EMBO reports

A peptide encoded by pri-miRNA-31 represses autoimmunity by promoting Treg differentiation

Hong Zhou, Fangzhou Lou, Jing BAI, Yang Sun, Wei Cai, Libo SUN, Zhenyao Xu, Zhaoyuan Liu, Lingyun Zhang, Qianqian Yin, Junxun ZHANG, Yuanyuan Gao, Zhikai Wang, Liman Niu, Xiaojie CAI, Siyu DENG, Hong Wang, Li Xia, Florent Ginhoux, Qun Li, and Honglin Wang
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(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.)

Dear Prof. Wang,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details, please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

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/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an

appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) 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: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that, where applicable, the number "n" for how many independent experiments were performed and the type of replicate (biological or technical), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please note our new reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

11) Please add a conflict-of-interest statement to the manuscript and order the manuscript sections like this (using these section names):
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods -Data availability section - Acknowledgements - Author contributions - Conflict of interest statement - References - Figure legends - Expanded View Figure legends.

12) Please add up to 5 key words to the title page.

Finally, please note that all corresponding and co-corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please do that for the co-corresponding author Qun Li. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

The manuscript entitled "Modulating immune disorder by a peptide nested in microRNA host gene to treat autoimmune disease" by Hong ZHOU and collaborators identified a short open reading frame encoded peptide produced from the microRNA31 host gene. they described its expression and function. They discovered a peptide, termed miPEP31, which is encoded by pri-miRNA-31. They provide evidences that miPEP31 is a cell-penetrating peptide both in vitro and in vivo. They show that miPEP31 is expressed in regulatory T cells (Treg) and promotes their differentiation. They also find that miPEP31 downregulates miR-31 expression, enhances induction of pTreg cells, and dramatically suppresses experimental autoimmune encephalomyelitis (EAE). Mechanistically, they identified miPEP31 acting as a transcriptional repressor to inhibit the transcription of miR31 and suggest that miPEP31 binds to the miR31 promoter, counteracting an unknown miR31 transcriptional regulator.

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etc...

I am not a specialist of Treg cells but the experiments provided on the biology of the miPEP31 look convincing. My main criticisms concern some interpretations and the conclusions drawn in this paper.
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Without these two experiments, the conclusions drawn are not experimentally supported. The authors can not exclude that miPEP31 is acting in parallel. Leaving the story without considering the possibility that miPEP31 act at a genome scale is not acceptable. I would rather suggest either performing these experiments or to modify the manuscript accordingly in order to be more precautious in their conclusions.

Other points:

- Please add more precisions to the mass spectrometry detection of the conserved sequence of miPEP31 in human. Change "Mass Spectrum" to "Mass spectrometry analysis" in the method section and in the figure EV4. Also, precise the expected sequence in amino acid of the human form of miPEP31. Does the entire predicted sequence match to the peptide identified? What was the false discovery rate used in the PEAKS analysis? What was the score of the identified peptide? Did the authors check that the observed peptide is unique to the miPEP31 human form? Was this peptide the only match for this MSMS spectrum? Were the mass spectrometry data deposited into public repository? Did the authors manage to detect any signal in western blot using the miPEP31 antibody from HCT116 lysate? What was the rationale behind performing two successive ultrafiltration steps after immunoprecipitation?

- p5 lane 16: GSM2778762, SRR6023964 : instead introduce the reference where these data were published please.

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Referee #2:

Zhou et al. show that the 44aa peptide miPEP31, encoded by pri-miRNA-31, induces differentiation of Foxp3+ regulatory T cells. The peptide is a transcriptional repressor that modulates miR-31 expression, which promotes differentiation of pTregs and inhibits EAE. Overall, the experiments are well laid out and the results are interesting. Peptides may become good candidates as therapeutic molecules since they are cheaper than other therapies such as monoclonal antibodies, are specific and are potentially safe biological agents. However, the work needs substantial improved statistical analysis and additional experiments to demonstrate the penetrance of the peptide in target cells in vitro and in vivo.

1. Figure 1F-G, in addition to the WB, immunohistochemistry of an embryo or neonate could have been performed to localize the expression of the peptide in lymphoid organs. Alternatively, expression of the peptide should be shown in some non-lymphoid tissue in the WB, to demonstrate that expression is restricted to lymphoid tissue.

2. Figure 2 A and B show representative images without any quantification. To demonstrate peptide expression in Foxp3+ and - cells, a number of experiments with sufficient sample size to show a statistically significant analysis should be performed. The same applies for Figures E and F, the data are representative and not quantified, the number of animals per experiment should also be shown together with the statistical analysis of the data.

3. Does miPEP31 have any effect on membrane expression of Treg cells suppression markers?

4. Figure 3A-D, although CD4+ T cells are mentioned in the results section, the experiments are performed with adherent NIH3T3 cells, making it easier for the peptide to penetrate the cell, these assays should have been done with a suspension cell line more similar to lymphocytes (Jurkat).

5. Figure 3D, how many samples have been analyzed? Statistical analysis is not shown in figure legend.

6. Figure 3E-G, the presence of FAM labeled miPEP31 is detected in CD4+ and - fixed cells, however this experiment does not determine the exact localization of the peptide, immunofluorescence of these cells would be needed to determine the localization of the peptide in these cells.

7. Figure 4, they show that positively charged Arg residues are those that favor peptide entry into the cell, in panel C there is no statistical analysis. Panel D shows representative images and there is also no quantification. It is not demonstrated whether the scramble peptide, which is used as a control in some experiments, has the same penetrability characteristics as the miPEP31 peptide. It is also not mentioned whether both peptides have similar physicochemical parameters.

8. Figure 5A_B, representative images and MFI are shown but quantification and statistical analysis are lacking.

9. Clinical score in figure 5C should be analyzed as two way ANOVA repeated measures. P value is shown in the graphic, what is the statistical significance?

10. Figure 5D, Histological scoring of this figure and statistical analysis are needed.

11. Figure 6A-B, the Q PCRs are normalized by the basal conditions, however they perform a tTest of each sample compared to the basal level. This is not correct, the statistical analysis should be an ANOVA.

12. Figure 6F should be completed with EAE score of WT mice +/- miPEP31 in parallel.

13. Figure 7, The statistical analysis is not specified in the figure legend, what do these P values correspond to? In panel 7C it is indicated that a t-Test has been performed, however an ANOVA should be performed instead, with the appropriate post-test.

14. Methodology. They perform RNA extraction with Trizol, this precipitation based method can lead to problems for the extraction of microRNAs, how do they make sure they are not losing smaller sized material such as microRNAs?
Intracellular labeling for IL-17 is done with the Foxp3 permeabilization kit that permeabilizes nuclei, this is not very suitable. Intracellular labeling for IL-17 should be performed with a gentler permeabilization for the plasma membrane, and both labelings should be performed separately.

