EMBO
reports

RKIP mediates autoimmune inflammation by positively regulating IL-17R signaling

Wenlong Lin, Ning Wang, Kangxing, Zhou, Fasheng Su, Yu Jiang, Jianan Shou, Huan Liu, Chunmei Ma, Youchun Qian, Kai Wang, Xiaojian Wang

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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   7 September 2017

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 support the publication of your paper in EMBO reports. Nevertheless, they all have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. In particular, the suggested controls should be used for the experiments in Figs. 6D/E (ref. #1), significance on disease outcome needs to be proven, endothelial cells should be employed (instead of HeLa), and interaction studies using endogenous proteins should be performed (ref. #2). Further, a mouse strain with abrogated IL17 activity should be used for comparison in the experiment shown in Fig. 1A (ref. #3), and the specificity of RKIP for IL17 should be established (refs. #2 and #3). Finally, data should be provided that demonstrate the findings also apply to humans. As the reports are below, I will not further detail them here, also as I think that all points should be addressed experimentally in the revised manuscript.

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 a 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 or rejection 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; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: 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 labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

We now 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. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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.

REFEREE REPORTS

Referee #1:

Lin and colleagues found that Raf kinase inhibitor protein (RKIP) is crucial for IL-17A signaling by regulating IL-17R-Act1 interactions on a SEFIR dependent manner. They performed EAE and intraperitoneally injection experiments to demonstrate the role of RKIP in non-hematopoietic cells by enhancing IL-17-induced signaling in these cells, promoting inflammatory cytokine production. The study is novel and interesting. However, detailed structure-function analysis is necessary to show which domain of RKIP is required for RKIP-SEFIR (from Act1 or IL-17R).

Minors,
1. Some of the histology images should be improved, such as Fig1 B-C.
2. The pull down of TARF3-Flag in Figure5 F was not efficient, thus the authors should be more cautious about whether TRAF3 could directedly interact with RKIP.
3 The authors may need to include purified IL-17R and Act1 (SEFIR mutant) as controls for Fig6D and 6E.

Referee #2:

Inhibitor Protein (RKIP) in regulating IL-17-induced inflammatory / autoimmune responses. Previous work from the authors established that RKIP functions to promote inflammation in mouse models of colitis and in anti-viral innate immune responses. In the present study the authors employed RKIP KO mice in the EAE model of multiple sclerosis and they show that the clinical score and inflammation is reduced in mice lacking RKIP. They further establish using adoptive transfer experiments that the effects are not due to loss of RKIP in the hematopoietic compartment, instead RKIP in non-hematopoietic cells is required to promote EAE. Moreover, the RKIP-1 dependent EAE is limited to Th17 T cell-mediated disease as RKIP deficiency does not affect inflammation induced by Th1 T cells. Interestingly, the induction of IL-17-driven cytokines is significantly ameliorated in RKIP-deficient primary astrocytes. In further exploring the possible mechanism of RKIP function in IL-17 signaling the authors provide some evidence that MAPK and NF-κB activation is affected by RKIP deletion or loss and that the IL-17R-Act1 complex is disrupted by loss of RKIP. Finally, further in vivo evidence of the role for RKIP in general IL-17-mediated inflammation is provided in separate experiments employing models of peritoneal and lung inflammation. Overall the study is interesting and the findings suggest that RKIP is novel component of the IL-17 signaling cascade. However, a number of weaknesses exist that significantly

diminish the overall impact of the study.

