Supplementary Information

Diabetes primes neutrophils to undergo NETosis which severely impairs wound healing

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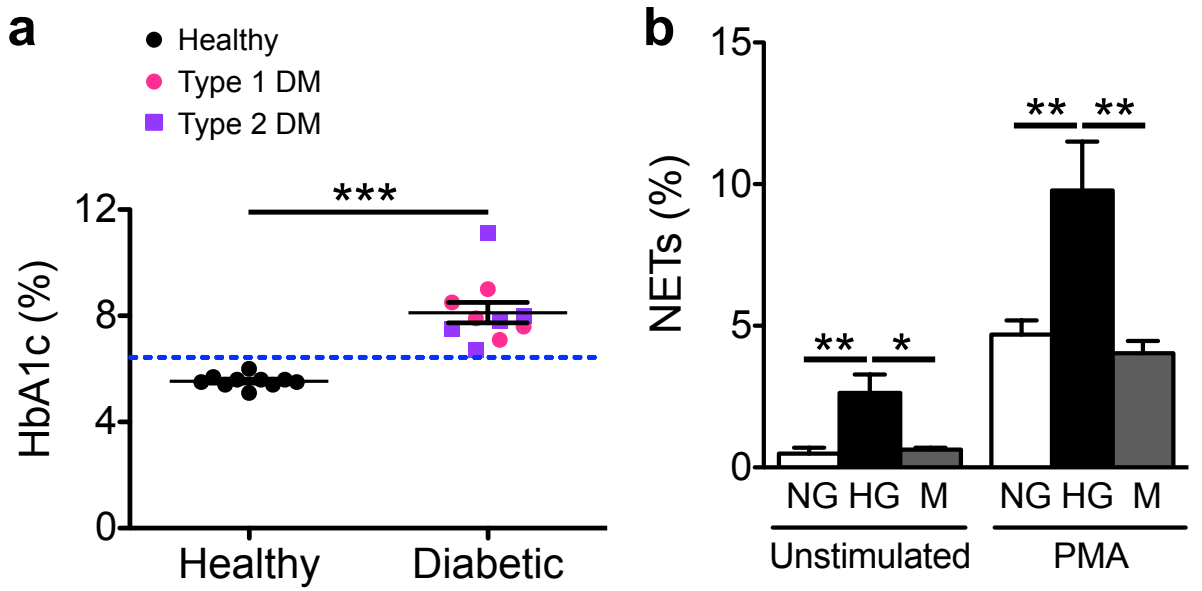
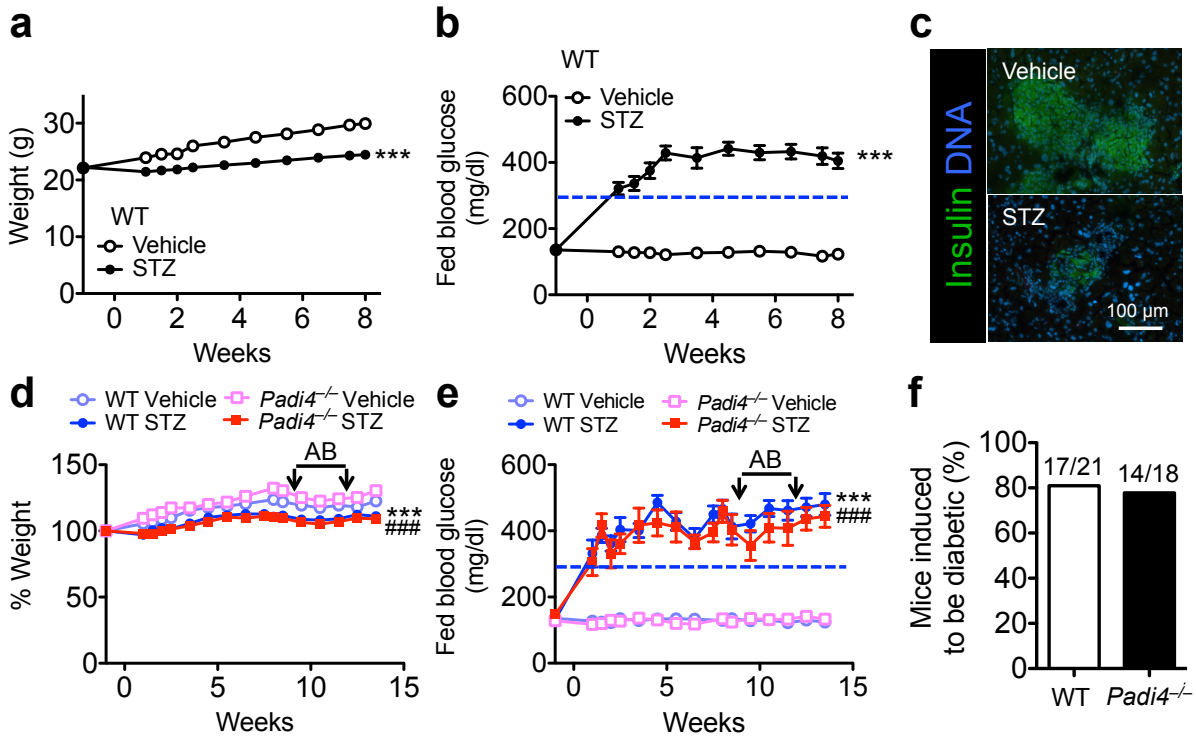
This supplement contains 10 supplementary figures and 1 supplementary table.

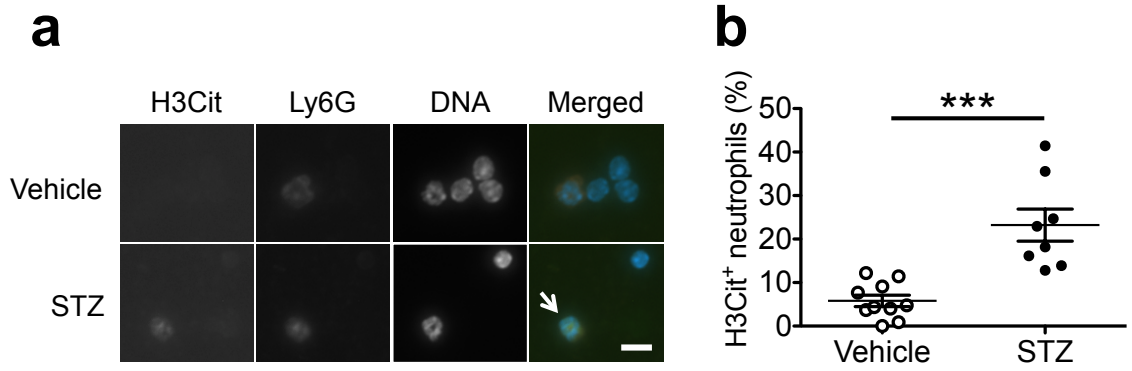
Supplementary figures



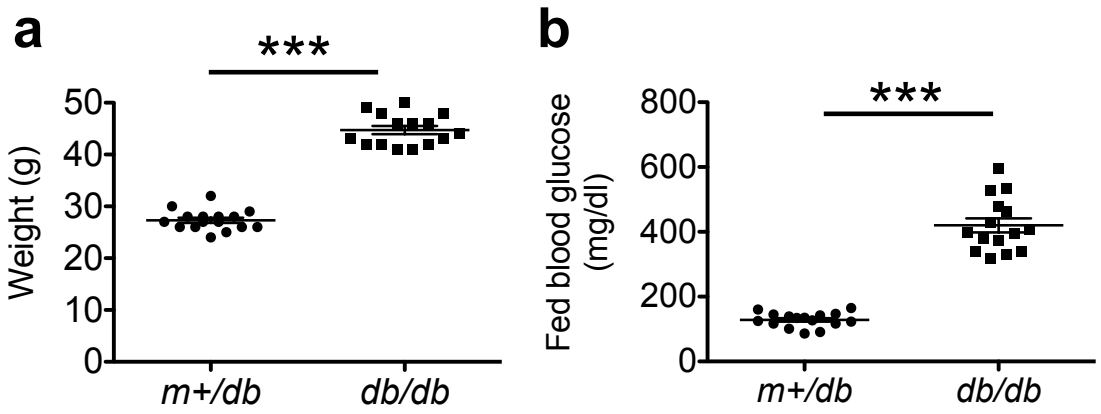
Supplementary Figure 1. HbA1c levels of recruited individuals and NET formation by healthy human neutrophils under hyperglycemic conditions *in vitro*. (**a**) All individuals with diabetes had HbA1c >6.5% (reference to Figure **1a–c**). n = 10 per group, ****P*<0.001, Mann-Whitney test. (**b**) High glucose (HG) enhances PMA (100 nM)-stimulated NET formation in neutrophils isolated from healthy individuals compared to neutrophils exposed to normal glucose (NG) or mannitol (M), osmotic control. n = 5 per condition, **P*<0.05, ***P*<0.01, repeated measures ANOVA.



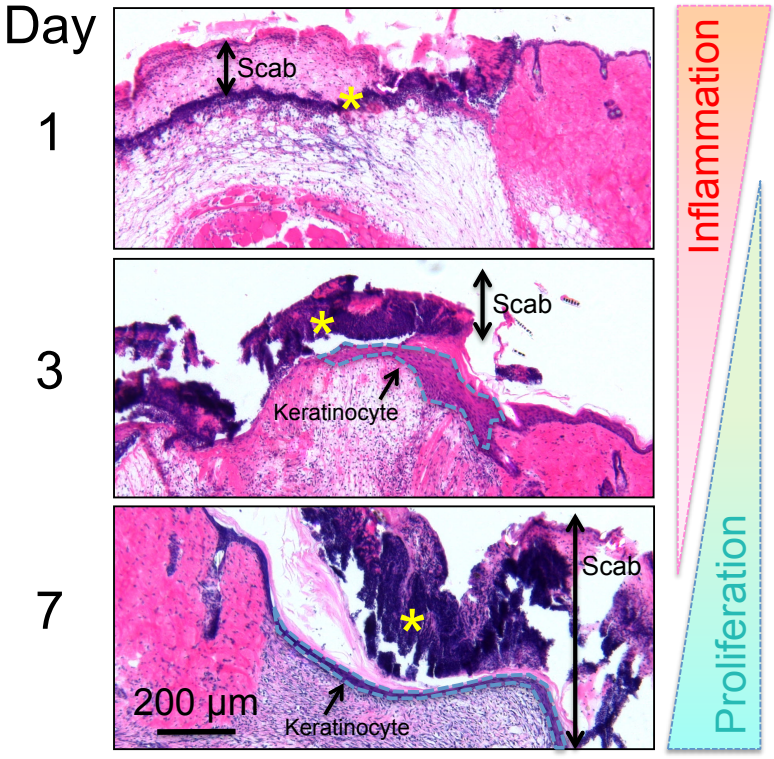
Supplementary Figure 2. Basic parameters of STZ-induced diabetes in WT and *Padi4*$^{-/-}$ mice. Mice were injected i.p. with vehicle or STZ (50 mg/kg per day) for 5 consecutive days. Body weight and fed blood glucose were examined starting 1 week after completion of injections. (**a**) STZ-treated mice gained less weight compared to the vehicle control. (**b**) Diabetes was defined as fed blood glucose >300 mg/dl (indicated by blue dotted line). STZ-treated mice became diabetic the first week after treatment. (**a**,**b**) n = 15 for vehicle, n = 13 for STZ, ***P<0.001 at all time points starting week 1 between vehicle and STZ, Student's t test. (**c**) Validation of diabetes induction. Representative immunofluorescence images showing a marked reduction of insulin-producing β cells and disrupted islet morphology in the pancreas of STZ-treated mice. (**d,e**) *Padi4*$^{-/-}$ mice attained body weight (**d**) and fed blood glucose levels (**e**) as similar to WT after STZ injection. AB indicates the period of antibiotic treatment (after wounding), which did not affect fed blood glucose levels in any group (**e**). (**d,e**) n = 7 for WT vehicle, n = 9 for WT STZ, n = 5 for *Padi4*$^{-/-}$ vehicle, n = 6 for *Padi4*$^{-/-}$ STZ, ***P<0.001 at all time points starting week 1 between WT vehicle and WT STZ, $^{###}P$<0.001 at all time points starting week 1 between *Padi4*$^{-/-}$ vehicle and *Padi4*$^{-/-}$ STZ, Student's t test. (**f**) P=1.00. Chi-square test indicates no difference between WT and *Padi4*$^{-/-}$ in diabetes inducibility using STZ.



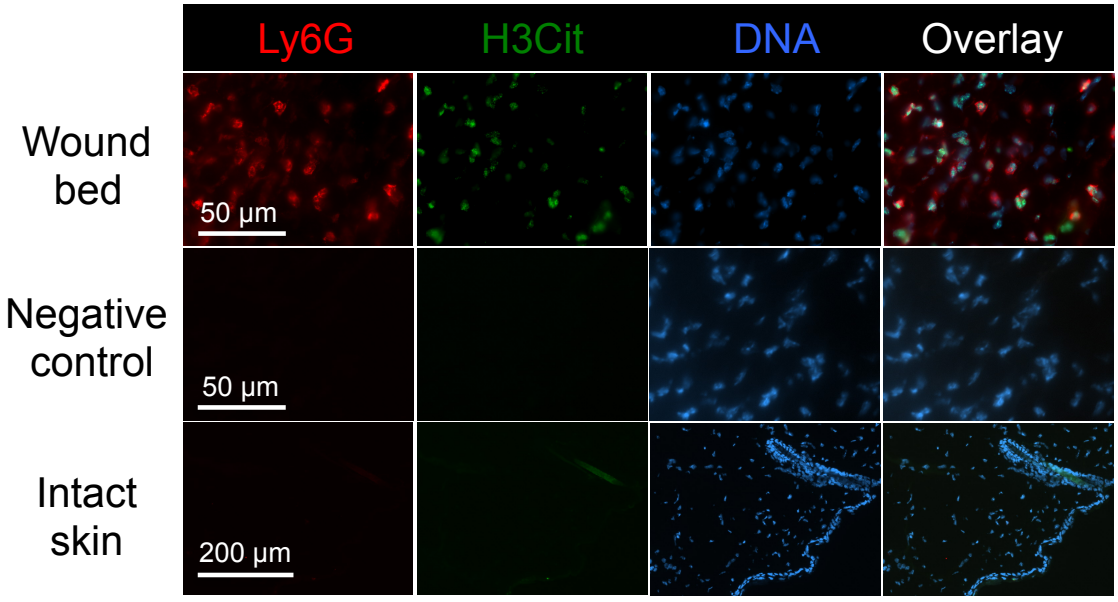
Supplementary Figure 3. Mouse whole blood cytospin was performed and cells were stained for H3Cit and Ly6G. (**a**) Representative images showing an example of an H3Cit and Ly6G double-positive cell in cytospin from STZ-induced diabetic mice (arrow). Scale, 10 μm. (**b**) More circulating neutrophils with elevated basal H3Cit level were detected in STZ-treated mice. n = 10 for vehicle, n = 8 for STZ, ***P<0.001, Student's t test.



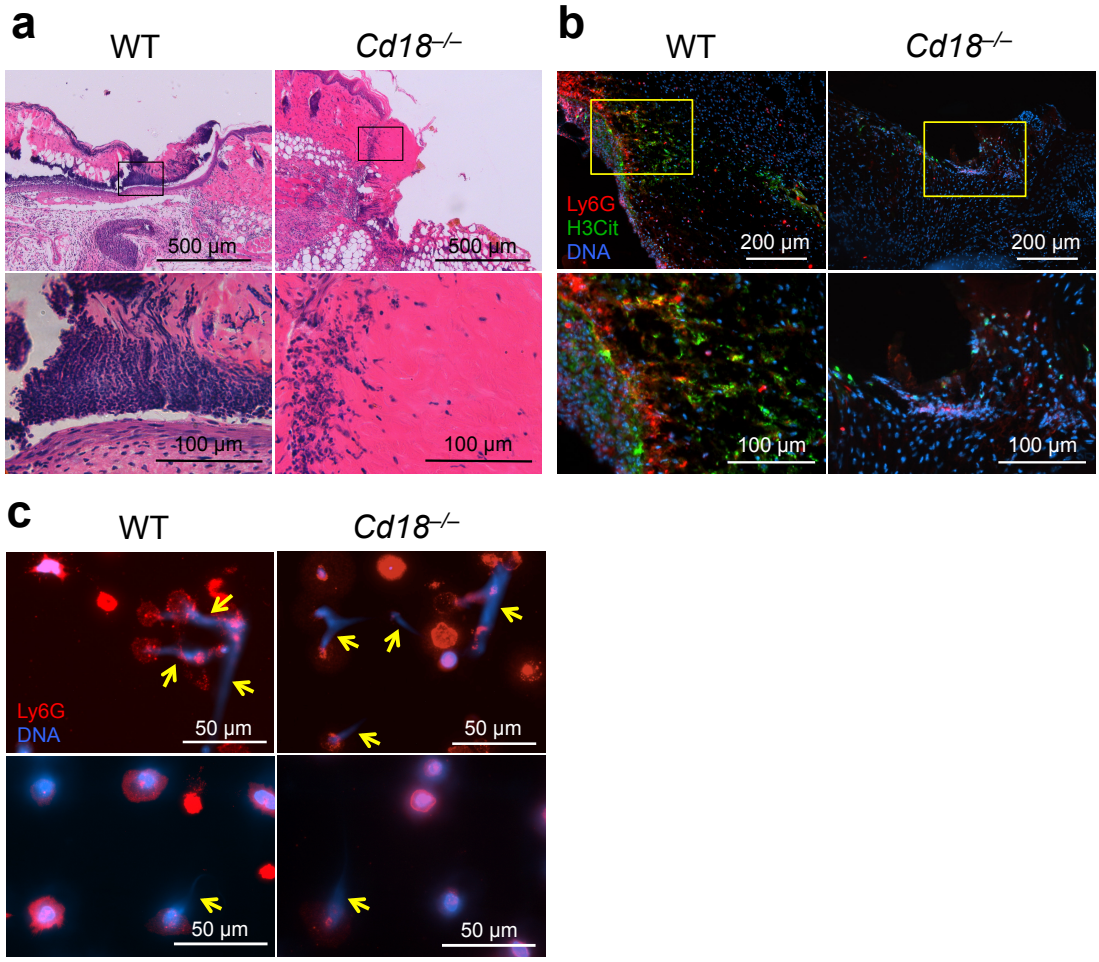
Supplementary Figure 4. Basic parameters of non-diabetic control *m+/db* and diabetic *db/db* mice. (**a**) Body weight and (**b**) fed blood glucose were significantly higher in 9 to 12-week old *db/db* mice. n = 16 for *m+/db*, n = 15 for *db/db*, ***P<0.001, Mann-Whitney test.



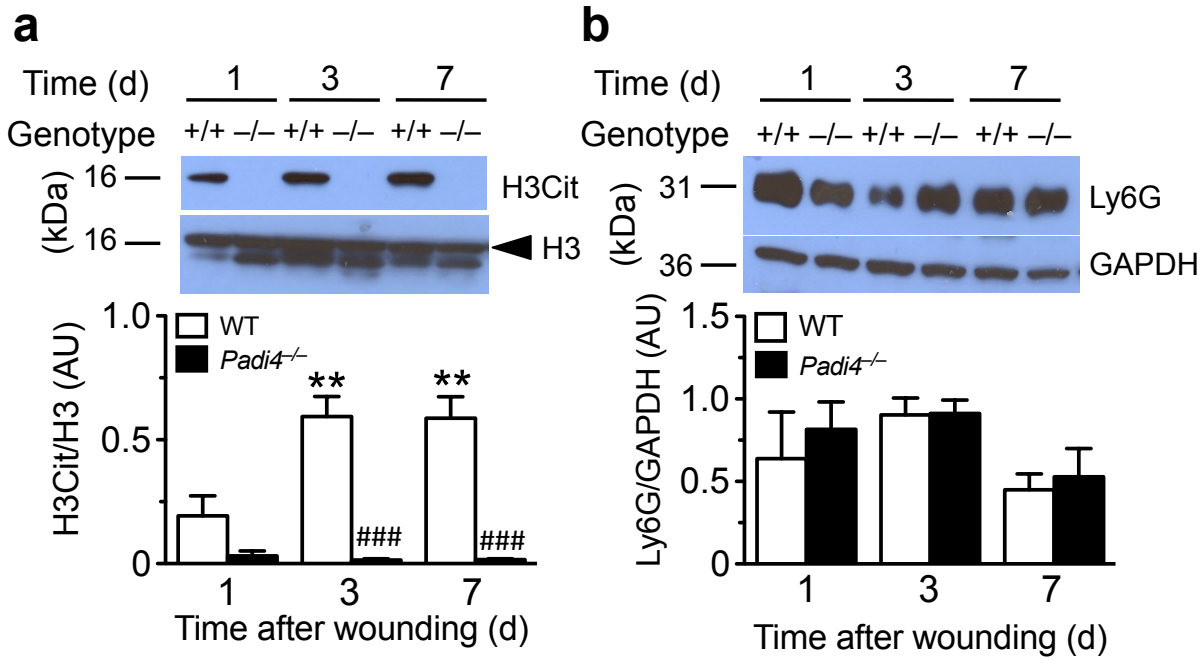
Supplementary Figure 5.
Inflammation and proliferation stages overlap. H&E images showed that leukocytes (asterisk) were rapidly recruited to the wounds during the inflammation stage of wound healing. Leukocytes concentrated immediately beneath the scab 1 day post wounding. A majority of neutrophils migrated into the scab on day 3 through 7, during which the proliferation stage of wound healing started and keratinocytes (blue dotted lines) migrated while proliferating into the wound bed from the shoulders of the wound to effect re-epithelialization.



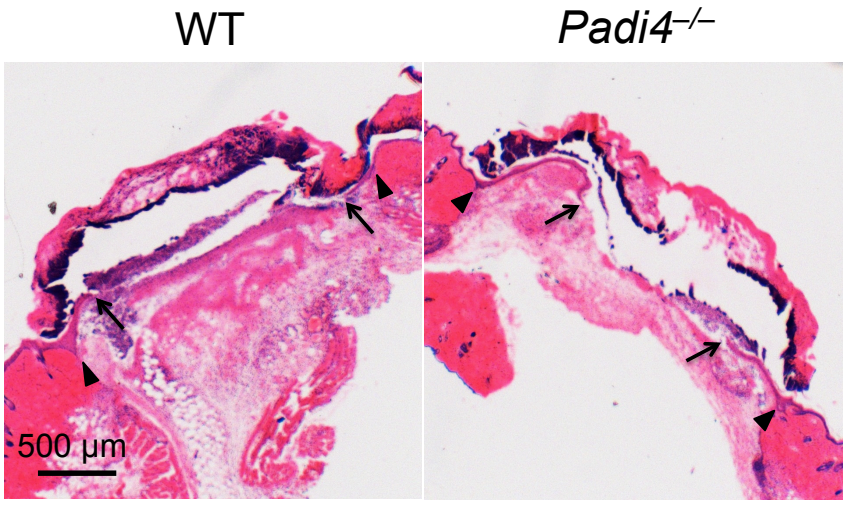
Supplementary Figure 6. Neutrophil H3Cit is only observed in the wounded skin. No neutrophils or H3Cit were detected in the surface layers of intact skin (The non-cellular red and green are non-specific signals of a hair follicle, bottom row). Ly6G and H3Cit signals were negative in the wound bed control omitting primary antibodies (reference to Figure **2b**).



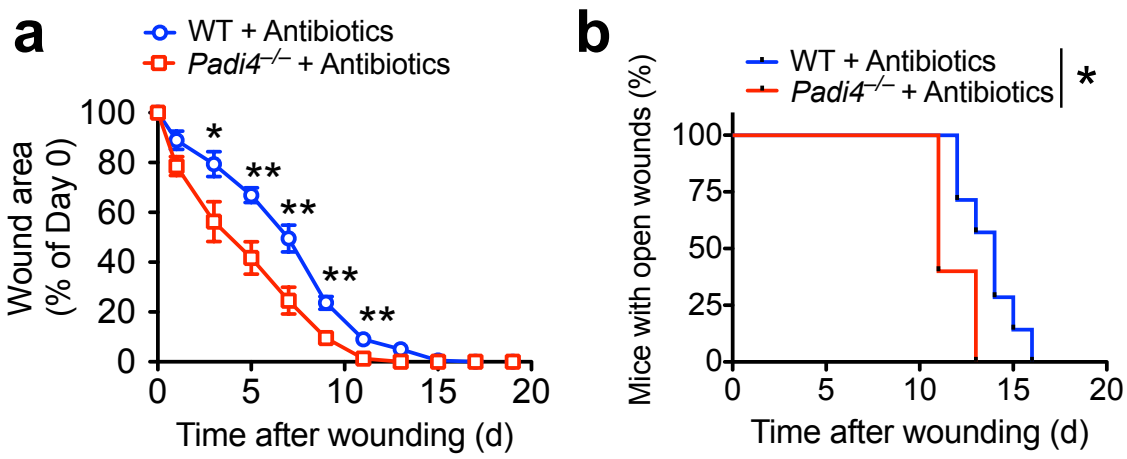
Supplementary Figure 7. *Cd18*$^{-/-}$ mouse neutrophils are able to produce NETs. (**a**) H&E staining showed that leukocyte recruitment to wounds was severely diminished in *Cd18*$^{-/-}$ mice. Lower panels (magnified view of black-boxed area of the upper panels) revealed extracellular DNA patterns in the scabs of *Cd18*$^{-/-}$ mice in areas with neutrophils. (**b**) Immunofluorescence microscopy confirmed a significant reduction of neutrophils (Ly6G, red) in the wounds of *Cd18*$^{-/-}$ mice. The few neutrophils present in the wounds were able to hypercitrullinate histone H3 (H3Cit, green). Lower panels are magnified images of the area enclosed by the yellow box in the upper panels. (**a**,**b**) All images were from wounds collected 3 days post injury. (**c**) Neutrophils were isolated from WT and *Cd18*$^{-/-}$ mice and subjected to LPS stimulation (25 µg/mL, 2.5 h) *in vitro*. Representative immunofluorescence images showed NET formation by both WT and *Cd18*$^{-/-}$ neutrophils (yellow arrows).



Supplementary Figure 8. H3Cit (**a**) is absent while neutrophil recruitment (**b**) is unaffected in wounds of *Padi4*$^{-/-}$ mice. Summarized Western blot data of Figure **3b**. +/+, WT; –/–, *Padi4*$^{-/-}$. Per order in the bar chart, n = 5, 8, 7 for WT groups, n = 5, 9, 7 for *Padi4*$^{-/-}$ groups, $^{**}P<0.01$ versus day 1 WT, $^{\#\#\#}P<0.001$ versus WT on respective day, Student's t test.



Supplementary Figure 9. H&E staining of wounds from WT and *Padi4*$^{-/-}$ mice 3 days post injury. Re-epithelialization was determined by the distance that keratinocytes migrated from the shoulder of the wounds (indicated by arrowheads) into the wound bed. Arrows indicate the keratinocyte migrating tip. Re-epithelialization occurred faster in *Padi4*$^{-/-}$ mice.



Supplementary Figure 10. Antibiotics do not abrogate the beneficial effect of PAD4 deficiency on wound healing. Under antibiotic treatment, *Padi4*$^{-/-}$ mice still fared better in terms of (**a**) wound area reduction and (**b**) days required for total wound closure. n = 7 for WT vehicle, n = 5 for *Padi4*$^{-/-}$ vehicle, **P*<0.05, ***P*<0.01 between groups on the same day or between curves, (**a**) Student's t test, (**b**) log-rank test.

Supplementary Table 1. Parameters of healthy individuals and individuals with diabetes analyzed using Mann-Whitney test

	Healthy individuals	Diabetic individuals	*P* value
Age (years)	39 ± 4	45 ± 5	0.520
Leukocyte count (K/μL)[a]	6.37 ± 0.55	6.37 ± 0.79	0.796
Platelet count (K/μL)[a]	280.80 ± 16.07	289.10 ± 13.87	0.605
HbA1c (%)	5.54 ± 0.07	8.12 ± 0.39	<0.001
Glucose (mg/dL)	91.10 ± 3.50	165.00 ± 26.80	0.004
Cholesterol (mg/dL)	176.80 ± 9.85	184.80 ± 13.69	0.631
Triglycerides (mg/dL)	97.90 ± 17.67	197.20 ± 61.73	0.121
HDL (mg/dL)	75.20 ± 9.74	61.30 ± 10.08	0.257
LDLC (mg/dL)[b]	82.10 ± 6.44	85.83 ± 12.87	0.957

All samples from the recruited individuals (10 per group) were analyzed for all the above lab tests, except the following –

[a]Lab test performed on samples from 9 healthy and 9 diabetic individuals.

[b]Lab test performed on samples from 10 healthy and 6 diabetic individuals.

Data analyzed separately between individuals with type 1 diabetes mellitus (T1DM) and their corresponding healthy controls

	Healthy individuals	T1DM individuals	*P* value
Age (years)	41 ± 7	41 ± 6	0.916
Leukocyte count (K/μL)[c]	6.13 ± 1.00	6.77 ± 1.70	1.000
Platelet count (K/μL)[c]	295.80 ± 19.88	304.30 ± 20.09	0.886
HbA1c (%)	5.68 ± 0.09	8.02 ± 0.33	<0.02
Glucose (mg/dL)	90.40 ± 3.98	210.60 ± 46.55	0.151

n = 5 per group except –

[c]Lab test performed on samples from 4 healthy and 4 diabetic individuals.

Data analyzed separately between individuals with type 2 diabetes mellitus (T2DM) and their corresponding healthy controls

	Healthy individuals	T2DM individuals	*P* value
Age (years)	37 ± 5	48 ± 8	0.421
Leukocyte count (K/μL)	6.56 ± 0.68	6.05 ± 0.70	0.691
Platelet count (K/μL)	268.80 ± 24.74	277.00 ± 19.17	0.421
HbA1c (%)	5.40 ± 0.08	8.22 ± 0.75	<0.02
Glucose (mg/dL)	91.80 ± 6.26	119.40 ± 4.91	<0.04

n = 5 per group