Answering reviewers

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 28272

Title: Altered pattern of TNFalpha production in peripheral blood monocytes from Crohn's Disease

Authors: Claudia Loganes, Alessia Pin, Samuele Naviglio, Martina Girardelli, Anna Monica Bianco, Stefano Martelossi, Alberto Tommasini, Elisa Piscianz

Reviewer's code: 03592313

Trieste, August 25, 2016

Dear Editor,

Thank you for giving us the possibility to revise our manuscript.
We have taken into account the reviewer comments and revised the manuscript in order to improve its readability. Moreover, we gave the manuscript to a native English speaker for editing. Any change to the text of the manuscript has been highlighted in yellow.

The answers to the reviewer suggestions are as following, highlighted in bold.

We hope that our revision will meet with your approval.

Thank you again for accepting our manuscript in the World Journal of Gastroenterology.

Reviewer comment:

The manuscript described that peripheral monocytes from CD express higher basal and stimulated TNFα than controls, regardless of NOD2 genotype and without a clear correlation with disease activity. In general, the results are very interesting. There are, however, some flaws that would require some revisions. These are reported below for the authors convenience.

1. *Except TNFα, how about other pro-inflammatory cytokines produced in monocytes of CD and UC?*

Author replay:

Clinical data, experimental analysis and animal models demonstrated that Crohn's disease (CD) is a chronic disease characterized by an infiltration of monocyte/macrophage cells and Type 1 T-helper lymphocyte (Th) cells in the inflamed mucosa. These cells are considered the major producers of tumor necrosis factor- α (TNFα), a key mediator of the inflammatory state in CD.

An increased TNFα expression was found in colonic biopsies derived from patients with CD in active disease compared to non-inflamed tissue in control subjects (*Murch SH, Gut. 1993*; *Breese EJ, Gastroenterology. 1994*).

For these reasons, we decided to evaluate the intracellular expression of TNFα in monocytes, since they are a feasible and a little invasive but, at the same time, a representative source of inflammatory cells.

Regarding other pro-inflammatory mediators, it is well established that the production of pro-inflammatory cytokines (such as IL1β and IL6) is enhanced in Inflammatory Bowel Disease (IBD), characterized by a dysregulated immune response (*Neurath MF, Immunology. 2014*; *Strober W, Gastroenterology. 2011*).

However, our study was not meant to analyze the network of cytokine produced in CD, but to exploit intracellular TNFα as the main readout for possible defects in the TLR-NFkB signaling machinery. Indeed, the assay that we performed is used in the clinics to screen for primary defects in this pathway (TLRs, IL1R, IRAK4, MYD88, IKBKG). Since the release of IL1β can be highly independent from NFkB, we cannot exclude different effects of *NOD2* genotypes on this cytokine, yet this has not been investigated in the present study.

2. *The average age of the patients is 13. Can they represent the adult suffering of CD and UC?*

Author replay:

In our opinion, this pediatric cohort cannot represent the adult subjects suffering from CD and UC. A study conducted by Pak et al. (*Pak S, J Pediatr Gastroenterol Nutr. 2012*) investigated different cytokine profiles in patients with CD, stratifying patients by age (pediatric and adult) and correlating results to age-matched controls. This study support our findings, highlighting an increased TNFα production in CD groups compared with healthy controls, although cytokine profile in adult patients differed from pediatric cohort.

On a clinical ground, the age at onset seems to influence disease outcome and response to therapy: early onset IBD seems to be more serious than that with a later onset, presenting an aggressive disease phenotype, a severe progression and poor responsiveness to most conventional therapies. Indeed, it has been suggested that the different phenotypes in adult- and pediatric-onset CD may reflect different etiopathogenetic mechanisms. (*Heyman MB, J Pediatr. 2005)*.

All these results suggest that a fundamental difference exists between adults and children in the immunological mechanisms of IBD pathogenesis.