1. The most significant effects on EAE of loss of RKIP are on the clinical scores; however, the effects are less than complete and it is difficult to determine from the data provided whether the outcome on disease is in fact significant. Many of the histograms shown suggest significance where it is not overtly apparent (or non-significance when changes in absolute numbers appear to be robust). Overall the data presented in Figs 1 and 2 do not strongly support the conclusions or narrative of the text.
2. HeLa are not endothelial cells so the conclusions drawn from these experiments that the findings represent vascular inflammation are incorrect.
3. The effects of RKIP loss or knockdown on inflammatory gene expression induced by IL-17 are convincing. However, the specificity for iL-17 has not been established. Parralel experiments determining whether TNF-, IL-1- or TLR-induced gene expression and signaling is affected should be performed
4. The signaling data in figure 5 are difficult to interpret. Overall the effects on IL-17-induced MAPK and NF-κB signaling intermediates are marginal at best
5. The interaction studies are over reliant on overexpression of exogenous proteins

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Referee #3:

In this work Lin et al have explored the role of Raf kinase inhibitor protein in Th17 responses. The authors find that RKIP-deficiency ameliorates the development of EAE and go on to propose that this is explained by RKIP acts by promoting IL17R-Act1 interaction, which positively regulated IL17R signaling. The work is interesting, and the conclusions are well supported by the data. This reviewer has a couple of suggestions to what could further improve the work.

1. Figure 1. The phenotype in Figure 1A should be compared to a KO mouse strain with abrogated IL17 activity. E.g. IL17R-/- or IL17-/- mice.

2. Figure 4. Is RKIP selective for IL17R in the inflammatory response? Wt and KO cells should be compared for induction of other cytokines and PRRs that stimulate the same genes through different signaling pathways. For instance TNFa, IL1, LPS.

3. The work would gain significantly if the authors were able to provide data demonstrating that the described phenomenon also applies in humans.

1st Revision - authors' response	2 February 2018

To Reviewer 1:

Thanks very much for your comments and suggestions regarding our manuscript entitled "RKIP mediates autoimmune inflammation by positively regulating IL-17R-Act1 interactions" (Manuscript ID EMBOR-2017-44951V1). According to your suggestions, we have added some new data and re-organized the manuscript. If you have any further questions, please inform us and we can discuss them further.

Minor points:

1. Question: *Some of the histology images should be improved, such as Fig1 B-C.*
Response: Thanks for your suggestion. We had retaken the photos and the new histological images with higher quality were provided in the revised manuscript **(Figure 1C-D)**.

2. Question: *The pull down of TARF3-Flag in Figure5 F was not efficient, thus the authors should be more cautious about whether TRAF3 could directly interact with RKIP.*

Response: According to your suggestion, we have improved the expression of Flag-TRAF3 and repeated this experiment. As shown in **Figure 5F** in the current manuscript, there was no interaction of TRAF3 with RKIP in HEK293T cells.

3. Question: *The authors may need to include purified IL-17R and Act1 (SEFIR mutant) as controls for Fig6D and 6E.*
Response: According to your suggestion, we have performed these experiments and the new data were shown in **Figure 6E and 6F** in the revised manuscript. GST-tagged RKIP protein can directly interact with HA-IL17R-FL or Flag-Act1-FL protein, but not with HA-IL17R-ΔSEFIR or Flag-Act1-ΔSEFIR protein.

To Reviewer 2:
Thanks very much for your comments and suggestions regarding our manuscript entitled "RKIP mediates autoimmune inflammation by positively regulating IL-17R-Act1 interactions" (Manuscript ID EMBOR-2017-44951V1). According to your suggestions, we have added some new data and re-organized the manuscript. If you have any further questions, please inform us and we can discuss them further.

Major points:

1. Question: *The most significant effects on EAE of loss of RKIP are on the clinical scores; however, the effects are less than complete and it is difficult to determine from the data provided whether the outcome on disease is in fact significant. Many of the histograms shown suggest significance where it is not overtly apparent (or non-significance when changes in absolute numbers appear to be robust). Overall the data presented in Figs 1 and 2 do not strongly support the conclusions or narrative of the text.*
Response: EAE is a complex mice MS model which was affected by many factors, including immune system and non-immune system. Th17 cells and IL-17R signaling play critical roles on the EAE pathogenesis. Many of the genes which have been reported to be involved in IL-17R signaling transduction regulate the EAE pathogenesis in a limited scale, which is hard to completely abrogate EAE symptom when they are deficient in mice (Huang et al, 2015; Kang et al, 2010; Kang et al, 2013; Ma et al, 2017; Xiao et al, 2013; Zhu et al, 2010). In our study, we found RKIP positively regulates the EAE pathogenesis in an IL-17R-signaling dependent manner. RKIP deficiency in mice partly ameliorates the EAE pathogenesis, but hardly abolishes the symptom, which was based on comprehensive analysis of the EAE clinical scores, H&E and LFB staining, and inflammation of CNS. A linear regression analysis based on the EAE clinical scores was performed as previous reported (Lee et al, 2012) (**Figure 1B, Figure 2B**). RKIP deficiency in mice, especially in non-hematopoietic cells, significant impairs the EAE symptom.
As you mentioned, "Many of the histograms shown suggest significance where it is not overtly apparent (or non-significance when changes in absolute numbers appear to be robust)".
In the current manuscript, we have provided the results from another independent experiment in **Figure 1E-1F and Figure 2F-2G**. Both the percentages and the absolute numbers of CNS-infiltrating CD11b+Gr-1+ neutrophils were significantly reduced in RKIP-KO mice compared to WT mice **(Figure 1E)**. Within the CNS-infiltrating $CD4^+$ T cell population, the percentages and absolute numbers of IL-17$^+$ Th17 cells and Foxp3$^+$ T reg cells in RKIP-KO mice were comparable to those in WT mice **(Figure 1F)**, while the numbers of Th1 (IFNγ$^+$ $CD4^+$ T) cells in RKIP-KO mice were slightly decreased compared to those in WT mice **(Figure 1F)**. And the percentage of CNS-infiltrating CD11b$^+$Gr-1$^+$ neutrophils were significantly reduced in the WT→KO group compared to that in the KO→WT and WT→WT groups **(Figure 2F)**. Moreover, the percentages and absolute numbers of IL-17$^+$ Th17 cells, IFN-γ$^+$ Th1 cells, and Foxp3$^+$ T reg cells in the CNS-infiltrating $CD4^+$ T cell population were comparable among the three groups **(Figure 2G)**.

2. Question: *HeLa are not endothelial cells so the conclusions drawn from these experiments that the findings represent vascular inflammation are incorrect.*
Response: Sorry for the mistake. We have corrected description of *"HeLa cells are endothelial cells" to "HeLa cells are epithelial cells".* HeLa cells have been widely used to investigate the IL-17R signaling (Zhu et al, 2010). We have deleted the statement of "vascular inflammation" in the revised manuscript.

3. Question: The effects of RKIP loss or knockdown on inflammatory gene expression induced by IL-17 are convincing. However, the specificity for iL-17 has not been established. Parralel experiments determining whether TNF-, IL-1- or TLR-induced gene expression and signaling is affected should be performed.

Response: Thanks for your suggestion. We have determined the effects of RKIP knockdown on signaling transduction and inflammatory cytokines expression induced by the TNF-α, IL-1β or LPS-treatment in HeLa cells. As shown in **Figure EV4A-4C** in the revised manuscript, RKIP silencing had no significant effects on TNF-α, IL-1β or LPS-induced the gene expression of cytokines or chemokines. The activated MAKP and NF-kB pathway induced by TNF-α, IL-1β or LPS was comparable in RKIP specific silenced or negative control transfected HeLa cells (**Figure EV4E-4G**). Collectively, these data suggest RKIP has no effect on TNF-α, IL-1β or LPS-induced signaling and gene expression of cytokines or chemokines.

4. Question: The signaling data in figure 5 are difficult to interpret. Overall the effects on IL-17-induced MAPK and NF-κB signaling intermediates are marginal at best.

