

1 **Neutrophil extracellular traps impair regeneration.**

2

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18

19 **Abstract**

20 Fibrosis is a major health burden across diseases and organs. To remedy, we study wound
21 induced hair follicle regeneration (WIHN) as a model of non-fibrotic healing that instead
22 recapitulates embryogenesis for de novo hair follicle morphogenesis after wounding. We have
23 previously demonstrated that TLR3 promotes WIHN through binding dsRNA, but the source of
24 which is still unclear. Here, we demonstrate that multiple distinct contexts of high WIHN all
25 show a strong neutrophil signature, and given the likelihood of nuclear dsRNA release during
26 the production of neutrophil extracellular trap (NETs), we hypothesized that neutrophils and
27 NETs might promote WIHN. Consistent with this, in addition to the presence of neutrophils
28 shortly after wounding, neutrophils remain within the wound after the barrier is reestablished,
29 where they produce extracellular traps (NETs) that likely release spliceosomal U1 dsRNA.
30 Contrary to our hypothesis, genetic models of neutrophil depletion show enhanced WIHN.
31 Pad4 null mice that are defective in NET production also augment WIHN. Finally, using single-
32 cell RNA sequencing, we identified a dramatic increase in neutrophil populations in the wound
33 beds of low regenerating Tlr3^{-/-} mice. Taken together, these results demonstrate that although
34 neutrophils are stimulated by a common pro-regenerative cue, their presence and NETs can
35 hinder regeneration.

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37

38 **Introduction**

39 After suffering a wound, the body initiates a well-coordinated physiological process to
40 restore homeostasis and reestablish the barrier. This spontaneous process comprises four

41 discrete phases: hemostasis, inflammation, proliferation, and remodeling¹. Although all of these
42 phases have been extensively studied, the molecular details as to why repair tends to result in
43 fibrotic scar tissue rather than complete regeneration have not been fully elucidated. This
44 tendency of wound repair to lead to fibrosis, and in some cases, hypertrophic scars, contributes
45 an enormous burden on human health¹⁻³. Inflammation and different components of the
46 immune system have been shown to contribute to regeneration in salamanders and zebrafish,
47 as well as to promote healing and maintain barrier function in mammals upon mucosal injury⁴⁻⁶.
48 Although the cellular effects of macrophages and T cells are well studied⁷⁻¹⁰, the role of
49 neutrophils in modulating regeneration remains elusive.

50 Unlike urodele salamanders, complete skin regeneration after wounding in mammals is
51 rare¹¹. It occurs via de novo hair follicle generation in mice and rabbits through a process that
52 mimics skin embryogenesis¹²⁻¹⁵. This process of de novo follicle neogenesis (Wound Induced
53 Hair Neogenesis; WIHN) was first fully characterized in mice after receiving full thickness
54 wounds¹⁶. These regenerated follicles establish a distinct stem cell population, express
55 characteristic differentiation markers, produce functional hair shafts, and can complete the hair
56 cycle. In addition to the hair follicles, sebaceous glands, specialized vascular and nerve
57 supports, and surrounding fat cells are regenerated^{16,17}.

58 Immediately after wounding, a robust inflammatory phase occurs, which allows the
59 ingress of keratinocytes and fibroblasts to proceed afterward. The early stage of wound healing
60 is defined by the dramatic recruitment of neutrophils, which are instrumental in providing
61 defense against microbial pathogens¹⁸⁻²². This is followed by an influx of macrophages (M ϕ 's)
62 that continue the phagocytic processes begun by neutrophils and aid in the transition to the
63 proliferative phase of wound healing²³⁻²⁵. While much is known about the importance of this
64 inflammatory phase for preventing and abrogating infections, less is known about how it
65 influences regenerative capacity or WIHN. Increasingly, macrophages have been shown to be
66 essential for WIHN via TNF-induced AKT/ β -catenin signaling⁸⁻¹⁰. However, the role of
67 neutrophils in modeling regeneration or WIHN is unclear.

68 Toll-like receptors (TLRs) are highly conserved single-pass membrane-spanning
69 receptors that recognize structurally conserved molecular components of invading microbes
70 and activate a cascade of inflammatory signaling pathways²⁶. Rather than simply recognizing
71 pathogen associated molecules, they can also initiate "sterile" inflammation upon recognizing
72 damage-associated molecular patterns (DAMPs), which are critical to recruit immune cells and
73 initiate wound healing²⁷. TLR3 is activated by dsRNA and has primarily been studied in the
74 context of viral infection²⁸. Mounting evidence shows that TLR3 also plays an important role in
75 wound repair²⁹⁻³⁷. Synthetic double strand RNA (dsRNA) polyriboinosinic-polyribocytidylic acid
76 (poly(I:C)) treatment dramatically increases WIHN in mice. Furthermore, wound-released
77 dsRNA activates TLR3 to promote hair follicle regeneration³⁴. The identity and source of the
78 physiological RNA remains an open and important question in the field. Notably, the dsRNA U1
79 spliceosomal small nuclear RNA (snRNA), may be an important endogenous RNA sensed via
80 TLR3^{32,33,38,39}. Specifically, UV damage releases U1 snRNA that stimulates cytokine production in
81 keratinocytes and increases barrier gene transcription^{32,33}.

82 In addition to phagocytosis and degranulation, neutrophils can produce extracellular
83 traps (ET), large extracellular web-like structures composed of decondensed chromatin bound
84 to various cytosolic and granule proteins^{21,22,40-42}. While originally recognized as a defense

85 mechanism against pathogens^{21,40,41}, they have also been found to mediate sterile
86 inflammatory processes^{43,44}. In the absence of infection, ETs can be stimulated in sterile tissue
87 environments through various cytokines^{41,45-47} and by activated platelets^{43,44}. Interestingly, ETs
88 are found within sterile wounds of mice and delay wound healing⁴⁸. Mechanistically, ETs are
89 formed by the rapid decondensation of the cellular chromatin, followed by the fragmentation
90 of the nuclear membrane and mixing of the nuclear and cytoplasmic compartments, before
91 being expelled from the cell. The ability of neutrophils to rapidly migrate to the wound site and
92 produce ETs, coupled with the nuclear localization of some dsRNA, made us question whether
93 neutrophils were a source of the dsRNA critical for WIHN. Interestingly, while there are
94 extensive studies on the DNA components released during ET formation^{22,42,49}, the RNA
95 components are poorly understood.

96 To probe how neutrophils influence wound regeneration and WIHN, we analyzed
97 multiple microarrays from distinct contexts of high regenerating mice and found a common
98 neutrophil signature. Using immunofluorescence and flow cytometry we found that neutrophils
99 remain in the wound bed, albeit at low levels, after the acute inflammatory phase, where they
100 produce NETs that contain the nuclear U1 dsRNA. To define how this influences regeneration,
101 we used antibody mediated neutrophil depletion to eliminate neutrophils from the wound bed
102 at select time points, but discovered that the technique is ineffective in the context of large full-
103 thickness wounds, which are necessary to initiate WIHN. However, using a neutrophil-specific
104 diphtheria toxin ablation model, we were able to deplete neutrophils in the wound bed and
105 found that—contrary to our initial hypothesis-- the absence of neutrophils enhances WIHN.
106 Eliminating neutrophil's ability to produce NETs by knocking out Pad4 also boosted WIHN,
107 confirming the negative influence of neutrophils on regeneration. Finally, we used single-cell
108 RNA sequencing to characterized WIHN deficient Tlr3^{-/-} mice and found that they have a
109 dramatically increased population of neutrophils in the re-epithelized wound bed, compared to
110 wild-type mice, likely contributing to their diminished regenerative capacity. These results
111 indicate that, while important for preventing infection, neutrophils and their NETs negatively
112 impact regeneration and WIHN. Although a common pro-regenerative signal might increase
113 neutrophil infiltration, neutrophils instead likely contribute to fibrosis.

114 115 **Results**

116 117 **Neutrophil signature present during skin regeneration**

118
119 To begin to characterize the role of neutrophils in WIHN, we performed bioinformatic
120 analysis on previous microarrays of multiple distinct high regenerating mouse models, probing
121 for innate immune and neutrophil signatures. First, we analyzed the proteome comparing the
122 center of the re-epithelialized wound bed (high WIHN) to the surrounding periphery/edge (low
123 WIHN) (Fig. 1a)³⁷. Gene ontology analysis revealed that, in the area of high WIHN, neutrophil
124 aggregation and other defense pathways against bacterium are enriched, characterized by an
125 abundance of antimicrobial and granular proteins, such as Neutrophil Elastase (Elane),
126 Cathelicidin (Camp), and Myeloperoxidase (Mpo) (Fig. 1b-c). In a second model system, high
127 WIHN Rnasel^{-/-} mice (manuscript in review), at the time of scab detachment, are also enriched
128 in genes associated with neutrophils. Neutrophil chemotaxis is the most significant upregulated

129 gene ontology category, with other chemotactic and inflammatory pathways up as well (Fig.
130 1d). Finally, we analyzed the gene expression changes between wounded specific pathogen free
131 (SPF) mice, which have increased regeneration and WIHN, when compared to germ free (GF)
132 mice (manuscript in review), at the time of scab detachment. Like the other two high
133 regeneration models, when compared to GF, SPF mice had elevated neutrophil chemotaxis and
134 immune response transcripts (Fig. 1e). Together, these disparate experimental contexts
135 demonstrate that neutrophil chemotaxis can correlate with high regeneration and WIHN.

136

137 **Neutrophils persist in the wound bed after the acute inflammatory phase, producing** 138 **extracellular traps**

139

140 Given the above correlations of high neutrophil infiltration to high WIHN, we first
141 characterized neutrophil infiltration in the wound beds of C57BL/6J mice after a large square
142 wound cut into the center of their backs, to the depth of fascia. As anticipated, neutrophils are
143 abundant in the acute phase of the wound healing process (1-3 days post wounding), as seen
144 by H&E staining (Fig. 2a) and immunofluorescence of Myeloperoxidase (MPO), a major
145 neutrophil granule protein (Fig. 2b). While neutrophils predominate at early time points, they
146 are still present in the wound bed as late as wound day 11, well after re-epithelization has
147 completed (Fig. 2c). In contrast, macrophages only accumulate starting at wound day 3 (Fig. 2b)
148 but remain a major component of the wound even after re-epithelization (Fig. S1). These
149 results show the dynamic changes of immune cell infiltration, but with an underappreciated
150 persistence of some neutrophils late in wound healing, during the time of morphogenesis.

151 Given the importance of dsRNA to promote WIHN, and the association of neutrophils
152 with high WIHN, we hypothesized that neutrophil extracellular traps (NETs) release of
153 neutrophil nuclear content might release dsRNA, in addition to dsDNA, from the nucleus to
154 promote WIHN. We visualized citrullinated histone H3 (H3Cit) to identify NETs as early as
155 wound day 3 (Fig. 2d). The production of the NETs is mediated by Pad4, an enzyme that
156 modifies the arginine residues on histones to citrulline, which changes their charge, leading to
157 massive chromatin decondensation^{50,51}. Consistent with this, NETs are virtually absent in mice
158 lacking Pad4, while abundant in the wound bed after the acute phase of wound healing in WT
159 mice (Fig. 2e). Although NETs are characterized by their extruded DNA, which forms a web-like
160 scaffold containing cytosolic and granular proteins, little is known about the RNA content within
161 them and how that might influence wound healing. Given that U1 small nuclear (sn) RNA is
162 proposed as a TLR3 agonist damage-associated molecular pattern important for skin barrier
163 repair^{32,33}, we visualized it by FISH. U1 snRNA is present throughout the wound beds of
164 C57BL/6J mice (Fig. 2f). Rather than the baseline nuclear appearance of unwounded tissue, U1
165 snRNA in the wound bed is markedly cytoplasmic, suggesting that its cellular localizations shifts
166 from the nucleus as a potential step towards cellular release. Consistent with this, we noted a
167 fine haze of signal extracellularly. Taken together, these data suggest a potential model where
168 neutrophils persist to the morphogenesis stage of wound healing and release extracellular traps
169 that contain U1 dsRNA to perhaps modulate WIHN.

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171

172 **Large full-thickness wounds dramatically reduce antibody-mediated neutrophil depletion**

173

174 To functionally assess how neutrophils modulate wound induced hair neogenesis
175 (WIHN), we sought to deplete neutrophils throughout the wound bed at select times. To
176 accomplish this, we adapted a widely used antibody mediated depletion scheme, where we
177 injected neutrophil specific antibodies intraperitoneally (Fig. 3a)⁵². Most experiments were
178 completed using a Ly6G positive monoclonal antibody (1A8 clone), which recognizes a 21-25kD
179 glycosylphosphatidylinositol (GPI)-linked differentiation antigen that is expressed by myeloid-
180 derived cells. While monocytes transiently express Ly6G during bone marrow development,
181 Ly6G expression in peripheral neutrophils directly correlates its level of differentiation and
182 maturation. The less specific anti-granulocyte receptor-1 (Gr-1) antibody (RB6-8C5 clone),
183 which recognizes both Ly6G and Ly6C and thereby also targets dendritic cells and
184 subpopulations of lymphocytes and monocytes, was also used in select experiments. To
185 prevent confounding flow cytometry measurements caused by epitope masking from the initial
186 Ly6G antibody injected during the antibody mediated neutrophil depletion experiments⁵³, a
187 distinct Ly6G antibody clone was used for analysis via flow cytometry. Injections were done one
188 day before and one day after large full-thickness wounds on the back of mice. The depletion
189 scheme focused on the early time points of the wound healing process because those were
190 shown to be most critical in dsRNA-Tlr3 enhanced WIHN³⁴.

