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Modeling Asthma and Influenza Co-morbidity in C57BL/6 Mice

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Corresponding Author:	Amali Samarasinghe University of Tennessee Health Science Center Memphis, TN UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	asamaras@uthsc.edu
Corresponding Author's Institution:	University of Tennessee Health Science Center
Corresponding Author's Secondary Institution:	
First Author:	Amali Samarasinghe
First Author Secondary Information:	
Other Authors:	Scott A Hoselton, M.S.
	Jane M Schuh, Ph.D.
	Jonathan A McCullers, M.D.
Order of Authors Secondary Information:	
Abstract:	Allergic asthma and influenza are both diseases of the pulmonary system that affect millions worldwide. During the 2009 influenza pandemic, asthma was identified as an underlying disease in hospitalized patients, although reasons for this increased susceptibility were unknown. Animal models are necessary to explore mechanisms of disease pathogenesis. However, models that could be used to study influenza virus infections in existing asthma are lacking. This protocol describes the development of mouse model systems of asthma and influenza comorbidity using an Aspergillus fumigatus-induced asthma model and the 2009 pandemic H1N1 influenza strain A/CA/04/2009. The host responses that occur in acute and chronic asthma can be explored by changing the timing of the influenza virus infection. These models suggest that the allergic host response to influenza virus depends on the state of the allergic airways and level of allergic inflammation.
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COLLEGE of MEDICINE

Department of Pediatrics
Children's Foundation Research Center, Room 402R
50 N. Dunlap Street
Memphis, TN 38163
Phone: (901) 287-5318
Fax: (901) 287-4478
E-mail: asamaras@uthsc.edu

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Immunology and Infection Editor
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One Alewife Center
Cambridge, MA 02140

Dear Dr. Zaman,

JoVE 53269R3: Revision 3

Please accept our revised manuscript titled, "Modeling Asthma and Influenza Co-morbidity in C57BL/6 Mice" for consideration for publication in *JoVE*. We have revised the manuscript based on the editorial comments and those of the two Reviewers. We thank you and the Reviewers for taking the time to help us improve the quality of our paper. All comments have been addressed in the manuscript (track changes) and Rebuttal Letter.

Thank you for your interest in our work and please let me know if you should need additional information to help with your decision.

Sincerely,

A handwritten signature in black ink, appearing to read "Amali E. Samarasinghe". The signature is stylized with a large, sweeping "S" at the end.

Amali E. Samarasinghe
Assistant Professor
Division of Infectious Diseases

TITLE: Modeling Asthma and Influenza Co-morbidity in C57BL/6J Mice

AUTHORS AND AFFILIATIONS:

Samarasinghe, Amali E.^{1,2}, Hoselton, Scott A.³, Schuh, Jane M.⁴, and McCullers, Jonathan A.^{5,6}

1. Department of Pediatrics, University of Tennessee Health Science Center, Memphis, TN, USA. asamaras@uthsc.edu
2. Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA.
3. Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND, USA. scott.hoselton@ndsu.edu
4. Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND, USA. jane.schuh@ndsu.edu
5. Department of Pediatrics, University of Tennessee Health Science Center, Memphis, TN, USA. jmccul10@uthsc.edu
6. Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA.

CORRESPONDING AUTHOR: Amali E. Samarasinghe, Ph.D.

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SHORT ABSTRACT:

Asthma and influenza are diseases affecting the pulmonary system that affects millions worldwide. The aim of this study was to develop a mouse model of asthma and influenza comorbidity to study the intersection of these two diseases in the same host.

LONG ABSTRACT:

Allergic asthma and influenza are both diseases of the pulmonary system that affect millions worldwide. During the 2009 influenza pandemic, asthma was identified as an underlying disease in hospitalized patients, although reasons for this increased susceptibility were unknown. Animal models are necessary to explore mechanisms of disease pathogenesis. However, models that could be used to study influenza virus infections in existing asthma are lacking. This protocol describes the development of mouse model systems of asthma and influenza comorbidity using an *Aspergillus fumigatus*-induced asthma model and the 2009 pandemic H1N1 influenza strain A/CA/04/2009. The host responses that occur in acute and chronic asthma can be explored by changing the timing of the influenza virus infection. These models suggest that the allergic host response to influenza virus depends on the state of the allergic airways and level of allergic inflammation.

INTRODUCTION:

Commonly referred to as a disease, asthma is a syndrome¹ that affects over 235 million of the world's population.² Allergic asthma is the most prevalent type of asthma and results from

exposures to environmental factors such as pollen, house dust mite and cockroach antigens, and fungi.³ Fungal antigens are common sensitizing allergens affecting 25% of persistent asthmatics and can cause life threatening asthma attacks.⁴ The development of an accurate model of allergic asthma is challenging because mice do not naturally develop asthma and there are differences between mouse and human respiratory systems.⁵ The selection of the mouse asthma model system is largely dependent on the experimental question since the output may vary with the antigen used. A fungal asthma model using *Aspergillus fumigatus* conidia used in native form and natural route of entry into the airways of mice, described in detail elsewhere,⁶⁻⁸ has been shown to elicit all the hallmarks of human disease including airways hyperresponsiveness, eosinophilic inflammation, mucus hypersecretion, and airway wall remodeling events.⁹

Influenza affects millions worldwide every year. Genetic alterations that occur in influenza viruses by antigenic drifts and shifts, and re-assortments in common hosts can result in a novel virus with the potential to cause a pandemic. The influenza pandemic of 1918 (Spanish Flu) claimed over 50 million lives mostly of young adults.¹⁰ The first influenza pandemic of the 21st century occurred in 2009. Although the 2009 influenza pandemic was not as severe as that of 1918, approximately 90 million people were infected¹¹ of which a little over 200,000 died.¹² Hospitalized patients included those with underlying diseases such as obesity, cardiovascular and metabolic disease, asthma, COPD, and diabetes.¹³⁻¹⁶ Although asthma was identified as a risk factor for severe influenza morbidity,^{17,18} asthmatics were less likely to be admitted to the intensive care unit and die compared to patients without asthma.^{19,20} The reasons for these seemingly counterintuitive findings are unknown. Therefore, a reliable animal model system was necessary to study the intersection of these two diseases.

Since other respiratory viruses such as rhinovirus and respiratory syncytial virus (RSV) have been shown to induce asthma, existing animal models of asthma and influenza explored whether influenza virus infections induces the development of asthma.²¹ The airways, lung parenchymal tissue, and the immune responses are different during acute allergic asthma exacerbations and chronic stable asthma. Therefore, models that can explore how influenza virus infections intersect allergic asthma in each situation are necessary to understand the underlying mechanisms of asthma and influenza comorbidities. This protocol describes a mouse model that can recapitulate these two scenarios.

PROTOCOL:

Ethics Statement. All experiments were approved by the Institutional Animal Use and Care Committee at St. Jude Children's Research Hospital.

Note: All described experiments in this protocol must be conducted under BSL-2 and ABSL-2 conditions since *Aspergillus fumigatus* and influenza A virus are BSL-2 agents.

1. Modeling Allergic Asthma in Mice (Figure 1)

1.1) Fungal sensitization

1.1.1) Re-constitute *A. fumigatus* antigen in sterile PBS at 1 mg protein/mL. Aliquot and store at -20°C.

1.1.2) House animals in a micro-isolator cages with autoclaved paper bedding under specific pathogen-free conditions with *ad libitum* food and water in a 12 hour light:dark cycle. Obtain C57BL/6J female mice at six weeks of age and acclimatize for one week. Start mice on the asthma protocol at seven weeks of age.

1.1.3) Dilute *A. fumigatus* antigen to 20 µg/mL in sterile PBS and add dropwise to an equal volume of Alum while stirring. Allow antigen to be adsorbed for 30 mins at room temperature with constant stirring.

1.1.4) Fill 1 mL luer-lock syringes with the mixture and deliver 0.1 mL intraperitoneally and 0.1 mL subcutaneously between the ears to each mouse. Administer PBS and Alum as a mock-sensitization to mice that serve as naïve controls.

1.1.5) Two weeks after the initial sensitization, dilute *A. fumigatus* antigen to a concentration of 200 µg/mL in sterile PBS and deliver 20 µL intranasally to each mouse. Repeat once weekly for a total of three intranasal administrations. Administer PBS as a mock-sensitization to mice that serve as naïve controls.

1.2) Fungal challenge

1.2.1) Prepare Sabouraud dextrose agar at 65 g/L per manufacturer's guidelines and sterilize in an autoclave at 121°C for 15 mins. Place in a water bath set at 50°C. to temper.

1.2.2) Once tempered, aseptically add 10 mL to 25 cm² canted neck, vented cap cell culture flasks. Lay the flasks flat and allow agar to set. Once set, recap flasks and store at 4°C until use.

1.2.3) Re-suspend the *A. fumigatus* culture aseptically by adding 5 mL of sterile PBS. Store overnight at 4°C. Prepare 60 µL aliquots for use and store at 4°C.

