THE
EMBO
JOURNAL

STAT4 activation by leukemia inhibitory factor confers a therapeutic effect on intestinal inflammation

Yanan S. Zhang, Dazhuan E. Xin, Zhizhang Wang, Xinyang Song, Yanyun Sun, Quanli C. Zou, Jichen Yue, Chenxi Zhang, Junxun M. Zhang, Zhi Liu, Xiaoren Zhang, Ting C. Zhao, Bing Su and Y. Eugene Chin

Review timeline:

Submission date:	9th Apr 2018
Editorial Decision:	28th May 2018
Revision received:	16th Aug 2018
Editorial Decision:	12th Oct 2018
Revision received:	26th Nov 2018
Editorial Decision:	5th Dec 2018
Revision received:	18th Dec 2018
Accepted:	11th Jan 2019)

Editor: Karin Dumstrei

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 28th May 2018

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see below both referees find the analysis interesting, but they also find that further data is needed to substantiate the major claims. They raise issues regarding the methodology, the STAT3/4 link and how STAT3/STAT4 binding site preference is linked to gene expression and colitis.

The referees raise valid points and the analysis would have to be extended along those lines for consideration here. Should you be able to do so then we would be able to consider a revised version. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the major concerns raised at this stage.

REFEREE REPORTS:

Referee #1:

Zhang et al provide an extensive data set that defines a role for STAT4 in LIF signaling during the regulation of Th17 development and IBD. They define a circuit where LIF acts on IEC through STAT3 to promote epithelial repair and in lymphocytes impairs Th17 development through activation of STAT4. Through these two pathways, LIF is proposed to have a protective effect on IBD.

The authors provide a vast amount of data which is an asset in providing a comprehensive picture, but a challenge in fitting all of the pieces together. Some of the points are fairly clear (phenotype in the Stat4-/- mice; treatment effects of LIF) which could have a significant impact, but some connections are not fully made and some conclusions are not fully substantiated. The following points should be addressed.

1. The data on the microbiome (Fig. 7) are not functionally linked to the rest of the paper. There is clearly an effect of Stat4-deficiency on specific bacteria, but while the LIF affects inflammation, the effects on the bacteria are strictly correlational. It is not clear if LIF altering the inflammation affects the bacteria or LIF alters bacteria through an inflammation-independent mechanism and this affects inflammation. Without this functional link, the data in the final figure is not satisfying and could even be left out. On a related note, more details need to be provided on the antibiotic treatment used in Fig. 1D/E.

2. The authors conclude that STAT4 is competing for STAT3 binding activity, but some additional experiments are required to fully conclude this. It is still possible that STAT4 is competing in activation, and that the effects observed are by altered STAT3 activity. First, the authors need to provide quantitation of western analysis of pSTATs in the various panel. Even better would be to provide intracellular staining for pSTAT3 and pSTAT4 which is far more quantitative. Second, in experiments parallel to Fig. 5L, the authors should compare STAT3 binding by ChIP in WT and Stat4-/- Th17 cells. Third, the authors should examine pSTAT3 in WT and Stat4-/- T cells in vitro and in vivo. This should include analysis of pSTAT4 in Th17 cells cultured with or without LIF.

3. In the analysis of T cell differentiation the authors need to consider the 'pathogenic' Th17 cells that are IL-17/IFNg-double positive cells from the in vivo models (Fig. 3C/D) and determine if there are any differences. Related, the authors should examine the culture conditions for 'pathogenic' Th17 (IL-6/IL-1/IL-23 but not TGFb) to see if LIF has an effect as well.

4. For Fig. 4B, STAT3 activation should be shown as well.

5. For Fig. 4C, another panel should include a condition where STAT3 and STAT4 are co-transfected.

6. Fig. 4I ideally should include a condition where both STAT3 and STAT4 are co-transfected.

7. The disease etiology in the DSS and Rag-transfer models is very different; the former thought to rely more on innate cells, while the latter is clearly T cell-dependent. Have the authors examined the effects of LIF on innate lymphoid cells? This should at least be discussed.

8. Nomenclature for gene names should not have hyphens or symbol characters.

9. Overall, the writing of the report needs to be improved greatly. There are many places where it is hard to understand sentences and what is actually being described or concluded.

Referee #2:

In their report Zhang et al describe a number of interesting findings. Their major observation is LIF-induced STAT4 activation and antagonism of activated STAT4 and STAT3. This, in turn, affected the differentiation of naïve CD4 T cells and the severity of intestinal inflammation in a mouse colitis model. To this reviewer's best knowledge, some of these claims are unprecedented, for example the antagonism of STAT3 and STAT4. STAT4's transcriptional activity is also demonstrated to be regulated by C-terminal serine phosphorylation, whereby serine phosphorylation dampens the transcription activation activity of STAT4. However, the biochemical analyses are not described in the paper, there is no description of the mass spectrometrical analysis and the source of the purified STAT4 protein remains unclear. Aside from these methodological shortcomings, which are probably just an oversight, the authors do not really explore the biological consequences of this phenomenon, in particular regarding the competition between STAT4 and STAT3 for chromatin binding. In fact,

this is my main criticism of this work, namely that the authors progress in sweeping steps but do not always provide a convincing mechanistic explanation to connect their advances. Another example is the differential binding of STAT3 and STAT4 to SIE (classical STAT binding element) and another binding site called AGG element. We are confronted with ChIP data but it remains unclear to this reviewer how the binding site preferences link to gene expression profiles, and in fact colitis severity.
The discussion is relatively brief and may expand a little more on related recent work on STAT4 in LIF signalling.
In conclusion, the authors have assembled an impressive assortment of experiments and make a number of novel and important claims. This work undoubtedly is of interest to a wide audience. In some places, as outlined above, a smaller step size, ie more in-depth biochemical investigation, would have helped to keep the story somewhat more coherent. I would also suggest that the authors attend carefully to the writing style, which is unsatisfactory at present.

1st Revision - authors' response 16th Aug 2018

Point by point responses to referees' comments:
Referee #1:
Zhang et al provide an extensive data set that defines a role for STAT4 in LIF signaling during the regulation of Th17 development and IBD. They define a circuit where LIF acts on IEC through STAT3 to promote epithelial repair and in lymphocytes impairs Th17 development through activation of STAT4. Through these two pathways, LIF is proposed to have a protective effect on IBD.

We appreciate the referee's positive comments on our study.

The authors provide a vast amount of data which is an asset in providing a comprehensive picture, but a challenge in fitting all of the pieces together. Some of the points are fairly clear (phenotype in the Stat4-/- mice; treatment effects of LIF) which could have a significant impact, but some connections are not fully made and some conclusions are not fully substantiated.

We are encouraged that the referee finds our study to be "an asset in providing a comprehensive picture". In our revised manuscript, we have included new data, and explained our findings more precisely to fully connect the results and substantiate all the conclusions.

The following points should be addressed.
1. The data on the microbiome (Fig. 7) are not functionally linked to the rest of the paper. There is clearly an effect of Stat4-deficiency on specific bacteria, but while the LIF affects inflammation, the effects on the bacteria are strictly correlational. It is not clear if LIF altering the inflammation affects the bacteria or LIF alters bacteria through an inflammation-independent mechanism and this affects inflammation. Without this functional link, the data in the final figure is not satisfying and could even be left out. On a related note, more details need to be provided on the antibiotic treatment used in Fig. 1D/E.

We appreciate the referee's suggestions and have carefully analyzed phenotypes of microbiota composition and inflammation in colitis mice treated with or without LIF to connect the findings explicitly. The reason we checked microbiota composition in our story, that's because we verified LIF promoted proliferation and repair of intestinal epithelia, while the integrity of intestinal epithelia is the key of commensal microbiota segregation(Mankertz & Schulzke, 2007; Peterson & Artis, 2014). It has been reported that, during DSS induced colitis, the outgrowth of Gamma-proteobacteria such as E. coli, are the main consequence of losing epithelial layer and dysbiosis of such bacteria then induces amplified inflammatory responses in the gut (Arthur et al, 2012; Lupp et al, 2007). Our data suggested that bacteria invaded into intestinal epithelia when using DSS to induce tissue damage on intestine, IECs then secreted LIF to promote repair of damaged epithelia as a negative feedback mechanism of controlling gut microbiome dysbiosis (**Fig 1A-H**). Conversely, autocrined LIF enhanced the intestinal barrier function by preventing E.coli invasion and

restored colonic microbiota dysbiosis in mice (**Fig 7A-F**), which are the main cause of pro-inflammatory cytokines secretion and overt inflammation during IBD pathogenesis (Grivennikov, 2013; Rakoff-Nahoum et al, 2004). Besides the transcriptional regulation LIF-induced on *Il17a/f* promoter in Th17 cells, it is also not surprising, pathogenic and non-pathogenic Th17 cells were less in colon due to decrease of IL-6 and IL-1β. Indeed, *Stat4*-deficiency exhibited huge impact on commensal microbiota composition in colon, growth of pro-inflammatory microbes increased dramatically in *Stat4*$^{-/-}$ mice. Since, *Stat4*$^{-/-}$ mice have more defects in immune system (Jacobson et al, 1995), and the positive effect of LIF on preventing bacterial invasion disappeared here, we inferred the inefficient immune response cannot help on clearance of invaded bacteria. Although we didn't explore the mechanism how STAT4 affects microbiome furthermore, we showed that the production of LIF was dependent on microbiota during colitis, as LIF protected host from severe colitis by different mechanisms in IECs and Th17 cells, which greatly affect gut microbiota homeostasis, it is worth to show that, in response to the gut microbiota dysbiosis, LIF serves as a negative feedback regulator to alleviate gut inflammation by restoring a normal gut microbiota community.
We have included the experiment procedures on antibiotics treatment in the Materials and Methods part. Briefly, the SPF wild-type mice were treated with a cocktail of antibiotics of 1mg/ml of neomycin, 0.5mg/ml of vancomycin, 1mg/ml of metronidazole and 1mg/ml of ampicillin for four weeks. Fresh antibiotics solution was supplied every week. Four weeks later, drinking water was further supplemented with 1mg/ml of streptomycin, 170μg/ml of gentamicin, 125μg/ml of ciprofloxacin, and 1mg/ml of bacitracin for another five weeks. With antibiotics treatment, more than 99% intestinal microbes were removed. Colitis was induced in the microbe-free mice nine weeks later as described in the section of DSS-induced colitis. Those mice were sacrificed on indicated day after DSS treatment, and the colon tissues were obtained for further analysis.

