nature research

Peer Review Information

Journal: Nature Immunology
Manuscript Title: TCF1 controls Treg functions that regulate inflammation, CD8 T-cell cytotoxicity, and severity of colon cancer
Corresponding author name(s): Khashayarsha Khazaie, Fotini Gounari, Majid Kazemian

Editorial Notes:

Redactions – unpublished data	Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A31353
Message: 9th Feb 2021

Dear Khash,

Thank you for providing your point-by-point response to the referees comments on your manuscript entitled, "TCF1 controls Treg functions that regulate inflammation, CD8 T-cell cytotoxicity, and severity of colon cancer". As noted previously, while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the current manuscript for publication, but would be very interested in considering a revised version that addresses these concerns.

We invite you to submit a substantially revised manuscript, however please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions.

Specifically, the revision should include new experiments to address:

(1) additional data analysis and interrogation of the role of TCF-1 in human colorectal cancer (analysis of existing scRNA-seq datasets)
(2) Correlate differential gene expression of TCF-1 with ChIP-seq binding dataset.
(3) Compare expression of TCF-1 in the APC mutant mice vs wild-type controls.

(4) Discuss role of microbiota in polyposis.

Please include the additional textual clarifications as indicated in your response letter. {also note that the NI style for phosphorylated proteins such as STAT5 is "p-STAT5"; please revise throughout.}

When you revise your manuscript, please take into account all reviewer and editor comments, please highlight all changes in the manuscript text file in Microsoft Word format.

If you choose to revise your manuscript taking into account all reviewer and editor comments, please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

The Reporting Summary can be found here:
https://www.nature.com/documents/nr-reporting-summary.pdf

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the

peer review and production process or after publication if any issues arise.

You may use the link below to submit your revised manuscript and related files:
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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Kind regards,

Laurie

Laurie A. Dempsey, Ph.D.
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Referee expertise:

Referee #1: human T cells

Referee #2: regulatory T cell biology

Referee #3: human colorectal cancer

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:

This is an interesting manuscript that investigates the role of TCF1 in Treg identity and function. Using TCF1-depleted mice, the authors show that TCF1-deficient Tregs maintain their core Treg gene expression signature but display diverse functional properties. They present data from ex vivo and in vitro assays – including scRNA-seq analyses - suggesting that Tregs lacking TCF1 are more potent in suppressing the viral antigen-specific CD8+ T cell cytotoxicity and T cell proliferation but are compromised in their ability to hinder the polarization of CD4+ T cells toward the pro-inflammatory Th1 and Th17 lineages. In a tumor model of polyposis, TCF1-depleted mice have a higher incidence of colon polyps and pre-invasive colon tumors.

The authors use diverse experimental approaches to support their findings and to assess the mechanisms for the enhanced suppressive activity of TCF1-deficient TregS during viral infection. They subsequently show that Apc (delta468) Foxp3creTcffl/fl mice have a high incidence of pre-invasive colon tumors. However, the functional properties of TCF1-deficient Tregs in the context of this tumor model are not investigated. Furthermore, what is the single-cell transcriptional profile of Treg populations in the TCF1 defective Apc (delta468) Foxp3creTcffl/fl mice? Does the adoptive transfer of Foxp3cre Tregs rescue the phenotype?

The manuscript could also benefit from a parallel investigation of the TCF1-mediated regulation of Treg phenotype and function in humans, for instance interrogating public scRNA-seq data for human colon cancer.

Minor comments:

- Annotation of the velocity maps in Fig. 4g with the major clusters of interest would allow a better visualization of the intercluster relations.

- Fig. S5: Include a graph that shows the differences in the expression of Maf.

- The manuscript is in need of extensive proofreading, as indicated by some examples below:

'MIF is the receptor for CXCR2 and CXCR4…': MIF is a soluble cytokine not a receptor.

'Of note, CXCR4 is a key chemokine for attracting…': CXCR4 is a chemokine receptor.

'…in naïve mice mice that had not been vaccinated': Presumably the authors refer to 'infected' as opposed to 'vaccinated' mice.

Some other typos throughout the text:
'classificataions', 'aand', 'ecodes', 'unplulsed', 'similarities conserved intercluster relations', 'signaling signaling', 'expresioon', 'associataed'

Reviewer #2:
Remarks to the Author:
This study examined the role of Treg cell functional regulation by TCF1. The authors revealed that TCF1 does not play a role in Treg core transcriptional signature but regulates the TGFβ receptor, Th17 and Wnt/Bcat signaling pathways. They also showed that TCF1 expression level plays a role in the control of Tregs plasticity. Moreover, the study also reveals that the deficiency of TCF1 in Tregs induces enhanced suppression by Treg cells of CD4/CD8 T cell Proliferation, but at the same time this deletion promotes inflammatory cytokine production by CD4/CD8 T cells. The proneness of TCF1-deficient Treg cells to inflammatory skewing is associated with a poorer outcome in a colorectal cancer model, indicating a role for TCF1 in tumor regulatory response.
Overall, while these studies are informative of the role of TCF1 in Treg cell function, they remain more phenotypic than mechanistic. As such, this study would benefit from deeper probing of these pathways in the phenotype of the TCF1-deficeint Treg cells to be able to better reflect on the role pf TCF1 in Treg cell biology.

Major comments:
- The authors claim that TCF7 plays his role by regulating TGFb, Wnt/Bcat and TH17 signaling however, their results didn't show the implication of those pathways. They should employ approaches such as Treg cell-specific deletion of a floxed beta catenin (B6(Cg)-Ctnnb1tm1Knw/J), Treg cell-specific expression of a previously described gain of function Ctnnb1ΔEx3, and/or Treg cell-specific deletion of TGFb receptors to recapitulate or rescue the phenotype. The authors should also decipher and explain how TCF7 regulate those pathways? For example, it is a direct transcriptional regulation?

- On the first part of this manuscript, the authors use single cell RNA seq analysis to reveal the impact of TCF7 deletion on Tregs subsets, however they didn't link this to the TCF7 expression on these different subsets. Does TCF7 (TCF1) specifically regulate the development of these subsets? What is the role of TCF7 on these subsets?

- Finally, the authors use a specific cancer model, however an important question is to know if this is a common mechanism for different cancer and also the role of TCF7 on this model should be decipher further: what is the role Wnt/Bcat on this model? What is the role of the different subsets describe in first part of the manuscript?

Minor comments:
- The authors represent p-Stat3 in the figures2, however they write P-Stat5 in the manuscript.
- The authors also define CXCR2 and CXCR4 as chemokine, however, they are Chemokine receptors.
- The also use sometimes Treg instead of TregS, and make several typos including cluser instead of Cluster, classificataions instead of classification, ecodes instead of encodes, exeptional instead of exeptional. Aand instead of and etc.
- The gating strategy in figure6i, seems that you cut a part of the populations.
- Several references are used twice.
- Figure legend 4: inversion between F and E
- The scale of the heatmap in figure1c (row min, row max) is not appropriate to appreciate the different between the different gene.
- In figure 2, they authors should represent a representative dot plot for the mLN Tregs for each population.

- The authors should represent level de TCF1 in the different subsets in Figure 3/4. Moreover, they didn't find or reveal Wnt/Bcat pathways on these pathway analysis. The authors should reconcile the results of figue1 and figure3/4.

- The gating strategy on figure 5c and f for the proliferating cells in confusing because the authors gates on the non-proliferating cells and also the gating includes Half of the cells that did one division.
- The text on the manuscript, on the figures and on the figure's legend are confusing. It's difficult to know what they did in figure6.
- Also, in figure 6, they are missing legend on the dot plot and fig6 K they represent CD4+ Ing+ IL17+ with a percentage around 40-60 however in the dot plot the percentage is around 8-15%.
-
- In figure S5, they authors should revealed or show the impact of the APC468 mutation on TCF1 expression on the different subsets.
-

Reviewer #3:
Remarks to the Author:
The manuscript of Osman et al investigates the role of TCF1 in regulating Tregs functions both under homeostatic conditions and under pathological experimental settings. The topic is original and highly significant in the field.
Authors performed a comprehensive set of experiments using an ad hoc generated mouse where TCF1 has been selectively ablated only in Foxp3 Tregs. Authors initially perform RNAseq to extensively characterize the phenotype of Treg subsets in the presence or absence of TCF1. Then, they evaluated the consequences of TCF1 ablation in different pathological conditions, including viral infections, Th1 and Th17 in vitro and in vivo polarization, as well as during tumor development.
The authors conclude that TCF1 ablation in Treg cells may have different functional outcomes according to the disease condition, i.e. during infections TCF1 deficient Tregs enhance their suppressive function towards cytotoxic CD8+T cells, while fail to suppress Th1 and Th17 polarization, and promote inflammation and polyps growth in a model of intestinal tumor progression.

The scientific question is of great interest, and the various experimental in vitro and in vivo models are relevant to address it and convincingly elucidate the multifaceted functions of TCF1 in Treg physiology. Statistics is solid, conclusions are appropriate for the data generated and the references are adequate to contextualise their findings. The manuscript is very well written rendering the story easy to follow.

Nevertheless, few clarifications are required to improve the manuscript. For instance, two main points should be better addressed:
i) Ablation of TCF1 in Treg cells poises them to acquire Th17-like features. Consistently, authors perform experiments to evaluate Treg functions in those conditions where they control or promote Th17-mediated pathologies progression. Nonetheless, both Th17 models proposed in the manuscript occur in the intestinal compartment, ie the small bowel for the Th17 polarization in vivo and the small bowel/colon for the polyposis.
It is however unclear if and how the microbiota might contribute in the induction or

support of this functional transition.
Given the key role of the microbiota in shaping Th17/Treg cell functions in the intestinal compartment, authors should evaluate how the modulation of the gut microbiota might influence the functional properties of TCF1-deficient and sufficient Treg cells.
Moreover, authors should evaluate the TCF1-dependent Treg-Th17 axis in different models of Th17-mediated pathologies which are (less) dependent on microbiota influence, or at least that are not occurring in the gut.
ii) While authors suggest that modulation of TCF1 activity might be utlised in the future as a switch for Treg functions for human pathologies, including cancer, they do not provide any clue if cells with similar phenotypes (without mentioning functions) are present in humans and if modulation of TCF1 in human Treg cells might truly enhance or blunt their suppressive functions.

Author Rebuttal to Initial comments

Point-by-point response

Reviewer #1

This is an interesting manuscript that investigates the role of TCF1 in Treg identity and function. Using TCF1-depleted mice, the authors show that TCF1-deficient Tregs maintain their core Treg gene expression signature but display diverse functional properties. They present data from ex vivo and in vitro assays – including scRNA-seq analyses - suggesting that Tregs lacking TCF1 are more potent in suppressing the viral antigen-specific CD8+ T cell cytotoxicity and T cell proliferation but are compromised in their ability to hinder the polarization of CD4+ T cells toward the pro-inflammatory Th1 and Th17 lineages. In a tumor model of polyposis, TCF1-depleted mice have a higher incidence of colon polyps and pre-invasive colon tumors.

The authors use diverse experimental approaches to support their findings and to assess the mechanisms for the enhanced suppressive activity of TCF1-deficient Tregs during viral infection. They subsequently show that Apc (delta468) Foxp3creTcffl/fl mice have a high incidence of pre-invasive colon tumors. However, the functional properties of TCF1-deficient Tregs in the context of this tumor model are not investigated. Furthermore, what is the single-cell transcriptional profile of Treg populations in the TCF1 defective Apc (delta468) Foxp3creTcf$^{fl/fl}$ mice? Does the adoptive transfer of Foxp3cre Tregs rescue the phenotype?

The manuscript could also benefit from a parallel investigation of the TCF1-mediated regulation of Treg phenotype and function in humans, for instance interrogating public scRNA-seq data for human colon cancer.

We thank the reviewer for positive and insightful comments. We agree with the reviewer that the environment of the tumor bearing mice could potentially change the properties of TCF1 deficient T_{reg}s, for example through TLR activation (PMID 25790134) and/or Wnt mediate induction of β-catenin signaling (PMID 33664518). These are interesting and important considerations that we have in part addressed by

examining WT Tregs in mice with polyposis (**Fig S6**). We are very interested and are investigating these mechanisms in detail, however feel that they are outside the scope of our current report which focuses on the role of TCF1 in Tregs.

Earlier reports from our lab (PMID: 19570783, 25855122) and others (PMID: 15899788) have shown that adoptive transfer of T_{reg}s from healthy mice (but not from mice with polyposis, or T_{reg}s with IL10 deficiency) to mice with polyposis or ablation of RORγt from Tregs in mice with polyposis rescues T_{reg} functions and attenuates polyposis. We have now highlighted with yellow this observation along with its citation in the third paragraph of the introduction.

