natureresearch

Peer Review Information

Journal: Nature Immunology
Manuscript Title: Type I Interferon Transcriptional Network Regulates Expression of Coinhibitory Receptors in Human T cells
Corresponding author name(s): Tomokazu S. Sumida, Asaf Madi

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Redactions – unpublished data	Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.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-A31326
Message: 30th Dec 2020

Dear David (and Vijay),
Apologies for the delay getting back to you - the holidays, short staffing and an inundation of COVID submissions has inevitably slowed things.

Thank you for submitting your manuscript to NI and including the responses to the three Referees- this was helpful. The topic is of potential interest so in principle we'd be willing to consider the manuscript for publication, however it would need to be (re-)reviewed which brings me to my next point. Since you didn't use the transfer link in the Nature decision letter we don't have the identity of the Referees or their confidential comments to the editors. Having this information can greatly assist with our decision making and moreover our knowing the Ref. identities would mean we could go back to them (generally the preferred option) or avoid them if they indicate outright rejection or are very negative about the data for whatever reason. I can request this material from Nature but I need the corresponding author to 'OK' this. So if you're happy for me to approach Nature for this please send me an email indicating as such. Note, if we don't go this route the only option is an entirely fresh round(s) of review with (likely) different Referees. The choice is yours.

A couple of quick observations re. your revision. You seem willing to do most of the requests which is good/appreciated. Most important of these I feel is the further

validation of perturbing STAT3/SP140 etc. Both protein and functional validations would be helpful here. You've declined to assess the receptor activity (Ref. 2 comment) though this would seem to be relatively straightforward(?)- especially if you've acceded to do the killing assays. Further showing the effects on more than one donor would be important I feel especially since at least 2 of the Refs. raise this issue.

Finally, I also have a more general/philosophical question which is raised by your findings. Given the differential effects of IFN on TIGIT vs. the other checkpoint molecules what's this telling us about their role physiologically? Seems an odd relationship that's surely telling us something - I for one would welcome a few insights here. Note, I'm not asking you to try to address this experimentally.

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 we go back to the original Refs. at Nature.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions (i.e. NI) 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.

naturereseach

You may use the link below to submit your revised manuscript and related files:
[REDACTED]

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.

Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

The Macmillan Building
4 Crinan Street
Tel: 212-726-9207
Fax: 212-696-9752
z.fehervari@nature.com

Author Rebuttal to Initial comments

Point-by-point responses to the reviewers' comments

Decision on Nature submission 2020-10-19559A

Referees' comments:

Referee #1 (Remarks to the Author):

Type 1 interferon (IFN-I) is known to modulate T cell immunity in viral infection, autoimmunity, and cancer, and has been implicated in the induction of T cell exhaustion in chronic viral infection. In this study Sumida et al describe a gene regulatory network activated in human CD8 and CD4 T cells in response to IFN-I. Using in vitro activated T cells stimulated in the presence or absence of IFN-I and a time course of RNAseq they observe three waves of transcriptional regulation that lead to the induction of inhibitory receptors and effector molecules. The authors show that IFN-I regulates co-inhibitory receptor expression on human T cells, inducing PD-1/TIM-3/LAG-3 while inhibiting TIGIT expression. The authors use perturbation of key transcription factors on human primary T cells to reveal both canonical and non-canonical IFN-I transcriptional regulators, and identify regulators that possibly control expression of co-inhibitory receptors. In the second part of the study the authors use their previously published single cell RNA-sequencing data from blood cells of subjects infected with SARS-CoV-2 and find that viral load was strongly associated with T cell IFN-I signatures. They found that the dynamic IFN-I response in vitro closely mirrored T cell features with acute IFN-I linked viral infection. Finally, the authors propose that Stat3 is a positive regulator of TIM3 and SP140 is a key regulator for differential LAG3 and TIGIT expression and suggest that transcription factors controlling the expression IFN-1 responsive genes may provide targets for improved immunotherapy.

Major issues

1. The study completely relies on an RNA sequencing to reconstruct relationships between transcription factors and their putative products. This is not sufficient. Although human T cells cannot be as readily manipulated as mouse T cells, they are just as accessible to molecular methods such as ATACseq and ChIPseq. Thus, the proposed relationships between regulators and their target genes should be examined directly rather than being deduced solely on RNAseq data.

Response: While we appreciate the reviewer's suggestions that ATAC-seq and ChIP-seq may theoretically be useful to understand upstream regulation of gene expression, there are several technical issues that preclude this approach. First, it is not feasible to perform multiple transcription factor (TF) ChIP-seq at multiple timepoints with human primary CD4$^+$ naïve T cells or CD8$^+$ naïve T cells as this would require an amount of blood that is simply not possible to collect from a human subject. Second, while ATAC-seq is technically feasible and is in common use in our laboratory, the information provided by ATAC-seq is limited. For example, motif analysis may provide some clues to reveal upstream regulatory TFs, but those data will

not necessarily reflect the direct binding of TFs. Footprint analysis may provide more direct evidence of TF binding, but it requires over 10 times deeper sequencing and as such it is not cost efficient to perform ATAC-seq for all timepoints. However, we have taken these comments to heart and have generated a new gene regulatory network by taking into account the following data: 1) our high-temporal resolution expression data, and 2) publicly available Protein-DNA Binding database that includes ChIP-seq data and chromatin accessibility data[1]. We believe our computational analysis can, to a large degree, overcome this issue and would be a useful platform for the community to explore the complex system. We have thus performed further validation experiments and confirmed that the key IFN-I regulators (SP140, BCL3, and STAT3) indeed control co-inhibitory receptors (PD-1, LAG-3, TIM-3, and TIGIT) as we have shown in the original manuscript (now the validation data are in Supplementary Figure 8). While we can perform the proposed ATAC-seq experiments, we would suggest that it would not give us extensive advantage over our system biology approach and our fundamental observation that IFN-I regulate co-inhibitory receptor expression in humans is of general interest.

2. This study lacks any confirmation experiments. For example, there is no evidence for STAT3 regulating TIM3 or SP140 regulating Lag3 or TIGIT. However, the authors state: "Analysis of the intermediate wave gene regulatory network demonstrated that SP140 is a bi-directional regulator for LAG3 and TIGIT under IFN-I response" This is simply not the case! The proposed relationships are purely correlative. The manuscript is full of conclusions based solely on RNAseq data and computational reconstruction of a regulator network, such as this: "BCL3 and STAT3 are highlighted as validated positive regulators on LAG3 and HAVCR2 respectively (Figure 5k)". There is just no validation! The data is in no way sufficient. Another example: "Indeed, our perturbation experiment demonstrated the critical roles of those 'Bridging TFs' in the regulation of ISGs and co-inhibitory receptors (Figure 4c)." The data in Fig. 4c may suggest that but they demonstrate nothing.

Response: We agree with these excellent suggestions, and we thank the reviewer for the comments. As requested, we performed the validation experiments and the results verified our observations (Supplementary Figure 8).

3. Perturbation experiments, using shRNA knock-down of putative transcriptional regulators were done solely at the systems level using RNAseq as a readout. While this may be a starting point, it surely cannot be the end. For example, the authors conclude that Stat3 positively regulates the expression of Havcr2 (TIM3) (page 7). This may or may not be true. There is simply no experiment that test this notion. Not even FACS plots of the perturbation experiment are shown. There is no mechanistic insight how Stat3 might regulate TIM3 expression or insight as to how IFN-I might induce Stat3 signalling in the first place. Based on published data this reviewer would indeed argue that it is unlikely that Stat3 signalling induces Tim3.

Response: We agree with the reviewer. Our validation data at protein level confirmed the extensive RNA-seq data-based findings (Supplementary Figure 8).

