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Dear Editors and staff,

Please find enclosed the revised manuscript entitled ***“Characterization of respiratory dendritic cells from equine lung tissues.”*** We appreciate the detailed comments and suggestions for improvement provided by the reviewers and prepared a revision of the manuscript. Below is a list of our specific responses to each of the reviewers’ suggestions. In the text of the revised manuscript, all changes addressing the reviewer comments are in red.

*Reviewer 1:*

*The stated objective of this paper is to isolate and characterize DC derived from lung monocytes and to compare these cells with blood-derived DC. While there is value in comparing tissue- derived vs blood-derived DC in the equine system, there are multiple inconsistencies in referring to these different cell types as well as a lack of critical evaluation experiments, which are outlined in more detail below.*

*Major comments*

*1. There is a general inaccuracy in reference to the different cell types, particularly tissue-resident lung dendritic cells and lung-derived dendritic cells, which leads to a conflict between the bulk of the introduction and the posed scientific question of whether tissue-resident DC have differing phenotype from blood-derived DC as well as the stated objective to assess the phenotype of lung-derived DC. Also, there is confusion regarding the comparison of these different populations. For example, blood mononuclear cells are compared to lung mononuclear cells, without accounting for the fact that these are likely very different cell populations. So concluding that lung MC have more cells expressing APC markers when lung MC are likely to be mostly macrophages whereas PBMC are mostly lymphocytes, seems to be an over interpretation. This is where phenotyping by CD3 or CD5 and CD14 (see comment 3 below) would allow assessment of differential expression of these markers blood versus lung monocytes/macrophages. In line with this, adherent PBMC and adherent lung MC are referred to as monocytes, but without demonstrating that these are indeed monocytes.*

**The purpose of this study was to find a method to isolate and culture DCs from lung tissues based on the fact that pathogens (including EHV-1) that infect via the respiratory tract are likely to encounter local dendritic cells rather than blood derived dendritic cells. It was not our intent to generate multiple different dendritic cell populations of different origins and extensively characterize them, but simply develop the technology to generate respiratory dendritic cells suitable for studying respiratory pathogens at suitable numbers. BALF were initially also collected, but isolated cell numbers were not suitable for use of *in vitro* studies. Currently the only described protocol to isolate equine dendritic cells with numbers suitable for cryopreservation and *in vitro* use, is the PBMC protocol described here with supplementation of GM-CSF and IL-4 [1, 2]. We adapted this protocol for lung tissues with minimal changes (slightly shorter incubation and omission of the Nycoprep). The changes made were necessary because cells lost viability with longer incubation and the Nycoprep step (which is used to limit cell debris in the PBMC protocol) did not show to improve quality of cells for the lung isolation protocol. We agree with the reviewer that some of the nomenclature chosen in our manuscript to describe the different cell types is confusing and have made changes to clarify our purpose (line 100-106) and nomenclature accordingly.**

*2. The introduction emphasizes the need to study tissue-resident DC, but the paper evaluated lung-derived DC, not primary tissue-resident DC. If the question is whether blood-derived DC can be used to adequately evaluate tissue-resident lung DC, then a third group of primary lung tissue-resident DC should be included and compared to both lung derived and blood derived DC.*

**The aim of this study was to find a method to isolate and culture DCs from lung tissues at sufficient numbers to perform *in vitro* studies with respiratory pathogens. Isolating DCs from BALF did not result in sufficient numbers for this purpose (data not shown) and so we chose to employ a protocol adapted from blood cells on lung tissues. We understand that our lung-derived DCs are mononuclear cell-derived DCs rather than primary tissue-resident DCs, and have corrected this accordingly in the manuscript. Please see line 97-106, line 329-337, and line 431-435.**

*3. There is also inaccurate or incomplete evaluation of the different cell types, which limits interpretation of results. For example, the discussion mentions that the MOS populations were likely of mixed cell type and yet there was no assessment of what cells were present and in what purity. There are at the minimum, readily available markers for equine monocytes (CD14) and T cells (CD3, CD5) which could have been employed to assess this and an evaluation of size on FSC/SSC could have helped assess population purity.*

**All cell populations were evaluated by FSC/SSC analysis, and this analysis revealed that there were multiple cell populations in the earlier steps of isolation (PBMCs, L-MCs, B-MOs and L-MOs), which was expected and has been reported numerous times prior [3-5]. It was not our scope to characterize these different cell populations, rather than to develop a protocol for isolation of respiratory equine dendritic cells, that we could use for future *in vitro* studies with EHV-1. Because of this, we focused this study on evaluating and comparing our population of L-DCs with B-DCs. Results of the FSC/SSC analysis were added in figure 1 and the content was added in Result 3.1.**

