Reviewer's report

Title: Successive influenza virus infection and Streptococcus pneumoniae stimulation alter human dendritic cell function

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Reviewer: Julie McAuley

Reviewer's report:

The authors have chosen to address the question as to whether dendritic cells contribute to the enhanced inflammatory response during consecutive infection with influenza, followed by S. pneumoniae. This viral-bacterial synergistic infection is a common cause of severe pneumonia resulting in hospitalisations and death. The data presented by the authors indicates that challenge of DCs with influenza virus and pneumococcus resulted in maturation of the DCs, upregulation of pro-inflammatory cytokines and down regulation of anti-inflammatory mediators in a time and dose dependent manner. The study however is limited to an in vitro viral-bacterial system and provides no mechanistic evidence of pathways activated in the presence of either pathogen. The data presented is novel as it uses human DCs and a human influenza isolate and may contribute to enhancing knowledge in the field of the detrimental effects of the exuberant responses to influenza followed by consecutive infection with bacterial pathogens. However, the authors must firstly address some major issues prior to publication:

Major Revisions

1. In the methods section in the abstract (and in the methods text), the authors mention that heat killed pneumococcus were used to mimic the viral pneumococcal infection. The results make no mention of whether these treated bacteria were utilized, but instead refer to the number of CFU that each system was exposed to, which indicates live bacteria were used. This needs to be clarified throughout the text as to what exactly was done.

2. It is not demonstrated whether the DCs infected with influenza yielded a productive infection. The methods section “virus preparation, titration and infection” indicates that DCs were infected with a particular MOI. However, it has been reported in literature that infection of DCs with some influenzas is abortive. This paper needs an experiment and a results section showing whether the particular strain used to infect DCs yields intact virions, or cannot produce virus. This would be a major consideration when interpreting results in a time course manner as is done by the authors.

3. In Figures 2a, 3a, and 4b for each time point listed, comparisons to mock infection must be made each time, not just a single mock infection, figures 3b, 3c, 5 and 6 have done this correctly.

4. Figure 2D, 5d, 5e, 5f, 5g, 5h and 6b are unclear. The way the axes are labeled
appears as though every treatment group has also received the indicated CFU of pneumococcus.

5. For Figure 1, n=4. How many times was this experiment repeated? Are the results indicative of several experiments? The first paragraph in the results section indicates marked increases, yet does not provide statistics. Figure 1 indicates fold-increase compared to mock control, whilst Figure 3 shows MFI – it would be good to keep a consensus between the graphs.

6. Figure 2C 6hour time point is identical to 2D 5x106. Whilst these treatment groups should be identical given the same conditions, were they truly different experiments and warrant graphing twice? Same argument for Figure 5A at 24hrs and 5G at 5x106 dose, as well as Figure 6A at 6hr and 6B at 5x106 dose.

7. The only time the methodology is clearly explained with respect to the different groupings is in the 2nd paragraph of the discussion. In the methods section under the heading “viral bacterial stimulation protocol” it is unclear as to which DC stimulation sentence refers to the concurrent or successive challenge. The sentence beginning with “To examine the dose effect of….” should include ‘6h post-infection with influenza’ at the end of the sentence. Similarly, “DCs treated with both influenza virus and pneumococcus” should include some terminology that the cells were infected concurrently. Also, there is no mention here as to whether the heat killed bacteria were used in any of the experiments as indicated in “bacterial preparation” and in the abstract.

8. The use of the word “exquisitely” in the first section of the results is incorrect.

9. In the results section “Successive challenge of influenza virus and pneumococcus dysregulated DC cytokine production” what is meant by negative dose response? What is meant by positive dose response?

10. In the 3rd paragraph of the section listed in point 8, there is overuse of the word synergistic and it is often used incorrectly. In this same paragraph, the discussion of the results indicate increasing bacterial dose increased the inflammatory response, whilst virus infection lowered the production of pro-inflammatory cytokines. However, figure 5 shows the most marked upregulation is when cells were infected with both virus and bacteria and not bacteria alone. It is unclear as to what the authors mean by “inverse dose response” and “direct dose response”.

11. In results section, last line of “successive challenge of influenza virus and pneumococcus caused a time related change in DC phenotype: the authors indicate there is a lack of change of DC activation markers with respect to dose, however this statement isn’t clear as to whether it didn’t change from the upregulated state, or whether it was not different from the mock controls.

12. Figure 2 indicates a marked upregulation of cell death at 6h post-co-infection with virus and bacteria, and Figure 5 indicates at this same time point and treatment regime that there is a marked increase in TNF, IFN and IL-2. There should be some discussion on the links between these inflammatory pathways and induction of cell death in the DCs and how these mechanisms could affect outcomes in vivo.
13. If heat treated bacteria were used in the successive and concurrent infection experiments were the results the same as for when live bacteria were used?

Minor Revisions:
1. 1st line in abstract Background, Introduction and Discussion should be “respiratory disease” not “respiratory diseases”
2. Monocyte DCs are commonly referred to in literature as MoDCs, not MDDCs. This nomenclature should be fixed in the text and wherever DCs are mentioned, it should state MoDCs.
3. The authors have often written “treated by” and “infected by”, this should be “treated with” and “infected with”.
4. 7th paragraph, 1st sentence in the discussion section: plastic means flexible, therefore the sentence needs changing.

Overall, the paper attempts to address whether DCs can induce the negative outcome of an exuberant inflammatory response when successively infected by influenza, followed by bacteria. It would have been nice to have included some mechanistic data as to how the bacteria and/or virus induced the effects observed (i.e. activation of TLR pathways?). Additionally, if the authors more clearly stated the reasons why they were looking at both cell death and inflammatory cytokine response would have given the paper more clarity. Perhaps they should state that they were examining the hypothesis that IAV induction of cell death in DCs rendered these cells ineffective? The reasons they are looking at both cell death and inflammation seems disjointed. The authors should also make the point that they are producing novel data that directly relates human DCs and a human influenza isolate, which makes the use of only an in vitro system somewhat more justifiable, as most data relating to this type of research is based on the mouse model. With completion of the major revisions and re-writing of the methods and results sections to increase clarity as suggested, I would recommend that this paper could be published in this journal.

**Level of interest:** An article of importance in its field

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests