Leveraging Neutrophil-Particle Interactions to Develop Therapeutics for Acute Inflammatory Diseases

by

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Dedication

To all the women who did science before me, making it possible for me to make a difference. Additionally, I'd like to dedicate my thesis to Dr. Vincent Starai at the University of Georgia who got me into research in the first place.

Acknowledgments

"A Ph.D. is a marathon, not a sprint," I remember Dr. Omolola Eniola-Adefeso declaring to the group of newly accepted graduate students, including myself, at orientation. I thought to myself, I'm a long-distance runner, and I can handle a long-painful race to the finish line of getting this degree. While this is true, I'd now beg to differ on how much longer this race was compared to any of the races I had run. However, like a marathon, a Ph.D. is also impossible to do without your cheerleaders, coaches, and support staff. My cheerleaders—Nick Renberg, Nick's family (Kathy, Paul, Karissa, and Elise), and my dogs Rubi and Ada truly got me through the hardest times emotionally. My best friend Kat Clancy has supported me throughout both my undergraduate and graduate degrees and am so thankful we chose to be lab partners in Biology 1107 so many years ago. I could have never imagined the amazing friendship that would blossom after that semester.

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The work described in this thesis would not have been possible without the above people (team). I am so proud of the work accomplished by my thesis, and I look forward to where this work goes next.

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List of Abbreviations

ALI Acute Lung Injury

ANOVA Analysis of variance

APC Antigen Presenting Cell

ARDS Acute Respiratory Distress Syndrome

AST Aspartate Aminotransferase

BALF Bronchoalveolar Lavage Fluid

BSA Bovine Serum Albumin

BV421 Brilliant Violet 421

CAM Cellular Adhesion Molecule

CD62-L L-selectin

CFU Colony Forming Unit

COX-2 Cyclooxygenase-2

CXCL1/KC Chemokine Ligand 1

Cy5.5 Cyanine5.5

DCM Dichloromethane

DI Deionized

DIC Distant Intravascular Coagulation

DLS Dynamic Light Scattering

DMSO Dimethyl sulfoxide

DVT Deep Vein Thrombosis

EDC N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride

ELISA Enzyme-Linked Immunosorbent Assay

FACS Fluorescence-activated cell sorting

FBS Fetal Bovine Serum

FDA Food and Drug Administration

FITC Fluorescein isothiocyanate

GFP Green Fluorescent Protein

HCl Hydrochloric Acid

HL-60 Human Leukemia-60

HMW High Molecular Weight

IACUC Institutional Animal Care & Use Committee

ICAM-1 Intercellular adhesion molecule 1

IgM Immunoglobulin M

IL-1 Interleukin-1

IL-12 Interleukin-12

IL-17 Interleukin-17

IL-6 Interleukin- 6

IL-8 Interleukin-8

INFγ Interferon gamma

IRB Internal Review Board

IV Intravenously

JNK c-Jun N-terminal Kinase

LA:GA Lactic to Glycolic Acid Ratio

LC-MS/MS Liquid Chromatography-Tandem Mass Spectrometry

LFA-1 Lymphocyte function-associated antigen 1

LMW Low Molecular Weight

LPS Lipopolysaccharide

LSD Least Significant Difference

MAC1 Macrophage-1

MAP Mean Arterial Pressure

MAPK Mitogen-Activated Protein Kinase

MCIRCC the Michigan Center for Integrative Research for Critical Care

MCP1 monocyte chemoattractant protein-1 (MCP-1/CCL2)

MFI Mean Fluorescent Intensity

MS Multiple Sclerosis

NADPH Nicotinamide Adenine Dinucleotide Phosphate

NaOH Sodium Hydroxide

NCI National Cancer Institute

NCL Nanotechnology Characterization Laboratory

NET Neutrophil Extracellular Traps

NF-κB Nuclear Factor-κB

NHS N-Hydroxysuccinimide

NLR Neutrophil to Lymphocyte Ratio

NOX NADPH Oxidase

NSAID Non-steroidal anti-inflammatory drugs

NΦ Neutrophil

PAMP Pathogen-Associated Molecular Patterns

PBA phenylboronic acid

PBS -/- Phosphate Buffered Saline (with no magnesium or calcium)

PerCP Peridinin-Chlorophyll-Protein

PLA Polylactic Acid

PLG Poly(lactide-co-glycolide)

PLGA poly(lactic-co-glycolic acid)

PMA Phorbol Myristate Acetate

Poly-A Salicylate-based Poly(anhydride-ester)

PRR Pattern Recognition Receptors

PS Polystyrene

PSGL-1 P-selectin glycoprotein ligand-1

ROS Reactive Oxygen Species

RPMI Roswell Park Memorial Institute

SA Salicylic Acid

SAPK Stress-Activated Protein Kinase

SEM Scanning Electron Microscopy

TNF-α Tumor necrosis factor alpha

VCAM-1 Vascular cell adhesion molecule 1

W/V Weight/Volume

WBC White Blood Cell

Abstract

Inflammatory diseases including acute respiratory distress syndrome (ARDS), sepsis, and deep vein thrombosis (DVT) are propagated by a systemic inflammatory response to the disease onset. Mortality rates of ARDS, sepsis, and DVT positively correlate with systemic inflammation, but there are no established curative protocols for systemic inflammation. In ARDS and sepsis, the inflammation in these diseases is propagated by neutrophils and neutrophil damage to tissues leading to organ failure. Neutrophils contribute to clot formation in DVT, high levels of neutrophil involvement can often lead to blood vessel blockage. Depletion of neutrophils can improve the outcome of inflammation in animal models but is not a practical solution for human patients. Particle-based therapeutics have been established as a method of redirecting neutrophils in inflammation, but little research has investigated effects of particle-based therapeutics on neutrophil physiology. Thus, the overarching goal of this research is to investigate anti-inflammatory properties of particle-based therapeutics both as neutrophil diversion tactics and as delivery vehicles for therapeutic agents.

Currently, the clinic utilizes IV-delivered particle-based therapeutics to treat cancer and as diagnostics. Side effects of these therapeutics include immunosuppression, implicating the immune system's role in clearing intravenously delivered particles and the ability to modulate circulating immune cells through this tactic. Further, IV-delivered particles in the clinic have been limited to liposomal and protein-based formulations. These formulations are inherently less stable compared to polymeric materials. Polymeric materials are a novel solution to the particle-based therapeutic world due to ease of mass production, material consistency, and stability. My

dissertation work has investigated a novel, degradable, polymeric particle system that targets circulating phagocytic immune cells and reprograms the cellular inflammation cascade. I first investigated the use of Poly-A particles as a therapeutic in acute localized inflammation *in vivo.* In this work, I found that Poly-A particles both modulate neutrophil accumulation and reprogram neutrophils to a quiescent state via inherent therapeutic properties. I next investigated the extent of neutrophil modulation via Poly-A particles in an *in vitro* model for NETosis, finding that Poly-A particles both reduce and slow the progression of NET formation.

After developing several i*n vitro* models for studying particle interactions with neutrophils, I employed my expertise in evaluating a polymeric material already prevalent in the clinic, PLGA. Importantly, it is necessary to choose materials that have minimal inflammatory impact, and PLGA must be optimized to minimize inflammatory side effects. Finally, I investigated the safety of an *in vivo* infusion of polymeric particles and found that Poly-A particles did not induce infusion reactions. Overcoming this major hurdle of safely infusing poly-A particles shows the clinic translatability of Poly-A as a therapeutic for inflammatory diseases. My work fills the knowledge gap of neutrophil-particle interactions and allows for the development of an innovative and dependable treatment approach. The findings of my dissertation illuminate a new perspective on treating conditions characterized by immune dysfunction and inspire the application of particles elsewhere.

Chapter 1 Introduction

1.1 Publication Information

The written text of this chapter is partially adapted from the published work "*Polymeric particle-based therapies for acute inflammatory diseases"* in Nature Reviews Materials with author list **Emma R. Brannon**, M. Valentina Guevara, Noah J. Pacifici, Jonathan K. Lee, Jamal S. Lewis & Omolola Eniola-Adefeso. The purpose of this chapter is to examine the current state of inflammatory diseases in the clinical setting as well as to investigate neutrophils as the proposed target for therapeutic development. Furthermore, I will discuss polymeric particles that have been investigated in the literature as therapeutics that target neutrophils in inflammatory diseases.

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1.2 Inflammatory Diseases of Interest and Neutrophil Engagement

1.2.1 Function and Role of Acute Inflammation

Inflammation is an essential part of the immune system's response against harmful stimuli, ranging from invading pathogens to physical trauma. Inflammation plays a crucial role in maintaining overall health via recognition, containment, and signaling of invading pathogens or toxins.[1,2] Unlike in homeostasis, exacerbated signaling and cellular responses can lead to tissue damage and organ dysfunction.[1-3] Uncontrolled inflammation is associated with numerous acute

and chronic diseases such as acute lung/liver injury, sepsis, asthma, inflammatory bowel disease, rheumatoid arthritis, and neurodegenerative diseases.[2,3] Acute inflammation, regulated by the innate immune system, is responsible for initial recognition and alarm to an inflammatory stimulus, and typically lasts on a scale of days to weeks. Chronic inflammation occurs from continual inflammation over months or years that fails to contain the inflammatory stimulus. Chronic inflammation is typically controlled by the adaptive immune system.[2-4]

The acute inflammatory response consists of a highly coordinated network of immune cells and molecules. Acute inflammation can either be initiated by pathogenic infections or sterilely via mechanical trauma, ischemia-reperfusion, or chemicals.[3] Pattern recognition receptors (PRR)s are expressed by innate immune cells to initially detect pathogens or cell injury. In the case of pathogens invading via punctured skin, oral, or inhaled routes, PRRs recognize pathogen-associated molecular patterns (PAMP)s triggering inflammatory responses.[1,5] The resident macrophages and dendritic cells are the first cells to interact with invading pathogens in the tissue space. As antigen presenting cells (APC)s, activated macrophages and dendritic cells will phagocytose foreign bodies and migrate to the lymph nodes where the processed antigen will be presented to lymphocytes to activate T-cell differentiation.[3] Additionally, macrophages, dendritic cells, and damaged endothelial cells release inflammatory cytokines to alert other immune cells and organ specific cells such as intestine, lung, or liver. At this stage, cytokines such as TNF-α, IL-1, INFγ, IL-6, IL-8, IL-12, and IL-17, migrate to the bloodstream to recruit other cell types into action, primarily neutrophils.[6]

During sterile inflammation, a mechanical injury, blood clot, or chemical irritant can cause damage on the cellular or tissue scale, initiating the release of inflammatory cytokines (TNF-α, IL-1) from resident immune or damaged endothelial cells. Additionally, damage-associated molecular

patterns (DAMP)s are released from injured cells, stimulating PRRs on resident immune cells and leading to inflammatory cytokine production for neutrophil recruitment.[1]

Neutrophils are the first white blood cells (WBC) recruited to the site of inflammation and play an important role in both pathogen clearance and inflammation resolution. Making up ~50-70% of the circulating WBC population in humans,[7] neutrophils locate the site of inflammation by following released cytokines/chemokines. As the neutrophils slowly roll along the endothelium, they identify changes in surface protein expression such as increased P/E-selectin.[2] Once at the site of inflammation, neutrophils firmly adhere to the endothelium to initiate transmigration. The neutrophils then shed PSGL-1 and CD62-L to accelerate transmigration to the infected tissue.[8,9] Interactions between the neutrophil's LFA-1 and the tissue's ICAM-1 allow for neutrophil transmigration to the inflamed tissue space. At the site of inflammation, neutrophils have several different mechanisms to contain pathogenic infections, including phagocytosis, release of reactive oxygen species (ROS), degranulation, and production of neutrophil extracellular traps (NET)s.[2] Some neutrophils may also travel to lymph nodes to stimulate T-cell differentiation.[10] After the neutrophils have begun to contain the infection, released granules, NETs, and apoptotic neutrophils recruit monocytes and adaptive immune cells to initiate inflammation resolution.[2,10] Both pathogenic and sterile inflammation begins to resolve as monocytes accumulate at the site of inflammation to clear out any remaining necrotic cells and apoptotic neutrophils in a process called efferocytosis.[1,11] The recruitment cascade and specific immune cell functions is shown in Figure 1-1.

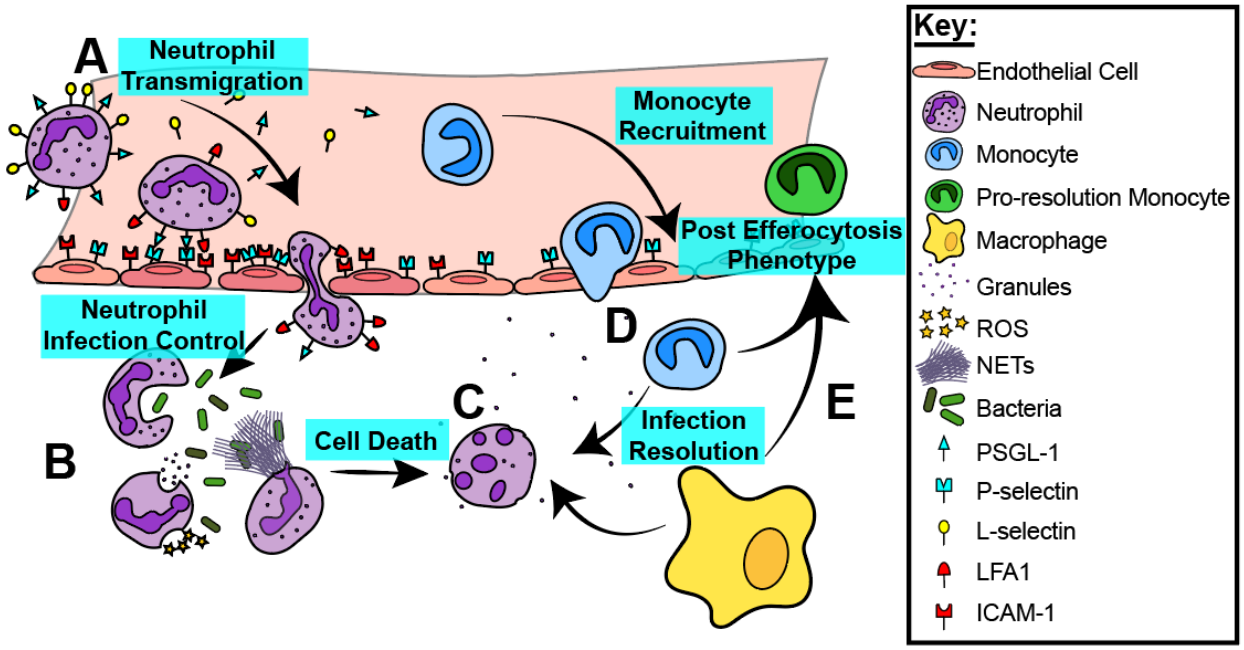


Figure 1-1 Key immune cells in inflammation resolution.

(A) Neutrophils slowly roll along endothelium via PSGL-1 and L-selectin expressed on the neutrophil and corresponding ligands on the endothelium. Once at the site of inflammation, LFA-1 expressed on the neutrophil locks with ICAM-1 on the endothelium to initiate transmigration to the infected or inflamed tissue space**. (B)** At the site of infection, neutrophils phagocytose pathogens or release ROS/granules/NETs to prevent pathogenic spread. **(C)** Neutrophils then commit to apoptosis**, (D)** initiating migration of other immune cell types. **(E)** Monocytes and macrophages clean up dead cellular materials (pathogens/neutrophils) and ultimately migrate to the liver/lymph nodes to remove and process pathogenic materials.

1.2.2 Progression of Acute Inflammation to Inflammatory Disease

In some cases, the acute inflammatory response fails to subside causing disease propagation. Neutrophils may continually accumulate and release cytotoxic agents, harming surrounding host cells in addition to the pathogens.[2] Prolonged neutrophil mobilization alongside host tissue damage leads to overwhelming release of inflammatory cytokines, resulting in a cytokine storm.[12] Detrimental effects of a cytokine storm include excess granulopoiesis and reduced lymphopoiesis.[13] This change in cellular composition is measured as the neutrophil to lymphocyte ratio (NLR) and has recently been identified as a key biomarker for acute

inflammatory diseases including sepsis, acute respiratory distress syndrome, and stroke. Greater neutrophil infiltration further promotes enhanced vascular permeability, allowing pathogenic spread to the bloodstream and ultimately systemic acute inflammation.[14]

In the bloodstream, complement pathways become activated in the presence of pathogens via the classical, lectin, or alternative pathways. Complement proteins such as C3, opsonize foreign bodies in the bloodstream, converting C3 to C3a and C5a. Both C3a and C5a function as anaphylatoxins causing further downstream inflammation via inherent pro-inflammatory properties.[15] Anaphylatoxins are small polypeptides that function as activating agents on granulocytes, mast cells, macrophages, and endothelial cells. Some of these effector functions include chemotaxis, ROS bursts, cytokine release, in addition to vasodilation and vascular permeability.[15] Inflammatory cytokines combined with complement activation can induce the expression of tissue factor on endothelial cells, initiating the coagulation pathway.[6,16] Once triggered by inflammation, the coagulation pathway is difficult to halt via normal negative feedback mechanisms such as antithrombin, activated protein C, or tissue factor pathway inhibitor. This can lead to distant intravascular coagulation (DIC) and eventually multi-organ failure if widespread.[6]

Similar to pathogenic infections, sterile recruits neutrophils to the injured site via immunomodulatory molecules. Unlike infections there are no pathogens to destroy, but neutrophils use similar tactics to contain the inflammation. Thus, the release of granules, ROS, and NETs can damage surrounding host cells. Sterile inflammation can become pathological when the inflammatory stimulus cannot be resolved such as in chronic autoimmune diseases or severe trauma.[1]

To date, most clinical treatments for inflammatory diseases center on infection control, pain management, and supportive care. Traditional therapeutic approaches such as steroidal and non-steroidal anti-inflammatory drugs are extensively used for the treatment of different inflammatory diseases.[17-20] New strategies, including the usage of monoclonal antibody therapies have recently emerged as promising agents to treat the damaging effects of inflammation.[21-25] Although there has been success with these anti-inflammatory therapies, there are still considerable limitations. In particular, these therapies increase risk of secondary infections, and lack of specific biodistribution, causing severe side effects.[19,26-31] To avoid some of these obstacles, advanced drug delivery systems have been investigated over the past decades to therapeutically target the inflammatory response. Hence, my thesis work focuses on the development of novel therapeutics that target and modulate neutrophil behavior in severe cases of inflammation.

1.2.3 Involvement of Neutrophils in the Propagation of Acute Inflammation

Several acute inflammatory diseases such as ALI, ARDS, sepsis, and DVT are propagated by an extreme acute immune response from neutrophils. As mentioned previously, the overzealous response from neutrophils can damage host tissue via secretion of proteolytic enzymes, ROS, and NETs.[32] While neutrophils function as innate immune cells to contain an infection, host cells can also succumb to damage from neutrophils. In ALI/ARDS, damage to the endothelium results in enhanced permeability and ultimately inflammation and disease propagation.[33] It has become clear that neutrophils play a strong role in the inflammatory response, especially in diseases intensified by a rampant acute inflammatory response. Moreover, researchers and clinicians have found that elevated blood neutrophil counts can be used as a biomarker to predict severe cases of sepsis and ARDS, including ARDS caused by COVID-19.[34-36] Sepsis has historically been a difficult disease

to diagnose, thus this new found emphasis on neutrophils can progress the field in more ways than one.

1.2.4 Lack of Consideration of Neutrophils in the Development of Therapeutics

Neutrophils have largely been under studied in the development of systemic therapeutics despite playing a large role in inflammation and comprising of the majority circulating WBCs.[35,37] Neutrophils are inherently difficult to work with due to their short lifespan and quick activation response once outside the body.[38] Furthermore, common *in vivo* models for inflammation typically utilize mice despite the numerous differences between mouse and human neutrophils.[39] Isolated human neutrophils from freshly drawn blood, is the next best choice in studying neutrophil behavior, but lacks the physiologic environment that neutrophils are naturally designed to respond to in inflammation.[2,38] For these reasons, much of my thesis work focuses on the development of assays, *in vivo* and *in vitro*, to fully understand and investigate neutrophils in the development of novel therapeutics.

1.3 Neutrophils as Therapeutic Targets

Despite the known involvement of neutrophils in inflammatory diseases, it was not until recently that neutrophils were considered as therapeutic targets. Strategies that inhibit neutrophil disease propagation include blocking neutrophil activation, derived mediators, or recruitment.[35] Due to redundancies within the immune cascade, however, it can be difficult to target neutrophil activation or derived mediators without also inhibiting other important immune functions. Thus, direct targeting methods must be used.

Preventing neutrophil accumulation at the site of injury, via neutrophil depletion techniques in animal models, preserves organ function, and prevents further damage to site of

injury.[40,41] While complete depletion of neutrophils is not an applicable approach to clinical diseases, other methods, such as neutrophil diversion may be suitable. In acute inflammation, particles interfere with neutrophil disease propagation via drug delivery or diversion. By injecting mice with 2 μm polystyrene particles post endotoxin induced ALI, neutrophils were diverted from the lungs to the liver, preventing lung damage.[42] More recent work evaluated neutrophil diversion by drug-free polymeric particles, treating inflammatory conditions including encephalitis, spinal cord injury, and sepsis.[43-45]

Degradable polymeric particles have been found to modulate the immune response in multiple sclerosis (MS), an inflammatory autoimmune disease of the nervous system.[43] Poly(lactide-co-glycolide) (PLG)-based nanoparticles with ranging molecular weights treated mice in an *in vivo* murine model of MS. Of the immune cells monitored throughout the study, neutrophils engulfed particles more readily than other immune cell types. Despite the belief that neutrophils play a minor role in MS, the alleviation of MS symptoms in particle treated mice suggests that neutrophils are involved in both innate and adaptive inflammatory responses.[43] By understanding neutrophil modulation in acute inflammatory diseases, future work could employ particles to treat more complex, chronic inflammatory diseases with neutrophil involvement.

To achieve a particle-based therapy for acute inflammation, it is important to develop a biocompatible and degradable system with optimized particle size, shape, and surface characteristics. All of these parameters have previously been reported to impact phagocytosis in macrophages, monocytes, and neutrophils.[43,46] Tuning particle composition in uptake modulation studies found that neutrophils preferentially uptake hydrophilic particles.[43] Neutrophils also phagocytose rod-shaped over sphere-shaped microparticles, suggesting the importance of shape when designing particles for treating acute inflammatory responses.[46] Importantly, materials used

to fabricate particles must be degradable to prevent accumulation of materials within the body. Degradation byproducts, however, can influence neutrophil behavior from the inside out, so much consideration should be taken when selecting materials for treating inflammatory diseases.[47] The following sections will discuss various types of degradable materials that can be used as particle-based therapeutics.

1.4 Optimized Materials for Targeting Neutrophils in Acute Inflammation

Optimizing particle material is an important design aspect in creating therapeutics for inflammatory diseases to limit the release of immunostimulatory byproducts and particle accumulation. Degradable polymeric materials are an ideal candidate in designing particle-based therapeutics for inflammatory diseases in that they can be easily modified, optimized, and produced in large quantities. Polymeric materials are known to heavily influence immune cell modulation and inflammatory signals. In preliminary research investigating neutrophil uptake of polystyrene particles revealed that the act of phagocytizing non-degradable polystyrene induced inflammatory neutrophil characteristics.[48] Thus, in optimizing particles to treat inflammation, it is incredibly important to utilize materials that inherently reduce inflammation. The following sections describe potential materials that can be used for developing the optimal particle-based therapeutics for treating inflammatory diseases.

1.4.1 PLGA Particles in the Literature

Much literature on treating inflammation with polymeric particles has centralized material development on Poly(lactic-co-glycolic acid) (PLGA). As a well characterized material, PLGA has been incorporated into several FDA approved particle-based therapeutics and thus is a seemingly safe material. The primary degradation byproduct of PLGA, lactic acid, is known to

have anti-inflammatory properties on both macrophages and dendritic cells.[43,45,49] In large quantities lactic acid can become an inflammatory agent, making it a less ideal material to use when optimizing polymeric particles to treat inflammation.[50] Despite this, PLGA particles can be used as a delivery mechanism of anti-inflammatory therapeutics, for example NSAIDs, to further reduce inflammation and overcome immediate side effects of PLGA degradation.[51]

1.4.2 Bio-Inspired Materials

Recent literature has quickly begun to demonstrate the many different approaches in designing anti-inflammatory polymeric materials. In treating neurological inflammation, a common marker for apoptotic cells, phospholipid phosphatidylserine, was used as the base in a particle formulation to reduce inflammation of activated microglial cells and macrophages.[52] While initial *in vitro* studies of polymerized phosphatidylserine particles have proven beneficial immunomodulatory capabilities, *in vivo* experiments have yet been completed to demonstrate anti-inflammatory effects in a disease model. While this material is innovative, it is important to remember that even simple, drug-free PLG-based particles have been shown to enhance recovery post neurological inflammation via immune cell diversion.[43] Materials should be optimized based on anti-inflammatory properties, but care must also be taken to ensure that inherent therapeutic properties of particles are maintained.

1.4.3 Bio Responsive Materials

Degradable polymeric particle systems can be designed to degrade at the site of inflammation via the incorporation of stimuli responsive properties. For example, vanillyl alcohol, a known antioxidant and anti-inflammatory agent, can be incorporated into copolyoxalate via hydrogen peroxide-sensitive peroxalate ester bonds. This process yields a polymer that can be formulated

into a particle-based therapeutic that degrades when exposed to nitric oxide, a heavily expressed molecule of inflammation.[50] Similarly, another group created a particles out of a polymeric blend of both ROS and pH sensitive polymeric linkers to achieve a dual responsive nanoparticle. Naproxen, an anti-inflammatory drug, was modified with a ROS responsive linker, phenylboronic acid (PBA), then conjugated onto dextran. Particles were then formulated out of a blend of the modified PBA-dextran and a pH-sensitive acetylated dextran.[53] As a novel material, much of the work completed is conceptual and has yet to transition from *in vitro* to *in vivo* studies,

1.4.4 Polymerized Anti-Inflammatory Compounds

Another innovative approach, polymerization of known anti-inflammatory compounds, has been heavily researched over the past few decades. Degradable polymer systems can be functionalized with a range of anti-inflammatory agents including aspirin, naproxen, and ibuprofen.[54,55] The resulting polymer can then be formulated into a particle via oil-water emulsion techniques.[56] Polymerized salicylic acid, termed PolyAspirn or Poly-A has potential in treating acute inflammation via targeting neutrophils and reprogramming inflammatory pathways from the inside out.[47]

1.4.5 Design Constraints for Translating Particle-Based Therapeutics to the Clinic

Polymeric materials are ideal for clinical translation due to their versatility and tunability for various applications.[37,57] For example, PLGA represents a polymer that can be tuned for specific applications to either induce or prevent an inflammatory response as shown in macrophages.[49] Particles-based therapeutics represent a simplistic approach to the complex problem of inflammatory diseases. It has been shown that non-degradable particles can simply divert neutrophils by existing in the bloodstream with circulating WBCs[42]—i.e. no targeting or

biomimetic materials needed. Important hurdles in scaling polymeric particle fabrication exist (sterility, scale-up, uniformity), reducing the applicability of complex materials (targeting ligands, biomimetic properties) that may enhance the difficulty of scaling. Furthermore, it is important to develop materials that are stable for long term storage with minimal technology required for stability. For example, the COVID-19 vaccine distribution faced many challenges due to the low temperature storage constraint.[58] Thus, it is ever important to focus on developing materials that fit these many constraints for scale-up, storage, and distribution.

Poly-A represents a material that has inherent anti-inflammatory properties and, in this work, has functionally shown its use in modulating neutrophils in inflammation. As a simple, polymeric formulation, it has the potential for scaled production, sterilization, and minimal storage requirements. The majority of my thesis work investigates Poly-A particles as a therapeutic while also examining potential pitfalls and toxicities.

1.5 Dissertation Framework

The work completed in this thesis aims to elucidate the effects of particle-based therapeutics on neutrophils. While neutrophils have been largely ignored in the literature, neutrophils are an incredibly important immune cell population to investigate in the development of particle-based therapeutics. Throughout my thesis, I used a range of *in vitro* and *in vivo* models to understand the physiological effects of Poly-A particles on neutrophils to translate their use across disease models and species.

In chapter 1 of my thesis I provided background information and motivation for the research discussed in chapters 3-6 shown in Figure 1-2. Specifically, I discussed the involvement of neutrophils in acute inflammation resolution alongside cases of disease progression fueled by neutrophil behavior. Neutrophils represent a unique target for inflammatory diseases as they are

the major circulating white blood cells and inherently interact with particle-based therapeutics. Lastly, I discussed various materials that can be used for particle-based therapeutic design and the importance of material optimization.

Chapter 2 describes the methods and materials used throughout my thesis work. This chapter is organized by study approvals, particle characterization, disease models inflammation, and statistical analysis. The following results chapters utilize the methods described in chapter 2.

In chapter 3 of my thesis, I *characterized the physiological effects of Poly-A particles on neutrophils in vitro and in vivo.* This aim of my thesis, published in Advanced Healthcare Materials,[47] focuses on the use of degradable particle systems to treat localized lung inflammation. Poly-A was found to have an additional therapeutic benefit over other degradable particles in treating localized lung inflammation in both the ALI and ARDS models. I then utilized whole

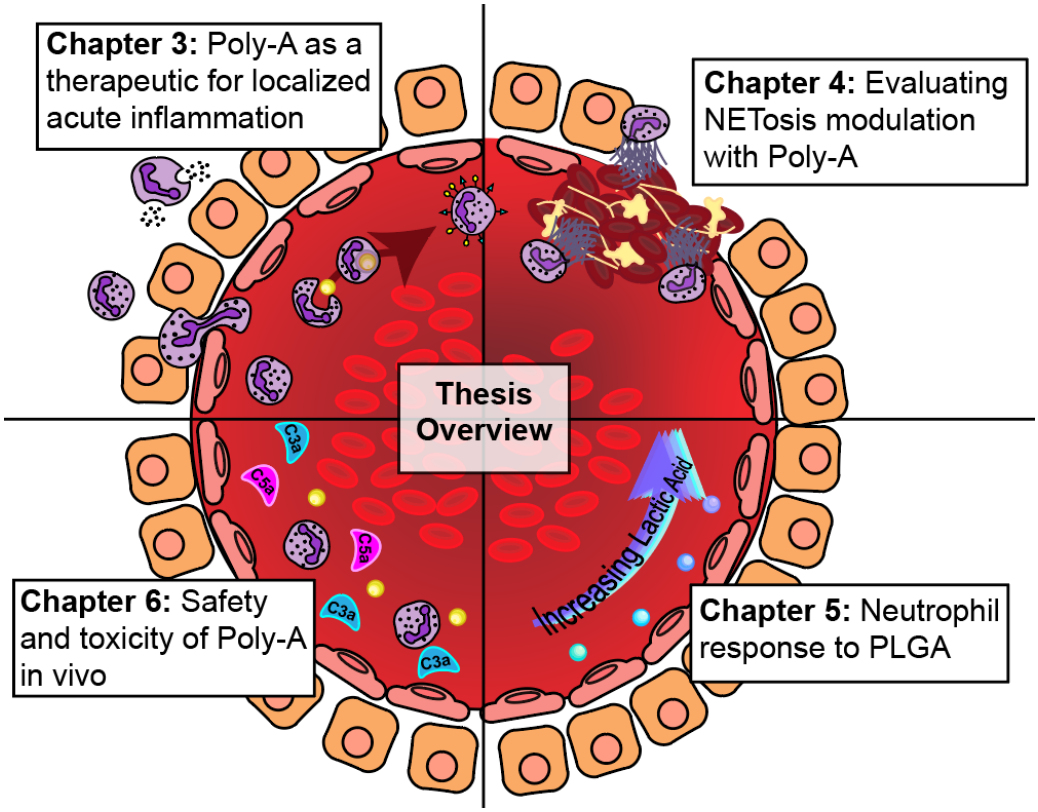


Figure 1-2 Visualization of research chapters discussed in this thesis.

human blood to characterize the benefit of Poly-A over other degradable particles. From this work, I found that phagocytosis of Poly-A particles modulates neutrophil surface protein expression reverting neutrophils to a less inflamed state. This research was the first to show the impacts of material selection on neutrophil activation and ultimately the importance of selecting materials for developing therapeutics for inflammatory diseases.

After discovering the effects of Poly-A particles on neutrophil surface protein expression, it was important to investigate other aspects of neutrophil activation. In chapter 4, *I developed and utilized an in vitro model for NETosis to investigate the effects of particle-based therapeutics.* Most current methods of NETosis assays only measure systemic effects of NETosis or are low throughput, limiting our ability to study many iterations of particle-based therapeutics. Using an automated microscope, EVOS M7000, I captured full-well scans along with 20x fluorescent images to provide both a systemic and a cellular understanding of NETosis modulation by particles. In this chapter, I found that Poly-A particles reduce NETosis in a dose-dependent manner. This work provides a foundation for others to investigate the effects of particles on NETosis in addition to providing insight into the importance of material optimization for designing particle-based therapeutics.

Once detailed characterization (*in vitro* and *in vivo*) of Poly-A particles was completed, I took a step back to evaluate other polymeric materials that are currently used in the clinic. In Chapter 5 *I investigate the effects of varying PLGA composition on neutrophil surface protein expression.* Particle formulations of PLGA have been used in the clinic since 1989 but are limited to local delivery.[59] Meanwhile, IV injected particle-based therapeutics (liposomal/colloidal) have been used clinically since 1995.[60] PLGA is a biocompatible, tunable, and scalable material, that has thus far been limited in application due to a lack of knowledge in understanding PLGA particle-

neutrophil interactions.[61] In this chapter, I fabricated 1-micron PLGA particles of varying composition and investigated neutrophil activation post-particle uptake via surface protein expression. From this work, I found that both the lactic to glycolic acid ratio and molecular weight play a role in neutrophil activation. This knowledge can be utilized to optimize PLGA-based therapeutics designed specifically for neutrophil modulation.

In chapter 6 *I investigated a clinically relevant pig model to evaluate the safety of Poly-A particles as an intravenous therapeutic.* As part of the Michigan Center for Integrative Research for Critical Care (MCIRCC), the Eniola-Adefeso lab collaborated with Dr. Hakam Tiba to scale up initial *in vivo* studies of Poly-A particles into a porcine model. Pigs are the ideal model for examining the safety of particle-based therapeutics due to their innate immune response to intravenous particles.[62,63] I investigated two different injection schemes and found a slower injection rate reduced risk of adverse reactions. The work from chapter 6 is promising for the transition of Poly-A from benchtop to the clinic.

Finally, chapter 7 discusses the outlook for particle-based therapeutics in the clinic along with hurdles that remain.

Chapter 2 Materials and Methods

2.1 Introduction

Data generated in chapters 3-6 utilized the following methods and materials. A combination of *in vitro* and *in vivo* methods was utilized to investigate particle-based therapeutics across species. Mouse models were used to initially determine the effectiveness of various particle-based therapeutics for treating inflammatory diseases. I then utilized healthy human blood for a range of *in vitro* models to characterize the functionality of Poly-A particles. *In vitro* and *In vivo* pig models were used to scale up Poly-A as an intravenous therapeutic and investigate the safety of a direct intravenous infusion. Finally, I employed human *in vitro* assays to further investigate changing the material of particle-based therapeutics and the resulting effects on neutrophil physiology.

2.2 Human and Animal Study Approvals

2.2.1 Murine Study Approvals

All protocols used in this work were approved by the University of Michigan Institutional Animal Care & Use Committee (IACUC) under PRO00010572. Additionally, all experimentation followed guidelines in accordance with guidelines set by the National Institute of Health. Mice (BALB/cj and C57BL/6) were purchased from Jackson Laboratories and kept in pathogen free housing.

2.2.2 Human Study Approvals

This work utilized healthy human blood collected under the protocol approved by the University of Michigan Internal Review Board (IRB-MED; ID #HUM00013973). Informed, written consent was collected from each donor prior to blood collection. Briefly, sterile filtered heparin loaded in a syringe or sodium citrate vacutainers were gathered prior to donor arrival. With the donor sitting down, a tourniquet was tied around the arm, the vein and surrounding area was swabbed with an alcohol wipe, and phlebotomy was performed via venipuncture. Once the previously determined blood volume was collected the tourniquet was removed and butterfly needle extracted. Immediately gauze was held at the incision site to allow for blood coagulation. The gauze was then replaced by a bandage and donor ID, blood volume, date was recorded to ensure donor payment.

