nature portfolio

Peer Review File

Type 1 interferon perturbates clonal competition by reshaping human blood development

Corresponding Author: Dr Anna Nam

Version 0:

Decision Letter:

26th Sep 2025

Dear Dr Nam,

First, please accept my apologies for the delay in returning this decision to you. Thank you for your patience.

Your Article, "Type 1 interferon perturbates clonal competition by reshaping human blood development" has now been seen by 3 referees. You will see from their comments copied below that 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 manuscript for publication, but would be very interested in considering a revised version that addresses their concerns.

We hope you will find the referees' comments useful as you decide how to proceed. If you wish to submit a substantially revised manuscript, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions.

If you choose to revise your manuscript taking into account all reviewer and editor comments, please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable 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:

*1) 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.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available here.
Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary: https://www.nature.com/documents/nr-reporting-summary.pdf
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.

Please be aware of our guidelines on digital image standards.

EXTENDED DATA FIGURES

When re-submitting your manuscript, please ensure that any supplementary figures and tables that are crucial to the manuscript’s conclusions are converted into Extended Data figures and tables to increase visibility of these data. Extended Data figures and tables are online-only (present in the online PDF and full-text HTML versions of the paper), peer-reviewed

display items that provide essential background to the article but are not included in the main article due to space constraints. A maximum of ten Extended Data display items (figures and tables) is permitted.

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

Link 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 Genetics or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

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

Nature Genetics 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.

Thank you for the opportunity to review your work.

Sincerely,

Safia Danovi, PhD
Senior Editor, Nature Genetics
ORCiD: 0009-0007-7822-5479

Referee expertise:

Referee #1: blood, single-cell multi-omics

Referee #2: HSCs, interferon

Referee #3: HSC biology

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

This is very comprehensive and exciting study on the effects of a clonally depleting therapy on a clonal disease, which should be of interest to the broad field of clonal evolution. The authors assemble a unique, clinically annotated patient cohort with serially treated samples, apply state-of-the-art single cell genomic assays and analytical strategies, complemented with challenging single-cell functional assays. The authors describe two major effects of IFN alpha on CD34+ HSPCs: the development of a new population (IMP) which seems to be biased towards inflammatory neutrophil differentiation, and an increase in lymphoid differentiation that balances the prominent mega-erythroid commitment typical of MPN. The authors go on an perform a deep characterization of the molecular drivers behind this process (NFkB signaling and PU.1 mediated differentiation biases). Finally, authors assess the specific effects of somatic CALR and JAK2 mutations in these differentiation shifts, and how they might counteract the effects of IFN alpha therapy. However, specific major concerns remain towards the proposed existence and physiological relevance of the IMP population, the granularity to which authors describe the inflammatory signaling pathways induced by IFN alpha treatment, and the overall interpretation of some of the claims made through the text.

Major comments:

1. Presence, identity and molecular drivers of the IMP population.
o The size of the IMP cluster is extremely small. Authors should state how many cells in total are included in the analysis in Figure 1f and Ext. Fig.2e, and comment on their ability to perform differential expression analysis in such a small group of cells.
o The physiological relevance of a population represents less than 1% of the cellular pool in CD34+ MPN cells (or 0.0001% of the MPN BM) is not completely clear. If the hypothesizes is that the main physiological relevance comes from an increased production of inflammatory neutrophils, authors should discuss the putative role of Neu1 population in IFN alpha

treated MPN.
o Whether IFN alpha directly induces the formation of IMPs is a critical part of the paper. The authors present only tangential evidence (Figure 6). Are IMPs present in IFN alpha treated patients that are no longer on active treatment? Does treatment of HSCs with IFN alpha lead to generation of the IMP population in-vitro, and does that lead to increased and more rapid neutrophil-biased differentiation? Do authors have any evidence that these cells by-pass a GMP differentiation state, as Fig.3b seems to suggest?
o The effect of somatic mutations in IMPs is underpowered and needs stronger support. The authors highlight a single patient (IFN02, Figure6) in which the presence of a double mutant CALR clone leads to lower IMP-related transcription. It is unclear how the mutant cell fraction changes in the IMP population (the low % of IMPs in Ext. Fig.14d makes it impossible to assess any changes). Where are IMPs in Ext. Fig.14g? Why do authors merge HSC1 and IMPs in Ext. Fig.14k? Do somatic mutations modify the neutrophil-differentiation bias of IMPs? (e.g. what is the proportion of mutant cells in the barplot in Fig.3g). Authors could also address this question by genotyping the resulting colonies from Figure 3b.
o Related to the point above, it seems like many patients included in Fig.6/Ext. Fig.12 and 14 don't present molecular responses (reduction of mutant cell frequency). Authors could compare the differential effect of somatic mutations in the IMP population in responders (IFN03, IFN04) versus non-responders (IFN07, IFN02) to substantiate their claims related to "changes to clonal dynamics could be predicted based on priming of clonal stem cells to differentiate into IMPs"

2. IFN alpha coordinates anti- and pro-inflammatory transcriptional programs

The terms "inflammation", "pro-inflammatory" and "anti-inflammatory" are used too broadly across the text, when the authors are referring to completely distinct inflammatory mechanisms such as IFN signaling, TNF alpha and/or NFkB-mediated signaling. In most cases, it is unclear which specific inflammatory pathway (rather than broad "inflammation") the authors are referring to. This lack of granularity should be clarified across the text and in figures.

o For example, authors find upregulation of IFN genes and an increase in inflammatory neutrophils. Therefore, this claim should be rewritten and clarified: "These findings indicated that isolated IFNα exerts an overall anti-inflammatory response in human HSPCs." (e.g. lines 213-222)
o HALLMARK genesets (Figure2d) lack sufficient granularity to distinguish the transcriptional effects of distinct inflammatory signaling pathways. For example, authors show downregulation of epithelial mesenchymal transition genes in hematopoietic stem cells, when the actual transcriptional signal is related to JUN, CXCL8 or CD44 expression (according to Table S4). The authors should use genesets specifically related to pro and anti-inflammatory pathways and interferon target genes, ideally in hematopoietic cell types.
o The physiological relevance of a putative anti-inflammatory program in cDCP is unclear (Figure2e), since authors show no changes in cDCP proportions upon IFN alpha treatment (Ext. Fig.3). The authors could choose to show instead another cell type in which an anti-inflammatory program could be relevant for the pathophysiology of the disease (e.g. lymphoid progenitors that are expanded upon IFN alpha treatment).
o Is the NFkB pathway that the authors describe canonical or non-canonical?

3. PU.1 is a master regulator of IFN alpha-induced differentiation.
- It is unclear why authors show mixed myeloid (GMP) and lymphoid (MLP-proB, preB) trajectories in the same pseudotime (Fig.5e). Authors should treat these lineages independently and should indicate whether the differences in TF motif accessibility shown are statistically significant.
- The model proposed in Fig.5f doesn't seem to be supported by the data. The left plot implies increased STAT5 accessibility in HSCs, but STAT5 doesn't appear in Fig.5c. The authors draw a line suggesting higher PU.1 levels lead to IMP development, but they don't show any data supporting this claim.

Conclusions and interpretation:

- Lines 267-268: "These data suggested that IFNα may induce an alternate path for accelerated neutrophil development from HSCs." INF alpha might bias HSCs towards neutrophilic differentiation, but the data doesn't probe an alternate differentiation pathway.
- Line 301: "These data implicated IMP development as a feature of HSC aging." This is too speculative. The text should focus on the effect of inflammation in IMPs, and not broadly as an aging-related phenomena.
- Lines 585–587 and lines 601-604: The "alternate route" of differentiation, and specially, prediction of clonal dynamics by IMPs, are overstated.

Minor comments:

Figure and text clarity. The authors do a fantastic job at presenting their complex dataset in an accessible format. However, there are specific figures and parts of the text that should be clarified:
o Figure 1c. Treatment status and somatic genotypes with so many cells are hard to visualize. I would suggest density subtraction plots to highlight treatment and genotype-specific regions.
o Extended Data Fig.2 e and f panels should be relabeled for clarify. What is the overall cell number stated below panel e? In Ext. Figure 2f, what does the y-axis label refer to?
o Figure 2c doesn't belong in Figure 2. The message of increased cell cycle and decreased quiescence (through reduced CXCR4 expression) could belong in Figure 1 of Ext. Fig.3. The same is true for Ext. Fig.4d. The boxplot displaying CD184 expression suggests no difference in expression despite a significant p-value. The authors could choose a different type of plot or remove it completely.
o In Ext. Fig.9 and Ext. Fig.10 authors should indicate where the motif of each TF is located within the peak.

o Lines 342-358 and Ext. Fig.10a/b. What is the relevance of MHC class II gene regulation? The authors should clarify.
o Lines 580-585. The relevance of a IMPDN gene signature in DNMT3A-mutated HSCs is unclear. Are these DNMT3A CH individual being treated with IFN alpha? Is there any evidence of increased IFN alpha in their plasma?
o Lines 626-628: the relevance of COVID-19 individuals' responses in the context of IFN alpha therapy is unclear and should be clarified.
Analytical:
- It is unclear the number of cells used for each analysis for each figure. Authors should label the number of cells per group in their boxplots across the manuscript.
- The results of CALR and JAK2 genotyping calls should be included in a supplementary figure, similarly to those related to transcriptome and chromatin accessibility quality controls. What's the accuracy of genotyping? Do the number of mutant cells match the expected VAF of the CALR and JAK2 mutation based on bulk sequencing assays? (which the authors have in Table S1).
- Code to generate main figures from the GEO data should be made available to readers by the time of publication.
- In GoT-ATAC, the first dimension was excluded (line 1569). This requires justification.
- Methods section on retrospective flow cytometry data should be rewritten and clarified. Why do the authors need to detect antibody panels in the first place and what do they mean by unused flow cytometry channels? Why do authors create ensembl FCS files?
- Table S6 – GSEA GMP states and GSEA IMP states "Table S11…"
- Supplementary TableS5 – please include the number of cells represented in each cell type. For example, there's an IMP DEA but untreated samples shouldn't have IMPs?

Reviewer #2 (Remarks to the Author):

In this study, Lama, Isakov et al describe serial single cell multiomic analysis of human bone marrow from patients with MPN, a mosaic condition involving expansion of a mutant HSC clone that promotes megakaryocyte/erythroid development. They use single cell analysis to compare the response by WT versus MUT clones to IFNa therapy, which is known to select for WT clones. They utilize transcriptome, ATAC, and CUT&RUN analysis to identify key TF pathways induced by IFNa and changes in differentiation trajectories in WT versus MUT subtypes that help to explain clonal selection. Specifically, they show that PU.1 is a critical transcription factor downstream of IFNa that promotes changes in differentiation trajectory, with emergence of a myeloid progenitor state called IMP. CALR mutant clones have increased chromatin accessibility for PU.1 and are therefore more prone to IMP differentiation, leading to a long term advantage for WT clones. The authors compared their data to patients who received hydroxyurea and to patients with JAK2 mutations to strengthen and validate their findings.

Overall this is a technically advanced and expansive study that provides new insight into mechanisms of IFNa activity and clonal selection. The study answers a number of long standing questions in the field. However, a number of claims are not strongly supported by the data and some areas of clarification are needed.

Specific comments:

1) It is interesting that downregulation of CXCR4 happened in response to IFNa, as this may provide mechanism of increased cell division. Was there a differential effect of CXCR4 downregulation when comparing WT and MUT cells?

2) Figure 2E: the authors state that: " These findings indicated that isolated IFNα exerts an overall anti-inflammatory response in human HSPCs."

This statement is inaccurate since interferon pathways (which are inflammatory) are massively upregulated; whereas the statement is just referring to TNFa.

3) Regarding GoT and derivation of neutrophil-only colonies on methylcellulose assay, the authors write (lines 285-286):
Overall, these data demonstrated that IFNα expanded the
inflammatory neutrophils (relative to other neutrophils), consistent with the induction of an
alternate route of inflammatory myeloid differentiation.

They imply that there is a direct route to neutrophil production. Where do the neutrophils lie on the pseudotime plot in 3a and is there any evidence of neutrophils that can derive more closely from IMPs?

4) Lines 290-292. The authors find that the inflammatory signature is higher in samples after 3-4 weeks off IFNa therapy "suggesting the reinforcement of the inflammatory signature following exposure"
This is hard to understand biologically. Why would an inflammatory signature increase after cessation of the therapy. Are they referring to the TNFa signature (similar to #2 above)? Use of the word "inflammatory" is really too general. Alternatively, can the authors assure that these patients did not have recurrence of disease or other confounding clinical variables at this time such as intercurrent viral infection?

5) Lines 300-301: The authors write: "These
data implicated IMP development as a feature of HSC aging."

This is a pretty strong statement to make based on a small sample set of people with MPN. This statement would need to be validated in an aging cohort.

6) Lines 363-366: “RFX3 overexpression (but not RFX2)
expanded the granulocytic colonies (CFU-G) and diminished the erythroid colonies (BFU-E)
compared to control-vector transduced CD34+ cells”
Does transient overexpression of RFX3 also confer this effect or does the RFX3 need to be continually expressed?

7) Figure 5a would be clarified by labeling cell types at the top.

8) Lines 426-427: The authors write that “IFNα directly upregulates the expression of PU.1”
Pu.1 has been shown to be induced by TNF and IL1 signaling (Etzrodt 2019, Chavez 2021). Dependence of this activation on TNF signaling was shown through inhibitors and loss of function studies. In order to claim that IFNa directly induces Pu.1, similar inhibitor and LOF studies are required. The activation seen here may be indirect through TNFa or IL1.

9) Figure 5f shows that the percentage of MkP and MEP correlates with the platelet count, which is not surprising. The authors also showed that people on IFNa tend to have lower MkP, but this was not analyzed as a continuous variable. Can you correlate IFN response with MkP percentage? Does the expression of Pu.1 also inversely correlate with the platelet count?

10) Lines 449-451/ Fig 6b:

“Computing proportions of progenitor cell types between wildtype and mutated cells separately showed that IFNα globally remodeled the differentiation toward lymphoid development, reducing the overall megakaryocytic bias in the mutated cells (Fig. 6b). “

Figure 6b does not seem to show what is described? Alternatively, a clearer explanation of this proportion is needed.

Further, the authors write that “Consistently, computation of the mutant cell frequency across the progenitor subsets before and after treatment revealed that the enrichment of the mutated cells in the myeloid compartments did not change following IFNα treatment (Extended Data Fig. 12f). These data revealed that somatic mutations may constrain IFNα-induced differentiation remodeling.”
This statement does not make sense logically. If the mutated cells in myeloid compartments did not change following IFNa treatment, that indicates that somatic mutations do NOT constrain IFNa-induced differentiation remodeling.

If PU.1 sites are more accessible in the setting of CALR mutation compared to WT. Wouldn’t you predict then that you would see a stronger effect of PU.1 induction by IFNa, with more lymphoid and less myeloid skewing? Wouldn’t you expect to see differences in Figure 6b?

11) Re CUT&RUN data (lines 487-9): The authors write that
“CALR mutations may alter the chromatin landscape of TF regulatory regions, which in turn modulates the downstream differentiation remodeling by IFNα and restrains lymphoid development in the mutated HSCs (Fig. 6f). “

There is some handwaving here – the authors claim that the CALR mutation alters the cooperative binding of PU1 with other TFs e.g. IRF4/8 to modulate myeloid and lymphoid differentiation. Whereas typically Pu1 promotes lymphoid and myeloid development at the expense of erythroid, here the authors say that in the setting of CALR mutation you still get enhanced myeloid differentiation but lymphoid is restrained? This is not shown by the data.

12) Figure 6f follows the prediction of Hormaechea et al (PMID: 33743191) which should be cited.

Reviewer #3 (Remarks to the Author):

This study uses single-cell multi-omics in MPN patients before and during IFNa therapy to dissect hematopoietic responses. The authors show that IFNa drives polarized hematopoietic remodeling expanding lymphoid progenitors while inducing a neutrophil committed IMP trajectory from HSCs. Further analysis reveals that IFNa suppresses TNFa and TGFb signaling, promotes cell-cycle entry, and rewires differentiation through RFX3 and PU1. Clonal dynamics were concluded to correlate with the propensity of mutant HSCs to adopt the IMP fate, suggesting that IMP resistance as a determinant of inflammatory clonal selection in MPN.

Major Comments:

The study design is elegant and the data compilation extensive.
The two major claims of the paper are that IFNa 1. Remodels hematopoietic differentiation and 2. alters clonal dynamics.

Remodeling of differentiation is reported along two lines: induction of an inflammatory myeloid progenitor (IMP) and induction of lymphoid priming. The evidence for the IMP subset being induced by IFNa and the molecular features of that are strong. The role for RFX2/3 in the IMP phenotype is validated with OE experiments that are compelling. What is not clear is whether this IMP population emerges in a subset of cells that is distinct from those that have lymphoid priming. The reasonable assumption is that the events are happening in cells beyond a myeloid/lymphoid branch but the OE experiments show an upregulation of IL-7R and raise the possibility that RFX2/3, a known master regulator, may affect the lymphoid priming.
This is of interest in part because lymphoid priming is unanticipated. Prior findings in the mouse showed reduced lymphoid differentiation with IFNa (https://doi.org/10.1084/jem.187.1.79) hypothesized due to reduced IL-7 signaling. It would be expected that reduced IL-7 signaling would also reduce the B cell progenitors measured here and yet these were increased. The molecular results regarding PU.1 and TCF3 accessibility are convincing and affirm a previously known relationship, but it is not clear if this could result in increased pro- and pre-B cells if IFNa impairs IL-7R signaling. Were TCF3 expression levels actually modified by IFNa? Did expression of its target and driver of lymphoid differentiation EBF1 change? Was there any effect on IL-7R expression or TSLP that can compensate? The use of K562 to show that IFNa can upregulate PU.1 are not terribly helpful as this is a very aberrant leukemia line; the data would be more compelling with primary CD34+ cells as were used in Figure 4. This could test the role of IL-7 in lymphoid priming as it could be held or added to the media. Realizing that these experiments are time consuming and may not be definitive, the authors should at least address the apparent paradox of IFNa leading to increased B progenitors when it has been reported to also decrease IL-7R signaling.

The second main conclusion of the paper is about clonal dynamics being altered by IFNa. The abstract states that they measured “IFNa effects on clonal stem cells from the admixed wildtype HSCs.” But, clonal effects on WT HSC are not detailed, as least as I could discern. The focus seems to be more about the relative mutant clone abundance compared to the admixture of normal clones comprising the HSC pool. The data for a distortion induced by IFNa turns out not to be in the relative abundance of myeloid or lymphoid progenitors in mutant or normal with IFNa. Rather, proliferation was increased in CALR mutant cells that was exacerbated by IFNa. Also, a patient with a defined subclone of mutant cells had lower IMP gene expression in association with a time related increase in subclone representation. This led to the hypothesis that IMP propensity after IFNa could influence the relative founder clone size. Three JAK V617F mutant patient samples showed that the 2 with clinical improvement from IFNa had a higher IMP signature and improved lymphoid progenitor counts. Further, 16 patients showed a lower mutant cell pool correlated with a higher IMP + HSC2/overall Mutant cell ratio. Therefore, the authors conclude that forming IMP induced by IFNa can reduce clonal overgrowth. This is a reasonable conclusion but sorting through this was unnecessarily complicated in part because of the incredibly dense prose used by the authors throughout the paper. For example, the authors state that “inflammation-induced cell cycle entry rates are decoupled from clonal dynamics.” In what sense? The data presented preceding the statement were about cell cycle entry only. Understanding the paper is made worse by the use of other imprecise language; for example using ‘clone size’ rather than ‘mutant cell frequency’ that was the apparent parameter measured. Statements like “IMP propensity” are made without clarity on how that was computed and validated. Please modify and clarify the language and please focus the narrative. The paper is almost unreadable as written.

The authors should also acknowledge that they are not assessing clones within the normal HSC population despite single cell data. Interferon is likely to have very clone specific effects on both normal and mutant cells. The results observed in normal HSC may be the select outgrowth of subclones that are predisposed to lymphoid or IMP differentiation. Was this measured? Can they state with assurance that the effects of IFNa are generalizable to all clones?

Further definition and validation of IMPs is needed. For example, are IMPs transcriptionally distinct from activated GMPs or instead a distortion of a continuum from HSCs. Are they consistently present across patients? Why is the IMP cluster not visible in Extended Data Fig1e? Does Cluster X consistently emerge across timepoints and genotypes? Table S2 shows not all patients have detectable IMPs. Does this variability undermine the claim that IFNa universally induces a pro-inflammatory trajectory? Could patient-specific factors (co-mutations, prior therapy, clinical response) explain this heterogeneity?

Cell death and dropout analysis is not discussed but may affect interpretation of the observations. While cell cycle activity is evaluated in HSPCs, which populations are specifically depleted or ablated by IFNa remains unclear; have apoptotic or stress signatures been observed in any specific clusters?

The RNA velocity plots currently display manually annotated directionality arrows; however, please include the raw velocity flow vectors in the supplementary materials to better support interpretation of lineage transitions. In addition, demonstrate that the IMP-to-neutrophil differentiation path is supported by velocity-derived transition probabilities. Given the observed size of the IMP population and its neutrophil commitment, please estimate the putative neutrophil output from IMPs (Neu1) relative to that from GMPs (Neu2), and clarify whether this aligns with the abundance and phenotype of mature neutrophils observed in PBMCs.

The data are largely correlative with only modest functional validation (RFX3 OE experiment). The conclusions should reflect that the usual means of increased and decreased expression to validate the relationships proposed for RFX2/3 and PU1 for example were not performed.

Please clarify how complete response (patients IFN01, 03, 05, 06, 07, 09, 10, 11, 12) compares with partial response cases. Correlating molecular findings, such as clonal contraction, lymphoid skewing, IMP signatures with clinical readouts such as platelets, hematocrit, spleen size, and symptom burden would add strength to the paper and add to its translational relevance.

