nature research

Peer Review Information

Journal: Nature Immunology
Manuscript Title: Distinct transcription factor networks control neutrophil-driven inflammation
Corresponding author name(s): Irina A Udalova

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A31167
Message: 22nd Dec 2020

Dear Professor Udalova,

Your Article, "Distinct transcription factor networks control neutrophil-driven inflammation" has now been seen by 2 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 interested in considering a revised version that addresses these serious 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.

Please do not hesitate to get in touch if you would like to discuss these issues further.

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

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

If revising your manuscript:

* Include a “Response to referees” document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a

compelling argument. This response will be sent back to the referees along with the revised manuscript.

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

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

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

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

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

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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

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

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

href="http://www.springernature.com/orcid">www.springernature.com/orcid.

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

Thank you for the opportunity to review your work.

Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

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Referee expertise:

Referee #1: Neutrophil development

Referee #2: Neutrophil function

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In study by Khoyratty et al. that entitled "Distinct transcription factor networks control neutrophil-driven inflammation" is a straightforward study, the authors investigated the transcriptional programming of neutrophils in an inflammatory model, i.e. air-pouch model of acute inflammation. The authors performed bulk RNA-seq and ATAC-seq on neutrophils in the bone marrow, blood, membrane and air pouch. From the transcriptomic and epigenomic analyzes, the authors concluded that neutrophil chromatin accessibility and transcriptional changes as neutrophil transit through different tissue compartments (from the bone marrow to blood, blood to inflamed tissues) signify a change in their functional capacity. Analysis of their ATACseq data also revealed the top binding transcription factor motifs for which the authors selected to study further, these include JunB, RelB, IRF5, RFX2, KLF6 and RUNX1. To dissect out the specific transcription factors that are involved in the maturation and activation of neutrophils, the authors employed a CRISPR/Cas9 system to knockout various transcription factors in myeloid (neutrophil) HOXB8 cell lines. Here, the authors showed that both KLF6 and RUNX1 were important for neutrophil maturation while IRF5, JUNB and RELB were shown to be important for activation and production of cytokines. Overall, this study provides information on the specific programming changes during their transition between tissue compartments, as well as how transcription factors regulate neutrophil function at inflamed sites. There are several key issues that the authors should address to strengthen the paper, and I have the following suggestions for authors' consideration:

Major comments:
1. Based on the air pouch acute inflammatory model, the authors concluded that transcriptional change at two intervals, one in the transition from the bone marrow to the blood, and then from the blood into the inflamed tissues, and changes were reflected in upregulation/downregulation of specific transcript and chromatin accessibility modules. However, without examining bone marrow and blood neutrophils (sorted the same way as described by the authors) in control mice, it is difficult to know whether the results reflect inflammation-induced changes, or it is a result of accelerated trajectory of neutrophil development. Perhaps the authors should examine published datasets that were obtained at homeostasis, which have been separated into their individual cell developmental states (see Xie et al. 2020, Nat. Immunol; Muench et al., 2020, Nature; Kwok et al., 2020, Immunity) to evaluate if their transcription factors or gene modules are present already at steady state.

2. One key technical issue that should be pointed out is the sorting strategy. While the authors acknowledged that neutrophils in the bone marrow and blood are composed of immature and mature subsets, the RNA-seq and ATAC-seq were performed on total Ly6GhiCD11b+ cells, which consist of both immature and mature neutrophils. The results obtained is an averaged expression and chromatin accessibility of both immature and mature subsets. How do the authors explain if these differences they observed is a tissue signature (BM v Blood) or simply a reflection of changes in the proportion of immature vs mature cells in different tissue compartments. This also raises a major question whether the study has identified inflammation specific TF changes or changes that occur naturally as neutrophils migrate from the bone marrow to the blood.

3. The authors utilized zymosan, a ligand for dectin-1 and TLR2 to drive neutrophil recruitment into the pouch cavity. As such, the author's findings for the bulk RNAseq and ATACseq is uniquely driven by inflammatory responses to zymosan alone via dectin-1 and TLR2 signaling. It is thus unclear if a similar transcriptional reprogramming process will also be initiated in other models of acute inflammation. This is an important question to address.

4. Figure 2B is rather confusing, more information should be provided to help the reader to understand. In addition, on page 7, "The ATAC-seq analyses identified two distinct remodeling events, the transition of neutrophils from BM to blood (411 opening and 341 closing peaks), and from blood to the inflamed tissue (2,294 opening and 1,645 closing peaks)", but the numbers stated by the authors do not match with what were shown in the Supplementary Fig 3A. Can the authors check.

5. It is unclear what Figure 2C and 2D is comparing. What differential expressed genes are represented here and what is the significance for performing this comparison?

6. Figure 4A, what is "neutrophils per filed (20X)"?

7. The authors performed RNA-seq for cell lines with CEBPb, RELB, IRF5 and JunB knockouts. They stated that cell lines were challenged with zymosan for 2 hours or left unstimulated. However, it is unclear what is being reflected in Figure 5 – whether it is a combination of two subsets, or just the inflammation-induced changes. If so, the authors can actually subset out the true DEGs from zymosan induced inflammation by doing a pairwise comparison with the unstimulated control, and examine the effects of

transcription factor knockout. Presentation in a heatmap/violin plots might be useful for this.

8. The authors define mature HoxB8 neutrophils based on CD101 expression. Can the authors be sure that CD101 expression is not affected by the lack of specific transcription factors ? Perhaps the authors should also examine cell nucleus morphology to complement and support their flow cytometric analysis in determining the differentiation stage of the HoxB8 neutrophils.

9. While the authors performed myocardial infarction model with the cre-Mrp8 x Jun fl/fl and showed that these mice have a reduction of infarction size, there is no analysis of the neutrophil development, neutrophil subset composition and their function in this mouse model. The authors should use primary neutrophils from this model to validate the in vitro functional data from the HoxB8 neutrophil cell line.

10. The authors need to discuss that they are not able to distinguish the changes they are looking at are true transcriptional changes induced by the inflammatory state, or whether the inflammatory state accelerates neutrophil maturation and mobilized into the periphery. For example, the expression of JunB is presented to be only activated in inflammatory settings. However, the authors need to rule out that JunB is already present and activated in mature neutrophils in the unchallenged state, since JunB expression might naturally come up as the neutrophil matures.

Minor comments:

1. The authors should check all figures to ensure good quality and consistent formatting.

2. Figure 1a, it is unclear what the three harvest points represent? No information is provided in figure legend or text.

3. Formatting with genes and protein symbols in the manuscript should be consistent. Mouse gene symbols should be italicized (e.g. Runx1) with the first letter capitalised. Mouse protein symbols should be capitalised (e.g. RUNX1). Knockouts are represented as Runx1-/- to show that the gene Runx1 is deleted on both alleles as proteins cannot be knocked-out by CRISPR/Ca9 systems. This formatting is sometimes observed (Figure 2E, Figure 5A and Figure 6A) but not in others.

Reviewer #2:
Remarks to the Author:
The authors present an interesting study on transcription factor networks and chromatin remodelling in neutrophils as they emigrate from the bone marrow, circulate, and eventually infiltrate into sites of inflammation. The authors identify transcription factors crucial to each of these stages of the neutrophil journey and in a proof-of-principle approach, he authors show that interference with JunB inflammation in a preclinical mouse model of MI can be alleviated. Overall, I am very positive towards this study as the authors use state of the art techniques, draw the right conclusions from their data and provide an important piece to the community. However, I have several general as well as technical concerns:

1- The authors should confirm that the differences at transcriptome level between MEM and the rest of conditions is not due to the digestion process of the tissue. This could explain why there are differences between air pouch exudate and membrane in RNA but not in chromatin accessibility. Also the authors should comment on how the small differences in handling of neutrophils isolated from different compartments may impact on the data.
2- The PCA analysis is performed on differentially expressed genes. This PCA should be done in all detected genes to define the differences between neutrophil states considering the whole transcriptome?
3- The authors show that the transitions from bone marrow to blood and blood to membrane are associated to upregulation or downregulation of certain groups of genes and this coincide with open/close chromatin peaks. Could the authors confirm some of these changes at protein level?
4- The authors provide an interesting correlation showing that most of the changes at transcriptome level (downregulation/upregulation) directly associate with remodeling of the chromatin (close/open). Is this evenly occurring among all states? Are there particular states where chromatin conformation does not relate with gene expression?
5- In LysM Runx1-/- mouse model, the authors show that this TF is involved in the neutrophil maturation within the bone marrow. However, no link is provided that these TFs drive the transition from bone marrow to blood. Are Runx1 or Klf6 involved in BM to blood neutrophil mobilization? Also, the authors should provide absolute counts of neutrophils in the bone marrow and blood.
6- Rfx2 and Relb are shown to be regulators of steady-state neutrophil apoptosis. Do neutrophils deficient for these TFs exhibit also increased apoptosis upon inflammation in vivo?
7- How the RNAseq from the Hoxb cells correlate with the genes found in the RNAseq of the different neutrophil states? As for Runx1 and Klf6, the authors should provide a causal link between these TFs and the transcriptional profile found in recruited neutrophils within the air pouch.
8- Do the authors have evidences of the existence of this regulatory network in human neutrophils?
9- Can the authors detail how they obtained the 1865 DE genes?
10- PCA for RNA analysis shows 3 points/population, except for MB (2 points). How can this come from 2 mice? Also, why has 1 MB point excluded from the analysis?
11- Did authors compare samples through paired analysis (BM BL MB AP for each mice)?
12- During their different analysis, authors switch from different p-values to adj p-values. Is there a statistical rational for that?

Author Rebuttal to Initial comments

Response to the reviewers: manuscript NI-A31167

We would like to thank the reviewers for their overall positive assessment of the manuscript and useful comments, which helped to improve and streamline the manuscript. We have addressed the reviewers' questions and concerns in our point-by-point response below. We substantially revised the manuscript and figures. The modified text is highlighted in the revised manuscript.

