.3. Drill two holes (1 ¼ inch diameter) on opposite sides most distant from each other of an 8.5 L airtight container (11.25 x 7.75 x 6 inch) with a lid (see **Figure 1** exposure chamber). The positioning of the holes is to be centered top to bottom and left to right.

1.2.4. Insert the threaded sides of the CPVC male adapter assembled before into each hole in the containers.

1.2.5. From the inside of the container, attach a 3/4 inch CPVC cap on the other side (Chamber smoke input) and a 3/4 inch CPVC Drip irrigation female adapter on one side (Chamber smoke output).

1.2.6. Drill five 3 mm holes on the top of the CPVC cap of the chamber smoke output in a quincunx (shower head) pattern. This will allow the cigarette smoke to enter the chamber with higher velocity and ensures that it spreads evenly inside the chamber in all directions.

1.3. Assembling cigarette chambers (makes up to 4 murine exposure units)

1.3.1. Take a one-hole rubber stopper (manufacturer size 8.5) and insert a 1/4 inch barbed Y connector on the wider side and a straight barbed fitting (8 mm opening) on the narrower side. The cigarette will be placed here during the smoking procedure (cigarette pedestal).

1.3.2. Connect one end of a 12 inch long medical grade vinyl pipe to one of the barbed connectors on the Y connector attached to the rubber stopper and the other end to a 1/4 inch fitting and insert the opposite side of this fitting on a one-hole rubber stopper (manufacturer size 1).

1.3.3. On another rubber stopper (manufacturer size 8.5), insert one 1/4 inch straight tubing connector on the wider side of the stopper and connect the outer end of the fitting to a 7 ft medical grade vinyl pipe.

1.3.4. Connect the two rubber stopper structures assembled before in steps 1.3.1–1.3.3 to an 8 inch x 1.75 inch glass cylinder from a laboratory glass drain tube.

1.4. Valve control system

1.4.1. The system is controlled by a rhythmic opening and closing of solenoid valves that simulate inhalation (puffing) of cigarette smoke and clean air. The system that controls the solenoid valves was commercially designed (see **Table of Materials**).

1.5. Assembling all components together (see Figure 1)

1.5.1. Mount four solenoid valves to the sides of the valve control system using 1 inch fasteners.

1.5.2. Connect the solenoid valves to the valve control system following the manufacturer's instructions.

1.5.3. Attach a 10–32 (M) threaded straight connector to the exhaust ("EXH") connection on the solenoid valve and a threaded port adaptor on the "IN" and "OUT" connections of the same solenoid valve.

1.5.4. Connect the flowmeter attached to the compressor to the solenoid valve through the "OUT" connection using a 7 ft medical grade vinyl tubing.

1.5.5. Connect the 7 ft vinyl tube assembled with the rubber stopper in step 1.3.3. on the "IN" connector on the solenoid valve.

1.5.6. Insert the small rubber stopper of the cigarette chamber on the chamber smoke input.

1.5.7. Connect the solenoid valve to the second connection of the barbed Y connector on the cigarette pedestal assembled in step 1.3.1.

[Place **Figure 1** here]

2. Cigarette smoke exposure

CAUTION: Avoid second- and third-hand exposure to cigarette smoke. Cigarette and exposure chambers should be used within a Class II Type B2 Laminar Flow Biological Safety Cabinets. Proper PPE should be worn while conducting the smoke exposure experiments (i.e., masks, gloves, hairnet, gown).

2.1. Setting pressure and airflow

2.1.1. Once all the components are assembled as shown in **Figure 1**, turn on the air compressor and wait for the safety alarm to turn off on its own.

2.1.2. Adjust the pressure of the air compressor to 40–50 psi by turning the knob on the pressure regulator.

2.1.3. Adjust the airflow from the air compressor to 5 L using the flowmeter.

2.1.4. Turn on the valve controller.

2.1.5. Adjust the digital timer on the valve controller to the PULSE-C (shown in the display as "Pu-c") operating mode by pressing the SET/LOCK key while holding down the UP key at the first digit of the timer. Then, press the UP key until the Pu-c mode is reached. Press the RESET key to set the displayed operating mode (i.e., Pu-C) as the working mode.

2.1.6. Press the SET/LOCK to change timer 1 (shown in the display as "T1").

2.1.7. Press the UP or DOWN keys to set T1 to 20 s.

2.1.8. Press the SET/LOCK to change timer 2 (shown in the display as "T2").

2.1.9. Press the UP or DOWN keys to set T2 to 3 s.

NOTE: Steps 2.1.5 through 2.1.9 are tailored to be used with the specific timer (see **Table of Materials**). For further instructions on other uses for this product, see its corresponding user manual.

2.2. Cigarette smoke treatment

NOTE: This system allows for the use of 1–4 murine exposure chambers at the same time.

2.2.1. Turn on the air compressor and wait for the safety alarm to turn off on its own.

2.2.2. Turn on the valve controller.

2.2.3. Transfer 5 mice into each of the four exposure chambers with airtight removable lids with a volume of 8.5 L. Place the four exposure chambers with mice within a Class II Type B2 Laminar Flow Biological Safety Cabinets.

2.2.4. Inside the laminar flow biological safety cabinet, light up a cigarette and insert the cigarette inside of the cigarette chamber. Use commercially available cigarettes which contain 15 mg/cig tar and 1.1 mg/cig nicotine[8] as compared to Kentucky 3RF4 research cigarettes (9.5 mg/cig tar and 0.73 mg/cig nicotine)[9].

