nature portfolio

Peer Review File

Lipid asymmetry disruption by XKR8 orchestrates the neutrophil extracellular traps formation and inhibits fungal infection

Corresponding Author: Dr Ning Wu

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Decision Letter:

26th Aug 2025

Dear Dr Wu,

Your Article, "Lipid asymmetry disruption by XKR8 orchestrates the neutrophil extracellular traps formation and inhibits fungal infection" has now been seen by 4 referees. You will see from their comments copied below that while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication, but would be very interested in considering a revised version that addresses these serious concerns.

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

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

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

If revising your manuscript:

* Include a “Response to referees” document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

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

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

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

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

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

Extended Data figures and tables are online-only (appearing in the online PDF and full-text HTML version of the paper),

peer-reviewed display items that provide essential background to the Article but are not included in the printed version of the paper due to space constraints or being of interest only to a few specialists. A maximum of ten Extended Data display items (figures and tables) is typically permitted. When re-submitting your manuscript, please ensure that any supplementary figures and tables that are more critical to the manuscript's conclusions are converted to Extended data to increase these data's visibility.

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

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

Link Redacted

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

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

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

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

Thank you for the opportunity to review your work.

Sincerely,
Paula

Paula Jauregui, PhD
Senior Editor
Nature Immunology

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
A. Summary of the key results- The manuscript, titled "Lipid asymmetry disruption by XKR8 orchestrates the neutrophil extracellular traps formation and inhibits fungal infection," describes a central and critical role for XKR8 in NET formation. The authors take a methodical approach to dissect this pathway and link it to other critical pathways, including plasma membrane lipid changes, RS production, and calcium fluxing. The biochemical description of this pathway is remarkable in three ways: unexpected, all work was performed without the benefit of an antibody to XKR8, and was performed in primary neutrophils, which are notoriously difficult to work with.

B. Originality and significance: if not novel, please include reference—Original and highly significant for neutrophil biology.

C. Data & methodology: validity of approach, quality of data, quality of presentation-- nicely written and well laid out.

D. Appropriate use of statistics and treatment of uncertainties – appropriate.

E. Conclusions: robustness, validity, reliability – conclusions are fully supported by the data provided.

F. Suggested improvements: experiments, data for possible revision—My major concern is the use of a C. albicans lung infection model. C. albicans is not a pathogen in mice or humans in the lungs. The system, while used by others in the field, is highly artificial. Here, the authors use these data to support a role for XKR8 in lung host defenses. But this model is so artificial that I do not support this conclusion. If C. albicans is desired, then a disseminated model of infection should be used. If a lung model is desired, then use another model that has human relevance. Additionally, there are cohorts of human

patients who have invasive candidiasis or pulmonary infections. It would be nice to know if humans have SNPs in XKR8 enriched in these patient populations over the general population. This indirect evidence would go a long way to establish human relevance.
Please add some baseline characteristics for the knockout mice that were introduced in this manuscript. Some general characterization of immune cells (including # of neutrophils and other subsets, lymph node structure, spleen size, and architecture) would assist in the conclusion that these KOs do not have a global effect on the immune system or mouse development.

Minor: Consider adding a sentence in the introduction about the physiological role of lipid asymmetry in neutrophils at homeostasis.

Many instances of “extend” when “extent” was intended.

G. References: appropriate credit to previous work?- acceptable.

H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction, and conclusions—well written.

Reviewer #2

(Remarks to the Author)
The authors identify XKR8 as a regulator of NET formation and delineate the mechanism by which it controls NETosis. This is a major step forward in our understanding of how NETs are made and also provides compelling new in vivo evidence for a role of NETs in two relevant disease models. The authors use robust NET assays and rigorous analyses. They use physiological NET stimuli and combine human and mouse neutrophil work, resulting in a well-executed, convincing and interesting study.

The following points should be addressed before publication:

Extended figure 1: do KO mice have normal circulating neutrophil counts?

Extended 2h,i, Quantification of NETs in WT, XKR8 KO and PAD4 KO BMNs. How was this quantified – citrullination or DNA/MPO imaging? Since multiple NET quantification techniques are used throughput the paper, each figure legend should specify the method.

Caspase 3 requirement: multiple papers showed that caspase inhibitors do not block NET release by human neutrophils. Eg:
https://pmc.ncbi.nlm.nih.gov/articles/PMC3193439/
https://elifesciences.org/articles/24437
Caspase 3 requirement could be a feature of mouse NETosis. The same caspase inhibitors should be tested with human neutrophils.

