EMBO Molecular Medicine

Lanatoside C activates the E3 ligase STUB1 to inhibit FOXP3 transcriptional activity and promote antitumor immunity

Qian Zhou, Tong Yang, Xixi Yu, Bo Li, Jin Liu, Yongxin Mao, Rongxiang Guo, Zhuo Feng, Li Zhou, Guandi Zeng, nan li, Jinxia Liang, lu liu, Pengju feng, Hong-Bing Shu, and Liang Chen

Corresponding authors: Liang Chen (chenliang@jnu.edu.cn) , Hong-Bing Shu (shuh@whu.edu.cn), Qian Zhou (zhouqian@jnu.edu.cn)

Review Timeline:

Submission Date:	6th Aug 24
Editorial Decision:	28th Aug 24
Revision Received:	27th Nov 24
Editorial Decision:	18th Dec 24
Revision Received:	29th Dec 24
Accepted:	3rd Feb 25

Editor: Jingyi Hou

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

28th Aug 2024

Dear Dr. Zhou,

Thank you again for submitting your work to EMBO Molecular Medicine. We have now heard back from the two referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find your study of potential interest. However, they raise a series of concerns, which should be convincingly addressed in a major revision of the present manuscript.

The referees' recommendations are relatively straightforward, so there is no need for me to reiterate the points listed below. All the issues raised by the reviewers need to be carefully addressed.

We would welcome the submission of a revised version within three months for further consideration. As you may already know, our editorial policy allows in principle a single round of major revision, and it is therefore essential to provide responses to the referees' comments that are as complete as possible.
EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published, we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

Kind regards,
Jingyi

Jingyi Hou
Editor
EMBO Molecular Medicine

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When submitting your revised manuscript, please carefully review the instructions that follow below. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

1) A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure). For guidance, download the 'Figure Guide PDF': (https://www.embopress.org/page/journal/17574684/authorguide#figureformat).

3) A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) A complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary

datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).

In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

7) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.). See also 'Figure Legend' guidelines: https://www.embopress.org/page/journal/17574684/authorguide#figureformat

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

See detailed instructions here:

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11) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting

- the medical issue you are addressing,

- the results obtained and

- their clinical impact.

This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

12) Author contributions: You will be asked to provide CRediT (Contributor Role Taxonomy) terms in the submission system. These replace a narrative author contribution section in the manuscript.

13) A Conflict of Interest statement should be provided in the main text.

14) Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one-sentences bullet points that summarizes the paper. Please write the bullet points to summarize the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion

of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

Please also suggest a striking image or visual abstract to illustrate your article as a PNG file 550 px wide x 300-600 px high.

15) All Materials and Methods need to be described in the main text using our 'Structured Methods' format, which is required for all research articles. According to this format, the Methods section includes a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section describing the methods using a step-by-step protocol format.

The Reagents and Tools Table can be downloaded from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#structuredmethods)

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

Treg cells are major obstacle in cancer therapy. In this study, the authors develope an interesting screening technique and found that Lanatoside C, an FDA-approved drug,could inhibit FOXP3 transcriptional activity and promote antitumor immunity,thus has potential translational values.

Referee #1 (Remarks for Author):

The potential interesting manuscript by Dr. Zhou and colleagues investigated the potential inhibitors of FOXP3 transcriptional activity by screening FDA-approved drugs, and identified Lanatoside C (Lac) as an effective disruptor of the FOXP3-RUNX1 complex. Functional assays revealed that Lac attenuated the differentiation and proliferation of Tregs as well as their inhibitory activities on CTLs. Furthermore, the authors explored the mechanisms through which Lac induces the degradation of RUNX1. Notably, they demonstrated the anti-tumor efficacy of Lac in two mouse models of lung cancer and highlighted its potential synergistic effect with ICB in delaying tumor growth. Overall, this study is well designed and the results obtained are interesting and convincing. The immunosuppressive function of Tregs is a major challenge in cancer therapy. Considering Lac is FDA-approved, its discovery as a FOXP3 inhibitor offers a unique advantage for expedited clinical application, and thus has potential translational values. I have only a few minor concerns.
1. In Figure 7, Lac dramatically enhanced PD-1 antibody to shrink tumors. However, their impact on reduced Tregs infiltration and enhanced expression of IFNγ and perforin (as shown with the flow cytometry data in Figure 7 and Figure S9) seems relatively mild. One possible reason could be due to the method used, since flow cytometry analysis might not truly represent the immune-microenvironment in situ tumors. The authors are encouraged to include the image data from Immunohistochemistry or IF staining on these samples.
2. The gating strategy and analysis in flow cytometry data could be optimized to get better separation on cell populations. Also, ELISA could be employed to determine the cytokine production in some experiments.
3. The dosage of Lanatoside C: High dose of Lanatoside C is toxic for patients in clinic. The authors should discuss whether the concentration of Lanatoside C used in this study could be related to clinic.
4. For clarity, the names of fluorescence dye in the flow data should be omitted.
5. The screen platform is interesting and useful. Could this technique be used for other model? The authors could add their view in the discussion.

Referee #2 (Comments on Novelty/Model System for Author):

The study's core finding that an FDA-approved drug, Lanatoside C, can inhibit Treg function and enhance anti-tumor immunity is quite exciting. However, several technical issues, especially the poor Foxp3 FACS staining, undermined the quality of the study. Most of the quality issues are fixable with a proper revision.

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reduced Treg activity. The main strength of the study is the identification of a new therapeutic potential for the FDA-approved drug Lanatoside C in promoting anti-tumor immunity. Some weaknesses, mostly involving Treg characterization, are also identified as listed below.

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6. Please add quantification to the Western-blot results in Fig. 3H and Fig. 4I. The differences in band intensity are not evident in these experiments.

7. The STUB1 co-IP bands look poor in Fig. 4D. Also, Lac treatment in this experiment did not degrade RUNX1 more compared to DMSO treatment. Is there an explanation?

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Reply: We thank Reviewer for finding our work interesting and convincing.

1. In Figure 7, Lac dramatically enhanced PD-1 antibody to shrink tumors. However, their impact on reduced Tregs infiltration and enhanced expression of IFNγ and perforin (as shown with the flow cytometry data in Figure 7 and Figure S9) seems relatively mild. One possible reason could be due to the method used, since flow cytometry analysis might not truly represent the immune-microenvironment in situ tumors. The authors are encouraged to include the image data from Immunohistochemistry or IF staining on these samples.

Reply: We thank Reviewer for these great suggestions! Following Reviewer's suggestion, we have checked Tregs infiltration ($CD4^+FOXP3^+$) and expression of IFNγ ($CD8^+IFN\gamma^+$) and perforin ($CD8^+$ $perforin^+$) through IF staining on these

samples. Our results argued that Lac drastically reduced the proportion of $CD4^+FOXP3^+$Tregs in immune-microenvironment in situ tumors (updated in new Fig. EV5B & EV5C). Unfortunately, Perforin and IFNγ staining didn't work well IF (Fig.1 to Reviewer). We agree with Reviewer that FACS by itself may underestimate the immune reaction in tumor in situ. However, due to limitation of reagents, we are unable to present data from IF.

