Diabetic Complications Consortium

Application Title: **Biofilm-modified macrophage (BAM) phenotype and function in diabetic wounds**

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1. Project Accomplishments:

The overall objective of this proposal was to gain an understanding on how does the diabetic wound environment influences BAM phenotype and function. The phenotype and function of macrophages in wounds infected with biofilm are not well understood. S. aureus (SA) is one of the four most prevalent bacterial species identified in chronic wounds. While there are numerous studies testing the efficacy of a specific treatment for management of SA biofilm, understanding of molecular mechanism explaining the pathogenicity of SA biofilm is scantly studied. The current work is the first to specifically address the biofilm component of SA pathogenicity by the comparative use of three isogenic mutant strains of SA. Importantly, each of these strains are known to possess varying degree of biofilm forming ability. Well characterized S. aureus USA300LAC (USA300) served as the model strain for wound infection. The biofilm forming capability of this strain is well documented. The isogenic mutant strains USA300::sarA (ΔsarA) and USA300::rexB (ΔrexB) were used as hypo- and hyper-biofilm forming mutants, respectively. Staphylococcal accessory regulator (sarA) is one of the global regulators implicated in biofilm formation. Biofilm forming capacity is compromised in sarA mutants, The Δrex B is a transposon mutant that was created by disruption of rexB, which encodes for an ATP-dependent helicase/nuclease subunit that is important in DNA repair of double-stranded breaks. We report the hyper-biofilm activity of this strain.

Major project accomplishments include:

a. That biofilm infection impairs granulation tissue collagen deposition leading to increased risk of wound recurrence as predicted by compromised tensile strength of the repaired tissue.
b. Biofilm infection significantly induces MMP-3 expression in macrophages. In the wound microenvironment, matrix metalloproteinases (MMPs) contribute to the breakdown of collagen.

2. Specific Aims:

1. **AIM 1. Determine if functionally active wound macrophage isolated from biofilm-infected diabetic wounds show $ECM_{degrade}$ phenotype causing wound collagen degradation.**

Results:

Macrophages were isolated and exposed to conditioned media (CM) from *in vitro* mature biofilms from the three isogenic strains of SA studied in this work. The exposure of macrophages to CM from hyper-biofilm forming ΔrexB resulted in increased expression of MMP-3 protein (**Figure 1**). To understand the molecular species released by SA biofilm bacteria that induces the $ECM_{degrade}$ phenotype of macrophages, we performed molecular weight separation of the CM. The studies clearly demonstrate that the unidentified factor in CM that induces MMP-3 is macrophages is >50 Kda in size (Figure 1). Proteomics studies are

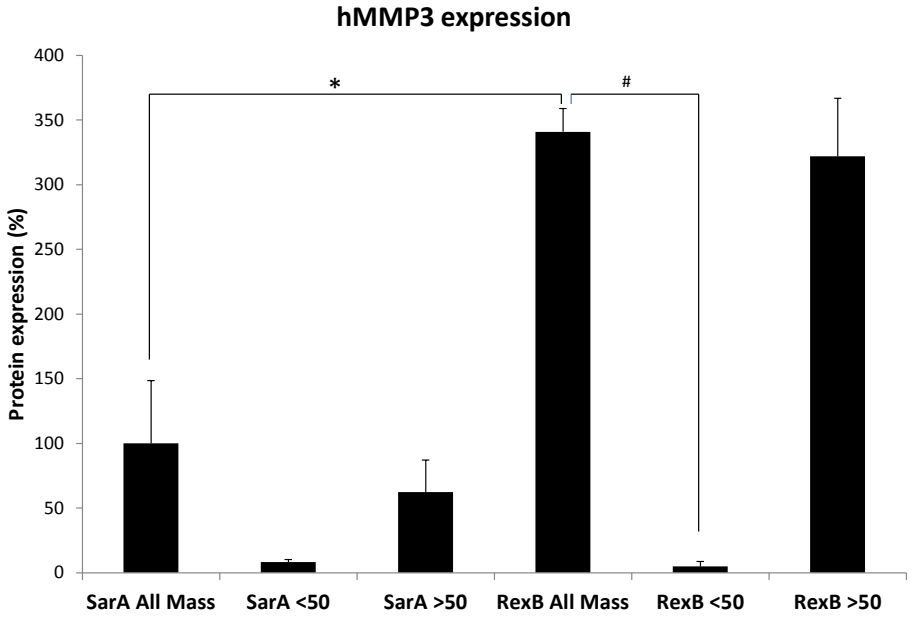

Figure 1. The exposure of human macrophages to conditioned media for from hyper-biofilm forming ΔrexB for 48h resulted in increased expression of MMP-3 protein as determined using ELISA. To understand the molecular species released by SA biofilm bacteria that induces the $ECM_{degrade}$ phenotype of macrophages, we performed molecular weight separation of the CM. The studies clearly demonstrate that the unidentified factor in CM that induces MMP-3 is macrophages is >50 Kda in size.

currently ongoing to characterize the identity of this unknown factor in conditioned media.

AIM 2. Investigate whether increased presence of $ECM^{degrade}$ wound macrophage phenotypes in biofilm-infected human diabetic wounds are associated with reduced wound collagen levels and increased wound recurrence.

To address this aim, we first performed a preclinical porcine study, where six 2" x 2" wounds were generated. A total of 10^8 CFU of S.aureus (SA) mutants sarA and rexB and wild type SA, USA300 was inoculated onto the wounds topically and dispersed across the surface with sterile spatula. Control wounds were inoculated with vehicle (PBS) only. The wounds were covered individually after bacterial inoculation and bandaged as described above.

Results: Wound closure, as determined by digital planimetry, was comparable among three types of infections studied (not shown). However, significant attenuation (~50%) of re-epithelialization was noted in whole wound cross-section histological images in hyper-biofilm forming ΔrexB infected wounds compared to ΔsarA or USA300 infections (not shown). Masson trichrome staining revealed a marked reduction in granulation tissue collagen contents (blue stain) in burn wounds infected with ΔrexB (hyper-biofilm) or USA300 infected wounds compared to group infected with ΔsarA (hypo-biofilm; **Figures 2A,B**). Hydroxyproline assay was performed to quantify collagen levels in wound-edge tissue. Significant loss of collagen in hyper-biofilm infected wounds was noted compared to hypo-biofilm infected SA mutant (**Figure 2C**). To test the functional significance of this observation, tensile strength of the repaired skin was studied in wounds infected with USA300, ΔsarA or ΔrexB. Compared to hypo-biofilm forming ΔsarA, biofilm infection by USA300 as well as ΔrexB significantly compromised the tensile strength of the repaired skin (**Figure 2D**). These data demonstrate that biofilm infection impairs granulation tissue collagen deposition leading to increased risk of wound recurrence as predicted by compromised tensile strength of the repaired tissue.

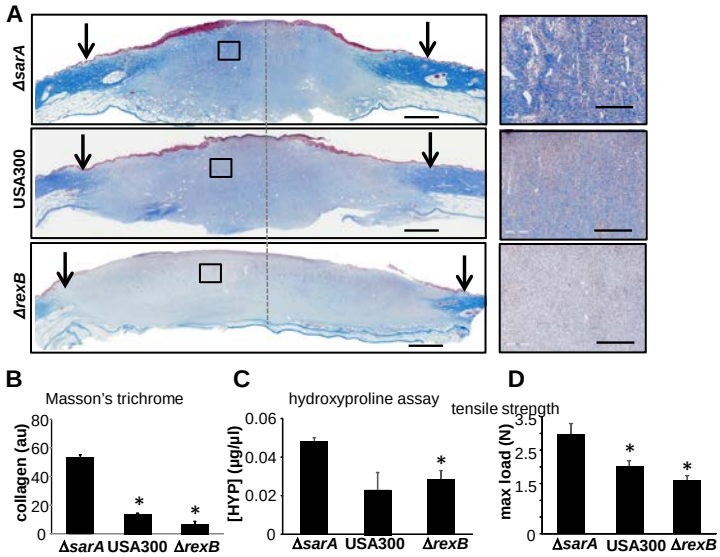

Figure 2. On day 3 post-burn, the porcine wounds were infected by isogenic strains of S. *aureus* USA300, USA300::*rexB* (*ΔrexB)* or USA300::*sarA (ΔsarA)*. **A,** Representative images of formalin-fixed paraffin- embedded (FFPE) biofilm infected day 35 burn wound biopsy sections (5 µm) were stained using Masson's Trichrome (MT). The MT staining results in blue-black nuclei, blue collagen, and light red or pink cytoplasm. Epidermal cells appear reddish. Scale bar = 200 µm. The edges of the wound have been shown with black/white arrows. Right panels are the zooms of the boxed areas within the images in the left panels. Scale bar, 50 µm. **B,** Bar graph shows quantitation of collagen abundance using MT stains. Data are mean±SEM (n=6), *, p<0.05 compared to *ΔsarA*. **C**, Granulation (d35 post-infection) tissue collagen content was determined using hydroxyproline assay. Data are mean±SEM (n=6), *, p<0.05 compared to *ΔsarA*. **D,** Two 8 x 16-mm full-thickness excisional wounds were made on the dorsal skin of C57BL6 mice. Each of the two wounds was topically infected with isogenic strains of S. *aureus* USA300, USA300::*rexB* (*ΔrexB)* or USA300::*sarA (ΔsarA).* The wounds were allowed to heal**.** Tensile strength of the healed wounds was measured on d20 post wounding**.** Data are mean±SEM (n=7), *, p<0.05 compared to *ΔsarA*.

Publications:

1. Sashwati Roy, Suman Santra, Sriteja Dixith, Amitava Das, Subhadip Ghatak, Piya Das Ghatak, Savita Khanna, Shomita Mathew-Steiner, Mithun Sinha, Britani Blackstone, Heather M. Powell, Valerie K. Bergdall, Daniel J. Wozniak and Chandan K. Sen. Staphylococcus aureus Biofilm Infection Compromises Wound Healing by Causing Deficiencies in Granulation Tissue Collagen. Annals of Surgery, *revision pending.*