*4. The methods for generating blood- and lung-derived DC are different, with different duration of stimulation and centrifugation steps, which prevents a direct comparison.***We agree that the duration of incubation with cytokines is important for DC differentiation. We cultured blood-derived DCs based on the protocol described previously for horse blood [1], in which the DCs would be cultured with cytokines for 7 days in total. We have tried to use the same protocol on lung-derived DCs, but cell survival declined rapidly after 5 days of incubation. Furthermore, we found that 5-day-culture for lung DCs resulted in sufficient DC differentiation. We have added additional description regarding this point in discussion. Please see line 399-405.**

*Specific comments:  
- Abstract: clarify lung adherent mononuclear derived DC, not lung-DC.*

**Changed in manuscript, background and result sections in Abstract.**

*- Background:  
\* Clarify which species information is described for. E.g. line 57-58, are these percentages for equine?*

**The statement has been changed in the manuscript in line 58. The percentage mentioned in the content is from a human study. Such information is still unclear in horses.**

*\* Double check the term "Cultivated", cultured or derived might be more appropriate in many locations*

**Changed in manuscript in line 58-66 and line 101.**

*\* Line 61: "appropriate" for what purpose?*

**The authors of the original paper cited in our manuscript intended to emphasize that the method could yield enough cell number for experiments. The sentence has been re-written in the manuscript in line 63-64.**

*\* Line 62: what is meant by "highly differentiated". Isn't it more relevant to distinguish between immature and mature DC? The protocol specifically derives immature DC?*

**According to the concepts of immunology based on human studies [6, 7], DCs patrolling throughout the host’s body are considered to be immature before exposure with antigens. Immature DCs are good at capturing antigens (endocytosis). Once they take up antigens and are exposed to danger signals, maturation will occur and mature DCs will function in antigen presentation and T cell activation (but no longer be as good at endocytosis as immature DCs). Since B-DCs derived from PBMCs were not treated with LPS, the DC population would be generally considered as immature DCs. However, phenotypically the blood-derived DCs showed higher expression of CD86 with lower endocytosis activity compared to lung-derived DCs, which likely implies that blood-derived DCs were more mature than lung-derived DCs and may have undergone at the minimum partial maturation during the 7 day incubation period. This has also been addressed in the manuscript in line 63-64, line 77-78, and line 396-399.**

*\* Line 63-65: not sure what the point here is. It is well known that cytokines are required to drive differentiation.*

**The statement has been removed to avoid confusion.**

*\* Line ~75-82 and throughout: Be careful to distinguish between references to tissue-resident DC and DC derived from tissue mononuclear cells*

**Changed in manuscript. The clarification has been made in line 329-335.**

*- Methods:  
\* Since the effect of URT viruses such as EHV on the phenotype of DC is emphasized and since the age range covers young horses at high risk of URT as well as old horses at risk of equine asthma, it would have been informative to do some active assessment of respiratory health such as BAL cytology and/or viral PCR.*

**All horses used for tissue collection were clinically respiratory healthy. The associations between DCs and EHV-1 or other respiratory pathogens were not an objective in this study, but such investigation is the goal of subsequent studies and the basis for the purpose of the current study.**

*\* A concentration of cytokines needs to be provided, ideally with units rather than grams (line 149).*

**The issue has been addressed in the manuscript in line 153-154.**

*\* What was the rationale to do an extra density centrifugation and longer duration of culture/stimulation with blood cells compared to lung cells? This is especially important in light of the statement made that low expression of DC phenotype markers is due to the tissue source and not due the duration of culture (ie 5days). The literature reports that a minimum of 6-7 days of culture is required for equine cells to differentiate into DC, in contrast to human cells.*

**We agree that the duration of incubation with cytokines is important for DC differentiation. We cultured blood-derived DCs based on the protocol described previously for horse blood [1], in which the DCs would be cultured with cytokines for 7 days in total. We have tried to use the same protocol on lung-derived DCs, but cell survival declined rapidly after 5 days of incubation. Furthermore, we found that 5-day-culture for lung DCs resulted in sufficient DC differentiation. We have added additional description regarding this point in Discussion (line 399-405). The additional density centrifugation step (which is used to limit cell debris in the PBMC protocol) did not show to improve quality of cells for the lung isolation protocol and caused a loss of total cells, which why we decided to omit it for the L-DC protocol.**