With regard to STAT3 signaling, it is one of the most central pathways induced by IFN-I[2,3], and there are several studies suggesting the role of STAT3 in inducing TIM-3 expression in T cells[4,5]. Our validation experiment with STAT3 knockdown clearly demonstrated that STAT3 positively regulates TIM-3, but negatively on TIGIT (Supplementary Figure 8).

4. The perturbation experiments do not control for potential defects in proliferation, cell death or activation. Thus, any of the readouts could be impacted on by a multitude of factors.

Response: We thank the reviewer for pointing this out. This issue is linked with next critique and is discussed below.

5. The study lacks any insight into the complex functions of IFN-I. How is the positive role of IFN-I in T cell effector function integrated with its negative role in inducing inhibitory receptor expression? How is TCR signalling linked to IFN-I signalling? For example, PD-1 is well known to be induced directly by TCR signalling and its expression level is directly controlled by TCR signalling strength. How does IFN-I fir[fit] into this picture? There would have been ample opportunity to perform sophisticated in vitro analyses to really understand these relationships. However, this was not attempted.

Response: We thank the reviewer for these comments, and completely agree that IFN-I has a versatile role in T cell biology including regulation of co-inhibitory receptors, effector function, and activation. To control for the contribution of T cell activation and proliferation over IFN-I response, we have determined the effect of IFN-I on the other T cell co-inhibitory receptors by assessing T cell activation markers (CD25, CD69, CD44), which were suggested by the reviewers, and proliferation (cell violet proliferation dye staining) simultaneously. There was no significant impact on T cell activation and proliferation by IFN-I treatment (Supplementary Figure 3a-c). Of note, the induction of LAG-3, PD-1, and TIM-3 and the suppression of TIGIT by IFN-β were consistently observed across the different cellular division states, further validating the fact that the IFN-β mediated changes on co-inhibitory receptor expressions were not affected by T cell proliferation (Supplementary Figure 3d). Next, to exclude the possibility that the effect of gene knockdowns might be confounded by T cell activation and proliferation, we further assessed the impact of gene knockdowns of co-inhibitory receptors (LAG-3, TIM-3, PD-1) in parallel with T cell activation status (assessed by activation marker CD69) and proliferation. Again, the effects of shRNA mediated intervention of SP140, STAT3, and BCL3 on co-inhibitory receptors were observed at each stage of cellular division, specifically at highly

divided state (Supplementary Figure 8a). We also confirmed that the activation status of T cells assessed by CD69 was not changed at each division state under shRNA mediated knockdown for SP140, BCL3, and STAT3 (Supplementary Figure 8c). Taken together, IFN-I induced changes of co-inhibitory receptor expressions and shRNA-based perturbation were confounded by neither T cell activation nor proliferation. We have added these new findings to the manuscript.

Further concerns

1. The RNAseq data on which the whole study is build derives from T cells from just a single donor! Given the high degree of variability in the human population, this is not acceptable. It is not even acceptable in inbred mice. It is unclear to this reviewer why this approach was chosen? It is considered a major flaw by this reviewer.

Response: After having spent over a decade investigating how common genetic variations observed in autoimmunity have profound effects in biologic function, we would suggest that the only way a complex network can be elucidated due to issues of gene expression kinetics is to deeply examine one individual subject. This is routinely done with rodent models, followed by replication of key observations in a larger population cohort, and examination of protein expression as we have performed.

Specifically, deep replication in a single, genetically defined subject to construct regulatory network is necessary because of individual variation in gene expression *kinetics*. To construct an extensive regulatory network, it is critical to replicate gene expression kinetics data. We indeed performed RNA-seq with different subjects in an attempt to construct the network; however we failed due to the slight shift of gene expression kinetics among the subjects, though the overall gene expression patterns were similar to each other. We thus selected the individual who showed stable response to IFN-β stimulation and repeated three experiment every two weeks to construct the network. As we demonstrated in Figure 2b, we captured well-replicated transcriptional kinetics that allowed us to construct the gene regulatory network. Although there is a high degree of individual variation in human, we have already demonstrated that the impact of IFN-I on TIM-3, LAG-3, PD-1, and TIGIT expression is highly conserved across many individuals (Figure 1, Supplementary Figure 2). Nevertheless, our validation experiments with multiple subjects confirmed that the regulators we identified with our network play the consistent role beyond the individual variation.

2. The single cell RNAseq data based on ten COVID19 patients has been published before by the same group (ref 32). While this is not a major problem is[it] impacts novelty. Novelty is also impacted by the multiple studies published over the last 6 months that have shown a tight link between the IFN-I response and the severity of COVID-19. This is really not new and the authors do not provide further insight than showing another correlation. Furthermore, the analysis of the

patient data is superficial. There is no indication that IFN-I is increased in certain patients or reduced in others. Again, only correlations are shown.

Response: We never claimed that the IFN-I response observed in T cells in COVID-19 patients are a "novel" finding. Specifically, as is typical in human *in vitro* work, we were concerned that our observations would represent a test-tube observation without *in vivo* evidence. We were struck by our early observations that there was a strong correlation between SARS-CoV-2 viral load and the type I IFN signature in T cells. This prospectively led us to hypothesize that if our *in vitro* observations were correct, then we would see a similar transcriptional signature, and the fundamental observation dissociating PD-1/TIM-3/LAG-3 module with the TIGIT module, in COVID-19 patients. In a rather remarkable series of experiments, the *in vitro* findings, again observed from one subject, were almost precisely confirmed *in vivo*. It is important to note that the paper referred to by the reviewer has yet to publish or accepted for publication.

To be more specific, we wanted to explore with this scRNA-seq data the correlation between ISG and co-inhibitory receptors. We found that some co-inhibitory receptors (*LAG3, PDCD1, HAVCR2*) showed positive correlation with ISGs but some other co-inhibitory receptors (*CD160, TIGIT, BTLA, LAIR1*) showed negative correlation with ISGs (Figure 5e). This pattern was also observed in an *in vitro* human T cell experiment with IFN-β treatment, suggesting our *in vitro* culture model can be compatible with *in vivo* conditions for SARS-CoV-2 infection in humans. Co-inhibitory receptors are expressed together as a module in exhausted T cells in cancer or chronic infection (i.e., PD1, TIM-3 and TIGIT), however, we demonstrated that the co-inhibitory module in T cells from COVID-19 patients are different. This fact prompted us to integrate *in vitro* gene regulatory network data into *in vivo* data, and we demonstrated enrichment of *in vitro* IFN-I response signature in a "Dividing T cells" cluster in acute SARS-CoV-2 infection (Figure 5h). Given that some studies reported stronger IFN-I responses is not necessarily associated with severe disease[6,7], we believe that our rich data and high quality IFN-I response gene regulatory network, which highlights the roles of non-canonical regulators overshadowed by conventional JAK-STAT regulators, are novel and of interest to the field.

3. Some of the conclusions or premises don't seem to be useful: "Given that expansion of dividing CD4+/CD8+ T cells are a unique characteristic of COVID-19 patients …" Surely, dividing T cells are a characteristic of any infection!

Response: We thank the reviewer for the comment. We have revised the description as "*Given that expansion of dividing CD4+/CD8+ T cells are a unique characteristic of acute viral infection, including COVID-19 patients …".* (page 11, line 250)

Referee #2 (Remarks to the Author):

This is a well written paper highlighting transcriptional regulatory network in driven by IFN to regulate checkpoint expression in human T cells. High resolution mapping of these networks over time course of T cell activation combined with other data sets (chip seq, in vitro TF perturbation) provides new insights into the key TFs and regulatory network that establish the interferon signature. Correlating these networks with in vivo COVID-19 data (while not novel) is a supports the validity of these findings, albeit other mediators could certainly be involved.

