Author’s response to reviews

Title: Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series

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Author’s response to reviews: see over
Dear Dr. Nazareno,

Thank you very much for your e-mail regarding our manuscript number 3572367131080173 entitled *Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series* by Delsing et al, and for your willingness to assess a revised version of our manuscript.

We thank the reviewers and the editors for their careful assessment of our manuscript. Please find attached a revised version of the manuscript and a point-by-point reply to the suggestions of the reviewers.

Regarding the editorial suggestions:
- we have clarified in the text that the Aspergillosis cases were proven, and whether they were acute or chronic
- we have corrected the text as suggested.
- We toned down the conclusion regarding the therapy implications of our data (end of Page 16).

The changes are highlighted in yellow in the revised manuscript. We believe that the changes suggested by the editors and the reviewers improved the quality of our manuscript, and we are looking forward to your decision regarding its suitability for publication.

Yours sincerely,

Mihai Netea
Point-by-point reply to the reviewers

Reviewer: Sunil Shaunak

Reviewer’s report:
This paper by Delsing et al describes the studies in 8 patients with life threatening invasive fungal infections who were treated with recombinant interferon-gamma (100 µg subcutaneously 3 times weekly) for only 2 weeks (i.e., 6 doses) in addition to standard antifungal therapy.

Comments on the clinical data set:
1) The important clinical observation is that 6 of the 8 patients treated with just a 6 doses of interferon-gamma over 2 weeks recovered from their fungal infection. The other two patients were already in the intensive care unit and were therefore likely to have been so critically ill at the time that interferon-gamma treatment was initiated that this therapeutic intervention was too late for them – this probably explains why they died. Given that this group of very sick patients is so difficult to study, the clinical outcome data is important enough to justify its publication.

   We thank the reviewer for this comment.

2) I agree with the authors that a multicentre trial is now needed to fully explore and to define the therapeutic efficacy of interferon-gamma immunotherapy in life threatening invasive fungal infections.

   We thank the reviewer for this comment.

3) I cannot see that Figure 1 adds anything useful to the information that could easily and succinctly be summarised in the paper’s text.

   While we agree with the reviewer that the information from the figure is summarized in the paper’s text, Figure 1 was made to illustrate that the case series described in our manuscript is part of a clinical trial and partly concerns patients that were deemed to benefit from IFN-gamma therapy as “last resort”. We believe that this figure helps the reader to understand more easily the nature of the data presented in this manuscript, and we would therefore propose to keep it. However, we are willing to remove the figure if the editorial board would consider that as more appropriate.

4) I cannot see that Figure 5 adds anything useful to information that could also be easily and succinctly summarised in the paper’s text.

   We agree with the reviewer that the leukocyte dynamics could be summarised in the paper’s text. However, this is one of the first reports in which the leukocyte numbers are monitored during the course of recombinant IFNγ treatment for invasive fungal infections. As on a individual level as well as on within the total patient group we observe specific trends that we would like to show in detail in a graph in the
online supplement rather than only discussing them. However, if the reviewer or editor disagrees, we are willing to remove this figure.

5) Figure 7 is a useful albeit brief summary. It would benefit from referring only to the patient clinical details. In my opinion, it is not useful to provide the baseline TNF-alpha or the baseline HLA-DR data in this figure.

As suggested by the reviewer we removed baseline TNFα and baseline HLA-DR from the table with patient characteristics as mean TNFα and mean HLA-DR does not contribute to the manuscript’s message, as there is considerable patient-to-patient variation. Originally the table was constructed to stratify each patient individually. Although this is in our opinion more informative it compromises patient anonymity and it was therefore adapted.

Comments on the in-vitro studies data set:

6) A. The authors suggest that the most important observation is the increase in HLA-DR expression on blood monocytes with interferon gamma therapy. It is not clear to me how the percentage calculation on the y-axis of Figure 6 has been determined. This should be clarified.