2.2.3 Porcine Study Approvals

Animal handling and sample collection was completed by the Tiba Research Laboratory at the University of Michigan. Protocols were approved by IACUC and followed the Guide for the Care and Use of Laboratory Animals as stated by the National Research Council (U.S.) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (U.S.), and National Academies Press (U.S.).

2.3 Particle Fabrication and Characterization

2.3.1 Polymeric Particle Fabrication

Poly-A polymers were provided by the Uhrich group at UC Riverside. PLGA polymers were purchased from Sigma. Polymeric particles were fabricated using an oil and water emulsification evaporation process as described previously.[47] Polymer dissolved in dichloromethane (DCM) was slowly injected into the emulsified oil phase, 1% (W/V) polyvinyl alcohol (PVA). The solution

was mixed for 2 hours, allowing for full evaporation of DCM and formation of particles. The particles were isolated from solution by centrifugation at 4000 rpm and washed 3x with DI water. Particle size was modulated by increasing the polymer concentration in DCM; 2- and 1- micron sized particles were fabricated using 4- or 1-mg/mL polymer/DCM, respectively.

2.3.2 Fluorescent Particle Fabrication

For fluorescent particles, polymer (either Poly-A or PLGA) was dissolved in DMSO and combined with N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC). After 5 minutes of stirring, N-Hydroxysuccinimide (NHS) was added to the Poly-A solution. The solution was rotated for 15 minutes, then Cy5.5 was added to the polymer solution and allowed to rotate overnight. The resulting polymer was isolated with dialysis over 2 days. The isolated polymer was vacuum dried. Particles labeled with Cy5.5 were fabricated by supplementing 5% of the polymer composition with polymer (either Poly-A or PLGA respectively) conjugated to Cy5.5. This conjugation/supplementation method was previously described by Brannon et al.[47]

2.3.3 Particle Characterization

Particle shape and size was confirmed by scanning electron microscopy (SEM). Dynamic light scattering (DLS) was used to measure surface charge of the particles. Particle concentration was determined using a hemocytometer on the same day as the experiment. Particle concentration was determined by counting dilute particle samples on a hemocytometer. Particle mass was determined by weighing out particle samples, resuspending particles in PBS -/-, and calculating the respective particle content with a hemocytometer.

2.3.4 Particle Degradation in PBS

A total of 1E7 particles were suspended in 10mL of PBS -/- at pH 7.2 and placed under rotation at 37°C. Periodically, the particles were centrifuged, and the supernatant was removed and replaced. The supernatant was then added to a 96-well plate, and the fluorescence intensity was measured (ex = 315 nm and em = 408 nm for salicylic acid). The cumulative fluorescence intensity at each time point is plotted.

2.3.5 Particle Degradation in Human Plasma

Particles were resuspended in human plasma at 1.25 E8 particles/mL. The particle samples were then rotated at 37°C for 6-, 20-, 30-hrs. The particles isolated from plasma via centrifugation at 4000 rpm and resuspended in DI water. The washed particles were then dried on glass coverslips for SEM imaging.

2.4 Particle Degradation in "Phagosome-Like" Environment

2.4.1 Degradation Experiment

Particles were counted and diluted to 8E8 particles/mL in buffer containing PBS without calcium or magnesium (PBS-/-) supplemented with 2% fetal bovine serum (FBS). This solution represented a protein rich biological fluid. In a beaker on a stir plate, 19.75 mL of buffer was combined with 0.250 mL diluted particles. A pH probe was set up to actively measure the pH of the system throughout the experiment. To imitate the phagosome environment of a neutrophil, the pH was raised to 8.5 using 0.1 M NaOH for the first 30 minutes of gentle agitation. Similar to a neutrophil phagosome, the pH was then reduced to 7.4 for the remainder of the experiment (1.5-hrs). The 2-hr experimental timeline was chosen based on a typical uptake experiment timeline (2-hrs total). The resulting particles were isolated and washed twice using centrifugation at 3000 G. The isolated

particles were spotted on a glass coverslip for SEM imaging. Particle size was determined using ImageJ. At least 200 particles were sized across 3 images to determine the average size.

2.4.2 Percent Volume Decrease Calculation

Samples exposed to basic conditions were compared to particles fabricated in the same batch that were stirred at constant neutral pH for 2-hrs. Percent volume decrease was first calculated by converting the average size to volume. The percent decrease was then calculated by dividing the difference between the control and basic pH sample volumes by the volume of the control and multiplying by 100. This process was done for at least 2 replicates for each particle type.

2.5 Murine Models of Inflammation

2.5.1 Biodistribution of Particles

C57BL/6J mice were intravenously injected with 2E8 Poly-A particles labeled with Cy5.5. Mice were euthanized 30 minutes post particle injection by CO_2 inhalation and biodistribution samples were harvested. We extracted blood via cardiac puncture, along with the lungs, liver, kidneys, and spleen. Whole organs were then scanned by an Odyssey CLx infrared imaging system (LI-COR). To scan blood samples, 100 µL of blood was plated in a clear bottom 96-well plate. For each mouse, the percentage of total signal in each compartment was calculated and reported as a Percent Injected Dose.

2.5.2 Acute Lung Injury (ALI)

Mice were briefly anesthetized with isoflurane and placed at a 45° angle to instill lipopolysaccharide (LPS) intratracheally. An approximate 30 mg/kg dose of particles (2E8 particles/mouse) was injected using a catheter either 1-, 2-, or 4-hrs post LPS instillation. Mice

were euthanized 2-hour post particle injection for neutrophil BALF analysis or 1-hr post particle injection for blood/liver analysis. Samples were collected immediately after CO_2 inhalation euthanasia.

2.5.3 Acute Respiratory Distress Syndrome (ARDS)

Pseudomonas aeruginosa was cultured overnight in Difco nutrient broth at 37℃, and concentration was determined by optical density absorbance at 600nm in comparison to an established growth curve. Each mouse was anesthetized by xylazine/ketamine intraperitoneal injection. A pipette was used to intranasally administer 30 µL of 2E5 CFU the lungs. Mice were euthanized via CO_2 inhalation for neutrophil migration characterization studies or after particle injections. Particles (2E8 particles/mouse) were injected 18-hrs post infection. Soluble aspirin was injected at 3.75 mg/mL in 200 µL PBS-/- with 10% DMSO as a control for completely degraded particles. Mice were euthanized 24-hrs post infection for particle studies.

2.5.4 Bronchoalveolar Lavage Fluid (BALF) Collection

After euthanasia, the chest cavity was exposed and the diaphragm was punctured to reveal heart and lungs. The trachea was exposed and a small opening was made for catheter insertion. PBS-/- was then flushed through the catheter to fill the lungs to capacity. The lungs were flushed to collect 3 mL total of BALF to remove leukocytes present in the lungs for analysis. BALF samples were centrifuged at 500G to harvest leukocytes. The supernatant was saved for ELISA analysis. BALF cells were counted with a hemocytometer with either trypan blue (ALI model) or Turks Diluting Fluid (ARDS model). For the ALI model neutrophil percentages were determined via flow cytometry after staining with CD45, CD11b, and Ly6G (BioLegend). For the ARDS model

samples were prepared with cytospin processing and stained to differentiate neutrophils from mononuclear cells.

2.5.5 Toxicity Study

A group of 9 mice was broken into 3 groups that received either saline, Poly-A (2E8/mouse), or soluble aspirin. For each group n=3. Mice were injected on day 0 and scored/weighed for a 5-day period. On day 5 blood, spleen, and liver were collected to evaluate neutrophil distribution in each compartment. Plasma from the blood was saved for an Aspartate Aminotransferase (AST) assay (Sigma, MAK055-1KT), and cytokine ELISAs.

2.5.6 Blood, Liver, and Spleen Dissociation

Blood was collected via cardiac puncture and one liver lobe was harvested. Blood samples were immediately placed on ice, FC blocked (TruStain FcX, BioLegend), stained, and lyse/fixed (eBioscience). Liver and spleen samples were rinsed with PBS -/- and placed into 5 mg/mL Type IV Collagenase supplemented with DNAse in PBS-/-. Samples were lightly chopped and incubated at 37°C for 15 minutes. A gentleMACS Dissociator (Miltenyi Biotec) was used to fully dissociate the organ samples. Each sample was strained with a 70 μm strainer (Miltenyi Biotec) and neutrophils were isolated using Lymphoprep (Cosmo Bio Usa Inc). Neutrophils were then FC blocked, stained, and fixed. Blood, liver, and spleen samples were stained with CD45, CD11b, Ly6G, PSGL, and CD62-L (BioLegend). All samples were run on an Attune flow cytometer for analysis.

2.5.7 ELISAs

ELISAs were used to evaluate protein concentration in BALF and plasma samples. BALF samples were evaluated for IgM, albumin, IL-10, KC, MCP1, MIP2, TNF-α, and IL-6. These ELISAs were

all purchased from Bethyl Laboratories. Plasma samples were evaluated for IL-6 (Invitrogen) and CXCL1/KC (R&D Systems).

2.5.8 Salicylic Acid Measurements in Mouse Plasma

Mice were injected with 2E9 Poly-A particles/mouse or free aspirin. Blood was collected via cardiac puncture at the time of euthanasia. The blood was centrifuged for 15 min at 2000*g* to isolate plasma. Salicylic acid was measured in plasma via liquid chromatography-tandem mass spectrometry (LC-MS/MS). This work was done by the Pharmacokinetics Core at the University of Michigan.

2.6 Human *in Vitro* Experiments

2.6.1 Whole Blood Uptake Experiments

Blood was collected via venipuncture into heparinized syringes from human donors, then aliquoted into duplicates. LPS (1 μg/mL) was added immediately to activate samples for 30 min at 37℃. Post activation, 1E6 particles were added, and samples are incubated for an additional 1.5 hours. Samples were then FC blocked, stained, and lyse/fixed (eBioscience). Samples were prepared for flow cytometry by washing with FACS buffer at 500 G for 5 min. Flow cytometry data was collected with an Attune flow cytometer.

2.6.2 Neutrophil Isolation

Blood was collected from healthy donors in sodium citrated vacutainers via venipuncture. Following protocols provided by the Knight lab at the University of Michigan. In a biosafety cabinet, blood was then layered in a 1 to 1 ratio over Ficoll-Paque Plus (cytiva) and centrifuged at 1440 rpm for 20 minutes at room temperature. The bottom layer containing granulocytes and

erythrocytes was collected and mixed with 20% (W/V) dextran supplemented with 0.15 M NaCl in a 1:2 ratio of dextran to cell volume. The sample volume was then increased with PBS-/- and the erythrocytes were allowed to settle for 40 minutes. The granulocyte supernatant was then isolated via centrifugation and the remaining erythrocytes were lysed using the hypotonic/hypertonic (0.2%; 1.8%) NaCl method. Neutrophils were then isolated via centrifugation and counted on a hemocytometer. Neutrophils purity (98.74%) was confirmed on flow cytometry by staining for CD45-BV605 and CD11b-FITC (BioLegend).

2.6.3 EVOS NETosis Microscopy

Glass bottom 96-well plates were purchased from Cellvis (P96-1.5H-N) and coated with 0.001% poly-l-lysine. Isolated neutrophils were seeded (1E5 cells per well) into a 96-well plate and incubated at 37 °C with or without particles in RPMI media supplemented with 10% donor specific plasma. After 1 hour of incubation, the media and any free-floating cells/particles were gently removed. Cells were then activated with RPMI supplemented with 10% donor specific plasma, 0.05 nM SYTOX Green, and either 100 μg/mL LPS from Escherichia coli O111:B4 or 20 nM Phorbol myristate acetate (PMA). Full wells were then imaged using an EVOS M7000 with an incubator attachment. Images were collected with a 4x objective using the GFP filter cube. The images were tiled and stitched to output full-well images. Additional images were captured using a 20x objective in the brightfield, GFP, and Cy5 channels. From these images neutrophil, SYTOX Green, particle overlays could be used to estimate NETosis probability using MATLAB image processing.

2.6.4 NETosis Image Processing

The images for each individual well and each of the three channels were loaded into the MATLAB workspace.[64] Images were then grouped by well number into an w_{ell} by $n_{channel}$ array. Each image was denoised using a Gaussian convolution. Mean fluorescent intensity (MFI) was calculated for the denoised images by dividing the sum of the pixel values by the number of pixels. Each channel, GFP, Cy5, and brightfield, were thresholded separately using functions from the MATLAB Image Processing Toolbox.[65] The threshold images were binary matrices that mapped the locations of objects (NETs, particles, or neutrophils) as a 1 for a location of the object and a 0 for empty locations. To ensure the thresholding of each channel was correctly performed, random wells were selected for quality control. For random wells, the threshholded images generated were overlayed against the original image to visually ensure the thresholding was done correctly. Each binary matrix was then analyzed for percent well coverage—calculated by dividing the count of nonzero pixel values by the number of pixels—and properties of clusters such as area, major diameter, minor diameter, and perimeter were outputted using the regionprops() function. The binary images were multiplied by a unique scalar for each channel such that the sum of all possible overlapping pixels in a well would result in a unique value (examples of scalars: 1, 4, 9 or 1, 10, 100). This allowed us to add the three channels of each well together to create a master overlay that contained the location information from each channel and all possible overlaps between channels (8 unique codes). The master overlay image matrix for each well was converted into a vector and the count of pixels of each unique code was recorded. All counts, percent coverage, and MFIs for each well were then exported to an Excel document.

2.7 *In Vitro* and *in Vivo* Porcine Models

2.7.1 In Vitro Particle Uptake

Pig blood was purchased from Scholl Slaughterhouse located in Blissfield, MI. Blood was collected in 500 mL containers pre-spiked with heparin to prevent blood coagulation. Blood was immediately transported (45-minute drive) to Ann Arbor, MI and used for uptake experiments. Briefly, blood was aliquoted into 100 µL aliquots and treated with a range of particle concentrations (1E5, 1E6, 1E7, 1E8 particles/mL) in triplicates. After 2 hours of incubation at 37°C, samples were prepared for flow cytometry.

2.7.2 Sample Staining for Flow Cytometry Analysis

Neutrophils were identified on flow cytometry using SWC1 (CD52), CD172a, and SWC8. Primary and secondary antibodies were diluted as suggested by vendors: SWC1 Primary 1:100 (Bio-Rad), SWC1 secondary 1:100 (BV421, Jackson Immuno); CD172a 1:10 (FITC Bio-Rad); and SWC8 Primary 1:20 (Bio-Rad), SWC8 Secondary 1:20 (PerCP Jackson Immuno). Each sample was treated with 0.1 µL SWC1 primary/secondary, 1 µL CD172a, and 2.5 µL SWC8 primary/secondary. To stain the samples, samples were first treated with primary antibodies and incubated on ice for 30 min. Samples were washed with 2 mL 1% BSA in PBS -/- at 400 G for 5 min. Samples were gently resuspended and stained with secondary antibodies for 30 minutes on ice. Samples were lysed/fixed with 1-step lyse/fix (eBioscience). Prior to flow cytometry, samples were washed twice in FACS buffer and resuspended in a final volume of 0.5 mL FACS buffer. Data was collected on an Attune flow cytometer.

2.7.3 Pig Dosage Calculation

In this model we based our scaled particle dosage from mouse to pig on blood volume. Yucatan pigs on average have 62.5 mL/kg of blood. Particle dosage was calculated the day of the experiment by multiplying 62.5 mL/kg by the pig's weight. Both the first and second pig used in this work weighed 43 kg and received 1E8 particles per mL of blood—2.69E11 particles total.

2.7.4 In Vivo Particle Infusion

Yucatan pigs underwent infusion of 67 mL Poly-A particles at 4E9 particles/mL in saline. In the first pig experiment, particles were infused at 10 mL/min into the left external jugular. Due to the adverse reaction to the particle infusion, we moved the infusion site and rate in the next experiment. The optimized infusion site and rate was found to be the lateral saphenous vein at 4 mL/min.

2.7.5 C5a ELISA

Complement protein C5a was measured in plasma samples via a pre-coated ELISA kit (BIOMATIK Cat# EKU03408-96T). This assay was specifically designed for measuring porcine C5a in a sandwich-based ELISA assay. Plasma samples were diluted in PBS -/- by a factor of 20.

2.7.6 Histology

Samples of small intestine (duodenum), lung, liver, kidney, spleen, and trachea were flash frozen in liquid nitrogen and were submitted for blinded histopathologic examination by ULAM in the In Vivo Animal Core.

2.8 Statistics and Data Analysis

Statistical analysis was performed using GraphPad Prism Software using either One-Way or Two Way ANOVA with Fisher's LSD Test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. n.s. indicates "not significant".

Chapter 3 Poly-Salicylic Acid Polymer Microparticle Decoys Therapeutically Treat Acute Respiratory Distress Syndrome

3.1 Publication Information

The work shown in this chapter is adapted from work published in Advanced Healthcare Materials titled "*Polysalicylic Acid Polymer Microparticle Decoys Therapeutically Treat Acute Respiratory Distress Syndrome*" with author list **Emma R. Brannon**, William J. Kelley, Michael W. Newstead, Alison L. Banka, Kathryn E. Uhrich, Colleen E. O'Connor, Theodore J. Standiford, Omolola Eniola-Adefeso. Additionally, art illustrating our work was selected for the cover of Advanced Healthcare Materials and displayed in the Chapter 3 appendix (Section 3.7).

Citation: Brannon, E. R., et al. (2022). "Polysalicylic Acid Polymer Microparticle Decoys Therapeutically Treat Acute Respiratory Distress Syndrome." Advanced Healthcare Materials **11**(7): 2101534.

3.2 Abstract

Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS) result from inflammation of the lungs and lead to high mortality rates, often as a secondary disease. Neutrophils play a large role in propagating ALI and ARDS yet no therapeutics in the clinic target inflammation that results from neutrophilic damage. Particle-based therapeutics are known to interact with neutrophils in the blood stream, thus in this work we examine the application of utilizing particles to treat inflammation localized to the lungs. Particles fabricated from

polymerized salicylic acid termed “PolyAspirin” or Poly-A were found to reduce neutrophil accumulation in the lungs in murine models of ALI and ARDS. Furthermore, we found that the therapeutic backbone of the polymer alters neutrophil surface protein expression, enhancing the diversion of neutrophils over other particle types. This work represents an entirely novel therapeutic strategy for ALI/ARDS, enhancing the treatment options for many systemic inflammatory diseases.