The paper's main finding, while exciting from a transcriptional regulatory network standpoint, falls short of having high impact. The functional consequences of these regulatory networks are not explored, either in vitro or indirectly in the COVID-19 data (e.g. correlation with outcomes).

Prior papers suggest type I interferon regulates expression on co-inhibitory receptors such as Lag-3 and TIGIT, albeit using difference metrics and technologies. The authors have also written a prior review on this general topic and therefore the concept has already been presented. http://dx.doi.org/10.1016/j.immuni.2016.05.001.

Response: In our review paper that is cited by the reviewer, we discussed IFN-γ (type 2 IFN) production and co-inhibitory receptors but barely touched upon the topic related to effect of type 1 IFN on co-inhibitory receptors. Only our study showing a defect in T cell TIM-3 expression in patients with MS that is referenced in the paper[8] was a clinical paper that showed treatment with either Copaxone or Avonex corrected the defect in TIM-3 expression. In one *in vitro* experiment, it was shown that Copaxone or IFN-β increased mRNA expression of TIM-3 in PBMC but not with purified CD4$^+$ T cells. Moreover, the study did not investigate the key co-inhibitory receptors PD-1, TIGIT, and LAG-3. In retrospect, those data in patients suggest a further human *in vivo* validation of our data, and we added that point to the discussion. Thus, this bidirectional regulation of co-inhibitory receptors by IFN-I is novel especially in human T cell field, and we believe this broader picture of co-inhibitory receptor regulation would be a valuable finding to the community.

While changes in the expression of some co-inhibitory molecules is striking, expression changes in others is less pronounced. Can the authors show that the function response of IFNb treated T cells are more susceptible to inhibition by the predicted counter ligands?

Response: Although we could argue that this request is not within the scope of our paper, per request by the reviewer, we performed the suggested experiment. We stimulated primary CD8$^+$ T cells with and without IFN-β under PD-L1 or PD-L2 and analyzed the production of Granzyme B and IFN-γ, which are well established functional cytotoxic cytokines in the context of viral

infection[8]. We observed the suppression of granzyme B production by ligation of PD-1, albeit IFN-β treatment didn't further facilitate this inhibition compared to control condition (Figure for Reviewer REDACTED). Nevertheless, T cell functional properties are controlled by complex combination of co-stimulatory and co-inhibitory receptor signals, which cannot be fully assessed with this limited *in vitro* experimental setting. T cell killing function in the context of viral infection should be better assessed by using the system where antigen specific T cell function can be determined *in vivo*, thus further studies with viral infection models are needed to answer this question. Again, this is not our focus of this study and we believe this should be investigated in future research.

[REDACTED FIGURE AND LEGEND]

It is concerning that the transcriptional profile of IFN-β response was based on data from only 1 individual. The authors state "To avoid inter-individual variation, we selected one healthy subject whose T cells exhibited a stable response to IFN-β, and repeated the experiment three times at a two-week interval for each experiment"…

How can that 1 person be representative if it is "normal" for there to be variations in responses from healthy controls?

Response: This concern that was raised by Reviewer #1 under "Further concerns", point #1.

Methods used are not clear, particularly for the lentiviral shRNA work. Computational methods are also very poorly written making it hard to follow some of the findings. Methods section needs more elaborate descriptions of the statistical/computational analyses.

Response: As suggested, we revised the Methods section with more detailed descriptions.

It would have been preferred to look at IFNβ responses in patients receiving Avonex as part of treatment for disease. Of course, there are many mediators that could be instrumental in altering T cell responses in COVID.

Response: Excellent point. We realized that we had performed this experiment[8]. It's discussed in the revised manuscript (page 14, line 321-325).

The observation of 2 potential interferon regulatory modules is interesting (Figures 3D-F), but little insights are provided on the potential biological relevance of these modules.

Response: To provide insights on the potential biological relevance of these modules, we have discussed the perturbation experiment that showed the bi-directional regulation on ISGs and co-inhibitory receptors (Figure 3d-f, Supplementary Figure 5b, c). We also report our investigation of T cell subsets where these two modules were differentially enriched in COVID-19 patients. The 'IFN-I regulator module 1' was more enriched in dividing T cells and effector T cells in both CD4^{+} and CD8^{+} T cell subsets as compared to 'IFN-I regulator module 2' (Supplementary Figure 7d). These data suggest that 'IFN-I regulator module 1' plays a pivotal role in those cell types which have stronger type 1 IFN response signature and higher expression of LAG-3, TIM-3, PD-1 but lower TIGIT in COVID-19 (Figure 5f-h). We discussed these observations and insights in the revised manuscript (page 11 line 240-243).

The role of inhibitory receptors in the interferon TF modules is a little bit lost as the figures progress. Three inhibitory receptors and their relations to the interferon modules are indeed shown in Figure 4[5]I and 4[5]K as well as some gene expression, but the current results do not suggest a solid general regulatory program of inhibitory receptor expression by type I interferons. It is just shown for these 3 receptors.

Response: We have shown the co-inhibitory receptor expression data for each cell type in COVID-19 T cells in Figure 5c and the correlation with ISGs in Figure 5e. We also demonstrated how these TF modules regulate co-inhibitory receptors in the biplot (Figure 3f) and the heatmap (Supplementary Figure 4c) where BTLA and CD160 were highlighted in addition to PD-1, LAG-3, TIM-3, and TIGIT. To validate the impact of type 1 IFN on BTLA and CD160, we performed flow cytometry analysis and confirmed that BTLA and CD160 were downregulated by IFN-β treatment as well as TIGIT (Supplementary Figure 2d) which is regulated by 'IFN-I regulator module1' in opposite directions from LAG-3, PD-1, TIM-3 (Figure 3f, Supplementary Figure 5c). These results support that our proposal that regulator modules control co-inhibitory receptors is solid.

Figure 1 has some repetitiveness.. why do we need both histograms and dot plots of the same data? – is it the same? Cut the histograms? Why not show dot plots for TIM/LAG3, TIM/PD1, TIM/TIGIT, and then combine the graphs for A, B, D.

Response: We have revised Figure 1 as recommended.

Figure 2, part B to supplemental data.

Response: We think Figure 2b is informative as it captures the correlation patterns across the time points, and so we would like to keep this figure as is.

Referee #3 (Remarks to the Author):

Summary of the key results:

In this manuscript, Sumida, Dulberg and Schupp et al. set out to define the transcriptional consequences of type I interferon sensing on CD8 T cell activation and its effects on the expression of co-inhibitory receptors such as PD-1, LAG-3 and TIM-3. They demonstrate that human T cells stimulated in vitro in the presence of IFNb express higher levels of PD-1, LAG-3 and TIM-3 but lower levels of TIGIT. They also find effector molecules such as IFNg to be unregulated. They then perform an RNAseq time course over 96 hours on human T cells stimulated with or without IFNb in culture. They find 3 major waves of transcriptional activation over time and show that many important T cell genes, including many key transcription factors, are differentially regulated by exposure to IFNb over time. They then use shRNA to knock down expression of 21 total TFs and use RNAseq of each TF knockdown to understand the impact that each TF is having on the response to IFNb. They construct a network model using their data and highlight the important TFs acting in the network within and between each transcriptional wave. Finally, to assess the utility of their network model the authors analyze scRNAseq data of CD4 and CD8 T cells from patients with SARS-CoV2 infection to determine if the transcriptional responses observed in response to IFNb in vitro are preserved in patients with Covid-19. They find enrichment for their in vitro stimulation-derived transcriptional signatures and then propose SP140, STAT3 and BCL3 as TFs that differentially modulate inhibitory receptor expression.

Major Comments:

The authors correctly identify type I interferon as an important modulator of T cell differentiation and function, and the data presented here represent the most comprehensive profiling of the transcriptional response of human T cells to type I interferon to date. The experiments are well-executed and the data are high quality. Indeed, the RNAseq profiling of human T cell activation following knockdown of 21 key IFN-regulated TFs is an extremely useful dataset for the community.

