Format for ANSWERING REVIEWERS

June 15th, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 3271-review.doc).

Title: ROLE FOR THE ADVANCED GLYCATION END PRODUCTS RECEPTOR IN CROHN'S DISEASE INFLAMMATION

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

1 Format has been updated

2 Revision has been made according to the suggestions of reviewers #1 and #2 as specified in the following point by point letter

3 References and typesetting were corrected

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

Yours sincerely,

Rachele Ciccocioppo

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REVIEWER #00504435:

We warmly thank the Reviewer for her/his constructive comments aimed at improving the quality of our manuscript. Here below, please find our point by point response.

Materials and Methods section:

1. Originally, we did not perform the negative control of immunohistochemistry using the isotype control antibody because we followed the instructions for using the goat anti-human polyclonal anti-RAGE antibody from R&D System as described in references no. 22 and 23, but only in the latter, the authors described the negative control (an anti-human IgG1). As requested, therefore, we have carried out the immunohistochemistry analysis for negative control by using an anti-human IgG1 isotype as primary antibody on seriate sections, as now specified in the Methods section.
2. We originally did not perform the double staining since the pattern of RAGE staining could have been cytosolic, membranous, or both, and then it would be hard to recognize two different markers. This is why we preferred to use seriate sections in evaluating the origin of $RAGE^+$ cells. As requested, therefore, we have now added some representative images of the CD138 and CD68 staining of $RAGE^+$ cells (see the new Figure 5), since plasma cells and macrophages are the main cellular populations resulting who resulted $RAGE^+$.
3. We fully agree with this remark, and we apologize for the unsatisfactory explanation of the grading given in the original version, which has now been corrected.
4. In this series of functional experiments, we did not use an isotype antibody as negative control since the primary anti-RAGE antibody is a polyclonal antibody, and therefore we would have had to use a panel of isotype antibodies that we did not check due to the limited number of cells available for each experiment.

Results section:

1. We fully agree with the Reviewer since a quantitative correlation between $RAGE^+$ cells and ulcerative lesions would add strength to our hypothesis. However, at present, it is very hard to answer this question because of the invariable presence of ulcers in all the surgical specimens from diseased areas used for immunohistochemistry, and the absence of them in the specimens from non-diseased areas. As a consequence, we gave this data in a descriptive manner. Having said that, we agree that this is an interesting issue to address in future studies.
2. We completely agree with this remark. Thus, we added Figure 7 showing the dot plot of the grading of RAGE expression at epithelial level with BMI.
3. As already shown (van Heel DA, et al. Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. Lancet 2005; 365: 1794-1796), MDP is a component of bacterial cell-wall peptidoglycan present in most bacterial species, which binds the intracellular receptor NOD2. The stimulation of NOD2, in turn, leads to nuclear-factor-kB activation, which represents an upstream molecule in the inflammatory cascade of Crohn's disease. With our experiments, therefore, we aim to reproduce what might happen *in vivo*, when mucosal immune cells are primarily activated by antigens of the gut microbiota. The use of a RAGE ligands would have predictably blocked TNF-α production as already shown by previous reports.

Discussion section

1. We are grateful to the Reviewer for raising this important issue. We have now distributed the comments better and, as requested, we have also added several hypothesis concerning a putative separate role of the RAGE expression in these two cellular populations, together with two new references (nos. 32 and 33). Finally, as regards the possibility of RAGE being involved in intestinal or skin ulcers, we did not find any specific reports; however, we found evidence of its involvement in the epithelial-myofibroblast transition preceding fibrosis.
2. We completely agree with the need for some speculation about the functional implication of a different pattern of RAGE expression between epithelial cells and *lamina propria* mononuclear cells. Specifically, we have now better characterized the cytosolic pattern into diffuse and granular, which may represent different functions, as stated in the new reference no. 32. Indeed, different cellular staining might reflect distinct functional roles of this decoy receptor, with the diffuse pattern probably indicating an activated status, and the granular pattern indicating a secretory pathway. We thank the Reviewer for giving us the opportunity to discuss this issue more broadly as now added to the revised version of the manuscript.

Tables

1. As for the reason why the number of patients described in Table 1 differs from that presented in Table 2-4, it depends on the fact that in some cases we did not have appropriate tissue for evaluating all the parameters needed for grading.
2. We can agree with this criticism, and the explanation lies in the fact that 100% indicated the sum of the values in the same line (for instance, the same grading or intensity or amount of positive cells) and not in the same column.

Figures

1. We fully agree with the Referee and we have now combined together Figures 1 and 6 in this revised version.
2. As requested, we have now indicated the values in Figure 5 more appropriately.
3. Again, in the Figure 5, we presented the blots of β-actin in full. We have to specify that the last sentence of the Referee appeared truncated, and we deduced that the words 'be added' were probably missing.

REVIEWER #00030998:

We warmly thank the Reviewer for his/her positive reaction to our manuscript, and for her/his helpful comments aimed at improving the quality of our work. Here below, our point by point response to the minor concerns raised.

1. We fully agree with the concern about the relatively small sample size used which was due to the need for apparently normal tissue at a certain distance from damaged tissue in patient group A, and of a high number of mucosal immune cells for functional experiments from biopsies of group B. As requested, we added a comment about the need for wider studies.
2. We thank the Reviewer for this valuable suggestion, and the terms 'inflamed' and 'non-inflamed' have been now substituted with 'diseased' and 'non-diseased', since we were referring to the macroscopic appearance of the tissue during surgical intervention. As a consequence, we did not have a histological degree of inflammation to correlate RAGE

expression with, since all the diseased tract presented a high degree of chronic inflammation.

3. Again, we thank the Reviewer for this comment. We have now specified the impact of clinical parameters better, such as disease duration, location, and behaviour.
4. As requested by the Reviewer, the medication that the patients were taking during the study have been specified (see Table 1), and no correlation with RAGE expression appeared evident. Moreover, we would like to underline that our study was mainly intended to address the issue of the involvement of RAGE in chronic inflammation rather than its correlation to clinical parameters as evaluated in a longitudinal manner in the same patient. However, we completely agree that this is a crucial point that needs further investigation.