Referee #3:

The work from Zhou et al. identifies miPEP31 as a peptide translated from pri-miRNA31. The authors had previously shown that miR31 negatively regulates peripherally induced Tregs and they now establish that miPEP31 selectively downregulates miR-31, thereby favoring the generation of pTregs. The relevance of these results are highlighted by the use of miPEP31 in vivo in the EAE model, where it ameliorates EAE severity.

In general the manuscript is well organized and clearly written, although it would benefit from being proofread for grammar mistakes. A very large amount of data is presented, some of which are not completely relevant to the story and could be removed from the main figures. Please see some additional comments below:

1. How does miPEP31 enter the cells?
2. Fig 2E. It would be desirable to repeat these experiments using cells from mice that are not immunized, to compare WT vs inflammatory conditions.
3. Is the increase in Foxp3+ cells observed in Figure 2 a result of an increase in newly generated Tregs, or is it due to increased proliferation (and therefore expansion) or the already existing pool of Tregs?
4. Fig 2E. Please include the number of experiments and mice per group analyzed and add the statistical analysis of the data.
5. Fig 2F Suppression assays were performed using naïve T cells as responder cells. Could the authors explain the rationale for that? It would be desirable to also repeat these experiments using memory T cells as responder population, and include the statistical summary of all experiments performed.
6. Fig 3A. This histogram does not prove that miPEP31 is intracellularly detected (it could be attached to the cell surface). Please define what "NC" is and how it was obtained.
7. Fig 3B. It would be desirable to include markers that identify cytoplasm and/or nucleus of the cells, to help locate GFP.
8. Fig 3A, B. Please compare the values obtained to the endogenous miPEP31 expression on Tregs.
9. While overall the experiments performed to prove that miPEP31 enters the cells are convincing, some of them, particularly the ones performed in NIH 3T3, could we moved to supplementary materials, to show in the main figures the results obtained in primary T cells.
10. Fig 5E. Could the authors include the frequency and numbers of Foxp3+ cells infiltrating the spinal cord?
11. Fig 6 Please repeat the experiments using primary CD4+ T cells and Foxp3+ Tregs instead of NIH 3T3.

POINT-TO-POINT REPLY TO THE REVIEWERS' COMMENTS

Changes in the revised manuscript text are underlined.

Reviewers' Comments:

Referee #1:

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Other typos are present such as...
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P9 lane 22 we here show.
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Re: *We indeed appreciate the reviewer's comments. We have made corrections according to your comments. Moreover, our manuscript has been proofread by AJE (American Journal Experts) to improve English writing.*

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acceptable. I would rather suggest either performing these experiments or to modify the manuscript accordingly in order to be more precautious in their conclusions.

Re: *Thanks a lot for the reviewer's comments. Indeed we have limited evidence to support the conclusion that miPEP31 selectively down regulates miR-31 without affecting other miRNAs. We followed the reviewer's instructions and revised the manuscript accordingly* ***(page 2 line 10, page 5 line 4, page 10 line 22, page 16 line 20 and page 19 line 13)***.

*Through promoter bashing experiments, we found a short conserved sequence (TCAAGG) on miR31hg that mediated miPEP31 responsiveness. We sought to identify the transcription factor that binds to this sequence, but unfortunately, after the screening of several transcription factors with high probability of binding (predicted by JASPAR), we failed to characterize this transcription factor precisely. Indeed, like other transcriptional repressors, miPEP31 is more likely to act at a genome-scale instead of only one target on miR-31hg. However, with limited information on the miPEP31-interacting transcriptional factor and DNA matrix, we have difficulties to investigate how miPEP31 globally regulates gene expression at the genome level, while our study focused on how miPEP31 regulates the expression of miR-31. Thus, we made our conclusion more precautious as the reviewer suggested, and addressed this critical point in the discussion (****page 17, line 13 - 17****).*

*These comments provided by reviewer are really helpful. miR-31 deficiency in $CD4^+$ T cells leads to increased T_{reg} cell differentiation (Zhang et al., 2015). No effect of miPEP31 on miR-31 KO T_{reg} is probably due to that the T_{reg} cells of miR-31 KO mice were already fully differentiated. In order to exclude this condition, we followed the reviewer's instruction and performed "epistatic experiment". We constructed plasmid expressing pri-miR-31 under constant promoter and transduced the plasmid into wild type $CD4^+$ T cells under T_{reg} cell differentiation condition in the presence of miPEP31 or scPEP. The differentiation of T_{reg} cells was promoted by miPEP31 compared with scPEP. However, the promoted differentiation of T_{reg} cells by miPEP31 was inhibited by transfecting of the constant promoter-driven miR-31 (****Figure 6G, H****). Furthermore, there is no significant difference on the therapeutic effects of miPEP31 and scPEP on CKO mice induced to develop EAE. These results indicate that miPEP31 induces T_{reg} cell differentiation through regulating miR-31.*

Other points:

Comment: Please add more precisions to the mass spectrometry detection of the conserved sequence of miPEP31 in human. Change "Mass Spectrum" to "Mass spectrometry analysis" in the method section and in the figure EV4. Also, precise the expected sequence in amino acid of the human form of miPEP31. Does the entire predicted sequence match to the peptide identified? What was the false discovery rate used in the PEAKS analysis? What was the score of the identified peptide? Did the

authors check that the observed peptide is unique to the miPEP31 human form? Was this peptide the only match for this MSMS spectrum? Were the mass spectrometry data deposited into public repository? Did the authors manage to detect any signal in western blot using the miPEP31 antibody from HCT116 lysate? What was the rationale behind performing two successive ultrafiltration steps after immunoprecipitation?

Re: *We have added more precise description of mass spectrometry detection in the figure legends and method sections (**page 24, line 2 - 14**). The expected sequence in amino acid of the human form of miPEP31 (**MIRNKCAFNERVCFPSLRWKERLWKSITTKRDGIAPVTRNWRGGKMLA**) was shown in figure EV4C. We found a fragment unique to the human miPEP31 (**SITTKRDGIAPVTRNWRGGKMLA**), but we did not find a peptide matching the entire predicted sequence. The scores of this identified peptide and other fragments with different coverage are shown as below:*

Peptide	*Unique*	*-10lgP*	*Length*
SITTKRDGIAPVTRNWRGGKMLA	*Y*	*42.7*	*24*
RDGIAPVTRN	*Y*	*29.17*	*8*
RDGIAPVTRNW	*Y*	*27.06*	*9*
TTKRDGIAPVTR	*Y*	*23.52*	*10*
ITTKRDGIAPV	*Y*	*21.55*	*9*
TKRDGIAPVTR	*Y*	*17.9*	*9*

The resulting sequences were searched through the UniProt Human Proteome database (downloaded on May 5th, 2018) with the mass error tolerance set at 10 ppm and 0.02 Da for parent and fragment, respectively. FDR estimation was enabled. Peptides were filtered for log10p > 20, and the proteins were filtered for log10p > 15 plus one unique peptide. For all experiments, these settings gave an FDR of < 1% at the peptide-spectrum match level.