2.2.5. Switch ON the valves on the valve controller that correspond to the chambers that are currently in use. The exposure is divided into 2 phases: (T1) clean air is pumped into the exposure chamber for 20 s and (T2) airflow causes the cigarette to burn and smoke from the cigarette chamber is pumped into the exposure chamber for 3 s. Allow the cigarette to burn out completely until it reaches the filter.

2.2.5.1. Adjust the timer settings to perform an average of ~10 puffs/cigarette over an ~4-min period. Note that the timer and system are easily customizable for enhancing or lowering CS dosing regimen according to the research needs of the investigators.

2.2.6. Remove the cigarette filter and dispose of it by placing the cigarette butt in a glass beaker with water to extinguish the flame and dampen the odor.

2.2.7. Make sure the cigarette chamber is closed again and without a cigarette. Let the machine pump clean air for 10 min. It is of utmost importance to maintain constant monitoring of the vertebrate animals that are exposed to CS. This exposing regimen is optimized for 5 female mice over 9-weeks old per exposure chamber.

2.2.8. Repeat steps 2.3.4 through 2.3.7 three times for a total of 4 cigarettes per chamber a day. This procedure is repeated 5 days a week for as long as the researcher needs for their experiments.

2.2.9. Remove the mice from the exposure chambers back into their corresponding cages.

2.2.10. Turn off the valve controller and the air compressor.

2.2.11. Remove the exposure and cigarette chambers and wash with water and soap to remove any residue of tar.

2.2.12. Let the chambers fully dry before using them again.

REPRESENTATIVE RESULTS:

One of the main hallmarks of CS exposure is emphysema that is characterized by the damage and destruction of air sacs (alveoli) in the lung. Thus, initial experiments focused on the DIY system's ability to provoke emphysematous changes in the lungs of female mice upon repeated whole-

body exposure to CS. The CS dosing regimen was chosen based on our prior publications in which we utilized the DIY system described here to treat mice with CS and study the molecular pathophysiology of emphysema[10–16]. Specifically, mice were exposed whole-body to the smoke of four commercial cigarettes with filter daily, with smoke-free intervals of 10 min in between each cigarette, five days a week for a duration of 4 months[10–16].

Hematoxylin and eosin (H&E)-stained lung histology showed the destruction of the alveoli in mice exposed to CS in comparison to Air treated mice (**Figure 2A**). In agreement, histomorphometric analysis of lung sections in a blinded fashion showed that the mean linear intercept (MLI) was significantly higher in mice exposed to CS as compared to Air controls (**Figure 2B**). As expected, WBIS to CS provokes a drop in body weight (**Figure 2C**). Consistent with the above observations CS-exposed mice also showed enhanced airway infiltration of immune cells as well as induction of Matrix metalloproteases 9 and 12 (*Mmp9* and *Mmp12*) gene expression, which are responsible for tissue damage (**Figure 2D,E**)[17]. Cotinine, a metabolite of nicotine and a biomarker for CS exposure, was detected to be significantly elevated in the serum of mice exposed to 4 months of CS but was undetectable in Air-exposed mice (**Figure 2F**).

There is an increasing appreciation of the multifaceted impact of CS exposure on the body's cells and tissues. A prior study showed that WBI exposure of mice to CS with a regimen of 6 h/day, 5 days/week for 9 months with 3R4F cigarettes led to an alteration in the hematopoietic stem cell niche[18]. Therefore, we tested the ability of this DIY system to alter bone marrow homeostasis utilizing our pre-established CS dosing regimen[10–16]. After exposure, we analyzed BM populations using flow cytometry (**Figure 3A**). In accordance with the expectations, treatment of mice with CS on this DIY system resulted in an alteration in bone marrow (BM) populations. Specifically, flow cytometric analysis showed a significant increase in hematopoietic stem and progenitor (HSPC) populations after 4 months of CS exposure as compared to Air controls (**Figure 3B**). Extending these observations, whole-body exposure to CS of mice utilizing a commercially available system (see **Table of Materials**) also showed an alteration in HSPC populations (**Figure 3C**). The dosing regimen and duration of CS exposure used in the commercial system and the prior publication on CS and hematopoiesis [18] were quite different than this DIY system suggesting that bone marrow homeostasis is exquisitely sensitive to a wide range of CS dosing and treatment regimens (**Figure 3C**). Overall, this data highlights that this DIY system is an affordable option that can be used to expose mice to CS under controlled conditions to reliably study its effects in a range of cells and tissues.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the connections of our WBIS for exposure to CS. This figure demonstrates how all components are assembled to form a working apparatus. The figure shows only one assembled smoking chamber of the four that the machine is capable of operating.

Figure 2: CS-mediated induction of airway inflammation and lung emphysematous changes of mice. (**A**) H&E stained lung sections from WT C57BL/6 mice exposed to Air or CS for 4 months. 4x magnification; inset 20x magnification. Scale bar 200 μM. (**B**) Mean linear intercept (MLI) as a

