nature portfolio

Peer Review File

Neutrophil single-cell analysis identifies a type II interferon-related subset for predicting relapse of autoimmune small vessel vasculitis

Corresponding Author: Dr Masayuki Nishide

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Brief Overview:
The manuscript by Nishide et al. uses multidimensional techniques to profile the transcriptome and surface proteins of neutrophils from healthy individuals and new onset patients with microscopic polyangiitis (MPA) at single cell resolution. The single-cell analysis resulted in classification of neutrophils into 7 subsets, with changes in the frequency of 3 subsets in patients compared to healthy controls: Neu_Mature decreased in patients; Neu_Immature and Neu_T2ISG increased. The Neu_T2ISG was characterized by expression of interferon γ(IFN-γ) response genes. Trajectory analysis indicates this population differentiates from Neu_Mature population. In vitro experiments showed priming of neutrophils with IFN-γ and TNF-α increased neutrophil extracellular trap (NET) formation induced with anti-myeloperoxidase (MPO) antibody. The increased proportion of the Neu_T2ISG subset was associated with worse symptoms, and in a larger cohort serum IFN-γ levels were elevated in patients at disease onset without treatment. Further analysis of prognosis suggests that serum IFN-γ levels may be a biomarker for disease relapse.

Major comments:
The ability to capture therapy-naïve patients for this study is commendable. Another main strength of the paper is the in-depth computational analysis of the gene expression and surface protein data sets to uncover neutrophil heterogeneity. This heterogeneity highlights an immune cell population not simply terminally differentiated but one that is much more dynamic and communicates with other immune cells. This makes this a strong manuscript which will be useful for researchers investigating neutrophil mediated disease or pathogenetic processes. However, there are several limitations that should be addressed.

1. Interestingly the Discussion lacks a limitation section. For instance, some limitations are sole inclusion of MPA patients which may limit conclusions to GPA forms of ANCA associated vasculitis, and the absence of a disease control comparator group. While including in-depth analysis of a disease control may be beyond the scope of this study, it is fair to acknowledge that neutrophil heterogeneity identified among patients with MPA in this study may differ in other autoimmune diseases.
2. The bulk of neutrophils are comprised of Neu_Mature, even when reduced in patients. The neutrophil heterogeneity identified in this study indicates increase in patients of two neutrophil populations (Neu_Immature and Neu_T2ISG). What does the significant, but relatively small increase in these other two populations mean to the disease pathology induced by ANCA mediated activation of mature neutrophils?
3. The data in Figure 3d-f are important to show that IFN-γ signaling can induce different neutrophil functions, i.e. cell surface expression, viability, and NET formation. These and other neutrophil functions have been shown to be activated by IFN-γ. (Reviewed in Ellis, TN and Beaman, BL. Immunology 2004 May; 112(1):2-12) In the Results section describing Figure 3 it states “IFN-γ priming induces a uniquely primed neutrophil subset that is sensitive to MPO-ANCA stimulation and readily triggers NET formation.” As the authors acknowledge MPO-ANCA is capable of inducing neutrophil oxidative burst, degranulation, NETosis, etc. Although a minor point, it appears the NET formation was induced with a commercial anti-MPO antibody, not patient MPO-ANCA. More significant, it is not clear how the IFN-γ treatment only or preferentially stimulates a subset of neutrophils. Co-staining with a marker that is enriched/unique to the Neu-T2ISG and detecting NETosis in those neutrophils would be more convincing.
4. Figure 4 depicts patterns of gene expression that differ among patients with MPA and 3 patients with more severe

symptoms were enriched for IFN-g response genes and Neu_T2ISG. The Results section states these 3 patients had “long duration between the onset of vasculitis symptoms and sample collection.” What is long duration and how might that contribute to interpretation of the results regarding differentiation and proportion of Neu_T2ISG in disease?
5. Serum IFN-g analysis and findings as a biomarker of relapse could have important implications for clinical management of disease. What was the time to relapse? Was time to relapse from disease onset, onset of induction therapy, or after remission was achieved? How was remission defined? BVAS or other disease severity index alone or BVAS plus clinical review for no sign of disease?

Other less major comments:
1. Paragraph on monocytes analysis is interesting, but is seems out of place or unnecessary. It might fit earlier in the manuscript demonstrating the validity of the analysis of neutrophils because using the approach described in this manuscript confirms the conclusions on monocytes in MPA reported previously with slightly different approach.
2. The module score for LDN in myelocyte population shown in Supplementary Figure 4 is unclear. Forgive me, but the Myelocyte cluster is not obvious compared to the other 6 clusters. Previous studies (Mhaonaigh, AU. et al. Frontiers in Immunology 2019. 10: Article 2603 and Jones, BE. et al. Kidney International 2020. 98: 744-757) on LDN/LDGs in patients with ANCA associated vasculitis identified both mature and immature neutrophils which indicates the LDN population is comprised of more than myelocytes.
3. IFN-γ/Th1 signaling has been shown to be elevated in patients with ANCA associated vasculitis (Csernok, E. et al. Arthritis and Rheumatism 1999. 42(4): 742-750), and AAV not thought to be a type I interferonopathy (Batten, I. et al. Scientific Reports 2021. 11(1): 8272). How do these prior findings impact the results presented in this manuscript?
4. In Figure 3C Neu_T2ISG labeled as “Signal” in box below circos plot. Should the label be “Receiver”?

Minor corrections
Line 357: “Neutrophil priming are …” ; change to “Neutrophil priming are is …”
Line 371: “degranuration” ; change to “degranulation”
Linee 431: “tretment” ; change to “treatment”

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The present manuscript entitled «Neutrophil single-cell analysis identifies a uniquely primed subset and provides insights into predicting relapse of autoimmune small vessel vasculitis» by the team of Masayuki Nishide attempts to distinguish neutrophil subsets of patients suffering from microscopic polyangiitis (MPA) in order to predict relapse.
This study follows a previous interesting study exploring two phenotypes of new-onset MPA based on monocyte subtypes (Nishide et al, 2023).
The team bases their research on scRNAseq data of blood leukocytes from six untreated patients with new-onset MPA. Authors found seven neutrophil subsets, of which two were increased in MPA patients. One of the increased subsets is characterized by IFNg expression. Cell-cell interaction analysis showed that expression of this subset was affected by IFNg from CD8+ T-cells. By experimental methods they found that IFNg primed neutrophils are sensitive to MPO-ANCA stimulation, which triggers NET formation. In a validation cohort they found IFNg to be increased in the serum of MPA-patients, which is associated with relapse.
This study is of importance because it explores new biomarkers for relapse in MPA.
The manuscript is well written and the complex topic is well explained. However, there are a few remarks that would ameliorate the manuscript and makes it easier for the reader to understand:

Major points:
- How are the two neutrophil groups Neutrophil_1 and Neutrophil_2 of Figure 1 related to or devided into the seven neutrophil subsets of Figure 2?
- Please indicate which patients in supplementary table 3 suffered from a relapse. In Figure 4d there are 9 relapses, but in supplementary table 3, there are only 7 patients with a worse disease. It is not clear, how worse is defined and how it differs from relapse.
- What is the ratio of differentially expressed genes to total genes found?
- Do the authors have information about GPA (granulomatosis with polyangiitis) patients as well? Do they show similar results?
- Assuming that all the patients in the study are of Japanese origin, do the authors think the results are also applicable to patients with other ethnic background?
- Did the authors also measure ROS production in addition to NET release? It would be interesting to know if the different neutrophil subsets produce different amounts of ROS as it is known for LDNs compared to NDNs. Also, it would be interesting to know if neutrophils from MPA patients produce less ROS in vitro compared to controls comparable to a study of peripheral blood neutrophils from GPA patients demonstrating a decreased ROS production in vitro compared to those from healthy controls (Amsler et al, Rheumatology, 2023).
- Can the authors comment on the occurrence or relevance of LDN (low density neutrophils) in their study?
- Did the authors perform any immunophenotyping of neutrophils from MPA patients to confirm the subsets found in the scRNAseq cohort?

- Please explain in more detail the similarities and differences of neutrophil subpopulations from MPA patients compared to the study of Montaldo et al (Nat immunol 2022) subtyping neutrophils in patients undergoing stress-induced myelopoiesis.
- Compared to the authors first study identifying two phenotypes of new-onset MPA, can the authors describe whether the subsets of neutrophils found in the current study overlap with the two former MPA-phenotypes and comment on the reasons why or why not?
- It would increase the value of the manuscript if a reference about neutrophils in vasculitis was added such as Aymonnier et al. The neutrophil: A key resourceful agent in immune-mediated vasculitis. Immunol Rev. 2023 Mar;314(1):326-356. doi: 10.1111/imr.13170. Epub 2022 Nov 21. PMID: 36408947. Or Michailidou et al. Role of Neutrophils in Systemic Vasculitides. Front Immunol. 2020 Dec 17;11:619705. doi: 10.3389/fimmu.2020.619705. PMID: 33391289; PMCID: PMC7774018.