2. The authors conclude that STAT4 is competing for STAT3 binding activity, but some additional experiments are required to fully conclude this. It is still possible that STAT4 is competing in activation, and that the effects observed are by altered STAT3 activity. First, the authors need to provide quantitation of western analysis of pSTATs in the various panel. Even better would be to provide intracellular staining for pSTAT3 and pSTAT4 which is far more quantitative. Second, in experiments parallel to Fig. 5L, the authors should compare STAT3 binding by ChIP in WT and Stat4-/- Th17 cells. Third, the authors should examine pSTAT3 in WT and Stat4-/- T cells in vitro and in vivo. This should include analysis of pSTAT4 in Th17 cells cultured with or without LIF.

We appreciate the referee's suggestions. We have considered the possibility that STAT4 is competing in activation, and decreasing the activation of STAT3. But based on our results, we found the presence of STAT4 did not attenuate STAT3 activation either in T cells or in ectopic STAT4-expressing cells (**Fig 1I, 4B and C; and EV4C and D**). Meanwhile, LIF stimulation promoted not only STAT4 homodimer formation but also STAT3-STAT4 heterodimer formation (**Fig 4I and EV4B**), following by translocation of STAT3 and STAT4 into nucleus (**Fig 4J and K; and EV4F**).
First, in the revised manuscript, we have included quantitation of western blot analysis of pSTATs in several panels (**Fig EV1C and D, EV4C and D**), and provided the intracellular staining of pSTAT3 and pSTAT4 in HeLa cells which were transfected with STAT3 and STAT4 constructs (**Fig EV4E**).
Second, we used wild-type or *Stat4*$^{-/-}$ CD4+ T cells differentiated by IL-6 and TGFβ to do ChIP-qPCR, and found without STAT4, LIF did not block STAT3's binding on SIE or AGG-elements any more (**Fig EV5E**).
Third, we examined pSTAT3 and pSTAT4 in wild-type or *Stat4*$^{-/-}$ Th17 cells differentiated in vitro (**Fig EV4C**), and pSTAT3 in the extract of spleen from wild-type or *Stat4*$^{-/-}$colitis model mice (**Fig EV4D**). LIF-induced STAT3 activation level was not jeopardized by STAT4 in vitro and in vivo.

3. In the analysis of T cell differentiation the authors need to consider the 'pathogenic' Th17 cells that are IL-17/IFNg-double positive cells from the in vivo models (Fig. 3C/D) and determine if there are any differences. Related, the authors should examine the culture

conditions for 'pathogenic' Th17 (IL-6/IL-1/IL-23 but not TGFb) to see if LIF has an effect as well.

We have performed the suggested experiments and found that, in mouse inflamed colons, pro-inflammatory Th17 cells accumulation were increased. Interestingly, we found injection of LIF reduced both IL-17A single positive and IL-17A and IFNγ double-positive Th17 cells accumulation in wild-type mice (**Fig 3C and EV3A**), which stands for non-pathogenic and pathogenic Th17 cells respectively. We further tested the inhibitory effect of LIF under two different Th17 cell induction condition, IL-6 and TGFβ primed non-pathogenic Th17 cells differentiation, IL-6 IL-23 and IL-1β induced pathogenic Th17 cells development. The results proved that LIF inhibited both pathogenic and non-pathogenic Th17 cells differentiation in wild-type $CD4^+$ T cells, however, LIF's inhibitory effect on Th17 cells vanished in *Stat4*-deficient $CD4^+$ T cells (**Fig 3G**).

4. For Fig. 4B, STAT3 activation should be shown as well.

We performed the experiments in Fig. 4B and tested STAT3 activation in this experimental set. STAT3 activation level was not interfered by STAT4 in primary T cells treated with LIF (**Fig 4B**).

5. For Fig. 4C, another panel should include a condition where STAT3 and STAT4 are co-transfected.

We included the condition this referee suggested in Fig. 4C and found that the presence of STAT4 displayed a minor effect on the interaction between LIFR and STAT3 (**Fig 4C**).

6. Fig. 4I ideally should include a condition where both STAT3 and STAT4 are co-transfected.

We co-transfected GFP-tagged STAT4 and RFP-tagged STAT3 to HeLa cells and found that STAT3 and STAT4 translocated to nucleus upon LIF treatment, they colocalized in the nucleus (**Fig EV4E**).

7. The disease etiology in the DSS and Rag-transfer models is very different; the former thought to rely more on innate cells, while the latter is clearly T cell-dependent. Have the authors examined the effects of LIF on innate lymphoid cells? This should at least be discussed.

We agree with the referee that the disease etiology in the DSS and Rag-transfer colitis models is very different. We checked IL-17A-producing innate lymphoid cells (ILCs) in lamina propria under our experimental set. We gated $CD45^+CD3^-CD127^+$ cells from LPLs, the results showed that LIF exhibited a minor influence on IL-17A producing ILCs from both wild-type and $Stat4^{-/-}$mice (**Figure for reviewers only 1**).