measure of interalveolar wall distance was measured using unbiased histomorphometry from mice treated by Air or CS. (**C**) Mice weights after 4 months of Air or CS exposure. (**D**) Total and differential cell counts from bronchoalveolar lavage (BAL) fluid of control (Air) versus CS treated mice. Total leukocytes (Total), macrophages (Mac), neutrophils (Neu), and lymphocytes (Lym). Relative expression of (**E**) *Mmp9* and (**F**) *Mmp12* mRNA quantified by real-time PCR from BAL fluid of Air or CS exposed mice and normalized to *Gapdh* expression. n = 4–5 mice/group. (**G**) Serum levels of cotinine in mice exposed to Air or CS were measured by ELISA 24 h after the last CS treatment; n = 7–8 mice/group. Statistical comparisons were done using (**B,C,D,E**) Unpaired *t*-test and (**F**) Welch's *t*-test. Data shown Mean ± SEM. **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 3: In line with expectations this DIY system can be used to study CS-mediated effects in the bone marrow of mice. (**A**) Gating strategies to identify HSPCs and HSCs by flow cytometry. Lineage markers include: Gr1, Mac1, B220, CD4, CD8, and Ter119. (**B**) Percentage of HSPC and HSCs in the whole bone marrow after CS exposure using this DIY system with the same 4-month regimen. (**C**) Percentage of HSPC and HSCs in the whole bone marrow after CS exposure using the commercially available system with the following exposure procedure: 24 3RF4 research cigarettes daily, 12 puffs/cigarette, 5 days a week for 4.5 weeks duration. (**B–C**) Mann-Whitney test; n = 5 mice/group. Data shown as Mean ± SEM. *p < 0.05.

DISCUSSION:

Here we provide the information required for the construction of an apparatus for WBIS of mice to CS. After installation of the system, it is critically important that investigators calibrate the system based on the delivered dose of nicotine or cotinine in animals. The apparatus contains a timer and pressure gauges that can be used to adjust cigarette puff volume, puff frequency, combined smoke exposure period, and rest intervals that animals receive between each cigarette. Furthermore, the actual number of cigarettes administered daily may vary depending on tar and nicotine content. Finally, it is imperative that any component exposed to cigarette smoke be cleaned on a regular basis to ensure proper smoke circulation and consistent exposure to the animals.

There are at least half a dozen commercial systems and protocols available for the treatment of mice with CS and air toxicants. However, the majority of equipment used for this purpose require commercial vendors or in-depth knowledge of electronics and/or electrical engineering for assembly. Some of those systems employ WBI regimens while others incorporate nose-only treatments, but these systems can cost up to $100,000 making them prohibitively expensive for most laboratories.

The advantage of this DIY system is the inherent simplicity in the manufacture, low cost (~$6,000), and versatility. Furthermore, the components necessary for the construction of this DIY apparatus are readily available from commercial retailers and supply chains. We acknowledge a limitation of the exposure protocol and equipment as the lack of dosimetry equipment to measure cigarette smoke constituents delivered into the mouse exposure chambers. However, the design of this system works in a controlled fashion and we showed that the levels of serum cotinine in this chosen smoking regimen are comparable to other murine models of CS-induced

emphysema[20,21]. Furthermore, this method has been shown to have applications beyond monitoring the effects of CS in the lungs and BM. Our group used this system to study how cigarette smoke affects the intestinal tissue[15]. We have also recently adapted this system to study the deleterious effects of exposure to electronic cigarettes on the lungs[22].

In summary, this apparatus represents an affordable and easy-to-build exposure system to study the vast array of detrimental effects of cigarette smoking.

ACKNOWLEDGMENTS:

AR, XH, and PE were supported by NIH grant R01HL140398 and a Gilson Longenbaugh Foundation grant. DEMM and KK were supported by the NIH grants R01HL136333 and R01HL134880 (KYK), and a grant from the Helis Medical Research Foundation. DEMM is also supported by the Howard Hughes Medical Institute (HHMI) Gilliam Fellowship for Advanced Study. PE is also supported by Training in Precision Environmental Health Sciences NIEHS T32 ES027801 Fellowship Program. JC and MF are supported by Tobacco Research Funds from the Department of Epigenetics and Molecular Carcinogenesis and by the Center for Epigenetics (Scholar Award to MF) at MD Anderson. FK and YZ are supported by NIH grants R01 ES029442-01 and R01 AI135803-01 as well as VA Merit grant CX000104. This project was supported by the Cytometry and Cell Sorting Core at Baylor College of Medicine with funding from the CPRIT Core Facility Support Award (CPRIT-RP180672), the NIH (CA125123 and RR024574), and the assistance of Joel M. Sederstrom.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

