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Supplementary Video 1. Live recording of NETosis in vitro. This video shows typical characteristics of the NETosis cascade imaged starting 1 hour after PMA stimulation (time expressed in hh:mm:ss). The neutrophil in the centre has a delobulated nucleus and is undergoing progressive and massive chromatin decondensation. As the fluorescence signal of Hoechst 33342 is proportional to DNA concentration, nuclear delobulation and chromatin decondensation determine a reduction in the blue fluorescence, which spreads as the nuclear area enlarges. While such process takes up to 1 hour, the last step is the abrupt release of NETs in the extracellular space, evidenced by the staining of the cell impermeable Sytox green dye labelling cell free dsDNA in the medium, which takes just a few minutes.

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NETosis delays diabetic wound healing in mice and in humans

ADDITIONAL MATERIAL DATA

Analysis of proteomic data. We previously developed and validated a proteomic platform for identification of biomarkers and new molecular mechanisms of diabetic wound healing (1). This was based on proteomic characterization of tissue lysates obtained from wound biopsies in patients ("discovery cohort") whose wound outcome could be defined as rapidly healing (RH, n=17) or non-healing (NH, n=11). Details of this protocol can be found elsewhere (1). Briefly, proteomic analysis was performed with iTRAQ labelling and mass spectrometry on MALDI-TOF/TOF and Orbitrap. After bioinformatic analysis of peptides and sequence coverage, data were filtered retaining only proteins identified by at least two unique peptides and with a false discovery rate <5%. Abundance of individual proteins was expressed as the NH/RH ratio, with confidence interval derived from technical triplicate over the background generated by swapping iTRAQ labels. The protein list and those upregulated or downregulated in NH versus RH have been published previously (1). We herein re-analyzed such data to evaluate whether proteins associated with NETs showed differential abundance in NH versus RH wounds. To this end, we built a custom pathway made up of proteins that have been associated with NETs. A PubMed search was run on Sept 2014 with search string ("NETs" or "neutrophil extracellular traps" or "NETosis"): two reviews of the literature, published in 2013 (2) and 2014 (3) provided the best and updated overview of NET-associated proteins. This list was matched with the list of proteins identified in diabetic wounds as described above. Proteins were divided into groups according to the belonging cellular compartment (nucleus, granules, cytoplasm, cytoskeleton, plasmamembrane). Individual protein relative expression was retrieved, and the average relative expression of each compartment was calculated.
To validate the measure of NET-associated proteins in individual wound lysates, we used samples collected from patients of a "validation cohort", who were divided according to the wound outcome at follow-up in those whose ulcer had worsened and in those whose ulcer was stable or healed. We measured tissue elastase content using the Polymorphonuclear Elastase Human ELISA Kit (Abcam).

Patients and follow-up. The protocol was approved by the Ethical Committee of the University Hospital of Padova and participants provided informed consent. Patients and controls were recruited at the Diabetes outpatient clinic of the University Hospital of Padova. Three groups of individuals were enrolled: controls without diabetes; patients with diabetes; patients with DFU. Exclusion criteria were age <18 or >85 years, active cancer, non-diabetic wounds (e.g. systemic sclerosis, venous ulcer), severe sepsis, immunosuppression or immunologic disorders, inability to provide informed consent; pregnancy or lactation. Patients with DFU had digital photographic documentation of the wound and classification according to PEDIS (4) and TUC (5). For all participants, we collected the following data: age, sex, BMI, HbA1c, diabetes duration, prevalence of hypertension (blood pressure >140/90 mm Hg on at least 2 occasions, or use of anti-hypertensive medications), dyslipidemia (total cholesterol >200 mg/dl [5.17 mmol/l] or LDL cholesterol >130 mg/dl [3.36 mmol/l] or triacylglycerol >150 mg/dl [1.69 mmol/l]), cigarette smoking (of 1 or more cigarette per day), coronary artery disease (past history of myocardial infarction or angina, or evidence of >50% coronary stenosis at a coronary angiography), peripheral vascular disease (instrumental evidence of significant stenosis of leg arteries), retinopathy (defined by ophthalmologic examination), neuropathy (based on history, clinical examination and eventually confirmed by electromyography), chronic renal failure (estimated glomerular filtration rate <60 ml min/1.73/m^2). We also recorded ongoing medications. Foot ulcers were classified as neuropathic based

on typical ulcer sites, absence of ischemia and normal transcutaneous O_2 pressure; ischemic in the presence of necrosis/gangrene without clinical and instrumental signs of neuropathy; neuroischemic in the presence of clinical/instrumental evidence of both ischemia and neuropathy. Fasting blood samples were drawn for the determination of circulating markers of NETosis. Patients were followed-up under routine ambulatory care for medications of the wound. We recorded the following events related to wound healing: definite healing; partial healing; worsening (defined as an increase in PEDIS/TUC compared to baseline); major/minor amputations; revascularization.

Circulating mono- and oligonucleosomes. The Cell Death Detection ELISAPLUS (Roche Diagnostics, IN, USA) was used. Serum samples, positive and negative controls were added into the microplate, covered with adhesive film, and incubated for 1h with the immunoreagent containing anti-histone-biotin, anti-DNA-POD, and incubation buffer. After washing, the reaction was run for 20 min avoiding direct exposure to light, then stopped by adding stop solution, and absorbance read at 490 nm wavelength on a spectrophotometer (BIO-RAD Model 680).

Circulating cell-free double strand DNA. The serum was diluted with an equal volume of saline and phenol was then added to the sample to be purified in a 1.5 ml microcentrifuge tube. Tubes were mixed vigorously until formation of an emulsion, and then centrifuged at 80% maximum speed for 1 min at room temperature. At the interface, a light coloured protein layer was visible. The top (aqueous) phase containing the DNA was carefully removed and transferred into a new tube. An equal volume of chloroform was added and the mixture shaken vigorously and centrifuged at max speed for 5 min at room temperature. Then, 1/10 volume of 3 M sodium acetate (pH 5.2) was added to the solution of DNA and mixed by vortexing. 2.5 vol of ice-cold 100% ethanol was added and mixed by flicking the tube several times, placed in dry ice for 5 min, spun for 25 min in a fixed-angle microcentrifuge at high speed at 4°C, and the supernatant removed. 1 ml of 70% ethanol was then added, the tubes inverted several times, and centrifuged at high speed at 4°C, the supernatant removed and air dried for 15 min. The DNA pellet was resuspended in 100 µl of ultrapure water. The quantification of dsDNA was carried out using the Qubit® 2.0 Fluorometer (Life Technologies).

Serum NET proteins. Serum elastase concentrations were measured using the polymorphonuclear Elastase Human ELISA Kit (Abcam), according to the manufacturer's instructions. Serum NGAL concentrations were measured using the ELISA kit (R&D Systems, cat# DLCN20), according to the manufacturer's instructions. Serum lactoferrin concentrations were measured using the ELISA kit (Novus Biological cat# KA1267), according to the manufacturer's instructions. Serum proteinase-3 concentrations were measured using the ELISA kit (Cusabio, cat# E13058h), according to the manufacturer's instructions, and c-ANCA with the Abnova ELISA kit (cat# KA1084).

Isolation of human neutrophils and stimulation of NETosis in vitro. Intact human neutrophils were isolated from anticoagulated whole blood. Non-target cells were removed by immunomagnetic depletion (MACSxpress Human Neutrophil Isolation Kit, Miltenyi Biotec) and simultaneously, erythrocytes were sedimented, yielding a high purity neutrophil population. To stimulate NOX-dependent NETosis, neutrophils were incubated with phorbol 12-myristate-13-acetate (PMA) 50 nM. To stimulate NOX-independent NETosis, neutrophils were incubated with the calcium ionophore a23187 0,25 mM, or ionomycin. After 2h of incubation, cells were fixed with 4% PFA for immunofluorescence analysis. In separate experiments, using neutrophils isolated from patients with DFU (n=14), patients with diabetes (n=11), or participants without diabetes (n=11), we used the Cayman NETosis assay to determine the activity of NET-bound neutrophil elastase. The assay is based on the enzymatic activity of neutrophil elastase in the culture medium that has been released from NETs through the action of S7 Nuclease. A

colorimetric assay employing a specific elastase substrate (N-methoxysuccinyl-Ala-Ala-Pro-Val p-Nitroanilide) was used after washing away non-NET associated elastase, as to measure only NET-associated elastase activity. The substrate is selectively cleaved by elastase to give a 4-nitroaniline product that absorbs light at 405 nm. To determine the activity of NET-bound MPO, we employed a modification of the same assay, measuring MPO peroxidase activity with a fluorometric assay (Cayman Chemicals) in culture medium after S7 Nuclease treatment and washing non NET-bound MPO. The reaction between hydrogen peroxide and ADHP (10-acetyl-3,7-dihydroxyphenoxazine) produces the highly fluorescent compound resorufin. Resorufin fluorescence can be analyzed with an excitation wavelength of 530-540 nm and emission wavelength of 585-595 nm.

Immunofluorescence. Coverslips with fixed cells were removed from the plates issued on drops kept on parafilm. After washing with PBS, the cells were permeabilized for 1 min with 0.5% Triton X100 in PBS, washed in PBS and blocked for 20 min with blocking buffer. Cells were then stained with a mouse anti-human PL2-3 mAb, directed against the subnucleosomal complex of Histone 2A, 2B and chromatin (kind gift of Volker Brinkmann, Max Planck Institute for Infection Biology, Berlin, Germany), with a polyclonal rabbit anti-human neutrophil elastase (Calbiochem 481001) in blocking buffer for 1h at 37°C. In parallel experiments, cells were stained with a polyclonal rabbit anti-human citrullinated (R2+R8+R17) histone H3. After washing with PBS the secondary antibodies were applied for 30 min (Cy3 goat anti-mouse, Alexa488 goat anti-rabbit, Jackson Laboratory). Cells were then washed with PBS and stained with Hoechst 33342 (100 ng/ml, Sigma-Aldrich), washed with PBS and mounted with Elvanol. Images were acquired with a Leica DM5000B microscope, equipped with a DFC300 FX CCD camera. Netting cells were semi-automatically identified by relating the fluorescence signals of the anti-chromatin antibodies to the signal of Hoechst 33342 (6). To this end, a customized procedure was run using ImageJ/Fiji software (1.48r, NIH, USA): a line of interest (LOI) was drawn with care to cross the cell borders intersecting 2 lobes of the nucleus. Relative intensity signal was plotted for each channel separately (Hoechst 33342, blue; Chromatin, red) against distance in pixels along the LOI. Cells undergoing NETosis were identified by a chromatin signal increase and spread out of the nuclear region.

Transmission electron microscopy. Isolated neutrophils were centrifuged at 3000g for 5 minutes and fixed in a paraformaldehyde 4% and glutaraldehyde 2% for 1h at 4°C. After fixation cells were washed twice in buffer solution pH 7.2 and then subjected to post-fixation with osmium tetroxide for 1h at 4°C. After alcohol dehydration cells were inclused in the EPON resin. Sections of the block were thinly sliced (approximately 10 nm) with an ultramicrotome (LKB Ultratome) and collected on a special screen. Finally, the screen and sample sections were coloured with uranyl acetate and then with lead nitrate. Images were recorded with a transmission electron microscope Tecnai 12 (FEI), operating at voltages ranging from 20 to 120 kV, and providing magnification ranging from 120X to 300,000X, equipped with a digital camera at high resolution TIETZ.

Animals. All procedures were approved by the local ethics committee and from the Italian Ministry of Health. Experiments were conducted according to the National Institutes of Health Principles of Laboratory Animal Care. C57Bl/6J mice from the in-house colony were used. Diabetes was induced with a single i.p. injection of streptozotocin (150 mg/kg, Sigma Aldrich) in citrate buffer pH 4.5. Glycemia was measured with a commercially available glucometer (Abbott) and animals with blood glucose ≥300 mg/dl were housed for 4 weeks before performing the experiments. To ensure PAD4 inhibition, Cl-amidine (Merck Millipore) was injected daily subcutaneously at 10 mg/kg (in DMSO) for 1 week before performing excisional wounds and continued throughout healing. Control animals received daily injection of the vehicle (DMSO).

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Wound healing in mice. Animals were sedated with inhaled isoflurane (Abbott) and the lateral portion of the tight was shaved with Veet® cream. After skin disinfection, a 4 mm-round excisional full-thickness wound was performed with a skin biopsy punch (Kai Medical, Japan). Digital photography of the wounds were taken at pre-specified time points with a reference ruler to estimate wound area, that was calculated with ImageJ software (NIH).

Mouse neutrophils isolation. *Skin neutrophils*. Animals were euthanized using our Institutional approved protocol and the wound with a surrounding portion of skin (epidermis+dermis) was excised. The tissue was minced and digested in 4 mL of trypsin-EDTA 0.25% (Sigma-Aldrich) and 0.08% collagenase type II (Worthington Biochemical Corporation) for 1 hour at 37°C. Four milliliters of 100% FBS were added and the suspension was filtered through a 70 micron cell strainer. Cells were centrifuged and the pellet suspended in PBS for flow cytometry analysis. *Bone marrow neutrophils*. Neutrophils were isolated according to Swamydas and Lionakis (7). Briefly animals were euthanized and bones from hind-limbs (femurs and tibiae) removed and placed into ice-cold RPMI 1640 (Life Techonologies). Under a culture hood, bones were flushed with RPMI supplemented with 10% FBS and 2mM EDTA. RBC were lysed by hypotonic shock with 0.2% NaCl then buffered with an iso-volume of 1.8% NaCl. After centrifugation neutrophils were isolated by density gradient centrifugation with Histopaque 1119 and 1077 (both from Sigma Aldrich) at 2000 RPM for 30 min. Neutrophils were collected at the interface of the Histopaque 1119 and 1077 layers and immediately used for experiments. To detect release of NETs by cultured neutrophils stimulated with PMA or ionomycin (as described above), 160 nM Sytox Green were added to 200 µl of culture medium in each well of a 96-well plate with 50,000 neutrophils. After mixing, the plate was incubated at 37°C for 30 min and the Sytox Green (excitation 485 nm/emission 535 nm) fluorescence was measured using a Victor Perkin Elmer plate reader.

Flow cytometry. Cells were stained with a monoclonal anti mouse Ly-6C/G-PE (Gr-1, eBiosciences) and 7-AAD (BD). After a wash with PBS, cells were fixed with PFA 2% for 12 minutes at 37°C, washed with PBS and permeabilized with methanol 90% for 30 minutes at +4°C. Then cells were stained with a rabbit anti-Histone H3 Citrulline (Ab5102, Abcam) and a rabbit anti-histone H4 citrullined (Merck Millipore) for 30' at +4°C. We then incubated cells with an Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) for 30 minutes at +4°C. Data were acquired using a FACS Canto instrument (BD Biosciences) and analyzed with FlowJo X (TreeStar Inc., USA).

PAD4 activity. PAD4 activity was assessed with a commercially available kit (Cayman Chemicals) in lithium-heparin plasma and in skin extracts. The skin was homogenized in lysis buffer (20 mM Tris–HCl [pH 7.4], 10 mM b-mercaptoethanol, 100 mM NaCl, and 10% glycerol, supplemented with Complete EDTA-free protease inhibitor mix [Roche Biochemicals, one tablet/50 ml]). After sonication, samples were centrifuged for 30 min at 13000g at 4°C. Supernatants were used immediately in the assay. Activity was normalized for protein concentration that was measured with a bicinchoninic acid-based kit according to the manufacture's instruction (Pierce™ BCA kit, ThermoFisher).

Multiphoton microscopy. *In vivo (intravital microscopy)*. Wild type C57BL/6J mice were sedated with zolazepam/ thylamine and xylazine, placed on a custom-made holder and positioned under an 25x objective optimized for multiphoton imaging (Olympus XLPLN25XWMP2, N.A. 1.05, W.D. 2 mm). Wounds were performed as previously described and assessed three days after wounding. 30 minutes before imaging mice were injected intravenously with 5 µl of Sytox green (Life Technologies), 3 µl of PE-conjugated anti-mouse Gr-1 (eBiosciences) and 50 µl of Hoechst 33342 (1 mg/ml, Sigma Aldrich).

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In separate experiments, to stain the vasculature, mice were injected with 50 µl of a 10 mg/dl solution of high molecular weight (150 kDa) FITC-conjugated Dextran via the tail vein.
In vitro. Neutrophils were isolated from peripheral blood of healthy donors with the MACSxpress® Neutrophil Isolation Kit (Miltenyi Biotech) according to the manufacture's instruction. To induce the formation of NETs, neutrophils were stimulated with 100 nM PMA (Sigma Aldrich) at 37°C 5% CO2 for two hours. Cells were stained with Hoechst 33342 (dilution 1:1000 from a 1 mg/ml solution) and 50 nm TMRM (Life Technologies) and just before imaging 100 nM Sytox green (Life Technologies) was added to the media to allow visualization of NETs. Experiments were performed with an 60x objective (Olympus LUMFLN60XW, N.A. 1.1, 1.5 WD).
In all experiments, we used a modular multiphoton microscope (Bergamo II, Thorlabs) equipped with 8 kHz resonant scanner, extended-field-of-view collection optics, 4 independent detection channels in the backward direction and laser-scanned Dodt contrast in the forward direction. The microscope was coupled to two synchronized pulsed laser beams generated by a Ti:Sapphire pump laser (Chameleon Ultra 2, Coherent) and an optical parametric oscillator (Camelion Compact OPO). Two-photon microscopy at 800 nm excitation was used to visualize Sytox Green and PE-conjugated anti-Gr-1 mAb while three-photon excitation at 800 nm was used for Hoechst 33342 visualization.

Statistical analysis. Data are expressed as mean±standard error, or as percentage where appropriate. Normality was checked using the Kolmogorov-Smirnov test. Non-normal variables were log-transformed before statistical analysis. Comparison of continuous variables between 2 or more groups were performed using Student's t test, or ANOVA, respectively. The post-hoc LSD test was used. Comparisons of categorical variables between 2 or more groups were performed using the chi square test. A multivariable logistic regression analysis was used to evaluate the association between NETosis biomarkers and wound healing independently from confounders. Differences in survival curves were checked using the Gehan-Breslow-Wilcoxon test. Statistical significance was accepted at $p<0.05$ and SPSS version 22.0 was used.

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