1.2.4) Eight days before the final intranasal administration of *A. fumigatus* antigen, culture *A. fumigatus* on Sabouraud dextrose agar for the fungal challenge. Add one well vortexed 60 µL aliquot of fungus in each agar flask and spread using a disposable inoculating loop. Incubate flasks at 37°C for 8 days.

1.2.5) Set up the inhalation challenge apparatus in a class II biological safety cabinet as described in detail by Schuh and Hoselton.⁷ Create holes in the fungal culture flasks, one on the rear top and one on the bottom of the flask immediately above the culture by using a cork-borer.

1.2.6) Attach the fungal culture flask to the regulated medical grade air supply through the bottom hole and to the inhalation chamber through the top hole. Cover the inhalation ports with laboratory tape.

1.2.7) Run medical grade air over the culture at 2 psi for 10 mins to coat the chamber. Prepare sterile anesthetic cocktail with 75 mg/kg ketamine and 25 mg/kg xylazine.

1.2.8) Inject the drug cocktail to three mice intraperitoneally and determine anesthesia by toe pinch reflex and lack of movement. Apply a drop of lubricating ointment in each eye to prevent drying out.

1.2.9) Turn off the air, and replace the fungal flask with a new one and open the ports of the inhalation chamber.

1.2.10) Place the anesthetized mice supine with their noses in the ports. Turn on the air over the culture and allow the animals to inhale the liberated fungal conidia for 10 mins.

1.2.11) Place each animal supine in a new cage and place the cage on a heating pad. Monitor animals for recovery from anesthesia marked by turning over and movement around the cage.

1.2.12) Repeat procedure after 14 days. Anesthetize animals that serve as naïve controls and place in a new cage.

2. Modeling Influenza in Mice

Note: Adjust the age, gender, and strain of mice and the dose of the virus according to the experimental goals.

2.1) Obtain female C57BL/6J mice at 14 or 17 weeks of age and acclimatize to the facility in micro-isolator cages with autoclaved paper bedding and *ad libitum* food and water in a 12 hour light:dark cycle. Number animals in each cage by tail mark or ear notch. One week later, weigh each mouse and record weight with date and time.

2.2) Thaw a stock of A/CA/04/2009 virus and dilute in sterile PBS at $\log_{10}4.305$ TCID₅₀/mL in a glass vial. Anesthetize mice with 2.5% isoflurane with oxygen in an anesthesia chamber within the class II biological safety cabinet.

2.3) Deliver 50 µL of virus intranasally to each mouse for a final dose of 1000 TCID₅₀ pH1N1 per mouse. Hold mouse supine until conscious and place in new micro-isolator cage. Administer PBS under anesthesia to naïve controls.

2.4) Record animal weights every 24 hours until experimental endpoints are reached.

Note: It is good practice to use the same stock virus tube(s) to infect all animals in the study and not use previously thawed tubes as the viral titer may decrease during repeated freeze-thaw

cycles.

3. Modeling Asthma and Influenza Co-morbidity in Mice (Figures 2 and 3)

3.1) To mimic acute asthma and influenza, infect animals one week after the second fungal challenge with pH1N1 as noted in section 2. To mimic chronic asthma and influenza, infect animals 4 weeks after the second fungal challenge.

4. Pulmonary function test

4.1) Weigh and record each mouse weight.

4.2) Perform pulmonary function test on each animal. Note: Of the various equipment available to measure pulmonary function in rodents, we have opted to use the *flexiVent* FX1 module and flexiware software as previously described.²²

4.2.1) Open the software and go to “Study Definition and Planning.” Click on “Create a new study” to launch the Study Definition Wizard.

4.2.2) Follow the Wizard to input details of the study: Title and Status, Objective and Hypothesis; Protocol; Subject (animal) Groups.

4.2.3) Create subjects by clicking on the mouse icon and add all necessary information on the animal including the Group Membership which will assign subjects to Subject Groups.

4.2.4) Verify all information and confirm to close the wizard.

4.3) After concluding the “Study Definition and Planning” session, open the “Experimentation Session” in the software. Follow the steps of the pop-up wizard to set-up the instrument.

4.3.1.) Select the Study (see 4.2.1) and the Experiment Template which depends on the instrument.

4.3.2) Add details as deemed necessary to Session Properties. The instrument will then be connected to the software.

4.3.3) Select the instrument module attached to the software when prompted and click OK.

4.3.4) Select a subject in the Study to Site 1 and enter the current weight of the animal when prompted.

4.3.5) Add 0.5 mL of saline to the finemist nebulizer and click OK when prompted to Prime the nebulizer (the generated mist will be visible). Attach the nebulizer to the instrument adaptor when complete.

4.3.6) Perform Channel Calibrations when prompted. Calibrate the Cylinder Pressure and Airway Opening Pressure following the software wizard using a manometer to apply known pressures.

4.3.7) At the conclusion of Channel Calibrations, the Tube Calibration Wizard will be initiated; attach an 18 g cannula to the Y-tubing adaptor and select the desired perturbations. Perform closed and open calibrations and verify that Calibration Results are void of errors.

4.3.8) Once the calibrations are complete, select whether to start recording continuous data when prompted. Click “Yes” to start the default ventilation pattern. The instrument is ready to gather data.

4.4) Prepare a stock acetyl- β -methylcholine (A β M) at 125 mg/mL in sterile saline; freeze aliquots at -20°C until use. Dilute the frozen stock of A β M to the desired doses in sterile saline (3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL, and 25 mg/mL).

4.5) Prepare anesthetics in sterile PBS before procedure. Administer 10 mg/kg body weight xylazine intraperitoneally and place mouse in its own cage to rest for 5 min. Administer 70 mg/kg body weight pentobarbital intraperitoneally and allow mouse to rest for 5 min.

4.6) Intubate mouse as previously described.²³

4.6.1) Briefly, place the anesthetized mouse supine on a slanted surgical board and fix in place with plastic tape over the forelimbs and tail.

4.6.2) Make a small incision between the upper thorax and lower jaw. Separate the salivary glands and thyroid gland to expose the trachea. Gently pass curved surgical forceps underneath the trachea and pull a piece of thread underneath.

4.6.3) Perform tracheostomy between two tracheal rings with sharp scissors taking care not to cut through the trachea.

4.6.4) Insert the 18 g cannula that was used to calibrate the instrument. Fasten the cannula to the trachea with thread and attach the mouse to the Y-tubing of the instrument adaptor with the cannula.

4.7) Perform a deep inflation to recruit the airways and help the animal succumb to the ventilator. Select the “mouse inhaled dose response v7.0” script with default ventilation at 150 breaths/minute.

4.8) Add saline followed by each incremental dose of A β M to the nebulizer when prompted to perform a dose response curve.

4.9) At the conclusion of the data acquisition after the highest A β M dose, disconnect the mouse from the ventilator and humanely euthanize the animal by performing a cervical dislocation. Proceed to tissue harvest.

4.10) Add saline to the nebulizer and activate the nebulizer to remove any remnant high dose A β M. Change the subject in the software program and proceed with next mouse beginning with a tube calibration (step 4.1-4.3).

5. Tissue harvest

5.1) Spray euthanized mouse with 70% ethanol and open the thoracic cavity. Open diaphragm and perform bronchoalveolar lavage (BAL) with 1 mL of sterile PBS injected through the 18 g cannula and gently draw out repeating once for a total of 2 mL.²⁴ Place the sample in ice.

5.2) Harvest the cardiac and diaphragmatic lobes of the right lung for RNA work, and azygous and apical lobes for protein work, place in separate 5 mL snap-cap tubes and snap freeze in liquid nitrogen.

5.3) Harvest the left lobe using blunt forceps and scissors taking care not to damage/slice through the lobe. Fix the lung *ex vivo* with 10% normal buffered formalin with a 30 g needle. Store in 10% normal buffered formalin until histological analyses.

Note: When animals are taken through the model for determination of viral burden in the lungs, harvest whole lungs after euthanasia and snap freeze in liquid nitrogen.

6. Tissue processing for Flow Cytometry

6.1) Centrifuge the BAL contents at 600 $\times g$ for 10 mins at 4°C and save the BAL fluid for protein assays at -80°C until use.

6.2) Perform red blood cell lysis if necessary noting that cells are more susceptible to death after viral infection. Perform red cell lysis by adding 8 parts cold distilled water and pulse-vortexing for 30 seconds followed by a one-minute incubation at room temperature. Add one part 10 \times PBS, mix to neutralize the water and centrifuge as above.

6.3) Re-suspend the BAL cells in 200 μ L of staining media (sterile PBS with 5% FBS) and enumerate cells by trypan blue dye exclusion assay to determine the cell concentration.

6.4) Cytospin 50,000 cells as follows:

6.4.1) Transfer the calculated volume of cells into a tube and bring volume up to 100 μ L with sterile PBS. Assemble the labeled slide and cytofunnel in the metal clip and place in the centrifuge.

6.4.2) Add the diluted cells into the cytofunnel and spin at $55 \times g$ for 5 minutes to affix the cells to the slides.

6.4.3) Perform differential staining by sequentially dipping the slides 20 times each in methanol, xanthene, and thiazine followed by distilled water and air-drying.

6.5) Add 1 μL of human gamma globulin for every 10,000 cells in the cell suspension in step 6.3 and incubate in ice for 30 mins. Adjust volume to 1 mL with staining media and centrifuge at $600 \times g$ for 10 mins at 4°C and aspirate supernatant. Re-suspend cells in 100 μL of staining media and prepare four pooled samples with 10 μL from each sample. Adjust volumes to 200 μL and centrifuge as above.

6.6) Re-suspend cells in 50 μL of antibody cocktail containing fluorochrome-conjugated antibodies diluted in staining media (see table of materials). Re-suspend one of the pooled samples in 50 μL of staining media, one pooled sample in 50 μL of isotype control antibodies, and the remaining pooled samples with diluted antibodies individually. Incubate samples in ice protected from light for 30 mins.

Note: The use of antibodies will be based on research interests and should be optimized previously for the experiment. Each antibody used should be used individually in a pooled sample for instrument set up. The use of bone marrow cells or splenocytes for single color controls may be necessary if the cell yield is low and is acceptable if fluorochrome-conjugated antibodies are made against an abundant marker such as B220.

6.7) Add 450 μL of staining media and centrifuge at $600 \times g$ for 10 mins at 4°C . Aspirate supernatant and repeat.

6.8) Aspirate supernatant and re-suspend cells in 50 μL of freshly prepared stabilizing fixative by gentle pipetting. Add 200 μL more of stabilizing fixative and strain through a 40 μm nylon mesh into a polystyrene FACS tube for analysis by flow cytometry.

Note: Whole lungs may be digested to make single cell suspensions and used to analyze cell populations by flow cytometry.

7. Tissue Processing for RNA Analysis

7.1) Place frozen lung lobes in ice within a fume hood, and perform standard Trizol extraction of RNA according to manufacturer's recommended protocol. After DNase treatment and purification of RNA, convert 1 μg of RNA to cDNA.

7.2) Using RNA-specific validated primer sets, measure changes in gene expression with quantitative real-time PCR and analyze data using the $2^{-\Delta\Delta\text{Ct}}$ method normalized to the internal housekeeping gene²⁵ validated for lungs.

8. Tissue processing for Viral Titration

8.1) Place frozen lungs in ice and add 1 mL of sterile PBS and mechanically homogenize tissue until entire tissue is pulsed. Centrifuge samples at 600 $\times g$ for 10 mins at 4°C and store supernatants in aliquots at -80°C until use.

8.2) Quantify virus using standard tissue culture infectious dose 50 (TCID₅₀) method²⁶ with MDCK.2 cells.

REPRESENTATIVE RESULTS:

The development and characterization of mouse models that recapitulated patient subsets with asthma and influenza is described. Such models are important in understanding host-pathogen interactions that occur during influenza virus infections in hosts that have underlying disease in the pulmonary system which alters the immune response to the invading virus.

Fungal asthma model

This model has been extensively tested and validated in both genders of BALB/cJ mice and C57BL/6J mice. The fungal asthma model was designed by exposing animals to small doses of whole allergen over a period of time and finally challenging the animals with unmanipulated clinically relevant allergen via the inhalation route thereby mimicking a natural allergen in a natural mode of entry as in humans (**Figure 1**). There will be no physical signs of illness in mice after this model and disease pathogenesis must be determined by tissue harvest and processing. In order to determine the success of the model, investigators should perform BAL to harvest cells in the airways and verify the presence of eosinophils and CD4⁺ T cells at days 3, 7, 14, and 28 after the second fungal challenge. Peribronchovascular inflammation will be apparent in hematoxylin and eosin staining with peak levels at day 3 tapering over time, but still apparent around the large airways at day 28. Mucus producing goblet cells can be visualized in the airways after periodic acid Schiff's staining. Airway wall remodeling events including subepithelial fibrosis and smooth muscle hyperplasia can be noted after trichrome staining starting as early as day 14. In addition, investigators are encouraged to quantify total IgE in the serum as a marker of allergy in these mice. The reader is encouraged to refer to previous publications which discuss this model of fungal asthma in more detail.^{6,7,9}

Models of asthma and influenza comorbidity

These models were established and characterized for both genders of C57BL/6J strain. The models were built using the fungal asthma model as a base. The difference between the two models is the state of the allergic airways at the time of viral infection. In the acute asthma and influenza model, mice are infected with the pH1N1 influenza strain one week after the second fungal challenge (**Figure 2**) at which time the eosinophilic inflammation is at its peak. Mice are infected with the pH1N1 influenza strain four weeks after the second fungal challenge in the chronic asthma and influenza model (**Figure 3**) at which time the allergic inflammation has

largely resolved and airway remodeling is apparent. Weight loss is a marker of influenza morbidity in mice and investigators must weigh animals before infection and everyday thereafter until the termination of the experiment to ensure disease morbidity.

Airway hyperresponsiveness in the models of comorbidity

Changes to the mouse airways and tissues can be measured using a computer controlled small animal ventilator system. While we only performed the SnapShot and Primewave perturbations, investigators can perform the PV Loop perturbations with this system if desired. It may not be possible to perform a dose curve after influenza infections in mice with preexisting asthma at early time points as the airways do not always return to baseline at the end of the script. Investigators may need to modify the script to add more maneuvers or opt to test one pre-selected dose of A β M. Data presented in **Figure 4** are representative of data generated from Primewave perturbations after 25 mg/mL A β M. Influenza infection results in increased airways resistance, tissue damping and elastance.

Cell infiltration into the airways differs between the two models of asthma and influenza comorbidity

Typically, the acute asthma and influenza model has more robust cell infiltration as the viral infection occurs at the peak of allergic inflammation. The reduced cell number in the chronic asthma and influenza model is likely because the viral infection occurs after the resolution of the allergic inflammation in the lungs. Eosinophils are prominent in the acute asthma and influenza model throughout the viral kinetic cycle, while neutrophils dominate the airways in the virus-only controls (**Figure 5**). Since viral clearance of influenza is dependent largely on the activation and recruitment of CD8⁺ T cells, their presence in the airways should be noted. Representative data show the large number of red blood cells in the airways after BAL in influenza infections as well as sloughed epithelial cells and vacuolated immune cells at 7 days after virus infection. More CD8⁺ T cells are recruited into the airways in the acute asthma and influenza model compared to the chronic asthma and influenza model (**Figure 5**). The reader is referred to our previous publication for a representation of cell types that can be analyzed using this model.²⁷ While our analysis was limited to the cells in the BAL compartment, whole lungs can be digested into single cell suspensions to analyze cell populations by flow cytometry.

Influenza virus infection causes weight loss in mice

Each animal should be weighed immediately before the infection and every 24 hours thereafter until the termination of the experiment. Weight loss will be noted in the control animals gradually over time with a peak weight loss at day 7. Animals in the acute asthma and influenza model will not lose weight while those in the chronic asthma and influenza model will (**Figure 6**). This is a contrasting yet consistent event. Both groups of animals in the models will have viral replication in the airways that is equivalent to the flu-only controls. There will be significantly less virus in the lungs of mice in the acute asthma and influenza comorbidity group at day 7 (**Figure 6**). The mean viral titer in the lungs of flu-only control animals at day 8 was

quantified at $\log_{10}4.366$ which is equivalent to the viral titers of acute asthma and influenza group at day 7 indicating that viral clearance was enhanced in acute asthma. Since disease pathogenesis was investigated, a non-lethal dose was selected based on a mean weight loss of 15% of original body weight. The weight loss cutoff was set at 30% in these studies per IACUC, and none of the animals reached this cutoff with the infectious dose used. The use of 20-times higher viral load of A/CA/04/2009 caused a maximum mean weight loss of 25% in control animals before recovery and mice in the co-morbidity group were protected.

Figure Legends:

Figure 1: Schematic representation of fungal asthma model. Mice are acclimatized for a week and injected subcutaneously (SQ) and intraperitoneally (IP) with *Aspergillus fumigatus* antigen adsorbed in Alum. After two weeks, *A. fumigatus* antigen diluted in PBS is administered intranasally (IN) once weekly for three weeks. After the final IN sensitization, anesthetized mice are exposed to live *A. fumigatus* conidia via inhalation (IH) route. Photographs show the inhalation chamber, arrangement of mice, and appearance of the fungal culture.

Figure 2: Schematic representation of acute asthma and influenza model. Mice are subjected to the fungal asthma model and infected with 1000 TCID₅₀ A/CA/04/2009 intranasally (IN) one week after the second inhalation (IH) challenge. Mice are weighed immediately before the viral infection and daily afterwards for nine days and sacrificed at predetermined time points after the viral infection. Age-matched female C57BL/6 mice are infected with virus and serve as the influenza-only controls.