However, despite the large amount of high quality data, the manuscript in its current form lacks to some degree in originality, fails to acknowledge or address previous data, and the significance of the findings for the field are not clear. The lack of clear and high-impact significance for the findings makes the current manuscript a better fit for high quality immunology-specific journals.

We appreciate that the reviewer evaluated our work as a well-constructed and supported by high-quality data. We will address each point raised by reviewer.

The effects of type I interferon on T cells have been extensively studies previously, both with regard to T cell activation (PMID: 15814665), survival (PMID: 9927514), effector function (PMID: 16585561), and even the expression of PD-1 (PMID: 21263073). The vast majority of these findings were not discussed and instead the authors focused on the observation that IFN blockade restores immune function in chronic viral infections in mouse models of LCMV and HIV. Importantly, work by the Honjo lab has previously demonstrated a direct role for the ISGF3 complex in driving the expression of PD-1 after IFNa treatment (PMID: 21263073) and the study that the authors cite showing that blockade of type I IFN in a mouse model of HIV improves T cell function (PMID: 27941243) demonstrated that PD-1 and TIM-3 expression were reduced on T cells in vivo following blockade of IFN. Thus, the manuscript is lacking in originality and impact and the authors could have done more to highlight previous work in this space and discuss how their work supports, refutes, or extends beyond previous studies.

Response: We thank the reviewer for the constructive and helpful comments, and agree with the comment that previous work, in particular Honjo's, has shown that IFN-I can induce PD-1 in mice, which is now discussed in revised manuscript (page 13, line290-295). As related to this point, our previous work in mice showed that IL-27 is the major factor that induces the PD-1/TIM-3/LAG-3 and TIGIT module and moreover, IL-27 acted differently in human T cells as compared to murine T cells (Supplementary Figure 2). Thus, and given that the response to IFN-I varies between mouse and human[9], it was imperative to thoroughly investigate human T cells and proteins to complement our previous work in rodents. Furthermore, our study not only focused on a single co-inhibitory receptor but examined how multiple co-inhibitory receptors are regulated by IFN-I using a system immunology approach which has not been applied in human T cells. These aspects contribute the originality of our work. Finally, we have added further discussion about the previous findings about IFN-I and co-inhibitory molecules and highlighted the importance of our approach and findings in the revised manuscript.

Further, there are some conceptual flaws to the work that raise questions about the relevance of these data for the inhibitory role of type I IFN in chronic viral infection and potentially also cancer. It would definitely be an interesting study to investigate the precise mechanisms by which T cell-specific IFN sensing contribute to immune suppression during chronic infection, and this reviewer agrees that despite some evidence that inhibitory receptor expression is reduced following IFN blockade, the current evidence is only correlative evidence and not direct. However, the experiment the authors did seems not well-suited to addressing this question because they performed an in vitro stimulation of human T cells in the presence of IFNb for 96 hours, which more accurately models IFN sensing during acute infection than chronic infection. Because it has been demonstrated that type I IFN can act as a co-stimulatory signal (PMID: 15814665), one explanation for the observations of enhanced inhibitory receptor expression are that the T cells stimulated with CD3/28 in the presence of IFNb are more activated and have acquired

exaggerated effector functions, and the expression of inhibitory receptors such as PD-1, TIM-3 and LAG-3 is high on activated T cells that are not yet dysfunctional. This would be consistent with the observation of enhanced IFNg production in extended data fig 1d. It could be true that the authors' in vitro model of IFNb stimulation of human T cells accurately models the effects of chronic IFN stimulation of T cells, and analyses could be done to make that case, but none are provided here.

Response: We appreciated the insightful comments. We agree that our *in vitro* T cell culture system would not perfectly mimic the environment or condition of chronic infection or cancer milieu *in vivo*. We added a discussion about this issue as a limitation of this study (page 14, line 321-325).

Regarding the T cell activation by IFN-I, we had examined T cell proliferation by using cell trace violet dye and demonstrated similar or less proliferation in IFN-β treated T cells than in the control (Supplementary Figure 3a-d). It is also clear that TIM-3 expression is higher in IFN-β treated cells as compared to control conditions within the same cell division stage, suggesting that TIM-3 induction by IFN-β is independent from T cell proliferation, and this is discussed above. As suggested by the reviewer in the later comment below, we further determined the expression of activation marker (CD44, CD25 and CD69) and the cellular division states by flow cytometry and found that there was no statistical difference between control and IFN-β treatment (Supplementary Figure 3b-d). Thus, our data indicate that the induction of PD-1, LAG-3, TIM-3 under IFN-β treatment is not confounded by T cell activation or proliferation, at least in our network analysis.

Finally, a major weakness of the manuscript in its current form is that the relevance of the network analysis in figure 4 is not clear. It is also not totally clear what the goal of the network analysis was, given that the title of the paper is about IFNb regulating the expression of inhibitory receptors on T cells, and this result was demonstrated already in figure 1. Indeed, in figure 3 the authors perturb IFN-regulated TFs using shRNAs and show which TFs appear to have an impact on the expression of specific inhibitory receptors. Given the clearly demonstrated effect of IFNb on inhibitory receptor expression in figure 1-3, the relevance of the network in fig 4 must be made more clear. What exactly do the authors want the reader to take away from this figure? Further, the hierarchy of TFs constructed from the network is neither clearly explained, nor validated with simple positive control observations. For instance, how do the authors explain the fact that STAT1 and STAT2 are lower in the hierarchy, yet they are direct targets of IFN-I signaling and will be phosphorylated by JAK1 following IFNAR binding to IFNb?

Response: We thank the reviewer for these comments. Although a focus of this work was to identify regulatory factors for co-inhibitory receptors, we also attempted to explore the foundation of the complex transcriptional regulatory circuit altered by IFN-I. Our goal was to

provide a roadmap to future investigations of the underlying TFs interactions correlated with the regulators as highlighted in our work. It is worth noting that besides the regulators that we explored here, we demonstrated that other TFs can play important roles in regulating co-inhibitory receptors and ISGs. Intriguingly, the regulators shown in the network are not necessarily directly linked with conventional JAK-STAT pathway. This global view of IFN-I driven regulatory circuits highlights the non-canonical regulators of IFN-I response in human T cells.

In addition, our network analysis provided in-depth insights into the characteristics of each TF during IFN-I response in human T cells. For example, the network captured the difference between core IFN-I regulators STAT1 and STAT2. As shown in Figure 4b (bottom heatmaps), STAT1 and STAT2 showed different scores by a hypergeometric test (HG) and centrality (Cent); STAT1 was higher in Cent and lower in HG, in contrast, STAT2 was higher in HG and lower in Cent. This suggests that STAT2 is specialized to regulate DEGs at early and intermediate phase of IFN-I response, while on the other hand, STAT1 has more broader roles controlling not only IFN-I response genes but also T cell survival and memory formation[10,11]. Indeed, STAT2 expression was not affected by CD3/CD28 stimulation but was induced by IFN-β (Supplementary Figure 4c). In contrast, STAT1 was induced by just CD3/CD28 stimulation though to a lesser degree than with IFN-β. Therefore, we believe that the whole picture view of IFN-I regulatory network depicted in Figure 4 provides valuable information to the reader and offers opportunities to explore the human T cell IFN-I response well beyond the scope of our investigations.

