BEHAVIORAL CHANGES OF MACROPHAGES AND NEUTROPHILS DURING BIOFILM INDUCTION: NEUTROPHIL AGGREGATE FORMATION IN RESPONSE TO BACTERIA

By

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Abstract

Staphylococcus aureus is a well-known pathogen, notable for its frequent biofilm formation on indwelling medical devices and its ability to evade host innate immune cells. Using different S. aureus UAMS-1 bacterial strains to infect PGRP-dsRed/CX3CR1-EGFP double transgenic mice, we observe the potential changes in behavior of macrophages and neutrophils during the induction of biofilm. We have visually identified neutrophil aggregates in infected tissues by means of two-photon imaging. Additionally, cell tracking calculations for velocity, displacement, and meandering have been conducted, suggesting variance between the infected and the non-infected models. Neutrophils of the infected model have been characterized by faster speeds, as well as greater displacement and less meandering, in comparison to the neutrophils of the non-infected model. Furthermore, the infected model suggested directionality of the neutrophils, specifically gathering at the aggregate. These results propose that neutrophils act accordingly in the presence of *S. aureus* infection and introduce a connection between the aggregates and behavior.

Table of Contents

Introduction

Too often have biofilms been observed on indwelling medical devices, causing many deaths by nosocomial infections. In aqueous environments, biofilm is formed when bacteria adhere to surfaces and form an organized community that is able to stick to different kinds of materials through intracellular adhesion. *Staphylococcus aureus (S. aureus)* is a gram-positive coccal bacterium that is known to be one of the main sources of biofilm on medical devices. In addition, *S. aureus* biofilm infections are also known to be resistant to antibiotics and facilitate biofilm persistence, as compared to *S. aureus* planktonic infections. Planktonic infections, which refer to single or suspended cells, express different characteristics and induce macrophage and neutrophil responses (1).

Neutrophils are the most abundant type of white blood cells; they are the first innate immune cells to arrive at the injury site and exhibit multiple mechanisms to clear foreign infections. These methods include phagocytosis, ingesting and digesting the pathogen, degranulation, releasing antimicrobial factors, and neutrophil extracellular traps (NETs), excreting chromatin and serine proteases to form extracellular matrices (2, 3). Due to its high motility, the neutrophils quickly assemble at the infection site, attracted by cytokines, such as tumor necrosis factor alpha (TNF-α), released by monocytes and macrophages already present in the tissue (2, 4). Without neutrophils, individuals are highly susceptible to infections. Furthermore, the macrophages, in addition to its production of pro-inflammatory or anti-inflammatory cytokines to initiate immune responses, can also destroy bacteria by phagocytosis (1, 5). The main difference between the two cells are that macrophages are recruited much later in the immune response, are much larger, and live much longer, as opposed to neutrophils, which die after devouring a pathogen.

Studies suggest that the biofilm interferes with these responses by polarizing macrophages toward anti-inflammatory phenotype and inducing cell cytotoxicity, which can result in many different fates for the cell. In other words, biofilm inhibits the activity of the immune cells, preventing phagocytosis of the bacteria (6). This is due to the distinct gene expression exhibited by biofilms, in comparison to planktonic cells. Biofilms are composed of self-produced extracellular matrixes that protects the pathogen inside. The structure and the metabolism of the biofilm also changes, depending on different environmental factors, such as nutrient availability. Further studies have shown biofilm to express different mechanisms that are either directed at neutrophils or disguise the biofilm. Quorum sensing molecules promote bacterial toxins to encourage neutrophil granulation and rapid cell death while the expression of lipooligosaccharide glycoforms prevent biofilm recognition by neutrophils and evade the response entirely (3, 5).