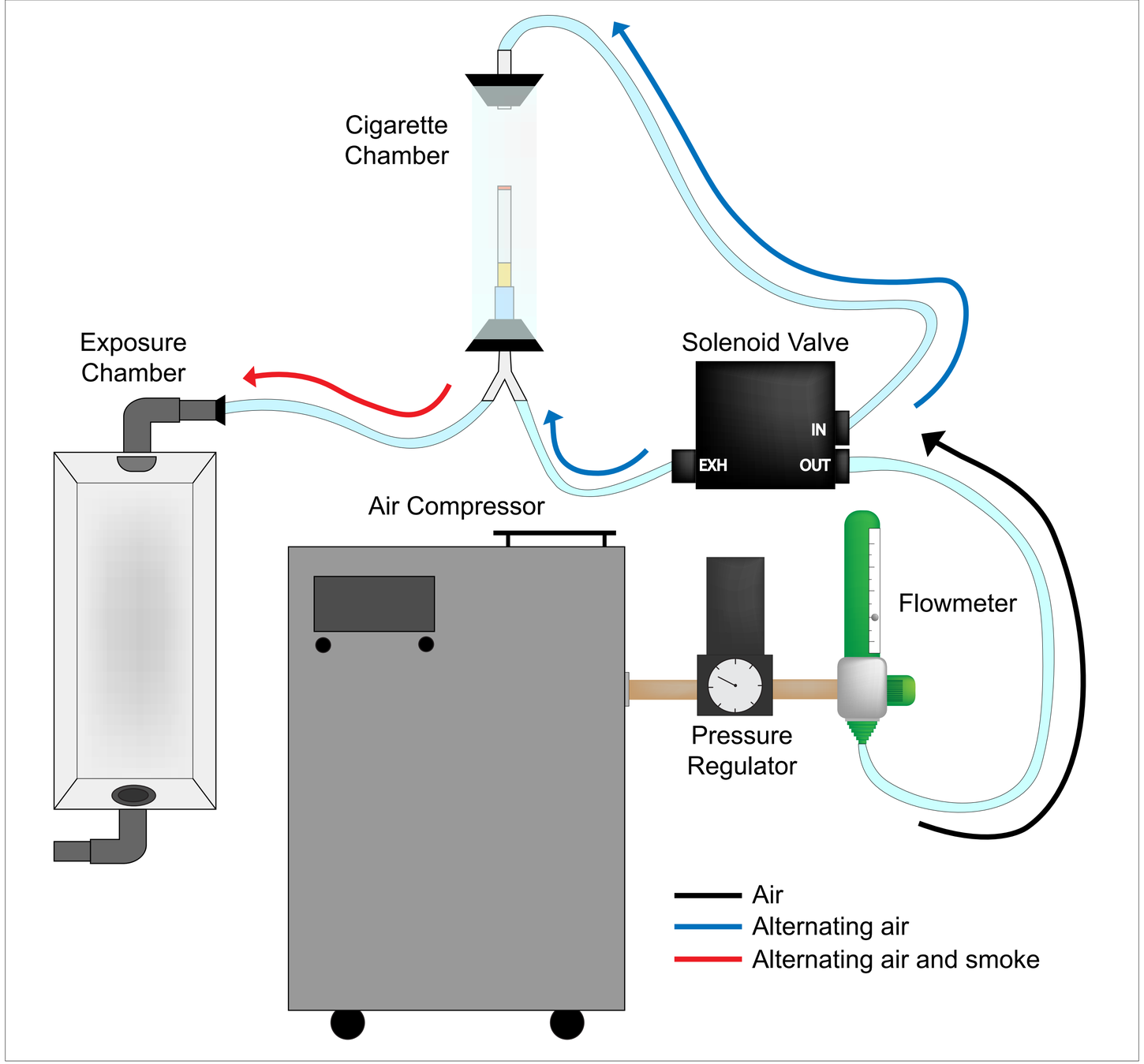

Figure 2

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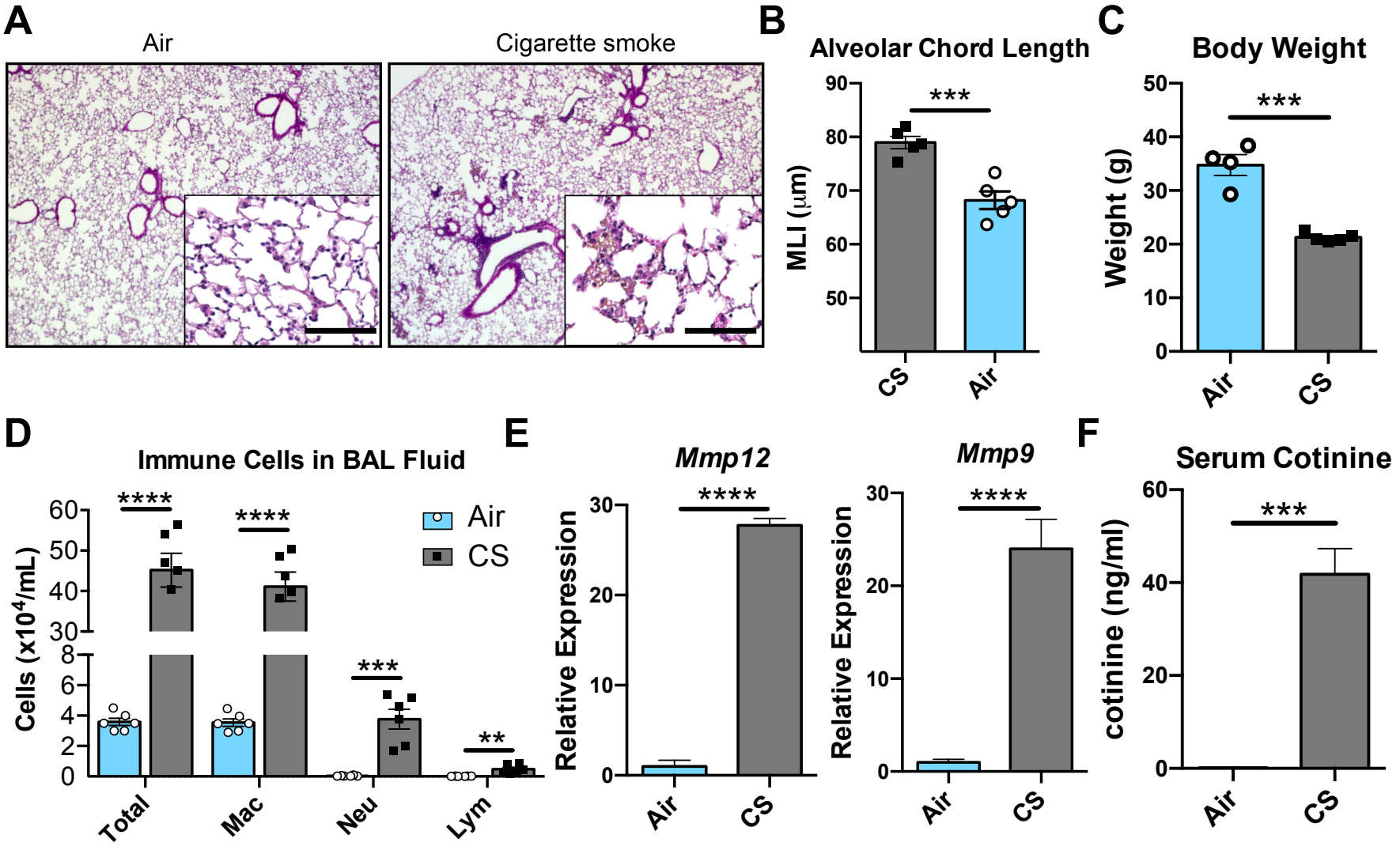

