Point-by-Point to Responses and Revisions

Revision summary

We are very grateful to the reviewers for their positive and constructive comments on our manuscript. We believe that their valuable suggestions have helped us greatly improve the manuscript. We have updated the manuscript accordingly and provide point-by-point responses to the reviewers' comments below. Note that changes in manuscript are highlighted in blue.

POINT BY POINT RESPONSES TO REVIEWERS:

Reviewer #1

The manuscript by Qu et al. deeply examines the role of neutrophils in endometrial hyperplasia (EH) and endometritis (EN) in human, mouse, and monkey. The novel findings include an estrogen responsive subgroup of neutrophils in EH. Their findings suggest specific mechanisms of action and that aspirin is able to block NET production which decreases EH in a monkey and mouse model. The work will be significant to the field to show the important role of neutrophils in disease development and as a possible therapeutic target. Some concerns about the data need to be addressed:

1. Typically in human uterus studies, the cut off is 44-45 years old. Women over 45 are considered peri-menopausal/menopausal. Many of the participants in this study were over 45. The age of the participants needs to be fully described and also indicated if the women are still cycling. One control sample is not in the same menstrual phase. The neutrophils will change based on cycle stage and should not be grouped with those in the proliferative phase. Some of the data analyses only have participants greater than 45 years of age. We also know that as women age, the immune system responses decreases. Please show if the older women cluster differently than the women who are younger. Indicate if the participants cluster together or independently. Indicate what age participants were used for the humanized mice and the neutrophil studies.

Response: Thank you very much for your valuable questions. The age of menopause in women is between 45 and 55 years old, and all participants involved in our study had regular menstrual cycles. As you mentioned, most of our normal endometrial samples are selected from the proliferative phase.The reasons for selecting endometrial samples from the proliferative phase are as follows: clinically, hysteroscopy is usually performed 3–5 days after the end of menstruation to observe endometrial lesions; therefore, in compliance with medical ethics, we obtained endometrium from the proliferative phase. Only one sample came from the secretory phase, but both this and the others are physiological endometrium rather than pathological endometrium. As suggested, we have presented the cellular distributions for samples from patients aged ≥45 years and <45 years. We observed no obvious differences in cellular distribution or in the proportions of various cell types. In addition, there was no statistically significant difference in age among the three groups (**Fig. R1**). Therefore, although our data include samples from both ≥45-year-old and <45-year-old individuals within the premenopausal population, this does not affect the main conclusions. Although the

normal group contained one secretory-phase sample that exhibited a high neutrophil proportion (**Fig. R2**), this did not affect the overall conclusion that the EH group had a statistically significantly higher neutrophil proportion than the normal group. In addition, to directly address whether older donors cluster differently, we colored the integrated UMAP by donor age. Cells from donors ≥45 and <45 years were intermixed and did not form age-segregated clusters, whereas disease-associated differences were evident (**Fig. R3**), indicating that the observed cellular heterogeneity is driven by disease state rather than by donor age.

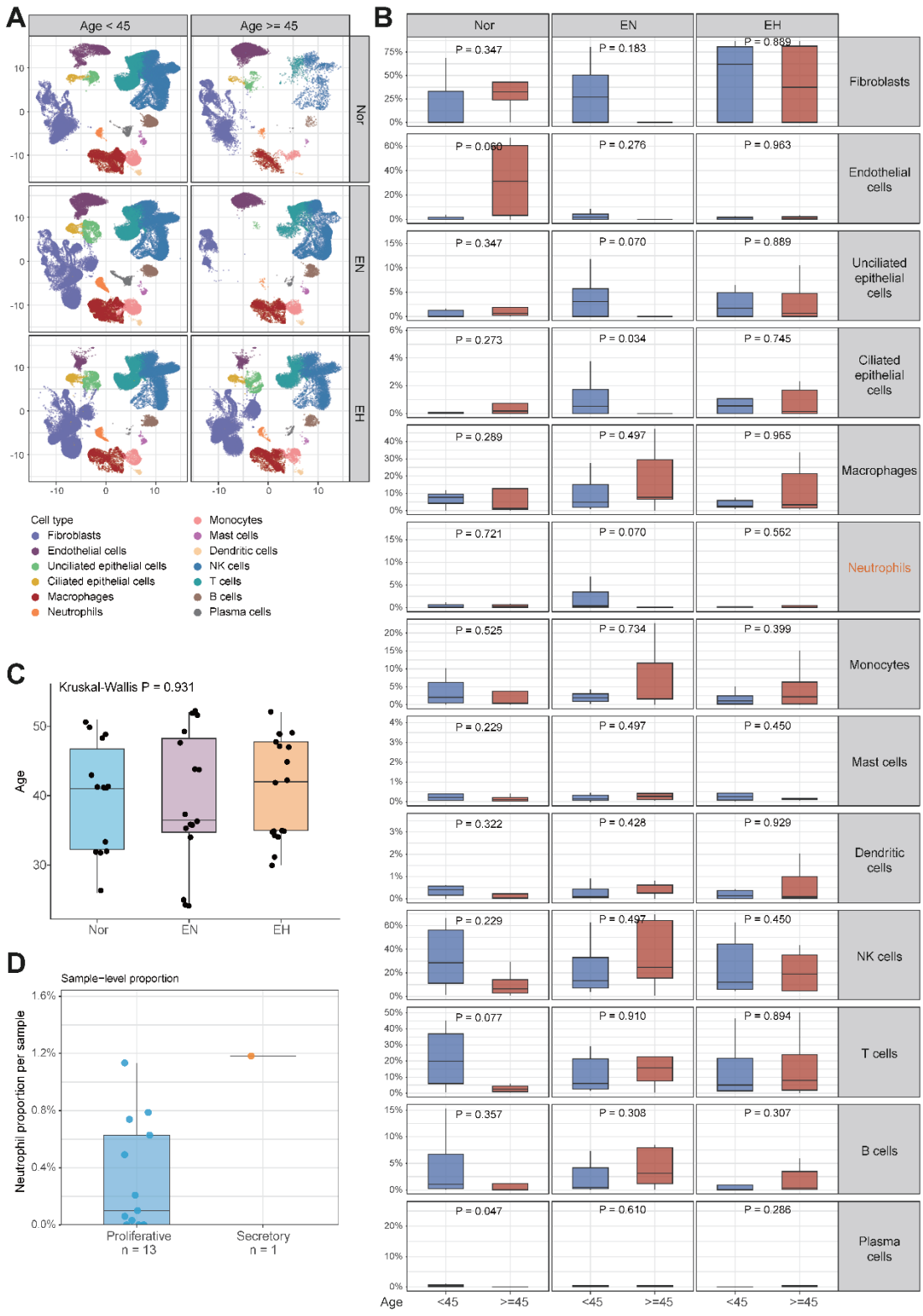


Fig. R1. Cell composition and neutrophil abundance across age strata and sample groups.

(A) UMAP plots showing the distribution of major cell types in samples from individuals aged <45 years and >=45 years across the Nor, EN, and EH groups. Each dot represents a single cell, and colors indicate annotated cell types, including fibroblasts, endothelial cells, unciliated epithelial cells, ciliated epithelial cells, macrophages, neutrophils, monocytes, mast cells, dendritic cells, natural killer cells, T cells, B cells, and plasma cells. **(B)** Boxplots comparing the sample-level proportions of each major cell type between the <45-year and >=45-year groups within Nor, EN,

and EH. P values are shown above each comparison. **(C)** Age distribution across the Nor, EN, and EH groups. The Kruskal-Wallis test showed no significant difference in age distribution among the three groups ($P = 0.931$). **(D)** Sample-level neutrophil proportions in proliferative and secretory endometrial samples. Points represent individual samples, and boxplots summarize the distribution within each phase.

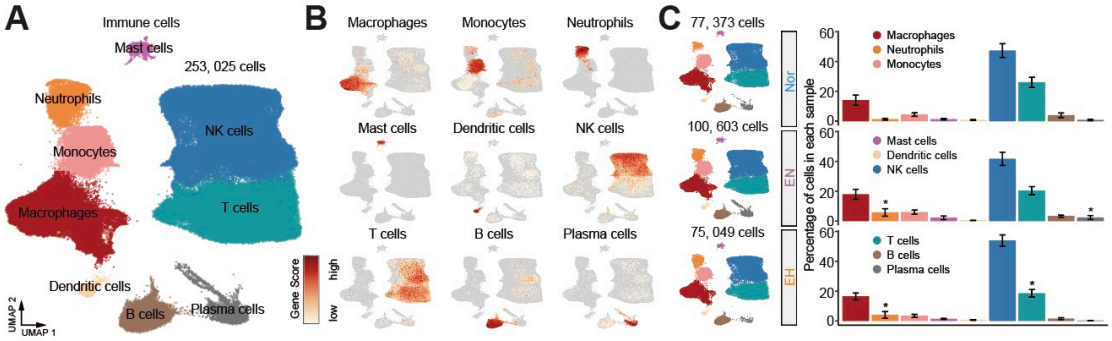


Fig. R2. The proportion of neutrophils is higher in the tissues of patients with EH.

(A) UMAP plot showing the subtypes of immune cells, colored by subtypes. **(B)** Feature plots showing the gene score of selected cluster-specific gene signatures. Cells with the highest gene score are colored red. **(C)** UMAP plots displaying the distribution of cells in different groups as colored by immune cell types (left) and bar plots showing the cell type abundance for different groups (right). The *p*-value was calculated using the Wilcoxon rank sum test. * $p < 0.05$.

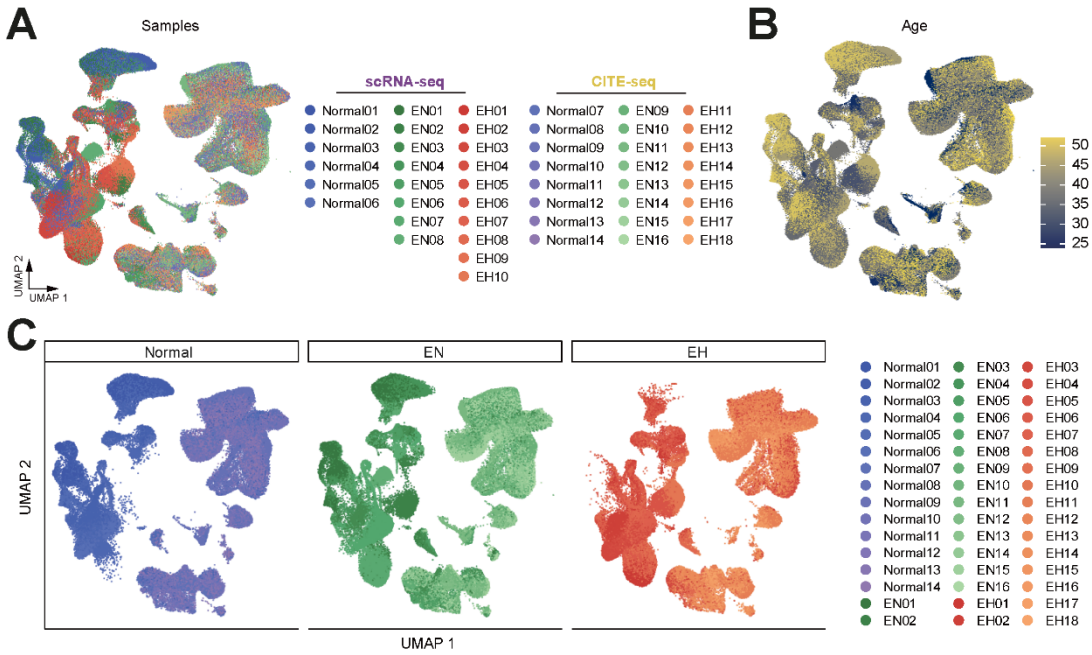


Fig. R3. Single-cell transcriptomic landscape of normal, EN, and EH tissues.

(A) UMAP embedding of integrated scRNA-seq and CITE-seq data. Cells are colored by individual sample IDs. The left panel shows samples from scRNA-seq, and the right panel shows samples from CITE-seq. **(B)** UMAP embedding of the same cells colored by age. The color gradient ranges from 25 (dark blue) to 50 (yellow). **(C)** UMAP embeddings split by disease status: Normal (left), EN (middle), and EH (right). Cells within each panel are colored by individual sample IDs as indicated in the legend on the right.

In this study, the method for constructing neutrophil-humanized mice is as follows: Immunodeficient NOD/ShiLtJGpt-Prkdcem26Cd52Il2rgem26Cd22 (NCG,

GemPharmatech Co. Ltd., T001475) mice were used for creation of human immune system (HIS) mice as previously described (STAR Protoc 3, 101217 (2022)). Briefly, we used CD34 Microbead Kit to purify human $CD34^+$ cells from fetal liver under clinical ethical approval from Drum Tower Hospital with informed consent (protocol# 2021-488-01). NCG pups within 7 days of birth were sub-lethally irradiated and engrafted with $CD34^+$ cells by intrahepatic injection. We used 8-week-old female mice for subsequent experiments.

2.The mouse studies were not cycle staged and euthanized in any particular cycle stage. The stage of cyclicity is important when comparing uterine phenotypes and infiltration of neutrophil subpopulations. Please address if ESR1 specific neutrophils were given to the mice or if a mixture was used. Please indicate the age of the participants and if you would think the neutrophils would act differently if they were neutrophils from EN or EH. The dose of estrogen used may inhibit cycling and so likely would the anti-Ly6G as the neutrophils are very important for estrous in the mouse. Please address the cyclicity in the mice that were not given estrogen (as the estrogen would likely stop their cycles and that would possibly be constant).

Response: Thank you for your insightful question. We will answer your questions one by one.

