Dear editors,

We really appreciate the editorial and reviewers' comments which aimed to improve the quality of our manuscript. We have now carefully revised the manuscript according to the comments. The revised parts are colored texts and filmable contents are highlighted. In addition, please also see the following point-to-point responses to the comments.

1. Editorial Comments:

1) Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have thoroughly proofread the manuscript.

2) Please make the title concise. Maybe: In vivo augmentation of the induction in gut homing regulatory T- cells.

We have modified the title accordingly.

3) Please provide an email address for each author.

All the email addresses have been added.

4) Please list the corresponding author separately.

Corresponding author is now listed separately.

5) Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

The Short Abstract/Summary has been rephrased accordingly.

6) Lines 69-90: Please reduce the usage of word "novel" to focus on the science rather than to present a technique as an advertisement.

We have removed most "novel" terms in the manuscript.

7) Please ensure the Introduction includes all of the following:

A. A clear statement of the overall goal of this method.

Yes.

B. The rationale behind the development and/or use of this technique.

Yes.

C. The advantages over alternative techniques with applicable references to previous studies.

Yes.

D. A description of the context of the technique in the wider body of literature.

Yes.

E. Information to help readers to determine whether the method is appropriate for their application.
Yes.

8) Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
The ethic statement has been added.

9) JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: ALDEFLUOR kit (STEMCELL Technologies), CM-RPMI, (RIA) by Heartland Assays, Inc. (Ames, IA), etc.
All the commercial languages have been removed.

10) Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.
Yes.

11) Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
Yes.

12) The Protocol should contain only action items that direct the reader to do something.
Yes.

13) The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.
Yes.

14) Please use complete sentences throughout the protocol steps.
Yes.

15) Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
See colored revisions.

16) 1.5: Either convert to table or use complete sentence to detail the same. If making table, please upload the table separately in .xlsx style to your editorial manager account and refer the table in the manuscript wherever applicable.
This part has been modified.

17) 2.1: How is the harvesting performed? Please include the details or include some citations for the same.
A citation has been included.

18) 4.2: Please detail how is this done.
Details have been added.

19) There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Filmable content has been highlighted.

20) Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
Yes.

21) Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows reprints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Copy right permission has been attached with revision submission.

22) As we are a methods journal, please ensure the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

A. ***Critical steps within the protocol.***
Yes.

B. Any modifications and troubleshooting of the technique.
Yes.

C. Any limitations of the technique.
Yes.

D. The significance with respect to existing methods.

Yes.

E. Any future applications of the technique.
Yes.

23) Please do not abbreviate the journal titles in the references section.
Yes.

24) Please sort the materials table in the alphabetical order.
Yes.

2. Reviewer 1:

1) Manuscript Summary: The manuscript by Bi at al describes a new method that can be used to induce CCR9+Foxp3+ Tregs using lentiviral in vitro transductions. The method is also used in a recent JI paper by authors.

2) Major Concerns:

A. It is unclear why author describe first the use of BMDCs but then switch to a DC-cell line. Please clarify and provide lentiviral transduction protocols for both, if different.
We used DC2.4 cells for mechanistic studies of DC-CYP-ALDH cells because DC2.4 cells are a cell line and are readily available. However, we used BMDCs for investigating the therapeutic effects of DC-CYP-ALDH cells for TNBS-induced colitis. The reason is that DC2.4 cells have a C57BL/6 background and the TNBS colitis is induced in Balb/c mice. The rationale for the choice of TNBS colitis is that it is mediated by Th1 cells and Th1 cells are recently shown to be critical in the pathogenesis of colitis.

The transduction protocol for both DC2.4 cells and BMDCs is the same.

B. It would be important to describe how the DC-identity of BM cells was or can be confirmed after the in vitro culture steps, ie what is the expect percentage of CD11c+ cells for example?
Normally, more than 90% of the DCs generated by our protocol are $CD11c^+$. DCs are routinely used in our studies[1,2].

C. In vivo experiments: How was the "gut-homing" of Tregs confirmed, now only the expression of CCR9 is determined in mesenteric lymphnode T cells. Did author confirm the gut homing by isolating the intra epithelial and/or lamina propria T regs? Were the proportions of CCR+ cells compared between mesenteric and inguinal/cervival lymph nodes? Were other gut homing markers for Tregs tested?
These information can be found in our JI paper[2]. Since this is a method paper, we are not including all the data published in our recent JI paper in this manuscript. We only requested the permission for reproducing the four figures presented in this paper.

To briefly address the reviewer's question, firstly, we confirmed the gut-homing by subcutaneous immunization with $OVA_{323-339}$-pulsed DC-CYP-ALDH cells followed by enumeration of I-A^d/$OVA_{323-339}$ tetramer$^+$ foxp3$^+$ and IL-10$^+$ Treg cells in intestines.

Secondly, we did not compare I-A^d/$OVA_{323-339}$ tetramer$^+$ foxp3$^+$ and IL-10$^+$ Treg cells between mesenteric and inguinal/cervical lymph nodes, which is an interesting experiment in the future to further define the specific homing capacity of the induced Treg cells.

Thirdly, other gut-homing receptors should be investigated as well in future studies.

D. How suppressive the Foxp3CCR9-positive Tregs are compared to CCR9-negative T regs? In Figure 4, it seems that CCR-negative Tregs tend to have have a higher expression of foxp3? Please include a discussion.

This is an interesting question. Currently, we have only demonstrated that DC-CYP-ALDH cells can increase the frequency (number) of gut-homing Treg cells in peripheral lymphoid tissues and intestines[2]. Consequently, regulatory function in intestines as a whole is enhanced because percent of Treg cells in intestines are increased. Figure 4 shows that intraperitoneal treatment with DC-CYP-ALDH cells, when compared to those with control treatments, significantly increased the percentage of CCR9$^+$foxp3$^+$ Treg cells in mesenteric lymph nodes, meaning that more Treg cells in the mesenteric lymph nodes are able to specifically home into intestinal tissues. Figure 4 further shows that most foxp3$^+$ T cells in mesenteric lymph nodes are CCR9$^-$ and therefore do not have gut-homing capacity. However, whether DC-CYP-ALDH cells can also enhance the regulatory function of each Treg cell per se (such as enhanced expression levels of foxp3 and/or IL-10) requires further investigation.

E. Experiment in Figure 2 is not sufficiently detailed. Please provide the a more detailed biochemical rationale in the method section. What are the DEAB+ and DEAB- cells is the facs blots, are they a mixture of two separate cell cultures or overlaid blots?

Sorry for the insufficient information that led to confusion in Figure 2. We have now provided detailed description of the protocol for the "Evaluation of the overexpressed RALDH2 in DC-CYP-ALDH cells" (section 4.2). "+DEAB" and "-DEAB" are overlaid two FACS plots in both BMDC and BMDC-CYP-ALDH panels, meaning that each panel contains FACS plots from two tubes in which one tube was added with DEAB and the other was not. "+DEAB" tubes are served as negative control for "-DEAB" tubes.

F. ***Did authors perform live gating in all their FACS analyses (FSC/SSC), if so please include this in the description and ideally show these blots, this is especially important for Figure 1 which now contains only a histogram.***

Yes. We routinely perform live gating for all FACS analyses. We now included this description in the figure legend.

3) Minor Concerns:

A. Please include a better description of thee custom made lenti-cyp-aldh plasmid, what is the backbone, is it commercially available?

We have now included more description of the custom made lenti-CYP-ALDH plasmid in "Materials". To address the reviewer's question, the backbone is Addgene's pRRL-SIN.cPPt.PGKGFP.WPRE lentiviral vector. The plasmid containing the CYP27B1 cDNA and the plasmid containing the ALDH1a2 cDNA are available from GeneCopoeia.

B. Can the generated lentivirus-containing supernatant infect humans, include necessary precautions if so.

All the reagents described in this protocol are for animal studies only and are not intended for human use.

3. Reviewer 2:

1) Manuscript Summary: This is a well written paper that covers an interesting and important topic building on the authors recent work in JI. It will be useful for researchers studying immune responses in the gut, regulatory T cell therapy or clinicians interested in IBD.

2) Major Concerns: The methods and presentation are sound and uncontroversial my concerns are

A. What does this add to the JI paper published earlier this year?

Our JI paper is more focused on describing the mechanisms of this technology. However, this JoVE paper will provide more detailed step-by-step description of the techniques that are used in the generation of DC-CYP-ALDH cells. Therefore, readers will find much easier to reproduce the technology by following the protocol described in this JoVE paper.

B. Could they look at using the same approach to generate gut homing Tregs in human cells? This should be straightforward and would extend the utility of the technique to those studying patient cells and those developing clinical grade Tregs. This should at least be covered in the discussion.

Yes, we are preparing for human studies. We have now added this discussion.

4. References:

1 Li, C. H. et al. Dendritic cells, engineered to overexpress 25-hydroxyvitamin D 1alpha-hydroxylase and pulsed with a myelin antigen, provide myelin-specific suppression of ongoing experimental allergic encephalomyelitis. FASEB J. doi:fj.201601243R [pii] 10.1096/fj.201601243R, (2017).

2 Xu, Y. et al. In Vivo Generation of Gut-Homing Regulatory T Cells for the Suppression of Colitis. J Immunol. 202 (12), 3447-3457, doi:10.4049/jimmunol.1800018 jimmunol.1800018 [pii], (2019).