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JoVE

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Dear Dr. Upponi, Dear Reviewers,

Please find below a point-by-point description of the changes made to the manuscript in response to your constructive comments. All changes in the manuscript have been made using the "track changes" function of Word.

Editorial comments:
•NOTE: Please download this version of the Microsoft word document (File name: 55095_R1_062916) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.

Answer: This version has been downloaded und used to make the required changes.

•There is an instance of unnecessary branding: 10.1.1 - Trizol
Answer: Instead of Trizol, we use the name of the solution as provided by the company: acid guanidinium thiocyanate-phenol-chloroform mixture.

•Formatting:
-Please use italics for Latin phrases (in vivo, in vitro, etc.).
Answer: The text has been checked and all latin phrases are now in italics.

-Please include spaces between numbers and units.

Answer: The text has been checked and where missing, spaces have been added.

•Length exceeds 2.75 pg of highlighted material and must be reduced accordingly.

The highlighted text has been shortened.

•Grammar: Please copyedit the manuscript for numerous grammatical errors. Such editing is required prior to acceptance, and should be performed by a native English speaker.
Answer: The text has been copyedited as requested.

-6.4.8, 7.4 – missing parenthesis
Answer: Parentheses have been added.

•Visualization: Section 6.4 is still discontinuous. We will not be able to film the section in detail unless additional steps are highlighted with a decrease in other highlighted material.
Answer: Highlighting section 6 has been modified. It is now more continuous and includes the entire tissue preparation but omits the actual staining protocol. However, since hematoxylin/eosin stainings are standard protocols published in a plethora of research papers, we think it will be

sufficient to state in the video that the prepared tissue can be used for hematoxylin/eosin stainings according to standard protocols as described in the manuscript in section 6.4".

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.
Answer: All figures shown in the manuscript have not been published before.

•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.
Answer: DOI was missing for reference 14 where it has been manually included.

Reviewers' comments:
Reviewer #1:
Minor Concerns:
1. It would be helpful to have more details on preparation of the DSS solution. What company provides the best DSS for such experiments? Do you recommend filtering the final DSS solution? What is duration of the experiment (days of DSS treatment)? How many times the DSS solution should be replaced with a fresh one during this time period?
Answer: We used DSS from Affymetrix. However, other companies such as ICN and Sigma also provide DSS. As with the DSS concentration, the ideal source of DSS has to be tested as well in each laboratory and caution needs to be paid when lots change. DSS dissolves easily in water and it is not necessary to feed sterile DSS, so we do not filter the solution. 7 days of DSS treatment induces severe colitis. If mild colitis needs to be analyzed, 3-4 days of treatment are recommended. As long as the DSS drinking water stays clear, it is not necessary to change the water; if it turns turbid, it needs to be replaced. These informations have been added to section 1.

2. It is not clear wheter the nutritional supplement should be administered just once, or every day during DSS colitis.
Answer: The nutritional supplement should be applied daily by gavage as described in our previous article (Vargas Robles, 2016, Ox Med Cell Longev). This has been clarified in section 2.5 of the protocol: "Apply nutritional supplements once daily by gavage during the course of the colitis experiment."

Reviewer #2:
Minor Concerns:
1) It would be good to go a little more into detail how DSS is inducing a colitis-like phenotype. This topic is only glanced over in the introduction.
Answer: More information has been added to the introduction on page 3: "DSS induces erosions in the mucosa, resulting in barrier dysfunction and increased intestinal epithelial permeability. The exact mechanism remains unknown; however, a study suggests that DSS interacts with medium-chain-length fatty acids to form nano-lipocomplexes that are able to enter epithelial cells and induce inflammatory signaling pathways." References 11 and 12 have been added for the interested reader.

2)Page 2, lines 85/86. Three times "well" sounds awkward

Answer: We agree. Lines 85-88 on page 2 now read: “Although the clinical signs of UC have been well described, the pathogenesis is still poorly understood.[1] It is accepted among experts that UC is a multifactorial disease, with genetic mutations and aberrant immune responses playing a major role.”

3) Page 2: Is the feeding of supplements a one time application or done more often?
Answer: Compare comment of reviewer 1. It should be applied daily and this has been clarified in section 2.5.

4) Page 2, 2.2 Somewhat confusing, the syringe is described as 1ml, 27G x 13 mm. The latter two numbers do not describe the syringe but probably a needle, which I assume is replaced by the feeding needle.
Answer: Sorry for the confusion. The sentence now reads: Fill a 1 ml syringe connected to a gavage needle (15 G x 50 mm) with the nutritional supplement mixture.

5) Page 7 line 287 ...mount THEM on
Answer: Thank you. This has been corrected (now line 303).

6) Page 10, line 444 STORE
Answer: Thank you. This has been corrected (now line 462).

Reviewer #3:
Minor Concerns:
1. Page 9, 9.2 homogenizer should be "homogenate"
Answer: We actually wash off remaining tissue from the tip of the homogenizing device to make sure that all tissue is included for sonication. This has been clarified in section 9.2.

2. Page 3, 1.2: DSS range should be changed to 2.5-5%
Answer: The range has been changed as requested in section 1.2.

3. Western Blot analysis of tissue should be added to methodology. In the example Fig 3C the text states mRNA data however immunoblotting is presented. Both could be shown.
Answer: Figure 3C actually represents PCR bands from an agarose gel where black and white have been reverted. This has been clarified in the figure legend. We agree that Western blots are means of analyzing protein production. However, since chemokines and cytokines are rather secreted proteins, analysis by ELISA with commercial kits is more frequently being used. Since we did not use Western blots neither in this nor in our previous paper, and given that Western blot is a very standardized technique; we do not think that it will be critical to include a detailed description of a Western blot protocol here.

4. For immunofluorescence studies other methods of fixation should be discussed and what is the advantage of ethanol fixation.
Answer: We have included a sentence in the discussion explaining the use of ethanol for fixation on page 15: “We use ethanol for fixation by dehydration because neither does it interfere with actin filaments (as methanol does) nor does it trigger autofluorescence (as PFA does)”.

5. For tissue processing and analysis, tissue localization ie. proximal versus distal colon should be added and discussed as the effects of DSS could vary along the colon.
Answer: We agree. In the figure legend, we mentioned that the images were taken from distal colon tissues. We also added this information in the results section on page 12. The images shown in figure 2A were taken from distal colon areas. This information has been added to the figure legend. To analyze the overall colon damage, "swiss rolls" of the entire colon should be prepared as described in section 5.8. A note has been added to section 5.8 to point this out.

6. For both MPO and ROS assays time points should be specified and the temporal differences should be discussed.
Answer: We clarified in sections 8.1 and 9.1 that tissues were collected after 7 days of DSS colitis. We did not perform time courses but such inflammation parameters usually increase over time.

7. Page 14 permeability assays; as has been shown by Parkos et.al in a number of recent manuscripts direct application of FITC dextran into ligated ileal (could be extended to proximal colon) loops is another localized way to measure intestinal permeability. Should be added to discussion.
Answer: Thank you. We have included such a paper of the Parkos group and described this method in the discussion on page 15, lines 643-654.

We hope that our revisions meet your expectations and that the manuscript is now suitable for publication in "JoVE".

Sincerely

Michael Schnoor (on behalf of all authors)