Reviewer #1:

Remarks to the Author:

In study by Khoyratty et al. that entitled "Distinct transcription factor networks control neutrophil-driven inflammation" is a straightforward study, the authors investigated the transcriptional programming of neutrophils in an inflammatory model, i.e. air-pouch model of acute inflammation. The authors performed bulk RNA-seq and ATAC-seq on neutrophils in the bone marrow, blood, membrane and air pouch. From the transcriptomic and epigenomic analyzes, the authors concluded that neutrophil chromatin accessibility and transcriptional changes as neutrophil transit through different tissue compartments (from the bone marrow to blood, blood to inflamed tissues) signify a change in their functional capacity. Analysis of their ATACseq data also revealed the top binding transcription factor motifs for which the authors selected to study further, these include JunB, RelB, IRF5, RFX2, KLF6 and RUNX1. To dissect out the specific transcription factors that are involved in the maturation and activation of neutrophils, the authors employed a CRISPR/Cas9 system to knockout various transcription factors in myeloid (neutrophil) HOXB8 cell lines. Here, the authors showed that both KLF6 and RUNX1 were important for neutrophil maturation while IRF5, JUNB and RELB were shown to be important for activation and production of cytokines. Overall, this study provides information on the specific programming changes during their transition between tissue compartments, as well as how transcription factors regulate neutrophil function at inflamed sites. There are several key issues that the authors should address to strengthen the paper, and I have the following suggestions for authors' consideration:

Major comments:

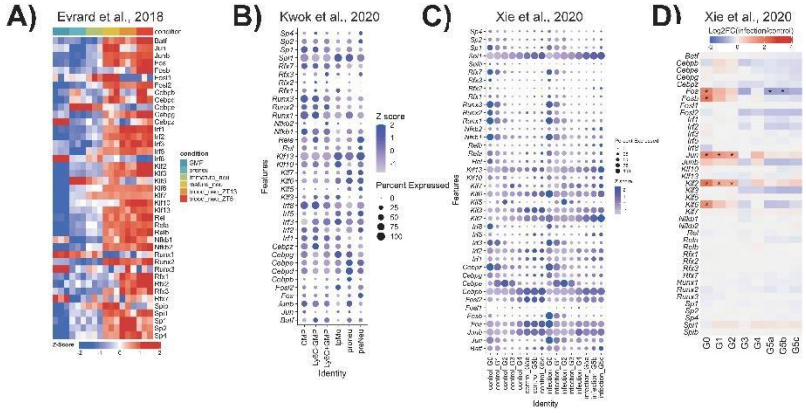
1. Based on the air pouch acute inflammatory model, the authors concluded that transcriptional change at two intervals, one in the transition from the bone marrow to the blood, and then from the blood into the inflamed tissues, and changes were reflected in upregulation/downregulation of specific transcript and chromatin accessibility modules. However, without examining bone marrow and blood neutrophils (sorted the same way as described by the authors) in control mice, it is difficult to know whether the results reflect inflammation-induced changes, or it is a result of accelerated trajectory of neutrophil development. Perhaps the authors should examine published datasets that were obtained at homeostasis, which have been separated into their individual cell developmental states (see Xie et al. 2020, Nat. Immunol; Muench et al., 2020, Nature; Kwok et al., 2020, Immunity) to evaluate if their transcription factors or gene modules are present already at steady state.

We thank the reviewer for suggesting to re-analyse the published datasets to examine the change of neutrophil specific transcription factors (TFs) during homeostasis and inflammation, which we have now done.

1. We used three published neutrophil RNA-seq datasets (Evrard et al. 2018, Immunity; Kwok et al. 2020, Immunity; Xie et al. 2020, Nat. Immunol) to examine the expression of the TFs identified and validated in this study, and other members of their families, during neutrophil maturation at homeostasis. The expression of *Runx1* was consistently highest at the earliest stages of neutrophil differentiation, whereas the expression of *Klf6, Cebpb, Rfx2, RelB, Irf5* and *JunB* increased with neutrophil maturation in the bone marrow and remained high in blood (**Rebuttal FigR1A,B,C**).

2. In addition, the Xie et al 2020, Nat. Immunol. dataset allowed us to directly compare the expression of the identified TFs during homeostasis and in bacterial challenge. *CEBPb, Runx1, Rfx2, Relb, Irf5* and *Junb*, remained unaffected by *E. coli* challenge (**Rebuttal FigR1C,D**). Expression of *Klf6* appeared to be induced by infection in G0 subpopulation only, but unaffected in other states (**Rebuttal FigR1D**). The data are now included in **Suppl Fig 4**.

We concluded that the mRNA expression of the identified TFs reflected chiefly on the stages of neutrophil maturation and was minimally affected by inflammation. This does not exclude the possibility of further post-translational protein modifications of these TFs in response to inflammation (see response to point 10 below).



Rebuttal Figure R1. Transcriptional change of neutrophil subpopulations. (**A**) Heatmap showing row-scaled expression of the signature TFs in bulk RNA-seq analysis of neutrophil populations in the bone marrow and blood (Evrard et al. 2018, Immunity). (**B**) Dot plot showing the scaled expression of signature genes for each scRNA-defined bone marrow neutrophil subpopulation, coloured by the average expression of each gene in each cluster scaled across all clusters (Kwok et al. 2020, Immunity). (**C**) Dot plot showing the scaled expression of signature TFs in each of Go to G5 clusters, mapped to progressively maturing neutrophils in scRNA-Seq analysis (Xie et al. 2020, Nat. Immunol), before and after intraperitoneal *E. coli* challenge, coloured by the average expression of each gene in each cluster scaled across all clusters. (**B,C**) Dot size represents the percentage of cells in each cluster with more than one read of the corresponding TFs. (**D**) Heatmap showing the log2[fold-change] in gene expression of the signature TFs between neutrophils isolated from control and *E. coli*-challenged mice (Xie et al. 2020, Nat. Immunol). The asterisks mean log2[fold-change] > 1 or <-1, padj<0.05 in corresponding cells.

2. One key technical issue that should be pointed out is the sorting strategy. While the authors acknowledged that neutrophils in the bone marrow and blood are composed of immature and mature subsets, the RNA-seq and ATAC-seq were performed on total Ly6GhiCD11b+ cells, which consist of both immature and mature neutrophils. The results obtained is an averaged expression and chromatin accessibility of both immature and mature subsets. How do the authors explain if these differences they observed is a tissue signature (BM v Blood) or simply a reflection of changes in the proportion of immature vs mature cells in different tissue compartments. This also raises a major question whether the study has identified inflammation specific TF

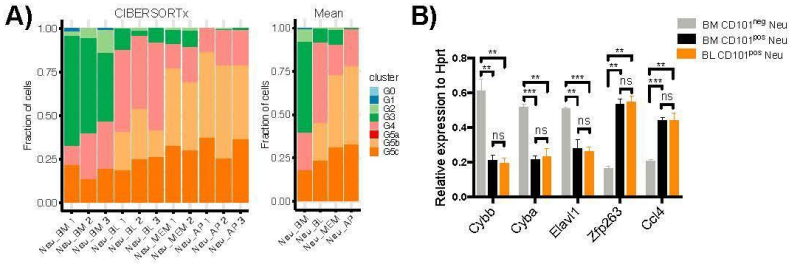
changes or changes that occur naturally as neutrophils migrate from the bone marrow to the blood.

We agree with the reviewer that the observed gene expression and chromatin differences at the BM-vs-Blood transition is likely to reflect the differences in neutrophil maturity. In fact, one could envisage that mobilization of mature neutrophils into circulation is the beginning of inflammatory response and the role for TFs controlling neutrophil maturation in this process is one of the findings we report in this manuscript. Indeed, we stated in the original discussion: "*One interesting result of our analysis was the capture of transcriptional changes related to neutrophil maturation, as underlined by the difference in chromatin landscape and expression profiles at the transition from the bone marrow to the blood (Figs 1, 2). This was likely to reflect on the presence of immature Ly6G$^+$CD101$^-$ neutrophils in the bone marrow samples (Suppl Fig 1), consistent with recently published studies (2, 11)*".

We have further strengthened this point during the revision:

1. We compared the scRNA-seq-defined neutrophil sub-populations G0 to G5, mapped to progressively maturing neutrophils (Xie et al. 2020, Nat. Immunol), with neutrophil samples in our study. Using the modified CibersortX analysis Xie et al showed that G2 correlated best with pre-neutrophils; G3 with immature neutrophils and G4 with mature neutrophils as identified in Evrard et al. 2018, Immunity at steady state. When we applied the same analysis to our dataset, it demonstrated that under acute inflammation BM-sorted neutrophils contained a significant proportion of G2-G3 cells, whereas blood (BL), membrane (MEM) and air pouch (AP) neutrophils were G4, G5b and G5c cells (**Rebuttal FigR2A**), indicating increased neutrophil maturation at the BM-to-Blood transition. The data were also consistent with our FACS analysis of BM and BL populations (**Suppl Fig S1G**). The data are now included in **Suppl Fig 1.**
2. We have sorted immature Ly6G$^+$CD101$^-$ and mature Ly6G$^+$CD101$^+$ neutrophils from the BM and the blood, and examined the mRNA expression of signature genes by qPCR. The result suggested that both the downregulation of BM-signature genes (*Cybb*, *Cyba*, *Elavl1*) and the upregulation of blood-signature genes (*Ccl4*, *Zfp263*), as defined by our DEG analysis in Fig 1, occurred between CD101$^-$ and CD101$^+$ neutrophils in BM, with equivalent expression of signature genes in CD101$^+$ neutrophils in BM and blood (**Rebuttal FigR2B**)

In summary, we appreciate the accurate prediction of the reviewer which we now integrate in the interpretation of our data. Overall, the observed transcriptional reprogramming at the BM-vs-Blood transition based on the sorting strategy used, captures the changes in neutrophil maturation and identifies TFs controlling these changes (**Figs 3,4**).



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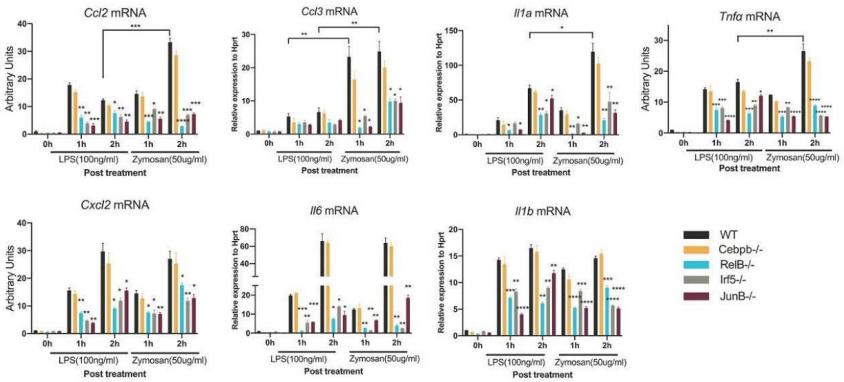
Rebuttal Figure R2. Extent of transcriptional difference between neutrophil subpopulation. (A) Correlation between indicated neutrophil samples with scRNA-seq-defined neutrophil populations reported by Xie et al. (1). Left: The fraction of each scRNA-seq defined clusters (G0-G5) in neutrophils samples in current study. Right: The mean fraction of indicated scRNA-seq defined clusters (G0-G5) in each group of neutrophil samples. (B) mRNA expression of BM- and blood-signature genes in $CD101^{neg}$ and $CD101^{pos}$ neutrophils sorted from BM and blood. Results are from three mice. ***p<0.001; **p<0.01; ns, not significant as determined by two-way ANOVA analysis with Tukey multiple comparison.

3. The authors utilized zymosan, a ligand for dectin-1 and TLR2 to drive neutrophil recruitment into the pouch cavity. As such, the author's findings for the bulk RNAseq and ATACseq is uniquely driven by inflammatory responses to zymosan alone via dectin-1 and TLR2 signaling. It is thus unclear if a similar transcriptional reprogramming process will also be initiated in other models of acute inflammation.

We thank the reviewer for raising this important point.

To examine whether neutrophil transcriptional reprogramming occurs in response to other inflammatory stimuli, we treated wild type and TF-deficient HoxB8 neutrophils with either LPS or zymosan and analysed the mRNA expression of inflammatory mediators, by qRT-PCR. We found that both LPS or zymosan induced expression of proinflammatory chemokines *Ccl2, Ccl3, Cxcl2* and cytokines *Il1a, Il1b, Il6, and Tnfa* (**Rebuttal FigR3**). Notably, RelB-deficient, Irf5-deficient and JunB-deficient neutrophils overall expressed lower levels of these mediators in response to either LPS or zymosan stimulation (**Rebuttal FigR3**).

These data indicate that that the overall transcriptional reprogramming observed in current study is not restricted to the one driven by dectin-1 and TLR2 signaling. We also noted that while induction of *Cxcl2, Il1b, Il6*, was indistinguishable between zymosan and LPS, induction of *Ccl2*, *Ccl3*, *Il1a*, and *Tnfa* appeared to be more robust in response to zymosan stimulation, emphasizing signal-specific differences as it has been extensively shown for other myeloid cells, e.g. macrophages.



Rebuttal Figure R3. Transcriptional reprogramming of neutrophils in response to zymosan and LPS. Cytokine and chemokine expression by HoxB8 neutrophils under LPS or Zymosan challenge. Statistical comparison was made by two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001 ****P<0.0001.

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4. Figure 2B is rather confusing, more information should be provided to help the reader to understand. In addition, on page 7, "The ATAC-seq analyses identified two distinct remodeling events, the transition of neutrophils from BM to blood (411 opening and 341 closing peaks), and from blood to the inflamed tissue (2,294 opening and 1,645 closing peaks)", but the numbers stated by the authors do not match with what were shown in the Supplementary Fig 3A. Can the authors check.

Fig 2B shows ATAC signal (FPKM) over differentially accessible peaks for each major remodelling events: BM-to-Blood (top) and Blood-to-Membrane (bottom). We show the peaks for which ATAC signal is increasing (i.e. chromatin is opening) or decreasing (chromatin is closing). We have amended the labels and provided more information in the revised text (**page 7**).

It appears that figures in the text were incorrect, and corresponded to the earlier version of the analysis. They have now been updated. We apologize for this oversight.

5. It is unclear what Figure 2C and 2D is comparing. What differential expressed genes are represented here and what is the significance for performing this comparison?

The purpose of these figures is to compare changes between the transcriptome and chromatin landscapes. Fold change in mRNA levels of differentially expressed genes is plotted against fold change in ATAC peaks located in promoters (< 2.5 k.b. from TSS) of the same genes. The original **Fig 2C** highlighted in red the genes that were significantly up- or down-regulated in both mRNA and ATAC-seq datasets. The original **Fig 2D** showed the gene ontology analysis of the up-regulated genes with opening promoters (red) and down-regulated genes with closing promoters (grey).

We have changed colours in **Fig 2C** and **Fig 2D**, to avoid any confusion. We now show genes which are both upregulated and located in the regions of opening ATAC-Seq peaks (increased chromatin accessibility) in red, genes which are both downregulated and located in the regions of closing ATAC-Seq peaks (decreased chromatin accessibility) - in blue.

6. Figure 4A, what is “neutrophils per filed (20X)”?

The annotation has been corrected, and technical detail of quantifying migrated neutrophils has been described in the Methods section.

7. The authors performed RNA-seq for cell lines with CEBPb, RELB, IRF5 and JunB knockouts. They stated that cell lines were challenged with zymosan for 2 hours or left unstimulated. However, it is unclear what is being reflected in Figure 5 – whether it is a combination of two subsets, or just the inflammation-induced changes. If so, the authors can actually subset out the true DEGs from zymosan induced inflammation by doing a pairwise comparison with the unstimulated control, and examine the effects of transcription factor knockout. Presentation in a heatmap/violin plots might be useful for this.

Fig 5 shows changes in expression of putative “target genes” in zymosan-stimulated cells only. We defined the “target genes” as: (1) the genes which expression is affected by the TF KO and (2) proximal to consensus binding motifs for the same TF, that are located in the regions of open chromatin identified by ATAC-Seq. We have clarified the description in the text of the manuscript (**page 15**) and in the **Fig 5 legend**.

Based on Reviewer’s suggestion, we now also show the relative expression of all Zymosan regulated genes in WT HoxB8 neutrophils (padj < 0.05, fold change > 1) from

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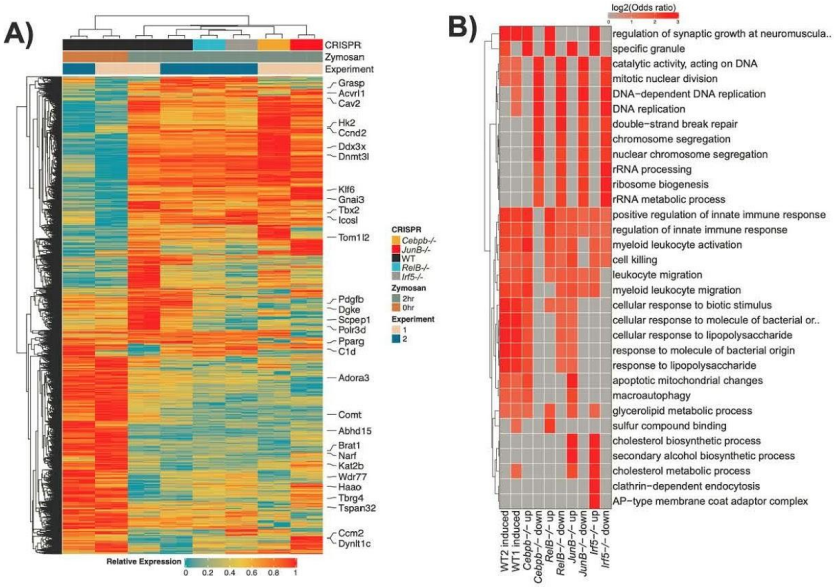
two independent experiments, which compare sets of transcription factor knock-out cells to WT controls. The heatmap highlights the global changes in gene expression in response to zymosan and the effect of each transcription factor knock-out (**Rebuttal FigR4A)**. We have produced the gene ontology analysis of the pairwise comparison of (1) zymosan-induced sample with the unstimulated control from the two experiments and (2) zymosan-induced TF knockout with zymosan induced corresponding WT control (**Rebuttal Fig R4B**). Analyses of global gene expression confirms the role for RELB, IRF5 and JunB in regulation of zymosan-induced immune genes and pathways and possible contribution to zymosan induction-independent cellular processes, e.g. DNA recombination and replication, ribosome biosynthesis. Since the analysis of global gene expression was not stratified by the increased likelihood of a gene being a direct target of a selected TF, the genes contributing to later GO categories may represent indirect targets of the selected TFs. We have included the global overview of gene expression in new **Suppl Fig 10**.



Rebuttal Fig 4 (**A**) Hierarchical clustering of all DEGs (padj < 0.05, $|\log_2 FC|>1$). Data are presented as heatmap normalized to the minimum and maximum of each row. (**B**) Gene ontology (GO) analysis showing the log2 odds ratio of genes regulated by specific TF knockout with the indicated GO annotation.

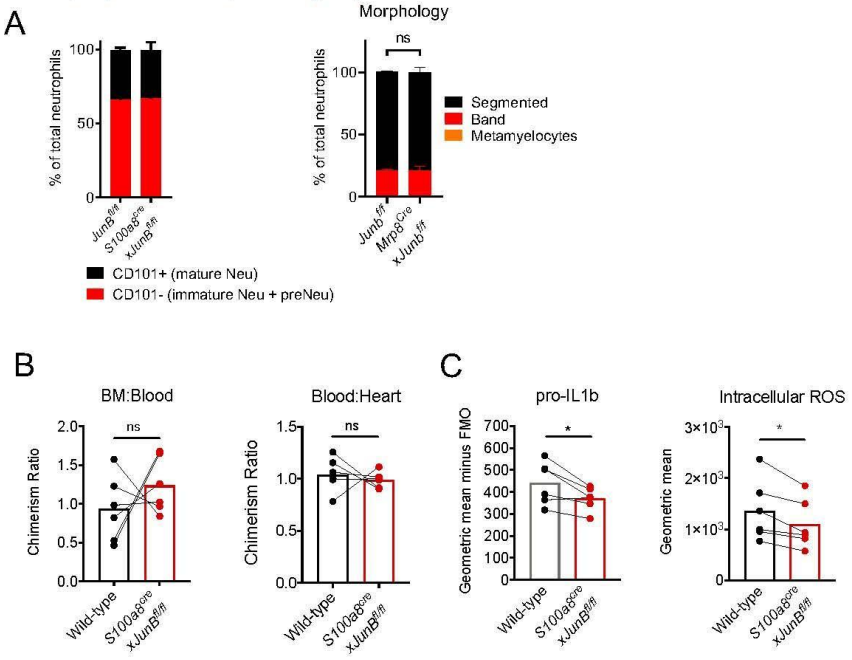
8. The authors define mature HoxB8 neutrophils based on CD101 expression. Can the authors be sure that CD101 expression is not affected by the lack of specific transcription factors? Perhaps the authors should also examine cell nucleus morphology to complement and support their flow cytometric analysis in determining the differentiation stage of the HoxB8 neutrophils.

We agree with the reviewer that neutrophil nucleus morphology assessment is a powerful way of monitoring neutrophil maturation. Neutrophil nucleus morphology has indeed been assessed (**Fig 3A**) and quantified (**Fig 3B**) in the original submission and is consistent with CD101 expression.

9. While the authors performed myocardial infarction model with the cre-Mrp8 x Jun fl/fl and showed that these mice have a reduction of infarction size, there is no analysis of the neutrophil development, neutrophil subset composition and their function in this mouse model. The authors should use primary neutrophils from this model to validate the in vitro functional data from the HoxB8 neutrophil cell line.

We have further analysed the *S110a8-cre x JunB fl/fl* model and included new data in new **Fig 7** and new **Suppl Fig 12**.

Firstly, we analysed the BM and blood of these mice at steady state and confirmed that they have no defect in neutrophil maturation (**Rebuttal Figure R5A**). Secondly, we generated mixed chimeric mice harboring both control and JUNB-deficient neutrophils by BM transplantation into WT recipient mice and confirmed their chimerism. 3h after induction of cardiac ischemic-reperfusion injury we analyzed neutrophils in the myocardia from either genotype by flow cytometry. Determination of the chimerism ratio in BM:Blood or Blood:Heart in JUNB-deficient and control neutrophils did not show any significant change between genotypes (**Rebuttal Figure R5B**), corroborating our finding that neutrophil recruitment to the site of inflammation is not affected by depletion of JUNB in neutrophils (**Fig 4c**). However, in agreement with our HoxB8 neutrophil *in vitro* and *in vivo* analyses (**Fig 5e, 6c, e**), we found consistent reductions both in intracellular levels of pro-IL1b and ROS in JUNB-deficient neutrophils compared to control neutrophils (**Rebuttal Figure R5C**). Overall, our new data suggest no alterations in development and migration in the JunB mutants, while inflammatory-related properties are specifically impaired.



Rebuttal Figure R5. Phenotypic assessment of JunB-deficient neutrophils *in vivo*. (A) percentages of neutrophil subsets (pre-neutrophils, immature and mature neutrophils) from $JunB^{fl/fl}$ and $S100a8^{cre/cre}xJunB^{fl/fl}$ mice, assessed by flow cytometry

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(left) and morphological assessment (right). Data are shown as means and SD derived from at least three mice from each group within one experiment. Statistical comparison was made by one-way ANOVA. ns, no significant difference. (B) The chimerism ratio of neutrophils in the blood (left), and heart (right) in mice subjected to permanent myocardial infarction 6 weeks after mixed bone marrow transplant. Data are shown as means and SD derived from three mice from each group within one experiment. Statistical comparison was made by paired student-t test. ns, no significant difference. (C) Determination of pro-IL1b (left panel) or ROS production (right panel) in WT and JunB-deficient neutrophils in mice subjected to permanent myocardial infarction 6 weeks after mixed bone marrow transplant. Data are shown as means and SD derived from three mice from each group within one experiment. Statistical comparison was made by paired student-t test; *$p<0.05$.

10. The authors need to discuss that they are not able to distinguish the changes they are looking at are true transcriptional changes induced by the inflammatory state, or whether the inflammatory state accelerates neutrophil maturation and mobilised into the periphery. For example, the expression of JunB is presented to be only activated in inflammatory settings. However, the authors need to rule out that JunB is already present and activated in mature neutrophils in the unchallenged state, since JunB expression might naturally come up as the neutrophil matures.

In this study we map two different transitions: (1) BM-to-Blood and (2) Blood-to-Membrane/Air Pouch.

The first transition is associated with neutrophil maturation, as discussed in response to points 1, 2 above. We agree with the reviewer that the role of inflammation in this transition is largely related to changes in neutrophil maturation and in proportion of immature to mature neutrophils in the BM and blood, as indicated in response to point 2 above. Consistently, the TFs that were highlighted to likely control this transition were shown to control neutrophil maturation (**Fig 3, 4**).

The second transition Blood-to-Membrane/Air Pouch is inked to neutrophils infiltrating the tissue, which in our model is caused by injection of zymosan into the air pouch. As both blood and membrane neutrophils comprised of mature neutrophils (**Rebuttal Fig R2**), zymosan enhances inflammatory and IFN responses in the cells reaching the tissue (**Fig 1c-e**). This is consistent with the conclusions of Xie et al 2020, Nat Immunol that "In relatively mature G4 and G5 neutrophils, bacterial infection triggered significant upregulation of cytokine production and secretion genes". The transcriptional changes observed in fully mature HoxB8 neutrophils, stimulated with zymosan *in vitro* (**Fig 6, Suppl Fig S10, Rebuttal Figure R3**), also confirm that these are true transcriptional changes induced by inflammatory stimuli.

In our study, the prediction of key TFs controlling neutrophil responses at the site of inflammation, was based on the increased availability of the binding motifs for their interaction with the DNA in the inflamed tissue. We did not use increase in TF mRNA expression as a selection factor. Thanks to the additional analysis conducted after the Reviewer's advice, we now confirm that mRNA expression of signal-induced TFs, such as RELB, IRF5, JUNB, increases as neutrophil mature, remain stable in mature neutrophils (BM, Blood, tissue) and is minimally affected by infectious challenge (**Rebuttal Fig R1**). Protein levels of JUNB also increase over neutrophil differentiation course (**Rebuttal Fig R6**). Importantly, zymosan stimulation does not alter the level of JUNB protein but results in post-translational modification of the protein, i.e.

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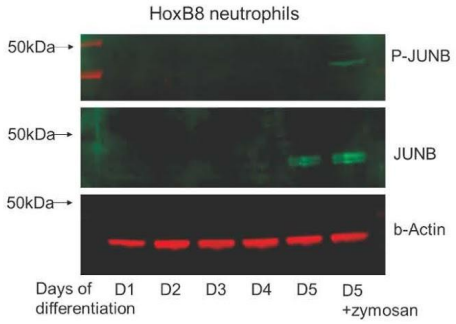
phosphorylation, indicating its activation (**Rebuttal Fig R6**) and supporting its participation in controlling transcriptional regulation in the inflamed tissue.



Rebuttal Figure R6. Post-translational activation of JUNB in neutrophils in response to zymosan. Western blot analysis of JUNB phosphorylation in response to zymosan stimulation in HoxB8 mature neutrophils.

Minor comments:

1. The authors should check all figures to ensure good quality and consistent formatting.

Many thanks for the suggestion and the quality of figures have been improved.

2. Figure 1a, it is unclear what the three harvest points represent? No information is provided in figure legend or text. The multiple points were representing multiple time points used in supplementary Fig 1 to describe the system. We have now simplified the graphics indicating only one point (4h) that was used in genomic sample collection.

3. Formatting with genes and protein symbols in the manuscript should be consistent. Mouse gene symbols should be italicized (e.g. Runx1) with the first letter capitalised. Mouse protein symbols should be capitalised (e.g. RUNX1). Knockouts are represented as Runx1-/- to show that the gene Runx1 is deleted on both alleles as proteins cannot be knocked-out by CRISPR/Ca9 systems. This formatting is sometimes observed (Figure 2E, Figure 5A and Figure 6A) but not in others.

Many thanks for the reviewer to point it out, and it has been corrected now.

