

Real Time Fluorescent Microscopy Assessment of PAD4-Mediated NETosis and NET Degradation by *S. aureus* Nuclease During Nidus Formation on Metal Implants

Youliang Ren^{1,2}, Karen L. de Bentley^{1,2,3}, Jason Weeks^{1,2}, Thomas Xue¹, Ye Shu¹, Allie Jia Hui Tay¹, Sashank Lekkala¹, Shu-Chi A. Yeh^{1,2}, Edward M. Schwarz^{1,2} and Chao Xie^{1,2*}

¹Center for Musculoskeletal Research, ²Department of Orthopaedics, ³Department of Pathology, University of Rochester School of Medicine and Dentistry, Rochester, NY USA. ⁴Department of Microbiology & Immunology, University of Rochester Medical Center, Rochester, NY, USA.

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INTRODUCTION: Bone infection remains a catastrophic outcome of orthopaedic surgery,¹ and *Staphylococcus aureus* is the most common pathogen in implant-associated osteomyelitis.² Currently, the direct host-pathogen interactions that govern nidus formation on the implant remain poorly defined. Additionally, there are no experimental systems that can directly quantify neutrophil and bacteria behavior on the implant in real time. Although the “Race for the Surface” hypothesis³ remains as dogma to explain the competition between host cells and bacteria for implant colonization immediately post-op, recent literature on soft-tissue infections demonstrated crucial roles for protein arginine deiminase 4 (PAD4)-dependent neutrophil extracellular traps (NET) and *S. aureus* Nuc degradation of NETs as dominant determinates of immunity and infection respectively.⁴ To test the hypothesis that PAD4 and Nuc are also operant during nidus formation on metal implants, we developed a novel *in vitro* real time fluorescent microscopy model with quantitative outcomes of bacteria growth and neutrophil swarming behavior on the implant surface. More specifically, we tested the hypotheses that: 1) neutrophils undergo both lytic and viable NET formation after phagocytosing bacteria on contaminated implants and PAD4 inhibition leads to exacerbated nidus formation and bacterial growth; and 2) Nuc deficient *S. aureus* fail to form nidi on implants and are rapidly cleared by phagocytic neutrophils.

METHODS: All animal studies were performed under protocols approved by the UCAR. Twelve-week-old CatchupTM-red mice that have red-fluorescent neutrophils were sacrificed to collect neutrophils from the tibia and femur via density gradient centrifugation-separation and added to cell culture wells containing etched sterile and *S. aureus* (EGFP+USA 300) contaminated titanium pins (Fig 1). Longitudinal scanning confocal microscopy with environment chamber (5%CO₂, 37°C) was performed for 6 hours, then SYTOX Blue was added to the co-culture to stain the nucleic acid of dead cell. Scanning electron microscopy (SEM) was performed to evaluate bacteria and biofilm formation and the morphological characteristics of neutrophils on pin surface. GSK484 (PAD4 inhibitor) was added to the culture dish to assess the role of NETosis, and *S. aureus* strain Nuc^Δ AH1680 EGFP was used to assess the role of NET degradation. The volume of bacteria and neutrophils was calculated by Imaris to evaluate the swarming ability of neutrophils in different groups. Data are presented as means ± standard error of mean, and statistical significance was determined using a two-way ANOVA, *p*<0.05 was considered significant.

RESULTS: SEM demonstrated that etching the implant surface (Fig1. A) guides robust *S. aureus* growth within the grooves (Fig1. B) and serves as a prospective region of interest (ROI) for real time fluorescent imaging (Fig1.C). SEM also confirmed NETosis following neutrophil addition to *S. aureus* contaminated pins (Fig. 1D-H). Real time fluorescent microscopy confirmed neutrophil swarming towards *S. aureus* on the pin, phagocytosis of bacteria and NETosis (Fig2A, A'). These results demonstrate that swarming neutrophils (Fig2. B) in the USA300 or Nuc^Δ AH1680 co-cultures show significantly higher travelling displacement and velocity than co-cultures treated with GSK 484 (Fig2. C-F). Longitudinal assessment of nidus formation demonstrated that large numbers of bacteria exist within the groove of the implant at 1hr, while neutrophils first swarm into the ROI at 3hrs and their numbers increase out to 6hrs producing a modest nidus with low bacteria counts and a predominance of host cells and biomaterial (Fig3 A). PAD4 inhibition results in dramatically increased *S. aureus* proliferation and reduction of swarming of neutrophils leading to a nidus that is primarily comprised of bacteria in static biofilm (Fig. 3B). In contrast, Nuc^Δ *S. aureus* that are present within implant groove at 1hr are efficiently cleared by large numbers of swarming phagocytic neutrophils by 6hrs, suggesting that degradation of NETs into biofilm eDNA is required for nidus formation (Fig3. C). SYTOX blue staining of these co-cultures confirmed the presence of NETs in and around the nidus, absence of NETs in GSK484 treated cultures, and robust NETs in and around the nidus formed by Nuc^Δ AH1680 EGFP (Fig3.A-C). Quantitative analyses confirmed the increase in bacteria volume and lack of neutrophil swarming in GSK484 treated cultures (Fig3 D, E).

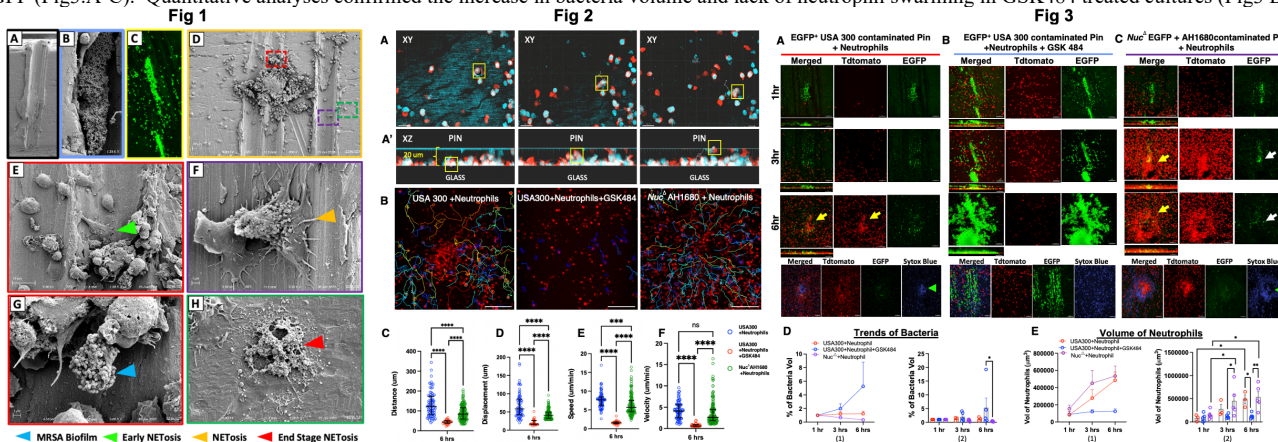


Figure 1. Validation of etch-guided nidus formation and NETosis in the *in vitro* model of the Race for the Surface. **Figure 2.** Real time fluorescent microscopy imaging of neutrophil migration and phagocytosis of *S. aureus* at the nidus with quantification of swarming behavior. **Figure 3.** PAD4 inhibition increases bacterial growth, eliminates NETs and decreases neutrophil swarming, during nidus formation that is Nuc dependent.

DISCUSSION: Elucidation of the pathogenic mechanisms that allow virulent bacteria to form incalculable biofilms and host defenses responsible for protection are critical for novel interventions for implant-associated osteomyelitis. Based on the success of longitudinal intravital imaging of the bone marrow (LIMB) in mice,⁵ this approach enables direct assessment of bacteria and host cell behaviors in real time. However, our initial LIMB studies identified that the prospective fixed ROI on a random region of the implant surface is a major limitation, and nidus formation is a stochastic process that may or may not occur in the ROI. Thus, our demonstration that surface etching guides nidus formation to the grooves allows for 100% success in the prospective ROI overcomes this obstacle. We also confirm the critical roles of NETs and their remodeling by *S. aureus* Nuc in the prevention and exacerbation of nidus formation on the implant respectively. Thus, future *in vivo* studies are warranted to see if these targets can be manipulated to inhibit implant-associated osteomyelitis.

SIGNIFICANCE: Here we describe the first *in vitro* real time imaging model of *S. aureus* nidus formation in the presence of phagocytic neutrophils on metal implants quantitative outcomes of bacteria and neutrophil behavior. As this model may recapitulate the initial colonizing events that lead to implant-associated osteomyelitis, this model can be used to characterize antimicrobial surfaces, and assess drug therapies. We also validate the critical roles of NETosis in limiting implant infections, and *S. aureus* nuclease in NET remodeling to generate eDNA for biofilm formation on the implant.

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