B. In addition, they refer to a paper by Meisel et al in 2009 (reference 30) to justify their splitting of the interferon gamma treated patients into those with <50% HLA-DR expression (and then defined as immuno-paralysed) and those with >50% HLA-DR expression (and then defined as immuno-competent.). I cannot find any reference in the Meisel et al (2009) paper which justifies using such a cut-off to draw Figure 6. The authors need to provide more clarification about this matter.

   A. On the Y-axis the percentage of monocytes that express HLA-DR is depicted (percent monocytes positive for HLA-DR), measured by flow cytometry. Please observe also the representative flow diagram for the HLA-DR measurement in the online supplement.

   B. The reviewer is correct. In the study by Meisel and coworkers the standardized quantitative “Quantibrite” test was used which analyses mHLA-DR in monoclonal antibodies (mAb) per cell. Mistakenly we used this reference to support our cut-off value of 50% to distinguish between immunoparalyzed and immunocompetent patients. We thank the reviewer for noticing this error, which we now corrected. In this respect, similarity to the 8000 mAbs/cell used as the cutoff value in the study of Meisel et al., The 50% cutoff used in our study is an arbitrary value. Nevertheless, we feel that it is justifiable to use this cut-off value in this case series. This value is more than 2 SDs below baseline mHLA-DR expression measured in 18 healthy volunteers in an earlier study of our group using the same methodology in the same laboratory (79 +/- 10 [mean +/- SD]) [1]. Therefore, a cut-off value of 50% is also arbitrary, but well below the lower bound of the 99% confidence interval of healthy immunocompetent volunteers, and therefore it is likely to represent immunoparalysis. We included the rationale for using the 50% cut-off value in the revised manuscript (page 5 line 25 6, line 1 - 3), and we
mentioned this as a limitation in the discussion section (page 16, line 22 and 25; page 17, line 1-4).


A. Figures 2, 3 and 4 relate to in-vitro stimulation of PBMCs from interferon gamma treated patients with a variety of exogenous ligands. It is not clear to me why the stimulation assays for IL-1 beta and TNF-alpha were performed for 24 hours only, whilst the stimulation assay for IL-17 and IL-22 were performed for 7 days, and the stimulation assay for IL-10 was then performed for 2 days. The reasons for this need to be explained in full by the authors.

The ex-vivo production of cytokines was assessed at timepoints where their production has been shown to peak, as assessed by several earlier studies performed in our laboratory. For monocyte derived cytokines such as IL-1 beta and TNFα the expression peaks at 24 hours and remains elevated when cells are stimulated for longer periods. While it takes 7 days before the T-cell cytokines IL-17 and IL-22 are produced in significant amounts. The cytokine IL-10 was measured at 48 hours because of limited amounts of culture supernatant and in our experience some stimuli induce IL-10 at 48 hours. An overview of the dynamics of Candida-induced cytokines can be found in the paper of van de Veerdonk et al [2]. We now included this information in our methods section. (page 6 line 22-24, page 7 lines 1 and 2)


B. With regard to their interpretation of the results as shown in Figures 2, 3 and 4, the authors state that there are increased ex-vivo responses for IL-1 beta and TNF-alpha, as well as for IL-17 and IL-22. My reading of the graphs leads me to conclude that increases in IL-1 beta and TNF-alpha are only seen on days 1 and 2 after starting interferon gamma therapy. In the case of IL-17 and IL-22, an increase is only seen on day 1 after starting interferon gamma therapy.

We agree with the reviewer that statistical significance compared to baseline is only observed at days 1 and 2 following initiation of IFN-γ therapy for IL-1 and TNF and at day 1 for IL-17 and we have corrected this in the text (Page 10, lines 6-10).

C. I am concerned that the authors have over-interpreted the data as shown in Figures 2, 3 and 4 as currently shown in the manuscript.
We toned down the interpretation of the changes in *ex-vivo* cytokine production.