Version 1:

Decision Letter:

6th May 2026

Dear Dr Nam,

Your Article, "Type 1 interferon perturbates clonal competition by reshaping human blood development" has now been seen by 3 referees. You will see from their comments below that while they find your work of interest, some important points are raised. We are interested in the possibility of publishing your study in Nature Genetics, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

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. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

Our aim is to assess the revisions editorially but, depending on your response, we might need to return to one or more reviewers. Please be assured that we'll only do this if necessary.

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:

*1) 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.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available
here.
Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary: https://www.nature.com/documents/nr-reporting-summary.pdf
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.

Please be aware of our guidelines on digital image standards.

EXTENDED DATA FIGURES

When re-submitting your manuscript, please ensure that any supplementary figures and tables that are crucial to the manuscript’s conclusions are converted into Extended Data figures and tables to increase visibility of these data. Extended Data figures and tables are online-only (present in the online PDF and full-text HTML versions of the paper), peer-reviewed display items that provide essential background to the article but are not included in the main article due to space constraints. A maximum of ten Extended Data display items (figures and tables) is permitted.

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

Link 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 four to eight weeks. If you cannot send it within this time, please let us know.

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

Nature Genetics 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,

Safia Danovi, PhD
Senior Editor, Nature Genetics
ORCiD: 0009-0007-7822-5479

Referee expertise:

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

The authors have done a fantastic job at addressing most of the comments in their revisions, and the manuscript has been substantially improved. With minor revisions, this reviewer would recommend publication in Nature Genetics.

The effect of somatic mutations in IMPs still presents some conceptual challenges as presented, and should be clarified before this reviewer can fully endorse the model in Figure6p. The authors use IFN02 patient as an example of resistance to IMP differentiation, specifically the CALR double mutant clone, that expands upon treatment and downregulates the IMPup program. However, in FigureS9g, as pointed out by the authors in their rebuttal, the vast majority of IMPs come from the "IFNa double MUT". How do authors define this population as IMPs if it's downregulating the IMP signature? These seems to argue against the proposed model by which double mutant cells resist IMP differentiation.

Figure 6 tries to merge three conceptually distinct arguments: (1) CALR mutations alter myeloid differentiation ratios through PU.1/CEBPA co-operative binding, (2) a double-mutant CALR clone downregulates the IMP signature and expands, and (3) the model is generalizable to JAK2 and DNMT3A mutations. In this reviewer's opinion, the figure could be greatly clarified by moving panels to supplementary material or creating an additional figure if the editorial requirements allow.

This reviewer would also encourage authors to integrate their several conceptual model figures (Figure2g, Figure5f, Figure6f, Figure6p) into a final single, unified model that would leave readers with an integrated summary of their findings.

There are places where the text reads convoluted, which the authors could consider simplifying and focusing (e.g. lines 137-147, 193-198, 235-239, 257-263, 306-307, 447-449, 518-521, 607-612).

In line 479, Fig. 11f should read 7f?

Reviewer #2 (Remarks to the Author):

The authors have responded fully to my comments and I have no further concerns.

Reviewer #3 (Remarks to the Author):

The revised manuscript is much improved. The evidence for the IMP state is strengthened, the RFX3 functional data are more convincing, and the authors now put greater caution in parts of the clonal discussion. These revisions are much appreciated. There remain only minor concerns with the presentation.

1. Central mechanistic claims:
Several issues acknowledged as beyond the scope of the present study are not peripheral, they relate directly to the proposed mechanism. The absence of PU.1 validation in primary CD34+ cells, the inability to track wildtype subclones, the lack of lineage-tracing evidence for IMP developmental origin and fate, and the absence of any quantitative assessment of IMP-derived neutrophil output leave the central model largely inferential. The authors should be more explicit in the main text about what the data can and cannot establish, rather than deferring these limitations to the discussion or supplement; clear statements about limitations would be more helpful to the field.

2. Functional validation:
The addition of RFX3 overexpression and IFNAR1-blocking experiments is a meaningful improvement. However, the

proposed regulatory roles of RFX3, PU.1, and related transcription factors are still supported more by association and partial perturbation than by a systematic demonstration of sufficiency and requirement. The standard approach of both gain and loss-of-function experiments for the key regulatory relationships proposed for RFX2/3 and PU.1 has not been performed. The conclusions should more clearly reflect this limitation.

Version 2:

Decision Letter:

Our ref: NG-A69964R1

1st Jun 2026

Dear Dr. Nam,

Thank you for submitting your revised manuscript "Type 1 interferon perturbates clonal competition by reshaping human blood development" (NG-A69964R1). It has now been seen again by Reviewer #1 whose comments are below. The reviewer finds that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Genetics, pending minor revisions to satisfy our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements soon. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Genetics Please do not hesitate to contact me if you have any questions.

Sincerely,

Safia Danovi, PhD
Senior Editor, Nature Genetics
ORCiD: 0009-0007-7822-5479

Reviewer #1 (Remarks to the Author):

The authors have addressed all remaining concerns and this reviewer believes the manuscript will be of great interest to the readership of Nature Genetics.

March 10th, 2026

Dear *Nature Genetics* editors and reviewers—

We thank the reviewers and editors for their enthusiasm for our work and thoughtful review regarding our manuscript (NG-A69964), by Lama, Isakov, and colleagues. We have revised the manuscript accordingly and hope to have fully addressed all the concerns and suggestions as detailed in our point-by-point description of the revision below.

In brief, the key changes to the manuscript are as follows:

1. We have made extensive textual changes to clarify several points raised by the reviewers. Specifically, we clarify (1) terminologies used (e.g. pro-inflammatory/anti-inflammatory), (2) our results in the context of prior work showing lymphoid differentiation defects upon IFNa treatment in mice, (3) and what our collective data shows regarding the newly identified inflammatory myeloid progenitors (IMP), including an extensive discussion on the evidence our data provides for the differentiation trajectories of IMPs and ultimately the need for an in vivo model of IMPs to track their differentiation via genetic barcodes, as a future directions. We address these points and several other important questions in the point-by-point discussion below.
2. We have included numerous new panels of analyses. Key highlights include (1) gene set enrichment using an immune-specific pathways that corroborate our findings using the Hallmark pathways, (2) RNA velocity measurements supporting the differentiation of IMPs to inflammatory neutrophils, (3) differential expression analysis between *CALR*-mutated versus wildtype IMPs in patients with molecular response versus resistance to substantiate the role of IMPs in clonal dynamics, and (4) additional correlations of single-cell multi-omics data with clinical measurements.
3. We thank the reviewers for appreciating that the body of work was already expansive with the main strengths of our experimental design in the innovative single-cell multi-omics methods applied to rare clinical samples, the results of which were corroborated with key follow-up experiments (e.g. single cell colony forming unit assay, RFX3 overexpression in primary CD34+ cells, and in vitro studies of PU.1 expression and regulation, including CUT&RUN). In this revised version, we have included an experiment in which RFX3 was overexpressed transiently in CD34+ cells and was sufficient to drive granulocytic differentiation (upon Reviewer #2's question). We also performed an experiment to show that blocking IFNa with an IFNa receptor (IFNAR1) antagonizing antibody inhibits the upregulation of PU.1 gene expression.

We hope that we have adequately addressed the reviewers' comments and welcome any further questions or comments.

Kind regards, The authors

Point-by-point response to reviewer comments:

Reviewer: 1

This is very comprehensive and exciting study on the effects of a clonally depleting therapy on a clonal disease, which should be of interest to the broad field of clonal evolution. The authors assemble a unique, clinically annotated patient cohort with serially treated samples, apply state-of-the-art single cell genomic assays and analytical strategies, complemented with challenging single-cell functional assays. The authors describe two major effects of IFN alpha on CD34+ HSPCs: the development of a new population (IMP) which seems to be biased towards inflammatory neutrophil differentiation, and an increase in lymphoid differentiation that balances the prominent mega-erythroid commitment typical of MPN. The authors go on an perform a deep characterization of the molecular drivers behind this process (NFkB signaling and PU.1 mediated differentiation biases). Finally, authors assess the specific effects of somatic CALR and JAK2 mutations in these differentiation shifts, and how they might counteract the effects of IFN alpha therapy. However, specific major concerns remain towards the proposed existence and physiological relevance of the IMP population, the granularity to which authors describe the inflammatory signaling pathways induced by IFN alpha treatment, and the overall interpretation of some of the claims made through the text.

We thank the reviewer for commenting positively on our work and appreciating the broad impact of the study findings that were made possible by the combination of unique clinical cohort of samples, innovative technologies, and functional studies. We are grateful for the constructive feedback that has served to improve the manuscript significantly.

Major comments:

1. Presence, identity and molecular drivers of the IMP population.

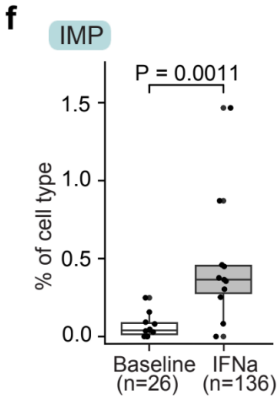
The size of the IMP cluster is extremely small. Authors should state how many cells in total are included in the analysis in Figure 1f and Ext. Fig.2e, and comment on their ability to perform differential expression analysis in such a small group of cells.

We thank the reviewer for highlighting this important point. This population is indeed small compared to other progenitor subgroups. Thus, our ability to profile 13 treated samples and over 65,000 CD34+ cells (across baseline and treated samples) have enabled us to identify this new population and determine its increase upon IFNa therapy, as displayed in Fig. 1f and former Extended Data Fig. 2e (current 1l). As suggested, we have included the total cell numbers on Fig. 1f and Extended Fig. 1l (pasted below). Across the samples, we identified 162 IMPs, which provided sufficient numbers of cells to perform differential expression analysis with GMPs in displayed in Fig. 1e. Initially, we had included just one treated time point per patient for those with two treated time points in Fig. 1f, but so as not to confuse the readers regarding the numbers listed in the text and figure, we included all the samples for Fig. 1f and maintained the paired sample analysis in the Extended Data Fig. 1l. We leveraged the ability to include IMPs from all the samples for the differential expression analysis via linear mixed modeling that accounts for batch effects from patient source and treatment status. In this revised version, we also included differential expression analysis between only IFNa-treated IMPs (n = 136 cells) and treated GMPs, which revealed similar results (Extended Data Fig. 1n, pasted below).

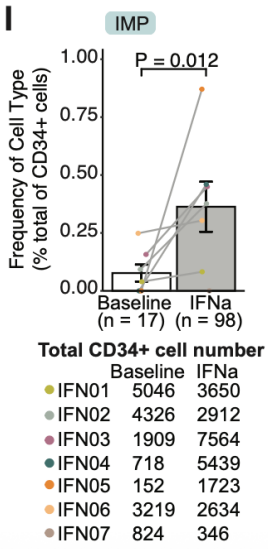
We have modified the Results text:

"To elucidate the identity of Cluster X, we performed differential expression analysis between Cluster X (n = 162 cells total) and GMPs via linear mixed model that explicitly models the effects of patient batch and treatment status (Fig. 1e, top; Supplementary Table 3, Methods)… Differential expression analysis of IFNα-treated IMPs versus GMPs highlighted the same differentially expressed genes and pathways (Extended Data Fig. 1n)."

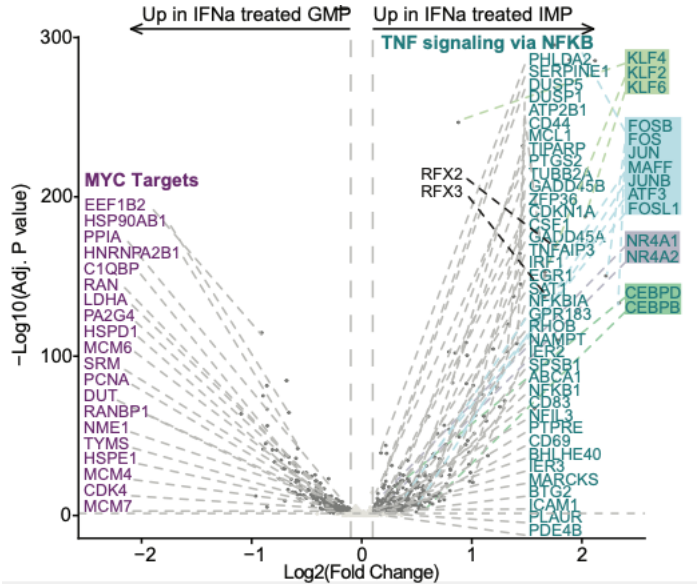
Fig. 1f, showing IMP percentage with total cell numbers added:



Extended Data Fig. 1l, showing paired samples of IMPs, with total CD34+ cell count listed:



Extended Data Fig. 1n, showing differential expression analysis between IFNa-treated IMPs vs GMPs:



The physiological relevance of a population represents less than 1% of the cellular pool in CD34+ MPN cells (or 0.0001% of the MPN BM) is not completely clear. If the hypothesizes is that the main physiological relevance comes from an increased production of inflammatory neutrophils, authors should discuss the putative role of Neu1 population in IFN alpha treated MPN.

We are grateful for the reviewer's important feedback. The reviewer rightly points out that IMPs represent a small population within the CD34+ pool. Nonetheless, we believe this is consistent with the data suggesting that IMPs are precipitously differentiating into the CD34-neg precursor states and therefore detected as a small population, as reflected in its transcription factor network with upregulation of immediate early response factors (AP1, KLF, NR4A) and emergency granulopoiesis TFs (CEBPB/CEBPD). Based on our data, the physiological relevance of IMPs are three-fold: (1) its generation indicates that IFNa treatment results in two opposing programs simultaneously within the same HSC population by enhancing the production of IMPs (with elevated AP-1/NF-kB) and downregulation of the same AP-1/NF-kB program in the other HSPC compartments (Fig. 2); (2) the impact of precipitous differentiation of IMPs from HSCs on perturbing clonal dynamics upon IFNa therapy (Fig. 6); and (3) generation of activated, pro-inflammatory Neu1 population (Fig. 3). Determining the putative role of Neu1 is complicated by the reduction in overall neutrophil counts in patients treated with IFNa. However, we may speculate that Neu1 may contribute to the well-established side effects of IFNa, such as flu-like symptoms (fever, malaise, chills) and development or exacerbation of autoimmune disorders (e.g. vasculitis and lupus) with known pathogenic roles of aberrant neutrophils (PMID: 33391289, 12642603). We also believe that IMPs and Neu1 may contribute to pro-inflammatory effects in inflamm-aging and autoimmune disorders (especially type 1 interferonopathies), further motivating our efforts to develop an in vivo model of IMPs. Thus, further studies are required to determine the functional role of Neu1.

We have linked the TF network of IMPs to their small population in the Results section:

"The upregulation of immediate early response and emergency granulopoiesis TFs suggested a precipitous differentiation pathway with rapid transitions to the subsequent CD34-negative precursor stages, potentially underlying the small size of this population in the $CD34^{+}$ compartment."

We have discussed the potential consequences of Neu1 and the need for further studies in the Discussion (pasted below):

"Further studies are also required to determine the role of IFNα-induced inflammatory neutrophils (Neu1). While its physiologic relevance to clinical response data is complicated by the overall reduction of neutrophil counts by IFNα,[118] we may posit that Neu1 may contribute to the known side effects of IFNα therapy, such as fever, malaise and other flu-like symptoms, as well as exacerbation or precipitation of autoimmune disorders, such as vasculitis or lupus nephritis wherein neutrophils may play a key role.[119-122] IMPs and Neu1 may also contribute to the pathologic processes of inflamm-aging and auto-inflammatory disorders (especially type 1 interferonopathies) in non-neoplastic conditions, further motivating our pursuit of an in vivo model of IMPs."

Whether IFN alpha directly induces the formation of IMPs is a critical part of the paper. The authors present only tangential evidence (Figure 6). Are IMPs present in IFN alpha treated patients that are no longer on active treatment? Does treatment of HSCs with IFN alpha lead to generation of the IMP population in-vitro, and does that lead to increased and more rapid neutrophil-biased differentiation? Do authors have any evidence that these cells by-pass a GMP differentiation state, as Fig.3b seems to suggest?

We thank the reviewer for these insightful questions regarding the role of IFNa in the formation of IMPs. We have addressed them point-by-point below:

Whether IFN alpha directly induces the formation of IMPs is a critical part of the paper. The authors present only tangential evidence (Figure 6). Are IMPs present in IFN alpha treated patients that are no longer on active treatment?

The main evidence we identified for the role of IFNa in inducing IMPs is the significant increase of IMPs upon a sustained and isolated treatment with IFNa, as shown in Fig. 1f. The presence of few IMPs in the baseline samples may be due to endogenous IFNa activity. The IMP cluster emerges only in the IFNa-treated samples, whereas it is not observed in baseline samples due to the low numbers of IMPs at baseline (Fig. 1g). This is in concordance with the absence of this population in prior publications of primary MPN bone marrow samples from patients not under IFNa therapy. IMPs were identified in all treated samples, except for IFN02 Year 2 and IFN07 Year 1 samples due to the low overall CD34+ cells captured (541 and 346 cells, respectively; potentially due to low number of cells banked initially or quality of the cryopreserved samples, which can be variable sample-to-sample). We have revised the Results section to include this point:

"Consistent with the identification of IMPs in this IFNα-treated cohort of patients, IMPs derived predominately from the IFNα-treated samples with elevated frequencies in the IFNα-treated versus baseline $CD34^+$ cells (Fig. 1f; paired samples in Extended Data Fig. 1l; overall $CD34^+$ cells per sample in Extended Data Fig. 1m, showing no difference between baseline and treated samples). Separately integrating the samples based on treatment status also confirmed the specificity of IMPs to IFNα-treated bone marrow (Fig. 1g). All IFNα-treated samples included IMPs except for IFN02 Year 2 and IFN07 Year 1 samples due to the small number of overall $CD34^+$ cells captured (541 and 346, respectively)."

We identified that IMPs were present in all three samples from patients who discontinued IFNa therapy for 2-3 weeks (0.3%-1.46%). However, these data do not dispute the role of IFNa in the formation of IMPs as we observed the presence of residual IFNa response signaling in these samples (albeit to a lower degree compared to cells under active IFNa therapy, Fig. 3h). Furthermore, following IFNa treatment, HSC1 displayed an upregulation of the HSC1 gene signature (i.e. genes upregulated in HSC1 vs HSC2/3), consistent with the presence of IMPs in the post-treated samples. We further clarify this in the Results section:

"To determine whether the IMP and HSC1 cell states may be maintained following discontinuation of IFNα therapy, we compared HSCs from baseline to post-treated samples (IFN04, IFN05, IFN06), not on active IFNα therapy (off therapy for ~3-4 weeks)…. With respect to the IFNα-regulated pathways, we observed a residual IFNα response signature and a slight downregulation of TNFα signaling via NF-κB pathway in the post-therapy samples (as expected), whereas TGFβ signaling was upregulated in the post-treated HSCs compared to both baseline and actively treated HSCs (Fig. 3h and Extended Data Fig. 3k).... Intriguingly, post-therapy HSC1 subset retained its elevated cell state program following IFNα exposure (i.e. differentially upregulated genes in HSC1 versus HSC2/3, Fig. 3h and Extended Data Fig. 3k). Consistent with the maintenance of the HSC1 gene signature and presence of residual IFNα signaling following therapy discontinuation, IMPs were identified in these post-therapy samples (0.3-1.46%, Supplementary Table 2)."

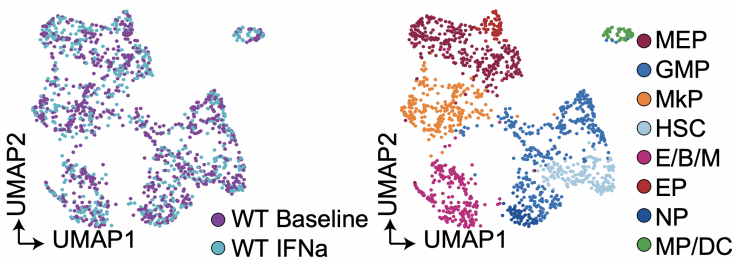
As IFNa has been shown to activate the NF-kB pathway in other cellular contexts (e.g. PMID: 11095741), IMPs may be induced directly by IFNa. However, we cannot exclude the possibility that IMP production is mediated by another molecule together with IFNa or subsequent to IFN signaling. We clarify these important points in the Discussion pasted below:

"Toward this end, future directions include developing an *in vivo* model of IMPs (mouse or xenograft) and genetically tracing their lineage trajectory. Developing an *in vivo* model of IMPs would also enable us to determine the mechanisms of IMP development and identify whether IFNα directly induces IMPs in a subset of poised HSCs or IMP generation is mediated by another molecule with or subsequent to IFNα activation."

Does treatment of HSCs with IFN alpha lead to generation of the IMP population in-vitro, and does that lead to increased and more rapid neutrophil-biased differentiation?

We thank the reviewer for these important questions related to the nature of IMPs. As suggested, we treated wildtype cord blood HSCs with IFNa (without RFX3 overexpression) for 24 hours using the same experimental condition as in the RFX3 experiment. We did not observe a distinct IMP-like population (reviewer only figure pasted below, representative of three replicates). This is not surprising, however, because the in vitro system cannot recapitulate the in vivo IFNa effects of inducing HSCs into cell cycle entry (PMID: 24493802) and a key distinction of IMPs from HSC1 is their induction into cell cycle (Extended Data Figs. 3c-e). While the mechanisms that underlie the differences between in vivo and in vitro effects are unknown, our data highlighted the downregulation of CXCR4, a key bone marrow niche factor, as potentially mediating the exit from quiescence (given the well-established literature on its role in maintaining HSC quiescence). Thus, these data suggested that HSCs may require the niche environment for their entry into cell cycle by IFNa. Nonetheless, we believe the specificity of IMPs to IFNa treatment in human strongly supports its role in the development of IMPs, as discussed above. Our lab is actively working on developing an in vivo model of IMPs which will enable us to functionally determine the mechanisms underlying IFNa-induction of IMPs.