3.3 Introduction

Acute, systemic inflammatory diseases are difficult to treat and are associated with high mortality rates. Among these, both acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are especially problematic and deadly; in a 2001 study, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) accounted for 41% of postoperative deaths,[66] while 2016 study revealed the worldwide mortality rate of ARDS alone to be as high as 40%.[67] Although methods of treating ARDS and ALI symptoms have improved, mortality rates remain high, establishing the need for new treatment methods and potential therapeutics.[68,69] Part of the difficulty with developing reliable therapeutics for ARDS lies in the various conditions that lead to ARDS, including pneumonia, systemic bacterial infection, severe burns, or viral infections.

Regardless of the primary cause, ARDS is characterized by an unrestrained innate inflammatory response, where a high count of neutrophils in the lung bronchoalveolar lavage fluid (BALF) in ARDS patients strongly correlates with disease severity and mortality.[33,70-73] This is largely due to the nature of the neutrophil response to acute inflammation; as inflammatory stimuli are detected, circulating neutrophils are rapidly recruited to the site of inflammation *via* the upregulation of cellular adhesion molecules (CAMs) such as E-Selectin, P-Selectin, ICAM-1, and VCAM-1 on the surface of endothelial cells. Ligands on the neutrophil surface bind to the

upregulated CAMs, allowing the neutrophils to roll along the endothelial surface, eventually resulting in firm adhesion and trans endothelial migration.[74-76] As neutrophils transmigrate into the alveolar space, they damage the lung endothelium and epithelium, eventually resulting in a "leaky" lung vasculature through which fluids, proteins, and pathogens can potentially migrate, exacerbating the inflammation.[33] Once present in the alveolar space, neutrophils release cytotoxic materials such as neutrophil extracellular traps (NETs), reactive oxygen species (ROS), and neutrophil elastase, all of which have been shown to contribute to the severity of ALI/ARDS.[77-81]

While over-stimulation of the immune system plays a major role in diseases such as ALI and ARDS, no therapeutics exist to treat the immune-related symptoms. Many clinical trials testing immunotherapeutic proteins have failed, potentially due to the complexity of immune-signaling agents, redundancies in the inflammatory pathway, and heterogeneity of ARDS and ALI in patients.[82] However, it has been shown in preliminary publications and research that circulating immune cells can also be blocked by intravenously-administered, drug-free, non-targeted polymeric microparticles in applications ranging from experimental autoimmune encephalitis (EAE), West Nile Virus, myocardial infarction, spinal cord injury, and ALI.[43,83-85] Thus, such particles can potentially be designed to prevent the excessive accumulation of rogue neutrophils in ALI/ARDS applications, ameliorating the potential for any inflammatory damage while allowing for disease resolution to take place.

Polymeric drug delivery particles such as poly(lactic-co-glycolic acid) (PLGA)-based particles have already achieved FDA approval and are used clinically for delivering active pharmaceutical agents in cancer applications.[86] Importantly, hydrolysis of PLGA-based polymeric particles produces biocompatible byproducts, lactic acid and glycolic acid.[87,88] Minimal systemic toxicity is expected from PLGA-based polymeric particles, but even minimal toxicity could be

harmful when treating an inflammatory disease.[88] For this reason, it is important to consider other polymers as the base of a polymeric particle treatment for inflammatory conditions.

In this work, salicylate-based poly(anhydride-ester) polymers ("PolyAspirin" or "Poly-A") were fabricated into micron-sized spheres and evaluated as a potential therapeutic for inflammation resolution. Poly-A degrades into the active component of aspirin, salicylic acid; it is hypothesized that Poly-A particles may confer therapeutic effects in ALI and ARDS.[89,90] Non-degradable polystyrene and degradable PLGA particles were used as controls to evaluate the therapeutic effect of Poly-A. In both an endotoxin and bacterial murine model of ALI, Poly-A particles significantly cleared neutrophils from the lungs while also reducing the inflammatory effects of ALI. From *in vitro* assays we found that phagocytosis of Poly-A reprograms neutrophils, changing the surface protein expression to a less inflammatory state. Together these *in vivo* and *in vitro* findings emphasize the importance in using anti-inflammatory materials for designing particle-based therapeutics to treat inflammation.

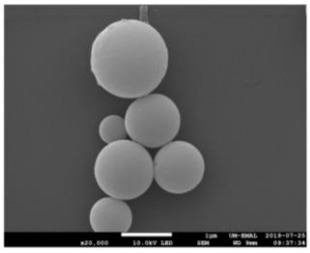
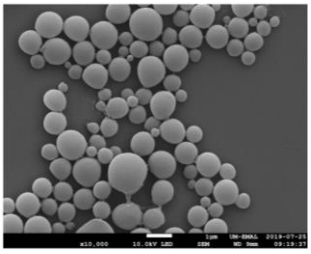
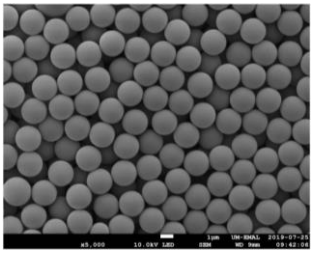
3.4 Results

3.4.1 Particle Characterization

The particles used in this work had smooth surfaces and similar surface charges as characterized by SEM and shown in Table 3.1. Both the Poly-A and PLGA particles were similar in size, averaging 0.9 and 0.8 μm in diameter. The polystyrene (PS) particles used in this work were twice the size of Poly-A and PLGA to compare the results from this work to results published by Fromen et al.[42] Particle degradation was characterized in PBS at 37°C. As shown in Figure 3-1 particles degrade slowly over the period of multiple days.

We conducted a biodistribution study to examine the predominant organs of particle accumulation post intravenous injection (Figure 3-2). From this study we found that both Poly-A and PLGA particles accumulate in the liver post injection. This result was expected based from previous work by Fromen et al. that showed particles injected intravenously accumulate in the liver.[42]

Table 3-1 Particle Characterization

	1 µm Poly-A	1 µm PLGA	2 µm PS
SEM Image			
Size	0.9 ± 0.30 µm	0.8 ± 0.30 µm	2.0 ± 0.16 µm
Zetapotential	-27.2	-31.8	-44.2

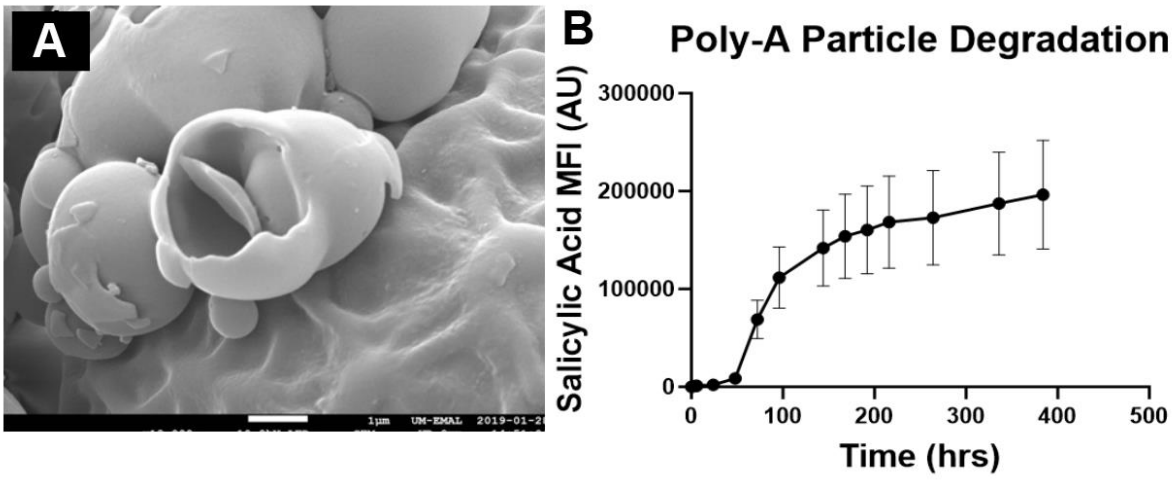


Figure 3-1 Degradation of Poly-A particles in PBS -/- at 37°C.

Particles were rotated at 37°C in PBS for 17 days to determine the extent of degradation. (**A**) SEM image of particles after 13 days of degradation. (**B**) Hydrolytic degradation profile of Poly-A particles as measured by the release of salicylic acid.

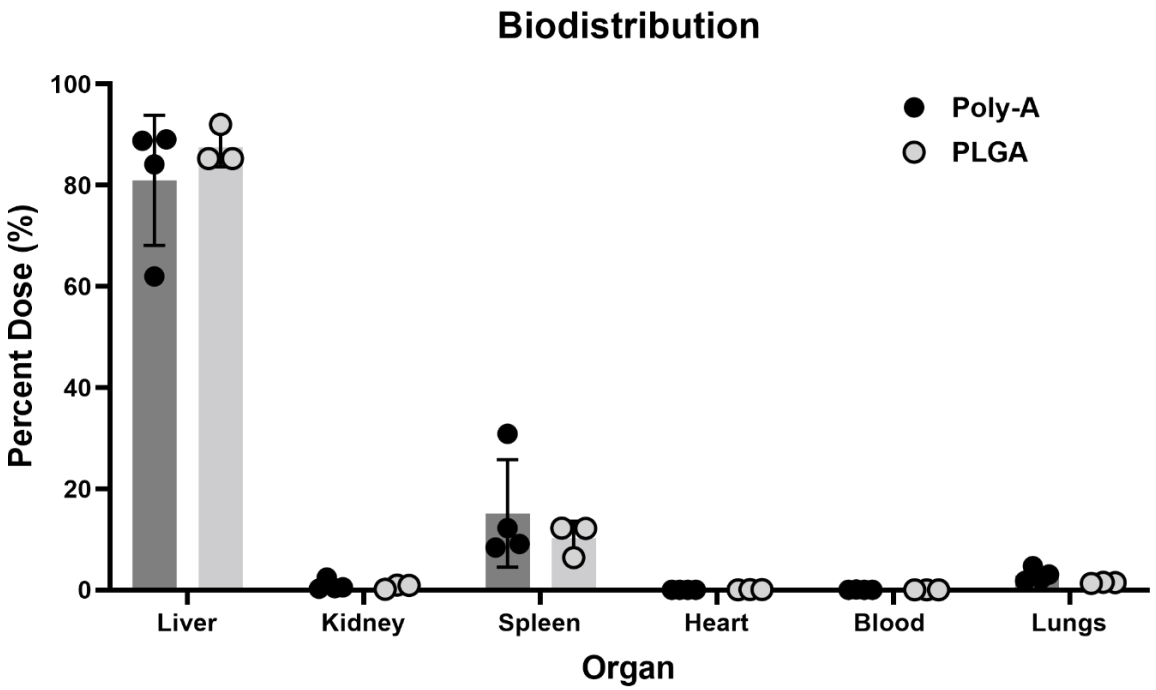


Figure 3-2 Biodistribution of Poly-A and PLGA particles in mice 30-minutes post-injection.

C57BL/6J Mice were injected with particles 2 hours after intratracheal LPS administration and sacrificed 30 minutes after particle injection. Particle distribution was quantified using a fluorescence signal.

3.4.2 Investigation of Various Particle Types in Treating ALI

Previous work by Fromen et al. has shown that drug-free particle-based divert neutrophils, preventing accumulation at sites of localized lung inflammation. In previous work, it was noted that C57BL/6 mice had a reduced inflammatory response to BALB/Cj mice, and the resulting reduction of neutrophils in the bronchoalveolar lavage fluid (BALF) was less extensive.[42] We hypothesized that by utilizing a particle-based material that has additional anti-inflammatory benefit, we would further reduce the neutrophil accumulation in the lungs. Prior to beginning these experiments, we characterized the influx of neutrophils into the BALF to ensure that the particle intervention was injected on the correct timeline for optimal neutrophil diversion (Figure 3-3). From these preliminary experiments, we found that C57BL/6 mice have a similar neutrophil

response to BALB/Cj, but the response is slower and neutrophil accumulation in the BALF peaks at a later timepoint—6-hrs post instillation (Figure 3-3D and E).

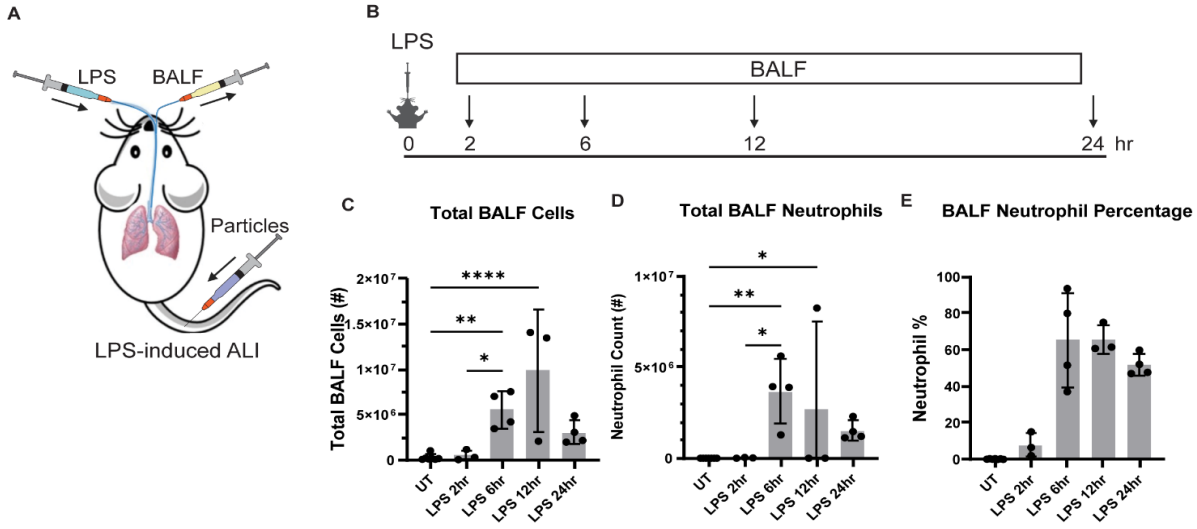


Figure 3-3 Characterizing the influx of neutrophils into the BALF of C57BL/6 Mice.

(A) Experimental overview of treatments and sampling. **(B)** Experimental timeline for characterizing BALF at different time points. Samples were taken 2-, 6-, 12-, and 24-hrs post LPS instillation **(C)** Total cell accumulation in BALF over time. **(D)** Neutrophil counts in the BALF at each time point**. (E)** Percentage of neutrophils in the BALF at each time point. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD Test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Based on the characterization curve, we chose to investigate multiple injection timelines to understand the best intervention scheme for particle-based therapeutics in diverting neutrophils (Figure 3-4). We injected particles (Poly-A, PLGA, or polystyrene) either 2-hrs or 4-hrs post LPS instillation and harvested BALF 2-hrs post injection. Comparing the injection timeline, we found that injecting earlier led to more significant reductions in total cells and neutrophils in the BALF (Figure 3-4C and D). Injecting later, 4-hrs post LPS, led to a loss in significant reduction of PS particle treatments on neutrophil accumulation in the BALF (Figure 3-4F). This loss in

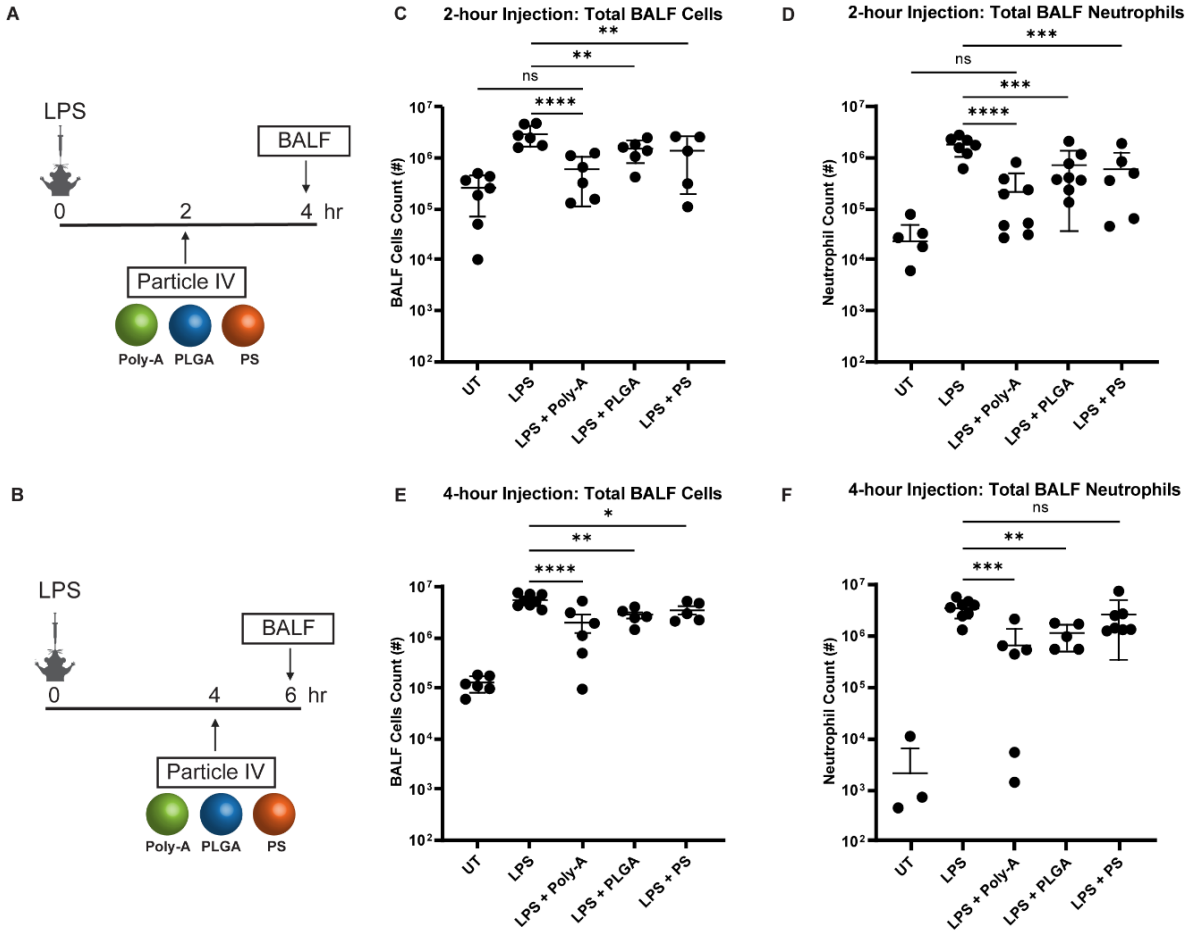


Figure 3-4 Modulation of neutrophil accumulation in the lungs with various particle types.