Minor points:
- Introduction: Line 105: remove the word “into” directly before “to”
- Results: In order to find the heatmap faster in the figures while reading the text, I suggest to add “Fig.4a” at the end of the first sentence on line 305 instead of line 311.
- Discussion: Line 371 and 374: “degranulation” instead of “degranuration”

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I jointly reviewed this manuscript with Reviewer #2. Please refer to reviewer #2 comments.

(Remarks on code availability)
I did not install the review code

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors provided thoughtful, thorough, and meticulous responses to reviewer comments. The additional gene expression analysis (included in Supplementary Table 2c), single-cell analysis (included in Supplementary Figure 9), and in vitro stimulation results (included in revised Figure 3, panels 3f and 3i) strengthens major conclusions of the manuscript regarding the importance and potential clinical implications of Neu_T2ISG neutrophil subset. The findings reported in this manuscript contribute new insights into neutrophil heterogeneity that will be important for future studies in patients with other forms of ANCA-associated vasculitis and different autoimmune diseases. In addition, the potential clinical implications related to IFN-γ levels, which stemmed from neutrophil profiling and bioinformatic analyses, deserve testing in additional patient cohorts.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have answered to the issues raised by the Reviewer and have provided additional data to further document their points.

(Remarks on code availability)
I do not have any specific expertise in evaluating the reliability and the accessibility of the code.

Our response to the Reviewer #1

We sincerely appreciate the opportunity to revise our manuscript. We have responded to the comments from Reviewer #1 in the following point-by-point manner. The line number corresponds to the number in the manuscript with tracked changes (Revised Manuscript Tracked Changed).

Brief Overview:

The manuscript by Nishide et al. uses multidimensional techniques to profile the transcriptome and surface proteins of neutrophils from healthy individuals and new onset patients with microscopic polyangiitis (MPA) at single cell resolution. The single-cell analysis resulted in classification of neutrophils into 7 subsets, with changes in the frequency of 3 subsets in patients compared to healthy controls: Neu Mature decreased in patients; Neu Immature and Neu T2ISG increased. The Neu T2ISG was characterized by expression of interferon γ(IFN-γ) response genes. Trajectory analysis indicates this population differentiates from Neu Mature population. In vitro experiments showed priming of neutrophils with IFN-γ and TNF-α increased neutrophil extracellular trap (NET) formation induced with anti-myeloperoxidase (MPO) antibody. The increased proportion of the Neu T2ISG subset was associated with worse symptoms, and in a larger cohort serum IFN-γ levels were elevated in patients at disease onset without treatment. Further analysis of prognosis suggests that serum IFN-γ levels may be a biomarker for disease relapse.

Major comments:

The ability to capture therapy-naïve patients for this study is commendable. Another main strength of the paper is the in-depth computational analysis of the gene expression and surface protein data sets to uncover neutrophil heterogeneity. This heterogeneity highlights an immune cell population not simply terminally differentiated but one that is much more dynamic and communicates with other immune cells. This makes this a strong manuscript which will be useful for researchers investigating neutrophil mediated disease or pathogenetic processes. However, there are several limitations that should be addressed.

<Our response>

We are grateful to you for perfectly summarizing the essence of our study and

recognizing the strength of focusing on therapy-naïve patients and their neutrophil heterogeneity at the single cell level.

1. Interestingly the Discussion lacks a limitation section. For instance, some limitations are sole inclusion of MPA patients which may limit conclusions to GPA forms of ANCA associated vasculitis, and the absence of a disease control comparator group. While including in-depth analysis of a disease control may be beyond the scope of this study, it is fair to acknowledge that neutrophil heterogeneity identified among patients with MPA in this study may differ in other autoimmune diseases.

<Our response 1>

We apologize for not including a Limitation section in the original manuscript and thank you for these remarks. As pointed out, the following limitations should be discussed; the patients included in this study were limited to MPA, therefore it is unknown whether the concept of this study can be applied to GPA. In addition, we addressed the lack of the neutrophil profiles of other disease controls and the patients recruited in this study were all Japanese, therefore racial differences cannot be considered.

<Revised Point 1>

-Discussion

We have added the following sentences.

(Line 469 - 477)

This study has several limitations. First, all patients recruited were Japanese, and potential racial differences were not evaluated. Second, the neutrophil profiles of other disease control groups were not analyzed, leaving it unclear whether the unique neutrophil profile identified in this study is specific to MPA. Importantly, a previous study demonstrated that a Th1 cytokine pattern, particularly IFN-γ expression, predominates in granulomatosis with polyangiitis (GPA) [41]. Although this study focuses on MPA and does not include GPA patients, the IFN-γ signaling pathway may represent a shared feature in the pathophysiology of other forms of ANCA-associated vasculitis.

2. The bulk of neutrophils are comprised of Neu_Mature, even when reduced in patients. The neutrophil heterogeneity identified in this study indicates increase

in patients of two neutrophil populations (Neu_Immature and Neu_T2ISG). What does the significant, but relatively small increase in these other two populations mean to the disease pathology induced by ANCA mediated activation of mature neutrophils?

<Our response 2>

Thank you for raising this important point. As you correctly noted, Neu_Mature constitutes the majority of neutrophils, while Neu_Immature and Neu_T2ISG are modestly, though significantly, increased in MPA. To address this, we conducted additional differential gene expression (DEG) analysis between MPA patients and healthy donors, with a particular focus on the Neu_Mature subset. This analysis revealed that characteristic genes identified in Neu_T2ISG (e.g., GBP1 and FCGR1A) are also upregulated in Neu_Mature.

These findings suggest that while the proportional increase in distinct populations as “Neu_Immature” and “Neu_T2ISG” is relatively small, the differences in gene expression are more broadly distributed across mature neutrophil subset, extending beyond distinct cell composition. We have added Supplementary Table 2c and results from this additional DEG analysis to the manuscript. Thank you again for your comment.

<Revised point 2>

-Results

We have added the following sentences.

(Line 246 - 248)

In addition, focusing on the Neu_Mature subset, which constitutes the majority of neutrophils, 22 IFN-γ response genes were still identified as DEGs characteristic of MPA patients (Supplementary Table 2c). These data suggest that the upregulation of IFN-γ response genes is broadly distributed across neutrophil subsets, representing a key feature of MPA patients.

-Supplementary Information

We have added the following Supplementary Table 2c and legend.

c

Neu_Mature DEGs MPA v.s. HD	
AC010894.3	IFITM3
AC244021.1	IL4R
ADM	ISG15
AIM2	LILRA5
AL691403.1	LINC00486
ANKRD44	LY6E
CD177	MED25
CLEC12A	MT2A
CST7	MYL12A
DSC2	OAS1
EIF2AK2	OAS2
EPSTI1	OAS3
FCGR1A	PARP9
FOLR3	RNF213
GBP1	RSAD2
GLIPR1	S100A12
GRINA	S100A8
H3F3A	S100A9
HERC5	SAMD9L
HIST2H2BE	SEC14L1
HLA.B	SERPING1
IFI16	SH3GLB1
IFI44	SIGLEC9
IFI44L	TLR5
IFI6	TNFAIP6
IFIH1	TNFSF13B
IFIT1	TRIM22
IFIT2	WWOX
IFITM1	XAF1
IFITM2	

(c) The top genes significantly upregulated in the Neu_Mature subset in MPA patients compared to healthy donors.

3. The data in Figure 3d-f are important to show that IFN-γ signaling can induce different neutrophil functions, i.e. cell surface expression, viability, and NET formation. These and other neutrophil functions have been shown to be activated by IFN-γ. (Reviewed in Ellis, TN and Beaman, BL. Immunology 2004 May; 112(1):2-12) In the Results section describing Figure 3 it states "IFN-γ priming induces a uniquely primed neutrophil subset that is sensitive to MPO-ANCA stimulation and readily triggers NET formation." As the authors acknowledge MPO-ANCA is capable of inducing neutrophil oxidative burst, degranulation, NETosis, etc. Although a minor point, it appears the NET formation was induced with a commercial anti-MPO antibody, not patient MPO-ANCA. More significant, it is not clear how the IFN-γ treatment only or preferentially stimulates a subset

of neutrophils. Co-staining with a marker that is enriched/unique to the Neu-T2ISG and detecting NETosis in those neutrophils would be more convincing.

<Our response 3>

Thank you for your thoughtful and detailed comments. We have addressed the following four points in response.

<Response 3-1>

We have cited the suggested reference (Ellis, TN and Beaman, BL, Immunology 2004) in the appropriate section of the manuscript to strengthen our discussion.

<Response 3-2>

As noted, MPO-ANCA induces various neutrophil functions, including oxidative burst, degranulation, and NETosis. In our original manuscript, we focused on NETosis only. In response to your comment, we have performed an additional ROS production assay and confirmed that IFN-γ-primed neutrophils are highly sensitive to ROS production in response to MPO-ANCA stimulation. The results of this assay and corresponding figures have been added to the revised manuscript.

