Format for ANSWERING REVIEWERS

October 28, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name4648-review.doc).

Title: Longitudinal analysis of inflammation and microbiota dynamics in a model of mild chronic dextran sulphate sodium-induced colitis in mice.

Authors: Luigia De Fazio, Elena Cavazza, Enzo Spisni, Antonio Strillacci, Manuela Centanni, Marco Candela, Chiara Praticò, Massimo Campieri, Chiara Ricci and Maria Chiara Valerii

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 4648

The manuscript has been improved according to the suggestions of reviewers:

Answer to reviewer's comments:

Referee #1
Mayor comments. At the end of the discussion should be added a paragraph of conclusions that reflect a more comprehensive conclusion of the abstract: DSS 1,5% for 9 days induced a mild colitis in which dysbiosis showed a pivotal role during the acute phase but chronic colitis occurred despite dysbiosis subsided.
Answer: As suggested, a concluding paragraph with conclusions has been added.

Referee#2
Despite the fact that the DSS-induced colitis model is quite common, the authors used a state of the art approach. The paper is highly interesting and has a strong potential for being used as a benchmark for further studies evaluating possible treatments of colitis.

Referee#3
Minor comments: Please add parameter to x-axis for all the figures.
Answer: X-axis has been labeled in all the figures.

Referee#4

Point 1) The authors underline to importance of the molecular weight of DSS in colitis induction. How was the MW of DSS in their experiment?
Answer: this data has been added in the material and method section.

Point 2) The authors discussed that in most human IBD studies the systemic level of cytokines are measured, while in experimental colitides usually the local cytokine profile changes are examined, thus leading to information loss on systemic inflammation. As in the case of DSS-induced colitis not just DSS itself, but the gut microbiota contribute to the inflammatory process, and, moreover, some cytokines act via autocrin or paracrin pathways in a local inflammatory milieu, it would be crucial to measure not just the systemic cytokine profiles but the local cytokine profile changes. The comparison of the

systemic and the local cytokine profiles would let us to take an exact insight into the immunopathogenesis of colitis.
Answer: we believe that focusing our attention on measuring circulating cytokines instead of those produced locally represents a step forward in view of using this model in preclinical studies and, as underlined by Referee#2, to propose this model as a benchmark for further studies that evaluate possible treatments of colitis. The determination of locally produced cytokines is very poorly reproducible and it does not allow to establish the true cytokine levels in the extracellular fluid. Evaluating cytokine secretion by quantifying their mRNA using real-time PCR (see Tokuyamaet al., Internat. Immunol. 2005; 17: 1023-34), does not allow to obtain the true level of any secreted cytokine. The ex-vivo cytokine secretion by tissue specimens (see Freire Bento et al., Biochem Pharmacol 2012, 84; 1459-69) needs to be standardized for the specimen's weight and the incubation time and in any case does not allow to establish the true amount secreted in vivo. Moreover, all this makes it very difficult to compare results obtained in different Labs (for example, trying to compare results obtained by Freire Bento et al., Biochem Pharmacol 2012, 84; 1459-69 with those obtained by Sha et al., Int. Immunopharmacol. 2013;15:23-9). The same happens for the indirect quantification of cytokines by IHC, since the acting cytokines are those secreted by cells and not those accumulated in their cytosol. Cytokines and chemokines act in precise ranges of concentration. It does not make sense reasoning about twice the amount of cytokine X if the amount is outside the concentration window in which the cytokine is really effective. With these biases, we do not consider it crucial to measure local cytokine profile changes. Moreover, in clinical practice the severity of colitis is associated with the level of circulating cytokines and the circulating cytokines themselves represent important targets for biological drugs (see for example Infliximab and Adalimumab).

Point 3) The authors did not examine the levels of type I interferons (especially IFN-alpha and -beta). It is known that stimulation of apical epithelial Toll-like receptor 9 results in accumulation of NF-kB inhibitors, thus blocking its activation, and in suppression of inflammation, partly due to the induction of type-I IFNs. In tissue regeneration type-I IFNs are considered as protective cytokines against colonic inflammation. It would be crucial to know the longitudinal changes of type I. IFNs as well. Some of the changes in the observed cytokine levels may be related to the colonic microbiome and its changes.

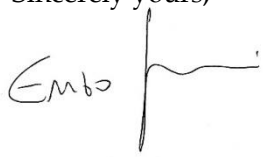
Answer: Certainly it is true that immune cells sense microbial products through the Toll-like receptor (TLR) family, which triggers the host's defense response through a wide array of mechanisms, including type 1 interferons (IFNs) secretion. Nevertheless, studying the interactions between the immune system and microbiota is outside the aim of this work. In addition, TLR-9 is only one of the many pattern recognition receptors involved in recognizing pathogen-associated molecular patterns. We should consider it in a much complex network including other pattern recognition receptor families. But with our data it is not possible to directly correlate cytokines expression with the microbiota structure.
Analogously to the reported capacity of pathobionts to nurture inflammation in the gut (Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009; 9:313-23), we can aspect that the observed deviations in the gut microbiota structure can foster changes in cytokine expression. However, much more study needs to be done to better clarify this point. These considerations have been added to the Discussion section, and a new reference (ref.34) has been added.

Point 4) It would enhance the strenght of the manuscript if the authors discussed the role of the microbiome-native immune system-cytokine profile axis in colitis induction and mucosal regeneration.
Answer: The interactions between microbiota and the immune system are very complex and remain, for a large extent, to be elucidated (see Kamada et al., Role of the gut microbiota in immunity and inflammatory disease; Nature Reviews Immunology 2013; 13:321-35). Mechanistic relationship between microbiota composition and colitis has not been described yet. Even the relationship between microbiota composition and mucosal regeneration remains to be established. Nevertheless, a short

discussion about this issue has been addressed in the Discussion section, and a new reference (ref. 35) has been added.

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

Sincerely yours,



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