Response: The intensity of phosphorylated proteins normalized to total protein or actin **in Figure 5** has been calculated using the Immage J software. And the numbers have been added under the phosphorylated proteins blots. As shown in **Figure 5**, RKIP deficiency in astrocytes or RKIP knockdown in HeLa cells reduced the intensity of phosphorylated MAPK and NF-κB signaling molecules, including TAK1, P65, JNK, ERK1/2 and P38, treated with IL-17A or IL-17F. Besides, RKIP silenced human glioblastoma-like epithelial cells U-87MG cells also showed impaired phosphorylated MAPK and NF-κB signaling molecules when compared to control transfectants (**Figure 5E**).

5. *Question: The interaction studies are over reliant on overexpression of exogenous proteins.*
Response: Thanks for your suggestion. We have immunoprecipitated endogenous RKIP using anti-RKIP antibody in the HeLa cells. As shown in **Figure 6A**, endogenous RKIP interacts with IL-17R and Act1, and this kind of interaction peaks at 10 min after IL17 stimulation.

To Reviewer 3:
Thanks very much for your comments and suggestions regarding our manuscript entitled "RKIP mediates autoimmune inflammation by positively regulating IL-17R-Act1 interactions" (Manuscript ID EMBOR-2017-44951V1). According to your suggestions, we have added some new data and re-organized the manuscript. If you have any further questions, please inform us and we can discuss them further.

1. *Question: Figure 1. The phenotype in Figure 1A should be compared to a KO mouse strain with abrogated IL17 activity. E.g. IL17R$^{-/-}$ or IL17$^{-/-}$ mice.*

Response: Yes, you are right. However, we haven't IL17R$^{-/-}$ or IL17$^{-/-}$ strain on hand. If we buy these stains from JAX and cross them with RKIP knockout mice, it will at least take one and half a year to get enough mice for experiment. Therefore, we used IL-17 blocking antibody to demonstrate whether the effect of RKIP deficiency on EAE development is due to the reduced IL-17R signaling rather than something else. As shown in **Figure 3G and 3H** in the revised manuscript, the injection of anti-α IL-17 antibody resulted in a significant inhibited EAE symptom, including clinical scores, inflammation and demyelination, both in RKIP WT and KO mice. The RKIP deficient mice exhibited much reduced EAE severity compared to WT mice, which was obliterated after the injection of IL-17-blocking antibody. Consistently, the induction of IL-17-induced genes, such as IL-6, CXCL2, and TNF-α, in CNS tissues were substantially attenuated in the KO mice compared to WT mice, and the expression of these genes in WT mice decreased to comparable levels in KO mice after treatment with IL-17 blocking antibody **(Figure 3I)**. Taken together, these data suggest RKIP regulates EAE pathogenesis is dependent on IL-17R signaling.

2. Question: Figure 4. Is RKIP selective for IL17R in the inflammatory response? Wt and KO cells should be compared for induction of other cytokines and PRRs that stimulate the same genes through different signaling pathways. For instance TNFa, IL1, LPS.
Response: According to your suggestion. RKIP$^{+/-}$ breeding cage was set up to get the 1-day-old neonatal mice for isolating astrocytes. Unfortunately, we failed to get the 1-day-old RKIP WT and KO littermates for 3 times over the past 5 month. Therefore, we determined the role of RKIP on the

TNF-α, IL-1β and LPS-induced signaling using the RKIP specific siRNA. As shown in **Figure EV4A-4C** in the revised manuscript, RKIP silencing had no significant effects on TNF-α, IL-1β or LPS-induced the gene expression of cytokines or chemokines. The activated MAKP and NF-kB pathway induced by TNF-α, IL-1β or LPS was comparable in RKIP specific silenced or negative control transfected HeLa cells (**Figure EV4E-4G**). Collectively, these data suggest RKIP has no effect on TNF-α, IL-1β or LPS-induced signaling and gene expression of cytokines or chemokines.