Reviewer only figure showing no IFNa-specific cluster in IFNa-treated CD34+ cells:



Do authors have any evidence that these cells by-pass a GMP differentiation state, as Fig.3b seems to suggest?

We thank the reviewer for this question. The follow data suggested that IMPs may represent a differentiation trajectory that is distinct from the conventional GMP differentiation route: the similarity of IMPs to HSC1 in their upregulation of TF network including RFX2/3, AP-1, and KLF factors (that is markedly distinct from GMPs) and RNA velocity prediction that IMPs develop from HSCs. Moreover, dimensional reduction by snATAC-seq revealed a contiguous cell state transition from HSCs to IMPs, revealing their epigenetic chromatin state similarity and distinction from GMPs. These data prompted us to perform the single cell CFU assay, as the same experiment was used to demonstrate that HSCs may beget megakaryocytic progenitors directly, bypassing the MEP stage (PMID: 26541609). The development of neutrophil-only cells of single cell CFU assay of IFNa-treated HSCs referenced by the reviewer thus supported the model wherein HSC display early lineage commitment to neutrophils. As another supportive evidence, overexpression of RFX3 induced an IMP-like state and upregulated CFU-G (rather than CFU-GM). Collectively, these data provided evidence for a distinct differentiation path of IMPs. We would like to further note that the emergence of the IMP population itself indicates that stem cells traverse a distinct differentiation route in transiting through the IMP stage. However, rigorously addressing the reviewer's questions regarding the differentiation paths of IMPs, compared to GMPs, would require an in vivo model that enables careful lineage tracing studies, which we are pursuing as future directions but beyond the scope of this current manuscript. This question has prompted us to put a question mark next to IMP in the model depicted in Fig. 3b, as IMPs may mediate early neutrophil commitment as indicated by the neutrophil only colonies but is not directly tested by the experiment. We have expounded upon this important point in the discussion:

"However, our results are distinct from our current understanding of trained immunity, as the identification of IMPs indicates that stem cells traverse an alternate route of differentiation. The following results from our data suggest that IMPs may represent an alternate path of myeloid differentiation distinct from GMPs: (1) the transcription factor network of IMPs was most similar to that of a subset of HSCs (HSC1) and markedly distinct from the GMP state under the same IFNα-activated microenvironment; (2) relatedly, RNA velocity measurements predicted their derivation from HSC1; and (3) by chromatin state, IMPs could be traced contiguously from HSCs and are discontiguous from GMPs on the snATAC-seq data-derived UMAP. These data provide useful corroborating evidence, as several known cell state transitions of this blood developmental system are represented in the UMAP embeddings of scRNA-seq and snATAC-seq data and are predicted by RNA velocity measurements. However, to definitively determine the origins and differentiation trajectories of IMPs, we would require a means to genetically lineage trace these cells (e.g. via lentivirally introduced molecular barcodes[116,117]). Toward this end, future directions include developing an *in vivo* model of IMPs (mouse or xenograft) and genetically tracing their lineage trajectory. Developing an *in vivo* model of IMPs would also enable us to determine the mechanisms of IMP development and identify whether IFNα directly induces IMPs in a subset of poised HSCs or IMP generation is mediated by another molecule with or subsequent to IFNα activation. Such an *in vivo* model would also facilitate a platform to causally determine the relationship between clonal dynamics and IMP differentiation rates.

The upregulation neutrophil lineage markers (e.g. *MPO*, *CSF3R*) and downregulation of antigen presenting molecules suggested a neutrophilic differentiation bias of IMPs. Concerted expression and chromatin accessible binding sites of immediate early response TFs and CEBPB/D, emergency granulopoiesis TFs, nominated IMPs as a means to rapidly generate inflammatory neutrophils (which harbor the IMP TF signature). Single cell colony output of neutrophil only colonies enriched in IFNα-treated bone marrow suggested an early uni-lineage commitment by HSCs, supporting this model. As additional support for a neutrophilic-biased differentiation program of IMPs, overexpression of RFX3 induced an IMP-like population and increased CFU-granulocytic

colonies. However, lineage tracing methods via an *in vivo* model of IMPs would allow us to trace the lineage outputs of IMP and the relative contributions of neutrophils from IMPs versus GMPs. Nonetheless, the identification of the IMP population itself indicates that HSCs traverse an alternate differentiation course (whether this route includes GMPs or not)."

The effect of somatic mutations in IMPs is underpowered and needs stronger support. The authors highlight a single patient (IFN02, Figure6) in which the presence of a double mutant CALR clone leads to lower IMP-related transcription. It is unclear how the mutant cell fraction changes in the IMP population (the low % of IMPs in Ext. Fig.14d makes it impossible to assess any changes). Where are IMPs in Ext. Fig.14g? Why do authors merge HSC1 and IMPs in Ext. Fig.14k? Do somatic mutations modify the neutrophil-differentiation bias of IMPs? (e.g. what is the proportion of mutant cells in the barplot in Fig.3g). Authors could also address this question by genotyping the resulting colonies from Figure 3b.

We thank the reviewer for raising these questions. The reviewer correctly points out that the dramatic expansion of the double mutant clone in IFN02 upon IFNa treatment prompted us to examine the transcriptional states of the wildtype, single mutant, and double mutant HSCs at baseline. This analysis revealed that the double mutant HSCs exhibited a downregulation of the IMP gene signature and upregulation of IFN signaling. These results, in turn, prompted us to ask whether the clonal changes upon IFNa may be explained by the propensity of mutant HSCs to differentiate into IMPs. The reviewer astutely points out that the frequency of IMPs in IFN02 in particular is quite small. As the vast majority of the IFNa-treated HSCs consist of the double mutant clone, the low frequency of IMPs is consistent with our model that the double mutant HSCs are resistant to IMP development. Because HSC1 expressed a transcription factor network characteristic of IMPs and the RNA velocity data indicated that HSC1 may be poised to IMP development, we tested the enrichment of the mutated cells in the IMP/HSC1 compartments (relative to mutant frequency in other HSPCs) when assessing the propensity of the mutated cells to differentiate into IMPs. We clarify this point in the Results section:

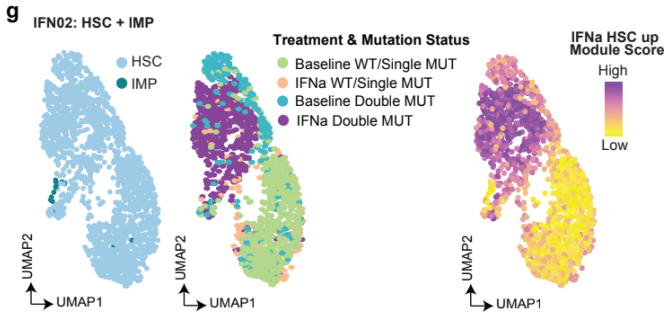
"We therefore evaluated the change in HSC mutant cell frequency between time points as a function of the propensity of mutated HSCs to develop into IMPs. Previously, we had observed that the megakaryocytic differentiation bias of *CALR*-mutated cells was evident in the increased frequency of the mutated cells in the MkP compartment relative to mutant cell frequency in the total $CD34^+$ population[26]; therefore, we determined the IMP differentiation bias or propensity of the mutated cells by computing the fold change of mutant cell frequency within IMPs and HSC1 (predicted to beget IMPs, Fig. 3a) relative to that in the total $CD34^+$ compartment (Extended Data Fig. 9i). Indeed, this measure of propensity of mutated HSCs to differentiate into IMPs could model clonal dynamics (Extended Data Fig. 9i)."

We agree that determining the proportion of the mutated cells in neutrophils would be informative and corroborate the results in HSC1/IMPs. However, neutrophils are known to have very low overall transcript counts, which prevents the use of GoT to genotype this cell type. Unfortunately, we did not save the colonies from the single cell CFU analysis in Fig 3e. We would like to note that were these studies feasible, they would be supportive but still not definitive, and again ultimately an vivo model would enable to functionally assess clonal outputs as a function of IMP development (included in the Discussion, pasted below). Nonetheless, we were able to further substantiate the role of somatic mutations in IMPs by performing the analysis suggested by the reviewer below.

In the Discussion:

"Such an in vivo model would also facilitate a platform to causally determine the relationship between clonal dynamics and IMP differentiation rates."

With respect to the question regarding where IMPs are in the former Extended Data Fig. 14g, we acknowledge the difficulty of visualizing IMPs in the full UMAP. We have therefore displayed them together with HSCs only, showing a distinct IMP cluster (Extended Data Fig. 9g):

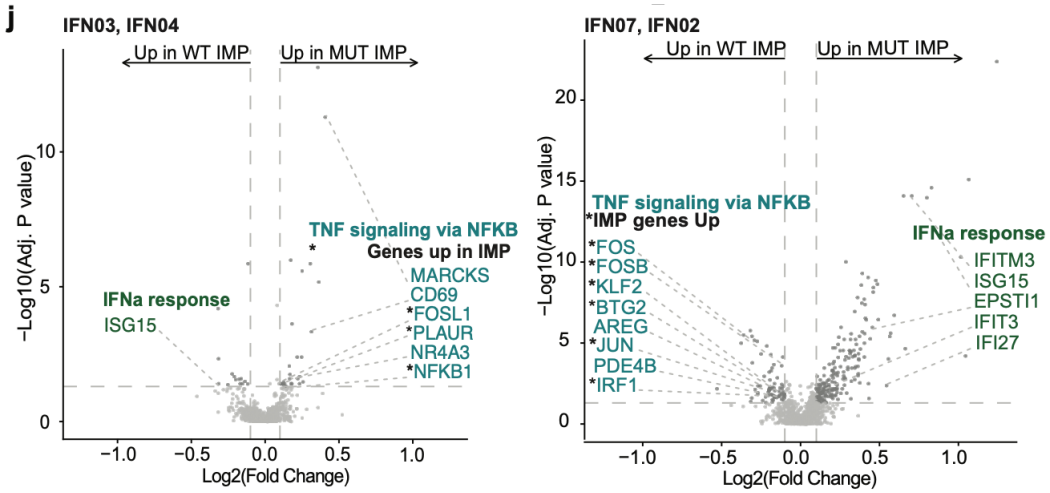


Related to the point above, it seems like many patients included in Fig.6/Ext. Fig.12 and 14 don't present molecular responses (reduction of mutant cell frequency). Authors could compare the differential effect of somatic mutations in the IMP population in responders (IFN03, IFN04) versus non-responders (IFN07, IFN02) to substantiate their claims related to "changes to clonal dynamics could be predicted based on priming of clonal stem cells to differentiate into IMPs".

We thank the reviewer for this suggestion. We have performed the exact differential expression analyses suggested by the reviewer. Mutated IMPs from patients that showed molecular resistance (IFN07, IFN02) downregulated the IMP TF signature (FOS, FOSB, JUN, KLF2, IRF1, NR4A1) and upregulated IFN signaling genes (ISG15, IFITM3, IFI27), whereas mutated IMPs from patients displaying molecular response (IFN03, IFN04) upregulated genes in the NF-kB pathway/IMP signature including FOSL1, NR4A3, NFKB1, and CD69 and downregulated ISG15. These data collectively supported the model wherein mutated stem cells that are poised transcriptionally to develop into IMPs get depleted, whereas mutated HSCs that are poised against IMP development expand upon IFNa therapy. We have included this analysis in Extended Data Fig. 9j (pasted below), and included the following text in the Results section:

"Consistent with this model, differential expression analysis between mutated and wildtype IMPs from patients that displayed clonal expansion upon therapy (IFN02, IFN07) revealed that mutated IMPs downregulated the IMP signature (e.g. *FOS*, *FOSB*, *JUN*, *KLF2*, *IRF1*, *NR4A1*) and upregulated IFN signaling genes (e.g. *ISG15*, *IFITM3*, *IFI27*), potentially reflecting the transcriptional poising against IMP development. The same analysis in IMPs from patients who displayed molecular response (IFN03, IFN04) revealed that mutated IMPs upregulated genes in the NF-kB pathway/IMP signature (including *FOSL1*, *NR4A3*, *NFKB1*, *CD69*) and downregulated *ISG15* (Extended Data Fig. 9j, Supplementary Table 32)."

Extended Data Fig. 9j, showing differential gene expression analysis between mutated and wildtype IMPs from patients with molecular response (left) and those with resistance (right):



2. IFN alpha coordinates anti- and pro-inflammatory transcriptional programs

The terms “inflammation”, “pro-inflammatory” and “anti-inflammatory” are used too broadly across the text, when the authors are referring to completely distinct inflammatory mechanisms such as IFN signaling, TNF alpha and/or NFkB-mediated signaling. In most cases, it is unclear which specific inflammatory pathway (rather than broad “inflammation”) the authors are referring to. This lack of granularity should be clarified across the text and in figures.

We thank the reviewer for highlighting the ambiguity in these terminologies. We fully agree that these terms required qualifications upon their first usage and have endeavored to be clear in their use throughout the manuscript. We have reserved ‘inflammation’ for general settings wherein IFNa would be one of many cytokines, such as infection. For other statements that specify a particular cytokine or downstream pathway we have specified those instead. Furthermore, cytokines involved in various inflammatory processes can function as ‘pro-inflammatory’ and ‘anti-inflammatory’, the latter to counterbalance the overactivation of the pro-inflammatory effects. In accordance with the immunology field at large (e.g. PMID: 30341437), we have used the term “pro-inflammatory”, as the common usage of this term to indicate those of signaling cascades induced by cytokines commonly implicated in aging, inflammatory disorders, and MPN (TNF, IL1, IL6, IL8), whereas anti-inflammatory effects would counter the activities of these cytokines, with IL10 as the classic example (key references included in the manuscript text pasted below). The key insight that our data revealed was that IFNa can act both as a pro-inflammatory cytokine via the induction of IMPs (with elevated NF-kB/AP-1) and as an anti-inflammatory cytokine dampening NF-kB/AP-1 pathways in the HSPCs. We have clarified the use of these terms in the introduction and endeavored to clarify the use of these terms “pro-inflammatory” or “anti-inflammatory” throughout the manuscript.

“MPNs are driven by somatic mutations in *CALR*, *JAK2* or *MPL* that override the highly regulated process of hematopoiesis resulting in an overproduction of one or more myeloid lineages, such as increased platelet production in essential thrombocythemia (ET) or red blood cells in polycythemia vera (PV).[14] Another key facet of ET and PV pathobiology includes elevation of pro-inflammatory cytokines implicated in inflamm-aging[15,16] and autoinflammatory disorders[17,18], such as TNFα, IL-1, IL-6 and IL-8 associated with NF-κB activity (in contrast to anti-inflammatory cytokines such as IL-10[19] that counterbalance these pro-inflammatory cytokines)[20-24]. Intriguingly, in the face of this pro-inflammatory cytokine milieu, antiviral cytokine IFNα therapy is the only clonally selective MPN treatment, often effecting molecular response.[12,13,25] Even in the absence of molecular response, IFNα treatment frequently induces normalization of the patients’ blood counts and amelioration of disease status.[12,13,25]”

For example, authors find upregulation of IFN genes and an increase in inflammatory neutrophils. Therefore, this claim should be rewritten and clarified: “These findings indicated that isolated IFNα exerts an overall anti-inflammatory response in human HSPCs.” (e.g. lines 213-222)

We have modified the text to be more specific in our terminology in accordance with the introductory definitions outlined above:

“These findings indicated that isolated IFNα functions like an anti-inflammatory cytokine in human HSPCs by dampening NF-κB and AP-1 signaling programs.”

HALLMARK genesets (Figure2d) lack sufficient granularity to distinguish the transcriptional effects of distinct inflammatory signaling pathways. For example, authors show downregulation of epithelial mesenchymal transition genes in hematopoietic stem cells, when the actual transcriptional signal is related to JUN, CXCL8 or CD44 expression (according to Table S4). The authors should use genesets specifically related to pro and anti-inflammatory pathways and interferon target genes, ideally in hematopoietic cell types.

We thank the reviewer for highlighting this important point. We have opted to focus on the Hallmark pathways as they have been carefully curated for core sets of genes that are canonical across cell types (PMID: 26771021). Hallmark pathways are frequently deployed in the gene set enrichment analyses of RNA-seq data in our hematopoiesis field (e.g. PMID: 33958784, 38917807, 37666991, 40409258). Furthermore, the highly specific enrichment of the IFNa pathway (including 18 canonical IFN genes in HSCs) is an indication that these gene sets are relevant to HSPCs. The reviewer makes an important point regarding the enrichment of the epithelial mesenchymal transition pathway, which is clearly not relevant to HSPCs. Upon closer examination of the genes

driving this pathway enrichment, we identified that many of these genes overlapped with the TGFb signaling program, which regulates fibrosis and extracellular matrix remodeling, similar to the program observed during EMT. Thus, we included this important clarification in the Results section:

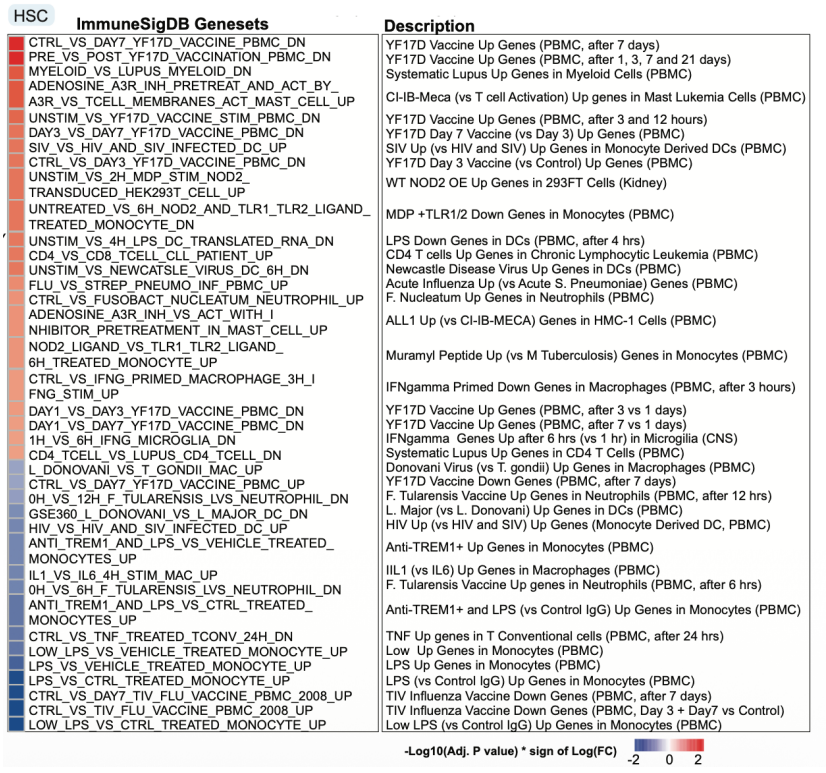
"Though not directly relevant to HSPCs, the epithelial mesenchymal transition pathway was also downregulated, in part due to the overlapping transcriptional program with TGFβ signaling in extracellular matrix remodeling and fibrosis (highlighting the downregulation of the same *TGFB1*, *SERPINE1*, *THBS1* genes as well as *VEGFA* and *CXCL8*; Supplementary Table 6)."

To corroborate the Hallmark pathway results, we have tested the gene sets of the immunological pathways in immune cells using ImuneSigDB, as suggested by the reviewer. These pathways consisted of highly specific experimental condition labels that we have endeavored to decode on the right column for ease of interpretation (pasted below). This analysis yielded similar key pathways including the upregulation of IFN and IFN-related conditions (e.g. viral, vaccine, and lupus–a type 1 interferonopathy) and downregulation of genes that are upregulated upon LPS and TNF treatment (upstream of NF-kB), further corroborating our results using the Hallmark pathways (Extended Data Fig. 2l, Supplementary Table 7).

"We focused on the canonical Hallmark pathways, a highly curated dataset relevant across cell types and frequently utilized in RNA-seq analysis of human hematopoiesis[53-56], and corroborated these results using ImmuneSigDB genesets derived from blood cells (Extended Data Fig. 2l, Supplementary Table 7)."

"These results were corroborated by gene set enrichment analysis with ImmuneSigDB pathways, showing downregulation of TNF target genes (Extended Data Fig. 2l, Supplementary Table 7)."

Extended Data Fig. 2l, showing gene set enrichment analysis using immune cell-specific gene sets:

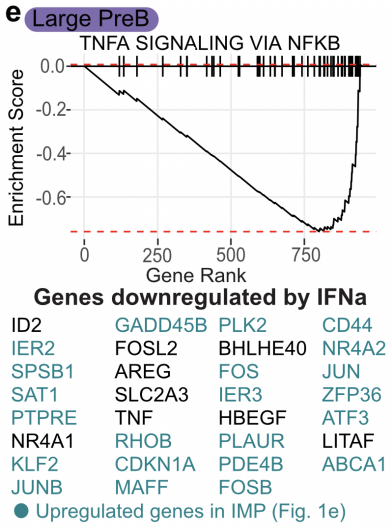


The physiological relevance of a putative anti-inflammatory program in cDCP is unclear (Figure2e), since authors show no changes in cDCP proportions upon IFN alpha treatment (Ext. Fig.3). The authors could choose to show instead another cell type in which an anti-inflammatory program could be relevant for the pathophysiology of the disease (e.g. lymphoid progenitors that are expanded upon IFN alpha treatment).