Figure 3

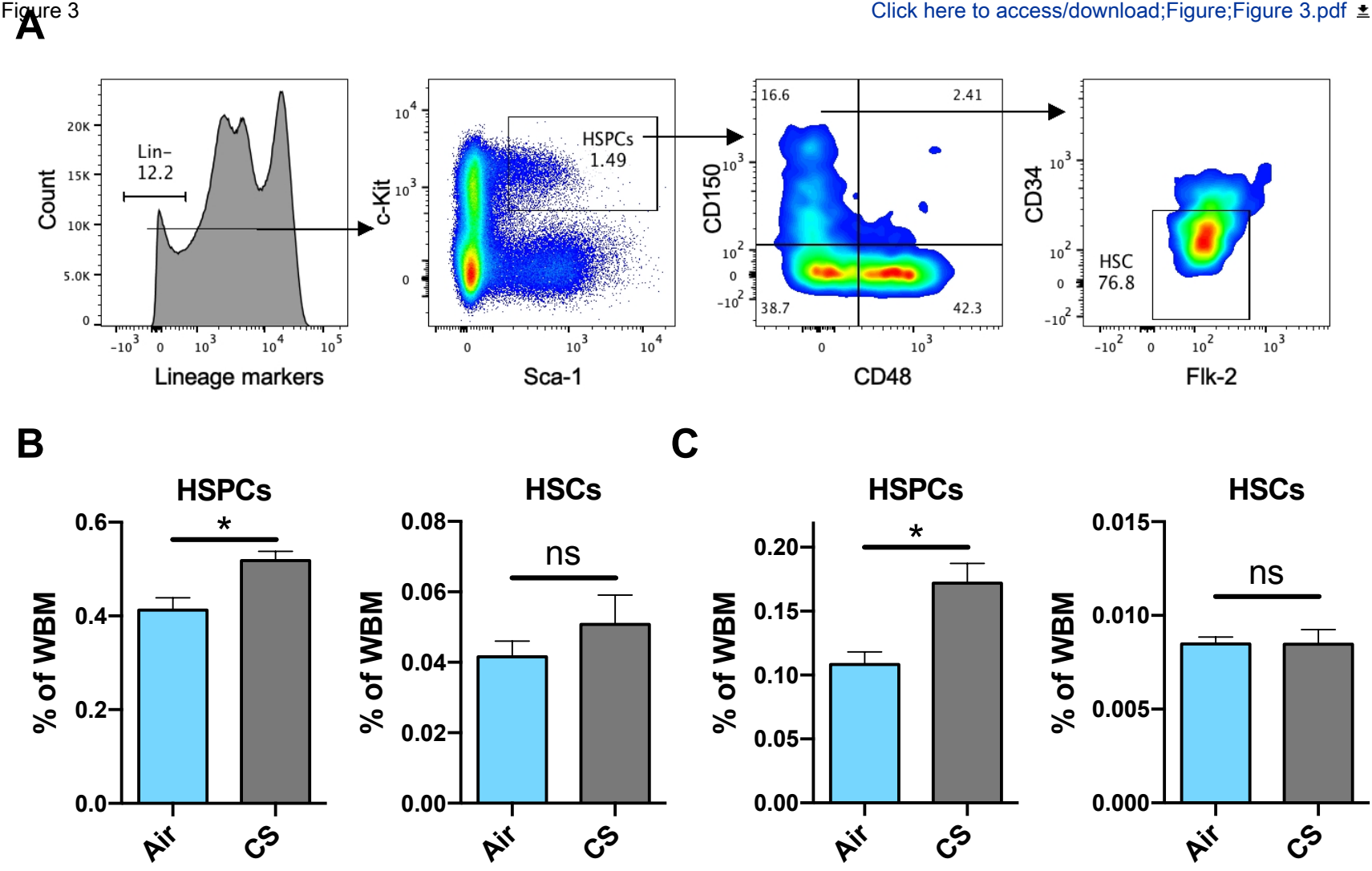

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xls

Name of Material/ Equipment	Company	Catalog Number
1 in fastener	Lowes	756990
1/4 in Barbed Y connector	VWR	89093-282
1/4 in straight tubing connector	VWR	62866-378
1/8 hex nipple	Lowes	877221
1/8 in threaded coupling fitting	Lowes	877208
1/8 in threaded male adapter nipple fitting	Lowes	877243
10/32 (M) threaded straight connector	Bimba	EB60
3/4 in 90-degree elbow CPVC fitting	Lowes	22643
3/4 in chlorinated polyvinyl chloride (CPVC) pipe	Lowes	23814
3/4 in CPVC cap	Lowes	23773
3/4 in CPVC Drip irrigation female adapter	Lowes	194629
3/4 in diameter CPVC male adapter	Lowes	23766
8.5 L airtight container with lid (11.25in x 7.75in x 6 in)	Komax	N/A
Glass drain tube (1.75 in diameter x 8 in length)	KIMAX	6500
Isonic Solenoid Valves	Bimba	V2A02-AW1
Marlboro Red 100's	Marlboro	N/A
Oxygen swivel barbed connector	Global Medical Solutions	RES002
Panasonic Timer LT4H-W	Panasonic	LT4HW
Pressure regulator	Allied Electronics and Automation	70600552
Rubber stopper # 1 (one hole)	VWR	59581-163
Rubber stopper # 8.5 (one hole)	VWR	59581-389
Scireq inExpose system	Scireq and Emka Technologies	N/A
Straight barbed fitting (8mm opening)	VWR	10028-872
Thread Sealant tape	Lowes	1184243
Threaded port adaptor	Bimba	P1SA1
Timeter Aridyne 2000 Medical Air Compressor	MFI Medical	AHC-TE20
Timeter flowmeter	Allied Healthcare Products	15006-03YP2