3. *From Fig1 we found that TLR1/2 participating in the production of TNFα in monocytes. Please explain the relationship and difference of TLR and NOD in CD and UC.*

Author replay:

The aim of our research was the analysis of the integrity of the different pathways of the innate immune system (TLRs and NOD2): we chose to analyse the production of TNFα triggered in monocyte by different stimuli, as a tool to depict the presence of possible alterations in different components of the TLR-NOD machinery for the sensing of PAMPs. This cytometric protocol was adapted from a clinically oriented technique used for the screening of primary immunodeficiency diseases such as Interleukin-1 receptor-associated kinase 4 deficiency (*Takada H, J Pediatr 2006*). The use of a different set of microbial stimuli was meant to examined the main innate immune signaling, such as the myeloid differentiation factor 88 (MyD88)-dependent pathway (shared by most TLRs), MyD-88-independent (activated by TLR3) pathway and NOD-dependent pathway, that lead all to the MAPK and NF-κB activation (*Jin HS, Journal of Bacteriology and Virology 2014*).

A differential expression of TLR members in IBD was already described by several studies in CD or UC patients (*Cario E, Infection and immunity, 2000; Hausmann M, Gastroenterology, 2002*), but to date there isn't a clear correlation of such TLR alterations with IBD susceptibility.

In our study, a higher percentage of monocytes producing TNFα was recorded in CD compared with healthy controls, after stimulation with Pam_3CSK_4, a synthetic lipopeptide TLR2 agonist. This observation should not be surprising, given the role of the *Mycobacterium avium* subspecies *paratuberculosis (*MAP) in the pathogenesis of CD, which shared some clinical features with the Johne's disease

caused by MAP. The presence of MAP in different CD tissue has been proven by several researches, but its role in the CD etiology remains to be defined.

The stimulation with the NOD2 ligand MDP-L18 led to a stronger response in CD and UC. While this behaviour did not find a clear explanation, it highlights the importance of NOD2 signalling in CD and not in UC. Indeed, this finding is coherent with the knowledge that NOD2 is the main genetic factor associated with CD. However, despite the huge amount of works on this topic, the mechanisms by which the NOD2 variants contribute to the pathogenesis of the disease remain controversial.

In the discussion, we added some comments on the role of NOD2, MAP and TLRs in CD.

4. *In Figure 4, there is a small mistake. "remissive Ulcerative Colitis (UC)"*

Author replay:

We do thanks the reviewer for the advice and we apologize for the inaccuracy.

We corrected the error in the final manuscript.

Editor Comment

Step 1. Please revise your manuscript according to the reviewers' comments.

Author replay: We revised our manuscript according to the reviewers' comments, correcting few sentences to make the manuscript more clear and precise. All corrections are highlighted in yellow in the revised manuscript. Here we reported the major corrections and modifications:

- **Grammar mistakes were corrected;**
- **The acknowledgment section was added;**
- **The discussion section was improved, including some new comments as suggested by the reviewer.**
- **Bibliography was improved.**

Step 2. Please update the manuscript according to the Guidelines and Requirements for Manuscript Revision-Basic study.

Author replay: We revised the manuscript according to the provided guidelines.

Step 3. Please provide the scientific research process

Author replay: The scientific research process was provided, as required.

Step 4. Please provide an Audio Core Tip.

Author replay: The audio core tip was provided as an mp3 format.

Step 5. Please subject the manuscript to CrossCheck analysis and the final title to Google Scholar search, and store screenshot images of the results.

Author replay:

We performed the plagiarism analysis using Turnitin, a plagiarism-prevention service used in our Institute. Turnitin gave a 38% of similarity and removing from the analysis the article title page and bibliography, the percentage of similarity became 16%. In addition, the Google Scholar search did not provide a similar title. The documents reporting the screenshot images of Google Scholar result and plagiarism analysis were provided.

Step 6. Please provide the files related to academic rules and norms.

Author replay: We provided all the required documents in PDF format.

Step 7. Please provide the approved grant application form(s) or funding agency copy of any approval document(s)/letter(s).

Author replay: The approved grant application form was provided, as requested.

Step 8. Please revise the language of your manuscript.

Author replay: The revised manuscript was carefully checked and corrected by a native English speaker.

Step 9. Please sign the Copyright Assignment form.

Author replay: The copyright assignment form was signed by all the authors and attached as PDF file.

Step10. Submit the revised manuscript and all related documents.

Author replay: We submitted the edited manuscript and all required documents.

Thank you and best regards

Sincerely yours,

Claudia Loganes

Department of Medicine, Surgery and Health Sciences

University of Trieste

Piazzale Europa 1, Trieste 34128, Italy

Tel: +39 040 3785422; e-mail: claudia.loganes@gmail.com