We thank the reviewer for this comment. We agree that it would be more relevant to include this data for lymphoid progenitors. As suggested, we have included the anti-inflammatory effect in large Pre-B cells (Fig. 2e) and moved the effects on cDCPs to supplementary figures (Extended Data Fig. 2m). We have modified the Results sections as follows:

“Furthermore, in contrast to the pro-inflammatory NF-κB-activated state of the IFNα-associated IMPs, we observed a downregulation of pro-inflammatory pathways, including the TNFα signaling via NF-κB pathway, across the stem and progenitor cells (Fig. 2e; Extended Data Fig. 2l and Supplementary Tables 6-7) and especially pronounced in the classic dendritic cell progenitors (cDCP; Extended Data Fig. 2m and Supplementary Table 6). Downregulated genes in the pro-inflammatory NF-κB-related pathways included the AP-1 subunits, *TNF*, and *CD44* in large pre-B cells (Fig. 2e) and *NFKB1*, *CXCL8* (encoding the pro-inflammatory IL-8), *IL1B* and its receptor *IL1R1* in cDCPs (Extended Data Fig. 2m and Supplementary Tables 5-6).”

Fig. 2e, showing genes downregulated by IFNa, that overlap with IMP signature genes:



Is the NFkB pathway that the authors describe canonical or non-canonical?

We thank the reviewer for raising this important question. We specifically observed the upregulation of NFKB1 (p50) and its regulator NFKBIA of the canonical NF-kB pathway (rather than NFKB2/p52 of the noncanonical pathway). We have included this distinction in the Results section:

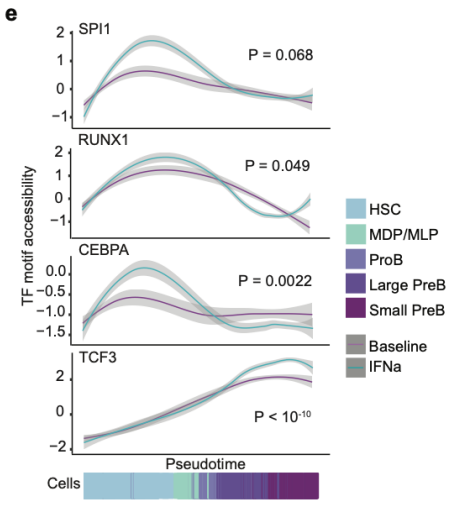
“Downregulation of *NFKB1* (p50) and its regulator *NFKBIA* suggested downregulation of the canonical NF-κB pathway (rather than the noncanonical pathway involving *NFKB2*/p52).[58]”

3. PU.1 is a master regulator of IFN alpha-induced differentiation.

It is unclear why authors show mixed myeloid (GMP) and lymphoid (MLP-proB, preB) trajectories in the same pseudotime (Fig.5e). Authors should treat these lineages independently and should indicate whether the differences in TF motif accessibility shown are statistically significant.

We thank the reviewer for this suggestion. As we are focusing on lymphoid differentiation, we have excluded GMPs from the pseudotime plot in Fig. 5e (pasted below) and have put statistics for IFNa versus baseline TF accessibility in the lymphoid progenitors, as these TFs were initially selected for this analysis because their binding site accessibilities were significantly upregulated in IFNa-treated HSCs (displayed in Fig. 5c).

Fig. 5e, showing differential IFNa vs baseline TF motif enrichment across lymphoid differentiation:



The model proposed in Fig.5f doesn't seem to be supported by the data. The left plot implies increased STAT5 accessibility in HSCs, but STAT5 doesn't appear in Fig.5c. The authors draw a line suggesting higher PU.1 levels lead to IMP development, but they don't show any data supporting this claim.

We thank the reviewer for allowing us to clarify these points. With respect to the question regarding PU.1, we did not identify PU.1 as being significantly differentially accessible when comparing IMPs to HSCs (potentially due to concurrent elevated PU.1 activity in HSCs themselves). However, when we compared the gene expression of IMPs to HSC1 we did observe the upregulation of its gene SPI1 (Extended Data Fig. 3c). Initially, we did not highlight this finding, but have modified the text in this revised version. Further, the regulatory peaks of RFX2/3 included the binding motif of PU.1, providing evidence for its role in upregulating these TF gene expressions (Extended Data Fig. 5a-c). We have pasted the relevant text below:

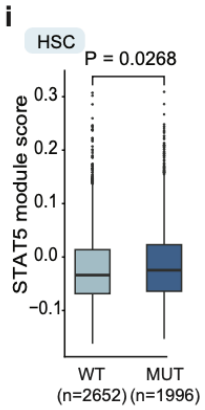
"To define the transcriptional state transitions from HSC1 to IMPs, we compared IMPs to HSC1 and identified a reinforcement of the *PU.1*, *RFX3*, *AP-1*, *CEBPB/D*, and *KLF* family TF expressions and downregulation of *NR4A2* (Fig. 3a, right, and Extended Data Fig. 3c; Supplementary Table 9; only IFNα-treated cells included in the differential expression analysis)"

"To determine which TFs upregulated the gene expressions of RFX2/3, we determined TF motifs present in the regulatory peaks that correlated with their gene expression (i.e., linked peaks analysis).[76] In the cis-regulatory region of RFX3, we identified the binding motifs of IRF1, FOSB and PU.1, which were overexpressed in IMPs (Extended Data Fig. 5a, b; Supplementary Table 15). Likewise, the most significant regulatory regions for RFX2 included motifs for PU.1, KLF factors, NR4A1/2 and AP-1 subunit factors (Extended Data Fig. 5a, c; Supplementary Table 15)."

The JAK2/STAT5 activity shown in the model in Fig. 5f was primarily based on our canonical understanding of MPN as being driven by overactivated JAK2/STAT5 pathway, as well as the important work that revealed mutant CALR induces JAK/STAT pathway (PMID: 26608331, 26951227, 26817954, 26668133). These studies revealed that the mutant CALR activates the thrombopoietin receptor (MPL), thereby activating the downstream JAK2/STAT5 pathway and the myeloid differentiation bias, as depicted in the model. STAT5 was not among the most differentially accessible TF motif between CALR mutant and wildtype cells, which was consistent with our published GoT data of CALR-mutated HSCs in which differential expression analysis of mutant and wildtype HSCs from the same individual did not yield JAK/STAT pathways as the most significant pathway (PMID: 31270458). To clarify this point, we have included the above references that revealed the upregulation of JAK/STAT targets by CALR mutations. We also included data showing that mutant CALR modestly upregulates STAT5 target genes (Wierenga_STAT5A_targets_group2, C2: Chemical and Genetic Perturbations in MsigDB; Extended Data Fig. 7i, pasted below):

"Altogether, these data indicated that IFNα therapy remodels hematopoietic differentiation hierarchy from the major bifurcation between lymphoid[93] versus granulo-monocytic + megakaryocytic-erythroid[94] (latter enhanced by JAK2/STAT5 activity in *CALR*-mutated MPN[95-98], Extended Data Fig. 7i), to the bifurcation at PU.1-regulated lymphoid + granulo-monocytic[99] versus megakaryocytic-erythroid commitments (Fig. 5f)."

Extended Data Fig. 7i, showing modest increase in STAT5 targets by CALR mutations in baseline HSCs:



Conclusions and interpretation:

- Lines 267-268: “These data suggested that IFNα may induce an alternate path for accelerated neutrophil development from HSCs.” INF alpha might bias HSCs towards neutrophilic differentiation, but the data doesn’t probe an alternate differentiation pathway.

We thank the reviewer for highlighting this point and regret that we could have been more precise in our interpretation. This prior sentence was referring to the single cell CFU data in which we observed neutrophil only colonies more frequently in the IFNa-treated samples. These data suggested an early neutrophilic lineage commitment by HSCs, rather than the typical passage through a bipotent progenitor stage (which would yield both monocytes and neutrophils). We have modified the sentence to reflect this particular point:

“These data suggested that IFNα may induce an early neutrophilic commitment by HSCs potentially via IMPs.”

- Line 301: “These data implicated IMP development as a feature of HSC aging.” This is too speculative. The text should focus on the effect of inflammation in IMPs, and not broadly as an aging-related phenomena.

We agree with the reviewer that the link with aging is beyond the scope of this work and have removed this text from the Results section.

- Lines 585–587 and lines 601-604: The “alternate route” of differentiation, and specially, prediction of clonal dynamics by IMPs, are overstated.

We thank the reviewer for the opportunity to expound upon this point. With respect to our usage of ‘alternate route of differentiation’, we would like to highlight the logical conclusion that identification of IMP itself indicates that the hematopoietic stem cells traverse through this alternate state along its differentiation path, and are therefore taking an ‘alternate route of differentiation.’ As the reviewer #3 pointed out, our model of clonal dynamics by IMPs is a reasonable conclusion based on our data showing that propensity for IMP development (transcriptionally and by mutant cell enrichment in IMP/HSC1) correlates with mutant clone size change following treatment. Nonetheless, since there are likely other contributing factors and it is not possible to demonstrate cause-and-effect using our human data, we have modified these statements accordingly:

“Altogether, our results supported a unified model of clonal dynamics wherein the resistance of HSC clones to differentiate into IMPs may contribute to clonal fitness and expansion in the stem cell niche under inflammation (Fig. 6p).”

“Instead, our data provided evidence for a model wherein IFNα-induced IMP route of differentiation from HSCs may provide the means to perturb steady-state clonal competition.”

We have also included the following in the Discussion to clarify the need for functional studies to determine causality.

“Developing an *in vivo* model of IMPs would also enable us to determine the mechanisms of IMP development and identify whether IFNα directly induces IMPs in a subset of poised HSCs or IMP generation is mediated by

another molecule with or subsequent to IFNα activation. Such an *in vivo* model would also facilitate a platform to causally determine the relationship between clonal dynamics and IMP differentiation rates."

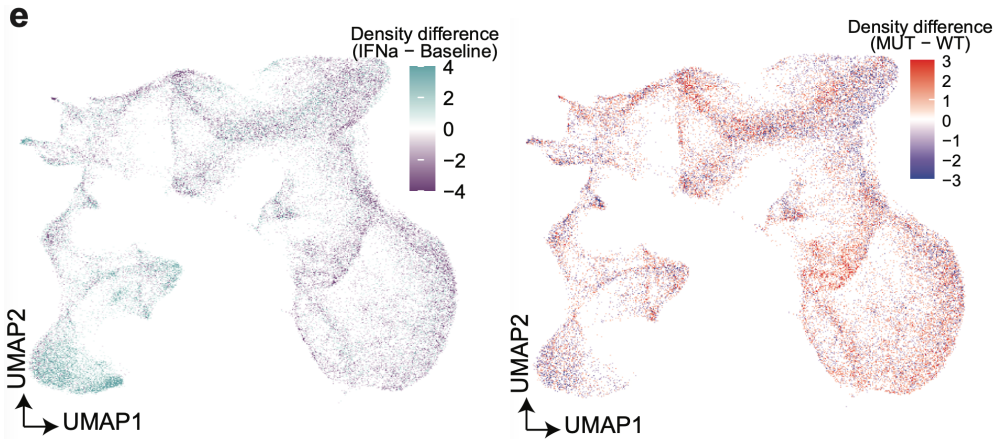
Minor comments:

Figure and text clarity. The authors do a fantastic job at presenting their complex dataset in an accessible format. However, there are specific figures and parts of the text that should be clarified:

We thank the reviewer for commenting positively on our data presentation. We have addressed each of the critiques that have served to improve the clarity of the work.

Figure 1c. Treatment status and somatic genotypes with so many cells are hard to visualize. I would suggest density subtraction plots to highlight treatment and genotype-specific regions.

We thank the reviewer for this suggestion. The primary function of Fig. 1c was to clearly demonstrate the multi-omics nature of our single cell data, with whole transcriptomics, treatment status and genotyping data for the same thousands of cells (while accurately depicting the associated cell numbers on these plots). We did not mean for the readers to derive any understanding of cell type enrichment from this data. Nonetheless, we agree that the density subtraction plots would be an effective visualization of our results and have included them in Extended Data Fig. 1e, highlighting the enrichment of IMPs and lymphoid progenitors in IFNa treated samples and the enrichment of mutated cells in the myeloid lineages:



Extended Data Fig.2 e and f panels should be relabeled for clarify. What is the overall cell number stated below panel e? In Ext. Figure 2f, what does the y-axis label refer to?

Thank you for this helpful suggestion. We had included overall CD34+ cell number per sample in Extended Data Fig 2e (current 1l) to indicate that the identification of IMPs in the treated samples was independent of the total CD34+ cells captured and to show that for IFN07, IMPs were not identified due to the low number of CD34+ cells. For Extended Data Fig. 2f (current 1m), the y-axis shows the total CD34+ cell count per sample, similarly to indicate that the enrichment of IMPs in treated samples was not because we captured more CD34+ cells in the treated samples compared to baseline. We modified the labels and added the following text to clarify:

"Consistent with the identification of IMPs in this IFNα-treated cohort of patients, IMPs derived predominately from the IFNα-treated samples with elevated frequencies in the IFNα-treated versus baseline $CD34^+$ cells (Fig. 1f; paired samples in Extended Data Fig. 1l; overall $CD34^+$ cells per sample in Extended Data Fig. 1m, showing no difference between baseline and treated samples)."

Extended Data Figs. 1 and m, with clear labels:

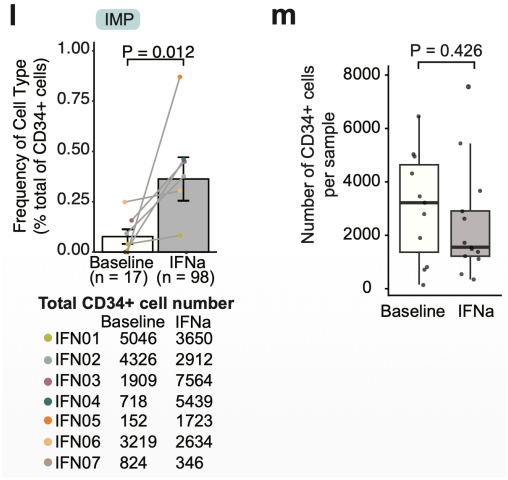
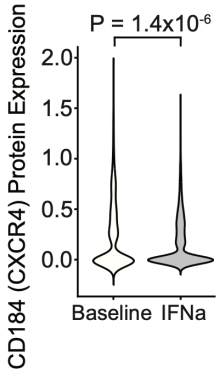


Figure 2c doesn't belong in Figure 2. The message of increased cell cycle and decreased quiescence (through reduced CXCR4 expression) could belong in Figure 1 of Ext. Fig.3. The same is true for Ext. Fig.4d. The boxplot displaying CD184 expression suggests no difference in expression despite a significant p-value. The authors could choose a different type of plot or remove it completely.

We thank the reviewer for this suggestion. We agree that CXCR4 expression analysis would go well with the cell cycle analysis in Fig 1. However, because the identification of CXCR4 downregulation by IFNa only emerged upon unbiased differential expression analyses shown in Fig. 2, we have opted to keep it in this section, as a logical progression of the analysis. We thank the reviewer for the suggestion to use a different plot and found that the violin plot showing the density of the cells at each expression level highlighted the predominance of cells with CXCR4 expression levels at 0 or <0.5 normalized counts in the IFNa-treated samples compared to baseline (current Extended Data Fig. 2i):



o In Ext. Fig.9 and Ext. Fig.10 authors should indicate where the motif of each TF is located within the peak.

We thank the reviewer for this suggestion. We have included the precise coordinates of all TFs within the peaks in the Supplementary Tables 15, 16. As it would be challenging to precisely indicate where all of these TFs are located, we have re-ordered the TFs on the figures to reflect their positional order in the peaks.

o Lines 342-358 and Ext. Fig.10a/b. What is the relevance of MHC class II gene regulation? The authors should clarify.

We thank the reviewer for the opportunity to clarify this emphasis on MHC class II gene regulation. To determine what genes may be differentially regulated during the HSC1 to IMP transition, we performed a differential gene expression analysis between HSC1 and IMPs. This analysis revealed that IMPs downregulated MHC class II genes (CD74, HLA-DPA1, HLA-DRB1, HLA-DPB1), which suggested a differentiation into myeloid effector cells (vs. antigen presenting cells). In combination with upregulation of MPO and G-CSF receptor gene expressions in IMPs and expansion of CFU-G upon RFX3 overexpression, it also supported a neutrophil differentiation bias, rather than toward antigen presenting cells such as those of the monocytic/dendritic lineages. Additionally, as previous studies have found that other members of the RFX TF family regulate the HLA-DR locus, we

hypothesized that RFX2/3 may also regulate HLA-DR expression in IMPs. We have clarified the motivation for highlighting the regulation of MHC class II genes:

"Furthermore, as other RFX members (i.e., RFX1 and RFX8) play key regulatory roles in MHC class II expression,[77-79] we hypothesized that RFX2/3 may play a role in downregulating the MHC class II genes in IMPs (as observed in Extended Data Fig. 3c), potentially highlighting their route to becoming myeloid effector cells, rather than antigen presenting cells."

o Lines 580-585. The relevance of a IMPDN gene signature in DNMT3A-mutated HSCs is unclear. Are these DNMT3A CH individual being treated with IFN alpha? Is there any evidence of increased IFN alpha in their plasma?

Thank you for the opportunity to clarify this point. Although individuals with DNMT3A-mutated CH are not currently treated in general, IFNa exposure from infection or other inflammation-related processes may alter the clonal dynamics of DNMT3A-mutated CH cells. As DNMT3A-mutant clones frequently expand in patients with MPN upon IFNa treatment (PMID:34507355), we tested whether DNMT3A-mutated CH HSCs may be poised with a transcriptional state that may reflect its resistance to IMP development by assessing the expression of genes downregulated in IMPs and identified that this was the case. As reviewer #2 pointed out, this finding was analogous to and supported by a work in mice showing that DNMT3A-mutated HSC resisted differentiation upon IFN exposure, supporting its clonal expansion (PMID: 33743191). We have modified the text to clarify this point:

"Further, we assessed whether this model may apply to clonal hematopoiesis under inflammatory settings with enhanced IFN signaling (e.g. infection), as *DNMT3A*-mutated clones are often resistant to and expand upon IFNα treatment in patients with MPN.[25] We examined the IMP^{DN} gene signature in *DNMT3A*-mutated HSCs from individuals with clonal hematopoiesis (data from our previous publication[48]). Consistently, the *DNMT3A*-mutated HSCs demonstrated increased expression of the IMP^{DN} gene signature compared to the admixed wildtype HSCs (Fig. 6o), suggesting that the *DNMT3A*-mutated HSCs may be poised to resist IMP development upon increased IFN activity due to various inflammatory settings, such as infection."

o Lines 626-628: the relevance of COVID-19 individuals' responses in the context of IFN alpha therapy is unclear and should be clarified.

We thank the reviewer for the opportunity to clarify this important point and apologize for the lack of clarity. We had meant to link our findings to a series of interesting papers published in Science, first of which demonstrated that the peripheral blood of patients with severe and critical COVID-19 showed evidence of impaired type 1 IFN response, with enhanced pro-inflammatory NF-kB activity associated with elevated TNF and IL-6 (PMID: 32661059). Subsequent studies corroborated this finding, showing that patients with an inborn error of type 1 IFN immunity (PMID: 32972995) and those with autoantibodies against type 1 IFN suffered life-threatening COVID-19 (PMID: 32972996). While the primary role of type 1 IFN is likely to control the virus itself, the absence of the anti-inflammatory effects of IFNa signaling may also contribute to the exaggerated pro-inflammatory immune response. We have clarified this point in the Discussion:

"Thus, our studies clarify that a predominant effect of sustained IFNα in human HSPCs is the downregulation of downstream pathways involved in TNFα signaling via AP-1 and NF-κB, consistent with amelioration of the pro-inflammatory state in MPN and multiple sclerosis upon type 1 IFN administration.[27,129] The observed downregulation of the pro-inflammatory NF-κB activity in HSPCs and lymphoid differentiation shift may also be consistent with prior observations that patients with deficiencies in type 1 IFN response (due to germline inborn errors of type 1 IFN or antibodies against IFNa) exhibit severe and life-threatening COVID-19.[133-135] The deficiency in type 1 IFN response was associated with elevated TNF and IL6 mediated via NF-kB.[133] Thus, while the primary role of type 1 IFN may be to control the virus itself, the exaggerated pro-inflammatory NF-kB-mediated response may also potentially be due to the lack of anti-inflammatory effects of IFNa. Coherently, *TNF* expression was demonstrated to be decreased in patients with MPN who received IFNα treatment.[136]

Analytical:

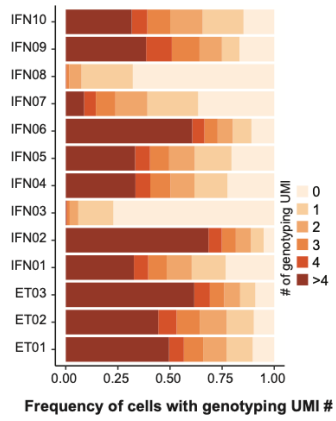
- It is unclear the number of cells used for each analysis for each figure. Authors should label the number of cells per group in their boxplots across the manuscript.

We thank the reviewer for this suggestion to enhance the clarity of the analyses. We have added cell numbers of boxplots on the figures or in the legend (due to space constraints) throughout the manuscript.

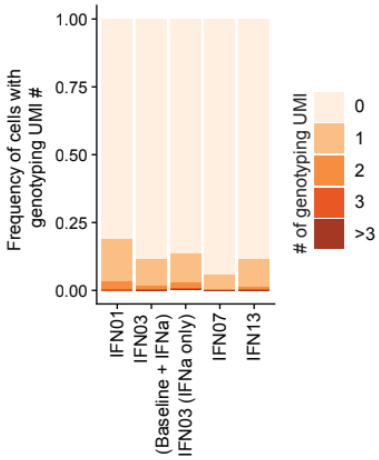
- The results of CALR and JAK2 genotyping calls should be included in a supplementary figure, similarly to those related to transcriptome and chromatin accessibility quality controls. What's the accuracy of genotyping? Do the number of mutant cells match the expected VAF of the CALR and JAK2 mutation based on bulk sequencing assays? (which the authors have in Table S1).