Page 11: authors claim “impaired Ca2+ signaling in XKR8 KO cells during NETs formation” but this hasn’t been shown. Can the authors demonstrate a reduced calcium signal linked to altered PM lipid tension?

HC067047 (TRPV4 inhibitor), Pyr3 (TRPC3 inhibitor), SKF96365 (TRPV2 inhibitor), 9-
Phenanthrol (TRPM4 inhibitor), JNJ-28583113 (TRPM2 inhibitor) and GsMT×4 (Piezo1 inhibitor) should also be tested in a NETosis assay with primary human neutrophils.

It is encouraging that agonists can reverse the NET inhibition in KO neutrophils, however it is unclear that these agonists are not simply NET inducers that act via an unrelated pathway. Authors should show NET results for the agonists in the absence of PMA, preferably in human primary neutrophils.

Engulfment of apoptotic neutrophils is an important polarization signal for macrophages and reduced neutrophil PS exposure may hyperactivate the macrophage IL-23/GCSF axis. Is there an elevated basal inflammatory state in the KO mouse strains? Please show cytokine levels in naïve mice.

Fig 6K: description in figure legend is unclear: two genotypes are listed but this is not shown in graph. It would be better to show data for both genotypes, treated +/- agonist

Reviewer #3

(Remarks to the Author)
This is an amazing manuscript that reveals how XKR8 orchestrates NET formation and represents a fundamental step change in our understanding. The data and approaches are convincing. I particularly like the KO and KI models which show

similar phenotypes. I have two comments:

1) It would be good to show the importance of XKR8 in NET formation and inflammation during a model autoimmune disease. This will provide even further justification of the broader importance of this discovery.

2) minor comment: in figures that indicate lung injury score (eg: fig 5d). Representative histology pictures should be included in the supplemental data.

Reviewer #4

(Remarks to the Author)
In this manuscript, the authors investigate the role of the Xkr8 lipid scramblase in the formation of neutrophil extracellular traps (NET). They show that the Xkr8 scramblase is highly expressed in neutrophils and that its activation following processing by caspase 3 leads to PS exposure, which promotes NET formation. They show that activation of Xkr8 alters membrane properties, which results in the activation of various Ca2+ mechanosensitive ion channels. Consistently, they show that inhibiting calcium signaling before lipid scrambling drastically reduces NET formation but that blocking the channels after NET formation does not. The authors also show that NET formation promoted by Xkr8 plays vital roles in neutrophil-induced immune responses in vivo and in resistance to fungal infections.

Overall, this is a very interesting manuscript, the experiments are well designed, executed and interpreted. I only have three concerns that might require some additional experimental work.

1) The authors show that KO of TMEM16F does not affect PS exposure in neutrophils (Fig. 1). However, they also conclude that the main effect of Xkr8 activation is the activation of Ca2+ channels and a consequent Ca2+ influx (Fig. 4). Further, they show that in Xkr8 KO cells, there is Ca2+ dependent PS exposure during NET formation. The first of these findings appears inconsistent with the other two. Indeed, if Xkr8 activation induces Ca2+ entry via mechanosensitive ion channels and Ca2+-dependent PS exposure is seen during NET formation, then one would expect that TMEM16F or another TMEM16 family member might also play a role in this process.

2) A related concern regards the proposed mechanism by which Xkr8 modifies membrane properties to activate multiple mechanosensitive Ca2+ channels. On one hand, I find the pleiotropic effects of Xkr8 very reasonable, since it is reasonable to expect that multiple proteins will be affected by a change in membrane properties. However, I find two aspects somewhat confusing.
a. Ca2+ signaling transduction pathways are tightly regulated and it is difficult to envision how the effects of such a broad-spectrum activation could be controlled in a cell.
b. I do not understand how the authors can see the effects of specific inhibitors and agonists of these channels. If Xkr8 activation promotes activity of many channels at the same time, then their signals should be additive and blocking/activating one of them at a time should not have major effects. Either the tested compounds have cross-effects among channels, or something else must be affected.

To address these concerns the authors should carry out additional experiments:

i. It is important to show that activation of these channels results in Ca2+ entry into the cells, and determine how the various tested compounds affect this entry. This is especially important since the authors propose that the Ca2+ effects are very long-lasting, up to several hours.

ii. Could the authors look at cell/animal strains where some of these channels have been genetically ablated to better identify which channels mediate the Ca2+ responses?
These experiments might also help explain the conundrum regarding the role of TMEM16F in PS exposure during NET formation.

3) The authors show that PS exposure precedes NET formation by several hours. However, the authors do not show whether PS externalization persists during or after NET formation. They assume it does, as they suggest membrane remodeling caused by Xkr8 activation induces Ca2+ entry in cells. However, it is important to directly demonstrate this is the case by measuring PS exposure inside the NETs. Once point to consider is that simply measuring persistence of the AnxV signal after treatment might not be sufficient, since AnxV binds to PS tightly and it could 'trap' it on the outer leaflet rather than being actively kept there by Xkr8.

Minor
1) Could the authors show representative images of the cells undergoing NET formation in WT and KO lines. This would be helpful visualize the morphological changes that are described in the text?
2) Pg. 4, line 23, “despite ubiquitously expressed” should be “despite being ubiquitously expressed”
3) Pg. 11, line 9 “Thus we intended to figure out how lipid scrambling activates subsequent Ca2+.” This seems a broken sentence, unless the authors mean to imply that lipid scrambling activates the Ca2+ ions.

Decision Letter:

Our ref: NI-A40695A

15th Jan 2026

Dear Dr. Wu,

Thank you for submitting your revised manuscript "Lipid asymmetry disruption by XKR8 orchestrates the neutrophil extracellular traps formation and inhibits fungal infection" (NI-A40695A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Immunology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

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

If you had not uploaded a Word file for the current version of the manuscript, we will need one before beginning the editing process; please email that to immunology@us.nature.com at your earliest convenience.

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

Sincerely,
Paula

Paula Jauregui, PhD
Senior Editor
Nature Immunology

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed my concerns. No further concerns.

Reviewer #2

(Remarks to the Author)
The authors have addressed all my comments. I support publication of this manuscript.

Reviewer #3

(Remarks to the Author)
I have no further comments and the authors have addressed all my comments, as well of those of the other reviewers

Reviewer #4

(Remarks to the Author)
The authors have addressed all of my concerns and I have no further requests.

Review round: 1

We thank all the reviewers for their positive comments and helpful advices to further enhance the findings in this study. We have performed additional experiments to address all the concerns raised by the reviewers. Below are our point-by-point responses to the reviewers' specific comments.

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

A. Summary of the key results- The manuscript, titled "Lipid asymmetry disruption by XKR8 orchestrates the neutrophil extracellular traps formation and inhibits fungal infection," describes a central and critical role for XKR8 in NET formation. The authors take a methodical approach to dissect this pathway and link it to other critical pathways, including plasma membrane lipid changes, RS production, and calcium fluxing. The biochemical description of this pathway is remarkable in three ways: unexpected, all work was performed without the benefit of an antibody to XKR8, and was performed in primary neutrophils, which are notoriously difficult to work with.

B. Originality and significance: if not novel, please include reference—Original and highly significant for neutrophil biology.

C. Data & methodology: validity of approach, quality of data, quality of presentation-- nicely written and well laid out.

D. Appropriate use of statistics and treatment of uncertainties – appropriate.

E. Conclusions: robustness, validity, reliability – conclusions are fully supported by the data provided.

We thank you for your supportive and encouraging comments on our work!

F. Suggested improvements: experiments, data for possible revision—My major concern is the use of a C. albicans lung infection model. C. albicans is not a pathogen in mice or humans in the lungs. The system, while used by others in the field, is highly artificial. Here, the authors use these data to support a role for XKR8 in lung host defenses. But this model is so artificial that I do not support this conclusion. If C. albicans is desired, then a disseminated model of infection should be used. If a lung model is desired, then use another model that has human relevance.

Response: We agree with the reviewer that the *C. albicans* lung infection model is artificial, although it has contributed substantially to our understanding of NETs biology (*e.g.* PMID: 25217981). As recommended, we additionally performed a disseminated *C. albicans* infection model using tail vein injection. In this setting, loss of XKR8 in neutrophils also markedly impaired the anti-fungal immunity, leading to significantly increased lethality in XKR8 KO mice (see revised Fig. 6k). Furthermore, intravenous administration of a sublethal dose of *C. alb* resulted in systemic infection, and XKR8-deficient mice exhibited substantially higher fungal burdens across

multiple organs, including the liver, lungs, spleen, and brain (see revised Fig. 6l). In addition, histological analysis of liver and lungs in infected mice demonstrated more severe tissue damage in XKR8 KO mice (see revised Extended Data Fig. 10f). Consistently, XKR8 2DA KI mice phenocopied the KO animals, demonstrating elevated *C. alb* loads in these organs within the disseminated infection model. These results further support that caspase-3-mediated activation of XKR8 in neutrophils plays a critical role in host defense against fungal pathogens *in vivo* (see revised Fig. 6m). Detailed results have been incorporated into the revised manuscript (page 15 line 13-21, Fig. 6k-m and Extended Data Fig. 10f).