Figure 3. Schematic representation of chronic asthma and influenza model. Mice are subjected to the fungal asthma model and infected with 1000 TCID₅₀ A/CA/04/2009 intranasally (IN) four weeks after the second inhalation (IH) challenge. Mice are weighed immediately before the viral infection and daily afterwards for nine days and sacrificed at predetermined time points after the viral infection. Age-matched female C57BL/6 mice are infected with virus and serve as the influenza-only controls.

Figure 4. Pulmonary function test representing differences between the two asthma and influenza comorbidity models. Intubated animals were attached to a computer controlled small animal ventilator at a default ventilation rate of 150 breaths/minute. Software generated data after 25 mg/mL acetyl-beta-methylcholine challenge were recorded. The average of the dataset for each animal was used to calculate the average and standard error of the mean in each group for each time point. Influenza infection caused an increased in airway resistance (A), tissue damping (B), and tissue elastance (C). Acute asthma adds further to these responses while chronic asthma causes a reduction in these parameters. * $p < 0.05$ by Unpaired Student's *t*-test with Welch's correction. This Figure has been modified from Samarasinghe *et al.*, *Immunology and Cell Biology*, 2014²⁷ and reproduced with permission from Nature Publishing Group.

Figure 5. Analysis of airways inflammation in the two asthma and influenza comorbidity

models. Cells from the bronchoalveolar lavage were enumerated, cytospun, and stained for flow cytometric analysis. Representative cytospin images from both models at the day 7 time point show that flu-only controls have sloughed airway epithelial cells in addition to immune cells and most cells are highly vacuolated indicative of cell death (A). The mean cell number for the groups by Trypan Blue dye exclusion assay (B) and the number of CD8⁺ T cells in the airways by flow cytometry (C) are shown. Data represent the mean and standard deviation in each group for each timepoint. *p<0.05 by Unpaired Student's *t*-test with Welch's correction. This Figure has been modified from Samarasinghe *et al.*, *Immunology and Cell Biology*, 2014²⁷ and reproduced with permission from Nature Publishing Group.

Figure 6. Determination of influenza morbidity in mice. Each mouse is weighed immediately prior to and every 24 hours after infection with 1000 TCID₅₀ A/CA/04/2009 influenza virus. Animals that are naïve to allergen and virus do not lose weight as well as mice in the acute asthma and influenza comorbidity model (A). A gradual reduction in body weight is observed in animals infected with the virus and those in the chronic asthma and influenza comorbidity model (B). Replicative influenza virus is present in the lungs from days 1-7 with viral clearance by day 9 (C&D). Data represent the mean and standard deviation in each group for each timepoint. *p<0.05 by Unpaired Student's *t*-test with Welch's correction. Flu=influenza; AA=Acute Asthma; CA=Chronic Asthma; ctr=control. This Figure has been modified from Samarasinghe *et al.*, *Immunology and Cell Biology*, 2014²⁷ and reproduced with permission from Nature Publishing Group.

Figure 7. Asthma and influenza comorbidity with a mouse adapted influenza A virus. Mice were taken through the fungal asthma model and infected with 1000 TCID₅₀ A/PR/08/1934 (PR8) influenza virus one week after the second fungal inhalation challenge. Age-matched animals were infected with the same dose of virus as the virus-only controls. Animals were weighed prior to and every 24 hours after infection for 12 days. The peak mean weight loss in mice in the comorbidity group was 8% compared to 27% in mice infected with virus alone. *p<0.05 by two-way ANOVA.

DISCUSSION:

The high incidences of allergic asthma and influenza increase the likelihood that these two diseases would occur simultaneously in the same individual. While some viruses are clearly demonstrated to be causative agents initiating the pathogenesis of asthma,^{28,29} the impact of influenza virus infections on either the development or exacerbation of asthma is unclear. The association and the importance of studying influenza viruses in the context of allergic disease became evident when asthma was identified as a risk factor for hospitalization during the 2009 influenza pandemic.¹³ The goal of this study was to develop a mouse model system that can recapitulate two subsets of patients with allergic asthma, those undergoing exacerbation and those with underlying disease.

Interestingly, animals infected during allergic asthma exacerbation with elevated eosinophilic inflammation and goblet cell metaplasia were protected against influenza morbidity marked by

no change in body weight and enhanced viral clearance. Mice that were infected after the resolution of airways inflammation and initiation of airway remodeling events were susceptible to influenza disease morbidity and had higher viral load in the lungs. There was clear evidence of airway epithelial damage in the chronic asthma and influenza model which was absent in the acute asthma and influenza model.²⁷ Influenza virus infection alone was sufficient to induce airways hyperresponsiveness in mice suggesting that influenza virus may cause airway reactivity and wheezing similar to other respiratory viruses. These data clearly show that the state of the allergic airways at the time of influenza virus infection determined the outcome of the host's response to the invading virus.

Influenza virus is not a natural mouse pathogen and as such, careful titrations should be performed to determine the dose which induces the disease state that one wishes to investigate. The virus strain used can be changed depending on the investigator's research question. In this study, the A/CA/04/2009 virus strain was selected because the interest was on how the 2009 pandemic influenza impacts pre-existing asthma. Using mouse adapted influenza A virus, A/PR/08/1934, demonstrated that the protective effect of acute allergic asthma was not unique to A/CA/04/2009 (**Figure 7**). When using a different strain of influenza virus, preliminary studies must be performed with influenza infections alone to determine the dose that results in a mean maximum weight loss of 15% which generally results in a non-lethal infection.

Similarly, mice do not develop asthma. In order to induce fungal allergic inflammation that results in the development of human disease characteristics, each step in the asthma model must be performed with precision because variations in allergen doses and exposure times may induce immune tolerance instead of allergic sensitization. Since the fungal asthma model takes eight weeks to complete, influenza-only control animals should be age-matched to be at 15 and 18 weeks of age at the time of infection. Viral disease pathogenesis is different in younger mice (typically 6-8 weeks at the time of viral infection) compared to older mice (16 weeks and above). Therefore, this model is not suitable to study the asthma and influenza comorbidity in younger mice. As shown here, there are differences in body weight change and viral replication and immune responses between 15 and 18 week old mice after influenza virus infection. These differences are likely because these ages fall at the exponential decline of thymic involution in mice.³⁰ The limitations of the current model system include the length of time (9 or 12 weeks to infection), the technical and environmental challenges of handling *A. fumigatus*, and the inability to study comorbidity in mice younger than 15 weeks. However, the make-up of this model system can be adapted using other allergen models (house dust mite or cockroach antigen) and viruses (various influenza A strains and RSV) thereby resulting in the ability to model asthma and influenza comorbidity in mice in a shorter duration of time.

Most existing mouse models of asthma and influenza were designed to investigate whether influenza virus can trigger allergic asthma.³¹ Seasonal influenza virus has been shown to trigger airway reactivity in mice by inducing the activation of type 2 innate lymphoid cells.²¹ In contrast, influenza virus infection prior to allergen challenge has reduced eosinophilia.³² Mice with house dust mite-induced asthma infected with influenza A virus³³ had increased

inflammation and mucus production which is a contrasting observation to the model described here. Therefore, the immune response of an allergic host to influenza virus may depend on the allergen and the timing of the viral infection in relation to the allergen. This model can be modified to investigate immune memory responses to influenza virus, responses to heterotypic viral infections, and also can be used to investigate co-infections with other respiratory pathogens. Our future studies are aimed to delineating pathways involved in bacterial co-infections of hosts with asthma and influenza.

While asthma was a risk factor for hospitalization during the 2009 pandemic, retrospective studies showed that asthmatics were less likely to require mechanical ventilation and develop pneumonia and other complications of influenza compared to non-asthmatics. Previous vaccinations, early medical care, and steroid therapies³⁴ have been proposed as reasons for this seemingly contradictory finding. The underlying mechanisms of viral infections in the airways of “healthy” hosts are not yet fully understood. Furthermore, the presence of virus in the airways does not necessarily mean that it is having a negative impact or causing disease as indicated by the presence of rhinovirus in healthy humans.³⁵ Host genetics, age, lung volume, nutritional status, and other underlying conditions may all have an impact on how an individual responds to influenza virus. This model of asthma and influenza comorbidity, suggest that infections during allergic inflammation may protect the host from virus-induced host pathology while infections of an allergic host with resolved inflammation but undergoing airway remodeling is less efficient at clearing the virus and more susceptible to virus-induced structural damage. These models can be used to investigate innate and adaptive immune responses as well as structural changes that occur in allergic hosts infected with influenza virus.

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DISCLOSURES:

The authors have nothing to disclose.

