nature portfolio

Peer Review File

*HIF-2*α-Dependent induction of miR-29a Restrains T_H1 Activity during T cell Dependent Colitis

Open Access This file is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. In the cases where the authors are anonymous, such as is the case for the reports of anonymous peer reviewers, author attribution should be to 'Anonymous Referee' followed by a clear attribution to the source work. The images or other third party material in this file are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors showed that HIF2a expressed in CD4 T cells induces miR-29a in the hypoxic intestine and suppresses T-bet expression, thereby reducing the Th1 response and inhibiting colitis. The authors also showed that in vivo delivery of miR-29a can suppress colitis using a mouse model. The authors clearly showed the importance of the HIF2a-miR-29a axis in suppressing the Th1 response using a variety of genetically engineered mice and pharmacological inhibitors. But I have a couple of questions as below.
Throughout this paper, the authors use Lck-Cre mice, so it is likely that HIF2a and miR-29a are also defective in CD8 T cells. CD8 T cells also produce a lot of IFNg and T-bet is known to be important for IFNg expression in CD8 T cells. I am also interested in HIF2a and miR-29a expression in CD8 T cells. In addition, is there any change in IFNg expression in CD8 T cells derived from Lck-Cre/HIF2a floxed mice or Lck-Cre/miR-29a mice?
In Figure S5, the authors show that in vivo delivery of miR-29a results in greater uptake by CD4 positive cells as well as CD4 negative cells, but is there a change in IFNg production in CD4 negative cells? The authors have shown that miRNAs are delivered to CD4 T cells in mesenteric lymph node, but is miR-29a delivered to CD4T cells in lamina propria? And is the production of IFNg in CD4T cells of lamina propria reduced?
In Figure 7, the authors use a TNFΔARE mouse model of colitis. Is TNFa expression in CD4 T cells also regulated by HIF2a-miR-29a-T-bet axis? If so, is TNFa produced by CD4 T cells as well as IFNg, which is suppressed by the HIF2a-miR-29a axis, important in the pathogenesis of colitis?

Reviewer #2 (Remarks to the Author):

In their manuscript, Czopik and colleagues investigate how the hypoxic environment in colitis regulates Th1 cell activity. Specifically, they have studied the T-cell intrinsic effects of Hif2a, and miR-29a. To this end, they have employed multiple mouse models of conditional deletion and stabilization of Hif2a and deletion of miR-29a in transfer as well as pharmacologically induced colitis. They found that Hif2a was upregulated in colitogenic T cells and that this upregulation was associated with elevated expression of miR-29a, a microRNA with an established function in curtailing Th1 polarization. Mechanistically, ChIP experiment suggested direct control of miR-29a expression by Hif2a. Overall, in this setting T-bet levels were reduced, in line with published data on miR-29a. Functions of Hif2a and miR-29a were corroborated using genetic models. Altogether, the authors propose that Hif2a-sensed hypoxia in Th1 cells reduces the proinflammatory state, thus preventing excessive disease. With exception of the well-established role of miR-29a, data presented in this study are novel and of high interest to an interdisciplinary scientific community. Furthermore, they describe an interesting and promising first step towards pharmacological intervention using miR-29a mimics. Throughout, experiments have been rigorously confirmed and the conclusions are largely justified by the experiments shown. Nevertheless there are a number of issues that need to be experimentally addressed, mostly related to establishing conclusive mechanistic links between the various molecular entities studied here, such as the link between Hif2a and miR-29a.

Major concerns:

1.) The authors‘ main conclusion that the hypoxic environment curtails Th1 responses mechanistically via a Hif2a/miR-29/T-bet axis is insufficiently supported by the data. Although correlations in gene expression levels, published literature regarding control of T-bet expression by miR-29 and weak binding of Hif2a to one site in the miR-29 promoter are supportive of this conclusion, solid evidence is missing. The authors should perform a “rescue” experiment to demonstrate mechanistic links, including analyzing colitis outcome in a cross of stabilized Hif2a transgenic mice to miR-29 deficiency. This experiment will reveal, whether these molecules act in conjunction, in parallel or independent of each other. A mechanistic link between miR-29 and suppression of the Th1 phenotype is well established. However, this link may be based on

regulation Tbet or Ifng or other yet uncharacterized targets. Ultimately evidence can only be obtained through decoupling of gene expression from miR-29 based regulation by genetic deletion of miRNA response elements in selected target genes. Such experiments are beyond the scope of this study. However, the authors should refrain from overstating a potential link between T-bet and miR-29 apart from what is known in the literature. They should remove the respective part of the "highlights" bullet points and also omit Fig. 4A.

2.) In Fig. 6 (miR-29 deficient mice) the authors have refrained from performing transfer colitis experiments but rather used the short-term TNBS model. This model is subject to a different interpretation, because here, the full complement of Th1 biased cells is already in place, whereas the transfer colitis model relies on a relatively homogeneous subset of T cells. For the sake of consistency the authors should also perform the transfer colitis experiment here.

3.) The authors have used Lck-Cre mice throughout for conditional deletion. It has been reported that the Cre transgene alone has effects on T-cell development and the composition of the peripheral T cell pool (Carow et al., JI 2016). Especially in light of the rather moderate effects shown in Hif2a deficiency, "Cre only" controls should be shown to exclude potential experimental artifacts.

4.) As stated correctly in the introduction, Th1-converted Th17 cells may contribute to colitis development. Did the authors, in addition to assess T-bet levels, co-stain for Rorgt or IL-17 to determine whether loss of Hif2a affects Th17 conversion?

Minor:

5.) The authors should thoroughly rephrase their figure legends. Throughout they are difficult to understand and in part lack critical information. The authors should also be more specific as to the statistical methods employed for each experiment.

6.) Fig. 1A appears to be mislabeled or is difficult to understand (see above). With regard to controls for the EF5 staining, the figure refers to isotype, whereas Materials and Methods refer to competed (pre-loaded) antibody. The authors need to make a precise statement.

7.) Fig. 2A: The scale bar refers to fold change over normoxia, but values for normoxia are shown within the scale as well. How are these data to be interpreted? The experiment has not been described in Materials and Methods at all. Were the listed miRNAs the only ones probed? Details with regard to the "T-cell focused miRNA array" need to be provided. What statistics were applied? Furthermore, data need to be deposited appropriately.

8.) Fig. 3F,G: The text in the Results section refers to 7 putative HREs, but only 3 are investigated here. Why were 4 HREs omitted from the study? The authors should also state clearly and discuss that Hif2a binding in these experiments is not impressive (i.e. barely above background).

9.) Fig. 4: In vitro treatments of T cells with miR-29 mimics and inhibitors are not described in Materials and Methods. Fig. 4I reports lack of up-regulation, but no baseline expression is shown.

10.) Fig. 7/S5: The authors should comment on why they employed the IP route for control of delivery, while using IV for functional experiments. In Fig. 7A, the time unit is missing.

11.) References 15 and 17 refer to the same paper.

Reviewer #3 (Remarks to the Author):

Czopik and colleagues hypothesize that T cell dependent stabilization of HIF molecules results in microRNA reprogramming in the setting of intestinal inflammation. Using a combination of quantitative flow cytometry and luciferase reporter assays, the Authors report hypoxia related

stabilization of HIF molecules during experimental colitis, this resulting in HIF-2α dependent upregulation of miRNA-29a and repression of Th1 cell immunity. Notably, HIF-2α deficient mice display enhanced T-bet and IFNγ levels further corroborating the link between HIF-2α and Th1 cell immunity during colitis. Of translational relevance, DOPC-mediated delivery of miRNA-29a results in control of Th1-mediated colitis.

The study is sound as it links expression of HIF-2α with miRNA-29a reprogramming and subsequent control of T effector immunity. The data are supported by experimental models of adoptive transfer, TNBS and DSS colitis, being therefore relevant to both acute and chronic intestinal inflammation; some of the findings have been generated using patients' samples, adding translational value to the overall study. The conclusions are based upon solid experimental evidence; data are logically presented, and methodological details are provided. In addition to the innovative link between HIF-2α, miRNA-29a reprogramming and control of Th1 immunity, the study proposed has important clinical implications for the treatment of human IBD.
My comments are as follows:
- Figure 2D: Are the differences in miRNA-29a expression between normoxic and hypoxic Th1, Th17 and Treg cells significant?
- Figure 2E: Was the impact of hypoxia tested on other T cell subsets? Are these changes present also when considering cells from IBD patients?
- Figure 3E: When referring to this panel in the text, please specify that only three of the reported HRE are shown.
- Legend to Supplementary Figure 2: Please indicate the correct mouse number. Further, there are only two panels in this figure, while the legend indicates panels 'K' to 'N'.
- Supplementary Figure 3: Please indicate at which time points differences are statistically significant.
- Figure 6B indicates that T-bet and IFNγ are partly modulated by miRNA-29a and that hypoxia or other factors related to it play a role in the modulation of Th1 molecules, independently of miRNA-29a. Can the Authors discuss these findings?
- Page 14, line 7 from the bottom: Please check whether reference to Supplementary Figure 4B is correct (it should be Supplementary Fig. 4C).
- Page 17, reference to figure 7I and 7J should be changed to Figure 7H and 7I, as Panel J is not present.
- Page 17, lines 6-10 "indeed a recent study of acute DSS model… suggesting additional protective roles for this microRNA". I would suggest moving this section to the discussion.
- Methods, in vitro cell culture: Can the Authors check the description of in vitro cell culture including cytokines and concentrations used? It looks like a higher TGF-β concentration was used for Th17 than for Treg cells.
- In vitro T cell proliferation assay: Can the Authors indicate the readout used and in which section proliferation results were presented?

Minor points:
- Figure 1A: Please review the corresponding legend, as this indicates that results from mesenteric lymph nodes are presented. However, it appears all compartments are shown.
- Supplementary Figure 1: Fulminant disease is indicated at 7 weeks post transfer. However, based on Figure 1, mice appear to gain weight at this time point. Please clarify.

We want to thank the Reviewers for their insightful remarks that have greatly improved the quality of our manuscript. We believe to have addressed all comments to the best of our ability. In our resubmission file we include a copy of the manuscript with all the changes highlighted and we also reproduce all relevant passages and figure panels.

Thank you for considering the edited version of our manuscript.

Sincerely,

Agnieszka Czopik, Ph. D.

Reviewer #1 (Remarks to the Author):

1) The authors showed that HIF2a expressed in CD4 T cells induces miR-29a in the hypoxic intestine and suppresses T-bet expression, thereby reducing the Th1 response and inhibiting colitis. The authors also showed that in vivo delivery of miR-29a can suppress colitis using a mouse model. The authors clearly showed the importance of the HIF2a-miR-29a axis in suppressing the Th1 response using a variety of genetically engineered mice and pharmacological inhibitors. But I have a couple of questions as below.

Thank you very much for your kind comments.

2) Throughout this paper, the authors use Lck-Cre mice, so it is likely that HIF2a and miR-29a are also defective in CD8 T cells. CD8 T cells also produce a lot of IFNg and T-bet is known to be important for IFNg expression in CD8 T cells. I am also interested in HIF2a and miR-29a expression in CD8 T cells. In addition, is there any change in IFNg expression in CD8 T cells derived from Lck-Cre/HIF2a floxed mice or Lck-Cre/miR-29a mice?

Thank you for this very thoughtful suggestion. While still not completely understood, the role of CD8 T cells in IBD is becoming better defined and appreciated. At the same time studies on the role of CD8 T cells in IBD exhibit sometimes contradictory outcomes, which might be reflecting the heterogeneity of the CD8 T cell subsets. The Lck Cre driver deletes miR-29a in both CD4 and CD8 T cell lineages and the role of miR-29a in CD8 T cell biology is indeed very interesting. The function of this micro RNA in CD8 T cells has been previously studied and described (1-3), including the direct effect of miR-29a expression on IFNγ production and Eomes signaling in these cells. These reports show that miR-29a-deficient CD8 T cells overproduce IFNγ and Eomes, and exhibit increase in proliferation rates as compared to normal CD8 T cells. Additionally, Velica et all (4) showed that ectopic overexpression of HIF-2α leads to a diminished production of IFNγ in the CD8 T cells.

To address the role of CD8 T cells in our model of TNBS colitis we pre-treated miR-29a$^{loxP/loxP}$ Lck Cre^{+} and Lck Cre^{+} controls mice with CD8-depleting monoclonal antibody injections followed by TNBS challenge as shown in Figure 1 (below).

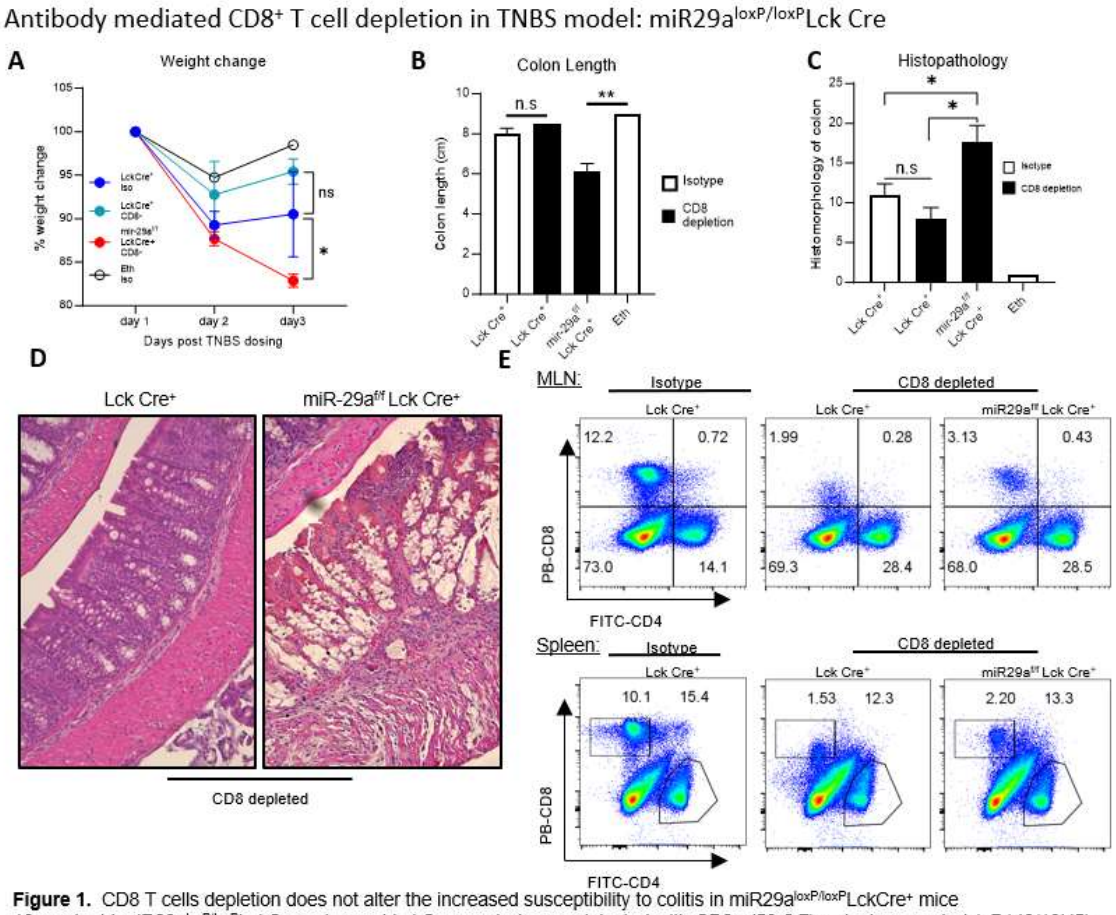


Figure 1. CD8 T cells depletion does not alter the increased susceptibility to colitis in miR29a$^{loxP/loxP}$LckCre+ mice
10 week old miR29a$^{loxP/loxP}$LckCre+ mice and LckCre+ controls were injected with CD8a (53-6.7) or Isotype control (eB149/10H5) rat monoclonal antibodies from eBioscience on day 0 and day 7. On day 0 mice were shaved on their back and sensitized with 1%TNBS in Acetone/Olive oil. On day 7 mice were challenged with intrarectal administration of 2.5% TNBS in 45% ethanol or ethanol only and weight change was recorded daily. Animals which lost less then 5% BW at 24h following TNBS challenge were excluded from further study as non-responders. **A**. Weight change chart shows weight loss in the course of colitis development; n=2-3 mice per group, 2-way ANOVA used to calculate p value. **B.** Mice were euthanized on day 3 and colon length was measured. **C**. Histopathology scores were obtained by a scientist blinded to the identity of the animals. **D.** Representative micrographs of H&E staining in the colon show infiltration of lymphocytes into the intestinal epithelium in LckCre+ and miR29a$^{loxP/loxP}$ Lck Cre+ mice with profound crypt necrosis in miR29a$^{loxP/loxP}$ Lck Cre+; original magnification 20x. **E.** Mesenteric lymph node (MLN) cells and splenocytes were isolated and stained for CD4 and CD8 markers and gated on live lymphocytes. Representative plots for each group are shown. **p<0.001 and *p<0.01 in two-tailed T-test.

Our results suggest that in the acute model of TNBS colitis CD8 T cell-depleted and CD8 T cell-sufficient animals develop colitis with a similar pathology and similar colon shortening phenotype. Additionally, depleting CD8 T cells in miR-29a$^{loxP/loxP}$ Lck Cre^{+} mice resulted in markedly enhanced disease phenotype compared to similarly depleted Lck Cre^{+} control mice, again preserving the difference observed previously in mice not depleted for CD8 T cells. We conclude that in our model the CD8 T cell contribution to the disease pathology is not readily detectable and may be eclipsed by the TH1 CD4 T cells and the IFNγ produced by this population. Because our colitis model appears not sufficiently sensitive to directly demonstrate the role of CD8 T cells, we are currently unable to attribute the observed changes of IFNγ production to that T cell subset and we feel that an in-depth investigation of HIF-2α and miR-29a function in CD8 T cells would be better suited for a more focused, separate study addressing CD8 T cell contribution in colitis in the context of HIFs and miR-29a deficiency.

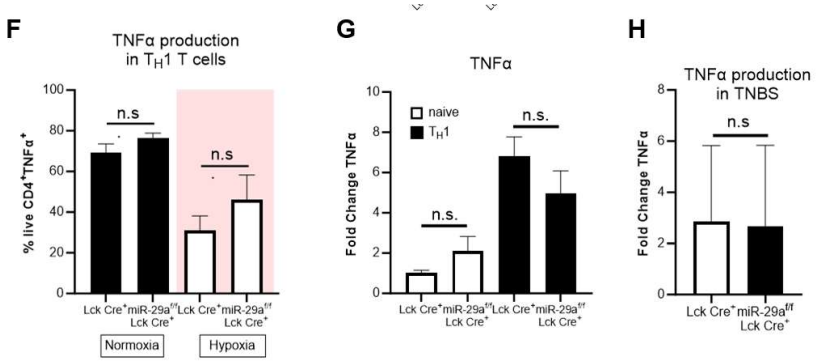
3) In Figure S5, the authors show that in vivo delivery of miR-29a results in greater uptake by CD4 positive cells as well as CD4 negative cells, but is there a change in IFNg production in CD4 negative cells? The authors have shown that miRNAs are delivered to CD4 T cells in mesenteric lymph node, but is miR-29a delivered to CD4T cells in lamina propria? And is the production of IFNg in CD4T cells of lamina propria reduced?

In our experiment tracing the miRNA uptake by lymphocytes, we injected the fluorescent tracer miRNA into colitic animals and subsequently harvested the MLN cells at different time points. We were able to localize T cells that have taken up the tracer miRNA, however, this tracer miRNA has no biological activity and therefore would not affect the production of cytokines in the T cells or in the $CD4^-$ cells in this experiment. We were able to show the indirect IFNγ-suppressive effect of administering miR-29a mimetic in the $CD4^+$ T cells in the context of TNBS colitis (Figure 7E) and in chronic ileitis (in Figure 7G).

Additionally, we were unable to localize microRNA tracer-positive T cells in the lamina propria in the TNBS colitis model, which may be due to the inherently short half-life of microRNA and the labeled microRNA derivative being used up and broken down inside the T cells which are likely undergoing cell division and activation at the same time. In conclusion we were unable to detect fluorescent T cells within the lamina propria by our assays but are able to enumerate the cells carrying labeled microRNA while they are still within the mesenteric lymph node.

4) In Figure 7, the authors use a TNFΔARE mouse model of colitis. Is TNFa expression in CD4 T cells also regulated by HIF2a-miR-29a-T-bet axis? If so, is TNFa produced by CD4 T cells as well as IFNg, which is suppressed by the HIF2a-miR-29a axis, important in the pathogenesis of colitis?

Thank you for these excellent suggestions. TNFα is a very important cytokine in the pathogenesis of colitis and in order to address these concerns, we have measured and reported in Figure S5 the levels of this cytokine in miR-29a-deficient T_H1 T cells cultured ex-vivo in normoxia and hypoxia (S5F), in naïve vs differentiated T_H1 T cells (S5G) and in vivo within TNBS colitis in miR-29a$^{loxP/loxP}$ Lck Cre$^+$ and Lck Cre$^+$ mice (S5H) within the colonic tissue on day 3 following the TNBS challenge (relevant panels of Figure S5 are reproduced) .



Our results suggest that TNFa is produced in similar levels in miR-29a$^{loxP/loxP}$ Lck Cre$^+$ and Lck Cre$^+$ mice or in isolated T cells. We conclude that cultured T cells produce similar levels of TNFα regardless of their miR-29a status, suggesting that this cytokine is not dependent on the hypoxia-miR-29a regulatory axis.

Reviewer #2 (Remarks to the Author):

In their manuscript, Czopik and colleagues investigate how the hypoxic environment in colitis regulates Th1 cell activity. Specifically, they have studied the T-cell intrinsic effects of Hif2a, and miR-29a. To this end, they have employed multiple mouse models of conditional deletion and stabilization of Hif2a and deletion of miR-29a in transfer as well as pharmacologically induced colitis. They found that Hif2a was upregulated in colitogenic T cells and that this upregulation was associated with elevated expression of miR-29a, a microRNA with an established function in curtailing Th1 polarization. Mechanistically, ChIP experiment suggested direct control of miR-29a expression by Hif2a. Overall, in this setting T-bet levels were reduced, in line with published data on miR-29a. Functions of Hif2a and miR-29a were corroborated using genetic models. Altogether, the authors propose that Hif2a-sensed hypoxia in Th1 cells reduces the proinflammatory state, thus preventing excessive disease. With exception of the well-established role of miR-29a, data presented in this study are novel and of high interest to an interdisciplinary scientific community. Furthermore, they describe an interesting and promising first step towards pharmacological intervention using miR-29a mimics. Throughout, experiments have been rigorously confirmed and the conclusions are largely justified by the experiments shown. Nevertheless, there are a number of issues that need to be experimentally addressed, mostly related to establishing conclusive mechanistic links between the various molecular entities studied here, such as the link between Hif2a and miR-29a.

Thank you very much for these kind and enthusiastic comments.

Major concerns:

1.) The authors' main conclusion that the hypoxic environment curtails Th1 responses mechanistically via a Hif2a/miR-29/T-bet axis is insufficiently supported by the data. Although correlations in gene expression levels, published literature regarding control of T-bet expression by miR-29 and weak binding of Hif2a to one site in the miR-29 promoter are supportive of this conclusion, solid evidence is missing. The authors should perform a "rescue" experiment to demonstrate mechanistic links, including analyzing colitis outcome in a cross of stabilized Hif2a transgenic mice to miR-29 deficiency. This experiment will reveal, whether these molecules act in conjunction, in parallel or independent of each other. A mechanistic link between miR-29 and suppression of the Th1 phenotype is well established. However, this link may be based on regulation Tbet or IFNg or other yet uncharacterized targets. Ultimately evidence can only be obtained through decoupling of gene expression from miR-29 based regulation by genetic deletion of miRNA response elements in selected target genes. Such experiments are beyond the scope of this study. However, the authors should refrain from overstating a potential link between T-bet and miR-29 apart from what is known in the literature. They should remove the respective part of the "highlights" bullet points and also omit Fig. 4A.

We thank the reviewer for these thoughtful comments and we completely agree with them. The mouse line LSL HIF2A dPA/miR29$^{loxP/loxP}$ Lck Cre suggested by the reviewer is certainly a great idea. However, deriving this triple-transgenic line has proven to be very challenging technically and requires a very long breeding time to accomplish. We have therefore made changes to our manuscript to reflect the suggestions made by the

reviewer to address the lack of solid mechanistic evidence of the Hif-2α control of miR-29a/T bet axis. We have deleted the "highlight" part and deleted Figure 4A, as suggested. We have also removed the mechanistic focus and toned down the claims of direct regulation of miR-29a by HIF-2α. Modified manuscript text is as below:

Page 2 (Highlights):

- *HIF-2α enhances miR-29a to limit T_H1 development in hypoxia*

Page 3

"Together, these findings reveal a previously unrecognized function for hypoxia-dependent induction of miR-29a in attenuating T_H1-mediated inflammation."

Page 11

"Together these studies point to a role for HIF-2α in the induction of miR-29a in $CD4^+$ T cells during ambient hypoxia exposure."

Page 11 (this text and the corresponding figure panel were deleted, as per suggestions)

~~*"Using in silico analysis, we confirmed miR-29a binding site for Tbet mRNA conserved across multiple species (Figure 4A)."*~~

Page 12

"Taken together, our results provide evidence that the increase in miR-29a expression in response to either hypoxia or pharmacological HIF stabilization in vitro, or in a genetic mutation that leads to HIF-2α stabilization in vivo allows for a coordinated reduction in T_H1 responses during colitis."

Page 20

"Mice with a T cell-deficiency in HIF-2α display heightened experimental colitis scores and enhanced T_H1 markers in T cells. Mice with a T cell intrinsic-deficiency of miR-29a show enhanced colitic profiles in T_H1-mediated colitis with concomitantly increased Tbet expression and cytokine production combined with enhanced mucosal injury score. Enhanced T_H1 phenotype of miR29a-deficient T cells leads to impaired T_H17 differentiation in the inflamed mucosa in the transfer colitis model."

Page 23

"Our genetic deletion data implicates HIF-2α as an enhancer of miR-29a in T cells during intestinal inflammation."

2.) In Fig. 6 (miR-29 deficient mice) the authors have refrained from performing transfer colitis experiments but rather used the short-term TNBS model. This model is subject to a different interpretation, because here, the full complement of Th1 biased cells is already in place, whereas the transfer colitis model relies on a relatively homogeneous subset of T cells. For the sake of consistency, the authors should also perform the transfer colitis experiment here.

We have performed the transfer colitis experiments and are reporting our observations in Figure 6, E-J and Figure S4E.

"To establish a role for miR-29a in T cells during intestinal inflammation we performed adoptive transfer of naïve CD4^{+} CD45RB^{high} T cells derived from miR-29a sufficient (Lck Cre+) and deficient (miR-29a$^{loxP/loxP}$ Lck Cre^{+}) mice into Rag1$^{-/-}$ recipients. Unexpectedly, both groups displayed similar weight loss (Figure S4E) and similar intestinal inflammatory scores (data not shown). We then hypothesized that T cell differentiation into effector subsets may be impaired in the absence of miR-29a, and to test this we prepared in-vitro skewed T_H1 T cells and adoptively transferred them into Rag1$^{-/-}$ recipients. At 9 weeks post transfer mice receiving miR-29a-deficient T cells lost significantly less weight (Figure 6E), although their colon inflammatory scores remained comparable to the controls (Figure 6F). Both groups displayed similar inflammatory changes in the colon (Figure 6G). Interestingly, analysis of T cells from the mesenteric lymph nodes showed that effector T cell differentiation is affected by the loss of miR-29a (Figure 6H). While miR-29a-deficient T cells efficiently retained their T_H1 lineage commitment and produced high amounts of IFNγ (Figure 6I) with notably higher survival rate under hypoxia (Figure S4C), the miR-29a-sufficient T cells were more proficient differentiating into IL-17A-producing T cells (Figure 6J). Indeed, purified CD4^{+} miR-29a-deficient T cells skewed in-vitro towards T_H17 show diminished survival and limited IL-17A production under hypoxia (Figure S4F, S4G and S4H). The loss of T_H1 identity and differentiation into IL-17A^{+} T cells within the hypoxic intestinal environment in the transfer colitis model has been described previously (5), and a report by Leppkes et al (6) identified the highly pathogenic role for IL-17A^{+} T cells in the transfer colitis model which results in a higher disease severity than IFNγ^{+} T cells alone. We conclude that in CD4^{+} T cells deletion of miR-29a relieves the restraint on Tbet expression and leads to better retention of T_H1 identity and higher IFNγ production concomitant with an impairment in IL-17A production resulting in a diminished disease severity."

Figure 6 E-J:

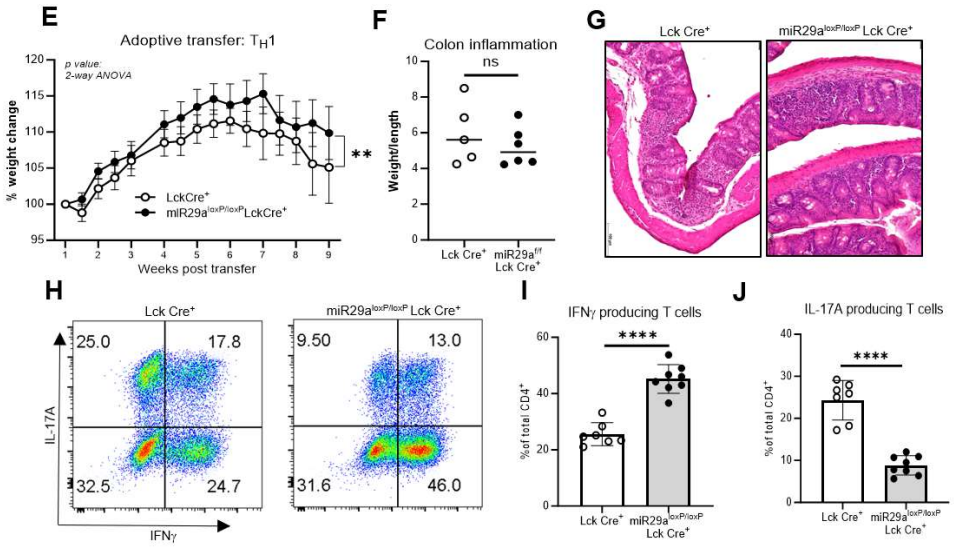
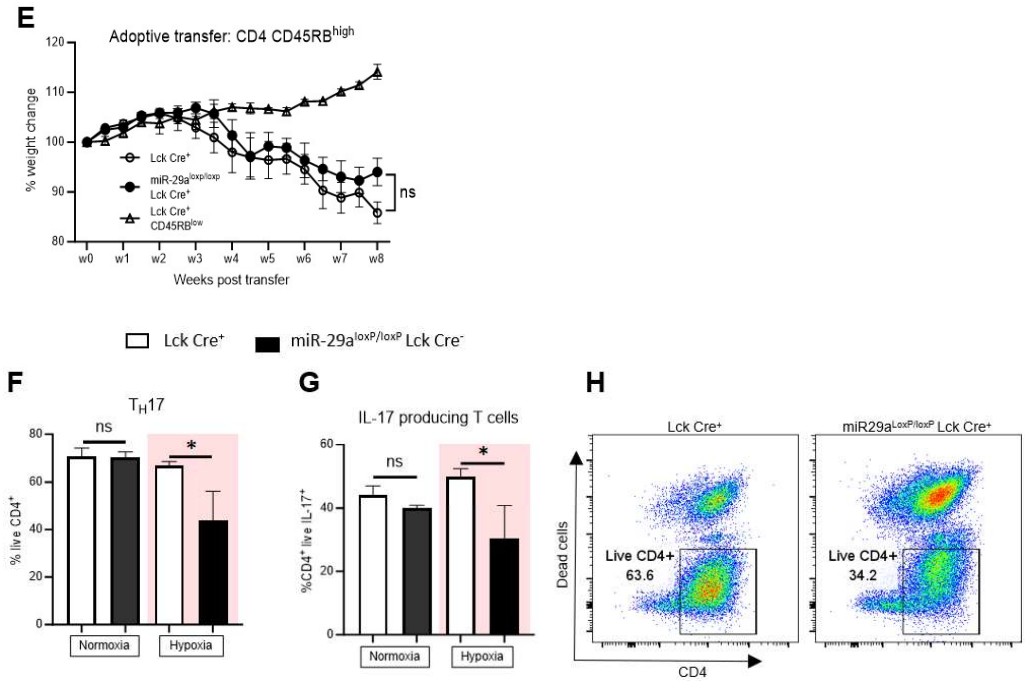


Figure S4E-H:

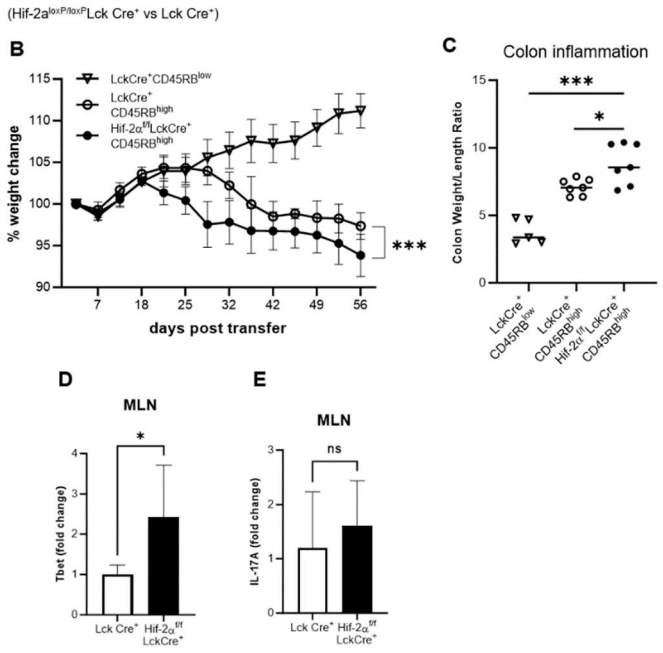


3.) The authors have used Lck-Cre mice throughout for conditional deletion. It has been reported that the Cre transgene alone has effects on T-cell development and the composition of the peripheral T cell pool (Carow et al., JI 2016). Especially in light of the rather moderate effects shown in Hif2a deficiency, "Cre only" controls should be shown to exclude potential experimental artifacts.

We have addressed this point by including Lck Cre$^+$ mice in Figure S3 B-E in comparison with HIF-2α-deficient mice. We report that inclusion of Lck Cre$^+$ controls did not change our experimental conclusions. Edited text now reads:

"Adoptive transfer of naïve CD4$^+$ T cells from Hif-2$\alpha^{loxP/loxP}$ Lck Cre$^+$ and Lck Cre$^+$ controls (to account for the effect of the Cre) resulted in enhanced weight loss and colonic inflammation in recipients of HIF-2α deficient cells (Figure S3B and S3C). "

Figure S3:



4.) As stated correctly in the introduction, Th1-converted Th17 cells may contribute to colitis development. Did the authors, in addition to assess T-bet levels, co-stain for Rorgt or IL-17 to determine whether loss of Hif2a affects Th17 conversion?

Thank you very much for this important comment. We have addressed it by including data in supplemental Figure S3 (above) and appending the text as follows:

"T cells in MLN from these mice showed increased expression of Tbet (Figure S3D), but no significant differences were noted in expression of IL-17A (Figure S3E)."

Minor:

5.) The authors should thoroughly rephrase their figure legends. Throughout they are difficult to understand and in part lack critical information. The authors should also be more specific as to the statistical methods employed for each experiment.

We appreciate this suggestion and have thoroughly edited the figure legends and included missing statistical methods. For brevity, we have not included the complete edited figure legends here but rather with the text of the manuscript, with all changes highlighted in color.

6.) Fig. 1A appears to be mislabeled or is difficult to understand (see above). With regard to controls for the EF5 staining, the figure refers to isotype, whereas Materials and Methods refer to competed (pre-loaded) antibody. The authors need to make a precise statement.

Thank you for pointing this out, and we have clarified the methods which are now in agreement with the figure and the legend:

"A matched isotype antibody was utilized as a negative staining control. Cells were stained and gated on: singlets, live, MHCII$^-$, CD3$^+$, CD4$^+$ and effector memory T cells were additionally gated on CD44$^+$ and CD62L$^-$."

7.) Fig. 2A: The scale bar refers to fold change over normoxia, but values for normoxia are shown within the scale as well. How are these data to be interpreted? The experiment has not been described in Materials and Methods at all. Were the listed miRNAs the only ones probed? Details with regard to the "T-cell focused miRNA array" need to be provided. What statistics were applied? Furthermore, data need to be deposited appropriately.

The Methods section has now been amended to include description of the limited T cell miRNA analysis and currently reads:

"Limited target q-PCR based array with miRNA specific primers from Qiagen (custom array) and Bio-Rad CFX384 system was used to measure changes in T cell-specific microRNAs shown in Figure 2A; data was then normalized to the expression of RNU6 and further compared to the average of normoxia samples to create the heatmap. RNA for the array was prepared from T cells stimulated with anti CD3/CD28 for 8h in normoxia and hypoxia."

We have also clarified the nomenclature of the array files and re-deposited the data.

8.) Fig. 3F,G: The text in the Results section refers to 7 putative HREs, but only 3 are investigated here. Why were 4 HREs omitted from the study? The authors should also state clearly and discuss that Hif2a binding in these experiments is not impressive (i.e. barely above background).

We limited our analysis to the 3 most proximal HREs as those 3 fulfill majority of the criteria (sequence context) for functional HREs and are located within 1Kb of the transcriptional start site. To clarify these considerations, we have amended the text to read as follows:

"We employed chromatin immunoprecipitation (ChIP) assay to address binding of HIF-2α to individual 3 proximal HREs that most closely conformed to the previously established criteria for functional HREs within 1kb upstream of the transcriptional start site(7). These studies demonstrate modest binding of HIF-2α to HRE#1 (Figure 3G first panel) but not to HRE#2 and HRE#3 (Figure 3G, panel 2 and 3) during ambient hypoxia exposure of CD4$^+$ T cells (4 more distal HREs were not tested in this assay)."

9.) Fig. 4: In vitro treatments of T cells with miR-29 mimics and inhibitors are not described in Materials and Methods.

We added a new section in Methods to address this:

"T Cell Culture and Transfection with miRNAs

T_H1 skewed (day 3) CD4+ T cells were rested overnight in fresh medium. Subsequently the T cells were transfected with 100nM miRNA mimetic or 300nM miRNA inhibitor (both from Dharmacon, Lafayette, CO) using Lipofectamine 3000 (Invitrogen), following manufacturer's recommendations. Twelve hours after transfection cells were re-stimulated with 5.0 µg/ml anti-CD3/anti-CD28 mAbs (eBioscience) and collected for further analysis 48 h later."

10.) Fig. 4I reports lack of up-regulation, but no baseline expression is shown.

To clarify the data presentation, we changed the text as follows:

"Conversely, T cells transfected with miR-29a mimetic downregulate Tbet upon in vitro activation under normoxia compared to control-transfected cells (Figure 4I)."

11.) Fig. 7/S5: The authors should comment on why they employed the IP route for control of delivery, while using IV for functional experiments. In Fig. 7A, the time unit is missing.

We have chosen the IV route as it required less handling time and no additional dilution steps for the mimetic in lipid emulsion preparation. The new text now reads:

"intraperitoneally (or IV, not shown, producing comparable results) injected Cy3-labeled and"

"were delivered to mice in a lipid emulsion of nanoparticles (iv, chosen as the preferred method requiring less handling time for the mimetic prior to injection; on days 1 and 3 post-TNBS"

Time unit (days) has been added to Figure 7A.

11.) References 15 and 17 refer to the same paper.

Thank you, we have removed the duplicate reference.

Reviewer #3 (Remarks to the Author):

Czopik and colleagues hypothesize that T cell dependent stabilization of HIF molecules results in microRNA reprogramming in the setting of intestinal inflammation. Using a combination of quantitative flow cytometry and luciferase reporter assays, the Authors report hypoxia related stabilization of HIF molecules during experimental colitis, this resulting in HIF-2α dependent upregulation of miRNA-29a and repression of Th1 cell immunity. Notably, HIF-2α deficient mice display enhanced T-bet and IFNγ levels further corroborating the link between HIF-2α and Th1 cell immunity during colitis. Of translational relevance, DOPC-mediated delivery of miRNA-29a results in control of Th1-mediated colitis.

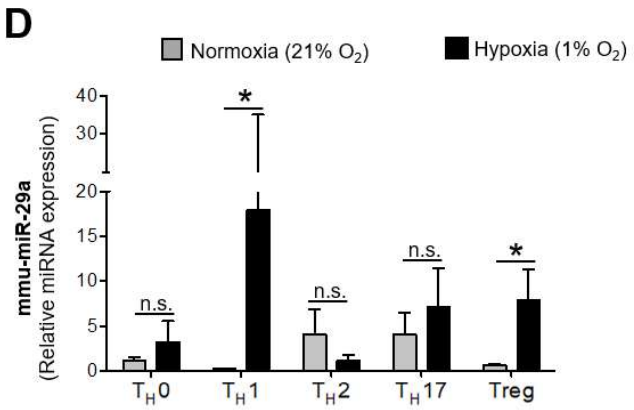
The study is sound as it links expression of HIF-2α with miRNA-29a reprogramming and subsequent control of T effector immunity. The data are supported by experimental models of adoptive transfer, TNBS and DSS colitis, being therefore relevant to both acute and chronic intestinal inflammation; some of the findings have been generated using patients' samples, adding translational value to the overall study. The conclusions are based upon solid experimental evidence; data are logically presented, and methodological details are provided. In addition to the innovative link between HIF-2α, miRNA-29a reprogramming and control of Th1 immunity, the study proposed has important clinical implications for the treatment of human IBD.

Thank you very much, we sincerely appreciate your kind words.

My comments are as follows:

1) Figure 2D: Are the differences in miRNA-29a expression between normoxic and hypoxic Th1, Th17 and Treg cells significant?

We have added the appropriate statistical designations to the Figure 2D to indicate statistical significance:



2) Figure 2E: Was the impact of hypoxia tested on other T cell subsets? Are these changes present also when considering cells from IBD patients?

Thank you for these very relevant questions. We have not followed the impact of hypoxia on other T cell lineages in this limited study but plan to follow up on this in the future work, especially considering the induction of miR-29a in the T_{reg} population under hypoxia. We have also not been able to secure T cell samples from IBD patients for our study.

3) Figure 3E: When referring to this panel in the text, please specify that only three of the reported HRE are shown.

We have specified as suggested and the new text now reads:

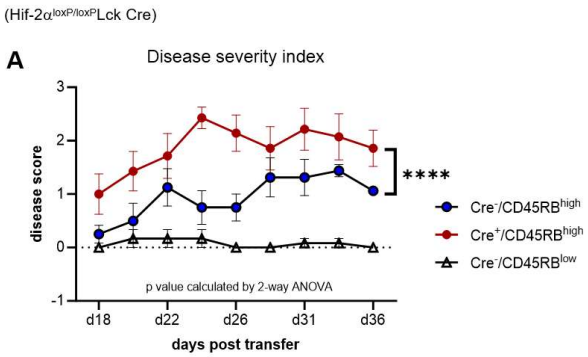
"Analysis of the nucleotide sequence within the miR-29b1-a proximal promoter identifies 7 potential Hypoxia Responsive Elements (HRE) and the 3 most proximal to the transcriptional start site are indicated on the diagram (Figure 3F)."

4) Legend to Supplementary Figure 2: Please indicate the correct mouse number. Further, there are only two panels in this figure, while the legend indicates panels 'K' to 'N'.

We have corrected the figure legend respectively.

5) Supplementary Figure 3: Please indicate at which time points differences are statistically significant.

We have recalculated the statistics and included the detailed description of the calculations in the figure legend:



"A. *Disease severity index as measured by the stool consistency and presence/absence of rectal prolapse was assayed every two days from day 18 until day 36 post-injection in each group of transfer recipients, (n=6-7mice/group, p value <0.0001, two-way ANOVA, column means over the 18d period of assessment, Sidak's multiple comparison test). "*

6) Figure 6B indicates that T-bet and IFNγ are partly modulated by miRNA-29a and that hypoxia or other factors related to it play a role in the modulation of Th1 molecules, independently of miRNA-29a. Can the Authors discuss these findings?

We appreciate the suggestion and have included the following in the discussion section of the manuscript:

"Several signal transduction pathways regulating miR-29 family of miRNAs have been described in the context of specific diseases(8). For example, a report by Chandiran et al highlight the role of Notch-1-mediated miR-29a repression in early T-cell differentiation towards T_H1(9), while others studied the role of AKT-MYC-dependent regulation of miR-29s expression in B cell malignancies(10, 11), underscoring the cell type-specific regulation of these important miRNAs in lymphocyte biology. While our work focuses specifically on the novel aspects of miR-29a activation in $CD4^+$T cells by tissue hypoxia, some of the previously identified mechanisms of miR-29a activation may also contribute to the phenotypes observed in our study."

7) Page 14, line 7 from the bottom: Please check whether reference to Supplementary Figure 4B is correct (it should be Supplementary Fig. 4C).

We have reorganized Figure S4 to accommodate Reviewers' requested experiments and matched the references to the correct panels.

8) Page 17, reference to figure 7I and 7J should be changed to Figure 7H and 7I, as Panel J is not present.

We have changed the wording accordingly and the new text reads:

"Upon conclusion of treatment with miR-29a mimetic $TNF^{\Delta ARE/+}$ mice were showing reduced number of IFNγ^+ $CD4^+$ T cells in the MLN as compared to control-mimetic treated mice (Figure 7G), and these animals developed significantly diminished histological changes in the ileum (Figure 7H and Figure 7I)."

9) Page 17, lines 6-10 "indeed a recent study of acute DSS model… suggesting additional protective roles for this microRNA". I would suggest moving this section to the discussion.

Thank you for the suggestion, we have moved the section to the Discussion.

10) Methods, *in vitro* cell culture: Can the Authors check the description of *in vitro* cell culture including cytokines and concentrations used? It looks like a higher TGF-β concentration was used for Th17 than for Treg cells.

We have reviewed and corrected the concentration of cytokines used in our culture:

"T_H17 were differentiated with recombinant TGFβ (3ng/ml; RnD systems), IL-23 (10ng/ml), IL-6 (20ng/ml; R & D systems), anti-IL-4 (10µg/ml; BioXCell) and anti-IFNγ (10µg/ml; BioXCell). Lastly, T_{reg} cells were differentiated with the addition of recombinant TGFβ (5ng/ml, RnD systems),"

11) *In vitro* T cell proliferation assay: Can the Authors indicate the readout used and in which section proliferation results were presented?

Thank you very much. We have removed that section of methods having realized that no such experiments are presented in this manuscript.

Minor points:

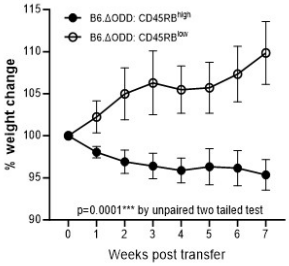
1) Figure 1A: Please review the corresponding legend, as this indicates that results from mesenteric lymph nodes are presented. However, it appears all compartments are shown.

The figure legend has been changed accordingly to reflect the graph:

***"A.** Flow cytometric sub-analysis of cellular profile within Cy5-labelled EF5 (nitroimidazole; injected IP-3h) naïve and effector memory CD4$^+$ T cells from the spleen, mesenteric lymph nodes and the colons."*

2) Supplementary Figure 1: Fulminant disease is indicated at 7 weeks post transfer. However, based on Figure 1, mice appear to gain weight at this time point. Please clarify.

Thank you for pointing this out; we have corrected the weight change data and also moved the graph to Figure S1B (below).



References

1. K. J. Yee Mon *et al.*, MicroRNA-29 specifies age-related differences in the CD8+ T cell immune response. *Cell Rep* **37**, 109969 (2021).
2. E. M. Wissink, N. L. Smith, R. Spektor, B. D. Rudd, A. Grimson, MicroRNAs and Their Targets Are Differentially Regulated in Adult and Neonatal Mouse CD8+ T Cells. *Genetics* **201**, 1017-1030 (2015).
3. E. Stelekati *et al.*, MicroRNA-29a attenuates CD8 T cell exhaustion and induces memory-like CD8 T cells during chronic infection. *Proceedings of the National Academy of Sciences of the United States of America* **119**, e2106083119 (2022).
4. P. Veliça *et al.*, Modified Hypoxia-Inducible Factor Expression in CD8(+) T Cells Increases Antitumor Efficacy. *Cancer immunology research* **9**, 401-414 (2021).
5. H. P. Liu *et al.*, TGF-β converts Th1 cells into Th17 cells through stimulation of Runx1 expression. *European journal of immunology* **45**, 1010-1018 (2015).
6. M. Leppkes *et al.*, RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology* **136**, 257-267 (2009).
7. R. H. Wenger, D. P. Stiehl, G. Camenisch, Integration of oxygen signaling at the consensus HRE. *Science's STKE : signal transduction knowledge environment* **2005**, re12 (2005).
8. M. Horita, C. Farquharson, L. A. Stephen, The role of miR-29 family in disease. *Journal of cellular biochemistry* **122**, 696-715 (2021).
9. K. Chandiran *et al.*, Notch1 primes CD4 T cells for T helper type I differentiation through its early effects on miR-29. *Molecular immunology* **99**, 191-198 (2018).
10. K. M. Smith *et al.*, miR-29ab1 deficiency identifies a negative feedback loop controlling Th1 bias that is dysregulated in multiple sclerosis. *Journal of immunology (Baltimore, Md. : 1950)* **189**, 1567-1576 (2012).
11. A. van Nieuwenhuijze *et al.*, Defective germinal center B-cell response and reduced arthritic pathology in microRNA-29a-deficient mice. *Cellular and molecular life sciences : CMLS* **74**, 2095-2106 (2017).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have properly addressed the reviewer's concerns.

Reviewer #2 (Remarks to the Author):

The authors have done an excellent job in addressing all concerns raised by the reviewer. The reviewer acknowledges the complexity of generating new mouse lines in a limited amount of time. The authors have sufficiently toned down their conclusions. Inclusion of the transfer colitis data in Figure 6 is indeed interesting.

Reviewer #3 (Remarks to the Author):

The Authors have addressed the points previously raised.

Please check the TGF-beta concentration in the text, when describing Th17 and Treg cell differentiation (lines 619-621).