<Our response 3-3>

As you correctly pointed out, in the original experiments we used commercially available anti-MPO antibodies. To address this limitation, we purified IgG from stored sera of MPA patients and repeated the experiments using patient-derived antibodies. The results confirm that TNF-α + IFN-γ-primed neutrophils are sensitive to NET formation induced by patient-derived MPO-ANCA. These findings and the data have been included in the revised manuscript.

<Our response 3-4>

We share your interest in determining whether a specific neutrophil subset, such as Neu_T2ISG, is preferentially sensitive to NET formation. Unfortunately, specific surface markers to definitively identify Neu_T2ISG are currently unavailable. To address this question as thoroughly as possible, we conducted additional single-cell analyses on *in vitro* stimulated neutrophils to investigate their differentiation into diverse populations. As a result, stimulation with a combination of TNF-α and IFN-γ induced a uniform subset resembling

Neu_T2ISG. By contrast, stimulation with TNF-α or IFN-γ alone gave rise to subsets with distinct characteristics. Given that the combination of TNF-α and IFN-γ particularly promotes ROS production and NET formation compared to the other conditions, it is reasonable to suggest that Neu_T2ISG is highly sensitive to these processes. While these findings do not directly prove that Neu_T2ISG is uniquely responsible for enhanced ROS production and NET formation, they suggest that this subset has a high potential to generate ROS and NETs. Supplementary Fig. 9 and results from these analyses have been included in the revised manuscript.

<Revised point 3>

-Results

We have added the following sentences.

(Line 317 - 320)

Upon subsequent stimulation with MPO-ANCA, TNF-α priming could enhance ROS production and NET formation, which was further enhanced by concurrent IFN-γ priming (Fig.3f, Fig. 3g, and Fig. 3h).

(Line 320 - 324)

Next, we purified serum IgG from MPA patients (patient IgG) and evaluated NET formation following stimulation with these immunoglobulins. As expected, priming with both IFN-γ and TNF-α induced significantly enhanced NET formation in response to patient IgG compared to priming with TNF-α alone (Fig. 3i).

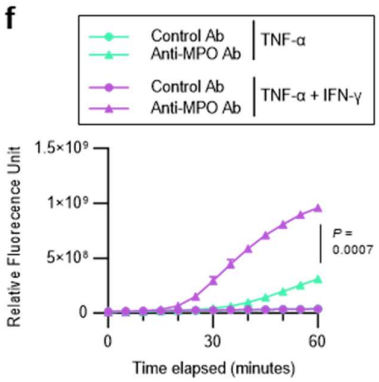
(Line 325 - 335)

To further investigate the characteristics of the TNF-α and IFN-γ-primed neutrophil subset, we conducted additional scRNA-seq analyses on in vitro stimulated neutrophils (Supplementary Fig. 9a and 9b). Stimulation with a combination of TNF-α and IFN-γ induced a uniform subset (Cluster1), resembling Neu_T2ISG (Supplementary Fig. 9c and 9d). In contrast, in vitro stimulation with TNF-α or IFN-γ alone gave rise to subsets with the distinct characteristics, which resembling Neu_Longlived (Cluster2) or Neu_T1ISG/Neu_T2ISG mixed (Cluster3) (Supplementary Fig. 9c and 9d). These findings suggest that priming with a combination of TNF-α and IFN-γ induces a uniquely primed neutrophil subset, which is highly sensitive to MPO-

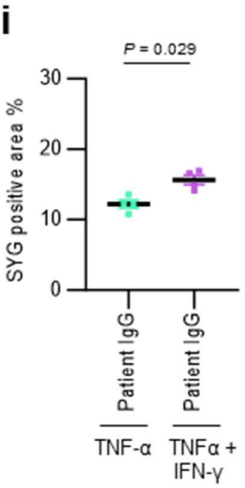
ANCA stimulation and readily triggers ROS production and NET formation.

-Main Figures

We have added the following Fig. 3f, 3i, and legend.



(f) Reactive oxygen species (ROS) production of neutrophils isolated from healthy donors. TNF-α–primed cells were treated with or without IFN-γ for 16 hours, followed by stimulation with anti-MPO antibody (10 μg/mL) for 1 hour. ROS levels were monitored every minute for a total of 60 cycles. Representative traces show the means with SEMs from a triplicate experiment.



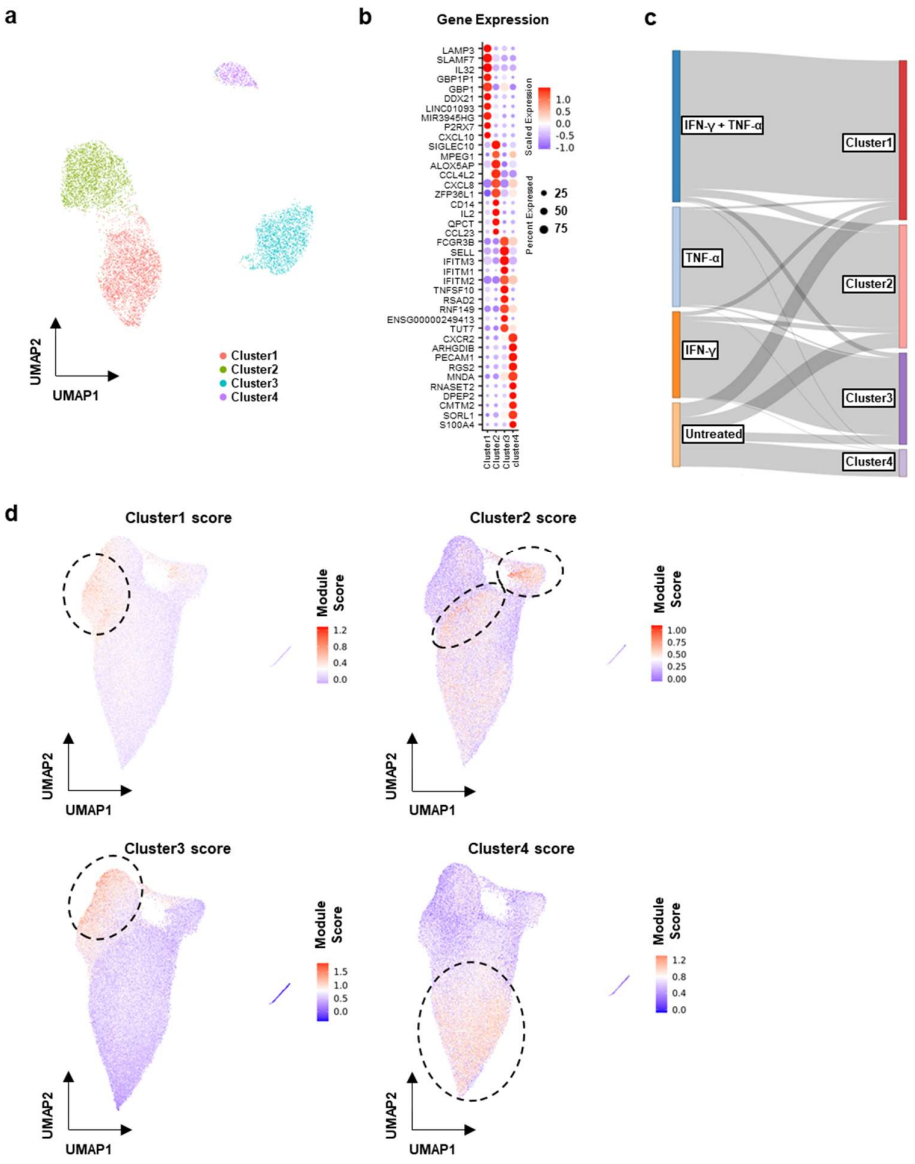
SYTOX green immunofluorescence analysis to detect neutrophil extracellular trap (NET) formation. TNF-α–primed cells were treated with or without IFN-γ for 16 hours, followed by stimulation with anti-MPO antibody (10 μg/mL) for 4 hours to induce NET formation. Scale bar: 100 μm. The area of SYTOX Green–

positive cells per field (green area) was calculated against the area of DAPI-positive neutrophils (blue area) in the same fields for quantitative analysis of NET formation induced by anti-MPO antibody **(h)** and purified IgG from MPA patients **(i)**.

-Supplementary Information

We have added the following Supplementary Fig. 9 and legends.

Supplementary Figure 9. scRNA-seq analysis of neutrophils stimulated *in vitro*.



(a) UMAP plots showing scRNA-seq data of neutrophils isolated from healthy

donors and stimulated with IFN-γ + TNF-α, TNF-α alone, IFN-γ alone, or left unstimulated. **(b)** Balloon plot showing highly expressed genes in each cell cluster. Balloon color indicates the averaged scaled expression of the indicated genes. Balloon size indicates the percentage of cells expressing the indicated genes. **(c)** Sankey plot showing the correspondence between the stimulation condition and each cell cluster. **(d)** Module scoring analysis of each cell cluster. Module scores were calculated using the highly expressed genes in each cluster. Each cell was colored according to the scaled module score. The cells with high module score are indicated by dashed circle.

-Discussion

We have added the following sentences.

(Line 455 – 457)

importantly, IFN-γ elicits a variety of responses from neutrophils such as increased ROS production and induction of antigen presentation[38],

-Methods

We have added the following sentences.

(Line 656 - 660)

Extracellular ROS were quantified using the Fluorimetric Hydrogen Peroxide Assay Kit (Sigma-Aldrich, Cat No. MAK165). A master mix was applied at 50 µL per well, and fluorescence was measured every minute for one hour at an excitation wavelength of 540 nm and an emission wavelength of 590 nm using a microplate reader preheated to 37°C.

(Line 666 - 668)

In the indicated experiment, serum IgG purification from MPA patients was performed using the Melon™ gel IgG purification kit (Thermo Fisher, Cat No. 45206).

-References

We have added the following reference.

38. Ellis, T.N. & Beaman, B.L. Interferon-gamma activation of polymorphonuclear neutrophil function. Immunology 112, 2-12 (2004).

4. Figure 4 depicts patterns of gene expression that differ among patients with

MPA and 3 patients with more severe symptoms were enriched for IFN-g response genes and Neu_T2ISG. The Results section states these 3 patients had "long duration between the onset of vasculitis symptoms and sample collection." What is long duration and how might that contribute to interpretation of the results regarding differentiation and proportion of Neu_T2ISG in disease?

<Our response 4>

We apologize for any ambiguity in the original description of the results. In this study, "long duration" refers to the extended period between the onset of vasculitis symptoms and the initiation of treatment. In the original manuscript, the duration was only shown in Fig. 4a; we have now explicitly detailed the duration for each patient in the Results section.

A key factor contributing to the increase in Neu_T2ISG with longer duration may be sustained myelopoiesis signaling. As shown in the heatmap (Fig. 4a), cases with longer durations, such as MPA-6, exhibit enrichment of genes associated with myelopoiesis (e.g., S100A8 and S100A9) in Gene Cluster 2), as well as IFN-γ response genes (T2ISG). Prolonged activation of these pathways may lead to an increase in longer-lived subsets such as Neu_T2ISG. While this remains speculative, we have included this interpretation in the revised Discussion section. Thank you again for raising this important point.

<Revised point 4>

-Results

We have added the following sentences.

(Line 357 - 359)

From a clinical point of view, MPA-4, MPA-5, and MPA-6 had a long duration between the onset of vasculitis symptoms and initiation of treatment (5 months, 7 months, and 13 months, respectively)

-Discussion

We have added the following sentences.

(Line 426 - 429)

Given that patients with a longer duration after the onset of vasculitis symptoms have a higher proportion of the Neu_T2ISG subset, chronic exposure to pathogens may induce continuous myelopoiesis and contribute to an increase

in longer-lived subsets such as Neu_T2ISG.

5. Serum IFN-g analysis and findings as a biomarker of relapse could have important implications for clinical management of disease. What was the time to relapse? Was time to relapse from disease onset, onset of induction therapy, or after remission was achieved? How was remission defined? BVAS or other disease severity index alone or BVAS plus clinical review for no sign of disease?

<Our response 5>

Thank you for highlighting the lack of important clinical details. We have reviewed and included the time to relapse for each patient in the revised Supplementary Table 3. In this study, the time to relapse was calculated from the initiation of induction therapy. The definition for "Remission" was based on established BVAS criteria; defined as a BVAS score of 0. We have clarified these points in the Figure Legends and Methods section.

<Revised point 5>

-Supplementary Information

We have added the following information to Supplementary Table 3.

“Time from starting treatment to relapse (months)”

-Methods

We have added the following sentences.

(Line 502 - 515)

Definition of disease activity status

The Birmingham Vasculitis Activity Score (BVAS) 2008 version 3 was used to rate MPA disease activity. The definitions for "New", "Remission", "Worse", and "Persistent" were based on established BVAS criteria; "New" was defined as the appearance of newly developed disease manifestations in BVAS. "Remission" was defined as a BVAS score of 0. "Worse" was defined as a worsening of existing disease activity within 4 weeks, accompanied by an increase in the BVAS score. "Persistent" was defined as a state in which the BVAS score remained ≥ 1 for 4 weeks or longer, with no changes in pre-existing symptoms or signs and no evidence of new disease activity. In this study, "Relapse" was defined as a transition from a “remission” or “persistent” state to a “worse” state. The clinical status of each patient was recorded, and the time to relapse was

calculated from the initiation of induction therapy, with December 31, 2023, serving as the study endpoint.

Other less major comments:

1. Paragraph on monocytes analysis is interesting, but is seems out of place or unnecessary. It might fit earlier in the manuscript demonstrating the validity of the analysis of neutrophils because using the approach described in this manuscript confirms the conclusions on monocytes in MPA reported previously with slightly different approach.

<Our response 6>

Thank you for this valuable suggestion regarding the organization of the manuscript. We have moved the PBMC results, including the monocyte analysis, to an earlier section of the Results.

<Revised point 6>

-Results

We have moved and modified the following sentences.

(Line 179 – 193)

We previously reported a unique $CD14^+$ monocyte population with an activated status related to the pathogenesis of MPA using Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-Seq) [17]. In the current Abseq analysis, monocytes were divided into five subsets: activated $CD14^+$ monocytes (CD14Mono_Activated), $CD14^+$ monocytes characterized by *VCAN* gene expression (CD14Mono_VCAN), $CD14^+$ monocytes characterized by *HLA* gene expression (CD14Mono_HLA), $CD16^+$ monocytes (CD16Mono), and classical dendritic cells (cDC) (Supplementary Fig.3a). Highly expressed genes and surface proteins in each cell population are shown in Supplementary Fig.3b and Supplementary Fig. 3c, respectively. The proportion of the CD14Mono_Activated subset was significantly higher in patients with MPA (Supplementary Fig. 3d). Differential abundance analysis showed that significantly upregulated neighbors ($\alpha < 0.05$) in patients with MPA compared to healthy controls were particularly enriched in the CD14Mono_Activated subset (Supplementary Fig. 3e and 3f). These findings are consistent with our previous observations[17].

2. The module score for LDN in myelocyte population shown in Supplementary Figure 4 is unclear. Forgive me, but the Myelocyte cluster is not obvious compared to the other 6 clusters. Previous studies (Mhaonaigh, AU. et al. Frontiers in Immunology 2019. 10: Article 2603 and Jones, BE. et al. Kidney International 2020. 98: 744-757) on LDN/LDGs in patients with ANCA associated vasculitis identified both mature and immature neutrophils which indicates the LDN population is comprised of more than myelocytes.

<Our response 7>

Thank you for raising this important point. The module score for LDN is strongly enriched in a small cluster of myelocytes located toward the right in the UMAP. First, we have highlighted these clusters with circles to improve visibility in the revised Supplementary Figure 5.

We also agree with previous studies indicating that the LDN population includes both mature and immature neutrophils in patients with ANCA-associated vasculitis. The original module scoring referred to previously reported[6] module 4, which highlights gene expression patterns specific to immature LDN populations. When using module 2, which reflects more broadly distributed LDN gene expression, we found characteristic LDN-associated gene expression in some parts of the Neu_Immature subset. These findings indicate that characteristic genes of LDN are not restricted to Myelocytes but are also present in Neu_Immature subsets. We have clarified these findings in the revised manuscript and added appropriate references.

<Revised point 7>

-Results

We have added the following sentences.

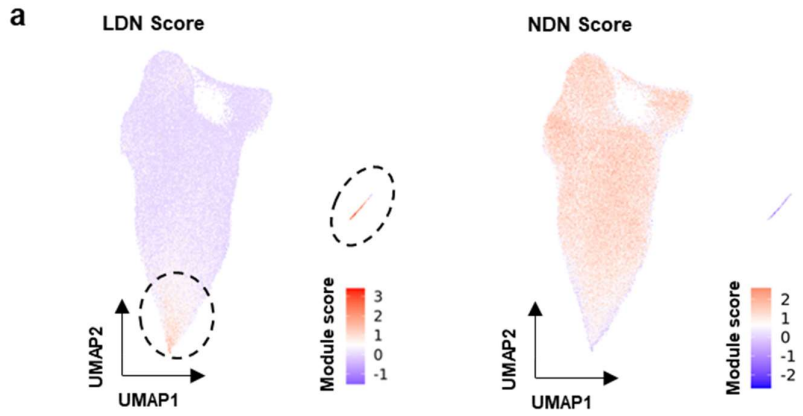
(Line 215 - 218)

The gene expression profile of the Myelocyte subset and some parts of the Neu_Immature subset aligned with that of low-density neutrophils (LDN), which are typically found in inflammatory and cancer pathologies[6] (Supplementary Fig. 5a).