We have deposited the mass spectrometry data to the Proteomics Identifications Database. Project Name: MS to detect peptides of human HCT116 cell lysate immunoprecipitated by anti-miPEP31; Project accession: PXD02913. Reviewer account details: Username: reviewer_pxd029131@ebi.ac.uk; Password: OZEKkGyu.

Unfortunately, we could not detect signal in western blot using the miPEP31 antibody from HCT116 lysate.

For small peptide detection, the relative abundance is very critical for the successful detection. The two successive ultrafiltration steps were often used in the small peptide detection by mass spectrometry to improve the relative abundance (Lou et al., 2020; Niu et al., 2020).

Comment: p5 lane 16: GSM2778762, SRR6023964: instead introduce the reference where these data were published please.

Re: *We followed the reviewer's comments and introduced the reference (**page 5 line 23**).*

Comment: p8 lane 18 F-miPEP31 ; does the authors mean FAM-miPEP31 ?

Re: *The F-miPEP31 means FAM-miPEP31 (**page 9, line 12**). We have made correction and checked the full text carefully again in the revised manuscript.*

Comment: p16 lane 22: "Other scaffold proteins may be involved in the interaction between miPEP31 and DNA, which needs our further study». I don't understand this sentence since the authors show that miPEP31 binds directly to DNA.

Re: *We thank the reviewer for pointing out the ambiguous sentence. We meant to say that miPEP31 could interact with other scaffold proteins and form complicated machinery to regulate gene expression. We clarified this in our revised manuscript (**page 18, line 11 - 12**).*

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Zhou et al. show that the 44aa peptide miPEP31, encoded by pri-miRNA-31, induces differentiation of Foxp3+ regulatory T cells. The peptide is a transcriptional repressor that modulates miR-31 expression, which promotes differentiation of pTregs and inhibits EAE. Overall, the experiments are well laid out and the results are interesting. Peptides may become good candidates as therapeutic molecules since they are cheaper than other therapies such as monoclonal antibodies, are specific and are potentially safe biological agents. However, the work needs substantial improved statistical analysis and additional experiments to demonstrate the penetrance of the peptide in target cells in vitro and in vivo.

Comment: 1. Figure 1F-G, in addition to the WB, immunohistochemistry of an embryo or neonate could have been performed to localize the expression of the peptide in lymphoid organs. Alternatively, expression of the peptide should be shown in some non-lymphoid tissue in the WB, to demonstrate that expression is restricted to lymphoid tissue.

Re: *We followed the reviewer's comments. In our revised manuscript, we detected the expression of miPEP31 in various tissues. We found that miPEP31 was wildly expressed. However, miPEP31 was highly expressed in immune related tissue (spleen, lymph node and thymus), liver, and intestine (**Figure 1G**), which is consistent with the expression of miR-31(Bardua et al., 2018; Liu et al., 2020; Tian et al., 2020; Zheng et*

al., 2021).

Comment: 2. Figure 2 A and B show representative images without any quantification. To demonstrate peptide expression in Foxp3+ and - cells, a number of experiments with sufficient sample size to show a statistically significant analysis should be performed. The same applies for Figures E and F, the data are representative and not quantified, the number of animals per experiment should also be shown together with the statistical analysis of the data.

Re: *We appreciate the reviewers' comment. The quantification of representative images of Figure 2 A, B, E and F are shown in revised Figure 2 C, H and J. The statistical analysis of the data is provided in the figure legends (**page 46, line 2 – 10 and page 46, line 13-15**).*

Comment: 3. Does miPEP31 have any effect on membrane expression of Treg cells suppression markers?

Re: *We induced T_{reg} cell differentiation with 10 μM synthetic scPEP or miPEP31 and detected the membrane expression of T_{reg} cell suppression markers by flow cytometry. The expression of suppression markers ICOS and LAG-3 were not affected by miPEP31 (**Figure EV1C, D**).*

Comment: 4. Figure 3A-D, although CD4+ T cells are mentioned in the results section, the experiments are performed with adherent NIH3T3 cells, making it easier for the peptide to penetrate the cell, these assays should have been done with a suspension cell line more similar to lymphocytes (Jurkat).

Re: *We appreciate the reviewers' comment. In our revised manuscript, we repeated these experiments with anti-cd3/28 activated $CD4^+$ T cells, which are more precise for the penetrating mechanism study. Our results showed that miPEP31 could also penetrate the membrane of $CD4^+$ T cells (**Figure 3A-D)**.*

Comment: 5. Figure 3D, how many samples have been analyzed? Statistical analysis is not shown in figure legend.

Re: *We added the statistical analysis in the figure legend of revised manuscript (**page 47, line 8 - 11**).*

Comment: 6. Figure 3E-G, the presence of FAM labeled miPEP31 is detected in CD4+ and - fixed cells, however this experiment does not determine the exact localization of the peptide, immunofluorescence of these cells would be needed to determine the localization of the peptide in these cells.

Re: *Following the reviewer's suggestion, we repeated the experiment of Figure 3A-D*

with $CD4^+$ T cells. In the revised Figure 3B, we found that FAM-labeled miPEP31 could penetrate into $CD4^+$ T cells. The fluorescence images showed that FAM-labeled miPEP31 localized in both cytoplasm and nucleus of $CD4^+$ T cells.

Comment: 7. Figure 4, they show that positively charged Arg residues are those that favor peptide entry into the cell, in panel C there is no statistical analysis. Panel D shows representative images and there is also no quantification. It is not demonstrated whether the scramble peptide, which is used as a control in some experiments, has the same penetrability characteristics as the miPEP31 peptide. It is also not mentioned whether both peptides have similar physicochemical parameters.