Reviewer #2:

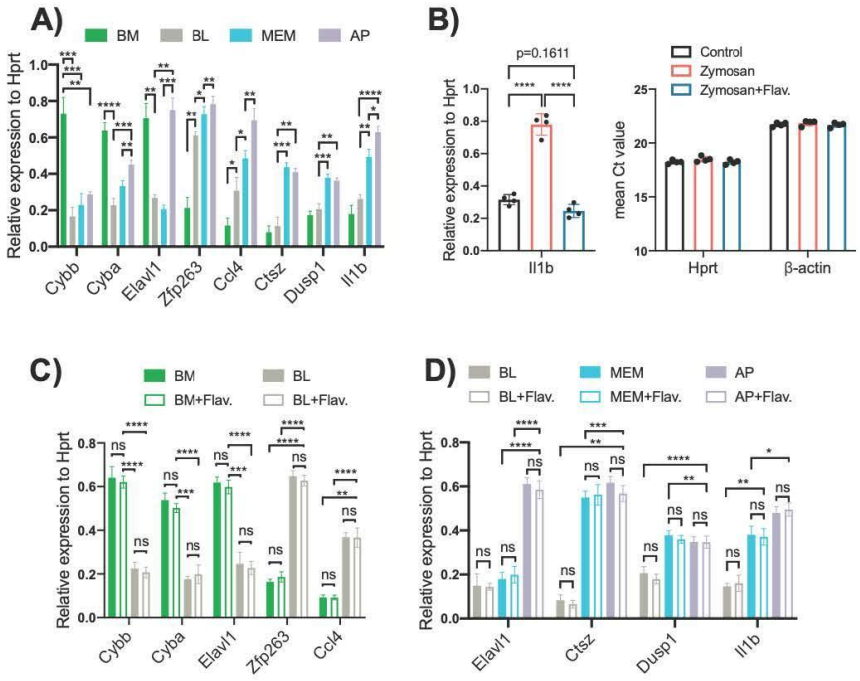
Remarks to the Author:

The authors present an interesting study on transcription factor networks and chromatin remodelling in neutrophils as they emigrate from the bone marrow, circulate, and eventually infiltrate into sites of inflammation. The authors identify transcription factors crucial to each of these stages of the neutrophil journey and in a proof-of-principle approach, the authors show that interference with JunB inflammation in a preclinical mouse model of MI can be alleviated. Overall, I am very positive towards this study as the authors use state of the art techniques, draw the right conclusions from their data and provide an important piece to the community. However, I have several general as well as technical concerns:

1- The authors should confirm that the differences at transcriptome level between MEM and the rest of conditions is not due to the digestion process of the tissue. This could explain why there are differences between air pouch exudate and membrane in RNA but not in chromatin accessibility. Also, the authors should comment on how the small differences in handling of neutrophils isolated from different compartments may impact on the data.

This is an important technical question and we thank the review for raising it. We have now conducted two experiments to clarify this issue:

1. We processed BM, BL, MEM and AP in an exactly identical manner, including incubation with the digestion enzymes at 37°C for one hour normally used for MEM samples only. When we analysed expression of tissue-signature genes (BM (*Cybb*, *Cyba*, *Elavl1*), BL (*Ccl4*, *Zfp263*), MEM/AP (*Ctsz*, *Dusp1*, *Il1b*)) by qPCR, we found that tissue processing did not introduce a major effect on gene expression (**Rebuttal FigR7A**), indicating that the tissue processing procedure did not bias our results.
2. Additionally, we investigated whether any gene expression was induced during tissue processing, by treating BM, BL, MEM, and AP neutrophils with an inhibitor of RNA polymerase II (flavopiridol) to prevent de novo gene transcription, followed by qRT-PCR analysis of mRNA expression of signature genes. The value of this approach was first validated in BM neutrophils treated *in vitro* with flavopiridol (2μM) and stimulated with zymosan. Flavopiridol prevented zymosan-induced expression of *Il1b* mRNA, without affecting the expression of the housekeeping gene *Hprt* and *β-actin* (**Rebuttal FigR7B**). Importantly, we found that flavopiridol failed to inhibit mRNA expression of tissue signature genes in neutrophils from BM (*Cybb*, *Cyba*, *Elavl1*), BL (*Ccl4*, *Zfp263*), MEM/AP (*Ctsz*, *Dusp1*, *Il1b*) (**Rebuttal FigR7C&D**), confirming that neutrophil transcriptional signature was existed before neutrophil isolation and was not affected by de novo gene expression induced by tissue processing procedure.

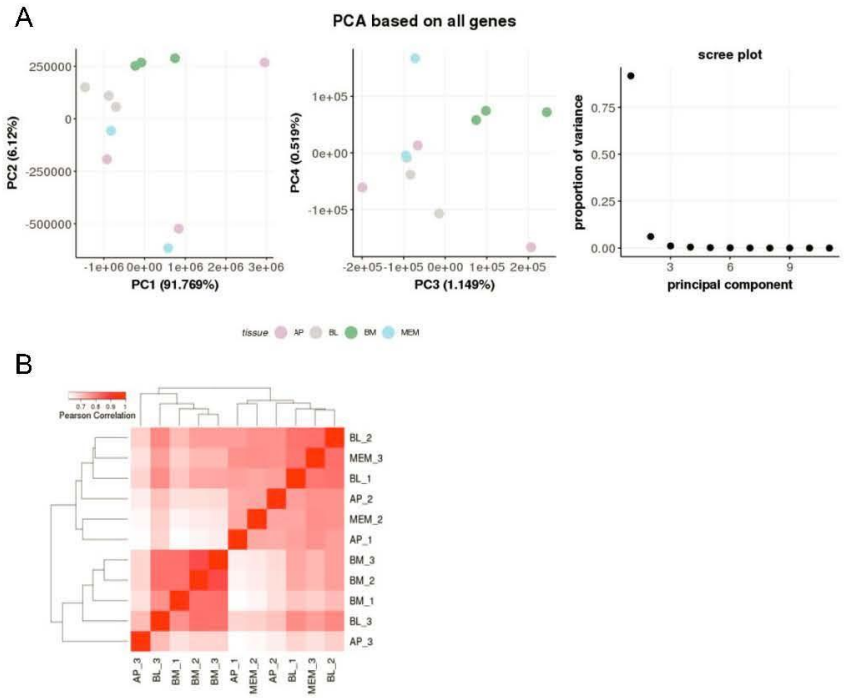


Rebuttal Figure R7. Neutrophil transcriptional signature exists before neutrophil isolation. (A) Tissue-signature gene expression in neutrophils bone marrow, blood, air pouch membrane and exudate and lung neutrophils measured by qPCR. Data are for 3 mice per condition. (B) Bone marrow neutrophils were isolated and incubated for 1h with vehicle or zymosan (25ng/ml) or zymosan with flavopiridol (2uM). Induction of Il1b mRNA levels and expression of the housekeeping gene *Hrpt* and *β-actin* were determined by qPCR. (B) mRNA expression of bone marrow- and blood-associated genes in bone marrow and blood incubated with digestion enzyme mix and flavopiridol. (A,B,C,D) Results are from 3-4 mice. Bar graphs show mean normalised gene expression ± SEM in the indicated genes. ***p<0.001; * p<0.05 as determined by two-way ANOVA (A,C,D) or one-way ANOVA with Tukey multiple comparison (B).

2- The PCA analysis is performed on differentially expressed genes. This PCA should be done in all detected genes to define the differences between neutrophil states considering the whole transcriptome?

We routinely perform PCS on genes that are differentially expressed (from a DESeq2 likelihood ratio test – reflecting significant changes across all samples) as the vast majority of genes in any experiment are not differentially expressed. Therefore, it is often necessary to filter genes with an appropriate statistical test to reveal the underlying structure of the data. Other genomics publications in the field use a similar strategy. For example, Evrad et al 2018, Immunity, conduct PCA on their bulk RNA-seq data "using the top 20% variable genes (as measured by standard deviation across samples) and then those that were significantly associated with a cell population (FDR-corrected ANOVA, q-value < 0.05) resulting in 4820 DEGs".

However, on the reviewer's request we have generated the PCA analysis on all detected genes (normalised TPMs). PC1 vs PC2 projection separates BM from BL from MEM and AP neutrophils, but separation of MEM and AP neutrophils is less clear (**Rebuttal Fig R8A**), compared to more restrictive DEG analysis (**Fig 1B**). We have additionally included a Pearson correlation matrix based on the same data (normalised TPMs of all detected genes) that shows the samples generally split into either more quiescent (BM, blood) or more active (tissue, air pouch) states (**Rebuttal Fig R8B**), broadly reflecting our results from more thorough analysis.



Rebuttal Fig 8. Transcriptional difference between different neutrophil states. (**A**) PCA of gene expression data from neutrophils recovered from bone marrow, blood, air pouch membrane and exudate in mice subjected to air pouch model and zymosan challenge for 4 hours. (**B**) A correlation matrix generated with Pearson's correlation coefficients represents similarities of gene expression between subsets (low similarity = white, high similarity = red).

3- The authors show that the transitions from bone marrow to blood and blood to membrane are associated to upregulation or downregulation of certain groups of genes and this coincide with open/close chromatin peaks. Could the authors confirm some of these changes at protein level?

Indeed, how much mRNA expression translates into protein production is an important question, which we have already addressed to a certain degree in the original manuscript.

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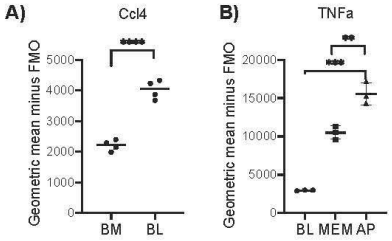
Firstly, using flow cytometry we showed that levels of pro-IL1b are increased in neutrophils transiting from blood into air pouch membrane and exudate (**Sup Fig1E**). Secondly, using protein arrays we assessed the secretion of inflammatory cytokines and chemokines, including IL-1b, by HoxB8 neutrophils deficient in selected TFs (**Fig 6**). Thirdly, using Western Blot we examined the production of caveolin 1, which mRNA expression was universally affected by RelB, IRF5 and JunB knock-outs (**Fig 5**).

Here we extend our protein analysis, by including two other proteins: TNF and CCL4. *Ccl4* was detected as a gene that was upregulated at the BM-to-Blood transition, a *Tnf* is progressively induced in neutrophils transiting from blood into air pouch membrane and exudate (**Fig 1**). Using flow cytometry we demonstrate higher levels of CCL4 protein in blood compared to BM neutrophils (**Rebuttal Fig R9A**), and higher levels of TNF in MEM and AP compared to BL neutrophils (**Rebuttal Fig R9B**). Altogether, these results reinforced the transcriptional change of transiting neutrophils during inflammation.



Rebuttal Figure R9. Differential expression of Ccl4 in neutrophils from bone marrow and blood. Geometric mean of Ccl4 (**A**) and TNF (**B**) expression in $CD11b^{+}Ly6G^{+}$ neutrophils. Data are shown as means and SD derived from three mice from each group within one experiment. Statistical comparison was made by Student's t-test, ****P<0.0001 (**A**) or One-way ANOVA, **P<0.001, ****P<0.0001 (**B**).

4- The authors provide an interesting correlation showing that most of the changes at transcriptome level (downregulation/upregulation) directly associate with remodeling of the chromatin (close/open). Is this evenly occurring among all states? Are there particular states where chromatin conformation does not relate with gene expression?

The global changes (BM to AP) correlate well, indicating that chromatin state is broadly aligned with mRNA levels. There are also matching changes from the BM to the blood, and blood to tissue in each dataset (**Fig 2**).

However, some of the granularity observed in the mRNA data is absent when studying the chromatin directly. The most obvious is the absence of chromatin remodelling at the final transition from the tissue (MEM) to air pouch (AP), with ongoing changes in mRNA expression. These data suggest that changes in gene expression at this final step of neutrophil migration does not require global alterations in transcriptional control.

5- In LysM Runx1-/- mouse model, the authors show that this TF is involved in the neutrophil maturation within the bone marrow. However, no link is provided that these TFs drive the transition from bone marrow to blood. Are Runx1 or Klf6 involved in BM to blood neutrophil mobilisation? Also, the authors should provide absolute counts of neutrophils in the bone marrow and blood.

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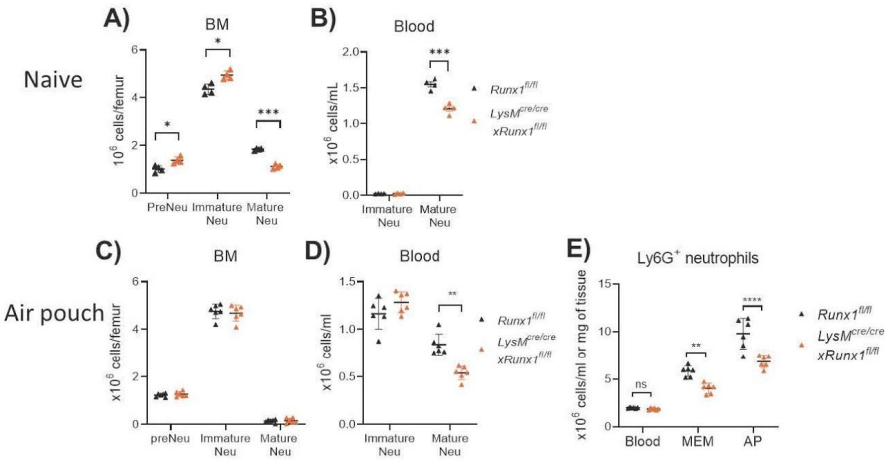
The Reviewer raised an interesting point whether the compromised neutrophil maturation in the BM will be reflected in the number of mature neutrophils in the blood.

We have shown in the original manuscript that a lower percentage (15% vs 24%) of Ly6G^{hi} CD101^{+} mature neutrophils was detected in the bone marrow of naïve *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ mice (**Fig 3f**). We now show the absolute counts of neutrophils from the same experiment, which confirm the reduction in mature neutrophil numbers in the BM of *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ mice (**Rebuttal Fig R10A**). We also show that absolute counts for mature neutrophils in the blood of *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ mice is reduced by a similar degree (**Rebuttal Fig R10B**). Thus, we concluded that RUNX1-deficiency reduces the number of mature neutrophils in circulation reflecting on their demoted maturation in the BM. These data are now included in **Suppl Fig 7**.

During the revision, we conducted new experiments, in which we subjected *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ and control *Runx1*$^{fl/fl}$ mice to the air pouch model of inflammation and characterized the proportion of mature and immature neutrophils in the BM and blood. Following induction with zymosan, mature neutrophils transit in their entirety to the blood (**Suppl Fig 1g, h**). Zymosan also induced egress of a significant number of immature neutrophils into the circulation (**Suppl Fig 1h**). We confirmed that zymosan induction leads to depletion of mature neutrophils in the BM in both *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ and control *Runx1*$^{fl/fl}$ mice and stimulates egress of a significant number of immature neutrophils into the circulation (**Rebuttal Fig R10C, D**). We observed a shift towards a lower number of mature neutrophil in the blood of *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ mice (**Rebuttal Fig R10D**), but the total number of neutrophils in circulation was comparable between *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ and control *Runx1*$^{fl/fl}$ mice (**Rebuttal Fig R10E**). Of importance, the recruitment of neutrophils to the air pouch membrane and exudate was significantly lower in *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ mice (**Rebuttal Fig R10E**), supporting our data on the reduced capacity of RUNX1-deficient neutrophils to trans-migrate *in vitro* and infiltrate the tissue *in vivo* (**Fig 4**).

We have included these data in the **revised Fig 4, Suppl Fig 7** and **new Suppl Fig 9**.



Rebuttal Figure R10. Runx1 deficiency inhibits neutrophil mobilization into tissue. (A,B) Quantification of indicated neutrophil subsets in the bone marrow (A) and blood (B) of naïve LysM$^{cre/cre}$ xRunx1$^{fl/fl}$ and Runx1$^{fl/fl}$ control mice. (C,D) Quantification of indicated neutrophil subsets in the bone marrow (C) and blood (D) of LysM$^{cre/cre}$ xRunx1$^{fl/fl}$ and Runx1$^{fl/fl}$ control mice subjected to the air pouch model of acute inflammation. (E) Quantification of total neutrophil numbers in the blood, air pouch membrane and exudate from mice subjected to the air pouch model and zymosan

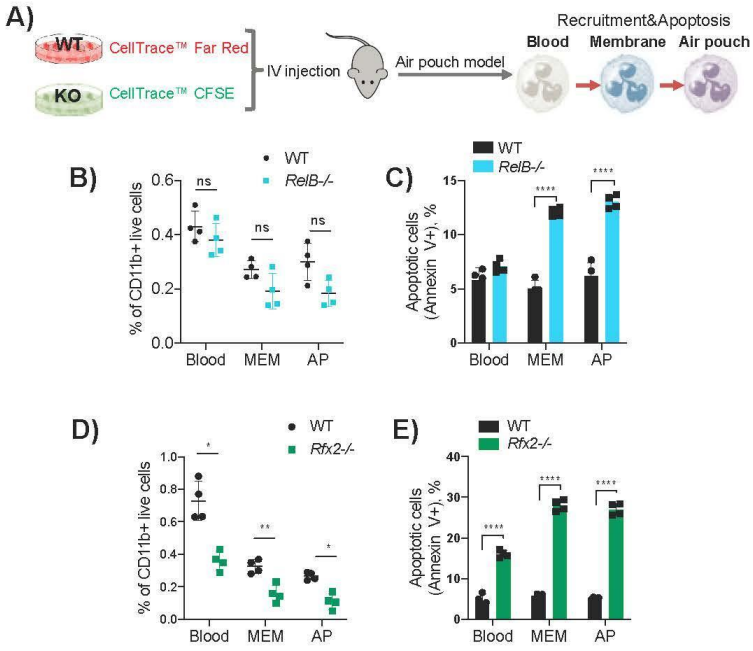
challenge. Data are shown as means and SD from four to six mice. Statistical comparison was made by two-way ANOVA, *P<0.05; **<0.01; ***<0.001; ****P<0.0001.

6- Rfx2 and Relb are shown to be regulators of steady-state neutrophil apoptosis. Do neutrophils deficient for these TFs exhibit also increased apoptosis upon inflammation in vivo?

We thank the Reviewer for prompting us to further investigate the effect of RFX2 and RELB deficiency on neutrophil survival in inflammation.

To assessing the effect of RFX2 and RELB on neutrophil survival upon inflammation *in vivo*, we performed additional adoptive transfer experiments, in which the equivalent mixtures of wild-type neutrophils and either RFX2-deficient or RELB-deficient HoxB8 neutrophils were intravenously injected into mice with an air-pouch created under their skin. The mice were then subjected to zymosan challenge and the apoptotic rate and recruitment of adoptively transferred neutrophils were assessed by flow cytometry (**Rebuttal Fig R11A**). As reported in **Fig 4C**, RELB-deficient neutrophils infiltrated the site of inflammation as efficiently as WT cells (**Rebuttal Fig R11B**), but showed an increased level of apoptosis at the site of inflammation (**Rebuttal Fig R11C**). In contrast, RFX2-deficient neutrophils were more apoptotic than WT cells already in the blood, and their rate of apoptosis increased further at the site of inflammation **(Rebuttal Fig R11E)**. Consequently, a significant reduction in recovery of RFX2-deficient cells was observed in all the compartments (**Rebuttal Fig R11D**). These results suggest the intrinsic requirement of RFX2 and RELb in maintaining neutrophil survival in vivo.

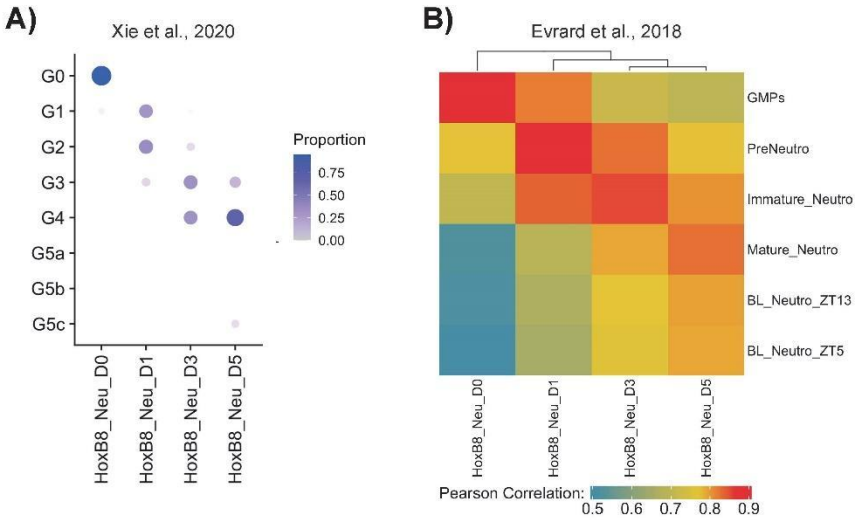
We have included these data in the **revised Fig 4** and **new Suppl Fig 9**.