The reviewer raises an important point regarding the parallel investigation of Tregs in human CRCs. As suggested by the reviewer, we have now analyzed scRNA-seq data from human colon cancers (please refer to point #1 response to editorial comment). In brief, we show that TCF1 is significantly downregulated in tumor infiltrating Tregs compared to adjacent normal tissue control Tregs. Moreover, these infiltrating Tregs gain of T_H17 associated characteristics, consistent with our observation in mice that loss of TCF1 (or it's downregulation) is beneficial for the tumor and detrimental to the host. The results of these analysis are included in **new Figure 8**.

Minor comments:
- Annotation of the velocity maps in Fig. 4g with the major clusters of interest would allow a better visualization of the intercluster relations.

We apologize for missing this. We have now annotated each cluster with a number that is consistent through the manuscript.

- Fig. S5: Include a graph that shows the differences in the expression of Maf.
Done. This is now included in new **Figure 4cd**.

- The manuscript is in need of extensive proofreading, as indicated by some examples below:

We apologize for the oversights.

'MIF is the receptor for CXCR2 and CXCR4…': MIF is a soluble cytokine not a receptor..

It is now corrected.

'Of note, CXCR4 is a key chemokine for attracting…': CXCR4 is a chemokine receptor.

It is now corrected.

'…in naïve mice mice that had not been vaccinated': Presumably the authors refer to 'infected' as opposed to 'vaccinated' mice.

It is now corrected.

Some other typos throughout the text:
'classificataions', 'aand', 'ecodes', 'unplulsed', 'similarities conserved intercluster relations', 'signaling signaling', 'expresioon', 'associataed'
It is now corrected.

Figure legend 4: inversion between F and E

It is now corrected. Annotations in Figure 4 now match the Figure Legend.

Reviewer #2:

This study examined the role of Treg cell functional regulation by TCF1. The authors revealed that TCF1 does not play a role in Treg core transcriptional signature but regulates the TGFβ receptor, Th17 and Wnt/Bcat signaling pathways. They also showed that TCF1 expression level plays a role in the control of Tregs plasticity. Moreover, the study also reveals that the deficiency of TCF1 in Tregs induces enhanced suppression by Treg cells of CD4/CD8 T cell Proliferation, but at the same time this deletion promotes inflammatory cytokine production by CD4/CD8 T cells. The proneness of TCF1-deficient Treg cells to inflammatory skewing is associated with a poorer outcome in a colorectal cancer model, indicating a role for TCF1 in tumor regulatory response.

Overall, while these studies are informative of the role of TCF1 in Treg cell function, they remain more phenotypic than mechanistic. As such, this study would benefit from deeper probing of these pathways in the phenotype of the TCF1-deficeint Treg cells to be able to better reflect on the role pf TCF1 in Treg cell biology.

We thank the reviewer for the succinct summary of our study and insightful feedback. We have now provided a more extensive assessment of these pathways. Specifically, we have now included TCF1 ChIP-seq data in Tregs and associated it with gene expression data at bulk and single cell levels and additionally probed these pathways in human colorectal cancers (please refer to the response to editorial comments). Below are point by point responses to specific comments.

Major comments:

- The authors claim that TCF7 plays his role by regulating TGFb, Wnt/Bcat and TH17 signaling however, their results didn't show the implication of those pathways. They should employ approaches such as Treg cell-specific deletion of a floxed beta catenin (B6(Cg)-Ctnnb1tm1Knw/J), Treg cell-specific expression of a previously described gain of function Ctnnb1ΔEx3, and/or Treg cell-specific deletion of TGFb receptors

to recapitulate or rescue the phenotype. The authors should also decipher and explain how TCF7 regulate those pathways? For example, it is a direct transcriptional regulation?

In our original manuscript, we show significant changes in TGFb signaling at the transcription and translation levels, by RNAseq and FACS analysis. We demonstrate the implication of this pathway by showing that a specific TGFbR inhibitor blocks Treg suppression of CD8 T-cell response to viral (TMEV) infection. Additionally, we have now included extensive analyses of TCF1 ChIP-Seq in Tregs and its correlation with TCF1 mediated changes in gene expression (please refer to our response to editorial comments). These analyses indicate that the probed pathways are in part directly transcriptionally regulated by TCF1.

We have also addressed some the questions raised by the reviewer in our now-published manuscript (PMID: 33664518), which was under-revision when we originally submitted the current manuscript. The published report documents that overexpression of β-catenin in Tregs upregulates RORγT and renders the Tregs pro-inflammatory. We have now referred to these findings in the third paragraph of the introduction and discussed this in the revised manuscript. Much more [REDACTED].

- On the first part of this manuscript, the authors use single cell RNA seq analysis to reveal the impact of TCF7 deletion on Tregs subsets, however they didn't link this to the TCF7 expression on these different subsets. Does TCF7 (TCF1) specifically regulate the development of these subsets? What is the role of TCF7 on these subsets?

This is an important question. We have probed the role of TCF1 in the development of individual T_{reg} subsets by comparing scRNAseq data across different mice (Foxp3Cre, Foxp3Cre TCF-$1^{fl/fl}$, Polyposis, WT) using differential gene/pathway/trajectory analyses (**Fig. 4 b,c & SFig 3b**).

To better answer the role of TCF1 in each T_{reg} subset, we have now compared the differential gene expression signature of each subset of TCF-1 deficient or sufficient T_{reg}s with ChIP-seq data showing binding of TCF1 to gene regulatory regions, as presented in new **Figure 4 d-f and 4g** (please also refer to our response to editorial comments above).

- Finally, the authors use a specific cancer model, however an important question is to know if this is a common mechanism for different cancer and also the role of TCF7 on this model should be decipher further: what is the role Wnt/Bcat on this model? What is the role of the different subsets describe in first part of the manuscript?

We agree that TCF1 is highly likely to modulate T_{reg} responses in other pathologies, including other cancers (PMID 26047578), autoimmune diseases (PMID 30374130), and chronic infections (PMID **21435589**). These studies are certainly important and need to be done. We hope that our current manuscript will provide the rationale for further investigations as suggested by the reviewer. The role of β-catenin was addressed in our recently accepted manuscript (PMID: 33664518). We have also directly analyzed Tregs from human

colorectal cancers showing the downregulation of TCF1 and enhancement of T_H17 characteristics in tumor infiltrating Tregs (**new Figure 8**) (please also refer to our response to editorial comments above).

Minor comments:
- The authors represent p-Stat3 in the figures2, however they write P-Stat5 in the manuscript.

Apologies for the unintended error, this is now corrected as it should have been pSTAT5. STAT3 is however also a target of TCF1 and transcriptionally activated upon TCF1 deletion.

- The authors also define CXCR2 and CXCR4 as chemokine, however, they are Chemokine receptors.

Apologies for the unintended error, this is now corrected.

- The also use sometimes Treg instead of TregS, and make several typos including cluser instead of Cluster, classificataions instead of classification, ecodes instead of encodes, exeptional instead of exeptional. Aand instead of and etc.

Apologies for the typos. These are now corrected.

- The gating strategy in figure6i, seems that you cut a part of the populations.

We have now used a wider gate.

- Several references are used twice.

Now Corrected.

- Figure legend 4: inversion between F and E

Annotations in Figure 4 now match the Figure Legend.

- The scale of the heatmap in figure1c (row min, row max) is not appropriate to appreciate the different between the

different gene.

We apologize for missing the citation to the raw data in the supplemental table. This visualization is common (and default) for GSEA type analyses.

- In figure 2, they authors should represent a representative dot plot for the mLN Tregs for each population.

Representative dot plots are now in new **Figure S2**.

- The authors should represent level de TCF1 in the different subsets in Figure 3/4. Moreover, they didn't find or reveal Wnt/Bcat pathways on these pathway analyses. The authors should reconcile the results of figue1 and figure3/4.

We apologize for missing to include this information. Levels of Tcf7 expressions are shown in new **Figures S3b and S6b**. We have also added analysis of TCF1 ChIP-seq data and its connection to both bulk and single cell data that further reconcile figue1 and figure3/4.

Wnt pathway was not detectable in our single cell data. We suspect that this is due to the limit of 10X single cell RNAseq in detecting low expressed transcripts, such as nuclear factors. Current pathway analysis methods have much better statistical power when using bulk RNA-seq. Thus, some of the pathways easily detected by bulk RNA-seq may not be detected in single cell data.

- The gating strategy on figure 5e and f for the proliferating cells in confusing because the authors gates on the non-proliferating cells and also the gating includes Half of the cells that did one division.

We now show gating for dividing cells, in the **new Figure 5f**.

- The text on the manuscript, on the figures and on the figure's legend are confusing. It's difficult to know what they did in figure6.

This is now improved and highlighted in yellow.

- Also, in figure 6, they are missing legend on the dot plot and fig6 K they represent CD4+ Ing+ IL17+ with a percentage around 40-60 however in the dot plot the percentage is around 8-15%.

This is now corrected.

- In figure S5, they authors should revealed or show the impact of the APC468 mutation on TCF1 expression on the different subsets.

Levels of Tcf7 expressions are shown in new **Figure S1b and S6b.**

Reviewer #3:

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Authors performed a comprehensive set of experiments using an ad hoc generated mouse where TCF1 has been selectively ablated only in Foxp3 Tregs. Authors initially perform RNAseq to extensively characterize the phenotype of Treg subsets in the presence or absence of TCF1. Then, they evaluated the consequences

of TCF1 ablation in different pathological conditions, including viral infections, Th1 and Th17 in vitro and in vivo polarization, as well as during tumor development.

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It is however unclear if and how the microbiota might contribute in the induction or support of this functional transition.
Given the key role of the microbiota in shaping Th17/Treg cell functions in the intestinal compartment, authors should evaluate how the modulation of the gut microbiota might influence the functional properties of TCF1-deficient and sufficient Treg cells.

Moreover, authors should evaluate the TCF1-dependent Treg-Th17 axis in different models of Th17-mediated pathologies which are (less) dependent on microbiota influence, or at least that are not occurring in the gut.
ii) While authors suggest that modulation of TCF1 activity might be utlised in the future as a switch for Treg functions for human pathologies, including cancer, they do not provide any clue if cells with similar phenotypes (without mentioning functions) are present in humans and if modulation of TCF1 in human Treg cells might truly enhance or blunt their suppressive functions.

We thank the reviewer for enthusiasm and critical comments. We agree with the reviewer that the connection with microbiota is very interesting. Indeed, RORγT$^+$ T$_{reg}$s are induced through interaction of CD4$^+$ T cells with gut microbiota. Interestingly, [REDACTED] We expect tumor growth to have a major role in altering the properties of microbiota induced Tregs. This is [REDACTED]. Nevertheless, we have now included a paragraph on microbiota in the Discussion section.

Regarding the relevance of our finding to human CRC, we have sourced the existing single cell RNA-sequencing dataset from treatment-naïve colorectal cancer patients (PMID: 31341169) and integrated the results into our manuscript (**new Figure 8**). Briefly, we found that TCF1 is downregulated in tumor infiltrating Tregs of CRC patients, when compared to Tregs from adjacent normal tissues or PBMC (please also see our response to editorial comments above).

In summary, in response to the insightful comments of the reviewers, we have performed a number of additional analyses that support and extend our original conclusions. We thank the reviewers for their careful reviews and comments.

Decision Letter, first revision:

Subject: Your manuscript, NI-A31353A

Message: Our ref: NI-A31353A

1st Jun 2021

Dear Dr. Khazaie,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "TCF1 controls Treg functions that regulate inflammation, CD8 T-cell cytotoxicity, and severity of colon cancer" (NI-A31353A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments and please make sure to upload your checklist.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "TCF1 controls Treg functions that regulate inflammation, CD8 T-cell cytotoxicity, and severity of colon cancer". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Immunology offers a Transparent Peer Review option for new original research

manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

Cover suggestions

As you prepare your final files we encourage you to consider whether you have any images or illustrations that may be appropriate for use on the cover of Nature Immunology.

Covers should be both aesthetically appealing and scientifically relevant, and should be supplied at the best quality available. Due to the prominence of these images, we do not generally select images featuring faces, children, text, graphs, schematic drawings, or collages on our covers.

We accept TIFF, JPEG, PNG or PSD file formats (a layered PSD file would be ideal), and the image should be at least 300ppi resolution (preferably 600-1200 ppi), in CMYK colour mode.

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Best regards,

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On behalf of

Laurie A. Dempsey, Ph.D.
Senior Editor
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ORCID: 0000-0002-3304-796X

Reviewer #1:
Remarks to the Author:
The authors have made significant efforts to improve the study and have addressed my concerns.

Reviewer #2:
Remarks to the Author:
I thank the authors for addressing all of my comments. The authors have fully answered my concerns, and as such I have no further issues with the manuscript.

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A31353B

Message: In reply please quote: NI-A31353B

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Laurie

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