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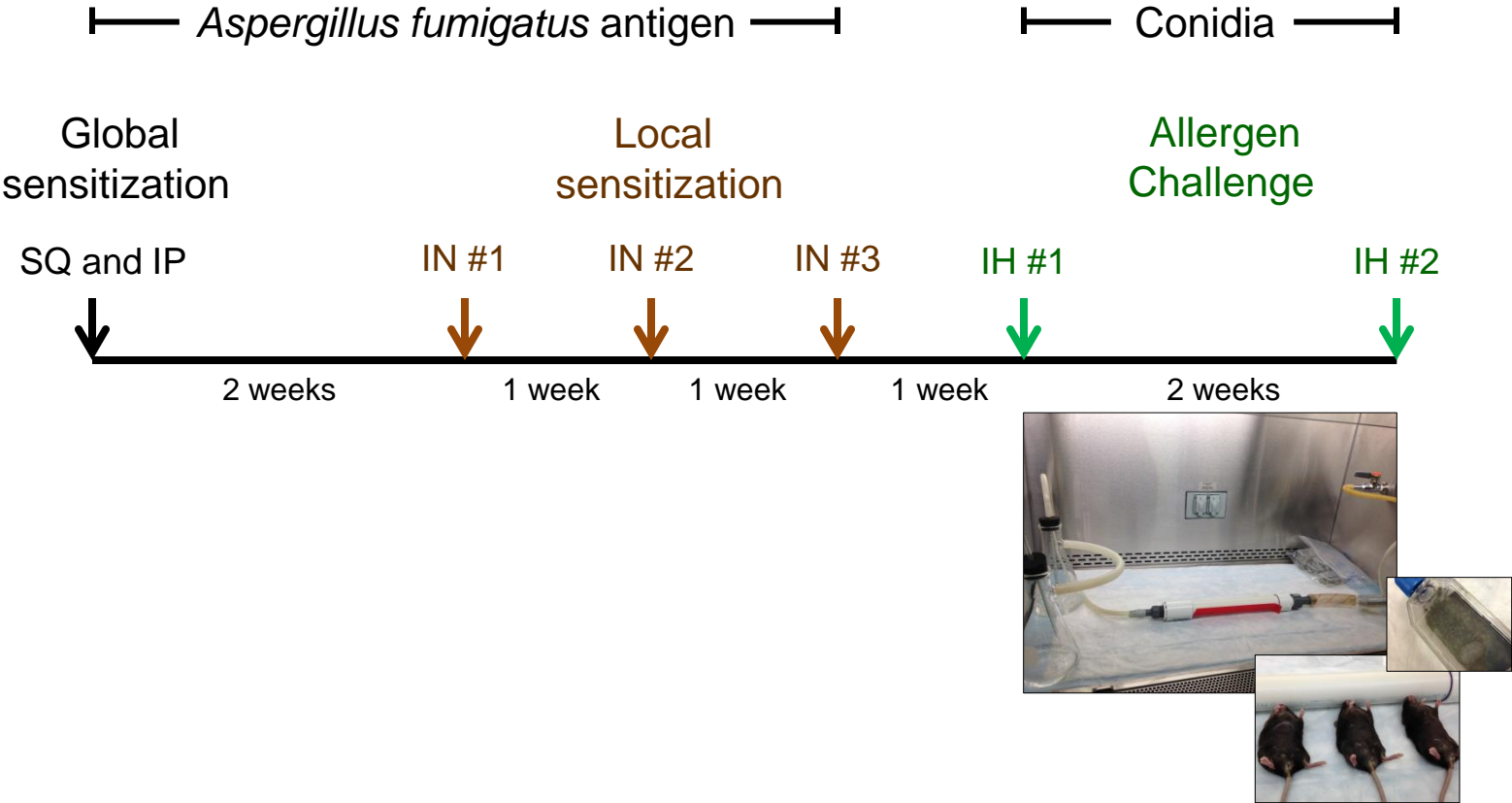


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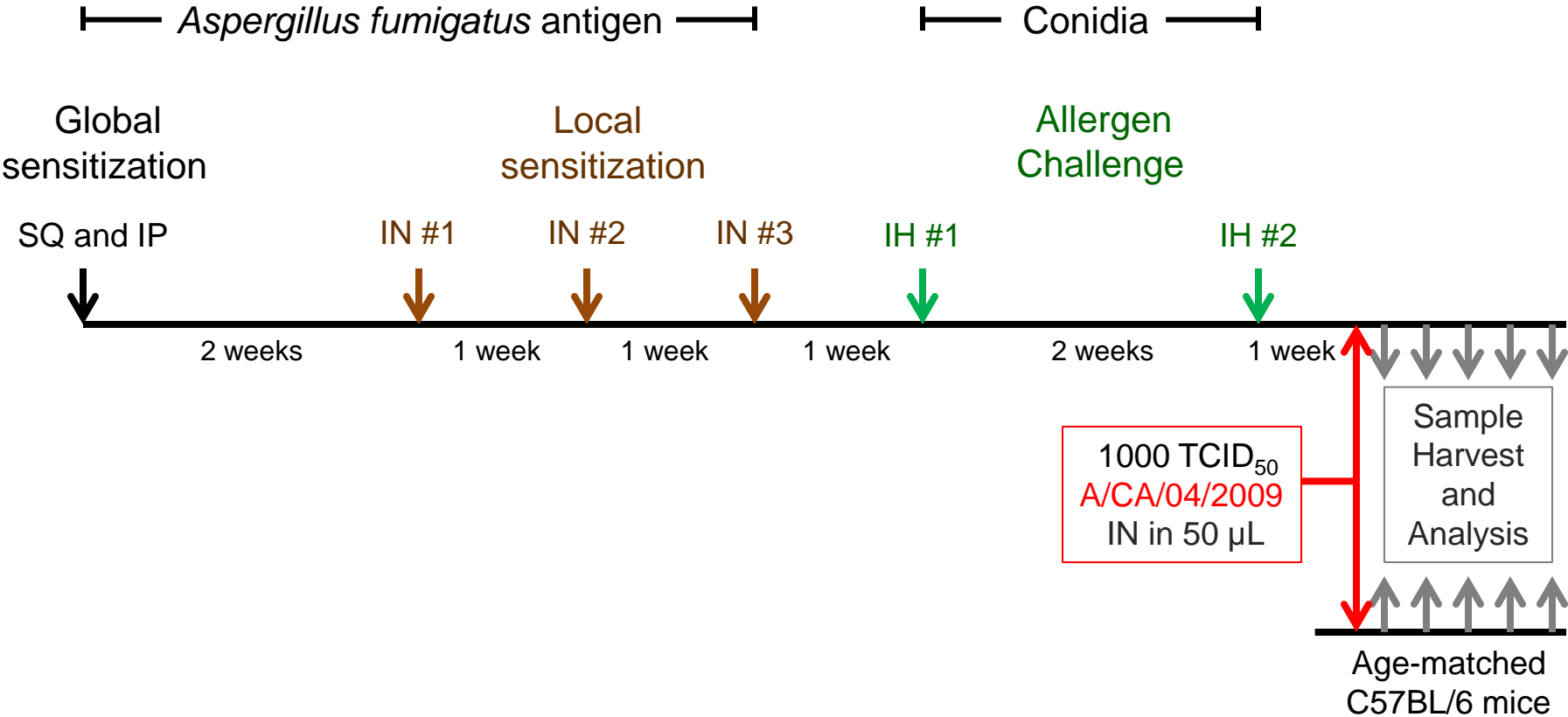


