METHODS

Patients and controls

We included 74 SLE subjects, who fulfilled ≥4 ACR[19] classification criteria and had different levels of disease activity according to the SLEDAI score[20]. We also included 77 age and gender-matched healthy controls. Patients with other autoimmune diseases (except for concomitant antiphospholipid syndrome), chronic viral infections or an infection within the two weeks prior to the blood draw were excluded. Demographic and clinical characteristics were recorded. Peripheral venous blood samples were obtained from all subjects. Anti-dsDNA antibodies were measured by ELISA, and C3 and C4 levels were measured by nephelometry. All subjects signed informed consent prior to their participation; the protocol was approved by the local ethics committee at Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ, Ref. 1243) and at the NIH (94-AR-0066).

Neutrophil isolation and induction of NETs

Neutrophils were isolated by density centrifugation through Ficoll-Hypaque, and then through dextran as previously described[21]. Erythrocyte lysis was performed with hypotonic saline solution (0.2%).

LDGs were isolated from peripheral blood mononuclear cells (PBMCs) obtained from SLE subjects. Positive selection was performed with magnetic columns (MACS, Miltenyi Biotec; Auburn, CA, United States), using anti-CD10 antibody (Ancell; Stillwater, MN, United States). Purity was verified through flow cytometry, and was >95%. Once neutrophils and LDGs were isolated, they were cultured in 24-well plates ($2x10^6$ cells/plate) with RPMI for one hour before assessing NETosis. In neutrophils derived from healthy controls, NETs were induced with lipopolysaccharide (LPS, 1 µg/ml; Sigma-Aldrich, St. Louis, MO, United States) stimulation for one hour. In neutrophils derived from SLE patients, NETs were either induced with LPS stimulation or they were formed without additional stimuli (spontaneous). In the case of LDGs, NETs were induced without additional stimuli. NETs were purified following micrococcal nuclease treatment as previously described[21] (Thermo Scientific; Waltham, MA, United States).

NET quantification

NETs were quantified by two approaches, Sytox green assessment using a plate reader and quantification by fluorescent microscopy, as previously described[22]

a) **Sytox Green quantification:** Extracellular DNA was quantified by NET incubation with Sytox Green (0.2 μM; Life Technologies, Eugene, OR, United States) for 90 minutes at 37°C in a 96-well black-bottom plate. Fluorescence was read at a 485/528 nm in a fluorescence spectrometer (Synergy, Biotek; Winooski, VT, United States).

b) **Immunofluorescence / confocal microscopy:** Once neutrophils were isolated and counted, they were seeded in poly-L-lysine-coated coverslips (approximately $1.5x10^6$ cells/ml) and incubated for 1 hour with RPMI (± LPS 1 μg/ml) to induce NETosis. Cells were fixed with 4% paraformaldehyde, then washed with PBS and blocked with porcine gelatin 0.2%. Neutrophils were incubated with rabbit, unconjugated anti-elastase antibody (1:500; Abcam; Cambridge,UK) and mouse, unconjugated anti-ubiquitin antibody (1:250, Santa Cruz Biotechnology, Dallas, Tx, United States) for 30 minutes at 37°C, washed and incubated with Alexa Fluor-conjugated secondary antibodies (Life Technologies, Carlsbad, CA, United States) for 30 minutes at 37°C. This was followed by incubation with Hoechst (1:100; Life Technologies, Carlsbad, CA, United States) for 10 minutes at room temperature to detect DNA. Coverslips were mounted in Prolong Antifade Reagent (Life Technologies, Carlsbad, CA, United States) and analyzed by confocal microscopy (Nikon A1R+ microscope, Nikon NIS Elements v 4.5 software; Tokyo, Japan). Colocalization of extracellular elastase and Hoechst was considered a NET, in association with the characteristic strand morphology. The percentage of netting neutrophils was calculated from an average of 6 fields, using 40x objective, as a percentage of the total number of neutrophils[23]. Colocalization analysis was based on pixel intensity spatial correlation method (Dunn,K, et al. Cell Phys 2001; Am J Physiol Cell Physiol. 2011; 300(4):C723-42).

Determination of ubiquitin content in NETs

Total ubiquitin content in NETs from SLE subjects and controls was assessed by enzyme-linked immunosorbent assay (ELISA; BlueGene Biotech, Shanghai, China), using 10 μg of NETs from each subject.

