



Jeremy Anderson,
Murdoch Children's Research Institute
Parkville, VIC 3052, Australia,
jeremy.anderson@mcri.edu.au

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Dear Ms. Željka Nikolić,

Re: pathogens-2270375, Response to Reviewers

We would like to thank the reviewers for their helpful feedback which has allowed us to improve the manuscript. We have made the relevant changes as Tracked Changes in the revised manuscript.

Reviewer 1:

Introduction

1. Line 43-45: Could the authors specify whether the diseases are more bacterial or viral?

Thank you for this comment. We have specified that they are susceptible to both bacterial and viral diseases. The line now reads:

‘Preterm infants have an increased susceptibility to severe bacterial and viral diseases. These include sepsis (1), necrotizing enterocolitis (2) and severe lower respiratory tract infections (3)’.

Please refer to lines 43-45, page 1 in the tracked changes version of the manuscript.

2. Line 54: TLR activation is not always associated with protection. It could be responsible for tissue injury and contribute to disease.

Thank you for this important comment. We agree and have re-worded this sentence as follows:

‘Upon recognition, this leads to the activation of NF- κ B to initiate inflammatory responses against pathogens which can be protective or harmful (4)’.

Please refer to lines 55-56, page 2 in the tracked changes version of the manuscript.

Methods

3. Line 84: please add a brief description for PBMC separation and preservation method.

Thank you for this comment. We have included a brief description of the PBMC/CBMC isolation and preservation methodology.

‘Briefly, CBMCs were isolated by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) at 400g for 30min without brakes. Isolated CBMCs were washed twice with PBS prior to being resuspended in freezing mix (90% fetal bovine serum (FBS) + 10% DMSO) and stored at -80°C for 24h in a cool cell (Sigma-Aldrich, St. Louis USA). This was then transferred to liquid

nitrogen. Sera was stored in -80°C. For CBMC samples, these were shipped to the Murdoch Children's Research Institute, Melbourne, Australia'.

Please refer to lines 87-93, page 2 in the tracked changes version of the manuscript.

4. Please indicate whether you measured the PBMC purity and vitality before stimulation.

While we did not measure PBMC purity, all samples had a >90% viability before stimulation. We followed standard procedures for PBMC isolation that we have used for multiple clinical studies which yield PBMCs with minimal contamination by granulocytes.

5. Line 105: is the volume of 900 mL correct? Do you mean μ L?

Thank you for noticing this error. It is meant to read 900 μ L, this has now been fixed.

6. Lines 107-108: why did the authors select different stimulation times (2 and 4 h) for bacterial and viral TLR ligands?

During initial testing of the NF- κ B kinetics, we found that cells responded slower to viral ligands, likely due to viral TLRs being intracellular, similar to a previous study (5). We have included this reference into the methodology on line 115 in the tracked changes version of the manuscript.

7. Please add a reference for selecting the TLR ligand concentration for the stimulation assay.

Various concentrations of TLRs are used for stimulation assays, depending on the outcome measured. For our study we used 2 μ g/ml of LTA, 10 μ g/ml of poly I:C, 10ng/ml of LPS, 2 μ g/ml of R848 and 2 μ M of CpG-ODN2216.

While studies typically use 1 μ g/ml of LTA we found 2 μ g/ml led to a better result especially from our CBMCs. Typical LPS stimulation concentrations vary from 1ng-10ng/ml. Poly I:C stimulations vary from 30ng/ml-10 μ g/ml. R848 stimulation varies from 10ng/ml-10 μ g/ml. CpG stimulation often vary from 1-5 μ M. Much of this information can be found on the product data sheets.

8. Line 117: is the volume of staining Ab (50mL) correct? The same for line 119.

Apologies for this error. This has now been changed to μ L where required.