(A-B) Dosing/harvest schedule for the 2-hour and 4-hour injections in C57BL/6 mice. **(C)** Total BALF cells and (**D**) neutrophils for the 2-hour injection time experiments for untreated (UT) mice, LPS-only, LPS + Poly-A (PA) particles, LPS + Poly lactic-co-glycolic acid (PLGA) particles, and LPS + polystyrene (PS) particles. **(E)** Total BALF cells and (**F**) neutrophils for the 4-hour injection time experiments. All particles were injected at 2 x 10^8 per mouse. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. n.s. indicates "not significant."

significance for the non-degradable control suggests the importance of degradation byproducts in modulating neutrophil behavior. Both Poly-A and PLGA significantly reduced neutrophil accumulation in both injection timelines as shown in Figure 3-4D and F. The reduction in neutrophil accumulation was more significant for Poly-A particles, and could be due to the enhanced anti-inflammatory properties of Poly-A. This difference in therapeutic benefit suggests that degradable materials with anti-inflammatory degradation products may provide additional benefit rather than neutrophil diversion alone.

3.4.3 Impact of Particle Material in Treating ARDS

Next, we sought to investigate particle treatments in a more complex model of lung inflammation, ARDS. To model ARDS we infected the lungs of C57BL/6 mice with live *P. aeruginosa.* Similar to our ALI experiments, we first characterized the neutrophil influx into the lungs over time (12-, 18-, 24-, 30-, 36-hrs) post infection (Figure 3-5).

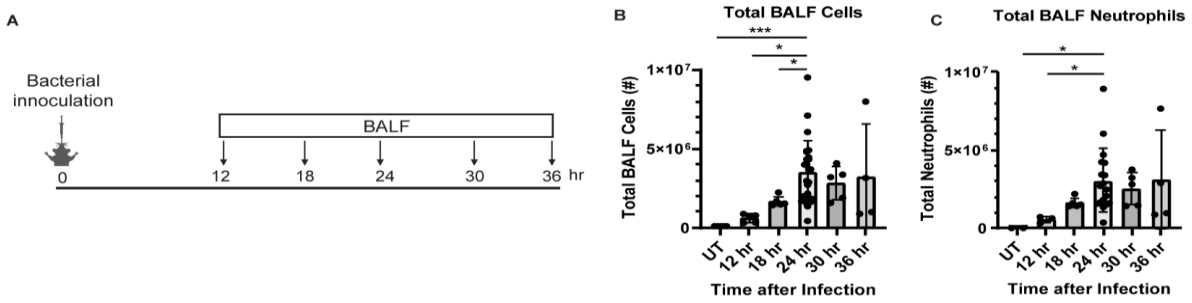


Figure 3-5 Characterization of P. aeruginosa lung infection in C57BL/6J mice.

*(*A) Representation of the timeline of bacterial infection and BALF sampling. (B) Total cells in BALF 12, 18, 24, 30, and 36 hrs post-infection. (C) Total neutrophils in BALF 12, 18, 24, 30, and 36-hrs post-infection. (D) Timeline for 6-hour particle injection and 24-hour BALF collection. (E) Total BALF cells and (F) neutrophils collected post 6-hour injection timeline. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD Test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

The neutrophil influx was found to peak in the BALF at 24-hrs post infection, with the neutrophil influx beginning at 12-hrs post infection. From the initial characterization study, we hypothesized that 18-hrs post infection would be the optimal time to inject particles for neutrophil diversion. Thus, we chose to inject either Poly-A, PLGA, or polystyrene particles 18-hrs post *P. aeruginosa* infection and evaluate the BALF 6-hrs post particle injection (Figure 3-6A). Using this treatment scheme, we found that only Poly-A particles reduced the accumulation of total cells and neutrophils in the BALF (Figure 3-6B and C). We measured lung damage by quantifying protein (IgM and albumin) leakage into the BALF. Poly-A lead to a significant reduction in both

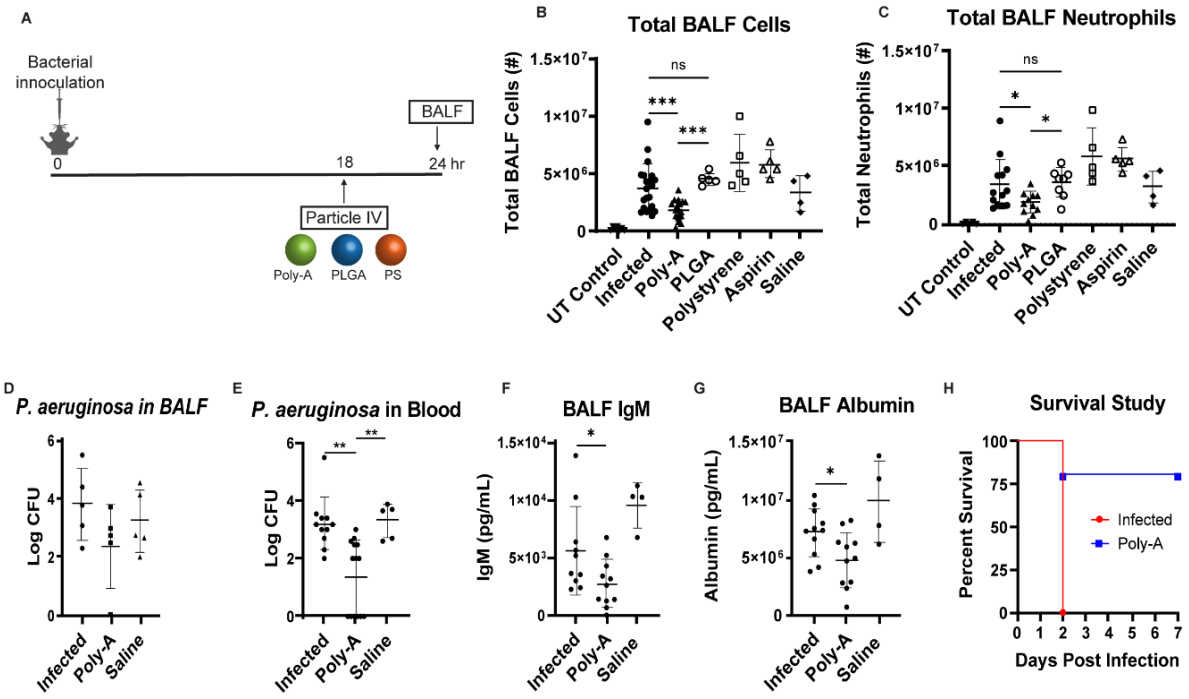


Figure 3-6 Impact of Poly-A particle injection in ARDS model.

(A) Representation of the timeline of particle treatment and BALF sampling relative to the time of P. aeruginosa infection in C57BL/6 mice. (**B**) Total cells and (**C**) neutrophils in the BALF 24 hours post-infection after 18-hr injection of Poly-A, PLGA, polystyrene, or soluble aspirin compared to infected mice with no treatment and saline controls. UT control implies mice with no LPS or particle treatment. **(D)** Bacterial CFU in BALF collected at 24 hours post-infection for mice treated with Poly-A or saline at 18-hr post-infection. **(E)** Bacterial CFU in blood collected at 24 hours post-infection for mice treated with Poly-A or saline at 18-hr post-infection. (**F**) IgM and (**G**) albumin level in BALF collected at 24 hours post-infection for mice treated with Poly-A or saline at 18-hr post-infection. **(H)** Post-infection survival (N=5 for each group) for P. aeruginosa infected mice with and without 18-hour Poly-A injection. All mice received 2 x 10^8 particles for every particle type. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

IgM and albumin in the BALF, suggesting a reduction in lung damage (Figure 3-6F and G). Reductions in blood CFU of *P. aeruginosa* further show that the lung vasculature remained intact for mice treated with Poly-A, as compared to saline treated mice. Importantly, in a survival study, we found that an 18-hour Poly-A particle injection significantly improved survival rates. Infected mice with no treatment died within 48 hours post-infection, while 80% of the mice receiving Poly-A injection lived out to one week. No bacterial CFU was detected in the blood or BALF samples of the surviving Poly-A treated mice, indicating recovery.

Next, we investigated whether the Poly-A particle treatment alters the BALF level of inflammatory markers typically predictive of human ARDS mortality.[91-94] We used ELISAs to measure the levels of IL-10, KC (CXCL1), MCP1 (CCL2), MIP2 (CXCL2), TNF, and IL-6 in the BALF (Figure 3-7). Poly-A particle injection significantly reduced the levels of KC, MCP1, TNF, and IL-6 by ~94%, ~81%, ~68%, and ~81%, respectively, as compared to infected controls. This result suggests that Poly-A particles' administration and the corresponding reduction of neutrophils in the airways alleviates the lungs' inflammatory response, both directly and indirectly, highlighting the Poly-A particles' potential as an effective therapeutic for ARDS.

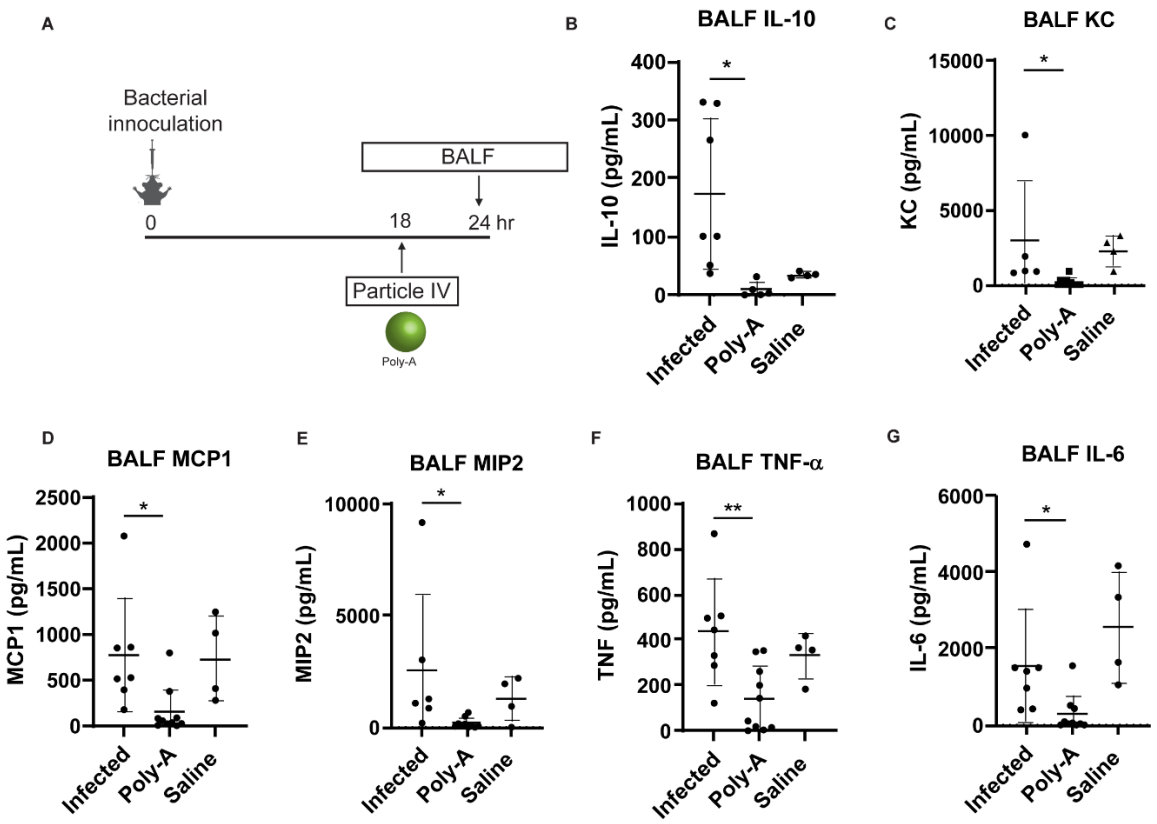


Figure 3-7 Cytokine and protein content in BALF of ARDS mice after Poly-A injection, as measured by ELISA.

(A) Schematic of Poly-A particle treatment and BALF protein sampling relative to the timing of bacterial infection. The concentration of (B) IL-10, (C) KC, (D) MCP1, (E) MIP2, (F) TNF-a, and (G) IL-6 in the BALF of P. aeruginosa infected mice. Mice were injected with either 2 x 10^8 Poly-A particles or saline at 18 hours post-infection, and samples were collected 24 hours post-infection. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$.

3.4.4 Impact of Particle Phagocytosis on Neutrophil Surface Protein Expression

Due to the success of Poly-A in preventing neutrophil accumulation in both ALI and ARDS, we sought to investigate the cellular mechanism of Poly-A's function. To do this we utilized whole blood from healthy donors to investigate particle material effects on neutrophil activation. More specifically, we exposed neutrophils to particles and measured the resulting changes in surface protein expression (Figure 3-8A). Uptake experiments in whole human blood showed that Poly-A particle internalization reverted neutrophils from an activated state to an un-activated state. We measured particle uptake (Figure 3-8B) and activation via surface protein expression of L-selectin (CD62L) and PSGL-1 on neutrophils (Figure 3-8C and D). Both CD62-L and PSGL-1 are shed

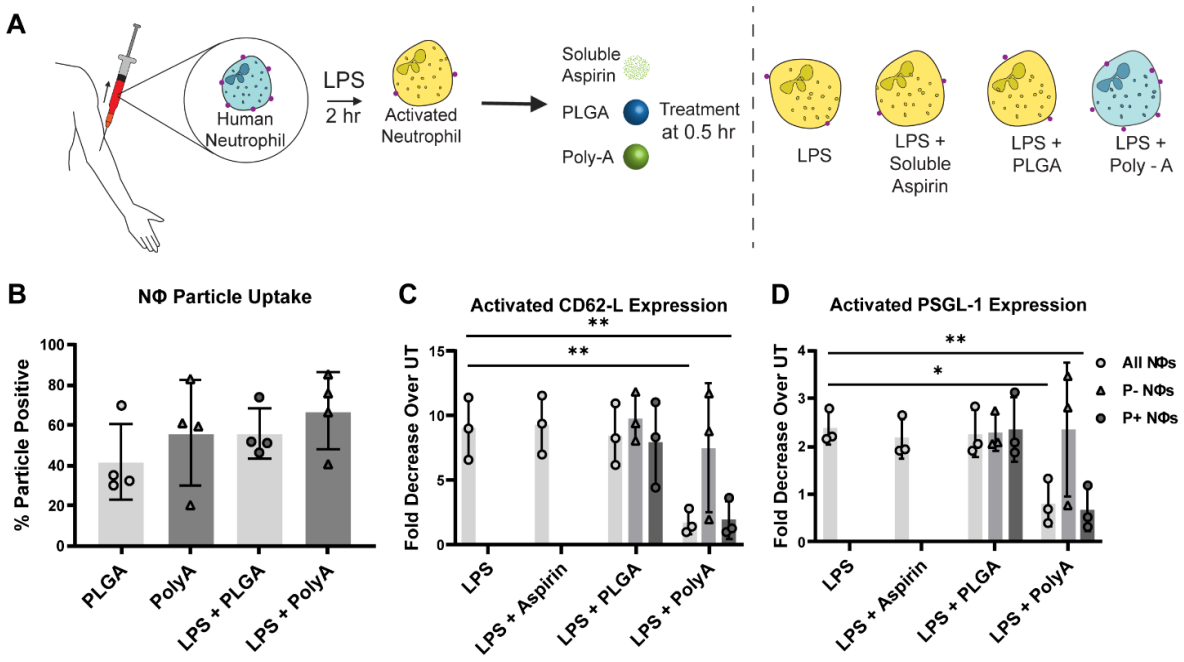


Figure 3-8 Impact of Poly-A particle phagocytosis on surface protein expression by human neutrophils.

(A) Schematic depiction of L-selectin surface expression (red dots) by neutrophils in naïve or LPS-activated cells with or without particle treatment. **(B)** The fraction of neutrophils that internalized particles in whole blood. (**C**) Fold decrease in L-selectin (CD62-L) surface expression by neutrophils (NΦ) in whole blood exposed to LPS only, LPS + Aspirin, LPS + PLGA, and LPS + Poly-A particles relative to neutrophils in untreated (UT), non-LPS activated blood. **(D)** Fold decrease in PSGL-1. Each 100 µL blood sample received 2E6 particles for every particle type 30 min after LPS activation, and the protein expression was evaluated at 2 hr after LPS activation. Three human donors were used for this experiment, n=3. P- NΦs = Particle negative neutrophils; P+ Nøs = Particle positive neutrophils. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$.

from the neutrophil surface during activation.[8,9] The shedding of CD62L and PSGL-1 helps neutrophils to transmigrate more easily to reach sites of inflammation *in vivo*.[8,9] Expression was plotted as fold change over untreated samples to normalize values across experiments. LPS induces a 10-fold decrease in CD62-L expression, but Poly-A positive LPS stimulated neutrophils had no fold change compared to untreated. Similarly, LPS induces a 2-fold decrease in PSGL-1 expression on neutrophils (NΦ*)s*. Poly-A positive neutrophils stimulated with LPS, however, have no fold decrease in PSGL-1 expression. PLGA particles were just as frequently phagocytosed by neutrophils as shown in Figure 3-8B but did not have the same anti-inflammatory effects. Soluble aspirin also failed to prevent CD62-L or PSGL-1 shedding. The particles may release a high localized concentration of salicylic acid within the neutrophil causing the observed anti-inflammatory effects. In addition to the anti-inflammatory changes in protein expression, we also observed a 4-fold increase in expression of LFA-1, a surface protein utilized for neutrophil transmigration (Figure 3-9). After observing such drastic changes in surface protein expression, we suspected that these changes influenced the increased neutrophil diversion from BALF seen in Poly-A treated ALI mice.[27]

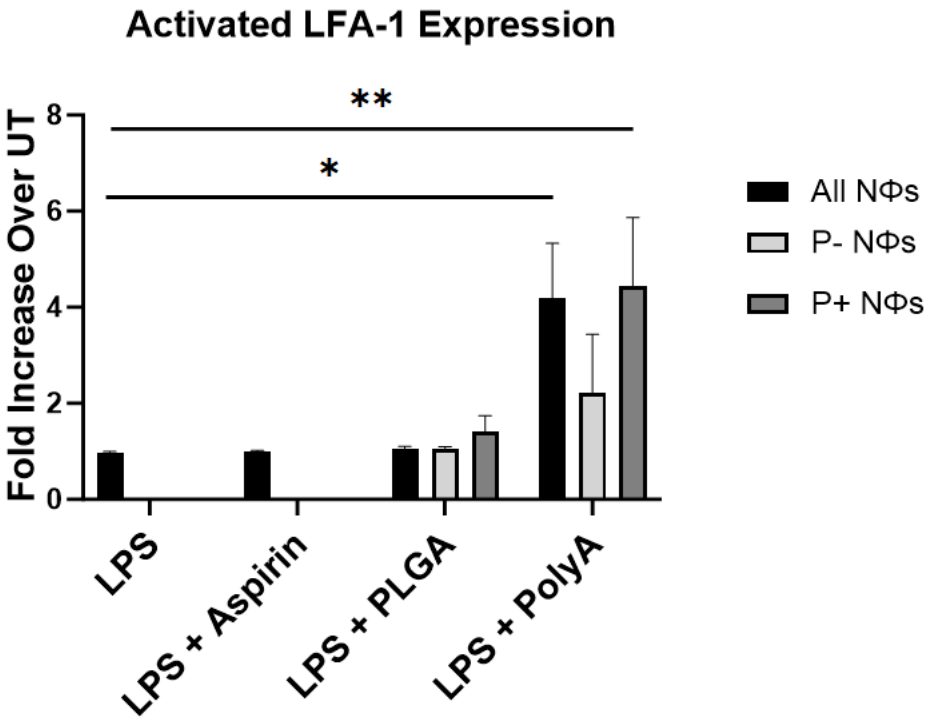


Figure 3-9 Neutrophil expression of LFA-1 in activation conditions with particle treatment.

Neutrophils were activated with LPS and treated with soluble aspirin, PLGA particles, or Poly-A particles. Flow cytometry was then used to evaluate the expression of LFA-1.

3.4.5 Understanding the Effects of Neutrophil Surface Protein Expression and Diversion of Neutrophils

We conducted a set of experiments to evaluate neutrophil diversion to the liver and surface protein expression of Poly-A positive neutrophils once in the liver (Figure 3-10A). As expected, particle treated mice had reduced circulating neutrophils compared to LPS only mice (Figure 3-10B). Poly-A treated mice had significantly greater neutrophil counts in the liver compared to untreated mice, unlike PLGA (Figure 3-10C). When looking at particle positive neutrophils in the liver, it was found that there was no difference between Poly-A and PLGA mice, suggesting that Poly-A influences overall neutrophil accumulation in the liver (Figure 3-10D). PLGA positive blood neutrophils had a significant fold decrease in PSGL-1 expression compared to LPS only (Figure 3-10E). In the liver, however, Poly-A particles had significant preservation of PSGL-1 expression compared to LPS only mice (Figure 3-10F).

While our *in vitro* human studies revealed significant preservation of CD62L on neutrophils post-phagocytosis of Poly-A particles. However, looking at neutrophils harvested from mouse blood or liver, we did not see enhanced expression of CD62L in Poly-A positive neutrophils (Figure 3-11).

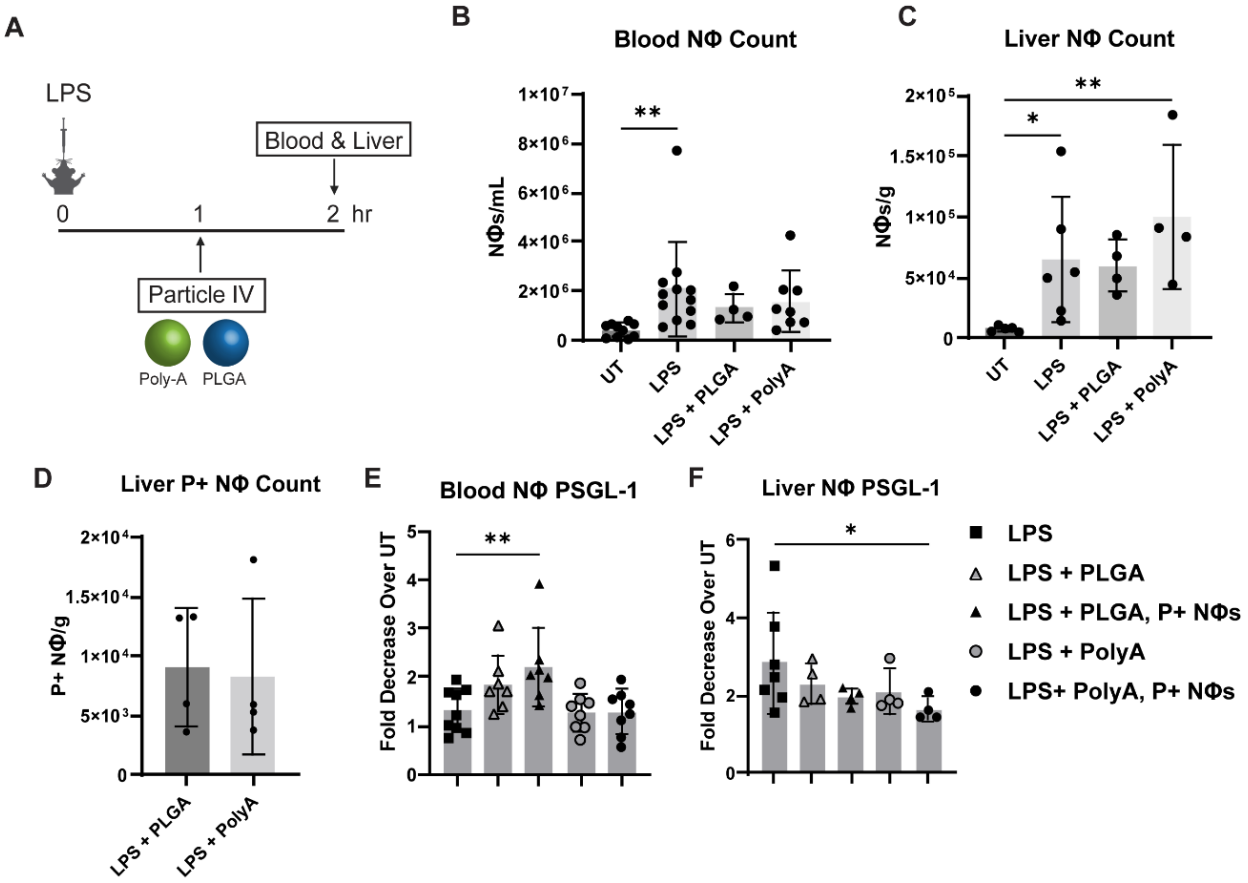


Figure 3-10 In vivo impact of Poly-A particles on neutrophil accumulation and surface protein.

(A) Schematic showing experimental timing and set up. (B) Neutrophil (NΦ) concentration in blood 2-hours post LPS instillation and 1-hour post-injection. (C) Neutrophil accumulation in the liver post LPS instillations and particle injections. (D) Particle Positive neutrophil accumulation in the liver. Fold decrease in PSGL-1 expression compared to untreated neutrophils in the (E) blood and (F) neutrophils isolated from the liver. P+ Nøs = Particle positive neutrophils. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$. N≥3 for this experiment.

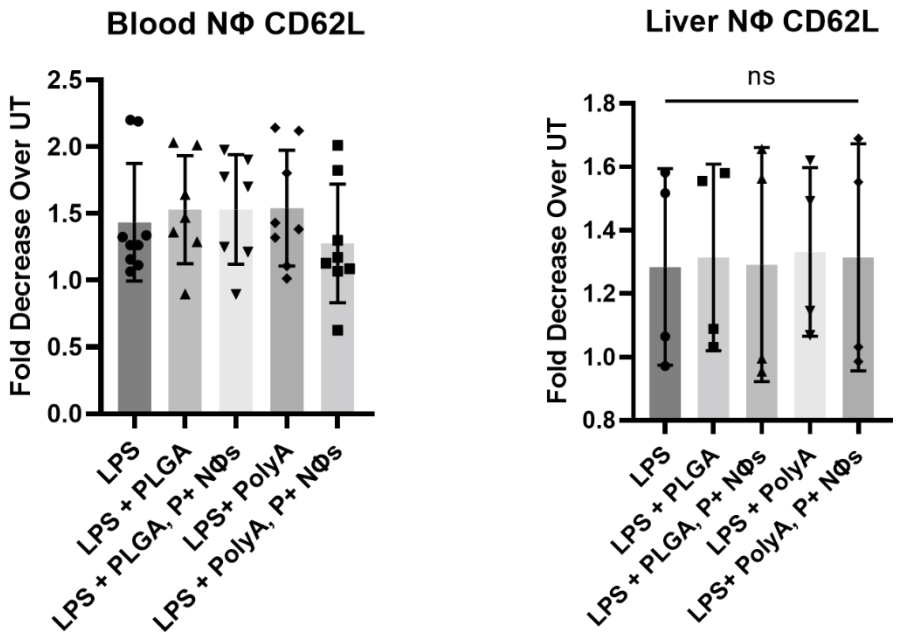


Figure 3-11 CD62L expression on mouse neutrophils harvested from blood and liver.

Particle-positive neutrophils in **(A)** blood, **(B)** liver, and **(C)** lungs. Fold decrease of L-selectin on **(G)** blood neutrophils and **(H)** neutrophils isolated from the liver *P+ Nøs = Particle positive neutrophils* Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD Test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. n.s. indicates "not significant."

3.5 Discussion

In this chapter, I investigated the functionality of Poly-A particles to treat inflammatory disease of the lungs: ALI and ARDS. We hypothesized that Poly-A particles would divert neutrophils from the site of inflammation through physical interactions, allowing local tissue immunity to clear the primary source of injury. Poly-A particles more effectively reduced neutrophil accumulation in the BALF over PLGA and polystyrene particles in both LPS- and *P. aeruginosa*-induced ALI/ARDS. In the case of *P. aeruginosa* only Poly-A particles significantly reduced neutrophils infiltration and overall lung damage of infected mice. Survival of mice infected with *P. aeruginosa* was enhanced with Poly-A particles treatment. This enhanced survival of mice treated with Poly-A particles could be due to reductions in lung inflammatory cytokines and lung damage, as indicated

by a reduction in IgM and albumin in the BALF.

From these initial *in vivo* studies, we hypothesized that Poly-A particles alleviate inflammation in ALI/ARDS by multiple mechanisms. We know from previous studies that particles function in diverting neutrophils via particle-neutrophil collisions that lead to neutrophil phagocytosis of particles in the bloodstream, ultimately diverting particle-laden cells to the liver.[85,95] This work further expanded the phenomena of particle-neutrophil diversion to include degradable particles, including both Poly-A and PLGA, in models of ALI/ARDS.

It is known that the exaggerated pro-inflammatory cycle seen in ARDS is propagated by neutrophils present in the airways as neutrophils contribute to cytokine release (TNF-α) that further induces other immune cells to an inflammatory state.[96] By reducing neutrophil lung infiltration we demonstrated a reduction in lung damage as measured by IgM and albumin—both IgM and albumin are major components of blood, not typically found in BALF unless the vasculature has become leaky due to lung damage.[33,73] Further, the reduction of *P. aeruginosa* CFUs in the blood supports the hypothesis that Poly-A prevented lung damage leading to leaky vasculature.

In addition to neutrophil diversion, Poly-A particles exert added therapeutic benefits in ALI/ARDS relative to other particle types. Neutrophils shifted to a quiescent state after uptake of Poly-A particles, as indicated by changes in crucial neutrophil cell adhesion molecules' expression. According to the literature, LPS stimulation induces rapid neutrophil shedding of L-selectin, thus by measuring L-selectin shedding we can understand the activation level of the neutrophil.[97-101] Similarly, a few studies have reported that PSGL-1 expression is downregulated in human neutrophils *in vivo* in response to systemic inflammation caused by endotoxin infusion in healthy volunteers and *in vitro* by LPS in anticoagulated blood.[102] Thus, the observed preservation of L-selectin and PSGL-1 expression on neutrophils that phagocytosed Poly-A

particles in LPS-activated human blood and PSGL-1 expression on neutrophils *in vivo* highlights an anti-inflammatory effect. In this work we found that Poly-A particles also induce enhanced expression of LFA-1. LFA-1 is necessary for neutrophils slow rolling and transmigration, but without the shedding of L-selectin and PSGL1, this function would be hindered.[103]

Interestingly, the measurable impact of Poly-A on human neutrophils expressed L-selectin was not observed in mouse neutrophils *in vivo.* Given that L-selectin expression can be readily perturbed, we suspect that the tissue dissociation process necessary to obtain single cells needed for flow cytometric analysis was enough to cause significant L-selectin shedding across all neutrophils in blood obtained from particle treated mice, limiting our ability to collect accurate measurements for *in vivo* assays.

Soluble aspirin did not impact neutrophil lung localization and adhesion molecule expression both *in vitro* and *in vivo,* suggesting the importance of the Poly-A particle form. While others have reported aspirin sheds L-selectin expression on neutrophils, these studies were conducted with isolated human neutrophils and aspirin at non-physiologically relevant concentrations, up to 1000 μg/mL.[104,105] Furthermore, aspirin itself was found not to be the culprit for L-selectin shedding.[106] Instead, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activation via aspirin at the plasma membrane induces superoxide anion production resulting in L-selectin downregulation.[106] Conversely, Poly-A particles are entirely phagocytosed; thus, we hypothesize that this may provide the necessary burst of salicylic acid needed to modify inflammation response in neutrophils. Intracellularly, the particles are phagocytosed into a phagosome, and unlike macrophages, neutrophil phagosomes are known to experience a pH increase to between 9 -10 up to 30 min after phagocytosis.[107] It is also known that Poly-A breakdown is accelerated in neutral basic environments, thus we suspect enhanced salicylic acid

release within neutrophils immediately after phagocytosis.[54] Given that Poly-A particles in solution *in vitro* did not release an appreciable amount of salicylic acid in the 2-hr period of the particle-blood assays, we do not expect extracellular activity of Poly-A; hence we saw no change in neutrophils that did not uptake Poly-A *in vitro*.

An interesting finding in this study is that the use of intravenously administered particles as a therapeutic for ALI/ARDS (and possibly other acute inflammatory conditions) depends heavily on the time of injection relative to the onset of inflammation, similar to other studies showing that the time of intervention in lung inflammation is crucial to treatment efficacy. Given that the inflammatory response in human ARDS occurs on an extended timeline compared to mice, it is likely that the therapeutic window in humans is also much more expansive. Additionally, patient BALF assessment is a diagnostic method of ALI and lung injury severity in the clinics, suggesting an avenue for identifying patients for Poly-A particle therapeutic.[108] Thus, intervention via Poly-A particle injection would be possible at the point of clinical presentation of ARDS.[109,110] Notably, the mapping of the Poly-A particle efficacy to the kinetics of neutrophil lung infiltration in both the LPS and *P. aeruginosa* ARDS model would suggest these particles would be efficacious in ARDS regardless of the underlying cause, including in respiratory viral infections. Indeed, a few recent studies have reported elevated levels of neutrophils and neutrophil extracellular traps in the blood and lungs of COVID19 patients, which is linked to poor prognosis.[111-115]

The current standard of care for ARDS patients is mechanical ventilation for gas exchange, which in some cases can further injure the lungs.[93,116] Microparticle-based neutrophil-blocking therapeutic approach for ARDS is promising in that it acts on a subset of cells involved in the triggering of cytokine storm in ARDS – neutrophils – directly. A significant concern for the

neutrophil-blocking approach with Poly-A is the possibility of propagating the primary disease with reduced presence of neutrophils. Nevertheless, our results showed a positive outcome, i.e., significantly reduced lung injury and mortality, with the Poly-A particle treatment even with an active bacterial infection present in the lungs. Furthermore, with redundancies in the inflammatory cascade single-molecule or cytokine blocking pharmaceuticals also have the potential of prolonging recovery due to non-specific binding or signaling.[33,117]

3.6 Conclusion

In conclusion, we have shown that Poly-A particles are a novel therapeutic that could greatly improve the current state of care for patients with acute inflammatory diseases. Future work investigating various particle parameters (e.g., concentration, size, shape, and polymer chemistry) and dosing strategy (single versus multiple treatments) may shed more light on the optimal formulation for maximum effect.[85,95,118,119] We expect that Poly-A particles would function as a tool to enhance current methods of treatment e.g., antibiotics or antivirals to enhance the therapeutic response. That is, the Poly-A particles could prevent neutrophil-associated lung damage while allowing antibiotics/antivirals to control infection. The impact of Poly-A particles may also extend beyond neutrophils. For example, previous works have shown that COX-2 inhibitors such as aspirin decrease the formation of neutrophil-platelet aggregates in inflammation, which may decrease cytokine production by neutrophils.[120-122] To fully establish the therapeutic mechanism of Poly-A particles in ALI/ARDS, further *ex vivo* and *in vivo* experiments investigating physiologic effects of Poly-A are necessary.

Lastly, the bulk of the results here are based on murine models of ALI/ARDS, which though are useful first tools for developing potential therapies, lack in complexity relative to humans. All the same, our preliminary observation of an anti-inflammatory impact of Poly-A

particles with human neutrophils and our prior work demonstrating a similar particle phagocytosis pattern in mouse neutrophils suggest the results obtained here are likely relevant in humans.[118] From this work, it is important to investigate other inflammatory pathways in correspondence to Poly-A treatment. We suspect that the inhibition of inflammatory pathways may apply to more than L-selectin shedding, i.e. NETosis. Future work confirming the safety of Poly-A particle infusions is necessary to ensure safe translation from initial *in vivo* mouse studies to the clinic.

