

Cathepsin C inhibition reduces neutrophil serine protease activity and improves activated neutrophil-mediated disorders



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I have read the manuscript titled "Cathepsin C inhibition reduces neutrophil serine protease activity and improves activated neutrophil-mediated disorders" by Nishibata et al. This manuscript dissects out the therapeutic potential of CatC inhibitors against in vitro and in an in vivo model of MPO-AAV to inhibit NET formation. They have specifically developed a new CatC inhibitor, MOD06051, for this purpose. I have the following comments/concerns regarding the manuscript:

1. Neutrophil swelling in absence of staining for NET markers such as citrullinated histones (H3Cit) or increased PAD4 expression could be the result of non-NET related death. Therefore the gold standard technique of identifying NETs should be used, if positive these would indicate early NET formation prior to expulsion of DNA.
2. Figure 3. Only FACS has been used to identify NETs. In vitro imaging should be done in conjunction with these experiments to demonstrate NETs and so the extent of inhibition can be visualised and quantitated. Supplementary figure two, does not show NET specific antibodies, so extracellular DNA could be from lytic or necrotic cell death.
3. Figure 4. In conjunction with the quantification the level of pathology in the kidney should be showed (so the extent of attenuation of injury can be visualised). Supplementary figure 4 only shows pathology of the MPO-AAV group, not what the attenuation of the pathology looked like after the inhibitor was used. Supplementary figure 5 uses H&E staining, when PAS staining should really be used to delineate the pathology (H&E not sensitive enough).
4. Figure 5. Glomerulonephritis is characterised by inflammation of the glomeruli therefore glomerular NETs should be shown with a clear indication of a NET versus a neutrophil not NETting, so readers can see the distinction between the two. The current image is not very convincing in 5b as no co-localisation of NET constituents is evident- the quantification indicates 10 NETs per high powered view- therefore there should be a representative image of this.
5. All the data has been presented as bar graphs. Individual data points would give a better representation of the data

Reviewer #2 (Remarks to the Author):

Nishibata et al. developed the cathepsin C inhibitor MOD06051 that inhibited CatC with an IC₅₀ of 1.5 nM in vitro without affecting other cathepsins. MOD06051 reduced neutrophil elastase (NE) in neutrophils differentiated from human CD34⁺ stem cells in vitro and in bone marrow and blood PMN from normal rats after 2-week treatment. It was next suggested that MOD06051 inhibits NETs formation because of NE inhibition. The authors then used an MPO-ANCA rat model to investigate MOD06051 in this vasculitis model. The hallmarks of vasculitis, namely, hematuria, and proteinuria were not affected by the study drug. Nevertheless, renal pathology and NGAL together with cortical NETs in the kidney were reduced with MOD06051. The topic is interesting. However, several key aspects of the study have been reported previously. For example, CatC- and NE-dependent NETs formation (DOI: 10.1083/jcb.201006052, DOI: 10.1172/JCI76009), NETs-dependent MPO-ANCA vasculitis in mice (DOI: 10.1073/pnas.1708247114, not cited). The novelty is that a pharmacological inhibitor was produced that inhibits these key events. I found several issues that need to be addressed by additional experiments.

comment 1:

To calculate the NE activity inhibition by MOD06051 (here assessed using a fluorogenic substrate and expressed as percentages between 0 to 100% in figure 1B or as mU/ml in 1C), a baseline is needed where all NE activity is abrogated in the investigated cell type. This can be achieved by using the appropriate cell type from NE KO animals or by an excess of inhibitors (alpha1-antitrypsin etc.). This is

an important issue, because otherwise the inhibition is difficult to evaluate. For example, in figure 1B it looks like the 3 mg per kilogram dose inhibited an NE activity by approximately 90% whereas the same dose in 1C suggests approximately 50% inhibition. In fact, the same concerns are valid for the PR3 data. No firm conclusions on the MOD06051 inhibition capacity can be derived from the current data.

Comment 2:

Along the same lines, if the CatC inhibition by MOD06051 was complete, then why do the authors find approximately 50% residual NE activity in blood PMN (Figure 1C)? If CatC was indeed completely inhibited other NSP-activating proteases would be at work. Thus, measuring the achieved CatC activity in parallel with the true NE activity (see comment 1) would help to understand this issue.

Comment 3:

In the data on inhibition of membrane-PR3 by MOD06051 are not convincing (figure 1e, f). I cannot see how many experiments were performed neither do I see standard deviations. The MFIs or geomean of the positive peak together with the percentage of the PR3-positive peak should be reported. In fact, there seems to be substantial PR3 left on the cell membrane, even in the presence of the 1 μ M inhibitor, whereas the same concentration abrogated PR3 activity completely (figure 1D).

Comment 4:

Detecting NETs by extracellular DNA only (DAPI or cytox) is not sufficient (e.g. DOI: 10.1111/cmi.12652). The investigators need more convincing evidence that the MPO-MPO-IGG immune complexes induce indeed NETs (e.g. MPO, NE co-staining). In addition, given the importance of NETs for this study, a second independent method to quantify NETs formation is required (e.g. DOI: 10.1083/jcb.201006052).

Comment 5:

The authors suggest reduced NETs deposition in the renal cortex. Stainings should be performed that reveal whether these NETs were restricted to the glomeruli and co-localize with glomerular neutrophils.

Comment 6:

Page 10, line 198: I disagree with the statement "affected glomeruli exhibiting necrotizing and crescentic glomerulonephritis (NCGN), characterized by crescent formation, and endocapillary proliferation... ". Vasculitis associated lesions are extra-capillary lesions. In fact, the investigators should report the extra-capillary lesions in more detail. I suggest assessing the percentage of glomeruli with necrosis and with crescents separately.

Comment 7:

The authors should provide data on the effect of MOD06051 on infiltrating cells in the kidney (e.g. flow cytometry). For example, reduction of NETs in the kidney could be a consequence of reduced infiltrating neutrophils e.g. in the glomeruli). It would be important to know whether the inhibitor reduces infiltrating myeloid cells and lymphocytes. Neutrophils and proinflammatory monocytes are considered important effector cells for the glomerulonephritis and can be identified by appropriate markers. Additionally, staining of kidney sections what establish a potential reduction of glomerular infiltrating cells.

Comment 8:

The investigators applied to the study drug in a prophylactic approach (i.e. before disease outbreak). Clinical translation would require starting treatment after signs of the disease were detected. For example, erythrocyturia could serve as a marker for the manifestation of glomerulonephritis and treatment would then start after the onset of erythrocyturia.

Reviewer #3 (Remarks to the Author):

In this manuscript the authors used a novel CatC inhibitor to suppress the cellular activity of neutrophil serine proteases, in cells from both human and rat, and reduce NET-forming cells from rat after ANCA stimulation. Further, in the presence of the CatC inhibitor they observed less NETs and reduced disease in a rat model of MPO-ANCA associated vasculitis.

Major comments:

The authors need to present more evidence on that MOD06051 inhibits NET-forming cells (undergoing NETosis) and/or release of NETs

1. The authors used FCM to measure sytox green positive neutrophils and morphological changes, to study NET-forming cells. I do appreciate the simplicity and relevance of this approach, but there are also uncertainties. In a previous publication that is referred to, PMA was used to induce NETosis and FCM results correlated with immunofluorescence data, but whether such a correlation can be observed during ANCA stimulation is not presented. This is relevant as sytox green staining by itself does not distinguish between cell death mechanisms with ruptured membranes, and whether the morphological changes observed represents NET-forming cells specifically is not clear to the reader. A comparison of morphological changes during different cell deaths using FCM could be presented in supplementary methods. A combination of sytox green positivity with morphological changes could perhaps be of help to distinguish between e.g., necrotic and NETotic cells.

2. In addition to FCM, it is important to also quantify what are actually released from the cells (rat and human). This would also include NETs released during vital NETosis. I agree with the authors that immunofluorescence imaging can be subjective, but there are reports on how this can be conducted in a more automated, objective, manner. Nevertheless, there are other alternatives as well, such as quantification of complexes of citrullinated H3 or MPO and DNA in the supernatants, which would also support the release of NETs from the cells.

3. The authors demonstrated that MOD06051 inhibited PR3 and NE activity in vitro in human CD34+ Bone Marrow Stem/Progenitor cells, but NETosis or released NETs were not measured. That was instead measured in human peripheral neutrophils when using sivelestat. Based on that MOD06051 would exert its effect during neutrophil development in the bone marrow it is not clear to me why NETosis and NETs were not measured for these cells, as this constitutes an important experiment.

4. The authors need to clarify why NETs in sup fig 2b and fig 5b were not stained for the same content. Also, If the authors want to include these figures as evidence of more NETs during disease, they must be quantified and compared with relevant controls (as in fig 5c), or otherwise be clearly stated that they are included for visualisation of NETs.

Minor comments:

1. It was recently published that also the CatC inhibitor BI-9740 significantly reduced NE and PR3 activity in human CD34+ bone marrow stem cells when differentiated to neutrophils, but this reference was not included in this manuscript. The authors need to discuss how this study relates- and is novel to other studies who have tried to delineate the importance of targeting CatC in ANCA-associated vasculitis, e.g., Jerke, Uwe; Eulenberg-Gustavus, Claudia; Rousselle et al, JASN 2022.

2. When describing FCM results, both NET-forming cells and NET formation is being used in the manuscript and needs to be consistent. Since the authors measure DNA within the cells, I suggest that NET-forming cells (i.e. cells undergoing NETosis) should be used, as NET formation is usually used to describe released NETs.

3. The authors mention that proteinuria was not prominent in this model, but this should be elaborated on as it is a relevant marker during disease and was described in the original publication of this disease model (as referred to as by the authors).

4. From how many healthy volunteers were neutrophils isolated in the experiments with sivelestat and avacopan?

5. For measurement of NET-forming cells in rats after ANCA-activation 3 rats/group were included, whereas 5 and 8 were included in other experiments. How come there is such a big discrepancy? The reason for this, and impact of, could be added to the discussion.

6. In the beginning and in the end of the discussion section, line 220-222, 302-305 and 320-321, it is not clear to the reader what was done on human cells and/or in rats. Except for the last words of line 321 the results are mentioned in general terms, but it is very important information in what system they were generated.

7. On line 58, I think another word than biophylactic could be used as I don't think the meaning of this is clear to the general audience.

8. Starting at line 66 I understand that the authors focus on describing NETosis where NE is involved in the signalling pathway. However, only one of the two references at line 67 mention the Raf-MEK-ERK signalling pathway, and that when using PMA during stimulation. As the authors use ANCA to stimulate neutrophils in this study, it would be interesting and relevant to add information about how they activate neutrophils. In this section, it could also be good to introduce lytic vs. suicidal NETosis, as the authors in the results section mention lytic NETs (which I think should be rephased to lytic NETosis or lytic NET-forming cells to match the phrasing in this manuscript).

Response to Reviewers' Comments

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Thank you very much for taking the time to review our manuscript. According to your kind suggestions, we have revised the manuscript. The revised portions are highlighted in red. We hope that the revised manuscript is now worthy of your acceptance.

1. Neutrophil swelling in absence of staining for NET markers such as citrullinated histones (H3Cit) or increased PAD4 expression could be the result of non-NET related death. Therefore the gold standard technique of identifying NETs should be used, if positive these would indicate early NET formation prior to expulsion of DNA.

We appreciate your constructive critique. For this revision, TNF- α /C5a-primed MPO-ANCA-IC-stimulated swollen neutrophils were subjected to immunofluorescent staining for Cit-H3. As shown in the revised Figure 3, these cells were positive for Cit-H3, thus suggesting that the swollen neutrophils are in the state of early NET formation prior to expulsion of DNA. Related descriptions were added to the Online methods (page 5), Results section (page 9), and the legend for Figure 3.

2. Figure 3. Only FACS has been used to identify NETs. In vitro imaging should be done in conjunction with these experiments to demonstrate NETs and so the extent of inhibition can be visualised and quantitated. Supplementary figure two, does not show NET specific antibodies, so extracellular DNA could be from lytic or necrotic cell death.

The revised Figure 3 visually demonstrated that treatment with 500 μ M sivelestat reduced the Cit-H3 intensity of human neutrophils primed with TNF- α or C5a followed by MPO-ANCA-IC stimulation. In the newly added Supplementary Figure 3b, we further demonstrated Cit-H3 and Sytox Green positivity of rat neutrophils primed with TNF- α or C5a followed by MPO-ANCA-IC stimulation. In addition, we documented an increase in MPO-DNA complexes in supernatants of rat neutrophils primed with TNF- α or C5a followed by MPO-ANCA-IC stimulation. These findings taken together suggest that the extracellular DNA observed in Supplementary Figure 3a (original Supplementary

Figure 2) was derived from neutrophils that underwent NETosis.

Related descriptions were added to the Online methods (pages 4–5), Results section (pages 8–9), and the legends for Figure 3 and Supplementary Figure 3.

3. Figure 4. In conjunction with the quantification the level of pathology in the kidney should be showed (so the extent of attenuation of injury can be visualised). Supplementary figure 4 only shows pathology of the MPO-AAV group, not what the attenuation of the pathology looked like after the inhibitor was used. Supplementary figure 5 uses H&E staining, when PAS staining should really be used to delineate the pathology (H&E not sensitive enough).

We have revised Figure 4 and Supplementary Figure 6 (original Supplementary Figure 5), accordingly.

4. Figure 5. Glomerulonephritis is characterised by inflammation of the glomeruli therefore glomerular NETs should be shown with a clear indication of a NET versus a neutrophil not NETting, so readers can see the distinction between the two. The current image is not very convincing in 5b as no co-localisation of NET constituents is evident- the quantification indicates 10 NETs per high powered view- therefore there should be a representative image of this.

In this study, NET deposition was observed in tubules in the renal cortex but not in glomeruli of AAV model rats (please see revised Figure 5b). Because NET deposition in glomeruli has been shown in human AAV (Kessenbrock *et al.* Nat Med 2009;15:623-5), further discussion is needed concerning this observed difference.

- 1) The severity of glomerulonephritis is different between human patients and the AAV model rats used here. Most AAV patients develop rapidly progressive renal dysfunction due to severe glomerulonephritis with massive proteinuria, whereas maximally 16.2% glomeruli were affected (Figure 4f) and proteinuria was not prominent (Figure 4c) in AAV model rats used in this study.
- 2) AAV model rats created in our laboratory have developed tubulo-interstitial damage rather than glomerulonephritis (Nonokawa *et al.* Arthritis Res Ther 2019;21:296), although the reason remains elusive.
- 3) NET deposition in tissues can be digested by DNase I (Hakim, *et al.* Proc Natl Acad Sci USA 2010;107:9813-8) and eliminated by macrophages (Nakazawa *et al.* J Autoimmun 2016;67:19-28).

In view of the above, we hypothesize that NETs deposited in sites of mild inflammation, such as glomeruli, might be easily removed *in vivo*, and that residual NET deposition was detected in severely damaged tubules in the renal cortex of AAV model rats. The CatC inhibitor MOD06051 can decrease NET-forming neutrophils in the blood (Figure 5a), regardless of the presence of ANCA (Figure 4a),

resulting in reduction of NET deposition in tissues and subsequent tissue destruction.

Because NETs deposited in tissues have already lost their form as neutrophils, NET constituents can appear to be scattered, not necessarily colocalized. In line with this, it is very difficult to count neutrophils which have released NETs in tissue sections; thus, we have counted areas with NET deposition in the renal cortex (we apologize for our previous statement that mislead you to “10 NETs were present per high-powered view”).

Figure 5b and the legend were revised and a related description was added to the Results section (page 11).

5. All the data has been presented as bar graphs. Individual data points would give a better representation of the data

We have revised the figures, accordingly. At the same time, we have reviewed statistical analyses and revised some figures that were not correctly labelled with a mark of significance.

Reviewer #2 (Remarks to the Author):

Nashibata et al. developed the cathepsin C inhibitor MOD06051 that inhibited CatC with an IC₅₀ of 1.5 nM in vitro without affecting other cathepsins. MOD06051 reduced neutrophil elastase (NE) in neutrophils differentiated from human CD34⁺ stem cells in vitro and in bone marrow and blood PMN from normal rats after 2-week treatment. It was next suggested that MOD06051 inhibits NETs formation because of NE inhibition. The authors then used an MPO-ANCA rat model to investigate MOD06051 in this vasculitis model. The hallmarks of vasculitis, namely, hematuria, and proteinuria were not affected by the study drug. Nevertheless, renal pathology and NGAL together with cortical NETs in the kidney were reduced with MOD06051. The topic is interesting. However, several key aspects of the study have been reported previously. For example, CatC- and NE-dependent NETs formation (DOI: 10.1083/jcb.201006052, DOI: 10.1172/JCI76009), NETs-dependent MPO-ANCA vasculitis in mice (DOI: 10.1073/pnas.1708247114, not cited). The novelty is that a pharmacological inhibitor was produced that inhibits these key events. I found several issues that need to be addressed by additional experiments.

Thank you very much for taking the time to review our manuscript. By addressing your constructive critiques, we have revised the manuscript. Schreiber's work (Proc Nat Acad Sci USA 2017;114:E9618-25) was introduced as a citation (Ref. no. 22) in the revised manuscript. The revised portions are highlighted in red. We hope that the revised manuscript is now worthy of your acceptance.

Comment 1:

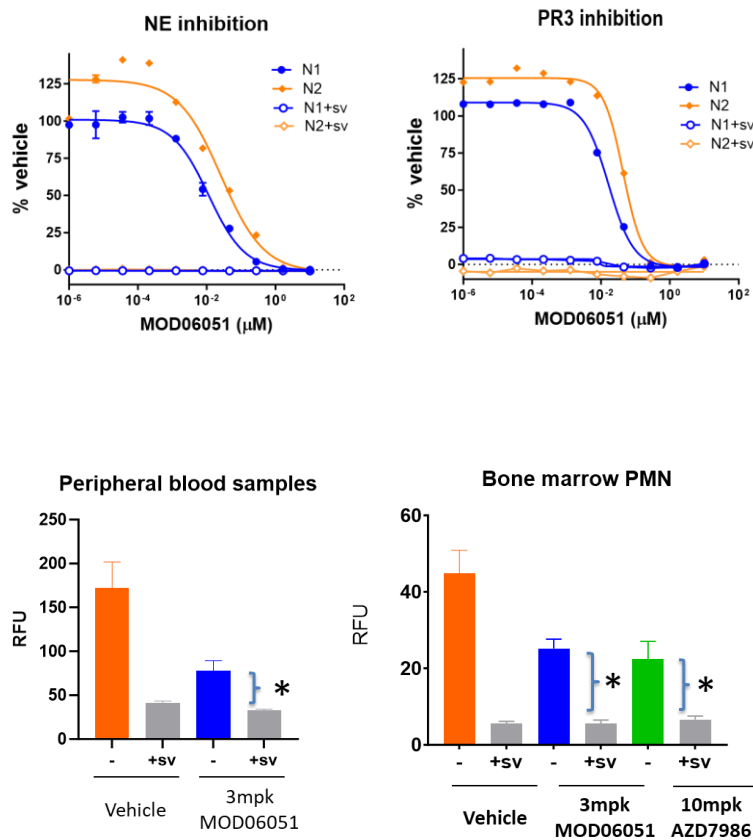
To calculate the NE activity inhibition by MOD06051 (here assessed using a fluorogenic substrate and expressed as percentages between 0 to 100% in figure 1B or as mU/ml in 1C), a baseline is needed where all NE activity is abrogated in the investigated cell type. This can be achieved by using the appropriate cell type from NE KO animals or by an excess of inhibitors (alpha1-antitrypsin etc.). This is an important issue, because otherwise the inhibition is difficult to evaluate. For example, in figure 1B it looks like the 3 mg per kilogram dose inhibited an NE activity by approximately 90% whereas the same dose in 1C suggests approximately 50% inhibition. In fact, the same concerns are valid for the PR3 data. No firm conclusions on the MOD06051 inhibition capacity can be derived from the current data.

Comment 2:

Along the same lines, if the CatC inhibition by MOD06051 was complete, then why do the authors find approximately 50% residual NE activity in blood PMN (Figure 1C)? If CatC was indeed completely inhibited other NSP-activating proteases would be at work. Thus, measuring the achieved CatC activity in parallel with the true NE activity (see comment 1) would help to understand this issue.

Thank you for asking these significant questions. We understand your points and prepared additional data and discussion materials to answer you.

As previously reported, NE activity in peripheral neutrophils was significantly suppressed in patients with genetic CatC deficiency (a.k.a. Papillon-Lefevre syndrome) who have almost null CatC activity (Klose *et al.* PLoS ONE 2021;16:e0261724). Therefore, we can reasonably expect a high level of suppression of NE activity at 3mpk dosing of MOD06051, which significantly suppresses CatC activity *in vivo*. As a positive control for most of the studies, we used AZD7986, a clinical stage CatC inhibitor which was reported to show significant suppression of both CatC and NE activities *in vivo* and in clinical trials (Doyle *et al.* J Med Chem 2016;59:9457-72; Palmer *et al.* Clin Pharmacol Ther 2018;104:1155-64). Also, we used sivelestat as an inhibitor for confirming specific NE activity in the samples. Although both MOD06051 and AZD7986 showed complete suppression of NE activity, the same as sivelestat, in the cellular NE assay using CD34⁺ HSC-derived neutrophils (shown in the below Reference Figure A), we observed that a minor fraction of fluorescent signals in the NE assay using *ex vivo* samples could not be suppressed by either CatC inhibitor but could be suppressed by sivelestat (please see the below Reference Figure B. The particular residual fractions mentioned in the above discussion are indicated by “*”). Actually, these analyses are based on the raw data of

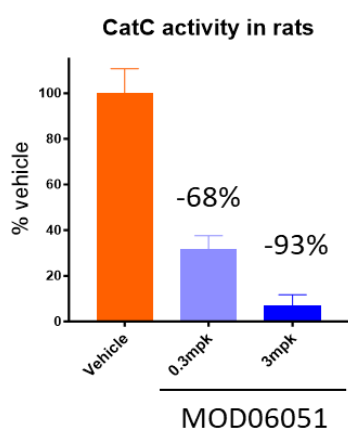


original Figures 1b and 1c in our manuscript). The reason for this relatively high background have not yet been uncovered, but non-specific digestion of the substrate by enzymes other than NE or another contaminant could be one possible explanation. Indeed, it is reported that not only NE and other NSPs such as CatG or PR3, which can be suppressed by CatC inhibition, but also leukocyte elastase, chymotrypsin and macrophage elastase can cleave the substrate. Also, non-enzymatic cleavage of the substrate was observed.

Since *ex vivo* peripheral blood and bone marrow-derived samples could be cruder than *in vitro*-differentiated neutrophils, we thought this could be why we observed much less background in the experiments using CD34⁺ HSC-derived neutrophils than in the ones used *ex vivo* samples.

From the above points, we concluded that the relative inhibition using AZD7986 as a positive control can be the best interpretation of the data we obtained.

We really appreciate your suggestion about the level of CatC inhibition *in vivo*. We performed an experiment in rats and a significant level of inhibition was confirmed as shown in the below Reference Figure C. 0.3 and 3mpk of MOD06051 showed 68% & 93% inhibition of CatC activity in rat whole blood, respectively.



Regarding the revision of the figures, we propose to set *in vivo* CatC inhibition as new Figure 1c and move the original Figure 1c to supplemental data (new Supplementary Figure 2) because the original Figures 1b and 1c are essentially the same (i.e., *in vivo* suppression of NE activity by MOD06051). Since the original Figure 1b showed dose-dependent efficacy of the compound rather than a single dose point as in the original Figure 1c, we'd like to keep the original Figure 1b rather than the original Figure 1c as part of the new main Figure 1. Also, we'd like to update the original Figures 1a and 1d with sivelestat data for each point to clearly show that MOD06051 significantly suppressed NE and PR3 enzymatic activity *in vitro* in CD34⁺ HSC-derived neutrophils.

Comment 3:

In the data on inhibition of membrane-PR3 by MOD06051 are not convincing (figure 1e, f). I cannot see how many experiments were performed neither do I see standard deviations. The MFIs or geomean of the positive peak together with the percentage of the PR3-positive peak should be reported. In fact, there seems to be substantial PR3 left on the cell membrane, even in the presence of the 1 μ M inhibitor, whereas the same concentration abrogated PR3 activity completely (figure 1D).

Thank you for asking these significant questions.

While it is well validated that the enzymatic activity of PR3 is almost completely controlled by the N-terminal dipeptide cleavage via CatC activity, the mechanism of surface presentation of PR3 is not fully elucidated yet. Although it is reported that the enzymatic activity of PR3 is significantly suppressed in neutrophils derived from Papillon-Lefevre Syndrome patients, who have almost null CatC activity, the surface expression level in neutrophils is only partially suppressed even after neutrophil activation when compared to healthy controls (Seren *et al.* J Biol Chem 2018;293:12415-28). Our results in this study (i.e., pharmacological inhibition of CatC led to almost complete suppression of PR3 activity but led to only partial suppression in PR3 cell surface inhibition in CD34⁺ HSC-derived neutrophils) are consistent with the previous findings described above.

Regarding the replication of the experiment, we repeated the experiments four times and obtained robust data as shown in the revised Figure 1f. MOD06051 showed dose-dependent suppression of PR3 surface expression.

In conclusion, we'd like to propose the revision of the figures as follows: For new Figure 1e: To overlay the data from whole dose points to clearly show the dose-dependent changes of PR3

expression signal distribution. For new Figure 1f: To show dose-dependent curve with mean $\pm$ SEM for each dose point.

Comment 4:

Detecting NETs by extracellular DNA only (DAPI or cytox) is not sufficient (e.g. DOI: 10.1111/cmi.12652). The investigators need more convincing evidence that the MPO-MPO-IGG immune complexes induce indeed NETs (e.g. MPO, NE co-staining). In addition, an given the importance of NETs for this study, a second independent method to quantify NETs formation is required (e.g. DOI: 10.1083/jcb.201006052).

We appreciate your constructive critique. For this revision, TNF- α /C5a-primed MPO-ANCA-IC-induced swollen neutrophils (human peripheral blood neutrophils) were subjected to immunofluorescent staining for Cit-H3 as a NETosis marker. As shown in the revised Figure 3, these cells were positive for Cit-H3, thus suggesting that the swollen neutrophils are in the state of early NET formation prior to expulsion of DNA. In addition, Cit-H3 intensity of neutrophils and the number of MPO-DNA complexes in supernatants also indicated NET formation was dose-dependently inhibited by sivelestat. Related descriptions were added to the Online methods (page 5), Results section (page 9), and the legend for Figure 3.

We further demonstrated Sytox Green and Cit-H3 positivity of rat neutrophils primed with TNF- α or C5a followed by MPO-ANCA-IC stimulation and the number of MPO-DNA complexes in supernatants in newly added Supplementary Figure 3b. These findings suggested that the extracellular DNA observed in Supplementary Figure 3a (original Supplementary Figure 2) was derived from neutrophils that underwent NETosis. Related descriptions were added to the Online methods (page 4), Results section (page 8), and the legend for Supplementary Figure 3.

Comment 5:

The authors suggest reduced NETs deposition in the renal cortex. Stainings should be performed that reveal whether these NETs were restricted to the glomeruli and co-localize with glomerular neutrophils.

In this study, NET deposition was observed in tubules in the renal cortex but not in glomeruli of AAV model rats (please see revised Figure 5b). Because NET deposition in glomeruli has been shown in human AAV (Kessenbrock *et al.* Nat Med 2009;15:623-5), further discussion is needed concerning this observed difference.

- 1) The severity of glomerulonephritis is different between human patients and the AAV model rats used here. Most AAV patients develop rapid progressive renal dysfunction due to severe glomerulonephritis with massive proteinuria, whereas maximally 16.2% glomeruli were affected

(Figure 4f) and proteinuria was not prominent (Figure 4c) in AAV model rats used in this study.

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In view of the above, we hypothesize that NETs deposited in sites of mild inflammation, such as glomeruli, might be easily removed *in vivo*, and that residual NET deposition was detected in severely damaged tubules in the renal cortex of AAV model rats. The CatC inhibitor MOD06051 can decrease NET-forming neutrophils in the blood (Figure 5a), regardless of the presence of ANCA (Figure 4a), resulting in reduction of NET deposition in tissues and subsequent tissue destruction. Figure 5b and the legend were revised and related descriptions were added to the sections of Results (page 11) and Discussion (page 15).

Comment 6:

Page 10, line 198: I disagree with the statement “affected glomeruli exhibiting necrotizing and crescentic glomerulonephritis (NCGN), characterized by crescent formation, and endocapillary proliferation... “. Vasculitis associated lesions are extra-capillary lesions. In fact, the investigators should report the extra-capillary lesions in more detail. I suggest assessing the percentage of glomeruli with necrosis and with crescents separately.

The sentence was revised. The rate of glomeruli exhibiting tuft necrosis and crescent formation was calculated separately and these data were newly presented as Figures 4g and 4h, respectively.

Comment 7:

The authors should provide data on the effect of MOD06051 on infiltrating cells in the kidney (e.g. flow cytometry). For example, reduction of NETs in the kidney could be a consequence of reduced infiltrating neutrophils e.g. in the glomeruli). It would be important to know whether the inhibitor reduces infiltrating myeloid cells and lymphocytes. Neutrophils and proinflammatory monocytes are considered important effector cells for the glomerulonephritis and can be identified by appropriate markers. Additionally, staining of kidney sections what establish a potential reduction of glomerular infiltrating cells.

We appreciate your important critique. For this revision, we have newly demonstrated the infiltration of neutrophils (MPO⁺ cells) and macrophages (CD68⁺ cells) in the kidneys.

The newly added Figure 5d shows that neutrophil infiltration into glomeruli was significantly suppressed by MOD06051 in a dose-dependent manner. Interestingly, although glomerular infiltration of neutrophils somewhat remained in AAV model rats administered with low-dose MOD06051, NET deposition in renal tissues was at a quite low level. Since neutrophils derived from Papillon-Lefevre syndrome patients, who have almost no CatC activity, showed no change or only a faint decrease in various readouts for chemotaxis (Roberts, *et al.* J Leukoc Biol 2016;100:433-444), it is not likely that pharmacological CatC inhibitor significantly suppresses chemotaxis of neutrophils *in vivo*. Taken together, we consider that MOD06051 principally suppresses NET formation rather than inhibits neutrophil recruitment.

Similarly, MOD06051 dose-dependent decrease in macrophage infiltration into and around glomeruli was demonstrated in the newly added Figure 5e.

Infiltration of other cells, including lymphocytes and proinflammatory monocytes, still remains to be addressed. Comprehensive and chronological analyses of inflammatory cells using high-throughput methods like FCM should be addressed in future studies to answer whether reduction of NETs in the kidneys could be a consequence of reduced infiltrating neutrophils.

Related descriptions were added to the Online methods (pages 7–8) and the sections of Results (page 11) and Discussion (page 15), and the legend for Figure 5.

Comment 8:

The investigators applied to the study drug in a prophylactic approach (i.e. before disease outbreak). Clinical translation would require starting treatment after signs of the disease were detected. For example, erythrocyturia could serve as a marker for the manifestation of glomerulonephritis and treatment would then start after the onset of erythrocyturia.

We agree that our approach in this study can be more prophylactic than therapeutic. However, although AAV patients can be diagnosed by glomerulonephritis or other symptoms with ANCA at the initial diagnosis, these patients usually experience long term repeated remission and exacerbation of symptoms. Therefore, medication is not necessarily administered only during the exacerbation stage but maintenance therapies are usually considered after remission. This means that the efficacy in prophylactic treatment can be still clinically meaningful. This issue has discussed in the revised manuscript (page 14).

Reviewer #3 (Remarks to the Author):

In this manuscript the authors used a novel CatC inhibitor to suppress the cellular activity of neutrophil serine proteases, in cells from both human and rat, and reduce NET-forming cells from rat after ANCA

stimulation. Further, in the presence of the CatC inhibitor they observed less NETs and reduced disease in a rat model of MPO-ANCA associated vasculitis.

Thank you very much for taking the time to review our manuscript. By addressing your constructive critiques, we have revised the manuscript. The revised portions are highlighted in red. We hope that the revised manuscript is now worthy of your acceptance.

Major comments:

The authors need to present more evidence on that MOD06051 inhibits NET-forming cells (undergoing NETosis) and/or release of NETs.

1. The authors used FCM to measure sytox green positive neutrophils and morphological changes, to study NET-forming cells. I do appreciate the simplicity and relevance of this approach, but there are also uncertainties. In a previous publication that is referred to, PMA was used to induce NETosis and FCM results correlated with immunofluorescence data, but whether such a correlation can be observed during ANCA stimulation is not presented. This is relevant as sytox green staining by itself does not distinguish between cell death mechanisms with ruptured membranes, and whether the morphological changes observed represents NET-forming cells specifically is not clear to the reader. A comparison of morphological changes during different cell deaths using FCM could be presented in supplementary methods. A combination of sytox green positivity with morphological changes could perhaps be of help to distinguish between e.g., necrotic and NETotic cells.

We appreciated your critical comments. We have demonstrated that FCM using Sytox Green can distinguish NET-forming or plasma membrane-ruptured neutrophils from apoptotic neutrophils (Masuda *et al.* Cytometry A 2017;91:822-9); however, this method cannot discriminate neutrophils undergoing NETosis from necrotic neutrophils. Therefore, to demonstrate that rat neutrophils primed with TNF- α or C5a followed by MPO-ANCA-IC stimulation underwent NETosis, these cells were subjected to immunofluorescent staining for Cit-H3 as a NETosis marker. The newly added Supplementary Figure 3b shows that rat neutrophils primed with TNF- α or C5a followed by MPO-ANCA-IC stimulation became positive for Cit-H3, thereby suggesting that these cells were NET-forming neutrophils. Furthermore, to quantify the level of NET formation objectively, NET-forming neutrophils were detected by FCM using Sytox Green. Sytox Green histograms in Supplementary Figure 3b demonstrated increase in NET-forming neutrophils by MPO-ANCA-IC stimulation consistent with Cit-H3 labelling. These findings suggested that FCM using Sytox Green could detect NET-forming neutrophils quantitatively. Related descriptions were added to the Online methods (page 4), Results section (page 8), and the legend for Supplementary Figure 3.

2. In addition to FCM, it is important to also quantify what are actually released from the cells (rat and human). This would also include NETs released during vital NETosis. I agree with the authors that immunofluorescence imaging can be subjective, but there are reports on how this can be conducted in a more automated, objective, manner. Nevertheless, there are other alternatives as well, such as quantification of complexes of citrullinated H3 or MPO and DNA in the supernatants, which would also support the release of NETs from the cells.

We appreciate your constructive comments. NET-derived MPO-DNA complexes in supernatants of human and rat neutrophils primed with TNF- α or C5a followed by MPO-ANCA-IC stimulation were assessed quantitatively with ELISA. Results shown in the revised Figure 3 and Supplementary Figure 3 suggested that these cells were NET-forming neutrophils. Related descriptions were added to the Online methods (page 4), Results section (page 8), and the legends for Figure 3 and Supplementary Figure 3.

3. The authors demonstrated that MOD06051 inhibited PR3 and NE activity in vitro in human CD34⁺ Bone Marrow Stem/Progenitor cells, but NETosis or released NETs were not measured. That was instead measured in human peripheral neutrophils when using sivelestat. Based on that MODO6051 would exert its effect during neutrophil development in the bone marrow it is not clear to me why NETosis and NETs were not measured for these cells, as this constitutes an important experiment.

We entirely agree with your critique. We unsuccessfully attempted to induce NETs in neutrophils differentiated from CD34⁺ Bone Marrow Stem/Progenitor Cells. Kuhikar *et al.* reported that NETs can be induced in neutrophils differentiated from CD34⁺ stem cells in peripheral blood and umbilical cord blood (Stem Cell Res 2021;50:102150). Because their neutrophil differentiation protocol is nearly identical to ours, the difference may be attributed to the source of cells; namely, bone marrow or peripheral blood. Although further investigation is needed, this limitation has been noted in the revised manuscript (pages 15–16).

4. The authors need to clarify why NETs in sup fig 2b and fig 5b were not stained for the same content. Also, If the authors want to include these figures as evidence of more NETs during disease, they must be quantified and compared with relevant controls (as in fig 5c), or otherwise be clearly stated that they are included for visualisation of NETs.

Original Supplementary Figure 2 (Supplementary Figure 3a in the revised manuscript) indicated that primed rat neutrophils could release NETs after stimulation with MPO-ANCA-ICs. For this revision, rat neutrophils primed with TNF- α or C5a followed by MPO-ANCA-IC stimulation were subjected to

NET staining. These cells were positive for Cit-H3, thus being identified as NET-forming neutrophils. Related descriptions were added to the Online methods (page 4), Results section (page 8), and the legend for Supplementary Figure 3.

In Figure 5c, the number of areas with NET deposition (recognized as MPO⁺ Cit-H3⁺ DAPI⁺ substances) under high-power fields of view in the renal cortex were counted and then compared to MOD06051-administered groups (low and high doses) and vehicle controls. The related description in the legend for Figure 5 was revised.

Minor comments:

1. It was recently published that also the CatC inhibitor BI-9740 significantly reduced NE and PR3 activity in human CD34⁺ bone marrow stem cells when differentiated to neutrophils, but this reference was not included in this manuscript. The authors need to discuss how this study relates- and is novel to other studies who have tried to delineate the importance of targeting CatC in ANCA-associated vasculitis, e.g., Jerke, Uwe; Eulenberg-Gustavus, Claudia; Rousselle et al, JASN 2022.

A description of how this study is novel and relates to other studies attempting to delineate the importance of targeting CatC in AAV was added in the Introduction section (page 5) by referring Jerke's paper (J Am Soc Nephrol 2022;33:936-947).

2. When describing FCM results, both NET-forming cells and NET formation is being used in the manuscript and needs to be consistent. Since the authors measure DNA within the cells, I suggest that NET-forming cells (i.e. cells undergoing NETosis) should be used, as NET formation is usually used to describe released NETs.

Regarding the terms, we have revised the manuscript thoroughly.

3. The authors mention that proteinuria was not prominent in this model, but this should be elaborated on as it is a relevant marker during disease and was described in the original publication of this disease model (as referred to as by the authors).

Most AAV patients develop rapidly progressive renal dysfunction due to severe glomerulonephritis with massive proteinuria, whereas maximally 16.2% glomeruli were affected (Figure 4f) and proteinuria was not prominent (Figure 4c) in AAV model rats used in this study. Therefore, we hypothesize that our model exhibits mild manifestation of AAV.

In this study, proteinuria was checked using dipsticks for human urine. Further investigation; e.g., rat albumin quantification, is needed to evaluate proteinuria precisely. This issue was included in the

Discussion section (page 16).

4. From how many healthy volunteers were neutrophils isolated in the experiments with sivelestat and avacopan?

Each assessment was based on three independent experiments. This issue was addressed in the legends for Figure 3 and Extended Data Figure 2.

5. For measurement of NET-forming cells in rats after ANCA-activation 3 rats/group were included, whereas 5 and 8 were included in other experiments. How come there is such a big discrepancy? The reason for this, and impact of, could be added to the discussion.

Number of rats enrolled in *in vivo* studies was determined based on predictive individual difference. Since the level of evidence is generally increased as the number increases, experiments using a small number of rats contain a limitation. This issue was discussed in the revised manuscript (page 16).

6. In the beginning and in the end of the discussion section, line 220-222, 302-305 and 320-321, it is not clear to the reader what was done on human cells and/or in rats. Except for the last words of line 321 the results are mentioned in general terms, but it is very important information in what system they were generated.

The related sentences were revised, accordingly.

7. On line 58, I think another word than biophylactic could be used as I don't think the meaning of this is clear to the general audience.

The term was revised.

8. Starting at line 66 I understand that the authors focus on describing NETosis where NE is involved in the signalling pathway. However, only one of the two references at line 67 mention the Raf-MEK-ERK signalling pathway, and that when using PMA during stimulation. As the authors use ANCA to stimulate neutrophils in this study, it would be interesting and relevant to add information about how they activate neutrophils. In this section, it could also be good to introduce lytic vs. suicidal NETosis, as the authors in the results section mention lytic NETs (which I think should be rephased to lytic NETosis or lytic NET-forming cells to match the phrasing in this manuscript).

