Author's response to reviews

Title: Host cytokine responses distinguish invasive and airway isolates from the Streptococcus Milleri/Anginosis Group

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Author's response to reviews: see over
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Dear Editors of BMC Infectious Diseases,

We would like to thank the reviewers for their constructive and helpful review of our manuscript entitled “Host cytokine responses distinguish invasive from airway isolates of the Streptococcus Milleri/Anginosis Group”. We have incorporated the majority of the suggestions from the reviewers and believe that the manuscript is much stronger for the revisions. Below you will find a point-by-point response to the concerns brought up by the reviewers.

Reviewer 1: Major Compulsory Revisions

1) It appears that the organisms are all washed and heat killed. This is important as responses to live bacteria may be quite different. It may be a necessity in order to study a 24 hour time point cytokine response, however, although clearly described in M&M, it would be appropriate to reflect this more clearly in the results and discussion. In particular with regard to interpretation, e.g. with regard to whether some strains could be actively suppressing cytokine production. (P.15 lines 320 – 322) the differences observed between isolates cannot relate to something actively secreted by these bacteria.

Because the different strains of SMG have very different growth rates, the organisms are all heat-killed and washed in order to eliminate bacterial growth in the 24 hour stimulation period. 24 hours was selected as a time point because maximal cytokine responses to the SMG were observed in preliminary time course experiments. This has been clarified in lines 171-173. In pilot experiments we found that immunostimulatory properties were only minimally different between live and heat-killed bacteria and that supernatants did not induce a strong response. We have not included this data as a supplementary figure; however we did mention it as “data not shown” in the text. The comment that the SMG could actively down-regulation cytokine production was removed and the interpretation of the TLR2 reporter results has been clarified in lines 329-337.
2) Page 8, line 178 – 179 and Table 1 / Fig 1a: What is the sensitivity of the assay for IL17A compared to the levels detected? Some indication as to whether this small difference is thought likely to be biologically significant, regardless of statistical significance, would be helpful.

The data were collected over multiple Luminex runs, with the limit of detection for IL17 ranging from 3-8 pg/ml. The median of each IL17 data set was consistently above the limit of detection. It should be noted that the values presented are averaged across three donors, and some donors produced levels of IL17A in the range of levels reported elsewhere considered physiologically relevant (~100 pg/ml). We recognize that a statistically significant result in IL17A production between isolates might not translate into a biological significance. The relevance of this finding requires further experimentation appropriate of future studies. We were cautious to conclude that the cytokine responses to the SMG are not sufficient to distinguish the three species, and do not make a point to conclude that IL17A is a differentiating feature. These comments are addressed in lines 280-286.

3) Figure 2: I am not experienced with the clustering technique, but visually the lowest two isolates (+C260 Invasive and M60R) do not look they should be clustered with the “low” group, when compared to C1051 Blood in the intermediate group for example. The discussion could be expanded a little in lines 199 – 202 to clarify further. This would not much change the conclusions from this figure however.

We agree with the reviewer that sometimes unsupervised clustering gives counter-intuitive results; however the strength of this methodology is that it is free from human bias. We also agree that the C260 and M60R isolates are unusual and by sight they appear demonstrate a profile more consistent with the “high” immunogenicity profile in Donor A and the “low” in Donor C. We commented further on the donor variability of these strains in lines 204-205.

4) Figure 3 shows median, interquartile ranges and outliers based on the Tukey method, and uses t-tests to examine significance. Are the data normally distributed? Perhaps a non-parametric statistical test might be appropriate.

The data are not normally distributed. The statistical analysis was changed to the non-parametric Mann-Whitney test as suggested. (All statistical analyses were changed to non-parametric tests).

Reviewer 1: Minor Compulsory Revisions

1) On the title page, does the square symbol beside the last 2 authors’ names denote equal contribution? Please indicate.

Thank you for noticing this! The authors contributed equally to this work and this has been denoted with an asterisk.
2) Table 1. Please alter to read “mean” or “median” rather than “average”

This table was removed as per reviewer#2’s suggestion.

3) Page 14, line 309 should refer to Supplementary Figure 1 (not 2)

Thank you for catching this. The figure identification has been changed appropriately.

4) Reference 31 is under review, may need to be updated and presumably should read “…analysis support…”

You are correct. The reference has been updated with the appropriate citation.

**Reviewer 1: Discretionary Reviews**

1) I wonder if the title might be better as “Host cytokine responses distinguish invasive from airway isolates of the Streptococcus Milleri/Anginosis Group”

Thank you for this suggestion - the title has been changed as suggested.

2) With regard to the underlying hypothesis, is there clinical evidence that there are separate disease causing strains and commensal strains, or can a commensal cause disease in the wrong setting? For example are there data on whether patients who get a SMG abscess carry the same strain “harmlessly”elsewhere?