Valve Control system	Shepherd Controls and Associates	N/A
Vinyl pipes	Vitality Medical	RES3007

Comments/Description

Listed as "Komax Biokips Large Bread Box | (280-oz) Large Storage Container"

Item was built-in the valve controller by Shepherd Controls & Associates
Also listed as "Norgren R07-100-RGKA"

Commercial system used for comparison with our DIY WBIS

Also listed as "Puritan Air Meter"

Company custom designed the valve control system for this model.

We are grateful for the opportunity to submit a revised version of our research article entitled “An affordable whole body inhalation system to study cigarette smoke exposure in mice” to JOVE. We thank the referees for the positive comments and excellent remarks concerning our manuscript. We hereby provide you with a detailed response to the comments mentioned by the reviewers. To fully address their remarks about “dosimetry” of mice with our DIY system we included quantification of cotinine from serum of mice.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise the title to be more concise. Suggestion: A whole body inhalation system to study cigarette smoke exposure in mice
3. Please do not include references in the Abstract.
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
5. Please spell out the journal titles in the references.

We have made the necessary changes in the revised manuscript.

Reviewer #1:

Manuscript Summary:

The authors describe the development of/an instruction on how to build a DIY animal exposure system and attempt to verify its functionality by demonstrating that using the DIY system, cigarette smoke exposures of a suitable mouse model results in the induction of biological responses comparable to the ones observed when a commercially available system is used.

The availability of affordable scientific instrumentation and/or instructions to build it is highly desirable in any field of research; it disconnects the ability of conducting research from the financial capacity of institutions, which increases the overall amount of research that can be conducted within institutions, countries and worldwide and hence increases the scientific progress and the robustness of the findings. In addition, it potentially eliminates conflicts of interest that may arise as soon as extensive funding is involved. This may increase the quality of the research conducted and increases the confidence in the results and the public and scientific acceptance.

The system appears to be well designed, and clearly the field of inhalation toxicology may benefit from its development. I therefore believe that the presented work is scientifically relevant and that the design of the exposure system should be published and made publicly available.

We are grateful to the reviewer for these positive comments and the recognition of the value of this work for the scientific community.

Major Concerns:

1) Affordable scientific instrumentation only reveals its advantages if there is a certain level of standardization realized. It is ultimately up to the scientific community to decide

which model of a DIY system is most widely used, but in any case, the instruction for building them should be clear/unambiguous, so every research group builds the same system.
Section 1 does at its current level of detail not meet this requirement as it leaves a couple of design options to the reader/the person building the system, for instance:
a) What are the exact dimensions of the exposer chamber (L x H x W)?
b) Where are the two holes exactly located in the chamber walls?
c) What is the exact location/geometrical arrangement of the 5 3mm holes in the CPVC cap?
To eliminate such ambiguities, it is proposed to include more detailed graphics, preferably one for each sub-part of the system in the written manuscript. 2D CAD drawings may be considered. (this request may be ignored if it is intended to share details 3D plans in the JoVE video, although I tend to prefer printable versions).

R1. As suggested by the reviewer we incorporated additional information to the manuscript to improve the description of assembly. For instance, we have now included more specific dimensions of the exposure chambers. These chambers are 11.25 x 7.75 x 6 in and have a volume of 8.5L. The location of the holes is centered on each side. We have added the specification that the five 3 mm holes on the CPVC cap are arranged on a quincunx pattern. We expect that the video portion of this manuscript will provide up-close details of each of the components to further clarify the final assembly. We added a statement that we will be happy to provide more detailed graphics/photos of each part in written form to any reader who contacts us.

2) From the written manuscript, it is not clear how the accumulation of smoke in the cigarette chamber is achieved, and how it is ultimately brought to the exposure chamber. This should be explained in more detail, unless it will be explained in detail in the video.

R2. This is an important point. Descriptive arrows have been added to Figure 1 to show the flow of smoke and/or air throughout the system. We also made changes to the text of the manuscript. Lastly, the video portion of this manuscript describes how smoke accumulates in the chamber.

3) The approach for system verification/validation is flawed:
3.1) It is unfortunate that the test exposures were not conducted under standardized conditions (applying standardized smoking regimes and using standardized test products). This drastically decreases the comparability to published literature and should be addressed in a more extensive discussion (put the system in a global context)

R3. We thank the reviewer for the comment. Using our smoke protocol, mice are exposed whole body to the smoke of four cigarettes (Marlboro Red 100's with filter), once a day, with smoke-free intervals of 10 minutes in between smoke exposures. We have shown that our DIY system promotes inflammation in the lung and alveolar damage/emphysema (Figure 2). We apologize if this was not clear in the initial submission, but we have published studies using this exact DIY system with the same regimen as far back as 2011 to study the pathophysiologic effects of cigarette smoke on lung emphysema after 4 to 5-months exposure. For example, we found that cigarette smoking produces reproducible effects on Th17 responses and furthermore we used our system to study the requirement of miR-22 (Lu 2015), Osteopontin (Shan 2012), and Pparγ (Shan 2014) in experimental emphysema. From our initial publication on the murine model of emphysema to the present, we have used commercially available

Marlboro Red 100s with filters, which has many advantages, including its clinical relevance, filters out larger particles, it is easily available, and epidemiologically is the most popular brand in the continental US but our system is versatile and accommodate virtually any cigarette.