Re: *We are grateful for this helpful comment. Statistical analysis for panel C was provided in the revised manuscript (**page 48, line 6 - 8**). The quantification of panel D was shown in Figure 4E. In our revised manuscript, we used imaging flow cytometry and confocal microscopy to study the exact location of FAM-labeled scPEP in $CD4^+$ T cells. We clearly demonstrated that FAM-labeled scPEP was able to penetrate into the nucleus of $CD4^+$ T cells **(Figure EV5F-H)**. scPEP is the scrambled peptide of miPEP31 with the same amino acid composition and positive charged residues. By circular dichroism analysis, similar helix structure was found in miPEP31 (59.2%) and in scPEP (50.5% **(Figure EV5A-D).** These data suggested that miPEP31 and scPEP have similar physicochemical parameters.*

Comment: 8. Figure 5A_B, representative images and MFI are shown but quantification and statistical analysis are lacking.

Re: *We appreciate the reviewers' comment. Statistical analysis and quantification of Figure 5 A, B was provided in the revised manuscript (**page 49, line 10**).*

Comment: 9. Clinical score in figure 5C should be analyzed as two way ANOVA repeated measures. P value is shown in the graphic, what is the statistical significance?

Re: *We followed the reviewer's comment. In our revised manuscript, we reanalyzed the clinical score in figure 5C using two-way ANOVA. The statistical result was shown in the figure legend (**page 49, line 11 - 18**).*

Comment: 10. Figure 5D, Histological scoring of this figure and statistical analysis are needed.

Re: *We followed the reviewer's comment, and histological scoring and statistical analysis of Figure 5D are provided in the **Figure 5E** of revised manuscript.*

Comment: 11. Figure 6A-B, the Q PCRs are normalized by the basal conditions,

however they perform a tTest of each sample compared to the basal level. This is not correct, the statistical analysis should be an ANOVA.

Re: *We followed the reviewer's comments. We have re-analyzed Figure 6A-B using one-way ANOVA (**page 50, line 8-11**).*

Comment: 12. Figure 6F should be completed with EAE score of WT mice +/- miPEP31 in parallel.

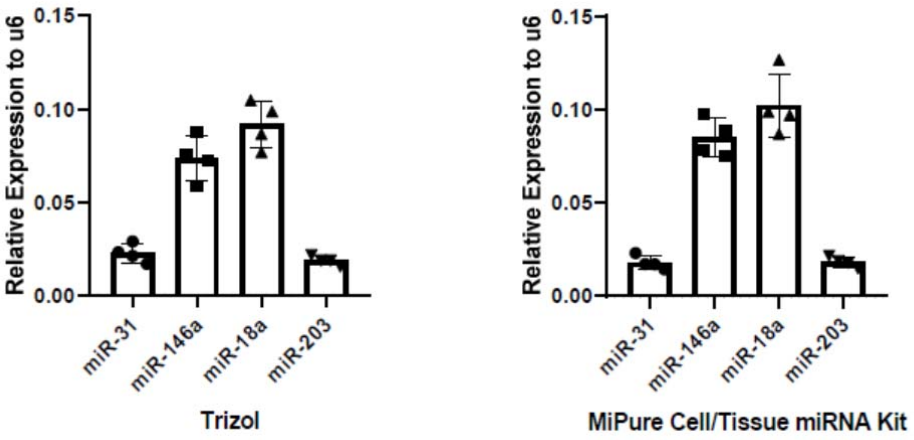
Re: *We followed the reviewer's suggestion, and completed the EAE score of WT mice with miPEP31 or scPEP in parallel (**revised Figure 6F**).*

Comment: 13. Figure 7, The statistical analysis is not specified in the figure legend, what do these P values correspond to? In panel 7C it is indicated that a t-Test has been performed, however an ANOVA should be performed instead, with the appropriate post-test.

Re: *We followed the reviewer's comment and modified it in the revised manuscript (**Page 52, line 13 – 15 and Page 53, line 1 - 2**).*

Comment: 14. Methodology. They perform RNA extraction with Trizol, this precipitation based method can lead to problems for the extraction of microRNAs, how do they make sure they are not losing smaller sized material such as microRNAs? Intracellular labeling for IL-17 is done with the Foxp3 permeabilization kit that permeabilizes nuclei, this is not very suitable. Intracellular labeling for IL-17 should be performed with a gentler permeabilization for the plasma membrane, and both labelings should be performed separately.

Re: *(1) We appreciate the reviewer's serious concern. In our study, we modified the standard Trizol RNA extraction method by adding a step of 4-hour incubation in -20 freezer during the isopropanol precipitation, which increases yield of small miRNAs. We compared the expression of 4 miRNAs extracted by Trizol or MiPure Cell/Tissue miRNA Kit (Vazyme, RC201), and the expressions profile of these miRNAs in $CD4^+$ T cells were comparable.*



Quantitative real-time PCR analyses of 4 miRNAs comparing different RNA extraction methods.

(2) In our study, we used different protocols for the permeabilization of T_{reg} and Th17 cells. When we stained nuclear transcriptional factors such as FoxP3, TF fixation/permeabilization buffer (ebiosceince, 00-5523-00) was used for the permeabilization of nuclei. Subsequent staining of FoxP3 was performed with intracellular permeabilization buffer (BD Biosciences, 554722) to maintain the penetrated state of nuclei. For the staining of IL-17a, cells permeabilizated and antibody was stained with intracellular permeabilization buffer (ebiosceince, 88-8824-00). So we used "The cells were fixed and permeabilized with the FoxP3 Staining Buffer Set (eBioscience, 00-5523-00) ***or*** *BD Cytofix/Cytoperm (BD Biosciences, 554722) and were stained with fluorescent antibodies." in the methods section. We also have clarified this in our revised method section (**page 20, line 15 - 18**).*

Referee #3:

The work from Zhou et al. identifies miPEP31 as a peptide translated from pri-miRNA31. The authors had previously shown that miR31 negatively regulates peripherally induced Tregs and they now establish that miPEP31 selectively downregulates miR-31, thereby favoring the generation of pTregs. The relevance of these results are highlighted by the use of miPEP31 in vivo in the EAE model, where it ameliorates EAE severity.

In general the manuscript is well organized and clearly written, although it would benefit from being proofread for grammar mistakes. A very large amount of data is presented, some of which are not completely relevant to the story and could be removed from the main figures. Please see some additional comments below:

Comment: 1. How does miPEP31 enter the cells?

Re: *Our results indicated that miPEP31 passively cross the cell membrane dependent on positively charged residues and α-helix structure which are common characteristics of Cell-Penetrating Peptides (CPPS) (Langel, 2021). There are 5 arginine residues and 4 lysine residues in miPEP31. These positively charged residues help miPEP31 across the negatively charged bilayer lipid membranes. Structure prediction by I-TASSER and circular dichroism both indicate that miPEP31 has an α-helix structure, which is a common element in CPPs. The penetration of miPEP31 across the cell membrane appears to be independent of the energy because at 4°C, FAM-miPEP31 could also enter the cell, while the nuclear transportation of miPEP31 is energy-dependent.*

Comment: 2. Fig 2E. It would be desirable to repeat these experiments using cells from mice that are not immunized, to compare WT vs inflammatory conditions.

