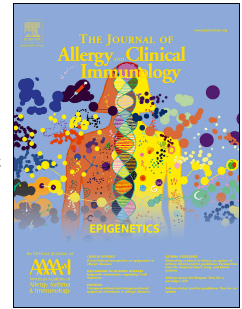


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**Tamoxifen restores extracellular trap formation in neutrophils from patients of chronic
granulomatous disease, in ROS-independent manner**

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Capsule Summary

Tamoxifen induces autophagy-dependent NET formation in CGD patient neutrophils and improves their antimicrobial activity. Given the genetic defect in their ROS-generation machinery, this study highlights repurposing of tamoxifen to potentiate host defense in CGD patients.

40

41 **To the Editor:**

42 Chronic Granulomatous Disease (CGD) is a primary immunodeficiency characterized by life-
43 threatening acute and recurrent bacterial and fungal infections (1). Mutations in one of the
44 several subunits of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme
45 complex, responsible for generation of reactive oxygen species (ROS) constitute the genetic
46 defects attributed to CGD. As ROS generation is a major antimicrobial defense mechanism of
47 neutrophils, defective oxidative killing of pathogens underlies the frequent and severe infectious
48 episodes in CGD patients. Therapeutic measures to correct the antimicrobial capacity of
49 neutrophils could be an attractive strategy for treating this devastating immune disorder.

50 In addition to oxidative killing, a recently established antimicrobial activity of neutrophils is the
51 formation of extracellular traps (NETs), which are decondensed chromatin fibrils coated with
52 granular proteases and histones (2). NETs can trap and kill extracellular pathogens, without the
53 need for phagocytosis, by placing them in close proximity to antimicrobial components.
54 Because NET formation (NETosis) has largely been reported to be dependent on reactive oxygen
55 species (ROS) generation by the NADPH oxidase complex, and neutrophils from CGD patients
56 are reported to be deficient in NETosis, impairing extracellular pathogen killing (2, 3) .
57 Consequently, gene therapy to restore NADPH oxidase function for potentiation of NET
58 formation is thought to be a viable therapy for infectious sequelae in this disease (3). However,
59 this approach is severely limited by reduced survival of gene-corrected cells, and has not
60 advanced beyond experimental salvage therapy. Here we report a new strategy to induce NETs
61 by treating CGD patient neutrophils with an FDA-approved drug tamoxifen (TMX). We show

that TMX does not require NADPH oxidase activity to induce NET formation and does so in a ROS-independent manner by activating autophagy.

We first compared NET formation in neutrophils freshly isolated from venous blood of healthy subjects and those from CGD patients. Purity was checked by flow cytometry and microscopy (Suppl. Fig. 1). As shown in Fig. 1A upper panel, in-vitro stimulation with LPS, an inflammatory TLR agonist from Gram-negative bacteria, live *Staphylococcus aureus* (SA), a common bacterial pathogen affecting CGD patients (1) or Phorbol myristate acetate (PMA, used as a positive control) generated NETs in healthy adult neutrophils within 4hrs of stimulation. These NETs, characterized as extruded DNA fibrils, were visualized using a fluorescent DNA stain Sytox Green. In contrast to healthy subjects, CGD patient neutrophils showed significantly impaired NET formation in response to all three stimuli (Fig. 1A, B &C). These observations confirm that CGD neutrophils exhibit impaired NET formation in response to inflammatory stimuli as well as bacterial infection. We then tested if TMX could restore this function in CGD neutrophils. Indeed, as shown in Fig. 1B, TMX treatment restored the NET formation in neutrophils from CGD patients upon LPS stimulation as well as bacterial infection, to the levels exhibited by healthy adult neutrophils (Fig 1C). Furthermore, TMX-induced NETs were restored in all patients tested, regardless of the antibiotic or steroid treatment that these patients were undergoing at the time of sample collection (Supplementary Table 1). TMX treatment significantly increased the extent of NET formation in healthy adult neutrophils as well. Importantly, CGD and healthy neutrophils exhibited significantly increased NET-mediated bacterial killing upon TMX treatment (Fig. 1D), albeit the effect was more pronounced in CGD. These data strongly supported the therapeutic potential of this drug. TMX-treated CGD neutrophils formed NETs despite an absent NADPH oxidase activity (confirmed by DHR test in these patients, data not shown). This suggested that TMX induces NET formation in a ROS independent manner. To confirm this, we measured cytosolic ROS in healthy human neutrophils stimulated with LPS

or PMA with or without TMX treatment. In line with previous reports, PMA induced robust oxidative burst in human neutrophils, while LPS stimulation showed moderate ROS formation (Fig. 2A) (E1). TMX treatment did not induce any ROS formation by itself. Interestingly, this drug significantly reduced the cytosolic ROS induced by PMA or LPS in neutrophils (Fig. 2A). These data show that TMX-mediated NET formation does not require NADPH oxidase activity.

We have recently reported that autophagy activation accelerates NET formation in murine neutrophils (E2). We thus examined whether TMX treatment induced NET formation via autophagy activation in human CGD neutrophils. Aggregation of nascent autophagy protein LC3, also called ATG8 or LC3-I) into LC3-PE complex (LC3-II) serves as a marker of autophagy activation and autophagosome formation (E3). Western blot analysis of unstimulated CGD and healthy human neutrophils showed quiescent autophagy as indicated by low or no processed LC3 II (Fig. 2B, NS). Treatment with LPS or TMX individually or in combination induced robust LC3 II processing in healthy subject neutrophils, indicating autophagy activation. In contrast, neutrophils from CGD patients showed minimal LC3 II upon LPS treatment suggesting impaired autophagy activation. TMX treatment with or without LPS led to a robust increase in the amount of LC3-II in human CGD neutrophils (Fig. 2B). This shows that, in the absence of ROS, autophagy activation is sufficient to induce NET formation. Because healthy adult and CGD neutrophils behaved in a seemingly similar fashion upon TMX treatment, we next utilized healthy neutrophils to examine whether autophagy is necessary for TMX-induced NET formation. Fig. 2C depicts immunofluorescence imaging of LC3 II puncta formation in neutrophils stimulated with LPS with or without TMX treatment in the presence or absence of NSC185058, a specific inhibitor of cysteine protease ATG4B which is required for LC3 II lipidation and puncta formation (E4). As previously observed, TMX treatment alone induced robust NET formation and also increased LPS-induced NET formation (Sytox Green stained

panels). This increase in NETosis correlated with enhanced numbers and size of LC3 II puncta (red stained panels) which localized to the nuclei (blue stained panels) of neutrophils stimulated with LPS and TMX. Importantly, treatment with the autophagy inhibitor NSC185058 almost completely inhibited NET formation induced by LPS or TMX, which directly correlated with absence of LC3 II puncta in these cells. Together, these data strongly suggest that autophagy activation is necessary and sufficient to induced NET formation upon TMX treatment.

In summary, we show here that Tamoxifen, an FDA approved drug in use for breast cancer treatment, rescues NET formation in neutrophils from human CGD patients, completely bypassing the requirement of NADPH oxidase-derived ROS. Our data of enhanced NET-mediated bacterial killing by CGD neutrophils demonstrates a novel therapeutic potential of TMX in this disease. Autophagy activation was found to be the underlying mechanism for TMX mediated NET formation. To the best of our knowledge, ours is the first report connecting TMX activated autophagy to its NET-inducing function in CGD. Autophagy-inducing action of TMX has been reported in cancer and is linked to the modulation of cholesterol biosynthesis by TMX (4). A detailed analysis of upstream autophagy components and their cross-talk, if any, with cholesterol biosynthesis in TMX-induced NET formation in CGD is a fascinating area of research currently being pursued in our lab. Of note, TMX-induced NET formation was inhibited by treatment with ATG4B inhibitor NSC185058 (Fig. 2B) which suppresses autophagosome generation, but not by inhibitors (wortmannin and LY294002) of class III PI3K activity essential for initiation of autophagy (data not shown). This suggests that TMX acts through ATG4 activation.

Defects in autophagy have been linked to systemic hyperinflammation and colitis frequently observed in CGD patients (5). Therefore, treatment of CGD patients with TMX could also

reduce inflammatory sequelae, in addition to improving antimicrobial activity by restoring NET formation. Since TMX is already FDA approved for treatment of another disease, our studies make this drug an attractive option for use in CGD patients. The promise of TMX is underscored by ongoing efforts to repurpose this drug for treatment of glioblastoma multiforme, desmoid tumors and bipolar disorders (6-8). It is also important to note that TMX exhibits thromboembolic and uterotrophic side effects of low, but significant increase in endometrial cancer at clinical doses approved for treating breast cancer (9). However these effects are attributed to estrogen-receptor dependent function of TMX, as opposed to its ER-independent effects at higher, albeit well-tolerated, doses (6). Future studies regarding the detailed mechanism of action and controlled trials would provide valuable information regarding efficacy and safety of TMX for its clinical use in CGD patients.

We are indebted to the patients and their families for providing us the opportunity to do this study. We thank Dr. Kol Zarembek at NIH and Sarah Abrahamson at UND for technical help with microscopy.

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Keywords: Neutrophils extracellular traps, Chronic Granulomatous Disease, Tamoxifen, Autophagy

Figure Legends

Figure 1. TMX treatment restores NET-formation in neutrophils from CGD patients. (A) Healthy adults or CGD patient neutrophils were stimulated with LPS, PMA or *S. aureus* (SA), with or without TMX pretreatment (B) followed by detection of NETs using DNA dye Sytox Green. Bar=200 μ . (C) Quantitation of NETting neutrophils upon indicated treatments. Bars are mean $\pm$ SE. n=5 for healthy adults; n= 3-8 for CGD patients. NS, no stimulation. Black asterisks indicate significant difference between healthy and CGD; green asterisks shows significant difference in healthy or CGD with or without TMX treatment. (*p < 0.05, **p < 0.01). (D) TMX-induced NETs improve antibacterial function of neutrophils. NETs were induced in neutrophils from healthy adults and CGD patients (n=6 each group) by TMX treatment followed

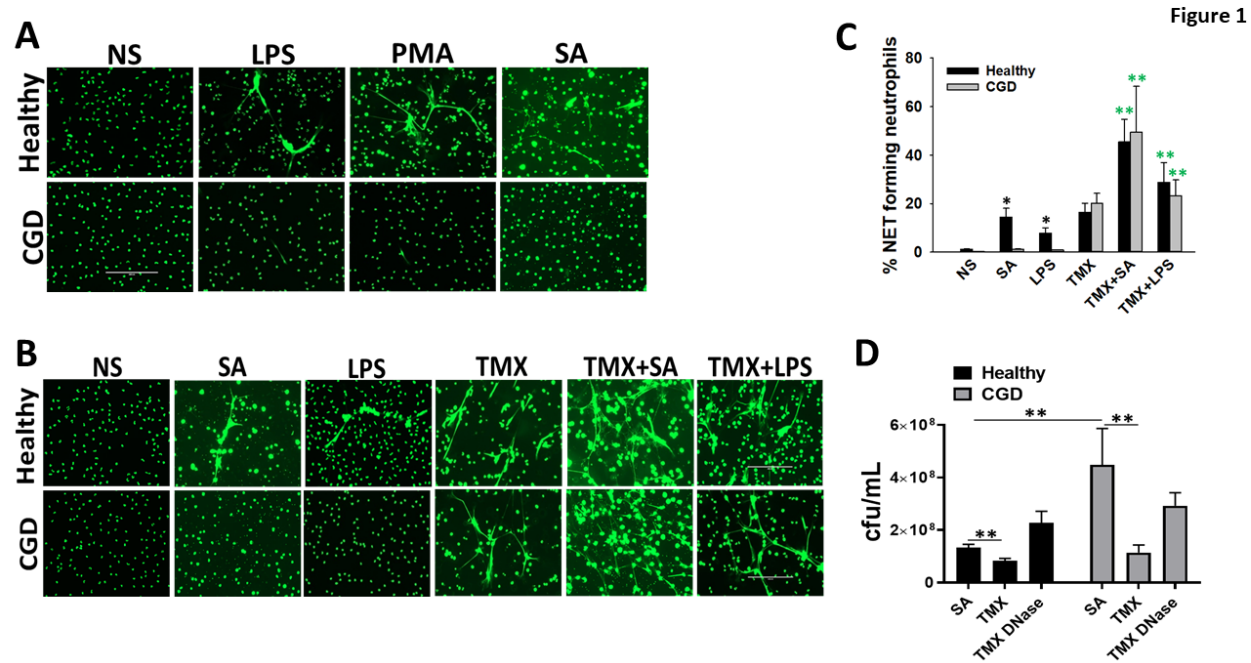
by incubation with *S. aureus* (SA) in the presence of cytochalasin D with or without DNase I for 30min. Data is from 3 independent experiments (**p < 0.01).

Figure 2. TMX induces NETs independent of ROS by autophagy activation. (A). Cytosolic ROS measurement in healthy adult neutrophils. n=3. (B). Western blots to show extent of autophagy activation by LC3 II processing in healthy adults or CGD patient neutrophils stimulated with LPS with or without TMX treatment. n=3 for each group. (C). Immunofluorescence analysis of autophagy activation depicted by LC3 II puncta (red) in neutrophils from healthy adults, upon stimulation with LPS or TMX alone or in combination, with or without ATG4 inhibitor NSC185058. Nuclei (Blue) are stained with 4',6-diamidino-2-phenylindole (DAPI). NET formation was analyzed in all samples by Sytox Green staining (Green). Representative images from n=3 humans are shown. Bar=200μ.

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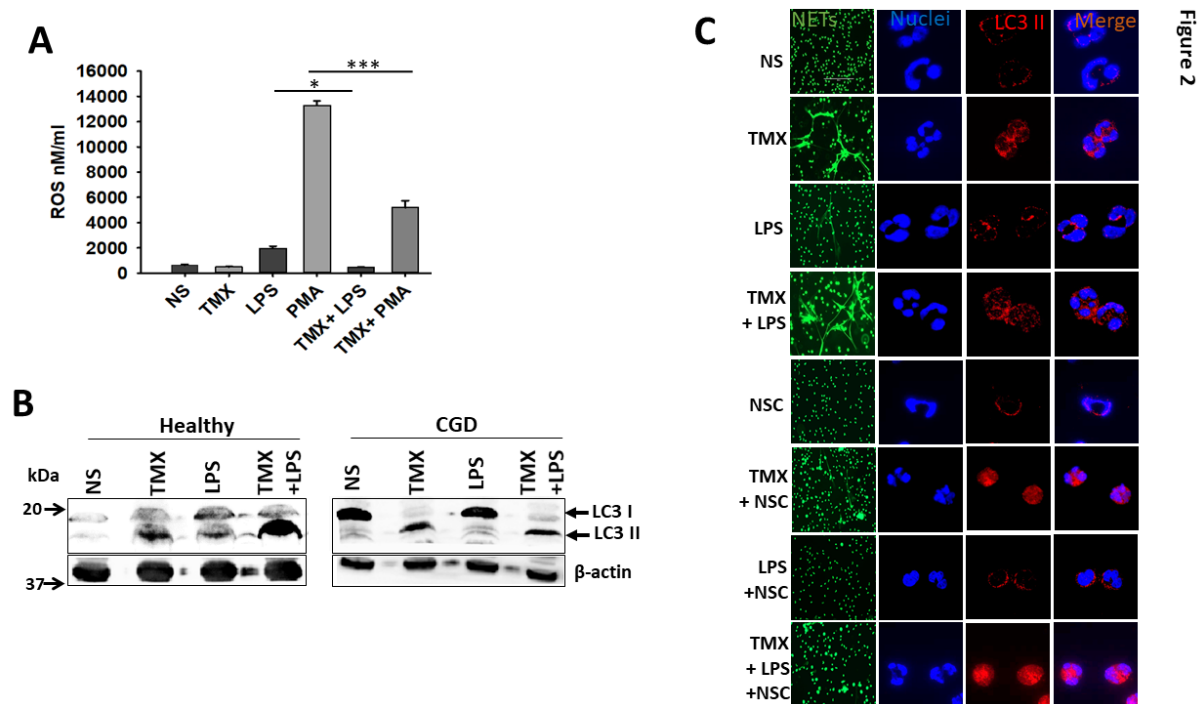


Figure 2

Online Repository

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Supplementary Information

Supplementary Figure 1. Purity of neutrophils isolated from human blood samples was assessed by flow cytometry using anti-human CD15 and CD16 antibodies as well as nuclear morphology (multilobed nuclei) assessed by Sytox Green Staining.

Supplementary Table 1. Clinical data for CGD patients enrolled in this study. Samples from CGD# 10-15 were used for NET-mediated bacterial killing assays.

Material and Methods

Human Subjects and isolation of blood neutrophils

Blood samples were obtained from healthy volunteers and CGD patients with informed consent at UND (North Dakota), NIH Clinical Center (Maryland). All procedures were approved by the UND and NIH Institutional Review Board. Supplementary Table 1 lists the clinical information of the CGD patients. Age range of healthy volunteers was 24-45 years and CGD patients was 18-48 years. Neutrophils were purified using Easy Sep Direct Human neutrophil isolation kit (STEMCELL Technologies). Purity of the neutrophils was ensured by flow cytometry analysis of CD15+CD16+ cells and by nuclear morphology (Supplementary Figure 1).

NET formation

Purified Human blood neutrophils were stimulated with Phorbol myristate acetate (PMA, 50nM, Sigma-Aldrich) or Ultrapure E. coli LPS (200 ng/ml, Sigma-Aldrich) for 2 hours at 37°C or were infected with *S. aureus* strain MRSA USA 300 at MOI 10 for 3hrs with or without TMX (5 µM, Sigma Aldrich) for 15-20 min. prior to simulation in 24 well plate. For some experiments, neutrophils were pretreated with NSC185058 (Cayman Chemical USA, 200 µM) for 30 min. Neutrophils were then cytocentrifuged, fixed with 4% paraformaldehyde, washed with PBS and NETs were stained using Sytox Green (Molecular Probe). Images were acquired using EVOS FL Cell Imaging System (Thermo Fisher Scientific Inc. USA). The percent NET formation was manually quantitated by dividing the number of NET-forming neutrophils by total number of cells in 8-10 random microscopic fields and multiplying the values by 100.

NET-mediated killing

Neutrophils purified from intravenous blood samples of healthy controls or CGD patients were plated in the presence of the phagocytosis inhibitor cytochalasinD (10 ug/ml; Sigma). NETs were induced by stimulation with 5uM TMX for 2h followed by addition of *S. aureus* strain MRSA USA 300 at MOI of 10. To ensure NET-specific bacterial killing, DNase I (125 U/ml; Invitrogen) was added in some wells for 15 min prior to addition of bacteria. After 30 min of incubation, well contents were scraped, serially diluted and plated on Trypticase Soy agar plates overnight to enumerate colony forming units (CFUs).

Measurement of ROS

Intracellular ROS were measured using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Thermo Fisher) as per the manufacturer's instructions following stimulation with PMA (50 nM) or LPS (200ng/ml) for 15 minutes with or without TMX (5µm). The amount of ROS was deduced by plotting against known concentrations of H₂O₂.

Western Blot analysis

For the detection of autophagy activation, neutrophils were plated in 90 mm dishes at the density of 10x10⁶ cells and were stimulated with LPS or TMX or both for 60 min. Cells were lysed with RIPA buffer containing protease and phosphatase inhibitors (Sigma Aldrich). Lysates were loaded on SDS-PAGE gels, transferred to polyvinylidene difluoride membranes and the blots were probed with anti-human LC3A/B Antibody (Cell Signaling Technology CST). Beta-actin (rabbit monoclonal anti-human antibody, Cell Signaling Technology CST) was used as loading control. Immunoreactivity was detected using super signal west Pico plus Chemiluminescent

substrate (Thermo Scientific) and analyzed on Bio Rad reader using Quantity one software and Chemi doc scanner.

Immunofluorescence microscopy

For detection of LC3 II puncta, neutrophils stimulated with LPS, TMX or both with or without NSC185058 treatment (as described above) were cytocentrifuged on glass slides and fixed with 4% paraformaldehyde. Immunostaining was performed using anti-human LC3 primary antibody (Cell Signaling Technology) followed by Alexa Fluor 546 conjugated goat anti rabbit secondary antibody (Thermo fisher scientific inc. USA).

Statistics

Statistical analyses were performed using the Student t test (SIGMA PLOT 8.0, Systat Software, San Jose, CA). A p value of ≤ 0.05 was considered statistically significant. NET-mediated bacterial killing was analyzed by a nonparametric Mann–Whitney U test.

Supplemental References

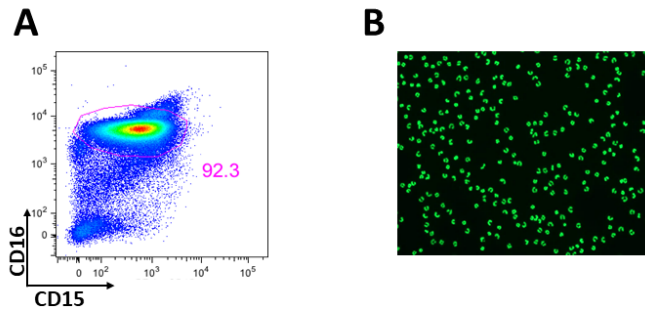
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Supplementary Figure 1



Supplementary Table 1. Clinical data for CGD patients enrolled in this study. Samples from CGD# 10-15 were used for NET-mediated bacterial killing assays.

CGD Patient #	Mutation	Age	Gender	Treatment
CGD-1	CYBB	33	M	posaconazole, isavuconazole, pioglitazone,
CGD-2	P47	27	M	posaconazole
CGD-3	CYBB	47	M	levofloxacin, voriconazole
CGD-4	CYBB	40	M	posaconazole
CGD-5	P47	26	F	itraconazole
CGD-6	CYBB	30	M	Steroids
CGD-7	P22	42	F	Steroids
CGD-8	P47	48	M	Steroids
CGD-9	CYBB	18	M	Steroids
CGD-10	p47	54	M	Steroids
CGD-11	p22	43	F	Steroids
CGD-12	p47	24	M	Steroids
CGD-13	p47	49	M	Azathioprine, itraconazole, bactrim fish oil, mesalamine
CGD-14	p47	56	M	Cardizem, dronabinol, temezepam MVA, fluticasone
CGD-15	p22	38	F	Azathioprine, Bactrim, itraconazole mesalamine