Proposed *in vitro* model of neutrophil swarming in a chronic, low-level inflammatory state

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ACADEMIC ABSTRACT

Chronic, low-grade inflammation is an underlying condition across a globally increasing number of debilitating diseases. These diseases include obesity, atherosclerosis, and diabetes and their resultant low-grade inflammation can be effectivity modeled with low dose stimulants such as lipopolysaccharide (LPS). While the innate immunity plays a significant role in fighting infectious disease, an initial exposure to low dose LPS hinders secondary infection clearance and pre-disposes murine models for fatal sepsis. Neutrophils are the most prevalent circulating innate immune cell and their homotypic aggregation, or swarming, is a key mechanism in clearing pathogens greater than 20 μm in size. We hypothesize that neutrophil swarming ability is altered when in a low dose LPS primed state; potentially leading to an overall altered innate immune response in the face of infection. However, an *in vitro* model does not currently exist to reliably quantify and compare neutrophil swarms across treatment groups. Here we propose a novel model utilizing fungal zymosan coated beads as a uniform target to which neutrophils may swarm.

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GENERAL AUDIENCE ABSTRACT

White blood cells are critical for our body's ability to fight off infection. The pathogens that cause infections come in many forms including fungus, viruses, and bacteria. However, in many debilitating inflammatory diseases such as heart disease and obesity, chronic inflammation prevents one's white blood cells from being able to properly fight off infection. In order to study white blood cell function without the variability that is analogous to living pathogens, we propose a model system that simulates an artificial pathogen target where both the target and the surrounding environment can be precisely controlled. This system can then be used to study white blood cell function, specifically how it may be impacted under inflammatory conditions.

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1. INTRODUCTION

A. Chronic, Low Level inflammation

Low dose endotoxemia and low grade inflammation are becoming in an increasingly prevalent health issue in the modern world in the wake of many adverse health conditions to include obesity, atherosclerosis, aging, and diabetes[1-3]. Alarmingly, these widespread and increasingly prevalent "modern" diseases of the western world are leading to additional health concerns beyond the diseases themselves. One of these additional health concerns is hindered pathogen clearance following infection[1]. Despite the large amounts of research for these conditions individually, there is relatively little known about the secondary effects of the resultant low grade inflammation on infection clearance. Therefore it is of increasing importance to understand the mechanisms by which these health conditions influence the innate immune system.

The hindrance of chronic disease states on infection clearance is clinically well known. A prime example of this phenomenon is 'diabetic foot' where the development of infected foot ulcers is the most common complication of diabetes with up to 34% of diabetics likely to be affected in their lifetime[4]. Diabetic patients' innate immune systems are first primed into the low grade inflammatory state and have subsequently reduced infection fighting ability which is one of the main factors that leads to the ulcer development and inability to clear the infection (Figure 1).

Another notable example is the increased susceptibility to infection due to aging. Elderly populations have a wide range of degenerative inflammatory states such as pulmonary hypoventilation, immobility, urinary retention, etc. These chronic conditions are now known to predispose the elderly to infection by commensal microbes with a sharp increase in both incidence and mortality over the average aged population[5]. Overall, there is a clear need for further research in link between chronic inflammation and its effect on infection clearance.

Low grade inflammation can be caused by many things such as stress, compromised mucosal barriers, or an altered microbiome. However, we will focus on endotoxemia because of its concurrent prevalence in a wide range of inflammatory disease states[1, 6]. Clinically, this presents as circulating low levels of bacterial endotoxin (lipopolysaccharide (LPS)) ranging between approximately 1-100 pg/mL in the blood where elderly patients have slightly elevated

levels of circulating LPS [7, 8]. This circulating, low level endotoxin has not been linked to any classical symptoms of endotoxemia (therefore considered subclinical) but is able to skew the immune system into a primed state where a very slight increase in pro-inflammatory cytokines is adequate for innate immune cell stimulation but not full maturation and later production of the anti-inflammatory signals required for a return to homeostasis (Figure 1)[1, 8]. Upon a secondary subsequent infection, the immune system no longer has the correct pro and anti-inflammatory cytokine balance to trigger the proper response. In contrast, an unprimed (healthy) immune system experiences a sharp increase in pro-inflammatory factors in the face of a robust infection which then triggers anti-inflammatory factors for complete resolution and return to a "normal" resting state. In this case, there are no residually elevated levels of either pro or anti-inflammatory cytokines post infection and the immune system is returned to a homeostatic state once again ready for proper infection clearance[1, 9-12].

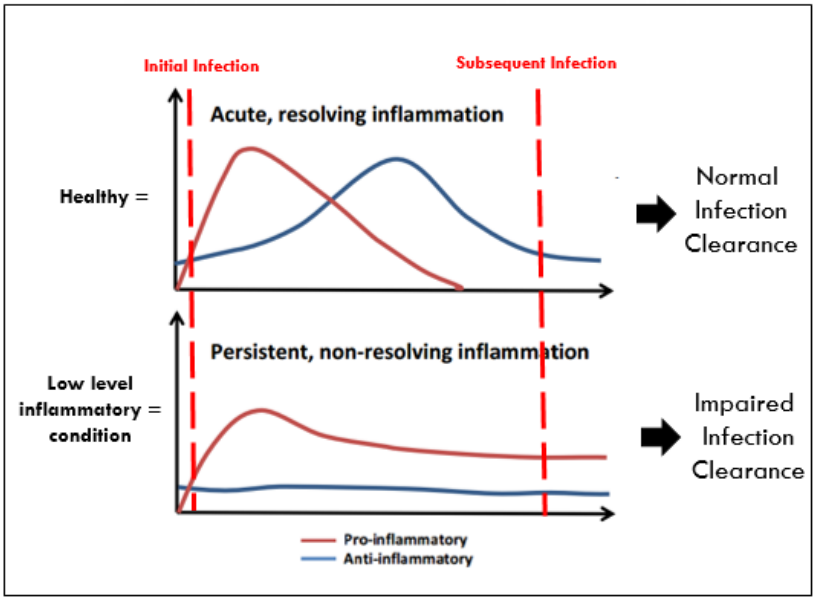

Figure 1. Development of non-resolving inflammation. Morris et al., 2014. *Front Immunol*. Adapted from original publication[1]. Used with permission of Liwu Li.

While the effects of low dose endotoxemia are well studied in monocytes and macrophages, the reduced infection clearance effect of low grade inflammation remains relatively unstudied in neutrophils[10, 11]. As a key player of the innate immune system, neutrophils are the most prevalent circulating innate immune cell and are the first responders to a site of infection[13]. Understanding their proper functionality, or lack thereof, is a key factor in understanding the human body's response to microbial infection when in a chronic, low level

inflammatory state. We hypothesize that if the innate immune system is primed into a low grade inflammation, the neutrophil response will be modulated away from normal antimicrobial functions such as pathogen clearance.

B. Neutrophil Antimicrobial Mechanisms in an Infection

Neutrophils are the most prevalent circulating innate immune cell and therefore play a fundamental role in overall infection clearance as both defenders against invading pathogens and inflammatory mediators[14]. While neutrophils play many roles within the body, they are best known for their function as the "first responders" to sites of microbial infection. A neutrophil's major anti-microbial mechanisms include (1) phagocytosis (2) homotypic aggregation (swarming) (3) degranulation and (4) neutrophil extracellular trap formation (NETosis) (Figure 2)[13-15]. As a commonly called professional phagocyte, they are most often noted as a crucial player in phagocytosing pathogens[13]. However, each of these defense mechanisms are equally important and why a neutrophil "decides" to preform one over the other is almost completely unstudied. Is phagocytosis vs swarming dependent on a size dependent decision-making system? Or do these trends follow activation of one Toll-like Receptor (TLR) versus another? By more fully understanding and elucidating the defensive strategies of neutrophils, we may be able to better understand overall innate immune system function and how it is modulated by low grade inflammation.

