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***Pseudomonas aeruginosa* uses T3SS to inhibit diabetic wound healing**

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ABSTRACT

Diabetic foot ulcers are responsible for more hospitalizations than any other complication of diabetes. Bacterial infection is recognized as an important factor associated with impaired healing in diabetic ulcers. *Pseudomonas aeruginosa* is the most frequently detected Gram-negative pathogen in diabetic ulcers. *P. aeruginosa* infection has been shown to impair healing in diabetic wounds in a manner that correlates with its ability to form biofilm. While the majority of infections in diabetic ulcers are biofilm associated, 33% of infections are nonbiofilm in nature. *P. aeruginosa* is the most prevalent Gram-negative pathogen in all diabetic wound types, which suggests that the deleterious impact of *P. aeruginosa* on healing in diabetic wounds goes beyond its ability to form biofilm and likely involves other factors. The Type III Secretion System (T3SS) virulence structure is required for the pathogenesis of all *P. aeruginosa* clinical isolates, suggesting that it may also play a role in the inhibition of wound repair in diabetic skin ulcers. We evaluated the role of T3SS in mediating *P. aeruginosa*-induced tissue damage in the wounds of diabetic mice. Our data demonstrate that *P. aeruginosa* establishes a robust and persistent infection in diabetic wounds independent of its ability to form biofilm and causes severe wound damage in a manner that primarily depends on its T3SS.

Diabetic foot ulcers are one of the leading causes of hospitalization for patients with diabetes in the developed world; leading to pain, suffering, and a poor quality of life.¹ About 15% of all diabetics develop foot ulcers at some point in their lives, of which ~20% become chronic. This accounts for 67% of all lower extremity amputations in the United States, and adds between \$9 and \$13 billion to the direct annual costs associated with diabetes itself in the United States.² Bacterial infection and a microbiome shift toward pathogenic bacteria, particularly *Staphylococcus aureus* and *Pseudomonas aeruginosa*, have long been recognized as important factors contributing to impaired healing in diabetic ulcers.^{3,4} While considerable attention has been given to the role of *S. aureus* in facilitating impaired healing in diabetic wounds, the impact of *P. aeruginosa* in diabetic wounds has been limited to two publications correlating impaired healing in diabetic wounds infected with *P. aeruginosa* with the bacteria's ability to form biofilm.^{5,6}

Although, the majority of infections in diabetic ulcers are polymicrobial and biofilm associated, 33% of infections are nonbiofilm and 23% are nonbiofilm and monomicrobial.^{7,8} *P. aeruginosa* is the most frequently detected Gram-negative pathogen in all infection types in diabetic

ulcers, including those that are nonbiofilm and/or monomicrobial in nature,^{7,8} and its presence in wounds correlates with a poor prognosis for healing.^{3,9,10} These findings suggest that *P. aeruginosa*'s deleterious effects on diabetic tissue repair goes beyond its ability to form biofilm and other *P. aeruginosa* virulence factors are likely involved, particularly in nonbiofilm associated wound infection settings.

P. aeruginosa strains are quite heterogeneous with respect to their genotype, the virulence factors they possess, and their physiological profiles. For example, while PAO1 and PAK strains are flagellated and biofilm formers, PA103 strain is nonflagellated and lacks the ability to form biofilm.^{11,12} One thing that is in common among all clinical virulent isolates is that they all require the type III secretion system (T3SS) virulence structure to cause infection.¹³ T3SS injectosome functions as a conduit, allowing *P. aeruginosa* to directly translocate multiple proteins, termed effector toxins, into the target host cytoplasm where they modify host cellular processes and advance *P. aeruginosa* infection.¹³ To date, four type III secreted effectors have been identified in *P. aeruginosa*: ExoU, ExoT, ExoS, and ExoY. ExoU is a potent phospholipase that induces necrotic cytotoxicity in eukaryotic cells.¹⁴

ExoS is a potent inducer of apoptosis.¹⁵ ExoT exerts several virulence functions, including induction of potent apoptosis and inhibition of cytokinesis in epithelial cells.^{16–19} Finally, ExoY is an adenylate cyclase that functions as an edema factor.²⁰

We have previously demonstrated that *P. aeruginosa* uses a variety of virulence strategies that involve the T3SS effector toxins' ability to inhibit wound healing of epithelial cell culture in vitro;^{16–19} however, the role of T3SS in tissue repair impairment in *P. aeruginosa*-infected diabetic wounds has not been examined. Using PA103, a strain of *P. aeruginosa* which lacks the ability to form biofilm,^{11,12} and C57B/6 and db/db mice models for normal and for type 2 diabetes,^{21,22} respectively, we evaluated the contribution of T3SS in establishing infection and in mediating *P. aeruginosa*-induced tissue damage in normal and diabetic wounds. Our data demonstrate that *P. aeruginosa* is capable of establishing robust and persistent infection in diabetic, but not in normal wounds. Consistent with this infection data, we found that *P. aeruginosa* causes severe tissue damage and inhibited wound repair in diabetic wounds—but not in normal wounds—in a manner, which is primarily dependent on T3SS.

MATERIALS AND METHODS

Bacteria preparation

PA103 and its isogenic T3SS mutant form (PA103 *pscJ-gent^R*) strains have been described previously.^{16,17} They were grown overnight in Luria-Bertani (LB) media at 37 °C in a nonshaking incubator. Bacterial counts were determined by optical density and confirmed by serial dilution and the determination of colony forming units (CFU) by plating on LB, followed by 48 hour incubation at 37 °C. Approximately 1,000 bacteria (resuspended in 10 µL phosphate buffered saline solution [PBS]) were added to normal and diabetic wounds.

Animal studies procedures

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). We have an approval from the Rush University Medical Center Institutional Animal Care and Use Committee (IACUC No: 10–094) to conduct research as indicated.

Animal wounding and infection studies

A sterile biopsy punch (3 mm-diameter) was used to punch through the full thickness of the back skin below the shoulder blades of diabetic mice C57BLKS-m *lepr^{db}* (db/db), or their normal littermates animals; C57BLKS-m *lepr^{db/+}* (db/+) or C57BL/6 mice. Bacteria was prepared as described above and added to the wounds within 10 minutes after wounding. Mice were housed in individual cages after wounding to prevent cross-contamination.

Bacteria burden evaluation

Wound tissues were harvested on days 1, 3, 6, and 10 after wounding and infection. Tissues were cut into small pieces by sterile scissors and digested with collagenase D (Roche

Diagnostics, 2 mg/mL) at 37 °C for 40 minutes. The total number of bacteria was determined by serial dilution and plating on LB agar plates.

Wound healing assessment

For wound healing assessment, transparency paper was used to trace the wound edges. The wound areas were subsequently calculated using NIH ImageJ software. Wound healing was assessed, as we described previously,²¹ at 24 hour intervals by digital photography and expressed as % wound remaining, calculated as the % of original wound area ([open wound area/initial wound area] X 100).

Histological analyses

For histological studies, wound tissues were harvested, fixed in 10% formalin for 48 hour, and embedded in paraffin. Wounds were transversely cut into 7-µm-thick sections from the middle part of the wound, and stained with hematoxylin and eosin (H&E), Masson's trichrome staining, and Sirius red, as we described.^{21,23} H&E and trichrome staining was visualized on a Nikon Eclipse Ti microscope using NIS-Elements AR software. Sirius red was visualized by polarized microscopy on a Zeiss AxioVert 200 microscope and AxioVision software.

Statistical analysis

Statistical analyses were performed by one-way analysis of variance, Bartlett's test for equal variances, or Student *t* test, using the Prism statistics software. Data are presented as mean ± standard error of the mean (SEM). *p*-values less than or equal to 0.05 were taken as significant.

RESULTS

PA103 establishes robust and persistent infection in diabetic wounds

PA103 is an ideal clinical strain for the studies involving the T3SS and the T3SS effector toxins. It is not flagellated and is incapable of forming biofilm, thus biofilm effects do not contribute to its pathogenesis.^{11,12} Moreover, PA103 is defective in *lasR*, thus the T3SS-independent factors, such as elastase, do not significantly contribute to pathogenesis in this isolate and the T3SS-mediated virulence functions predominate.²⁴

To assess the role of T3SS in mediating *P. aeruginosa*-induced damage in wound, we first conducted a time course infection studies to determine if PA103 is able to colonize and persist in normal and/or diabetic wounds. We used a sterile biopsy punch (3-mm diameter) to punch through the full thickness on the back skin below the shoulder blades in C57B (Normal) and db/db (diabetic) mice. These wounds received ~1,000 CFU wild-type PA103 or its isogenic T3SS mutant PA103 *pscJ-gent^R* (These strains have been previously described).^{17,18,25}

Tissue samples were collected on days 1, 3, 6, and 10 after wounding (~1 mm tissue from the wound edges) and evaluated for bacterial colonization after homogenization by serial dilution and plating, as previously described.²⁶ In normal wounds, both wild-type (PA103) and T3SS mutant (PA103 T3SS-) strains were able to colonize and were

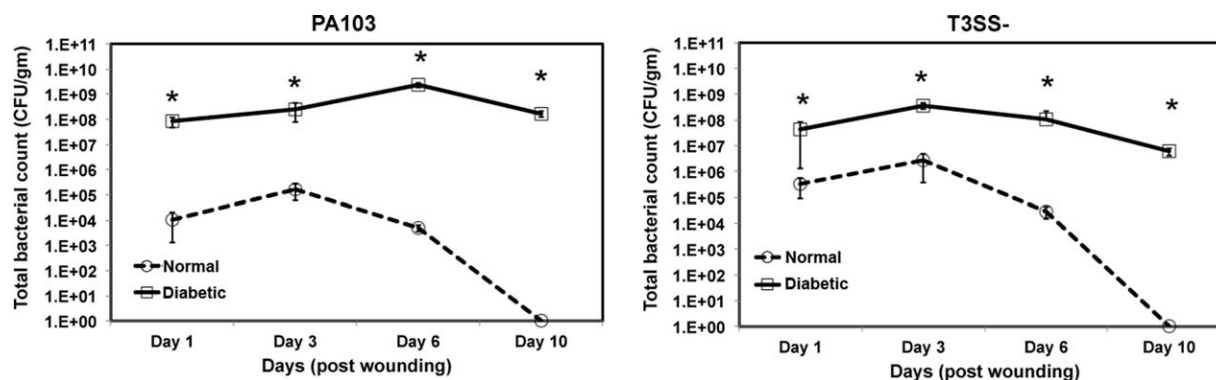


Figure 1. *P. aeruginosa* establishes robust infection in diabetic wound. Normal and diabetic wounds were infected with 1×10^3 of wild-type *P. aeruginosa* (PA103) or T3SS mutant PA103 *pscJ-gent^R* (T3SS-). Bacterial burden was determined by serial dilution and plating at indicated times after infection and is shown as the mean $\pm$ SEM (* Represents significance with $p < 0.0001$, Student *t* test; $n = 18$ [6 mice, 3 wounds/mouse]).

detected until day 6 postinfection but by day 10, all bacteria had been cleared and no bacteria could be detected (Figure 1). In contrast, wild-type and T3SS mutant bacteria were able to cause similar infection and persist in diabetic wounds (Figure 1). The bacterial burden in diabetic wounds were generally several log orders more than their counterparts in normal wounds (Figure 1, $n = 18$, $p < 0.0001$). These data demonstrated that *P. aeruginosa* is capable of causing robust and persistent infection in diabetic wounds in a manner that is independent of *P. aeruginosa*'s ability to form biofilm.

PA103 exacerbates tissue damage in diabetic wounds in a T3SS-dependent manner

We next evaluated the role of T3SS in mediating tissue damage in *P. aeruginosa* infected normal or diabetic

wounds. Normal and diabetic wounds were infected with PA103, PA103 *pscJ-gent^R* (T3SS-), or treated with PBS (Mock). We monitored healing daily by digital photography, as we described previously.²¹ Regardless of the strain, all infected normal wounds healed at rates similar to the wounds in the mock group, indicating that PA103 at this titer did not impact the healing processes in normal animals (Figure 2A and B).

In contrast, diabetic wounds infected with wild-type PA103 became substantially exacerbated compared with PBS treated and the T3SS mutant-infected db/db wounds, indicating that PA103 exacerbates wound healing in a manner that depends on its T3SS (Figure 3A and B). Of note, infection with PA103 or PA103 *pscJ-gent^R* (T3SS-) did not affect the weight of normal or diabetic mice during these studies (data not shown). This is consistent with previous reports, indicating that wound infection with PAO1

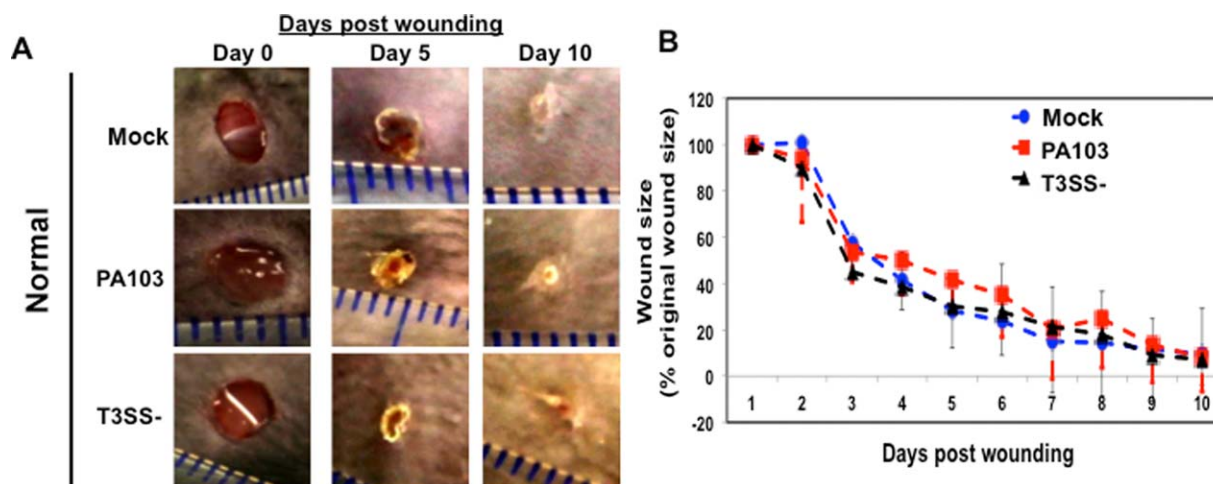


Figure 2. PA103 does not impact healing in normal skin. C57B (normal) wounds were either treated with PBS (Mock) or infected with 1×10^3 wild-type *P. aeruginosa* (PA103) or T3SS mutant PA103 *pscJ-gent^R* (T3SS-). Wound healing was monitored daily by digital photography. Representative images from the indicated timepoints are shown in (A) and the corresponding results are shown as mean $\pm$ SEM in (B) ($n = 6$ mice per group). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

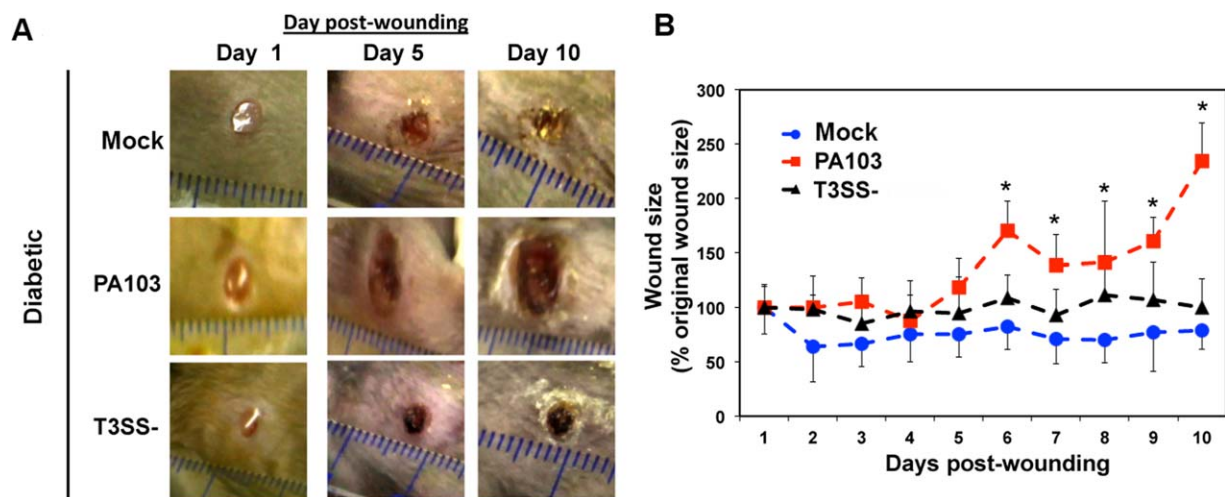


Figure 3. PA103 exacerbates damage in diabetic wound. db/db diabetic wounds were either treated with PBS (Mock) or infected with 1×10^3 wild-type *P. aeruginosa* (PA103) or T3SS mutant PA103 *pscJ-gent^R* (T3SS-). Wound healing was monitored daily by digital photography. Representative images from the indicated timepoints are shown in (A) and the corresponding results are shown as mean $\pm$ SEM in (B) ($n = 6$ mice per group). * Indicates significance with $p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(a biofilm-producing strain) does not affect the animal weight.^{5,6}

To further corroborate these findings, we stained the day 10 normal and diabetic wound tissues with H&E, as described.²¹ Regardless of the treatment, day 10 normal wounds were fully reepithelialized, exhibiting contraction, epidermal thickening, substantial granulation associated with scar formation, and loss of hair follicles (Figure 4A). In contrast, PA103 infected diabetic wounds showed minimal reepithelialization and were substantially inflamed as quantified by the number of leukocytes present in the wound (Figure 4B, $n = 24$, $p < 0.05$). Diabetic wounds infected with the T3SS mutant strain also showed increased inflammation relative to the PBS treated wounds, although their inflammation levels were still significantly less than PA103 treated diabetic wounds. Of note, wounds treated with PBS or infected with the T3SS mutant were reepithelialized, but they did not exhibit epidermal thickening or contraction.

Prompted by heightened inflammation in diabetic wounds infected with PA103 or the T3SS mutant strain (Figure 4B), and given the adverse impact of inflammation on collagen deposition and the remodeling phase of wound healing,²⁷ we evaluated the impact of *P. aeruginosa* infection on collagen deposition and connective tissue regeneration in normal and diabetic day 10 wounds by Masson's Trichrome staining, as described.²³ Consistent with the H&E staining results, normal wounds showed similar collagen depositions for all wounds regardless of treatment, as assessed by aniline blue densitometry of the wound area (Figure 5A). In contrast, diabetic wounds infected with wild-type PA103 or the T3SS mutant strain showed minimal collagen deposition (Figure 5, $n = 18$, $p < 0.05$). The connective tissues in these wounds were poor in collagen bundles, as shown by Masson's Trichrome light and diffuse blue staining. Mock treated diabetic wounds showed

modest but reduced collagen compared with normal wounds (Figure 5B, $n = 18$, $p < 0.05$). We confirmed the deleterious impact of *P. aeruginosa* on collagen deposition by Sirius red staining in day 10 wounds (Supporting Information Figure S1).

DISCUSSION

Given that *P. aeruginosa* is the most frequently detected Gram-negative bacterial pathogen even in nonbiofilm associated planktonic infections in diabetic foot ulcers,^{7,8,28} and given that *P. aeruginosa*'s presence in wounds correlates with a poor prognosis for healing,^{9,10} we postulated that there are likely other virulence factors that contribute to *P. aeruginosa* pathogenesis within the diabetic wound. In this communication, we focused on the role of T3SS as one such virulence factor that could potentially contribute to *P. aeruginosa*-induced damage in diabetic wounds. Our rationale for these studies was based on the understanding that T3SS is a major virulence structure that is required for pathogenesis of all *P. aeruginosa* strains studied thus far,^{13,29} and our previous reports which demonstrated the importance of T3SS effector toxins in mediating *P. aeruginosa* inhibition of wound repair in epithelial cell culture.^{16–18}

In this report, we demonstrate for the first time that *P. aeruginosa* can establish a robust and persistent infection in diabetic wounds in a manner that does not depend on its ability to form biofilm (Figure 1). We also demonstrate for the first time that the T3SS plays a major role in mediating *P. aeruginosa*-induced tissue damage in diabetic wounds in vivo (Figures 2–5). We found that diabetic wounds generally contained several log order more *P. aeruginosa* bacteria than normal wounds (Figure 1). The impaired antimicrobial defenses in diabetic wounds are at least in part due to delayed leukocyte infiltration in

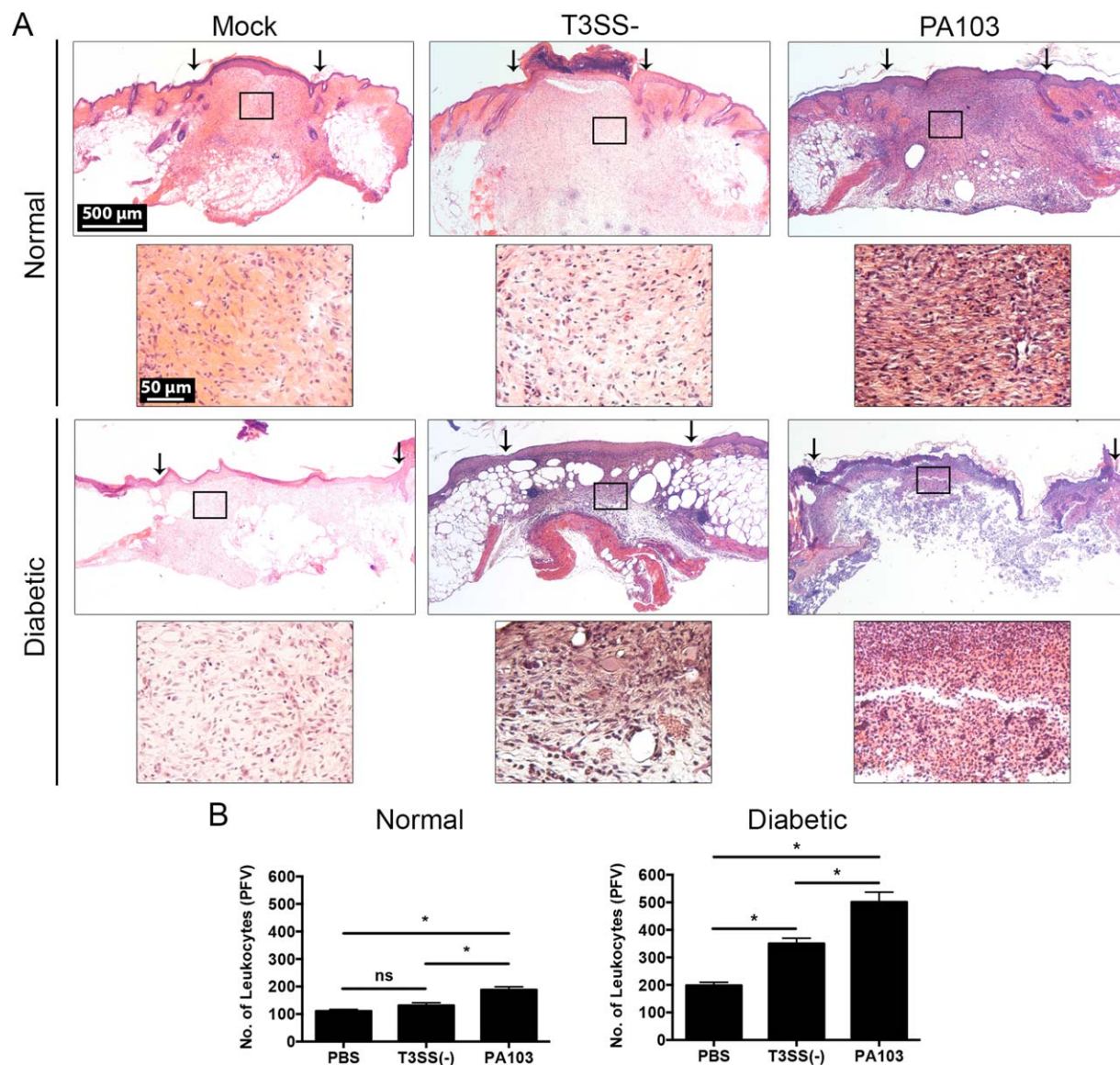


Figure 4. Histological analysis of normal and diabetic wounds infected with *P. aeruginosa*. Skin wounds were harvested from day 10 wounds of normal and diabetic mice which were either treated with PBS (Mock) or infected with 1×10^6 wild-type *P. aeruginosa* (PA103) or T3SS mutant PA103 *pscJ-gent^R* (T3SS-). (A) Wounds were fixed and stained with H&E. Wound edges are indicated by black arrows. (B) The corresponding tabulated number of leukocytes (per field of view) is shown as the mean $\pm$ SEM. ($n = 18$, 3 wounds per treatment and two fields of view assessed. *indicates significance with $p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

diabetic wounds early after injury²¹ and impaired bacterial killing by diabetic neutrophils due to reduced reactive oxygen species production resulting from impaired NADPH oxidase activity.³⁰

Persistent, nonresolving inflammation is a hallmark of human diabetic foot ulcers and is one of the well-recognized underlying factors contributing to impaired healing in diabetic chronic wound.^{31,32} Increased apoptosis in diabetic wounds has been reported and correlates with unresolved inflammation in the diabetic wound environment.³³ *P. aeruginosa*'s T3SS effectors, ExoS, and ExoT are potent

inducers of apoptosis in various cell types.^{15,18,19} Therefore, *P. aeruginosa* colonization could perpetuate persistent inflammation in diabetic wounds by increasing apoptosis in diabetic wound environment. We are currently investigating the role of T3SS effector toxins in inflicting damage in diabetic wounds.

The importance of the T3SS in mediating *P. aeruginosa* pathogenesis in diabetic wound provides us with a potential target for therapy. Small molecules or antibodies that can interfere with the T3SS structure and/or its function could potentially serve as a therapeutic means to enhance wound

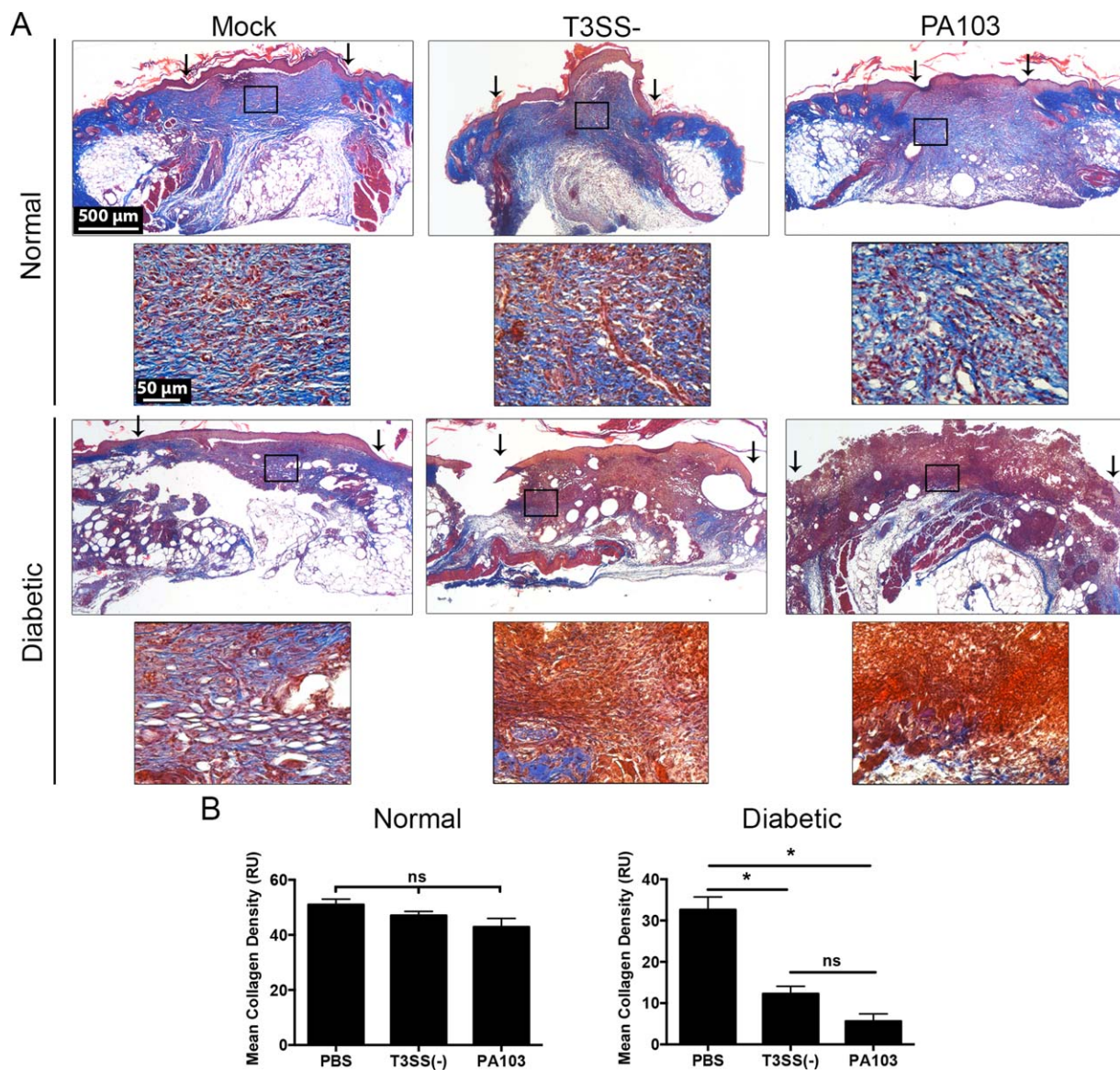


Figure 5. Analysis of collagen deposition of normal and diabetic wounds infected with *P. aeruginosa*. Skin wounds were harvested from day 10 wounds of normal and diabetic mice which were either treated with PBS (Mock) or infected with 1×10^6 wild-type *P. aeruginosa* (PA103) or T3SS mutant PA103 *pscJ-gent^r* (T3SS-). (A) Wounds were fixed and stained with Masson's trichrome stain. Wound edges are indicated by black arrows. (B) The amount of collagen was analyzed by densitometry for aniline blue using ImageJ and shown as the mean $\pm$ SEM. ($n = 18$, 3 wounds per treatment and two fields of view assessed. * indicates significance with $p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

healing in diabetic ulcers. Salicylidene acylhydrazides small molecules, INP0400, INP0401, and INP0402, have been successfully used to block T3SS-mediated secretion in a number of Gram-negative pathogens including *Yersinia*, *Chlamydia*, *Shigella*, and *Salmonella* species.^{34,35} Moreover, the plant auxin molecules indole-3-acetic-acid sodium salt, indole-3-butyric acid potassium salt, and 1-naphthalacetic acid potassium salt have been used to block T3SS expression and assembly in *P. aeruginosa*.³⁶ Finally, antibodies against the T3SS translocon components have also been

used as immunotherapy to treat infections by various Gram-negative pathogens including *P. aeruginosa*.³⁷

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Conflicts of Interest: None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Supporting Information Figure S1. Determination of collagen deposition in normal and diabetic wounds infected with *P. aeruginosa*. Skin wounds were harvested from day 10 wounds of normal and diabetic mice, which were either treated with PBS (Mock) or infected with 1×10^6 wild-type *P. aeruginosa* (PA103) or T3SS mutant PA103 *pscJ-gent^R* (T3SS-). Wounds were fixed and stained with Sirius red stain. Polarized light microscopy was used to visualize the birefringence of early and late collagen fibers, thereby distinguishing type III fibers (green birefringence) from type I fibers (yellow–orange birefringence), respectively.