Additionally, there are cohorts of human patients who have invasive candidiasis or pulmonary infections. It would be nice to know if humans have SNPs in XKR8 enriched in these patient populations over the general population. This indirect evidence would go a long way to establish human relevance.

Response: We appreciate this suggestion from the reviewer. We analyzed datasets from the IEU OpenGWAS database, including 20 pneumonia-related and 2 *Candida* infection GWAS from European populations. After removing weak instrumental variables ($F < 10$), 15 SNPs associated with serum XKR8 expression ($P < 1\times10^{-5}$) were used for Mendelian randomization (MR) analysis. Although five datasets showed statistically significant associations ($P < 0.05$), the odds ratios were inconsistent in direction. Additional analyses - including mediator MR using neutrophils and MR with available XKR8 pQTL data - also did not yield definitive causal evidence. The limited number of XKR8-associated SNPs and insufficient phenotypic resolution in pneumonia datasets likely reduced statistical power. Overall, these results do not support a strong causal relationship between XKR8 and invasive candidiasis or pulmonary infection.

Although we did not obtain strong evidence from SNP-based analyses, this may reflect the nature of XKR8 function rather than the absence of a biological role. XKR8 is ubiquitously expressed and is essential for apoptosis, a process that is evolutionarily conserved and therefore exhibits limited genetic variability. As a multi-pass membrane protein that becomes active upon caspase-3-mediated cleavage, it is also challenging to capture with high-confidence pQTL data, particularly in human samples. Despite these constraints, the consistent results observed in both human and mouse primary neutrophils in our study support the conclusion that XKR8-mediated lipid scrambling plays a fundamental role in NETs formation across species.

Please add some baseline characteristics for the knockout mice that were introduced in this manuscript. Some general characterization of immune cells (including # of neutrophils and other subsets, lymph node structure, spleen size, and architecture) would assist in the conclusion that these KOs do not have a global effect on the immune system or mouse development.

Response: We thank the reviewer for the helpful suggestion. We have now included all requested baseline information for the XKR8 KO mice used in this study. No differences were observed between WT and XKR8 KO mice in the evaluated parameters (see revised Extended Data Fig. 2d and Supplementary Table 1). These findings indicate that loss of XKR8 does not affect the baseline immune repertoire.

Minor: Consider adding a sentence in the introduction about the physiological role of lipid asymmetry in neutrophils at homeostasis.

Response: Thank you for your suggestion. We have added a brief paragraph in the introduction describing the physiological role of lipid asymmetry in neutrophils under homeostatic conditions. Please see page 3, line 10-15 in the revised manuscript.

Many instances of "extend" when "extent" was intended.

Response: We have done all the necessary corrections in the revised manuscript.

G. References: appropriate credit to previous work? - acceptable.

H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction, and conclusions—well written.

Reviewer #2 (Remarks to the Author):

The authors identify XKR8 as a regulator of NET formation and delineate the mechanism by which it controls NETosis. This is a major step forward in our understanding of how NETs are made and also provides compelling new in vivo evidence for a role of NETs in two relevant disease models. The authors use robust NET assays and rigorous analyses. They use physiological NET stimuli and combine human and mouse neutrophil work, resulting in a well-executed, convincing and interesting study.

Thank you very much for your highly positive comments on our study!

The following points should be addressed before publication:

Extended figure 1: do KO mice have normal circulating neutrophil counts?

Response: Yes. We systematically analyzed the immune repertoire and did not detect differences between WT and XKR8 KO mice, including comparable circulating neutrophil counts. These findings suggest that XKR8 does not play a significant role in immune cell development (Supplementary Table 1).

Extended 2h,i, Quantification of NETs in WT, XKR8 KO and PAD4 KO BMNs. How was this quantified – citrullination or DNA/MPO imaging? Since multiple NET quantification techniques are used throughput the paper, each figure legend should specify the method.

Response: Most NETs quantification in mouse BMNs was performed by measuring the area of chromatin DNA stained with DAPI or Hoechst 33342 (see page 4, line 10-12, and details in the Methods section, and Extended Data Fig. 1a). As the reviewer noted, our manuscript includes multiple complementary approaches for assessing NETs formation, including measurements of histone citrullination (for example, chromatin DNA area in Extended Data Fig. 3i and the ratio of

citrullinated histone to DNA in Extended Data Fig. 3j). In line with this helpful suggestion, we have revised the manuscript to clearly specify the quantification method used in each figure legend throughout.

Caspase 3 requirement: multiple papers showed that caspase inhibitors do not block NET release by human neutrophils.
Eg:
https://pmc.ncbi.nlm.nih.gov/articles/PMC3193439/
https://elifesciences.org/articles/24437
Caspase 3 requirement could be a feature of mouse NETosis. The same caspase inhibitors should be tested with human neutrophils.

Response: Thank you for raising this important point. We also noted this discrepancy between mouse and human neutrophils during our study. It is noteworthy that neutrophils isolated from human peripheral blood exhibit faster and more robust NETs formation than mouse BMNs when stimulated with 100 nM PMA, consistent with both the literature and our own data. We therefore hypothesized that this PMA dose might be excessively strong for human neutrophils, thereby masking the inhibitory effects of caspase-3 inhibitors.
To address this, we reduced the PMA concentration to 5 nM. At this lower dose, NETs formation in human neutrophils became comparable to that observed in mouse BMNs (approximately 30% after 4 hours of stimulation). Under these conditions, the caspase-3 inhibitors used in our mouse experiments, including QVD, Emricasan, zVAD, effectively suppressed NETs formation in human neutrophils as well (see revised Extended Data Fig. 5e). These results support the conclusion that the requirement of caspase-3 activity is a shared feature of NETosis in both mouse and human neutrophils.

Page 11: authors claim "impaired Ca2+ signaling in XKR8 KO cells during NETs formation" but this hasn't been shown. Can the authors demonstrate a reduced calcium signal linked to altered PM lipid tension?

Response: This is a valid point. As suggested, we evaluated intracellular calcium levels by the calcium indicator Indo-1 in WT and XKR8 KO BMNs following PMA stimulation. XKR8 KO BMNs exhibited lower intracellular Ca^{2+} levels than WT controls at 2 h treated with PMA, which corresponds to the time point at which XKR8-mediated lipid scrambling becomes evident (see revised Extended Data Fig. 7e).
To further examine the relationship between calcium levels, lipid scrambling, and altered PM lipid tension, we compared intracellular calcium levels in Annexin V^+ and Annexin V^- BMNs during PMA-induced NETs formation. Notably, among viable cells (gated on PI^- populations), Annexin V^+ BMNs displayed significantly higher intracellular Ca^{2+} levels than Annexin V^- cells (see revised Extended Data Fig. 7f).
These findings further support the notion that altered PM lipid tension by lipid scrambling contributes to elevated Ca^{2+} in BMNs during NETs formation. Please see details in page 11, line 18-22 in revised manuscript and Extended Data Fig. 7e,f.

HC067047 (TRPV4 inhibitor), Pyr3 (TRPC3 inhibitor), SKF96365 (TRPV2 inhibitor), 9-

Phenanthrol (TRPM4 inhibitor), JNJ-28583113 (TRPM2 inhibitor) and GsMT×4 (Piezo1 inhibitor) should also be tested in a NETosis assay with primary human neutrophils.

Response: We agree with the reviewer. As requested, we examined the effects of the Ca^{2+} channel inhibitors mentioned in primary human neutrophils. Consistent with our findings in mouse BMNs, HC067047 (TRPV4 inhibitor), Pyr3 (TRPC3 inhibitor), 9-Phenanthrol (TRPM4 inhibitor), JNJ-28583113 (TRPM2 inhibitor), and GsMT×4 (Piezo1 inhibitor) effectively blocked the PMA-induced NETs formation in primary human neutrophils, with the exception of SKF96365 (TRPV2 inhibitor) (see revised Extended Data Fig. 8c). Notably, SKF96365 even enhanced PMA-elicited NETs formation, likely due to off-target effects (see revised Extended Data Fig. 8c). Additional details are provided in page 12, line 20-22 of the revised manuscript and Extended Data Fig. 8c.

It is encouraging that agonists can reverse the NET inhibition in KO neutrophils, however it is unclear that these agonists are not simply NET inducers that act via an unrelated pathway. Authors should show NET results for the agonists in the absence of PMA, preferably in human primary neutrophils.

Response: This is a critical point! As requested, we evaluated whether the agonists CBD, Probenecid, GSK1016790A, Yoda1, and GSK1702934A could induce NETs formation in the absence of PMA. None of these agonists were able to trigger NETs in primary human neutrophils, with the exception of GSK1702934A (see revised Extended Data Fig. 8g). We further examined their effects in WT and XKR8 KO BMNs, and similarly, none of these agonists were able to induce NETs in mouse neutrophils without PMA stimulation (see revised Extended Data Fig. 8h). Together, these results indicate that these agonists do not act as NETs inducers via an unrelated pathway in either human or mouse neutrophils, except for GSK1702934A. Please see page 13, line 1-3 and Extended Data Fig. 8g,h in the revised manuscript for more details.

Engulfment of apoptotic neutrophils is an important polarization signal for macrophages and reduced neutrophil PS exposure may hyperactivate the macrophage IL-23/GCSF axis. Is there an elevated basal inflammatory state in the KO mouse strains? Please show cytokine levels in naïve mice.

Response: We fully agree that assessing the basal inflammatory state in XKR8 KO mice is essential. To address this, we performed a multiplex cytokine assay on serum from naïve WT and XKR8 KO mice. The results demonstrated that XKR8 KO mice do not exhibit elevated basal inflammation (see revised Extended Data Fig. 2e). Notably, although XKR8 has been implicated in lupus-like autoimmune disease in the MRL background, this phenotype is not observed in C57BL/6 mice (PMID: 29440417). It remains possible that aged XKR8 KO mice may develop elevated basal inflammation due to the impaired clearance of apoptotic neutrophils, however, such outcomes may also be mouse strain-dependent. Please see details in page 5, line 4-6 in revised manuscript and Extended Data Fig. 2e.

Fig 6K: description in figure legend is unclear: two genotypes are listed but this is not shown in graph. It would be better to show data for both genotypes, treated +/- agonist

Response: Thank you for your advice. We have modified the figure legend to make it more clear. We have added the results for both genotypes treated +/- agonist GSK1016790A. The administration of GSK1016790A significantly reduced the fungal burden to the similar level in WT controls. See more details in revised Fig. 6n and Extended Data Fig. 10h.

Reviewer #3 (Remarks to the Author):

This is an amazing manuscript that reveals how XKR8 orchestrates NET formation and represents a fundamental step change in our understanding. The data and approaches are convincing. I particularly like the KO and KI models which show similar phenotypes.

Many thanks for your positive comments!

I have two comments:
1) It would be good to show the importance of XKR8 in NET formation and inflammation during a model autoimmune disease. This will provide even further justification of the broader importance of this discovery.

Response: Thank you for this insightful suggestion. We agree that assessing whether XKR8-mediated NETs formation contributes to autoimmune disease is of substantial importance. Given the established role of NETs in pathogenesis of collagen antibody-induced arthritis (CAIA), a mouse model of rheumatoid arthritis (PMID: 39143217), we evaluated the contribution of XKR8 in this context. Notably, XKR8 KO mice exhibited markedly attenuated disease compared with WT controls, as reflected by reduced clinical scores and diminished paw thickness (see revised Fig. 5l-n). These results demonstrated that XKR8-mediated NETs formation plays a functional and pathogenic role in autoimmune disease. Please see page 14, line 16-21 of the revised manuscript and Fig. 5l-n for details.

2) minor comment: in figures that indicate lung injury score (eg: fig 5d). Representative histology pictures should be included in the supplemental data.

Response: Thank you for pointing this out. As suggested, we have now added representative histology pictures with the statistics in Fig. 5d and Fig. 5i.

Reviewer #4 (Remarks to the Author):

In this manuscript, the authors investigate the role of the Xkr8 lipid scramblase in the formation of neutrophil extracellular traps (NET). They show that the Xkr8 scramblase is highly expressed in neutrophils and that its activation following processing by caspase 3 leads to PS exposure, which promotes NET formation. They show that activation of Xkr8 alters membrane properties, which results in the activation of various Ca2+ mechanosensitive ion channels. Consistently, they show that inhibiting calcium signaling before lipid scrambling drastically reduces NET formation but that blocking the channels after NET formation does not. The authors also show that NET formation promoted by Xkr8 plays vital roles in neutrophil-induced immune responses in vivo and in resistance to fungal infections.

Overall, this is a very interesting manuscript, the experiments are well designed, executed and interpreted. I only have three concerns that might require some additional experimental work.

Thank you for your supportive comments!

1) The authors show that KO of TMEM16F does not affect PS exposure in neutrophils (Fig. 1). However, they also conclude that the main effect of Xkr8 activation is the activation of Ca2+ channels and a consequent Ca2+ influx (Fig. 4). Further, they show that in Xkr8 KO cells, there is Ca2+ dependent PS exposure during NET formation. The first of these findings appears inconsistent with the other two. Indeed, if Xkr8 activation induces Ca2+ entry via mechanosensitive ion channels and Ca2+-dependent PS exposure is seen during NET formation, then one would expect that TMEM16F or another TMEM16 family member might also play a role in this process.

Response: Thank you for highlighting this important point! We would like to clarify the following: First, loss of TMEM16F did not affect PS exposure during PMA-induced NETs formation, suggesting that Ca^{2+} signals during PMA-induced NETs may not be sufficient to activate TMEM16F (Fig. 1c). Second, XKR8-mediated lipid scrambling functions upstream to activate mechanosensitive Ca^{2+} channels, which are required for the subsequent propagation of NETs, including PAD4 activation. While Ca^{2+} influx downstream of XKR8-mediated lipid scrambling could, in principle, activate TMEM16F, such activation is unlikely to influence XKR8-dependent NETs formation, as the signaling cascade has already progressed beyond this checkpoint. In other words, even if TMEM16F becomes activated following XKR8-mediated lipid scrambling, this would still favor the NETs procession rather than impede it. Third, XKR8 KO neutrophils did not display impaired PS exposure in response to A23187 treatment (see revised Extended Data Fig. 5i).

To further delineate TMEM16F's role, we assessed NETs formation induced by PMA and A23187 in BMNs lacking XKR8, TMEM16F, or both. TMEM16F deficiency had no effect on PMA-induced NETs formation, whereas A23187-induced NETs formation was partially reduced in TMEM16F KO BMNs (see revised Extended Data Fig. 3k). Given that TMEM16F is a Ca^{2+}-activated scramblase and can be activated by A23187, these results indicate that TMEM16F contributes to A23187-induced NETs formation but does not participate in PMA-induced NETs. Importantly, XKR8 and TMEM16F double KO BMNs exhibited NETs defects comparable to those observed in XKR8 KO BMNs (see revised Extended Data Fig. 3k).

Collectively, these data show that XKR8-mediated lipid scrambling primarily functions to activate mechanosensitive Ca^{2+} channels, providing the requisite Ca^{2+} signals to drive the progression of NETs formation. In contrast, TMEM16F-mediated lipid scrambling plays a selective role in A23187-induced NETs formation and plays a minor or negligible role in other NETs induction scenarios examined in this study.

2) A related concern regards the proposed mechanism by which Xkr8 modifies membrane properties to activate multiple mechanosensitive Ca2+ channels. On one hand, I find the pleiotropic effects of Xkr8 very reasonable, since it is reasonable to expect that multiple proteins

will be affected by a change in membrane properties. However, I find two aspects somewhat confusing.
a. Ca2+ signaling transduction pathways are tightly regulated and it is difficult to envision how the effects of such a broad-spectrum activation could be controlled in a cell.
b. I do not understand how the authors can see the effects of specific inhibitors and agonists of these channels. If Xkr8 activation promotes activity of many channels at the same time, then their signals should be additive and blocking/activating one of them at a time should not have major effects. Either the tested compounds have cross-effects among channels, or something else must be affected.

To address these concerns the authors should carry out additional experiments:

i. It is important to show that activation of these channels results in Ca2+ entry into the cells, and determine how the various tested compounds affect this entry. This is especially important since the authors propose that the Ca2+ effects are very long-lasting, up to several hours.

Response: We agree that testing Ca^{2+} entry by these compounds is important. First, we monitored the transient Ca^{2+} influx induced by various Ca^{2+} channel agonists in WT and XKR8 KO BMNs. CBD (TRPV2 agonist), GSK1702934A (TRPC3 agonist), and Yoda1 (Piezo1 agonist) elicited strong, moderate, and mild Ca^{2+} influx, respectively, with comparable responses in WT and XKR8 KO BMNs (revised Extended Data Fig. 8d). In contrast, Probenecid and GSK1016790A did not trigger detectable Ca^{2+} influx within five minutes of treatment (revised Extended Data Fig. 8d).
Next, we evaluated intracellular Ca^{2+} level at two hours post PMA treatment, with or without these agonists, using the Ca^{2+} indicator Indo-1. XKR8 KO BMNs exhibited significantly lower Ca^{2+} levels compared with WT cells (revised Extended Data Fig. 8e). Notably, treatment with mechanosensitive Ca^{2+} channels agonists (CBD, Probenecid, GSK1016790A, Yoda1, and GSK1702934A) restored Ca^{2+} levels in XKR8 KO BMNs to those observed in WT cells (revised Extended Data Fig. 8e), thereby effectively rescuing NETs formation upon PMA stimulation (Fig. 4f). These results indicate that sufficient intracellular Ca^{2+} downstream of XKR8-mediated lipid scrambling is required for NETs formation. Importantly, sustained Ca^{2+} elevation may not be necessary. Once the signaling cascade is initiated, subsequent NETs formation proceeds independently of Ca^{2+}, as evidenced by chelation experiments at three hours post PMA treatment, which failed to inhibit NETs (Fig. 4b).