3.7 Appendix

3.7.1 Cover Illustration from Published Manuscript

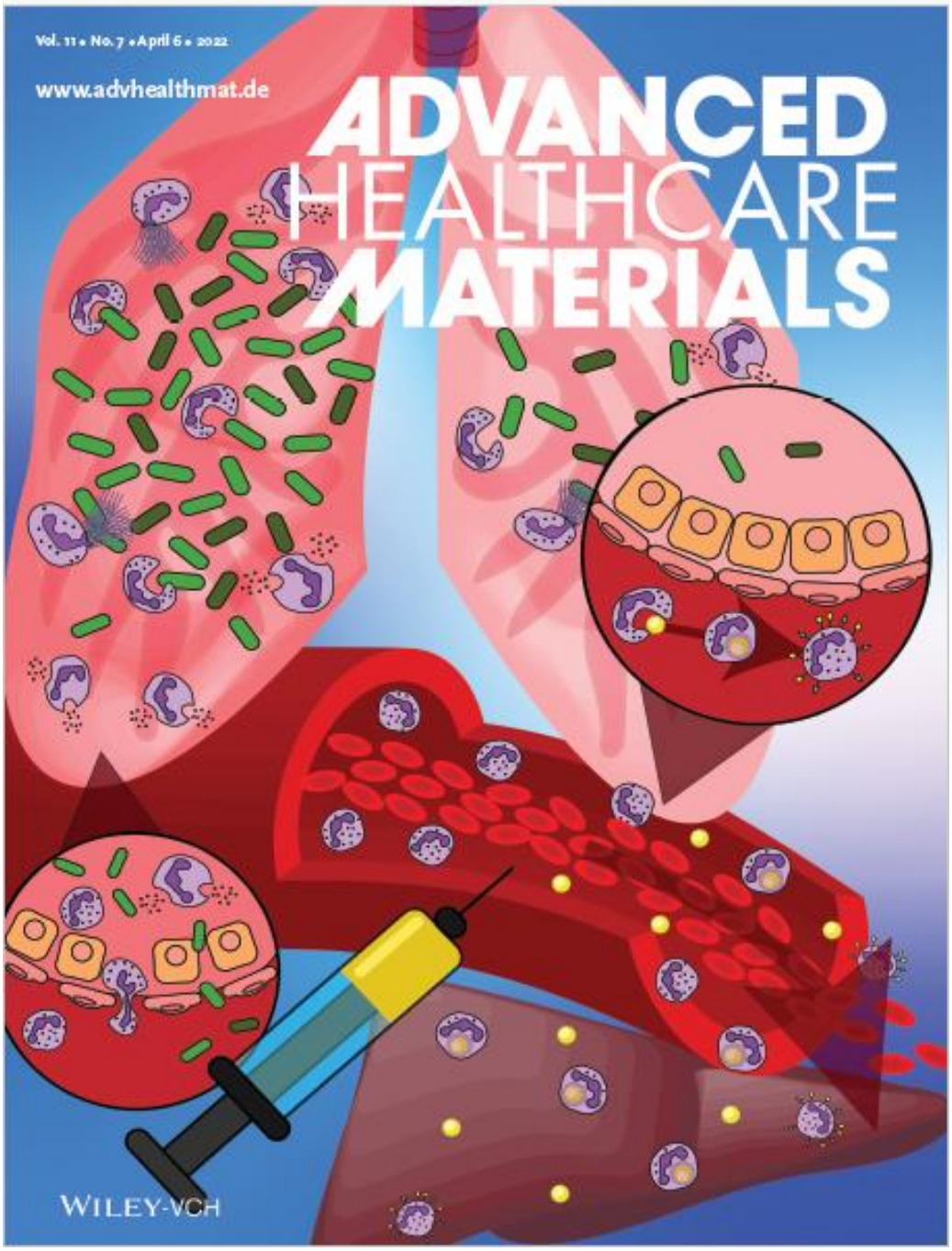


Figure 3-12 Cover illustration published in Advanced Healthcare Materials.

Brannon, E. R., et al. (2022). "Polysalicylic Acid Polymer Microparticle Decoys Therapeutically Treat Acute Respiratory Distress Syndrome." Advanced Healthcare Materials **11**(7): 2101534.

Chapter 4 NETosis Modulation and Reduction via Polymerized Salicylic Acid Microparticles

4.1 Publication Information

The work discussed in this chapter is not yet published but is submitted for publication with the title “Polymerized salicylic acid microparticles reduce the progression and formation of human neutrophil extracellular traps (NET)s” with author list **Emma R. Brannon**, Logan D. Piegols, Xueqi Chu, M. Valentina Guevara, Kathryn E. Uhrich, and Omolola Eniola-Adefeso. The content of this chapter has been adapted from the manuscript that will be submitted for publication to fit the purpose of this chapter.

Citation: Brannon, E. R., et al. (2024). " Polymerized salicylic acid microparticles reduce the progression and formation of human neutrophil extracellular traps (NET)s." Submitted to Advanced Healthcare Material

4.2 Abstract

Neutrophils can contribute to inflammatory disease propagation via innate mechanisms intended for inflammation resolution. For example, neutrophil extracellular traps (NET)s are necessary for trapping pathogens but can contribute to clot formation and blood flow restriction (ischemia). Currently, there are no therapeutics in the clinic that directly target NETs despite the known involvement of NETs contributing to mortality and increased disease severity. Vascular-deployed particle-based therapeutics are a novel and robust alternative to traditional small-molecule drugs

by enhancing drug delivery to cells of interest. To translate particle-based therapeutics to the clinic, high-throughput *in vitro* assays can be used to understand physiological effects on targeted cell populations, which is necessary for therapeutic optimization. In this work, we designed a high-throughput assay to investigate NETosis and functionally test immunomodulatory behavior of particle-based therapeutics. Briefly, we found that not only does polymeric composition play a role, but particle size also influences rates of NETosis. Salicylate-based poly(anhydride-ester) polymeric (Poly-A) particles were found to functionally inhibit NETosis, in addition to having other anti-inflammatory capabilities as shown in previous work. Particle-based therapeutics are a novel solution to treating inflammatory diseases, and it is necessary to utilize high throughput models to optimize safe and effective particle properties.

4.3 Introduction

Neutrophils, the primary circulating white blood cells in humans, play a major role in modulating the response to acute inflammation. As the first responder to inflammation, neutrophils contain invading pathogens/inflammatory agents via phagocytosis, the release of cytokines, granules, reactive oxygen species (ROS), and neutrophil extracellular traps (NET)s. [2,37,123,124] In severe inflammation, neutrophils can propagate inflammation by over-accumulating at sites of inflammation and damaging healthy tissue with granules/ROS/NETs.[2,125,126] This neutrophil dysfunction occurs in several inflammatory diseases —sepsis, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), deep vein thrombosis (DVT), and more —leading to further disease propagation.[125,126]

The release of NETs (NETosis) is a vital innate immune response in controlling infections/inflammation by supporting thrombi formation along the endothelium, effectively capturing unwanted pathogens.[2,124] However, excessive thrombosis, in this case,

thromboinflammation, can lead to vascular occlusion and ischemia.[124,127] Enhanced NETosis has been shown to correlate with disease severity in thrombo-inflammatory diseases along with ALI, ARDS, sepsis, and SARS-CoV-2 infection.[124,128] The increasing awareness of NET involvement in inflammatory diseases has prompted investigations of potential small molecule therapeutics that target NETosis via neutrophils, NETs, or mitochondria.[124,129] Localized lung injury/inflammation from chronic obstructive pulmonary disease (COPD), asthma, and ARDS has been shown to benefit from inhalation treatment of small molecule therapy, predominately neutrophil elastase inhibition or DNase treatment.[124,130] However, small molecule therapeutics become less effective in systemic inflammation. For example, sivelestat—an inhibitor of neutrophil elastase, has shown promise in reducing disseminated intravascular coagulation (DIC) but has failed in clinical trials due to low efficacy.[131,132]

Particle-based therapeutics are an innovative solution to drug targeting for the treatment of inflammatory diseases propagated by neutrophils and NETosis.[37] Small molecule therapeutics can be loaded into particle-based drug delivery systems to enhance efficacy in systemic inflammation. Okeke et al. has shown that loading sivelestat into lipid-based nanoparticles can enhance efficacy and prevent NETosis in an *in vivo* endotoxin shock sepsis model, resulting in improved survival rates.[131] While lipid-based particles are biocompatible and maintain cargo stability, lipid-based particles are limited by structural stability and low drug loading. Polymeric-based particles allow for high tunability/optimization and can easily be scaled from the bench to industrial scale. Poly(lactic-co-glycolic acid) (PLGA) is a biocompatible polymer that has been used in several FDA-approved particle formulations.[59] In macrophages and dendritic cells, PLGA can have anti-inflammatory effects in high doses, but inflammatory effects at low doses.[37,49] However, the literature has not thoroughly investigated the effects of various polymeric materials on neutrophils

post-interaction. Initial studies from Brannon et al. found that degradable salicylic acid (SA) based poly(anhydride-ester) polymeric (Poly-Aspirin or Poly-A) particles can alter neutrophil function, reducing L-Selectin and PSGL-1 shedding—implying reduced activation. [47]

When tuning particle-based therapeutics for clinical purposes, it is important to optimize the material to minimize unintended side effects and enhance pharmacological outcomes. Materials such as cationic lipids, polystyrene, and graphene oxide are known to induce NETosis, thus would not be the optimal choice when designing particle-based therapeutics that interact with neutrophils in diseases with known negative NETosis impact.[133,134] Polymeric particle formulations (PLGA, Polydopamine, polyethylene glycol) used in combination with DNase surface conjugation can target and lyse NETs. Despite this, the literature has not yet investigated optimized material properties for reducing NETosis.[135,136] While surface conjugated formulations have succeeded in animal models, much is still unknown concerning effects of degradable polymeric particles on neutrophil physiology. To understand the effects of various material properties on NETosis, it is imperative that a high throughput screening approach is utilized.

NETosis therapeutic screening assays are often tedious, and, in many cases, assay sensitivity is sacrificed with enhanced data collection and analysis. Unlike traditional small molecule therapeutics, particle-based therapeutics are dependent on cellular uptake for optimal drug-delivery. For this reason, changes in neutrophil physiology may be missed with the current high-throughput assays used for small molecule therapeutics. Plate reader assays are common for measuring NETosis, however, plate readers assume the entirety of a sample will have a homogenous response rather than a response dependent on particle phagocytosis. Microscopy assays require intensive, time-consuming analysis procedures and results may be biased due to the user-dependent nature of image analysis and cell counting.[137,138] Further, neutrophil isolation

technique (anticoagulant, gradient, RBC lysis), NET inducing agent, and assay type are all compounding factors that can result in the wide variability observed in neutrophil response and resulting NETosis.[139-141] Thus, a sensitive yet high throughput assay would improve the current state of NETosis assays and enhance consistency from scientist to scientist.

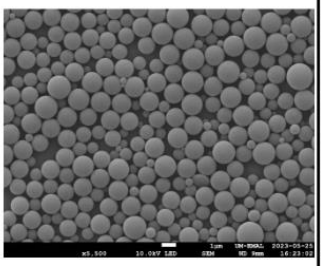
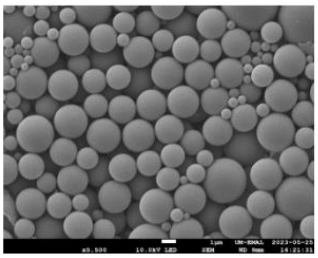
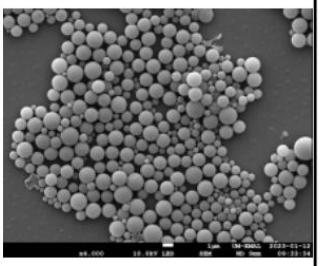
In this work, we developed a high throughput microscopy screening assay for evaluating primary neutrophil NETosis in response to particle-based therapeutics. Images were gathered using an EVOS M7000, and then analyzed via MATLAB to provide rapid, ample, and unbiased results. From this work we generated a systemic analysis from full well scans of a 96-well plate, a single cell analysis from 20x fluorescent overlay images, and a region property analysis to understand NET shape and size. Results from these experiments show Poly-A microparticles inherently reduce NETosis on both a systemic and single neutrophil level. In comparison, PLGA particles were found to induce a baseline level of NETosis, elevating the importance of high-throughput assays to understand the neutrophil response to various particle-based therapeutics.

4.4 Results

4.4.1 Developing an in Vitro NETosis Assay for Particle-Based Therapeutics and Measuring Systemic NET Formation

Particle size and smooth surface morphology was confirmed using SEM as shown in Table 4-1. A neutrophil isolation protocol adapted from prior work by Knight and colleagues was utilized for this work. Microscopy assays are the most sensitive method of measuring NETosis, thus we employed an automated fluorescent microscope, the EVOS M7000. Using the automation features

Table 4-1 Particle Characterization for Particles used in NETosis Assay.

	1 µm Poly-A	2 µm Poly-A	PLGA
SEM Image			
Size	1.16 ± 0.36 µm	1.81 ± 0.70 µm	1.16 ± 0.34 µm
Zetapotential	-31.5 ± 2.47	-28.5 ± 1.13	-31.7 ± 0.61

of the EVOS M7000, we captured full well scans at 1-, 2-, 3-, and 5-hrs post activation with either PMA or LPS in a 96-well plate (Figure 4-1). Figure 4-1A shows images captured using the EVOS 5-hrs post activation. Primary neutrophils isolated from healthy donors were incubated with SYTOX Green—a marker for extracellular DNA—allowing us to visualize and quantify percent coverage of NETs in each well. Percent well coverage of NETs was quantified using the MATLAB Image Processing Toolbox to threshold the SYTOX Green signal, which we use as a location identifier for NET locations, and then calculate percent well coverage.[65]

Neutrophils were activated with either LPS or PMA and NET formation was tracked over a 5-hour period via percent coverage. Figure 4-1B shows that PMA, but not LPS, induces NET formation compared to unactivated neutrophils in terms of percent well coverage. When treating neutrophils with a therapeutic, either 1 μm Poly-A or the particle equivalent dose of salicylic acid, we found significant impacts on NET formation with particle treatment but not soluble SA treatment. Poly-A particles significantly reduced NET percent coverage in unactivated conditions (100% of donors), LPS activated (90.9% of donors), and PMA activated (63.6% of donors) as shown in Figure 4-1C. Poly-A particles degrade to release salicylic acid (SA). Concentrated salicylic acid (5 mM or 0.691 mg/mL) is known to reduce nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) expression which leads to a reduction in NETosis.[142,143] When treating neutrophils with a particle equivalent dosage of salicylic acid (0.05 mg/mL) we did not see a significant change in the percent well coverage of NETs. We suspect from this work that the 1 μm Poly-A particles release a bolus dose of salicylic acid once phagocytized, but the equivalent dose of free SA is not enough to impact the cell from a suspension. Importantly, salicylic acid in the plasma is toxic above 0.3 mg/mL, less than half the dosage of salicylic acid in suspension necessary to inhibit NETosis.

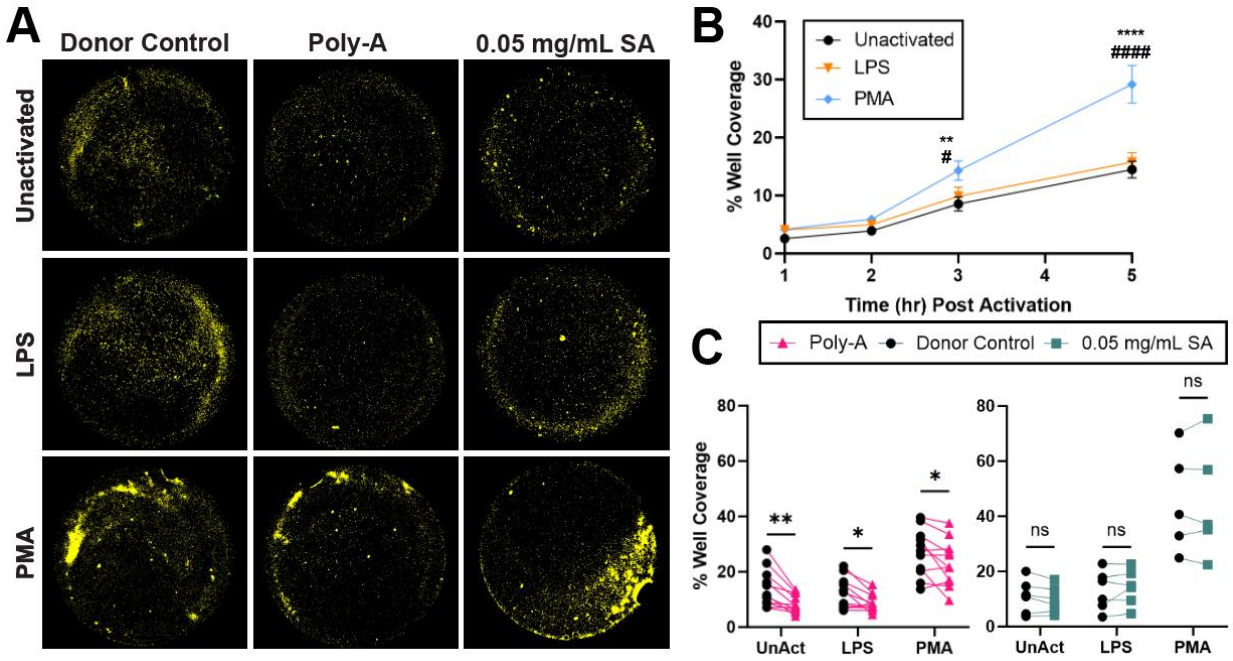


Figure 4-1 Characterizing and quantifying NETosis with SYTOX Green in whole wells with either Poly-A or free salicylic acid treatment.

(A) Images captured 5-hrs post activation with an EVOS M7000 microscope showing a visual reduction in NET coverage for samples treated with Poly-A particles. (B) Percent well coverage of NETs over the course of the experiment with various activating agents. (C) Percent well coverage at 5-hrs post activation of individual donors representing each data point treated either with Poly-A particles or particle equivalent dosage of salicylic acid (SA). Statistical analysis was completed in Prism with ANOVA testing. (B) followed a 2way ANOVA where (*) denotes statistical significance between unactivated and PMA-activated groups and # denotes significance between LPS- and PMA-activated groups. (C) followed 2way ANOVA testing with repeated measures for each donor. For both (B) and (C) Fisher's LSD test was used and the following symbols denote p-value not significant (ns), $p<0.05$ (*, #), $p<0.01$ (**, ##), $p<0.001$ (***,###), $p<0.0001$ (****, ####).