We thank the reviewer for these suggestions. We have included data for genotyping UMI counts (number of unique genotyping transcripts captured) per cell across the samples for GoT (Extended Data Fig. 1b) and GoT-ATAC (Extended Data Fig. 4f), and number of genotyping DNA fragments for GoT-ChA (Extended Data Fig. 10f). In our previous manuscript that described the GoT method, we had performed a species-mixing experiment with murine cells expressing mutant CALR transgene and the human cells expressing wildtype CALR, providing ground truth data to determine the accuracy and optimize the analytic pipeline, and thereby validating this approach for CALR mutations (PMID: 31270458). Similarly, the authors of GoT-ChA performed a cell line experiment to demonstrate the accuracy of genotyping JAK2 mutations by determining the frequency of mutant and wildtype cells in the cell lines with known mutational status (PMID: 38720070). In primary samples, accuracy can also be estimated in settings where mutations are restricted to the cancerous cells that are transcriptionally distinct from admixed non-neoplastic cells. For example, in our recent manuscript where we describe a genotyping method in probe-based scRNA-seq, we called GoT-Multi (PMID: 41075791), we applied GoT-Multi to primary large B-cell lymphomas and could determine the rate of false positive calls in non-neoplastic T-cells or myeloid cells (as the lymphoma driver mutations were restricted to the B-cell lymphoma cells). However, determining the accuracy of genotyping is challenging in MPN, as mutant and wildtype cells are present in all cellular compartments and cannot be distinguished from each other by gene expression. Nonetheless, as we are replicating the same experimental and analytical procedures from the published methods manuscripts, we anticipate a similar rate of high accuracy in these samples. Further, in our original GoT manuscript, we identified a clear enrichment of CALR-mutant cells in the myeloid progenitor compartments (i.e. megakaryocytic-erythroid and granulomonocytic progenitor lineages) and relative depletion in the lymphoid progenitor lineages (that we validated by performing ddPCR for CALR mutations in sorted HSPC compartments, PMID: 31270458). This enrichment was recapitulated in our data (Fig. 6a,b; Extended Data Fig. 8e), supporting the accuracy of these GoT experiments.

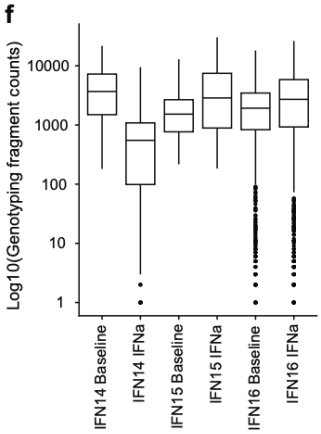
Extended Data Fig. 1a (right), showing proportions of cells binned based on number of genotyping UMI in GoT-IM:



Extended Data Fig. 4f, showing proportions of cells binned based on number of genotyping UMI in GoT-ATAC:

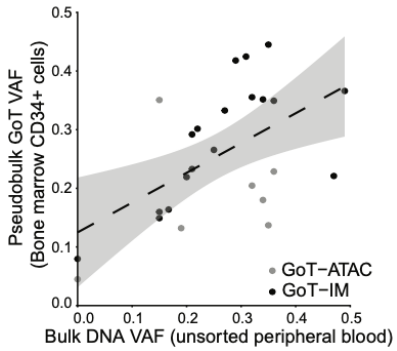


Extended Data Fig. 10f, showing genotyping fragment counts per genotyped cells:

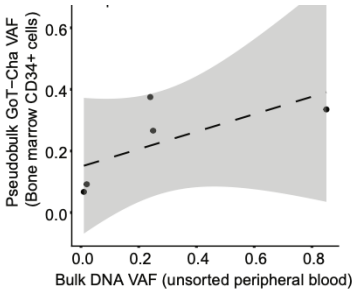


We agree that comparing the expected mutant cell frequency based on VAF from bulk sequencing versus based on single cell genotyping is an important assessment that provides evidence of the accuracy of these methods. As CALR mutations are known to be heterozygous, we divided the number of mutant cells by half to determine the pseudobulk VAF (Extended Data Fig. 4g, pasted below). A notably caveat is that bulk sequencing was performed on peripheral blood without cell fractionation, as compared to single cell genotyping on CD34+ cells for GoT-IM and GoT-ATAC, which would impact the VAFs since the mutant cell frequencies vary in different cell types. Still, the two mutant cell frequency measurements were well correlated (P = 0.01). As JAK2 mutations are frequently present as both homozygous and heterozygous clones within the same neoplasm (PMID: 22898600), we leveraged the ability of GoT-ChA to make homozygous and heterozygous calls to determine the pseudobulk VAF, which correlated well with the bulk DNA VAF on unfractionated peripheral blood cells (VAFs of 5 of 6 samples available, Extended Data Fig. 10g, pasted below).

Extended Data Fig. 4g, showing correlation of bulk VAF versus pseudobulk VAF derived from GoT-IM and GoT-ATAC:



Extended Data Fig. 10g, showing correlation of bulk VAF versus pseudobulk VAF derived from GoT-IM and GoT-ATAC:



- Code to generate main figures from the GEO data should be made available to readers by the time of publication.

We agree with the reviewer and will upload the code used for generation of main figures by the time of the publication.

- In GoT-ATAC, the first dimension was excluded (line 1569). This requires justification.

We thank the reviewer for this point. In this revised version, we have included the justification that the first dimension was removed per standard practice given that it captures technical variation due to sequencing depth rather than biological differences (PMID: 34725479).

"The first LSI component was removed as it represents technical variation due to sequencing depth rather than biological differences.[162]"

- Methods section on retrospective flow cytometry data should be rewritten and clarified. Why do the authors need to detect antibody panels in the first place and what do they mean by unused flow cytometry channels? Why do authors create ensembl FCS files?

Thank you for the requests for clarification. We regret that we should have been clearer in our description of this analysis, especially related to the nature of the clinical flow cytometry files that were retrospectively analyzed. In the clinical flow cytometry lab, several panels of antibodies are utilized for each sample. Therefore, we wrote a software that detects the flow cytometry markers of interest to select the files with the relevant antibody panels for further downstream analysis. By unused flow cytometry channels, we meant that if a flow cytometer has 10 fluorescence channels but only 8 channels were utilized for fluorescent antibodies, the additional two channels were excluded. We agree that such a detail would create confusion and is beyond the scope of the methods section and therefore removed. We further expound upon the creation of the ensemble FCS files. Ensemble FCS files were used to make the UMAPs consistent across all the FCS files (such that cells of a given type co-localize within the UMAP dimensional reduction space for each FCS file). The ensemble FCS file is a concatenation of all the FCS files corresponding to a given antibody panel, and thus contains a sampling of most of the range of immunophenotypes in the data files. With this, a UMAP embedding can be calculated and applied to every FSC file. Thus, all the FCS data map to the same UMAP embedding and the cells with the same immunophenotype will co-localize in the UMAP embedding. We have modified the relevant parts of the Methods section to simplify and clarify the description of the work:

"Custom software was developed using python, FlowKit[171] and umap-learn[155] to process the raw clinical flow cytometry data files (FCS files). Data processing involved selecting the antibody panels of interest (i.e. the ones with CD34, TdT, CD19 antibodies), as more than one antibody panel may have been run on a given sample. A UMAP embedding was created that corresponded to the antibody panel and could be applied to each individual FCS file so that cells of a given immunophenotype were co-localized in the UMAP plots for all of the FCS files. The ensemble UMAP was calculated using a subsampling of each of the FCS files in order to present the full spectrum of immunophenotypes to the UMAP algorithm for calculating the embedding. The various channels

used in the UMAP embedding included the forward scatter height (FSC-H), side scatter height (SSC-H), and channels corresponding to each of the fluorescent markers. The normalization factors and UMAP embeddings were then applied to all the individual files. Modified FCS files were created that included the UMAPs as additional channels for subsequent evaluation and gating using FlowJo software (v10.8.1, FlowJo LLC, Ashland, Oregon, USA).

Using FlowJo, appropriate gates were determined based on the clusters in the UMAP plots and their expression of protein markers. Additional standard gating was also performed using the original data channels. UMAP gates were determined following exclusion of doublets and non-viable cells via standard flow cytometry gating approaches. Once the UMAP gates were determined upon sufficient segregation of cell populations and verified to encompass cells of the same or similar type, they were applied using a template to all the FCS files of the given antibody panel."

- Table S6 – GSEA GMP states and GSEA IMP states "Table S11…"

We thank the reviewer for a careful review. We have corrected it in the new updated supplementary tables.

- Supplementary Table S5 – please include the number of cells represented in each cell type. For example, there's an IMP DEA but untreated samples shouldn't have IMPs?

We have included the cell numbers. We agree that most of the IMPs are derived from treated samples, and that the few IMPs in the baseline samples underpower the analysis and have removed this from the table.

Reviewer #2 (Remarks to the Author):

In this study, Lama, Isakov et al describe serial single cell multiomic analysis of human bone marrow from patients with MPN, a mosaic condition involving expansion of a mutant HSC clone that promotes megakaryocyte/erythroid development. They use single cell analysis to compare the response by WT versus MUT clones to IFNa therapy, which is known to select for WT clones. They utilize transcriptome, ATAC, and CUT&RUN analysis to identify key TF pathways induced by IFNa and changes in differentiation trajectories in WT versus MUT subtypes that help to explain clonal selection. Specifically, they show that PU.1 is a critical transcription factor downstream of IFNa that promotes changes in differentiation trajectory, with emergence of a myeloid progenitor state called IMP. CALR mutant clones have increased chromatin accessibility for PU.1 and are therefore more prone to IMP differentiation, leading to a long term advantage for WT clones. The authors compared their data to patients who received hydroxyurea and to patients with JAK2 mutations to strengthen and validate their findings.

Overall this is a technically advanced and expansive study that provides new insight into mechanisms of IFNa activity and clonal selection. The study answers a number of long standing questions in the field. However, a number of claims are not strongly supported by the data and some areas of clarification are needed.

We thank the reviewer for commenting favorably on the technical advancements and highlighting the expansive nature of this work. We are grateful to the reviewer for expressing that these discoveries have addressed long standing questions in the field. We are also grateful for the thoughtful critiques that have served to improve the manuscript significantly. We hope to have addressed each critique fully.

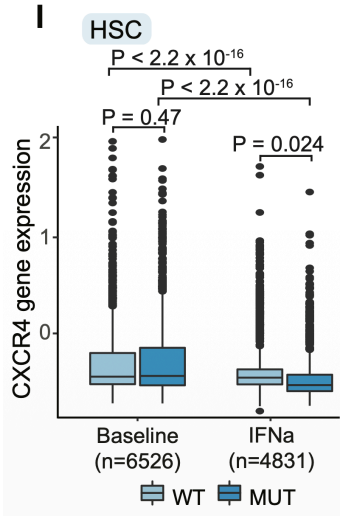
Specific comments:

1) It is interesting that downregulation of CXCR4 happened in response to IFNa, as this may provide mechanism of increased cell division. Was there a differential effect of CXCR4 downregulation when comparing WT and MUT cells?

We thank the reviewer for this compelling question. We also found the downregulation of CXCR4 to be quite interesting and consistent with the activation of HSCs into cell cycle entry. The reviewer's question keenly asks whether the differential rates of cell cycle entries observed in mutated versus wildtype HSCs are consistent with the CXCR4 expression. Indeed, we observed that CXCR4 expression was more significantly decreased in the

mutated HSCs compared to wild type upon IFNa treatment, consistent with the higher rates of cell cycle entry by mutated HSCs. We have included this important analysis in the Results sections:

"Across the patients regardless of clonal dynamics, IFNα induced greater rates of cell cycle entry of CALR-mutated HSCs compared to the admixed wildtype counterparts (Fig. 6h and Extended Data Fig. 8k). Consistently, IFNα-treated mutated HSCs downregulated *CXCR4* to a greater extent compared to wildtype HSCs (Extended Data Fig. 8l)."



2) Figure 2E: the authors state that: " These findings indicated that isolated IFNα exerts an overall anti-inflammatory response in human HSPCs." This statement is inaccurate since interferon pathways (which are inflammatory) are massively upregulated; whereas the statement is just referring to TNFa.

We thank the reviewer for the opportunity to clarify our language throughout the manuscript. Indeed, IFNa is one of the inflammatory cytokines, and thus our use of 'pro-inflammatory' and 'anti-inflammatory' required clear qualifications upon their first usage. Cytokines involved in various inflammatory processes can function as 'pro-inflammatory' and 'anti-inflammatory', to counterbalance the overactivation of the pro-inflammatory effects. Specifically, we have used the term "pro-inflammatory", as a widely used terminology to indicate those of signaling cascades induced by cytokines commonly implicated in aging, inflammatory disorders, and MPN (e.g. TNF, IL1, IL6, IL8), whereas anti-inflammatory cytokines would counter the activities of these cytokines, e.g. IL10 as the classic example (e.g. PMID: 30341437). The insight that our data highlighted was that IFNa can act both as a pro-inflammatory cytokine via the induction of IMPs and as an anti-inflammatory cytokine dampening NF-kB/AP-1 pathways in the HSPCs. We have clarified this important point in the introduction and qualified these adjectives throughout the manuscript. The introduction now includes:

"MPNs are driven by somatic mutations in *CALR*, *JAK2* or *MPL* that override the highly regulated process of hematopoiesis resulting in an overproduction of one or more myeloid lineages, such as increased platelet production in essential thrombocythemia (ET) or red blood cells in polycythemia vera (PV).[14] Another key facet of ET and PV pathobiology includes elevation of pro-inflammatory cytokines implicated in inflamm-aging[15,16] and autoinflammatory disorders[17,18], such as TNFα, IL-1, IL-6 and IL-8 associated with NF-κB activity (in contrast to anti-inflammatory cytokines such as IL-10[19] that counterbalance these pro-inflammatory cytokines)[20-24]. Intriguingly, in the face of this pro-inflammatory cytokine milieu, antiviral cytokine IFNα therapy is the only clonally selective MPN treatment, often effecting molecular response.[12,13,25] Even in the absence of molecular response, IFNα treatment frequently induces normalization of the patients' blood counts and amelioration of disease status.[12,13,25]"

Within the Results section:

"These findings indicated that isolated IFNα functions as an anti-inflammatory cytokine in human HSPCs by dampening NF-κB and AP-1 transcriptional programs."

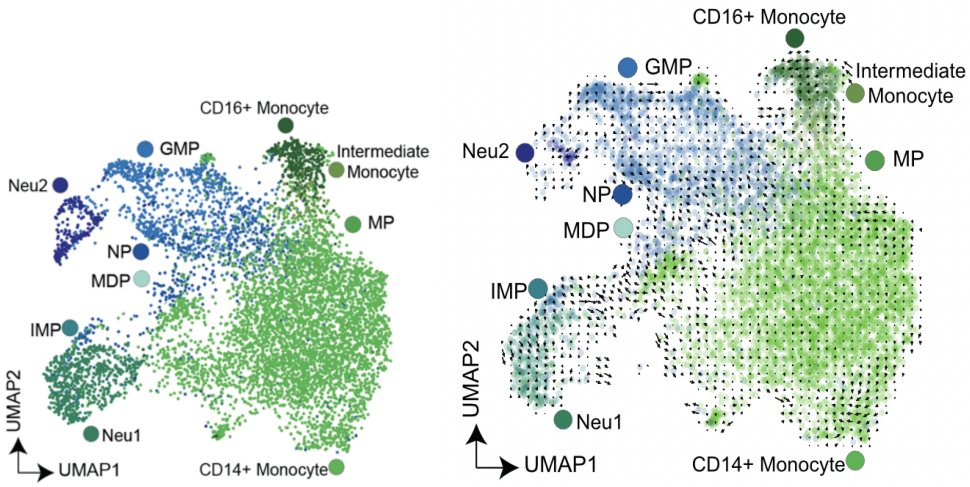
"Given the induction of IMP differentiation with upregulation of AP-1 and NF-κB targets, these findings suggested that isolated IFNα initiates both *anti-inflammatory* (AP-1/NF-κB^{low}) and *pro-inflammatory* (AP-1/NF-κB^{high}) states – both occurring in parallel within the same individual's hematopoiesis."

3) Regarding GoT and derivation of neutrophil-only colonies on methylcellulose assay, the authors write (lines 285-286): "Overall, these data demonstrated that IFNα expanded the inflammatory neutrophils (relative to other neutrophils), consistent with the induction of an alternate route of inflammatory myeloid differentiation." They imply that there is a direct route to neutrophil production. Where do the neutrophils lie on the pseudotime plot in 3a and is there any evidence of neutrophils that can derive more closely from IMPs?

Thank you for this important question and for the opportunity to clarify our results. To address a similar question of which mature myeloid cell may cluster with IMPs, we had previously integrated myeloid progenitor cells and mature myeloid cells Extended Data Fig. 3j (pasted below, left), where we observed that IMPs co-clustered with the inflammatory neutrophils (Neu1), whereas the conventional neutrophils (Neu2) clustered with GMPs and neutrophil progenitors (NP). Following the reviewer's suggestion, we performed additional RNA velocity analysis on this data (pasted below, right). RNA velocity further revealed that IMPs were predicted to lead to inflammatory neutrophils. These findings provided evidence for derivation of inflammatory neutrophils from IMPs. We have included these Results:

"Consistently, unsupervised co-embedding of the myeloid progenitors with the mature myeloid compartment revealed that IMPs clustered with the Neu1 subset, and by RNA velocity measurements, IMPs were predicted to differentiate into Neu1 (Extended Data Fig. 3j)."

Extended Data Fig. 3j, showing co-clustering of IMP and Neu1, and RNA velocity vectors from IMPs pointing to Neu1:



While these data provide important evidence in primary human data, we acknowledge that further studies are required to trace the lineage output of IMPs. We are currently working on establishing an in vivo model of IMPs (mouse and xenograft) whereby we can retrace the origins and outputs of this novel IMP population. We have included the following in the Discussion:

"The upregulation neutrophil lineage markers (e.g. *MPO*, *CSF3R*) and downregulation of antigen presenting molecules suggested a neutrophilic differentiation bias of IMPs. Concerted expression and chromatin accessible binding sites of immediate early response TFs and CEBPB/D, emergency granulopoiesis TFs, nominated IMPs as a means to rapidly generate inflammatory neutrophils (which harbor the IMP TF signature). Single cell colony output of neutrophil only colonies enriched in IFNα-treated bone marrow suggested an early uni-lineage commitment by HSCs, supporting this model. As additional support for a neutrophilic-biased differentiation program of IMPs, overexpression of RFX3 induced an IMP-like population and increased CFU-granulocytic colonies. However, lineage tracing methods via an *in vivo* model of IMPs would allow us to trace the lineage outputs of IMP and the relative contributions of neutrophils from IMPs versus GMPs. Nonetheless, the

identification of the IMP population itself indicates that HSCs traverse an alternate differentiation course (whether this route includes GMPs or not)."

4) Lines 290-292. The authors find that the inflammatory signature is higher in samples after 3-4 weeks off IFNa therapy "suggesting the reinforcement of the inflammatory signature following exposure". This is hard to understand biologically. Why would an inflammatory signature increase after cessation of the therapy. Are they referring to the TNFa signature (similar to #2 above)? Use of the word "inflammatory" is really too general. Alternatively, can the authors assure that these patients did not have recurrence of disease or other confounding clinical variables at this time such as intercurrent viral infection?

We regret that we could have been clearer in our text. Indeed, we were referring specifically to the HSC1/IMP 'pro-inflammatory' signature with upregulation of NF-kB/AP-1. Following IFNa discontinuation, the HSC1 population expressed higher NF-kB/AP-1 transcriptional program than HSC1 from baseline (pre-treated) bone marrow, suggesting a positive reinforcement of this cell state upon exposure. This finding was consistent with the observation that HSC1/IMP frequencies showed a trending increase with age (i.e. with more time and opportunity for exposure to various inflammatory insults involving IFNa). We have clarified this text accordingly:

"Intriguingly, post-therapy HSC1 subset retained its elevated cell state program following IFNα exposure (i.e. differentially upregulated genes in HSC1 versus HSC2/3, Fig. 3h and Extended Data Fig. 3k). Consistent with the maintenance of the HSC1 gene signature and presence of residual IFNα signaling following therapy discontinuation, IMPs were identified in these post-therapy samples (0.3-1.46%, Supplementary Table 2). HSC1 and IMP frequencies at baseline showed a trending increase with age (Fig. 3i), and HSC1 exhibited a higher aging gene signature compared to the other HSCs (Extended Data Fig. 3l), suggesting accumulation of HSC1 and IMP over time with chronic inflammation.[69]"

However, the reviewer raises an important point that there may be other confounding factors that may have influenced these findings. While we do not have information on the status of potential viral illnesses, we checked the disease state of these patients and identified similar clinical findings (i.e. blood counts, spleen size, bone marrow cellularity) during active and post IFNa therapy, suggesting no significant change in their disease status. We included this important point in the Results text:

"There was no evidence of disease rebound upon discontinuation with respect to the patient's blood counts or other parameters such as spleen size or bone marrow cellularity (Supplementary Table 1)."

5) Lines 300-301: The authors write: "These data implicated IMP development as a feature of HSC aging." This is a pretty strong statement to make based on a small sample set of people with MPN. This statement would need to be validated in an aging cohort.