D. The authors conclude with the following sentence “Biomarkers of impaired anti-fungal immunity should be described in order to identify patients who will benefit most from immuno-stimulatory therapy”. I would refer the authors of the paper of Armstrong-James et al entitled “Renal allograft recipients fail to increase interferon gamma during invasive fungal diseases” that was published as a brief communication in the Amer J Transplantation (2012; 12: 3437 -40). A blood based assay is described in this paper which could be used to identify those patients who could benefit from interferon gamma therapy. The authors should reference this paper at the end of their discussion.

We would like to thank the reviewer for this suggestion and we included the paper earlier in our discussion in a section where we discuss the need for biomarkers to determine which patients would benefit from adjunctive immunotherapy (page 15 lines 12-19). We also rephrased our concluding sentence to state that further investigation of biomarkers is required.
Reviewer: Richard Hotchkiss

Reviewer's report:
General comments
The purpose of this investigation was to examine the efficacy IFN gamma in patients with fungal sepsis. The investigation examined effects of IFN-gamma on the ability of immune effector cells to restore cytokine production and to increase HLA-DR expression. This is an important area of study and there is a growing belief that administration of agents that boost patient immunity will improve outcome in sepsis. This study is an important contribution to the field. Overall the methods appear reasonable. There are some issues that need to be addressed however.

Minor essential revisions:
1) The authors make a point about HLA-DR expression being an important aspect of the study and show that IFN-gamma can increase HLA-DR. The investigators also state that examination of monocyte HLA-DR expression was not different if performed immediately or within 24 hrs. The authors did 5 samples to test this fact. This seems like a small number of samples to test and there is presentation of the data. This investigator is a little skeptical of this data as HLA-DR is known to vary quickly. Also, in the supplementary methods, there is no mention of standardization using isotype control beads or the Quantibrite method that is recommended. The authors need to address this point and note key limitations in this regard.

   We agree with the reviewer that Quantibrite is perhaps the most reliable tool for the quantification of antigens. However, if the flow cytometer is stable and calibrated, the MFI and S/N ratios can be used also for this purpose. Our flow cytometer is regularly checked on stability of the optics and flow system, as part of our quality control system to guarantee that the flow cytometer produces the same fluorescence intensity with the same materials at any time. Changes in MFI are used extensively in various publications. In our hands the monocyte HLA-DR expression did not change within 24 hours after blood withdrawal. To be sure that no change occurred, we have compared the expression immediately after withdrawal and after 24 hours in 5 separate experiments with different blood samples. We did not find significant differences when the samples were immediately stored at 4°C. Nevertheless, we agree with the reviewer about this limitation, and we added a note on this in the manuscript (Page 17, lines 1-4).

2) Why do the authors believe that IFN-gamma led to decreased absolute lymphocyte counts?

   We are not sure we understand the reviewer correctly. As depicted in Figure 4, we observed an increase in lymphocyte counts after IFN therapy.

3) The investigators should show a representative flow diagram for the HLA-DR measurement.
A representative flow diagram is now depicted in supplemental Figure 2 in the online supplement.
Editor's comment:
"I agree with the reviewers that this manuscript has merit but requires significant revisions as they have nicely outlined. In addition I recommend the following

1) Proofread to correct grammatical errors.

   We have revised the manuscript to correct grammatical errors.

2) Clarify if aspergillosis cases were proven or probable and if they were acute or chronic.

   The aspergillosis cases were all proven and we included this information to the manuscript. Additionally we stated whether the cases of aspergillosis were acute or chronic. (page 8, lines 13-15 and 24-25, page 9, lines 2-4 )

3) Would tone down the conclusion that treatment "represents a promising intervention to improve outcome." I don't think there is enough data to support this statement. Would emphasize that clinical trials are needed to assess the impact of this intervention on clinical outcome."

   We toned down the statement. Furthermore we emphasized that trials are required to study the effect on outcome in more detail. (page 17 lines 9-12)