With regard to the hierarchical network, we agree with the reviewer that the previous representation of TF interaction could be confusing and, to some degree, misleading. Thus, we replaced the hierarchical network with a new and simplified backbone network which represents the interactions of each TF. And related, we observed that the downregulated TFs served a more central role in the network than the upregulated TFs, suggesting the stronger impact of downregulated TF in IFN-I response (Figure 4c, d, Supplementary Figure 6a). Specifically, at the late phase, most TFs in the network were also downregulated. To validate the function of downregulated TFs, we focused on STAT5A because it was also shown to regulate co-inhibitory receptors (Figure 5k). Indeed, overexpression of STAT5A in human primary $CD4^+$ T cells inhibited the IFN-β induced co-inhibitory receptor expressions (Supplementary Figure 8c), indicating that STAT5A negatively regulates PD-1 and its downregulation is necessary to elicit IFN-I response[12] and IFN-I mediated enhancement of co-inhibitory receptor expression. Furthermore, we found TGF-β signaling pathways and associated genes (i.e. *SKI*, *SMAD3, IKZF2*) were enriched in those downregulated regulatory TFs at each time wave (Figure 4b; bottom heatmap, Supplementary Figure 6b), suggesting counter regulatory mechanisms between IFN-I response and TGF-β signaling in human T cells[13,14]. Given that STAT5 and TGF-β signaling are the core components to drive Treg differentiation and function and that inhibition of Treg function via IFN-I signal is important to

elicit effector T cell function[15,16], it would be reasonable to suppress those Treg associated TFs during IFN-I response to mount effective T cell actions against pathogens. Together, these observations with our network analysis provide in-depth insights of T cell IFN-I response with highly relevance to viral infection including COVID-19.

Also, the validation of the authors' findings in patients with SARS-CoV2 infection is correlative. While it does appear that the expression of inhibitory receptors is correlated with ISGs as the authors predicted, the fact remains that higher type I IFN in patients is clearly correlated with higher viral load and higher viral load could drive the expression of multiple inhibitory receptors because of chronic high-level antigen exposure. A better and more convincing validation of the authors' findings would be using an in vitro T cell killing assay. Since the authors have a good system for lentiviral infection of human T cells, this could be done with a tumor antigen-specific TCR or viral-specific TCR and tumor cells (PMID: 28783722) and would also enable the authors to functionally validate some of their findings from the TF knock down experiments. For instance, does knocking down the TFs such as SP140, STAT3 or BCL3 enhance the function of T cells in vitro because of reduced inhibitory receptor expression?

Response: While this is a very interesting suggestion, assessing T cell killing capacity is beyond the scope of this study. Furthermore, it is technically challenging to transduce both specific TCR and shRNA into primary human naïve T cells before TCR stimulation. Instead, we analyzed the perturbation data to determine if SP140/STAT3/BCL3 knockdown affected T cell cytotoxic signatures by using gene enrichment analysis. BCL3 and SP140 knockdown datasets captured some aspects of T cell cytotoxic properties as can be indicated by the GSEA enrichment score for six gene signatures related to cytotoxic/effector function. In contrast, we did not observe consistent effects of STAT3 on cytotoxic signatures (Reviewer Figure REDACTED). These data indicate that SP140 and BCL3 are likely to regulate T cell cytotoxic function possibly via altering co-inhibitory receptor expressions. However, given the multifaceted role of these regulators on T cell function, and that its function is sometimes context dependent, we believe further confirmation with *in vivo* functional studies is required.

Specific comments:

Figure 1

-no controls showing the expression of activation markers. The IFNb stimulated cells could just be activated more strongly.

Response: We thank the reviewer for this insight which we addressed above. We have assessed the activation marker expression (CD25, CD44, and CD69) on T cells, and demonstrated that the T cells activation status was not different between control versus IFN-β treatment (Supplementary Figure 3c). Thus, we believe the effect of IFN-I on human T cells, at least under our experimental conditions, was not strongly cofounded by T cell activation signature that are known to induce PD-1, TIM-3, LAG-3 expression.

-no unstained condition or isotope control is shown for the expression of PD-1, TIM-3 and LAG-3 in figure 1 or extended data figure 1.

Response: As suggested, we included the isotype control data in revised Figure 1 and Supplementary Figure 2.

Figure 3

-regulatory module 1 and 2 definition seems arbitrary based on PC1. There is quite a bit of variation by PC2 in regulatory module 1 and this could represent 2 different biological states based on high vs low PC1. Have the authors explored this?

Response: We thank the reviewer for raising this point. As suggested, we further explored these differences by looking directly on those genes that are differentially affected by the TFs in the PC2 high versus the TFs in PC2 low. The regulatory module 2 was further divided into two modules with potentially different roles (Reviewer figure REDACTED). However, this subdivision of IFN-I regulatory module 2 was not directly associated with ISGs or co-inhibitory receptor expression, we think this result does not add more values on our manuscript.

-PC biplot is difficult to understand. More explanation should be dedicated to what the authors are attempting to show here. The highlighted genes are also cherry picked.

Response: To make these biplots easier to understand, we have elaborated the heatmaps in Supplementary Figure 5b, c.

Regarding the critique for "cherry picked" genes in the biplots, we would like to clarify that the genes shown are only DEGs and so some genes that did not met DEG criteria (see Method section; page 26, line 623-630) were excluded. In addition, the ISGs plotted in Figure 3e is the gene set established by the other researchers[18] and so are not biased by our selections.

-PC plots are underselling the work the authors have done. I would perhaps show some changes in gene regulation for some interesting TF KDs as a heat map, at least in the extended data so that readers can get a more granular sense for what is significantly changing as a result of perturbing key IFN-regulated TFs.

Response: The requested heatmaps are included in Supplementary Figure 5b, c.

Figure 4

-not enough explanation of the ranking under the network diagrams. There is a rank row, but then rows for centrality and hypergeometric test. How all of this is contributing to the ranking and what the "normalized value" is representing in each case is not clear. Please provide a more clear explanation and this should be included in the figure legend because the figure is not interpretable without it.

Response: Thank you for pointing this out. We have revised the figure legend as follows:

Original: “Middle row; heatmaps representing a ranking of the TFs based on ‘Cent’ stands for centrality and ‘HG’ stands for hypergeometric test. Bottom row; hierarchical backbone networks. Red circles represent up-regulated TFs, blue circles represent down-regulated TFs.”

Revised: “Middle row: heatmaps representing a ranking of the TFs based on their centrality, connectivity and gene-target enrichment in the corresponding regulatory network. ‘Cent’ stands for centrality, which is a parameter that is given to each node, based on the shortest path from the node to the other nodes in the network. It represents how central and connected a node is in the rest of the network. ‘HG’ stands for hyper-geometric, the value in the heatmap is the $-\log_{10}(\mathrm{P.value})$ of a hypergeometric enrichment test of gene-targets to each TF in the network. The rank column is an average of both HG and Cent values, after score rescaling (0-1).”

-TFs being highlighted in text do not seems to correlate with their rank in the middle panel heat maps. Myc and Tbet are both discussed for the intermediate wave, for instance, but are on opposite ends of the ranking. Authors should make clear how the reader should interpret these rankings.

Response: We thank the reviewer for pointing this out. It was misleading to highlight the low ranked regulators in the text. MYC showed higher Cent score but a very low HG score,

indicating its extensive role on regulating downstream genes but limited unique contribution to this specific IFN-I network. In the revised manuscript, we focused on highly ranked regulators (i.e. STAT1 and STAT2) (page 8, line 171-179).

-No discussion of STAT1 or 2, despite the fact that they appear to be important upregulated TFs and the stimulus used was type I interferon.

Response: Thank you for raising this point. STAT1 also turned out to be an important regulator for ISGs and co-inhibitory receptors according to our perturbation data (Figure 3d-f, revised Supplementary Figure 5b, c). However, the impact of STAT1 knockdown was moderate compared to that of STAT3 knockdown, and that is why we did not discuss STAT1 in the previous version. As we agree that STAT1 and STAT2 are important factors on IFN-I signaling, we discussed these two genes in the revised version (page 8, line 171-179).