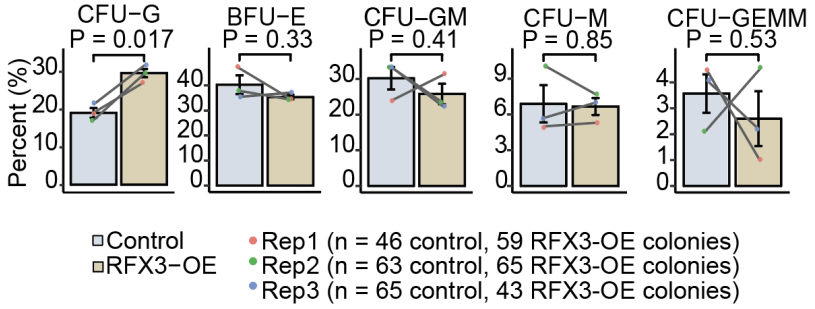
We thank the reviewer for this feedback, and agree this assertion is beyond the scope of this current work. We have removed this statement from the text.

6) Lines 363-366: "RFX3 overexpression (but not RFX2) expanded the granulocytic colonies (CFU-G) and diminished the erythroid colonies (BFU-E) compared to control-vector transduced CD34+ cells." Does transient overexpression of RFX3 also confer this effect or does the RFX3 need to be continually expressed?

We thank the reviewer for this interesting question of whether CFU-G biased differentiation requires a sustained RFX3 expression, or whether a transient expression in the early progenitors would be sufficient. To test if transient overexpression of RFX3 confers similar effects to sustained expression, we performed nucleofection of cord blood CD34+ cells with RFX3-overexpressing and control plasmids, a standard method for inducing transient expression of a gene of interest. Transient overexpression of RFX3 in CD34+ cells led to expansion of granulocyte colonies compared to control, as we observed in lentiviral transduction (Extended Data Fig. 6d). Erythroid colonies, however, did not show a consistent diminution. These results suggest that even transient overexpression of RFX3 may be sufficient to induce increased granulocytic differentiation (consistent with its upregulation in CD34+ IMPs), but more continuous overexpression may be needed to impact erythroid differentiation rates. We have included the figure pasted below (Extended Data Fig. 6d) and the following text:

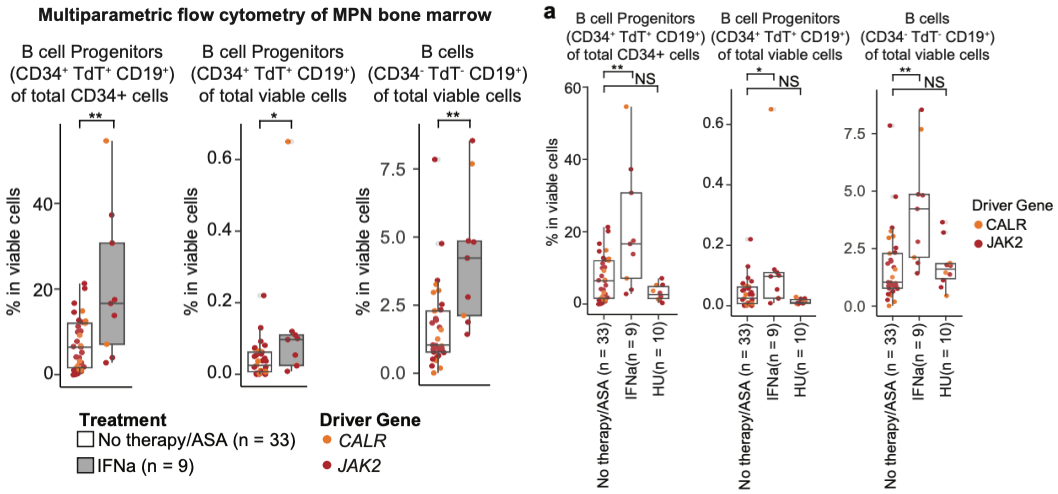
"Transient expression of *RFX3* of CD34$^+$ cells via transfection increased the frequencies of CFU-G, consistent with its upregulation in the early CD34$^+$ HSPC compartment, but did not consistently impact BFU-E proportions potentially indicating that decrease in erythroid colony frequencies may be a secondary effect (Extended Data Fig. 6d)."

Extended Data Fig. 6d, showing CFU-G increase upon transient overexpression of RFX3 in CD34+ cells:



7) Figure 5a would be clarified by labeling cell types at the top.

We agree that labeling the cell types would enhance clarity and have done so for this figure (pasted below, left) and its corresponding Extended Data Fig. 7a (pasted below, right).



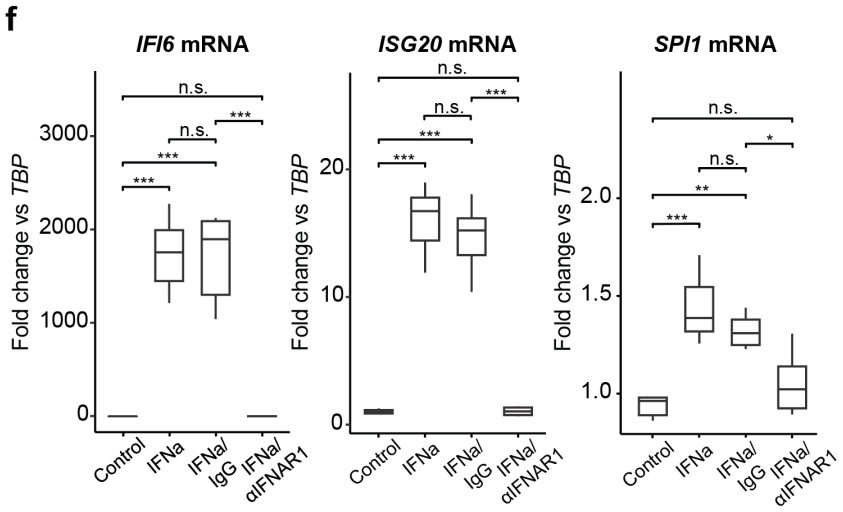
8) Lines 426-427: The authors write that "IFNα directly upregulates the expression of PU.1" Pu.1 has been shown to be induced by TNF and IL1 signaling (Etzrodt 2019, Chavez 2021). Dependence of this activation on TNF signaling was shown through inhibitors and loss of function studies. In order to claim that IFNa directly induces Pu.1, similar inhibitor and LOF studies are required. The activation seen here may be indirect through TNFa or IL1.

We are grateful for this important point. Our studies provided support for PU.1 upregulation by IFNa, e.g. in vivo increase in PU.1 RNA upon IFNa treatment, in vitro increase in PU.1 RNA/protein expression by IFNa treatment, and regulatory regions of PU.1 with IRF motifs, etc. Nonetheless, we agree that we had not performed additional inhibitor or LOF studies to further support the claim that IFNa directly induces the expression of PU.1. We have addressed this point by modifying the Results text (pasted below). We also performed an additional study to further substantiate the role of IFNa in upregulating PU.1 by treating K562 cells with an IFNAR1-inhibiting antibody prior to IFNa treatment. We identified that while IFNa significantly increased PU.1 mRNA levels as we demonstrated before, addition of anti-IFNAR1 led to a reduction of PU.1 mRNA, comparable to control levels (P = 0.03, n = 6 replicates), supporting the role of IFNa in inducing PU.1 expression have included the following figure (Extended Data Fig. 7g).

"IFNα treatment of a hematopoietic cell line (K562) in vitro upregulated PU.1 gene expression, which was inhibited by an IFNα receptor (IFNAR1) antibody (Extended Data Fig. 7g). Consistently, IFNα treatment

increased PU.1 protein expression (Extended Data Fig. 7h). These data supported the role of IFNα in upregulating the expression of PU.1."

Extended Data Fig. 7g, showing inhibition of IFNa-induced PU.1 upregulation by IFNAR1 inhibitor:

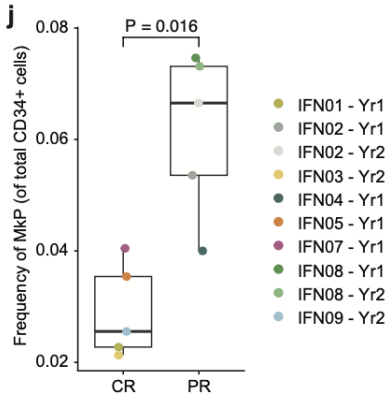


9) Figure 5f shows that the percentage of MkP and MEP correlates with the platelet count, which is not surprising. The authors also showed that people on IFNa tend to have lower MkP, but this was not analyzed as a continuous variable. Can you correlate IFN response with MkP percentage? Does the expression of Pu.1 also inversely correlate with the platelet count?

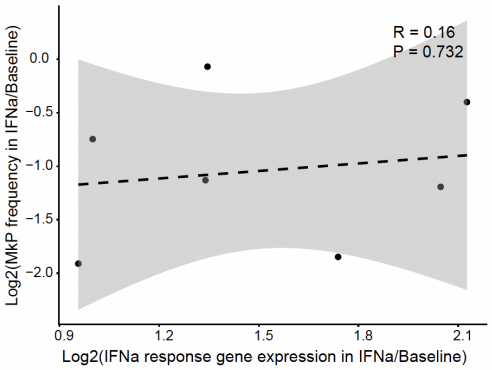
We thank the reviewer for these insightful questions. We have included the suggested analyses. We examined the proportion of MkP in patients with partial versus complete clinical response to IFNa and identified that MkP proportion was higher in patients with partial clinical response as compared to those with complete response (Extended Data Fig. 7j). To address the question of whether the degree of IFNa response correlates with MkP frequencies, we computed the fold change in IFNa response gene set upregulation by HSCs following therapy vs. baseline and fold change in proportion of MkP during treatment vs baseline, in our attempt to overcome batch effects. However, IFNa response did not correlate with change in MkP percentage, likely indicating that this cohort size is underpowered to overcome the inter-patient variabilities for this question. Upon the reviewer's question, we also identified that PU.1 expression correlated negatively with the patients' platelet counts, further supporting the role of PU.1 in the clinical outcome (Extended Data Fig. 7k). We have modified the Results section accordingly:

"Consistently, the proportions of MkP during therapy were higher in the patients with partial clinical response as compared to those with complete response (P = 0.016, Wilcoxon rank sum, Extended Data Fig. 7j). In support of the key role of PU.1 impacting clinical outcome, mean PU.1 expression level within the early HSPC compartment correlated negatively with the patient's platelet counts (Extended Data Fig. 7k)."

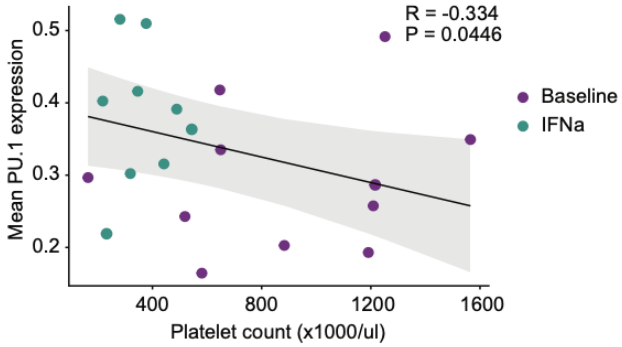
Extended Data Fig. 7j, showing MkP proportions in CD34+ cells in patients with complete vs. partial response:



Reviewer only figure showing, no correlation between fold change in interferon response gene expression and fold change in proportion of MkP during treatment vs baseline:

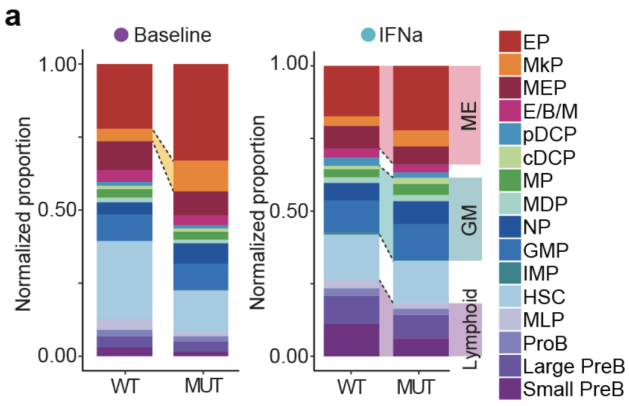


Extended Data Fig. 7k, showing negative correlation between platelet counts and PU.1 expression in early HSPCs:



10) Lines 449-451/ Fig 6b: “Computing proportions of progenitor cell types between wildtype and mutated cells separately showed that IFNα globally remodeled the differentiation toward lymphoid development, reducing the overall megakaryocytic bias in the mutated cells (Fig. 6b). “Figure 6b does not seem to show what is described? Alternatively, a clearer explanation of this proportion is needed.

We regret that this confusion was wrought by a typographical error. We had meant to refer to Fig. 6a, which demonstrates that both wildtype and mutated HSPCs show an expansion of the lymphoid progenitor compartment compared to their counterparts at baseline, with a corresponding decrease in the megakaryocytic fraction in the mutated HSPCs. The appropriate figure is pasted here, and the text changed to refer to Fig. 6a.



Further, the authors write that “Consistently, computation of the mutant cell frequency across the progenitor subsets before and after treatment revealed that the enrichment of the mutated cells in the myeloid

compartments did not change following IFNα treatment (Extended Data Fig. 12f). These data revealed that somatic mutations may constrain IFNα-induced differentiation remodeling." This statement does not make sense logically. If the mutated cells in myeloid compartments did not change following IFNa treatment, that indicates that somatic mutations do NOT constrain IFNa-induced differentiation remodeling.

We thank the reviewer for highlighting the need to clarify this point further in our text. Indeed, the myeloid bias was maintained following IFNa therapy, even though the overall lymphoid expansion was observed in both mutated and wildtype HSPCs. If the effects of IFNa were similar on both the wildtype and mutated HSPCs, we would anticipate that the proportions of progenitor subtypes would be synchronized upon IFNa (i.e., with similar proportion of lymphoid progenitors between wildtype and mutated HSPCs). Instead, what we observe is that the lymphoid differentiation is constrained in the mutated stem cells. We have clarified this important point in the following text:

"However, the lymphoid expansion was constrained in the mutated compartment due to the relative expansion of the granulo-monocytic and megakaryocytic-erythroid progenitors compared to wildtype HSPCs (Fig. 6a-b). Consistently, computation of the mutant cell frequency across the progenitor subsets before and after treatment revealed that the enrichment of the mutated cells in the myeloid compartments was maintained following IFNα treatment (Extended Data Fig. 8e). Since the proportion of lymphoid progenitors would be similar across the genotypes if IFNα signaling was received similarly by the wildtype and mutated HSCs, the constrained lymphoid bias in the mutated cells indicated that somatic mutations may alter the outcome of IFNα-induced differentiation remodeling."

If PU.1 sites are more accessible in the setting of CALR mutation compared to WT. Wouldn't you predict then that you would see a stronger effect of PU.1 induction by IFNa, with more lymphoid and less myeloid skewing? Wouldn't you expect to see differences in Figure 6b?

We thank the reviewer for the opportunity to clarify this point. We had previously included the following sentence to indicate that PU.1 cooperates with other transcription factors:

"Importantly, the IRFs,[86,87] RUNX1,[88] CEBPA[89-91] and TCF3[92] have been demonstrated to co-regulate target gene expression with PU.1."

We should have further specified the key points that PU.1 with CEBPA has been shown to regulate granulo-monocytic development (PMID: 17671233; 7565736; 18424555), whereas PU.1 with TCF3 positively regulates lymphoid development (PMID: 33951726). We have therefore added this key point following the sentence pasted above:

"Specifically, PU.1 and CEBPA have been demonstrated to co-regulate granulo-monocytic development[89-91], whereas PU.1 with TCF3 regulates lymphoid differentiation[92]."

Thus, the enhanced accessibility of PU.1 and CEBPA in the CALR mutated cells indicated a potential mechanism by which the granulo-monocytic skewing was observed. Part of the confusion may have also derived from the fact that we did not address the aspect that the megakaryocytic-erythroid bias is maintained under treatment as well. This is likely due to the sustained activity of CALR mutations in aberrantly activating the thrombopoietin receptor (MPL). We modified the text to enhance the clarity of this key result:

"We also identified that the chromatin accessibility of PU.1 and CEBPA were increased in the mutated cells (Fig. 6c; Supplementary Table 25). These data suggested that *CALR* mutations alter the chromatin state of key lineage specifying TF binding sites, skewing the lineage-modulating activities of IFNα. Specifically, since PU.1 has been demonstrated to cooperate with CEBPA for granulo-monocytic differentiation[89], these data indicated that IFNα-induced PU.1 activities may be skewed toward granulo-monocytic (versus lymphoid) development via enhancements of PU.1 and CEBPA co-activity in the mutated HSPCs, compared to wildtype HSPCs. Maintenance of the megakaryocytic-erythroid bias under IFNα treatment was likely due to the pathogenic activity of mutant CALR in aberrantly activating the thrombopoietin receptor (MPL)[95-98]."

11) Re CUT&RUN data (lines 487-9): The authors write that "CALR mutations may alter the chromatin landscape of TF regulatory regions, which in turn modulates the downstream differentiation remodeling by IFNα and restrains lymphoid development in the mutated HSCs (Fig. 6f).

There is some handwaving here – the authors claim that the CALR mutation alters the cooperative binding of PU1 with other TFs e.g. IRF4/8 to modulate myeloid and lymphoid differentiation. Whereas typically Pu1 promotes lymphoid and myeloid development at the expense of erythroid, here the authors say that in the setting of CALR mutation you still get enhanced myeloid differentiation but lymphoid is restrained? This is not shown by the data.

We thank the reviewer for highlighting the need to clarify these points. This comment is related to the prior question in comment #10. We had meant to highlight the key point that the activity of PU.1 is skewed away from lymphoid development due to the enhanced CEBPA activity in CALR-mutated cells, thereby promoting PU.1 + CEBPA-mediated granulo-monocytic development.

We have clarified this point further in the Results text:

"These data demonstrated that *CALR* mutations enhance PU.1 binding activities and alter the preferential cooperating TF partners of PU.1, namely, CEBPA/B toward granulo-monocytic development. Overall, these results suggested that *CALR* mutations may alter the chromatin landscape of TF regulatory regions, which in turn modulates the downstream differentiation remodeling by IFNα and restrains lymphoid development in the mutated HSCs (Fig. 6f)."

12) Figure 6f follows the prediction of Hormaechea et al (PMID: 33743191) which should be cited.

We thank the reviewer for highlighting this work. Based on the content of this manuscript, we believe that the reviewer may be referring to Fig. 6o/p. We agree that this work is consistent with our model of wherein clones that resist differentiation expand within the stem cell population upon an inflammatory insult such as infection. Although IMPs were not specifically identified, this work also found that several key transcription factors of IMPs, such as AP-1 complex genes and NR4A1 were significantly downregulated in Dnmt3a-null HSCs upon M. avian infection, which is also consistent with our model. We have highlighted these parallels in the Results:

"Consistently, the *DNMT3A*-mutated HSCs demonstrated increased expression of the IMP^{DN} gene signature compared to the admixed wildtype HSCs (Fig. 6o), suggesting that the *DNMT3A*-mutated HSCs may be poised to resist IMP development upon increased IFN activity due to various inflammatory settings, such as infection. These results were consistent with a prior study in a mouse model of *DNMT3A*-mutated clonal hematopoiesis subjected to *M. avian* infection that elicited an IFN-driven inflammatory response.[110] Although IMPs were not specifically identified, *Dnmt3a*-knocked out HSCs resisted differentiation and expressed reduced levels of IMP transcription factors, such as AP-1 subunits and *Nr4a1*.[110] Altogether, our results supported a unified model of clonal dynamics wherein the resistance of HSC clones to differentiate into IMPs may contribute to clonal fitness and expansion in the stem cell niche under inflammation (Fig. 6p)."

Reviewer #3 (Remarks to the Author):

This study uses single-cell multi-omics in MPN patients before and during IFNa therapy to dissect hematopoietic responses. The authors show that IFNa drives polarized hematopoietic remodeling expanding lymphoid progenitors while inducing a neutrophil committed IMP trajectory from HSCs. Further analysis reveals that IFNa suppresses TNFa and TGFb signaling, promotes cell-cycle entry, and rewires differentiation through RFX3 and PU1. Clonal dynamics were concluded to correlate with the propensity of mutant HSCs to adopt the IMP fate, suggesting that IMP resistance as a determinant of inflammatory clonal selection in MPN.

Major Comments:

The study design is elegant and the data compilation extensive.

The two major claims of the paper are that IFNa 1. Remodels hematopoietic differentiation and 2. alters clonal dynamics.

Remodeling of differentiation is reported along two lines: induction of an inflammatory myeloid progenitor (IMP) and induction of lymphoid priming. The evidence for the IMP subset being induced by IFNa and the molecular

features of that are strong. The role for RFX2/3 in the IMP phenotype is validated with OE experiments that are compelling. What is not clear is whether this IMP population emerges in a subset of cells that is distinct from those that have lymphoid priming. The reasonable assumption is that the events are happening in cells beyond a myeloid/lymphoid branch but the OE experiments show an upregulation of IL-7R and raise the possibility that RFX2/3, a known master regulator, may affect the lymphoid priming.

This is of interest in part because lymphoid priming is unanticipated. Prior findings in the mouse showed reduced lymphoid differentiation with IFNa (https://doi.org/10.1084/jem.187.1.79) hypothesized due to reduced IL-7 signaling. It would be expected that reduced IL-7 signaling would also reduce the B cell progenitors measured here and yet these were increased. The molecular results regarding PU.1 and TCF3 accessibility are convincing and affirm a previously known relationship, but it is not clear if this could result in increased pro- and pre-B cells if IFNa impairs IL-7R signaling.

Were TCF3 expression levels actually modified by IFNa? Did expression of its target and driver of lymphoid differentiation EBF1 change? Was there any effect on IL-7R expression or TSLP that can compensate?