Thank you very much for your constructive comments. The citation was corrected. Although the pathway whereby ANCA activates neutrophils has not been revealed completely, common molecules, such as ROS and PAD4, are involved in the formation of PMA- and ANCA-induced NETs (Nakazawa *et al.* Front Pharmacol 2021;12:684886). This issue was addressed in the Introduction section (page 5). The term “lytic NETs” was revised.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I have read the revised manuscript titled "Cathepsin C inhibition reduces neutrophil serine protease activity and improves activated neutrophil-mediated disorders" by Nishibata et al. The authors have diligently replied and revised to both my comments and the other two reviewers. I still have a few concerns that need to be addressed.

My primary concern with the model of MPO-AAV is that there are no NETs in the glomeruli. Kessenbrock et al (2009), O'Sullivan et al (2015), Schrieber et al (2017) all report in both human and mouse studies that NETs are a prominent feature of glomerular pathology. It is difficult to state the inhibitor is reducing NETs if you can't demonstrate them in areas of glomerular injury.

Figure 2A Figure 2b shows sytox green positive neutrophils between the vehicle control and MOD06051 treated neutrophils. To determine specificity in this method is there a control for neutrophils (not simulated, no treatment)? This would give the readers an idea of the baseline activity of neutrophils.

Figure 3 shows both in A and B citrullinated histones in red. This experiment should really be conducted, with MPO or NE to demonstrate they are neutrophils, secondly to demonstrate that it is a NET you need to include a merged image showing co-localisation and DNA strands in an untreated well, so you can demonstrate you have reduced NETs with the treatment.

Figure 4 Would benefit from a schematic outlining how the animal model is conducted and timing of treatment.

Figure 5:

D MPO + cells are shown but it is unclear where they are without a DAPI counterstain to show that this picture is within the cortex (or even in the kidney).

E has no accompanying figure legend (it is incorrectly labelled as D in the figure legend) In this new figure E it would help readers if you were to indicate by a image heading what the stainings is showing eg macrophages or neutrophils as it is not really clear in the current form.

Reviewer #2 (Remarks to the Author):

Nashibata et al. provide a revised manuscript. I appreciate that the authors performed new experiments and analyses. However, some of my criticisms that I mentioned in my initial evaluation remain in my opinion:

1) Little new information because several aspects of NETs and CatC inhibition in general and in AAV and were already shown by others (ref. DOI: 10.1083/jcb.201006052, DOI: 10.1172/JCI76009), NETs-dependent MPO-ANCA vasculitis in mice (DOI: 10.1073/pnas.1708247114, not cited), CatC inhibition reduces PR3 antigen /DOI: 10.1681/ASN.2021081112). New but weak are the animal model data (see comment 3)

2) A second independent method to actually quantify NETs formation was requested but not provided in the revised manuscript (e.g. assessment of nuclear area as in DOI: 10.1083/jcb.201006052)

3) AAV is a necrotizing crescentic glomerulonephritis – not a primary tubulo-interstitial disease. The authors suggested reduced NETs deposition in the renal cortex. I requested stainings that reveal whether these NETs were restricted to the glomeruli and co-localize with glomerular neutrophils. The authors do not provide these data. Therefore, I see no data that clearly establish glomerular NETs linked to glomerulonephritis. Thus, the importance of reducing glomerular NETs to prevent glomerulonephritis is not convincingly demonstrated in the glomerulonephritis AAV model used by the authors.

Reviewer #3 (Remarks to the Author):

Dear authors,

Thanks for this new version of the manuscript. Although I think the manuscript is improved, more clarity is needed for some important parts of the manuscript.

Major comments:

1. It is stated in the methods section that neutrophils were subjected to Cit-H3 staining after 3 hours of ANCA stimulation. In Fig 3, it seems that Cit-H3 can be detected, co-localised with DNA, after this time but it does not seem that these structures are released out of the cells (based on that the DNA looks very similar in unstimulated cells). Is that correct? It is important to show that NET-forming cells contain Cit-H3 but more importantly, with the presence of NETs in tissues in mind, to prove that these structures are also released. The ELISA-data suggests that these structures are released but does not rhyme with what is currently shown for the immunofluorescence data in Fig 3, which I assume is from the same time point.
2. Sup Fig 3a shows some structures suggested to be NETs, and additional staining in sup Fig 3b was done to prove this. However, no such strands are displayed in sup Fig 3b. The authors need to show that extracellular DNA structures induced by ANCA, such as in sup Fig 3a, contain Cit-H3 and DNA.

Response to Reviewers' Comments

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Thank you very much for taking the time to evaluate our revised manuscript. We appreciate your critical comments to further improve the manuscript.

My primary concern with the model of MPO-AAV is that there are no NETs in the glomeruli. Kessenbrock et al (2009), O’Sullivan et al (2015), Schrieber et al (2017) all report in both human and mouse studies that NETs are a prominent feature of glomerular pathology. It is difficult to state the inhibitor is reducing NETs if you can’t demonstrate them in areas of glomerular injury.

For this revision, all kidney sections were subjected to NETs staining again. For this analysis, rather than using frozen tissue sections as in the original analysis, we used formalin-fixed paraffin-embedded tissue sections. We could detect NET-forming neutrophils in the glomeruli of MPO-AAV rats and demonstrated a suppressive effect of MOD06051. These results are shown as new Figures 5b and 5c.

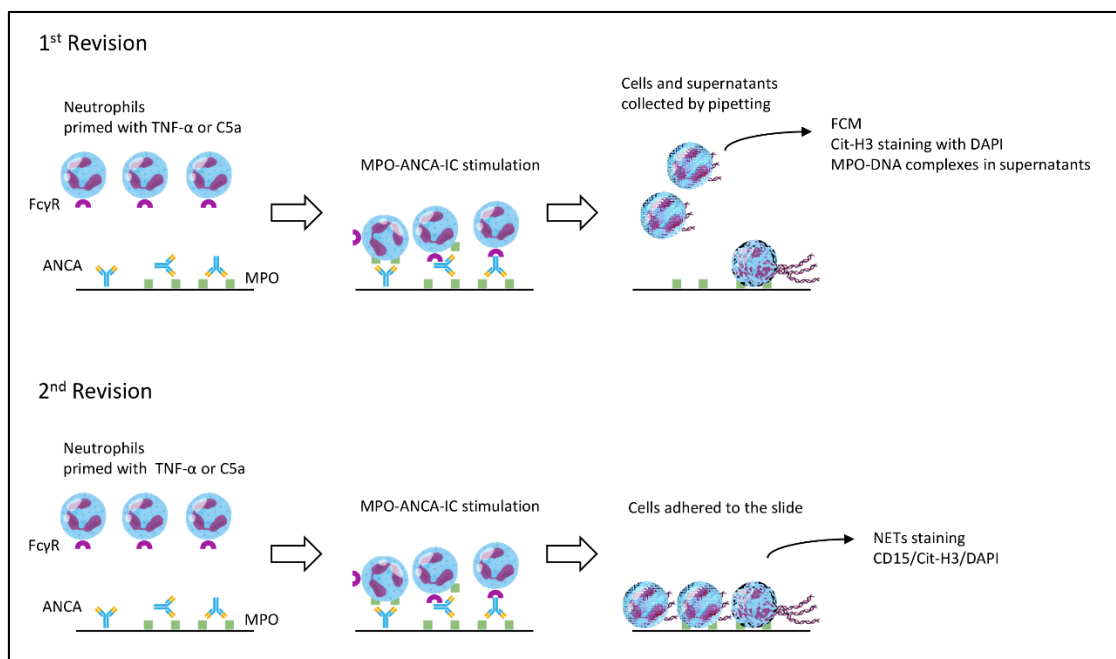
Figure 2A Figure 2B shows sytox green positive neutrophils between the vehicle control and MOD06051 treated neutrophils. To determine specificity in this method is there a control for neutrophils (not stimulated, no treatment)? This would give the readers an idea of the baseline activity of neutrophils.

The requested data was originally included as Supplementary Figure 3b. In order to thoroughly explain the data, the figure was moved to Figure 2c in this revision. The related description was revised for clarity (pages 9–10, lines 185–193).

Figure 3 shows both in A and B citrullinated histones in red. This experiment should really be conducted, with MPO or NE to demonstrate they are neutrophils, secondly to demonstrate that it is a NET you need to include a merged image showing co-localisation and DNA strands in an untreated well, so you can demonstrate you have reduced NETs with the treatment.