-description of the backbone hierarchical layout in methods is not clear enough. Please provide more explanation of what is being done here and how results should be interpreted. Not clear what the significance of this part of the figure is. There appears to be no central message here.

Response: As we discussed above, we decided to remove the hierarchical network in the revised version. Instead, we generated a simplified backbone network that represents the interaction of regulators (TFs) at each transcriptional wave. The interpretation of the new backbone network is discussed above and described in the revised manuscript (page 8, line 183-195).

-Lines 174-179, I disagree. Are the authors claiming that this hierarchy suggests that down regulation of sox4 is necessary for the activation of STAT1/2, which are literally direct downstream targets of type I interferon? The significance of anything in this hierarchy is questionable.

[Description in lines 174-179; These data suggest that loss of suppression of TFs at higher hierarchy contributes the activation of downstream effector TFs under IFN-I response, which was also observed in the intermediate and late regulatory network. The elucidation of this backbone network enables us to shed light on the regulatory interactions within each component of the transcriptional network, providing further depth to the extent of interactions within the network.]

Response: We agree with the reviewer and removed the hierarchical backbone network.

-line 191-193 - is there a significant overlap between the bridging TFs and the TFs that were perturbed? No evidence to support this claim is shown.

Response: Fifteen out of nineteen perturbed TFs were categorized as bridging TFs. Also, fifteen out of thirty-six bridging TFs were perturbed TFs. We consider this to be a significant overlap.

Figure 5

-the analysis the authors are citing of their COVID-19 patient single cell RNAseq is a pre print and has not completed peer review at this point.

Response: Correct - It is under revision at *Nature Communications*.

-The authors end the paper on suggested roles for SP140, STAT3 and BCL3, all of which were included in the list of TFs that were perturbed with shRNAs in figure 3. Because the authors have RNAseq data from those conditions, the logic here is a little circular. However, it would be nice to experimentally validate that knockdown of these TFs has the predicted impact on the cell surface expression of PD-1, TIM-3, LAG-3, TIGIT. If this experiment is done, activation markers such as CD25, CD69, CD44 should be used to determine if the effects are specific to the transcriptional programs highlighted or are also compromising T cell activation.

Response: This is again a good point. As suggested, we performed further validation experiments and demonstrated that the roles of the three regulatory TFs on PD-1, TIM-3, LAG-3, and TIGIT are verified at the protein level and are not confounded by T cell activation nor proliferation (Supplementary Figure 8).

Minor comments:

-typo in extended data 5d title

Response: We have corrected the typo.

-typo in 5h y axis

Response: We have corrected the typo.

Reference

1. Yevshin, I., Sharipov, R., Kolmykov, S., Kondrakhin, Y. & Kolpakov, F. GTRD: a database on gene transcription regulation-2019 update. *Nucleic Acids Res* **47**, D100–D105 (2019).

2. Darnell, J. E., Kerr, I. M. & Stark, G. R. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**, 1415–1421 (1994).

3. Leonard, W. J. & O'Shea, J. J. Jaks and STATs: biological implications. *Annu Rev Immunol* **16**, 293–322 (1998).

4. Zhu, C. *et al.* An IL-27/NFIL3 signalling axis drives Tim-3 and IL-10 expression and T-cell dysfunction. *Nat Commun* **6**, 6072 (2015).

5. Che, K. F. *et al.* p38 Mitogen-activated protein kinase/signal transducer and activator of transcription-3 pathway signaling regulates expression of inhibitory molecules in T cells activated by HIV-1-exposed dendritic cells. *Mol Med* **18**, 1169–1182 (2012).

6. Zhang, Q. *et al.* Inborn errors of type I IFN immunity in patients with life-threatening COVID-19. *Science* **370**, (2020).

7. Bastard, P. *et al.* Autoantibodies against type I IFNs in patients with life-threatening COVID-19. *Science* **370**, (2020).

8. Urban, S. L., Berg, L. J. & Welsh, R. M. Type 1 interferon licenses naïve CD8 T cells to mediate anti-viral cytotoxicity. *Virology* **493**, 52–59 (2016).

9. Mestas, J. & Hughes, C. C. W. Of mice and not men: differences between mouse and human immunology. *J Immunol* **172**, 2731–2738 (2004).

10. Quigley, M., Huang, X. & Yang, Y. STAT1 Signaling in CD8 T Cells Is Required for Their Clonal Expansion and Memory Formation Following Viral Infection In Vivo. *The Journal of Immunology* **180**, 2158–2164 (2008).

11. Hofer, M. J. *et al.* Mice Deficient in STAT1 but Not STAT2 or IRF9 Develop a Lethal CD4+ T-Cell-Mediated Disease following Infection with Lymphocytic Choriomeningitis Virus. *Journal of Virology* **86**, 6932–6946 (2012).

12. Wellbrock, C. *et al.* STAT5 contributes to interferon resistance of melanoma cells. *Curr Biol* **15**, 1629–1639 (2005).

13. Grunwell, J. R. *et al.* TGF-β1 Suppresses the Type I IFN Response and Induces Mitochondrial Dysfunction in Alveolar Macrophages. *J Immunol* **200**, 2115–2128 (2018).

14. Thomas, D. A. & Massagué, J. TGF-β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* **8**, 369–380 (2005).

15. Srivastava, S., Koch, M. A., Pepper, M. & Campbell, D. J. Type I interferons directly inhibit regulatory T cells to allow optimal antiviral T cell responses during acute LCMV infection. *J Exp Med* **211**, 961–974 (2014).

16. Gangaplara, A. *et al.* Type I interferon signaling attenuates regulatory T cell function in viral infection and in the tumor microenvironment. *PLoS Pathog* **14**, e1006985 (2018).

17. Jaiswal, H. *et al.* The NF-κB regulator Bcl-3 restricts terminal differentiation and promotes memory cell formation of CD8+ T cells during viral infection. *PLOS Pathogens* **17**, e1009249 (2021).

18. El-Sherbiny, Y. M. *et al.* A novel two-score system for interferon status segregates autoimmune diseases and correlates with clinical features. *Sci Rep* **8**, 5793 (2018).

Decision Letter, first revision:

Subject: Decision on Nature Immunology submission NI-A31326A
Message: 6th Jul 2021

Dear David,

Thank you for your letting detailing how you would respond to the concerns of the Referees - this was helpful. I've now had the chance to discuss the manuscript and response letter with the colleagues and we are happy to take the manuscript forward. We therefore invite you 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.

I'd just like to flag a few issues that will need consideration:

-We'll re-designate this as a Resource. You don't need to do anything - we can make the change at our end. The format is slightly different i.e. there's no limit to the number of Extended Data Figures (though try not be excessive i.e. ideally not > ~12 or so.

-Unfortunately the Editors are in agreement about the concern of Ref. 1 re. the the inferences based on scRNAseq alone and would ideally have the ATACseq included in the analysis (we can agree that CHIPseq is not realistic). Given what is largely a more descriptive study, the inclusion of this extra data might help to alleviate this Referee's concern.

-Including the titration data on would be helpful (currently it seems you might only have it as rebuttal only).

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.

When 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.

* Please include a revised version of any required reporting checklist. It will be available to referees to aid in their evaluation of the manuscript goes back for peer review. They are available here:

Reporting summary:
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.

Please use the link below to submit your revised manuscript and related files:
[REDACTED]

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.

We hope to receive your revised manuscript within two weeks. 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.

Please do not hesitate to contact me if you have any questions or would like to discuss

these revisions further.