This is a great question and is one of our long-term experimental goals. There is limited evidence to suggest that there are separate disease causing strains and commensal strains of the SMG. A previous study from our lab (Grinwis et al., J Clin Micro 2010) investigated whether strains from invasive infections differ from commensal (or rather CF airway) strains based on phenotypic properties, such as enzymatic activities and biochemical properties. Certain “biotypes”, groups of isolates that shared phenotypic properties, were more likely to include invasive isolates, however we believe it’s more likely a case of the wrong strain in the wrong person at the wrong time. The Madrenas lab has argued that in the case of *Staphylococcus aureus*, a commensal in the wrong setting can be pathogenic. For example, they’ve found that macrophages and monocytes elicit an IL10 response that might dampen an immune response and allow for colonization in the nasopharynx. However, if *S. aureus* enters the bloodstream, an IL10 response can be detrimental, as higher levels of circulating IL10 are correlated with an increased risk of death from bacteremia, presumably through down-regulation of an immune response required for clearance. In addition, they have found that dendritic cells elicit a pro-inflammatory response to *S. aureus*, which could drive inflammation during an infection such as a skin abscess. In the case of the SMG, it appears that disease-causing and commensal strains induce opposing cytokine responses, however, whether these responses truly dictate the pathogenic potential of an isolate is unclear. It
could be the case that upon dissemination from a mucosal site, surface-molecule expression is altered, and consequently, an isolate becomes immunostimulatory. It is difficult to conclude either at this time, as there is no data on whether strains causing invasive infections were previously carried at mucosal sites in that individual. We are currently investigating these unanswered questions in ongoing studies.

3) Page 12 lines 255 – 256 / Fig 4a: Four isolates in the “Low” group and one in the “intermediate” group do seem to give more robust TLR2 responses. Do they have anything in common with each other? The text states a 1 – 13 fold induction, but the figure looks like a maximal 11 fold change.

Thank you for catching this error. The text was changed to read a fold induction of 1-11 (line 257). There is no obvious connection between the four isolates (i.e. species, source of infection), although this is an area of ongoing work in our labs.

4) The data on donor specific IL-17A vs IL-23 bias in responses are very interesting and it might be worth including Suppl. Fig 1 as a main figure.

Thank you! We agree that this is a very interesting phenomena and have included this figure as a main figure, as suggested.

Reviewer 2: Minor Compulsory Revisions

1) L50: The wording is unclear and may be interpreted as female respiratory tracts are colonized.

   Line 50 (now 49) was edited to prevent this misunderstanding!

2) L116: Please check the wording of this sentence.

   Line 116 (now 118) was edited to avoid confusion.

3) L240: Please expand on what is meant by phenotypically “active” and “non-active”. This was not clear.

   Line 240 (now 240-241) was edited to provide a clearer definition of a “biotype” and the difference between phenotypically “active” and “non-active”.

4) L250: The title to this results section should be re-worded to reflect the findings.

   Agreed. The title of this results section was edited to appropriately reflect the data presented in the section.
5) The data presented in Figure 1 is shown on a logarithmic scale – I believe this was done to include all the data on the same graph. It may be better to present each cytokine on an individual graph and this may show the IL17 differences better. Alternatively, the graph needs to be enlarged. Table 1 appears to repeat the data shown in Figure 1.

You are correct; the data was plotted on a logarithmic scale to include all data on one graph. We agree that this makes it difficult to read, and re-plotted the data such that each cytokine is plotted on a separate graph. Table 1 is a summary of what’s shown in Figure 1, which we included to clarify values that were difficult to see in the previous version and since it is repetitive, it was removed. Thank you for this helpful suggestion.

6) The resolution of Figure 2 needs to be increased.

Thank you for pointing this out. The resolution of the figure (now Figure 3) was increased.

7) The supplementary figures should have figure legends.

The supplementary figure legends are provided in Supplementary File 3. We apologize for any confusion.

Reviewer 3: Major Compulsory Revisions

1) …it appears that data are not normally distributed (Table 1 and Fig. 1). This is required in order to apply the parametric statistics used here.

You are correct; the data are not normally distributed. We applied the appropriate non-parametric statistical tests in the revised manuscript. This did not alter the conclusions made from the data.

2) I find that the number of donors for the PBMC studies would need to be tripled in order to make solid conclusions, and in order to apply the statistical methods used in this study.

We understand the limitations using few donors in this study given the occurrence of human variability in immune responses, and, as per editorial suggestion, we added a comment to address this in lines 348-351. Ultimately, the argument we wanted to make was that within a single individual, there is variability in the response to the SMG, depending on the strain. We found that this was the case in the three individuals we tested. We feel this is an important finding since it is likely the case for additional bacterial commensals/pathogens, yet typically a model strain is selected for studies. We anticipated donor variability in the cytokine responses to the SMG, but found remarkable similarity in some cases (i.e. donors A and B). Based on our findings, we hypothesize that the strain-specific cytokine
response elicited by an individual could be the determinant of whether the strain is recognized as a commensal or pathogen.

Editorial Review:

1) Please clarify if the clinical isolates used were collected as part of standard patient care and if any ethical approval was required for your use.

The isolates used were collected as standard patient care. This information has been included in the M&M line 110.

Sincerely,

Dawn M.E. Bowdish, on behalf of the authors
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Department of Pathology & Molecular Medicine
McMaster Immunology Research Centre