9. Line 120: please add the composition of the permeabilisation buffer.

The permeabilization buffer is a commercial product from BD which we have used according to the manufacturer's instructions. We have revised this sentence to read as follows:

'Cells were then washed with 1mL of permeabilisation buffer (1:10 diluted in distilled water; BD Bioscience, San Diego, CA, USA) followed by 1mL of flow buffer and centrifuged between washes at 400g, 4°C for 4mins'.

Please refer to lines 129-132, page 3 in the tracked changes version of the manuscript.

Results

10. *TLR expression in preterm and term infants*: please use “in” instead of “on” for TLR expression because some TLR are expressed intracellular.

Thank you for this suggestion, we have changed ‘on’ to ‘in’.

11. Figure 1: are the cells here stimulated with bacterial TLR ligands? If not please correct the title of the figure.

Thank you for this comment. We have fixed the title, it now reads as follows:

‘Baseline TLR expression in preterm infants, term infants, and adults’.

Please refer to line 177, page 5 in the tracked changes version of the manuscript.

Discussion

12. Did the authors compared the blood immune cell composition between preterm and term and adults? This could be included in the discussion and interpretation of the results.

Thank you for this comment. We have previously published the differences in the cellular populations of the preterm and term infant cohort (6). We have now included discussion of these results.

‘Our study also found that cytokine responses were mostly similar between preterm and term infant in response to bacterial and viral TLRs, consistent with previous studies (7, 8). This is despite the reduced frequencies in mDCs and classical monocytes that we have previously reported (6)’.

Please refer to lines 287-288, page 10 in the tracked changes version of the manuscript.

13. What about the expression of TLR in blood lymphocytes like gd T cells and may these cells produce cytokines in response to TLR stimulation? The authors may include this point in the discussion of the correlation between NFkB activation and cytokine response to viral ligands.

Thank you for this suggestion. While this would be very interesting to explore, we did not include $\gamma\delta$ T-cells in our analysis as these cells are very low in frequency at birth (9). Therefore, we have chosen not to comment as we do not know how they may differ in this situation.

14. Would the authors suggest doing the stimulation on purified cell populations instead of the whole PMBC?

Thank you for this valuable suggestion. We have included this in the discussion as future directions as it is beyond the scope of our study.

‘Further studies using purified immune cell populations may be valuable to accurately observe cell specific responses in an isolated setting’.

Please refer to lines 335-337, page 11 in the tracked changes version of the manuscript.

References:

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3. Townsi N, Laing IA, Hall GL, Simpson SJ. The impact of respiratory viruses on lung health after preterm birth. *Eur Clin Respir J*. 2018;5(1):1487214.
4. Anthoney N, Foldi I, Hidalgo A. Toll and Toll-like receptor signalling in development. *Development*. 2018;145(9).
5. Fitzgerald KA, Kagan JC. Toll-like Receptors and the Control of Immunity. *Cell*. 2020;180(6):1044-66.
6. Anderson J, Thang CM, Thanh LQ, Dai VTT, Phan VT, Nhu BTH, et al. Immune Profiling of Cord Blood From Preterm and Term Infants Reveals Distinct Differences in Pro-Inflammatory Responses. *Front Immunol*. 2021;12:777927.
7. Lavoie PM, Huang Q, Jolette E, Whalen M, Nuyt AM, Audibert F, et al. Profound lack of interleukin (IL)-12/IL-23p40 in neonates born early in gestation is associated with an increased risk of sepsis. *J Infect Dis*. 2010;202(11):1754-63.
8. Sadeghi K, Berger A, Langgartner M, Prusa AR, Hayde M, Herkner K, et al. Immaturity of infection control in preterm and term newborns is associated with impaired toll-like receptor signaling. *J Infect Dis*. 2007;195(2):296-302.
9. Jalali S, Harpur CM, Piers AT, Auladell M, Perriman L, Li S, et al. A high-dimensional cytometry atlas of peripheral blood over the human life span. *Immunol Cell Biol*. 2022;100(10):805-21.