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Reduced Bioactive Microbial Products (PAMPs) Contribute to Dysregulated Immune Responses And Impaired Healing in Infected Wounds in Diabetic Mice

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Abstract

Diabetic chronic ulcers are plagued with persistent non-resolving inflammation. However, diabetic wound environment early after injury suffers from inadequate inflammatory responses due to reductions the proinflammatory cytokines levels. Diabetic neutrophils have known impairments in bactericidal functions. We hypothesized that reduced bacterial killing by diabetic neutrophils, due to their bactericidal functional impairments, results in reduced bioactive bacterial products, known as pathogen associated molecular patterns (PAMPs), which in turn contribute to reduced signaling through TLRs, leading to inadequate production of proinflammatory cytokines in infected diabetic wound early after injury. We tested our hypothesis in db/db type 2 obese diabetic mouse wound infection model with *Pseudomonas aeruginosa*. Our data indicate that despite substantially higher levels of infection, TLR4-mediated signaling is reduced in diabetic wound early after injury, due to reduced bioactive levels of lipopolysaccharide (LPS). We further demonstrate that topical treatment with LPS enhances TLR4 signaling, increases proinflammatory cytokine production,

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AUTHOR CONTRIBUTIONS

Conceptualization: SHS; Formal Analysis: SHS, RR, FM, JZ; Funding Acquisition: SHS, JZ; Investigation: RR, FM, JZ; Methodology: RR, FM, JZ; Resources: SHS, TMK, JR; Supervision: SHS; Validation: RR, FM, JZ; Visualization: RR, FM, JZ; Writing - Original Draft Preparation: SHS, RR; Writing - Review and Editing: SHS, RR, JZ, FM, TMK, JR.

CONFLICTS OF INTEREST

Rush University Medical Center has filed a patent (International Application Number: PCT/US19/41112).

Dr. Sasha Shafikhani is the listed inventor on this application.

Prof. Jochen Reiser declares the following interests unrelated to this work: Consultancy: Walden Biosciences, Visterra, Mantra Bio, Aclipse Therapeutics, GLG, Guidepoint, Merck, Reata, Novateur; Ownership Interest: Walden Biosciences; Research Funding: Walden Biosciences; Honoraria: Visterra, Mantra Bio, Aclipse Therapeutics, GLG, Guidepoint, Merck, Reata, Novateur; Patents or Royalties: JR is an inventor on issued and pending patents pertinent to novel methods and treatments for proteinuric kidney diseases and stands to gain royalties from future commercialization.; JR is also a scientific co-founder of Walden Biosciences, a biotechnology company; Parts of JR's intellectual property has been outlicensed to Miltenyi Biotech; Advisory or Leadership Role: Walden Biosciences, Co-chair Scientific Advisory Board; and Other Interests or Relationships: Nephcure Kidney International.

restores leukocytes trafficking, reduces infection burden, and stimulates healing in diabetic wound. We posit that LPS may be a viable therapeutic option for the treatment of diabetic foot ulcers if it is applied topically after the surgical debridement process which is intended to reset chronic ulcers into acute fresh wounds.

Keywords

Diabetic Wound; Wound healing; PMN; Neutrophils; Leukocytes; Toll-like receptors (TLRs); TLR4; Chemokine; Cytokine; Proinflammatory responses; Lipopolysaccharide (LPS); Pseudomonas aeruginosa; Infection

INTRODUCTION

Diabetic foot ulcers (DFUs) account for 2/3 of all lower extremity amputations and cost between 9 to 13 billion dollars in care annually in the United States alone (Brem and Tomic-Canic, 2007, Raghav et al., 2018, Sen et al., 2009). Persistent non-resolving inflammation plagues chronic diabetic ulcers (Bjarnsholt et al., 2008, Blakytny and Jude, 2006, Dasu et al., 2010, Menke et al., 2007, Wetzler et al., 2000). Ironically, early after injury and during the acute phase of healing, the diabetic wound environment is completely different in that it suffers from inadequate inflammatory responses, characterized by reduced TLR signaling; reduced proinflammatory cytokines, (e.g., CCL2, IL-1β, and TNF-α); and reduced inflammatory leukocytes (Ishida et al., 2019, Nguyen et al., 2013, Roy Ruchi et al., 2022, Roy R. et al., 2022, Wood et al., 2014).

Neutrophils are the first inflammatory leukocytes migrating into the wound where they kill invading pathogens by a variety of mechanisms and jumpstart subsequent stages of inflammatory and non-inflammatory responses that are needed to fortify tissue's antimicrobial defenses and to initiate healing processes (Brinkmann et al., 2004, Diegelmann and Evans, 2004, Dovi et al., 2004, Kim et al., 2008). Despite excessive neutrophils in chronic diabetic ulcers, infection is rampant in DFUs, and this comorbidity has been attributed to impairments in diabetic neutrophil's bactericidal functions (Gallacher et al., 1995, Repine et al., 1980, Roy R. et al., 2022).