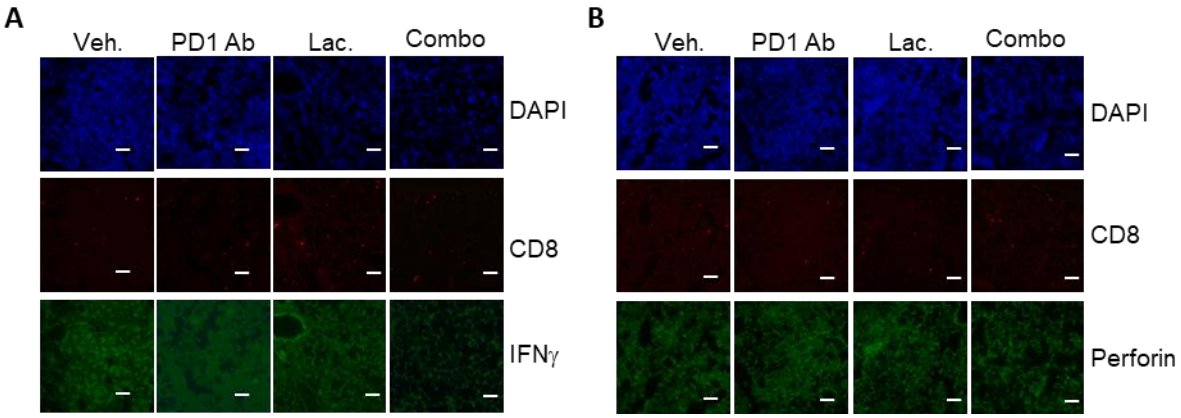

Fig.1 to Reviewer

A & B: Immunofluorescence analysis of expression of IFNγ and perforin by $CD8^+$T cells. $KRAS^{G12D}$ bearing lung cancer mouse received indicated treatments for 2 weeks. Part of lung tissues were fixed for Immunofluorescence analysis expression of IFNγ (A) and perforin (B) by $CD8^+$T cells. Scale bar: 100 μm.

2. The gating strategy and analysis in flow cytometry data could be optimized to get better separation on cell populations. Also, ELISA could be employed to determine the cytokine production in some experiments.

Reply: Thanks so much for your suggestions. The gating strategy and analysis in flow cytometry data are optimized. A new FOXP3 antibody (FJK-16s clone from eBioscience) and its Foxp3 staining buffer (00-5523-00, eBioscience) were also employed to repeated some key in vitro assay to get better separation on cell

populations (New Fig. 1D-G & Fig. EV1B & D& F& H & Fig.2 to Reviewer).

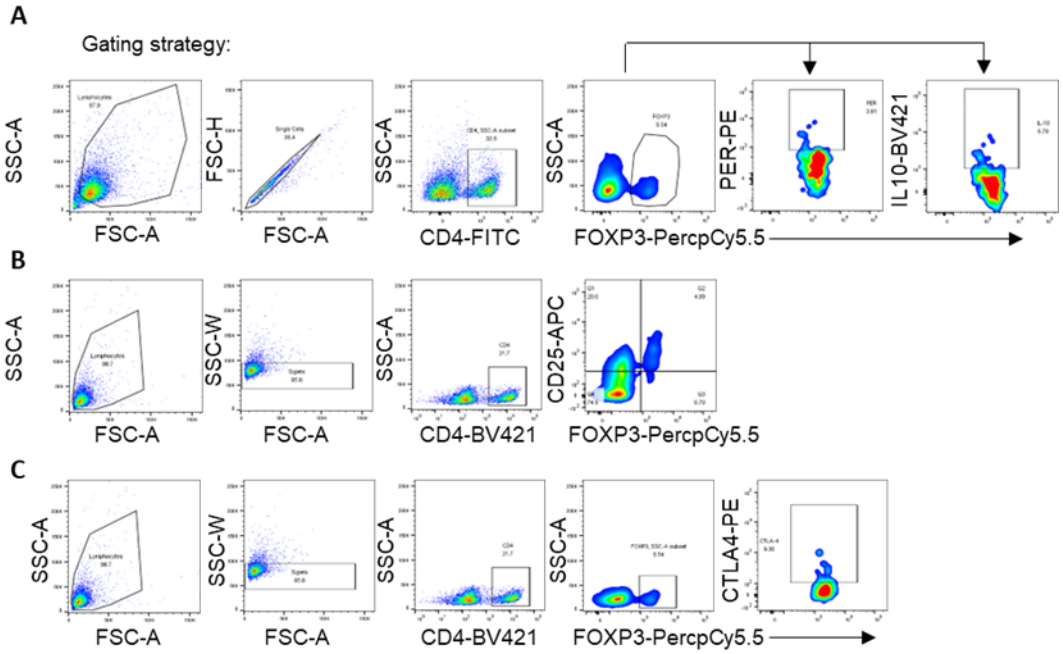

Fig.2 to Reviewer

A-C: Gating strategy for analyzing the expression of Perforin, IL10, CD25 or CTLA4 from mouse spleen or lymph node. New FOXP3 antibody (FJK-16s clone from eBioscience) and Foxp3 staining buffer (00-5523-00, eBioscience) were used to stain FOXP3 according to the manufacturer's manual.

Following Reviewer's suggestion, ELISA was employed to determine expression of IL10 secreted by Treg cells. Our results suggested that Lac. significantly suppress IL10 expression by Tregs (New Fig. 1H & Fig.3 to reviewer).

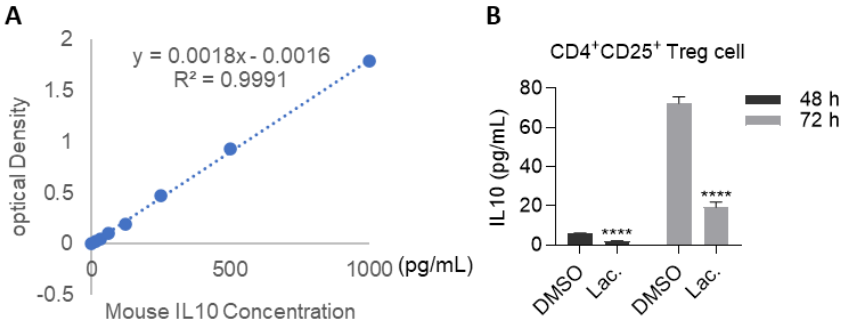

Fig.3 to Reviewer

A: Mouse IL10 ELISA standard curve; B: ELISA analysis of the impact of Lac on IL10

expression by Tregs. Tregs ($FOXP3^{+}CD25^{+}$) cells were treated without or with Lac for 48 or 72 hours before ELISA analysis.

3. The dosage of Lanatoside C: High dose of Lanatoside C is toxic for patients in clinic. The authors should discuss whether the concentration of Lanatoside C used in this study could be related to clinic.

Reply: We agree that Lanatoside C dose in our study is rather high (6mg/kg every two days). Our current paper is a proof of principle. If lucky enough, our work was supported for clinical translation, dosage will be carefully titrated in Stage I trial. Moreover, Lac could be administered into tumor directly or through help from nano-materials. We have discussed this issue in page 20 (line 445-line 452).

4. For clarity, the names of fluorescence dye in the flow data should be omitted.

Reply: Thanks so much for these suggestions. We have removed the names of fluorescence dye in the flow data.

5. The screen platform is interesting and useful. Could this technique be used for other model? The authors could add their view in the discussion.

Reply: We thank Reviewer for offering us the opportunity to highlight the potency and convenience of our screen platform. Actually, we have successfully applied similar platform in measuring the inhibitory function of small molecular chemicals on interaction between PD-1 and SHP2 (EMBO Molecular Medicine. 2020;12(6):e11571).

We have now discussed the potency and applicability of this platform in Discussion part (page17, line 384-line 394).

Referee #2 (Comments on Novelty/Model System for Author):

The study's core finding that an FDA-approved drug, Lanatoside C, can inhibit Treg function and enhance anti-tumor immunity is quite exciting. However, several technical issues, especially the poor Foxp3 FACS staining, undermined the quality of the study. Most of the quality issues are fixable with a proper revision.

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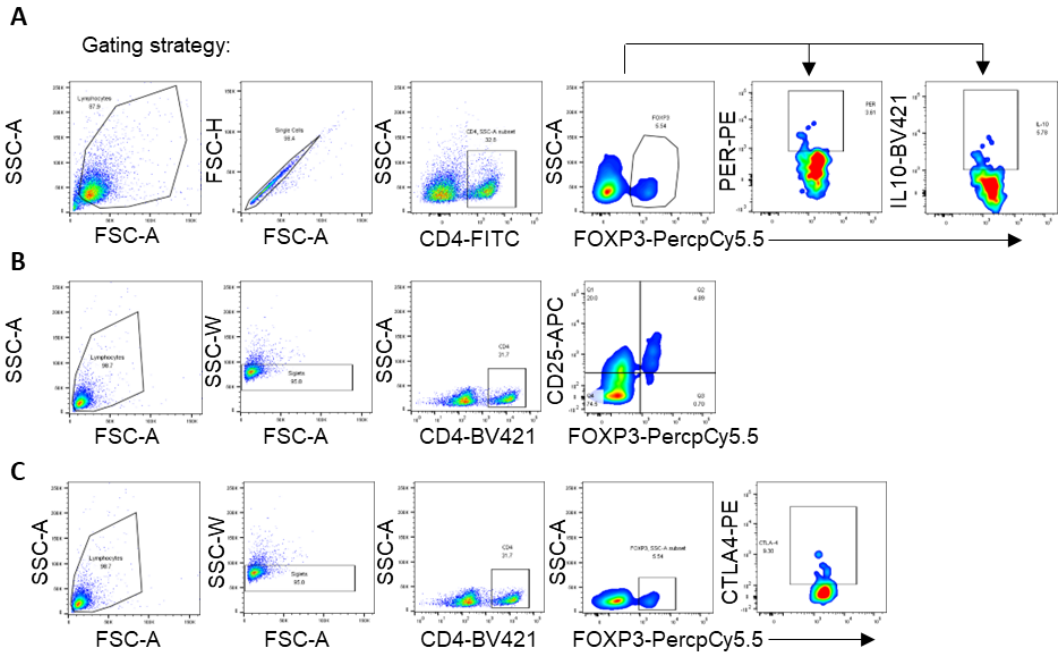
understandable that not all Foxp3 stains need to be repeated, but some of the key in vitro assays should be redone with proper Foxp3 staining to enhance the quality of the overall study.

Reply: Thanks so much for these great suggestions! A new FOXP3 antibody (FJK-16s clone from eBioscience) and its Foxp3 staining buffer (00-5523-00, eBioscience) were employed to repeat key in vitro assays. The new system greatly improved the FOXP3 staining (New Fig. 1D-G & Fig. EV1B & D & F& H) and better FOXP3^{+} population resolution (Fig.2 to Reviewer). Our current data clearly showed that Lac treatment inhibited CD25, CTLA4, Perforin (PER) and IL10 expression in Tregs.



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2. The authors showed very nice data on how Lac binds to STUB1's TPR domain.

However, it is not clear how the interaction between Lac and STUB1 can enhance the ubiquitination and degradation of RUNX1.

Reply: Our data (Fig. 4D) showed that Lac treatment enhanced ability of STUB1 to bind RUNX. Please refer to lane 1 and lane 2 in the experiment: MG132 stabilized RUNX1; In this case, Lac enhanced the ability of RUNX1 to immunoprecipitate STUB1.

We believe that binding of Lac to STUB1's TPR domain altered the structure of STUB1, such that the interaction between STUB1 and RUNX1 is enhanced. We tried to run a molecular simulation on that. However, due to our limited capacity on the simulation, we fail in these attempts. Therefore, we are unable to show the detailed structural information on this point. We hope that Reviewer is persuaded by our data that Lac binds STUB1 to form a STUB1/Lac complex and that this STUB1 complex has enhanced binding ability to RUNX1 for ubiquitylation.

3. RUNX1 is important for the development of multiple lymphoid and myeloid cell lineages. For example, thymic T cell development is disrupted in RUNX1 knockout mice. Mutations in RUNX1 could lead to leukemia. The current study has not adequately addressed the safety issue of Lac treatment. At a minimum, the authors need to analyze the composition of the thymic and spleen T cell subsets after two weeks of Lac treatment.

Reply: Thanks so much for pointing this important point to us. Following Reviewer's suggestion, we checked the composition of T cell subsets in thymic and spleen after

two weeks of Lac treatment.

We found that the spleens were larger in LAC treatment group compared to the Vehicle group (Fig. 3 to Reviewer A). We saw a decrease in proportion of $CD4^+$and $CD8^+$cells in spleens. However, the ratio of $CD8^+$ to $CD4^+$ cells remain unchanged (Fig. 3 to Reviewer B & C). Importantly, we saw that LAC treatment significantly reduced the proportion of TREG ($FOXP^+CD25^+$). We also noticed a decrease in TH1 ($CD4^+IFN\gamma^+$) and TH2 ($CD4^+IL4^+$) populations in spleens. In contrast, percentage of TH17 ($CD4^+IL17A^+$) cells remains constant (Fig. 3 to Reviewer D-G) in spleens.

We also checked the alteration of T cell subsets in thymus. We found that the percentage of double positive ($CD4^+CD8^+$) thymocytes were reduced. In contrast, CD8 single positive ($CD8^+$) thymocytes were increased and CD4 single positive ($CD4^+$) thymocytes had no remarkable change (Fig. 4 to Reviewer H & I). Taken together, our data showed that 2 weeks treatment with LAC reduced Treg percentage while have limited impact on overall immune components.

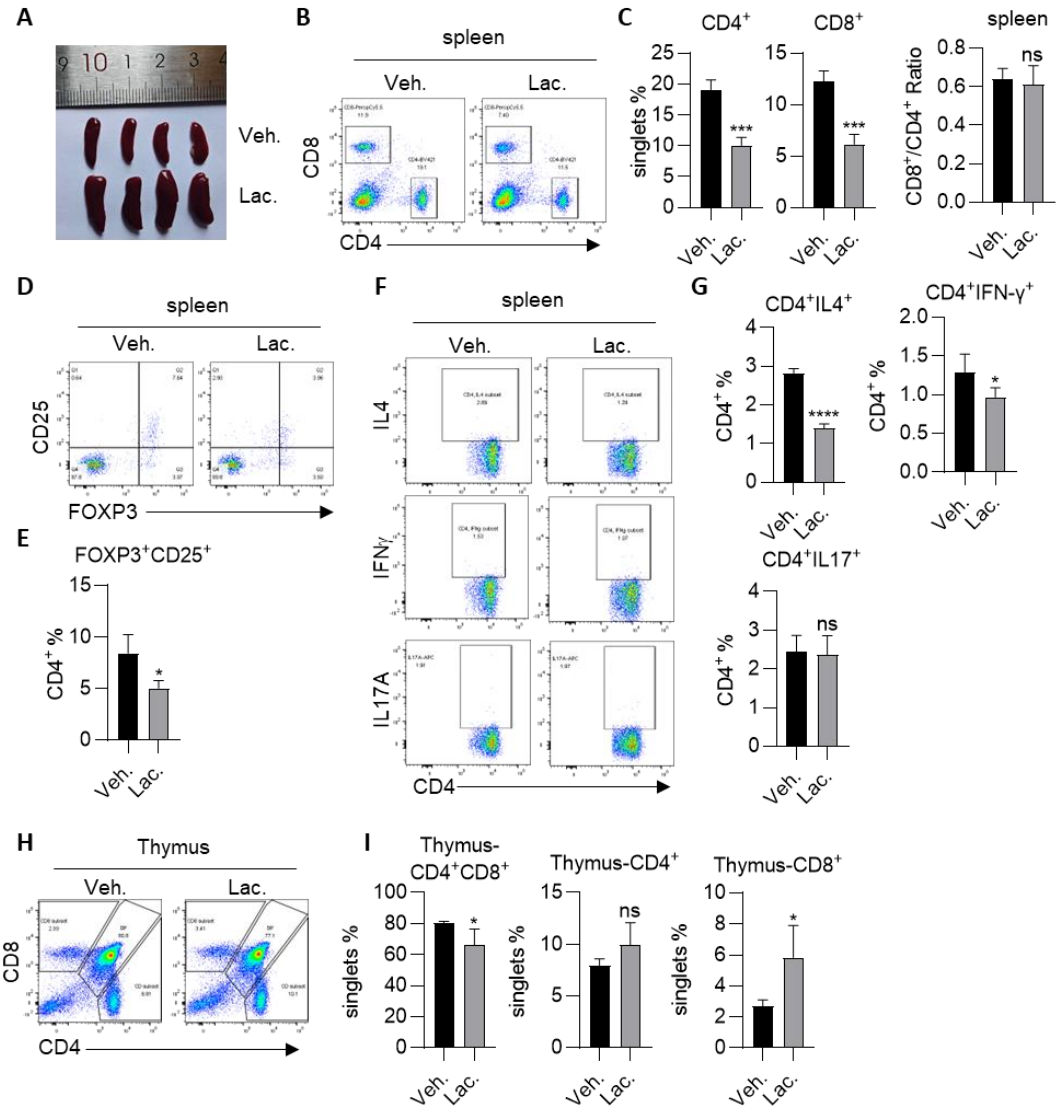

Fig. 4 to Reviewer

Mice (C57BL/6J) were randomized for treatment (n=4) with PBS or Lac for two weeks.

A: Spleens were dissected to photograph; B: FACS analysis of proportion of $CD4^+$and $CD8^+$cells in spleen after 2 weeks of Lac treatment; C: Statistics of Fig. 3 to Reviewer B; D-G: FACS analysis of proportion of TREG ($FOXP^+CD25^+$), TH1 ($CD4^+IFNG^+$), TH2 ($CD4^+IL4^+$) and TH17 ($CD4^+IL17A^+$) cell populations in the spleen after 2 weeks of Lac treatment (D & F). Statistics of Fig. 3 to Reviewer D & F (E & G); H: FACS analysis of proportion of $CD4^+$ SP, $CD8^+$ SP and $CD4^+CD8^+$ DP cells in thymus after 2 weeks of Lac treatment; I: Statistics of H.

4. In Fig. 1C, Foxp3 mRNA should be quantified along with CD25, CTLA4, and IFNg.

Reply: Thanks so much for this great suggestion. We conducted qRT-PCR to quantify

Foxp3 mRNA along with *Cd25*, *Ctla4*, *Il10* and *Ifn*γ. We found that Lac potently inhibited the transcription of *Foxp3*3, *Il10*, *Cd25* and *Ctla4*, but not *Ifnγ* (new Fig. 1C).

5. For CFSE measurement of cell division in Fig. 1I and Fig. 2A, all divided cell populations should be included (all peaks except the one on the most right side) instead of cells with the most divisions (the peak on the most left side). Cell divisions can also be quantified using the division index and proliferation index calculated by the Flowjo software.

Reply: We thank Reviewer for pointing out this important issue to us. Follow Reviewer's suggestion, Division index (DI) and proliferation index (PI) were calculated by the FlowJo software. Results were updated in New Fig. 1I & EV1L & 2A & EV2A.

6. Please add quantification to the Western-blot results in Fig. 3H and Fig. 4I. The differences in band intensity are not evident in these experiments.

Reply: Thanks so much for bringing this point to our attention. We quantified the bands against β-actin. The results are updated in New Appendix Fig. S3G (Fig. 3H) & Fig. 4J (Fig. 4I). Our results indicated that Lac treatment shortened the half-life of both ectopically expressed and endogenous RUNX1.

7. The STUB1 co-IP bands look poor in Fig. 4D. Also, Lac treatment in this experiment

did not degrade RUNX1 more compared to DMSO treatment. Is there an explanation?

Reply: We thank Reviewer for pointing this out to us. In order to clarify the degradation of RUNX1, we extended the treatment time of Lac and repeated this experiment (New Fig. 4D). Our results clearly showed that Lac promoted the association between RUNX1 and STUB1.

8. Please show statistics on the FACS data in Fig. 7G-7J since the differences in these experiments were not evident in some cases.

Reply: Thanks so much for this great suggestion. The statistics on the FACS data of Fig. 7G-7J were included in New Fig. EV5A & EV5F & EV5G.

18th Dec 2024

Dear Liang,

Thank you for submitting your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the two referees who re-assessed your work. As you will see, the referees are now supportive, and I am pleased to inform you that we will be able to accept your manuscript pending the following amendments:

1. Please remove the Authors' Contribution section from the manuscript file.

2. The current synopsis image is too large. Please provide the figure as a PNG file 550 px wide x 300-600 px high.

3. Appendix needs to be uploaded as a PDF file.

4. ORCIDs should be removed from page 33.

5. Methods
- "Materials and Methods" should be renamed to "Methods".
- Remove redundant information already included in the Reagents and Tools table, such as Reagents, Experimental instruments and equipment.
- Please rewrite the "Plasmid constructs" section to include more details. Remove the related information in Reagents and Tools table.
- Ethics statements should be removed from page 32 and integrated into the relevant sections on animal experiments in the "Methods" section.
- For animal work, confirm that all experiments were performed in accordance with relevant guidelines and regulations. Gender, age and genetic background must be indicated, along with housing conditions.

6. "Conflict of interest" should be renamed to "Disclosure Statement and Competing Interests" and placed after "Acknowledgments".

7. "The paper explained" is too brief. Please expand this section and incorporate it into the manuscript file. You may refer to any of our published articles for guidance on the appropriate length and structure.

8. The Synopsis and Bullet points are also too concise. Please refer to any of our published article for an example.

9. Data availability:
- Before submitting your revision, the mass spectrometry datasetes produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'.
- Please update the Data availability information in the Author checklist accordingly.

10. EV tables and datasets
- The current Tables EV1, 3, and 4 are quite large. Please update them to EV Datasets and upload them as Excel tables. Include the legends within the Dataset in a separate sheet labelled 'Legend'. Also, update their callouts (in the manuscript file and Reagents and Tools table) to Dataset EV#.
- Please update the callouts of the remaining EV tables accordingly.
- There are Chinese characters in the current Table EV3 - please correct this.

11. Funding: the text should be removed from the Comments box, and each funder and its grant number should be provided as separate entries (More Funders option): Guangdong basic and applied basic research foundation(2024A1515030238), Guangzhou Science and Technology program City-University Joint Funding Project (2024A03J0596), Open Project Funded by the MOE Key Laboratory of Tumor Molecular Biology (202302)

12. Source data:
- Source data for EV and Appendix figures need to be combined into one zip folder.
- For main figures: all SD need to be gouped into zip folders and uploaded as one folder per figure, using the nomenclature "manuscriptID_SourceDataForFigure x"
- Please provide numerical data as individual .xls (including a tab describing the data) or as .csv files (including a separate README file) instead of .PRISM, .pzfx, or .fcs file.

13. Please address the following issues in figure legends:
- Please note that the legend for fi gure 1J is missing in themanuscript. This needs to be rectifi ed.
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Please feel free to let me know if you have any questions. I look forward to seeing a revised form of your manuscript soon.

Kind regards,
Jingyi

Jingyi Hou
Editor
EMBO Molecular Medicine

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***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

This study is well designed and the results obtained are interesting. The immunosuppressive function of Tregs is a major challenge in cancer therapy. Considering Lac is FDA-approved, its discovery as a FOXP3 inhibitor offers a unique advantage for expedited clinical application, and thus has potential translational values.

Referee #1 (Remarks for Author):

The authors addressed most of the concerns raised by the Reviewers and have revised the manuscript accordingly.

Referee #2 (Remarks for Author):

The revised manuscript has fully addressed the reviewer's concerns. Congratulations to the authors for their excellent work!

The authors addressed the remaining editorial issues.

3rd Feb 2025

Dear Liang,

Happy Chinese New Year! I am pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

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If you have any questions, please do not hesitate to contact the Editorial Office.

Thank you for sending this interesting work to EMBO Molecular Medicine!

Kind regards,
Jingyi

Jingyi Hou
Senior Editor
EMBO Molecular Medicine

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Journal Submitted to: EMBO Molecular Medicine	The EMBO Journal - Author Guidelines
Manuscript Number: EMM-2024-20400	EMBO Reports - Author Guidelines
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Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Dataset EV2, page23 (line 497-line 513) in Manuscript

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Table EV2

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	page25 (line 545-line 552) in Manuscript
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
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Please detail **housing and husbandry conditions**.	Yes	page26 (line 562-line 570) in Manuscript

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Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	page32 (line 693-line 722)
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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Dataset EV3

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	The details were described as in Figure legends.
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	The details were described as in Figure legends.

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
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Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	page26 (line 562-line 570) in Manuscript
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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

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If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	