**Rebuttal Letter to JoVE53269R2**

Dear Dr. Zaman,

We thank the Editor and the Reviewers for reading our manuscript and helping us improve the quality of our work. Please find herewith our responses to the Editorial and Reviewer comments. All Editorial and Reviewer comments are in blue text while our responses are in black.

**Editorial comments:**  
  
\* All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made formatting changes and minor copy edits to your manuscript. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. **Please use this updated version for any future revisions**.  
We have used the most recent version available on the JoVE submission site to make our corrections. All changes are in red in the “track changes” option of Word.

•Additional detail is required:

-Section 2: Please specify what BSL level is needed for these experiments.

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.

•Branding: 4.2 - flexiVent FX1, flexiware – These should be mentioned as an example of software that could be used.

We have noted this as suggested on page 5.

\* Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.  
We have done so, thank you.

\* If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

While this statement was already made in the Figure Legends when images were re-used, we have changed the verbiage to make it more noticeable.   
  
\* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

DOI were not included in two articles by endnote. We have manually added them (reference 24 and 32) and believe the list to be completed as required.   
  
**Reviewers' comments:**  
  
**Reviewer #1:**   
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 fumigates 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 log104.3 (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