Standardization of smoking regimens is indeed desirable but historically has been a difficult proposition. For example, European, Australian, and Asian, North American laboratories may use commercially available machines or homemade system(s) specific to each region or country. Furthermore, the "dosing" and duration of whole-body cigarette smoking regimens used by labs around the world range in length and intensity depending on the system used. Whole-body exposure systems vary widely, in regimen and dosing, ranging from 4 to 10 cigarettes per-day over a range of 3 to 6-months of exposure. Nose-only exposure systems are also marketed as quicker alternatives, however they cost over $100,000 to set up. The goal of our study is to introduce a new highly customizable affordable system for exposing mice to air toxicants such as CS. We have added additional context to the discussion including that investigators who use our DIY system can use our suggested dosing as a starting point or they can easily adjust dosing for example by administering upwards of 10-cigarettes per day or shortening/lengthening the duration of treatment and/or switching to unfiltered Kentucky 3RF4 cigarettes all of which can be done easily with our DIY system. Furthermore, the timer and pressure can be easily adjusted to adjust puffs/cigarette for dosing intensity adjustments. Ultimately each lab will need to empirically carry out their own pilot studies after assembly of our system.

3.2) Although there is no doubt that the system technically works, I believe the authors fail to demonstrate that for the animals, the exposures in the DIY system are equivalent to the exposures in the commercially available system:
3.2.1) The exposure durations are not the same for the two tests. Exposures in the DIY system were conducted for 16 weeks, exposures in the commercial system for 5 weeks. It is well possible that the effects observed in the DIY system would not have arisen under a shorter, but more intensive exposure regime.

R4. We have published work demonstrating that our DYI exposure system results in emphysema in mice exposed 3 to 4 months of smoke (Lu 2015, Shan 2012, Shan 2014). In this repost, we did not perform a side-by-side comparison with the commercial system but as noted, the dosing and regimen of our DIY system is completely different. Further, the commercial system shown in Figure 3C is not intended to demonstrate equivalence to our DIY system, especially given the large differences in dosing. The perfect comparison would require access to two machines by the same laboratory and standardization of downstream methodologies not only in the bone marrow but also in the lungs with parameters such as MLI, BALF cell differentials, and qPCR to be carried out by same individuals & same reagents in identical manner. Our inclusion of bone marrow experiments done in DIY and SciReq systems (Figure 3) is meant to indicate that both regimens are sufficient to promote bone marrow hematopoietic progenitor cell expansion after cigarette smoke exposure. Our bone marrow data is also consistent with a prior study that showed a 9-months' whole-body CS exposure altered bone marrow homeostasis (Siggins 2014). Thus, the comparison of the two systems is not meant to show equivalent exposures but rather to demonstrate that the system is sufficient to induce physiologic changes that have been reported in other systems. We cannot comment on whether our DIY or SciReq elicits a stronger effect and the molecular mechanisms are beyond the scope of this manuscript. Furthermore, our DIY system has

been extensively validated by us for molecular and immunologic mechanisms of lung emphysema in mice and humans (see above). However, we have also removed language from the manuscript indicating the term “validation”.

3.2.2) Exposures in the DIY system were not conducted with the same test product as the exposures in the commercial system. Although it is unlikely that the changing the product would have

R5. Please see above.

3.2.3) No details on the smoking regime applied in the commercially available system are listed. If the smoke was not generated under the same settings (puff volume, puff frequency, puff number per cigarette/cigarette butt length), a 1-to-1 comparison is flawed

R6. We have included additional details on the SciReq system dosing and parameters in the revision. Indeed the dosing and regimen is completely different compared to our DIY system. We believe that our comparative bone marrow HSPC data in Figure 3 (one panel for DIY and one panel for our SciReq system) mainly highlights that whole-body exposure to cigarette smoke has a predictable effect on the bone marrow compartment as has been previously shown in another study (Siggins 2014). The most important aspect highlight that our DIY system can be used to study not only emphysematous changes in the lung but also peripheral effects in other organ systems. A comparison of the strength of one approach vs the other by including data on the same axis was not done because the ages of the mice are not identical at the time of euthanasia and chronological age has a robust effect on baseline HSPC and HSC numbers.

These are rather profound differences in the experimental procedures applied for the test and the reference, the possibility that the observed biological responses have the same physiological appearance, but different molecular causes can under these circumstances not be ignored.
Proposed action:
♣ Option 1 (preferred): A repetition of either the test or the reference exposure under aligned conditions is, to obtain conclusive evidence that the DIY system is equivalent to the commercial system (preferred option) alternatively
♣ Option 2: Elimination of the claim that the system is equivalent to commercial systems and limitation to the fact that the system is of low costs but still allows exposing animals under controlled, repeatable conditions. Data generated using the commercially available system could be omitted in this case, but dosimetry data need to be included to demonstrate (amongst other parameters) the stability of the smoke concentration in the chamber or the relevance of the achieved concentrations.
♣ Option 3: Substantiation of the claim that the system is equivalent to the commercial system by including literature on how mice respond to cigarette smoke generated and exposed under various conditions. This will require a detailed dosimetry in the system to be established.

