

Peer Review File

Piezo1 expression in neutrophils regulates shear-induced NETosis



Open Access This file is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. In the cases where the authors are anonymous, such as is the case for the reports of anonymous peer reviewers, author attribution should be to 'Anonymous Referee' followed by a clear attribution to the source work. The images or other third party material in this file are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript describes a study into the role of Piezo1 and fluid shear stress mechanotransduction in neutrophil NETosis. This is an interesting area of study, and Piezo1 has been shown to be important in T cells and other leukocytes. Also, there is a long history of neutrophil responses to fluid shear stress. My specific comments are given below.

1. Before jumping right into the topic of NETosis, the authors should briefly summarize the body of literature related to neutrophil mechanotransduction in fluid shear stress. For instance, neutrophils have been shown to modulate their sensitivity to activation to chemical activators like fMLP and PAF in the presence of shear stress.
2. PLB-985 cells are now accepted as a better cell line model for neutrophils, compared to differentiated HL-60 cells. By the time HL-60 cells show significant expression of important neutrophil markers like L-selectin, they are dying and generally not looking too healthy.
3. For experiments with primary human neutrophils, the authors should specify how many hours post blood collection and how many hours post isolation they were used. Generally 4 hours is considered the maximum after isolation, before they start dying.
4. Why only use 10 min of fluid flow instead of varying this parameter to gain an understanding of the temporal dependence of these observations? In previous studies of neutrophil mechanotransduction, the responses varied with time between 15 and 120 minutes.
5. Most Piezo1 studies also use the pharmacological inhibitor GxMTx4, but this study relies solely on the knockdown in HL-60 cells (which doesn't apply to the primary neutrophils).
6. Quantifying the percent circular nuclei seems like an unnecessary way to treat a continuous variable as a binary variable and lose information in the process. The authors could instead quantify the shape factor or circularity of the cell nucleus... but in either case this is a puzzling metric for a multi lobed neutrophil nucleus, which is famously nonellipsoid in shape, unlike other cell types.
7. The authors should quantify the percentage of adherent platelets that are activated, perhaps by staining for surface P-selectin, or by examining their morphology under high magnification.
8. Why didn't the authors use ratiometric imaging of calcium influx instead of a single channel measurement? The use of dual labels is generally more accurate.
9. The neutrophil membrane is more ruffled with microvilli projections, and not as smooth as most

cell types. It has been shown that certain adhesion receptors like L-selectin, and PSGL-1 exist primarily at the tips of microvilli. Where is Piezo1 located on the membrane surface? On microvilli or everywhere equally?

Reviewer #2 (Remarks to the Author):

General comments

This is a well-written manuscript with good organization and clear, logical flow. The authors attempt to answer a scientifically and clinically relevant question regarding regulation of NET formation by Piezo1. There is compelling evidence for shear stress-induced NETosis, however it is unclear why the authors did not continue to use the same shear stress dose that they proved to be biologically relevant in their dose response experiments (Figure 1). However, while the authors show that Ca^{2+} signaling and mechanosensitive ion channels are important for NETosis, the methods are not robust enough to determine the role of Piezo1 in shear stress induced NETosis, which is a key conclusion of the paper.

Major Comments

1. This paper does not definitively link Piezo1 activation with shear stress-induced NETosis. The authors show a reduction in NETosis with ruthenium red, GdCl_3 , and GSMTx4. However, these inhibitors are non-specific for Piezo1 and target many mechanosensitive cation channels (Figure 4b). The evidence for Piezo1-dependent NETosis is weaker. The authors induce shear-stress NETosis in HL-60 cells $\pm$ Piezo1 siRNA. Cell death is extremely high in both the static and shear stress conditions. NETosis is only marginally higher (~20%) in the control siRNA shear stress condition, however, only normalized NET area was measured, not cH3, which is more specific for NETosis.
2. It is unclear why there is different shear stress doses used for different read outs (80 dyne/cm² for NETosis imaging, 0.2-10 dyne/cm² for Ca^{2+} signaling), concern about induction of cell death at 80 dyne/cm² but should be repeated for all experiments.
3. Conclusions about the effect of ATP on NETosis is mixed and the authors state that there is an additive effect of shear stress and ATP, but their data does not support.
4. Additional scientific methods need to be shown in order to clearly show (i) platelets adhering to neutrophils, (ii) calcium influx in addition to immunofluorescence images (need to clearly state in methods how max calcium intensity was performed), (iii) Piezo1 knockdown needs to be quantified by western blot and the lanes properly labeled) or another method, and (iv) other standard measures of NETosis in dHL-60 cells as showed in human neutrophils.
5. Interpretation of actin cytoskeleton contribution to NET formation is not accurate to just look at displacement, which is usually a readout for migration. Need to consider looking at actin assembly or actin subtypes (F/G actin)

Minor Comments

1. The authors describe both classical and alternative pathways for NETosis (page 3, lines 62-71), however, it is unclear if this paper examines one or both pathways.
2. The shear stress condition is well described in the methods, however, treatment for the static condition is unclear.

3. In general, the methods section leaves out key methods, such as protein quantification, and reagents, such as Piezo1 antibody.
4. There may be non-specific cell death induced by the shear stress treatment. How do you quantify the contribution of non-specific cell death vs. NETosis? If requires cith3 then all NETosis readouts should include cith3 and not just chromatin morphology as a readout of NETosis, such as in Figures 4, 5, and 6.
5. I think there may be a typo on page 12, line 292. The range for shear stress is listed as 0.2-20 dyne/cm², but the experiment uses 0.2-10 dyne/cm².
6. In Figure 4a, authors show that Ca²⁺ is required for Ca²⁺ signaling. I would also include the effect of abrogating Ca²⁺ signaling on NET formation.
7. I would add discussion about why inhibition of cytoskeletal rearrangement is protective against NETosis (Figure 6), when in the introduction inhibition of the cytoskeleton is one mechanism of NETosis (page 3, lines 62-64).
8. I am not sure of the significance of presenting shear stress-induced motility and phagocytosis in the context of the rest of the paper.

We thank the Reviewers for their positive and highly constructive comments. We believe in addressing these comments and performing the requested changes the manuscript has been substantially improved. Below we provide a point-by-point response and also submit a revised manuscript with marked changes.

Reviewer #1

(Remarks to the Author):

This manuscript describes a study into the role of Piezo1 and fluid shear stress mechanotransduction in neutrophil NETosis. This is an interesting area of study, and Piezo1 has been shown to be important in T cells and other leukocytes. Also, there is a long history of neutrophil responses to fluid shear stress. My specific comments are given below.

- 1. Before jumping right into the topic of NETosis, the authors should briefly summarize the body of literature related to neutrophil mechanotransduction in fluid shear stress. For instance, neutrophils have been shown to modulate their sensitivity to activation to chemical activators like fMLP and PAF in the presence of shear stress.**

We appreciate the Reviewer's comment. We have updated the manuscript and have added the relevant literature on the shear stress sensitivity of neutrophils as given below:

Page 3, line 8:

Neutrophils are mechanosensitive, and hemodynamic shear stress regulates their activation e.g. during inflammation. Under static conditions, neutrophils have a spread-out cytoplasmic morphology, can form pseudopods, and migrate on glass slides, while upon exposure to shear stress, they retract their pseudopods and form a rounded morphology [4, 5]. Furthermore, shear stress increases the sensitivity of neutrophils to platelet-activating factor and consequently the shedding of L-selectin into the extracellular space. Furthermore, shear stress increases the activation of the integrin $\alpha M\beta 2$ on neutrophils and modulates neutrophil morphology [6].

- 2. PLB-985 cells are now accepted as a better cell line model for neutrophils, compared to differentiated HL-60 cells. By the time HL-60 cells show significant expression of important neutrophil markers like L-selectin, they are dying and generally not looking too healthy.**

Previous comparative studies between HL-60 and PLB-985 cells have confirmed that both cell lines can be differentiated into neutrophils after treatment with different agents, including DMSO. However, after differentiation with PMA and opsonized zymo-san, PLB-985 cell line develops higher level of oxidative stress compared to HL-60 cells (DOI 10.1002/bio.1091). Furthermore, RNAseq analysis of these two cell lines before and after differentiation with DMSO, has shown a significant similarity between the cell line and primary human neutrophils (doi: 10.1186/s12864-018-4957-6). Therefore, based on the available literature we initially decided to use HL-60 cell line as a cell model for this study. It should also be noted that the other advantage of the HL-60 cell line is that it is commercially available as a standardized cell model and could be purchased at reasonable costs in our region. We hope that the Reviewer can accept our use of HL-60

cells. We believe despite having limitations these cell lines serve our specific purpose for confirming the role of Piezo1 in shear induced NETosis.

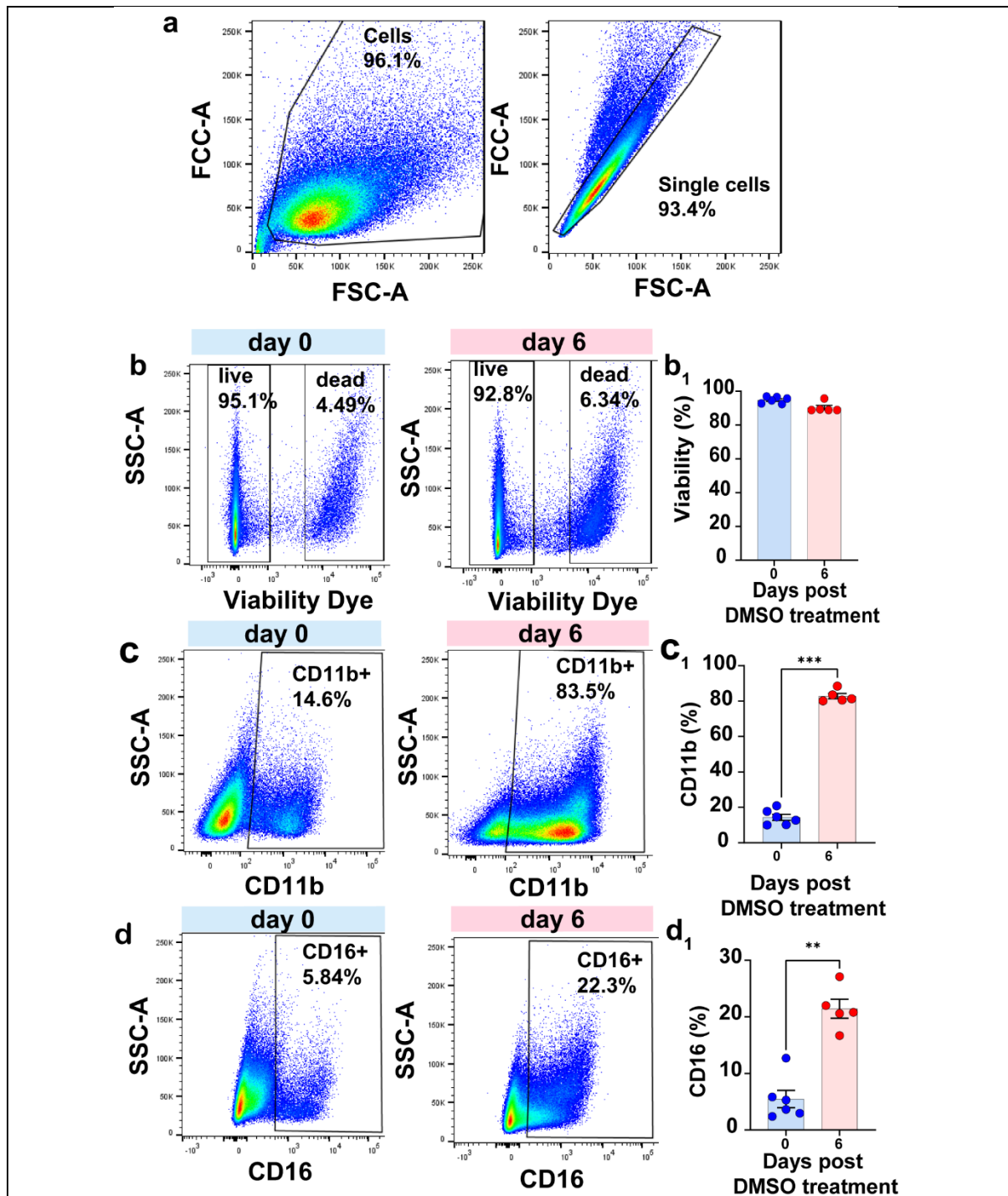
In our hands, we found that differentiation of HL-60 cells for 6 days leads to significant increase in the expression of CD11b and CD16, both typical neutrophil markers, without any effect on cell viability.

In response to the Reviewer's concern, we included the following data and text in the revised manuscript:

Page 15, Line 6:

These cells can be cultured for a long time and differentiated into neutrophils using DMSO treatment. In this study, we differentiated HL-60 cells using 0.1% DMSO for 6 days (dHL-60) and confirmed the expression of the neutrophil markers CD11b and CD16. It should be noted that under this condition, we did not observe a significant decline in cell viability (**Supplementary Figure 1**).

Supplementary Figure 1:



Supplementary Figure 1. Gating strategy and flow cytometry analysis of HL60 cells differentiation with DMSO. (a) Representative gating strategy for HL-60 cells. (b-d) representative flow cytometry plots and (b₁-d₁) bar graphs of HL-60 cells before and post 6 days of differentiation assessed for viability (b-b₁), expression of (c-c₁) CD11b and (d-d₁) CD16. Data is analyzed with paired Student's t-test. **P<0.01, ***P<0.001.

3. For experiments with primary human neutrophils, the authors should specify how many hours post blood collection and how many hours post isolation they were used. Generally, 4 hours is considered the maximum after isolation, before they start dying.

We thank the Reviewer for noting this missing information. After isolation, neutrophils were used after a short time (20 min) for further experiments, as described below:

Page 26, line 4:

After isolation, neutrophils were rested for 20 min and processed for further experiments.

4. Why only use 10 min of fluid flow instead of varying this parameter to gain an understanding of the temporal dependence of these observations? In previous studies of neutrophil mechanotransduction, the responses varied with time between 15 and 120 minutes.

In this study, we aimed to investigate the effect of exposure of circulatory neutrophils to high levels of shear stress on their capacity to form NETs once they adhere to the lumen. The experiments are designed accordingly to address this aim.

In our preliminary experiments, we found that exposure to less than 10 min of shear stress does not affect the ability of neutrophils to form NET. Furthermore, doubling the shear stress exposure time results in the loss of many activated cells due to their adhesion to the tubing. Therefore, a 10 min shear stress exposure time is selected for assessment of NETosis in this study.

For the subsequent steps, after exposure to shear stress, neutrophils were incubated on a substrate pre-coated with collagen for 3 hours. This amount of time was enough to allow the formation of detectable NETs in the positive control group.

Page 26, Line 19:

The 10 min exposure time is based on our preliminary experiments. Exposure for less than 10 min did not reliably yield a significant response. On the other side, doubling the shear stress exposure time results in the loss of many activated cells due to their adhesion to the tubing.

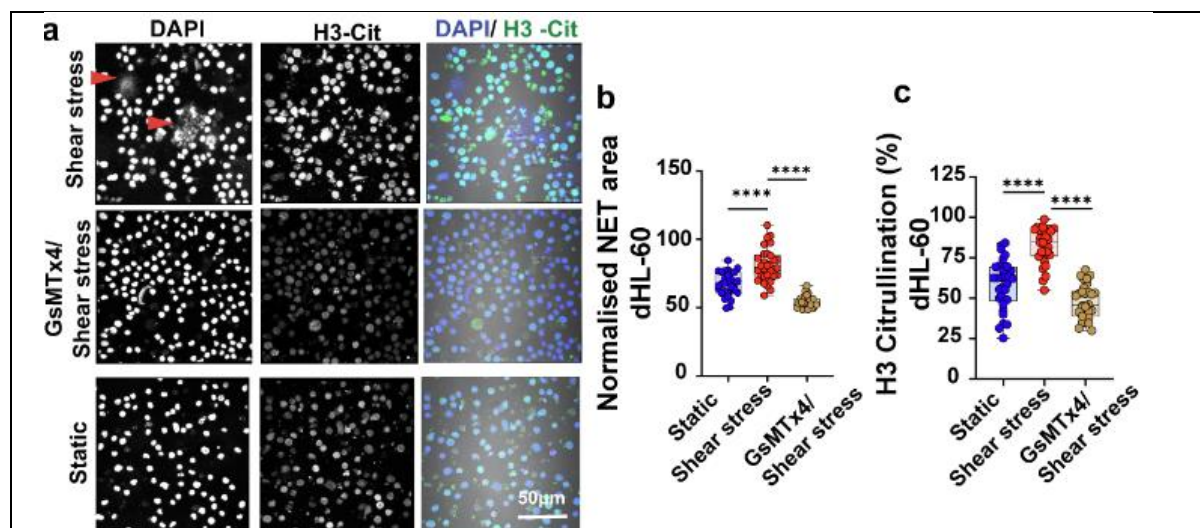
5. Most Piezo1 studies also use the pharmacological inhibitor GsMTx4, but this study relies solely on the knockdown in HL-60 cells (which doesn't apply to the primary neutrophils).

In direct response to the Reviewer's comment and to further confirm the contribution of Piezo1 in dHL-60 cells and in addition to the siRNA gene knockdown assay, we have now performed additional experiments using the pharmacological inhibitor of mechanosensitive ion channels, GsMTx4, as described below:

Page 11, Line 32:

Furthermore, we found that pre-treatment of neutrophils with GsMTx4 blocks the shear-induced NETosis and H3 citrullination in dHL-60 cells, confirming a role of mechanosensitive ion channels (**Figure 5a-c**).

Page 16, Figure 5 a-c:



Page 16, Line 3: (a-c) Representative fluorescence images and bar graphs showing the degree of NETosis and H3 citrullination (H3-Cit) in dHL-60 cells under static conditions or after shear stress in the presence or absence of GsMTX4. Red arrows in **a** showing NET release.

6. Quantifying the percent circular nuclei seems like an unnecessary way to treat a continuous variable as a binary variable and lose information in the process. The authors could instead quantify the shape factor or circularity of the cell nucleus... but in either case this is a puzzling metric for a multi lobed neutrophil nucleus, which is famously nonellipsoid in shape, unlike other cell types.

We agree with the Reviewer that there are alternative methods to assess the chromatin decondensation of neutrophils. Our goal was to use clear measures of nuclear circularity and neutrophil activation. Therefore, we have used the percentage of circular nuclei. To provide a more selective measure of neutrophil activation, we have assessed the effect of shear stress on NET area and now also include the percentage of H3 positive cells, as an additional marker of NETosis, in addition to the NET area measurements.

7. The authors should quantify the percentage of adherent platelets that are activated, perhaps by staining for surface P-selectin, or by examining their morphology under high magnification.

To directly follow the Reviewer's suggestion, we have now performed additional experiments and have assessed platelet activation after adhesion to NETs using P-selectin staining and assessment of platelet shape change, as described below:

Page 7, Line 4:

After shear stress stimulation of neutrophils at 80 dyne/cm² for 10 cycles (each cycle lasting 1 min), we allowed them to rest for 3 h inside a chamber slide, thereby facilitating the formation of NETs. Subsequently, the chamber slides containing neutrophils, were perfused with platelets for 10 min followed by several washing steps to remove non-adhering neutrophils. We measured the NET area covered by activated platelets using P-selectin staining and assessed changes of the platelet morphology (**Figure 2a**). Exposure of neutrophils to shear stress significantly increased platelet adhesion and average platelet cluster size over the NET area ($P < 0.001$) compared to the static control group. Platelet shape change, a prerequisite for platelet aggregation, was confirmed by assessing the platelet morphology after interaction with neutrophils exposed to shear stress or

resting neutrophils. Platelets exposed to shear stress-treated neutrophils exhibited a higher percentage of platelets with filopodia compared to those interacting with resting neutrophils (**Figure 2b**).

Page 8, Figure 2:

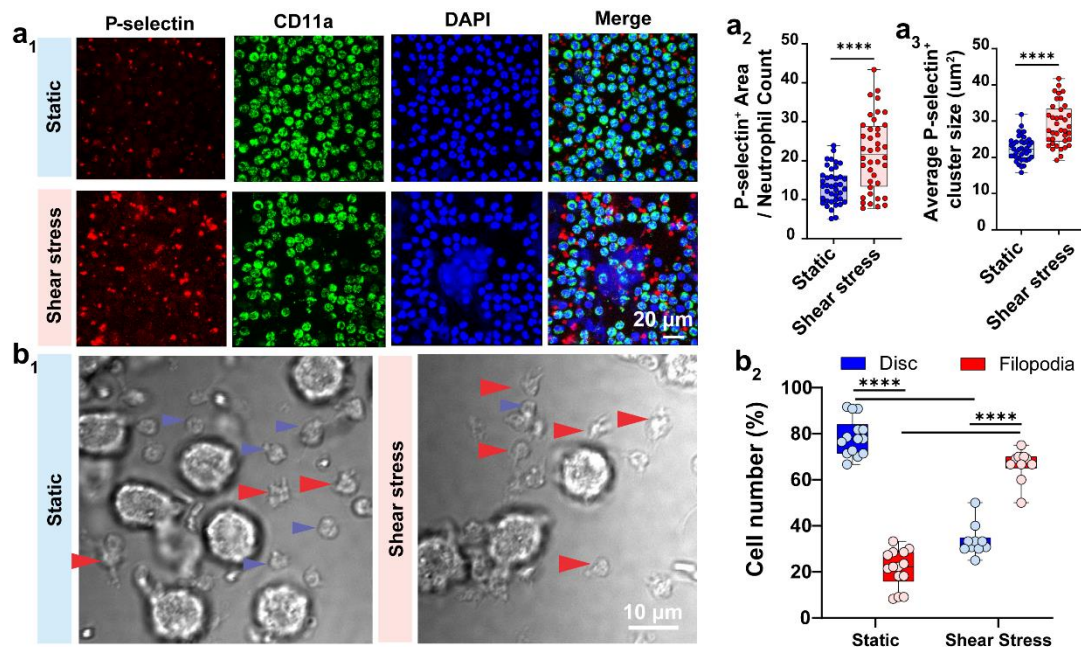


Figure 2. Shear stress induces NETosis and provides a scaffold for platelets to adhere to. (a₁) Representative confocal images of neutrophils under static conditions or exposure to shear stress of 80 dyne/cm² and incubated with platelets. Platelets are stained with P-selectin (red), neutrophils are stained with CD11a (green), and DNA is stained with DAPI (blue). (a₂-a₃) Bar graphs show the average area covered by P-selectin positive platelets normalized to the total neutrophil area and average P-selectin cluster size. (b₁) Representative confocal images and (b₂) a bar graph showing the effect of shear stress on platelet shape. Red arrows highlight platelets with filopodia, and blue arrows highlight platelets with discoid morphology. Box plots depict the minimum and maximum values (whiskers), the upper and lower quartiles, and the median. The length of the box represents the interquartile range. The graph represents data obtained from at least 3-4 independent donors. ***P<0.001.

Page 21, Line 23:

Here, we found that shear-induced NETosis can provide a scaffold for initial platelet adhesion, triggering further NETosis, platelet shape change and aggregation, and activation of downstream pathways.

8. Why didn't the authors use ratiometric imaging of calcium influx instead of a single channel measurement? The use of dual labels is generally more accurate.

We acknowledge the Reviewer's comment. Fluo-4 am is a well-accepted calcium-sensitive dye that has been used by us and others to measure changes in the [Ca²⁺]_i using standard confocal microscopy platform and standard 488 nm lasers and filter setting

that is available in our research facility. The ratiometric dye requires a special filter setting that we do not have access to. We apologize for this limitation.

9. The neutrophil membrane is more ruffled with microvilli projections, and not as smooth as most cell types. It has been shown that certain adhesion receptors like L-selectin, and PSGL-1 exist primarily at the tips of microvilli. Where is Piezo1 located on the membrane surface? On microvilli or everywhere equally?

We agree with the Reviewer that this is an interesting question. A recent study by Yang et al. in Nature Comm has demonstrated that Piezo1 is present on the microvilli structure of different cell types including HEK293 cells (doi: 10.1038/s41467-022-35034-6), although its direct expression on microvilli of neutrophils is not shown, it is also expected to be present to microvilli's projection as well as other parts of the neutrophils' membrane.

The discussion section has been updated to include further discussion on the membrane expression of Piezo1:

Page 22, Line 14:

The curve structure of Piezo1 is reported to play an important role in the activation of this ion channel by mechanical stress [66-69]. Furthermore, it is reported that membrane curvatures, such as present in microvilli, also regulate the distribution of Piezo1 on the plasma membrane of different cell types [70].

Reviewer #2 (Remarks to the Author):

General comments

This is a well-written manuscript with good organization and clear, logical flow. The authors attempt to answer a scientifically and clinically relevant question regarding regulation of NET formation by Piezo1. There is compelling evidence for shear stress-induced NETosis, however it is unclear why the authors did not continue to use the same shear stress dose that they proved to be biologically relevant in their dose response experiments (Figure 1). However, while the authors show that Ca^{2+} signaling and mechanosensitive ion channels are important for NETosis, the methods are not robust enough to determine the role of Piezo1 in shear stress induced NETosis, which is a key conclusion of the paper.

Major Comments

- 1. This paper does not definitively link Piezo1 activation with shear stress-induced NETosis. The authors show a reduction in NETosis with ruthenium red, GdCl_3 , and GSMTx4. However, these inhibitors are non-specific for Piezo1 and target many mechanosensitive cation channels (Figure 4b). The evidence for Piezo1-dependent NETosis is weaker. The authors induce shear-stress NETosis in HL-60 cells $\pm$ Piezo1 siRNA. Cell death is extremely high in both the static and shear stress conditions. NETosis is only marginally higher (~20%) in the control siRNA shear stress condition, however, only normalized NET area was measured, not CitH3, which is more specific for NETosis.**

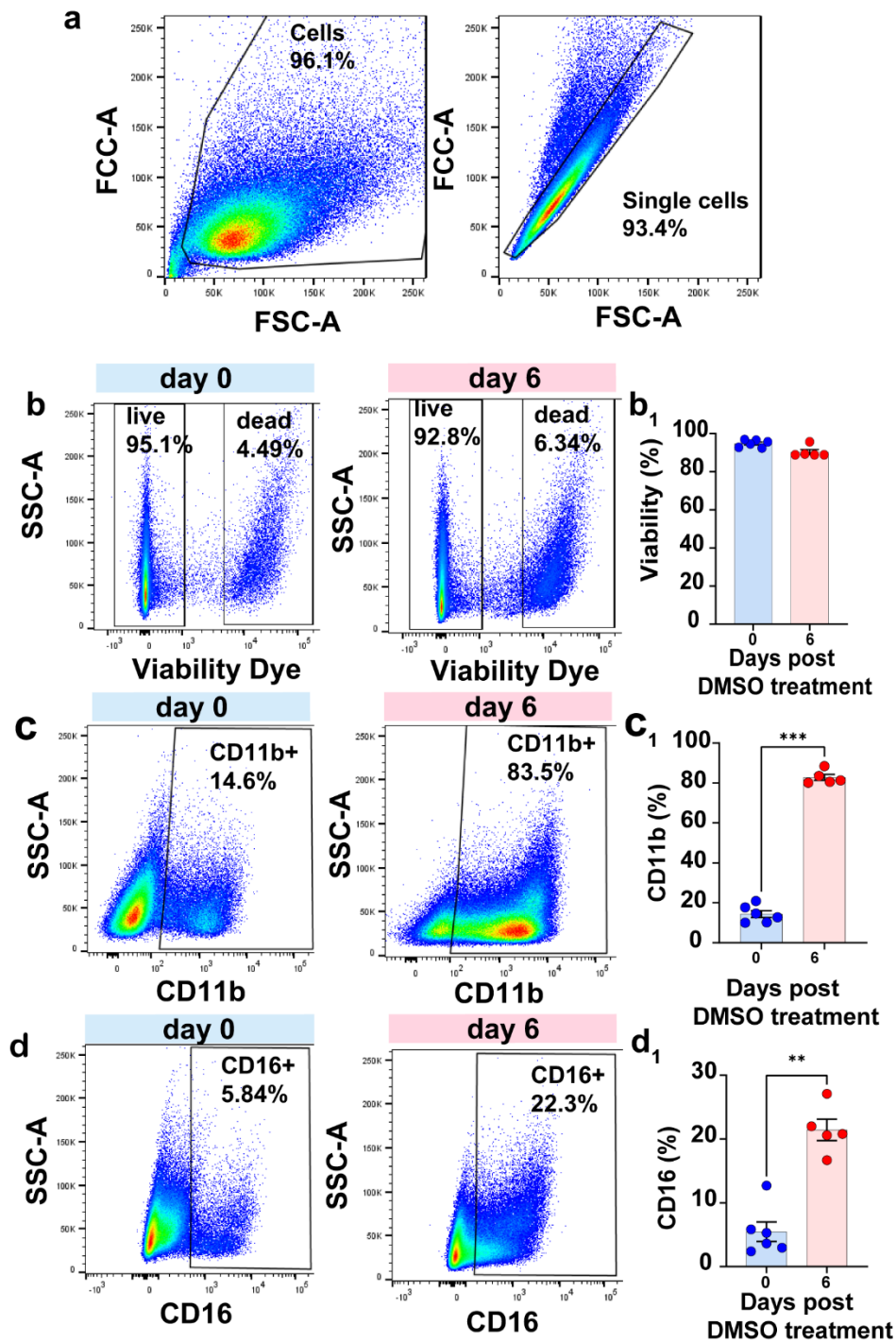
In direct response to the Reviewer's comments, we have conducted additional experiments to confirm the role of Piezo1 in HL-60 cells formation of NETs, by assessing CitH3, as described below.

Furthermore, in our preliminary assessments, we optimised the differentiation of HL60 cells so that after differentiation the viability of these cells is more 90%. Data from dHL-60 cells with more than 90% viability is now included in the revised manuscript.

Page 15, Line 7:

These cells can be cultured for a long time and differentiated into neutrophils using DMSO treatment. In this study, we differentiated HL-60 cells using 0.1% DMSO for 6 days (dHL-60) and confirmed the expression of the neutrophil markers CD11b and CD16. It should be noted that under this condition, we did not observe a significant decline in cell viability (Supplementary Figure 1).

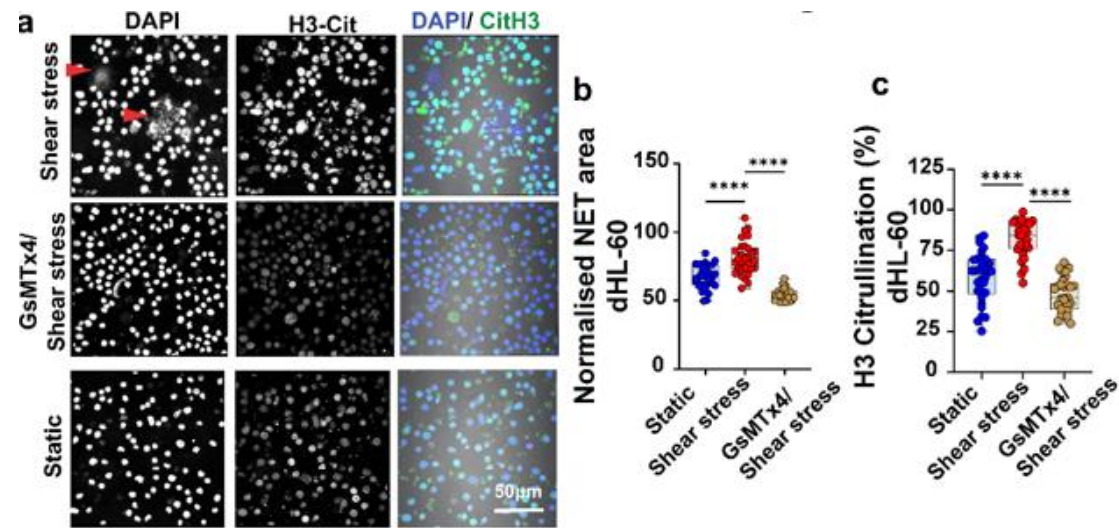
Supplementary Figure 1:



Supplementary Figure 1. Gating strategy and flow cytometry analysis of HL60 cells differentiation with DMSO. (a) Representative gating strategy for HL-60 cells. **(b-d)** representative flow cytometry plots and **(b₁-d₁)** bar graphs of HL-60 cells before and post 6 days of differentiation assessed for viability **(b-b₁)**, expression of **(c-c₁)** CD11b and **(d-d₁)** CD16. Data is analyzed with paired Student's t-test. **P<0.01, ***P<0.001.

First, we confirmed that shear stress induces NETosis in dHL-60 cells. For this, we exposed dHL-60 cells to 10 cycles of shear stress followed by 3 h of static rest to allow them to form NETs. Using this approach, we found that shear stress induces NETosis and increases the CitH3 in dHL-60 cells, similar to primary neutrophils. Furthermore, we found that pre-treatment of neutrophils with GsMTx4, blocks shear-induced NETosis and H3Cit in dHL-60 cells, confirming the role of mechanosensitive ion channels (**Figure 5a-c**).

Page 16, Figure 5:

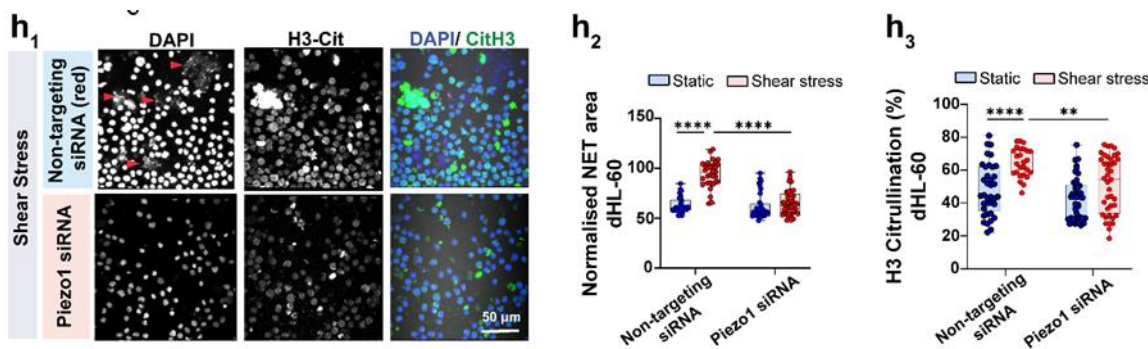


Page 16, Line 4: (a-c) Representative fluorescence images and bar graphs showing the degree of NETosis and H3 citrullination (H3-Cit) in dHL-60 cells under static conditions or after shear stress stimulation in the presence or absence of GsMTX4. Red arrows in **a** showing NET release.

Page 15, line 25:

Furthermore, we found that partial knockdown of Piezo1 in dHL-60 cells blocked the capacity of neutrophils to form NETs and reduces the percentage of cells with H3 citrullination compared to their static control group, while exposure of dHL-60 cells treated with scrambled siRNA to shear stress led to a 1.2 ± 0.1 fold increase in NET area and 1.6 ± 0.1 fold increase in percentage of cells with H3 citrullination compared to the static control group (**Figure 5h₁₋₃**).

Page 16, Figure 5h:



Page 16, line 11:

(h₁) Representative confocal images and (h₂₋₃) bar graphs showing the degree of NETosis and H3 citrullination in dHL-60 cells treated with Piezo1 siRNA or non-targeting siRNA, stained with DAPI (blue) and H3 citrullination antibody (green) in response to shear stress.

2. It is unclear why there is different shear stress doses used for different read outs (80 dyne/cm² for NETosis imaging, 0.2-10 dyne/cm² for Ca²⁺ signalling), concern about induction of cell death at 80 dyne/cm² but should be repeated for all experiments.

We thank the Reviewer for this very relevant question. To assess the change in intracellular calcium levels, we immobilized neutrophils inside a microfluidic channel precoated with Cell-Tak™ and measured changes in intracellular calcium. Any shear stress higher than 20 dyne/cm² led to the detachment of cells, preventing the assessment of changes in intracellular calcium levels. This method revealed the sensitivity of these cells to shear stress, with an increase in intracellular calcium in a dose-dependent manner in response to changes in shear stress.

Due to the technical limitations described above, we are unable to measure calcium signalling under 80 dyne/cm². However, we demonstrated that neutrophils could sense and respond to an elevation in shear stress (Figure 3a-c).

For the assessment of the effect of shear stress on NETosis (Figure 1b), we exposed neutrophils to different levels of shear stress. We observed a detectable level of NET formation within our experimental time at 80 dyne/cm², prompting us to use this level of shear stress for the remainder of the study to identify signalling pathways leading to NET formation.

3. Conclusions about the effect of ATP on NETosis is mixed and the authors state that there is an additive effect of shear stress and ATP, but their data does not support.

This is an interesting question. We found that shear stress stimulation does not change the NET area in response to ATP (Figure 1E); however, it increases the percentage of H3-positive cells (Figure 1f), and this data is consistent with a previous report.

The discussion has been updated accordingly, as below:

Page 21, line 8:

Here, we found that shear stress enhances H3 citrullination in response to ATP, a marker of neutrophil activation. However, shear stress does not increase the degree of NETosis in response to ATP, and this difference could be attributed to the timing of the experiment. This finding aligns with a previous report that documented an increase in H3 citrullination and PAD4 expression in response to ATP, but not in the context of NETosis [53].

4. Additional scientific methods need to be shown in order to clearly show (i) platelets adhering to neutrophils, (ii) calcium influx in addition to immunofluorescence images (need to clearly state in methods how max calcium intensity was performed), (iii) Piezo1 knockdown needs to be quantified by western blot and the lanes properly labeled) or another method, and (iv) other standard measures of NETosis in dHL-60 cells as showed in human neutrophils.

We followed the Reviewer's well justified suggestions as following:

(i) Additional experiments have been performed to confirm platelet adhesion to NETs, as described below:

Page 7, line 4:

After shear stress stimulation of neutrophils at 80 dyne/cm² for 10 cycles (each cycle lasting 1 minute), we allowed them to rest for 3 h thereby facilitating the formation of NETs. Subsequently, the cells were perfused with platelets for 10 min. We measured the NET area covered by activated platelets using P-selectin staining and assessed platelet morphology changes (**Figure 2a**). Exposure of neutrophils to shear stress significantly increased platelet adhesion and average platelet cluster size over the NET area ($P < 0.001$), compared to the static control group. Platelet shape change, a prerequisite for platelet aggregation, was confirmed by assessing platelet morphology after interaction with neutrophils exposed to shear stress or resting neutrophils. Platelets exposed to shear stress-treated neutrophils exhibited a higher percentage of platelets with filopodia compared to those interacting with resting neutrophils (**Figure 2b**).

Page 8, Figure 2:

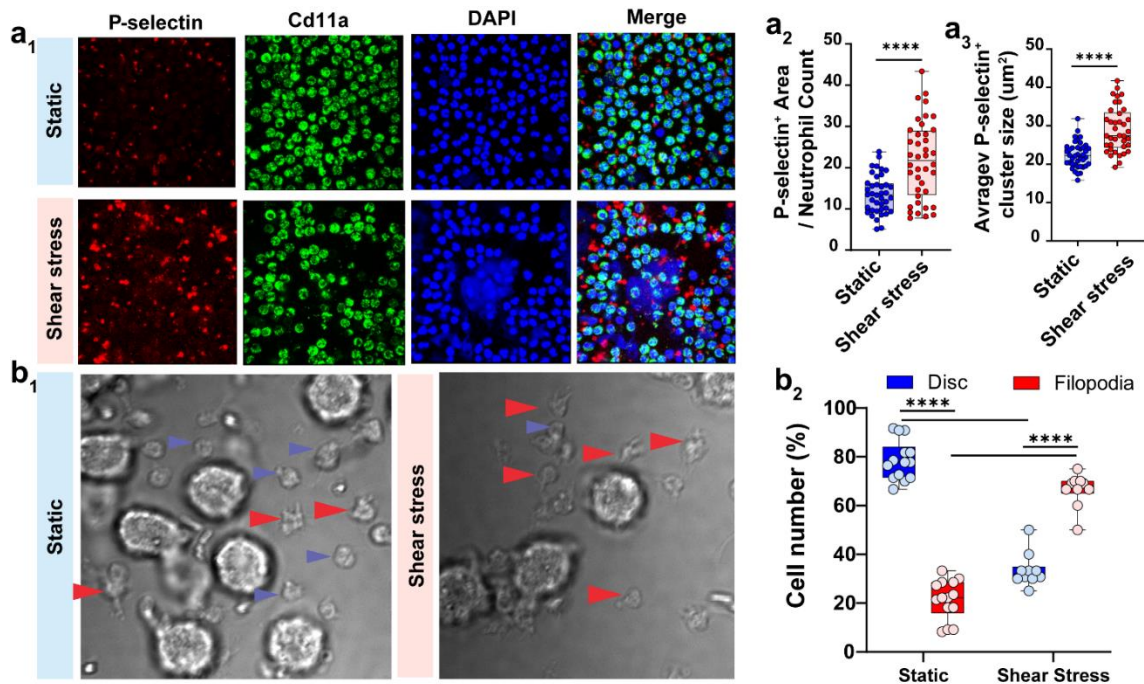


Figure 2. Shear stress induces NETosis and provides a scaffold for platelets to adhere to. (a₁) Representative confocal images of neutrophils under static conditions or exposure to shear stress of 80 dyne/cm² and incubated with platelets. Platelets are stained with P-selectin (red), Neutrophils are stained with CD11a (green), and DNA is stained with DAPI (blue). (a₂-a₃) Bar graphs show the average area covered by P-selectin positive platelets normalized to the total neutrophil area and average P-selectin cluster size. (b₁) Representative confocal images and (b₂) a bar graph showing the effect of shear stress on platelet shape change. Red arrows highlight platelets with filopodia and blue arrows highlight platelets with discoid morphology. Box plots depict the minimum and maximum values (whiskers), the upper and lower quartiles, and the median. The length of the box represents the interquartile range. The graph represents data obtained from at least 3-4 independent donors. ***P<0.001.

(iii) The Method section is updated to describe how max calcium intensity is quantified, as described below:

Page 27, line 3:

To assess the change in [Ca²⁺]_i levels, individual regions of interest (ROIs) were drawn around neutrophils using NIS Element software, and changes in fluorescence intensity across the experiment's duration were measured. Subsequently, the maximum value of fluorescence intensity of each ROIs measured for each experiment was selected to evaluate the cellular response to shear stress and the effectiveness of different treatment conditions in inhibiting the increase in [Ca²⁺]_i levels in response to shear stress.

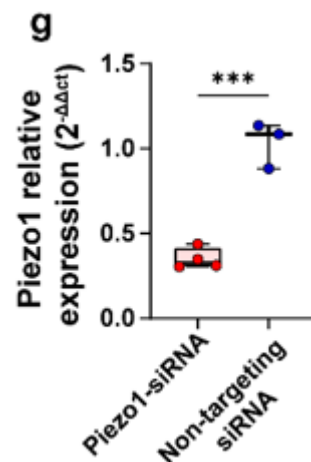
(iv) *Piezo1* knockdown in dHL-60 cells was confirmed using qPCR, as described below.

Page 28, line 11:

Quantitative Polymerase Chain Reaction

To assess *Piezo1* expression, RNA was extracted using the RNeasy Mini Kit (QIAGEN). The purity of RNA was then assessed using a NanoDrop (Thermo Fisher Scientific), and complementary DNA (cDNA) was synthesized with the SuperScript IV VILO Master Mix kit (Invitrogen). Subsequently, qPCR was conducted using the TaqMan Master Mix kit (QIAGEN) and the following primers: Hs00207230_m1 for *PIEZO1* and Hs00609296_g1 for *HMBS* (Thermo Fisher Scientific), serving as the internal control. The QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) was employed for qPCR, and all assays were performed according to the suppliers' instructions.

Page 16, Figure 5g:

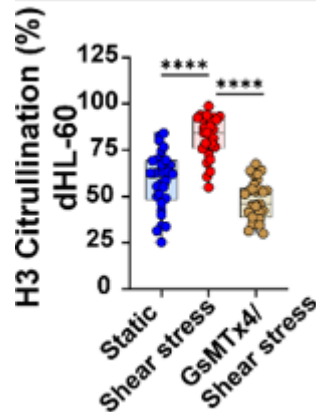


Page 16, Line 9:

(g) qPCR data confirming the knockdown of *Piezo1* in dHL60 cells treated with *Piezo1* siRNA compared to non-targeting siRNA

(iv) *H3* citrullination is assessed in dHL-60 cells before and after exposure to shear stress

Page 16, Figure 5c:



Page 16, Line 2:

a-c) Representative fluorescent images and bar graphs showing the degree of NETosis and H3 citrullination (H3-Cit) in dHL-60 cells under static conditions or after shear stress stimulation in the presence or absence of GsMTx4. Red arrows in **a** showing NET release.

5. Interpretation of actin cytoskeleton contribution to NET formation is not accurate to just look at displacement, which is usually a readout for migration. Need to consider looking at actin assembly or actin subtypes (F/G actin)

We directly followed the Reviewer's suggestion. Here, to further assess the effect of shear stress on actin dynamics in neutrophils, we assessed the disassembly and assembly of actin using G/F actin staining.

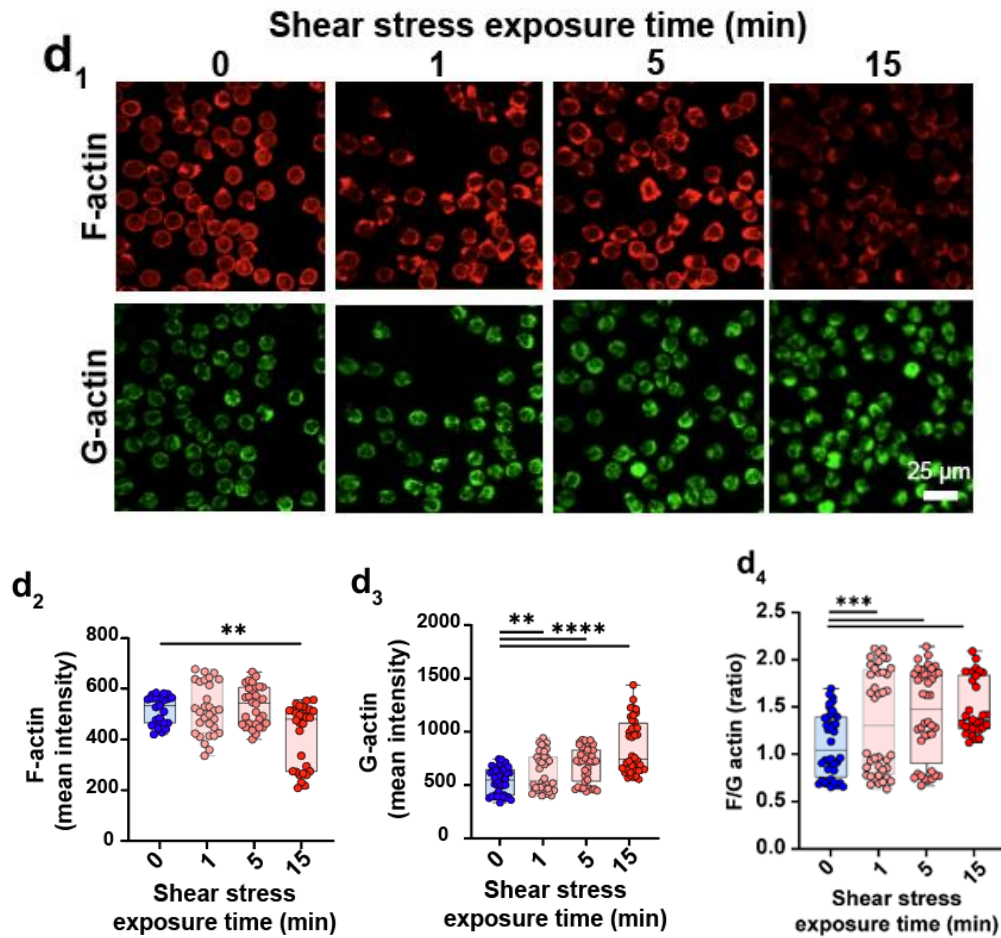
Page 29, line 16:

To assess actin dynamics, following each experiment, neutrophils were immediately fixed with 4% paraformaldehyde in PBS for 1 h on ice. Subsequently, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed with PBS, and stained with Alexa Fluor 488 DNase I to label G-actin and Alexa Fluor 568 phalloidin to label F-actin.

Page 17, Line 18:

Thus, to further confirm the effect of shear stress on cytoskeleton remodeling in neutrophils, we subjected them to shear stress (80 dyne/cm²) for 1, 5, and 15 min. We assessed the impact of shear stress exposure time on actin cytoskeleton organization by measuring the intensity and ratio of G-actin to F-actin. During the initial 5 min of shear stress exposure, the intensity of F-actin remains stable but by the 15-min mark, it significantly declined compared to baseline levels (P<0.01). Concurrently, the intensity of G-actin increased in a time-dependent manner following shear stress stimulation compared to baseline (P<0.01). Analysis of the G/F-actin ratio revealed a gradual increase during the first 15 min of shear stress exposure (**Figure 6 d₁₋₃**). Overall, this experiment suggests that shear stress exposure triggers the assembly, disassembly, and remodelling of the actin cytoskeleton.

Page 19, Figure 6d:



Page 19, Line 6:

(**d₁**) Representative confocal images of neutrophils exposed to shear stress for different duration of time and stained with Alexa Fluor 488 DNase I to label G-actin (green) and Alexa Fluor 568 phalloidin to label F-actin (red). Representative graphs showing the intensity of (**d₂**) F- actin, (**d₃**) G-actin and (**d₄**) F/G actin ratio.

Page 23, Line 6:

These previous data combined to our data presented here, change in the intensity and ratio of G to F actin and increase in cell motility as an independent marker of cytoskeleton remodelling, suggests that a functional actin cytoskeleton exists in the beginning of the NETosis process.

Minor Comments

- 1. The authors describe both classical and alternative pathways for NETosis (page 3, lines 62-71), however, it is unclear if this paper examines one or both pathways.**

The discussion section has been updated to directly address this important Reviewer comment as below:

Page 28, Line 12:

In conclusion, we demonstrate for the first time that Ca^{2+} influx via Piezo1 regulates NETosis in neutrophils subjected to shear stress. Furthermore, here we conclude that shear stress-induced NETosis was part of the classical pathway of NETosis, as this process took at least 3-4 h, was accompanied by nuclear and cell membrane rupture, which consequently leads to cell death.

- 2. The shear stress condition is well described in the methods, however, treatment for the static condition is unclear.**

We apologize for this. The Method section has been updated, and the treatment of the static group is now well described, as below:

Page 26, line 22:

The static control group was rested inside the chamber slide for the duration of the shear stress stimulation, under otherwise the same conditions.

- 3. In general, the methods section leaves out key methods, such as protein quantification, and reagents, such as Piezo1 antibody.**

We apologize for this. The Method section is updated and further information is provided as below:

Page 26, line 22:

The static control group was rested inside the chamber slide for the duration of the shear stress stimulation, under otherwise the same conditions. To assess the role of extracellular Ca^{2+} in shear induced NETosis, neutrophils were exposed to shear stress in the presence or absence of 2mM EGTA in the cell culture media. Followed by that, cells were collected and incubated inside the chamber slide coated with Cell-Tak™ (Cat# CLS354240, Corning, USA) for 3 h to allow them to adhere and form NETs.

Page 27, line 3:

To assess the change in $[\text{Ca}^{2+}]_i$ levels, individual regions of interest (ROIs) were drawn around neutrophils using NIS Element software, and changes in fluorescence intensity across the experiment's duration were measured. Subsequently, the maximum value of fluorescence intensity of each ROIs measured for each experiment was selected to evaluate the cellular response to shear stress and the effectiveness of different treatment conditions in inhibiting the increase in $[\text{Ca}^{2+}]_i$ levels in response to shear stress. For all

images, the amount of decondensed nuclei, total cell count, NET area assessment, and percentage of H3 positive cells were assessed using ImageJ.

Page 28, Line 1:

Western blot analysis

For extraction of whole cellular protein, primary neutrophils or HL-60 cells were rinsed with cold PBS and lysed with RIPA buffer, on ice for 30 min. The protein densitometry analysis was performed using Pierce BCA protein assay kit following the supplier's instructions. Western blot experiments were performed using standard protocols and as described in previously [28]. Rabbit anti-Piezo1 antibody (Novus biological, NBP1-78537) or mouse anti-GAPDH antibody (Santa Cruz, sc-166574 #2118S) were used as primary and mouse anti-rabbit HRP (Santa Cruz biotechnology, #sc-2357) or mouse IgG kappa binding protein HRP (Santa Cruz biotechnology, #sc-516102) were used as secondary antibodies.

Page 28, Line 12:

To assess Piezo1 expression, RNA was extracted using the RNeasy Mini Kit (QIAGEN). The purity of RNA was then assessed using a NanoDrop (Thermo Fisher Scientific), and complementary DNA (cDNA) was synthesized with the SuperScript IV VILO Master Mix kit (Invitrogen). Subsequently, qPCR was conducted using the TaqMan Master Mix kit (QIAGEN) and the following primers: Hs00207230_m1 for PIEZO1 and Hs00609296_g1 for HMBS (Thermo Fisher Scientific), serving as the internal control. The QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) was employed for qPCR, and all assays were performed according to the suppliers' instructions.

Page 29, line 16:

To assess actin dynamics, following each experiment, neutrophils were immediately fixed with 4% paraformaldehyde in PBS for 1 h on ice. Subsequently, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed with PBS, and stained with Alexa Fluor 488 DNase I to label G-actin and Alexa Fluor 568 phalloidin to label F-actin. Images were acquired using a Nikon A1 confocal microscope (Nikon, Japan) using a 60× objective lens with oil immersion.

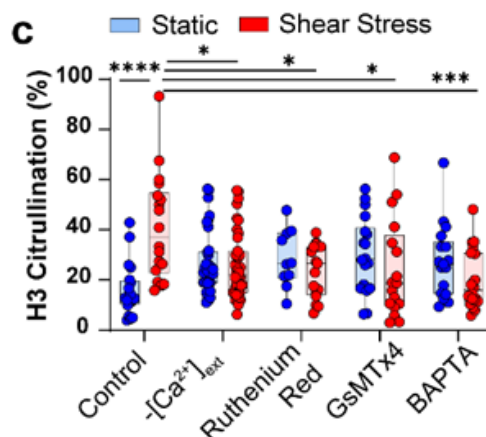
- 4. There may be non-specific cell death induced by the shear stress treatment. How do you quantify the contribution of non-specific cell death vs. NETosis? If requires citH3 then all NETosis readouts should include citH3 and not just chromatin morphology as a readout of NETosis, such as in Figures 4, 5, and 6.**

We directly followed the Reviewer's suggested and included analyses of the change in H3 citrullination in the Result section of all the respected results (figures), as bellow:

Page 11, line 6:

Furthermore, removal of calcium from the media using EGTA or pretreatment of neutrophils with ruthenium red and GSMTx4 eliminated the shear-induced NETosis and Citrullination of H3, confirming that Ca^{2+} influx via mechanosensitive ion channels controls shear-induced NETosis (**Figure 4b-c**).

Page 13, Figure 4c:



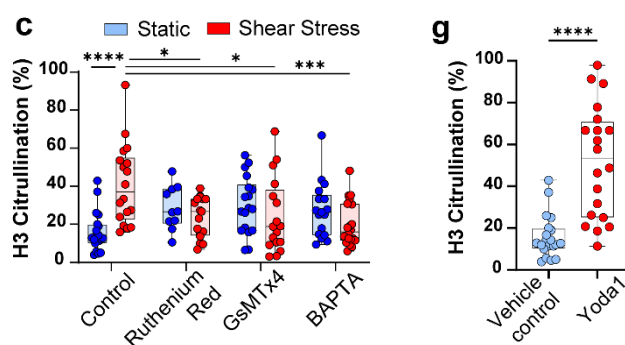
Page 13, Line 3:

and (c) percentage of cells H3 citrullination in response to shear stress of 80 dyne/cm² in the presence or absence of extracellular Ca²⁺ (2mM EGTA), ruthenium red (30 μM), GaCl₃ (30 μM), GsMTx4 (20 μM), thapsigargin (1 μM), and BAPTA (10 μM).

Page 11, Line 23:

We found that treatment of neutrophils with Yoda1 for 3 h led to a significant increase in NET area (P<0.001, **Figure 4g**), citrullination of H3 (P<0.001, **Figure h**) and a consequent increase in platelets adhering to NETs (P<0.001, **Figure 4i**).

Page 18, Figure 4c and g:



Page 13, line 3:

(c) percentage of cells with H3 citrullination in response to shear stress of 80 dyne/cm² in the presence or absence of ruthenium red (30 μM), GaCl₃ (30 μM), GsMTx4 (20 μM), thapsigargin (1 μM), and BAPTA (10 μM).

Page 14, line 4:

(g) percentage of cells H3 citrullination

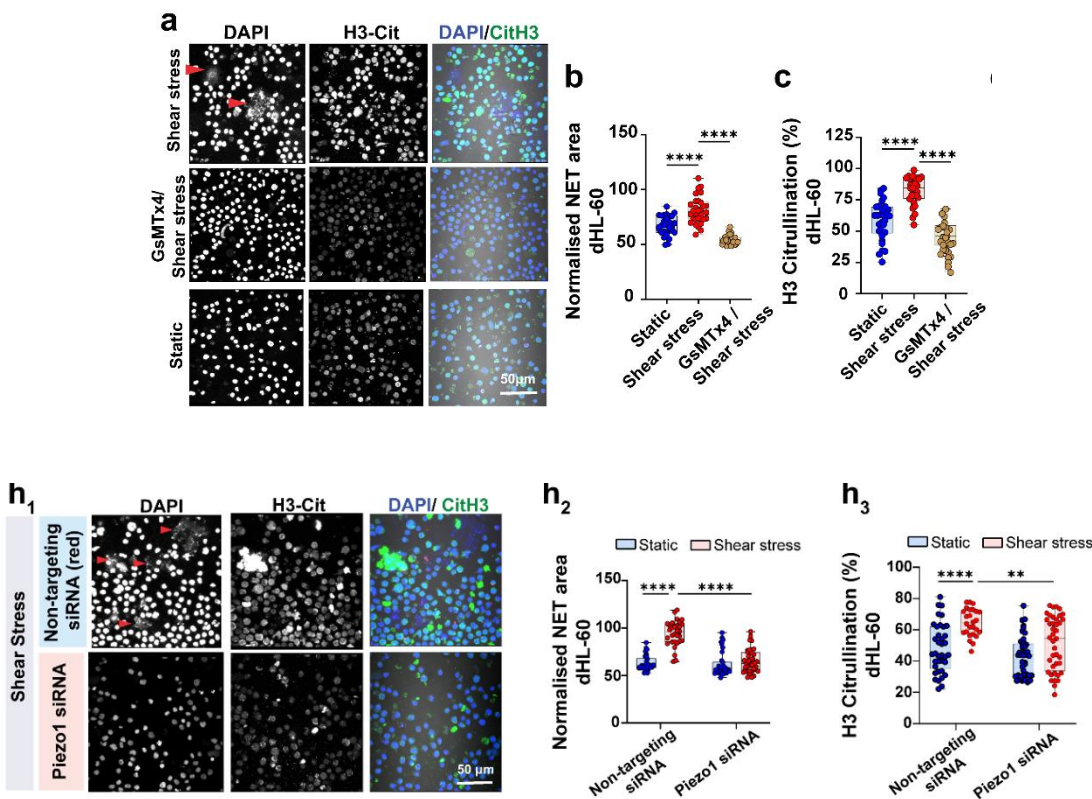
Page 15, line 14:

Furthermore, we found that pre-treatment of neutrophils with GsMTx4, blocks shear induced NETosis and H3 citrullination in dHL-60 cells, confirming the role of mechanosensitive ion channels (**Figure 5a-c**).

Page 15, line 25:

Furthermore, we found that partial knockdown of Piezo1 in dHL-60 cells blocks the capacity of neutrophils to form NETs and reduces the percentage of cells with H3 citrullination compared to their static control group. While exposure of dHL-60 cells treated with scrambled siRNA to shear stress led to a 1.2 ± 0.1 fold increase in NET area and 1.6 ± 0.1 fold increase in percentage of cells with H3 citrullination compared to their static control group (**Figure 5h₁₋₃**).

Page 16, Figure 5a-c and h₁₋₃:



Page 16, line 3:

(a-c) Representative fluorescence images and bar graphs showing the degree of NETosis and H3 citrullination in dHL-60 cells under static conditions or after shear stress stimulation in the presence or absence of GsMTX4.

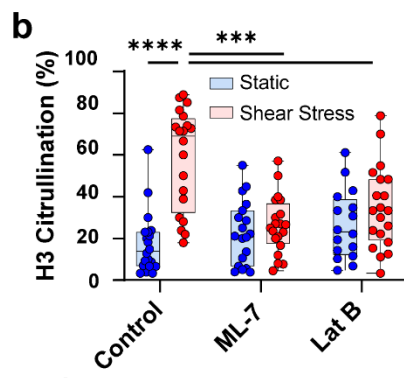
Page 16, line 12:

(h₁) Representative confocal images and (h₂₋₃) bar graphs showing the degree of NETosis and H3 citrullination in dHL-60 cells treated with Piezo1 siRNA or non-targeting siRNA, stained with DAPI (blue) and H3 citrullination antibody (green) in response to shear stress.

Page 17, line 12:

We found that both treatments reduced the NET area and H3 citrullination of neutrophils in response to shear stress ($P < 0.001$, **Figure 6a-b**).

Page 19, Figure 6b:



Page 19, line 2:

Graphs show (a) the normalized NET area and (b) H3 citrullination of neutrophils pretreated with ML-7 or latrunculin B under static conditions or shear stress.

5. I think there may be a typo on page 12, line 292. The range for shear stress is listed as 0.2-20 dyne/cm², but the experiment uses 0.2-10 dyne/cm².

We apologize for this typo, which is now corrected on page 12, as below:

Page 9, line 6:

For this experiment, we immobilized neutrophils inside a microfluidic channel and exposed them to different shear stress levels of 0.2, 2, and 10 dyne/cm².

6. In Figure 4a, authors show that Ca²⁺ is required for Ca²⁺ signaling. I would also include the effect of abrogating Ca²⁺ signaling on NET formation.

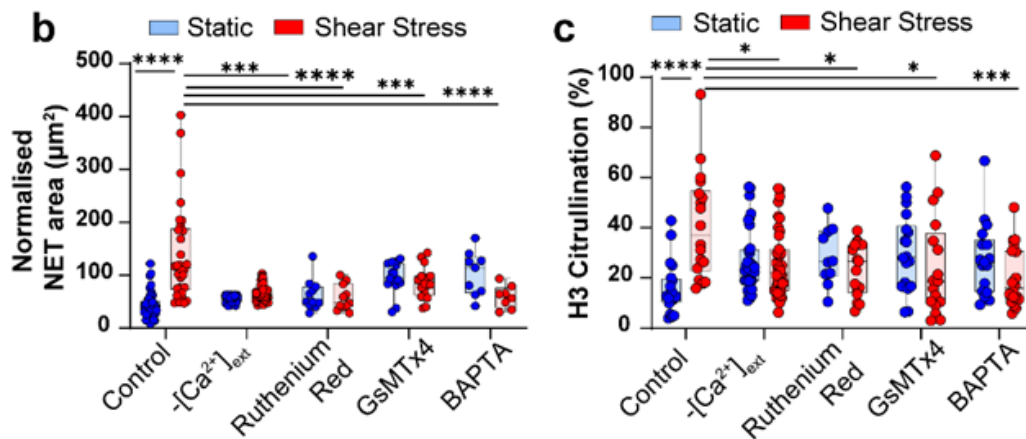
Directly following the Reviewer's suggestion, we have now performed additional experiments to assess the role of Ca²⁺ in the extracellular space on the ability of neutrophils to form NETs, as described below:

Page 11, line 6:

Furthermore, removal of calcium from the media using EGTA or pretreatment of neutrophils with ruthenium red and GSMTx4 eliminated the shear-induced NETosis and

Citrullination of H3, confirming that Ca^{2+} influx via mechanosensitive ion channels controls shear-induced NETosis (**Figure 4b-c**).

Page 13, Figure 4:



Page 13, Line 2:

(b) Percentage of area covered by NETs and **(c)** percentage of cells H3 citrullination in response to shear stress of 80 dyne/cm² in the presence or absence of extracellular Ca^{2+} (2 mM EGTA), ruthenium red (30 µM), GaCl₃ (30 µM), GsMTx4 (20 µM), thapsigargin (1 µM), and BAPTA (10 µM).

7. I would add discussion about why inhibition of cytoskeletal rearrangement is protective against NETosis (Figure 6), when in the introduction inhibition of the cytoskeleton is one mechanism of NETosis (page 3, lines 62-64).

We thank the Reviewer for this comment. The discussion section has now been updated and the contribution of actin dynamics in early stages of NETosis has been discussed, as given below:

Page 22, Line 32:

This is consistent with earlier reports showing that functional actin cytoskeleton is required for the early stage of NETosis. For example, PMA treatment of neutrophils rapidly increases the actin polymerization, where it reaches a peak in 60 min and then gradually decreases to the baseline at 180 min [71]. Furthermore, another study has revealed that neutrophil elastase degrades F-actin 30 min post exposure of neutrophils to candida albicans [73]. These previous data combined with our data showing an increase in cell motility as an independent marker of cytoskeleton remodelling, suggests that a functional actin cytoskeleton exists in the beginning of the NETosis process.

8. I am not sure of the significance of presenting shear stress-induced motility and phagocytosis in the context of the rest of the paper.

We followed the Reviewer's suggestion and have removed the Result section of the phagocytosis to focus more on NETosis.

Mortality data are additional experiments, that can indirectly confirm the effect of shear stress on cytoskeleton remodeling and are required to further confirm the contribution of cytoskeleton elements in NET formation in response to shear stress.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed my prior comments. I appreciate the new experiments and discussion. I have no further comments and it seems ready for publication from my perspective.

Reviewer #2 (Remarks to the Author):

The authors have responded adequately to a number of the previous suggestions. Specifically, they responded satisfactory to most of my Major Comments. However, I still have major concerns regarding the authors' NEW Figure 5a and 5h immunofluorescence data as the background does not appear consistent and merged colors are not clear on the new images. I recommend that they fix these images and redo the analysis before accepting the publication. All of my other comments have been addressed satisfactorily.

Reviewers' comments

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed my prior comments. I appreciate the new experiments and discussion. I have no further comments and it seems ready for publication from my perspective.

Reviewer #2 (Remarks to the Author):

The authors have responded adequately to a number of the previous suggestions. Specifically, they responded satisfactory to most of my Major Comments. However, I still have major concerns regarding the authors' NEW Figure 5a and 5h immunofluorescence data as the background does not appear consistent and merged colors are not clear on the new images. I recommend that they fix these images and redo the analysis before accepting the publication. All of my other comments have been addressed satisfactorily.

The immunofluorescence images in Figures 6a and 7a (old Figure 5a and 5h) have been updated. The brightfield channel has been removed from the merged images to improve visibility. This change has not affected the findings of these result sections.

In addition, Figure 4 and 6 has been divided into two figures to address the word limits of the figure legends and figure size limits respectively. The figure numbering has been corrected accordingly. Figure 4c3 has been updated, and the same number of cells per experimental group has been analyzed and presented. This has not affected the statistics.

Figure 8d4 is now presented as the F/P ratio to better demonstrate the decline in actin depolymerization. Raw data for this paper is presented in the source data file. The data availability statement has also been updated. All figure legends have been updated, and all queries from the editorial office have been addressed. We have used the Figshare link to share the movies associated with Figures 3a-b and 4e1-e2.