In this assay, neutrophils isolated from human peripheral blood were primed with TNF- α or C5a and

then stimulated with immobilized MPO-ANCA-IC on a slide with chambers. Thereafter, cells and supernatants in the chamber were collected by pipetting and the cells were subjected to flow cytometry and NETs staining, and MPO-DNA complexes in the supernatants were measured by ELISA. Neutrophils which completely underwent lytic NET formation adhered to the bottom of the chamber and thus could not be harvested, and therefore, citrullinated histone H3 (Cit-H3)-positive cells did not appear as releasing DNA. For this revision, we reproduced the experiment and cells adhered to the slide were subjected to NETs staining (please see the experimental image below). For NETs staining, CD15 was adopted as a neutrophil marker instead of MPO (anti-MPO antibody was not suitable for this purpose because MPO was immobilized on the slide).



We have successfully demonstrated that the cells were neutrophils by positive CD15 staining, and also the extracellular DNA induced by MPO-ANCA-IC stimulation was positive for Cit-H3 staining (see revised Figures 3a and 3c and Extended Data Figures 2a and 2c). In addition, colocalization of those three indicators was confirmed. The effects of sivelestat and avacopan on MPO-ANCA-IC-induced NET formation, evaluated with DAPI-positive DNA area per neutrophil as an indicator, were demonstrated in the revised Figures 3a and 3c and Extended Data Figures 2a and 2c, respectively.

Figure 4 Would benefit from a schematic outlining how the animal model is conducted and timing of treatment.

We have revised Figure 4 according to the advice.

Figure 5:

D MPO + cells are shown but it is unclear where they are without a DAPI counterstain to show that this picture is within the cortex (or even in the kidney).

We have replaced the photos by those with DAPI counterstain.

E has no accompanying figure legend (it is incorrectly labelled as D in the figure legend) In this new figure E it would help readers if you were to indicate by a image heading what the stainins is showing eg macrophages or neutrophils as it is not really clear in the current form.

We have revised Figure 5 and its legend.

Reviewer #2 (Remarks to the Author):

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Thank you very much for taking the time to evaluate our revised manuscript. We appreciate your critical comments to further improve the manuscript.

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We agree with your critical comments. We have introduced the previous evidence (page 5, lines 103–107) and humbly assessed the achievements of this study (page 6, lines 115–119).

2) A second independent method to actually quantify NETs formation was requested but not provided in the revised manuscript (e.g. assessment of nuclear area as in DOI: 10.1083/jcb.201006052)

We reproduced the experiment to revise Figure 3 and Extended Data Figure 2. At this time, after MPO-ANCA-IC stimulation, cells adhered to the slide were subjected to NETs staining. We have

successfully demonstrated that extracellular DNA induced by MPO-ANCA-IC stimulation was positive for Cit-H3 and the shown effects of sivelestat and avacopan on MPO-ANCA-IC-induced NET formation by evaluating DAPI-positive DNA area per neutrophil. Sivelestat inhibited MPO-ANCA-IC-induced NET formation in both TNF- α - and C5a-primed neutrophils (revised Figure 3), whereas avacopan inhibited MPO-ANCA-IC-induced NET formation in C5a-primed neutrophils but not in TNF- α -primed neutrophils (revised Extended Data Figure 2).

3) AAV is a necrotizing crescentic glomerulonephritis – not a primary tubulo-interstitial disease. The authors suggested reduced NETs deposition in the renal cortex. I requested stainings that reveal whether these NETs were restricted to the glomeruli and co-localize with glomerular neutrophils. The authors do not provide these data. Therefore, I see no data that clearly establish glomerular NETs linked to glomerulonephritis. Thus, the importance of reducing glomerular NETs to prevent glomerulonephritis is not convincingly demonstrated in the glomerulonephritis AAV model used by the authors.

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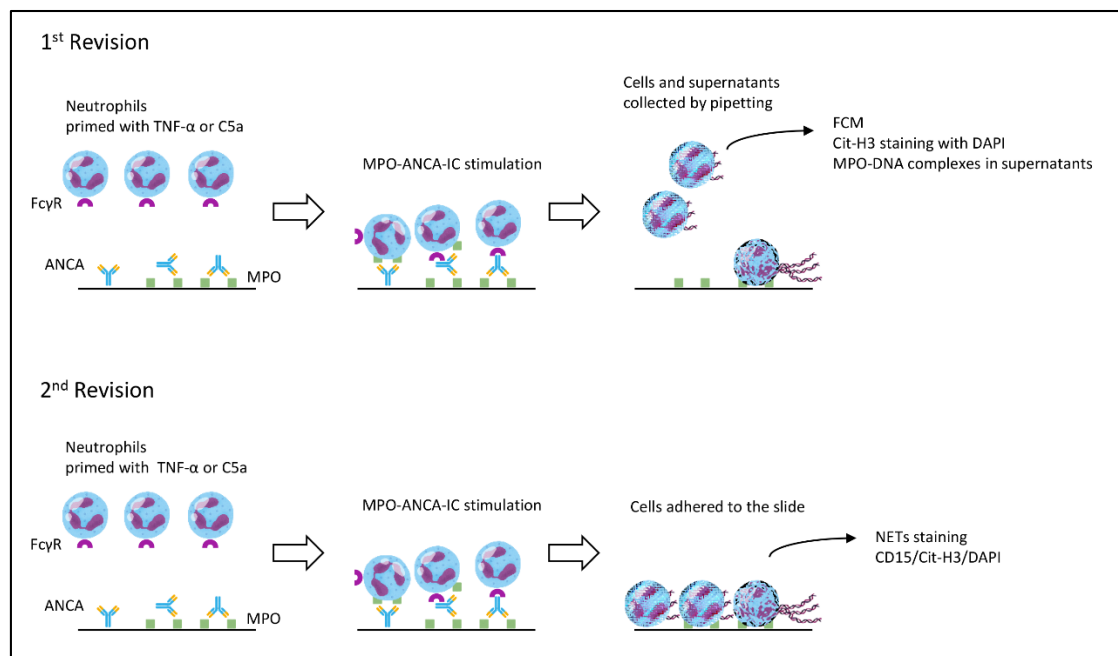
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We have successfully demonstrated that extracellular DNA induced by MPO-ANCA-IC stimulation was positive for Cit-H3 (see revised Figures 3a and 3c and Extended Data Figures 2a and 2c).

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To address this issue, neutrophils isolated from a non-treated normal rat were primed with TNF- α or C5a and then stimulated with immobilized MPO-ANCA-IC on a slide with chambers. Thereafter, cells

adhered to the slide were subjected to staining for Cit-H3. We have successfully demonstrated the colocalization of Cit-H3 and extracellular DNA (revised Supplementary Figure 3).

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

Nashibata et al. provide a revised version of their manuscript on cathepsin C to inhibit neutrophil serine proteases (NSP). The authors performed new experiments and show now in paraffin-embedded kidney sections NETs in affected glomeruli in the rat AAV model. The cathepsin C inhibitor MOD06051 reduced glomerular NETs and glomerular necrosis and crescents. In addition, the authors provide better NETs characterization by Cit-H3 detection and quantification. These new data improved the study.

I have no further questions for the authors.

Reviewer #3 (Remarks to the Author):

Dear authors,

Thank you for this improved version of the manuscript.

In fig 5c, which shows NETs/neutrophil in glomeruli, there are only one cell releasing NETs, or none, in the pictures of glomeruli. How does this relate to the numbers shown in the graph in 5c, which says almost 10% on average for pathology. Also, how many different glomeruli did you count to reach the average NETs/neutrophils in glomeruli? That should be added to the methods section for clarity.

I have been also asked to comment on the concerns raised by reviewer #1 and the author responses to these.

These are all important questions. I agree it is a problem that they cannot show any NETs in glomeruli, which is why I also asked that question in my previous response. Also, how did they reliably quantify something that is present to such small extent?

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I have no further questions for the authors.

Thank you very much for taking the time to review our manuscript. We really appreciate your constructive critiques to improve the manuscript.

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Thank you for this improved version of the manuscript.

