16/01/20

Dr. Ze-Mao Gong
Science Editor, Editorial Office
Baishideng Publishing Group Inc

Dear Dr. Gong,

We are grateful for the opportunity to revise our paper (ESPS Manuscript NO: 23521) entitled "Oral tolerance is inducible during active dextran sulfate sodium induced colitis", and the helpful comments of your reviewers. We are willing to publish in the *World Journal of Gastrointestinal Pharmacology and Therapeutics.*

We attach a version showing the tracked changes and, separately list our point-by-point responses. We feel that the comments have allowed us to improve the paper and hope you convey our gratitude to the reviewers.

Yours sincerely,

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According to the reviewer's comment, we make change the manuscript.

Reviewer #00159305

1.) Please, take care at abbreviations, some are missing (e.g., MLN in abstract)

Answer: We check the manuscript about abbreviations and modified it.

- Abstract RESULTS section lane 5: MLN
- Coretip section lane 2: DSS
- Coretip section lane 4: MLN (spell out)
- Coretip section lane 7: UC (spell out)
- COMMENT section lane 1: IBD
- COMMENT section lane 3: Crohn (spell out)
- COMMENT section lane 4: UC
- COMMENT section lane 4: DSS

2.) A few more aspects or additional references should be provided for the description of the colitis induction.

Answer: We added the 2nd sentence "DSS administration was performed as previously described with slight modification[11,20] " in the Materials and method induction of colitis section.

3.) On the histological analysis please provide some supplementary control data, pictures on how the specimens looked.

Answer: We modified the Fig 1F and added the information about Fig 1F in the Figure legend.

4.) Discussion section: can you please speculate in a addition phrase about the relevance of another inflammation-related process (e.g oxidative stress) in the context of inflammatory bowel diseases in the

present literature?

Answer: We added the sentences about the nitric oxide (NO) in the 12th paragraph in the discussion section and added the reference 45, 46, and 47.

5.) Why is Acknowledgments section empty? Should it be deleted if there is no one to acknowledge.

Answer: We added the sentence "The authors would like to thank Dr. Kokaze A for a review of biostatistics analysis.
".

6.) English should be double check : for example, some phrases are incomplete "The purpose of this study was to investigate whether oral tolerance is inducible in the active phase of DSS colitis, and how cytokines and regulatory cells, such as Foxp3+ T cell and B10 cells, which are associated with oral tolerance change during colitis in mesenteric lymph nodes (MLN) and the spleen…???"

Answer: We had the English grammar of the revised version checked by a native speaker. All new change points were highlighted in yellow.

Reviewer #00000085:

1.) Can the authors justify the use of ovalbumin and not colon extract proteins ?

Answer: We did not try another proteins. But it is well known that ovalbumin (OVA) is the allergen of allergy to egg in BALB/c mice. For example, Brandt et al. show in J Clin Invest (2003) that the intragastric OVA challenges in OVA/alum-sensitized BALB/c mice induces antigen-specific diarrhea. In addition, OVA have been used as representative antigens, since the oral tolerance was first reported(＊1). Previously, we also had evaluated oral tolerance with

specific antibody using OVA as antigen（＊2）. Because the purpose of this study is to investigate whether oral tolerance is inducible in the active phase of DSS colitis. We used OVA as antigen which is established to evaluete oral tolerance. We did not evaluate whether CEP is effective for active colitis, it is necessary to further study.

（＊1） J.Infect.Dis. 1911. 9:147

（＊2）Effect of intestinal microbiota on the induction of regulatory CD25+ CD4+ T cells. Clinical & Experimental Immunology 2008; 153:127-35

2.) Can the authors provide evidence that the Ova- specific T cell is also decrease following oral therapy ?