Re: *We followed the reviewer's comment and repeated these experiments using cells from not immunized mice. We found that the percentage of T_{reg} cells was higher in non-immunized mice than that in inflammatory conditions. In both conditions, miPEP31 increases the percentages of T_{reg} cells in vitro (**Figure 2G**).*

Comment: 3. Is the increase in Foxp3+ cells observed in Figure 2 a result of an increase in newly generated Tregs, or is it due to increased proliferation (and therefore expansion) or the already existing pool of Tregs?

Re: *We analyzed the proliferation marker Ki67 on FoxP3+ cells treated with miPEP31 or scPEP. We found that the expression of Ki67 was much higher in miPEP31-treated cells than in scPEP-treated, indicating that the promoted differentiation of T_{reg} is due to increased proliferation (**Figure 2F**).*

Comment: 4. Fig 2E. Please include the number of experiments and mice per group analyzed and add the statistical analysis of the data.

Re: *We followed the reviewer's comment and modified it in the revised manuscript **(Page 46, line 11-15)**.*

Comment: 5. Fig 2F Suppression assays were performed using naïve T cells as responder cells. Could the authors explain the rationale for that? It would be desirable to also repeat these experiments using memory T cells as responder population, and include the statistical summary of all experiments performed.

Re: *In vitro suppression assays are now widely used to determine the suppressive capacity of T_{reg}. The first in vitro assays to measure regulatory T-cell function were*

described by two groups over a decade ago (Takahashi et al., 1998; Thornton and Shevach, 1998). With this knowledge, murine T_{conv} and T_{reg} can be separated using CD4 and CD25 as a marker. However, through detecting CD45RB expression, naïve T_{conv} can be separated from memory T_{conv} and T_{reg}, resulting in better purity of both populations (Collison and Vignali, 2011). A similar strategy can be utilized by staining cells with CD44 and CD62L, with $CD44^{low}$ and $CD62L^{high}$ populations representing naïve T_{conv} cells (Collison and Vignali, 2011). In our original submitted manuscript, we performed the T_{reg} suppression assay with naïve T_{conv} cells to determine the suppressive capacity of T_{reg} cells. As the reviewer suggested, we repeated these experiments using memory T cells as responder population. We found that miPEP31 does not affect the suppression of memory T cells expansion by T_{reg} (Figure EV1A, B). The statistics analysis of the experiments above was performed ***(page 46, line 22 - 23 and page 54, line 2 - 10).***

Comment: 6. Fig 3A. This histogram does not prove that miPEP31 is intracellularly detected (it could be attached to the cell surface). Please define what "NC" is and how it was obtained.

Re: *These comments are really helpful. Using only flow cytometry, we cannot exclude that miPEP31 might attached to the cell surface. We thus performed other experiments to confirm the penetrating ability of miPEP31.*

(a) *We used imaging flow cytometry and confocal microscopy to study the exact location of FAM-labeled miPEP31 and scPEP. We demonstrated that FAM-labeled miPEP31 and scPEP were able to locate into the nucleus of $CD4^+$ T cells* ***(Figure 3E-G and Figure EV5F-H)****. Confocal microscopy was used to further confirm the cell penetrating property of scPEP in $CD4^+$ T cells* ***(Figure 3B)****.*

(b) *We used Histone H2A and Cytochrome C as proper controls to indicate the nuclear and cytoplasmic distribution in the cell fractionations. With these controls we were able to characterize nuclear and cytoplasmic proteins, and further demonstrated that miPEP31 is a cell penetrating peptide* ***(Figure 3C, D)****.*

(c) *We incubated $CD4^+$ T cells with FAM-labeled miPEP31 at 4˚C or 37˚C, and found at 4˚C, miPEP31 enters the cytoplasm but not the nucleus, while it enters both cytoplasm and nucleus at 37˚C, suggesting that miPEP31 crosses cell membrane through an energy-independent mechanism* ***(Figure 4D)****.*

In fig 3A, "NC" refers to negative control cells which were not treated with FAM-miPEP31.

Comment: 7. Fig 3B. It would be desirable to include markers that identify cytoplasm and/or nucleus of the cells, to help locate GFP.

Re: *We followed the reviewer's comment and repeated Fig 3B with primary T cells. In*

*the revised manuscript we used dapi to indicate the nucleus location, and found that FAM-miPEP31 could enter both cytoplasm and nucleus (**Figure 3B**).*

Comment: 8. Fig 3A, B. Please compare the values obtained to the endogenous miPEP31 expression on Tregs.

Re: *To detect the values of exogenous penetrated miPEP31 compared to endogenous miPEP31 level in $CD4^+$ T cells and T_{reg} cells, we measured the miPEP31 levels in cell lysates from $CD4^+$ T cells and T_{reg} cells treated with or without miPEP31. Our results showed that treatment with exogenous synthesized miPEP31 significantly increases the miPEP31 levels (**Figure 3D**).*

Comment: 9. While overall the experiments performed to prove that miPEP31 enters the cells are convincing, some of them, particularly the ones performed in NIH 3T3, could we moved to supplementary materials, to show in the main figures the results obtained in primary T cells.

Re: *We followed the reviewer's comment and repeated the experiments with primary T cells (**Figure 3A-D and Figure 6 A-C**).*

Comment: 10. Fig 5E. Could the authors include the frequency and numbers of Foxp3+ cells infiltrating the spinal cord?

Re: *We followed the reviewer's comment and included the frequency and numbers of FoxP3+ cells infiltrating the spinal cord in the revised manuscript (**Figure 5I**).*

Comment: 11. Fig 6 Please repeat the experiments using primary CD4+ T cells and Foxp3+ Tregs instead of NIH 3T3.

Re: *We followed the reviewer's comment and repeated the experiments with primary $CD4^+$ T cells (**Figure 6A-C**).*

REFERENCES

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Lou, F., Sun, Y., Xu, Z., Niu, L., Wang, Z., Deng, S., Liu, Z., Zhou, H., Bai, J., Yin, Q., *et al.* (2020). Excessive Polyamine Generation in Keratinocytes Promotes Self-RNA Sensing by Dendritic Cells in Psoriasis. Immunity *53*, 204-216.e210.

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T-cell generation by repressing retinoic acid-inducible protein 3. Nature communications *6*, 7639.

Zheng, J., Zhou, H., Yang, T., Liu, J., Qin, T., Gu, X., Wu, J., Zhang, Y., Wang, H., Tang, Y.*, et al.* (2021). Protective Role of microRNA-31 in Acetaminophen-Induced Liver Injury: A Negative Regulator of c-Jun N-Terminal Kinase (JNK) Signaling Pathway. Cellular and molecular gastroenterology and hepatology.