Rebuttal Figure R11. RFX2 and RELB knockout neutrophils exhibit increased apoptosis upon inflammation *in vivo*. (**A**) Experimental set-up of adoptive transfer experiments, and the recruitment and apoptosis of transferred WT and KO HoxB8 neutrophils were assessed by flow cytometry. Percentages of wild-type and RELB-deficient (**B**) or RFX2-deficient (**D**) HoxB8 neutrophils in the blood, air pouch membrane and exudate. Percentages of apoptotic wild-type and RELB-deficient (**C**) or RFX2-deficient (**E**) neutrophils in the same compartments.

7- How the RNAseq from the Hoxb cells correlate with the genes found in the RNAseq of the different neutrophil states?

We compared gene expression profiles of HoxB8 neutrophils across the differentiation/maturation days (D0 to D5) with scRNA-defined neutrophil subpopulations reported by Xie et al. Nat Imm 2020, using their pipeline. HoxB8 myeloid progenitors (D0) are mainly G0 cells while HoxB8 D1 neutrophils were a mixture of G1-G3 cells and HoxB8 D3 cells to be G2-G4 cells. Upon maturation, the HoxB8 D5 neutrophils contained a substantial number (~80%) of mature neutrophils (G4 cells) (**Rebuttal Figure R12A**). We also used the Pearson correlation analysis to compare the gene expression in HoxB8 neutrophils with neutrophil subtypes reported by Evrard et al. Immunity, 2018, and revealed that GMPs, preneutrophils, $CD101^{neg}$ immature neutrophils and $CD101^{positive}$ mature neutrophils were correlated with HoxB8 D0, D1, D3 and D5 neutrophils, respectively, consistent with our phenotypic analysis of HoxB8 neutrophils (**Rebuttal Figure R12B**). Therefore, HoxB8 neutrophils exhibited a gene expression matching different neutrophil differentiation stages.

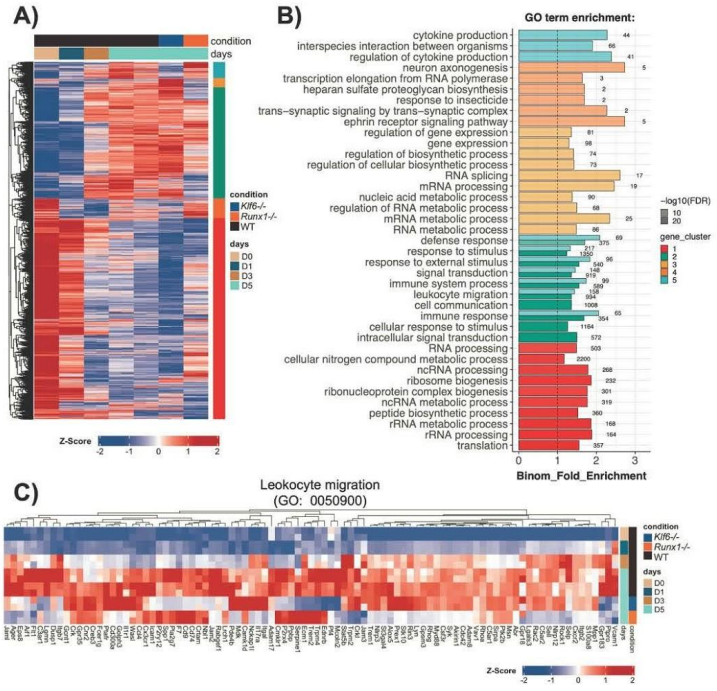


Rebuttal Figure R12. Correlation of HoxB8 neutrophils with previously defined neutrophil subpopulations. (**A**) The fraction of pre-defined scRNA-seq-defined neutrophil populations (G0-5 cells) reported by Xie et al, Nat Imm, 2020, in indicated HoxB8 neutrophils. (**B**) Heatmap showing Pearson correlation of HoxB8 neutrophils with the neutrophil subtypes reported by Evrard et al, Immunity 2018.

As for Runx1 and Klf6, the authors should provide a causal link between these TFs and the transcriptional profile found in recruited neutrophils within the air pouch.

We followed up on the Reviewer's advice to provide a causal role of Runx1 and Klf6 in neutrophil migration and recruitment to the site of inflammation by conducting a new RNA-seq analysis of gene expression in WT HoxB8 neutrophils throughout their differentiation and maturation in vitro (day 0, 1, 3 and 5), together with RUNX1- and KLF6-deficient HoxB8 neutrophils at the end of the maturation process (day 5). Hierarchical clustering of the differentially expressed genes identified five clusters, which encompassed genes downregulated (cluster 1: red and cluster 4: orange) or upregulated with maturation (cluster 2: green, cluster 3: yellow and cluster 5: blue). Genes in clusters 2 and 5 were downregulated in *Runx1-/-* neutrophils, or *Klf6-/-* neutrophils, respectively (**Rebuttal Fig R13A).** GO annotation analysis of gene expression revealed that these two clusters encompassed transcriptional programs of immune responses, cytokine production and leukocyte migration (**Rebuttal Fig R13B**). Genes in clusters 4 and 3 were upregulated in *Runx1-/-* neutrophils, or *Klf6-/-* neutrophils, respectively, with programs capturing metabolic and biosynthetic processes (**Rebuttal Fig R13B**). *Runx1* knockout inhibits the transcription of *Cxcr2*, *Sell*, *S100a8*, while *Klf6* knockout suppresses the mRNA expression of *Vcam1*, *Cd9*, *C3ar1* (**Rebuttal FigR13C**), all of which are genes that encode important cell adhesion and chemotaxis molecules. These results collectively suggest that Runx1 and Klf6 control the expression of genes involved in leukocyte migration. They also highlight differences in RUNX1- and KLF6- controlled transcriptional networks, supporting our observation of the likely independent function of the two pathways (**Fig 3g**).

We have included these data in the **new Suppl Fig 8**.



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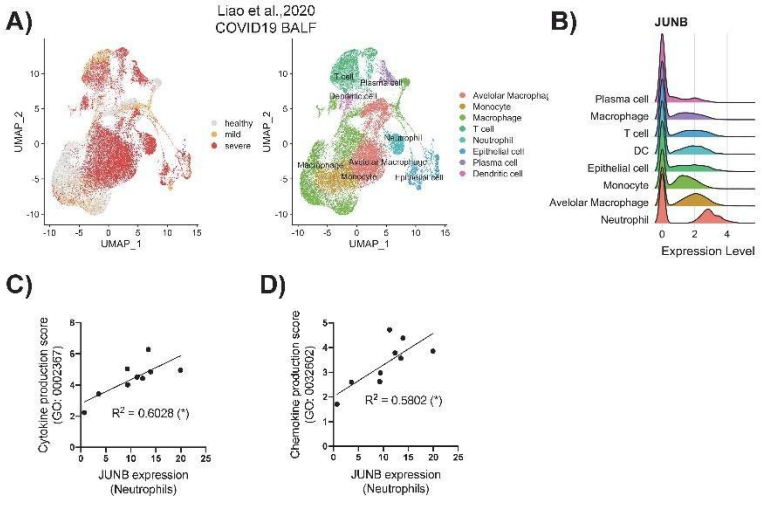
Rebuttal Figure R13. RUNX1 and KLF6 in transcriptional control of neutrophil migration. (A) Hierarchical clustering of all differentially expressed genes (LRT test padj < 0.01, log_2FC >1), based on Manhattan distances using the Ward method. Data are presented as heatmap normalised to the minimum and maximum of each row. (B) Gene ontology (GO) analysis, showing the top 10 enriched GO categories for each cluster from (A). (C) Leukocyte-migration-related gene expression in wild-type, *Klf6-/-*, *Runx1-/-* HoxB8 neutrophils.

8- Do the authors have evidences of the existence of this regulatory network in human neutrophils?

To determine whether similar transcriptional network exists in human disease, we analysed published scRNA-seq of bronchoalveolar lavage fluid (BALF) from healthy and COVID-19 patients (Liao et al, Nat Med 2020). The authors reported the presence of neutrophils in their scRNA-Seq dataset and noted that neutrophil number increases significantly in severe COVID-19 patients. We followed their Seurat-pipeline to streamline cell clustering along major populations. Using FeaturePlot data and differentially expressed marker genes among clusters, we identified 8 cell lineages. Macrophages (LYZ, CD68), alveolar macrophages (CD68, SIGLEC1), monocytes (CD14), neutrophils (FCGR3B), T cells (CD3E, TRAC), plasma cells (IGHG4, MZB1, CD19), dendritic cells (CLEC9A, CD1A) and epithelial cells (KRT18, KRT19) were identified (**Rebuttal Fig R14A**). We then examined expression of JUNB across these cell populations and found that it was preferentially expressed in neutrophils (**Rebuttal Fig R14B**). We then correlated JUNB expression with the average expression of genes encoding cytokines (**Rebuttal Fig R14B**) and chemokines (**Rebuttal Fig R14C**). While correlative, these data align with our findings in mice showing that JUNB may also regulate cytokine and chemokine expression in human neutrophils.



Rebuttal Figure R14. Signature TFs in COVID19 neutrophils. (A) The UMAP presentation of cell clusters was divided in bronchoalveolar lavage fluid (BALF) from healthy donors, mild, and severe COVID19 patients. (B) Violin plot of JUNB expression in indicated cell cluster. The correlation of JUNB to with the average expression of

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genes related to cytokine production (GO: 0002367) (C) and chemokine production (GO: 0032602) (D). One-tailed Spearman's test was used for the correlation analyses.

9- Can the authors detail how they obtained the 1865 DE genes?