Answer: In this study, we can not evaluate the cell numbers OVA-specific T cell, because there is no OVA-specific tetramer for BALB/C mice. In previous literature (*1), the cell number of OVA specific T cell from DO11.10 TCR Tg mice was evaluated in process of oral tolerance. DO11.10 TCR Tg mice are OVA-specific αß-TCR Tg mice with a BALB/C background. OVA-specific αß-TCR recognize the 323-339 peptide fragment of OVA. OVA specificity was evaluated using Anti-DO11.10 TCR antibody. The cell numbers of specific TCR-high CD4-positive T cells in peripheral lymphoid tissues markedly decreased in oral tolerance group but not in the control group. However, total numbers of CD4-positive T cells in thymus, spleen, and lymph nodes were not affected by the oral treatment. These data indicated that tolerance induction in Th2 cells was mainly due to down-regulation of TCR and not clonal deletion.

To the best of our knowledge, there is no similar report that the cell numbers of OVA-specific Tcell in process of oral tolerance for BALB/C mice.

(*1) Eur. J. Immunol. 1998. 28: 134-142

additional explanation

We examine indirectly whether OVA-specific $CD4^+$ T cell is decrease following oral therapy using Flow cytometric analysis of intracellularly stained cells.

Methods are following. OVA-mice were given intragastrically 5mg/day OVA on day1, 2, 3 and 4 and control mice which were given intragastrically PBS on day1, 2, 3 and 4. Single cell suspensions were prepared from the spleen and the MLN from OVA-mice and control mice. $CD4^+$ Tcell is purified using CD4 isolation kit (Miltenyo Biotec, Germany). Antigen presenting cells (APC) were prepared from intraperitoneal cells from mice which were not given any OVA. CD4+ Tcell and APC were co-culutured with the 323-339 peptide fragment of OVA (Medical & Biological) for 24 hours. We added BreffederinA for last 4 hours. These cells were analyzed with flow cytometry.
The antibodies used in this study were anti-CD16/CD32 Ab as an Fc-blocker, BV650 conjugated anti-CD3e Ab, FITC conjugated anti-CD4 Ab (BioLegend), BV421 conjugated anti-CD25 Ab, Alexa647 conjugated anti-IL-4 Ab (BD Biosciences, San Diego, CA, USA).
Dead cells were detected with the Zombie Red Fixable Viability Kit (BioLegend) according to the manufacturer's recommended protocol. Following staining of surface antigens, intracellular IL-4 staining was performed using the Transcription Factor Buffer set (BD Biosciences) according to the manufacturer's recommended protocol. All cells were acquired on the LSRFortessa flow cytometer (BD Biosciences)

Result is following. The frequencies of $CD3^+CD4^+IL4^+$ cell/$CD3^+CD4^+$ (%) from both OVA-mice and control-mice were 0%. Whereas the frequency of $CD3^+CD4^+IL4^+$ cell/$CD3^+CD4^+$ (%) from positive control was 1%. Positive control was $CD3^+CD4^+$ cells from the spleen of control-mice stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO, USA) and 1 μg/ml Brefeldin A (BioLegend) for 4h in 24-well flat-bottom plates.

One study showed that the IL-10 production of OVA-specific Tcell from DO11.10 TCR Tg mice increase in the process of oral tolerance but IL-10 production of OVA-specific Tcell from BALB/C mice did not change. (*2)
Our results and this previous study(*2) suggested that OVA-specific T cell from BALB/C could not be evaluated not only directly using OVA-specific tetramer but also indirectly using the 323-339 peptide.
(*2) Immunol Lett. 2009 Jun 30;125(1):7-14.

Additionally, we changed several sentences about statistical data. Because Our manuscript must include a statement affirming statistical review was performed by a biomedical statistician. Professor. Kokaze whom we consult for statistical methods instructed to change the statistical methods from Studend's t-test to Mann-Whitney U-test.
Most of results did not change except for the expression of IL-6 in the spleen. So, IL-6 in the spleen section was changed.
We rewrite all data was changed from mean ± SEM to median and interquartile range.
for example
from (DSS(+): 0.39±0.05 vs. DSS(-): 1.00±0.11, P<0.01)
to (DSS(+): 0.42 (0.31-0.53) vs. DSS(-): 1.00 (0.84-1.39)
All new change points about statistical data were highlighted in this color.

We add Terminology section following that.
Terminology: Oral tolerance is a phenomenon in which systemic immunity is suppressed relative to orally administered antigens. Oral immunotherapy has been applied for various immune disorders.