(1) The mouse studies were not cycle staged and euthanized in any particular cycle stage. The stage of cyclicity is important when comparing uterine phenotypes and infiltration of neutrophil subpopulations.

Clinically, EH is caused by long-term high-level estrogen stimulation combined with relatively low progesterone. In this study we established an estrogen-induced EH model based on published references (Adv Sci (Weinh). 2024 Nov;11(41):e2403038; Am J Pathol. 2010 Mar;176(3):1443-51). To ensure a uniform starting point, before the formal experiment we synchronized the mice by strictly screening estrous stage using vaginal cytology, and only mice in proestrus were enrolled for estrogen induction, because at this stage the endometrium is in a physiological proliferative state with the most sensitive and consistent response to estrogen. Continuous estradiol administration then maintains the uterus in a prolonged “persistent proliferation”-like state. We apologize that the Methods did not describe this synchronization in sufficient detail and have now added it.

(2) Please address if ESR1 specific neutrophils were given to the mice or if a mixture was used.

We did not administer any exogenous neutrophils to the mice. *Esr1*$^{fl/fl}$ or *S100a8*^{Cre}-*Esr1*$^{fl/fl}$ mice were given a daily subcutaneous injection of 50 μg/kg estradiol for 21 days to to establish a mouse model of EH.

(3) Please indicate the age of the participants and if you would think the neutrophils would act differently if they were neutrophils from EN or EH.

We have fully supplemented the patient information involved in the sequencing, including age. Based on current knowledge, neutrophils arising from different disease

backgrounds may differ functionally. We note that in this study we did not isolate neutrophils directly from patient endometrial tissue; the human-cell functional assays used peripheral-blood neutrophils, and the disease-specific behavior was interrogated through the single-cell atlas and the genetic mouse models.

(4) The dose of estrogen used may inhibit cycling and so likely would the anti-Ly6G as the neutrophils are very important for estrous in the mouse. Please address the cyclicity in the mice that were not given estrogen (as the estrogen would likely stop their cycles and that would possibly be constant).

Continuous administration of estrogen indeed suppresses the estrous cycle, keeping the mice in a constant estrogen-dominated state (mimicking EH). This is exactly what our model requires to simulate the persistent estrogen exposure seen in patients with EH. More importantly, the main focus of this study is to investigate the impact of neutrophil depletion on EH rather than on the estrous cycle. We compared the 'estrogen + anti-Ly6G' group with the 'estrogen + anti-Ly6G +Elacestrant' group – both groups received estrogen treatment, which by itself already suppresses the estrous cycle. Therefore, even if anti-Ly6G has a minor effect on the cycle, this effect is balanced between the two arms and does not interfere with the intergroup comparison. It is worth mentioning that the evaluation criteria for EH include uterine appearance, uterine weight, gland-to-stroma ratio, etc., rather than the estrous cycle.

3. CD66b is a marker for granulocytes. Most will be neutrophils, but the marker will also include eosinophils in the mixture. Were eosinophils subtracted from the neutrophil population to ensure purity.

Response: We thank the reviewer for this important comment. We fully acknowledge that CD66b is a pan-granulocyte marker expressed on both neutrophils and eosinophils. In our study, we did not perform additional exclusion of eosinophils from the $CD66b^+$ population. However, we would like to clarify the following points: although CD66b is indeed expressed on eosinophils, it is classically used as a neutrophil marker given that approximately 95% of $CD66b^+$ cells in peripheral blood are neutrophils. In the endometrial tissue, eosinophils represent an extremely rare population compared with neutrophils, further minimizing the potential impact of eosinophil contamination on our analysis. We have carefully re-examined our scRNA-seq data and also extensively consulted published single-cell transcriptomic datasets of human endometrial tissue from other groups (*Nat Med. 2020 Oct;26(10):1644-1653*; *Genome Med. 2026 Apr 7;18(1):68*; Nat Commun. 2022 Oct 22;13(1):6300). Indeed, we confirmed that eosinophils are extremely scarce in endometrial tissue. Therefore, we believe that this does not materially affect our major conclusions.

4. Unpaired two-tailed Student's t tests are not the appropriate statistical tests to run. There are three groups; therefore, one-way ANOVA should be run. The comparisons need to be made across all groups and statistics shown comparing each group to each group.

Response: Thank you for pointing out our mistake. Statistical analyses were performed

by using GraphPad Prism 9.0. Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to evaluate statistical significance for group comparisons. Differences at $*p < 0.05$ and $**p < 0.01$ were accepted as statistically significant. We have added the relevant description in the Statistical analysis section of the Methods.

5. For identification of neutrophil subsets, please explain why a resolution of 0.2 was used for clustering.

Response: Thank you for this important comment. To determine an appropriate clustering resolution for neutrophil subtyping, we systematically evaluated a range of resolutions from 0.1 to 0.4 rather than selecting the parameter arbitrarily. As shown in the newly added analysis (**Fig. R4A**), the clustering tree demonstrated that at lower resolutions (0.1–0.15), several transcriptionally distinct neutrophil populations were merged, whereas increasing the resolution beyond 0.2 resulted in further subdivision of existing clusters without introducing clearly distinct transcriptional states. At a resolution of 0.2, five neutrophil subsets were resolved and their cluster identities remained largely stable across neighboring resolutions. We further evaluated the quality and distinctiveness of these five clusters. First, all five neutrophil subsets showed consistently high ROGUE scores, indicating high within-cluster transcriptional purity (**Fig. R4B**). Second, comparison of the top 100 marker genes among the five subsets showed very limited overlap between clusters, with only 0–16 shared marker genes between any pair of subsets and low pairwise Jaccard similarities ($\leqslant 0.09$) (**Fig. R4C**).

These results indicate that the five clusters identified at resolution 0.2 represent transcriptionally distinct and internally homogeneous neutrophil states rather than artificial subdivisions caused by over-clustering. Therefore, we selected a resolution of 0.2 as a balance between preserving biologically meaningful neutrophil heterogeneity and avoiding excessive fragmentation of closely related cell states.

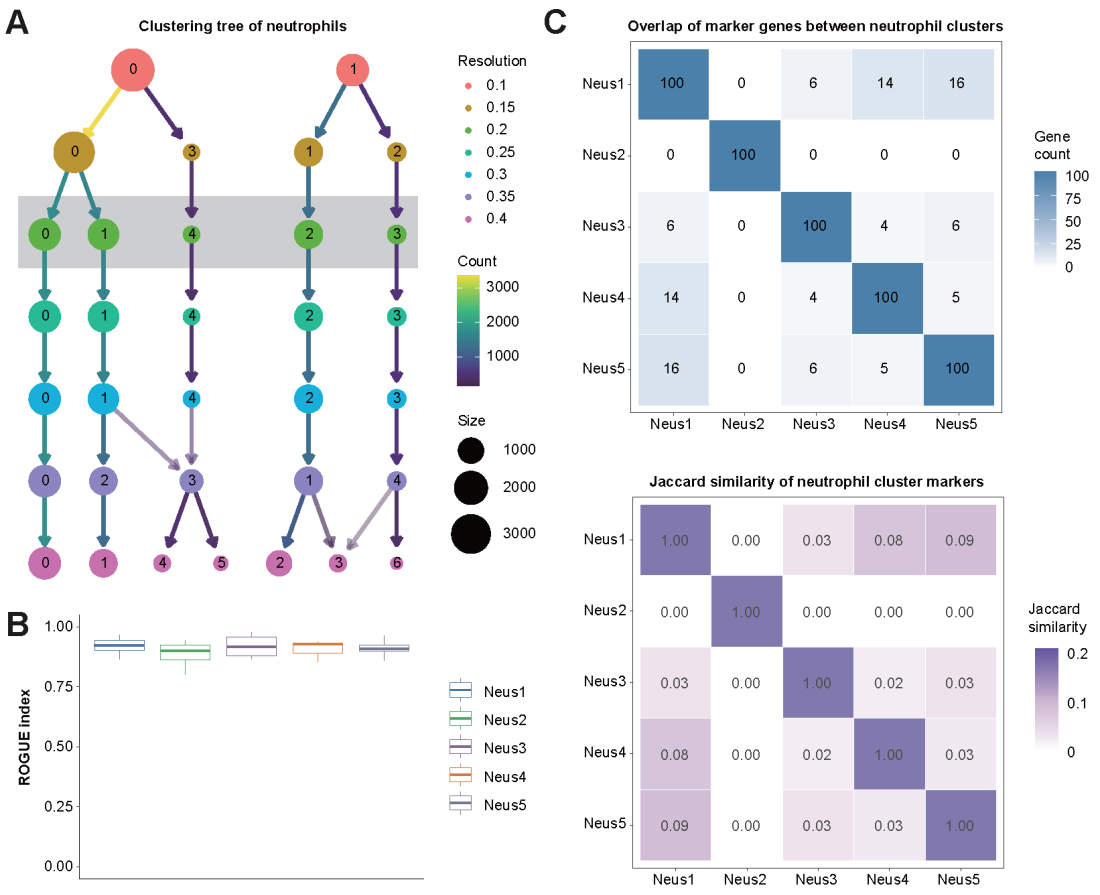


Fig. R4. Selection and validation of neutrophil subset clustering resolution.

(A) Clustering tree of neutrophils across resolutions from 0.1 to 0.4. Node size and color indicate the number of cells in each cluster, and edges show the relationships of clusters between adjacent resolutions. The shaded region highlights the resolution of 0.2, at which five neutrophil subsets were identified; this structure remained stable across the neighboring resolutions of 0.25 and 0.3. **(B)** ROGUE index values for the five neutrophil subsets identified at resolution 0.2, showing high within-cluster transcriptional homogeneity. **(C)** Heatmaps showing pairwise overlap of marker genes among the five neutrophil clusters. The upper heatmap shows the number of overlapping marker genes between cluster pairs, and the lower heatmap shows the corresponding Jaccard similarity. Limited marker-gene overlap and low Jaccard similarity values support the transcriptional distinctiveness of the five neutrophil subsets.

6. Please indicate why 50 nM E2 was used when 10 nM is typical for cell culture studies.

Response: The concentration of estrogen administration varies depending on the cell type and treatment time point. We referred to a previously published paper (Front Immunol. 2016 Dec 5;7:565), in which estrogen at 20 ng/mL (approximately 73.4 nM) was shown to induce NET formation. Based on this concentration range, we also used different concentrations of estrogen to induce NET formation and found that 50 nM estradiol had the most pronounced effect at 2 hours (**Fig. R5**).

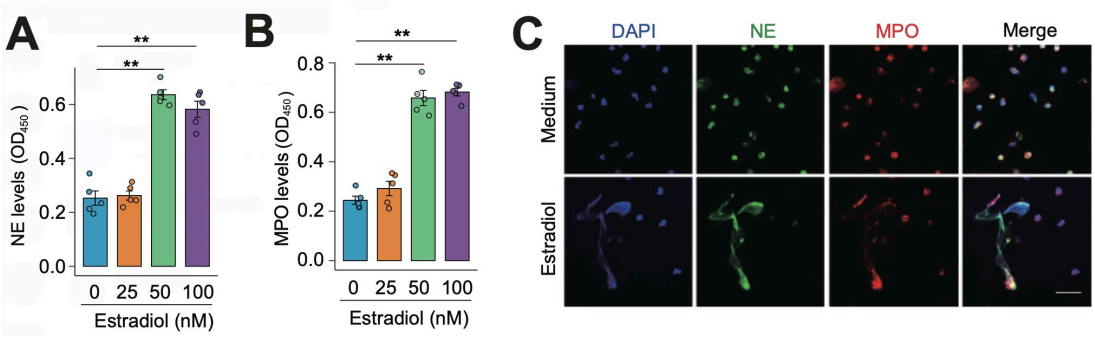


Fig. R5. Estrogen induces NETs formation *in vitro*.

(A-B) Purified human neutrophils were incubated with 25-100 nM estradiol at 37℃ for 2 h. The OD_{450} value reflects the levels of myeloperoxidase (MPO) or neutrophil elastase (NE). (n=5). ***P*<0.01. **(C)** Purified human neutrophils were incubated with 50 nM estradiol at 37℃ for 2 h. Cells were stained with NE (green), MPO (red) and DAPI (blue). Scale bar: 40 μm.

7. In figure 1, it is unclear why only a few cells were examined for cell communication and the communication was not shown as bidirectional signaling. For example, CellChat would allow for full communication of the cells in the uterus and the immune system.

Response: Thank you very much for your suggestion. Based on your advice, we comprehensively analyzed the interactions between immune cells and non-immune cells (**Fig. R6**). Since endometrial epithelial cells are the primary effector cells in endometrial hyperplastic diseases, we focused on the interactions between immune cells and endometrial epithelial cells. We noticed that the interactions of macrophages and neutrophils with Unciliated epithelial cells were significantly enhanced especially neutrophils (**Fig. R7**). Therefore, we believe that neutrophils are more worthy of further investigation

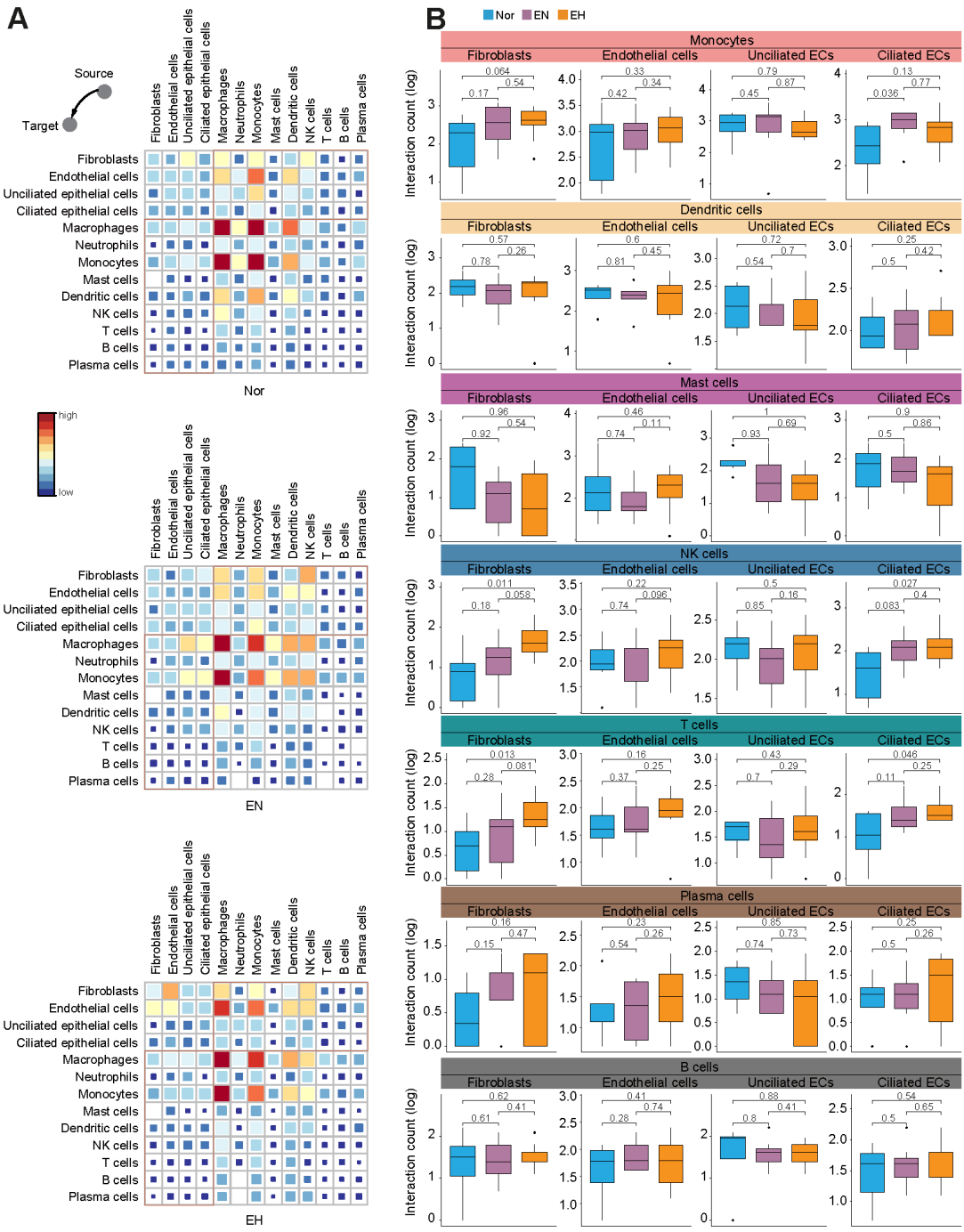


Fig. R6. Dynamics of cell-cell interaction networks in each group.

(A) A Heatmap illustrating the cell interaction patterns in Nor, EN, and EH samples. **(B)** Boxplots showing the number of predicted cell–cell interactions directed from individual immune cell populations to non-immune cell populations across Nor, EN, and EH groups. The *p*-value is calculated using the Wilcoxon rank sum test.

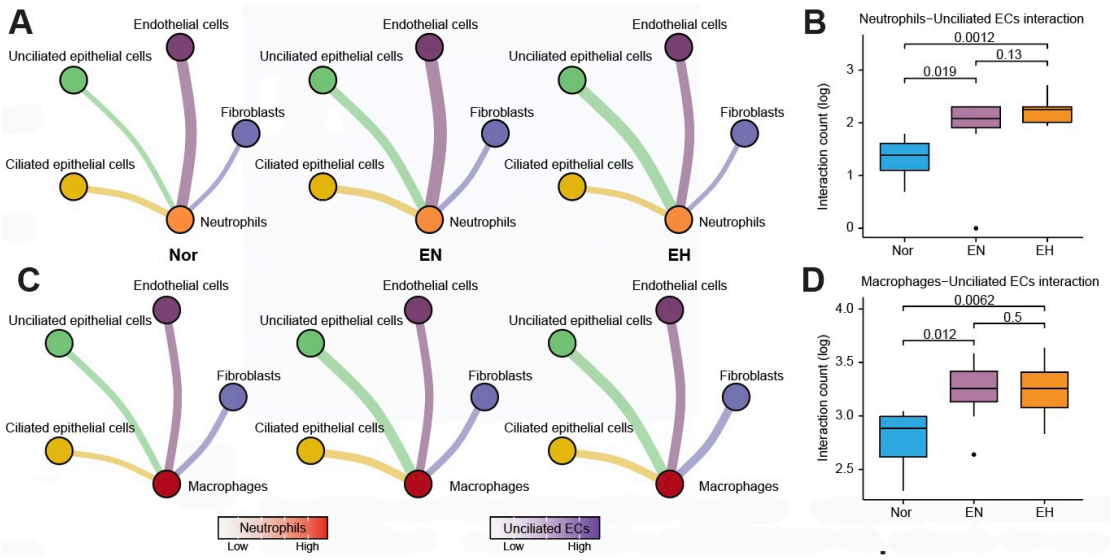


Fig. R7. Dynamics of cell-cell interaction networks in each group.

(A) A Heatmap illustrating the cell interaction patterns in Nor, EN, and EH samples. **(B)** Boxplot indicating cell interaction counts between immune cells and non-immune cells respectively. The *p*-value is calculated using the Wilcoxon rank sum test.

8. Figure 2, what do the arrows represent? in the E2 treated cKO, there is a lot of myometrium present in the image, show less myometrium as the focus is on glands and endometrium. The solvent cKO appears to have far more glands than shown in the graph—update to show true representation. The cycling and cycle stage will also be important as the gland numbers are different depending on cycle stage.

Response: We sincerely thank the reviewer for these careful and constructive comments regarding the histological presentation and the potential influence of the estrous cycle. First, we apologize that the meaning of the arrows was not sufficiently described in the original manuscript. The arrows indicate hyperplastic endometrial glands, and this information has now been clarified in the revised figure legend. Images at different magnifications are now provided to better illustrate both the overall uterine architecture and detailed histological changes (**Fig. R8**). The higher-magnification views were selected to focus primarily on the endometrium and endometrial glands while minimizing the proportion of myometrium.

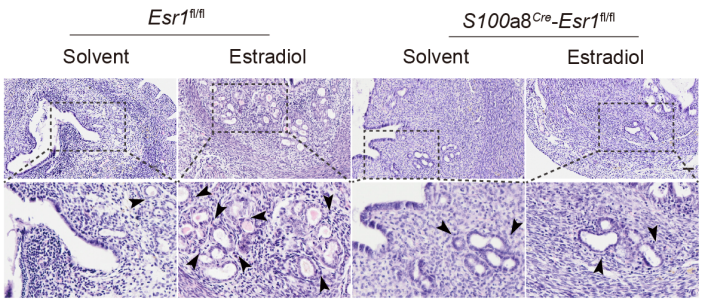


Fig. R8. *Esr1*$^{+}$ neutrophils are main pathogenic cells in the development of EH.

Representative images of H&E staining (n=4 or 5, scale bar, 50 μm). The arrows indicate hyperplastic endometrial

glands.

Before the formal experiment, we strictly screened the mice. Mice in the proestrus stage were selected for estrogen-induced EH, because at this stage the endometrium is already in a physiological proliferative state, exhibiting the most sensitive and consistent response to estrogen signals, thereby providing a uniform starting point for subsequent induction. This method make the uterus in a prolonged "persistent proliferation"-like state. Continuous administration of estrogen indeed suppresses the estrous cycle, keeping the mice in a constant estrogen-dominated state (mimicking EH). This is exactly what our model requires to simulate the persistent estrogen exposure seen in patients with EH. More importantly, the main focus of this study is to investigate the impact of neutrophil depletion on EH rather than on the estrous cycle.

9. In Figure 5, indicate if cycling is restored by the treatment. Indicate if the cycling is misregulated in these mice.

Response: Thank you for your insightful question. Continuous administration of estrogen indeed suppresses the estrous cycle, keeping the mice in a constant estrogen-dominated state (mimicking EH). This is exactly what our model requires to simulate the persistent estrogen exposure seen in patients with EH. In Figure 5, we aimed to explore the role of NETs in EH rather than on the estrous cycle. We compared the 'estrogen' group with the 'estrogen + GSK484' group – both groups received estrogen treatment, which by itself already suppresses the estrous cycle. It is worth mentioning that the evaluation criteria for EH include uterine appearance, uterine weight, gland-to-stroma ratio, etc., rather than the estrous cycle.

10. Figure 6, ameliorates is a strong word to use for the title of the figure.

Response: We agree with the reviewer that the word “ameliorates” may be too strong for a figure title. Accordingly, we have revised the title of Figure 6 to use a more neutral term. The original title, “Aspirin ameliorates EH in cynomolgus monkey through inhibiting NETs via HMGN2 acetylation,” has been changed to “Aspirin attenuates EH in cynomolgus monkey through inhibiting NETs via HMGN2 acetylation.” We believe this modification better reflects the experimental data and avoids overstatement.

11. Methods—mice need to be named as their scientific names and crosses.

Response: Following your advice, we have described this in detail in the Methods section.

12. In S2, none of the markers are picking up neutrophils. CD14 should pick up a large population of neutrophils, but it is not. It is unclear if the samples in C cluster as they do are due to age or due to disease state. The coloring used for normal in scRNA seq and CITE-seq are difficult to follow. Could purples/blues be used for normal, greens for EN, and reds/orange for EH? This would help address the location based on disease more clearly. The other part to help would also be the age of the donor, but also cycle stage.

Response: Regarding the concern that CD14 did not label the neutrophil population: CD14 is predominantly expressed by monocytes and macrophages, where it functions as the LPS co-receptor, whereas human neutrophils are characteristically CD14-low/negative. The absence of CD14 signal on the neutrophil cluster is therefore expected and does not indicate a failure to capture neutrophils. In our CITE-seq panel, neutrophils were reliably identified at the protein level by CD15, CD16 (FCGR3B) and CD66b, and were further confirmed at the transcriptomic level by canonical markers including *FCGR3B*, *CSF3R* and *S100A8/S100A9* (**Fig. R9**). To distinguish whether the clustering in the original panel reflected donor age or disease state, we now display the integrated embedding colored by donor age and split by disease status (**Fig. R3**); cells did not segregate according to age, whereas disease-associated shifts were apparent, indicating that the clustering is driven by disease rather than by donor age. As suggested, we have redrawn the relevant figures to eliminate ambiguity.

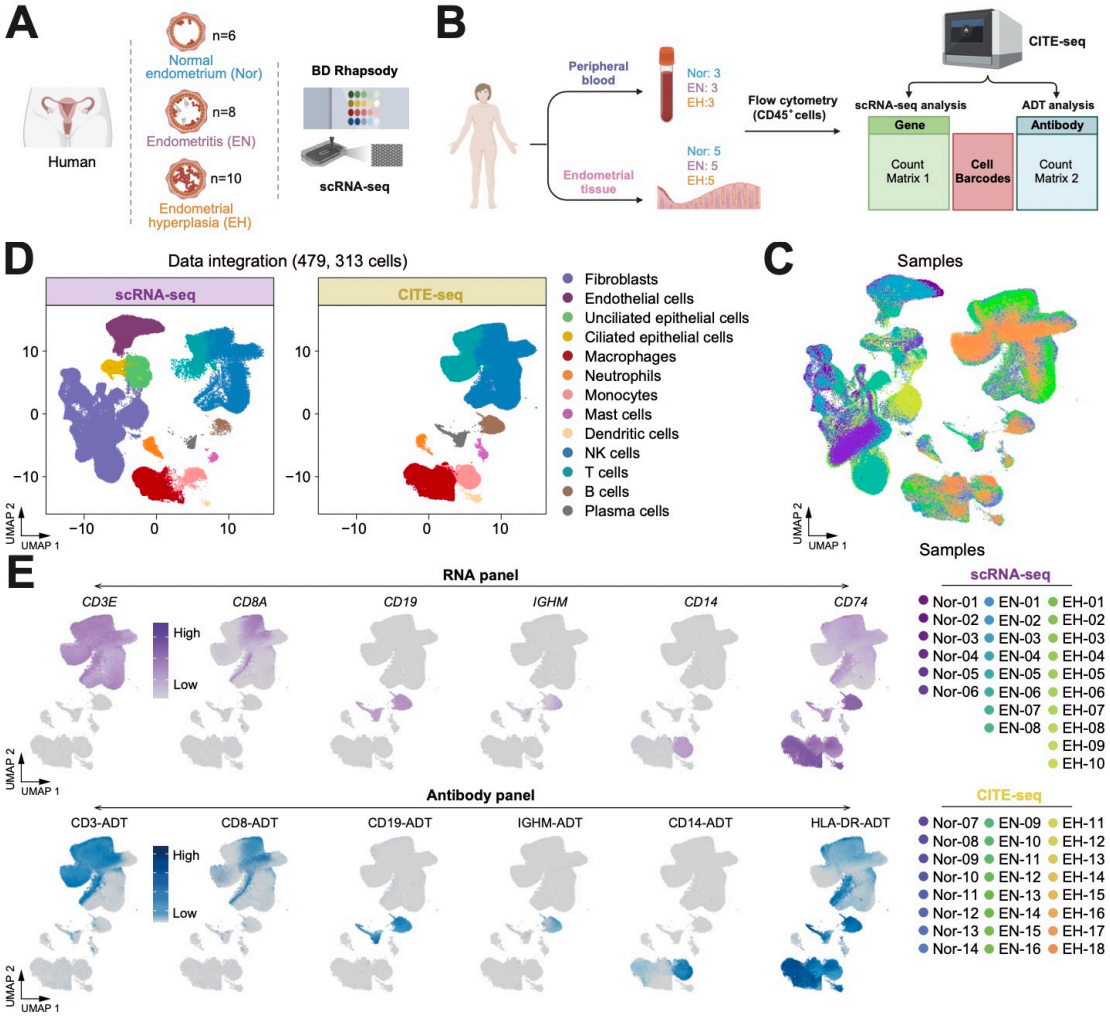


Fig. R9. CITE-seq assisted cell type identification. Related to Figure 1.

(A) Workflow for sample collection and scRNA-seq base on BD Rhapsody. **(B)** Workflow for sample collection and CITE-seq. **(C)** UMAP plot showing the distribution of samples. **(D)** UMAP plot showing the distribution of cell types by datasets. **(E)** Normalized expression of selected mRNAs and surface proteins highlighted on the UMAP space from **(D)**. Top: expression of mRNAs encoding surface markers widely used to identify main cell types.

Bottom: expression of the corresponding surface proteins.

13. Please indicate why CD15 was used as the IF marker in S3 instead of CD66b used for the flow cytometry. CD15 might not be the best marker to capture neutrophil presence.

Response: Thank you for your suggestion. We performed CD66b staining on endometrial tissue. We found that the proportion of $CD66b^+$ cell in EH tissue was higher than that in the normal group **(Fig. R10)**.

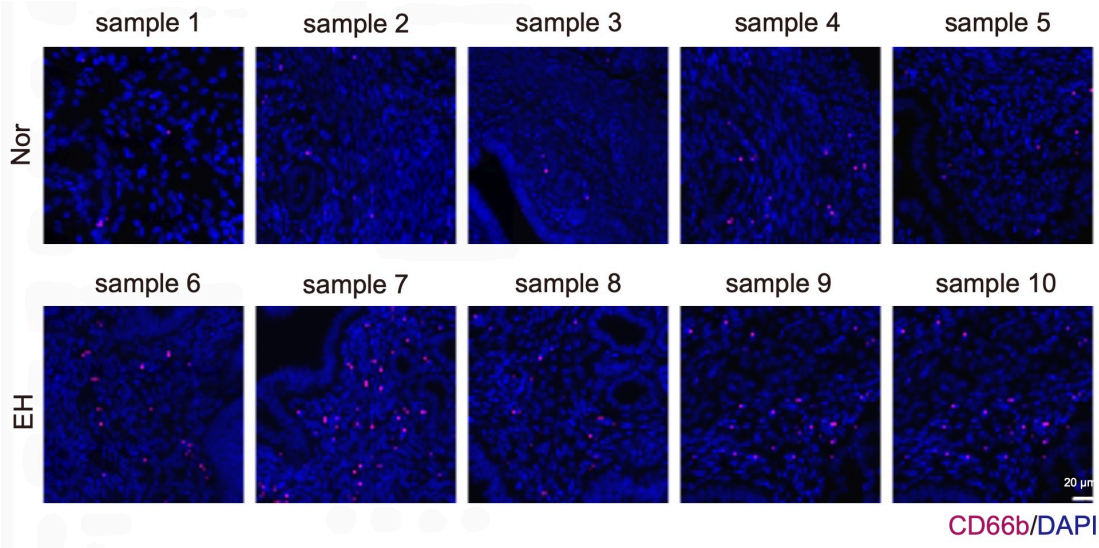


Fig. R10. The proportion of neutrophils increases in EH.

Human endometrial tissues were stained with CD66b (Red) and DAPI (blue). Scale bar: 20 μm.

14. Discussion: The discussion focuses on EH and not EN. The EN needs to be included in the discussion. The discussion is also a rehashing of the results and needs to be written with the intent to be a discussion of what the data could mean or do mean translationally. For line 348, KEGG analysis of the pathway to show what was enriched would be helpful. For line 354, why were other subsets not examined? Even if the proportions were not different, the gene expression could be very different and show significant changes taking place. Like 385, NETS serve as a bridge is a very important discussion point and this area should be expanded as to why it would be important—what could it mean for disease, drug targeting, treatments?

Response: Thank you for these valuable suggestions. Thank you for these valuable suggestions. We have substantially revised the Discussion. Specifically: (i) we added a dedicated paragraph on EN, contrasting the TGM2+ (Neus2), infection-associated, TLR4-linked neutrophil program of EN with the $ESR1^+$ (Neus4), estrogen-driven, HMGN2-dependent program of EH, and discussing what the sterile-vs-infectious distinction means for differential diagnosis and treatment selection. (ii) For the line-348 point, we now include the KEGG enrichment result and name the specific enriched pathways rather than referring to them generically. (iii) For the line-354 point, we clarify that we prioritized $ESR1^+$ neutrophils because the proportions of the other subsets did not change significantly, but we now explicitly acknowledge that their gene

expression could still differ, present this as a limitation, and note it as a direction for future work. (iv) We expanded the 'NETs as a bridge' point into a translational paragraph discussing the ERα–HMGN2–NET–NKCC1 axis as a non-hormonal drug-targeting opportunity, the rationale for repurposing aspirin, and the implications for EH and other estrogen-driven disorders. The revised text is rewritten to interpret the data translationally rather than restate the Results. Please check it in the revised manuscript. Thank you.

Minor comments.

15. Line 185 states that no murine neutrophils are present—the graphs show differently.

Response: Thank you for your suggestion. We have corrected the previous inaccurate wording.

16. Line 237, need an s on neutrophil

Response: Thank you very much for your valuable questions. We have corrected the language errors.

17. Fig S22, the title is awkward.

Response: Thank you for your suggestion. We have revised the title to 'Aspirin binds to HMGN2 protein'.

18. Please check final documents for English and word choices.

Response: Thank you very much for your valuable questions. We have corrected the language errors throughout the manuscript.

Reviewer #2

The hypothesis of this study is that a neutrophil subpopulation expressing Estrogen Receptor 1 (ESR1) promotes hyperplastic endometrium (EH) development; and that this occurs via neutrophil extracellular trap (NET) binding to the Sodium-Potassium-Chloride Cotransporter 1 (NKCC1), resulting in an imbalance of ion homeostasis, endometrial epithelial proliferation, and EH.

The experimental approach includes 8 models for analysis:
1. human endometrial tissues representing normal, endometrial hyperplasia (EH), and endometritis (EN) from donor women.
2. NOD/ShiLtJGpt Prkdcem26Cd52Il2rgem26Cd22 mice to create human immune system (HIS) mice (Fig. 2)
3. neutrophil cell specific Esr1 knockout mice (Figs. 2 & 3)
4. Hmgn2 knockout mice (Fig. 3)
5. Neutrophil-specific Pad4 knockout mice (Fig. 4)
6. epithelial cell-specific Slc12a2 (NKCC1 protein) knockout mice (Fig. 5)
7. female cynomolgus monkeys (Fig. 6)
8. Timm8b knockout mice (Fig. S13)

Overall, the presentation is clear, and the study provides interesting findings on the role of chronic estrogen treatment on NET formation and putative mechanisms that promote EH and EN development.

Key novel findings include: i) the identification of ESR1+ neutrophils in EH tissue, ii) estradiol (E2)-mediated NETs are promoted by ERα binding to the HMGN2 promoter region, iii) NETs bind to NKCC1 to cause an ion imbalance and promote endometrial cell proliferation and EH, and iv) aspirin appears to inhibit NET formation and modestly inhibit the number of estradiol-induced hyperplasic glands.
However, there are a number of general concerns about the robustness and quality of the data that dampen the enthusiasm of this study. These concerns are outlined below.

1. The American Institute of Aging indicates that American women begin the transition to menopause between 45 and 55 years of age, with the average age of natural menopause at 52 years of age. In contrast, a Chinese study defines early menopause as occurring between 40 to 45 years of age, and natural menopause occurring between 48 to 50 years of age. While the current study collected donor samples from women between the ages of 25 to 54, approximately 50% percent of women were 45 to 54 years old, placing them well into the transition to, or already at, natural menopause. Therefore, a concern is that variables such as the stage of menopause, estrogen levels, etc., were not included in this study. It is unclear on how these variables impact the multiomics analysis as well as uterine histopathology, the function of ESR1+ neutrophils, NET production, and putative pathways that promote EH and/or EN.

Response: Thank you very much for your valuable questions. The age of menopause in women is between 45 and 55 years old, and all participants involved in our study had

regular menstrual cycles. As you mentioned, most of our normal endometrial samples are selected from the proliferative phase.The reasons for selecting endometrial samples from the proliferative phase are as follows: clinically, hysteroscopy is usually performed 3–5 days after the end of menstruation to observe endometrial lesions; therefore, in compliance with medical ethics, we obtained endometrium from the proliferative phase. Only one sample came from the secretory phase, but both this and the others are physiological endometrium rather than pathological endometrium. As suggested, we have presented the cellular distributions for samples from patients aged ≥45 years and <45 years. We observed no obvious differences in cellular distribution or in the proportions of various cell types. In addition, there was no statistically significant difference in age among the three groups **(Fig. R11)**. Therefore, although our data include samples from both ≥45-year-old and <45-year-old individuals within the premenopausal population, this does not affect the main conclusions.

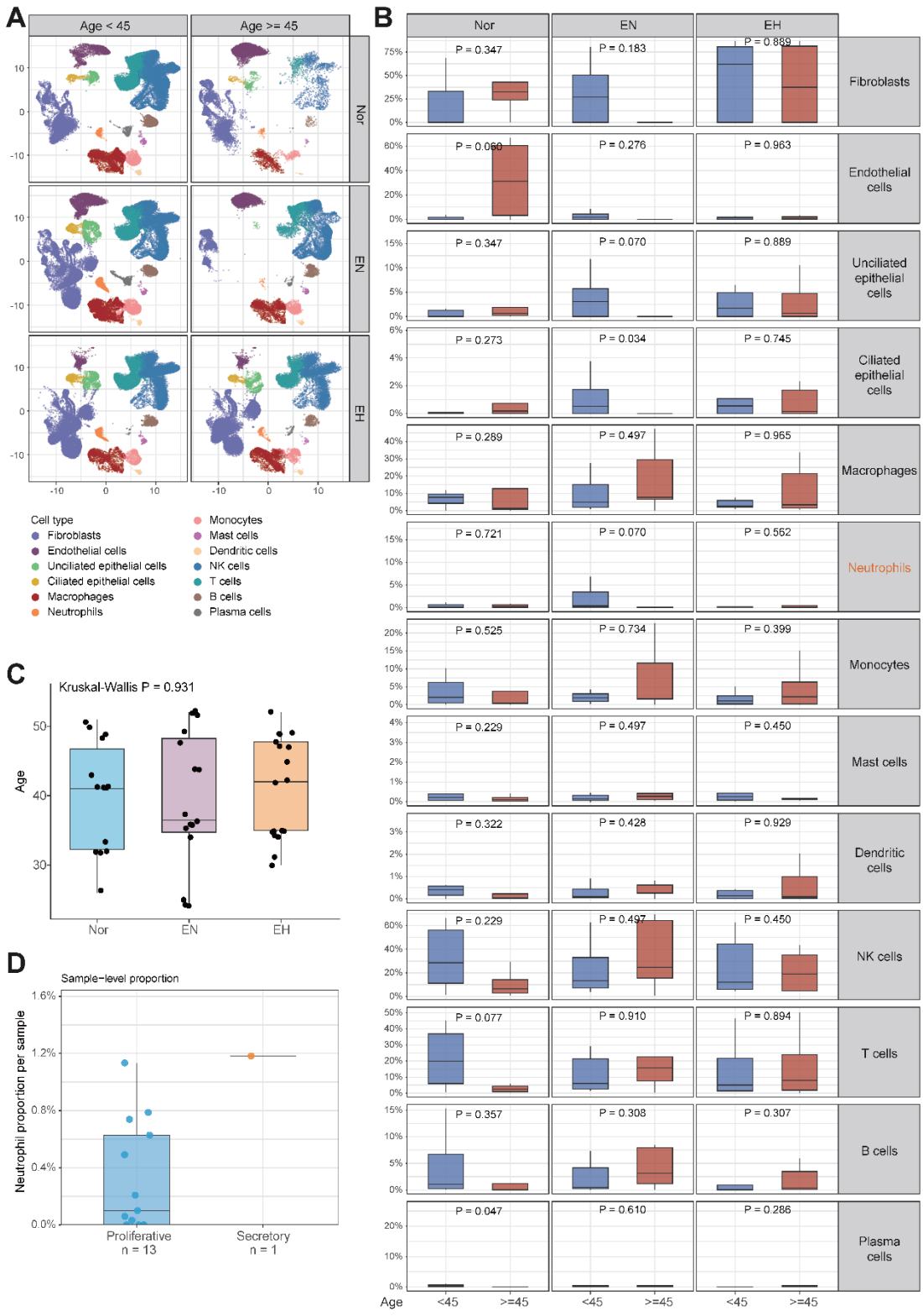


Fig. R11. Cell composition and neutrophil abundance across age strata and sample groups.

(A) UMAP plots showing the distribution of major cell types in samples from individuals aged <45 years and >=45 years across the Nor, EN, and EH groups. Each dot represents a single cell, and colors indicate annotated cell types, including fibroblasts, endothelial cells, unciliated epithelial cells, ciliated epithelial cells, macrophages, neutrophils, monocytes, mast cells, dendritic cells, natural killer cells, T cells, B cells, and plasma cells. **(B)** Boxplots comparing the sample-level proportions of each major cell type between the <45-year and >=45-year groups within Nor, EN,

and EH. P values are shown above each comparison. **(C)** Age distribution across the Nor, EN, and EH groups. The Kruskal-Wallis test showed no significant difference in age distribution among the three groups (P = 0.931). **(D)** Sample-level neutrophil proportions in proliferative and secretory endometrial samples. Points represent individual samples, and boxplots summarize the distribution within each phase.

2. The single cell multiomics analysis provides new information on the different blood cell subtypes in EH and EN compared to normal endometrial tissues; and identifies ESR1^{+} neutrophils as a new neutrophil subtype following "chronic" estrogen treatment. However, the overall analysis of the data in many figures is not always robust or rigorous.

Using Fig. 1F as a general example of a lack of rigor in data analysis:

a. Neutrophil:unciliated cell interactions were compared, however the arrow heads showing the direction(s) of the interactions were not included. These interactions could be bidirectional, with one interaction being dominant over the other. (Please note that the standard nomenclature in the literature is "non-ciliated", not unciliated.)

Response: Thank you very much for your suggestion. We have corrected the incorrect writing. Network diagrams showing directed interactions from neutrophils or macrophages (immune cells) to non-immune cells, including endothelial cells, unciliated epithelial cells, ciliated epithelial cells, and fibroblasts, in Nor, EN, and EH samples (**Fig. R12**). We have revised the figure legend to reduce ambiguity.

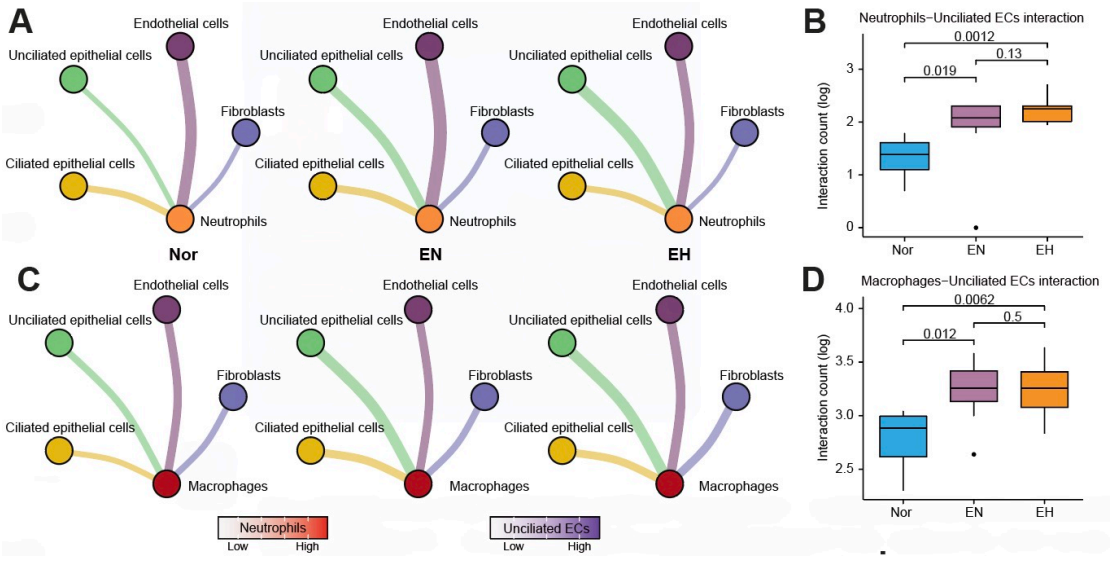


Fig. R12. Dynamics of cell-cell interaction networks in each group.

(A,C) Network diagrams showing directed interactions from neutrophils or macrophages (immune cells) to non-immune cells, including endothelial cells, unciliated epithelial cells, ciliated epithelial cells, and fibroblasts, in Nor, EN, and EH samples. **(B,D)** Boxplot indicating cell interaction counts between immune cells and non-immune cells respectively. The p-value is calculated using the Wilcoxon rank sum test.

b. Including the other blood cell subtypes in the cell-cell interaction analysis would have been more rigorous in demonstrating that neutrophils are the primary blood cells interacting with non-ciliated cells.

Response: Thank you very much for your suggestion. Based on your advice, we

comprehensively analyzed the interactions between immune cells and non-immune cells **(Fig. R13)**. Since endometrial epithelial cells are the primary effector cells in endometrial hyperplastic diseases, we focused on the interactions between immune cells and endometrial epithelial cells. We noticed that the interactions of macrophages and neutrophils with Unciliated epithelial cells were significantly enhanced especially neutrophils **(Fig. R12)**. Therefore, we believe that neutrophils are more worthy of further investigation

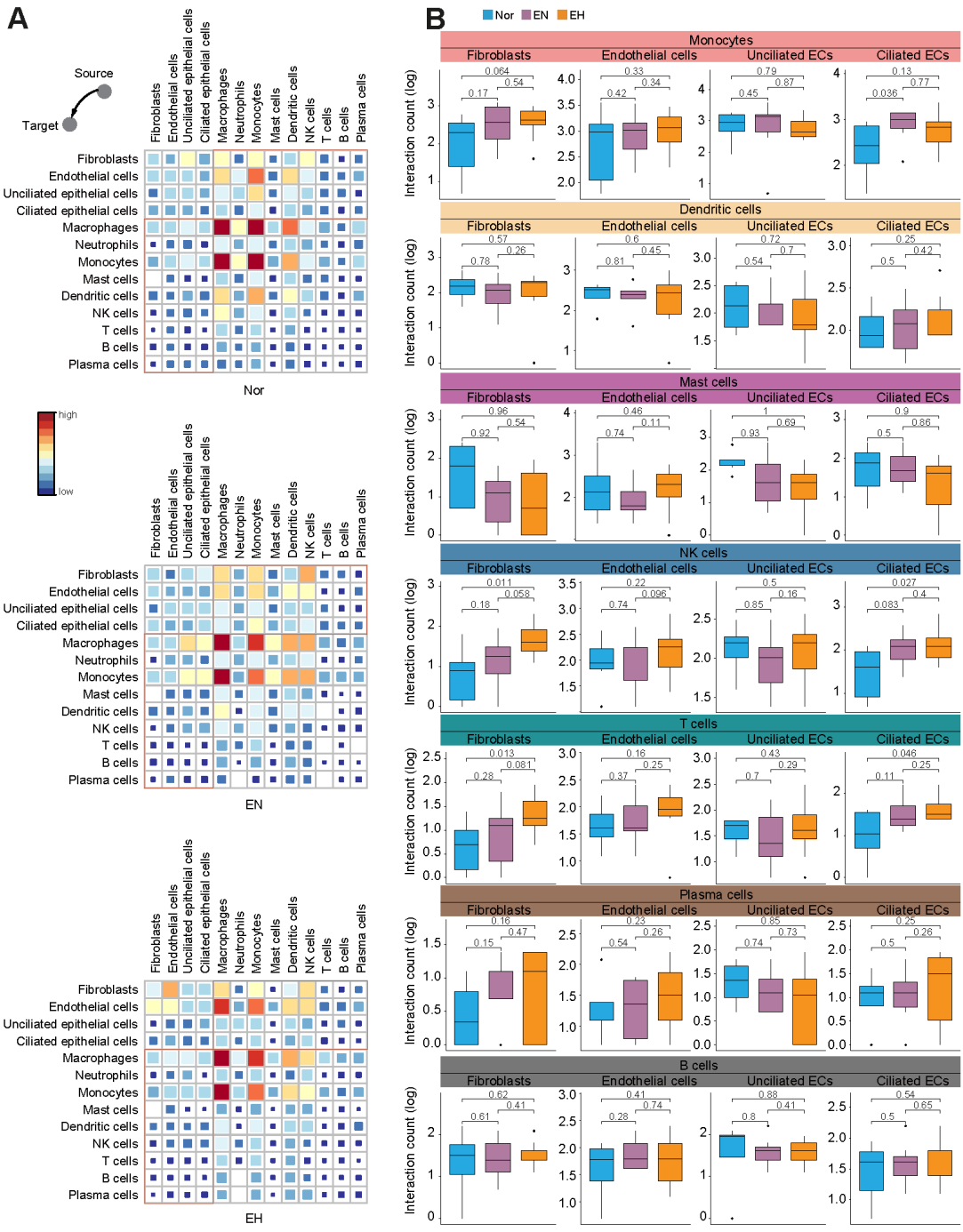


Fig. R13. Dynamics of cell-cell interaction networks in each group.

(A) A Heatmap illustrating the cell interaction patterns in Nor, EN, and EH samples. **(B)** Boxplot indicating cell interaction counts between immune cells and non-immune cells respectively. The *p*-value is calculated using the Wilcoxon rank sum test.

c. Ciliated and non-ciliated epithelial cells, fibroblasts and endothelial cells were basically not characterized. Their inclusion would have provided further insight into the impact of neutrophil activity on uterine histopathology, since there are numerous non-ciliated epithelial subtypes in the uterus, and potentially only one of these subtypes could be responsible for promoting EH development. In addition, chronic estradiol treatment is reported to induce ciliogenesis as well as hyperplasia, hypertrophy, and ESR1 expression in multiple cell types. Therefore, a significant shift in epithelial subpopulations or neutrophil/epithelial cell interactions occurring in EH and/or EN compared to normal endometrium would not have been captured.

Response: We thank the reviewer for this insightful comment. We agree that epithelial, fibroblast, and endothelial cell heterogeneity is important for understanding uterine histopathology. In our study, we annotated ciliated epithelial cells, unciliated epithelial cells, fibroblasts, and endothelial cells as major cell types. Our primary focus was to define the neutrophil subsets and the NET-mediated mechanism underlying EH. We specifically examined the interaction between neutrophils and unciliated epithelial cells, which was the dominant epithelial population interacting with neutrophils in the EH group. We further identified NKCC1 as the NET-DNA receptor on unciliated epithelial cells and demonstrated that epithelial cell-specific *Slc12a2* deletion attenuated estrogen-induced EH. These functional data support a key role for unciliated epithelial cells in NET-driven EH. We acknowledge that a more detailed dissection of epithelial subtypes was not performed in this study. We have added this as a limitation in the Discussion. However, our functional experiments, including epithelial-specific *Slc12a2* knockout, directly support the importance of unciliated epithelial cells in NET-mediated EH.

d. Fig. 1H does not include an EN group and a rationale for this omission was not provided.

Response: Thank you for your suggestion. In light of the fact that EH (EH) and EN (EN), as common gynecological diseases, have long plagued women. Although EN EN is also a disease that deserves serious attention, the well-established antibiotic treatment has brought great hope to patients with EN. Therefore, we focus more on the changes in neutrophils in the disease state of EH rather than on EN. We will add this limitation in the Discussion. For completeness, the EN group is nonetheless included alongside Normal and EH in the cell–cell interaction analyses (**Fig. R12 and Fig. R13**), so that the neutrophil–epithelial interaction landscape of EN can be directly compared.

3. A further concern is that the seven animal models used in determining the role of neutrophils and NETs formation in response to supraphysiological levels of E2 are greatly underutilized. In many cases, only uterine weight and uterine gland/stromal ratios are determined. A suggestion would be to develop a rigorous strategy for using a

few select mouse models in combination with a treatment regime that selectively targets estrogen activity, NET formation, pathways of interest, etc. to address the premise of this manuscript.

Response: We sincerely thank the reviewer for this insightful and constructive suggestion. We agree that a more comprehensive characterization of the uterine phenotype and a more systematic evaluation of NET formation across the different mouse models would strengthen the mechanistic framework of our study. In response to this concern, we have substantially expanded the histopathological and molecular characterization of the uterine phenotype in the revised manuscript. In addition to uterine weight and the gland-to-stroma ratio, we performed immunofluorescence staining for E-cadherin and PCNA in the uterine tissues from the different mouse models to further evaluate alterations in endometrial epithelial architecture and proliferative activity (**Fig. R14**). Moreover, we performed quantitative histomorphometric analysis of H&E-stained uterine sections and measured endometrial thickness to provide a more comprehensive assessment of the severity of EH (**Fig. R15**). In addition, we found that aspirin not only inhibits endometrial hyperplasia but also significantly inhibits NETs formation (**Fig. R16**).

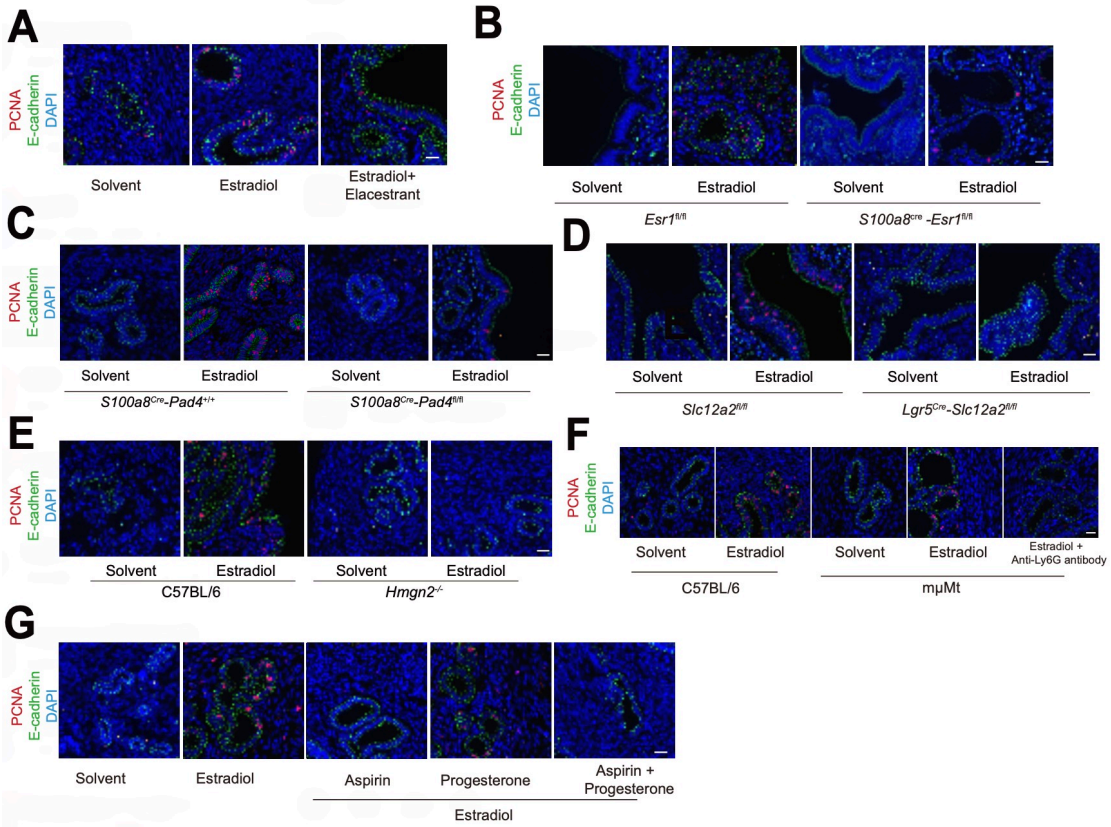


Fig. R14. Comprehensive histopathological assessment of EH in different mouse models.

(A- G) Representative immunofluorescence images showing PCNA (red) and E-Cadherin (green) staining in uterine sections from the indicated mouse models and experimental groups. Scale bars, 20 μm.

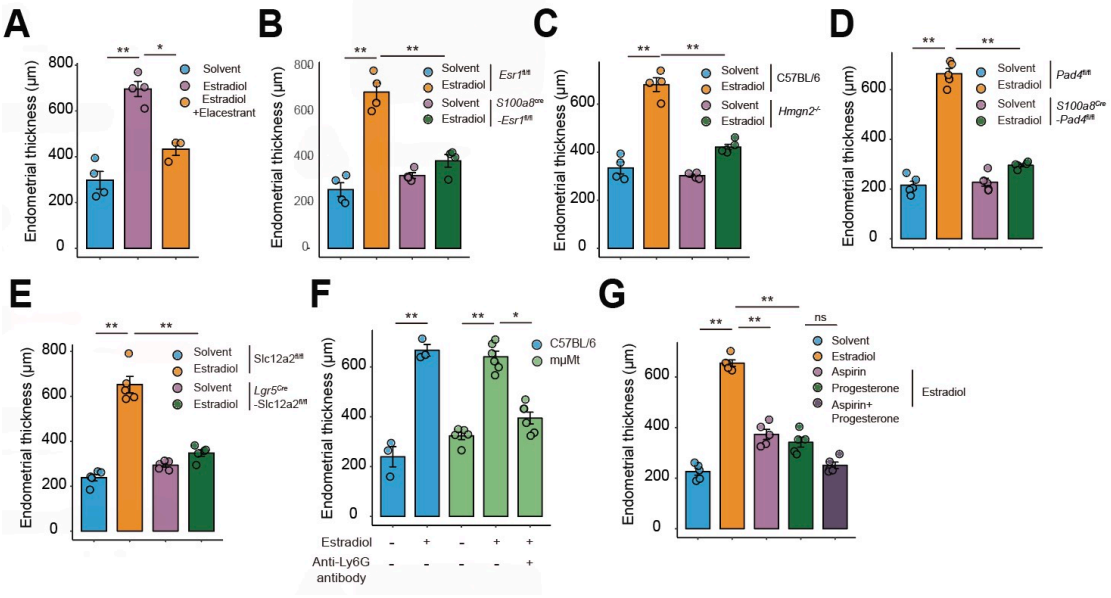


Fig. R15. Comprehensive histopathological assessment of EH in different mouse models.

(A-G) Quantification of endometrial thickness based on H&E-stained uterine sections from the indicated groups. Endometrial thickness was determined by measuring the perpendicular distance from the luminal epithelial surface to the endometrium–myometrium interface. The mean value for each mouse was used for statistical analysis.

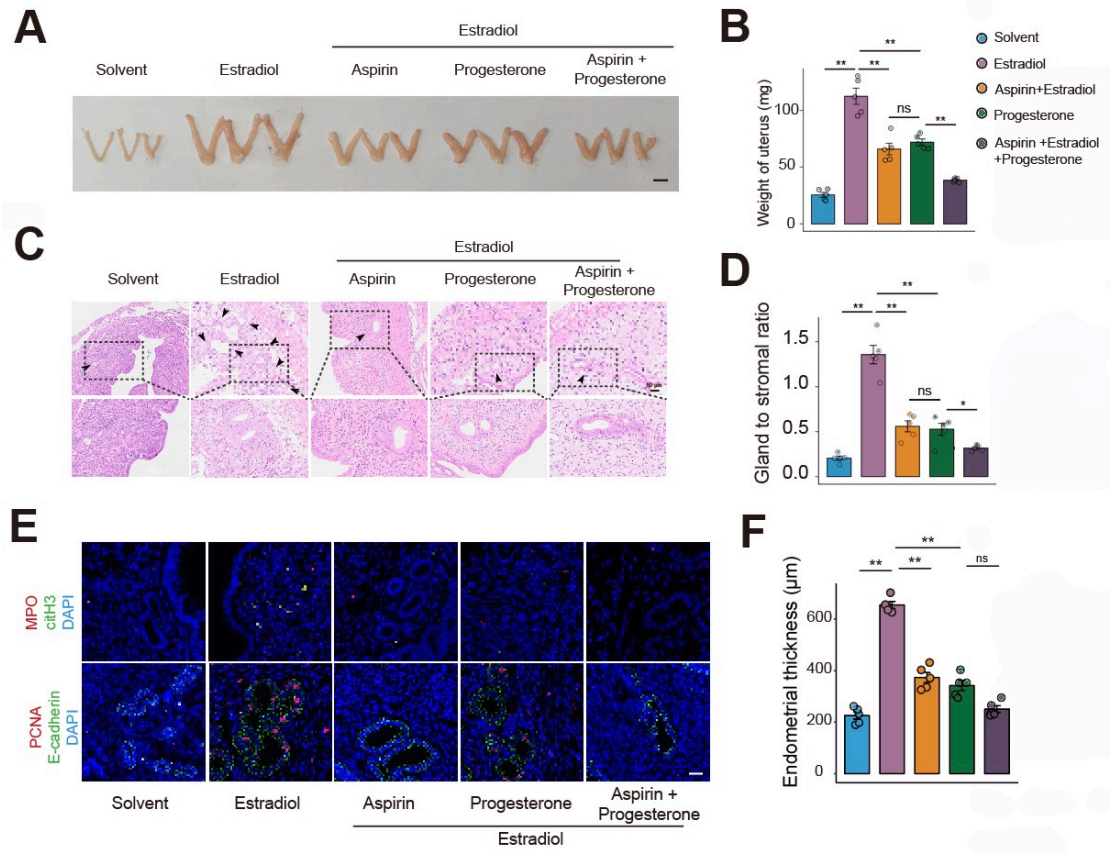


Fig. R16. Aspirin inhibit estrogen-induced NETs formation *in vivo.*

(A) C57BL/6 female mice were administered 50 μg/kg estradiol to induce endometrial hyperplasia. To evaluate therapeutic interventions, mice received either aspirin (5 mg/kg, intraperitoneal injection) or progesterone (2 mg, subcutaneous injection), followed by estradiol administration. Representative macrophotographs of uterus in each

group (n=5). Scale bar, 0.5 cm. **(B)** Uterus weight of each group (n=5), $^{**}P<0.01$. **(C)** Representative images of H&E staining (scale bar, 50 μm). **(D)** Statistics of the area ratio of gland to stroma according to H&E images (n=5), $^{**}P<0.01$, $^{*}P<0.05$. **(E)** Mice endometrial tissues were stained with PCNA or MPO (Red), citH3 or E-cadherin (green) and DAPI (blue), (n=3), Scale bar: 20 μm. **(F)** Statistics of endometrial thickness according to H&E staining images (n=4 or 5); $^{**}p < 0.01$.

4. Female animals were treated -/+ supraphysiological levels of E2 -/+ a secondary treatment of some kind. This treatment approach does not consider that females have an estrus cycle, and therefore all the data collected and analyzed in this manuscript only represents responses in asynchronous cycling mice.

Response: We thank the reviewer for this thoughtful comment and fully agree that estrous stage is an important variable. We would like to clarify that our animals were in fact synchronized: before estrogen induction, all mice were staged by vaginal cytology and only proestrus animals were enrolled, providing a uniform proliferative starting point across all models. We apologize that this synchronization step was omitted from the original Methods and have now added it. Clinically, EH is driven by long-term high-level estrogen combined with relatively low progesterone. Our estrogen-induced model artificially maintains high E2, which suppresses or arrests the normal estrous cycle and keeps the uterus in a prolonged "persistent proliferation" state; under these supraphysiological conditions, residual cycle-stage variability has minimal impact on the readouts. Because every estrogen-treated group starts from the same synchronized proestrus baseline and is then held in the same estrogen-dominated state, the multiomics, uterine-weight and histopathology data are normalized to a common starting point and are directly comparable across the mouse models and, in principle, to the monkey model. We now discuss, as a limitation, that we did not additionally profile physiological diestrus-transition animals, which would be an interesting avenue for characterizing neutrophil dynamics under normal cycling.

Endogenous levels of estrogen, cellular $ESR1^{+}$ expression, neutrophil numbers, and the number of ciliated and non-ciliated cells, are all dynamically regulated during the different phases of the estrus cycle. For example, estrogens are high and neutrophils are low/absent in the proestrus phase; while estrogens are low and neutrophils are high and associated with uterine epithelial cells primarily during the transition to diestrus.

Response: Thank you very much for your comment. In this article, we focus more on the effects of neutrophils on EH under pathological conditions rather than on changes in neutrophils under physiological conditions. Under such high concentrations of hormones, the normal estrous cycle of mice is typically effectively suppressed or even arrested, keeping the uterus in a prolonged "persistent proliferation"-like state. Therefore, in this context, cycle stage variability has minimal impact on the experimental results.

Synchronizing the mice in the estrus cycle and using mice in proestrus and transition to diestrus phases in combination with chronic E2 treatment of mice in proestrus would have provided a more physiological and rigorous analysis of the development of EH in

response to chronic E2 treatment. This approach would have also normalized (based on estrus phase) all of the multiomics analysis, uterine weights, and histopathology in response to various treatments in one mouse model and enabled a direct comparison between all of the different mouse models used in this manuscript. Even the monkey model could have been directly compared if the females had been synchronized to the same estrus phases as the mice.

Response: You raised a professional question. The mouse estrous cycle consists of four stages: estrus, metestrus, diestrus, and proestrus. Before the formal experiment, we strictly screened the mice. Mice in the proestrus stage were selected for estrogen-induced EH, because at this stage the endometrium is already in a physiological proliferative state, exhibiting the most sensitive and consistent response to estrogen signals, thereby providing a uniform starting point for subsequent induction. This method make the uterus in a prolonged "persistent proliferation"-like state. We sincerely apologize that we did not write the methodology section in such detail. We have made relevant additions to the methodology section.

5. Analysis of the micrographs showing uterine histopathology can be confusing. For example, the panels seem to show different regions of the uterus with some sections containing only endometrium while others contain endometrium and myometrium or possible myometrium only. The robustness and rigor of analyzing uterine histopathology and uterine gland/stroma rations would have been considerably strengthened by normalizing the tissue sections as follows: cutting sections from the same regions of the uterine horn in all experiments and cutting cross sections so that the uterine lumen, endometrium and myometrium are obvious and that any changes in the endometrium and endometrial glands in response to treatment are clearly visible. Measuring endometrial thickness would have also evaluated/confirmed responsiveness to E2 treatment.

Response: We sincerely thank the reviewer for this important comment regarding the rigor and standardization of uterine histopathological analysis. In response to this concern, we carefully re-evaluated the uterine histopathology and standardized the analysis across all experimental groups. Specifically, uterine horns were collected from the same anatomical region, and the maximal transverse section was selected for paraffin embedding and histological analysis. We selected transverse sections in which the uterine lumen, endometrium, and myometrium could be clearly distinguished, thereby allowing consistent evaluation of endometrial morphology and thickness across groups. In addition, representative images were selected from enlarged views of the endometrial regions to provide a clearer visualization of the treatment-induced histopathological changes, as shown in the revised figure **(Fig. R17)**.

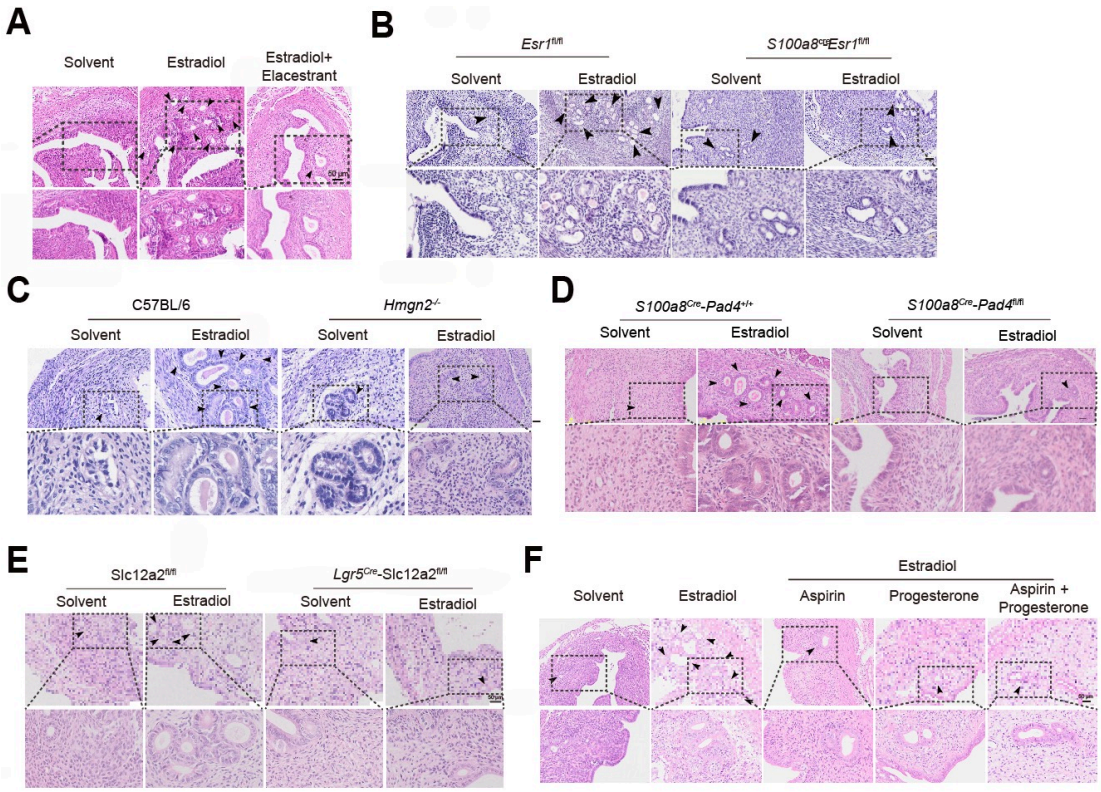


Fig. R17. Comprehensive histopathological assessment of EH in different mouse models.

(A-F) Representative images of H&E staining from the indicated groups. Scale bar, 50 μm. The arrows indicate hyperplastic endometrial glands.

6. In Fig.3, pseudotime analysis determined that module K3 representing disease progression was enriched for integrated stress response signaling, p38MAPK signaling, and response to steroid hormone. However, estrogen-induced NET formation and the TLR4 signaling pathway were investigated in Fig. S11B-D. It seems that determining the link between stress response and p38MAPK signaling and NET-related pathways would have been a more obvious avenue to peruse than TLR4. A rationale for specifically selecting TLR4 signaling would clarify this strategy.

Response: We sincerely thank the reviewer for the insightful comment. The reviewer pointed out that pseudotime analysis identified enrichment of integrated stress response, p38MAPK signaling, and steroid hormone response in module K3, suggesting that investigating these pathways might be a more direct approach. Regarding why we chose to investigate the TLR4 signaling pathway in Fig. S11B–D, our rationale is based on the following two considerations:(1) Pathways inferred from pseudotime analysis reflect transcriptional correlations, not necessarily functional mechanisms. Although module K3 shows enrichment of p38MAPK and integrated stress response-related genes, this only indicates transcriptional changes during disease progression. In fact, these pathways could be activated downstream of other upstream events. (2) TLR4 is the key pattern recognition receptor for LPS, and LPS-induced NET formation is a well-established pathway. In infectious inflammation (e.g., EN, EN), the LPS-TLR4 axis is a major driver of NETosis. Since our study compared sterile inflammation (EH)

and infectious inflammation (EN) side by side, we first wanted to determine whether estrogen-induced NET formation also depends on this canonical infectious pathway. Our experimental results showed that estrogen did not upregulate TLR4 expression, and TLR4-related genes were not significantly enriched in the EH group. This strongly suggests that estrogen-driven NET formation operates through a TLR4-independent, novel mechanism, thereby highlighting the specificity of the ERα–HMGN2 axis. In summary, our initial focus on the TLR4 pathway was aimed at excluding the possibility that estrogen induces NETs via the classical infectious pathway, providing essential prerequisite evidence for the discovery of the new mechanism. Once again, we thank the reviewer for the constructive suggestion, and we will systematically evaluate the roles of p38MAPK and integrated stress response pathways in estrogen-induced NET formation in our follow-up studies.

7. In most of the figures, there are a number of experiments that do not include treatment groups that would have verified the conclusion(s) drawn from the data. For example, in Figure 5F, ion content was determined following treatment -/+ E2 -/+ NETs, and in Fig. S14, endometrial epithelial cells and organoids were treated -/+ E2 -/+ NETs, with the conclusions that E2+NETs in combination increased ion content, cell proliferation and organoid size. Both of these experiments would have benefited by the addition of groups treat with an E2 -/+ a NET inhibitor to verify these conclusions.

Response: Thank you for your suggestion. The reason we did not add NETs inhibitors is that NETs do not form spontaneously in the cell and organoid culture systems. In these systems, our main focus is to evaluate the effects of exogenous NETs on the relevant signaling pathways. We extracted NETs and added them as exogenous stimulants to the culture system of epithelial cells or organoids, rather than inducing NET formation within the culture environment. We understand your expectation for more rigorous experimental design. To better address your concerns, we administered the NET inhibitor GSK484 or the NET scavenger Dnase I in mice and then evaluated the improvement in EH (**Fig. R18&19**). We found that inhibiting NETs ameliorates EH.

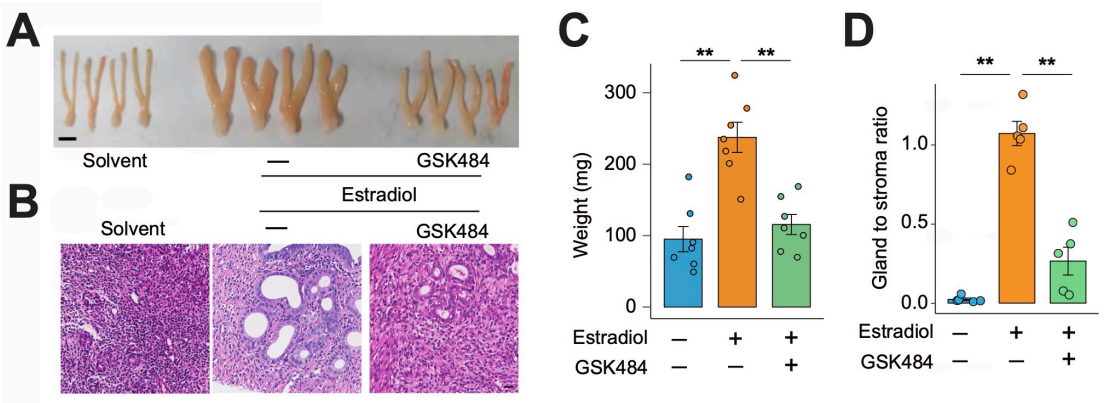


Fig. R18. GSK484 improves estrogen-induced EH in mice.

(A) Female C57BL/6 mice were treated with estradiol (50 μg/kg, s.c.) with or without GSK484 (4 mg/kg, i.p.) for 21 days. Representative macrophotographs of uterus in each group (n=4, scale bar, 0.5 cm). **(B)** Representative images of H&E staining (n=5, scale bar, 80 μm). **(C)** Uterus weight of each group (n=7). **(D)** Statistics of the area ratio of gland to stroma according to H&E staining images (n=5). $^{**}P<0.01$.

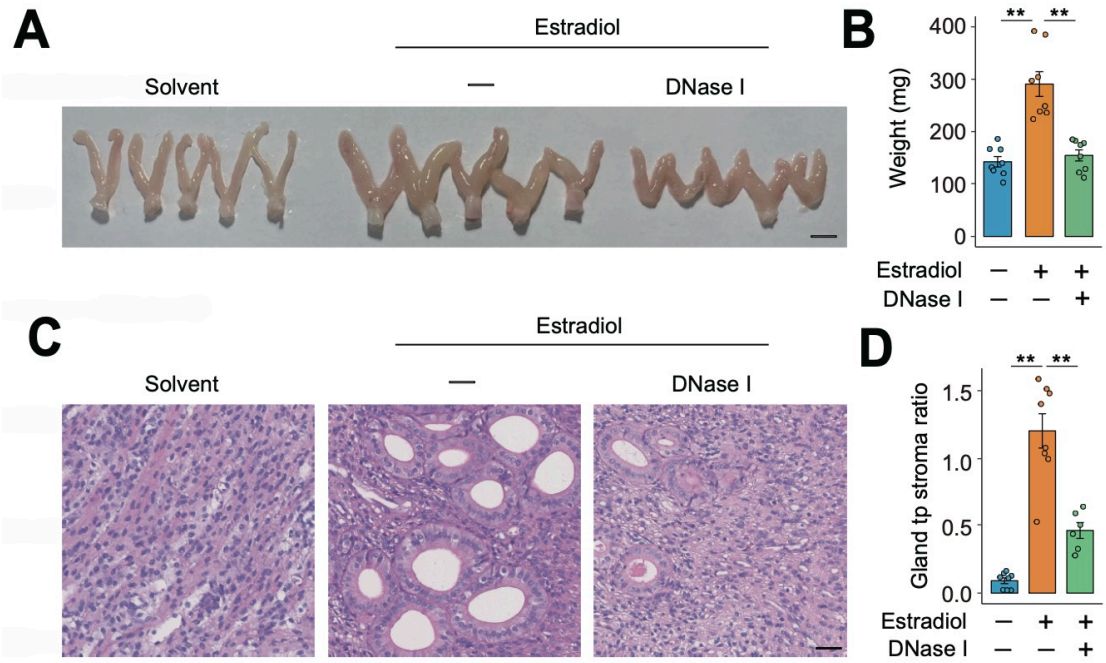


Fig. R19. DNase I ameliorated estrogen-induced EH in mice.

(A) C57BL/6 female mice were treated with 50 μg DNase I intraperitoneally three times per week during 50 μg/kg estrogen treatment. Representative macrophotographs of uterus in each group (n=5, scale bar, 0.5 cm). **(B)** Uterus weight of each group (n=5). **(C)** Representative images of H&E staining (n=5, scale bar, 80 μm). **(D)** Statistics of the area ratio of gland to stroma according to H&E staining images (n=5). ***P*<0.01.

8. Figure legends would be more informative if they consistently include basic information, such as inhibitor or drug concentrations, and whether the tissues/cells being analyzed are mouse or human, especially if several animal models are included in the same figure.

Response: Thank you for this helpful suggestion. We have systematically revised every figure legend to consistently state: (i) the species and source of each tissue or cell sample (human, mouse, or cynomolgus monkey); (ii) the exact concentration/dose and duration of each drug, inhibitor or stimulus; (iii) the genotype of each mouse model where multiple models appear in one figure; and (iv) the sample size (n) and the statistical test used.

Specific Comments

1. In the Materials and Methods section, please list all the cell lines by name and the sources they were purchased from.

Response: Thank you for your suggestion. We have added the relevant description in the Materials and Methods section.

2. Please describe briefly but succinctly how the endometrial gland to stromal ratios were determined.

Response: Thank you for your professional question. In our method, within a fixed area, we delineate the total area of all glandular epithelium and the entire endometrial

area separately. The gland-to-stroma ratio is then calculated using the formula: Gland/Stroma ratio = Total glandular area / (Total endometrial area – Total glandular area).

4. Minor grammatical errors exist infrequently throughout the manuscript. These, however, do not detract from readability.

Response: Thank you for your suggestion. We have corrected the grammatical errors throughout the manuscript.

Reviewer #3:

The well-written manuscript submitted by Qu et al. entitled "Estrogen-triggered neutrophil extracellular traps fuel sterile inflammation via NKCC1" uses rigorous cutting edge genomic and single-cell transcriptomics with spatial tissue transcriptomics of endometrial tissue to show that a population of ESR1+ neutrophils produce extracellular traps in a sterile inflammatory milieu which alter endometrial epithelial cell ion homeostasis leading to EH, a significant gynecological problem. Further the authors show that use of aspirin in a non-human primate model of EH ameliorates the disease phenotype. Strengths of this manuscript include identification and rigorous characterization of a novel ESR1+ neutrophil subset capable of NET formation in response to estrogen, mechanistic characterization of estrogen-responsive NET formation as a driver of pathogenic endometrial cell hyperplasia, rigorous NET quantitation methods applied across experiments, comparison and contrast of two distinct endometrial disorders, EH (sterile) and EN (bacterial infectious inflammation), and identification of NKCC1 as a effector molecule/receptor for NETs in disrupting endometrial ion homeostasis leading to hypertrophy. Further, the authors use mouse, non-human primate, and human clinical tissue samples appropriately and with intent to signal robust translational potential for these findings.

The following are minor comments offered to improve a strong manuscript:

1. Figure 3. The authors provide only qualitative data regarding NET formation and omit the quantitative data, choosing instead to include the quantitative data supporting estrogen induction of NET formation in ESR1+ neutrophils in Supplemental Figure S12. I would recommend including these strong data in the actual manuscript Figure 3.

Response: Thank you for your suggestion. We have revised Figure 3 accordingly **(Fig. R20)**. Please check it.

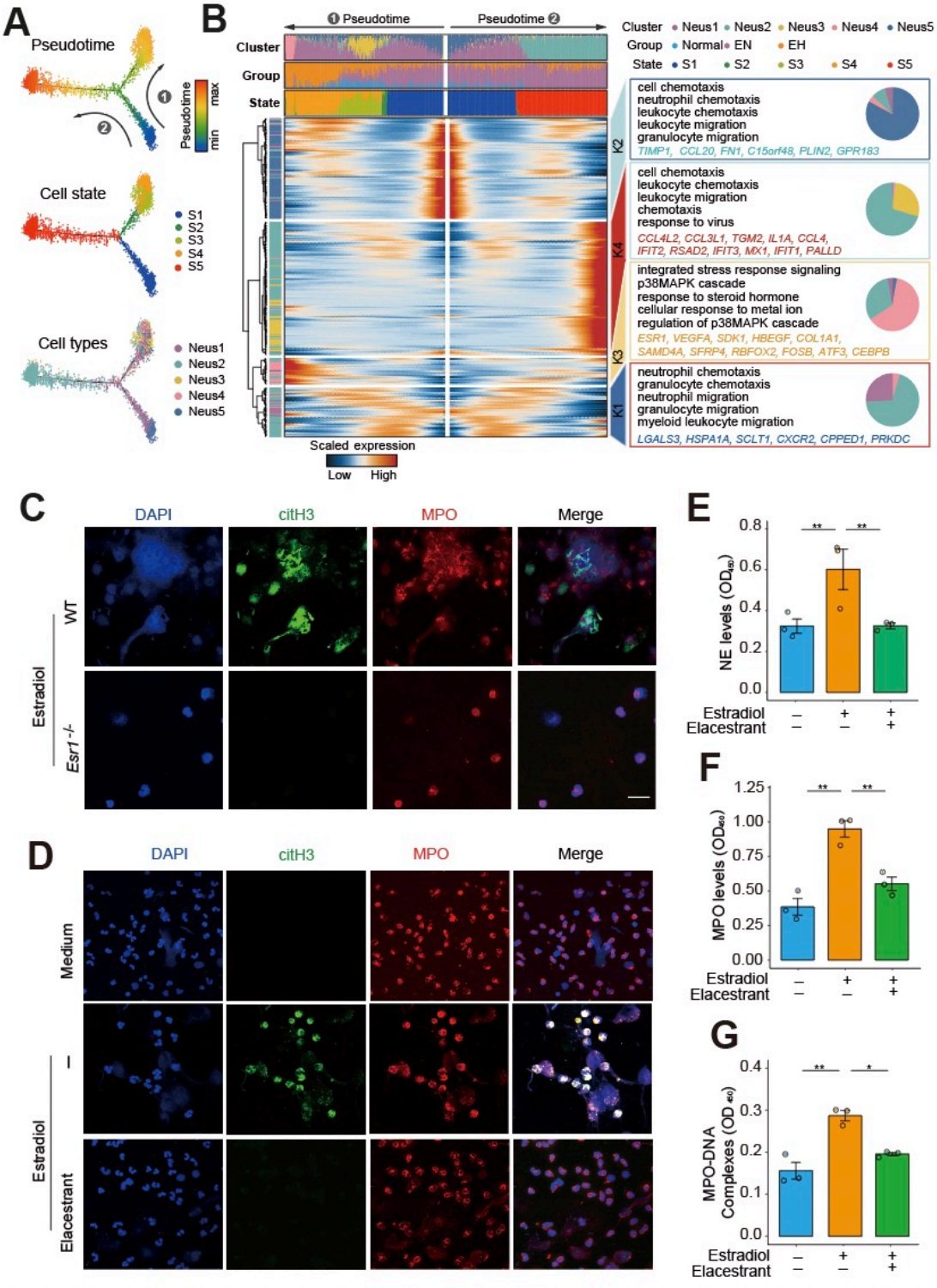


Fig. R20. Estrogen induces the binding of ERα to HMGN2 and promotes NET formation.

(A) Semisupervised pseudotime trajectory of neutrophil subtypes inferred by Monocle2. Trajectory is colored by pseudotime, cell states, and cell subtypes. **(B)** Heatmap showing the scaled expression of differentially expressed genes across pseudotime from (h). Genes and functional pathways (listed to the right of the heatmap) are assigned to specific cell states based on their expression levels. Bar plots above the heatmap are scaled diagrams of different cell states and cell clusters during pseudotime differentiation trajectory. Pie plots on the right showing the distribution of cell subtypes harboring differential genes across different modules. **(C)** Neutrophils were obtained from peripheral

blood of WT mice or E*SR1* KO mice. Neutrophils were incubated with 50 nM or medium at 37℃ for 2 h. Neutrophils were stained with citH3 (green), MPO (red) and DAPI (blue). Scale bar: 20 μm. **(D-G)** Neutrophils were incubated with 50 nM estradiol and/or 4 μM elacestrant at 37℃ for 12 h. OD_{450} value reflects the level of MPO, NE or MPO-DNA Complexes; ***P*<0.01. Neutrophils were stained with citH3 (green), MPO (red) and DAPI (blue). Scale bar: 20 μm.

2. In Figure 4F, exogenous NETs were applied as a stimulus to endometrial organoids with and without estrogen. Did the authors have a NETs-only control for those experiments? If so, those details should be shared in the figure.

Response: We thank the reviewer for this valuable comment. We fully agree that a "NETs-only" control in the organoid experiment (Figure 4F) would provide a more complete picture of the direct effect of NETs on endometrial organoids.In our experimental design, the primary aim of Figure 4F was to test whether exogenous NETs could augment estrogen-driven organoid growth, mimicking the pathological context of estrogen-induced hyperplasia. Therefore, we included three groups: vehicle control, estrogen alone, and estrogen plus NETs. We did not include a NETs-only group in this particular organoid assay. We would like to clarify that we have already examined the direct proliferative effect of NETs alone in endometrial epithelial cell lines (**Fig. R21**). These cell-based experiments consistently showed that NETs alone can promote epithelial cell proliferation. Although organoids represent a more complex system, we believe the cell-level data, together with the robust in vivo and pharmacological evidence (e.g., NET inhibition ameliorates EH) (**Fig. R22&23**), sufficiently support our main conclusions.

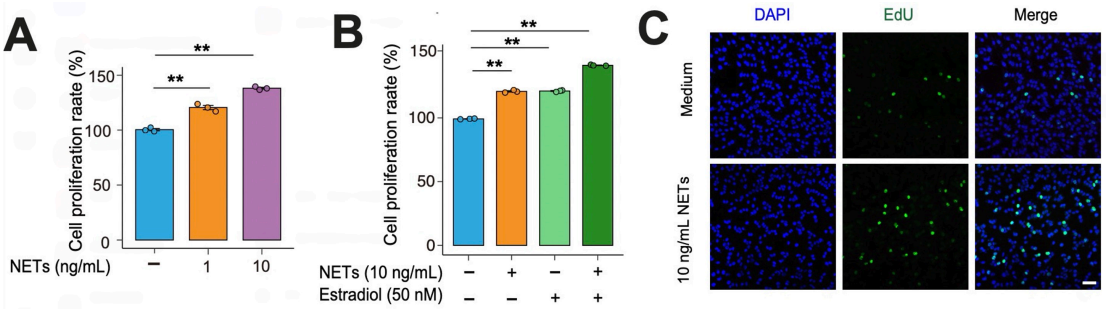


Fig. R21. NETs directly promote epithelial cell and endometrial organoid proliferation. Related to Figure 4.

(A-C) Endometrial epithelial cells were treated with indicated concentration of NETs or estradiol for 24 h. Then the cell proliferation levels were measured by MTT (a-b) or EdU staining (c). ***P*<0.01.

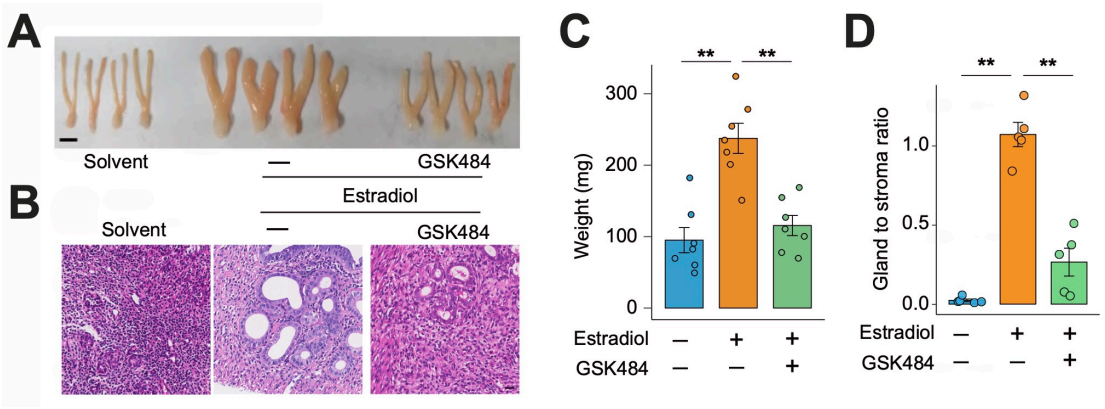


Fig. R22. GSK484 improves estrogen-induced EH in mice.

(A) Female C57BL/6 mice were treated with estradiol (50 μg/kg, s.c.) with or without GSK484 (4 mg/kg, i.p.) for 21 days. Representative macrophotographs of uterus in each group (n=4, scale bar, 0.5 cm). **(B)** Representative images of H&E staining (n=5, scale bar, 80 μm). **(C)** Uterus weight of each group (n=7). **(D)** Statistics of the area ratio of gland to stroma according to H&E staining images (n=5). ***P*<0.01.

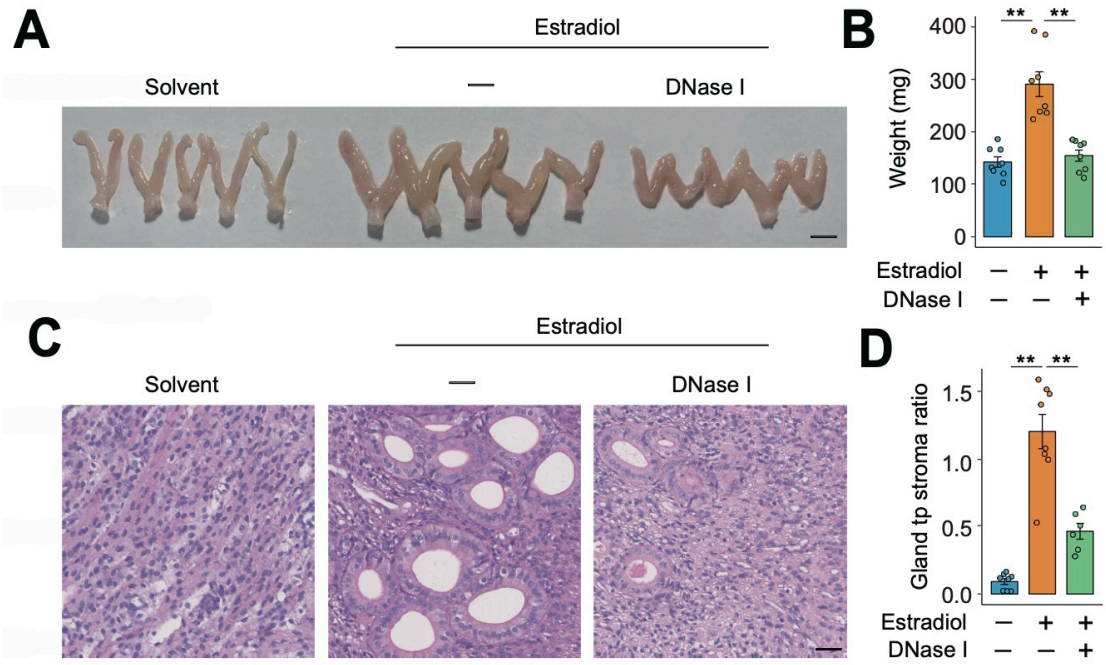


Fig. R23. DNase I ameliorated estrogen-induced EH in mice.

(A) C57BL/6 female mice were treated with 50 μg DNase I intraperitoneally three times per week during 50 μg/kg estrogen treatment. Representative macrophotographs of uterus in each group (n=5, scale bar, 0.5 cm). **(B)** Uterus weight of each group (n=5). **(C)** Representative images of H&E staining (n=5, scale bar, 80 μm). **(D)** Statistics of the area ratio of gland to stroma according to H&E staining images (n=5). ***P*<0.01.

3. S100a8 is expressed in many inflammatory cells of the myeloid lineage (typically highest in neutrophils and monocytes and less well expressed in macrophages and dendritic cells). Can the authors comment on the possible effects in this system on other cell types in the S100a8Cre-Pad4fl/fl mice?

Response: We thank the reviewer for this important and insightful comment. We fully agree that S100a8 (MRP8) is expressed in multiple myeloid subsets, particularly

neutrophils and inflammatory monocytes, and to a lesser extent in some macrophages and dendritic cells. Our primary use of this mouse model was to demonstrate that inhibition of NET formation ameliorates estrogen-induced EH. To better address your concerns, we administered the NET inhibitor GSK484 or the NET scavenger Dnase I in mice and evaluated the improvement in EH (**Fig. R22&23)**. We found that inhibiting NETs ameliorates EH. Because these pharmacological approaches do not depend on S100a8-Cre expression yet reproduce the genetic phenotype, the effect can be ascribed to NETs.Taken together, these animal experiment results confirm that inhibition of NET formation improves estrogen-induced EH.

4. Minor grammatical errors exist infrequently throughout the manuscript. These, however, do not detract from readability.

Response: Thank you for your suggestion. We have corrected the grammatical errors throughout the manuscript.