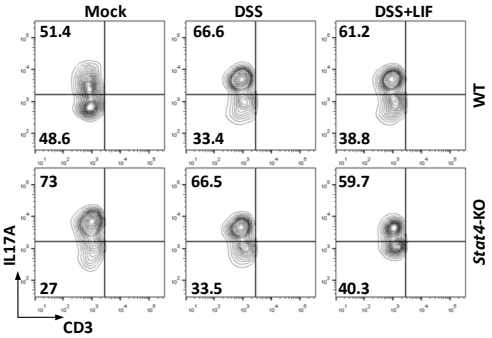

Figure for reviewers only 1. FACS staining of isolated LPLs from the colon of WT or Stat4-KO colitis mice received PBS or LIF treatment on day10 (n=4 per group) to analyze the amount of IL-$17A^+$ILCs.Data are representative of two independent experiments.

We included the following content in the discussion part.
Recent studies discovered innate lymphoid cells (ILCs) control innate immunity at mucosal surfaces and mediate experimental innate immune-mediated colitis (Buonocore et al, 2010). ILCs are a growing family of immune cells, including IFNγ-secreting ILC1 cells; IL-5, IL-9 and IL-13-secreting ILC2 cells; IL-17, IL-22 and IFNγ-secreting ILC3 cells and IL-10

and TGF-β1-secreting ILCregs (Eberl et al, 2015; Wang et al, 2017). In human, IL-17-producing ILC3 was noted in the inflamed mucosa of patients with Crohn's disease but not in patients with ulcerative colitis (UC) (Geremia et al, 2011). Other reported studies have shown that IL-17-producing Th17 cells in colon lamina propria increased in both Crohn's disease and ulcerative colitis (Kobayashi et al, 2008). The characters of DSS-induced mice colitis model resemble human UC (Wirtz et al, 2017). Even though, severe combined immunodeficiency (SCID) and *Rag*$^{-/-}$ mice develop severe intestinal inflammation under DSS-challenge, indicating DSS-induced acute colitis can progress without the help of adaptive immune cells (Dieleman et al, 1994). However, T cells have been demonstrated to accumulate in inflamed mucosa over time in DSS-induced colitis mice. It's not surprising that we also found Th17 cells accumulation in inflamed colon. But LIF only inhibited IL-17A^{+} Th17 cells accumulation obviously, not IL-17A^{+} ILC3 cells accumulation. IL-23 and IL-1β are responsible for IL-17A-producing ILC3 cells development, and RORγt is the main transcription regulator of ILC3 cells, while IL-6 is not required, which is different from Th17 prime condition. Our results demonstrated LIF's inhibitory effect on Th17 cells functioned through STAT4 and STAT3's reciprocal regulation on Il17a/f genes. We inferred that without STAT3 involvement during ILC3 cells development, LIF could not alter *Il17* genes expression, that's why there was no major difference of ILC3 cells accumulation in our colitis model.

8. Nomenclature for gene names should not have hyphens or symbol characters.

We deleted hyphens and symbol characters in gene names appeared in the revised manuscript and figures.

9. Overall, the writing of the report needs to be improved greatly. There are many places where it is hard to understand sentences and what is actually being described or concluded.

We appreciate the referee's suggestions and have carefully reorganized and rewrote the manuscript. The revised manuscript has been proofread by a native English speaker.

Referee #2:

In their report Zhang et al describe a number of interesting findings. Their major observation is LIF-induced STAT4 activation and antagonism of activated STAT4 and STAT3. This, in turn, affected the differentiation of naïve CD4 T cells and the severity of intestinal inflammation in a mouse colitis model. To this reviewer's best knowledge, some of these claims are unprecedented, for example the antagonism of STAT3 and STAT4. STAT4's transcriptional activity is also demonstrated to be regulated by C-terminal serine phosphorylation, whereby serine phosphorylation dampens the transcription activation activity of STAT4. However, the biochemical analyses are not described in the paper, there is no description of the mass spectrometrical analysis and the source of the purified STAT4 protein remains unclear. Aside from these methodological shortcomings, which are probably just an oversight, the authors do not really explore the biological consequences of this phenomenon, in particular regarding the competition between STAT4 and STAT3 for chromatin binding. In fact, this is my main criticism of this work, namely that the authors progress in sweeping steps but do not always provide a convincing mechanistic explanation to connect their advances. Another example is the differential binding of STAT3 and STAT4 to SIE (classical STAT binding element) and another binding site called AGG element. We are confronted with ChIPdata but it remains unclear to this reviewer how the binding site preferences link to gene expression profiles, and in fact colitis severity.

We appreciate the referee's positive comments on our study and suggestions. In our revised manuscript, we have included description of the mass spectrometrical analysis in detail to the Materials and Methods part. Briefly, HEK293T cells were transfected with Myc-tagged STAT4 and treated with LIF for 30 minutes. Immunoprecipitated STAT4 from the above HEK293T cells was separated via SDS-PAGE. Coomassie-blue-stained STAT4 band was excised from the gel for protease digestion, followed by mass spectrometry analysis with a Thermo LC-MS/MS System.