While infection is generally detrimental to wound healing processes, low sub-infective levels of bacteria actually accelerates wound healing by boosting inflammatory responses in wound via activation of TLRs by microbial products – known as pathogen associated molecular pattern molecules (PAMPs) (Chen and DiPietro, 2017, Dasu and Isseroff, 2012, Edwards and Harding, 2004, Laato et al., 1988, Portou et al., 2015, Soboll et al., 2006). We hypothesized that diabetic neutrophils' impaired ability to kill invading pathogens results in reduced bioactive PAMPs, which in turn contribute to reduced TLR expression and signaling in infected diabetic wounds early after injury, thus rendering diabetic wounds more prone to infection. We define "bioactive" PAMPs as free and soluble forms of microbial products that can interact with and trigger signaling through TLRs, not when these ligands are present in structural complexes (e.g., bacterial cell wall) within the live microorganisms, thus unable to interact with TLRs. We tested our hypothesis, using type 2 obese diabetic mice (db/db) wound model of infection with *Pseudomonas aeruginosa* which is the most

prevalent Gram-negative pathogen in DFUs and has been shown to colonize and cause tissue damage in this model (Ge et al., 2002, Goldufsky et al., 2015, Kirketerp-Moller et al., 2008, Roy R. et al., 2022).

RESULTS

TLR signaling is dampened in diabetic wound despite more infection.

We generated full-thickness excisional wounds in diabetic and non-diabetic mice and challenged these wounds with PA103 *P. aeruginosa* bacteria, which is shown to cause severe infection and wound damage in diabetic mice (Goldufsky et al., 2015). In line with our previous report, db/db wounds contained significantly more bacteria than normal wounds, confirming diabetic wound's vulnerability to increased infection (Supplementary Figure S1). NETosis (neutrophil extracellular trap formation) plays an important role against infection and it has been shown to be dysregulated in diabetic wounds during the chronic phase, contributing to impaired healing in diabetic mice and humans (Fadini et al., 2016, Wong et al., 2015). However, NETosis was not affected in infected or uninfected diabetic wounds as compared to normal wounds, suggesting that impaired NETosis is not responsible for the increased vulnerability to infection in diabetic wounds, at least during the acute phase of healing early after injury (Supplementary Figure S2).

We next assessed the impact of *P. aeruginosa* infection on the expression of TLR4, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and Interleukin-6 (IL-6) - which have all been shown to play key roles in immune defenses against *P. aeruginosa* and in wound healing (Awasthi et al., 2019, Faure et al., 2004, Ishida et al., 2006, Lin et al., 2003, Munir et al., 2020, Suga et al., 2014, Teng et al., 2009). Data indicated that these proteins were all substantially reduced in diabetic wounds early after injury (day 1), as assessed by Western blotting after normalizing the data to GAPDH loading control to account for the reduced leukocytes trafficking into diabetic wound early after injury (Figure 1a–b). Of note, the baseline expression of these proteins at the time of surgery (day 0) trended higher in diabetic skin than normal skin, although the data did not reach statistical significance (Supplementary Figure S3a–b). We corroborated these data by mRNA transcription analysis using RT-PCR after normalizing the data to 18S to account for the reduced leukocytes trafficking into diabetic wound early after injury. Data indicated that the mRNA levels of TLR4, NF-κB, and IL-6 were all significantly reduced in infected diabetic wounds early after injury (Figure 1c). Further supporting these data, immunohistochemical (IHC) analysis of TLR4 expression in normal and diabetic day 1 wound indicated that TLR4 expression is significantly diminished in diabetic wound early after injury (Figure 1d–e and Supplementary Figure S3c). Similarly, the expressions of IL-1β and TNF-α inflammatory cytokines, and myeloperoxidase (MPO) - a marker for primarily activated neutrophils (Klebanoff, 2005) - were also significantly reduced in day 1 diabetic wounds (Supplementary Figure S4). These data indicated that despite more infection, inflammatory responses are dampened in diabetic wounds early after injury.

Reduced bioactive PAMPs contribute to dampened inflammatory responses in infected diabetic wound.

To assess the role of reduced bioactive PAMPs as a potential contributor to reduced proinflammatory markers in diabetic wounds during the acute phase of healing early after injury, we first determined the levels of bioactive lipopolysaccharide (LPS) – (an important *P. aeruginosa* PAMP and a ligand for TLR4 (Huang et al., 2006)) – in normal and diabetic infected wounds on day 1 using the HEK-Blue hTLR4 reporter cells (Materials & Methods). Consistent with our hypothesis, the bioactive LPS levels were significantly reduced in diabetic wounds (Supplementary Figure S5). To increase PAMPs in diabetic wounds, we next facilitated bacterial killing by Polysporin gel which contains Bacitracin and Polymyxin B bactericidal antibiotics (Spann et al., 2003) after infection,. Despite reducing infection by nearly 3 log-orders (Supplementary Figure S6a), Polysporin-treated diabetic wounds contained significantly more neutrophils as assessed by histological analysis (Supplementary Figure S6b–c).

We reasoned if reduced bioactive PAMPs contributed to dampened expression of TLR4 and reduced production of proinflammatory cytokines in infected diabetic wounds, treating these wounds with a PAMP - which in the case of TLR4 is LPS (Huang et al., 2006) – should enhance proinflammatory environment in diabetic wounds. To test our hypothesis, we treated diabetic wounds with either LPS (100μg/wound) or PBS after wounding and prior to infection and assessed its impact on inflammatory environment and infection control in diabetic wounds. As expected, LPS topical treatment significantly increased the bioactive LPS levels in diabetic wounds (Supplementary Figure S7). Consistent with our hypothesis, LPS treatment resulted in significant increases in TLR4 expression in day 1 infected diabetic wounds as assessed by H&E histological analysis, by mRNA transcriptional analysis using RT-PCR, and by protein expression assessment using Western blotting (Figure 2a–e and Supplementary Figure S8a). Similarly, LPS treatment significantly increased the expression of NF-κB, IL-6, IL-β, and TNF-α in day 1 infected diabetic wounds (Figure 2c–e and Supplementary Figure S9a–b). LPS treatment also significantly increased the leukocytes influx as assessed by H&E staining (Figure 2f–g and Supplementary Figure S8b), and neutrophils influx as assessed by histological analysis using the neutrophil marker Ly6G and MPO using ELISA (Figure 2h–j and Supplementary Figure S8c, and Supplementary Figure S9c). Importantly, LPS treatment significantly reduced infection in diabetic wounds (Figure 2k and Supplementary Figure S9d). Interestingly, LPS treatment also significantly reduced the infection burdens in diabetic wounds challenged with *Staphylococcus aureus* USA300 (Supplementary Figure S10a), or diabetic wounds co-infected with USA300 and PA103 (Supplementary Figure S10b), suggesting that LPS treatment may have potential applications in the management of mono- and poly-microbial infections in diabetic wounds.

LPS-induced increases in inflammatory responses and infection control in diabetic wound is primarily dependent on neutrophils.

We recently demonstrated that increased expression of immunosuppressive IL-10 is a major cause of dampened TLR signaling and inflammatory responses in uninfected diabetic wounds early after injury (Roy Ruchi et al., 2022). We wondered if LPS treatment increased TLR signaling and inflammatory responses in diabetic wounds by reducing IL-10 in these

wounds. We first assessed IL-10 expression levels in infected normal and diabetic wounds. Data indicated that IL-10 levels were also significantly elevated in diabetic wounds infected with *P. aeruginosa* (Supplementary Figure S11). However, LPS treatment did not reduce IL-10 levels in day 1 diabetic wounds (Figure 3a), indicating that LPS treatment overcomes IL-10's immunosuppressive effects on TLR signaling and inflammatory responses in diabetic wounds.

Neutrophils play a critical role in combatting infection against bacterial pathogens including *P. aeruginosa* (Andrews and Sullivan, 2003, de Oliveira et al., 2016, Hirche et al., 2008, Koh et al., 2009, Kurahashi et al., 2009). Of note, LPS-treated normal and diabetic wounds showed a trend toward increased NETosis but the differences were not statistically significant (Supplementary Figure S12). To assess the role of neutrophils in the enhanced infection control in the LPS-treated diabetic wounds, we depleted db/db mice of neutrophils by anti-Ly6G antibody (Materials and Methods) and assessed the impact of neutrophil depletion on *P. aeruginosa* infection control in the LPS-treated diabetic wounds. Anti-Ly6G reduced neutrophils in the circulation by 93.1% as assessed by flow cytometry where neutrophils were identified as $CD45^{+}Ly6C/G^{hi}CD11b^{hi}$ (Atzeni et al., 2002, Roy R. et al., 2022), and in wound by 90.9% as assessed by histology (Figure 3b–d and Supplementary Figure S13a). Importantly, neutrophil-depletion abrogated the LPS's beneficial effects in boosting antimicrobial defenses against *P. aeruginosa*, as manifested by significant reductions in the production of proinflammatory markers MPO, IL-1β, and TNF-α, and ~1.3 log-order more bacteria in diabetic wounds (Figure 3e–h). These data indicated that LPS treatment increases infection control against *P. aeruginosa* by stimulating the neutrophil response in diabetic wounds. Of note, the baseline neutrophil contents of normal and diabetic skin at the time of surgery (day 0) were similar (Supplementary Figure S13b–d).

Topical treatment with LPS treatment does not lead to persistent non-resolving inflammation in diabetic wound.

To assess the long-term impact of LPS treatment on inflammatory responses in diabetic wound, we evaluated the dynamics of IL-1β and TNF-α proinflammatory cytokines that are reported to be reduced in diabetic wounds during the acute phase of healing early after injury but elevated in old chronic diabetic ulcers (Jeffcoate et al., 2005, Mirza et al., 2013, Roy Ruchi et al., 2022, Yan et al., 2016). Of note, low but comparable levels of IL-1β and TNF-α were detected in normal and diabetic skin prior to surgery (Supplementary Figure S14). Data indicated that while IL-1β and TNF-α expression continued to increase in the PBS-treated db/db infected wounds as the diabetic wounds aged, in the LPS-treated diabetic infected wounds, IL-1β and TNF-α were significantly higher during the acute phase of healing early after injury (day 1 for IL-1β and days 1 and 3 for TNF-α) but declined significantly in the old wounds, particularly at day 10 (Figure 4a–b). Similarly, the LPS-treated diabetic infected wounds contained significantly higher levels of neutrophils and leukocytes early after injury, (peaking on day 1 for neutrophils and day 3 for leukocytes), but declined significantly over time, reaching their lowest levels in day 10 wounds (Figure 4c–f, Supplementary Figure S15). In contrast, leukocyte and neutrophil contents of PBS-treated infected diabetic wounds were the lowest on day 1 but continued to increase, reaching their peaks on day 10.

Topical treatment with LPS stimulates healing in diabetic wound, despite infection.

Persistent inflammation dampens fibroblasts and myofibroblast functions in diabetic wounds, leading to reduced extracellular matrix (e.g., collagen and elastin) deposition, reduced scar tissue formation, and impaired healing in diabetic chronic ulcers (Augustine et al., 2014, Diegelmann and Evans, 2004, Yue et al., 1986). *P. aeruginosa* further reduces collagen deposition and exacerbates wound damage in diabetic mice by increasing the inflammatory environment in diabetic wound during the chronic phase at day 10 (Goldufsky et al., 2015). We assessed the impact of LPS treatment on fibroblasts, myofibroblast, extracellular matrix deposition, scar tissue formation, and wound healing. Data indicated that LPS treatment significantly increased fibroblast, myofibroblast, elastin and collagen connective tissue regeneration on day 10 in diabetic wounds, as assessed by IHC using their respective markers; Vimentin, α-SMA, Elastin, and Masson's Trichrome staining (Figure 5a–h and Supplementary Figure S16). Importantly, LPS treatment significantly stimulated healing in diabetic infected wounds, as assessed by digital photography (Figure 6a). Corroborating these data, H&E histological analyses demonstrated that LPS-treated wounds were significantly more re-epithelized, exhibited more epidermal thickening, and contained more scar tissues than the PBS-treated infected diabetic wounds (Figure 6b–d and Supplementary Figure S17).

DISCUSSION

In this report, we hypothesized that reduced bacterial killing by diabetic neutrophils, due to their bactericidal functional impairments, results in reduction in bioactive bacterial PAMPs, (e.g., LPS), which in turn contributes to dampened TLR expression/signaling and reduced inflammatory environments in diabetic wounds early after injury, making these wounds vulnerable to infection and impaired healing. Consistent with our hypothesis, our data showed that despite substantially more bacteria, bioactive LPS levels were significantly reduced in diabetic infected wounds early after injury, leading to reduced TLR4 expression and proinflammatory cytokine production in *P. aeruginosa*- infected diabetic wounds early after injury. We further demonstrated that topical treatment with bioactive LPS significantly increased TLR4 expression and proinflammatory cytokine production in diabetic wounds early after injury.

Encouragingly, LPS treatment also reduced the infection burdens of *P. aeruginosa* and *S.aureus* in single and co-infection models by 90–95%, suggesting that LPS treatment may have a broad application potential in the management of polymicrobial infections in diabetic wounds. This finding raises the question as to how diabetic neutrophils can combat infection in LPS-treated diabetic wounds if they have impaired bactericidal functions. We posit that diabetic neutrophils are not completely defective in their antimicrobial functions and LPS treatment may at least partially overcome neutrophil's bactericidal defects by increasing their numbers in diabetic wounds. Afterall, nearly half of diabetic wounds do not become infected (Armstrong et al., 2017).

We did not evaluate the effect of LPS treatment on biofilm formation during infection, This is a limitation of these studies, given the importance of biofilm infections which account for

55–67% of all reported infections in diabetic foot ulcers (Macdonald et al., 2021, Malik et al., 2013, Ramakant et al., 2011, Saseedharan et al., 2018).

High fraction of inspired oxygen (FiO_2) has been shown to reduce infection by activating neutrophils at surgical sites (Kroin et al., 2016). Thus, it would be interesting to assess the additive and/or synergistic impact of LPS and FiO_2 combination therapy on infection control in diabetic wounds.

Increased NETosis in chronic wounds is associated with non-healing diabetic ulcers in humans and impairs healing in type 1diabetic mice (Fadini et al., 2016, Wong et al., 2015). However, NETosis levels in day 1 wounds of db/db type 2 diabetic mice and non-diabetic control mice were comparable. This apparent contradiction may be due to differences in animal models (type 1 *vs.* type 2), or a limitation of db/db mice as a diabetes model.

Non-resolving and persistent inflammation are a hallmark of diabetic chronic ulcers (Bjarnsholt et al., 2008, Blakytny and Jude, 2006, Menke et al., 2007, Wetzler et al., 2000). However, our data indicate that diabetic wounds will not necessarily become persistently inflamed if inflammatory responses can be jumpstarted in them during the acute phase of healing early after injury. Encouragingly, LPS topical treatment substantially improved healing in diabetic wounds even in the presence of *P. aeruginosa* infection which has been shown to severely exacerbate tissue damage and prevent healing in diabetic wounds (Goldufsky et al., 2015, Zhao et al., 2010, Zhao et al., 2012).

Given that diabetic ulcers are already in a hyper-inflammatory state, one might question the therapeutic potential of LPS in these wounds. We posit that LPS could have real therapeutic potential in diabetic wound care if applied topically after the surgical debridement process, which is performed weekly or biweekly as a standard-of-care in the clinics to reset the chronic non-healing diabetic ulcers into an acute and fresh wounds (Cardinal et al., 2009, Golinko et al., 2008, Lebrun et al., 2010). We posit that after surgical debridement, diabetic wound environment is more likely to resemble acute wound in diabetic mice in need of inflammatory responses than chronic ulcer environment which is plagued with non-resolving inflammation. Consistent with this notion, the neutrophil and macrophage contents have been reported to be reduced in the normal skin at the edge of DFUs in diabetic patients with pathogenic non-healing DFUs (Sawaya et al., 2020).

MATERIALS & METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING:

Further information and requests for reagents may be directed to, and will be fulfilled by, the Lead Contact, Sasha Shafikhani (Sasha_Shafikhani@rush.edu).

PROCEDURES RELATED TO ANIMAL STUDIES:

We have an approval from the Rush University Medical Center Institutional Animal Care and Use Committee (IACUC No: 18–037) to conduct research as indicated. All procedures complied strictly with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal

Resources, National Academy of Sciences, Bethesda, MD, USA). We obtained 8-weeks old C57BLKS-m *lepr*^{db} (db/db) diabetic mice and normal non-diabetic littermate control mice db/+ (heterozygous) and C57BL/6 from the Jackson Laboratories (Bar Harbor, ME). These Mice were allowed to acclimate to the environment for 1 week prior to experimentation. Wounding and wound infection were carried out as we described previously (Goldufsky et al., 2015, Mohamed et al., 2022b, Wood et al., 2014). We used *Pseudomonas aeruginosa* (PA103) and *Staphylococcus aureus* USA300 bacterial strains for infection in this study. These strains has been described previously and has been shown to colonize and exacerbate wound damage in db/db wounds (Goldufsky et al., 2015, Pastar et al., 2013, Shafikhani and Engel, 2006, Wood et al., 2015). Infection levels in wounds were evaluated by determining the number of bacteria - colony forming unit (CFU) per gram of wound tissues, as described (Goldufsky et al., 2015, Kroin et al., 2015, Kroin et al., 2016, Kroin et al., 2018, Roy R. et al., 2022). Lipopolysaccharides (LPS) was added at 100ng per wound, prior to infection. LPS dose was chosen based on its demonstrated efficacy to reduce surgical site infection in normal mice (Mahmud et al., 2022). The number of animals needed for these studies was determined by the power analysis, based on the primary outcome: 80% power, α=0.05 in the 2-tailed student's *t*-test for means and an effect size=1.4, as we described (Kroin et al., 2015, Kroin et al., 2016, Mohamed et al., 2022b, Roy Ruchi et al., 2022, Roy R. et al., 2022, Wood et al., 2014).

HISTOPATHOLOGICAL EVALUATION:

Wound healing assessments were conducted as previously described (Goldufsky et al., 2015, Hamilton et al., 2021, Roy Ruchi et al., 2022, Wood et al., 2014). Leukocytes infiltration to the wound bed were performed as we described previously (Goldufsky et al., 2015, Kroin et al., 2016, Kroin et al., 2018). Neutrophils trafficking into wounds were assessed by immunohistochemical (IHC) analysis using anti-Ly6G antibody clone RB6–8C5 (Abcam) (Lucas et al., 2010, Roy R. et al., 2022). The histological data were normalized per wound surface area. Wound tissues' Myeloperoxidase (MPO) contents were assessed by ELISA (Gupta et al., 2017, Mahmud et al., 2022).

WESTERN BLOT ANALYSES:

We performed Western immunoblotting on tissue lysates, using the indicated antibodies after normalization to GAPDH loading control as described (Gupta et al., 2017, Kroin et al., 2016, Mohamed et al., 2022a, Shafikhani and Engel, 2006, Shafikhani et al., 2008, Wood et al., 2011).

BIOACTIVE LPS MEASUREMENTS IN WOUND:

Bioactive LPS in wound was determined by serial dilution and plating 24h after infection using HEK-Blue LPS detection kit 2 from InvivoGen following manufacturer's protocol. Data were normalized to infection levels.

ANTIBODIES (FOR WESTERN BLOTTING):

Anti-NF-κB (Cat. No. 8242), was purchased from Cell Signaling Technologies. Anti-TLR4 (Cat. No. 293072), and anti-IL-6 (Cat. No. 57315), were purchased from Santa Cruz Biotechnology. GAPDH (Cat. No. 1094-I-AP) antibody Rabbit Polyclonal from Proteintech.

NETosis:

NETosis levels in wound were determined, as described (Surolia et al., 2021), using Citrullinated Histone H3 (Cit-H3) ELISA kit (Cayman Chemical). Data were normalized to bacteria levels.

REAGENTS:

IL-1β and TNF-α ELISA kits were from Thermo Fisher. ELISA was performed as we discussed (Hamilton et al., 2021, Mahmud et al., 2022, Mohamed et al., 2022a, Roy Ruchi et al., 2022). Hematoxylin & Eosin were obtained from Thermo Fisher; Myeloperoxidase (MPO) Mouse ELISA Kit, Collagenase D, and LPS were obtained from Sigma Aldrich; Masson's Trichrome and Elastic connective tissue stains, and anti-α-SMA and anti-Vimentin antibodies were from Abcam. Polysporin was from Johnson & Johnson Consumer Inc; & sterile Petrolatum gel was from Fisher Scientific.

GENE TRANSCRIPTION ANALYSIS:

Gene expression at mRNA level was assessed by real-time polymerase chain reaction (RT-PCR), using gene-specific primer pairs by the Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR System as described (Roy Ruchi et al., 2022, Shafikhani, 2002, Shafikhani et al., 1997, Wood et al., 2014). The data were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH or 18S.

NEUTROPHIL DEPLETION STRATEGY:

Neutrophils were depleted in db/db mice as described previously (Roy R. et al., 2022). Briefly, db/db mice were injected intraperitoneally with 400μg anti-Ly6G antibody or IgG2a isotype control from Bio X Cell, a week before wounding and infection. Additional two consecutive doses of antibodies were administered with 100μg at 36 and 12 hours prior to wounding and infection experiment. Neutrophil depletion was confirmed by flow cytometry analysis (FACS). FACS analysis of PBMC was performed using fluorescence conjugated anti-CD45 (clone 30F11, #564225), anti-CD3ε (clone 500A2, #152316), anti-CD19 (clone 6D5, #115528), anti-NK1.1 (clone PK136, #108730), anti-CD11b (clone M1/70, #557397), anti-Ly6G (clone 1A8, #17–9668-82), LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (#L34966) and an isotype control antibody (TruStain, #101320). Compensation control was achieved with appropriate counting beads according manufacturer instruction and the acquisitions were performed by BD LSRFortessa equipped with four lasers and 18-instrument analyzer.

STATISTICAL ANALYSIS:

Statistical analyses between groups were conducted by One-way ANOVA with additional post hoc testing, and pair-wise comparisons between groups were performed or by unpaired

Student's *t*-test, using the GraphPad Prism software. Data are presented as Mean ± SEM. *P*-values less than or equal to 0.05 were considered as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

No datasets were generated or analyzed during this study.

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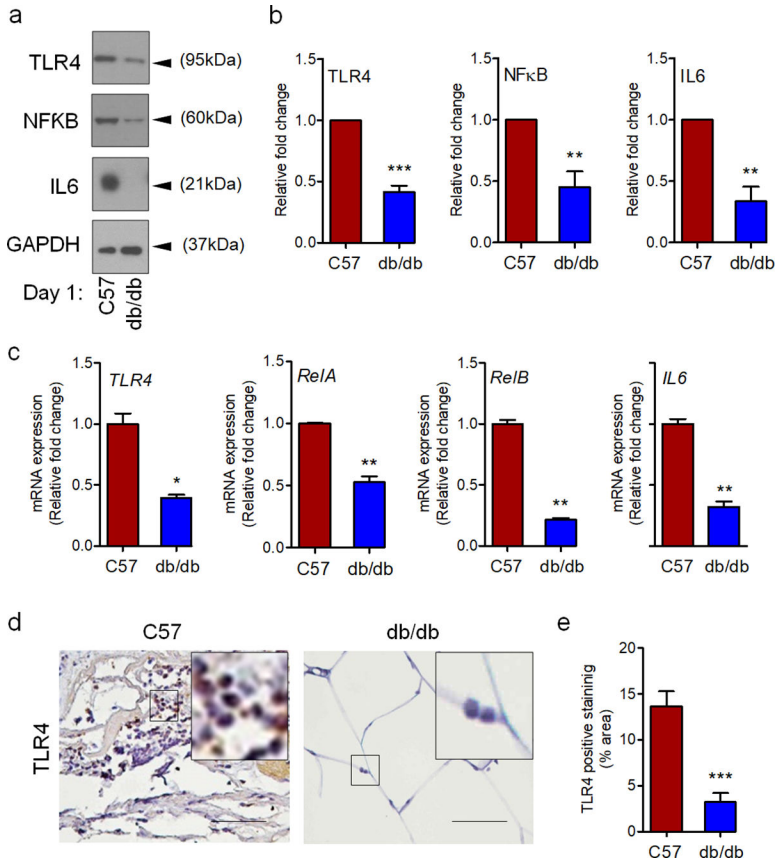

Figure 1. TLR signaling is dampened in diabetic wound despite more infection.

(a-e) Normal (C57) and diabetic (db/db) mice wounds were infected with PA103 (10^3 CFU/wound). (a) 24h after infection (day 1), wound tissues were assessed for protein expression of the indicated genes by Western blotting. (b) Densitometry analysis of Western blots is plotted as the mean ± SEM. (c) The mRNA levels of indicated genes in day 1 wounds were assessed by RT-PCR. (d) Histological analysis of TLR4 in infected wounds. Representative regions in wounds extending into the dermis. Scale bars=50μm. The square within each image is the magnified insert in the images. (e) The corresponding data of histological analysis are shown as the mean ± SEM. (N=4 mice/group for (a-c); N=4 mice/group, ≥9 random fields/wound/mouse for (d-e). *p<0.05, ** p<0.01, ***p<0.001, Student's *t*-test).

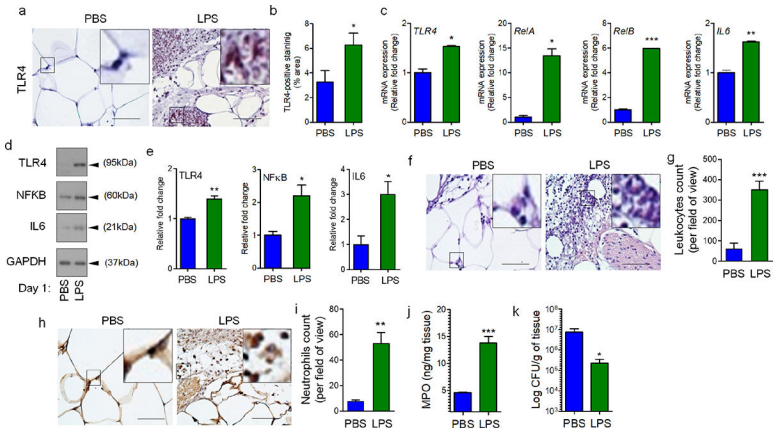

Figure 2. Topical treatment with LPS increases antimicrobial defenses in diabetic wound.

(a-k) db/db diabetic wounds were either treated with PBS or LPS (100 ng per wound) prior to infection. Day 1 wounds were assessed for TLR4 expression by immunohistochemistry (a-b); for mRNA expression of TLR4, NF-κB (*RelA* and *RelB* subunits), and IL-6 by RT-PCR (c); for the protein contents of the same genes by Western blotting (d-e); for their leukocyte content by H&E staining (f-g); for neutrophil content by histological analysis using anti-Ly6G antibody (h-i); for MPO content by ELISA (j); and for their infection levels by CFU determination (k). Scale bars=50μm. The tabulated data are shown as the mean ± SEM in (b, g, and i). (N=4 mice/group. *p<0.05, ** p<0.01, ***p<0.001, Student's *t*-test).

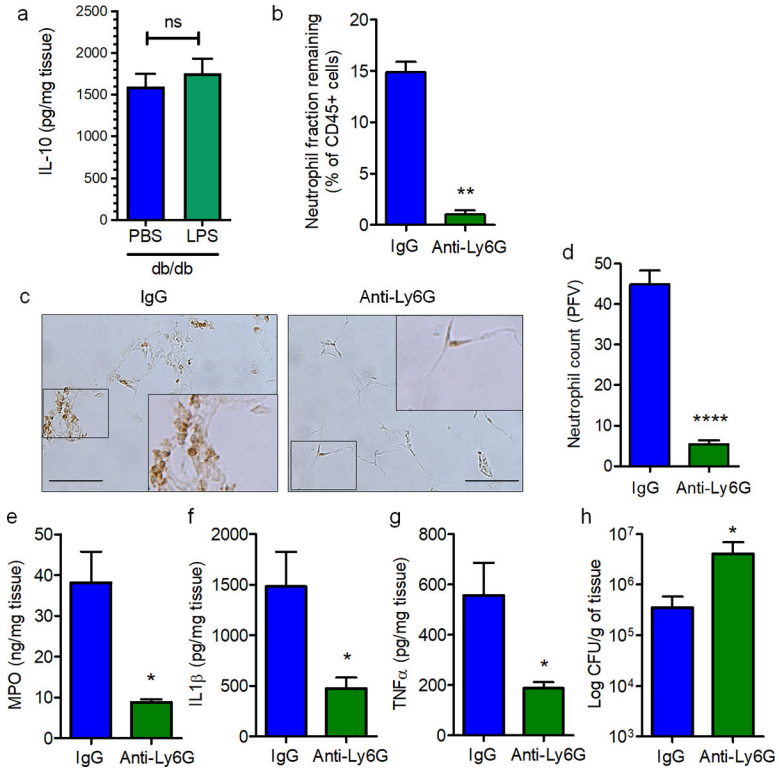

Figure 3. LPS enhances infection control in diabetic wound by increasing the neutrophil response in diabetic wounds.

(a) PBS and LPS-treated day 1 db/db infected wounds were assessed for their IL-10 contents by ELISA. (b) The neutrophil contents in the blood of anti-Ly6G or IgG-treated db/db mice were assessed by flow cytometry. (c-h) Anti-Ly6G or IgG-treated db/db diabetic mice were treated with PBS or LPS prior to infection. (c-d) Day 1 wounds were assessed for their neutrophil contents by immunohistochemistry using anti-Ly6G antibody (c) and the corresponding data are shown as the mean ± SEM in (d). (The scale bars are 50uM). (e-h) Day 1 wounds were assessed for MPO using ELISA (e); for IL-1β and TNF-α by ELISA (f-g); and for their bacterial contents by CFU (h). (N=4 mice/group. *p<0.05, ** p<0.01, ***p<0.001, Student's *t*-test).

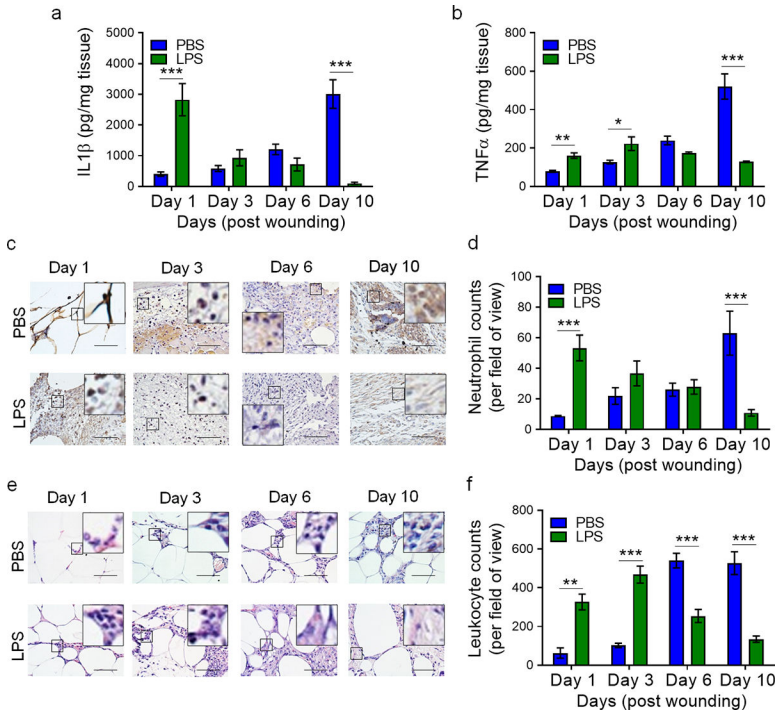

Figure 4. LPS treatment does not lead to persistent non-resolving inflammation in diabetic wound.

(a-f) At indicated timepoints, PBS and LPS-treated day 1 db/db infected wounds were assessed for IL-1β and TNF-α by ELISA (a-b); for neutrophils by IHC, using anti-Ly6G (c-d), and for leukocytes, using H&E staining (e-f). Representative regions in the wounds extending in the dermis are shown in (c & e) and the corresponding data are shown as the mean ± SEM in (d & f) respectively. The scale bars are 50μm. (N=4 mice/group; ≥5 random fields/wound/mouse. *p<0.05, ** p<0.01, ***p<0.001; One-way ANOVA with additional post hoc testing).

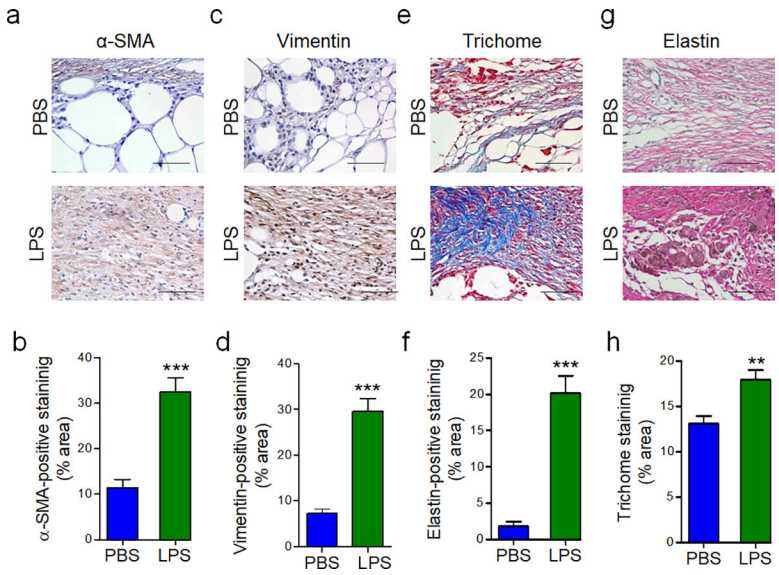

Figure 5. Topical treatment with LPS stimulates healing in diabetic wound, despite infection.

(a-d) At indicated timepoints, wound healing was assessed in the PBS and LPS-treated db/db infected wounds by digital photography (a) and by the assessment of the degree of re-epithelization, scar tissue (ST), and epidermal thickening (ET), using H&E staining (b). Black scale bars are 500μm. (c-d) The tabulated data associated with re-epithelialization (c) and scar tissue (d) are shown as mean ± SEM. (N=4 mice/group, *p<0.05, ** p<0.01, ***p<0.001. Statistical analyses between groups were by One-way ANOVA with additional post hoc testing, and pair-wise comparisons between groups were by unpaired Student's *t*-test). (e) A segment of PBS and LPS-treated re-epithelized wounds in day 10 is shown to highlight their epidermal thickening (ET).

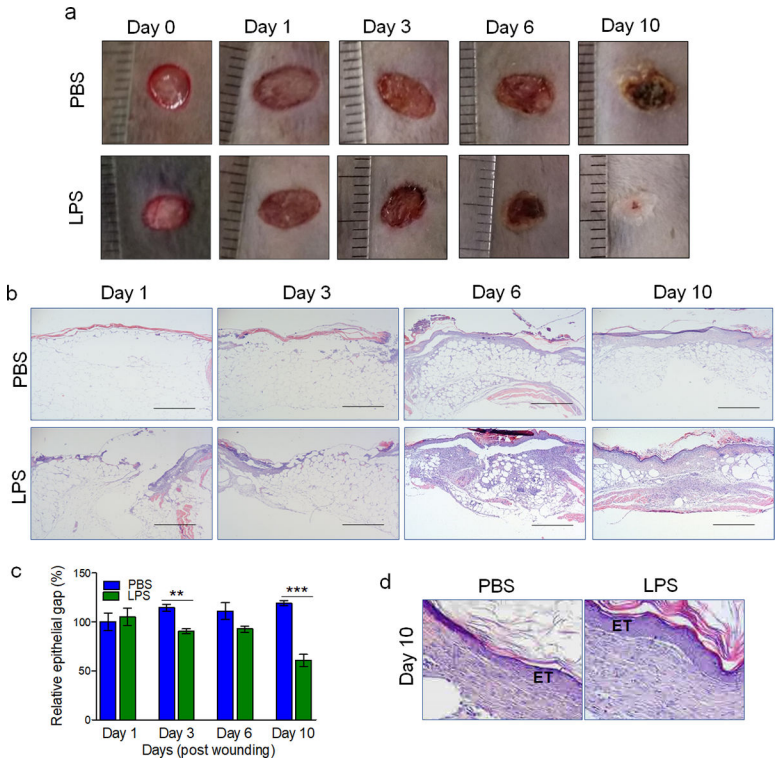

Figure 6. Histological healing assessment of PBS- and LPS-treated and infected diabetic wounds.

(a-d) PBS or LPS-treated db/db wounds were collected on day 10 and assessed for healing indicators; fibroblasts, myofibroblasts, elastin, or cartilage, using their respective markers, Vimentin, α-SMA, Elastin, and Masson's Trichrome staining respectively. (Cartilage levels were determined by aniline blue densitometry). Representative regions from underneath the wounds extending into the dermis are shown in (b and d) and the tabulated data are shown as mean ± SEM in (c). (N=4 mice/group, ≥9 random fields/wound/mouse; *p<0.05, ** p<0.01, ***p<0.001; Student's *t*-test). The scale bars are 50μm.