Dear Prof. Wang,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees #1 and #2 have remaining concerns and/or suggestion to improve the manuscript I ask you to address in a final revised version of the manuscript. Please carefully address the points by referee #2 regarding statistics, sample number and controls. Please also provide a point-by-point-response addressing these points.

Moreover, I have these editorial requests:

- Please provide a more comprehensive title (of not more than 100 characters including spaces), also mentioning miR-31.

- Please provide an abstract written in present tense throughout.

- Please add up to 5 key words to the title page.

- Please use abbreviations for the author contributions (like HW, HZ etc. ...). It seems author Junxun Zhang is missing. Please check.

- It seems there are no callouts for Figs. 1H, 6D+E, and EV2H. Please check and make sure that each figure panel is called out in a sequential manner.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main and EV figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

- Please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- As they are significantly cropped, please provide the source data for the few Western blots shown in the manuscript (including the EV and Appendix figures). The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the final revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

- Please upload Table S1 as separate file named Table EV1 and change the callouts in the text accordingly.

- Please update the data availability section with the correct accession codes and make sure these are public upon publication of the study.

- Please remove the sentence 'Correspondence and requests for materials should be addressed to Dr. Honglin WANG (honglin.wang@sjtu.edu.cn)' form the conflict-of-interest statement. This information is already provided on the title page and has nothing to do with COI.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with a few changes and queries we ask you to include in your final manuscript text. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study.
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

I am in general happy with the modifications introduced.

I still have suggestions for minor modifications to be introduced prior publication:
- The authors indicate in the mat and met that Peptides were filtered for log10p R 20 but they show in EV4 panel C a chromatogram with a peptide detected using log10p R 19,21 only.
Correct this mistake please.

- p 6 lane 2. 5'UTR is usually used for coding genes. I would rather prefer the term 5'leader sequence.

- Introduction: lane 12. The authors indicate Magny et al 2013 as a ref of sORF peptides in drosophila. The first experimental demonstration in arthropods is from Savard J et al, Cell 2006 ; in Drosophila from : Kondo et al., NCB 2007 and Galindo et al, PloS Biol 2007 ; Hanyu-Nakamura et al 2008.

-Introduction lane 14 & 15 : the review from plaza et al 2017 PMID: 28759257 is more recent and complementary to the ref indicated.

- p11 lane 2 : other description of miPEP autoregulation are : Chen et al, Plant Physiol. 2020 PMID: 32241877 ; sharma et al, Nat Plants 2020 PMID: 32958895.
- p11 lane 17 : ...Conditional expression...

- p14, Discussion lane 16 ; instead Lauressergues et al 2015, Hanada et al Genome Res. 2007 PMID: 17395691 is one of the first description. As well Anderson (even correct) is less appropriate than Ingolia 2011.

-15 lane 9 : However spliced pri-miR exluding the miR were discovered, that allowed the transcript to be exported into the cytoplasm (Chang et al, 2015 PMID: 26290535). This should be precised.

- p17 lane 17 : which needs our further research work.

Referee #2:

In the revised version of the article, Zhou et al. have partially completed the required experiments, but unfortunately it does not meet the requirements for publication. The statistical analysis, although some figures have been introduced, is not correct.

Although they have included the N of the experiments and the number of experiments they have done, the statistical analysis is very poor. For panels 2A and B you have done t tests, are these populations normal? For panels 2E and F in the new Fig. G, H, I and J it is indicated that you have done ANOVA, what post-test have you done? Without these data we cannot know if they are normal or non-normal distributions, which is key to be able to evaluate the statistical data. In addition, the statistics shown in panels 2E and F in the new figure G, H, I and J are not complete since they only compare against time zero (also not indicated in the figure), and in the new figure I and J again there are no statistics.

The expression data of suppression markers in Tregs cells are inconclusive, only one representative experiment with two of these receptors, ICOS and LAG-3, is shown, insufficient to determine that the peptide has no effect on the receptor repertoire of Tregs.

The peptide penetrance data in primary cells are inconclusive. The WB in Figure 3C lacks the actin, so we do not know if the nuclear and cytoplasmic extracts are contaminated.
The statistics in the 3D panel are confusing again and furthermore the data should be represented as the ratio nucleo/total extract .

The analysis of the new 4C panel is confusing and it is not understood which ANOVA statistical treatment has been applied, normality of the populations and post-test. It is striking that between the NC and F-NrTP group there is no significant difference.

Figure 5 C does not specify whether the ANOVA is a repeated measure or not, nor the post-test.

To complete the data in Figure 6C, the data from the WT animals in Figure 5C have been superimposed. The correct way to

present these data is to repeat experiments with the animals in parallel with the same experimental conditions.

Other aspects of the work that need to be improved before publication:

Since it has been shown that the expression of the peptide is not restricted to lymphoid tissue, what possible adverse effects might this have on a possible treatment. Some reflection on this in the text would be necessary.

Referee #3:

The authors have addressed all my suggestions satisfactorily and I have no further comments.

POINT-BY-POINT RESPONSE TO THE REVIEWERS' COMMENTS

Reviewers' Comments:

Referee #1:
I am in general happy with the modifications introduced.

I still have suggestions for minor modifications to be introduced prior publication:

Comment: The authors indicate in the mat and met that Peptides were filtered for log10p R 20 but they show in EV4 panel C a chromatogram with a peptide detected using log10p R 19,21 only.
Correct this mistake please.

Re: *We appreciate the reviewer's comments. We have made corrections according to your comments* ***(page 26, line 16)****.*

Comment: p 6 lane 2. 5'UTR is usually used for coding genes. I would rather prefer the term 5' leader sequence.

Re: *We have replaced "5'UTR" with "5' leader sequence" in the manuscript according to your comments* ***(page 7, line 4)****.*

Comment: Introduction: lane 12. The authors indicate Magny et al 2013 as a ref of sORF peptides in drosophila. The first experimental demonstration in arthropods is from Savard J et al, Cell 2006 ; in Drosophila from : Kondo et al., NCB 2007 and Galindo et al, PloS Biol 2007 ; Hanyu-Nakamura et al 2008.

Re: *We appreciate the reviewer's comments. The citation has been updated as suggested* ***(page 4, line 11-13)****.*

Comment: Introduction lane 14 & 15: the review from plaza et al 2017 PMID: 28759257 is more recent and complementary to the ref indicated.

