Anti-*S. aureus* mAbs Against Autolysin and Pore-Forming Toxins Enhance Vital NETosis and Phagocytosis by Cytoplasts Demonstrated with Real Time Fluorescent Microscopy and Imaging Cytometry

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Introduction: Patients with osteomyelitis have low protective antibody levels against *Staphylococcus aureus* and can benefit from passive immunization with anti-*S. aureus* antibodies. However, all *S. aureus* vaccine candidates have failed in clinical trials, highlighting the need for novel assays to evaluate these therapies mechanistically. Neutrophils defend against bacterial infections by generating neutrophil extracellular traps (NETs) via lytic NETosis, where neutrophils explode and release DNA, or vital NETosis, where neutrophils extrude DNA but survive as anuclear cytoplasts capable of phagocytosis. As *S. aureus* remodels NETs into biofilm during chronic osteomyelitis, cytoplasts are critical for combating soft-tissue infection. We hypothesized that monoclonal antibody (mAb) enhancement of vital NETosis reduces nidus formation on implants. To this end, we developed an in vitro longitudinal confocal imaging system to visualize neutrophil swarming and phagocytosis on an infected implant, and an imaging cytometry assay to quantify cytoplasts and their phagocytic capacity. We tested two mAbs with known in vivo efficacy: anti-glucosaminidase (Gmd) that enhances *S. aureus* clustering and opsonophagocytosis[1]; and anti-pore-forming toxins (PFT) that neutralize five toxins (α-hemolysin, Panton-Valentine leucocidin, LukED, HlgAB, HlgCB)[2].

Methods: For the longitudinal confocal imaging model, titanium wires were incubated with $EGFP^+$ USA300, a community-acquired methicillin-resistant *S. aureus* (MRSA). The implants were scratched to guide nidus (bacterial aggregation) formation, which served as a defined region of interest for longitudinal imaging. We co-cultured these implants with $tdTomato^+$ neutrophils isolated from the long bones of male and female Catchup mice[3]. The antibodies (50 µg/mL) were added at the beginning of the co-culture. We performed 3D time-lapse imaging at 1-, 3-, and 6-hours using a laser scanning confocal microscope equipped with an environmental chamber (n = 4/group). The volumes of neutrophils (tdTomato/red), *S. aureus* (EGFP/green), and their co-localization (yellow) were analyzed using Imaris.

For imaging cytometry, planktonic *S. aureus* was incubated with bone marrow cells from Catchup mice in a similar co-culture system at an MOI of 1. At 2 hours, an aliquot was diluted to measure CFUs, and the remaining cells were fixed with 4% PFA and stained with Hoechst to visualize DNA. The samples were analyzed on Cytek Amnis ImageStream imaging cytometer (n = 3/group). Cytoplasts were identified as $tdTomato^+$ and $Hoechst^-$ cells.

Results: Confocal imaging showed that neutrophil volume increased in the antibody-treated groups compared to the untreated control (**Fig. 1A, B**). Extracellular DNA labeled using SYTOX blue was also lower in the antibody-treated groups, indicating reduced lytic NETosis (**Fig. 1A, inset**). The co-localization of neutrophils and *S. aureus*, a surrogate for phagocytosis, was higher in the anti-PFT group. In addition, the diameter of the co-localized clusters was the highest in the anti-Gmd group, supporting the idea that anti-Gmd treatment results in *S. aureus* megacluster formation (**Fig. 1C**). Imaging cytometry analysis also showed trends toward higher neutrophils in the antibody-treated groups. However, neutrophil phagocytosis was reduced in the antibody-treated groups, potentially due to a mismatch between murine FcR and the humanized Fc on the antibodies. Interestingly, we observed an increase in cytoplasts in the combined anti-Gmd + anti-PFT group concomitant with reduced CFUs at 6 hours (**Fig. 2B**). In addition, the number of phagocytic cytoplasts and their phagocytic capacity, measured as the mean fluorescence intensity of GFP, were also increased in the anti-PFT and the combined treatment groups (**Fig. 2A, C, D**).

Conclusions/Discussion: Confirming the mechanism of action of mAbs in cocktail passive immunizations is critical for their success in clinical trials. Here, we developed two novel imaging outcomes that can quantify swarming, persistence, and opsonophagocytosis by neutrophils and cytoplasts in vitro. We also utilized these outcomes to demonstrate the predicted mechanisms of action of anti-Gmd and anti-PFT mAb and their synergistic activity, resulting in reduced bacterial nidus formation on titanium implants. Furthermore, these data support our hypothesis that promoting vital NETosis enhances bacterial clearance. Future studies will extend these experiments with human neutrophils.

Significance/Clinical Relevance: We developed confocal and imaging cytometry assays to rationally select candidate mAbs for a cocktail passive immunization against *S. aureus* based on their distinct mechanisms of action. Through this approach, we showed that combined anti-Gmd and anti-PFT therapy synergistically reduces CFUs on titanium wires by increasing vital NETosis and cytoplast phagocytic capacity.

References: [1] Varrone, J.J. et al. J. *Orthop Res. (2014)*. [2] Kailasan, S. et al. *Mabs. (2022)*. [3] Hasenberg, A. et al. *Nature Methods (2015).*

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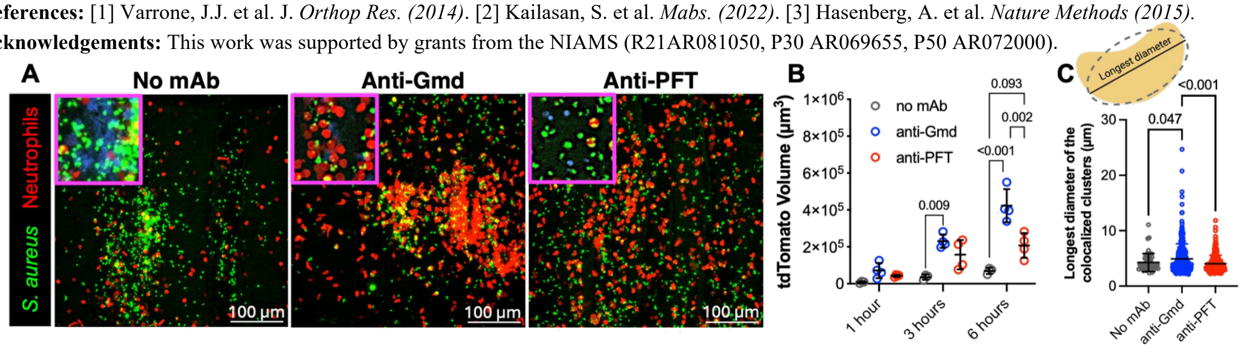

Figure 1. (A) Representative images of no mAb, anti-Gmd, and anti-PFT treated groups. Insets show lower extracellular DNA (blue) in the antibody-treated groups. (B) Neutrophil volume, and (C) longest diameter of the co-localized (yellow) clusters. *p-values from two-way ANOVA tests (n = 4).

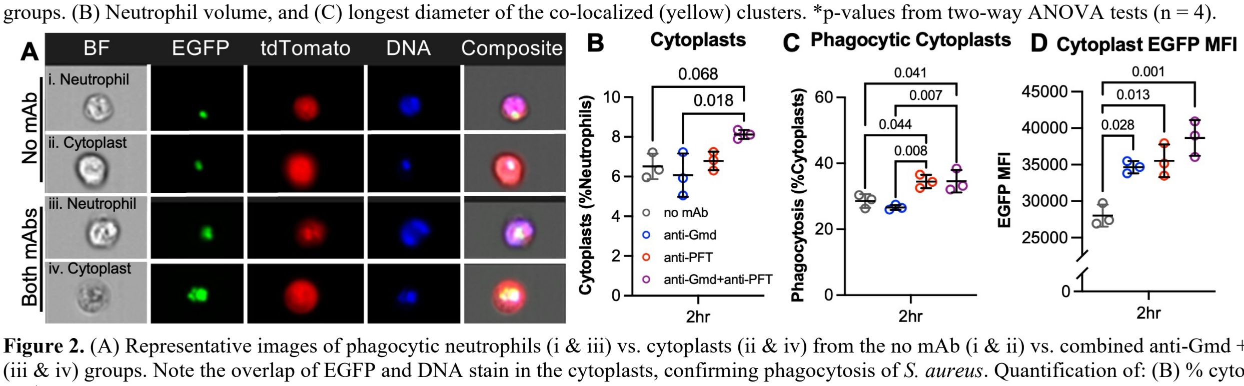

Figure 2. (A) Representative images of phagocytic neutrophils (i & iii) vs. cytoplasts (ii & iv) from the no mAb (i & ii) vs. combined anti-Gmd + anti-PFT (iii & iv) groups. Note the overlap of EGFP and DNA stain in the cytoplasts, confirming phagocytosis of *S. aureus*. Quantification of: (B) % cytoplasts of tdT^+ cells; (C) % phagocytic cytoplasts; and (D) mean fluorescence intensity (MFI) of phagocytosed clusters. *p-values from one-way ANOVA tests (n =3).