*\* How were the cells detached from the plate for analyses? And at what point were B-MOS and L-MOS analyzed?*

**More information has been added in Methods section (line 147-150 and line 166-168). B-MOS and L-MOS were collected after 4 hrs incubation to separate adherent from non-adherent cells. For analysis cells were collected by using Versene EDTA solution for detachment.**

*\* Why wasn't there a viability marker included in the flow analyses? Were blocking steps used in order to reduce high background labeling with the isotypes? Or alternatively, using the isotype at the same concentration as the test antibody can reduce background and better interpretation of test antibody expression. So besides using isotypes at similar concentrations as test antibodies, the flow analyses would greatly benefit from dead cell and doublet cell exclusion, as well as showing negative/unstained cells to show what is autofluorescence and what is non-specific binding of the isotypes. Also, the wording to describe the flow cytometry results should be adjusted: "mean values of expression" generally refer to MFI or degree of expression within cells, but only the percentage of cells positive or negative for a marker were evaluated?*

**All immunofluorescent staining procedures were established previously in our lab [8], and didn’t conduct blocking steps, but used isotype controls at similar concentrations as test antibodies. A line adding this information was inserted into the Methods (line 194). We did not include a viability marker in our assays, but employed gating when conducting flow cytometry analysis based on FSC/SSC dot plots to eliminate cell debris, which was FSC-low and SSC-low. We feel that the isotype binding is acceptable and easily distinguishes non-specific binding from positive signals, but appreciate the suggestion about how to reduce the background in immunofluorescent staining. We did examine the autofluorescence of unstained cells. As figures shown below (representative example), unstained cells were all negative for autofluorescence.**

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**The wording for the flow cytometry assays was adjusted accordingly in the manuscript, Methods 2.6.**

*\*Were there experiments performed to confirm cross-reactivity of the CD163, CD204, and Bla36 antibodies with the correct equine epitopes? Are there references for the distribution of Bla36 in macrophages and monocytes? In general, the choice of these antibodies and their purpose should be better explained, including references (include this information in Table 1). Also, why did the authors not use commercially available equine leukocyte markers that could have been used to identify cell types such as T cells, monocytes (e.g. CD14), and DC (many of those have been used in equine DC phenotyping context before)? This would give much better specificity for phenotyping DC and assessing the purity of the cultures.*

**The cross-activity of human Bla36 and CD163 antibody on equine cells has been determined and confirmed previously [9, 10]. The purpose of using CD163, CD204, and Bla36 was to see if the cultured DCs have some features of histiocytes. We didn’t conduct further flow cytometry about B or T cell markers because our focus was to identify the phenotype of the isolated DCs, not to characterize the lymphocytes contained in PBMCs, which has been previously done [3, 4].**

*\* What was the rationale for using the Duncan multiple range test, as this test is not all that commonly used.*

**The test has been changed to ANOVA plus post-hoc Tukey’s multiple comparison tests.**

*- Results:  
\* Section 3.1. The description of cell morphology is a major result, yet it is based completely on a subjective basis. Some more objective analyses is needed, especially since it is stated that the morphology of blood and lung derived cells is similar but the FSC of the flow results is different between these cells.*

**We added FSC/SSC plots as our Fig. 1 and the description was made in Results 3.1.**

*\* Line 262-3: it is mentioned that cells become more uniform over time, but no data on a time course is given.*

**No time course was experiments were performed, but cells were evaluated at different differentiation steps. FSC/SSC plots have been added to Fig. 1.**

*\* Line 264: Your data shows markedly fewer PBMC expressing MHCII than several other papers. In general, equine PBMC are reported to have >80% of cells to be MHCII+, which is markedly different from other species. This discrepancy should be discussed.*

**Expression of MHC class II on equine PBMCs varies widely from approximately 20% to 80% [5, 11-13]. A previous study has shown that the diversity of MHC class II may be due to age and variance of MHC haplotypes [13].**

*- Discussion:  
\* Line 302: it is stated that an identical protocol was used, but the methods described were not identical?*

**The content has been changed in our manuscript to avoid misunderstanding (line 336-337). An explanation regarding adjustments to the protocol for L-DC isolation has already been described above. The second density centrifugation with Nycoprep on blood-derived DC isolation was based on a previously described protocol [1]. This step is typically performed to remove cell debris and it showed limited help for purity of cells with no significantly beneficial for DC differentiation in our hands. Therefore, we didn’t apply this step on our lung-derived DCs. Overall the isolation protocol for lung-derived DCs we applied in this study is very similar to the one for B-DCs, with minor justified modifications.**

