Diabetes primes neutrophils to undergo NETosis, which impairs wound healing

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Wound healing is impaired in diabetes, resulting in significant morbidity and mortality. Neutrophils are the main leukocytes involved in the early phase of healing. As part of their antimicrobial defense, neutrophils form extracellular traps (NETs) by releasing decondensed chromatin lined with cytotoxic proteins. NETs, however, can also induce tissue damage. Here we show that neutrophils isolated from type 1 and type 2 diabetic humans and mice were primed to produce NETs (a process termed NETosis). Expression of peptidylarginine deiminase 4 (PAD4, encoded by Padi4 in mice), an enzyme important in chromatin decondensation, was elevated in neutrophils from individuals with diabetes. When subjected to excisional skin wounds, wild-type (WT) mice produced large quantities of NETs in wounds, but this was not observed in Padi4−/− mice. In diabetic mice, higher levels of citrullinated histone H3 (H3Cit, a NET marker) were found in their wounds than in normoglycemic mice and healing was delayed. Wound healing was accelerated in Padi4−/− mice as compared to WT mice, and it was not compromised by diabetes. DNase 1, which disrupts NETs, accelerated wound healing in diabetic and normoglycemic WT mice. Thus, NETs impair wound healing, particularly in diabetes, in which neutrophils are more susceptible to NETosis. Inhibiting NETosis or clearing NETs may improve wound healing and reduce NET-driven chronic inflammation in diabetes.

NETs were originally recognized as a host defense mechanism in which neutrophils release their nuclear and granular contents to contain and kill pathogens. Bacterial endotoxins, such as lipopolysaccharides (LPS), stimulate the release of NETs that form extensive webs of DNA coated with cytotoxic histones and microbial proteases. A prerequisite for NETosis is modification of arginine residues of histones to citrulline by PAD4, which changes the charge of the histones, leading to massive chromatin decondensation. NETs also form during sterile inflammation. NETs are a key scaffold in pathologic thrombi, and they fuel cardiovascular, inflammatory, autoimmune and thrombotic diseases in mice and humans.

Under diabetic conditions, neutrophils produce more superoxide and cytokines than in normoglycemic conditions. Tumor necrosis factor-α, which primes neutrophils for NETosis, is increased in diabetic individuals. The diabetic microenvironment may thus facilitate NETosis. To test whether diabetes predisposes neutrophils to NETosis, we isolated neutrophils from fresh whole blood obtained from individuals with either type 1 or type 2 diabetes whose glycated hemoglobin (HbA1c) was >6.5%, indicating mild prolonged hyperglycemia (Supplementary Fig. 1a). Neutrophils from these individuals were indeed more susceptible than healthy controls to NETosis when stimulated with the calcium ionophore ionomycin (Fig. 1a,b). PAD4 is a calcium-dependent enzyme that is key in mediating NETosis. Western blotting revealed a fourfold upregulation of PAD4 protein expression in the neutrophils from individuals with diabetes as compared to healthy controls (Fig. 1c), which should favor chromatin decondensation. Neutrophils from type 2 diabetics have elevated basal calcium levels. A direct correlation between intracellular calcium levels and fasting serum glucose levels has also been reported. Calcium flux is necessary for efficient NET formation, as it promotes production of reactive oxygen species (ROS) and PAD4-mediated chromatin citrullination. In addition, NETosis has been shown to metabolically require glucose. Therefore, elevated glucose, as seen in diabetes, may participate in NETosis on many levels. Our present findings are complemented by a recent report showing that circulating NET-related biomarkers, nucleosomes, cell-free double-stranded DNA and neutrophil elastase, are increased in the sera of individuals with type 2 diabetes, and nucleosomes positively correlate with these individuals’ Hba1c levels.

Because frequent hyperglycemia is common in both type 1 and type 2 diabetes, as indicated by the higher HbA1c levels seen in the diabetic cohorts compared to the healthy controls (Supplementary Fig. 1a and Supplementary Table 1), we hypothesized that high glucose alone may contribute to neutrophil priming. We therefore isolated neutrophils from healthy donors and pre-incubated them in media with normal (5.5 mM) or high (22 mM) glucose concentrations before stimulation with ionomycin or phorbol 12-myristate 13-acetate (PMA), which triggers ROS production. We found that both ionomycin and PMA
Diabetes or high glucose concentrations in vitro prime human and mouse neutrophils to undergo NETosis. (a,b) Combined (a) and separate (b) data showing the percentage of NET production by unstimulated and ionomycin-stimulated peripheral neutrophils isolated from fresh whole blood of healthy individuals (black circles, n = 10) and individuals with diabetes mellitus (DM) (pink circles, type 1 DM, n = 5; purple squares, type 2 DM, n = 5). (c) Western blot analysis of PAD4 expression in neutrophils from healthy or diabetic individuals (top) and quantification of PAD4 expression, normalized to GAPDH expression (bottom). AU, arbitrary units. n = 6 for healthy control, n = 6 for diabetic individuals. (d) Percentage of NET production by neutrophils from healthy individuals that were exposed to normal glucose (NG, 5.5 mM), high glucose (HG, 22 mM) and mannitol (M, 16.5 mM plus 5.5 mM glucose) in vitro.

We then examined the susceptibility to NETosis in diabetic mouse models, as well as the role of PAD4 and the impact of NETs on diabetic wound healing. Immunostaining of fresh blood cells from streptozotocin (STZ)-induced diabetic mice (a model of type 1 diabetes) (Supplementary Fig. 2a–c) revealed an approximately fourfold increase in H3Cit17 neutrophils when compared to normoglycemic mice (Supplementary Fig. 3). About 4.5-fold more isolated neutrophils from diabetic mice were H3Cit17, and ~2% produced NETs after incubation in vitro without stimulation, whereas <0.2% NETs were seen in the normoglycemic controls (Fig. 1e). LPS further stimulated more neutrophils from the STZ-induced diabetic mice to be H3Cit17 and form NETs compared to vehicle-treated normoglycemic diabetic and control mice (Fig. 1d and Supplementary Fig. 1b). Thus, the increased susceptibility of diabetic neutrophils to NETosis is at least in part due to elevations in blood glucose. Our observations differ from earlier reports17,18 possibly owing to different methods of neutrophil isolation. By using the less-activating Histopaque and Percoll gradients compared to dextran sedimentation19–21, we found a clear priming effect by diabetes or hyperglycemia on NETosis.

Figure 1. Diabetes or high glucose concentrations in vitro prime human and mouse neutrophils to undergo NETosis. (a,b) Combined (a) and separate (b) data showing the percentage of NET production by unstimulated and ionomycin-stimulated peripheral neutrophils isolated from fresh whole blood of healthy individuals (black circles, n = 10) and individuals with diabetes mellitus (DM) (pink circles, type 1 DM, n = 5; purple squares, type 2 DM, n = 5). (c) Western blot analysis of PAD4 expression in neutrophils from healthy or diabetic individuals (top) and quantification of PAD4 expression, normalized to GAPDH expression (bottom). AU, arbitrary units. n = 6 for healthy control, n = 6 for diabetic individuals. (d) Percentage of NET production by neutrophils from healthy individuals that were exposed to normal glucose (NG, 5.5 mM), high glucose (HG, 22 mM) and mannitol (M, 16.5 mM plus 5.5 mM glucose) in vitro. n = 5 per condition. (e,g,h) Percentage of cells that were hypercitrullinated at histone H3 (H3Cit17, left) and produced NETs (right) in neutrophils isolated from streptozotocin (STZ)-induced diabetic mice (n = 12 for vehicle, n = 10 for STZ) (e), db/db diabetic mice (n = 7 for m/db; n = 8 for db/db) (g) and normoglycemic WT mice whose neutrophils were exposed to different glucose concentrations in vitro (n = 10 per medium condition) (h). US, unstimulated. (f) Representative immunofluorescence images of isolated neutrophils from vehicle- or STZ-treated mice. Neutrophils marked by anti-Ly6G antibody. GADPH serves as a loading control. Data are mean ± s.e.m. (*P < 0.05, **P < 0.01, ***P < 0.001). (a–e,g) Mann-Whitney t-test. Data are means ± s.e.m.

Supplementary Fig. 2. NETs are present in the wounds of WT mice. (a) Representative western blot of the time course for H3Cit appearance in wounds after skin injury (left) and quantification of levels of H3Cit to histone H3 (right). AU, arbitrary units. Ctrl, control unwounded skin; H3, histone H3. **P < 0.01 versus Ctrl, Student’s t-test, n = 3 for Ctrl, 1 and 4 h; n = 5 for 1, 3, 7 and 14 d. Blot is representative of three independent experiments. Data are mean ± s.e.m. (b) Immunofluorescence images of the wound bed immediately beneath the scab 3 d after injury. Scale bar, 50 μm. (c) Representative confocal images of four wounds 3 d after injury. Area enclosed by the yellow box is magnified and shown on the right. Scale bars, 100 μm (left), 50 μm (right). (d) Western blots of wounds collected 3 d after injury from mice with defective leukocyte recruitment (Cd18−/−, left) and mice depleted of neutrophils using an anti-Ly6G antibody (right, representative of n = 7). IgG, IgG isotype control for the anti-Ly6G antibody. GAPDH serves as a loading control.
mice (Fig. 1e,f). Thus, as in humans, diabetes has inflammatory or metabolic components that predispose mouse neutrophils to NETosis. Although there is no specific anti-mouse PAD4 antibody to evaluate whether PAD4 protein expression is increased by diabetes, neutrophil priming could also be attributable to increased PAD4 activity as indicated by elevated histone H3 citrullination (Fig. 1e and Supplementary Fig. 3). Similar NETosis assays were performed with neutrophils from genetically modified db/db mice (Supplementary Fig. 4), a model of type 2 diabetes. A higher proportion of these neutrophils were H3Cit high and formed NETs when compared to neutrophils from normoglycemic control mice (Fig. 1g), indicating that enhanced NETosis is a common phenomenon in mouse diabetes regardless of the type or etiology, as we observed in the human condition. LPS stimulated more high glucose-exposed neutrophils from normoglycemic WT mice to become H3Cit high and produce NETs when compared to those exposed to normal glucose or mannitol (Fig. 1h), indicating a possible priming role of high glucose. Thus the mouse models of diabetes represent well the human condition with respect to susceptibility to NETosis and induction of PAD4 activity.

Depletion of neutrophils in mice was previously shown to accentuate re-epithelialization of uninjured diabetic wounds22. Because NETs can be injurious to tissues23, we asked whether NETs form in wounds and affect healing. We examined excisional wounds24 from normoglycemic WT mice. H&E staining confirmed that re-epithelialization of leukocytes, mainly neutrophils, overlaps with the keratinocyte proliferation stage that leads to re-epithelialization (Supplementary Fig. 5). Therefore, neutrophils or NETs could interfere with healing. Analysis of wounds revealed an increased amount of H3Cit that peaked from 3 to 7 d after wounding (Fig. 2a). Immunofluorescence images of wounds 3 d after injury showed that H3Cit+ neutrophils were present in the wound bed immediately beneath the scab (Fig. 2b and Supplementary Fig. 6). Confocal microscopy substantiated the presence of NETs in skin wounds. Externalized DNA colocalized with H3Cit in areas associated with intense staining of the neutrophil membrane marker Ly6G (Fig. 2c). Of note, H3Cit and neutrophils were absent in the surface layers of unwounded skin (Supplementary Fig. 6). Skin expresses PAD isoforms 1–3 (ref. 25), which could citrullinate extracellular proteins in the scab. To verify the cellular source of H3Cit, we subjected CD18 (β2 integrin)-deficient (CD18−/−) mice, which are defective in leukocyte recruitment, to wounding. In these mice, both H3Cit and Ly6G were undetectable by western blotting in wounds 3 d after injury (Fig. 2d), when H3Cit was maximal in the WT wounds (Fig. 2a), thus suggesting that H3Cit is of leukocyte origin. H&E staining and immunofluorescence microscopy showed that the few Cd18−/− neutrophils present in these wounds were H3Cit+ and produced NETs (Supplementary Fig. 7a,b). Indeed, Cd18−/− neutrophils produced NETs efficiently in vitro (Supplementary Fig. 7c), showing that β2 integrins were not required for NETosis. Wounds from WT mice with depleted neutrophils also showed markedly reduced H3Cit (Fig. 2d). Thus, our data indicate that neutrophils are the source of the H3Cit present in the wounds.

To establish the role of NETs in wound healing, we compared wounds of WT to Pad4−/− mice. Prominent extracellular DNA structures observed by H&E were absent in Pad4−/− scabs (Fig. 3a, top), as were the H3Cit and extracellular chromatin patterns seen in WT mice by confocal microscopy (Fig. 3a, bottom). In contrast to the robust H3Cit signals in WT wounds, no H3Cit was detectable in wounds from Pad4−/− mice despite normal neutrophil recruitment (Fig. 3b and Supplementary Fig. 8). Unlike in neutrophil recruitment-defective P- and E-selectin double mutants that have opportunistic infections26 and impaired wound healing24, wounds in Pad4−/− mice did not show overt signs of infection (Fig. 3c) and healed faster than wounds in WT mice (Fig. 3c,d). This is probably because other neutrophil functions such as phagocytosis11, degranulation and ROS production27 are intact in Pad4−/− neutrophils, so that these neutrophils are fully capable of performing other host defense mechanisms. About 80% of Pad4−/− mice had all wounds healed on day 14 compared to only 25% of WT controls (Fig. 3e). The beneficial effect of PAD4 deficiency on wound healing was observed early after injury (Fig. 3d), indicating that NETs might impair the onset of initial healing processes such as re-epithelialization. In line with this hypothesis, re-epithelialization progressed threefold faster in Pad4−/− mice compared to WT mice (Fig. 3f and Supplementary Fig. 9). Immunofluorescence staining of wounds for Ki67 (a proliferation marker) and TUNEL (an indicator of apoptosis) was not different between WT and Pad4−/− mice 3 d after wounding (data not shown). It is thus possible that keratinocyte migration is affected and further investigation is needed to prove it. Although WT and Pad4−/− neutrophils also express PAD2 and PAD3 (ref. 11), our data demonstrate that PAD4, the only nuclear PAD, is essential for the histone H3 citrullination and NETosis in skin wounds. Coudane et al.28 reported that PAD4 is the main PAD isoform detected in scabs of wounds from WT mice, and that PAD2 is unnecessary for
citrullination of scab proteins as observed in PAD2-deficient mice, further strengthening the unique deimination role of PAD4 in the wounds.

We next examined whether NETs interfere with diabetic wound healing. Type 1 diabetes was induced in WT and Padi4−/− mice by STZ, and 8 weeks later these mice were subjected to wounding. Changes in body weight, fed blood glucose and diabetes induction rate were similar between the two genotypes (Supplementary Fig. 2d–f). As expected, diabetic WT mice healed more slowly than normoglycemic controls (Fig. 4i). All normoglycemic WT mice healed by day 16, whereas ~20% of diabetic mice still had open wounds on day 19 (Fig. 4h). On day 7, diabetic Padi4−/− mice healed ~35% faster than diabetic WT mice (Fig. 4d). By day 15, all diabetic Padi4−/− mice were completely healed (Fig. 4c). Notably, diabetes did not impair wound healing in Padi4−/− mice (Fig. 4c,i), which underscores NETs as the major determinants delaying healing in the diabetic mice. Higher H3Cit levels were detected in wounds of STZ-induced diabetic WT mice compared to the normoglycemic WT mice 1 d after wounding (Fig. 4g). Enhanced NETosis in diabetic animals recapitulates our in vitro observations (Fig. 1e,f), further supporting the role of NETs in the delay in diabetic wound repair. Antibiotics, provided to mimic the medical regimen of diabetic patients with chronic wounds, did not abolish the beneficial effect of PAD4 deficiency (Supplementary Fig. 10).

NETs and histones directly induce epithelial and endothelial damage25. A high concentration of neutrophil elastase, a component of NETs3, can cause degradation of the wound matrix and delay healing29. Such a toxic environment produced by NETs may explain the slower keratinocyte repopulation in the wound beds of WT mice. Because PAD4 is not expressed in the skin25, its negative effect on wound healing is most likely due to infiltrating neutrophils. The use of NETosis to defend against microbes may not be very effective during wound healing, as Staphylococcus species, which are very abundant in diabetic wounds30, degrade NETs to escape trapping31.

Pre-digestion of NETs with DNase 1 accelerated their clearance by macrophages in vitro22. We thus tested whether systemic DNase 1 treatment could accelerate wound healing in diabetic mice that were maintained on antibiotics. Without DNase 1 treatment, wound healing was faster in diabetic Padi4−/− mice, as assessed by 28% more reduction in wound area (Fig. 4h, top) and 41% more re-epithelialization (Fig. 4h, bottom) compared to the diabetic WT mice as examined on day 3 after wounding. Administration of DNase 1 reduced wound area faster by >20% and enhanced re-epithelialization by >75% in diabetic WT mice, an extent similar to that of DNase 1-treated normoglycemic WT mice (Fig. 4h). DNase 1 treatment did not further improve wound healing in diabetic Padi4−/− mice (Fig. 4h). These data indicate that NETs are the major source of extracellular DNA that hinders wound healing. Such beneficial effects of DNase 1 were not confined to diabetic wounds. Three days after wounding, wound areas in normoglycemic mice treated with DNase 1 were smaller than in those treated with vehicle (Fig. 4i, top). Re-epithelialization was also enhanced by 54% in the DNase 1–treated group (Fig. 4i, bottom), whereas neutrophil recruitment was not affected (data not shown).

Our results indicate that plasma DNase 1 activity may regulate wound healing. Less functional polymorphisms of DNASE1 exist in the human population33,34. These polymorphisms or the presence of inhibitors impairing DNase 1 function35 predisposes individuals to cardiovascular
and autoimmune disease, probably because DNA (NETs) are not dismantled and removed in a timely manner.33–35 Wound healing could be similarly affected in individuals with decreased DNase 1 activity. Topical treatment with an ointment containing fibroinolysin and DNase (Elase) is sometimes used clinically for wound debridement. In addition to removing necrotic tissue, our findings suggest that the DNase component may also cleave NETs to enhance wound recovery.

In summary, our data demonstrate that diabetes activates neutrophils to overproduce PAD4 and NETs and identify NETs as a key factor delaying wound healing. PAD4 inhibition and cleavage of NETs by DNase I could be novel therapeutic approaches to wound resolution, not only in diabetes, but also in wounds resulting from aseptic procedures such as surgeries of normoglycemic patients. We further validate the importance of PAD4 in human disease and report the upregulation of PAD4 in individuals with diabetes, thus providing a new rationale for developing specific PAD4 inhibitors.36 Although NETs were first postulated to limit infection,1 a lack of NETs did not worsen bacteremia and co-wrote the manuscript.

**ONLINE METHODS**

**Animals.** All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Boston Children’s Hospital. Cd18−/− mice and Pad4−/− mice were on a C57BL/6j background and were routinely crossed to WT mice from the Jackson Laboratory (Bar Harbor, ME). Age- and sex-matched control mice included the WT littermates of the two strains and C57BL/6j mice purchased from the Jackson Laboratory. Except the wound healing time course experiment, wherein both male and female normoglycemic WT and Pad4−/− mice were used (Fig. 3d,e), male mice were used in all other experiments. Normoglycemic mice were used for experiments at age 8–10 weeks while STZ-induced diabetic mice and their vehicle-treated controls were used 8 weeks after successful induction. Nine-week-old male diabetic db/db mice and their normoglycemic control m+/db mice were purchased from Jackson Laboratory and used between 9 and 12 weeks of age. All mice were fed standard lab diet and maintained under standard laboratory conditions free of specific pathogens. The number of mice in each experiment is indicated in the figure legends. Sample size was chosen on the basis of previous experience with the animal strains and animal models. Investigators were not blinded to the genotypes of animals.

**Human blood cell samples.** The study was approved by the Institutional Review Board of Boston Children’s Hospital and Joslin Diabetes Center, and it conformed to the principles outlined in the Declaration of Helsinki. We obtained blood samples from ten healthy individuals (two males, eight females, age range 23–63 years old) and ten diabetic subjects (five males: two had type 1 DM; one had type 2 DM; three had type 1 DM, two had type 2 DM; age range 23–64 years old) after their written informed consent. Subjects with diabetes were recruited only if they were below 70 years old, not on steroid or other immunosuppressive medications, not presenting any signs of active infection (fever, high leukocyte count and diagnosis of infection), without diagnosis of cancer in the past 5 years and without overt heart failure. For in vitro high glucose NETosis assay, blood samples were obtained from five healthy individuals (three males, two females, age range 29–63 years old). Sample size was chosen on the basis of previous in vitro experience with human neutrophil samples. No statistical method was used to pre-determine the sample size.

**Induction of diabetic mouse model.** Mice were induced to be diabetic using multiple low-dose injections of streptozotocin (STZ). 6–8-week-old male C57BL/6j or Pad4−/− mice were randomized into treatment groups of either vehicle or STZ according to their blood glucose levels and body weight at baseline. Mice were fasted for 5 h and then injected with vehicle or STZ (intra-peritoneal (i.p.) injection, 50 mg/kg per day, pH 4, dissolved in 0.1 M sodium citrate buffer) for 5 consecutive days. Fed blood glucose level was measured starting 1 week afterward. Mice with fed blood glucose level above 300 mg/dl were considered diabetic and used for further experiments. Pancreatic islets were collected via the retro-orbital venous plexus. Red blood cells were lysed using rabbit secondary antibody (1:1,500, Invitrogen, cat. no. A11008) and Hoechst 33342 (1:10,000, Invitrogen, cat no. H3570). Images were acquired on an Axiovert 200M wide-field fluorescence microscope (Zeiss) coupled to an AxioCam MR3 monochromatic CCD camera (Zeiss) using a Zeiss Plan-Neofluar 20×/0.4 Corr Ph2 objective lens with the Zeiss AxioVision software (version 4.6.3.0). Percentages of H3Cit^high cells and NETs were determined from five or six non-overlapping fields per well and the average was taken from duplicates or triplicates for each condition in every experiment. Exposure time for H3Cit and DNA were identical for all treatments within the same experiment. Spread NETs were counted in a single channel for DNA. Images of this channel were exported in black-and-white for better contrast for quantification.

**Human neutrophil isolation and NETosis assay.** Blood was drawn from healthy individuals or diabetic subjects into EDTA-coated tubes. Neutrophils were isolated using Histopaque-1119 (Sigma) and Percoll Plus (GE Healthcare) gradients as described, a method that cause minimal activation of neutrophils during isolation. Purity of cells was >95% as determined by Wright-Giemsa staining. For experiments involving high glucose, neutrophils were resuspended in glucose-free HEPES-buffered RPMI medium supplemented with 22 mM glucose (5.5 mM normal), 22 mM (high) or 5.5 mM mannitol (osmotic control) and 2% heat-inactivated FBS. Neutrophils were plated at 10,000 cells per well in 96-well Cellbind plates (Corning). After incubation in the respective media for 1 h, cells were stimulated with LPS (Sigma) at indicated concentrations for 2.5 h. For experiments that did not involve high glucose, cells were resuspended in HEPES-buffered RPMI medium (11 mM glucose) supplemented with 2% heat-inactivated FBS, plated at 10,000 cells per well and incubated with LPS (4 μM) or PMA (100 nM) for 2.5 h. For experiments involving high glucose, cells were resuspended in glucose-free RPMI medium supplemented with 2% LPS and heat-inactivated FBS, plated at 10,000 cells per well and incubated with LPS (4 μM) or PMA (100 nM) for 2.5 h. For experiments involving high glucose, cells were resuspended in glucose-free RPMI medium supplemented with 2% LPS and heat-inactivated FBS, plated at 10,000 cells per well and incubated with LPS (4 μM) or PMA (100 nM) for 2.5 h.

**Measurement of basal H3Cit on mouse cytopsin.** Mouse whole blood was collected via the retro- orbital venous plexus. Red blood cells were lysed using ACK (ammonium-chloride-potassium) lysing buffer. After centrifugation, cells were resuspended in 7.5% BSA in PBS and spun at 1600 rpm for 4 min onto slides. They were instantly fixed with 4% PFA at 4°C overnight, and then stained using rabbit polyclonal anti-H3Cit (1:1,000, Abcam, cat. no. ab5103) and rat monoclonal anti-mouse Ly6G (1:500, BD Pharmingen, cat. no. 551459), followed by Alexa Fluor-conjugated secondary antibodies (1:1,500, Alexa Fluor 488 goat anti-rabbit IgG (H+L), Invitrogen, cat. no. A11008; 1:1,500, Alexa Fluor 555 goat anti-rat IgG (H+L), Invitrogen, cat. no. A21434) and Hoechst 33342 (1:10,000, Invitrogen, cat no. H3570). Images were acquired on an Axiovert 200M wide-field fluorescence microscope (Zeiss) coupled to an AxioCam MR3 monochromatic CCD camera (Zeiss) using a Zeiss Plan-Neofluar 20×/0.4 Corr Ph2 objective lens with the Zeiss AxioVision software (version 4.6.3.0). H3Cit^high neutrophils were determined by thresholding analysis using ImageJ software (NIH).

**Mouse neutrophil isolation and NETosis assay.** Peripheral blood neutrophils were isolated using Percoll (GE Healthcare) gradients as described, purity of cells was >90% as determined by Wright-Giemsa staining. Neutrophils were resuspended in HBSS (with calcium, magnesium and 5.5 mM glucose) for experiments involving high glucose; otherwise they were resuspended in HEPES-buffered RPMI medium. Neutrophils were plated at 50,000 cells per well in 96-well glass-bottomed plates and stimulated with Klebsiella pneumoniae LPS (Sigma) at indicated concentrations for 2.5 h. For high- glucose experiments, neutrophils were isolated from normoglycemic mice and pre-incubated for 1 h in media with normal (5.5 mM) or high (22 mM) glucose concentrations. A concentration of 22 mM corresponds to 396 mg/dl, which is similar to the fed blood glucose level in STZ-induced mice 8 weeks after induction (376.3 ± 26.9 mg/dl). Mannitol (16.5 mM in medium with 5.5 mM glucose) was used as an osmotic control. LPS (in respective medium) was added and neutrophils were further incubated for 2.5 h. Cells were then fixed in 2% PFA, permeabilized, blocked, stained with anti-H3Cit (1:1,000, Abcam, cat. no. ab5103), Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:1,500, Invitrogen, cat. no. A11008) and Hoechst 33342 (1:10,000, Invitrogen, cat. no. H3570). Images were acquired on an Axiovert 200M wide-field fluorescence microscope (Zeiss) coupled to an AxioCam MR3 monochromatic CCD camera (Zeiss) using a Zeiss Plan-Neofluar 20×/0.4 Corr Ph2 objective lens with the Zeiss AxioVision software (version 4.6.3.0). Percentages of H3Cit^high cells and NETs were determined from five or six non-overlapping fields per well and the average was taken from duplicates or triplicates for each condition in every experiment. Exposure time for H3Cit and DNA were identical for all treatments within the same experiment. Spread NETs were counted in a single channel for DNA. Images of this channel were exported in black-and-white for better contrast for quantification.

**Wounding and macroscopic healing assessment.** Full-thickness excisional wounds were made on the dorsal skin of mice under aseptic conditions as described. Mice were anesthetized with ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively, i.p.). Hair was removed and the skin was cleaned with 70% ethanol and Betadine. A fold of the dorsal skin was then picked up along the midline, placed over dental wax and punched through with a 4-mm disposable sterile biopsy punch (Miltex) such that two wounds were generated...
in one punch. The procedure was repeated; thus, four wounds were made per mouse. The mice were housed individually after wounding. In experiments involving diabetic mice, all mice were provided ad libitum with antibiotics (2.5% Sulfadiazine) in drinking water. Wounds were digitally photographed using a Sony Camcorder and total wound areas were calculated using ImageJ software. Wound area was expressed as a percentage compared to the area on day 0 when the wounds were made.

Western blot analysis. Levels of H3Cit and Ly6G of mouse wounds and PAD4 expression in human neutrophils were quantified by western blot. After collection of mouse wounds or isolation of human neutrophils, the samples were snap frozen and homogenized in RIPA buffer supplemented with protease inhibitor cocktails (Sigma) on ice. After centrifugation at 20,000g for 20 min at 4°C, the protein content of the supernatant was determined by bicinchoninic acid protein assay and an equal amount of protein per sample was resolved on gradient gels (4–20% Tris-Glycine gels, Lonza or Bolt 4–12% Bis-Tris Plus gels, Life Technologies) and electrophotoblot on PVDF membranes, which were then incubated with primary antibodies (rabbit polyclonal anti-H3Cit, 1:1,000, Abcam, cat. no. ab5103; rabbit polyclonal anti-H3, 1:5,000, Abcam, cat. no. ab1791; rat monoclonal anti-mouse Ly6G, 1:500, BD Pharmingen, cat. no. 551459; mouse monoclonal anti-human PAD4, 1:2,000, Abcam, cat. no. ab128086) at 4°C overnight and subsequently with appropriate HRP-conjugated secondary antibodies (1:10,000, goat anti-rabbit IgG (H+L)-HRP conjugate, BioRad, cat. no. 170-6515; 1:10,000, goat anti-mouse IgG (H+L)-HRP conjugate, BioRad, cat. no. 170-6516; 1:5,000, goat anti-rat IgG (H+L)-HRP conjugate, Invitrogen, cat. no. A10549) for 2 h at room temperature. The blots were developed with enhanced chemiluminescence substrate (Thermo Scientific, cat. no. 32106). Equal loading was confirmed by probing for GAPDH (1:40,000, Ambion, cat. no. AM4300). Blots were quantified using ImageJ software.

Immunofluorescence wide-field and confocal microscopy. Localization of H3Cit and neutrophils in the wounds were examined by immunofluorescence microscopy. Wounds were dissected, cut in half and instantly embedded in OCT. The tissue was cryosectioned into 10-µm and 20-µm sections for wide-field and confocal immunofluorescence microscopy, respectively. The sections were post-fixed in zinc fixative (100 mM Tris-HCl, 37 mM zinc chloride, 23 mM zinc acetate, 3.2 mM calcium acetate), permeabilized and incubated with primary antibodies against H3Cit (1:1,000, Abcam, cat. no. ab5103) and Ly6G (1:500, BD Pharmingen, cat. no. 551459) at 4°C overnight, and then with Alexa Fluor–conjugated secondary antibodies (1:1,500, Alexa Fluor 488 goat anti-rabbit IgG (H+L), Invitrogen, cat. no. A11008; 1:1,500, Alexa Fluor 555 goat anti-rat IgG (H+L), Invitrogen, cat. no. A21434) for 2 h at room temperature. Hoechst 33342 (1:10,000, Invitrogen, cat. no. H3570) was used to stain for DNA. Images were acquired on an Axiovert 200M wide-field fluorescence microscope (Zeiss) coupled to an AxioCam MR3 monochromatic CCD camera (Zeiss) using a Zeiss plan-Apochromat 63x/1.4 oil differential interference contrast (DIC) objective lens with the Zeiss AxioVision software (version 4.6.3.0), or on the Olympus IX 81 FV1000 LSM confocal microscope using UPLAPO 20x/0.70 or UPLAPO 60x/1.20 water objective lens with the Olympus Fluoview software (Version 3.1.2.2).

Histological examination. NET formation in wounds and re-epithelialization were examined in H&E-stained sections. Wounds were cut in half, fixed overnight in zinc fixative and embedded in paraffin. The tissue was sectioned at 10 µm and stained with H&E. Images were acquired on an Axioplan light microscope coupled to a color Zeiss Achromat 63x/0.95 water objective lens (to observe for NETs) or Olympus UPlanFl 4x/0.13 objective lens (for re-epithelialization analysis) with the Zeiss AxioVision software (version 4.6.3.0).

Neutrophil depletion. Neutrophils of 10-week-old WT mice were depleted 1 d before wounding by i.v. injection of a specific anti-neutrophil antibody (ultra-low-endotoxin and azide-free rat anti-Ly6G, 1A8 clone, Biolegend, cat. no. 127632) at a dose of 5 µg/g mouse. Control mice were injected with rat IgG isotype control. The mice were re-dosed at 2.5 µg/g mouse 2 d after the first injection. Circulating levels of neutrophils were evaluated by flow cytometry (BD FACSCanto II) using a FITC-conjugated rat monoclonal anti-mouse neutrophil antibody (1:300, anti-7/4, Abcam, cat. no. ab53453) and analyzed using FlowJo software. About 80% of circulating neutrophils were depleted throughout the 3-d wound healing period.

DNase 1 treatment. Normoglycemic and diabetic WT mice, randomized by blood glucose levels before assigning to treatments, were injected with 10 µg i.v. and 50 µg i.p. DNase 1 (dornase alfa, Genentech) 30 min before wounding and then 50 µg i.p. every 12 h until wound collection on day 3. Control mice were injected with vehicle (8.77 mg/ml sodium chloride and 0.15 mg/ml calcium chloride)40.

Statistical analysis. Data are presented as mean ± s.e.m. of at least two independent experiments, and were analyzed using Mann-Whitney test, two-tailed Student’s t-test (unpaired), Kruskal-Willis test followed by Dunn’s post-test, or repeated measures ANOVA with Bonferroni’s post-test, where appropriate. The percentage of mice with total wound closure and the rate of diabetes induction between WT and Pad4−/− were analyzed with two-tailed Fisher’s exact test of contingency tables. The percentage of mice with open wounds was analyzed with the log-rank test after constructing the Kaplan-Meier curves. All analyses were performed using GraphPad Prism software (Version 5.0). Results were considered significant when P < 0.05.