Furthermore, our data suggest that the multiple Ca^{2+} channels may function in an additive, "digital" rather than "analog" manner. Lipid scrambling mediated by XKR8 likely activates mechanosensitive channels locally, resulting in only partial activation of any single channel type. The engagement of multiple Ca^{2+} channels therefore ensures a robust and reliable Ca^{2+} signal sufficient to drive NETs formation. Please see page 12, line 25-30 and Extended Data Fig. 8d,e in revised manuscript for more details.

ii. Could the authors look at cell/animal strains where some of these channels have been genetically ablated to better identify which channels mediate the Ca2+ responses?
These experiments might also help explain the conundrum regarding the role of TMEM16F in PS exposure during NET formation.

Response: Yes! We performed CRISPR-Cas9-mediated genetic ablation of TRPV2, TRPC3, Piezo1, and TRPV4 in HL-60 cells. After differentiation into neutrophil-like cells using ATRA, we assessed their capacity to undergo NETs formation upon PMA stimulation. Notably, loss of any of these mechanosensitive channels substantially impaired PMA-induced NETs formation compared with parental control cells, without affecting PS externalization, demonstrating that these channels play an essential role in NETs induction (see revised page 13 line 9-11 and Extended Data Fig. 8j,k).

3) The authors show that PS exposure precedes NET formation by several hours. However, the authors do not show whether PS externalization persists during or after NET formation. They assume it does, as they suggest membrane remodeling caused by Xkr8 activation induces Ca2+ entry in cells. However, it is important to directly demonstrate this is the case by measuring PS exposure inside the NETs. Once point to consider is that simply measuring persistence of the AnxV signal after treatment might not be sufficient, since AnxV binds to PS tightly and it could 'trap' it on the outer leaflet rather than being actively kept there by Xkr8.

Response: Thank you for highlighting this important point regarding scramblase biology in the context of NETs formation. First, similar to apoptotic lipid scrambling, activation of XKR8 during NETs formation induces broad phospholipid scrambling, including PS, PE, and SM, and represents a critical early step in the NETosis cascade. However, once this checkpoint is successfully passed, continued XKR8 activity may be no longer required, analogous to apoptotic lipid scrambling after plasma membrane permeabilization. Second, XKR8-mediated lipid scrambling activated by casp-3 is known to be persistent in apoptosis, accompanied with the inhibition of flippase by casp-3 (PMID: 29400998). Third, to directly determine whether PS externalization persists during NETs formation, we performed sequential Annexin V staining. Neutrophils were washed with 1xbinding buffer after Annexin V-FITC staining at 2 hours following PMA stimulation. The washed, Annexin V-FITC-labeled cells were then rested for 20 minutes at 37°C and subsequently subjected to a second round of Annexin V-APC staining. Notably, Annexin V-APC readily labeled the previously Annexin V-FITC$^+$ population, demonstrating that XKR8-mediated lipid scrambling persists during the resting period (see revised Supplementary Fig. 2d). Together, our results demonstrate once activated, XKR8-mediated lipid scrambling is sustained during NETs formation. Although essential for initiating the NETotic program, continued XKR8 activity becomes dispensable once downstream signaling events have progressed beyond this checkpoint. Please see page 9, line 18-21 and Supplementary Fig. 2d in revised manuscript for more details.

Minor
1) Could the authors show representative images of the cells undergoing NET formation in WT and KO lines. This would be helpful visualize the morphological changes that are described in the text?

Response: Sure. We added the representative images of the BMNs undergoing NET formation from WT and XKR8 KO mice. Please see revised Extended Data Fig. 3b.

2) Pg. 4, line 23, "despite ubiquitously expressed" should be "despite being ubiquitously expressed"

Response: Corrected.

3) Pg. 11, line 9 "Thus we intended to figure out how lipid scrambling activates subsequent Ca2+." This seems a broken sentence, unless the authors mean to imply that lipid scrambling activates the Ca2+ ions.

Response: Thanks! We have rewritten the sentence.