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The graph in Figure 5c represents the percentage of NET-forming cells in neutrophils infiltrating into glomeruli and shows that about 10% neutrophils in glomeruli are undergoing NETosis in the pathology (vehicle) group. Since approximately three neutrophils infiltrated per glomerulus in the pathology (vehicle) group as shown in Figure 5d, calculations indicate that 0.3, i.e., 0 to 1, NET-forming neutrophils were present in the glomerulus; thus, this is consistent with the photo findings in Figures 5b and 5c. To calculate the percentage, more than 30 neutrophils infiltrating into glomeruli were counted. Although we did not record how many glomeruli were counted at the time, as approximately three, two, and two or one neutrophils were infiltrated into the glomerulus in the pathology (vehicle) group, low-dose (MOD06051) group, and high-dose (MOD06051) group, respectively (as shown in Figure 5d), it is estimated that about 10, 15, and 15–30 glomeruli were counted in each group.

I have been also asked to comment on the concerns raised by reviewer #1 and the author responses to these.

These are all important questions. I agree it is a problem that they cannot show any NETs in glomeruli, which is why I also asked that question in my previous response. Also, how did they reliably quantify something that is present to such small extent?

We appreciate your critical comments. To assess the involvement of NETs in glomerular lesions of MPO-AAV model rats, we have modified the staining protocol for NETs and successfully demonstrated that some neutrophils infiltrating into glomeruli underwent NETosis in the revised manuscript (version NCOMMS-23-35469B). However, the amount was smaller than that previously shown in patients with MPO-AAV (Kessenbrock et al. *Nat Med* 15: 623-625, 2009; O'Sullivan et al. *Kidney Int* 88: 1030-1046, 2015). We consider this is a limitation of animal models of MPO-AAV. Maximally 16.2% glomeruli were affected in MPO-AAV model rats used in this study, and this is consistent with the original paper demonstrating that less than 20% glomeruli showed glomerulonephritis on average (Little et al. *Am J Pathol* 174, 1212-1220, 2009). We have demonstrated that a few neutrophils underwent NETosis in the glomerulus in MPO-AAV model rats (Figures 5b and 5c), and this is equivalent to the MPO-ANCA transfer mouse model (Schreiber et al. *Proc Nat Acad Sci USA* 114, E9618-E9625, 2017). In this mouse model, approximately 20% glomeruli showed crescent formation, and no or few neutrophils were positive for phosphorylated MLKL (which means undergoing NET formation) in the glomerulus. For reference, the related figures are quoted below from the Schreiber paper.

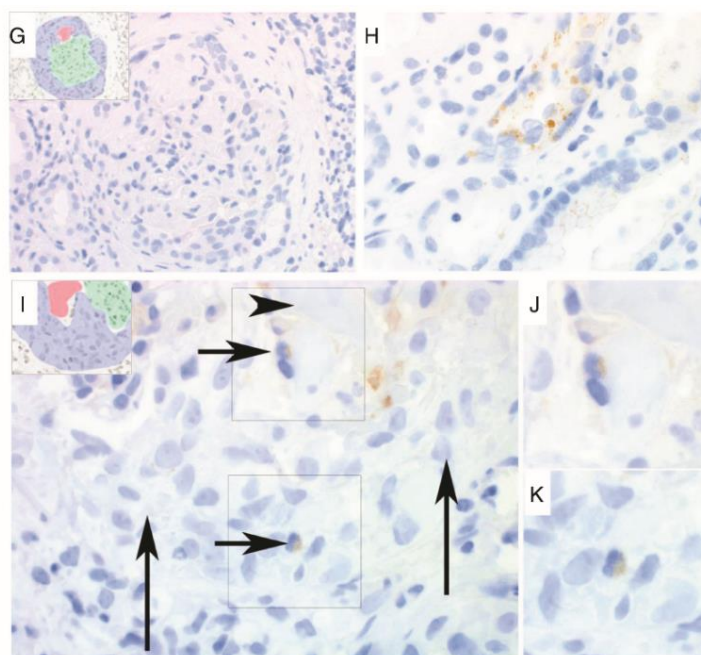
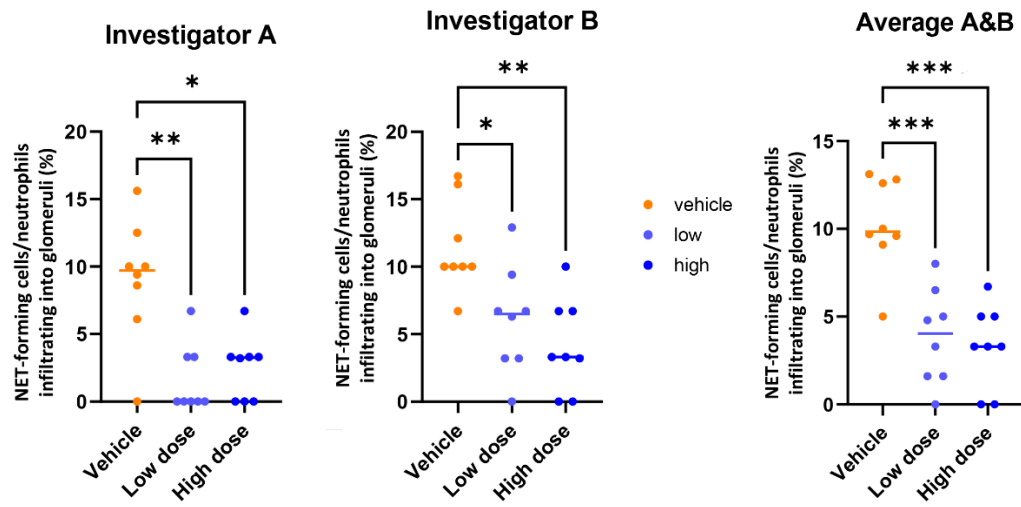


Figure 5. (G–K) Exemplary micrograph from a biopsy with active anti-MPO-ANCA-associated NCGN shows fibrinoid necrosis and a cellular crescent; the Insets (G) shows the extent of the crescent in blue, necrosis in red, and tuft in green color. No resident mesangial cells, endothelial cells, podocytes, or intracapillary or infiltrating leukocytes are positive (brown) for p-MLKL. (H) Some of the few tubular segments with epithelial positivity for p-MLKL. (I) Biopsy with active anti-MPO-ANCA-associated NCGN and cytoplasmic positivity for p-MLKL in two neutrophils, recognizable by their typical segmented nuclei (short arrows). The colors in the Left Inset are as above. The neutrophil in the Top Right Inset (arrow) is adjacent to a fibrinoid necrosis area (arrowhead) (I and J). The neutrophil in the Bottom Right Inset is adjacent to a fresh, cellular crescent (long arrows). Both neutrophils in the Right Insets are magnified in the corresponding two frames (J and K) to better appreciate nuclear morphology.

(We highlighted important sentences)

Although the exact reason why the extent of NET deposition in glomeruli is smaller in murine models than in humans remains unknown, one possible explanation is the difference in the activity of DNase I in serum, which digests NETs (Hakkim et al. Proc Nat Acad Sci USA 107: 9813-9818, 2010). It has been demonstrated that serum DNase I activity is significantly decreased in patients with MPO-AAV compared with healthy individuals (Nakazawa et al. J Soc Am Nephrol 25: 990-997, 2014). In contrast, there is no such evidence in rats and mice. Therefore, the amount of NET deposition in murine MPO-AAV models might be underestimated because NETs could be digested by DNase I *in vivo*. Concerning these issues, limitation and discussion were added in the revised manuscript (version NCOMMS-23-35469C; page 18, last paragraph).

For the previous manuscript (version NCOMMS-23-35469B), one investigator (investigator A) counted and evaluated the NET-forming cells in the neutrophils infiltrating into glomeruli. To improve the reliability of quantification, this time another investigator (investigator B) performed evaluation of the same sections independently to investigator A. As aforementioned, more than 30 neutrophils in 10–30 glomeruli were counted to calculate the percentage of NET-forming cells. Since approximately 100 glomeruli are present per section, the probability of both investigators A and B counting the same glomeruli show be reasonably low. Nevertheless, the results were well-reproduced and the differences between vehicle and compound-treated groups in each evaluation were statistically significant as shown in the figure below. Therefore, this revision (version NCOMMS-23-35469C) adopted the mean values of investigators A and B evaluations, improving the statistical confidence. The previous Figure 5c was replaced by this figure and the precise method was added to the Online Methods (page 8, lines 9–12).



* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

We hope that the revised manuscript (version NCOMMS-23-35469C) is now worthy of your acceptance.

REVIEWERS' COMMENTS

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Dear authors,

Thanks for the latest and improved version of the manuscript. I have no further comments.