-Supplementary Information

We have added the following Supplementary Fig. 5a and legend.

(a) Module scoring analysis of the neutrophil subpopulations. Module scores



were calculated using the averaged expression levels of the genes sets, referring to previously reported neutrophil gene expression profiles. Each cell was colored according to the scaled module score, with cells exhibiting high module scores indicated by dashed circles. LDN; low density neutrophils, NDN; normal density neutrophils.

-Discussion

We have added the following sentences.

(Line 410 - 412)

The Neu_Immature subset contains low-density neutrophils, which is typically observed in acute inflammatory conditions and in autoimmune diseases such as RA[30], SLE[31], and ANCA-associated vasculitis[32-34].

-References

We have added the following reference.

33. Ui Mhaonaigh, A., et al. Low Density Granulocytes in ANCA Vasculitis Are Heterogenous and Hypo-Responsive to Anti-Myeloperoxidase Antibodies. Front Immunol 10, 2603 (2019).

3. IFN-γ/Th1 signaling has been shown to be elevated in patients with ANCA associated vasculitis (Csernok, E. et al. Arthritis and Rheumatism 1999. 42(4): 742-750), and AAV not thought to be a type I interferonopathy (Batten, I. et al. Scientific Reports 2021. 11(1): 8272). How do these prior findings impact the results presented in this manuscript?

<Our response 8>

Thank you for highlighting these relevant studies. The first study (Csernok et al., Arthritis and Rheumatism 1999) demonstrates that a Th1 cytokine pattern, particularly IFN-γ expression, predominates in GPA. While our current study focuses on MPA and does not include GPA patients, the IFN-γ signaling pathway identified in both studies may represent a common feature in the pathophysiology of ANCA-associated vasculitis. We have now included this point in the Discussion section.

The second study (Batten et al., Scientific Reports 2021) showed that the systemic type I IFN response is not a major driver of AAV pathogenesis. This finding aligns with our results, as we observed no significant differences in the Neu_T1ISG population between MPA patients and healthy donors. However, it is worth noting that in our previous report, we identified a subset of monocytes in several MPA patients that exhibit characteristics of type I IFN activation. As shown in the heatmap (Fig. 4a), certain patients (e.g., MPA-3 and MPA-4) in this study exhibited mildly elevated T1ISG expressions in neutrophils. This suggests that while type I IFN signaling in neutrophils may not be a dominant driver, it could still play a role in a certain category of AAV patients. We have further discussed this point in the revised manuscript.

<Revised point 8>

-Discussion

We have added the following sentences.

(Line 432 - 441)

In regard to type I IFN, we observed no significant differences in the Neu_T1ISG population between MPA patients and healthy donors. This finding is consistent with previous reports indicating that systemic type I IFN responses are not major drivers of AAV pathogenesis[36]. However, our previous study identified a subset of monocytes in MPA patients with characteristics of type I IFN activation[17]. Similarly, in the current study, a few patients (e.g., MPA-3 and MPA-4) exhibited mildly elevated type I IFN signaling in neutrophils. These findings indicate that while type I IFN signaling in neutrophils may not be a predominant factor, it could still play a role in disease pathology in a specific subset of MPA patients.

(Line 473 - 475)

Importantly, a previous study demonstrated that a Th1 cytokine pattern, particularly IFN-γ expression, predominates in granulomatosis with polyangiitis

(GPA)[41].

-References

We have added the following references.

36. Batten, I., et al. Investigation of type I interferon responses in ANCA-associated vasculitis. Sci Rep 11, 8272 (2021).

41. Csernok, E., et al. Cytokine profiles in Wegener's granulomatosis: predominance of type 1 (Th1) in the granulomatous inflammation. Arthritis Rheum 42, 742-750 (1999).

4. In Figure 3C Neu T2ISG labeled as "Signal" in box below circos plot. Should the label be "Receiver"?

<Our response 9>

Thank you for pointing this out. In Fig. 3c, the label "Signal" was changed to "Receiver", and "Ligand" was changed to "Sender". Supplementary Fig. 8 was also modified.

Minor corrections

Line 357: "Neutrophil priming are …" ; change to "Neutrophil priming are is …"

Line 371: "degranuration" ; change to "degranulation"

Linee 431: "tretment" ; change to "treatment"

<Our response 10>

Thank you for pointing out these typos. We have corrected them as noted.

Our response to the Reviewers #2 and #3

We sincerely appreciate the opportunity to revise our manuscript. We have responded to the comments from Reviewers #2 and #3 in the following point-by-point manner. The line number corresponds to the number in the manuscript with tracked changes (Revised Manuscript Tracked Changed).

The present manuscript entitled «Neutrophil single-cell analysis identifies a uniquely primed subset and provides insights into predicting relapse of autoimmune small vessel vasculitis» by the team of Masayuki Nishide attempts to distinguish neutrophil subsets of patients suffering from microscopic polyangiitis (MPA) in order to predict relapse. This study follows a previous interesting study exploring two phenotypes of new-onset MPA based on monocyte subtypes (Nishide et al, 2023). The team bases their research on scRNAseq data of blood leukocytes from six untreated patients with new-onset MPA. Authors found seven neutrophil subsets, of which two were increased in MPA patients. One of the increased subsets is characterized by IFNg expression. Cell-cell interaction analysis showed that expression of this subset was affected by IFNg from CD8+ T-cells. By experimental methods they found that IFNg primed neutrophils are sensitive to MPO-ANCA stimulation, which triggers NET formation. In a validation cohort they found IFNg to be increased in the serum of MPA-patients, which is associated with relapse. This study is of importance because it explores new biomarkers for relapse in MPA. The manuscript is well written and the complex topic is well explained. However, there are a few remarks that would ameliorate the manuscript and makes it easier for the reader to understand:

<Our response>

We appreciate the reviewers #2 and #3 for recognizing the significance of our study and its potential implications for future clinical applications. We are particularly grateful for their constructive feedback, and have addressed their comments in the following point-by-point responses.

Major points:

- How are the two neutrophil groups Neutrophil_1 and Neutrophil_2 of Figure 1 related to or devided into the seven neutrophil subsets of Figure 2?

<Our response 1>

Thank you for this insightful question. In our analysis, the first step targeted the entire white blood cell population and broadly divided neutrophils into two groups: Neutrophil_1 and Neutrophil_2. In the second step, we performed a more detailed subdivision of neutrophils, which led to the identification of the seven neutrophil subsets. To clarify the relationship between these groups, we have indicated how Neutrophil_1 and Neutrophil_2 correspond to the seven subsets. Specifically, Neutrophil_1 corresponds to subsets Neu_Immature, Neu_Mature, Neu_Aged, Neu_T1ISG, and Neu_T2ISG, while Neutrophil_2 corresponds to subset Neu_Longlived. This relationship is shown using Sankey plot in the revised Extended Data Figures for improving readability.

<Revised point 1>

-Results

We have added the following sentences.

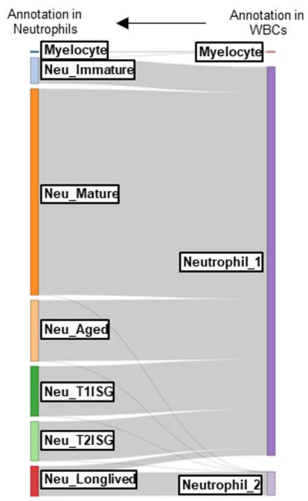
(Line 205 - 208)

Specifically, the Neutrophil_1 subset shown in Fig. 1b corresponds to the Neu_Immature, Neu_Mature, Neu_Aged, Neu_T1ISG, and Neu_T2ISG subsets, while the Neutrophil_2 subset corresponds to the Neu_Longlived subset (Supplementary Fig. 4a).

-Supplementary Information

We have added the following Supplementary Fig. 4a and legend.

a



(a) Sankey plot showing the correspondence between the clustering in **Fig.1b** and the clustering in **Fig.2a**.

- Please indicate which patients in supplementary table 3 suffered from a relapse. In Figure 4d there are 9 relapses, but in supplementary table 3, there are only 7 patients with a worse disease. It is not clear, how worse is defined and how it differs from relapse.

<Our response 2>

Thank you for highlighting the need for additional clinical details. We have reviewed and included information on which patients experienced relapse and the time to relapse for each patient in the revised Supplementary Table 3. Figure 4d highlights relapse events observed during the follow-up of "New" patients, indicating that 9 out of 24 "New" patients experienced a relapse in their clinical course. This distinction has been clarified in the revised manuscript.

The definitions for "New", "Remission", "Worse", and "Persistent" were based on established BVAS criteria; "New" was defined as the appearance of newly developed disease manifestations in BVAS. "Remission" was defined as a BVAS score of 0. "Worse" was defined as a worsening of existing disease activity within 4 weeks, accompanied by an increase in the BVAS score. "Persistent" was defined as a state in which the BVAS score remained ≥ 1 for 4 weeks or longer, with no changes in pre-existing symptoms or signs and no evidence of new disease activity. In this study, "Relapse" was defined as a transition from a “remission” or “persistent” state to a “worse” state.

Thus, "Worse" is a momentary status defined by BVAS, whereas “Relapse” refers to the transition in which a patient experiences a shift to the “Worse” status at some point during the clinical course. We have clarified these definitions in the Methods section.

<Revised point 2>

-Results

We have added the following sentences.

(Line 371 - 372)

In this study, among 24 new-onset patients assessed, the top six patients with the highest serum IFN-γ concentrations all experienced relapses (Fig. 4d).

-Supplementary Information

We have added the following information to Supplementary Table 3.

“If new, has the patient relapsed?”

“Time from starting treatment to relapse (months)”

-Methods

We have added the following sentences.

(Line 502 - 515)

Definition of disease activity status

The Birmingham Vasculitis Activity Score (BVAS) 2008 version 3 was used to rate MPA disease activity. The definitions for "New", "Remission", "Worse", and "Persistent" were based on established BVAS criteria; "New" was defined as the appearance of newly developed disease manifestations in BVAS. "Remission" was defined as a BVAS score of 0. "Worse" was defined as a worsening of existing disease activity within 4 weeks, accompanied by an increase in the BVAS score. "Persistent" was defined as a state in which the BVAS score remained ≥ 1 for 4 weeks or longer, with no changes in pre-existing symptoms or signs and no evidence of new disease activity. In this study, "Relapse" was defined as a transition from a “remission” or “persistent” state to a “worse” state. The clinical status of each patient was recorded, and the time to relapse was calculated from the initiation of induction therapy, with December 31, 2023, serving as the study endpoint.

- What is the ratio of differentially expressed genes to total genes found?

<Our response 3>

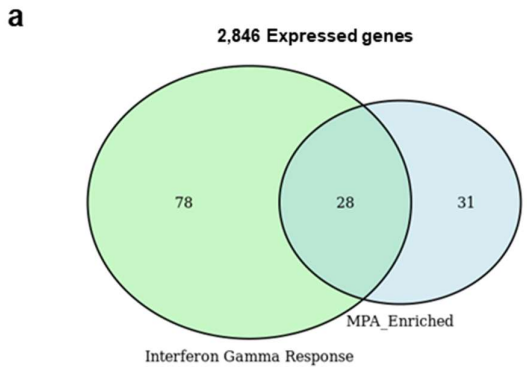
In response to this important question, a total of 2,846 genes detected in more than 10% of all neutrophils were analyzed for gene expression variation. Among these, 59 genes were identified as differentially expressed genes (DEGs), including 28 genes associated with IFN-γ signaling. These findings confirm increased IFN-γ signaling in neutrophils. The total number of genes analyzed has been described in the main text, and a Venn diagram illustrating the overlap of T2ISGs within the DEGs has been added to the Supplementary Fig. 6a.

<Revised point 3>

-Results

We have added the following sentences.
(Line 240 - 244)
Consistently, out of a total of 2,846 genes detected in more than 10% of all neutrophils, 59 genes were identified as differentially expressed genes (DEGs) in comparisons between neutrophils from MPA patients and healthy donors, including 28 genes associated with IFN-γ signaling (Supplementary Fig. 6a).

-Supplementary Information
We have added the following Supplementary Fig. 6a and legend.



(a) Venn diagram showing differentially expressed genes in comparisons between neutrophils from MPA patients and healthy donors. Out of 2,846 expressed genes (with expression rate > 0.1), 59 genes were upregulated (average log2 fold change > 0.25) in MPA patients including 28 genes associated with IFN-γ signaling.

- Do the authors have information about GPA (granulomatosis with polyangiitis) patients as well? Do they show similar results?
- Assuming that all the patients in the study are of Japanese origin, do the authors think the results are also applicable to patients with other ethnic background?

<Our response 4>
We apologize for not including a Limitation section in the original manuscript and thank the reviewer for these remarks. As pointed out, the following limitations should be discussed; the patients included in this study were limited to MPA, therefore it is unknown whether the concept of this study can be applied to GPA. In addition, we addressed the lack of the neutrophil profiles of other disease controls and the patients recruited in this study were all Japanese,

therefore racial differences cannot be considered.

<Revised Point 4>

-Discussion

We have added the following sentences.

(Line 469 – 477)

This study has several limitations. First, all patients recruited were Japanese, and potential racial differences were not evaluated. Second, the neutrophil profiles of other disease control groups were not analyzed, leaving it unclear whether the unique neutrophil profile identified in this study is specific to MPA. Importantly, a previous study demonstrated that a Th1 cytokine pattern, particularly IFN-γ expression, predominates in granulomatosis with polyangiitis (GPA) [41]. Although this study focuses on MPA and does not include GPA patients, the IFN-γ signaling pathway may represent a shared feature in the pathophysiology of other forms of ANCA-associated vasculitis.

- Did the authors also measure ROS production in addition to NET release? It would be interesting to know if the different neutrophil subsets produce different amounts of ROS as it is known for LDNs compared to NDNs. Also, it would be interesting to know if neutrophils from MPA patients produce less ROS in vitro compared to controls comparable to a study of peripheral blood neutrophils from GPA patients demonstrating a decreased ROS production in vitro compared to those from healthy controls (Amsler et al, Rheumatology, 2023).

<Our response 5>

Thank you for these insightful comments. In response, we have made the following three improvements:

<Response 5-1>

As noted, MPO-ANCA induces ROS production in addition to NET release. We have performed an additional ROS production assay and confirmed that IFN-γ-primed neutrophils are highly sensitive to ROS production in response to MPO-ANCA stimulation. The results of this assay and corresponding figures have been added to the revised manuscript.

<Our response 5-2>

We share your interest in whether different neutrophil subsets produce varying amounts of ROS. Unfortunately, no specific surface markers currently exist to definitively isolate neutrophil subsets identified in this study. To address your concerns as thoroughly as possible, we performed single-cell transcriptome analyses on *in vitro* stimulated neutrophils to investigate whether the differences of ROS production are linked with diverse populations. As a result, stimulation with a combination of TNF-α and IFN-γ induced a uniform subset resembling Neu_T2ISG. By contrast, stimulation with TNF-α or IFN-γ alone gave rise to subsets with distinct characteristics. Given that the combination of TNF-α and IFN-γ particularly promotes ROS production compared to the other conditions, it is reasonable to suggest that Neu_T2ISG is highly sensitive to these processes. While these findings do not directly prove that Neu_T2ISG is uniquely responsible for enhanced ROS production, they suggest that this subset has a high potential to generate ROS. Supplementary Fig. 9 and results from these analyses have been included in the revised manuscript.

<Response 5-3>

It is interesting to investigate whether the production of ROS by neutrophils in patients with MPA differs from that in controls. Unfortunately, in this study, neutrophils from MPA patients were promptly processed and used for scRNA-seq analysis, so there is no supply for additional ROS experiments. We hope that this will be recognized as an issue to be addressed in the future.

<Revised point 5>

-Results

We have added the following sentences.

(Line 317 - 320)

Upon subsequent stimulation with MPO-ANCA, TNF-α priming could enhance ROS production and NET formation, which was further enhanced by concurrent IFN-γ priming (Fig.3f, Fig. 3g, and Fig. 3h).

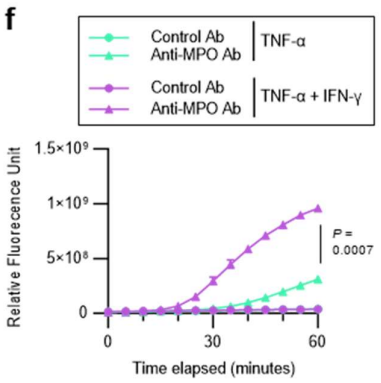
(Line 325 – 335)

To further investigate the characteristics of the TNF-α and IFN-γ-primed neutrophil subset, we conducted additional scRNA-seq analyses on in vitro stimulated neutrophils (Supplementary Fig. 9a and 9b). Stimulation with a combination of TNF-α and IFN-γ induced a uniform subset (Cluster1),

resembling Neu_T2ISG (Supplementary Fig. 9c and 9d). In contrast, in vitro stimulation with TNF-α or IFN-γ alone gave rise to subsets with the distinct characteristics, which resembling Neu_Longlived (Cluster2) or Neu_T1ISG/Neu_T2ISG mixed (Cluster3) (Supplementary Fig. 9c and 9d). These findings suggest that priming with a combination of TNF-α and IFN-γ induces a uniquely primed neutrophil subset, which is highly sensitive to MPO-ANCA stimulation and readily triggers ROS production and NET formation.

-Main Figures

We have added the following Fig. 3f and legend.

