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Dear Dr. Yuan Qi:

Please find enclosed the revised manuscript in Word format (file name: 32249-Revised manuscript.doc).

Title: Overexpression of fibrinogen-like protein 2 protects against T cell-induced colitis

Authors: Agata Bartczak, Jianhua Zhang, Oyedele Adeyi, Achiya Amir, David Grant, Reginald Gorczynski, Nazia Selzner, Andrzej Chruscinski, Gary A Levy

Name of Journal: *World Journal of Gastroenterology*

ESPS manuscript NO: 32249

We are pleased that the reviewers found our manuscript to be "very well written and confirms the action of fibrinogen-like 2 protein (FGL2) in inflammatory bowel diseases model." We also appreciate that the reviewer commented, "Collectively, the studies presented confirm that FGL2 is an important immunosuppressive effector and may be explored in inflammatory bowel disease (IBD) treatment. The study was perfectly designed and the methodology is sound." Furthermore, we are grateful that the reviewers provided helpful and constructive suggestions to improve the manuscript.

We have addressed the concerns of the reviewers by providing additional data and clarifying the mechanism of action of FGL2.

The following are point-by-point responses to the reviewer's concerns:

Reviewer #1

1. It should be described the clinical signs of colitis that were evaluated. There is no figure or description of these signs.

Thank you for pointing out this oversight. We have now included the following sentence on page 12 in the results section to indicate what clinical signs of colitis were monitored as follows: "Mice were monitored for signs of disease including, lethargy, piloerection, hunching, dehydration and weight loss."

2. It should be necessary to show the alteration of the expression of FGL2 after cell transfer.

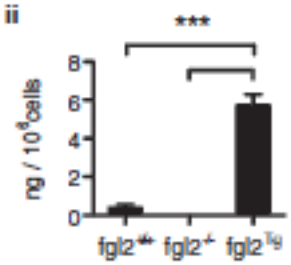
Thank you for this suggestion. We measured plasma concentrations of FGL2 following transfer of Treg into *Rag1*$^{-/-}$ mice. Interestingly, we found that there was no change in plasma levels of FGL2 following transfer of *fgl2*^{Tg} Treg. We have included these data as a new Figure 3D and discuss this on page 12 in the results section. These data strongly support the concept that the adoptively transferred Treg limit inflammation by raising the local FGL2 concentration (in the inflamed colon) as opposed to increasing the concentration systemically.

Reviewer #2

Collectively, the studies presented confirm that fibrinogen-like 2 protein (FGL2) is an important immunosuppressive effector and may be explored in inflammatory bowel disease (IBD) treatment. The study was perfectly designed and the methodology is sound.

1. However, I did not find any figure showing FGL2 expression in any of the cell populations studied.

Thank you for your suggestion. We previously showed that *fgl2*^{Tg} Treg produce more FGL2 than *fgl2*$^{+/+}$ Treg or *fgl2*$^{-/-}$ Treg (Bartczak et al., AJT, 2016). The figure from this paper showing the relevant data requested is shown below. We added a sentence in the results section of the manuscript on page 12 referencing this result.



We have also added a figure to this manuscript showing the production of FGL2 from stimulated and unstimulated *fgl2*^{Tg} CD4^{+} T cells (new Figure 1B). In this figure, stimulated *fgl2*^{Tg} CD4^{+} T cells secrete 4-8-fold more FGL2 than *fgl2*$^{+/+}$ CD4^{+} T cells in culture supernatants. The difference is even greater for unstimulated CD4+ T cells as quiescent *fgl2*$^{+/+}$ T cells secrete nearly undetectable amounts of FGL2.

2. Related with this, I don't see an explanation to understand the mechanism of action of FGL2 on lymphocytes. It is expressed on the cell surface to interact with any receptor in the same cell, or in other cells, or it is secreted (by these or other cells) to act through its receptors in the T cell subsets, or through cell-to-cell contacts? I would like to understand better this issue.

We have now revised the discussion on pages 17-18 to include more details about the mechanism by which FGL2 inhibits T cell proliferation.

We propose that FGL2 inhibits T cell proliferation indirectly by inhibiting the activation of APCs (Chruscinski Rambam Maimonides Med J. 2015). The rationale for this is two-fold. First, the receptor for FGL2, FcγRIIB, is expressed by APCs (DCs, macrophages and B cells) but not by T cells (Nimmerjahn and Ravetch Immunity 2006). When T cells are stimulated with anti-CD3 and anti-CD28 in the absence of APCs, the addition of recombinant FGL2 does not inhibit T cell proliferation (unpublished results). Second, we have shown that FcγRIIB is expressed on the surface of APCs, and recombinant FGL2 could directly inhibit the maturation of DCs as well as induce B cell apoptosis. When APCs FcγRIIB receptor knockout mice were used, recombinant FGL2 did not have any effect on these cells (Liu EurJImm 2008).

Based on these data, we hypothesize that FGL2 secreted from either *fgl2*^{Tg} Treg or Teff induce a tolerogenic phenotype APCs in inflamed tissue and potentially in the draining lymph node. For example, the increased expression of FGL2 by *fgl2*^{Tg} Teff may inhibit

the maturation of DCs encountered by them that in turn, have a reduced capacity for the activation and expansion of the same Teff.

Reviewer #3

In either case, the possible mechanism is that soluble FGL2 in cell culture or in vitro could suppress effector T cell function. This mechanism should be tested either by adding soluble FGL2 or adding tissue culture supernatant from transgenic cells to effector T cells. The alternate possibility is that FGL2 alters Treg function, which in turns inhibits effector T cells. The other comment relates to the description of the T cell and Treg infiltrates into the MLN and colon. Whether FGL2 blocks migration, survival, or proliferation has not been thoroughly tested in this paper. Thus, the conclusions should simply reflect the presence of fewer cells and the discussion might elaborate on the possible mechanisms.

We thank the reviewer for their comments. We believe that the mechanism of action of FGL2 does not involve the direct interaction between FGL2 and Teff cells. As mentioned in the reply to Reviewer #2, the receptor for FGL2, FcγRIIB, is not expressed on the surface of T cells but rather on antigen presenting cells including macrophages, B cells and endothelial cells. In the absence of APCs, FGL2 fails to inhibit T cell proliferation *in vitro*. In contrast, T cell proliferation is inhibited in both MLR and Treg suppression assays (where APC are present) using either *fgl2*^{Tg} cells or treating cultures with recombinant FGL2 (Bartczak AJT 2016, Shalev JI 2008). We have now updated the discussion to include more details about this mechanism of action.

We believe that we have now addressed the concerns of both reviewers and hope that this now makes this paper acceptable for publication in *World Journal of Gastroenterology*.

Sincerely,

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