Re: *We appreciate the reviewer's comments. The citation has been updated as suggested* ***(page 4, line 15-16)****.*

Comment: p11 lane 2: other description of miPEP autoregulation are : Chen et al, Plant Physiol. 2020 PMID: 32241877 ; sharma et al, Nat Plants 2020 PMID: 32958895.

Re: *We appreciate the reviewer's comments. The citation has been updated as suggested* ***(page 12, line 3-4)****.*

- p11 lane 17: ...Conditional expression...

Re: *The wording has been edited as suggested (**page 12, line 20**).*

Comment: p14, Discussion lane 16 ; instead Lauressergues et al 2015, Hanada et al Genome Res. 2007 PMID: 17395691 is one of the first description. As well Anderson (even correct) is less appropriate than Ingolia 2011.

Re: *We appreciate the reviewer's comments. The citation has been updated as suggested (**page 15, line 18-19**).*

Comment:15 lane 9: However spliced pri-miR exluding the miR were discovered, that allowed the transcript to be exported into the cytoplasm (Chang et al, 2015 PMID: 26290535). This should be precised.

Re: *We appreciate the reviewer's comments. We have added this to the discussion (**page 16, line 14-16**).*

Comment: p17 lane 17: which needs our further research work.

Re: *The text has been edited as suggested (**page 18, line 23**).*

Referee #2:

In the revised version of the article, Zhou et al. have partially completed the required experiments, but unfortunately it does not meet the requirements for publication. The statistical analysis, although some figures have been introduced, is not correct.

Comment: Although they have included the N of the experiments and the number of experiments they have done, the statistical analysis is very poor. For panels 2A and B you have done t tests, are these populations normal? For panels 2E and F in the new Fig. G, H, I and J it is indicated that you have done ANOVA, what post-test have you done? Without these data we cannot know if they are normal or non-normal distributions, which is key to be able to evaluate the statistical data. In addition, the statistics shown in panels 2E and F in the new figure G, H, I and J are not complete since they only compare against time zero (also not indicated in the figure), and in the new figure I and J again there are no statistics.

Re: *We indeed appreciate the reviewer's comments, and we improved the statistical analysis in our data as suggested by the reviewer. In the new revised version of manuscript, the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g.*

SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends. We also added complete statistical test to all diagrams.
*We are sorry for the inadequate description in the figure legend of Figure 2A-C. We now provide a more detailed figure legend in the revised version (**page 44, line 2-7**). In the new Figure 3C, we extend the sample number to meet the statistical requirements.*

*The detailed statistical analysis for the new Figure 2G, H, I and J has been provided in the figure legend (**page 44 line 18- page 45 line 3**).*

Comment: The expression data of suppression markers in Tregs cells are inconclusive, only one representative experiment with two of these receptors, ICOS and LAG-3, is shown, insufficient to determine that the peptide has no effect on the receptor repertoire of Tregs.

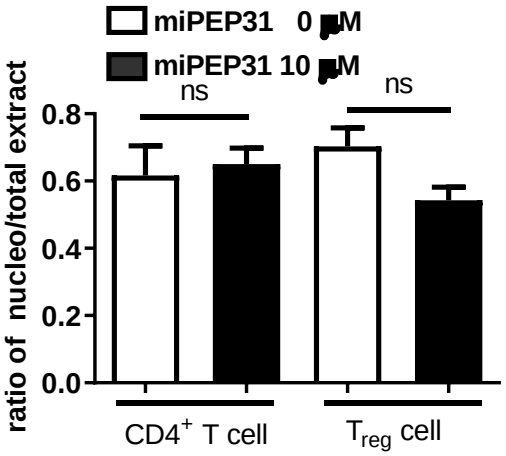
Re: *We appreciate the reviewer's comments. We further detected the expression of GITR and CTLA-4 besides ICOS and LAG-3 with statistical analysis (**Figure EV1C-G**). These data suggest that the suppressive receptors of T_{reg} cells were not affected by miPEP31.*

Comment: The peptide penetrance data in primary cells are inconclusive. The WB in Figure 3C lacks the actin, so we do not know if the nuclear and cytoplasmic extracts are contaminated.

Re: *We used cytochrome C which exists in the cytoplasm as an indicator of whether the nuclear and cytoplasmic extracts are contaminated. We also detected actin as the reviewer suggested.*

Comment: The statistics in the 3D panel are confusing again and furthermore the data should be represented as the ratio nucleo/total extract.

Re: *In 3D panel, we reanalyzed the data, and used nonparametric one-way ANOVA, Kruskal–Wallis (miPEP31 10 μM versus 0 μM) to analyze whether treatment of synthetic miPEP31 significantly increases the miPEP31 concentration compared with non-treated group in both nucleo/total extract of $CD4^+$ T cell and T_{reg} cells. As the reviewer suggested, we reformatted the 3D, and represented as the ratio nucleo/total extract (below). However, the distribution ratio between nucleo and total extract seems unchanged after the treatment of synthetic miPEP31, which is probably not informative enough to present in the figures.*



Comment: The analysis of the new 4C panel is confusing and it is not understood which ANOVA statistical treatment has been applied, normality of the populations and post-test. It is striking that between the NC and F-NrTP group there is no significant difference.

Re: *We appreciate the reviewer's comments. We reformatted 4C panel and reanalyzed the data with nonparametric one-way ANOVA, Kruskal–Wallis (F-NrTP versus NC and F-miPEP31 versus NC or mutant F-miPEP31). Detailed statistical analysis has been provided in the figure legend (**page 47, line 5-8**).*

Comment: Figure 5 C does not specify whether the ANOVA is a repeated measure or not, nor the post-test.

Re: *Statistics were performed by two-way ANOVA and Tukey post hoc test. Detailed statistical analysis has been provided in the new figure legend (**page 48, line 15-18**).*

Comment: To complete the data in Figure 6C, the data from the WT animals in Figure 5C have been superimposed. The correct way to present these data is to repeat experiments with the animals in parallel with the same experimental conditions.

Re: *We have induced additional EAE mouse model, and treated with miPEP31 or scPEP under the same experimental condition and completed the figure 6F with new data.*

Comment: Other aspects of the work that need to be improved before publication:

Since it has been shown that the expression of the peptide is not restricted to lymphoid tissue, what possible adverse effects might this have on a possible treatment. Some reflection on this in the text would be necessary.

Re: *We appreciate the reviewer's comments, and we have discussed this in the text* ***(page 20, line 11-13)****.*

Referee #3:

The authors have addressed all my suggestions satisfactorily and I have no further comments.

Dear Prof. Wang,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the 2 referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study. However, referee #2 has final suggestions and requests to improve the manuscript, I ask you to incorporate in a further revised version of the manuscript. Please also provide a final point-by-point-response to these points.