R7. We appreciate the flexible recommendations and made corrections to the manuscript. Our intention is not to make any assumptions about the equivalency of our system to the commercial system (please see above answers) but to merely introduce a relatively affordable and reliable system to treat mice in a controllable manner to study the effects of air toxicants on organ systems. The design of our system does not allow easy measurements of particulate matter. But achieve an objective measure of our

smoking protocol, we quantified cotinine in the serum of cigarette smoke-exposed mice by ELISA. While cotinine could not be detected in serum of air-exposed mice, the serum cotinine levels after 16 weeks smoke exposure (see new Figure 2F) were comparable to published cotinine levels in other smoke models (Ha et al., PLoS One 2015; Moreno-Gonzales et al., Nat Comm. 2013). Also, the cotinine level in human active smokers is >10 ng/ml and can reach up to 500 ng/ml in heavy smokers.

4) There is a complete lack of dosimetry
4.1) Data on smoke concentration, inlcuding particle density, nicotine concentration and a set of relevant other, toxicologically well described smoke constituents, in the exposure chamber (the overall challenge/smoke burden the mice were exposed to), but preferably also in the cigarette chamber should be provided. A comparison between the two systems and to literature data (and a comparison between Marlboro red and 3R4F) is highly recommended, as otherwise a comparison to literature data on the biological responses is not conclusive (respones do not need to be identical to the literature data, but differneces shuld be attributable to the dosimetry).

R8. Please see above regarding the inclusion of cotinine serum measurements from our DIY system. We have also reported cotinine serum measurements of cigarette smoke treated mice using our DIY system in the past (Madison 2019).

4.2) Data on the delivered dose (referring to tissue absorbed doses, the dose ending up in the animals blood/tissues) are missing. Relevant exposure markers (e.g. nicotine, cotinine) levels at least in the mouse serum and urine, should be reported and compared between the two systems and put into relation to each other and to literature data. Evidently this would require repetition of the exposures. If a detailed animal free dosimetry (as requested above) can be established and put into a close relation to literature values, the absence of the quantification of the delivered dose may be acceptable. Alternatively, the claim of equivalence to commercially available systems may be removed and a new claim may be formulated, stating that a low-cost system, which allows conducting stable and repeatable exposures resulting in biological responses that are in line with literature-based expectations, was developed.

R8. Please see the above response.

If the above-mentioned issues can be addressed and resolved I would recommend to consider the manuscript for publication.

Minor Concerns:
Overall, the manuscript is well written and very focused. The text may merely benefit from a proofreading for eliminating a couple of slips of the pen (e.g. abstract, line 66: remove the extra 'system', section 1.1.1: 'pipe into eight 4 segments', line 198: 'eoxin', and a couple more)

R9. We made corrections to the manuscript.

Reviewer #2:
Manuscript Summary:
This paper describes a protocol for assembling a cigarette smoke (CS) exposure apparatus that can be assembled with do-it yourself components. It is designed to provide a substitute for research CS exposure devices currently in use in the research

community that are available from limited manufacturers. A limited comparative analysis using the DIY apparatus vs a commercial available apparatus (SCIREQ) is presented.

Major Concerns:
The major point of consideration is whether the device as described can effectively act as a substitute for current research WBIS used in academic labs, and produce equivalent results. Some comparative responses are shown in the display data indicating comparable results with respect to measured parameters related to bone marrow cell compositions. A key question is whether emphysema response is equivalent. Side by side comparison of emphysema response would have been supportive. If not possible, the authors should describe how the emphysema response at 4 months CS compares to published studies that document emphysema generation in 4-6 months. Is the degree of airspace enlargement shown greater or equivalent to that reported in published studies?

R10. We thank the reviewer for the suggestions. As detailed above we have published extensively using our DIY system at 4-5-months to elicit emphysematous changes in mice. Airspace enlargement and other parameters of emphysema are comparable to those we and others have reported in the past. Our low cost system can thus be used to reliably elicit biological changes in line with published literature in the lung and other systems. We have incorporated these points into the Discussion accordingly.

Minor Concerns:
CS exposure apparatus are commonly used to model COPD/emphysema in mice. Given the importance of COPD as a public health burden a few additional sentences on the incidence and prevalence of COPD and its rank as a world health burden can be added to the introduction.

R11. We thank the reviewer for the suggestion and have included a statement in the Introduction.

Marlboro reds are used as the test cigarette, which is not standard practice. What is the reason for selecting Marlboro? Are other labs using this now instead of Kentucky research cigarettes? Is some information available on the tar and nicotine content of these?

R12. Please see above regarding choice of Marlboro Red 100s. The tar and nicotine contents of these cigarettes are 15 mg/cig of Tar and 1.1 mg/cig of nicotine according to Federal Trade Commission. Kentucky 3R4F contains 9.5 mg/cig tar and 0.726 mg/cig nicotine.

What accounts for the higher baseline of HSBCs in air treated controls for the DIY apparatus?

R13. Please see above. The relative and absolute number of HSPCs and HSCs populations in the bone marrow are impacted by aging. Specifically, HSPC and HSC numbers increase with age. The DIY and commercial system studies made use of mice of different ages, and this accounts for baseline differences between the two methods. For this reason they are presented in separate bar graphs and Figure panels.

What is the relative cost savings? How much does one DIY system cost?

R14. This is an excellent question. The approximate value of our DIY system in the continental USA is $6,000 for parts. We included this information in the manuscript.

WBIS: This abbreviation should be defined in the abstract also.

R15. This has been corrected.