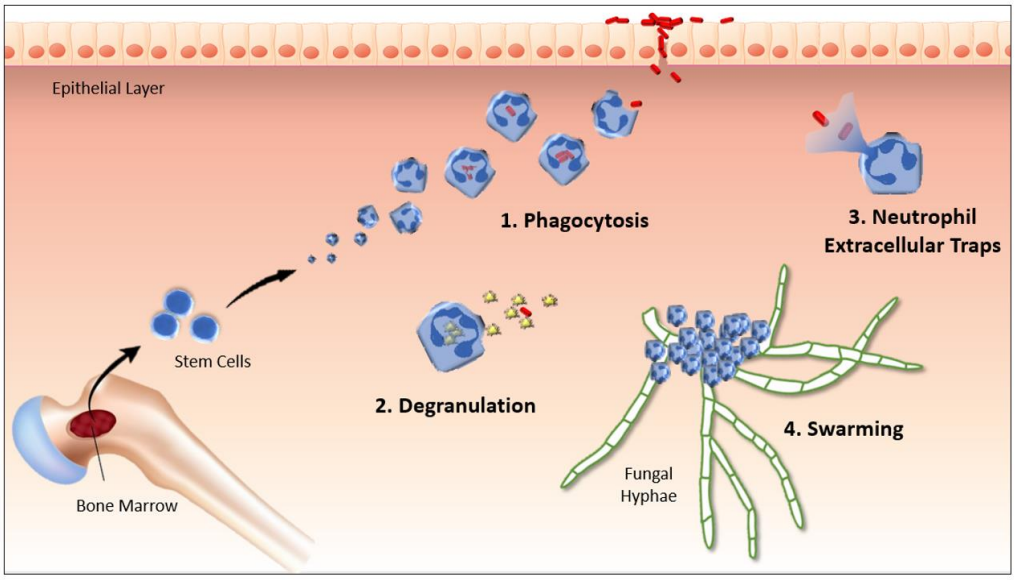

Figure 2. Neutrophil antimicrobial mechanisms of Action. Neutrophils originate as hematopoietic stem cells in bone marrow. After maturating, the main antimicrobial mechanisms include (1) Phagocytosis (2) Degranulation (3) Neutrophil Extracellular Traps (NETosis) and (4) Swarming.

Currently, neutrophils are considered relatively simplistic without the clearly defined subtypes and variations many other immune cells are known for. However, preliminary research and imaging shows that there are some differences in which of these defensive mechanisms a neutrophil will perform (*unpublished previous Li lab research*). This potentially shows that within a population of cells, some neutrophils may be predisposed to fulfill one function or utilize one of these anti-microbial mechanisms over another. Furthermore, the alteration of the ratio between each of these potential groups due to inflammation could lead to modulated infection clearance since each mechanism is likely more ideal for one type of pathogen versus another.

Immune cells such as neutrophils, macrophages, and dendritic cells use a class of receptor proteins called Toll-Like Receptors (TLRs) to recognize fundamentally constant microbial molecules, also known as Pathogen Associated Molecular Patterns (PAMPs)[11]. PAMPs are small biological molecules that are highly conserved within classes of microbes and can be recognized by the outer membrane bound TLR's on immune cells. Upon recognition of a PAMP, cell surface TLRs trigger an intracellular signal transduction pathway via the activation of downstream proteins and can lead to the production of reactive oxygen species, cytokine production, cell lifespan regulation, or other inflammatory processes[16]. The TLR signaling pathway eventually leads to the differential regulation of inflammatory reactions via transcription events. Therefore, regulatory proteins along this signaling cascade that have been identified as possible sources of altered cellular processes in inflammation are important foci of study. Thus the examination of both positive and negative regulatory proteins along this route may provide important clues into how processes such as neutrophil overall pathogen clearance ability may be modulated under chronic, low-level inflammation – either through the effectivity of the anti-microbial mechanism or appropriate decision making between mechanisms.

The TLR family includes thirteen different receptors all bearing the pro-inflammatory functions while each binding a different ligand, i.e. a specific PAMP[17]. Each of the individual TLRs bind specific families of PAMP ligands which are widely shared and highly conserved molecular patterns specific to microbes but unique from host molecules. Two of the most prominent neutrophil TLRs are TLR2 and TLR4. These two receptors respectively recognize Gram-positive and Gram-negative bacteria and are highly studied and well characterized motifs[16, 18]. TLR2 recognizes peptidoglycan, the major component of Gram-positive cell walls[19].

Fungal pathogens are typically associated with the zymosan PAMP, a repeating beta-glucan chain, and are also recognized by TLR2[19]. One of the most prolific bacterial associated molecular markers is the endotoxin lipopolysaccharide (LPS). LPS is comprised of a base sugar and lipoprotein structure that, while it may vary slightly between bacterial species, it can still be recognized by TLR4 to signal a Gram-negative bacterium's presence[16, 19-21]. Therefore by utilizing a classic PAMP, we can simulate each pathogen type based on its primary molecular coating.

Generally, neutrophil phagocytosis is optimal for particles approximately 0.5-8 μm in size – the size of the average bacterium since the particle in question would need to be small enough to be successfully endocytosed[22]. On the other hand, neutrophil swarming (homotypic aggregation) is most commonly seen in fungal infections where large hyphal branches can grow up to hundreds of microns in length[23]. Between phagocytosis and swarming, we hypothesize there is a size dependent decision-making mechanism by which the overall neutrophil population can "pick" the most energy efficient route of action. For example, if a single cell could successfully overtake and clear a pathogen (via phagocytosis) it would not be efficient for that same cell to rapidly recruit other neutrophils to the pathogen target. However, if that same cell recognized a fungal hyphae, it would presumably be "forced" to expend the additional energy for recruitment due to the failed phagocytosis. There is a potential case to be made that because bacterial pathogens typically fall within the phagocytic size range and are recognized by TLR4, that the trigger for this is purely TLR-based. Swarming is almost exclusively seen with fungal pathogens which are recognized via TLR2. While the question of how other neutrophil defense mechanisms such as NETosis and degranulation factor into this decision making matrix is valid, phagocytosis and swarming were initially focused on for their prominence in a neutrophil's anti-microbial arsenal[13, 15].

2. NEUTROPHIL SWARMING AND MODEL DEVELOPMENT

A. Neutrophil Swarming and Existing Models

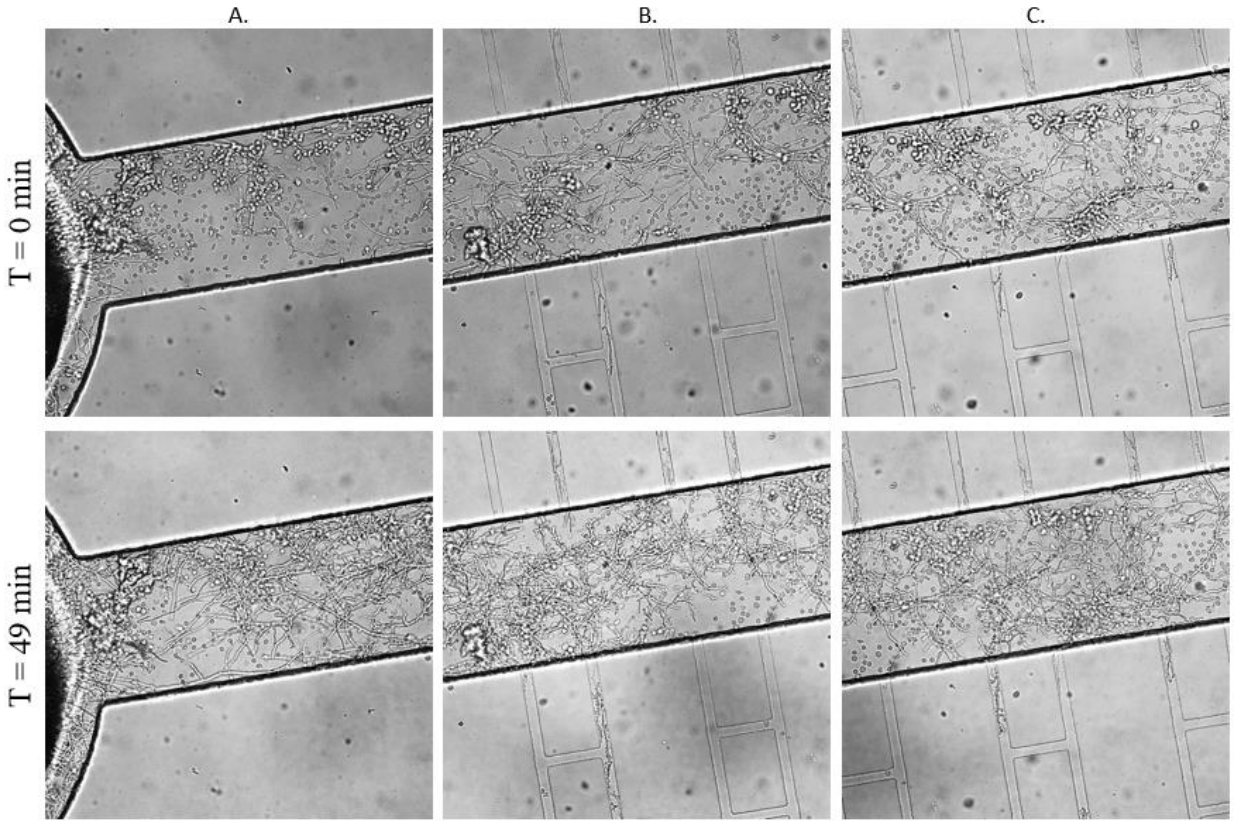

Figure 3. Neutrophil swarming to fungal hyphae. Wild Type C57BL/6 neutrophils in RPMI/10% FBS/HEPES/GM-CSF were co-incubated with *Aspergillis* for 8h at 32ºC. *Aspergillis* was allowed to hyphae from its spore form overnight. Microfluidic device primed with fibronectin to allow for cell migration. (A-C) Brightfield images of neutrophils and hyphae at three separate fields within a single microfluidic device, each field was imaged every 10 minutes for 8 hours. Images at t=0min and 49min shown (40x).

Neutrophil homotypic aggregation is the rapid and coordinated recruitment and clustering of cells around a target pathogen. Akin to the swarming of insects where the collective migration of many individuals creates a much larger cluster, neutrophil aggregation has adopted the swarming name. Swarming has recently emerged as an additional important mechanism of neutrophils to combat early stage microbial infection[15, 24]. In this mechanism, neutrophils activate swarming en masse via a positive feed forward production of Leukotriene B_4 (LTB_4) which acts much like a quorum sensing molecule – where the increasing upregulation of the LTB_4 chemical signal rapidly recruits additional cells to the target[15, 24]. Once recruited to the site

of infection, the massed neutrophils control the growth of the pathogen by releasing cytotoxic species and eventually dissipate once the inflammatory target has been resolved[15].

As demonstrated in Figure 3, the growth of the root-like *Aspergillis* fungal network spurs swarming migration in this closed environment *in vitro* microfluidic chamber. The neutrophils can be seen attacking the hyphae in coordinated aggregations and not simply moving to the closest target.

Previously, a majority of the work studying this mechanism has been *in vivo*; specifically, in mouse ears where fluorescent cells can be imaged in real time through the thin tissue. These studies primarily focus on the dynamics of neutrophil swarming migration where varying treatment groups were not used or necessarily appropriate[25]. This type of model and others also utilize large fungal hyphae as the swarm target since it has been well characterized that neutrophils will typically only swarm to large targets (>20 μm, i.e. fungal hyphae) and phagocytose smaller targets (>5 μm, i.e. bacteria) in order to maintain energy efficiency[26]. Fungal hyphae typically start as spores approximately 10 μm in size and can potentially grow upwards of 100 μm in a long, branched root-like fashion[23]. While the use of live fungal hyphae indeed induces the swarming phenotype desired for the study of singular swarms, it does not allow for the reliable comparison of swarming ability/swarms across treatment groups of cells. Additionally, the *in vivo* nature of these studies (while they do provide a realistic environment in which the neutrophils may migrate) does not allow for the finely tuned control of experimental parameters or quantification of secreted cytokines.

B. Model Development

Several other *in vitro*, non-hyphae based assays have been employed to analyze neutrophil swarming. However, many of these studies center around neutrophil roles in tissue reconstruction with an intention to model neutrophil migration dynamics – not necessarily comparing overall swarming ability across treatment groups[24]. Another study, touted as the first *in vitro* model of neutrophil swarming, relies on the manual counting of attached neutrophils and does not provide identical targets between treatment groups[27]. This collection of existing neutrophil swarming assays, both *in vivo* and *in vitro*, highlights the need for a new method of neutrophil swarming characterization.

While a classical pathogen associated molecular pattern (PAMP) is essential to induce neutrophil recognition in infection settings, the particle size dynamics of the PAMP target and how it relates to neutrophil decision making between phagocytosis and swarming are unclear at best. Based on this previous literature, it is most ideal to use target that is (1) coated in a fungal PAMP and (2) of a reasonably adequate size to invariably induce cellular homotypic aggregation. Thus it may be reasonably assumed that a zymosan target greater than 20 μm is "pre-optimized" for recognition by neutrophils and subsequent swarming induction.

Therefore, we propose a novel *in vitro* model of neutrophil swarming that utilizes fungal PAMP coated spherical beads as uniform targets for the reliable quantification and comparison of swarms across cellular treatment groups or genetic knockout lines (Figure 4). By using a spherical target of uniform shape and size for all treatment groups, swarm sizes can be quantified for the comparison of overall swarming ability. These PAMP coated beads effectively become uniformly shaped artificial pathogen targets.

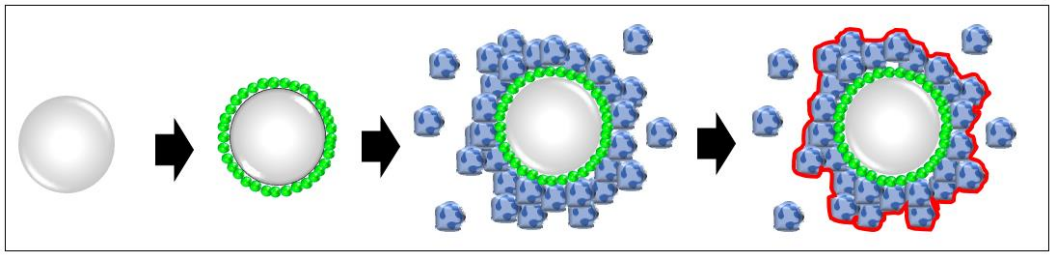

Figure 4. **Schematic of neutrophil-bead swarm model process.** A pre-silane coated glass bead is coated in fluorescent particles to simulate the size and PAMP nature of a live pathogen, the bead target is co-incubated with neutrophils, neutrophils swarm to the bead, and the swarm size can be quantified.

The use of a fungal PAMP is most likely the best microbial molecular pattern for neutrophil recognition in a swarming context since in *in vivo* infections, a fungal pathogen is the most likely common immunological threat above the size threshold previously mentioned. Also, the spherical bead shape is a distinct improvement from a live fungal hyphae target where neutrophils cluster along its long arms potentially without a singular point of focus and whose growth is difficult to regulate (Figure 3). Furthermore, fungal hyphae must be grown to specification at the exact time of primary neutrophil isolation which are classically difficult to work with due to their relatively short life spans following purification. The likelihood of growing identical hyphae in separate chambers / dishes to then match to "ready" neutrophils is not only extremely unlikely, but frustrating at best. Therefore by using PAMP coated beads

instead of live microbes, it reduces variation between experiments and simplifies the overall experimental process.

Advantages of this artificial pathogen model via a PAMP coated bead include the versatility of coating options, reliability of quantification, ease of imaging versus *in vivo* models, precise control of the experimental environment, and autonomy from live microbes.

C. Bead Coating

Silane coating, or silanization, was chosen to attach the organic PAMP coating molecule to the inorganic soda lime glass surface of the bead. This is a commonly used, low cost industrial practice that utilizes the silane functional group to make a charged end available to which covalent bonds may form (Figure 5A) [28, 29]. The practice of silanization specifically refers to coating a mineral surface such as glass or metal oxide with silane organofunctional alkoxysilane molecules whose hydroxyl groups can displace the alkoxy groups on the mineral surface. This results in the creation of a covalent bond between the silane molecule and the surface while its organofunctional end remains available (Figure 5B) [28, 29]. This process was used to attach the PAMP organic particles for bead coating to the bead's glass surface (Figures 4, 5, and 8).

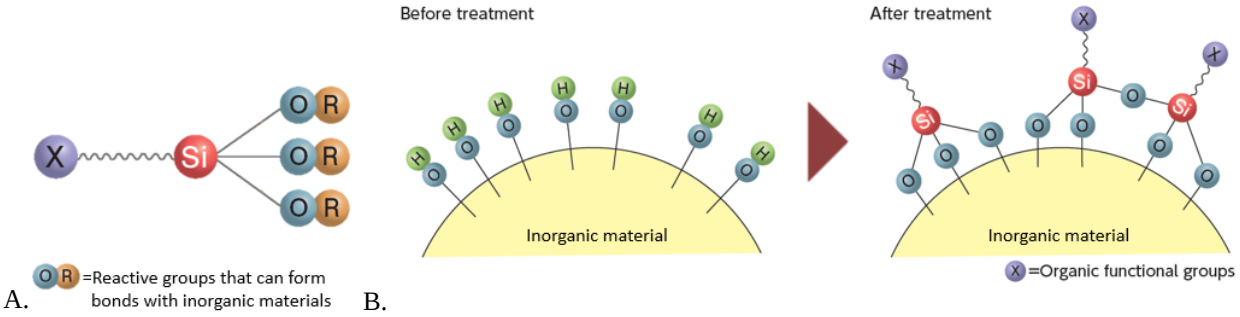

Figure 5. Silanization. (A) Silane molecule. (B) Silanization is a mineral surface treatment that allows for the attachment of available organofunctional groups via a silane intermediary. Shown is an unreactive inorganic surface to which organic functional groups are attached via silanization. Permission granted for figure adaptation from the Shin-Etsu Silicone guide to Silane Coupling Agents[28].

In order to validate the hypothesis that neutrophils require both a fungal PAMP as well as a large target size to initiate swarming, the target beads were also coated in a bacterial PAMP, a self-antigen, and were left uncoated. Since the established method of inducing low grade inflammation utilizes super low doses of lipopolysaccharide (LPS), a Gram positive bacterial PAMP was specifically chosen since the cell wall is mainly comprised of peptidoglycan and teichoic acid versus LPS as I n Gram negative bacteria. This selection helps to avoid interference

between the coating material and LPS priming[18]. Thus, fluorescently labelled *Staphylococcus aureus* (a Gram positive bacteria) bioparticles were chosen since they would accurately represent an actual bacterial pathogen while being able to be coated onto the bead[30].

To provide confirm that neutrophil swarming was pathogen dependent, two control measures were included. First, bovine serum albumin (BSA) was chosen as a quintessential self-antigen molecular marker. BSA is a stable, non-reactive protein that binds to nonspecific sites on immune cells without triggering inflammatory or other antimicrobial mechanisms[31]. Next, beads were also left completely uncoated to ensure that the silanated glass surface would not trigger an anti-microbial response. By using a wide variation of coating materials representative of each class of pathogen a neutrophil may encounter as well as differently sized beads, a broad range of questions may be answered such as whether neutrophil swarming is TLR dependent, size dependent, or both.

3. METHODS

Cell Prep: Bone marrow derived neutrophils were obtained from mice euthanized by isofluorine inhalation and cervical dislocation. Whole bone marrow was obtained from tibia and femur lavage using RPMI on ice, isolated using the BioRad EasySep magnetic kit, and resuspended in RPMI/c + 1 ng/mL GM-CSF, 1:100 Normal Mouse Serum (NMS), and 10 nM Leukotriene B4 (LBT4). Due to relatively short life span, overnight treated cells were recounted for accurate cellular concentrations immediately preceding bead inoculation. Neutrophils were then transferred to a 6-well glass bottom dish (Cellvis) that had been pre-coated in 6 μg/ml fibronectin in PBS overnight and inoculated with coated 30 μm polystyrene beads at a MOI of 1 bead: 200 cells (beads: cells). Culture was gently pipet mixed and swirled to ensure homogenous distribution and incubated for 60 min, 37ºC 5% CO_2. Neutrophils were fixed and stained with 1:500 dilution of DAPI.

Bead Coating: Pre-silane coated soda lime 35 μm glass beads (Cospheric) were coated with FITC zymosan bioparticles, pHrodo red *S. aureus* bioparticles, and fluorescently conjugated bovine serum albumin (BSA) (ThermoFisher) as appropriate. The coating material was first suspended in a 0.005% TWEEN in PBS to create a coating solution. Pulsing sonication was used to homogenize zymosan particles and the BSA in solution to ensure best coating results (Supp. Figure 1). Once homogenized, approximately 50 mg of beads (dry weight) were added to 1mL of coating solution and incubated for 1 hour, 27ºC rotating. Coating incubation practices must allow for constant movement of the beads with the coating material in solution to allow for uniform coating (Supp. Figure 2). Bead preparations were stored at 4ºC and re-imaged prior to each use to verify coating integrity.

Image Analysis: Images were taken through an Olympus fluorescent microscope using the Olympus DP Controller Software. Original magnification of images was 10x; FITC and DAPI filtered images were compiled and analyzed using ImageJ. Collected data includes total number of swarms per field, swarm size (area), and total fluorescence of each swarm (associated cells / swarm). To measure the area (μm^2) of each swarm, an outline was manually traced around the periphery of each swarm to include all immediately attached cells. Total fluorescence of each swarm was quantified in ImageJ by creating a binary tone image and using the ImageJ measuring tool to quantify the area of the binary space reflective of the swarm size. Automatic measurements are in pixels, therefore the pixel: μm ratio was predetermined and used to set the

ImageJ measurement unit pixel distance (microscope and imaging program specific). Swarms were identified as three or more sequential neutrophils touching a bead, i.e. if there were three individual neutrophils on three different sides of a bead it would not count as a swarm. All fields were imaged under DAPI, FITC / TRITC (depending on the bead coating material fluorescence), and brightfield. Brightfield imaging was to ensure that aggregated zymosan viewed under FITC were co-localized with (coated onto) a bead and not random clumping (Figure 6).

Exact glass bead size may vary when received from the manufacturer. To control for bead diameter variation, the diameter of the bead for each swarm was also measured by manually measuring the diameter in ImageJ in triplicate and finding the average.

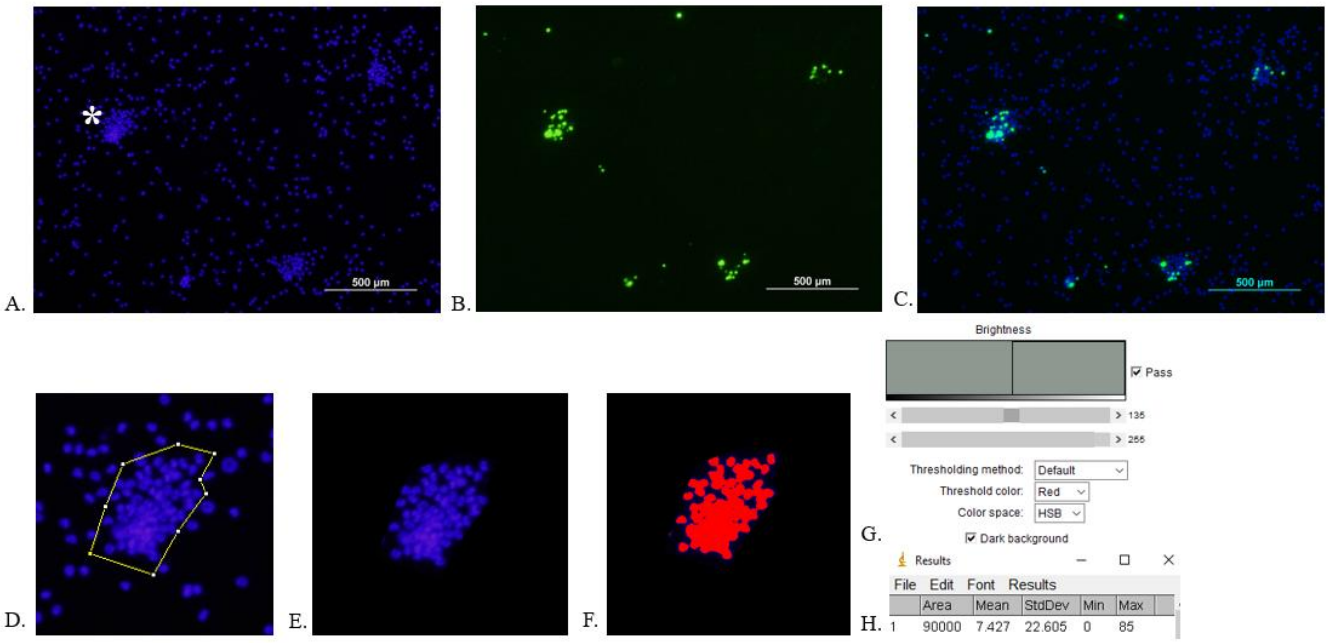

Figure 6. ImageJ quantification method. (A-C) Neutrophil swarm to zymosan coated beads imaged under (A) DAPI (B) FITC and then (C) overlayed (DAPI + FITC). (D) Manually drew an area around the selected swarm as indicated by an asterik in panel (A), neutrophils were only included if they were immediately attached to the main swarm. (E) This selection was copied into a new window and (F) turned into a binary image by (G) adjusting the color threshold until all DAPI fluoresnce was covered; this setting is kept constant throughout the experiment. (H) ImageJ measurement tool is used to find the area.

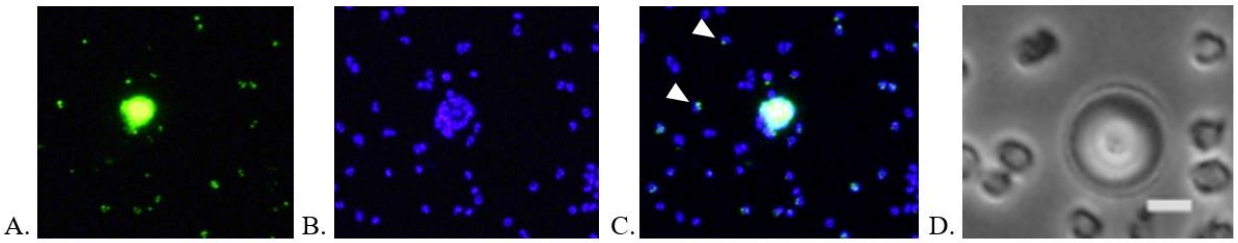

Figure 7. **Neutrophils swarming to zymosan covered bead.** A 30 µm polystyrene bead was coated in FITC labelled zymosan. Shown is the field imaged under (A) FITC, (B) DAPI, and the (C) FITC+DAPI overlay. Arrows indicate phagocytosed zymosan particles (D) Neutrophils and an uncoated bead; brightfield, scale bar is 20 µm. Bone marrow derived neutrophils were isolated from 8-9 week gender matched C57BL/6 mice using the BioRad EasySep magnetic kit and suspended in RPMI/10% FBS/ HEPES/1:100 NMS + 1 ng/mL GM-CSF + 10 nM LTB4. Neutrophils were mixed with coated beads at a ratio of 1 bead: 200 cells in a 6 ug/mL fibronectin coated 6 well glass bottom dish. Culture was gently mixed for homogenous distribution and incubated for 60 min, 37ºC 5% CO2. Neutrophils were fixed and stained with 1:500 DAPI. Images were taken with a fluorescent microscope at 10x original magnification and compiled and analyzed with ImageJ.

As shown in Figure 7, untreated wild type murine neutrophils will readily swarm to the target, but will preferentially phagocytose zymosan particles that are small enough to do so (Figure 7C, phagocytosed zymosan indicated by white arrows). An uncoated bead will not trigger antimicrobial mechanisms from the untreated neutrophils (Figure 7D). While an initial dose of the swarm initiating cytokine LTB_4 was included in the swarming assay media to immediately induce swarming behavior, it should be noted that although LTB_4 is present, neutrophils will not swarm without an adequate target (Figure 7D, 8E).

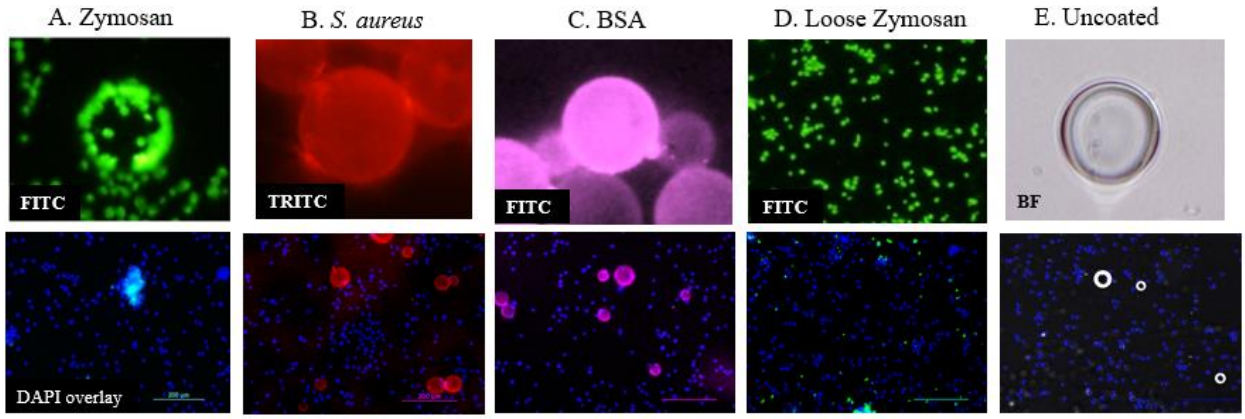

Figure 8. **Coating variations of glass beads**. 20-35 µm silanated glass beads were incubated with each respective coating material in 0.005% TWEEN +PBS. (A) Coated with FITC lablled zymosan bioparticles. (B) Coated with TRITC lablled *S. aureus* bioparticles. (C) Coated with TRITC lablleled BSA and falsed colored to magenta. (D) Loose zymosan bioparticles, no glass beads. (E) Glass bead only, uncoated and imaged under bright field. Each target particle has the respective neutrophil swarming interaction shown underneath; DAPI labelled neutrophils coincubated with the particle for 2 hours. Rows imaged at 100x and 10x, respectively. Scale bar shown is for 100 µm. Cells fixed post incubation.

Pre-silane coated soda lime glass beads were coated with various organic PAMP particles, chosen to best represent the respective pathogen type (Figure 8). The major pathogen types used in this coating trial were fungus and bacteria since viral pathogens do not carry the same large size potential or trigger the variety of anti-microbial mechanisms shown in Figure 1. Wild type, untreated neutrophils were co-incubated with the beads for 2 hours in glass bottom plates which allowed for imaging without disturbing neutrophil or bead orientations (Figure 8).

Zymosan, a primary fungal PAMP, can be seen coating a 35 μm bead in Figure 8A with the associated DAPI stained neutrophils swarming around it. A *Staphylococcus aureus* bioparticle was chosen as the bacterial PAMP representative due to its Gram positive outer membrane (Fig 8B). This was especially important since Gram negative bacteria's primary outer membrane PAMP is comprised LPS, a purified preparation of which is also used to induce *in vitro* inflammation. Therefore to avoid interference or skewing of the overall *in vitro* LPS levels, a Gram positive bacteria bioparticle was chosen. Notably, the neutrophils swarmed to the fungal PAMP bead but did not swarm to the bacterial PAMP bead (Figures 8A).

Neutrophils were also exposed to silanated beads coated with the representative "self" antigen Bovine Serum Albumin (BSA) (Figure 8C). As expected, neutrophils did not swarm or associate with the BSA beads. As an additional negative PAMP control, neutrophils were given completely uncoated soda lime glass beads and no swarming occurred (Figure 8E). Conversely, as a size control to study if the neutrophil swarming was simply fungal PAMP dependant, neutrophils were co-incubated with loose zymosan approximately 1-2 μm in size. In respsonse to the loose zymosan bioparticles, neutrophils phagocytosed the particles and did not swarm (Figure 8D).

Previous coating trials during method development included using a polystyrene bead and various solvents in which to coat the beads with each organic molecule (*unshown*). A preparation of 0.005% TWEEN in PBS proved to be the best solvent for allowing each bioparticle type to remain in solution, not clump during the coating process, and coat the pre-silanated beads (Supp. Figure 3). The successful coating of beads with these two biological markers, one a whole bacterial cell derivative (the *S. aureus* particle) and one a protein (BSA), suggests that this silanation process may be used to coat the beads in an array of other PAMPs, damage associated molecular patterns (DAMPs), or other potential immune cell targets.

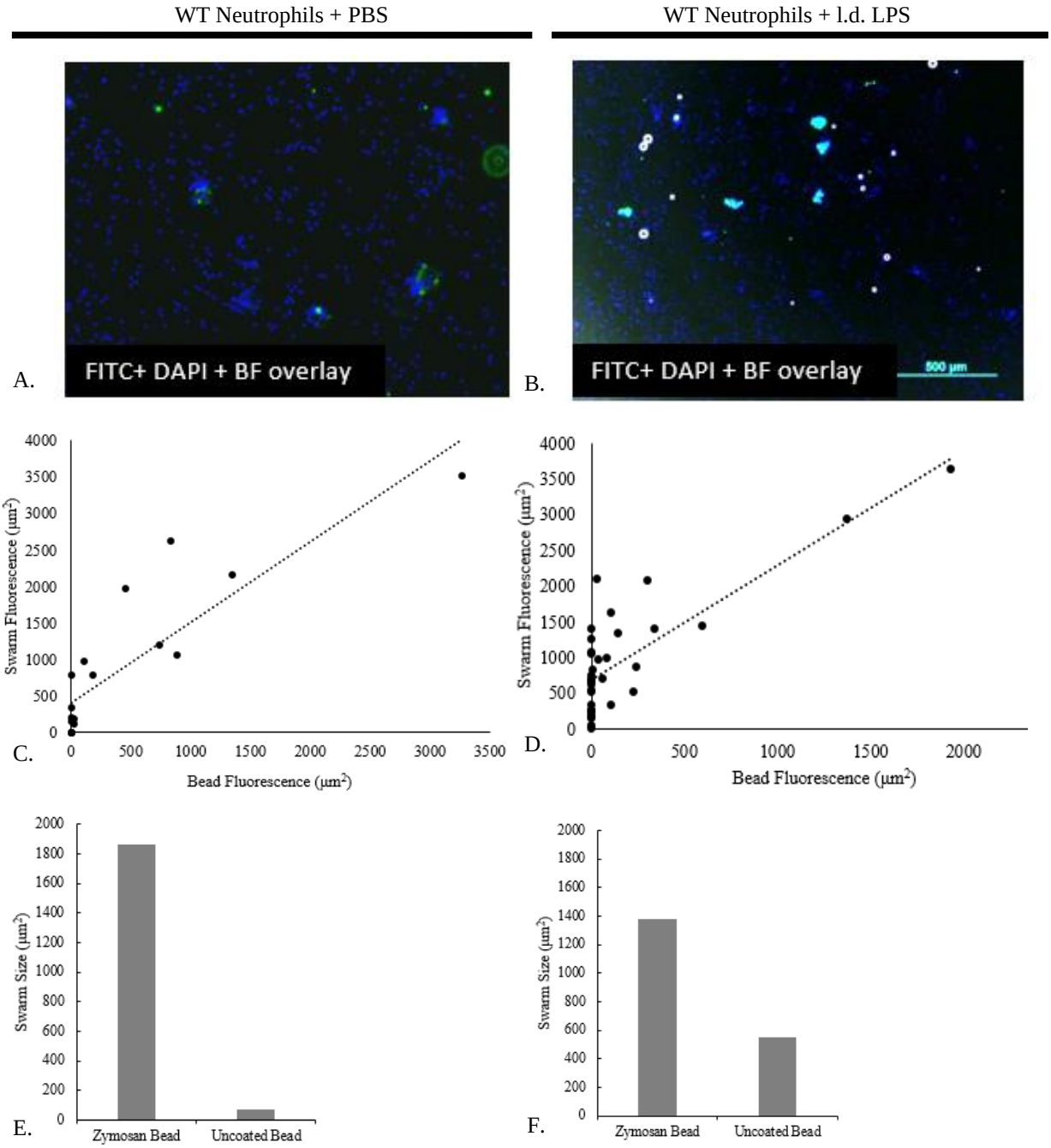

Figure 9. **Overswarming activity of "excited" neutrophils to Zymosan coated beads**. Bone marrow derived neutrophils were isolated from 8-9 week gender matched C57BL/6 mice using the BioRad EasySep magnetic kit and suspended in RPMI/10% FBS/ HEPES/1:100 NMS + 1 ng/mL GM-CSF + 10 nM LTB4 and treated with either (A, C, E) PBS or (B, D, F) 100 pg/mL LPS. Neutrophils were mixed with coated beads at a ratio of 1 zymosan bead: 200 cells/ Neutrophils were fixed and stained with 1:500 DAPI. Images were taken with a fluorescent microscope (FITC, DAPI, and BF) at 10x original magnification and compiled and analyzed with ImageJ. To measure the area (μm^2) of each swarm, an outline was manually traced around the periphery of each swarm to include all attached cells.

To study the effect of chronic, low-level inflammation on neutrophil swarming, wild type neutrophils were co-incubated with zymosan coated beads at an multiplicity of infection (MOI) of 1:200 and allowed to swarm for 2 hours (Figure 9A, 9B). The zymosan beads have a size range of approximately 20-35 μm and have a corresponding increase in target fluoresence once coated in the FITC zymosan. Unsurprisingly, neutrophil swarm sizes increase linearly as the size of the beads increase regardless of the treatment (Figure 9C, 9D). However, once induced into a chronic, low-level inflammatory state using 100 pg/mL of LPS, the treated neutrophils seemed to have a greater repsonse to smaller fluorescent targets (either due to small bead size or partial fluorescent coating) (Figure 9E, 9F).

5. CONCLUSION

A. Neutrophil Size Dependent Decision Making

This preliminary research has shown that once neutrophils have migrated to the site of infection, the cells may undergo a decision making mechanism to apprehend the pathogen or non-self-antigen based on particle size. This particle size dynamic would ideally lead to a more energy efficient approach where extra cells are not recruited to help if it is able to be phagocytosed by a single cell (Figure 7C). While this dynamic has not been well studied or characterized, this phenomenon has been clearly seen in both this preliminary research and other studies[22].

Typically, particles less than 10 μm in size (roughly the size of 1-2 bacteria where *Escherichia coli* is an approximately 5 μm cocci for reference) may be phagocytosed by a single neutrophil while 2-3 μm is the optimal size of a microparticle for phagocytosis[22]. However, when non-self-antigens (i.e. PAMPs) are presented on a particle that is much larger in size, neutrophils must alter their strategies accordingly. In these cases, the neutrophils may swarm to the target in the attempt to effectively fight the pathogen at hand (Figure 7).

We hypothesize there is a size threshold based neutrophil anti-microbial decision making process. When particles are of a standard bacterial size (<5 μm) the neutrophils will phagocytose them, when the pathogen is slightly too large the neutrophils will undergo "frustrated phagocytosis" leading to NETosis, and when pathogens are too large for effective NETosis then swarming is activated[32]. In support of this hypothesis, at least a single NET would be seen around pathogen targets along the 5+ μm "too large to phagocytose" threshold. While this was not yet observed in this study, "frustrated phagocytosis" is a well-documented phenomenon[33]. Using a gradient of bead sizes, model system may be adapted to further elucidate this size threshold based decision making process where neutrophils preform one antimicrobial defense mechanism versus another (Figure 10).

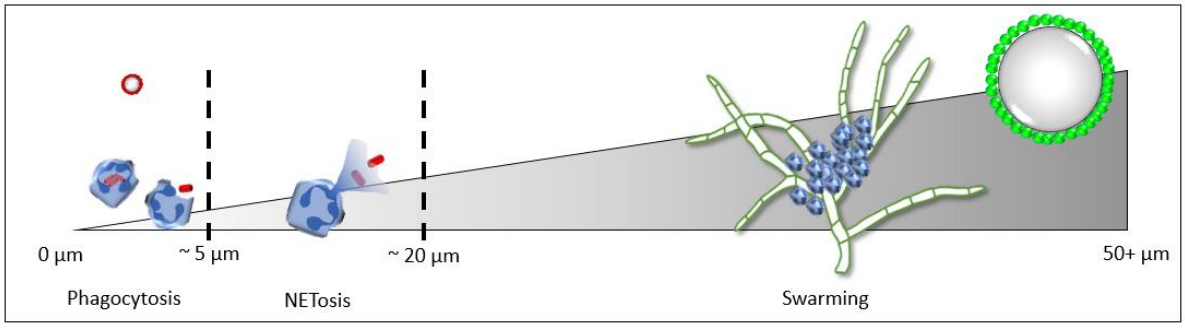

Figure 10. Neutrophil Size Dependent Decision Making. Proposed neutrophil anti-microbial mechanism decision making based on pathogen target size. Hashed lines indicate potential size thresholds at which the cell does one mechanism versus another.

B. Proposed "Excited" Neutrophil State

The results shown in Figure 9 suggest a novel neutrophil state where once cells are induced into a chronic, low-level inflammatory, there is a greater response to the fungal PAMP target beads than the untreated cells. This over response suggests an "excited" state where the pre-existing low-level inflammation causes primed neutrophils to overreact or over respond to the pathogen targets. While there is still a linear relationship between larger targets and the neutrophil swarm sizes, the over response to smaller targets is characterized by the increase of swarm sizes to targets of low fluorescence / size in Figures 9E and 9F. Here, the increase in neutrophil response is markedly higher under inflammatory conditions (Figure 9F).

This is an incredibly interesting phenomenon because it may suggest that the low level inflammation neutrophils are so "excited" by their pre-existing condition that its causing this over response when finally presented with a target. Furthermore, this "excited" state may support well known clinical phenomena where patients with non-antigenic implants have a subsequent inflammatory reaction[34].

C. Neutrophil Swarming Model Conclusions

Here, we present a novel *in vitro* method of studying neutrophil anti-microbial responses to include neutrophil swarming. By using a uniformly shaped pathogen target, we are able to precisely control what each neutrophil treatment group is exposed to for reliable quantification. Additionally, neutrophil treatments and extracellular chemical signaling such as LTB_4 can also be precisely measured within the *in vitro* environment. This model was centered around using uniformly shaped pathogen targets which are spherical glass beads coated in organic particles.

These PAMP coated beads can then simulate an actual pathogen target while providing a single point around which neutrophil swarming may be measured.

We would also like to introduce the potential for an "excited" neutrophil state. Neutrophils that have been induced into the chronic, low-level inflammatory state may become "excited" causing them to over respond to pathogen targets or even uncoated targets (Figure 5). While further research is needed to establish this novel state, this preliminary observation also supports the overall modulation of neutrophil response under inflammatory conditions. This was the opposite of our initial hypothesis that neutrophil swarming would be hindered by low-level inflammation, but provides an even more exciting explanation to altered neutrophil behavior.

Future directions for this model include validating an ImageJ / Fiji script to automate the quantification process (although manual review of measurements would still likely be required) or implanting the PAMP coated beads into a live mouse to combine the benefits of a controlled "pathogen" target and the whole body response of a live animal model. Another potential future direction would be the incorporation of this artificial pathogen bead system in cancer research by coating the beads in tumor cells to create a precisely sized tumor. Many current *in vitro* tumor models rely on a mound of tumor cells stuck to the bottom of a tissue culture plate – this alternative would allow neutrophils to interact and associate with a three dimensional tumor target on all sides[35]. There are huge number possibilities for this artificial pathogen model and we hope it will go on to aid researchers across the biomedical field.

D. Summary

Proper infection fighting ability by neutrophils is a crucial component of the human body's innate immune response. However, a globally increasing number of chronic diseases leads to non-resolving low grade inflammation which primes neutrophils away from properly functioning in the face of a microbial invasion.

There are many proteins along the TLR pathways that act as potential mediators of leukocytic microbial killing ability and therefore may provide interesting insight into neutrophil defense mechanisms. This model allows for the use of gene knockout cell lines, gene knockout primary murine cells, or primary human cells to further study the effects of potentially relevant proteins. By studying neutrophil swarming, we hope to gain a better understanding of overall neutrophil anti-microbial mechanisms in low grade inflammation. Furthermore, we hope to

restore effective bacterial killing in primed leukocytes using an immunological homeostatic regulator therapy. Overall, this research question has an important potential impact by aiding in the better understanding of the mechanisms behind infection clearance in chronic, low- level inflammation. Immunotherapy is at the forefront of modern medical treatment and through better understanding the mechanisms by which immune cells can fight threats, we may be able to restore or even strengthen the overall immune system.

E. Overall Model – Summary Infographic

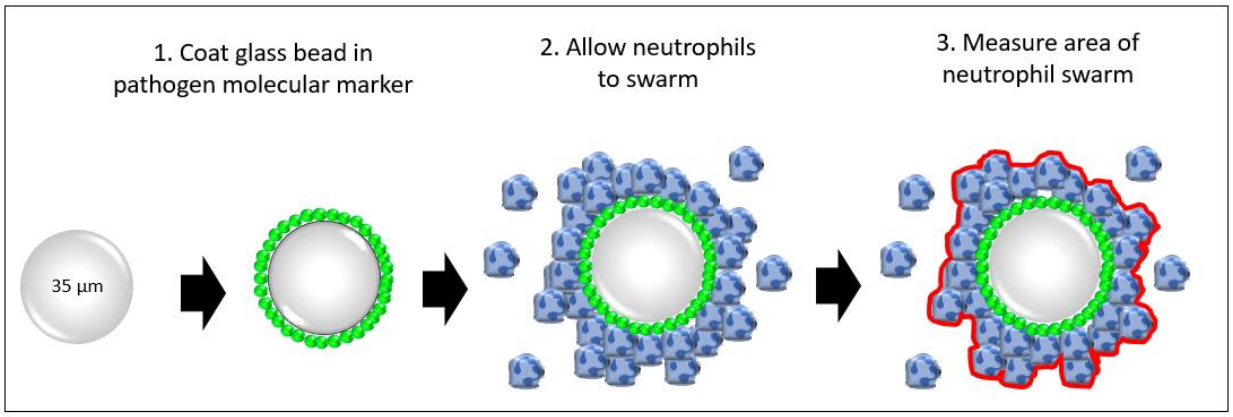

Figure 11. Summary infographic of the neutrophil-bead swarm model.

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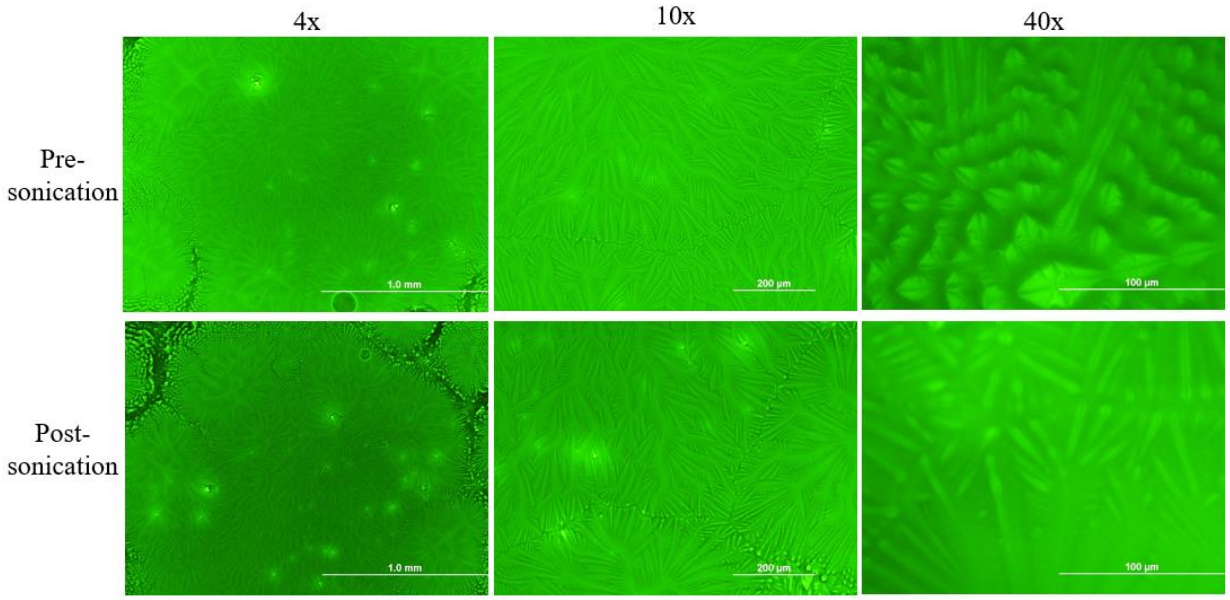
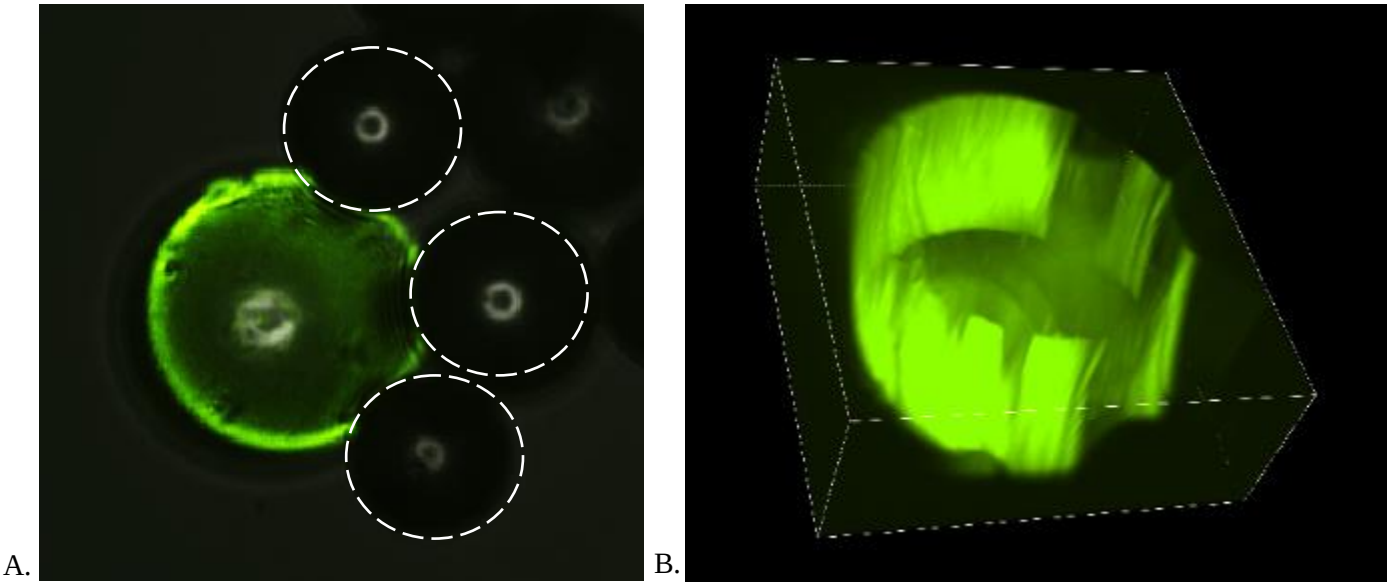
SUPPLEMENTARY FIGURES

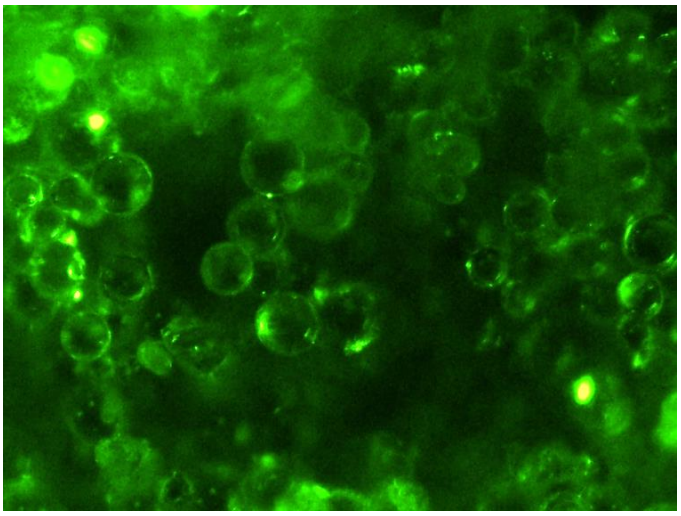


Supplementary Figure 1. 3.4 mg/mL FITC BSA in PBS pre and post sonication. Imaged at 4x, 10x, and 40x. 4x scale bar = 1.0 mm, 10x scale bar = 200 µm, 100x scale bar = 100 µm.

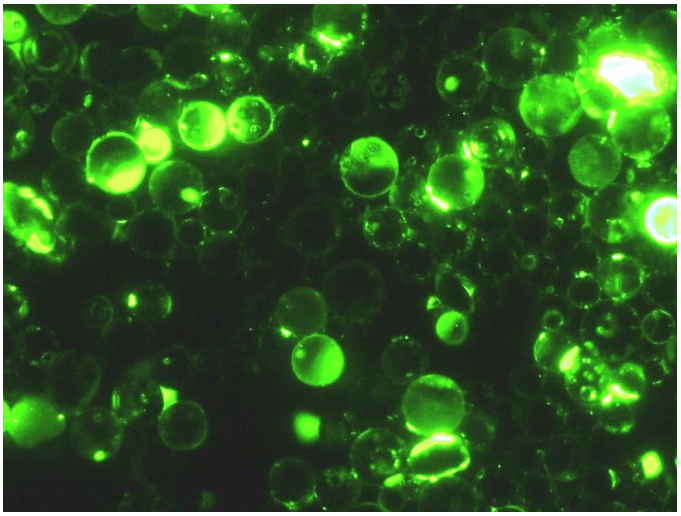


Supplementary Figure 2. Pre-silanated glass bead coated in FITC BSA demonstrating how adjacent beads affect bead coating uniformity (A) white dashed circles illustrate adjacent beads (B) a Z-stack image of the same bead. Imaged at 100x.

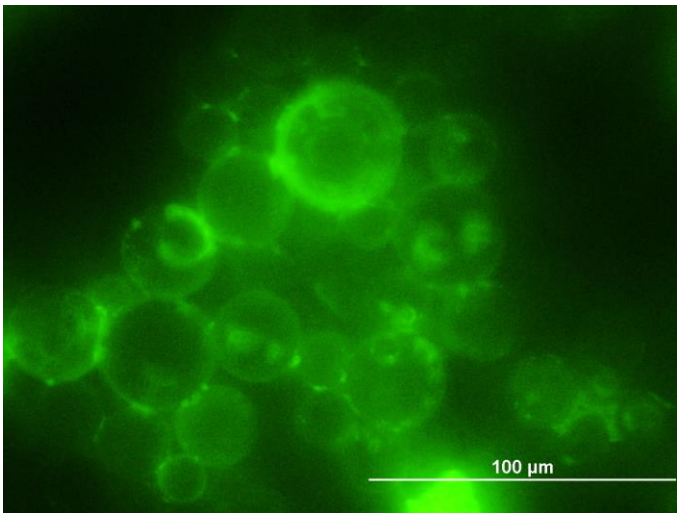
A. Toluene



B. Xylene



C. 0.005% TWEEN in PBS



Supplementary Figure 3. Pre-silanated soda lime glass beads in various solvent trails for FITC BSA coating. (A) Toluene (B) Xylene (C) 0.005% TWEEN in PBS. Imaged at 40x.