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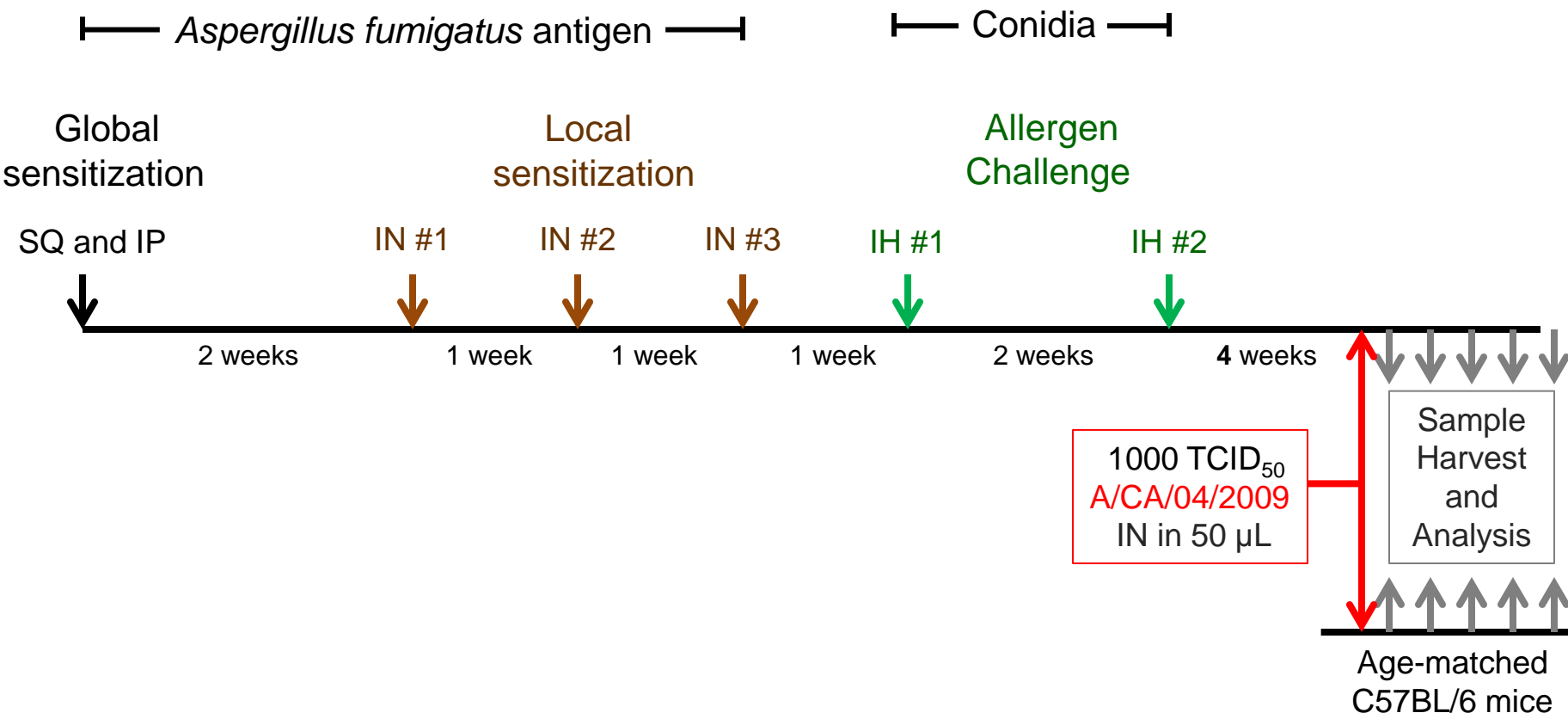


Figure 4

Acute asthma + Influenza

Chronic asthma + Influenza

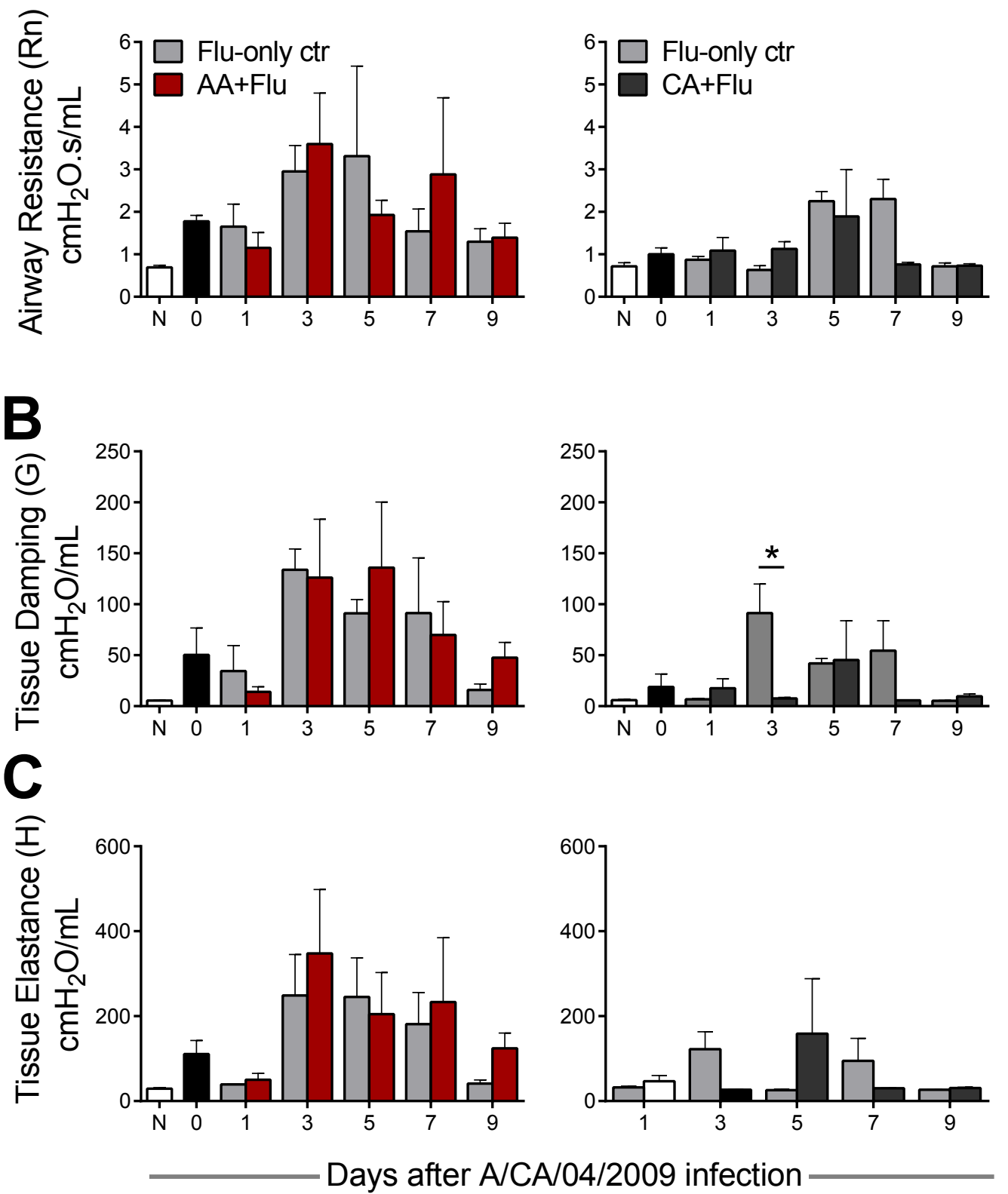


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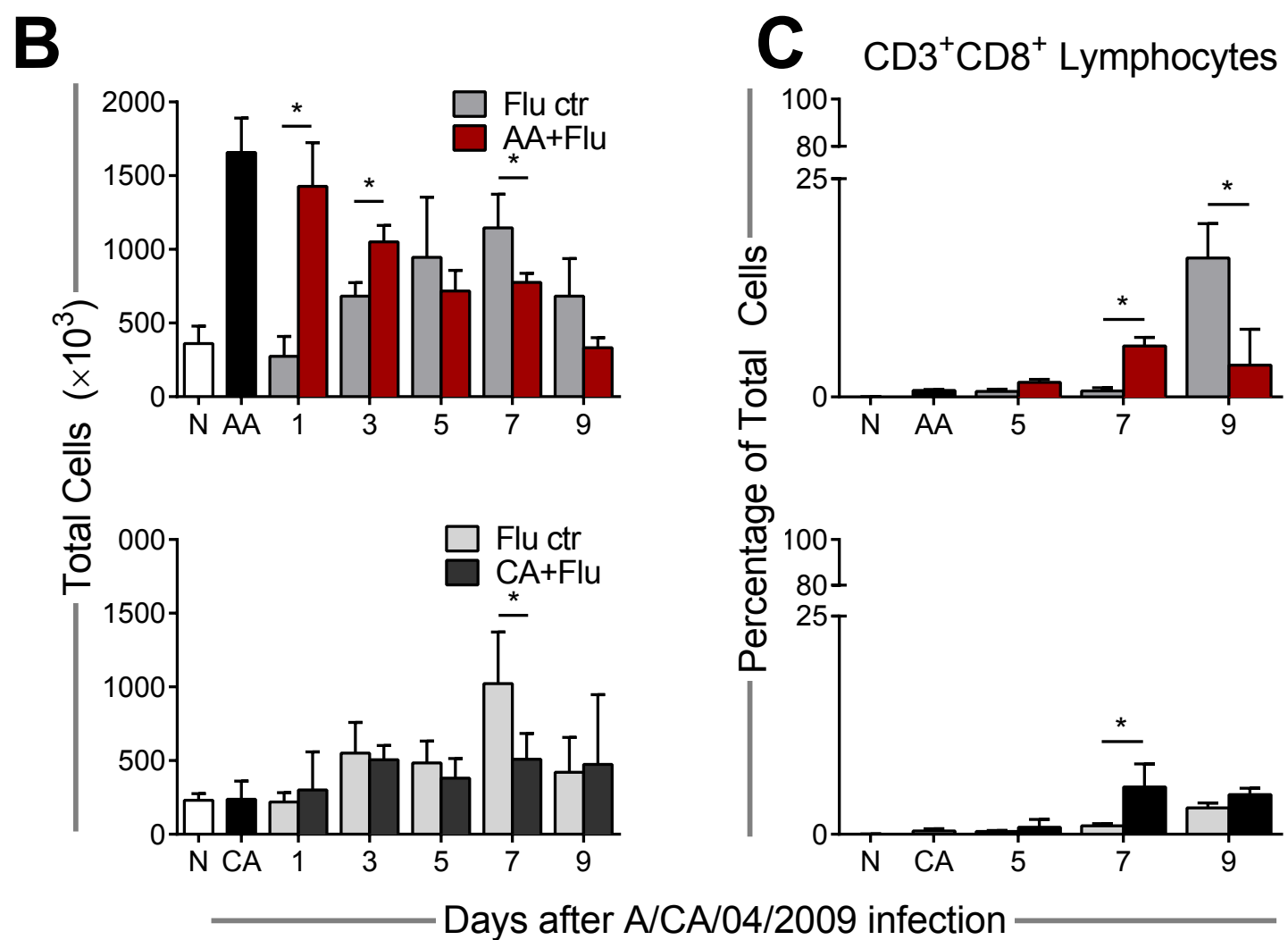
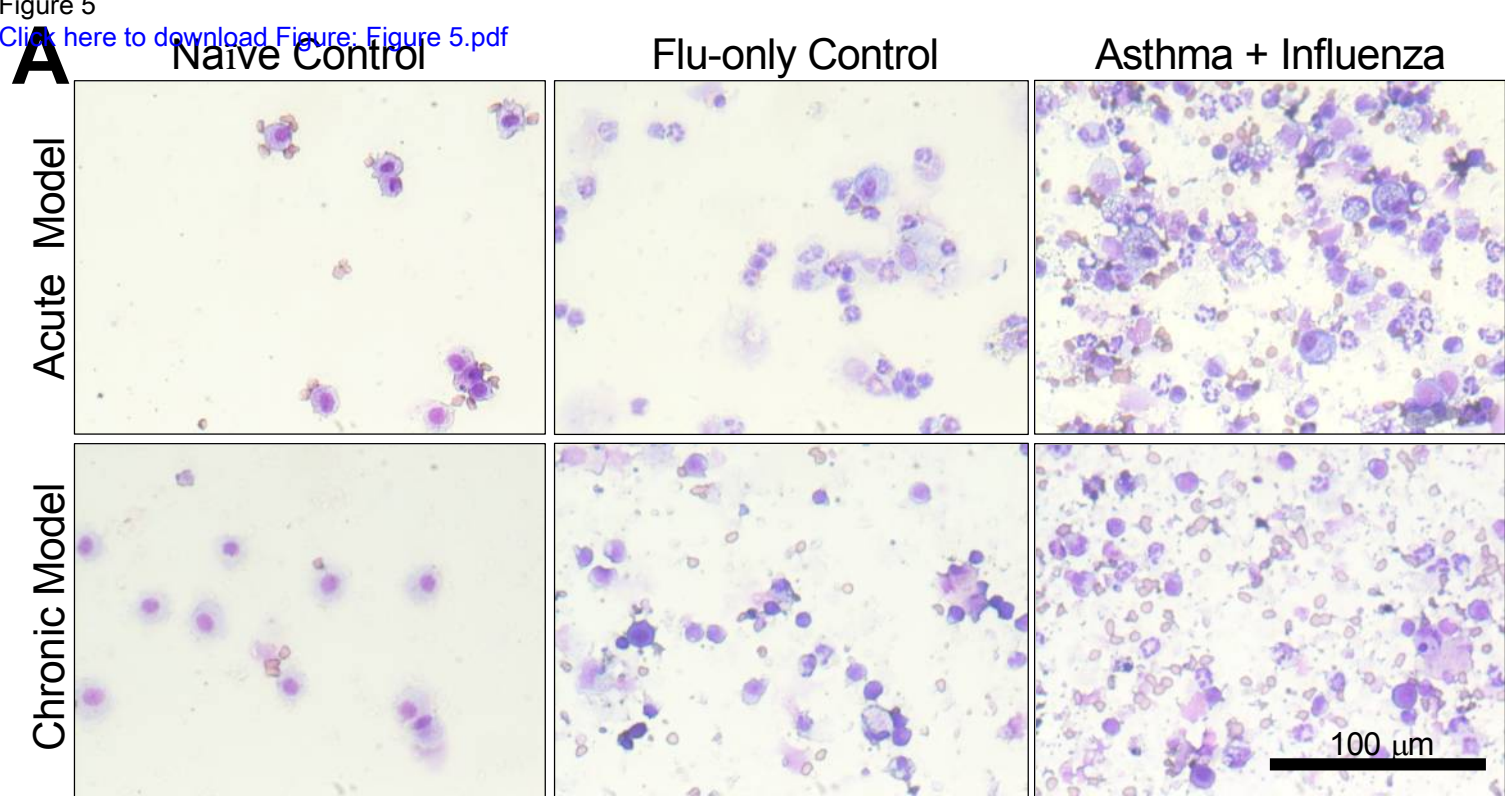


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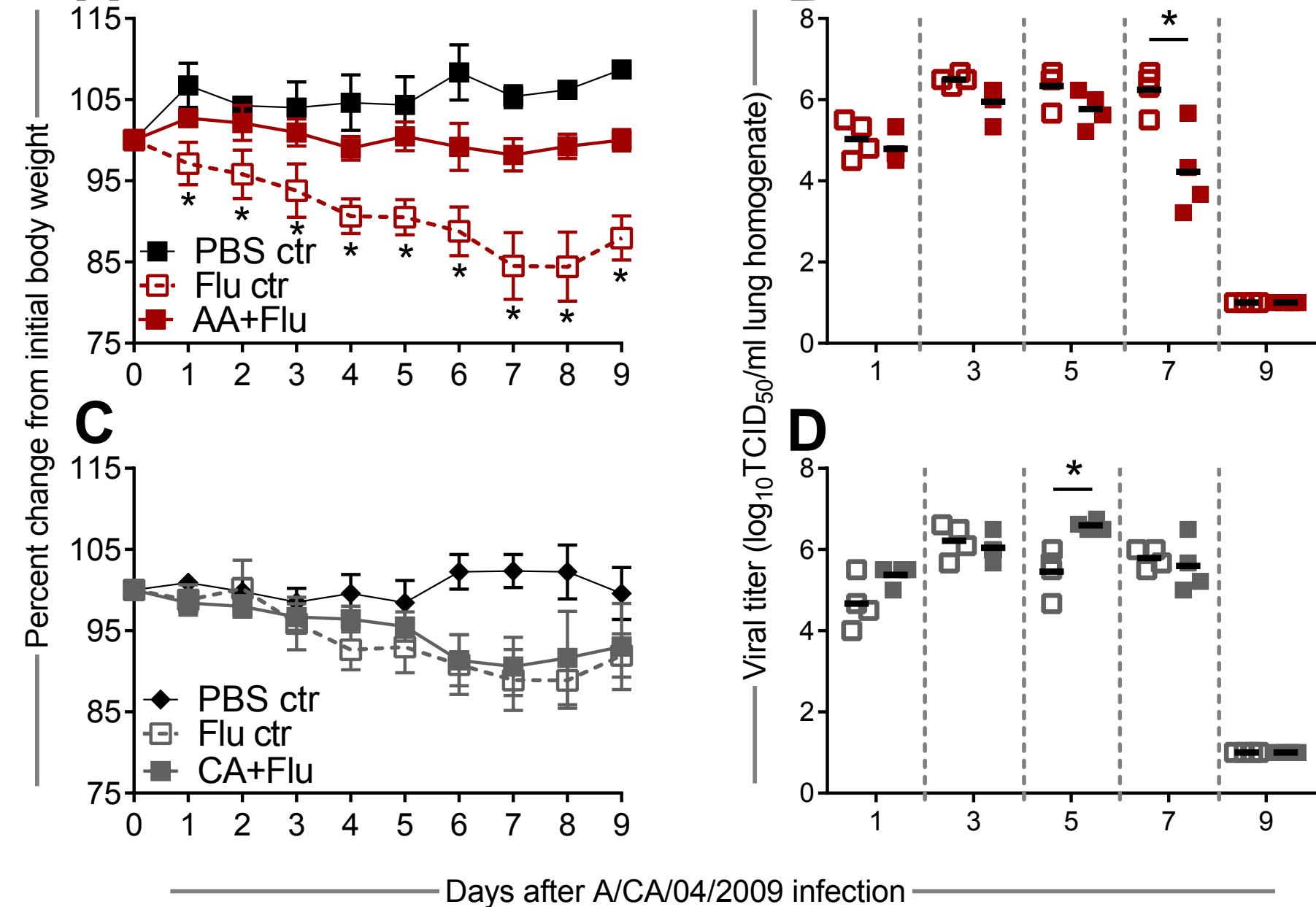
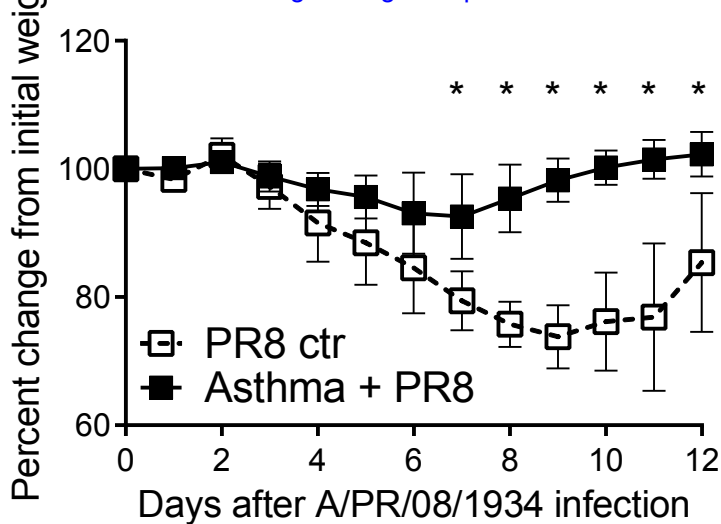


Figure 7

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
<i>Aspergillus fumigatus</i> antigen	Greer Labs	XPM3D3A25	
<i>Aspergillus fumigatus</i>	ATCC	NIH 5233	
Imject Alum	Pierce	77161	
Difco Sabouraud dextrose agar	BD	210950	
Spore-Klenz	Fisher Scientific	04-324-20	
Ketamine	Hospira	0409-2051-05	
Xylazine	Akorn Inc.	59399-110-20	
Isothesia (Isoflurane)	Butler Schein	05260-04-09	
Euthasol	Virbac	710101	
Acetyl-β-methylcholine	Sigma Aldrich	A2251	
<i>flexiVent</i> FX1	SCIREQ	FV-FXM1	
Nebulizer (standard mist)	SCIREQ	ANP-1100	
flexiWare Software	SCIREQ	version 7.0.1 rel B	
Quik-Dip 3 Part Differential Stain	Mercedes Medical	MER 1002	
Human gamma globulin	Sigma Aldrich	G4386	
Rat anti-mouse CD3ε-PE-Cy7	BD Biosciences	552774	Clone: 145-2C11; Diluted 1:50
Hamster anti-mouse CD8α-FITC	BD Biosciences	553031	Clone: 53-6.7, Diluted 1:50
FITC Rat IgG2a	BD Biosciences	553929	Diluted 1:50
PE-Cy7 Armenian Hamster IgG	BD Biosciences	400922	Clone: HTK888; Diluted 1:50
Stabilizing fixative	BD Biosciences	338036	Freshly prepare
MDCK.2	ATCC	CRL-2936	
TRIzol Reagent	Life technologies	15596-026	
Chloroform	IBI Scientific	IB05040	
iScript	Bio-Rad	170-8891	
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Dear Dr. Zaman,

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•Additional detail is required:

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We have included this in the text on page 2.

-6.4.2 – Centrifuge speeds should be listed in terms of centrifugal g force where possible.

We have converted the cytospin speed to g force on page 8.

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This manuscript builds on earlier work by the authors published in 2014 in Immunology and Cell Biology (Samarasinghe et al., Immunol Cell Biol. 2014 May-Jun;92(5):449-59. doi: 10.1038/icb.2013.113. Epub 2014 Jan 28). In the earlier study, the authors described development of a mouse model of allergic asthma mediated by *Aspergillus fumigatus* and its influence on influenza comorbidity. Based on their comorbidity model, they concluded that acute allergic airway disease is protective against influenza while chronic asthma is detrimental to the host during infection with the 2009 pandemic influenza A virus. In the current method manuscript, they explain in greater detail the procedures used in this prior work. My major concern is the re-use of previously published data. For example:

- 1) Figure 4 of this manuscript is a somewhat different representation of the same data shown in Figure 2C of the Immunology and Cell Biology paper,
- 2) Figure 5 B&C is the same as Figure 3A&B of the Immunology and Cell Biology paper and
- 3) Figure 6A-D is the same as Figure 2B of the Immunology and Cell Biology paper.

The previously published results are not appropriately cited. I would highly recommend that the authors at least acquire permission from the original journal (Immunology and Cell Biology) for the re-use of published data.

We thank the Reviewer for his/her concern and dedication to scientific integrity. According to *ICB* guidelines, the author maintains the right to the paper. However, I had obtained a written consent to re-use figures from ICB before we began to work on this paper. I have included a copy of this e-mail in the Supplemental Document section of the submission site. Also, we have extended the citations in the Figure Legends to draw the readers' attention to this detail.

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

This manuscript describes the development of complex protocol to model asthma exacerbations in human. In particular, a model for allergic airway inflammation induced by *Aspergillus fumigatus* is combined with the 2009 pandemic H1N1 influenza strain A/CA/04/2009. The authors describe the analysis of pulmonary function as well as airway inflammation in two separate asthma models (acute, chronic) in combination with influenza.

Major Concerns:

This protocol is clear and well-written. In general it contains sufficient detail and is largely based on a recent good quality publication from the same group (Samarasinghe, et al., Immune Cell Biol 2014). However, the protocol is very specific and thus the general applicability is difficult to judge. In this context, I have a few major concerns.

- The authors should explain how the protocol can be adapted for other influenza strains, e.g. the X31 or PR/8 that are also often used in mouse models.

We have added morbidity data with A/PR/08/1934 as a supplemental figure to show that the protection mediated by acute asthma holds true for mouse adapted influenza A virus as well. We have added more text to elaborate this point in the manuscript on page 13.

- The manuscript should give recommendations for adaptation to models for other allergens and should also explain limitations.

We thank the Reviewer for raising this point. We are not experienced in other allergen model systems such as house dust mite or cockroach antigen models. We have done preliminary studies with the ovalbumin model although we were unable to get chronic allergic responses with the OVA model as the inflammation resolves when OVA challenge is ceased. Therefore, we cannot practically provide recommendations for adaptations to other allergens. However, we did add text to note the possibility for those already working with these model systems (page 13).

- It is generally known that there can be large differences between batches of allergen or virus preparations. How are these tested? What is the virus titer that the authors recommend based on? The maximum weight loss is ~15 % at day 7-8. Because there is quite some variation, how do the authors deal with mice that have > 20% weight loss?

This is indeed true. We tackle this potential variable by always purchasing the fungal antigen from Greer, and we purchase large quantities from the same lot number to have sufficient antigen for repeat experiments. The fungal antigen is dissolved to 1 mg/mL by protein quantity provided by the manufacturer.

We avoid mutations from occurring in the virus by growing large batches of virus in MDCK.2 cells instead of eggs. We have found that A/CA/04/2009 mutates in three antigenic sites in the hemagglutinin binding pocket when it is cultured in eggs. Culturing the virus in MDCK.2 cells prevents mutations from occurring in the HA and NA proteins. Once our large stocks (500 mL) are grown and sequence verified to be void of mutations, we freeze them down in aliquots thereby having sufficient virus from the same batch to use for a series of experiments.

The recommended viral titer was based on preliminary studies with influenza infection alone wherein we detected a mean weight loss of 15% in control mice. Our stock of A/CA/04/2009 virus does not lead to fatal infections even at $\log_{10} 4.3$ (20,000 virus particles per

mouse). Since the SJCRH weight loss cut off was set to 30%, we noted that the few animals that reached a nadir of 20% also recovered by the next 24 hours.

- The immunological analysis is quite limited. It would be helpful to provide FACS staining details for all individual myeloid cell populations in BAL. Also a method to make single-cell suspensions from lungs should be included.

We thank the Reviewer of his/her comment. We showed the grating strategy and cocktails of antibodies used for markers in our *Immunology and Cell Biology* paper. Since this is a methods paper, we only discussed the identification of CD8⁺ T cells as an example of cell staining and analysis. In our previous publication we did not analyze cell population in whole lung which is the reason we omitted a method for preparing single cell suspensions from lung tissue. We use the Miltenyi GentleMACS method to prepare single-cell suspensions from lungs and spleens. If the Reviewer and Editor would like us to add a method for this, we are certainly able to. We added text to indicate this possibility (pages 8 and 10).

Minor Concerns:

Figure 1. The authors describe an analysis of BAL at several time points after the second fungal challenge (3, 7, 14, 28 days). It would be helpful if this is included in the figure.

This is a valid point raised by the Reviewer. The reason we have omitted these data is because we have already published the development of the fungal asthma model as indicated in the citations in the text (page 9) and because this manuscript was focused on the co-morbidity model.

Figure 6. To monitor virus clearance as accurately as possible, it is recommended to also include day 8 (Fig. 6B and 6D).

We agree with the Reviewer. While we have not titrated the virus in the lungs of asthma and influenza animals at Day 8, we have titered virus in the flu-only control mice at Day 8. When infected with 1000 TCID₅₀/mL A/CA/04/2009 C57BL/6 mice will have an average of log₁₀4.366±0.557 TCID₅₀/mL of lung homogenate. This is equivalent to the viral titer found in the co-morbidity group at Day 7. We have included this in the manuscript text (page 11). While we are able to perform this quantification at Day 8 in the comorbidity group, we would need more time than granted by the revision to do so as we will need to take mice through the model.

Best Regards,

Amali E. Samarasinghe