*\* Line 331: In contrast to what the authors state, several of these markers are available in conjugated format, so there is no limit on reagents for dual or triple staining. Please remove this statement.*

**The statement was removed to avoid misunderstanding.**

*\* line 336-338: this sentence is confusing: do the authors state that the cells in their PBMC samples exhibit a monocytic cell type? PBMC consist primarily of lymphocytes.*

**Equine PBMCs contain a portion of monocytes that varies depending on individual horse, however we agree the majority of PBMCs should be lymphocytes. Also, CD44 could be expressed not only on monocytes but also on lymphocytes. This has been changed in the manuscript. Please see line 246-249, line 343-346, and line 371-377.**

*\* Paragraph 344-377: please either re-work this paragraph or remove. The methods used, and experiments done, in this study do not adequately assess the requirements for DC maturation or the specific role of GM-CSF and IL4. Was there a group of DC cultured without GM-CSF and IL4?  And where the B-MOS and L-MOS cultured for 5-7 days before analysis? If correct for the latter, please rewrite the methods accordingly to avoid confusion that these cells were not analyzed immediately after the 2-4 hours of adherence to the plate.*

**The question has been addressed above.**

*\* Line 352:  what does the word "inactivated" reflect? Immature? Naïve? Please change accordingly.***The question has been addressed above.**

*Reviewer 2:*

*In this manuscript, the authors isolated and characterized lung DCs (L-DCs) and compared their features with blood DCs (B-DCs). The authors speculate that L-DCs are likely in an earlier differentiation stage compared to B-DCs. The manuscript is well-written; however, I do have some comments and suggestions for revision before acceptance.  
  
Comments/Revisions:  
  
1. Line 83: Please insert" the outcome of infectious diseases such as bacteria, fungi, parasites or viruses" and add references (Dendritic Cell Control of Immune Responses: Penelope A. Morel and Lisa H. Butterfield. Frontiers in Immunology, 2015; The role of dendritic cells in the innate recognition of pathogenic fungi (A. fumigatus, C. neoformans and C. albicans). Ramirez-Ortiz ZG, Means TK. Virulence, 2012).*

**Changed in the manuscript and the citations have been added (line 83).**

*2. Line 99: This sentence must be redone since there are other ways of infections.*

**Changed in the manuscript. Please see line 97-99.**

*3. Line 111-113: I would suggest redone these sentences.*

**Changed in the manuscript. Please see line 110.**

*4. Line 113: Please delete "sexes" and replace with "gender"*

**Changed in the manuscript. Please see line 112.**

*5. Line 114: Please delete "ranged in age from 2 years to 23 years" and replace with "ranged in age from 2-23 years".*

**Changed in the manuscript. Please see line 113.**

*6. Line 114-115. Ethical considerations must be included in the text. In addition, you MUST give the name and number of the protocol followed by your institution in this type of procedure.*

**Inserted into the manuscript. Please see line 113-116.**

*7. Line 116: Please delete "a piece of lung tissue" and replace by "a sample of..."*

**Changed in the manuscript. Please see line 117.**

*8. Please, after these lines, add brand companies with their countries as well.*

**Changed in the manuscript.**

*9. Line 213-217: Please include additional information such as "how many pathologist evaluated the density of immunolabelled cells and the magnification used"*

**Changed in the manuscript. Please see line 225-227.**

*10. Line 224: Please change "at P < 0.05" by "at p <0.05".*

**Changed in the manuscript. Please see line 237, 316, and 317.**

*11. Line 228-331: Please in the following sentence "experiments using dual or triple staining of classical DC markers such as CD86, CD172a and CD14 could be performed to further answer some of these questions..." add CD11c since this also has been used as an important marker to study the canine immune system.*

**Changed in the manuscript. Please see line 368.**

*12. In conclusion, please delete "DCs are known to have anti-tumor and anti-viral immune function [46, 47]. It might be possible to use DCs as therapeutic agents for neoplastic or viral diseases and characterizing..."and start with "In our study...". Also rewrite the last phrase since a lot of work must be done before this affirmation.*

**The Conclusion has been re-written, please see line 425-435.**

**References**

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13. Barbis DP, Bainbridge D, Crump AL, Zhang CH, Antczak DF. Variation in expression of MHC class II antigens on horse lymphocytes determined by MHC haplotype. Vet Immunol Immunopathol. 1994;42:103-114.