Although, we found phosphorylation on three serine residues of STAT4 via mass spectrometrical analysis, with site specific anti-phosphorylated serine antibodies, we confirmed that STAT4 S714 site was the only phosphorylated serine residue in LIF-treated $CD4^+$ T cells (**Fig 4F**). Based on co-IP and luciferase reporter assay, we proved STAT4 S713 phosphorylation dampened its transcription activity (**Fig 4G and H; 5G and H**).
We agreed with the referee that we progressed in sweeping steps. In the revised study, we performed more experiments to connect our advances.
First, to prove LIF-induced STAT4 serine phosphorylation affects the competition between STAT4 and STAT3 for chromatin binding, we constructed *Il17a* promoter sequence containing the six AGG and SIE-elements to luciferase reporter vector. We found wild-type STAT4 strongly inhibited STAT3's activity on initiating *Il17a* transcription upon LIF treatment, but the STAT4 S713A mutant and SPXX repeats-deleted construct exhibited less inhibition on STAT3's activity, suggesting LIF-induced non-canonical phosphorylation on STAT4 serine residue was involved in Th17 cells regulation (**Fig 5N**).
Second, STAT3 is a positive transcription factor for both *Il17a* and *Il17f* promoters. Both STAT3 and STAT4 can bind to the classical STAT-binding element SIE. Our group previously reported that STAT3 also binds to the newly defined AGG-element "AGGXXXAGG". But SIE-element is more active than AGG-element in luciferase reporter activation.STAT4 alone could drive SIE or AGG-element to initiate transcription, but the transcriptional activity of STAT4 was much lower than STAT3 (**Fig 5I and J**). By performing ChIP-qPCR in Th17 cells, we found LIF treatment decreased STAT3 but increased STAT4 binding on SIE and AGG elements (**Fig 5L**). However, without STAT4, LIF did not block STAT3's binding on SIE or AGG-elements any more (**Fig EV5E**). Evidence of STAT4 binding correlated with less binding of STAT3 significantly at SIE and AGG elements suggesting that STAT4 directly interfered with the binding ability of STAT3 in these loci. With less STAT3 on SIE and AGG-elements and more STAT4 on the inefficient AGG-elements, *Il17a/f* genes transcription decreased, leading to less accumulation of pro-inflammatory Th17 cells in colon lamina propria and prevention of overt inflammation. It means LIF-STAT4 signaling promoted colitis remission by balancing IL-17A-producing Th17 cells differentiation in intestine.
Finally, we proved that LIF-activated STAT3 promoted YAP expression in both RNA and protein level in IECs. YAP accumulation is involved in epithelial regeneration during inflammation (Cai et al, 2010; Taniguchi et al, 2015). In Yap promoter region, there are not classical STAT-binding site, but there are multiple AGG-elements. Although, it's been reported activated gp130 promotes YAP signaling independent of STAT3 (Taniguchi et al, 2015), we found STAT3 was able to induce Yap promoter which containing two AGG-elements, activation under LIF treatment through luciferase assay (**Fig 6E-I**). In inflamed colon, LIF dramatically enhanced epithelial cell proliferation (**Fig 6A and EV6A**). Mucosal healing predicts sustained remission and is a key treatment goal in IBD (Neurath, 2014). Based on reported knowledge and our findings, we have good reason to conclude that LIF-induced molecular mechanism is closely related to amelioration of colitis inflammation.

The discussion is relatively brief and may expand a little more on related recent work on STAT4 in LIF signalling.

IL-6 family cytokines are actively involved in JAK-STAT signaling pathway. Except for STAT3, LIF can activate STAT1 in many different cell types (Durbin et al, 1996; Fujio et al, 1997; Jenab & Morris, 1998). Until recently, Brenner's group reported LIF is elevated in both human and mouse models of arthritis and drives transcription and activation of STAT4 in fibroblasts leading to sustained release of inflammatory mediators including IL6, IL-1β, IL-11 and others (Nguyen et al, 2017). They demonstrated LIFR and STAT4 formed a molecular complex together with JAK1 and TYK2 kinases, controlled STAT4 activation and binding to Il6 promoter Obviously, fibroblasts-mediated inflammation is distinct from leukocytes. In CD4+ T cells, we noted that LIF can activate STAT4 in addition to STAT3. STAT4 is phosphorylated not only on canonical Y693 site, but also on S713 within the C-terminal transcription regulation domain. Surprisingly, S713 phosphorylation negatively remodels STAT4 transcription activity, and it did not help on the competing with STAT3 for promoter binding.

In conclusion, the authors have assembled an impressive assortment of experiments and make a number of novel and important claims. This work undoubtedly is of interest to a wide audience. In some places, as outlined above, a smaller step size, ie more in-depth biochemical investigation, would have helped to keep the story somewhat more coherent. I would also suggest that the authors attend carefully to the writing style, which is unsatisfactory at present.

We are encouraged that the referee finds our study to be “novel and important”. In our revised manuscript, we have included new data to address the raised concerns and carefully reorganized the writing.

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2nd Editorial Decision 12th Oct 2018

Thank you for submitting the revised manuscript. I am sorry for the delay in getting back to you with a decision, but I have now received the input back from the referees.