And thus, the main goal of this project is to study the changes in behavior of macrophages and neutrophils during the induction of biofilm formation. In this report, we characterize the speed, displacement, meandering index, and an aggregate formed by the neutrophils in response to the bacteria. Double transgenic PGRP-dsRed/CX3CR1-EGFP mice were utilized, in which neutrophils specifically fluoresce red and the macrophages and bacteria fluoresce green. The reporter fluorescent proteins mark the cytoplasm of each cell type, allowing for distinct recognition to enable cell tracking in the mice. We were able to follow neutrophil movement within the mouse ear in the infected and non-infected model, suggesting a close relationship between the behavior of the neutrophils and the bacteria.

Methodology

Animals:

All mice used were bred under the University of California, Riverside vivarium under specific pathogen-free conditions; all mice were handled in accordance with the Institutional Animal Care and Use Committee and National Institutes of Health guidelines. PGRP-dsRed/CX3CR1-EGFP double transgenic mice were used.

Catheter Implant and Two-photon Imaging:

Staphylococcus aureus UAMS-1 is preloaded into a small silicon catheter before implant into the mouse ear. Remove mouse ear hair with Nair. An incision is then made on the dorsal side of the mouse ear, and the catheter is inserted **(Fig. 1)**. After three days, the mouse is imaged live with two-photon microscopy.

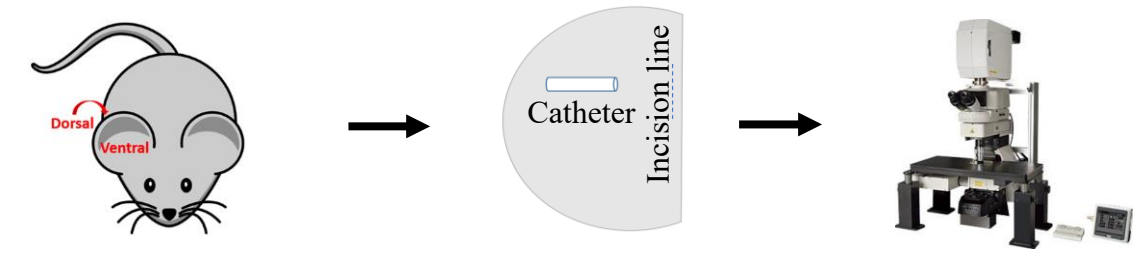

Figure 1. Catheter Implant

Cell Tracking:

All neutrophils were tracked using the software, Volocity. Different cell types were identified by its respective fluorescence, neutrophils with ds-Red and macrophages with EGFP. Due to excessive background noise, Volocity's automated tracking algorithms to

follow moving objects could not be utilized. Manual tracking was performed instead to create track objects by manually marking each individual object at each timepoint. The measurements of the neutrophil at each timepoint are then taken relative to the centroid of the region of interest (ROI) that marks the object and are collectively gathered to form one measurement item for accurate analysis.

Several parameters to outline behavior were defined: velocity, displacement, meandering index, and vector. Velocity represents the average speed of the neutrophil over the track. Displacement represents the average straight line distance from the position of the first timepoint to the last. Meandering index (MI) represents the measure of the neutrophil's deviation from a straight line, with values 0 to 1. A MI of 1 indicates a perfectly straight line; smaller values of MI represent more meandering in the track. Vector is the quantity represented by an arrow that indicates both magnitude and direction.

Statistical Analysis:

All results and data were graphed in the software, GraphPad Prism 6. Two-tailed tests were performed, resulting in p-values of less than 0.05 that were considered significant.

Results

Double transgenic reporter mice allow for visual identification of neutrophils *in vivo*.

PGRP-dsRed, expressing the red fluorescent protein dsRed, crossed with the CX3CR1-EGP, expressing the green fluorescent protein EGFP, enabled us to differentiate between the various cells among the mouse ear. As expected, neutrophils fluoresced red, but several other parts of the mouse ear such as the hair follicles also fluoresce red. The two were easily distinguishable due to the difference in size and shape. Hair follicles were big and unmoving while neutrophils were smaller and generally had some form of movement. Langerhans cells and macrophages also fluoresced green as expected. As with neutrophils and hair follicles, Langerhans cells and macrophages were also easily distinguishable by size. Langerhans cells displayed an elongated morphology while macrophages tend to have a circular shape. Visually identifying each of these cells played a role in determining the relationship of these cells to the presence of bacteria.