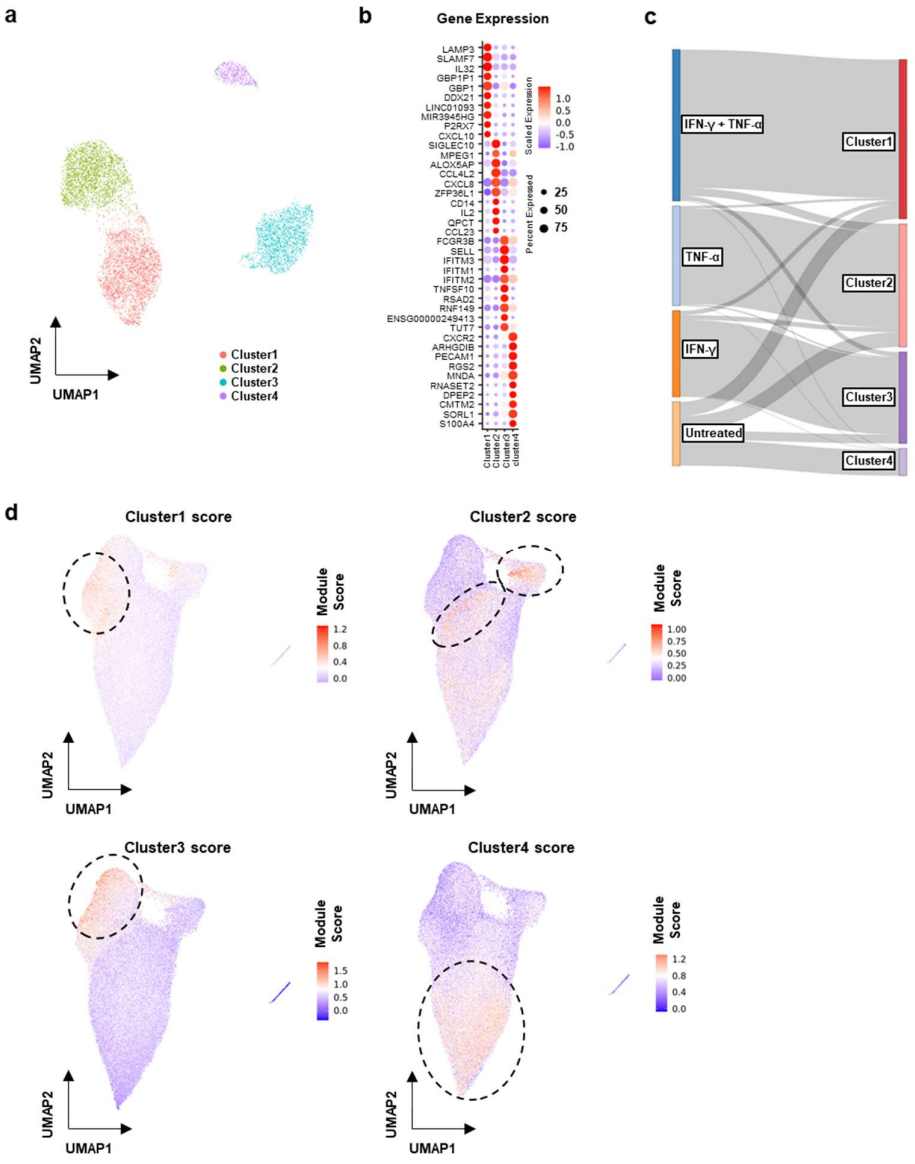


(f) Reactive oxygen species (ROS) production of neutrophils isolated from healthy donors. TNF-α–primed cells were treated with or without IFN-γ for 16 hours, followed by stimulation with anti-MPO antibody (10 μg/mL) for 1 hour. ROS levels were monitored every minute for a total of 60 cycles. Representative traces show the means with SEMs from a triplicate experiment.

-Supplementary Information

We have added the following Supplementary Fig. 9 and legends.

Supplementary Figure 9. scRNA-seq analysis of neutrophils stimulated *in vitro*.



(a) UMAP plots showing scRNA-seq data of neutrophils isolated from healthy donors and stimulated with IFN-γ + TNF-α, TNF-α alone, IFN-γ alone, or left unstimulated. **(b)** Balloon plot showing highly expressed genes in each cell cluster. Balloon color indicates the averaged scaled expression of the indicated genes. Balloon size indicates the percentage of cells expressing the indicated genes. **(c)** Sankey plot showing the correspondence between the stimulation condition and each cell cluster. **(d)** Module scoring analysis of each cell cluster. Module scores were calculated using the highly expressed genes in each cluster.

Each cell was colored according to the scaled module score. The cells with high module score are indicated by dashed circle.

-Methods

We have added the following sentences.

(Line 656 - 660)

Extracellular ROS were quantified using the Fluorimetric Hydrogen Peroxide Assay Kit (Sigma-Aldrich, Cat No. MAK165). A master mix was applied at 50 µL per well, and fluorescence was measured every minute for one hour at an excitation wavelength of 540 nm and an emission wavelength of 590 nm using a microplate reader preheated to 37°C.

- Can the authors comment on the occurrence or relevance of LDN (low density neutrophils) in their study?

<Our response 6>

Thank you for raising this important point. The module score for LDN is strongly enriched in a small cluster of myelocytes located toward the right in the UMAP. First, we have highlighted these clusters with circles to improve visibility in the revised Supplementary Figure 5a. These findings are consistent with previous studies indicating that the LDN population predominately arises from premature neutrophils. However, another study suggests that LDN includes both mature and immature neutrophils in patients with ANCA-associated vasculitis. The original module scoring referred to previously reported[6] module 4, which highlights gene expression patterns specific to immature LDN populations. When using module 2, which reflects more broadly distributed LDN gene expression, we found characteristic LDN-associated gene expression in some parts of the Neu_Immature subset.

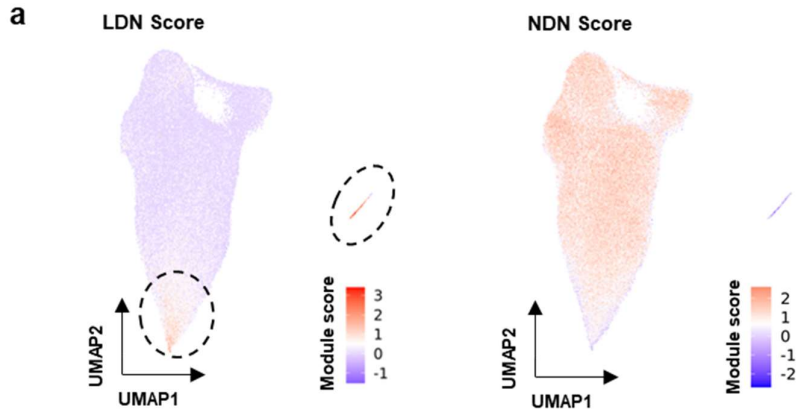
These findings indicate that the occurrence of the LDN population is primarily associated with premature neutrophils. However, LDN characteristic genes are not restricted to the Myelocyte subset but are also present in the Neu_Immature subset. We have clarified these findings in the revised manuscript.

<Revised point 6>

-Results

We have added the following sentences.
(Line 215 - 218)
The gene expression profile of the Myelocyte subset and some parts of the Neu_Immature subset aligned with that of low-density neutrophils (LDN), which are typically found in inflammatory and cancer pathologies[6] (Supplementary Fig. 5a).

-Supplementary Information
We have added the following Supplementary Fig. 5a and legend.



(a) Module scoring analysis of the neutrophil subpopulations. Module scores were calculated using the averaged expression levels of the genes sets, referring to previously reported neutrophil gene expression profiles. Each cell was colored according to the scaled module score, with cells exhibiting high module scores indicated by dashed circles. LDN; low density neutrophils, NDN; normal density neutrophils.

- Did the authors perform any immunophenotyping of neutrophils from MPA patients to confirm the subsets found in the scRNAseq cohort?

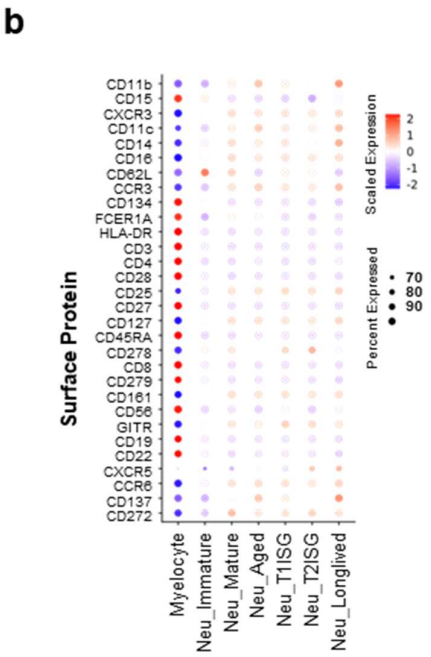
<Our response 7>
Thank you for this important question. In this study, we employed the Abseq method, which enables simultaneous acquisition of cell surface molecule data on neutrophils. We have added Supplementary Fig. 4b, which shows all surface proteins detected by Abseq analysis in each neutrophil subset. The expression levels of CD15 and HLA-DR are high in the Myelocyte subset, while CD62L is high in the Neu_Immature subset, suggesting these may serve as phenotypic

markers. However, as far as we analyzed using Abseq, no specific surface markers were identified to definitively isolate the remaining neutrophil subsets. As you kindly recognized, one of the strengths of single-cell analysis is its ability to identify subsets that are challenging to distinguish using conventional surface marker phenotyping. We have included these points in the revised Discussion section.