*3. **Question:** The work would gain significantly if the authors were able to provide data demonstrating that the described phenomenon also applies in humans.*
Response: According to your suggestion, we have employed the human glioblastoma-like epithelial cells, U-87MG cells, which was used to investigate the IL-17R signaling (Zhu et al, 2010), to demonstrate if RKIP can regulate the IL-17R signaling in human brain tissue derived cells. As shown in **Figure 4E and Figure 5E** in the revised manuscript, RKIP silencing in U-87MG cells significantly inhibited the IL-17A-induced MAPKs and NF-κB signaling pathways and the expression of cytokines.

Response Reference
Huang G, Wang Y, Vogel P, Chi H (2015) Control of IL-17 receptor signaling and tissue inflammation by the p38alpha-MKP-1 signaling axis in a mouse model of multiple sclerosis. *Science signaling* **8:** ra24

Kang Z, Altuntas CZ, Gulen MF, Liu C, Giltiay N, Qin H, Liu L, Qian W, Ransohoff RM, Bergmann C, Stohlman S, Tuohy VK, Li X (2010) Astrocyte-restricted ablation of interleukin-17-induced Act1-mediated signaling ameliorates autoimmune encephalomyelitis. *Immunity* **32:** 414-425

Kang Z, Wang C, Zepp J, Wu L, Sun K, Zhao J, Chandrasekharan U, DiCorleto PE, Trapp BD, Ransohoff RM, Li X (2013) Act1 mediates IL-17-induced EAE pathogenesis selectively in NG2+ glial cells. *Nature neuroscience* **16:** 1401-1408

Lee Y, Awasthi A, Yosef N, Quintana FJ, Xiao S, Peters A, Wu C, Kleinewietfeld M, Kunder S, Hafler DA, Sobel RA, Regev A, Kuchroo VK (2012) Induction and molecular signature of pathogenic TH17 cells. *Nature immunology* **13:** 991-999

Ma C, Lin W, Liu Z, Tang W, Gautam R, Li H, Qian Y, Huang H, Wang X (2017) NDR1 protein kinase promotes IL-17- and TNF-alpha-mediated inflammation by competitively binding TRAF3. *EMBO reports* **18:** 586-602

Xiao Y, Jin J, Chang M, Chang JH, Hu H, Zhou X, Brittain GC, Stansberg C, Torkildsen O, Wang X, Brink R, Cheng X, Sun SC (2013) Peli1 promotes microglia-mediated CNS inflammation by regulating Traf3 degradation. *Nature medicine* **19:** 595-602

Zhu S, Pan W, Shi P, Gao H, Zhao F, Song X, Liu Y, Zhao L, Li X, Shi Y, Qian Y (2010) Modulation of experimental autoimmune encephalomyelitis through TRAF3-mediated suppression of interleukin 17 receptor signaling. *The Journal of experimental medicine* **207:** 2647-2662

2nd Editorial Decision   2 March 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study. Please find their reports enclosed below.

As you will see, all three referees support the publication of your manuscript in EMBO reports. However, referee #1 points out one concern that has not been addressed during the revision, i.e. to identify the domain in RIPK that mediates the observed interactions. After cross commenting with the other referees, we do not think that any further experiments employing over-expressed mutant proteins to narrow down an interaction domain in RKIP are necessary to establish or confirm the association of these proteins. However, in case you have such data, or can provide this in a timely

manner, we would ask you to include these in the final revised manuscript.

Further, I have these editorial requests:

I would suggest a shortened title:
RKIP mediates autoimmune inflammation by positively regulating IL-17R signaling

The abstract is currently too long. Please shorten the abstract to not more than 175 words, and provide it written in present tense.

EMBO press does not permit citation of "data not shown". All data referred to in the paper should be displayed in the main or Expanded View figures. Thus, please add these data, or remove the call-out.

Please format the references according to EMBO reports style. See:
http://embor.embopress.org/authorguide#referencesformat

Please add a conflict of interest statement to the manuscript text (next to the acknowledgements).

Please provide all the Western blot panels with higher resolution, and with as unprocessed or modified images as possible. Several of the panels presently show compression artefacts, or are of low quality, fuzzy and out of focus.