191 Unexpectedly, on wound day 3, after receiving two IP injections of either Ly6G or Gr-1
192 antibodies, using our careful detection strategy, there was no apparent neutrophil depletion in
193 the wound bed, as measured via flow cytometry (Fig. 3b). Depletion was also ineffective in
194 other tissues after wounding, including the spleen and liver (Fig. S3). Looking one day earlier
195 (wound day 2), neutrophil depletion was still ineffective in the wound bed, but there was a
196 statistically significant depletion in the blood of the same mice (Fig. 3c). Although significant,
197 the neutrophil depletion was less than 50%, far less efficient than the previous reports^{52,54,55}.
198 An important distinction between ours and previous work is that we employed depletion in the
199 context of large wounds to fascia, prompting us to question whether large area wounding
200 globally modifies neutrophil blood trafficking dynamics to result in depletion failure. Indeed,
201 neutrophil depletion was very efficient in the blood of unwounded mice who had received an IP
202 injection of the Ly6G (1A8 clone) antibody 24 hours earlier (Fig. 3d), but this depletion was
203 abolished after wounding the mice (Fig. 3e). To explore this further, we repeated the injection
204 scheme in Fig. 3a. With and without wounding mice, we examined the neutrophil depletion in
205 the blood and wound bed at wound day 2. Consistent with our previous experiments, in the
206 context of wounding, the Ly6G (1A8) neutralizing antibody achieved less than 50 percent
207 neutrophil depletion in the blood. In contrast, mice that were not wounded, achieved
208 substantially more efficient depletion in the blood (Fig. 3f). Unwounded skin does not typically
209 contain neutrophils, so the Ly6G (1A8 clone) antibody injection had no effect (Fig. 3g).
210 Wounding caused dramatic recruitment of neutrophils to the wound bed, as shown earlier (Fig
211 3b-c, e). Ly6G antibody injection could not overcome this to deplete neutrophil from the wound
212 bed (Fig. 3g).

213 A previous report had success with antibody-mediated neutrophil depletion in a
214 wounding model where small 2cm excisions were made⁵⁵, leading us to question whether Ly6G
215 (1A8 clone) antibody injection was more effective in smaller, full-thickness wounds. To address
216 this, mice were injected with Ly6G (1A8 clone) antibody and then wounded with large wounds

217 (1.25x1.25cm²), like the previous experiments, or smaller wounds (0.6x0.6cm²) (Fig. 3h).
218 Neutrophil depletion efficacy was then assessed at wound day two in the blood and wound
219 bed. Mice that had been given small wounds had significant neutrophil depletion in both the
220 blood and wound bed, while those with the large wounds did not (Fig 3i-j). Taken together,
221 these data suggest that antibody mediated neutrophil depletion, while efficient in certain non-
222 wounding contexts, is not effective after the large full-thickness wounds necessary to induce
223 WIHN—perhaps because of the unusually robust stimuli of neutrophil recruitment in this
224 context.

225

226 **Neutrophils inhibit wound induced hair neogenesis**

227

228 Despite the minimal inhibition of neutrophils using conventional antibody depletion
229 with large wounds, we sought to begin testing how neutrophils might modulate WIHN. We
230 used the same antibody depletion scheme described (Fig 3a) but allowed the mice to recover
231 for 21 days before quantifying WIHN via confocal scanning laser microscopy (CSLM) (Fig 4a)⁵⁶.
232 Antibody injection did not affect wound closure speed (Fig. S4). Despite minimal neutrophil
233 depletion within the wound beds of mice post Ly6G (1A8 clone) injection (Fig 3b, c, e, g), the
234 number of regenerated hair follicles was increased, suggesting that neutrophils may hinder
235 WIHN (fold = 3.94) (Fig 4b).

236 To surmount the difficulties with meager neutrophil depletion using Ly6G antibodies,
237 we generated a transgenic mouse model for selective and inducible ablation of neutrophils
238 upon injection of diphtheria toxin (DT)⁵⁷. MRP8-Cre mice expressing Cre recombinase under the
239 control of the neutrophil-associated human MRP8 promoter⁵⁸⁻⁶⁰ were crossed with ROSA-iDTR^{KI}
240 mice, which have a Cre-inducible simian DT receptor (DTR)⁶¹. This generated mice with DTR
241 expression restricted to neutrophils that suffer cell death selectively after the injection of DT.
242 Two injections, one before wounding and one a day after, had no effect on control mice
243 (PMN^{WT} mice: MRP8-Cre⁻, ROSA-iDTR^{KI}) but substantially reduced neutrophil numbers in the
244 blood (fold = -4.33) and wound beds (fold = -4.27) of PMN^{DTR} (MRP8-Cre⁺, ROSA-iDTR^{KI}) mice at
245 wound day 2 (Fig. 4c-d). We next followed the mice after neutrophil ablation in the early phase
246 of wounding to find WIHN was substantially elevated (fold = 3.23) (Fig. 4e). The ablation of
247 neutrophils later in the wound healing process yielded the same results (Wound days 6, 8, and
248 10) (Fig. S5). Antibody mediated depletion (1A8 clone) at these later time points had no effect
249 on WIHN, suggesting that although effective in the blood, it is not able to target neutrophils
250 already present within the wound bed (Fig. S6). After overcoming the technical hurdles above,
251 these data suggest that neutrophils have a detrimental effect on the regeneration of hair
252 follicles.

253 To further explore this possibility, we sought to try a gain-of-function approach, rather
254 than the loss-of-function approach above. We injected purified neutrophils from the bone
255 marrow of mice into the wound beds of mice during the re-epithelialization process. Although
256 not significant, likely due to the high variability of WIHN, the addition of 200,000 neutrophils
257 reduced the number of regenerated hair follicles (fold = -0.73) (Fig. 4f).

258 Finally, given the presence of NETs late in wound healing (Fig 2e), we sought to test the
259 role of NETs in hair follicle neogenesis directly. We, therefore, tested WIHN in NET-deficient
260 Pad4 KO mice, as employed in Figure 2e. In the absence of Pad4 and with dramatically reduced

261 NETs, WIHN is enhanced (fold = 2.47, $p = 0.026$) (Fig. 4g). This suggests that NETs play a role in
262 reducing the regenerative capacity of mice during the wound healing process. This correlates
263 with the capacity of NETs to damage tissue in diseases such as small vessel vasculitis⁶², systemic
264 lupus erythematosus⁶³⁻⁶⁷, rheumatoid arthritis⁶⁸, and psoriasis⁶⁹.
265

266 **Single-cell RNAseq correlation of neutrophils with poor WIHN**

267 Double-stranded RNA sensing, mediated by Tlr3 and downstream effector pathways Il-
268 6/ Stat3, has been shown to be critical for WIHN, with Tlr3^{-/-} mice having substantially less
269 regenerated hair follicles than their wild-type controls³⁴. Given that TLR3 dsRNA sensing has
270 been shown to be critical for neutrophil recruitment and NET production in a model for acute
271 lung injury (ALI) and glomerulonephritis (GN), we wondered if Tlr3^{-/-} mice paradoxically have
272 increased neutrophil levels, contributing to lower WIHN^{70,71}. As anticipated, Tlr3^{-/-} mice had
273 substantially less WIHN than their WT controls (fold = -3.75, $p = 0.0218$) (Fig. 4h, Fig. S7a). We
274 performed single-cell RNA sequencing on wildtype and Tlr3^{-/-} re-epithelialized wound beds of
275 mice 10 days post wounding and prior to morphogenesis. Approximately 8014 sequenced cells
276 met standard quality control metrics and were further analyzed in the Seurat R package⁷².
277 Unsupervised clustering and UMAP non-linear dimensional reduction identified 18 cell clusters.
278 Seurat generated conserved and differentially expressed genes to assign cluster identities. We
279 found the neutrophil cluster to be substantially increased in the Tlr3^{-/-} mice (Fig. 4i, Fig. S7b).
280 This disparity is evident when looking at select differentially expressed neutrophil associated
281 genes that are all elevated in Tlr3^{-/-} mice (Fig. 4j). Together, these data suggest that elevated
282 neutrophil levels within Tlr3^{-/-} mice correlate with their deficiency in WIHN.

283 **Discussion**

284 The wound healing process is a careful balance of interconnected steps that must weigh
285 the benefits of quick barrier repair, which leads to fibrous scarring, and more complete
286 regeneration, that restores function and appearance. The role inflammation plays in
287 regeneration and scarring is still being elucidated, but increasing evidence suggests that neither
288 excess nor lack of inflammation supports regeneration. Fgf9-producing γ - δ T cells are critical for
289 WIHN, infiltrating into wound bed immediately before re-epithelialization and onset of hair
290 follicle regeneration⁷. Macrophages have also been shown to be important in the process, with
291 their ablation eliminating WIHN⁸⁻¹⁰. Additionally, the injection of the dsRNA mimic poly(I:C), as
292 early as 3 days after wound, dramatically enhances WIHN³⁴. Intriguingly, spiny mice (*Acomys*)
293 have a dramatically reduced inflammatory response post wounding, with less cytokines and
294 virtually no macrophages until late in the process, when compared to laboratory mice (*Mus*),
295 despite having substantially improved regeneration^{73,74}. Consistent with this idea of a complex
296 network of inflammatory cells influencing regeneration, we show that neutrophils persist in the
297 wound bed after the acute inflammatory phase and—despite multiple correlations to the
298 contrary—have a detrimental effect on regeneration.

299 Using histological and flow cytometry techniques, we show that while abundant
300 immediately after wounding, neutrophils remain in low levels within the wound bed after re-

301 epithelization. These late stage neutrophils produce NETs, which are eliminated in mice that are
302 deficient in Pad4, an enzyme critical for chromatin decondensation and NET formation^{50,51}.
303 Critically, Pad4^{-/-} mice have increased WIHN, when compared with WT mice. Furthermore,
304 antibody mediated and selective genetic neutrophil ablation dramatically boost WIHN. Single-
305 cell RNA sequencing also revealed that mice deficient in dsRNA sensing Tlr3, who have severely
306 reduced WIHN, have substantially more neutrophils present in the re-epithelized wound bed,
307 immediately preceding regeneration. All these data suggest a model where neutrophils play an
308 important role in defense against bacterial pathogens, but if they persist within the wound bed
309 too long after barrier repair, regeneration is hindered. In the future, it will be interesting to see
310 if selectively targeting NETs (e.g., PAD4 inhibitors, DNase I, N-acetylcysteine) enhance
311 regenerative wound repair.

312 When attempting to deplete neutrophils to assess their effects on WIHN, we also
313 discovered that the commonly used antibody depletion methods were not effective. Two
314 neutrophil-specific antibodies, which recognize different epitopes of the GPI-linked
315 differentiation antigen Ly6G, are commonly injected to rapidly deplete circulating neutrophils.
316 The most widely used clone (1A8) specifically recognizes Ly6G, while the anti-granulocyte
317 receptor-1 (Gr-1) antibody (RB6-8C5 clone) recognizes a heterodimer of Ly6G and Ly6C, making
318 it partially target monocytes. Both antibodies efficiently depleted neutrophils in unwounded
319 animals. When large full-thickness wounds (1.25x1.25cm²) were used, which are necessary to
320 initiate WIHN, depletion levels in the blood were drastically reduced, while having negligible
321 effects on neutrophils within the wound bed. Depletion efficacy was restored if smaller wounds
322 were used (0.6x0.6cm²), albeit at reduced levels when compared to unwounded mice. These
323 results suggest that wounding has a dramatic effect on recruiting neutrophil precursors for
324 mobilization and release, overwhelming the standard ability of depletion antibodies to work
325 effectively. This highlights the importance of rigorously testing antibody mediated depletion
326 methods in mouse models, to ensure they are achieving appreciable depletion. Furthermore, it
327 is important to carefully design neutrophil detection methods to assess depletion efficacy,
328 particularly with flow cytometry. Robust depletion can be erroneously detected if similar Ly6G
329 clones are used for both depletion and detection, due to epitope masking⁵³. This can occur
330 since identical monoclonal clones are sometimes given different clone numbers. Antibody
331 manufacturers are increasingly manipulating antibodies to reduce undesirable features like
332 non-specific binding, without affecting the epitope-binding variable region, yet renaming the
333 clones, necessitating substantial effort to ensure non-overlapping epitope binding by both
334 depletion and analytical flow antibodies.

335 Our work generates important areas of future investigation. One question is unraveling
336 the paradox of why neutrophil infiltration signatures correlate with high WIHN, but neutrophils
337 inhibit WIHN. One possible model is that a common upstream cue or factor both promotes
338 WIHN and promotes neutrophil infiltration, but the latter serves to limit WIHN in favor of
339 decreasing infection risk. Defining this common upstream signal will be important for future
340 work. It will also be interesting to define the function of neutrophils within the re-epithelized
341 wound since the barrier has been restored. Another important question is whether nuclear
342 RNAs released in NETs have any function besides the general theorized one for released DNA.
343 Though NETs inhibit WIHN, nuclear RNA release in NETs might have functions in separate

344 physiological processes that will yield important insights. A final question is the identity of the
345 neutrophil mobilization factor whose strength correlates to wound size. Potential signals
346 include products of complement activation (C5a) or a number of small molecular weight C-X-C
347 chemokines, which are recognized by Cxcr1 and Cxcr2^{75,76}.

348 In summary, we here demonstrate a novel role for NETs and neutrophils to inhibit
349 regeneration. Future studies will be important to further understand the biology of
350 regeneration and test the capacity for neutrophil inhibition to promote regenerative healing.

351

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367

368 **Methods**

369 *Mouse Lines*

370 All wild-type and control mice used for in vivo experiments were on the C57BL/6J background.
371 All mice were age-matched and co-housed until 3-weeks of age. *Pad4* knockout mice were
372 purchased from the Jackson Laboratory (B6.Cg-Padi4^{tm1.1K^{mow}}/J, 030315). The diphtheria toxin
373 (DT) mediated neutrophil ablative mice were generated by crossing ROSA26iDTR (C57BL/6-
374 Gt(ROSA)26Sor^{tm1(HBEGF)Awai}/J, 007900) and MRP8-Cre-ires/GFP (B6.Cg-Tg(S100A8-cre,-
375 EGFP)1Ilw/J, 021614) from the Jackson Laboratory to get heterogeneous mice and genotyped
376 according to their specifications. Mice who genotyped positive for Cre (MRP8-Cre+; ROSA-
377 iDTR^{KI}) were considered PMN^{DTR} mice, while those that were negative for Cre (MRP8-Cre-;
378 ROSA-iDTR^{KI}) were PMN^{WT} littermate controls⁵⁷. Tlr3 knockout mice (B6N.129S1-Tlr3^{tm1Flv}/J,
379 009675) and C57BL/6NJ controls (005304) were purchased from the Jackson Laboratory. All
380 mice were bred and housed at an American Association for the Accreditation of Laboratory
381 Animal Care (AAALAC)-compliant facility, and all experimental procedures were reviewed and

382 approved by the Johns Hopkins University Institutional Animal Care and Use Committee
383 (IACUC).

384

385 *Wound Induced Hair Neogenesis (WIHN) Assay*

386 All in vivo experimental surgical procedures were performed as previously
387 characterized^{7,16,34,37,77}. Briefly, after exposure to anesthesia (Baxter, Isoflurane), the dorsal side
388 of 3-week-old (21 days) male and female mice were shaved. Surgical scissors were used to
389 excise 1.25x1.25cm² of skin, creating wounds deep into the fascia. Approximately 3 weeks after
390 wounding (~21 days), neogenic hair follicles in the re-epithelialized skin tissue were quantified
391 using reflectance confocal scanning laser microscopy (CSLM) as previously published^{34,37}.

392

393 *Neutrophil Depletion*

394 C57BL/6J mice were depleted with 100 or 500µg of anti-Ly6G (Bio-X-Cell, 1A8 clone, BP0075-1)
395 via intraperitoneal injection (IP) injection one day prior to and one day after wounding mice^{52,54}.
396 Select experiments also used 200µg of anti-Gr1 (Bio-X-Cell, RB6-8C5 clone, BP0075). IgG2a (Bio-
397 X-Cell, BE0089) and IgG2b (Bio-X-Cell, BP0090) isotype controls were used for Ly6G or Gr1
398 experiments, respectively. DT depletion was done with PMN^{DTR} and PMN^{WT} littermate control
399 mice that were IP injected with 250ng DT (Sigma-Aldrich). The injections were primarily done
400 one day before and after wounding, or at wound days 6, 8, and 10.

401

402 *Flow Cytometry*

403 Flow cytometry was used to assess neutrophil depletion. Blood was collected via retro-orbital
404 sinus bleeds, and red blood cells were lysed RBC lysis buffer (BioLegend, 420301). Wound beds
405 were surgically removed, and cell suspensions were prepared by digesting the tissue in a
406 cocktail consisting of Liberase TL (Roche, 5401020001) and DNase I (Sigma, DN25) in RPMI 1640
407 (Gibco, 11875093). Cells were washed and then Fc blocked (BioLegend, 101320), before
408 staining with an antibody cocktail (Extended Table 1). Finally, cells were washed and
409 resuspended in FACs buffer containing Propidium Iodide (Miltenyi, 130-093-233). All flow
410 cytometry experiments were performed on a BD LSR II, and downstream analysis of data was
411 performed using FlowJo.

412

413 *Neutrophil Extracellular Trap measurement*

414 Wound beds were surgically removed at wound days 2 and 7, using a 6mm biopsy punch to
415 remove excess tissue. Cell suspensions were prepared by digesting the tissue in a cocktail
416 consisting of Liberase TL (Roche, 5401020001) and DNase I (Sigma, DN25) in RPMI 1640 (Gibco,
417 11875093). Cells were washed, and then Fc blocked (BioLegend, 101320), before staining with
418 an antibody cocktail containing anti-MPO (Abcam, ab208670, 1:500) or respective isotype
419 control (Abcam, ab172730, 1:500). Cells were then stained with a secondary Alexa Fluor 647
420 antibody (Abcam, ab150083, 1:2000). On the final wash, SYTOX green was added
421 (ThermoFisher, S7020, 1:1000). This was performed on a BD LSR II, and downstream analysis of
422 data was performed using FlowJo.

423

424 *3'-end single-cell RNA-sequencing:*

425 The re-epithelialized wounds beds (wound day 10) of a *Tlr3*^{-/-} and a C57BL/6NJ control mouse
426 were excised, and cell suspensions were prepared by digesting the mouse skin tissue in a
427 cocktail consisting of Liberase TL (Roche, 5401020001) and DNase I (Sigma, DN25) in RPMI 1640
428 (Gibco, 11875093). Propidium iodide and DAPI positive dead cells were removed via cell sorting
429 with a BD FACSAria II. Single-cell libraries were prepared via a 10× Genomics Chromium Single-
430 Cell Platform, followed by sequencing using Illumina NovaSeq 6000. The results were run
431 through Cell Ranger pipeline software for sequence alignment and basic filtering. GEM
432 generation, barcoding, cDNA amplification, library preparation, quality control, and sequencing
433 were performed at the Genomics High Throughput Sequencing facility at Johns Hopkins School
434 of Medicine.

435
436 Downstream analysis, after the Cell Ranger pipeline, was done using the Seurat R package. A
437 standard pre-processing workflow was done, removing low quality cells or doublets, filtering
438 unique feature count over 3750 and below 200, as well as filtering out cells with higher than 5%
439 mitochondrial counts. This resulted in 4150 WT and 5648 *Tlr3*^{-/-} cells for downstream
440 bioinformatics. Expression matrices then underwent normalization, scaling, principal
441 components analysis, and subsequent t-SNE analysis using Seurat packages. Seurat was then
442 used to generate conserved genes, differentially expressed genes, feature plots, dot plots, and
443 ridge plots. Cell clusters were then defined querying conserved genes and differentially
444 expressed genes against the Immgen gene expression database (www.immgen.org) using the
445 interactive tool “My Gene Set.”

446

447 *Histology*

448 Biopsies from mouse skin tissue were removed and fixed in 4% paraformaldehyde overnight
449 and then transferred to 70% ethanol. Samples were then submitted to the Johns Hopkins
450 Oncology Tissue Services Core facility where they were embedded in paraffin. Tissue sections
451 were obtained at 4µm thickness and mounted onto glass slides, followed by hematoxylin and
452 eosin (H&E) staining.

453

454 *Immunofluorescence and immunohistochemistry*

455 Immunofluorescence microscopy was performed on de-paraffinized tissue sections that
456 received heat-induced antigen retrieval using Target Retrieval Solution (Agilent Dako, S169984-
457 2). After washing and permeabilization in TBS-T universal buffer (0.2% Triton X-100 in tris-
458 buffered saline), sections were blocked at room temperature in 5% goat, donkey, or fetal
459 bovine serum with 1% bovine serum albumin. Tissue sections were then incubated overnight at
460 4°C with primary antibodies (Extended Table 2) in Antibody Diluent (Agilent Dako, S080983-2).
461 Following a wash step, sections were incubated in fluorescent-dye conjugated secondary
462 antibodies diluted in antibody diluent for 1 hour at room temperature. After final washing,
463 sections were mounted with VECTASHIELD® Hardset™ Antifade Mounting Medium with DAPI
464 (Vector Laboratories, H-1500) for nuclear staining. All imaging was done on a DFC365FX (Leica)
465 at 20x and 40x magnifications.

466

467

468 *U1 in situ hybridization*

469 U1 in situ probes were designed and ordered in the Stellaris Probe Designer (Biosearch
470 Technologies) (Extended Table 3). Tissue sections were de-parafinized and stained following
471 Biosearch Technologies Stellaris RNA FISH protocol for Formalin-Fixed Paraffin-Embedded
472 Tissue. Briefly, tissue sections were washed in Wash Buffer A (Biosearch Technologies, SMF-
473 WA1-60), before adding 200µl hybridization buffer (Biosearch Technologies, SMF-HB1-10)
474 containing the U1 probe and covering the tissue with a glass coverslip. The slides were then
475 incubated overnight in a humid box at 37°C. Slides were then immersed in Wash Buffer A in the
476 dark at 37°C for 30 minutes, allowing the coverslips to float off. Slides were then washed for 5
477 minutes with Wash Buffer B (Biosearch Technologies, SMF-WB1-20), before sections were
478 mounted with VECTASHIELD® Hardset™ Antifade Mounting Medium with DAPI (Vector
479 Laboratories, H-1500) for nuclear staining. All imaging was done on a DFC365FX (Leica) at 63x
480 magnifications.

481

482 *Microarray, RNA-seq and proteomic analysis*

483 Proteins from the wound center and wound edges were analyzed by proteomics, as previously
484 described³⁷. Briefly, we used Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) for
485 protein profiling and obtained the protein expression level by the MS Amanda algorithm.
486 Standard bioinformatics procedures were performed, including standardization of gene
487 expression, the definition of differentially expressed genes, and GO enrichment analysis. For
488 both Rnasel-/- and WT mice, total RNA was isolated from mouse tissue at the time of scab
489 detachment from the wound (~10 days post-wounding). RNA was submitted to the JHMI Deep
490 Sequencing & Microarray core facility and profiled using the Affymetrix Clariom™ S mouse array
491 platform, according to the manufacturer's protocols. Gene chips were scanned, generating CEL
492 pixel intensity files, which were processed and analyzed using Partek® Genomics Suite™
493 software, and the Robust Multichip Analysis (RMA) algorithm was used for normalization.
494 For SPF and GF analysis, total RNA from early wound bed skin (~WD12) was submitted to the
495 JHMI Transcriptomics and Deep Sequencing Core. The 1.0ST exon sequencing of mouse RNA
496 was performed according to the manufacturer's standard protocol. The raw affymetrix CEL data
497 was standardized using Robust Multichip Analysis (RMA) algorithm for comparison.

498

499 *Quantification and Statistical Analysis*

500 All in vivo and in vitro experiments were performed in at least individual instances. Univariate
501 statistical analysis was performed using Student's t-test, and multivariate analysis was
502 performed using ANOVA. All statistical analyses and graphical representations were generated
503 using GraphPad Prism software. Statistical significance is defined as p-values <0.05 derived
504 from the standard error of mean calculations.

505

506

507 **Extended Table 1**

Name	Host	Fluorophore	Manufacturer/Product #
MHCII (IA/IE)	Rat	BV421/Pacific Blue	BioLegend/107631

CD3	Rat	BV510/AmCyan	BD/740147
Ly6C	Rat	FITC	BD/553104
Ly6G	Rat	PE	BioLegend/127607
CD45	Rat	PE-Cy5.5	Invitrogen/35-0451-82
CD115	Rat	PE-Vio770	BioLegend/135523
CD11c	Hamster	APC	BioLegend/117310
CD11b	Rat	APC-Cy7	BD/557657

508

509 **Extended Table 2**

Name	Host	Dilution	Company/ Product #
MPO	Goat	1:200	Abcam/ab208670
F4/80	Rat	1:200	Abcam/ab6640
Ly6G	Rat	1:200	BioXCell/BP0075-1
H3Cit	Rabbit	1:500	Abcam/ab5103
Alexa Fluor® 488 Anti-Goat IgG (H+L)	Rabbit	1:1000	Invitrogen/A27012
Alexa Fluor® 488 Anti-Rabbit Ig (H+L)	Goat	1:1000	Invitrogen/A-11008
Alexa Fluor® 594 Anti-Rabbit IgG (H+L)	Goat	1:1000	Invitrogen/A-11037

Alexa Fluor® 488 Anti-Rat Ig (H+L)	Goat	1:1000	Invitrogen/A-11006
Alexa Fluor® 594 Anti-Rat Ig (H+L)	Donkey	1:1000	Invitrogen/ A-21209

510

511 **Extended Table 3**

Sequence Name	Sequence
U1 snRNA_1	cccctgccaggtaagtat
U1 snRNA_2	caccttcgtgatcatggt
U1 snRNA_3	aagcctcgccctgggaaa
U1 snRNA_4	acatccggagtgcaatgg
U1 snRNA_5	gggaaatcgcaggggtca
U1 snRNA_6	cagtcgagtttcccacat
U1 snRNA_7	ccccactaccacaaatta
U1 snRNA_8	aggggaaagcgcgaacgc

512

513

514 **Figure Legends**

515

516 **Figure 1: Neutrophil signature correlates with high skin regeneration in multiple models**

517 **a.** Schematic of hair neogenesis preferential localization to wound center (high WIHN) rather
518 than wound edge (low WIHN). **b.** Proteomic gene ontology (GO) analysis of the top 100 genes
519 wound center versus wound edge in wild type mice (at scab detachment) shows a
520 predominance in innate immune response pathways and neutrophil signatures. **c.** Abundance
521 ratios of genes from select GO terms highlighted in b. show enrichment of antimicrobial and
522 granular proteins, labeled in red. **d.** GO analysis of the top 200 genes from high WIHN *Rnase1^{-/-}*
523 versus wild-type mice at scab detachment shows a predominance of neutrophil and innate
524 immune cell chemotaxis pathways. Inset graphs show the gene fold expression changes for
525 genes present in that category. **e.** Neutrophil chemotaxis and innate immune categories are
526 included in Gene Ontology (GO) enrichment analysis of the top versus bottom 500 differentially
527 expressed genes between Specific pathogen-free (SPF; high WIHN) and germ-free (GF; low
528 WIHN) mice on ~WD12 (scab detachment) wound beds. (n=3 independent animals per group).
529 Inset graphs show the gene fold expression changes for genes present in that category.

530

531

532 **Figure 2: Neutrophils persist in wound bed after the acute inflammatory phase, producing**
533 **extracellular traps**

534 **a.** Neutrophils are present in the wound beds of C57BL/6J mice at early time points, visible in
535 representative hematoxylin and eosin [H&E] staining. Red arrows show select neutrophils,
536 evident by their multilobular nuclei. The black dashed line signifies the migrating epithelial
537 front. Black scale bar = 50 μm . **b.** Neutrophils predominate throughout the wound beds of
538 C57BL/6J mice on wound days 1 and 3, visible in prominent MPO immunofluorescence (green).
539 Few macrophages are present (Red, F4/80). Red arrows show select neutrophils. The white
540 dashed line signifies the dorsal edge of the wound bed. White scale bar = 200 μm . **c.** Percent
541 neutrophil (Ly6G+ cells from total CD45+ cells) levels are consistent in the blood throughout the
542 wound time course, but drop in the wound bed at wound day 11, as measured by FACS. **** p
543 < 0.0001 , as calculated by two-way ANOVA. n.s., not significant. N = 2 vs 4. Results are
544 representative of at least two independent experiments. **d.** Citrullinated histone H3 (H3Cit, red)
545 co-localized with Ly6G+ neutrophils (green), beginning at wound day 3 in the wound beds of
546 immunofluorescence stained C57BL/6J mice, indicating the formation of extracellular traps. Red
547 arrows show select neutrophils. The white dashed line signifies the dorsal edge of the wound
548 bed. White scale bar = 200 μm . **e.** Neutrophil extracellular trap positive cells (MPO+, SYTOX
549 green +) are present at late wound time points, but are absent in the wound beds of PAD4-/
550 mice, as measured by FACS **** p < 0.0001 , as calculated by two-way ANOVA. n.s., not
551 significant. N = 7 vs 4. Results are representative of at least two independent experiments. **f.**
552 Cytoplasmic U1 snRNA is present in the wound bed of C57BL/6J mice, while it localized
553 exclusively in the nuclei of unwounded controls, as visualized by representative FISH. White
554 arrows show select cells with nuclear or cytoplasmic U1 snRNA. The white dashed line signifies
555 the dorsal edge of the wound bed. The solid white line delineates a hair follicle. White scale bar
556 = 80 μm

557
558

559 **Figure 3: Large full-thickness wounds dramatically reduce antibody-mediated neutrophil**
560 **depletion**

561 **a.** Schematic of neutrophil depletion via intraperitoneal injection (IP) of Ly6G antibody (1A8
562 clone, 500 μg), Gr1 (Ly6G/Ly6C, RB6-8C5 clone, 200ng), or isotype control. IP injections are done
563 one day before and one day after wounding C57BL/6J mice with 1.25 cm^2 square wounds. Mice
564 were then sacrificed wound day 2 or 2 for FACS analysis of blood or wound bed (WB). **b.**
565 Antibody mediated depletion is not successful in the wound bed. Representative flow plots
566 from the wound beds (wound day 3) of mice treated as in **a.** The percent neutrophils (CD11b+,
567 Ly6c+) present in these samples are presented to the right. The difference between samples is
568 statistically insignificant, as calculated by the two-tailed Student's t-test. N = 2. Results are
569 representative of at least two independent experiments. **c.** Antibody mediated. Neutrophil
570 depletion is marginally successful in the blood, but not the wound bed, two days after
571 wounding. The percent neutrophils (CD11b+, Ly6c+), at wound day 2, present in the blood and
572 wound beds of mice treated as in **a.** (Ly6G, 1A8). ** p < 0.0011 , as calculated by two-way
573 ANOVA. n.s., not significant. N = 4. Results are representative of at least two independent
574 experiments. **d.** Neutrophil depletion from the blood is successful 1-day post injection (Ly6G,
575 1A8), in the absence of wounding, via FACS. The neutrophil depletion is statistically significant.

576 ****p < 0.0001, as calculated by two-tailed Student's t-test. N = 4 vs 6. **e.** The efficacy of
577 neutrophil depletion from the blood diminishes after 1.25x1.25cm² wounds are inflicted. The
578 percent neutrophils (Gr1+, Ly6c+) in the blood at wound day 2, via FACs. The neutrophil
579 depletion is statistically insignificant. n.s., not significant. N = 2 vs 3. Results are representative
580 of at least three independent experiments. **f.** Neutrophil depletion from the blood is more
581 pronounced in unwounded mice. The percent neutrophils (Gr1+, Ly6c+) present in the blood at
582 wound day 2 of mice IP injected with Ly6G antibody (1A8, 500 µg), with or without wounding,
583 via FACs. ****p < 0.0001 vs **p=0.0038 as calculated by two-way ANOVA. N = 6 vs 7. **g.**
584 Neutrophil depletion from the skin is ineffective, regardless of wound status. The percent
585 neutrophils (Gr1+, Ly6c+) present in the wound bed or skin at wound day 2 of mice IP injected
586 with Ly6G antibody (1A8, 500µg), with or without wounding. The neutrophil depletion is
587 insignificant in both cases, as calculated by two-way ANOVA. n.s., not significant. N = 6 vs 7. **h.**
588 C57BL/6J mice were given full thickness wounds on the center of their backs. The normal large
589 wound (LW) is 1.25x1.25cm², while the smaller wound (SW) is 0.6x0.6cm². **i.** Neutrophil
590 depletion from the blood is successful on small, but not large wounded mice. Mice wounded
591 with small or large wounds (**h.**) were IP injected with Ly6G antibody (1A8, 500µg) one day
592 before and after wounding. Blood was extracted on wound day 2 and analyzed by flow
593 cytometry for percent neutrophils (Gr1+, Ly6c+). *p = 0.0362 as calculated by two-way ANOVA.
594 n.s., not significant. N = 2 vs 3 and 2 vs 2. Results are representative of at least two independent
595 experiments. **j.** Neutrophil depletion from the wound bed is successful on small, but not large
596 wounded mice. Mice wounded with small or large wounds (**h.**) were IP injected with Ly6G
597 antibody (1A8, 500µg) one day before and after wounding. Wound beds were collected on
598 wound day 2 and analyzed by flow cytometry for percent neutrophils (Gr1+, Ly6c+). Depletion
599 was significant in the small wound, but not the large wound setting. *p = 0.0458 as calculated
600 by two-way ANOVA. n.s., not significant. N = 2 vs 3 and 2 vs 2. Results are representative of at
601 least two independent experiments.

602

603

604 **Figure 4: Neutrophils inhibit wound induced hair neogenesis**

605 **a.** Schematic of neutrophil depletion via IP of Ly6G antibody (100µg) or isotype control. IP
606 injections are done one day before and one day after wounding C57BL/6 mice with
607 1.25x1.25cm² square wounds. Wound induced hair neogenesis (WIHN) was measured 21 days
608 after wounding via confocal scanning laser microscopy (CSLM). **b.** Neutrophil targeted Ly6G
609 antibody injected mice exhibit increased WIHN (CSLM, images; fold = 3.94, p = 0.011, N = 10 vs
610 9). In each image, the dash red box indicates the area of hair follicle regeneration. **c.** Diphtheria
611 toxin (DT, 250ng) injection in heterozygous ROSA26iDTR/MRP8-Cre-ires mice (PMN^{DTR} and
612 PMN^{WT}), following the injection scheme in **a.**, successfully depletes neutrophils from the blood
613 in mice with large wounds. Fold = -4.33. **p = 0.0098. N = 2 vs. 3. Results are representative of
614 at least two independent experiments. **d.** Mice treated as in **c.** are depleted of neutrophils in
615 their wound beds at wound day 2. Fold = -4.27. **p = 0.0069. N = 2 vs. 3. Results are
616 representative of at least two independent experiments. **e.** PMN^{DTR} mice IP injected with
617 diphtheria toxin (DT, 250ng) on wound days -1 and 1 exhibit increased WIHN (CSLM, images;
618 fold = 3.23, ***p = 0.0010, N = 14 vs 6). In each image, the dash red box indicates the area of
619 hair follicle regeneration. **f.** Regenerated hair follicles after the injection of 50k-200k purified

620 neutrophils underneath the scab at WD7-8. (CSLM, images; n.s., not significant, N = 9 vs 3 vs 6).
621 In each image, the dash red box indicates the area of hair follicle regeneration. **g.** Pad4^{-/-} mice
622 defective in extracellular traps exhibit increased WIHN (CSLM, images; fold = 2.47, p = 0.026, N
623 = 10 vs 19). In each image, the dash red box indicates the area of hair follicle regeneration. **h.**
624 Tlr3^{-/-} mice exhibit decreased WIHN (fold = -3.75 p = 0.0218, N = 8 vs 10). **i.** The presence of
625 increased neutrophils correlates with decreased WIHN in TLR3^{-/-} mice. scRNA-seq t-SNE plot
626 shows differences between WT (red, 4150 cells) and Tlr3^{-/-} (blue, 5648 cells) wound beds at
627 wound day 10. The plots were generated via Seurat. The neutrophil cluster is circled in red.
628 Percent of neutrophils are graphed to the right. **j.** Neutrophil associated gene expression is
629 more pronounced within the neutrophils of Tlr3^{-/-} mice, compared to WT. Generated in Seurat
630 with RidgePlot function.

631

632 **Supplementary Figure Legends**

633

634 **S1:** Macrophage (F4/80) levels are largely absent from the blood and low in the wound bed
635 during the early phase of healing, but increase dramatically at wound day 11, as measured by
636 FACS. ***p < 0.004, as calculated by two-way ANOVA. n.s., not significant. N = 2 vs 4. Results
637 are representative of at least two independent experiments.

638

639 **S2:** Ly6G 1A8 antibody clone (Bio-X-Cell) masks the Ly6G epitope, preventing detection by the
640 Ly6G REA526 clone (Miltenyi, engineered 1A8 clone) and partially preventing detection by the
641 Gr1 RB6-8C5 clone (Bio-X-Cell) antibodies. Blood was extracted from a single C57BL/6J and
642 stained sequentially with combinations of two antibodies, to test Ly6G epitope masking, before
643 detecting by flow cytometry. If only stained with the Ly6G (REA526) or Gr1 (RB6-8C5), the
644 neutrophil population is detected normally (13.7% or 13.2%, respectively). If you first stain with
645 Ly6G (1A8 clone), followed by Ly6G (REA526) or Gr1 (RB6-8C5), you get varying degrees of Ly6G
646 antibody masking. Being a derivative of the 1A8 clone, the REA526 Ly6G binding site is almost
647 completely blocked by the prior incubation with the Ly6G 1A8 clone (1.10%), making the pair
648 unusable for neutrophil depletion experiments. Prior incubation with the Ly6G 1A8 clone
649 followed by Gr1 (recognized an epitope of Ly6G/Ly6C) shifts the neutrophil population to the
650 left, but they remain distinct from the negatively stained cells (12.7%). With careful gating, this
651 makes depletion with the Ly6G (1A8 clone) antibody and detection with the Gr1 (RB6-8C5)
652 antibody possible. The neutrophil population is boxed in red.

653

654 **S3:** Antibody mediated Neutrophil depletion is not efficient in the blood, spleen, liver, or wound
655 bed, two days after wounding. The percent neutrophils (CD11b+, Ly6c+), at wound day 2,
656 present in the blood, spleen, liver, and wound beds of mice IP injected with Ly6G antibody
657 (500µ, 1A8) one day before and one day after given 1.25x1.25cm² full thickness wounds. The
658 neutrophil depletion is statistically insignificant, as calculated by two-way ANOVA. n.s., not
659 significant. N = 4 vs 4, except for the Blood and Wound bed samples, which were 7 vs. 7.

660

661 **S4:** The wound closure rate was not affected by neutrophil depletion via IP of Ly6G antibody
662 (100µg) or isotype control. IP injections were done one day before and one day after wounding

663 C57BL/J6 mice with 1.25x1.25cm² square wounds. N = 4 vs 4. Results are representative of
664 three independent experiments.

665
666 **S5.** PMN^{DTR} mice IP injected with diphtheria toxin (DT, 250ng) on wound days 6, 8, and 10
667 exhibit increased WIHN (CSLM, images; fold = 3.28, **p = 0.0085, N = 13 vs 5).

668
669 **S6: a.** Schematic of neutrophil depletion via IP of Ly6G antibody (500µg) or isotype control. IP
670 injections are done on wound day 8 and 10, after wounding C57BL/J6 mice with 1.25x1.25cm²
671 square wounds. Wound induced hair neogenesis (WIHN) was measured 21 days after wounding
672 via confocal scanning laser microscopy (CSLM). **b.** Neutrophil depletion in the blood is
673 successful on wound day 9, one day after antibody injection (Ly6G, 1A8), via FACs. The
674 neutrophil depletion is statistically significant. ****p < 0.0001, as calculated by two-tailed
675 Student's t-test. Fold = -87.9. N = 2 vs 3. **c.** Late stage Ly6G antibody injected mice exhibit
676 normal WIHN (CSLM, images; n.s., not significant, N = 3 vs 3). In each image, the dash red box
677 indicates the area of hair follicle regeneration.

678
679 **S7: a.** Tlr3^{-/-} mice exhibit decreased WIHN (representative CSLM images, N = 8 vs. 10). In each
680 image, the dash red box indicates the area of hair follicle regeneration. **b.** Neutrophil associated
681 genes used to identify the neutrophil cluster in UMAP non-linear dimensional reduction, via
682 Seurat R package, of WT and Tlr3^{-/-} mice.

683

684

685

686 **Works Cited:**

687

688

- 689 1. Diegelmann, R.F. & Evans, M.C. Wound healing: an overview of acute, fibrotic and
690 delayed healing. *Frontiers in bioscience : a journal and virtual library* **9**, 283-289 (2004).
- 691 2. O'Connell, J.B. The economic burden of heart failure. *Clinical cardiology* **23**, III6-10
692 (2000).
- 693 3. Chung, F., *et al.* Assessing the burden of respiratory disease in the UK. *Respiratory*
694 *medicine* **96**, 963-975 (2002).
- 695 4. Godwin, J.W., Pinto, A.R. & Rosenthal, N.A. Macrophages are required for adult
696 salamander limb regeneration. *Proceedings of the National Academy of Sciences of the*
697 *United States of America* **110**, 9415-9420 (2013).
- 698 5. Kyritsis, N., *et al.* Acute inflammation initiates the regenerative response in the adult
699 zebrafish brain. *Science* **338**, 1353-1356 (2012).
- 700 6. Taniguchi, K., *et al.* A gp130-Src-YAP module links inflammation to epithelial
701 regeneration. *Nature* **519**, 57-62 (2015).
- 702 7. Gay, D., *et al.* Fgf9 from dermal gammadelta T cells induces hair follicle neogenesis after
703 wounding. *Nature medicine* **19**, 916-923 (2013).

- 704 8. Kasuya, A., Ito, T. & Tokura, Y. M2 macrophages promote wound-induced hair
705 neogenesis. *Journal of dermatological science* **91**, 250-255 (2018).
- 706 9. Rahmani, W., *et al.* Macrophages Promote Wound-Induced Hair Follicle Regeneration in
707 a CX3CR1- and TGF-beta1-Dependent Manner. *The Journal of investigative dermatology*
708 **138**, 2111-2122 (2018).
- 709 10. Wang, X., *et al.* Macrophages induce AKT/beta-catenin-dependent Lgr5(+) stem cell
710 activation and hair follicle regeneration through TNF. *Nature communications* **8**, 14091
711 (2017).
- 712 11. Seifert, A.W., *et al.* Skin shedding and tissue regeneration in African spiny mice
713 (*Acomys*). *Nature* **489**, 561-565 (2012).
- 714 12. Billingham, R.E. & Russell, P.S. Incomplete wound contracture and the phenomenon of
715 hair neogenesis in rabbits' skin. *Nature* **177**, 791-792 (1956).
- 716 13. Breedis, C. Regeneration of hair follicles and sebaceous glands from the epithelium of
717 scars in the rabbit. *Cancer research* **14**, 575-579 (1954).
- 718 14. Kligman, A.M. & Strauss, J.S. The formation of vellus hair follicles from human adult
719 epidermis. *The Journal of investigative dermatology* **27**, 19-23 (1956).
- 720 15. Lacassagne, A. & Latarjet, R. Action of methylcholanthrene on certain scars of the skin in
721 mice. *Cancer research* **6**, 183-188 (1946).
- 722 16. Ito, M., *et al.* Wnt-dependent de novo hair follicle regeneration in adult mouse skin after
723 wounding. *Nature* **447**, 316-320 (2007).
- 724 17. Plikus, M.V., *et al.* Regeneration of fat cells from myofibroblasts during wound healing.
725 *Science* **355**, 748-752 (2017).
- 726 18. Kim, M.H., *et al.* Dynamics of neutrophil infiltration during cutaneous wound healing
727 and infection using fluorescence imaging. *The Journal of investigative dermatology* **128**,
728 1812-1820 (2008).
- 729 19. Guo, S. & Dipietro, L.A. Factors affecting wound healing. *Journal of dental research* **89**,
730 219-229 (2010).
- 731 20. Segal, A.W. How neutrophils kill microbes. *Annual review of immunology* **23**, 197-223
732 (2005).
- 733 21. Brinkmann, V., *et al.* Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532-1535
734 (2004).
- 735 22. Papayannopoulos, V. Neutrophil extracellular traps in immunity and disease. *Nature*
736 *reviews. Immunology* **18**, 134-147 (2018).
- 737 23. Acosta, J.B., *et al.* The pro-inflammatory environment in recalcitrant diabetic foot
738 wounds. *International wound journal* **5**, 530-539 (2008).
- 739 24. Singer, A.J. & Clark, R.A. Cutaneous wound healing. *The New England journal of*
740 *medicine* **341**, 738-746 (1999).
- 741 25. DiPietro, L.A. Wound healing: the role of the macrophage and other immune cells. *Shock*
742 **4**, 233-240 (1995).
- 743 26. Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* **140**, 805-
744 820 (2010).
- 745 27. Huebener, P. & Schwabe, R.F. Regulation of wound healing and organ fibrosis by toll-like
746 receptors. *Biochimica et biophysica acta* **1832**, 1005-1017 (2013).

- 747 28. Kawai, T. & Akira, S. Toll-like receptor and RIG-I-like receptor signaling. *Annals of the*
748 *New York Academy of Sciences* **1143**, 1-20 (2008).
- 749 29. Cavassani, K.A., *et al.* TLR3 is an endogenous sensor of tissue necrosis during acute
750 inflammatory events. *The Journal of experimental medicine* **205**, 2609-2621 (2008).
- 751 30. Lin, Q., *et al.* Impaired wound healing with defective expression of chemokines and
752 recruitment of myeloid cells in TLR3-deficient mice. *Journal of immunology* **186**, 3710-
753 3717 (2011).
- 754 31. Lin, Q., *et al.* Toll-like receptor 3 ligand polyinosinic:polycytidylic acid promotes wound
755 healing in human and murine skin. *The Journal of investigative dermatology* **132**, 2085-
756 2092 (2012).
- 757 32. Bernard, J.J., *et al.* Ultraviolet radiation damages self noncoding RNA and is detected by
758 TLR3. *Nature medicine* **18**, 1286-1290 (2012).
- 759 33. Borkowski, A.W., *et al.* Toll-like receptor 3 activation is required for normal skin barrier
760 repair following UV damage. *The Journal of investigative dermatology* **135**, 569-578
761 (2015).
- 762 34. Nelson, A.M., *et al.* dsRNA Released by Tissue Damage Activates TLR3 to Drive Skin
763 Regeneration. *Cell stem cell* **17**, 139-151 (2015).
- 764 35. Zhu, A.S., Li, A., Ratliff, T.S., Melsom, M. & Garza, L.A. After Skin Wounding, Noncoding
765 dsRNA Coordinates Prostaglandins and Wnts to Promote Regeneration. *The Journal of*
766 *investigative dermatology* **137**, 1562-1568 (2017).
- 767 36. Lai, Y., *et al.* Commensal bacteria regulate Toll-like receptor 3-dependent inflammation
768 after skin injury. *Nature medicine* **15**, 1377-1382 (2009).
- 769 37. Kim, D., *et al.* Noncoding dsRNA induces retinoic acid synthesis to stimulate hair follicle
770 regeneration via TLR3. *Nature communications* **10**, 2811 (2019).
- 771 38. Liu, Y., *et al.* Tumor Exosomal RNAs Promote Lung Pre-metastatic Niche Formation by
772 Activating Alveolar Epithelial TLR3 to Recruit Neutrophils. *Cancer cell* **30**, 243-256
773 (2016).
- 774 39. Zhang, L.J., *et al.* Antimicrobial Peptide LL37 and MAVS Signaling Drive Interferon-beta
775 Production by Epidermal Keratinocytes during Skin Injury. *Immunity* **45**, 119-130 (2016).
- 776 40. Chow, O.A., *et al.* Statins enhance formation of phagocyte extracellular traps. *Cell host &*
777 *microbe* **8**, 445-454 (2010).
- 778 41. Mohanan, S., Horibata, S., McElwee, J.L., Dannenberg, A.J. & Coonrod, S.A. Identification
779 of macrophage extracellular trap-like structures in mammary gland adipose tissue: a
780 preliminary study. *Frontiers in immunology* **4**, 67 (2013).
- 781 42. Boe, D.M., Curtis, B.J., Chen, M.M., Ippolito, J.A. & Kovacs, E.J. Extracellular traps and
782 macrophages: new roles for the versatile phagocyte. *Journal of leukocyte biology* **97**,
783 1023-1035 (2015).
- 784 43. Rossaint, J., *et al.* Synchronized integrin engagement and chemokine activation is crucial
785 in neutrophil extracellular trap-mediated sterile inflammation. *Blood* **123**, 2573-2584
786 (2014).
- 787 44. Mauerer, N., *et al.* Activated platelets present high mobility group box 1 to neutrophils,
788 inducing autophagy and promoting the extrusion of neutrophil extracellular traps.
789 *Journal of thrombosis and haemostasis : JTH* **12**, 2074-2088 (2014).

- 790 45. Keshari, R.S., *et al.* Cytokines induced neutrophil extracellular traps formation:
791 implication for the inflammatory disease condition. *PloS one* **7**, e48111 (2012).
- 792 46. Gupta, A.K., Giaglis, S., Hasler, P. & Hahn, S. Efficient neutrophil extracellular trap
793 induction requires mobilization of both intracellular and extracellular calcium pools and
794 is modulated by cyclosporine A. *PloS one* **9**, e97088 (2014).
- 795 47. Wong, K.W. & Jacobs, W.R., Jr. Mycobacterium tuberculosis exploits human interferon
796 gamma to stimulate macrophage extracellular trap formation and necrosis. *The Journal*
797 *of infectious diseases* **208**, 109-119 (2013).
- 798 48. Wong, S.L., *et al.* Diabetes primes neutrophils to undergo NETosis, which impairs wound
799 healing. *Nature medicine* **21**, 815-819 (2015).
- 800 49. Delgado-Rizo, V., *et al.* Neutrophil Extracellular Traps and Its Implications in
801 Inflammation: An Overview. *Frontiers in immunology* **8**, 81 (2017).
- 802 50. Wang, Y., *et al.* Histone hypercitrullination mediates chromatin decondensation and
803 neutrophil extracellular trap formation. *The Journal of cell biology* **184**, 205-213 (2009).
- 804 51. Wang, Y., *et al.* Human PAD4 regulates histone arginine methylation levels via
805 demethylination. *Science* **306**, 279-283 (2004).
- 806 52. Daley, J.M., Thomay, A.A., Connolly, M.D., Reichner, J.S. & Albina, J.E. Use of Ly6G-
807 specific monoclonal antibody to deplete neutrophils in mice. *Journal of leukocyte*
808 *biology* **83**, 64-70 (2008).
- 809 53. Ribechini, E., Leenen, P.J. & Lutz, M.B. Gr-1 antibody induces STAT signaling,
810 macrophage marker expression and abrogation of myeloid-derived suppressor cell
811 activity in BM cells. *European journal of immunology* **39**, 3538-3551 (2009).
- 812 54. Carr, K.D., *et al.* Specific depletion reveals a novel role for neutrophil-mediated
813 protection in the liver during *Listeria monocytogenes* infection. *European journal of*
814 *immunology* **41**, 2666-2676 (2011).
- 815 55. Dovi, J.V., He, L.K. & DiPietro, L.A. Accelerated wound closure in neutrophil-depleted
816 mice. *Journal of leukocyte biology* **73**, 448-455 (2003).
- 817 56. Fan, C., *et al.* Characterization and quantification of wound-induced hair follicle
818 neogenesis using in vivo confocal scanning laser microscopy. *Skin research and*
819 *technology : official journal of International Society for Bioengineering and the Skin* **17**,
820 387-397 (2011).
- 821 57. Reber, L.L., *et al.* Neutrophil myeloperoxidase diminishes the toxic effects and mortality
822 induced by lipopolysaccharide. *The Journal of experimental medicine* **214**, 1249-1258
823 (2017).
- 824 58. Passegue, E., Wagner, E.F. & Weissman, I.L. JunB deficiency leads to a myeloproliferative
825 disorder arising from hematopoietic stem cells. *Cell* **119**, 431-443 (2004).
- 826 59. Elliott, E.R., *et al.* Deletion of Syk in neutrophils prevents immune complex arthritis.
827 *Journal of immunology* **187**, 4319-4330 (2011).
- 828 60. Abram, C.L., Roberge, G.L., Pao, L.I., Neel, B.G. & Lowell, C.A. Distinct roles for
829 neutrophils and dendritic cells in inflammation and autoimmunity in motheaten mice.
830 *Immunity* **38**, 489-501 (2013).
- 831 61. Buch, T., *et al.* A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation
832 after toxin administration. *Nature methods* **2**, 419-426 (2005).

- 833 62. Kessenbrock, K., *et al.* Netting neutrophils in autoimmune small-vessel vasculitis. *Nature*
834 *medicine* **15**, 623-625 (2009).
- 835 63. Hakkim, A., *et al.* Impairment of neutrophil extracellular trap degradation is associated
836 with lupus nephritis. *Proceedings of the National Academy of Sciences of the United*
837 *States of America* **107**, 9813-9818 (2010).
- 838 64. Lande, R., *et al.* Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-
839 peptide complexes in systemic lupus erythematosus. *Science translational medicine* **3**,
840 73ra19 (2011).
- 841 65. Garcia-Romo, G.S., *et al.* Netting neutrophils are major inducers of type I IFN production
842 in pediatric systemic lupus erythematosus. *Science translational medicine* **3**, 73ra20
843 (2011).
- 844 66. Villanueva, E., *et al.* Netting neutrophils induce endothelial damage, infiltrate tissues,
845 and expose immunostimulatory molecules in systemic lupus erythematosus. *Journal of*
846 *immunology* **187**, 538-552 (2011).
- 847 67. Leffler, J., *et al.* Neutrophil extracellular traps that are not degraded in systemic lupus
848 erythematosus activate complement exacerbating the disease. *Journal of immunology*
849 **188**, 3522-3531 (2012).
- 850 68. Khandpur, R., *et al.* NETs are a source of citrullinated autoantigens and stimulate
851 inflammatory responses in rheumatoid arthritis. *Science translational medicine* **5**,
852 178ra140 (2013).
- 853 69. Hu, S.C., *et al.* Neutrophil extracellular trap formation is increased in psoriasis and
854 induces human beta-defensin-2 production in epidermal keratinocytes. *Scientific reports*
855 **6**, 31119 (2016).
- 856 70. Gan, T., *et al.* TLR3 Regulated Poly I:C-Induced Neutrophil Extracellular Traps and Acute
857 Lung Injury Partly Through p38 MAP Kinase. *Frontiers in microbiology* **9**, 3174 (2018).
- 858 71. Liu, Q., *et al.* Toll-Like Receptor 3 Signaling Contributes to Regional Neutrophil
859 Recruitment in Cultured Human Glomerular Endothelial Cells. *Nephron* **139**, 349-358
860 (2018).
- 861 72. Satija, R., Farrell, J.A., Gennert, D., Schier, A.F. & Regev, A. Spatial reconstruction of
862 single-cell gene expression data. *Nature biotechnology* **33**, 495-502 (2015).
- 863 73. Jiang, T.X., Harn, H.I., Ou, K.L., Lei, M. & Chuong, C.M. Comparative regenerative biology
864 of spiny (*Acomys cahirinus*) and laboratory (*Mus musculus*) mouse skin. *Exp Dermatol*
865 **28**, 442-449 (2019).
- 866 74. Brant, J.O., Yoon, J.H., Polvadore, T., Barbazuk, W.B. & Maden, M. Cellular events during
867 scar-free skin regeneration in the spiny mouse, *Acomys*. *Wound Repair Regen* **24**, 75-88
868 (2016).
- 869 75. Trujillo, G., *et al.* Neutrophil recruitment to the lung in both C5a- and CXCL1-induced
870 alveolitis is impaired in vitamin D-binding protein-deficient mice. *Journal of immunology*
871 **191**, 848-856 (2013).
- 872 76. Metzemaekers, M., Gouwy, M. & Proost, P. Neutrophil chemoattractant receptors in
873 health and disease: double-edged swords. *Cell Mol Immunol* **17**, 433-450 (2020).
- 874 77. Lim, C.H., *et al.* Hedgehog stimulates hair follicle neogenesis by creating inductive
875 dermis during murine skin wound healing. *Nature communications* **9**, 4903 (2018).

- 876 78. Kim, S., *et al.* Simple cell culture media expansion of primary mouse keratinocytes.
877 *Journal of dermatological science* **93**, 135-138 (2019).
878

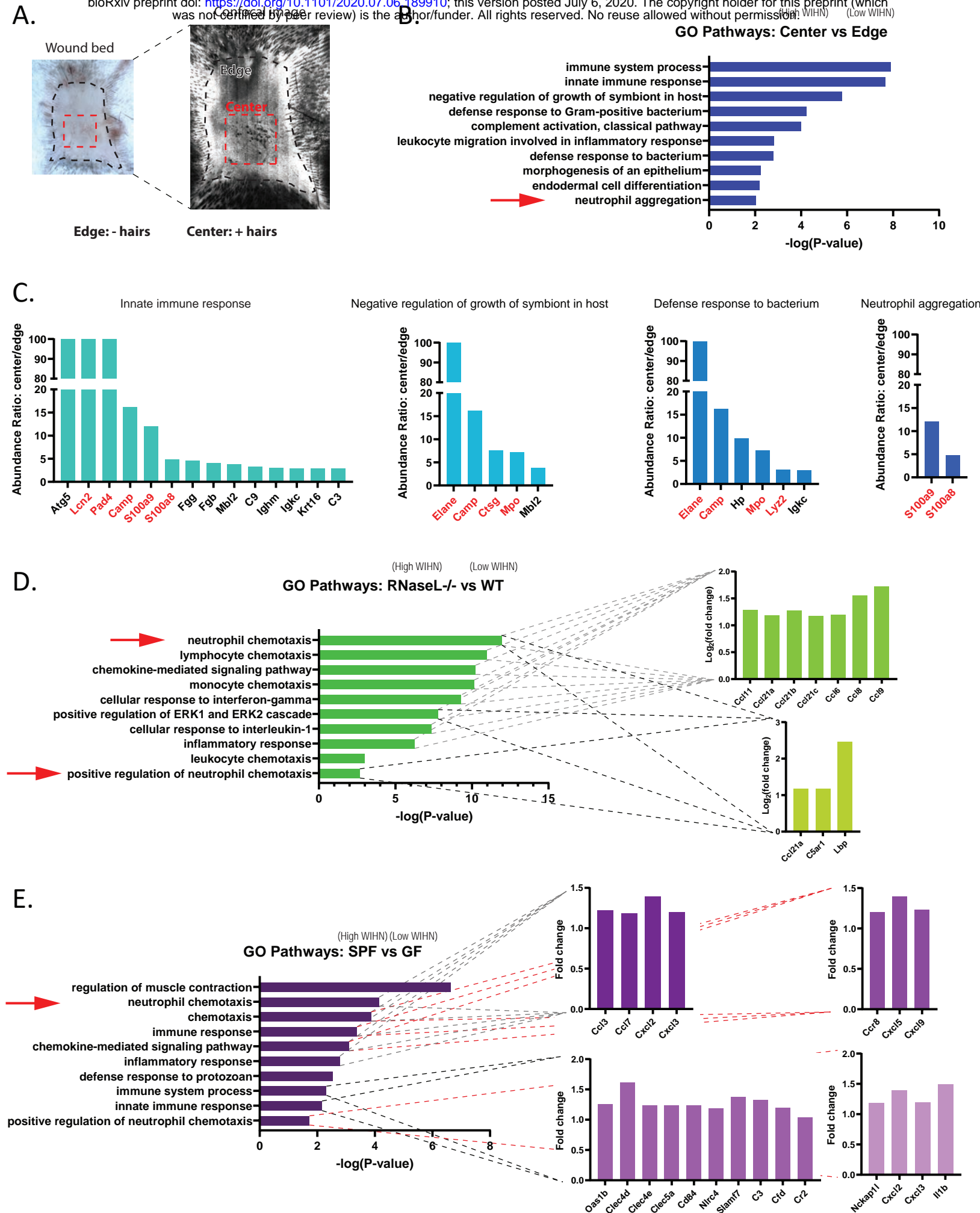


Figure 1: Neutrophil signature correlates with high skin regeneration in multiple models

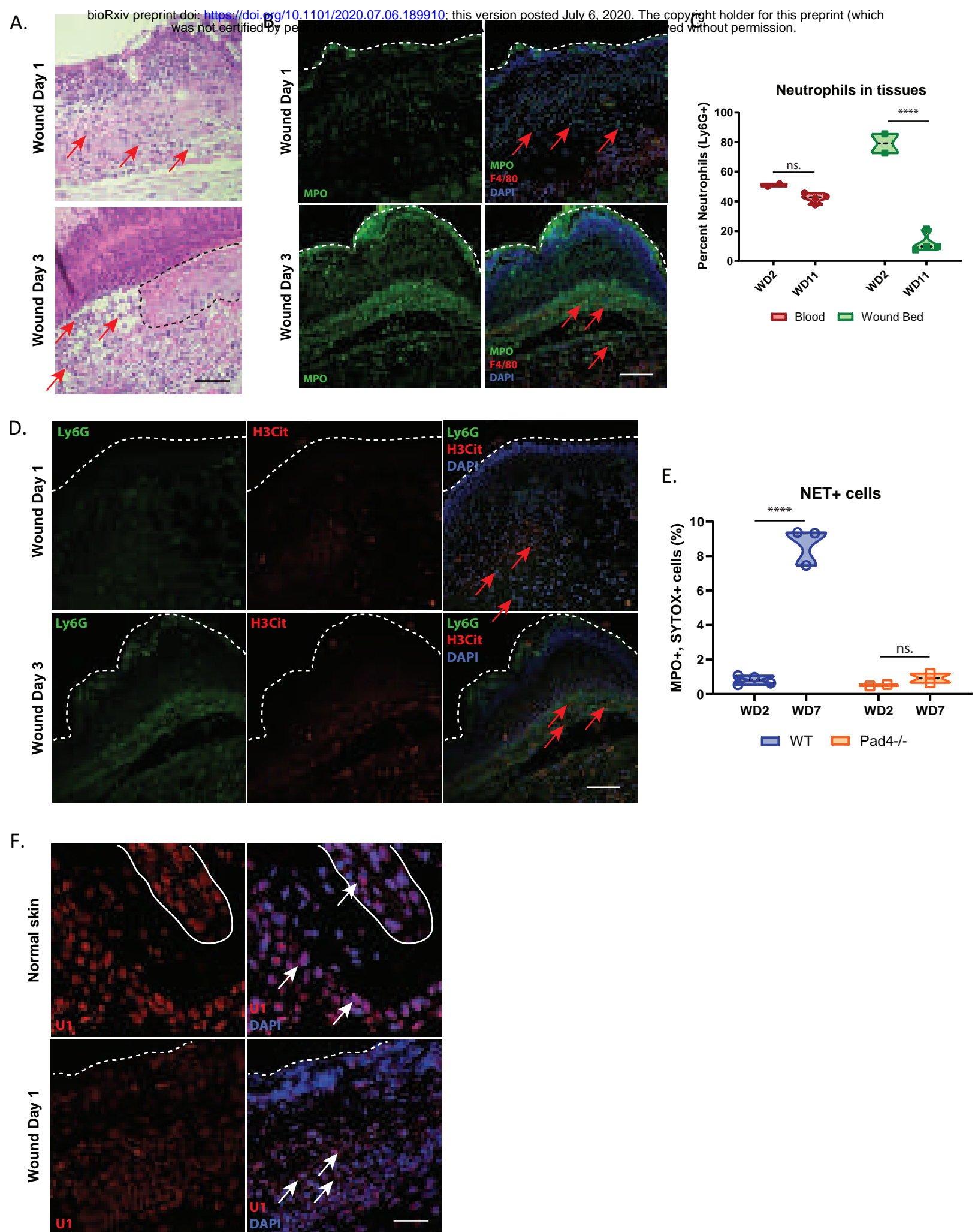


Figure 2: Neutrophils persist in wound bed after the acute inflammatory phase, producing extracellular traps

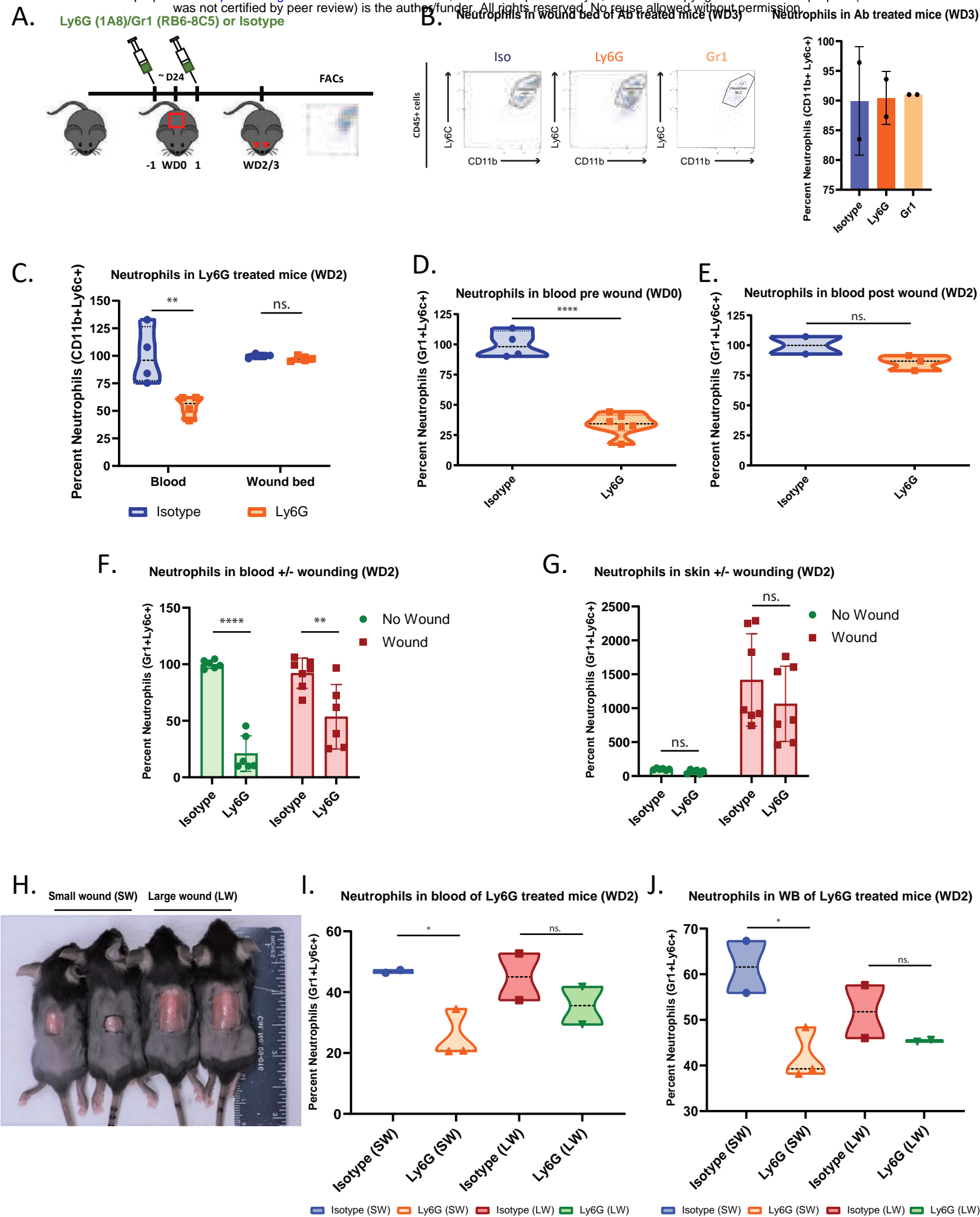


Figure 3: Large full-thickness wounds dramatically reduce antibody-mediated neutrophil depletion

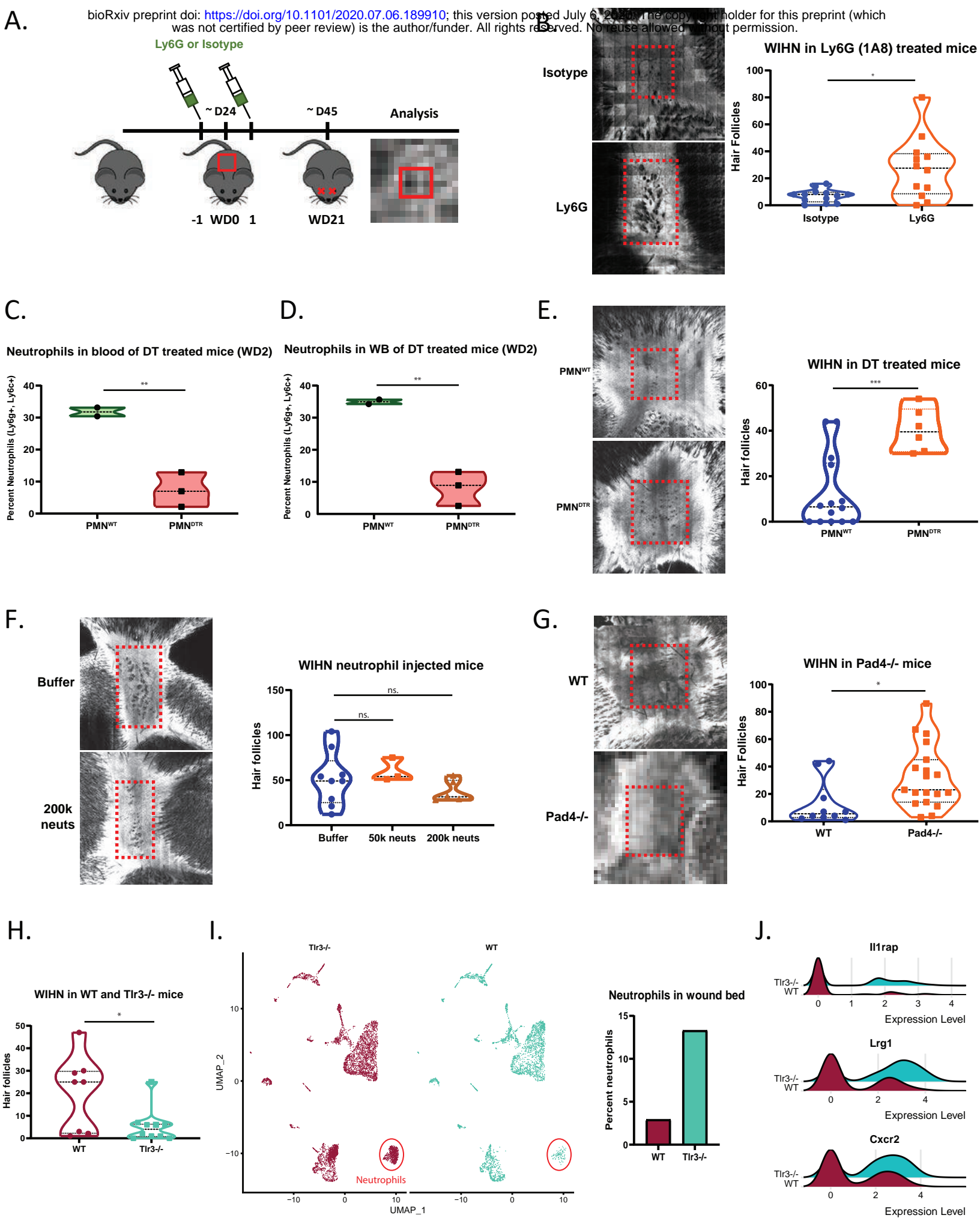
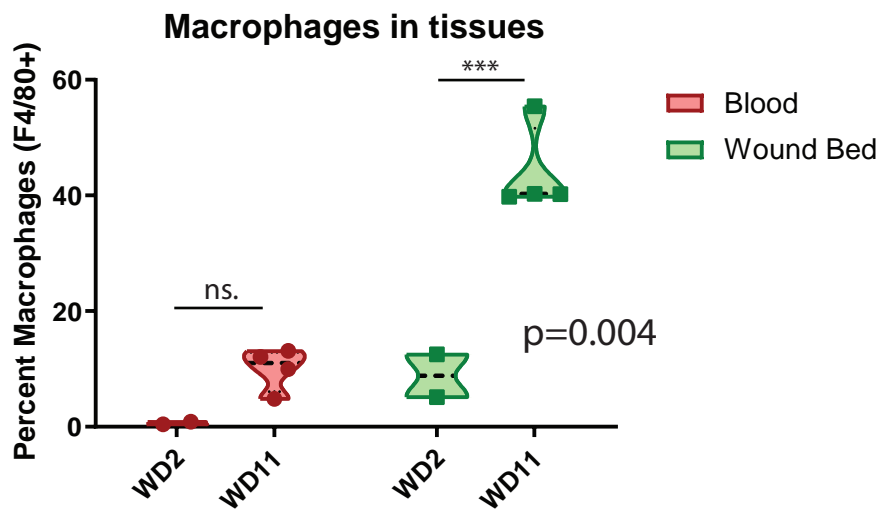


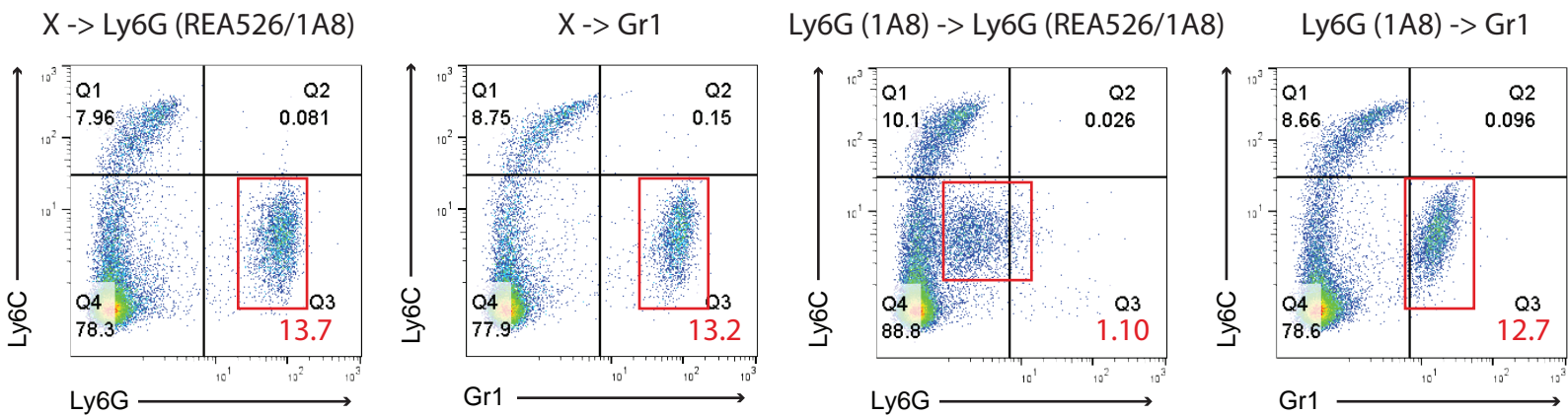
Figure 4: Neutrophils inhibit wound induced hair neogenesis

S1



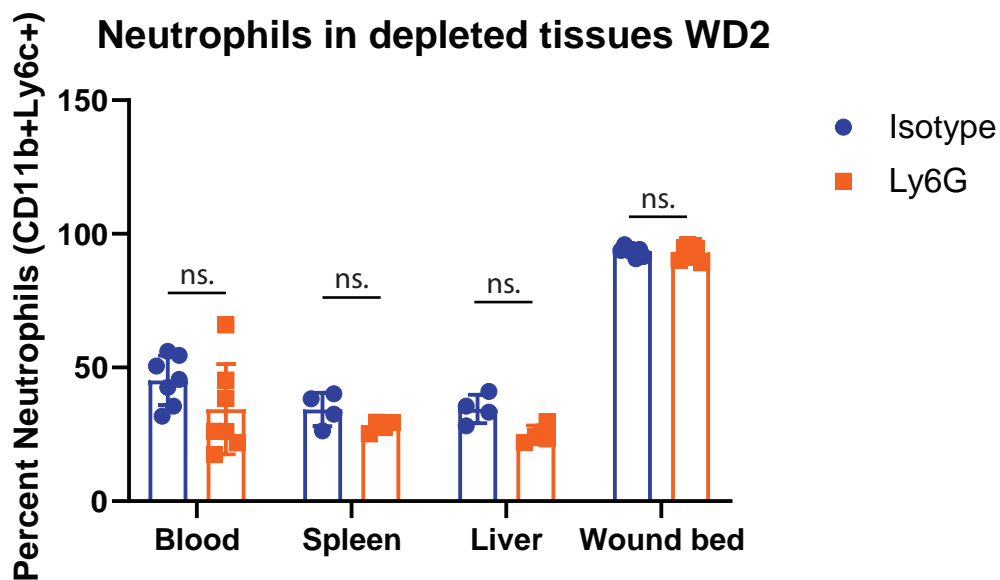
S1: Macrophage (F4/80) levels are largely absent from the blood and low in the wound bed during the early phase of healing, but increase dramatically at wound day 11, as measured by FACS. *** $p < 0.004$, as calculated by two-way ANOVA. n.s., not significant. N = 2 vs 4. Results are representative of at least two independent experiments.

S2



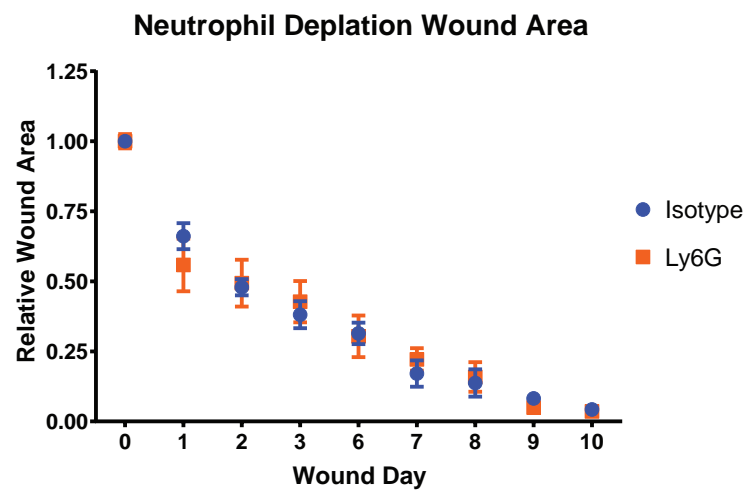
S2: Ly6G 1A8 antibody clone (Bio-X-Cell) masks the Ly6G epitope, preventing detection by the Ly6G REA526 clone (Miltenyi, engineered 1A8 clone) and partially preventing detection by the Gr1 RB6-8C5 clone (Bio-X-Cell) antibodies. Blood was extracted from a single C57BL/6J and stained sequentially with combinations of two antibodies, to test Ly6G epitope masking, before detecting by flow cytometry. If only stained with the Ly6G (REA526) or Gr1 (RB6-8C5), the neutrophil population is detected normally (13.7% or 13.2% respectively). If you first stain with Ly6G (1A8 clone), followed by Ly6G (REA526) or Gr1 (RB6-8C5), you get varying degrees of Ly6G antibody masking. Being a derivative of the 1A8 clone, the REA526 Ly6G binding site is almost completely blocked by the prior incubation with the Ly6G 1A8 clone (1.10%), making the pair unusable for neutrophil depletion experiments. Prior incubation with the Ly6G 1A8 clone followed by Gr1 (recognized an epitope of Ly6G/Ly6C) shifts the neutrophil population to the left, but they remain distinct from the negatively stained cells (12.7%). With careful gating, this makes depletion with the Ly6G (1A8 clone) antibody and detection with the Gr1 (RB6-8C5) antibody possible. The neutrophil population is boxed out in red.

S3



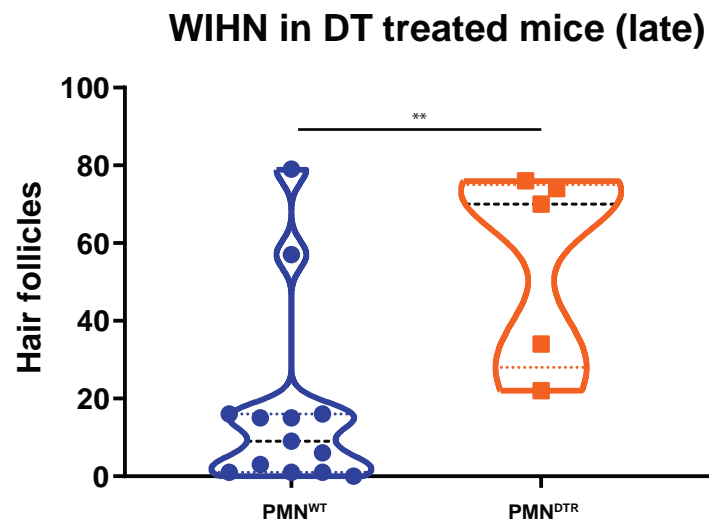
S3: Antibody mediated Neutrophil depletion is not efficient in the blood, spleen, liver, or wound bed, two days after wounding. The percent neutrophils (CD11b+, Ly6c+), at wound day 2, present in the blood, spleen, liver, and wound beds of mice IP injected with Ly6G antibody (500 μ , 1A8) one day before and one day after given 1.25x1.25cm² full thickness wounds. The neutrophil depletion is statistically insignificant, as calculated by two-way ANOVA. n.s., not significant. N = 4 vs 4, except for the Blood and Wound bed samples, which were 7 vs 7. S3: Antibody mediated Neutrophil depletion is not efficient in the blood, spleen, liver, or wound bed, two days after wounding. The percent neutrophils (CD11b+, Ly6c+), at wound day 2, present in the blood, spleen, liver, and wound beds of mice IP injected with Ly6G antibody (500 μ , 1A8) one day before and one day after given 1.25x1.25cm² full thickness wounds. The neutrophil depletion is statistically insignificant, as calculated by two-way ANOVA. n.s., not significant. N = 4 vs 4, except for the Blood and Wound bed samples, which were 7 vs 7.

S4



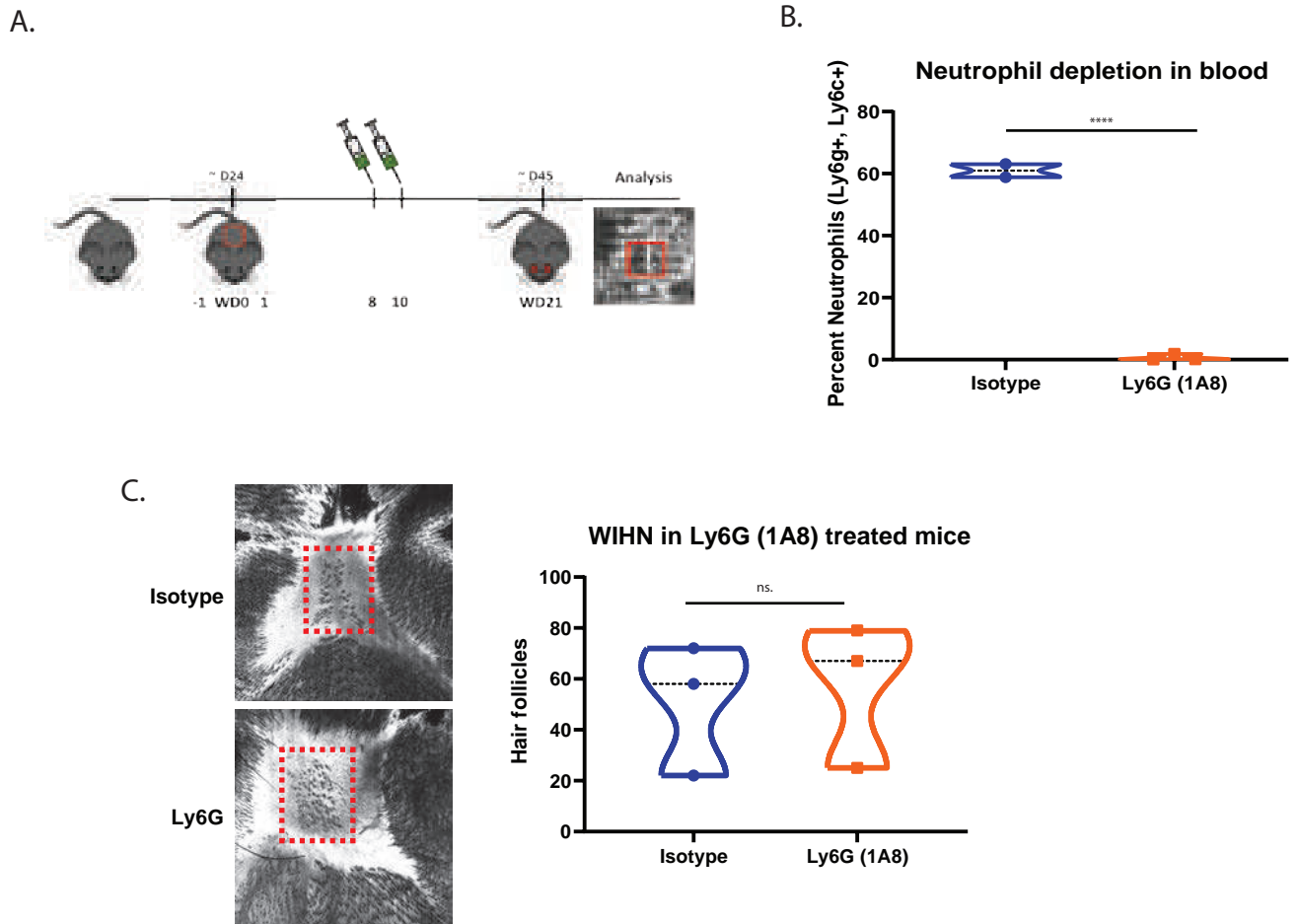
S4: The wound closure rate was not affected by neutrophil depletion via IP of Ly6G antibody (100 μ g) or isotype control. IP injections were done one day before and one day after wounding C57BL/J6 mice with 1.25x1.25cm² square wounds. N = 4 vs 4. Results are representative of three independent experiments.

S5



S5. PMN^{DTR} mice IP injected with diphtheria toxin (DT, 250ng) on wound days 6, 8, and 10 exhibit increased WIHN (CSLM, images; fold = 3.28, **p = 0.0085, N = 13 vs 5).

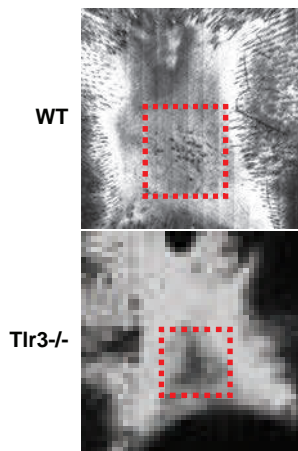
S6



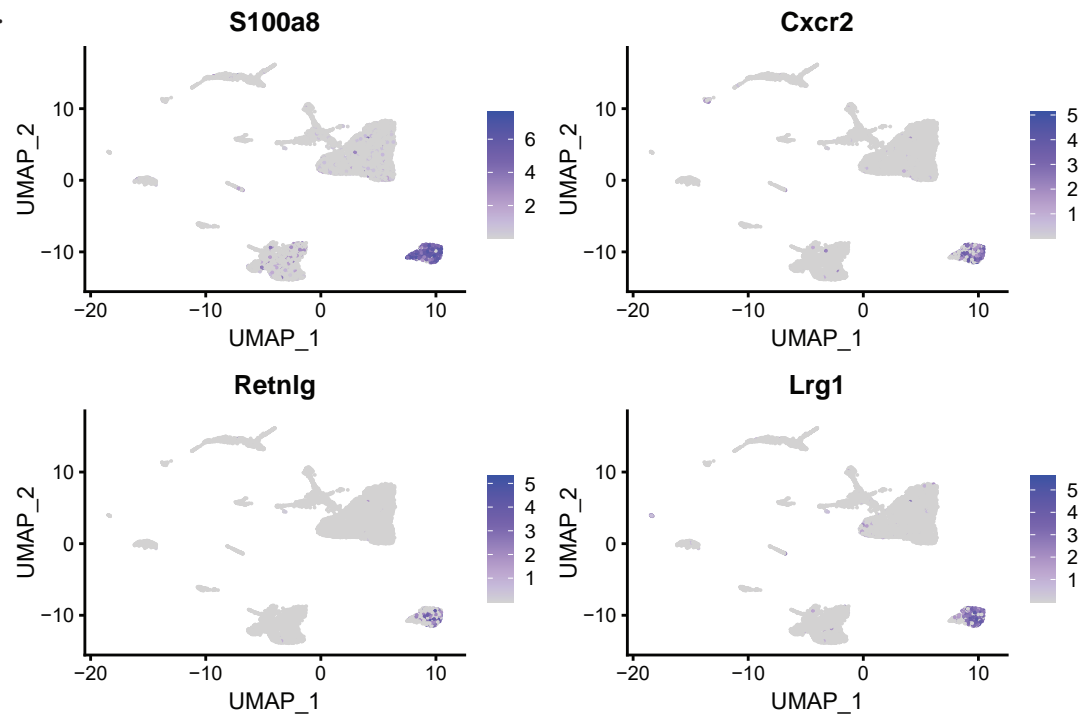
S6: a. Schematic of neutrophil depletion via IP of Ly6G antibody (500µg) or isotype control. IP injections are done on wound day 8 and 10, after wounding C57BL/J6 mice with 1.25x1.25cm² square wounds. Wound induced hair neogenesis (WIHN) was measured 21 days after wounding via confocal scanning laser microscopy (CSLM). b. Neutrophil depletion in the blood is successful on wound day 9, one day after antibody injection (Ly6G, 1A8), via FACs. The neutrophil depletion is statistically significant. ****p < 0.0001, as calculated by two-tailed Student's t test. Fold = -87.9. N = 2 vs 3. c. Late stage Ly6G antibody injected mice exhibit normal WIHN (CSLM, images; n.s., not significant, N = 3 vs 3). In each image, the dash red box indicates the area of hair follicle regeneration.

S7

A.



B.



S7: a. *Tlr3*^{-/-} mice exhibit decreased WIHN (representative CSLM images, N = 8 vs 10). In each image, the dash red box indicates the area of hair follicle regeneration. b. Neutrophil associated genes used to identify the neutrophil cluster in UMAP non-linear dimensional reduction, via Seurat R package, of WT and *Tlr3*^{-/-} mice.