<Revised point 7>

-Supplementary Information

We have added the following Supplementary Fig. 4b and legend.



(b) Balloon plot showing highly expressed genes surface proteins in each cell population. Balloon color indicates the averaged scaled expression of the indicated genes/proteins. Balloon size indicates the percentage of cells expressing the indicated genes/proteins.

-Discussion

We have added the following sentences.

(Line 339 - 406)

Distinguishing various neutrophil states using conventional surface marker

phenotyping has been challenging. In this study, single-cell transcriptome and surface antigen analyses of neutrophils from treatment-naïve patients with new-onset MPA revealed seven subtypes of neutrophils. High expression levels of CD15 and HLA-DR were observed in the Myelocyte subset, while CD62L was highly expressed in the Neu_Immature subset, suggesting these markers may be indicative of these specific subsets. However, no specific surface markers were identified to definitively isolate the remaining neutrophil subsets. From a trascriptomic perspective, a characteristic feature of neutrophils from MPA patients is the increased proportion of immature neutrophils (Neu_Immature) and a uniquely primed neutrophil subset, neutrophils characterized by type II ISGs (Neu_T2ISG).

- Please explain in more detail the similarities and differences of neutrophil subpopulations from MPA patients compared to the study of Montaldo et al (Nat immunol 2022) subtyping neutrophils in patients undergoing stress-induced myelopoiesis.

<Our response 8>

Thank you for highlighting this important study. To elucidate the similarities and differences between the neutrophil subpopulations in our study and those reported in Nat Immunol, 2022, we conducted additional module score analysis of representative genes. As a result, the module scores for the Precursors, Early Immature, and Immature subsets described in the referenced study closely align with the Myelocyte and Neu_Immature subsets identified in our study. Additionally, neutrophils from patients treated with G-CSF or those with pancreatic invasive ductal adenocarcinoma (PDAC) gave rise to various subsets of mature neutrophils, including those resembling the Neu_T1ISG and Neu_T2ISG subsets. Furthermore, the characteristics of neutrophils from hematopoietic stem cell transplantation (HSC-T) patients strongly resembled those of our Neu_T1ISG and Neu_T2ISG subsets. These findings suggest that neutrophils under stress-induced activation and those from MPA patients share commonalities, particularly in the interferon response of their mature subsets. We have added these results in the revised manuscript.

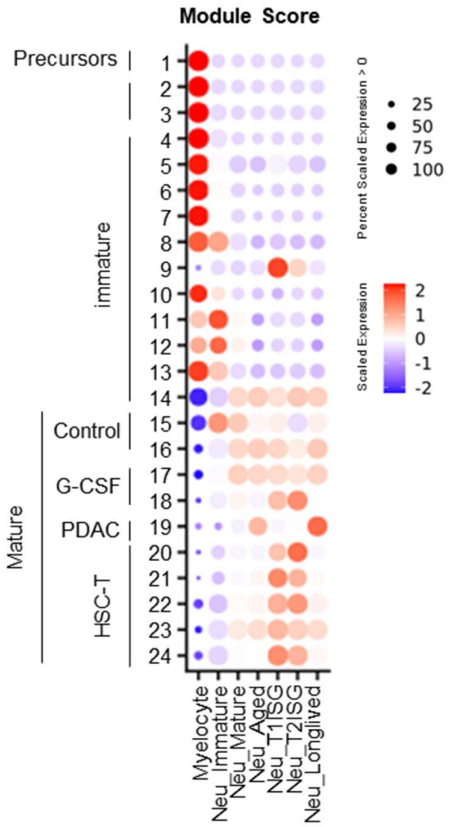
<Revised point 8>

-Results

We have added the following sentences.
(Line 225 - 232)
Additional module score analysis revealed that neutrophils from patients treated with G-CSF or those with pancreatic invasive ductal adenocarcinoma gave rise to various subsets of mature neutrophils, including those resembling the Neu_T1ISG and Neu_T2ISG subsets (Supplementary Fig. 5b). The characteristics of neutrophils from hematopoietic stem cell transplantation patients closely resembled those of the Neu_T1ISG and Neu_T2ISG subsets, suggesting that neutrophils under stress-induced activation and those from MPA patients share commonalities, particularly in the interferon response of their mature subsets (Supplementary Fig. 5b).

-Supplementary Information
We have added the following Supplementary Fig. 5b and legend.

b



(b) Balloon plot showing the averaged modules for each neutrophil

subpopulation, referring to previously reported gene expression profiles of stress-induced activated neutrophils. G-CSF; granulocyte colony stimulating factor, PDAC; pancreatic invasive ductal adenocarcinoma, HSC-T; hematopoietic stem cell transplant.

- Compared to the authors first study identifying two phenotypes of new-onset MPA, can the authors describe whether the subsets of neutrophils found in the current study overlap with the two former MPA-phenotypes and comment on the reasons why or why not?

<Our response 9>

In our previous study, PBMCs were analyzed and classified into three groups: MPA-MONO, characterized by high monocyte activation markers and a high relapse rate; MPA-IFN, characterized by a type I IFN signature; and a group not classified into either of these categories. As described in the Methods, MPA-1, -3, -4, and -6 from the current study were also analyzed in the previous study. According to the prior classification, MPA-3 and -4 belong to the MPA-IFN group, while MPA-6 corresponds to the MPA-MONO group.

In the current study, we found that MPA-3 and -4 exhibit slightly elevated T1ISG expression even in neutrophils, consistent with their classification as MPA-IFN in the previous study. MPA-6 shows elevated expression of genes related to myelopoiesis, such as those in Gene Cluster 2, along with increased T2ISG expression. These findings align with the MPA-MONO classification from the prior study. Although the primary cell types analyzed differ between the studies (PBMCs vs. neutrophils), we identified shared features, including upregulated T1ISG or myelopoiesis-related signaling, across both cell types. This observation suggests a potential common mechanism underlying the pathogenesis of these phenotypes, which we have discussed in the revised manuscript.

<Revised point 9>

-Discussion

We have added the following sentences.

(Line 426 - 429)

Given that patients with a longer duration after the onset of vasculitis symptoms have a higher proportion of the Neu_T2ISG subset, chronic exposure to

pathogens may induce continuous myelopoiesis and contribute to an increase in longer-lived subsets such as Neu_T2ISG.

(Line 432 - 441)
In regard to type I IFN, we observed no significant differences in the Neu_T1ISG population between MPA patients and healthy donors. This finding is consistent with previous reports indicating that systemic type I IFN responses are not major drivers of AAV pathogenesis[36]. However, our previous study identified a subset of monocytes in MPA patients with characteristics of type I IFN activation[17]. Similarly, in the current study, a few patients (e.g., MPA-3 and MPA-4) exhibited mildly elevated type I IFN signaling in neutrophils. These findings indicate that while type I IFN signaling in neutrophils may not be a predominant factor, it could still play a role in disease pathology in a specific subset of MPA patients.

- It would increase the value of the manuscript if a reference about neutrophils in vasculitis was added such as Aymonnier et al. The neutrophil: A key resourceful agent in immune-mediated vasculitis. Immunol Rev. 2023 Mar;314(1):326-356. doi: 10.1111/imr.13170. Epub 2022 Nov 21. PMID: 36408947. Or Michailidou et al. Role of Neutrophils in Systemic Vasculitides. Front Immunol. 2020 Dec 17;11:619705. doi: 10.3389/fimmu.2020.619705. PMID: 33391289; PMCID: PMC7774018.

<Our response 10>

Thank you for pointing out important references. We have cited the suggested references in the appropriate section of the manuscript to strengthen our discussion.

<Revised point 10>

-References

We have added the following references.

26. Aymonnier, K., Amsler, J., Lamprecht, P., Salama, A. & Witko-Sarsat, V. The neutrophil: A key resourceful agent in immune-mediated vasculitis. Immunol Rev 314, 326-356 (2023).

27. Michailidou, D., Mustelin, T. & Lood, C. Role of Neutrophils in Systemic Vasculitides. Front Immunol 11, 619705 (2020).

Minor points:

- Introduction: Line 105: remove the word “into” directly before “to”
- Results: In order to find the heatmap faster in the figures while reading the text, I suggest to add “Fig.4a” at the end of the first sentence on line 305 instead of line 311.
- Discussion: Line 371 and 374: “degranulation” instead of “degranuration”

<Our response 11>

Thank you for pointing out these typos. We have corrected them as noted.