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.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

The Macmillan Building
4 Crinan Street
Tel: 212-726-9207
Fax: 212-696-9752
z.fehervari@nature.com

Referee expertise:

Referee #1: Human immunology, IFN biology

Referee #2: T cell immunology

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
This study takes a comprehensive systems biology approach to examine the effects of IFN-I on human T cells, using predominantly an in vitro approach with primary T cells with validation against in vivo data sets from COVID19 patients. One focus is on inhibitory receptor expression, which is of interest given the previously proposed role of IFN-I in T cell exhaustion. Overall the work is well performed, with a complex and detailed bioinformatic analysis.

Strengths of the study include use of primary human T cells, testing of multiple time points, and perturbation using shRNA of a core set of 21 transcription factors to gain insight into their function in the T cell IFN response. Interesting findings include

discordant regulation of inhibitory receptors, identification of SP140 as a regulator of differential LAG3 and TIGIT expression, construction of IFN-I regulatory networks, identification of a late phase IFN-I response at 48-96 hr, and segregation of TFs into two modules of regulators.

Weaknesses of the study include that despite a detailed bioinformatic analysis the study remains at a descriptive level. It was difficult for me to link these findings to an increased understanding or advance in the role of IFN-Is in T cell biology, and why IFN-Is can have both activating and inhibitory functions. There is little mechanistic insight into the regulation of the late phase response, the interesting induction of non-ISGs, and the segregation of TFs. The dependence on solely gene expression profiling (albeit including scRNAseq) is also limiting, and the study could benefit from use of parallel techniques such as ATACseq to gain greater mechanistic insight.

Some more specific issues include use of only one relatively high concentration of IFN-b (500 U/ml) – IFN-Is can have different, and even opposing effects based on concentration. Also, if I understood Fig. 3 c and d correctly, some TFs that were minimally if at all knocked down by shRNAs (e.g. STAT1 and STAT3) were included in the analysis and seemingly had an effect?

Reviewer #2:
Remarks to the Author:
In this revised manuscript, Sumida, Dulberg and Schupp et al. have provided additional evidence that activation markers are not induced by IFNb treatment in vitro, have provided additional clarity and methodological detail about the network diagram in figure 4, removed the hierarchical network, and provided flow cytometry validation that knockdown of their candidate IFNb regulated TFs reduces the expression of inhibitory receptors.

Overall the manuscript is improved from the first submission and my concerns regarding activation status and the validity of the hierarchical network have been addressed.

I believe it was important to provide the flow cytometry validation currently in supplementary figure 8, and I applaud the authors for performing these experiments. However, I would like to point out that some of the data presented slightly undermine the authors' predictions about the IFNb-driven role of these TFs in regulating inhibitory receptor expression. For instance, for both SP140 and STAT3, the role of these TFs in driving the expression of LAG-3 and TIM-3 does not appear IFNb-specific. There is a general loss of LAG-3 and TIM-3 expression after knocking down these TFs and IFNb treatment appear to upregulate their expression in even in the SP140/STAT3 knockdown setting. These data appear to argue that while these TFs do play a role in regulating the predicted inhibitory receptor, this effect appears to be at last partially (STAT3-TIM-3) or completely (SP140-LAG-3; STAT5a-PD-1) independent of IFNb treatment. I note that the effect of BCL3 on LAG-3 and PD-1 does appear to blunt the upregulation induced by IFNb. These data do somewhat call into question whether the analyses the authors have presented is of use for understanding the specific transcriptional regulation of inhibitory receptors induced by IFNb. Perhaps this is why the authors have chosen to included the data as supplementary figure 8 despite now ending the paper on these data, which is a bit unusual.

Specific comments:

Line 78-85 - Why is proliferation only shown for CD4 T cells in supplementary figure 3? While I appreciate that the authors have conducted many experiments and analyses in both CD4 and CD8 T cells, the subset where the co-expression of inhibitory receptors is most appreciated as sign of dysfunction are CD8 T cells. This analysis should have been done with either both CD4 and CD8 or just CD8 T cells.

Line 95-97 - Because a single donor is being used, please provide information about the donor such as age, gender, and any known health conditions that the authors have on the patient.

Author Rebuttal, first revision:

Point-by-point responses to the reviewers' comments

Referees' comments:

Reviewer #1 (Remarks to the Author) This study takes a comprehensive systems biology approach to examine the effects of IFN-I on human T cells, using predominantly an in vitro approach with primary T cells with validation against in vivo data sets from COVID19 patients. One focus is on inhibitory receptor expression, which is of interest given the previously proposed role of IFN-I in T cell exhaustion. Overall the work is well performed, with a complex and detailed bioinformatic analysis.

Strengths of the study include use of primary human T cells, testing of multiple time points, and perturbation using shRNA of a core set of 21 transcription factors to gain insight into their function in the T cell IFN response. Interesting findings include discordant regulation of inhibitory receptors, identification of SP140 as a regulator of differential LAG3 and TIGIT expression, construction of IFN-I regulatory networks, identification of a late phase IFN-I response at 48-96 hr, and segregation of TFs into two modules of regulators.

Weaknesses of the study include that despite a detailed bioinformatic analysis the study remains at a descriptive level. It was difficult for me to link these findings to an increased understanding or advance in the role of IFN-Is in T cell biology, and why IFN-Is can have both activating and inhibitory functions. There is little mechanistic insight into the regulation of the late phase response, the interesting induction of non-ISGs, and the segregation of TFs. The dependence on solely gene expression profiling (albeit including scRNAseq) is also limiting, and the study could benefit from use of parallel techniques such as ATACseq to gain greater mechanistic insight.

Response: First, as the editor suggested, we agree with switching our content type from Article to Resource which may in part address the reviewer's concern that our study is descriptive. Having said that, we would suggest that the issue of being "descriptive" is

commonly applied to studies of human biology. While the original observation was made *in vitro* with the surprising finding where IFN-I induced the co-inhibitory cassette of TIM-3, PD-1, and LAG-3 while inhibiting TIGIT, this study discovered the underlying mechanistic pathway and confirmed those data at the protein level. More importantly, as we learned that the SARS-CoV-2 virus infection triggers a potent type I IFN response, we used our detailed *in vitro* observations to provide a predictive model that explained observations in humans related to T cell function and co-inhibitory receptor expression. Moreover, one of the strengths of our study is the value for genome-wide gene expression kinetics of IFN-I response on human T cells and the constructed regulatory network, which has never been explored. Thus, we appreciate that the reviewer found value in our manuscript from this aspect. In our manuscript, we narrowed down the candidate regulators focusing on the regulatory mechanisms of co-inhibitory receptor expression and identified SP140, STAT3, and BCL3 as key regulatory factors by not only gene expression level but also with protein validation experiments. Thus, our elucidation of the IFN-I regulatory network provides, we believe, an important resource for IFN-I related T cell biology in human infection, cancer, and autoimmunity.

As requested by the reviewer, we performed ATAC-seq at each time wave with three replicates. First, we found the temporal changes of chromatin accessibility by IFN-β treatment; chromatin accessibility was suppressed at the early phase (2h) but then enhanced at the late phase (72h) by IFN-β treatment (Supplementary Figure 5). Furthermore, the footprint analysis identified the temporal shift of TF bindings over time; specifically, the enriched bindings of IRFs/STATs at the early phase and shifted to AP-1 family bindings at the later phase during IFN-I response (Figure 3g, h, Supplementary Figure 6d). This observation is consistent with our transcriptional network analysis highlighting IRF1 as one of the upregulated regulators in the early and intermediate network, albeit downregulated regulator at the late phase (Figure 4b, c, Supplementary Figure 7a), further supporting our findings based on the regulatory network. We thank for the reviewer's suggestion and believe that additional ATAC-seq data will enhance the value of our manuscript as a Resource paper.