We thank the reviewer for the positive feedback regarding our experimental design and for highlighting the strengths of the evidence provided for IMPs and their regulation. We also thank the reviewer for this interesting and important discussion. We agree that there are outstanding questions regarding how RFX2/3 may affect lymphoid priming and will continue our investigations beyond this manuscript to clarify the role of RFX2/3 in IFNa-mediated hematopoietic remodeling. We have included this specific point regarding upregulation of IL-7R upon RFX3 overexpression in the Discussion:

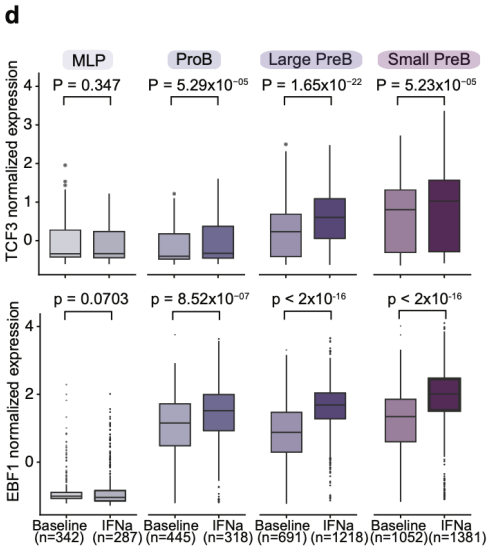
"Furthermore, while RFX3 appeared to play a key role for IMP differentiation downstream of the granulo-monocytic-lymphoid divide, *RFX3* overexpression upregulated *IL-7R*, suggesting its impact on lymphoid differentiation. Further studies are required to determine other potential roles of RFX3 in hematopoiesis."

We thank the reviewer for highlighting this important work in mice showing that IFNa reduced lymphoid differentiation. As the effects of IFNa are highly context dependent, the differences may be due to the distinct conditions in the two studies, particularly with respect to the developmental stages at which hematopoiesis is examined in the two studies (newborn versus adult). We include these contrasting results in the Discussion:

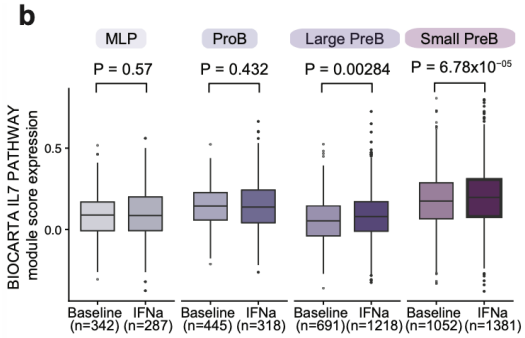
"A study in newborn mice identified that IFNα treatment impaired lymphoid differentiation at pro-B and pro-T-cell stages, potentially due to impaired IL-7 signaling[43]. In our data, IL-7 signaling was not impaired by IFNα treatment. Such paradoxical differences may potentially be due to experimental setup-related distinctions, such as differing developmental stages (newborn versus adult) at which hematopoiesis was probed, reflecting the highly context dependent nature of IFNα effects. "

With respect to the specific questions regarding TCF3, EBF1, IL-7R and TSLP gene expressions in our data, we observed that IFNa induces the upregulation of TCF3 and EBF1 gene expression in the B-lineage progenitors, corroborating the role of TCF3 in IFNa-induced lymphoid development (Extended Data Fig. 7d, pasted below). EBF1 expression was similarly upregulated by IFNa (middle panel). TSLP expression was essentially undetected in the bone marrow cells. IL-7R was detected but at a low level that was not altered by treatment. Therefore, we assessed the downstream targets of IL-7 signaling (Biocarta) and observed no impairment in the expression of IL-7 signaling related genes (Extended Data Fig. 2b). We include the following gene expression results in the revised manuscript and the corresponding text:

"Consistently, *TCF3* gene itself and its target *EBF1* expressions were also upregulated in the B-lymphoid progenitors by IFNα (Extended Data Fig. 7d)."



"Interestingly, IFNα also expanded the proportion of lymphoid progenitors within the HSPC compartment, an unexpected finding as IFNα has been shown to impair lymphoid development in newborn mice.[43] As this work suggested that IL-7 signaling may be impaired by IFNα, we tested IL-7 signaling gene set expression (Biocarta) and identified no deficiencies in the expression of genes downstream of IL-7-signaling (Extended Data Fig. 2b)."



The use of K562 to show that IFNa can upregulate PU.1 are not terribly helpful as this is a very aberrant leukemia line; the data would be more compelling with primary CD34+ cells as were used in Figure 4. This could test the role of IL-7 in lymphoid priming as it could be held or added to the media. Realizing that these experiments are time consuming and may not be definitive, the authors should at least address the apparent paradox of IFNa leading to increased B progenitors when it has been reported to also decrease IL-7R signaling.

We thank the reviewer for suggesting these studies as a future direction. Indeed, while primary CD34+ are more representative than K562 cells, we reasoned that IFNa-induced upregulation of PU.1 is likely a fundamental process that would be preserved in hematopoietic cell lines, serving as a cost-effective reagent for both gene and protein expression analysis. We also agree that resolving the role of IL-7 signaling in the context of IFNa treatment would be important to address in light of the prior work. Nonetheless, as the reviewer points out, conducting these studies definitively would require a considerable number of experiments and would be beyond the scope of this current work. As suggested, we have highlighted the contrasting results in both the Results and Discussion sections, as noted above.

The second main conclusion of the paper is about clonal dynamics being altered by IFNa. The abstract states that they measured "IFNa effects on clonal stem cells from the admixed wildtype HSCs." But, clonal effects on WT HSC are not detailed, as least as I could discern. The focus seems to be more about the relative mutant clone abundance compared to the admixture of normal clones comprising the HSC pool. The data for a distortion induced by IFNa turns out not to be in the relative abundance of myeloid or lymphoid progenitors in mutant or normal with IFNa. Rather, proliferation was increased in CALR mutant cells that was exacerbated by IFNa. Also, a patient with a defined subclone of mutant cells had lower IMP gene expression in association with a time

related increase in subclone representation. This led to the hypothesis that IMP propensity after IFNa could influence the relative founder clone size. Three JAK V617F mutant patient samples showed that the 2 with clinical improvement from IFNa had a higher IMP signature and improved lymphoid progenitor counts. Further, 16 patients showed a lower mutant cell pool correlated with a higher IMP + HSC2/overall Mutant cell ratio. Therefore, the authors conclude that forming IMP induced by IFNa can reduce clonal overgrowth. This is a reasonable conclusion but sorting through this was unnecessarily complicated in part because of the incredibly dense prose used by the authors throughout the paper. For example, the authors state that "inflammation-induced cell cycle entry rates are decoupled from clonal dynamics." In what sense? The data presented preceding the statement were about cell cycle entry only. Understanding the paper is made worse by the use of other imprecise language; for example using 'clone size' rather than 'mutant cell frequency' that was the apparent parameter measured. Statements like "IMP propensity" are made without clarity on how that was computed and validated. Please modify and clarify the language and please focus the narrative. The paper is almost unreadable as written.

The reviewer's summary of the key findings precisely highlights the points we had intended to convey. We thank the reviewer for highlighting texts that could be improved in their clarity, and regret any confusion the imprecise language may have caused. We have edited the texts to improve their clarity.

Specifically, we removed the use of 'clone size' from the text. We also changed "clonal" to "mutated" in the abstract. While "clonal" often refers to the neoplastic populations with myeloid neoplasm driver mutations (e.g. in 'clonal' hematopoiesis), it is also frequently used to describe non-neoplastic HSC clones, as the reviewer has highlighted by his critique. To avoid any confusion, we edited the abstract as follows:

"We studied HSCs, progenitors and immune cells from patients with myeloproliferative neoplasms (MPN) at baseline and following interferon-α (IFNα) treatment, the only therapy to deplete mutated stem cells. We deployed single-cell multi-omics methods that distinguish the IFNα effects on mutated stem cells from the admixed wildtype HSCs, with respect to their differentiation, transcriptomes, immunophenotypes, and chromatin accessibility."

We simplified the sentence that the reviewer specifically highlighted:

"These data indicated that inflammation-induced cell cycle entry rates cannot fully predict clonal dynamics."

We have clarified the definition and rationale behind the computation of mutated cells' 'propensity' to develop into IMPs:

"We therefore evaluated the change in HSC mutant cell frequency between time points as a function of the propensity of mutated HSCs to develop into IMPs. Previously, we had observed that the megakaryocytic differentiation bias of *CALR*-mutated cells was evident in the increased frequency of the mutated cells in the MkP compartment relative to mutant cell frequency in the total $CD34^+$ population[26]; therefore, we determined the IMP differentiation bias or propensity of the mutated cells by computing the fold change of mutant cell frequency within IMPs and HSC1 (predicted to beget IMPs, Fig. 3a) relative to that in the total $CD34^+$ compartment (Extended Data Fig. 9i). Indeed, this measure of propensity of mutated HSCs to differentiate into IMPs could model clonal dynamics (Extended Data Fig. 9i)."

The authors should also acknowledge that they are not assessing clones within the normal HSC population despite single cell data. Interferon is likely to have very clone specific effects on both normal and mutant cells. The results observed in normal HSC may be the select outgrowth of subclones that are predisposed to lymphoid or IMP differentiation. Was this measured? Can they state with assurance that the effects of IFNa are generalizable to all clones?

We thank the reviewer for raising the intriguing point that IFNa may be selecting for specific clones within the CALR-wildtype HSC population that are primed for lymphoid and/or IMP development. Addressing this question would require us to identify non-pathogenic somatic variants within the CALR-wildtype HSCs, determine their transcriptional states and lineage output at baseline, and track how IFNa impacts its clonal dynamics, just as we have done for the CALR mutated clones. Such an analysis is limited by (1) the small sampling of the bone marrow biopsies (relative to the whole HSC pool) and (2) sparsity of single cell data to detect variants; these limitations exclude the ability to identify the same variants in both baseline and treated samples at sufficient

sensitivity to address this question. The primary evidence we have is in the CALR-mutated HSC population, which we believe may be applicable to CALR-wildtype HSCs, as both CALR-mutated and wildtype HSCs displayed similar phenotypic responses to IFNa (i.e. IMP/lymphoid differentiation, increased cell cycle rates, downregulation NF-kB/AP-1 network in HSPCs, etc). The finding that CALR-mutated HSCs display IMP and lymphoid differentiation shifts upon IFNa exposure lends support to the role of IFNa in altering their differentiation state, rather than selecting for clones that are already primed. Furthermore, the clone with two CALR mutations in patient IFN02 displayed a marked megakaryocytic bias at baseline (Extended Data Fig. 9b) and downregulated the IMP transcriptional program but displayed lymphoid differentiation skewing and expanded by IFNa treatment. This case provided evidence for our model that HSC clones that are primed against IMP development get selected for upon IFNa therapy. Nonetheless, with respect to lymphoid priming, we agree that these data do not exclude the possibility that IFNa may also select for clones with lymphoid differentiation bias at baseline in parallel, particularly in the CALR-wildtype population. We have included this important point in the Discussion:

"It is possible that IFNα may be selecting for clones that are already lymphoid biased, particularly in the *CALR*-wildtype HSC compartment. The selection of the *CALR*-double mutant clone from patient IFN02 that displayed marked megakaryocytic differentiation bias and its concurrent shift toward lymphoid differentiation upon IFNα demonstrated that IFNα is able to shift the differentiation trajectories of HSCs. Nonetheless, due to the technical limitations of tracking the clones within the wildtype HSC pool, we cannot exclude the possibility that IFNα is concurrently selecting for HSC clones that are already lymphoid biased."

Further definition and validation of IMPs is needed. For example, are IMPs transcriptionally distinct from activated GMPs or instead a distortion of a continuum from HSCs. Are they consistently present across patients? Why is the IMP cluster not visible in Extended Data Fig1e? Does Cluster X consistently emerge across timepoints and genotypes? Table S2 shows not all patients have detectable IMPs. Does this variability undermine the claim that IFNa universally induces a pro-inflammatory trajectory? Could patient-specific factors (co-mutations, prior therapy, clinical response) explain this heterogeneity?

We thank the reviewer for this important feedback. We would like to address these questions in two sections below:

-Are IMPs transcriptionally distinct from activated GMPs or instead a distortion of a continuum from HSCs?

In the initial submission, we had performed differential expression analysis between IMPs and GMPs, which revealed that NF-kB signaling with AP-1 and RFX2/3 were upregulated in IMPs, whereas GMPs displayed MYC activities. To clarify their distinction in the setting of IFNa therapy, in this revised version we also included differential expression analysis between IFNa-treated IMPs and GMPs, which revealed similar results (Extended Data Fig. 1n). Thus, GMPs and IMPs within the same IFNa-elevated microenvironment displayed markedly distinct transcriptional states, and in fact opposing cell states with elevated NF-kB/AP-1 in IMPs and downregulated NF-kB/AP-1 in GMPs (Fig. 2d). While we cannot exclude the possibility that IMPs derived from GMPs, our data supported the trajectory of IMPs from HSCs that are primed toward IMP development, given their transcriptional and epigenetic (by chromatin accessibility) similarity to HSCs and marked distinction from GMPs. Current efforts include the development of the in vivo model of IMP development (mouse or xenograft) to precisely trace their lineage, relative to GMPs, to address this important question. We expound upon this important point in the Discussion:

"The following results from our data suggest that IMPs may represent an alternate path of myeloid differentiation distinct from GMPs: (1) the transcription factor network of IMPs was most similar to that of a subset of HSCs (HSC1) and markedly distinct from the GMP state under the same IFNα-activated microenvironment; (2) relatedly, RNA velocity measurements predicted their derivation from HSC1; and (3) by chromatin state, IMPs could be traced contiguously from HSCs and are discontiguous from GMPs on the snATAC-seq data-derived UMAP. These data provide useful corroborating evidence, as several known cell state transitions of this blood developmental system are represented in the UMAP embeddings of scRNA-seq and snATAC-seq data and are predicted by RNA velocity measurements. However, to definitively determine the origins and differentiation trajectories of IMPs, we would require a means to genetically lineage trace these cells (e.g. via lentivirally

introduced molecular barcodes[116,117]). Toward this end, future directions include developing an *in vivo* model of IMPs (mouse or xenograft) and genetically tracing their lineage trajectory. ”

-Are they consistently present across patients? Why is the IMP cluster not visible in Extended Data Fig1e? Does Cluster X consistently emerge across timepoints and genotypes? Table S2 shows not all patients have detectable IMPs. Does this variability undermine the claim that IFNa universally induces a pro-inflammatory trajectory? Could patient-specific factors (co-mutations, prior therapy, clinical response) explain this heterogeneity?

We regret that we could have been clearer with respect to Extended Data Fig. 1e (current 1f). As the ET01, ET02, ET03 samples were included from our prior study (PMID: 31270458), we included a separate dimensional reduction and cell type labeling to demonstrate the consistency of these published data to the newly generated data. These patients were not on any therapy at the time and therefore were among the non-treated samples, hence explaining why IMPs were not detected and supporting the specificity of IMP expansion to IFNa treatment. We clarified this in the Results:

“We integrated across the individual 24 samples to define the cell identities of the CD34^{+} HSPCs consistently across individual sampling (after analytically segregating the cells by time-point from the same experiments, Fig. 1c and Extended Data Fig. 1b-e; separate UMAPs of previously published ET01-03 with no IFNα exposure (Extended Data Fig. 1f) and samples generated for this study (Extended Data Fig. 1g); Methods).[33]”

IMPs were present across patients in the IFNa-treated conditions regardless of genotypes (CALR-mutated, JAK2-mutated, or WT), with the exception of IFN02 Year 2 and IFN07 Year 1 samples, which could be explained by the capture of very few overall CD34+ cells (541 and 346 CD34+ cells total, respectively). The reviewer raises an important point that while IMPs were detected consistently in IFNa-treated samples (except for the ones with few CD34+ cells noted above), the frequency of IMPs was highly variable across patients. We agree that there are several potential factors including those noted by the reviewer that may underlie the heterogeneity. We modified the Results to clarify both of these points:

“All IFNα-treated samples included IMPs except for IFN02 Year 2 and IFN07 Year 1 samples due to the small number of overall CD34^{+} cells captured (541 and 346, respectively). However, the frequency of IMP was heterogeneous across patient samples, potentially due to patient-specific factors (e.g. co-mutations, treatment history, prior infections, clinical response, etc).”

Cell death and dropout analysis is not discussed but may affect interpretation of the observations. While cell cycle activity is evaluated in HSPCs, which populations are specifically depleted or ablated by IFNa remains unclear; have apoptotic or stress signatures been observed in any specific clusters?

We thank the reviewer for highlighting this important point. We focused on cell cycle rates because notable manuscripts attributed the induction of HSCs into cell cycle as mediating clonal dynamics in the setting of IFNa treatment (Essers et al, Nature, 2009; Mullally et al, Blood, 2013; Mosca et al, Blood, 2021) or general inflammation (Heyde et al, Cell, 2021). Thus, we reserved these rare samples for our single-cell multi-omics studies that accurately determined cell cycle rates as a function of mutation status (along with several other measurements enabled by detection of transcriptomes and protein expression). In our pathway enrichment analysis through GSEA, we did not observe an enrichment of the apoptosis pathway (which includes the caspase gene family) in any of the clusters. We have included this negative finding in the Results:

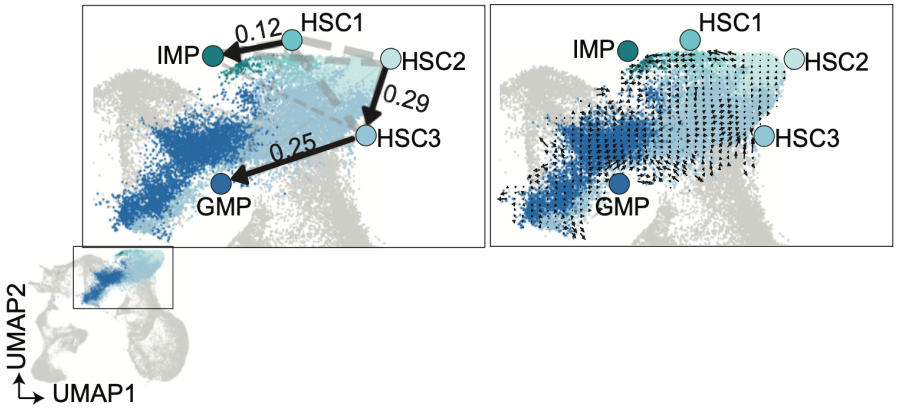
“The analysis also confirmed the upregulation of cell cycle-related pathways (G2M check point and E2F targets) as well as MYC targets (Fig. 2d and Supplementary Table 6), corroborating a previous study that reported enhanced MYC protein expression during IFNα-induced cell cycle entry of mouse HSCs.[57] Notably, the apoptosis gene set was not differentially regulated (Supplementary Table 6).”

Determining the apoptosis status in these cryopreserved samples is also confounded technically by cell death due to procedures of cryopreservation and subsequent thawing. The inability to rigorously detect the contributions of cell death in modulating clonal dynamics in primary samples is indeed a limitation, and we have noted it in the Discussion:

“Another parameter we could not rigorously assess was the contribution of apoptosis to clonal dynamics in primary HSCs, due to the reservation of these rare samples for the single-cell multi-omics studies and the challenges of deconvoluting biological versus technical sources of cell death in these cryopreserved samples.”

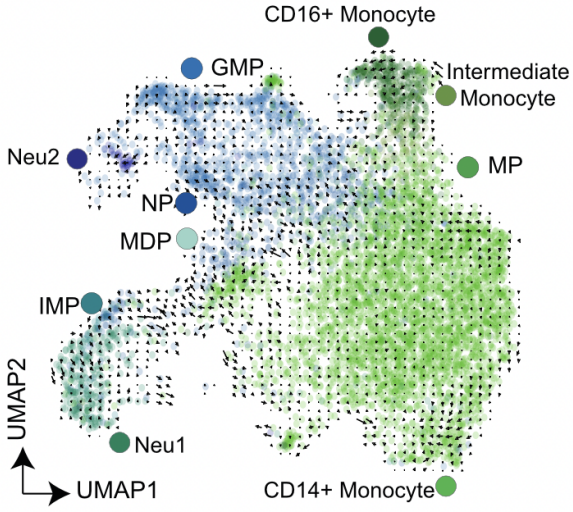
The RNA velocity plots currently display manually annotated directionality arrows; however, please include the raw velocity flow vectors in the supplementary materials to better support interpretation of lineage transitions. In addition, demonstrate that the IMP-to-neutrophil differentiation path is supported by velocity-derived transition probabilities. Given the observed size of the IMP population and its neutrophil commitment, please estimate the putative neutrophil output from IMPs (Neu1) relative to that from GMPs (Neu2), and clarify whether this aligns with the abundance and phenotype of mature neutrophils observed in PBMCs.

We thank the reviewer for the important recommendation. We would like to highlight that these directionality arrows were not manually drawn, but rather from partition-based graph abstraction (PAGA) analysis that computes connectivity and directionality of cell states by incorporating both data topology and RNA velocity data (PMID: 30890159). To alleviate any concerns, we have included the raw velocity flow vectors in Extended Data Fig. 3b (pasted below):



As requested, we have performed RNA velocity analysis on data that combines myeloid progenitors and mature myeloid cells, which predicted the differentiation of IMPs into Neu1 (current Extended Data Fig. 3j):

“Consistently, unsupervised co-embedding of the myeloid progenitors with the mature myeloid compartment revealed that IMPs clustered with the Neu1 subset, and by RNA velocity measurements, IMPs were predicted to differentiate into Neu1 (Extended Data Fig. 3j).”