Moreover, I have these editorial requests I also ask you to address:

- I would suggest this simplified title:
A peptide encoded by pri-miRNA-31 represses autoimmunity by promoting Treg differentiation

- Please have your final manuscript text be carefully proofread by a native speaker. There are a few typos and grammatical errors present.

- The blots in Fig. 7I/J have rather low resolution. Could this be improved?

- Please add a link to the deposited data in the data availability section. Please also add this to the author checklist (box F18).

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement'.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Yours sincerely ,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors addressed all my concerns; I am OK with the modifications introduced.

Referee 2:

Although the statistics have improved in this latest revised version, there are still some aspects of the statistics that need to be reviewed before publication;

- There are some inconsistencies throughout the paper, as all the distributions are indicated to be non-normal (K Wallis test) is this possible?
- The new statistical analysis of the clinical scores indicates that it is a 2 way ANOVA, but again it is not clear if it was analyzed with repeated measures?
- The correct statistical analysis in Figure EV1C-G should be a t-test of each marker in the experimental group with peptide or without peptide, not an ANOVA.
- Regarding the 3D panel, the correct representation of these results is core/total extract ratio, and should be changed in the figure. However, the statistical analysis should not be between with/without peptide, but if the ratio is above 1 in this case the peptide would be sublocalized in the nucleus. Once the ratios have been correctly calculated the correct analysis would be a t-test between CD4 and Treg.

POINT-TO-POINT REPLY TO THE REVIEWERS' COMMENTS

Changes in the revised manuscript text can be found with track changes.

Dr. Honglin WANG
Shanghai Jiao Tong University School of Medicine
Shanghai Institute of Immunology
280 Chongqing South Road
Shanghai 200025
CHINA

Dear Prof. Wang,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the 2 referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study. However, referee #2 has final suggestions and requests to improve the manuscript, I ask you to incorporate in a further revised version of the manuscript. Please also provide a final point-by-point-response to these points.

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- Please add a link to the deposited data in the data availability section. Please also add this to the author checklist (box F18).
- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement'.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Yours sincerely ,
Achim Breiling
Senior Editor
EMBO Reports

Reviewers' Comments:

Referee #1:

The authors addressed all my concerns; I am OK with the modifications introduced.

Referee #2:

Although the statistics have improved in this latest revised version, there are still some aspects of the statistics that need to be reviewed before publication;

Comment: There are some inconsistencies throughout the paper, as all the distributions are indicated to be non-normal (K Wallis test) is this possible?

Re: *We thank for the serious concern from the reviewer. We run the normality test by GraphPad with D'Agostino-Pearson to see if distributions of the data in our manuscript are normal. For the data meet the normality assumption, ordinary one-way ANOVA was performed. If the normality test failed, for homoscedastic data the Kruskal–Wallis test was performed. Alternatively, we use Brown-Forsythe and Welch's ANOVA for heteoscedastic data.*

Comment: The new statistical analysis of the clinical scores indicates that it is a 2 way ANOVA, but again it is not clear if it was analyzed with repeated measures?

Re: *The statistical analysis used for the clinical scores is repeated-measures two-way ANOVA and Tukey post hoc test. We have clarified this in the manuscript (Page 47, Line 17; Page 49, Line 7).*

Comment: The correct statistical analysis in Figure EV1C-G should be a t-test of each marker in the experimental group with peptide or without peptide , not an ANOVA.

Re: *We followed the reviewer's comments and reanalyzed Figure EV1C-G with t-test as suggested (Page 52, Line 14).*

Comment: Regarding the 3D panel, the correct representation of these results is core/total extract ratio, and should be changed in the figure. However, the statistical analysis should not be between with/without peptide, but if the ratio is above 1 in this case the peptide would be sublocalized in the nucleus. Once the ratios have been correctly calculated the correct analysis would be a t-test between CD4 and Treg.

Re: *We appreciate the reviewers' comment. We reformatted Figure 3D as suggested. In the new Figure 3D, the core/total extract ratio in CD4$^+$ T cells and T_{reg} with or without miPEP31 treatment was shown. The core/total extract ratio in all groups is about 1.5 indicating that miPEP31 is more likely concentrated in the nucleus. Our results also indicated that the increased miPEP31 seems unbiased in nucleus and cytoplasm in CD4$^+$ T cells and T_{reg} after the miPEP31 treatment (Page 9, Line 6).*

Prof. Honglin Wang
Precision Research Center for Refractory Diseases
Shanghai General Hospital
650 Xinsongjiang Road
Shanghai 200025
China

Dear Prof. Wang,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

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Corresponding Author Name: Honglin Wang & Qun Li
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2021-53475V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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 χ2 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For each type of experiment, we performed a pilot study. We then used the freely available statistical power analysis program G Power (version 3.1.9.2; Faul, F., Erdfelder, E., Buchner, A., & Lang, A.-G. 2009) to determine the minimal sample size needed in each set of experiments. We used the criteria of a p-value of a maximum of 0.05 and a power of at least 0.8. For each experiment in which animals were involved a minimal sample size of 3 animals per experimental condition was used. Each experiment was repeated at least 4 times to ensure no technical replication.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For each experiment in which animals were involved a minimal sample size of 3 animals per experimental condition was used. Each experiment was repeated at least 3 times to ensure no technical replication.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from the analysis with the exception of pre-established criteria.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For each experiment, animals were divided into groups on the basis of their age, sex, and genotype. For EAE, only females were used as stated in the Methods section.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were randomly assigned to different groups just taking age, sex and genotype as relevant matching criteria.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The experimenter was blinded to the genotype of animals used, identification numbers were assigned to animals prior to genotyping and were used during the experiment. Usually, littermates from different genotypes were caged together after weaning and subjected to the same treatment blinding genotype to the experimenter.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal studies were carried out with genotype blind to the experimenter.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, D'Agostino & Pearson and Shapiro-Wilk normality tests were assessed and non-parametric tests were used when not present or comparing ranks of scale.
Is there an estimate of variation within each group of data?	Yes

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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies citation is included in the Material and Methods section .
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines used in ths study were mycoplasma negative.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We have reported strain of mice used including genetic modifications. Details housing and husbandry conditions of wild type animals used in this study has been described in the Materials and Method section. Please see page 24 and 25.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	A statement of compliance with ethical regulations and committee approving breeding and maintenance of wild type animals has been described in the Method section (page 24-25)
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Maintence and breeding of wild type animals used in this study have been carried out as per the ARRIVE guidelines (page 24-25)

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. 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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Mass spectrometry data: Proteomics Identifications Database PXD029131.The deposited data can be achieved by the link: https://www.ebi.ac.uk/pride/archive/projects/PXD029131.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA