

Letter to the Editor

Defective nuclear entry of hydrolases prevents neutrophil extracellular trap formation in patients with chronic granulomatous disease

To the Editor:

Neutrophil antimicrobial activity depends on phagocytosis, neutrophil extracellular trap (NET) formation (NETosis), and cytokine production. NETosis is dependent on NADPH oxidase (NOX2)-driven reactive oxygen species (ROS) production, nuclear access of neutrophil elastase (NE), and histone degradation.¹ ROS production is deficient in patients with chronic granulomatous disease (CGD), resulting in impaired NETosis and recurrent severe bacterial and fungal infection.²

To shed light on the poorly understood steps downstream of ROS resulting in NETosis, several groups have recently explored macroautophagy,³⁻⁸ a cellular degradation pathway for cytoplasmic constituent delivery to lysosomes, which reshapes membrane compartments and therefore could be involved in hydrolase access to the nucleus. However, in all of these studies, no direct correlation of macroautophagic activity and NETosis

could be reported, and only pharmacologic inhibition of phosphatidylinositol 3 (PI3)-kinase activity was used to compromise macroautophagy. Therefore we decided to revisit the regulation of macroautophagy and ROS production by PI3-kinase inhibition during NETosis in neutrophils from patients with CGD and healthy donors (HDs).

NETosis was induced by phorbol 12-myristate 13-acetate (PMA; Fig 1, A) or *Candida albicans* (Fig 1, B) stimulation together with PI3-kinase inhibitors in neutrophils from HDs and patients with CGD, and DNA release was followed over time by using SYTOX assays. Both stimuli resulted in NETosis in neutrophils from HDs. However, only inhibition of PI3-kinases through 3-methyladenine (3-MA) caused a significant decrease in NETosis induced by both stimuli. Previously, nuclear NE translocation was suggested to initiate chromatin decondensation and subsequent NETosis.¹ NE's nuclear translocation, as assessed by means of confocal microscopy, was also reduced with 3-MA after opsonized yeast and PMA incubation (see Fig E1, A, and the Methods section in this article's Online Repository at www.jacionline.org). Therefore the PI3-kinase inhibitor 3-MA and, to

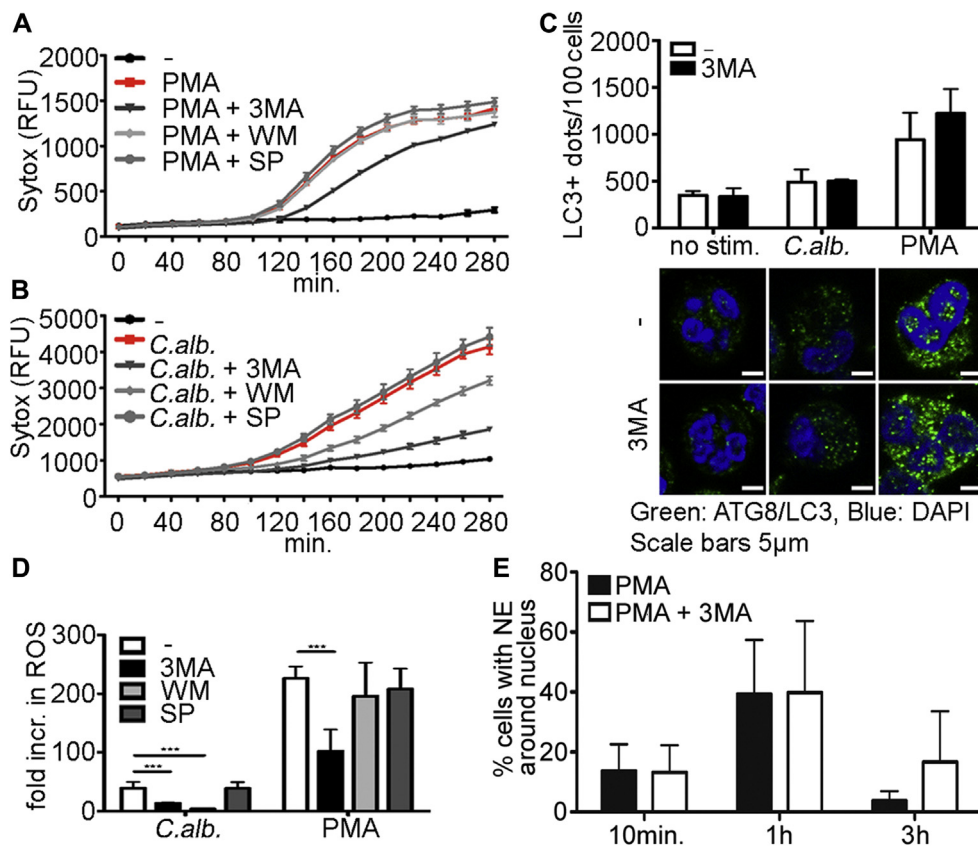


FIG 1. PI3-kinase inhibition decreases NETosis and ROS production without altering macroautophagy. **A** and **B**, SYTOX-based NET assay in PMA-stimulated (Fig 1, A) or *Candida albicans*-stimulated (Fig 1, B) neutrophils with or without PI3K inhibitors ($n = 3$, mean $\pm$ SD). **C**, Quantification of macroautophagy in neutrophils from HDs after 1 hour of stimulation (mean $\pm$ SD). Neutrophils of 6 HDs ($n \geq 100$ cells/well) are shown as triplicates from 2 independent HDs. Scale bars = 5 μ m. **D**, ROS production after 30 minutes of incubation, as indicated (mean $\pm$ SD). *** $P < .001$. **E**, Quantification of NE signal around nucleus of PMA-stimulated neutrophils with or without 3-MA (mean $\pm$ SD). Means $\pm$ SDs pooled from 4 HDs are shown. RFU, Relative fluorescence units; SP, spautin-1; WM, wortmannin.

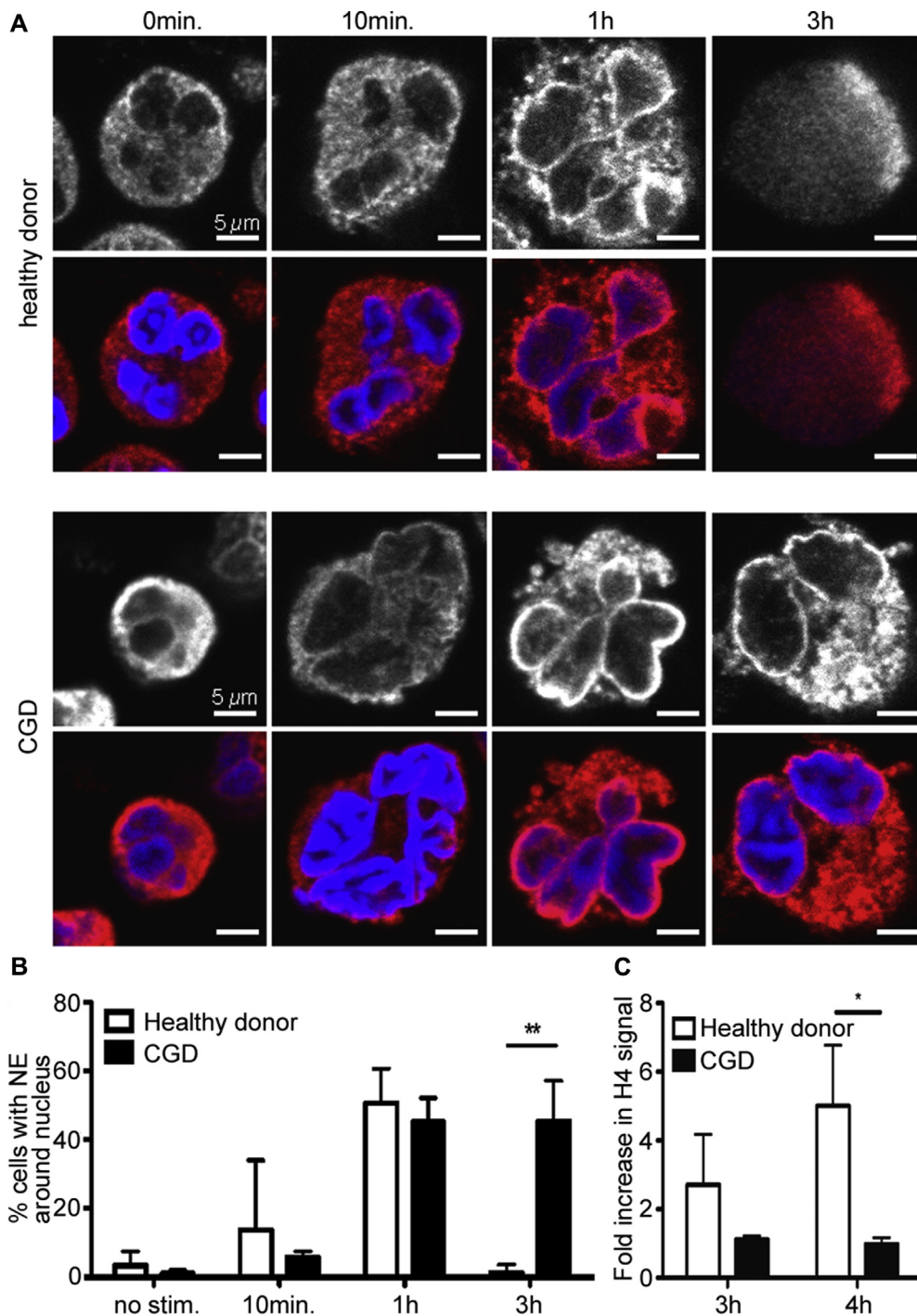


FIG 2. NE nuclear entry requires ROS for histone H4 degradation during NETosis. **A**, PMA-stimulated neutrophils from HDs or patients with CGD were analyzed for NE (red; black/white) and DAPI (blue) staining by means of immune fluorescence microscopy. Values from 2 HDs and 3 patients with CGD are shown. Scale bars = 5 μ m. **B**, NE signal around the nucleus of PMA-stimulated neutrophils (mean $\pm$ SD). ** $P < .01$, t test. **C**, Quantification of fold decrease in H4 levels in neutrophils from 9 HDs and 6 patients with CGD with or without PMA. * $P < .05$, t test.

a lesser extent, other PI3-kinase inhibitors, such as wortmannin and spautin-1, can inhibit NETosis.

Because 3-MA is classically used as a macroautophagy inhibitor, we investigated accumulation of autophagosomes in neutrophils treated with opsonized yeast and PMA with or without 3-MA through immunofluorescent quantification of dots

positive for light chain 3B (LC3B), which becomes attached to autophagosome membranes. Interestingly, on 1 hour of stimulation, 3-MA did not seem to effectively reduce autophagosome formation when compared with that seen in untreated cells (Fig 1, C). In addition, membrane association of LC3B, which was detected as LC3-II levels by means of Western blotting, was

not significantly different with or without 3-MA (see Fig E1, B). Along these lines, neutrophils of NETosis-incompetent patients with CGD induced macroautophagy to a similar extent as those from HDs after stimulation with opsonized yeast or PMA (see Fig E2 in this article's Online Repository at www.jacionline.org). In contrast, PI3-kinase inhibition with 3-MA consistently reduced both yeast- and PMA-induced ROS production (Fig 1, D), suggesting that the negative effect of 3-MA on NETosis might be related to the ineffectiveness of cells to generate ROS rather than blocking macroautophagy. Together, these results point to a macroautophagy-independent but ROS-dependent mechanism of PI3-kinase to control NETosis in human neutrophils.

To further investigate the steps leading to NETosis, we looked into the kinetics of NE localization. Confocal microscopy of NE in PMA-treated neutrophils from HDs and patients with CGD confirmed the absence of extracellular or intranuclear NE staining in neutrophils from patients with CGD (Fig 2, A and B). One hour after PMA stimulation, cells from patients with CGD and HDs recruited NE to the perinuclear region. Whether this NE is granule bound or released into the cytosol and physically associated with the nuclear membrane could not be determined. Three hours after PMA treatment, NE was still accumulating around cell nuclei of patients with CGD, whereas in HDs NE had already migrated into the nuclear region. Similarly, 3-MA-treated cells showed a trend toward stabilization of NE staining in the perinuclear area 3 hours after PMA treatment (Fig 1, E, and see Fig E3 in this article's Online Repository at www.jacionline.org).

As a second measure of neutrophil hydrolases' access to the nucleus, we assessed proteolysis of histone H4 (Fig 2, C, and see Fig E4 in this article's Online Repository at www.jacionline.org). After PMA activation, neutrophils from HDs initiated NETosis, and H4 was degraded to allow DNA decondensation (see Fig E4, A), whereas in cells from patients with CGD, H4 levels remained stable 3 and 4 hours after PMA induction (see Fig E4, B). This indicates that neutrophils deficient in phagosome-associated NOX2-mediated ROS production are unable to induce NE nuclear translocation but can nevertheless accumulate NE around their nuclei.

To confirm that the classical macroautophagy machinery was not involved in the transport of vesicular NE to the nucleus, we assessed perinuclear colocalization of LC3B and NE in neutrophils isolated from HDs using confocal microscopy. As previously observed (Fig 2), NE translocated to the area around the nucleus 1 hour after stimulation with PMA (see Fig E1, C and D), whereas LC3B did not accumulate there. These results indicate that NE trafficking to the nucleus does not depend on autophagosomes.

In contrast, previous studies suggested that PI3-kinase inhibition affects NETosis through compromising macroautophagy.³⁻⁸ However, most of these studies did not analyze the effect of PI3-kinase inhibition on ROS production.^{4-6,8} Moreover, although PI3-kinase inhibition with 3-MA was reliably able to downmodulate DNA release after NETosis stimulation, autophagosome-associated LC3-II levels could not be decreased in some of these studies, and *vice versa*, the PI3-kinase inhibitor wortmannin was able to compromise macroautophagy in some studies but did not affect DNA release.^{3,4,7,8} In addition, macroautophagy stimulation by means of inhibition of the mammalian target of rapamycin increased DNA release after NETosis stimulation, but it was not analyzed whether macroautophagy

inhibition would block this synergistic effect.⁶ Thus these previous studies and ours demonstrate that 3-MA reliably diminishes DNA release during NETosis but that LC3-II-coupled autophagic membranes are not decreased in most studies,⁷ whereas 3-MA compromises NOX2-dependent ROS formation.

PI3 phosphates are a class of phospholipid that coordinate the membrane localization and function of many proteins in the cell. These effector proteins usually contain the lipid-binding domains FYVE or PX. For example, the PX domain of the NOX2 p40phox subunit was described to be important for effective ROS production on phagocytosis of opsonized bacteria.⁹ Accordingly, PI3-kinase inhibition in neutrophils might prevent correct assembly of NOX2 and therefore diminish the oxidative burst in these cells, affecting their NETotic response. Indeed, we observed diminished ROS production on 3-MA treatment. Thus we favor the hypothesis that PI3-kinase inhibition compromises NETosis by inhibiting ROS formation to prevent neutrophil hydrolase access to the nucleus for DNA decondensation. If ROS formation represents the central axis for triggering NETosis in neutrophils, interfering with this pathway should be harnessed clinically.

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METHODS

Antibodies and cell dyes

For Western blotting, anti-ATG8/LC3 antibody (clone 5F10) was purchased from Nanotools (Teningen, Germany), and anti-histone H4 antibody (clone 62-141-13) was obtained from Millipore (Temecula, Calif). Directly labeled anti- β -actin (horseradish peroxidase [HRP]) antibody was obtained from Abcam (Cambridge, United Kingdom). For immunofluorescence staining, anti-ATG8/LC3 was received from MBL (Des Plaines, Ill), and anti-NE was kindly provided by Arturo Zychlinsky's laboratory (Berlin, Germany). Secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 555 were purchased from Invitrogen (Carlsbad, Calif). The nucleic acid stains SYTOX green and 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) and the redox-sensitive probe Amplex UltraRed were provided by Invitrogen.

Cell preparation

Blood was drawn from HDs (Zurich Blood Center) and 6 patients with CGD (University Children's Hospital Zurich) after obtaining informed consent from patients or parents in accordance with the Declaration of Helsinki and local ethical provisions. Neutrophils were isolated by means of density gradient centrifugation on Ficoll-Hypaque. The lower layer containing granulocytes was subjected to hypotonic lysis of red blood cells, followed by ice-cold PBS washing. Cells were resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated FBS and used immediately after isolation. For testing the production of ROS, cells were kept in PBS throughout the assay.

NET induction

Neutrophils were left unstimulated or treated with 100 nmol/L PMA or opsonized *Candida albicans* yeast. The *C. albicans* yeast-locked mutant strain Δ hgc1 was grown overnight at 30°C in Sabouraud medium and subcultured to reach the exponential growth phase. For opsonization, 2×10^7 yeast cells were washed in PBS, resuspended in 1 mL of 20% human plasma (isolated from the top layer after Ficoll-Hypaque gradient centrifugation), and incubated at 37°C for 20 minutes. Cells were again washed in PBS and added to the respective neutrophils at a 1:1 ratio. Where indicated, 30 minutes before 100 nmol/L PMA or opsonized *C. albicans* stimulation, cells were treated with the following PI3-kinase inhibitors: 5 mmol/L 3-MA, 50 nmol/L wortmannin or 1 μ M spautin-1.

NETosis occurred within 2 to 3 hours after stimulation. Neutrophils (5×10^4) were seeded per well in flat-bottom 96-well plates incubated with the appropriate stimuli and 1 μ mol/L SYTOX green to quantify the kinetics of NETosis. Fluorescence values were measured with a Tecan Infinite M200 Pro fluorometer (extinction 485 nm/emission 520 nm at 37°C; Tecan, Männedorf, Germany) every 20 minutes for a total of 280 minutes (mean $\pm$ SD; 3 independent assays with different donors). In addition, NETosis was assessed by means of quantification of the number of cells with positive nuclear staining for NE. Cells ($n \geq 100$ /well) were counted from confocal pictures by using ImageJ software (National Institutes of Health, Bethesda, Md).

Immunofluorescence and microscopy

For immunofluorescence staining, 4×10^5 neutrophils were seeded per well on poly-lysine-treated 8-well chamber slides (Ibidi, Planegg, Germany) and stimulated as indicated for 1 hour. After treatment, cells were fixed in 3% paraformaldehyde for 20 minutes at 4°C, permeabilized with 0.5% Triton-X100 for 1 minute at room temperature, and then incubated with the Image-iT FX signal enhancer (Invitrogen) and stained with the indicated antibodies followed by the appropriate secondary reagent. All washes were performed in PBS supplemented with 1% fish skin gelatin and 0.02% saponin. Slides were counterstained with DAPI and mounted with 50% glycerol in PBS. Cells were visualized through a $\times 63$ magnification 1.4 NA oil immersion lens with an inverted CLSM Leica SP5 confocal microscope (Leica, Wetzlar, Germany). For quantification of the fluorescence signal of the different antibodies, part of the perinuclear region was selected to calculate the intensity values by using ImageJ software. Data are expressed as dot plots, with median values displayed as a horizontal red line.

Measurement of ROS

Production of ROS by neutrophils was assessed with the cell-permeable fluorescent probe Amplex UltraRed. Neutrophils were seeded in flat-bottom 96-well plates, 2×10^5 cells/well, and stimulated with opsonized *C. albicans* (1:1 ratio) or 100 nmol/L PMA and with a mixture of 25 μ mol/L Amplex UltraRed and 0.5 U/mL HRP for 30 minutes at 37°C. Fluorescence values were analyzed in a Tecan Infinite M200 Pro fluorometer (extinction 530 nm/emission 580 nm).

Western blot analysis

At the indicated times, $6 \times$ Laemmli buffer with 1% β -mercaptoethanol was added to the neutrophils at a final concentration of $1 \times$ to obtain protein extracts. Cells were scraped off the wells and sonicated for 30 seconds at 50% intensity. Resulting cell lysates were frozen at -80°C for later immunoblotting. For Western blot analysis, cell lysates were boiled for 5 minutes, resolved by means of SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. For detection of primary antibodies, HRP-conjugated secondary antibodies and the ECL Femto detection system were used. Membranes were visualized in a Vilber Lourmat Fusion FX imaging system (Vilber Lourmat, Marne-la-Vallée, France) or exposed to films, and densitometry was performed with ImageJ software.

Histone degradation assay

Neutrophils were seeded at a density of 5×10^5 cells/well in 48-well plates. After stimulation with PMA for the indicated times, cell lysates were generated, and H4 degradation was analyzed by means of immunoblotting.

Statistical analysis

Where indicated, unpaired Student *t* tests were performed with GraphPad Prism Software (Version 5.0a; GraphPad Software, La Jolla, Calif).

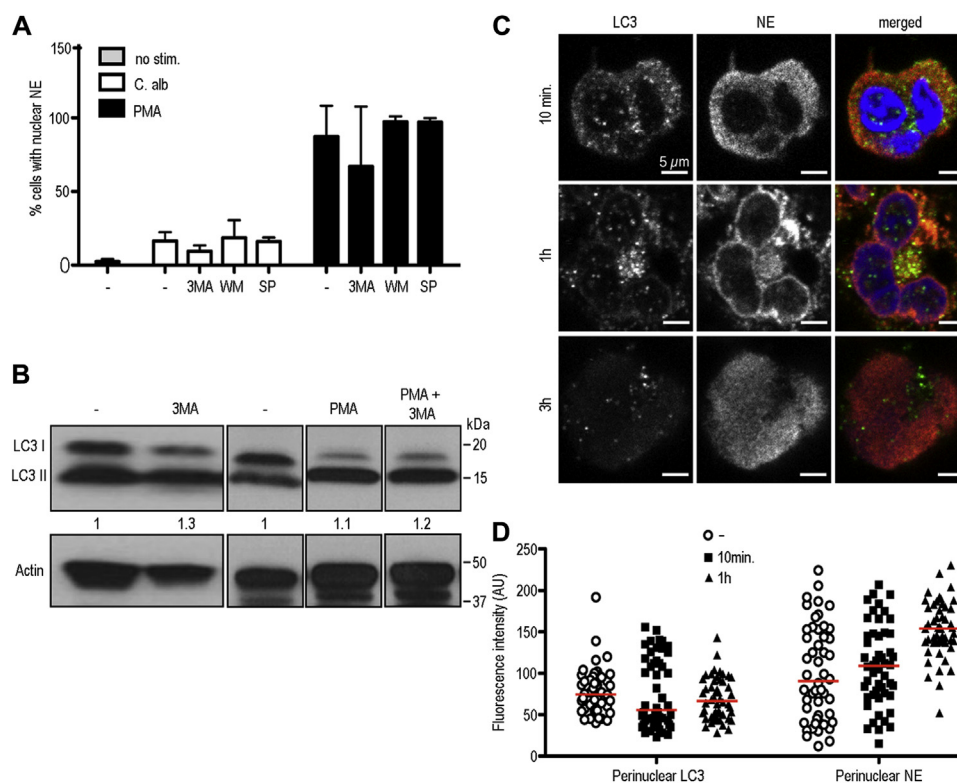


FIG E1. PI3-kinase inhibition blocks NE release but minimally affects macroautophagy in human activated neutrophils. **A**, Quantification of extracellular or nuclear NE in neutrophils with or without 3-MA, wortmannin (*WM*), and spautin-1 (*SP*) with or without stimulation with opsonized *C. albicans* (1:1 ratio) or PMA for 3 hours (pooled data from 4 HDs; 75 cells/donor; mean $\pm$ SD). **B**, Neutrophils from HDs were treated with or without 3-MA and with or without PMA, and LC3-II levels were assessed by means of Western blotting (1 blot of ≥ 3). Numbers below the blot represent the fold increase of LC3-II compared with nonstimulated control cells after normalization to corresponding actin levels. **C**, Neutrophils from HDs were incubated with PMA for 10 minutes, 1 hour, or 3 hours; fixed; permeabilized; and stained for ATG8/LC3 (green), NE (red), and DAPI (blue). Representative confocal pictures of 1 of 50 cells analyzed are shown. Data are from 3 HDs. **D**, Quantification of fluorescence intensity for ATG8/LC3 and NE signals in the perinuclear region of neutrophils from HDs ($n = 50$). Cells were left unstimulated or treated with PMA at the indicated time points. Pooled data are from 3 HDs.

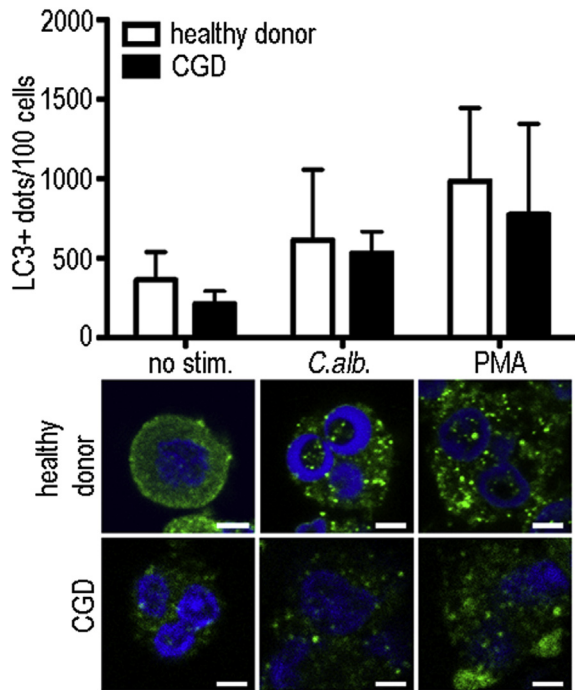


FIG E2. Intact macroautophagy in neutrophils from patients with CGD. Quantification of ATG8/LC3⁺ dots (ATG8/LC3 green, DAPI blue) on neutrophils ($n \geq 100$; mean $\pm$ SD) from 3 HDs and 4 patients with CGD after 1 hour of stimulation. Scale bars = 5 μ m.

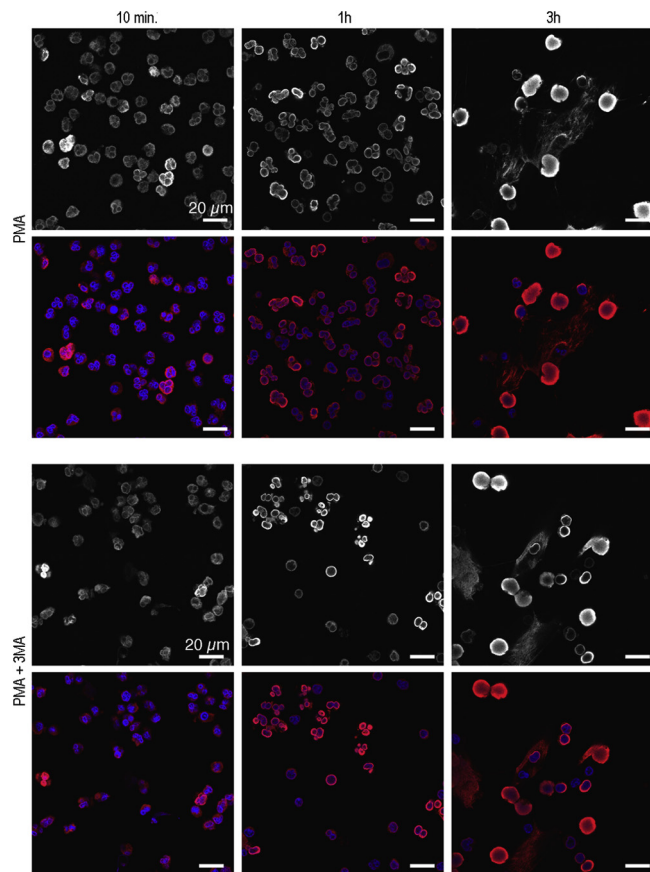


FIG E3. PI3-kinase inhibition prevents NE access to the nucleus of neutrophils. Immunofluorescence analysis of PMA-stimulated neutrophils from HDs ($n = 4$ HDs) with or without 3-MA, NE (red, merged color signals; black/white, confocal pictures), and DAPI (blue). Quantification is shown in Fig 1, E. Scale bars = 20 μ m.

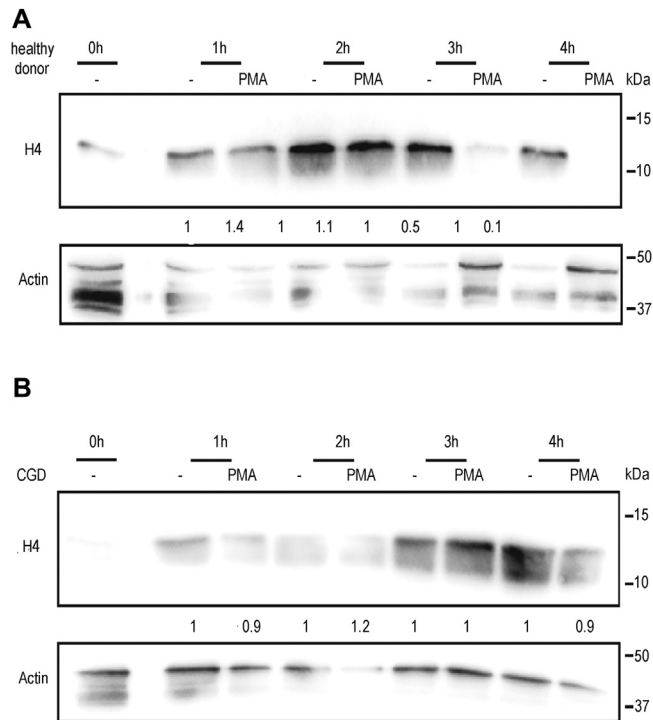


FIG E4. ROS triggers nuclear histone degradation. Western blot analysis (1/6 blots) of histone H4 in neutrophils from HDs (**A**) or patients with CGD (**B**) with or without PMA. Numbers indicate the fold increase of histone H4 compared with that nonstimulated control values (normalized to actin). Quantification is shown in [Fig 2, C](#).