The reviewer raises a fascinating question of whether the putative neutrophil subtype outputs could be estimated based on IMP and GMP proportions. To meaningfully address this question, we would need to understand not only their starting frequency but also several other factors, including the duration of time for neutrophils to develop

from GMPs and IMPs and be released from the bone marrow (as these rates during homeostasis versus IFNa may differ drastically (PMID: 24751955)), the number of cell divisions the cells undergo before the post-motitic differentiation stage, and turnover rates of the neutrophil subsets. Thus, without understanding these parameters, it would be merely speculation to predict the proportions of neutrophil output from IMPs and GMPs. Nonetheless, this is an important question that we may be able to address via an in vivo model of IMPs with lineage tracing. We include this point in this Discussion:

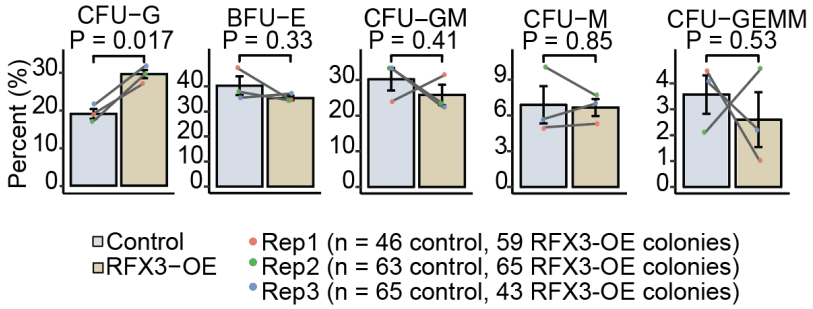
"Nonetheless, lineage tracing methods via an *in vivo* model of IMPs are again required to determine the lineage outputs of IMP and the relative contributions of neutrophils from IMPs versus GMPs."

The data are largely correlative with only modest functional validation (RFX3 OE experiment). The conclusions should reflect that the usual means of increased and decreased expression to validate the relationships proposed for RFX2/3 and PU1 for example were not performed.

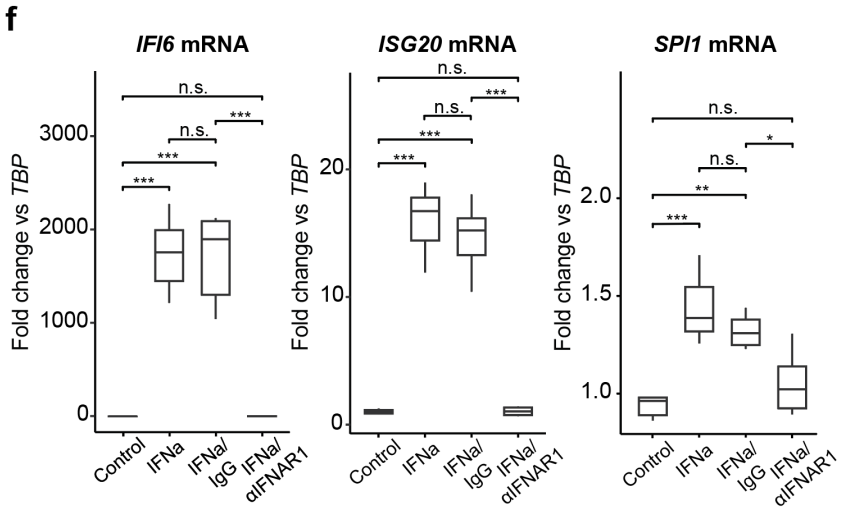
We agree with the reviewer that strength of the manuscript lies in the application of the single-cell multi-omics methods on primary bone marrow cells, serially sampled from MPN patients treated with IFNa. While we have endeavored to follow up on key aspects of these findings through additional studies (e.g. RFX3 OE, single cell CFU analysis, CUT&RUN analysis of PU.1), we agree that the usual experiments to determine sufficiency and requirement have not been extensively conducted to validate all of the key findings. We would like to highlight, however, we have included two additional functional studies in response to the other reviewers' questions. We have addressed the sufficiency of transient RFX3 expression in inducing granulocytic bias (Extended Data Fig. 6d, pasted below), and the use of IFNAR1-blocking antibody to substantiate the role of IFNa in inducing PU.1 (Extended Data Fig. 7g, pasted below). We have also acknowledged this point in the Discussion:

"Limitations of our study include the limited functional studies to determine the sufficiency and requirements of key factors such as PU.1 and RFX3 in mediating IFNα-induced phenotypes."

Extended Data Fig. 6d, showing CFU-G increase upon transient overexpression of RFX3 in CD34+ cells:



Extended Data Fig. 7g, showing inhibition of IFNa-induced PU.1 upregulation by IFNAR1 inhibitor:

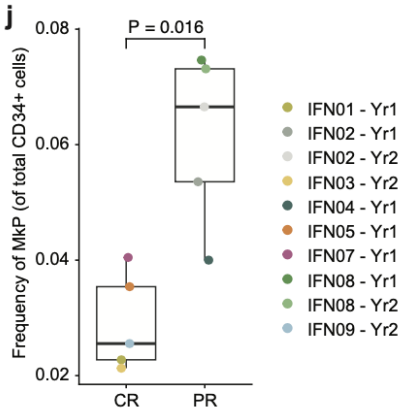


Please clarify how complete response (patients IFN01, 03, 05, 06, 07, 09, 10, 11, 12) compares with partial response cases. Correlating molecular findings, such as clonal contraction, lymphoid skewing, IMP signatures with clinical readouts such as platelets, hematocrit, spleen size, and symptom burden would add strength to the paper and add to its translational relevance.

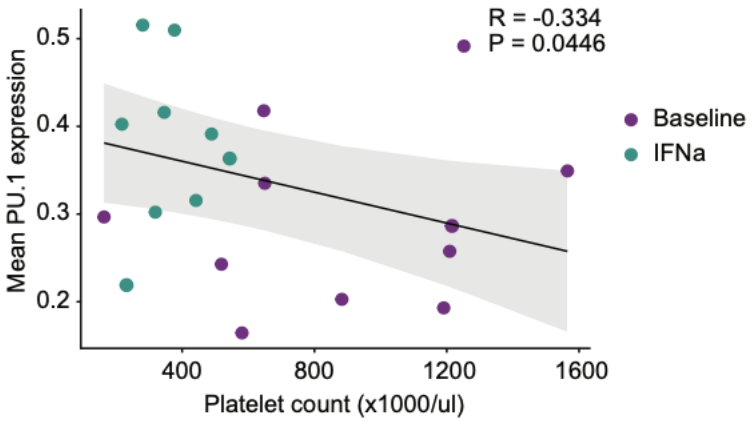
We thank the reviewer for the suggestion to correlate the molecular findings to the clinical readouts to strengthen the translational relevance of this manuscript. As the main cohort of patients we focus on are patients with CALR-mutated essential thrombocythemia, we had focused on their platelet counts and molecular response. In the initial draft, we showed that the proportion of MkP/MEPs could model the patient's platelet count. We also focused on the impact of IMP development on molecular response. We agree that such analyses could be expounded further to increase the translational relevance. Specifically, we have included data to show that patients who exhibited complete response harbored a significantly lower proportion of MkPs (of CD34+ cells) compared to patients who exhibited partial response (Extended Data Fig. 7j). We also identified that PU.1 expression in early HSPCs correlated negatively with the patients' platelet counts, further supporting the role of PU.1 in the clinical outcome (Extended Data Fig. 7k). We have modified the Results section accordingly:

"Consistently, the proportions of MkP during therapy were higher in the patients with partial clinical response as compared to those with complete response (P = 0.016, Wilcoxon rank sum, Extended Data Fig. 7j). In support of the key role of PU.1 impacting clinical outcome, mean PU.1 expression level within the early HSPC compartment trended negatively with the patient's platelet counts (Extended Data Fig. 7k)."

Extended Data Fig. 7j, showing MkP proportions in CD34+ cells in patients with complete vs. partial response:



Extended Data Fig. 7k, showing negative correlation between platelet counts and PU.1 expression in early HSPCs:

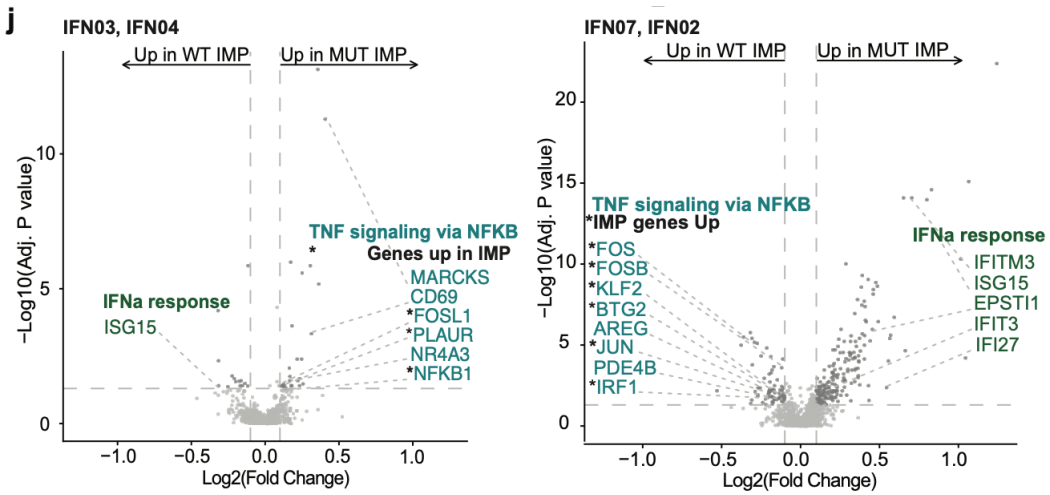


To determine whether the transcriptional state of CALR-mutated IMPs (vs. wildtype IMP) can predict molecular response, we performed differential expression analysis between mutated and wildtype IMPs in patients with molecular response (IFN03, IFN04) and those whose clones expanded (IFN07, IFN02). Mutated IMPs from

patients that showed molecular resistance (IFN07, IFN02) downregulated the IMP TF signature (FOS, FOSB, JUN, KLF2, IRF1, NR4A1) and upregulated IFN signaling genes (ISG15, IFITM3, IFI27), whereas mutated IMPs from patients displaying molecular response (IFN03, IFN04) upregulated genes in the NF-kB pathway/IMP signature including FOSL1, NR4A3, NFKB1, and CD69 and downregulated ISG15. These data collectively supported the model wherein mutated stem cells that are poised transcriptionally to develop into IMPs get depleted, whereas mutated HSCs that are poised against IMP development expand upon IFNa therapy. We have included this analysis in Extended Data Fig. 9j (pasted below), and included the following text in the Results section:

"Consistent with this model, differential expression analysis between mutated and wildtype IMPs from patients that displayed clonal expansion upon therapy (IFN02, IFN07) revealed that mutated IMPs downregulated the IMP signature (e.g. *FOS*, *FOSB*, *JUN*, *KLF2*, *IRF1*, *NR4A1*) and upregulated IFN signaling genes (e.g. *ISG15*, *IFITM3*, *IFI27*), potentially reflecting the transcriptional poising against IMP development. The same analysis in IMPs from patients who displayed molecular response (IFN03, IFN04) revealed that mutated IMPs upregulated genes in the NF-kB pathway/IMP signature (including *FOSL1*, *NR4A3*, *NFKB1*, *CD69*) and downregulated *ISG15* (Extended Data Fig. 9j, Supplementary Table 32)."

Extended Data Fig. 9j, showing differential gene expression analysis between mutated and wildtype IMPs from patients with molecular response (left) and those with resistance (right):



May 8th, 2026

Dear *Nature Genetics* editors and reviewers—

We thank the reviewers and editors for their careful review of our revised manuscript (NG-A69964R1), by Lama, Isakov, and colleagues. We are pleased to have addressed the main critiques. In this revised version, we have addressed the remaining minor comments from Reviewers #1 and #3 in the point-by-point response below.

Sincerely,

The authors

REVIEWER #1
The authors have done a fantastic job at addressing most of the comments in their revisions, and the manuscript has been substantially improved. With minor revisions, this reviewer would recommend publication in Nature Genetics.

We thank the reviewer for commenting favorably on our revisions and for the remaining minor suggestions for improvement. We hope to have fully addressed the reviewer's concerns in this current updated draft.

The effect of somatic mutations in IMPs still presents some conceptual challenges as presented, and should be clarified before this reviewer can fully endorse the model in Figure6p. The authors use IFN02 patient as an example of resistance to IMP differentiation, specifically the CALR double mutant clone, that expands upon treatment and downregulates the IMPup program. However, in FigureS9g, as pointed out by the authors in their rebuttal, the vast majority of IMPs come from the "IFNa double MUT". How do authors define this population as IMPs if it's downregulating the IMP signature? These seems to argue against the proposed model by which double mutant cells resist IMP differentiation.

We thank the reviewer for allowing us to clarify this important point. The key point is that although the double mutant HSC clone overall displayed a transcriptional poising against IMP development relative to other clones, there is sufficient cell state heterogeneity within the double mutant population such that some of the double mutant HSCs will differentiate into IMPs upon IFNa signaling, albeit at a reduced rate compared to other HSC clones. In other words, the double mutant HSC pool overall is less prone to IMP differentiation compared to other HSCs, resulting in its expansion upon chronic IFNa therapy. To allay any concerns, we have included a panel showing the upregulation of the IMP gene signature and transcription factors (including RFX2/3) in IMPs from IFN02 to demonstrate that IMPs are indeed formed upon IFNa stimulation albeit at a relatively reduced frequency (Extended Data Fig. 9h, pasted below).

We have modified the Results text to clarify this point. The new texts are highlighted in blue:

"As the IMP^{UP} signature has a significant overlap with the $IFN\alpha^{DN}$ gene signature, the downregulation of the $IFN\alpha^{DN}$ gene signature (including AP-1 and other immediate early response

factors) in the double mutant cells suggested that the double mutant HSCs may be on average less prone to IMP development relative to the other clones. Consistent with this hypothesis, the double mutant HSCs expressed lower *IMP*^{UP} and higher *IMP*^{DN} signatures overall, although heterogeneity in these gene programs resulted in IMP production (Extended Data Fig. 9h,i). As the double mutant clone was selected by IFNα treatment, these data raised the hypothesis that the resistance of mutated stem cells to differentiate rapidly into IMPs (relative to other clones) may contribute to its clonal fitness upon IFNα treatment."

…

"Although an overall bias against IMP development was observed, sufficient cell state heterogeneity within mutated HSCs enabled the induction of IMPs upon IFNa, albeit at decreased frequencies compared to wildtype."

Extended Data Fig. 9h, showing IMP gene scores and IMP-specific TFs enriched in IMPs from patient IFN02:

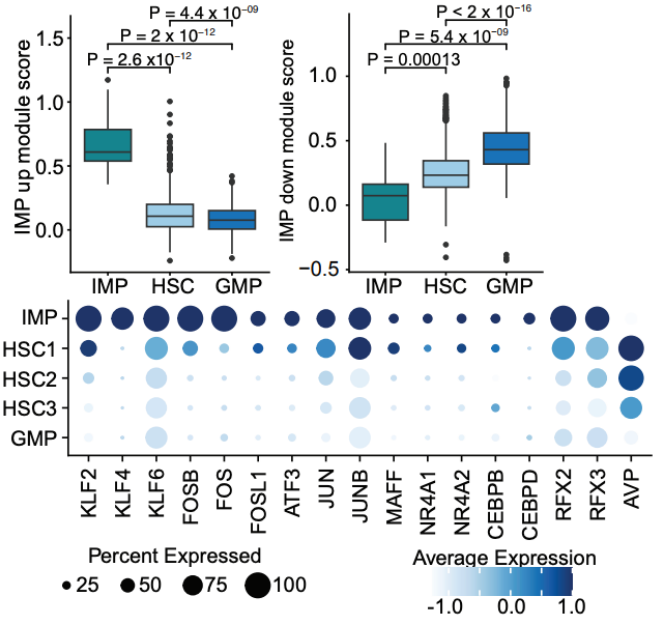
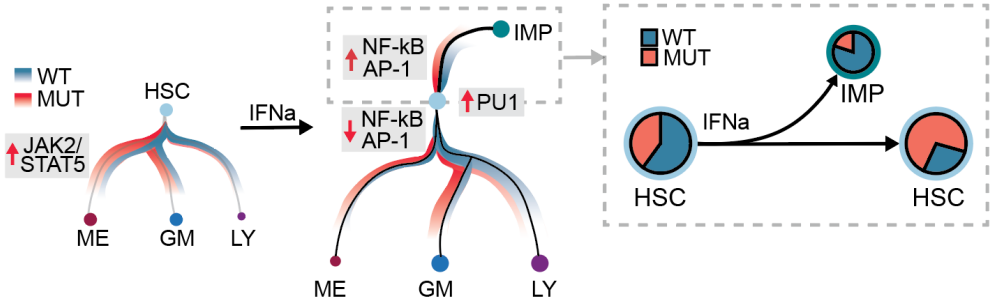


Figure 6 tries to merge three conceptually distinct arguments: (1) CALR mutations alter myeloid differentiation ratios through PU.1/CEBPA co-operative binding, (2) a double-mutant CALR clone downregulates the IMP signature and expands, and (3) the model is generalizable to JAK2 and DNMT3A mutations. In this reviewer's opinion, the figure could be greatly clarified by moving panels to supplementary material or creating an additional figure if the editorial requirements allow.

We thank the reviewer for this suggestion. We have separated this figure into two, with data on CALR mutational impact on IFNa treatment effects in Figure 6, and the remaining data on the role of IMP in clonal dynamics in Figure 7.

This reviewer would also encourage authors to integrate their several conceptual model figures (Figure2g, Figure5f, Figure6f, Figure6p) into a final single, unified model that would leave readers with an integrated summary of their findings.

We thank the reviewer for this suggestion. We have integrated the conceptual models into one cohesive figure (Fig. 7g, pasted below):



There are places where the text reads convoluted, which the authors could consider simplifying and focusing (e.g. lines 137-147, 193-198, 235-239, 257-263, 306-307, 447-449, 518-521, 607-612).

We thank the reviewer for highlighting texts that could be simplified. We have abbreviated the texts where possible without losing the intended meaning. These lines are highlighted in light green in the manuscript.

In line 479, Fig. 11f should read 7f?

We are grateful for the reviewer's careful reading of the manuscript. We have corrected this error.

REVIEWER #2

The authors have responded fully to my comments and I have no further concerns.

We thank the reviewer again for their prior critiques and are pleased to have addressed the reviewer's comments fully.

REVIEWER #3 (Remarks to the Author):

The revised manuscript is much improved. The evidence for the IMP state is strengthened, the RFX3 functional data are more convincing, and the authors now put greater caution in parts of the clonal discussion. These revisions are much appreciated. There remain only minor concerns with the presentation.

We thank the reviewer's positive review of our revised manuscript. We hope to have now addressed these remaining concerns by addressing the limitations of our study in the Results section, in addition to a fuller explanation in the Discussion we had included in the initial revision.

1. Central mechanistic claims:
Several issues acknowledged as beyond the scope of the present study are not peripheral, they relate directly to the proposed mechanism. The absence of PU.1 validation in primary CD34+ cells, the inability to track wildtype subclones, the lack of lineage-tracing evidence for IMP developmental origin and fate, and the absence of any quantitative assessment of IMP-derived

neutrophil output leave the central model largely inferential. The authors should be more explicit in the main text about what the data can and cannot establish, rather than deferring these limitations to the discussion or supplement; clear statements about limitations would be more helpful to the field.

We thank the reviewer for this comment. We agree that directly qualifying our data and their limitations within the Results section would enhance clarity. As we demonstrated that IFNa upregulates PU.1 gene expression and activity (by chromatin accessibility) in CD34+ cells in human, these findings motivate us to determine the regulation and function of PU.1 upon IFNa treatment via functional studies in HSCs. We also include in the Results section the necessity of an in vivo IMP model for lineage tracing, which in turn will allow us to assess their differentiation trajectories and quantify their contributions. We further acknowledge that the technical inability to trace the wildtype clones is another limitation of the study. We have added the following corresponding texts in the Results section:

"While the role of PU.1 in regulating lymphoid differentiation is well-established, genetic loss-of-function studies of PU.1 in primary HSCs would establish its requirement in lymphoid differentiation upon IFNα treatment."

"Overall, these data demonstrated that IFNα expanded the inflammatory neutrophils (relative to other neutrophils), consistent with the induction of inflammatory myeloid differentiation. These findings motivate the development of an in vivo model of IMPs to precisely lineage trace their differentiation trajectories and quantify the myeloid output."

"We note that due to technical limitations of genotyping individual clones within the wildtype compartment, we could not assess IFNα effects on distinct wildtype clones, but instead wildtype cells in aggregate served as the ideal *CALR*-wildtype control."

2. Functional validation:
The addition of RFX3 overexpression and IFNAR1-blocking experiments is a meaningful improvement. However, the proposed regulatory roles of RFX3, PU.1, and related transcription factors are still supported more by association and partial perturbation than by a systematic demonstration of sufficiency and requirement. The standard approach of both gain and loss-of-function experiments for the key regulatory relationships proposed for RFX2/3 and PU.1 has not been performed. The conclusions should more clearly reflect this limitation.

We thank the reviewer for appreciating the additional experiments included in the revised manuscript. We agree that the evidence we provide in human cells regarding the roles of RFX3 and PU.1 motivates the careful execution of studies to demonstrate sufficiency and requirement of the regulatory networks identified in our current manuscript. We have addressed this through the following texts in the Results section:

"While the role of PU.1 in regulating lymphoid differentiation is well-established, genetic loss-of-function studies of PU.1 in primary HSCs would establish its requirement in lymphoid differentiation upon IFNα treatment."

“These data highlighted RFX3 as a key regulator of IMP development, further motivating the development of an in vivo IMP model to establish the regulatory role of RFX3 in IMP development via gain and loss-of-function studies.”