Staphylococcus aureus UAMS-1 strain is non-fluorescent and thus cannot be seen. However, its presence was identified by analyzing the behaviors the neutrophils exhibit in response to it. Using the two-photon microscope, the interaction between neutrophils and *Staphylococcus aureus* was clearly observed, indicating change in behavior when introduced to bacteria. Compared to the non-infected model, neutrophils of the infected model appeared to travel faster and farther in the direction of the forming aggregate.

Neutrophils in the non-infected model exhibit no sign of direction and have decreased average velocity and displacement.

Because neutrophils are typically the first to respond among the numerous innate immune cells, we focused on its role to attack the *S. aureus* infection site. First, videos of the non-infected model were collected at sites near the catheter implant and neutrophils were manually tracked. **Figure 2** shows each individual neutrophil and the specific path it took during the entire time frame (**Fig. 2A)** and where the videos were taken in respect to the catheter **(Fig. 2B).**

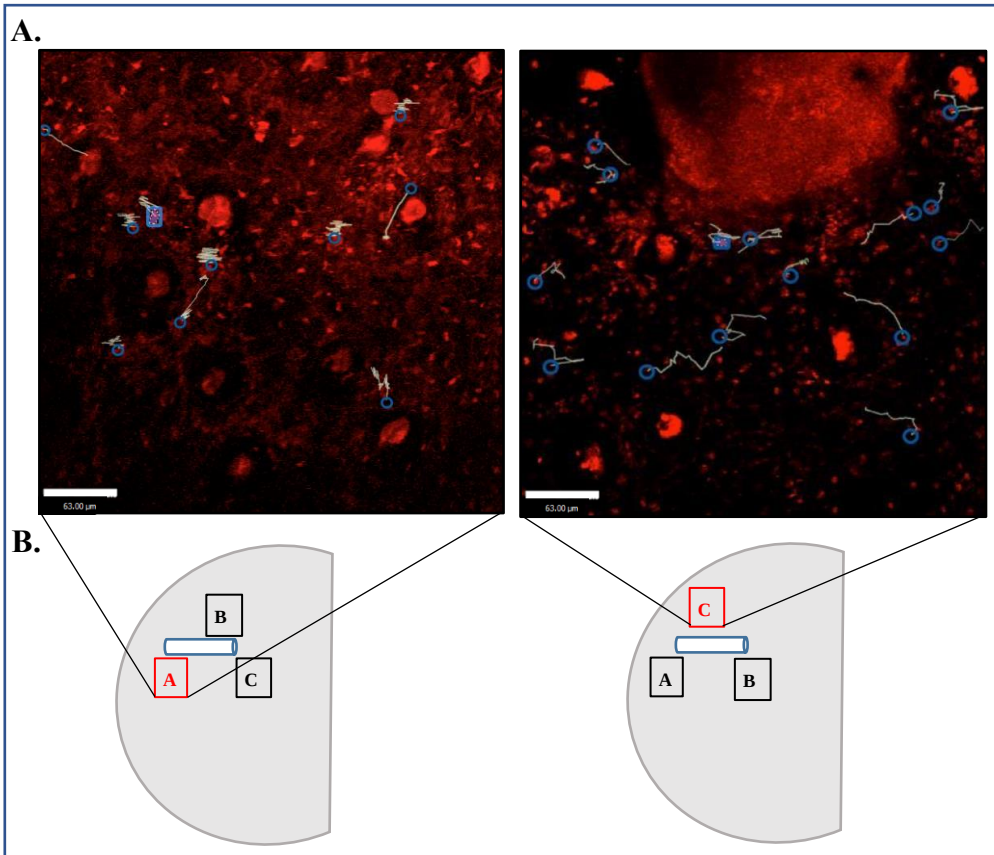

Figure 2. Neutrophils of non-infected model express random movement. (A) PGRP-dsRed, a transgenic reporter mouse strain, expresses red fluorescent protein in neutrophils to characterize movement. The neutrophil circled in blue represents the first timepoint collected; the highlighted line represents the path the cell takes to the last time point collected. (B) The area, in respect to the catheter, where the video was recorded is delineated by the red letter.

The neutrophils in the non-infected model were characterized by random movement and showed no action towards an explicit direction. Since the non-infected model carried no *S. aureus* in the catheter, this behavior was expected. Neutrophils, however, still did appear in response to the incision or the injury site. The average velocity in the non-infected models ranged from 0.032μm to 0.055μm, with a median of 0.050μm. The average displacement ranged from 15μm to 40μm, with a median of 25μm. The average meandering index ranged from 0.26 to 0.63, with a median of 0.42. These numbers suggest that when not in presence of bacteria, neutrophils appeared to have a relaxed behavior, aimlessly wandering to find any sort of infection to digest.

Neutrophil aggregates form in response to *S. aureus.*

In the infected model, distinct behavior was expressed by the neutrophils in the presence of *S. aureus.* The neutrophils seemed to be gathering at a forming aggregate, something we have not seen in previous models. Videos again were collected at the sites near the catheter implant and the neutrophils were manually tracked. **Figure 3** shows the path of each individual neutrophil as it travels toward the forming aggregate, delineated by a white arrow (**Fig. 3A)** in respect to the site of the catheter implant **(Fig. 3B).** By contrast, the neutrophils in this model were characterized by increased velocity and displacement and exhibit clear distinction of direction towards the aggregate. The average velocity in the infected model ranged from 0.054μm to 0.094μm, with a median of 0.070μm. The average displacement ranged from 39μm to 111μm, with a median of 58μm. The average meandering index ranged from 0.66 to 1, with a median of 0.72. These numbers suggest

that in the presence of bacteria, neutrophils appear to have an active behavior, directly traveling towards the infection site to clear out the invaders.

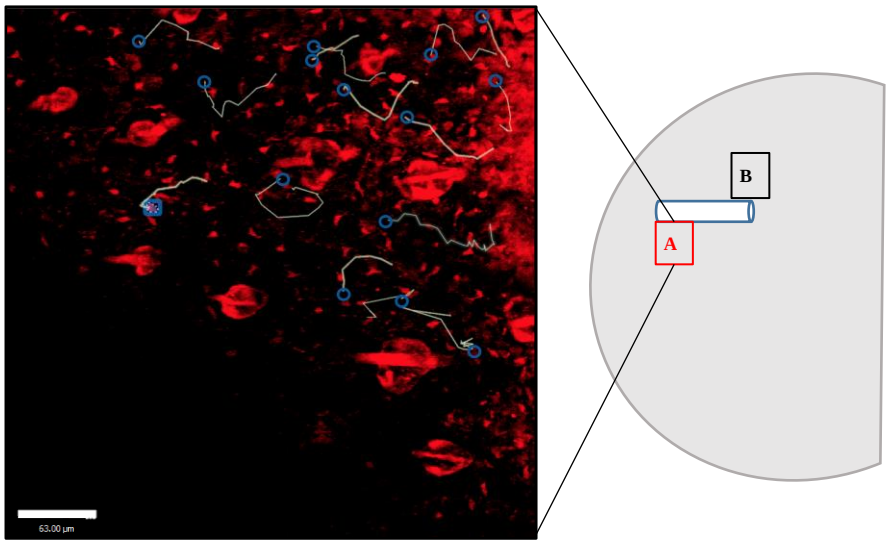

Figure 3. Neutrophils of infected model express direction to form aggregate. (A) PGRP-dsRed (red) transgenic mice were used to visualize neutrophils. *S. aureus* UAMS-1 strain is non-fluorescent and thus, cannot be seen. The neutrophil circled in blue represents the first timepoint collected; the highlighted line represents the path the cell takes to the last time point collected. (B) The area, in respect to the catheter, where the video was recorded is delineated by the red letter.

Neutrophil behavior is significantly different in the infected model vs. the non-infected model.

Figure 4 shows the quantification of the data when compared with each other. The velocity in the infected model is greater than in the non-infected model (**Fig. 4A).** The displacement is much higher in the infected model than in the non-infected model (**Fig. 4B).** The infected model exhibits an increase in meandering index when compared to the non-infected model (**Fig. 4C).** The differences between the infected model and the non-infected model were considered significant by a two-tailed test, with a p-value < 0.05.

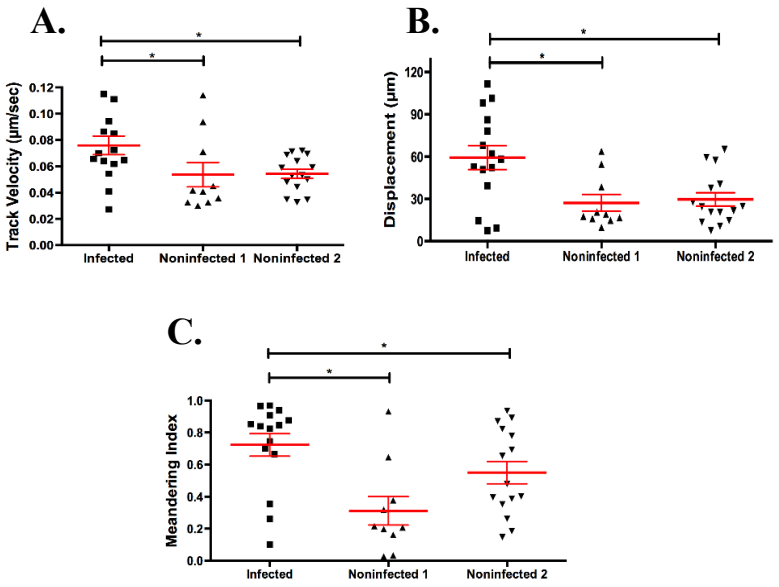

Figure 4. Quantification of infected models vs. non-infected models shows difference in behavior. (A) Mean neutrophil velocity (depicted as mean with SEM) is greater in the infected model. (B) Mean neutrophil displacement (depicted as mean with SEM) is greater in the infected model. (C) Mean neutrophil meandering index (depicted mean with SEM) is greater in the infected model.
*, statistically significant difference between bracketed groups, $p<0.05$

Conclusion

Both biofilm and neutrophils have been a topic of research for many years, and numerous studies have analyzed the mechanisms between them, such as phagocytosis and NETosis (7). In our current case, we found that tracking the neutrophils allowed us to further define the behaviors of the neutrophils in response to bacteria. Our discoveries suggest a close relationship between the formation of an aggregate, the neutrophils, and *Staphylococcus aureus.*

This study is the first to quantify the behavior of the neutrophils, revealing more insights on its role in biofilm persistence. Behavior was defined as speed, displacement, meandering index, and vector. Three key components were established to distinguish between the infected model and the non-infected model: An aggregate formed in response to the growth of bacteria in the infected model. The neutrophils in the infected model showed an increase in velocity and displacement when compared to the non-infected model. Higher meandering index was exhibited by the infected model, inferring directionality towards the forming aggregate.

Our findings suggest that neutrophils could possibly be forming these aggregates in response to the *S. aureus* in order to better fight off the infection as a group. It raises questions about the role of macrophages after the bacteria is cleared out by the neutrophils. Macrophages are rarely seen in day three of the experiment, but appear in day four. Additionally, no sign of aggregates can be seen at day four, further suggesting distinct roles between the two different cell types. Possible transition from neutrophil recruitment to macrophage recruitment analysis, *in vivo* or *in vitro*, may be the key to establishing defined roles of the cells in biofilm induction.

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