Diabetic Complications Consortium

Application Title: S. aureus strain-level diversity in diabetic wound neutrophil phenotypes

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1. Project Accomplishments:

We screened 221 *Staphylococcus aureus* isolates from diabetic foot ulcer (DFU) for production of staphyloxanthin (Stx), a pigmented virulence factor that allows *S. aureus* to resist oxidative stress. We are investigating the role of Stx in neutrophil recruitment and function in diabetic wound healing. Our findings include:

- High Stx production by *S. aureus* isolated from DFU was associated with poor healing outcomes.
- Wounds infected with high Stx producing strains (Stx↑) had greater neutrophil chemotaxis to the site of tissue injury than control or low producing Stx strains (Stx↓).
- Stx↑ isolates delayed wound healing more than Stx↓ isolates in a diabetic mouse model of excisional wound healing.
- Wounds treated with a *S. aureus* mutant for staphyloxanthin production, CrtM, mirrored wound healing kinetics of Stx↓ infected wounds.
- IRB approval and protocol optimization for in vitro neutrophil assays has been completed.

2. Specific Aims:

AIM 1: Investigate the role of *S. aureus* strain-level variation in mediating neutrophil interactions and their association with clinical DFU outcomes.

Results: We are still collecting data from these experiments, as obtaining human neutrophils through the hospital posed a number of challenges during COVID restrictions. We now have IRB approval and trained staff to perform phlebotomy so that we can collect blood (and neutrophils) independently. In vitro phagocytosis and survival assays are currently underway. We obtained Stx mutants (CrtM and CrtN) from the Nebraska Transposon library, generated on a USA300 LAC background. We confirmed that these mutants do not produce Stx and will be suitable controls for the experiments to examine the role of Stx in neutrophil function and wound healing. We have also obtained and optimized an inhibitor of Stx, thymol. These tools allow us to isolate the role of Stx in our models.

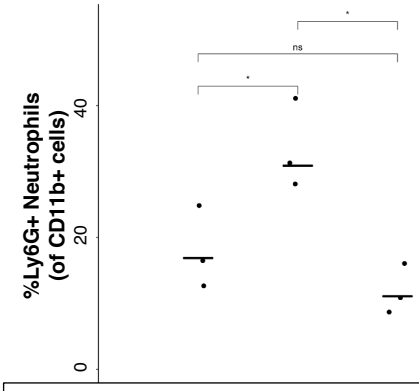

Figure 1: Neutrophil recruitment by staphyloxanthin high and low producing strains of S. aureus in an acute wound model.

To evaluate neutrophil chemotaxis, we performed an in vivo assay in a murine acute wound model, where a hole is punched in the ear and then exposed to *S. aureus*. After 48 hours, the ear is collected, digested, and flow cytometry performed to quantify how different *S. aureus* strains impact neutrophil recruitment. We observed that the Stx↑ recruited significantly more CD11b+ Ly6G+ neutrophils to the tissue, as compared to control and the Stx↓ strain (Figure 1).

AIM 2: Determine the *in vivo* contribution of *S. aureus* strain-level diversity on neutrophil phenotypes, dynamics, and healing in the murine diabetic wound.

Results: We hypothesized that DFU *S. aureus* strains with high Stx production *in vitro* would delay diabetic wound healing *in vivo*. To determine the importance of *in vitro* Stx production for diabetic wound healing, we performed excisional wound healing assays in diabetic mice (*Lepr*$^{db/db}$; db/db). *S. aureus* DFU clinical isolates were selected and used to infect diabetic wounds. We selected clinical isolates from the same patient, but collected at different time points. These two isolates, Stx↑and Stx↓, were phylogenetically almost identical, yet exhibited divergent phenotypes with respect to staphyloxanthin production. We observed that Stx↑ significantly delayed wound healing during the entire time course, as compared to control (PBS-treated) wounds and Stx↓ (Figure 2). At day 14, these differences were most apparent for clinical isolates and the CrtM Stx mutant (Figure 3), as well as the differences in healing between male and females. Females were significantly impaired in wound healing as compared to males (Figure 3). Tissue samples have been collected from these experiments and studies are underway to evaluate histology, neutrophil infiltration, and RNAseq gene expression analysis.

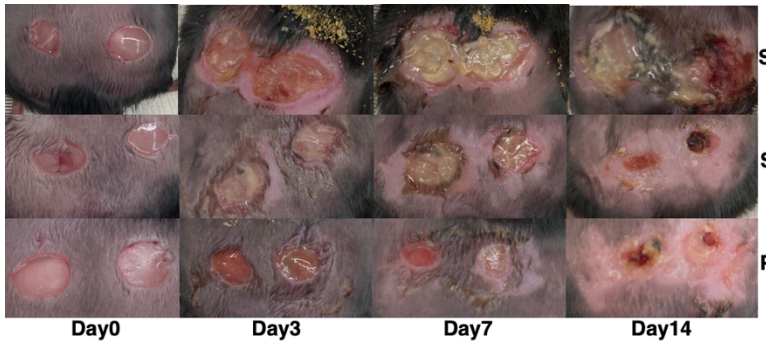

Figure 2: Representative diabetic wounds (8 mm, excisional) during the time course of healing as indicated below the photos. Staphyloxanthin high and low producing strains are compared to PBS in the lower panel.

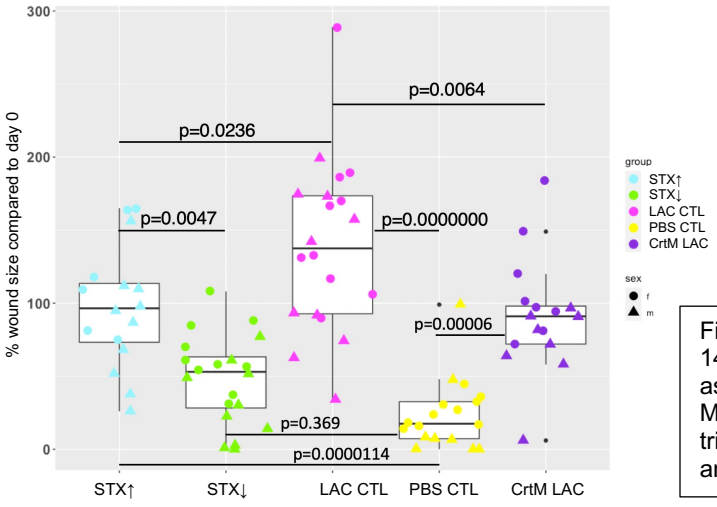

Figure 3: Wound closure quantified at day 14 post wounding. Wound size is expressed as percentage of day 0 wound size (y-axis). Male and female mice are indicated by triangles and circles, respectively. P-values are indicated on the graph.

3. Publications:

We have not published any findings from this project at this time. However, we expect to publish our findings within the next year.