Some more specific issues include use of only one relatively high concentration of IFN-b (500 U/ml) – IFN-Is can have different, and even opposing effects based on concentration. Also, if I understood Fig. 3 c and d correctly, some TFs that were minimally if at all knocked down by shRNAs (e.g. STAT1 and STAT3) were included in the analysis and seemingly had an effect?

Response: We performed the experiment to examine the effect of different concentrations of IFN-β on human primary CD4$^+$ T cells (now shown in Supplementary Figure 3d). We haven't seen the opposite effects by different dose of IFN-β treatment for those key co-inhibitory receptor expressions.

Regarding the efficiency of STAT1 and STAT3 knockdown, we repeatedly achieved more than 60% knockdown for both STAT1 and STAT3. It is not surprising to see a significant impact on the regulatory circuit by 60% knockdown, thus we would not consider this as a critical issue.

Reviewer #2 (Remarks to the Author)

In this revised manuscript, Sumida, Dulberg and Schupp et al. have provided additional evidence that activation markers are not induced by IFNb treatment in vitro, have provided additional clarity and methodological detail about the network diagram in figure 4, removed the hierarchical network, and provided flow cytometry validation that knockdown of their candidate IFNb regulated TFs reduces the expression of inhibitory receptors. Overall the manuscript is improved from the first submission and my concerns regarding activation status and the validity of the hierarchical network have been addressed.

I believe it was important to provide the flow cytometry validation currently in supplementary figure 8, and I applaud the authors for performing these experiments. However, I would like to point out that some of the data presented slightly undermine the authors' predictions about the IFNb-driven role of these TFs in regulating inhibitory receptor expression. For instance, for both SP140 and STAT3, the role of these TFs in driving the expression of LAG-3 and TIM-3 does not appear IFNb-specific. There is a general loss of LAG-3 and TIM-3 expression after knocking down these TFs and IFNb treatment appear to upregulate their expression in even in the SP140/STAT3 knockdown setting. These data appear to argue that while these TFs do play a role in regulating the predicted inhibitory receptor, this effect appears to be at last partially (STAT3-TIM-3) or completely (SP140-LAG-3; STAT5a-PD-1) independent of IFNb treatment. I note that the effect of BCL3 on LAG-3 and PD-1 does appear to blunt the upregulation induced by IFNb. These data do somewhat call into question whether the analyses the authors have presented is of use for understanding the specific transcriptional regulation of inhibitory receptors induced by IFNb. Perhaps this is why the authors have chosen to include the data as supplementary figure 8 despite now ending the paper on these data, which is a bit unusual.

Response: This is an excellent point, and thus we have already taken this potential issue into account for our perturbation analysis. As we stated in the manuscript (Method section, “Heatmap of perturbed TFs”), we attempted to control the effect of gene perturbation by normalizing the changes in gene expression by IFN-β treatment and perturbation. Indeed, *LAG3* and *HAVCR2* mRNA expression were blunted by SP140 and STAT3 knockdown (Figure REDACTED), supporting their specific role on IFN-β mediated effect. However, as the reviewer pointed out, we agree that the effect of SP140 and STAT3 knockdown on cell surface LAG-3 and TIM-3 protein levels were not clear enough to claim their role as IFN-β specific. This discrepancy between mRNA and protein expression is of course well known as post-translational events are important for protein expression. Moreover, it is possible that SP140

and STAT3 may have impact on post-transcriptional regulation for LAG-3 and TIM-3. While this is an interesting point to be studied further, we would respectively suggest that the detailed mechanism for post-transcriptional regulation of this process is beyond the scope of the present manuscript. .

[REDACTED FIGURE AND LEGEND]

With regard to the effect of STAT5A on PD-1, we agree with reviewer. Since STAT5A was identified as a key transcription factor through regulatory network analysis, it doesn't necessarily have to be IFN-β specific regulatory factor. As we found that STAT5A is relatively novel in the regulation of co-inhibitory receptor expression, we would prefer to retain those data. Instead, we discuss these limitations in the revised version as followings: Our regulatory network takes into account all differentially expressed genes by IFN-β treatment and we further ranked the key regulatory factors for the enrichment score under treatment with IFN-β. However, this does not exclude the possibility that they play an important role regulating certain genes or perhaps the same genes not under IFN-β treatment (page 14, line 335-339). Indeed, one of the challenges and limitations with the methods using system biology approach is to consider the highly complex post-transcriptional regulations, leading to unexpected discrepancy between mRNA and protein expression.

Specific comments: Line 78-85 - Why is proliferation only shown for CD4 T cells in supplementary figure 3? While I appreciate that the authors have conducted many experiments and analyses in both CD4 and CD8 T cells, the subset where the co-expression of inhibitory receptors is most appreciated as sign of dysfunction are CD8 T cells. This analysis should have been done with either both CD4 and CD8 or just CD8 T cells.

Response: As requested, $CD8^+$ T cells proliferation assay has been included in the revised manuscript (Supplementary Figure 3b). We didn't observe difference in cellular division by IFN-β treatment in $CD8^+$ T cells as well.

Line 95-97 - Because a single donor is being used, please provide information about the donor such as age, gender, and any known health conditions that the authors have on the patient.

Response: As requested, the information is provided in Method section.

Decision Letter, second revision:

Subject: Your manuscript, NI-RS31326B

Message: Our ref: NI-RS31326B

7th Jan 2022

Dear Dr. Hafler,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "Type I Interferon Transcriptional Network Regulates Expression of Coinhibitory Receptors in Human T cells" (NI-RS31326B). 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 "Type I Interferon Transcriptional Network Regulates Expression of Coinhibitory Receptors in Human T cells". 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.

If your image is selected, we may also use it on the journal website as a banner image, and may need to make artistic alterations to fit our journal style.

Please submit your suggestions, clearly labeled, along with your final files. We'll be in touch if more information is needed.

Nature Immunology has now transitioned to a unified Rights Collection system which will allow our Author Services team to quickly and easily collect the rights and permissions required to publish your work. Approximately 10 days after your paper is formally accepted, you will receive an email in providing you with a link to complete the grant of rights. If your paper is eligible for Open Access, our Author Services team will also be in touch regarding any additional information that may be required to arrange payment for your article.

You will not receive your proofs until the publishing agreement has been received through our system.

Please note that <i>Nature Immunology</i> is a Transformative Journal (TJ). Authors may publish their research with us through the traditional subscription access route or make their paper immediately open access through payment of an article-processing charge (APC). Authors will not be required to make a final decision about access to their article until it has been accepted. Find out more about Transformative Journals.

If you have any questions about costs, Open Access requirements, or our legal forms, please contact ASJournals@springernature.com.

<B>Authors may need to take specific actions to achieve compliance with funder and institutional open access mandates. For submissions from January 2021, if your research is supported by a funder that requires immediate open access (e.g. according to Plan S principles) then you should select the gold OA route, and we will direct you to the compliant route where possible. For authors selecting the subscription publication route our standard licensing terms will need to be accepted, including our self-archiving policies. Those standard licensing terms will supersede any other terms that the author or any third party may assert apply to any version of the manuscript.

Please use the following link for uploading these materials: [REDACTED]

If you have any further questions, please feel free to contact me.

Best regards,

Elle Morris
Senior Editorial Assistant
Nature Immunology
Phone: 212 726 9207
Fax: 212 696 9752
E-mail: immunology@us.nature.com

On behalf of

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

The Macmillan Building
4 Crinan Street
Tel: 212-726-9207
Fax: 212-696-9752
z.fehervari@nature.com

Reviewer #1:
Remarks to the Author:
The authors have adequately addressed the points raised during review.

Reviewer #2:
Remarks to the Author:
This is a second re-review. I have reviewed the authors' responses to previous reviewer comments and I believe that their responses and the modifications made to the the manuscript are sufficient. All my questions and concerns are addressed and I support publication.

Final Decision Letter: