June 19, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 11356-review.doc).

Title: *Clostridium difficile* infection aggravates colitis in interleukin10-deficient mice

Authors: Mi Na Kim, Seong-Joon Koh, Jung Mogg Kim, Jong Pil Im, Hyun Chae Jung, Joo Sung Kim

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 11356

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 the reviewers

1) Reviewer: 1 (01441415)

Comments to authors:

This manuscript by Kim et al. reported a novel model of C difficile infection in IL-10-/- mice as an IBD-like model. This model has considerable potentials to explore the pathophysiological role of C difficile infection in human IBD, the underlying mechanism and following therapeutic directions. However, this manuscript involves several concerns to be dissolved before acceptance for publication. Although the authors used IL-10-/- mice to mimic human IBD in this study, histological assessments in Fig. 5 and 6 revealed no derangements compared with WT mice without C difficile infection. It is strongly recommended to mention the solid rationale why the authors selected IL-10-/- mice, among several IBD animal models, in this study.

Reply: Thank you very much for the helpful comment. Indeed, various mouse models have been used to investigate inflammatory disease, including those induced by chemicals, immune cell transfer or gene targeting. For the experiments in this study, we chose to use mice deficient in IL-10, which spontaneously develop IBD which gradually progresses over several months. These mice rapidly develop severe, chronic

IBD after treatment with piroxicam, a non-steroidal anti-inflammatory drug that acts on endogenous prostaglandins, which are important inhibitors of the development of intestinal inflammation[1]. Therefore, we hypothesized that these mice would similarly respond to *C. difficile* infection. As expected, we observed that the development of acute colitis was indeed amplified in IL-10$^{-/-}$ mice. The purpose of the study was not to validate these mice as a model of IBD, therefore the lack of initial signs of inflammation in the untreated group is not of concern. What we identified was an exacerbation of histopathologic features after infection, including epithelial cell damage, inflammatory cells infiltration, and submucosal edema. This information has now been included in the Discussion section of the revised manuscript.

In the comparison group of Figure 6C, "WT" should be "WT + C. difficile", right?

Reply: Thank you very much for your comment. The label was indeed incorrect in the original version. The figure is now designated Figure 5, and the corresponding treatment labels have been revised.

The statistical analysis was performed in the assessments of body weight change in Figure 3? The figure presented the change ratio, is there any difference in baseline body weight between WT and IL-10-/- mice?

Reply: Thank you very much for your insightful comment. The baseline body weights were not different among the groups, and we have therefore added this statement to the corresponding Results section. Statistical analysis between the groups indicated that IL-10$^{-/-}$ mice exhibited a significantly greater weight loss than their WT counterparts in response to *C. difficile* infection.

The authors suggest that C. difficile infection has pivotal role for continuous aggravation of IBD. In Fig. 3, regardless of genetic backgrounds, body weight has recovered to the levels of control groups. This recovery implies that the aggravation of intestinal inflammation induced by C. difficile infection is just acute phenomenon, but chronic. How the authors interpret the data?

Reply: We greatly appreciate your astute observation and critical comment. IBD is a chronic, relapsing inflammatory disease. We agree that the *C. difficile*-induced inflammation causes an acute exacerbation of IBD. However, further studies are needed to evaluate the long-term effect of this acute inflammation, as well as its impact on the chronic development of IBD in IL-10$^{-/-}$ mice. We have consequently modified portions of the Discussion section to highlight the effect of *C. difficile* infection on acute colitis.

2) Reviewer: 2 (01434943)

Comments to authors:

This is a relatively straight forward experimental study describing an exacerbation of colitis by C. difficile. Attention to English grammar is required throughout the manuscript. Readability requires improvement. Specific comments follow: TITLE: Concise and descriptive. ABSTRACT: Are the values mean +/-SD or SEM? This should be stated.

Reply: Thank you very much for your comment. The revised manuscript has been critically edited by AmEditor Inc., an English language scientific editing company. Additionally, the values presented are indeed mean ± standard error. This information has been clarified in the Abstract and in the Statistical Analysis sub-section of the Methods section.

The Results section has several grammatical errors. CORE TIP: Appropriate apart from grammar. INTRODUCTION: A succinct summary of the work. METHODS: Well described and appropriate. RESULTS: Why were the cytokine studies confined to IFN-g, IL-12 and IL-23?. Further justification is required.

Reply: We greatly appreciate your careful review and comments. As indicated above, the revised manuscript has been carefully edited for grammar. The cytokines used in this study were selected based on previous works demonstrating their role in inflammation. For example, IFN-γ was implicated in *C. difficile* toxin A-mediated enteritis[2], and other studies have documented the role of IL-12 and IL-23 in chronic inflammation in IL-10$^{-/-}$ mice[3,4]. We have included this justification in the Discussion section in the revised manuscript.

The 4 lines in figure 3 are difficult to discriminate.2 dotted lines (eg for IL-10KO) should be used.

Reply: We apologize for the difficulty in reading the Figure. The graph in the revised version has been appropriately modified to more clearly distinguish the groups.

Was the histology scoring blinded?.

Reply: We apologize for not including this information in the original version. Histopathologic examination was indeed performed by an independent pathologist who was blinded to the study methods, and this information has been added to the relevant Methods sub-section.

IFN-g expression in figure 6 is strange. There is virtually no variation in 6B but huge variation in 6A and even more in 6C. Why is this so?. Were there outliers?.

Reply: We appreciate the careful scrutiny of our study's data. The figure corresponding to cytokine expression in colonic tissues has been re-designated as Figure 5. The differences in variability are likely due to the magnitude of the observed changes (fold-changes) among the various groups.

DISCUSSION: A good description. Some discussion of other compounds that exacerbate experimental colitis is warranted. For example, Geier found that the prebiotic FOS also tended to exacerbate experimental colitis. REFERENCES: Appropriate

Reply: We greatly appreciate your suggestion and have therefore included a paragraph concerning additional treatments/compounds that may exacerbate experimental colitis. We have included a reference to the suggested study on prebiotic fructo-oligosaccharide[5], as well as other references for additional studies examining pre- and probiotic treatments[6,7], in the Discussion section.

3) Reviewer: 3 (00030205)

Comments to authors:

This is an interesting study with new insight in to inflammtion related to clostridium d. Some aspects need revision.

1. The sample size is small and non-parametric statistics should be used.

Reply: Thank you very much for your comment. For the *in vivo* studies, 10 mice were included in each condition. The data were analyzed with a non-parametric Mann-Whitney *U*-test, which has been indicated in the Methods section of the revised manuscript.

2. The characteristics of the mice groups need to be described in the methods and not only in the figures.

Reply: Thank you very much for your helpful comment. We have added the group assignment and sample size information to the Methods section of the revised manuscript.

3. The relevance for clinical IBD management need a thorough discussion

Reply: We appreciate your astute suggestion. Infection with *C. difficile* has emerged as a significant clinical challenge for IBD patients, as it both mimics and precipitates IBD flares. It is therefore essential that clinicians are vigilant in identifying this infection[8]. The establishment of a mouse model of *C. difficile*-aggravated IBD will allow for further investigation into mechanisms and therapeutic treatments. The clinical relevance of our results is of great importance, and we have therefore included statements relating to this fact in the Introduction and Discussion sections.

References:

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3 Davidson NJ, Hudak SA, Lesley RE, Menon S, Leach MW, Rennick DM. IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *Journal of immunology (Baltimore, Md : 1950)* 1998; **161**(6): 3143-3149 [PMID: 9743382]

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3 References and typesetting were corrected

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

Sincerely yours,

Joo Sung Kim, MD, PhD

Department of Internal Medicine and Liver Research Institute

Seoul National University College of Medicine

103 Daehak-ro, Chongno-gu, Seoul 110-744, South Korea

Fax: +82-2-743-6701, Tel.: +82-2-740-8112

E-mail: jooskim@snu.ac.kr