As all the Western blot panels show significantly cropped images, we ask you to provide the original source data for these. The source data will be published in a separate source data files online along with the accepted manuscript and will be linked to the relevant figure (with the aim of making primary data more accessible and transparent to the reader). Please submit the source data (scans of the entire gels or blots) together with the 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.

Panel 3I is shown before 3H in Fig. 3. Please change this order in the figure.

It seems that Figure EV4E and 4F shows the same data.

For Figure EV2E and EV2F, please provide for the 100x panels images that contain all the area that is then shown in the 200x panels. Then indicate which subset of the 100x image is shown in the 200x images.

Please put similar styled scale bars to all microscopic images without any writing on them (simple white or black bars). Please provide the size information in the respective figure legend.

Finally, please be sure that the figures comply with our guidelines:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

Further, you have added the sentence "Similar results were obtained in two/three independent experiments. The error bars are means {plus minus} SEM values." to the end of each figure legend, which does not always make sense (e.g. for Fig. 6 and 7 that only shows Western blots without any error bars). For panel EV2G you indicate that these data show results using 3 (WT) and 4 (KO) mice, respectively, but then you state again "... Similar results were obtained in two independent experiments." This does not really make sense. Therefore, please go through the figure legends again and provide the correct information about replicates and statistical testing for each panel.

Further, statistical testing of experiments with n=2 makes also not sense. Please remove the p-values for all diagrams showing data using only one replicate (n=2). Finally, please indicate in all panels if the difference shown is not significant (many diagrams miss this information presently - e.g. 1E/F).

For statistics please see also: http://embor.embopress.org/authorguide#statisticalanalysis

Finally, please show your summary scheme presently in the Appendix as EV figure (it would be fine to have 6 EV figures). Please upload the table with the primer information as EV table (EV Table 1).

Then update all the call outs for these items in the manuscript text, and remove the appendix.

Regarding the author checklist, we ask you to add more detailed information, in particular for the boxes 1a, 1b, 4a, 5, 7, 8 and 9.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three 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 about 400 pixels) that can be used as part of a visual synopsis on our website.

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.

REFEREE REPORTS

Referee #1:

The authors did not address my major concern on which domain of RKIP is required for inteaction with Act1 and IL-17RA. Thus the conclusion is not convincing if not explain well how RKIP promotes Act1-IL-17R inteaction instead of interfering the interaction.

In the text, the authors should define IL-17 and IL-17R clearly. Otherwise, they have to use the specific gene symbols such as IL-17A, IL-17F, IL-17RA, IL-17RC for each experiment. IL-17R usually refers to IL-17RA/IL-17RC complex. There is no such IL-17RA/F. It is suggested specific gene symbols be used in all experiments including the figure labels and legends.

Referee #2:

The authors have responded to all three reviewers' critiques by performing new experiments. My major concern that the specificity for IL-17 signaling had not been established has been addressed and the authors now show in new data that RKIP knockdown does not affect TNF, IL1 or LPS signaling and gene expression. Figure 5 has been improved by the addition of densitometry data and the inclusion of the endogenous IP in Fig 6A alleviates concerns about the complete reliance on overexpression for the interaction studies. Overall the manuscript is significantly improved and the impact of the findings are elevated by the additional data.

Referee #3:

I find that the authors have addressed my points of criticism in a satisfactory manner.

2nd Revision - authors' response   9 March 2017

1. As you will see, all three referees support the publication of your manuscript in EMBO reports. However, referee #1 points out one concern that has not been addressed during the revision, i.e. to identify the domain in RIPK that mediates the observed interactions. After cross commenting with the other referees, we do not think that any further experiments employing over-expressed mutant proteins to narrow down an interaction domain in RKIP are necessary to establish or confirm the association of these proteins. However, in case you have such data, or can provide this in a timely manner, we would ask you to include these in the final revised manuscript.
Response: RKIP (187aa) is a conserved adaptor protein which contains PBP domain covering from aa30-aa170. Therefore, we haven't constructed any RKIP mutants to explore which domain is required for RKIP-SEFIR (from Act1 or IL-17R).