Protein content of NETs was also evaluated by Western Blot, by loading equal amounts of protein (100-200 µg) in a polyacrylamide gel (4-15%). Transfer was made to nitrocellulose membranes. Anti-total ubiquitin P4D1 (Santa Cruz Biotechnology; Santa Cruz, CA, United States), FK2 (Enzo Biochem; Farmingdale, NY, USA), anti-K48 (to recognize polyubiquitinated proteins through lysine 48; Cell Signaling, Danvers, MA, United States), and anti-K63 (to recognize polyubiquitinated proteins through lysine 63; Cell Signaling, Danvers, MA, United States) were used. The corresponding secondary antibodies were used and images were developed using chemiluminiscence with ECL Western Blotting Substrate (Thermo Fisher, Carlsbad, CA, United States). Samples were analyzed with a digital image analyzer with a CCD camera (Chemidoc MP, Biorad; Hercules, CA, United States) and quantified by densitometry with ImageLab software (Biorad, Hercules, CA, United States).

Immunoprecipitation

Immunoprecipitation of NET lysates (400 µg) was performed with the magnetic microsphere technique (Dynabeads, Invitrogen; Waltham, MA, United States), using anti-total ubiquitin (P4D1, Santa Cruz Biotechnology, Dallas, Tx, United States). Immunoprecipitates were analyzed by Western Blot, as described above, in order to identify potential ubiquitination substrates, such as MPO (Dako, Agilent; Santa Clara, CA, United States).

Anti-ubiquitinated MPO antibody detection

This was performed by an in-house ELISA. Briefly, recombinant non-ubiquitinated MPO (R&D systems; Minneapolis, MN, United States) and purified ubiquitinated human MPO (Lee Biosolutions; Maryland Heights, MO, United States) were used at a 100 ng/ml concentration (Supplementary Fig. 1). Both forms of MPO were diluted in PBS and added to 96-well plates and incubated overnight. Wells were washed and blocked with PBS + 10% FBS. Study subjects' serum samples were added to each well (100 µl; 5 µl of serum in 500 µl of PBS + 10% FBS) and incubated overnight. Wells were washed and anti-IgG human antibody (Inova Diagnostics; San Diego, CA, United States) was added and left for a 30-minute incubation. Substrate (TMB chromogen, INOVA Diagnostics, San Diego, CA, United States) was then added and absorbance was read at 450 nm (Sunrise/Tecan; Männedorf, Switzerland). We normalized obtained values by an optical density (OD) index, and the cutoff value we determined as positive was three standard deviations above the mean OD index value found in healthy controls.

Calcium flux assay

PBMCs from SLE subjects and healthy controls were isolated through density centrifugation by Ficoll-Hypaque. $CD14^+$ cells were purified by positive selection with magnetic columns (CD14 Microbeads, MACS, Miltenyi Biotec). Cells were incubated in 96-well plates ($1x10^6$ cells/well) with RPMI/FBS and M-CSF (50 ng/ml; R&D Systems) for 5 days. When morphological differentiation to macrophages was documented, cells were stimulated with recombinant human ubiquitin (1 μg; BioVision, Milpitas, CA, United States) or with NETs (50 μg) derived from healthy controls or SLE neutrophils or from lupus LDGs in the presence or absence of a CXCR4 inhibitor (AMD 3100, 3.3 μM; Tocris Bioscience, R&D Systems). NETs obtained after exposure to the E1 ubiquitin-activating enzyme inhibitor (PYR-41, Sigma N2915; 50μM (Yang Y, Kitagaki J, Dai RM, *et al*. Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics. *Cancer Res* 2007;67(19):9472–81)) were also used for macrophage stimulation. Intracellular calcium flux in macrophages was measured with the Fluo-4 NW Calcium Assay Kit (Invitrogen), and fluorescence was read at 494/516 nm (FLUOstar Omega, BMG Labtech; Ortenberg, Germany).

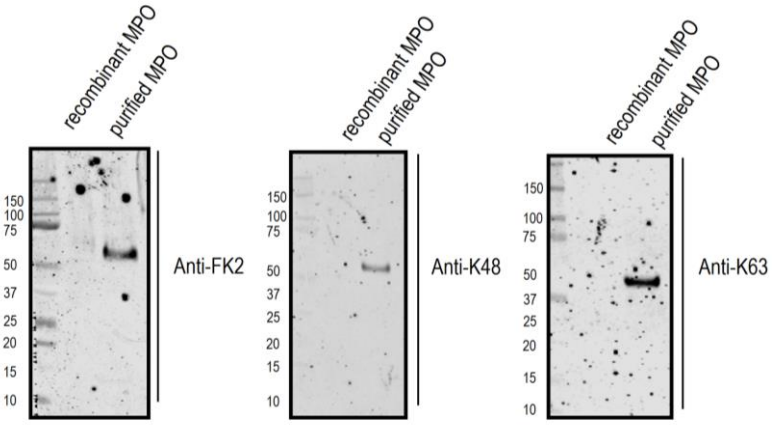
Assessment of cytokine synthesis

Monocyte-derived macrophages were isolated from SLE subjects and healthy controls, as described above, and seeded in 24-well plates. Macrophages were stimulated with NETs (50 μg) purified from SLE patients for 6 hours. We also stimulated a subset of cells with chloroquine (50 μM, 30 minutes prior to NETs addition) or CXCR4 inhibitor (3.3 μM) prior to the addition of NETs. Other macrophages were only stimulated with LPS (100 ng/ml). Cytokine release from macrophages (IL-6, IL-10 and TNF-α) was measured by ELISA (Becton Dickinson, Franklin Lakes, NJ, for IL-6; eBioscience, San Diego, CA for IL-10 and TNF-α). None of the patients included for the isolation of monocyte-derived macrophages were under treatment with any antimalarial.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (or standard error of the mean) or median and interquartile range, depending on sample distribution. Comparisons between groups were made by Student's T test or Mann Whitney U for independent or paired samples, according to the sample distribution. Association between categorical variables was assessed by Chi-square test. Tukey's multiple comparison was used to analyze differences in calcium flux. Spearman or Pearson coefficients were used for correlation. A *p* value under 0.05 was considered statistically significant. Analysis was performed with the support of the SPSS software v. 19.0.

Supplementary figure S1



Ubiquitination of MPO displays different patterns. Polyubiquitinated protein expression in two different sources of MPO (recombinant and purified from human neutrophils) was analyzed by Western Blot. Analysis of mono and polyubiquitinated proteins (FK2), polyubiquitinated proteins dependent on lysine 48 (K48) and polyubiquitinated proteins dependent on lysine 63 (K63) was performed. Purified MPO has both mono and polyubiquitinated proteins. This assay was performed in duplicate.

Supplementary table S1. Clinical and serological features of SLE subjects

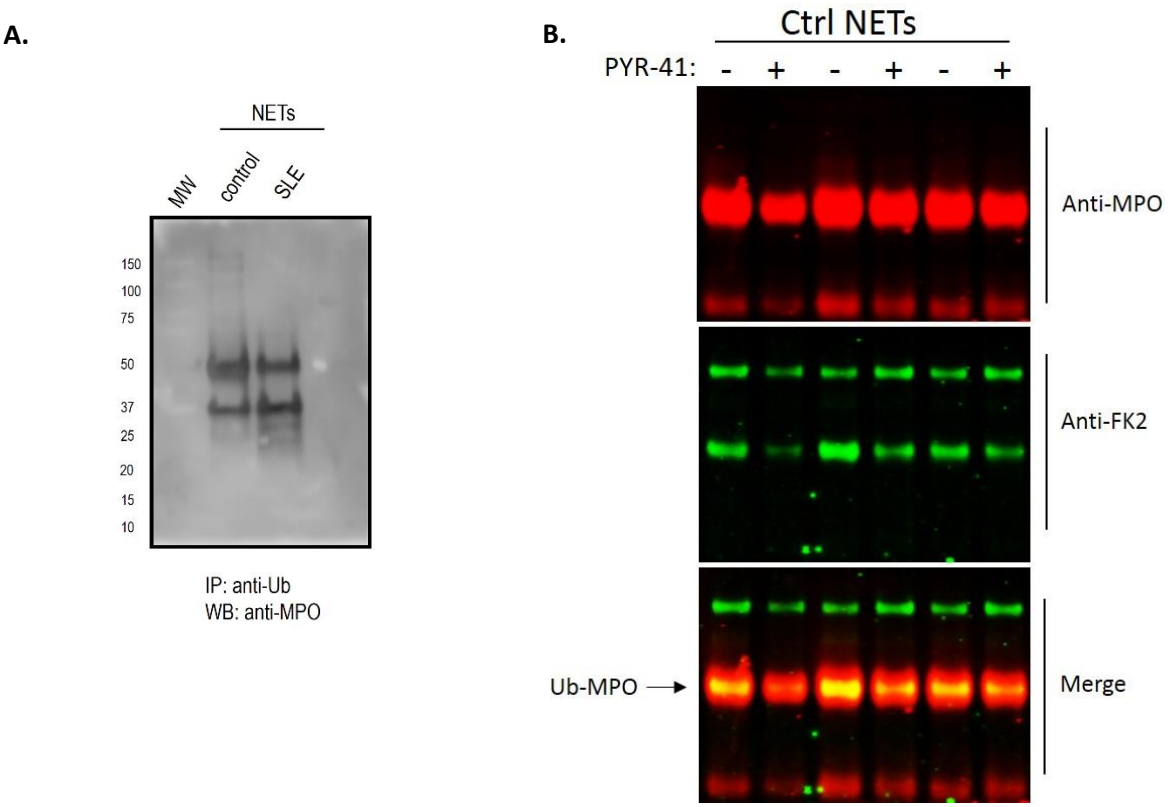
Characteristic	**Mean (± SD)**
SLEDAI score	15.67 ± 9.55
Anti-dsDNA antibodies (mg/dl)[a]	511.28 ± 1257
C3 (mg/dl)[b]	55.07 ± 27.33
C4 (mg/dl)[c]	10 ± 5
Clinical disease features	**Frequency (n,%)**
Renal	50 (68%)
Mucocutaneous	27 (36%)
Articular	18 (24%)
Hematologic	15 (20%)
Serositis	15 (20%)
Constitutional	11 (15%)
Vasculitis	9 (12%)
Disease onset	35 (47%)
Immunosuppressive treatment	27 (36%)

[a] Upper limit of normal: 9.6 mg/dl
[b] Lower limit of normal: 87 mg/dl
[c] Lower limit of normal:19 mg/dl

Supplementary table S2. Numbers and characteristics of subjects in each individual experiment.

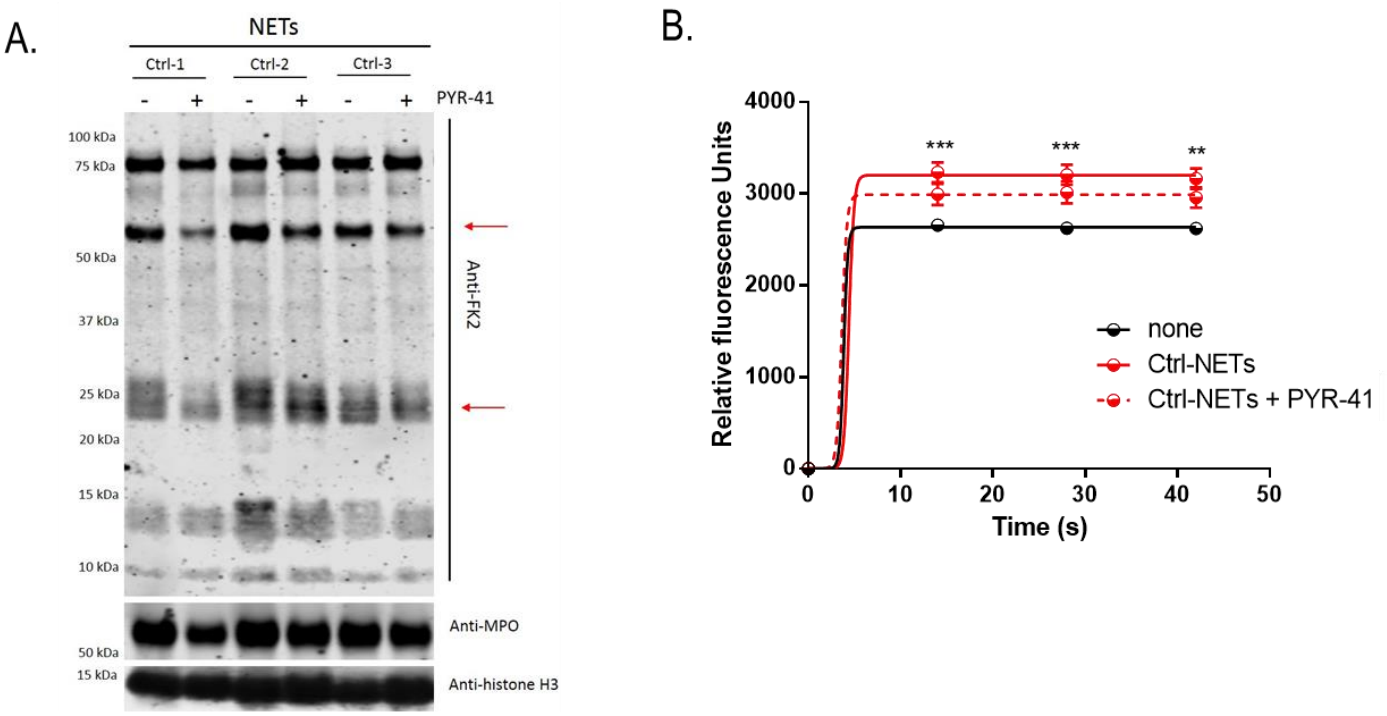
Experiment	Healthy controls	SLE subjects	SLEDAI (Mean ± SD)	Immunosuppressive treatment (n, %)
Neutrophil and NETs Immunofluorescence	50-56 (N=7)	50-56 (N=7)	21.5 ± 6.9	1 (14.2%)
Ubiquitin ELISA	8-12 (N=5)	8-12 (N=5)	4 ± 2	2 (40%)
Ubiquitin (K48 and K63) Western Blot	1-6; 61-74 (N=20)	1-6; 61-74 (N=20)	19.85 ± 7.62	7 (35%)
Immunoprecipitation	16-18; 53-60 (N=11)	16-18; 53-60 (N=11)	18 ± 12.1	4 (36.3%)
MPO ELISA	20-74 (N=55)	1-6; 20-70 (N=57)	11.1 ± 11.53	15 (26.3%)
Macrophages / calcium flux	7-15, 75-77 (N=12)	7-15 (N=9)	4.2 ± 2.39	5 (50%)
Macrophages / cytokines	16-21; 63-70; 71-74 (N=18)	16-21; 63-70; 71-74 (N=18)	14.83 ± 9.14	7 (38.8%)

Supplementary figure S2.



Supplementary figure S2. NETs contain ubiquitinated MPO. Immunoprecipitation with anti-ubiquitin and Western Blot with anti-MPO were performed in NETs from SLE and healthy controls. Interaction between MPO and ubiquitin is shown. **A.** A representative IP image is shown from 11 samples per group from which IP assays were performed. MW=molecular weight. **B.** Ubiquitination of MPO was corroborated by Western Blot in the presence or absence of the E1 activating enzyme inhibitor (PYR-41).

Supplementary figure S3



**p<0.01; ***p<0.001

Assessment of ubiquitin content in NETs and calcium flux in macrophages upon treatment with a ubiquitin-activating enzyme inhibitor. Neutrophils from healthy controls were incubated in the presence or absence of the E1 ubiquitin-activating enzyme inhibitor (PYR-41). NETs were stimulated with LPS (1 µg /mL) and harvested at 4 hours. **A.** NETs were resolved in a SDS-PAGE and probed for mono- and poly ubiquitination (FK2), MPO and total histone H3. Arrows indicate the only proteins that show reduced ubiquitination after inhibition. **B.** Monocyte-derived macrophages from healthy controls were incubated with 50 µg of NETs generated in the presence or absence of PYR-41. Calcium flux was measured using the Fluo-4 NW Calcium Assay Kit. Results represent the mean +/- SEM of 6 independent experiments. No significant differences were found in macrophages calcium flux upon treatment with NETs derived from PYR-41 exposed neutrophils in comparison to those without PYR-41 exposure.