These were obtained from a likelihood ratio test with DESeq2 comparing all experimental conditions, with an adjusted p value of < 0.1

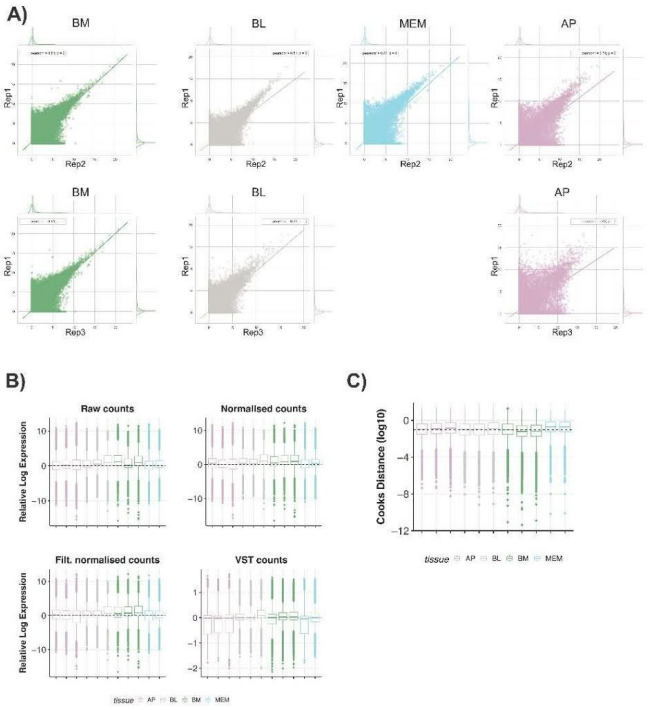
10- PCA for RNA analysis shows 3 points/population, except for MB (2 points). How can this come from 2 mice? Also, why has 1 MB point excluded from the analysis?

1 membrane sample was dropped due to low sequencing quality

11- Did authors compare samples through paired analysis (BM BL MB AP for each mice)?

We have conducted paired and statistically relevant analyses through each compartment using all of the biological replicates to ensure that our findings are robust and not the product of biological variance. We have not, however, compared each compartment between individual mice, as this would invalidate the assumptions made by the DESeq2 algorithm and not yield informative results. Our choice of analysis is robust to biological variation and well documented and referenced in the RNA-seq literature.

The quality control assessment of small-bulk RNA-seq data is shown in **Rebuttal Fig R15**.



Rebuttal Figure R15. Pairwise comparison and quality control for small bulk RNA-seq. (**A**) Pairwise comparison & Pearson correlation of replicate samples. Log transformed total counts (TPMs). (**B**) Relative log expression of raw, normalised, filtered normalised, and variance stabilised counts from DESeq2 analysis. (**C**) Cook's distance showing detected outliers from DESeq2

Due to the nature of SmartSeq2 sample preparation and the low cell numbers used, the total number of detected genes are lower than for bulk mRNA-seq (10,000 – 15,000 genes), and there is more variability between replicates for lowly expressed genes. However, the relative log expression plot indicates that the normalisation applied by DESeq2 is of good quality, and the Cooks distance indicates that there are few outliers and none of the samples are individually driving the fitted coefficients for specific genes (**Rebuttal Fig R15**).

12- During their different analysis, authors switch from different p-values to adj p-values. Is there a statistical rational for that?

They're all adjusted p-values (we apologize for the typos which have been removed).

Decision Letter, first revision:

Subject: Your manuscript, NI-A31167A
Message: Our ref: NI-A31167A

17th May 2021

Dear Dr. Udalova,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "Distinct transcription factor networks control neutrophil-driven inflammation" (NI-A31167A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

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

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

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

In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Distinct transcription factor networks control neutrophil-driven inflammation". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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

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

Best regards,

Elle Morris
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On behalf of

Zoltan Fehervari, Ph.D.
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Reviewer #1:
Remarks to the Author:
First of all, the authors should be commended for their efforts in revising the manuscript. I have few points that would like the authors to address, which i think will further strengthen the already very nice manuscript.

Fig 2b - While the authors have provided clarifications, the authors should consider making a histogram to show the ATAC-seq data over the 2.5kb between each of the compartment. The authors should also consider to move Suppl Figure 3C to main Figure 2. Together, this will further strengthen the message of Figure 2.

Page 13 - "Strikingly, the number of neutrophils mobilized into the site of inflammation (air pouch membrane and exudate) was significantly lower in the LysMcre/cre xRunx1fl/fl mice compared to control Runx1fl/fl mice (Fig 4d). This is consistent with the reported reduced migratory capacity of neutrophil progenitors in tissue (2)." - This statement should be modified, while previous report has suggested that neutrophil progenitors do not have effective migratory function, in the context of Fig 4d, the low total number of neutrophils at the site of inflammation is most likely attributed to the lower circulating mature neutrophils (Suppl Fig 9C).

Suppl Figure 12 - Can the authors clarify why for the analysis of S100A8Cre x JunB fl/fl

mice did not separate immature vs preNeu in the Cd101- population.

Reviewer #2:
Remarks to the Author:
The authors have gone a long way to answer my questions and indeed I have only minor comments left which I think should be discussed in the manuscript possibly in a 'limitation' section.

1. I am still not convinced that their processing does not impact on neutrophil signatures in different compartments. Based on marker analysis the authors here provided evidence that some marker genes do not seem to be affected - however, this may not hold true on a global scale and hence, I believe it is fair to point such limitation out in the discussion.

2. The CD11b+Ly6G+ neutrophil population in the bone marrow is not a homogenous population but in fact consists of neutrophils of different maturity; unless the authors gated for very mature neutrophils (CXCR2+, Ly6Ghi, CD101+) the authors should point out that differences between mature blood neutrophils and bone marrow neutrophils may at least in part be due to the fact that in the bone marrow not only mature neutrophils were gated for.

Author Rebuttal, first revision:

We are grateful to the reviewers for acknowledging our work during the revision and for further minor suggestions on how to improve the clarity of the message.

Reviewer #1

(Remarks to the Author)
First of all, the authors should be commended for their efforts in revising the manuscript. I have few points that would like the authors to address, which i think will further strengthen the already very nice manuscript.

Fig 2b - While the authors have provided clarifications, the authors should consider making a histogram to show the ATAC-seq data over the 2.5kb between each of the compartment. The authors should also consider to move Suppl Figure 3C to main Figure 2. Together, this will further strengthen the message of Figure 2.

We have modified Fig 2 according to reviewer's suggestions. An overall histogram of ATAC-seq signals is now presented in Fig 2C, while the previous expanded version is moved to Extended Data Fig 3B.

Page 13 - "Strikingly, the number of neutrophils mobilized into the site of inflammation (air pouch membrane and exudate) was significantly lower in the LysMcre/cre xRunx1fl/fl mice compared to control Runx1fl/fl mice (Fig 4d). This is consistent with the reported reduced migratory capacity of neutrophil progenitors in tissue (2)." - This statement should be modified, while previous report has suggested that neutrophil progenitors do not have effective migratory function, in the context of Fig 4d, the low total number of neutrophils at the site of inflammation is most likely attributed to the lower circulating mature neutrophils (Suppl Fig 9C).

We have modified the statement.

Suppl Figure 12 - Can the authors clarify why for the analysis of S100A8Cre x JunB fl/fl mice did not separate immature vs preNeu in the Cd101- population.

In response to the reviewer's original question, we investigated a potential effect of JunB deficiency on neutrophil maturation. Using two complementary analyses (FACS analysis and morphological cell assessment we demonstrated that there is no significant difference in neutrophil maturation between *JunB*$^{fl/fl}$ and *S100a8*^{cre}*JunB*$^{fl/fl}$ mice. The data are now presented in Extended Data Fig 6. The markers to distinguish preNeu and immature neutrophils (c-Kit and Cxcr4) were not included in the FACS panel for this specific revision experiment.

(Remarks to the Author)
The authors have gone a long way to answer my questions and indeed I have only minor comments left which I think should be discussed in the manuscript possibly in a 'limitation' section.

1. I am still not convinced that their processing does not impact on neutrophil signatures in different compartments. Based on marker analysis the authors here provided evidence that some marker genes do not seem to be affected - however, this may not hold true on a global scale and hence, I believe it is fair to point such limitation out in the discussion.

We have performed extensive work to address the question raised by the reviewer and think that it is unlikely that tissue processing has significantly impacted our neutrophil signatures.

However, we are happy to point out in the discussion on a possible limitation of cell isolation and further development of spatial genomics as a way to avoid such issues. We have included a corresponding short sentence.

2. The CD11b+Ly6G+ neutrophil population in the bone marrow is not a homogenous population but in fact consists of neutrophils of different maturity; unless the authors gated for very mature neutrophils (CXCR2+, Ly6Ghi, CD101+) the authors should point out that differences between mature blood neutrophils and bone marrow neutrophils may at least in part be due to the fact that in the bone marrow not only mature neutrophils were gated for.

The point has been raised by Reviewer 1 during the revision and has already been extensively addressed in our response to them, as well as in the text of the manuscript. For example, we point out in the main text that "the difference in chromatin landscape and expression profiles at the transition from the bone marrow to the blood was likely to reflect on the differences in proportion of immature Ly6G+CD101- and mature Ly6G+CD101+ neutrophils in the bone marrow and blood samples".

Final Decision Letter:

In reply please quote: NI-A31167B

Dear Dr. Udalova,

I am delighted to accept your manuscript entitled "Distinct transcription factor networks control neutrophil-driven inflammation" for publication in an upcoming issue of Nature Immunology.

The manuscript will now be copy-edited and prepared for the printer. Please check your calendar: if you will be unavailable to check the galley for some portion of the next month, we need the contact information of whom will be making corrections in your stead. When you receive your galleys, please examine them carefully to ensure that we have not inadvertently altered the sense of your text.

Acceptance is conditional on the data in the manuscript not being published elsewhere, or announced in the print or electronic media, until the embargo/publication date. These restrictions are not intended to deter you from presenting your data at academic meetings and conferences, but any enquiries from the media about papers not yet scheduled for publication should be referred to us.

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About one week before your paper is published online, we shall be distributing a press release to news organizations worldwide, which may very well include details of your work. We are happy for your institution or funding agency to prepare its own press release, but it must mention the embargo date and Nature Immunology. Our Press Office will contact you closer to the time of publication, but if you or your Press Office have any enquiries in the meantime, please contact press@nature.com.

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Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor

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