Further, I have these editorial requests:

1. *I would suggest a shortened title: RKIP mediates autoimmune inflammation by positively regulating IL-17R signaling*

Response: Thanks for your suggestion. We have replaced the title with "RKIP mediates autoimmune inflammation by positively regulating IL-17R signaling" in revised manuscript.

2. *The abstract is currently too long. Please shorten the abstract to not more than 175 words, and provide it written in present tense.*

Response: Thanks for your suggestion. We have shortened the abstract to 175 words, and provide it written in present tense in revised manuscript.

3. *EMBO press does not permit citation of "data not shown". All data referred to in the paper should be displayed in the main or Expanded View figures. Thus, please add these data, or remove the call-out.*

Response: According to your suggestion, we have removed the call-out of "data not shown", and cited a reference on page 8 in revised manuscript.

4. *Please format the references according to EMBO reports style. See:*
http://embor.embopress.org/authorguide#referencesformat

Response: We have formatted the references according EMBO reports style.

5. *Please add a conflict of interest statement to the manuscript text (next to the acknowledgements).*

Response: We have added a conflict of interest statement to the manuscript text (next to the acknowledgements) on page 28 in revised manuscript.

6. *Please provide all the Western blot panels with higher resolution, and with as unprocessed or modified images as possible. Several of the panels presently show compression artefacts, or are of low quality, fuzzy and out of focus.*

Response: We have provided all the Western blot panels with higher resolution in Figure 5,6 and EV Figure 4,5.

7. *As all the Western blot panels show significantly cropped images, we ask you to provide the original source data for these. The source data will be published in a separate source data files online along with the accepted manuscript and will be linked to the relevant figure (with the aim of making primary data more accessible and transparent to the reader). Please submit the source data (scans of the entire gels or blots) together with the 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.*

Response: We have submitted the source data (scans of the entire gels or blots).

8. *Panel 3I is shown before 3H in Fig. 3. Please change this order in the figure.*

Response: We have changed this order in the Figure 3.

9. *It seems that Figure EV4E and 4F shows the same data.*

Response: Sorry for this mistake, we have provided the correct data in Figure EV4E in revised figure.

10. *For Figure EV2E and EV2F, please provide for the 100x panels images that contain all the area that is then shown in the 200x panels. Then indicate which subset of the 100x image is shown in the 200x images.*

Response: we have retaken the photos and the new images were shown in revised manuscript.

11. *Please put similar styled scale bars to all microscopic images without any writing on them (simple white or black bars). Please provide the size information in the respective figure legend.*

Response: According to your suggestion, we have put similar styled scale bars to all microscopic images without any writing on them (simple white in immunofluorescence images or black bars in H&E and LFB images) and provide the size information in the respective figure legend in the revised manuscript.

12. *Finally, please be sure that the figures comply with our guidelines:*
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

Response: All the figures comply with the guidelines.

13. *Further, you have added the sentence "Similar results were obtained in two/three independent experiments. The error bars are means {plus minus} SEM values." to the end of each figure legend, which does not always make sense (e.g. for Fig. 6 and 7 that only shows Western blots without any error bars). For panel EV2G you indicate that these data show results using 3 (WT) and 4 (KO) mice, respectively, but then you state again "... Similar results were obtained in two independent experiments." This does not really make sense. Therefore, please go through the figure legends again and provide the correct information about replicates and statistical testing for each panel.*

Response: Sorry for this mistake, we have gone through the figure legends again and provide the correct information about replicates and statistical testing for each panel.

14. Further, statistical testing of experiments with n=2 makes also not sense. Please remove the p-values for all diagrams showing data using only one replicate (n=2). Finally, please indicate in all panels if the difference shown is not significant (many diagrams miss this information presently - e.g. 1E/F).
Response: Sorry for this misleading, all the data shown in figure are representative of three or two independent experiments with similar results. And we have added all panels with ns if the difference shown is not significant in revised figures.

15. Finally, please show your summary scheme presently in the Appendix as EV figure (it would be fine to have 6 EV figures). Please upload the table with the primer information as EV table (EV Table 1). Then update all the call outs for these items in the manuscript text, and remove the appendix.
Response: Thanks for your suggestion. We have showed our summary scheme presently as EV figure 6 and update all the call outs for these items in the manuscript text, and remove the appendix.

16. Regarding the author checklist, we ask you to add more detailed information, in particular for the boxes 1a, 1b, 4a, 5, 7, 8 and 9.
Response: we have added more detailed information, in particular for the boxes 1a, 1b, 4a, 5, 7, 8 and 9.

17. In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three 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 about 400 pixels) that can be used as part of a visual synopsis on our website.
 Response: According to your suggestion, we have added your requested items as bellows:
Summary of the manuscript
IL-17/IL-17R signaling plays important roles in various autoimmune diseases. This study shows RKIP participates in the pathogenesis of IL-17-mediated autoimmune diseases and inflammation by directly binding to IL-17RA and Act1, and promoting the formation of IL-17RA-Act1 complex, which is required for the downstream signaling and cytokine production.
Bullet points highlight
1. RKIP participates in the EAE pathogenesis through IL-17R-mediated signaling and inflammation.
2. RKIP positively regulates the IL-17-induced inflammation in vitro and in vivo.
3. RKIP directly binds IL-17R and Act1 and promotes the formation of IL-17R-Act1 complex.

18: In the text, the authors should define IL-17 and IL-17R cl early. Otherwise, they have to use the specific gene symbols such as IL-17A, IL-17F, IL-17RA, IL-17RC for each experiment. IL-17R usually refers to IL-17RA/IL-17RC complex. There is no such IL-17RA/F. It is suggested specific gene symbols be used in all experiments including the figure labels and legends.
Response: we have use the specific gene symbols such as IL-17A, IL-17F, IL-17RA in all experiments including the figure labels and legends in the revised manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ⬇

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xiaojian Wang
Journal Submitted to: The EMBO reports
Manuscript Number: EMBOR-2017-44951V1

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.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Minimal group sizes for EAE model and IL-17-induced in vivo studies were determined by using power calculations with the DSS Researcher's Tookit with an α of 0.05 and power of 0.8.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Yes. The statement about sample size estimate were called out in Figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA. Our data contain all samples from analysis.
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.	Yes. Sex and age mached mice were randomized treatment.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Yes.The clinical scores of EAE mice were performed in double-blinded manner.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes.Statistical significance between two experimental groups was assessed using unpaired two-tailed Student's t-test.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. statistical analyses were performed using GraphPad Prism 5.0.
Is there an estimate of variation within each group of data?	NA
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).	Yes, on page 22 of Article Materials and Methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes, astrocytes were prepared from 1-day-old neonatal mice as previously described [10, 42]. HeLa and HEK293T cells were obtained from American Type Culture Collection (ATCC). Cells were all tested for mycoplasma and they were 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.	Yes, the decription about mouse was shown on page 21 in the Materials and Methods section. RKIP knockout (RKIP-/-, RKIP-KO) mice were provided by Professor John Sedivy of Brown University, and 5~8-week-old RKIP-KO mice and their littermates with a C57BL/6 background were used in this study. The mice were maintained and bred in specific pathogen free (SPF) conditions.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Yes, the statement was shown on page 19 in the Materials and Methods section. Animal care and experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals with approval from the Scientific Investigation Board of Zhejiang University, Hangzhou.
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.	Yes

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	NA
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).	Yes
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.	Yes. We have learned the documents of biosecurity and research restrictions, and our study is carried out under these guidelines.