The referees appreciate that the analysis has been strengthened. However, they still raise remaining concerns.

Should you be able to address the remaining points raised then we can offer to consider a revised version. You can use the link below to upload the revised version.

REFEREE REPORTS:

Referee #1:

The authors have done a very good job of addressing the previous concerns. There are a couple of points that came up in the re-review.

In the response to my previous point 2 the authors refer to quantitation in panels EV1C and D that are not present in the supplemental materials, and a ChIP assay in panel EV5E that is different from the data presented in the panel. These data should be included if available and cited appropriately.

The authors note that a native English speaker reviewed the text. I find that hard to believe upon re-reading the discussion, particularly the new paragraph added. Further editing is needed to make the text clearer. Perhaps this could be done by the journal staff.

Referee #2:

The revisions presented by Zhang et al. unfortunately have not substantially improved the manuscript. Important experimental details remain unclear, e.g. the immunoprecipitated STAT4 used for mass spectrometry appears to be mouse protein, although it was transfected in a human cell line; no detailed information is given about transfection experiments and the cDNA constructs used including mutants. Another example is the differential STAT4 activation in immune and epithelial cell (Figs. 1l, j). The claim that in epithelial cells LIF activates only STAT3 (but not STAT4) is unfounded, because STAT4 activation has not been tested. By way of explanation the authors point to reduced STAT4 protein expression and refer to Fig. 1k but it shows mRNA expression. Figs. 1l, j show protein data, but use different loading controls (tubulin or actin), which precludes direct comparisons. Contrary to the authors claim the manuscript still contains numerous grammatical errors that make it difficult to understand. The abstract has been rewritten, but this has made things worse rather than better.
I acknowledge and appreciate the authors' sincere efforts to bring this manuscript, which reports a number of interesting observations, to a higher standard. I am afraid, though, that the dots are still not connected well enough to give a clear and convincing picture.

2nd Revision - authors' response 26th Nov 2018

Please see next page.

Point by point responses to referees' comments:

Referee #1:

The authors have done a very good job of addressing the previous concerns. There are a couple of points that came up in the re-review.

In the response to my previous point 2 the authors refer to quantitation in panels EV1C and D that are not present in the supplemental materials, and a ChIP assay in panel EV5E that is different from the data presented in the panel. These data should be included if available and cited appropriately.

We appreciate the referee's positive comments on our study and we do have included those data Referee #1 mentioned in the revised manuscript. During the first round of revision, we conducted more work to strengthen our points and reorganized our figures and expanded figures again and again. When we got the decision letter from the editor, we suddenly realized that we submitted another version of response letter, and the citation in it was not consistent with the figures that we submitted simultaneously. But the incorrect citation only appeared in the response letter, not in the manuscript. We are so sorry for this mistake, so we re-explained these two points here with appropriate citation.

First, in the previously revised manuscript, we have included quantitation of western blot analysis of pSTATs in several panels (**Fig 1I and J, lower panel; and EV4C and D**).

Second, we used wild-type and *Stat4*$^{-/-}$ CD4^{+} T cells primed by IL-6 and TGFβ to do ChIP-qPCR, and found that without STAT4, LIF did not block STAT3's binding on SIE or AGG-elements any more (**Fig 5K**).

The authors note that a native English speaker reviewed the text. I find that hard to believe upon re-reading the discussion, particularly the new paragraph added. Further editing is needed to make the text clearer. Perhaps this could be done by the journal staff.

We are sorry that the last version of the revised manuscript is not satisfactory. We appreciate the referee's suggestions and have carefully edited the manuscript again and again. The revised manuscript has been proofread by a native English speaker.

Referee #2:

The revisions presented by Zhang et al. unfortunately have not substantially improved the manuscript. Important experimental details remain unclear, e.g. the immunoprecipitated STAT4 used for mass spectrometry appears to be mouse protein, although it was transfected in a human cell line; no detailed information is given about transfection experiments and the cDNA constructs used including mutants. Another example is the differential STAT4 activation in immune and epithelial cell (Figs. 1l, j). The claim that in epithelial cells LIF activates only STAT3 (but not STAT4) is unfounded, because STAT4 activation has not been tested. By way of explanation the authors point to reduced STAT4 protein expression and refer to Fig. 1k but it shows mRNA expression. Figs. 1l, j show protein data, but use different loading controls

(tubulin or actin), which precludes direct comparisons. Contrary to the authors claim the manuscript still contains numerous grammatical errors that make it difficult to understand. The abstract has been rewritten, but this has made things worse rather than better.

We are sorry that in the revised manuscript we neglected the important information Referee #2 pointed out, and we appreciate the Referee's suggestion. We have included the experimental details clearly and done more work to improve the comparability between Fig 1I and J in our re-revised manuscript.

First, we indeed transfected the myc-tagged STAT4 construct of mouse origin into human cell line HEK293T to prepared sample for mass spectrometry analysis. We have included the information in detail to the Materials and Methods part. We also confirmed that whether endogenously expressed STAT4 in mouse $CD4^+$ T cells undergoes serine phosphorylation upon LIF treatment. We immnuoprecipitated STAT4 protein from the lysate of $CD4^+$ T cells which were isolated from mouse spleen and treated with LIF for 30 minutes. The immunoprecipitated STAT4 was separated via SDS-PAGE and then digested for mass spectrometry analysis. The results suggest that LIF can induce serine phosphorylation on the SPXX motifs of STAT4 in mouse $CD4^+$ T cells (**Figure for reviewers only 1**), and the finding is consistent with the data shown in **Fig 4E**. Because STAT4 is highly conservative in human and mouse (**Fig 4D**), we believe that LIF can induce serine phosphorylation on the SPXX motifs of STAT4 protein of human origin as well.

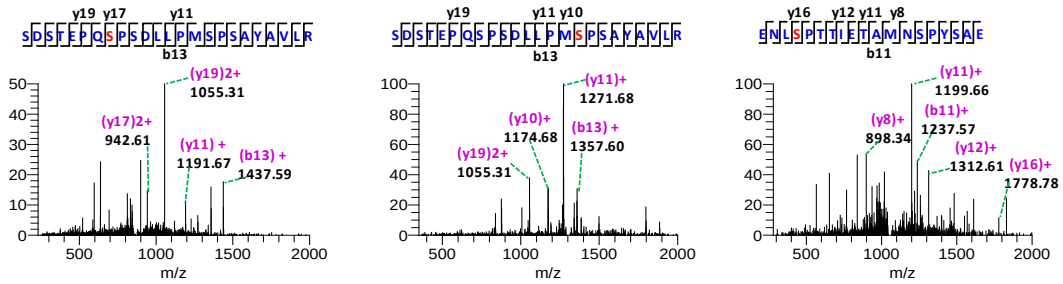

Figure for reviewers only 1. The mass spectrometry analysis of purified endogenous STAT4 protein from mouse $CD4^+$ T cells revealed the phosphorylation of STAT4 at serine residues in the C terminus.

Second, we have included description of the constructs we used in our study in detail to the Materials and Methods part. Briefly, a myc-tagged STAT4 construct was subcloned from a STAT4 construct of mouse origin which is a courtesy from Dr. Mark H. Kaplan. The other tagged wild-type and mutant STAT4 constructs were subcloned from the above myc-tagged STAT4 construct. Myc- and flag-tagged STAT3 constructs are of mouse origin. The full length and deleted constructs of LIFR are of human origin. All of the constructs were transfected into the cell lines we used in our experiments with Lipofectamine 2000 (Invitrogen).

STAT4 proteins are highly conservative in human and mouse. Since we conducted all of the pathological study in mice, therefore we used STAT4 constructs of mouse origin only in our experiments.

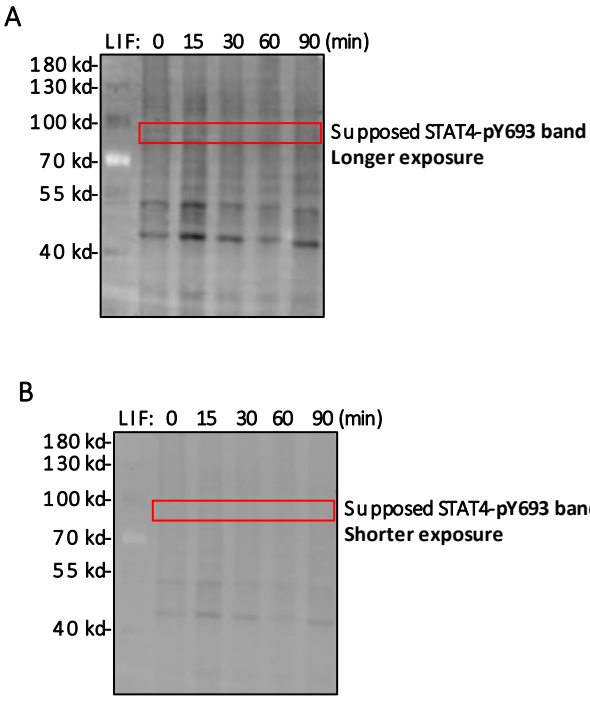

Figure for reviewers only 2. A, B Immunoblot analysis of STAT4 activation in the cell lysate of IECs treated with LIF (20 ng/ml) at different time points. C The intensity analysis of STAT4 protein level in CD4$^+$ T cells and IECs.

Third, we have tested the level of STAT4 activation in IECs with the antibody against phosphorylated STAT4 Y693 (**Figure for reviewers only 2A and B**). The molecular weight of STAT4 is 89 kd, but the signal is extremely weak around the supposed molecular weight on the membrane. We appreciate the Referee's suggestion about using the loading control for the comparison of protein data. So, we repeated the experiment shown in **Fig 1I**, and used β-actin as loading control which is the same as the loading control we used in **Fig 1J**. The old data have been replaced in our re-revised figures. Based on the statistical analysis of STAT4 expression intensity in CD4$^+$ T cells and IECs, we found that STAT4 protein level is much lower in IECs than in CD4$^+$ T cells (**Figure for reviewers only 2C**). In **Fig 1K**, it shows STAT4 mRNA expression in IECs, but the mRNA expression of STAT4 is also much lower in IECs than in CD4$^+$ T cells. Those data suggested that STAT4 mRNA and protein expression are rare in IECs, and LIF barely induce STAT4 activation in IECs.
Finally, we carefully edited the manuscript and corrected the grammatical errors. The revised manuscript has been proofread by a native English speaker.

I acknowledge and appreciate the authors' sincere efforts to bring this manuscript, which reports a number of interesting observations, to a higher standard. I am afraid, though, that the dots are still not connected well enough to give a clear and convincing picture.

We are encouraged that the Referee appreciates our efforts and finds our observations to be "interesting". As we illustrated in the cartoon (**Fig EV7C**), when the chemical DSS breaks intestinal barrier and invade into epithelium, IECs secret LIF as a negative feedback mechanism. We proved that LIF plays dual function in preventing colitis pathology, one is decreasing intestine inflammation by inhibiting Th17 accumulation via STAT4 activation in

colon, the other is promoting intestinal repair via STAT3 activation. Then the repaired intestinal epithelium keeps proinflammatory bacteria out. So far, we provided sort of a panorama of LIF's prevention of mouse colitis progression. For the molecular mechanisms, there are still lots of study we should have explored. We are pursuing to solve the remaining questions in our following study.

3rd Editorial Decision 5th Dec 2018

Thanks for submitting your revised version. Your study has now been re-reviewed by referee #1 whose comments are provided below. As you can see the referee appreciates that the added changes and support publication here. I am therefore very happy to accept the manuscript for publication here. Before I can send you the formal acceptance letter we just need to sort the following things out:

- We need the ChIP and the RNA seq data should be deposited in an appropriate database and that the accession numbers are provided in the manuscript

- We need 5 keywords

- We need a running title

- You can only have 6 EV figures - the rest would have to be added to the appendix. Would it be possible to combine EV1 and EV2? I also think it would be nice to have the model figure - currently in the EV7 figure into the main figures. Could you maybe add a figure 8? What are your thoughts on this? Would you also upload individual EV figure files? Thanks

- We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

- We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Could you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- It would also be good if you could provide me with a summary figure that I can place in the synopsis. The size should be 550 wide by 400 high (pixels).

- Our publisher has also done their pre-publication check on the manuscript and have made some comments in the figure legends. Please see attached word document. Would you please incorporate their changes when you submit the revised version.

I have provided you with a link below so that you can upload the revised files.

REFEREE REPORTS:

Referee #1:

The authors have addressed the previous comments and the writing has improved in the revision.

3rd Revision - authors' response 18th Dec 2018

The authors have made all requested editorial changes.

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:Y. Eugene Chin
Journal Submitted to:The Embo Journal
Manuscript Number:EMBOJ-2018-99595R

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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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?	Whenever possible power calculations have been used to estimate the number of animals needed to detect differences in the measured parameters on the basis of past experience and literature with a power of 90% and a type Ⅰ error rate of 5%.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See above.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	All animal experiments underwent randomization at entry. Age- and gender-matched mice were randomly allocated to each experimental arm through blinding the experimenter to animals. Animals were randomly assigned to treatment groups and during analysi.
For animal studies, include a statement about randomization even if no randomization was used.	All animal experiments underwent randomization at entry. Age- and gender-matched mice were randomly allocated to each experimental arm through blinding the experimenter to animals. Animals were randomly assigned to treatment groups and during analysi.
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.	Materials and Methods (Page 17)
4.b. For animal studies, include a statement about blinding even if no blinding was done	Materials and Methods (Page 17)
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.T-test was used to compare variances.
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

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

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

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

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).	pSTAT4Y693 (BD Biosciences, 554002); pSTAT4S721 (Santa Cruz, sc-28296); STAT4 (Santa Cruz, sc-486 and sc-398228); pSTAT3Y705 (Santa Cruz, sc-7993-R); STAT3 (Santa Cruz, sc-8019); YAP (Cell Signaling, 17074); LIFR (Santa Cruz, sc-659); HA (Santa Cruz, sc-7392); Myc (Santa Cruz, sc-40); Flag (Sigma, F1804); Histone H3 (Cell Signaling, 4499); pERK (Cell Signaling, 4370); Tubulin (Sigma, T619); b-actin (Sigma, A1978); Ki67 (Cell Signaling, 9449); Cleaved Caspase 3 (Cell Signaling, 9664); CD4-FITC (BD Bioscienc, 553047); CD4-APC (ebioscience,17-0041); IFNg-Percp cy5.5 (BD Biosciences, 560660); IFNg-PE-cy7 (BD Bioscience, 561040); IL17A-PE (ebioscience, 12-7177); Foxp3-PE (BD Bioscience, 560414); IL4-PE (BD Bioscience, 554435); CD45-Percp-cy5.5 (Biolegend, 103132); CD3-APC (BD Bioscience, 561826); CD127-FITC (ebioscience, 11-1271-81)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T cells, SW480 cells and DLD-1 cells were purchased from the Cell Bank of the Chinese Academy of Sciences. All cell lines were regularly tested for mycoplasma contamination.

* 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.	Materials and Methods (Page 15)
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Materials and Methods (Page 15)
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.	The manuscript complies with the guidelines.

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).	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