Elucidating the Mechanism of Anti-IsdB Antibody-Mediated *S. aureus* Sepsis and Death following Surgical Site Infection in a MRSA Implant-Associated Osteomyelitis Model

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**INTRODUCTION:** *Staphylococcus aureus* (*S. aureus*) is the most common pathogen in orthopaedic surgical site infections (SSI), which leads to sepsis and death in 10% of patients.¹ Results from a recent phase 2 clinical trial demonstrated that vaccination against the iron-regulated surface determinant protein B (IsdB) is associated with increased multiorgan failure and septic death in SSI patients,² and suggests that immunity against IsdB is pathogenic. Consistently, we also found that anti-IsdB antibody titers were associated with sepsis and death in a pilot study of patients with *S. aureus* infected total joint replacements.³ Thus, we hypothesize that *S. aureus* induces non-neutralizing anti-IsdB antibodies to invade leukocytes without triggering a bacterial respiratory burst, and in so doing, creates "Trojan horse" blood cells that disseminate the pathogen in an immune prevailed environment.⁴ Since hemoglobin (Hb) is the primary target of IsdB,⁵ and bacterial HB-Haptoglobin (Hp) complexes in bleeding surgical wounds are normally cleared by CD163-mediated endocytosis by M2 phenotype macrophages without a respiratory burst,⁶ we further hypothesize that this is the anti-IsdB-induced immune escape pathway used by *S. aureus* septic infections. Thus, we tested these hypotheses in vitro, and in an established murine model of *S. aureus* implant-associated osteomyelitis.⁷

**METHODS:** Monoclonal antibodies (mAb): An IgG1 irrelevant mAb (placebo), and a novel non-neutralizing anti-IsdB mAb (1.5) were produced and purified from cultured hybridoma cell lines. In vivo experiments: All studies with mice were performed on IACUC approved protocols. 6-week-old female BALB/c mice were passively immunized with 1mg (40 mg/kg i.p.) of placebo or anti-IsdB1.5 mAb one day before septic implant challenge. Following immunization, the mice were challenged with a translational implant coated with 5x10⁵ CFU of bioluminescent methicillin-resistant *S. aureus* (MRSA) strain (USA300 LAC+:lux), and longitudinal bioluminescent imaging (BLI) was performed as previously described.⁸ Mice were sacrificed on day 14, and internal organs (liver, kidney & spleen) were analyzed for CFU and histopathology as previously described.⁹ Generation of Hb-Hp complex: 25 µg/ml of Hb and 35 µg/ml of Hp were mixed and incubated at 37⁰C for 1hr.⁴ Co-immunoprecipitation assay: Recombinant IsdB was incubated with Hp, Hp-Hb complex or a combination at 37⁰C for 2hrs, and they were immunoprecipitated with anti-IsdB mAb and protein A beads. Proteins bound to the beads were analyzed via immunoblotting with anti-Hp mAb. In vitro experiments: Opsonophagocytosis assays with mouse macrophage RAW264.7 cells were performed with modifications as previously described. Briefly, GFF² *S. aureus* (MSSA strain (UAMS-1) was opsonized with irrelevant (negative control) or anti-IsdB IsdB1.5 mAb in the absence or presence of recombinant IsdB for 2hrs. Then, the opsonized bacteria were added to RAW cells (MOI=50) separately or together in the absence or presence of Hb, Hp or Hp complex for 3hrs and analyzed by fluorescence microscopy. *S. aureus* survival assay was examined using the same condition. Briefly, RAW cells and GFP² UAMS1 were co-cultured with these conditions in serum free DEMEM with gentamycin for 2 days.

**RESULTS:** Remarkably, a small fraction of the anti-IsdB immunized mice demonstrated MRSA dissemination, as evidenced by BLI (Figure 1) and CFU assays. Moreover, sepsis was evident from the pale gross pathology of the kidneys, which also had histopathology features of tubular necrosis. As this did not occur in any of the placebo treated mice, and we have never observed this in any mice challenge in this *S. aureus* infection model (n=5,000 over 13 years of study), we consider this to be a significant effect of anti-IsdB1.5 mAb. To test our molecular hypothesis, we performed in vitro binding studies, which demonstrated that anti-IsdB1.5 mAb facilitates a multi-molecular complex that includes *S. aureus* protein A (SpA), IsdB and the Hb-Hp complex (Figure 2). Thus, anti-IsdB1.5 mAb has the potential to tether *S. aureus* to the Hb-Hp complex via membrane bound SpA binding to its Fc domain. Using real time fluorescence imaging of lysotracker-red label RAW cells incubated with GFP² methicillin-susceptible *S. aureus* (UAMS-1), we found that this multi-molecular complex subverts bacterial opsonophagocytosis, and leads to a significant intracellular colonization of the RAW cells (n=4, P ≤ 0.0001), (Figure 3). In survival assays using gentamycin to kill extracellular bacteria in the RAW cell cultures, we found that all of the individual components of the multi-molecular complex (SpA, anti-IsdB1.5 mAb, IsdB & Hb-Hp) are required for intracellular colonization on day 2 (n=4, P ≤ 0.05), (Figure 4).

**DISCUSSION:** Although numerous *S. aureus* vaccine trials based on production of antibodies have been performed; all of them have failed. Most notably, a large phase 2 clinical trial of an IsdB active vaccine has to be terminated early due to sepsis and multiorgan failure after the surgery.⁷ Thus, elucidating the mechanism by which *S. aureus* evades host immune system is critical towards the development of a novel strategy for eradication of *S. aureus* infections and septic death following SSI. Here we demonstrate a possible mechanism by which IsdB immunization leads to *S. aureus* dissemination and multiorgan failure by usurping anti-IsdB antibodies to enter and persist in M2 macrophages (Trojan horses) via a multi-molecular complex that includes SpA, anti-IsdB, IsdB and the Hb-Hp complex. The Trojan horse macrophages that are colonized at the surgical site of infection can then migrate to internal organs and die, releasing large numbers of live *S. aureus* that would induce multiorgan failure and death of the host. Ongoing studies are aimed at evaluating the role of CD163 mediated endocytosis of tethered *S. aureus* in this process of Trojan horse formation, using anti-CD163 blocking mAb and macrophages from conditional-inducible CD163 KO mice (Cd163tm1(KOMP)Vegl) in vitro and in vivo.

**SIGNIFICANCE:** Based on the recent clinical findings that immunity against IsdB is associated with increased multiorgan failure and septic death,²,² here we demonstrate a potential mechanism by which *S. aureus* usurps anti-IsdB antibodies to colonize macrophages and lead to sepsis in a murine model of implant-associated osteomyelitis. This mechanism may be responsible for the death of ~10% of orthopaedic patients that contract implant-associated osteomyelitis from *S. aureus*.


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