4.4.2 Characterizing the Impact of Particles on Individual Neutrophils

To further investigate the effects of particle-based therapeutics on NETosis, we developed a method to also measure NETosis on a cellular level. In addition to imaging our 96-well plate with a 4x objective, we took 20x images in each well (**Figure 4-2**). Each 20x image was taken randomly within the well, utilizing the EVOS M7000 automation features. Three channels were used to collect information on neutrophils—brightfield, particles—Cy5.5, and NETs—SYTOX Green (Figure 4-2A). MATLAB was then used to convert individual channel images into binary matrices, overlay the binary matrices, and convert the data into a final coded matrix that is exported into Excel, as described in methods and materials. From this analysis, we gained sub-cellular resolution

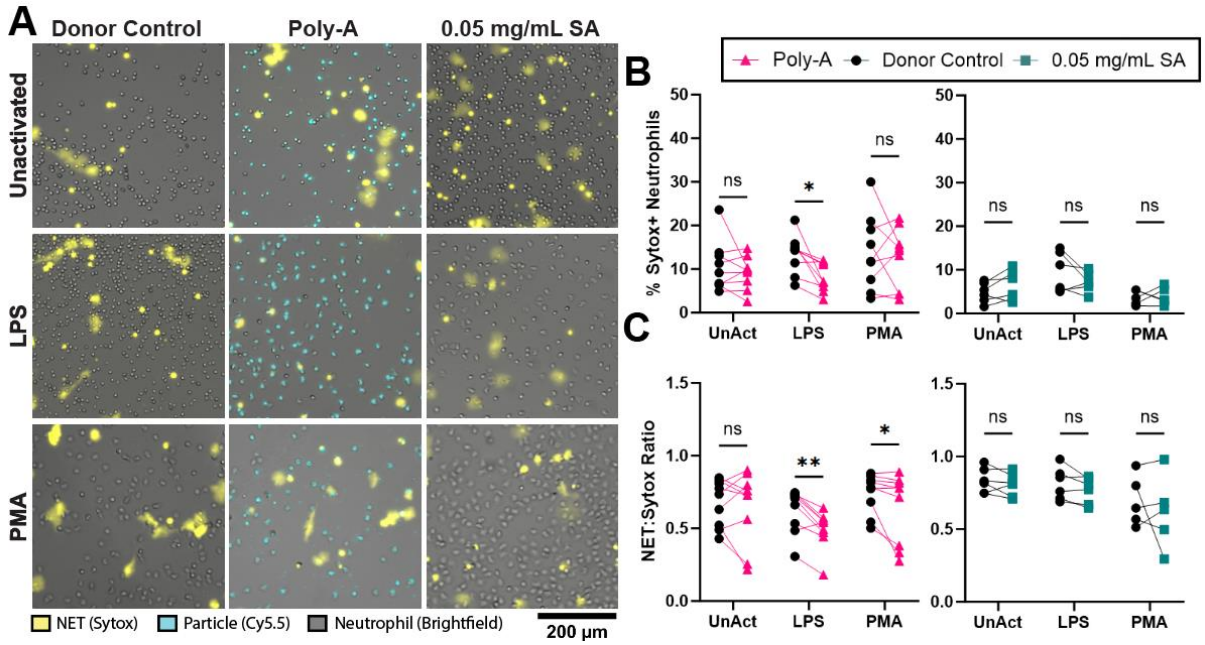


Figure 4-2 Quantifying NETosis on a single cell level via fluorescent imaging 1.5-hrs post activation.

(A) 20x images overlaying NETs, Poly-A particles, and neutrophils were collected and cross-analyzed pixel by pixel in MATLAB. (B) Percentage of SYTOX stained neutrophils in donor control, neutrophils overlayed with Poly-A particles, or SA treated samples. Poly-A data excludes neutrophils that did not overlap with particles (C) Ratio of NET area to SYTOX stained area by size analysis. Brightfield and Cy5.5 channels are not included in NET:SYTOX analysis. Statistical analysis was completed in Prism with ANOVA testing, following 2way ANOVA testing with repeated measures for each donor. For both (B) and (C) Fisher's LSD test was used and the following symbols denote p-value not significant (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****)

of neutrophil, particle, NETs, and all overlap permutations, allowing us to quantify NETosis related to particle uptake.

Neutrophils were dosed with a 16:1 ratio of 1 μm Poly-A particles and incubated for 1 hour prior to activation to allow for particle uptake and neutrophil adhesion. Particle phagocytosis was measured via neutrophil/Cy5.5 pixel overlay and found to be 84% throughout the experiment (Figure 4-3). Images collected 1.5-hrs post activation showed Poly-A particle association with individual neutrophils may have the greatest impact in the LPS activation condition, whereas unactivated and PMA activated visually have similar rates of NETosis (Figure 4-2A). To quantify this visual trend, we evaluated the SYTOX Green positive neutrophil pixels. Poly-A significantly reduced SYTOX Green stained neutrophils in LPS activation – on average 13.48% of untreated neutrophils were SYTOX positive versus 8.24% of neutrophils in the Poly-A treated sample (Figure 4-2B). The particle equivalent dose of soluble salicylic acid had no influence on the percentage of neutrophils stained with SYTOX Green.

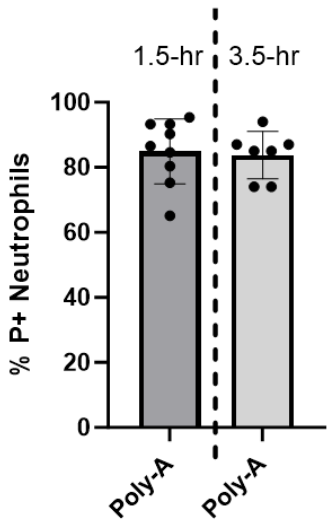


Figure 4-3 Particle uptake as measured by EVOS M7000 and detected on MATLAB.

Next, we sought to investigate SYTOX Green region properties in only the GFP channel. SYTOX Green is an impermeable membrane dye of a healthy cell but can penetrate the cell membrane in the early stages of NETosis prior to NET expulsion.[144] In order to quantify the extent of NETosis, we used a region properties analysis to differentiate between neutrophil-sized (non-expelled DNA) and greater than neutrophil-sized (expelled DNA, NET) SYTOX Green stained objects. We then calculated a ratio of NET pixels to total SYTOX Green pixels to quantify the number of neutrophils that have completed NETosis versus those that are beginning to NET (Figure 4-2C). Poly-A particles significantly reduced the NET:SYTOX ratio in both LPS and PMA conditions, suggesting that the particles are slowing down the NETosis process. Conversely, soluble SA did not reduce the ratio of NET:SYTOX.

4.4.3 Investigating the Impacts of a Traditional Degradable Particle System on NETosis

As a control, we evaluated PLGA – a degradable polymer often used as a drug carrier. The Poly-A polymer used in the previous section was low molecular weight (10.3 kDA), thus we chose a PLGA polymer similar in molecular weight. Specifically, we investigated the effects of a low molecular weight 50:50 PLGA (5-10 kDa) on NETosis (Figure 4-4).

From the full well analysis, we found that PLGA induces a baseline level of NETosis in all conditions 5-hrs post activation (Figure 4-4B). At an earlier time point (1.5-hrs) and on the cellular level we found that unactivated neutrophils treated with PLGA were more likely to be SYTOX+. This suggests that the baseline induction of NETosis by PLGA particles is most prominent in unactivated conditions but not in LPS or PMA activation at early time points (Figure 4-4D). While some donors treated with PLGA and activated with LPS or PMA have increased levels of SYTOX+ staining, donor to donor variation results in a non-significant trend. Looking at the ratio of NET to total SYTOX we find no trend when treating with PLGA particles. By 1.5 hours post

activation, most donors have NET:SYTOX ratios at the ceiling of the measurement, approximately 1 (Figure 4-4E). This means the ratios of SYTOX+ pixels of neutrophils that are actively expelling NETs is approximately equivalent to the total number of SYTOX+ pixels, i.e. all the neutrophils that are SYTOX+ have full NETs.

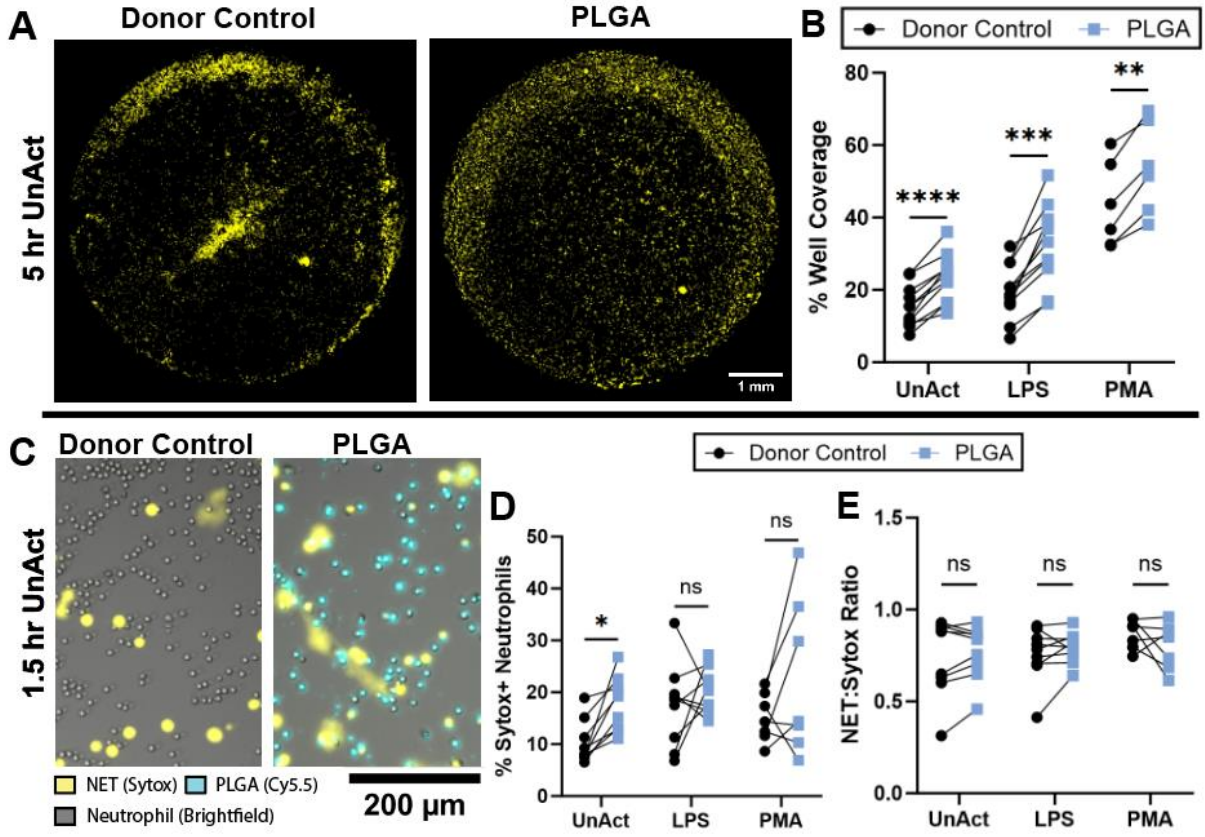


Figure 4-4 Non-therapeutically loaded PLGA particles induce baseline levels of NETosis.

(A) Full well scans of unactivated primary human neutrophils at 5-hrs with or without PLGA particle treatment. (B) Quantification of well coverage (%) by SYTOX Green staining. (C) Images taken at 20x magnification for cross-analysis of PLGA particles, SYTOX staining, and neutrophils. (D) Percent of particle-positive neutrophils that are stained with SYTOX Green plotted in comparison to donor controls (E) Ratio of NET area to SYTOX stained area by size analysis, not including brightfield and Cy5.5 channels. Statistical analysis was completed in Prism with ANOVA testing, following 2way ANOVA testing. Fisher's LSD test was used and the following symbols denote p-value not significant (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

4.4.4 Exploring 1 μm Poly-A Dosage Impacts on Particle Uptake and NETosis Reduction

The first two sets of data investigated 1 μm Poly-A at a high dosage of 16:1 particles to neutrophils to achieve maximum particle uptake and uniformity within the sample. We next wanted to investigate the effects of Poly-A particles in physiologically relevant doses in addition to global effects in a single well from varying levels of particle uptake. To achieve this goal, we titrated the dose of particles among our samples to artificially modulate particle uptake. Reducing the particle dosage by 75% (4:1 particles to neutrophils) resulted in a loss of NETosis reduction on the global level (Figure 4-5) as measured by percent well coverage. An 8:1 ratio of 1 μm Poly-A to neutrophils resulted in a significant reduction of percent well coverage in LPS activation but not in unactivated conditions.

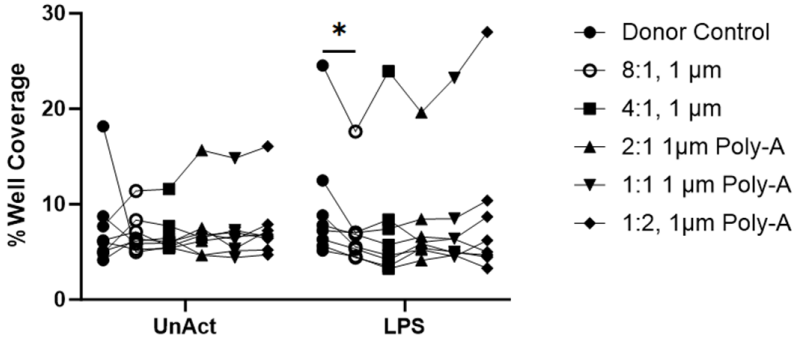


Figure 4-5 Percent well coverage of NETs with decreasing ratio of Poly-A to neutrophils

Statistical analysis was completed in Prism with ANOVA testing, following 2way ANOVA testing with repeated measures for each donor. For both (B) and (C) Fisher's LSD test was used and the following symbols denote p-value not significant (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****)

Overlay images were used to understand the overall population effects of 1 μm Poly-A dosage on a cellular level (Figure 4-6). Particle uptake was modulated by titrating the particle-to-neutrophil ratio, allowing us to analyze particle-negative and particle-positive populations within the same sample. Figure 4-6A displays increased particle uptake with increasing particle-to-neutrophil ratios. We quantified the rates of particle association with neutrophils, as shown in Figure 4-6B. The 2:1, 4:1, and 8:1 ratios correspond to 16.96%, 31.92%, and 56.20% uptake on average. Within the particle-positive neutrophil populations, we found all dosages to have equivalent rates of SYTOX Green staining. This suggests that as long as a neutrophil phagocytizes

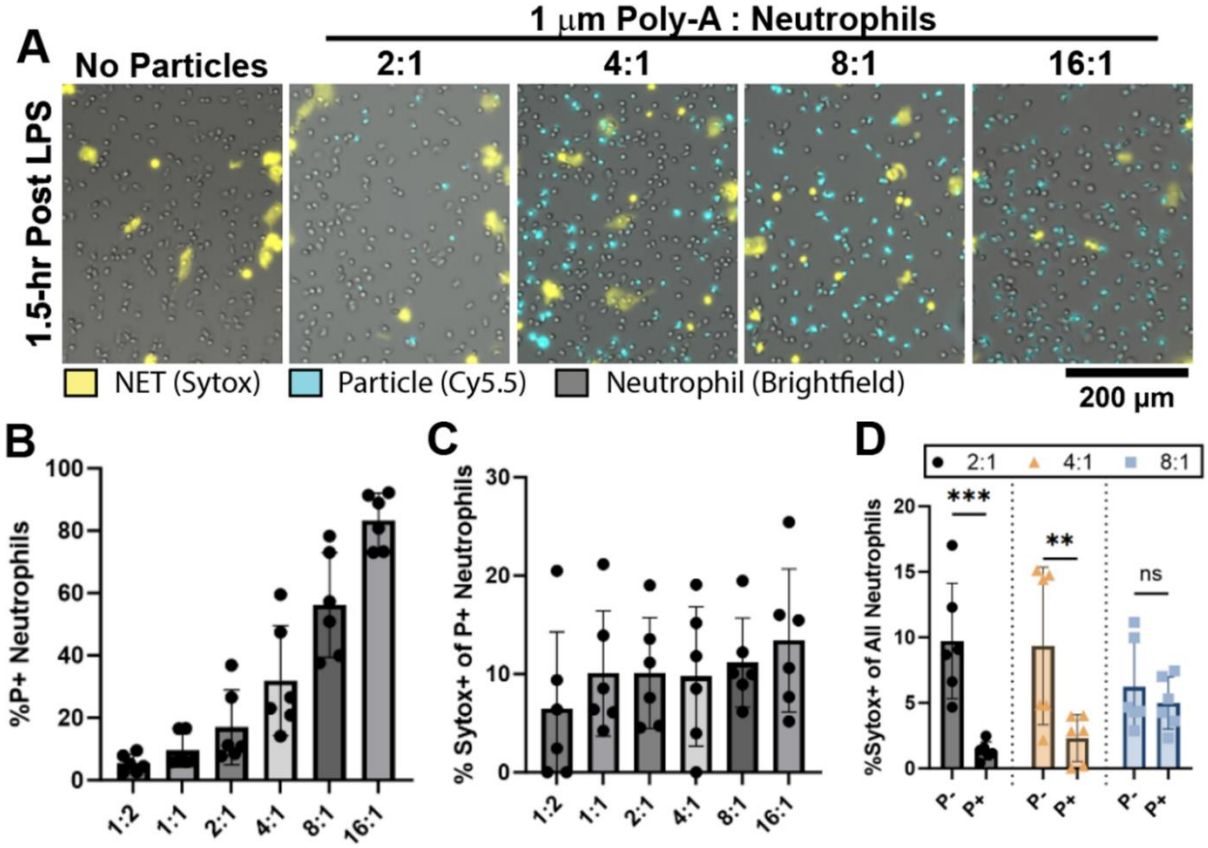


Figure 4-6 Dosage study investigating the limits of NETosis inhibition with 1 μm Poly-A.

(A) Images taken at 20x magnification for cross-analysis of Poly-A particles, SYTOX staining, and neutrophils. (B) Percent particle positive neutrophils with increasing particle dose. (C) Percent of particle positive neutrophils that are stained with SYTOX green. (D) Percentage of neutrophils that are stained with SYTOX green of the entire neutrophil population broken down into either particle positive or particle negative. Statistical analysis was completed in Prism with ANOVA testing, following 2way ANOVA testing. Fisher's LSD test was used, and the following symbols denote p-value not significant (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

a particle, it will have the same NET response despite dosage (Figure 4-6C). Looking at the entire neutrophil population we can quantify the rates of SYTOX Green staining in either particle-negative (P-) or particle-positive (P+) populations. Interestingly, we found that P+ neutrophils in the lower dosed groups (2:1 or 4:1 ratio) yielded significantly reduced SYTOX Green staining compared to the P- neutrophils. At the higher dosage (8:1 ratio) P+ and P- neutrophils had similar rates of SYTOX Green staining, suggesting that P+ neutrophils may influence P- neutrophil rates of NETosis in higher dosages (Figure 4-6D).

4.4.5 Enhancing Poly-A Therapeutic Effect by Increasing Particle Size to 2 μm.

Polymeric particles are versatile in that size and shape can be easily modulated to optimize therapeutic design by application. Human neutrophils phagocytize particles of different sizes/shapes at different rates, as shown by Safari et al.[46] Thus, it is important to conduct assays across various particle sizes to fully understand the material properties. In this work, we found that 2 μm Poly-A particles are more efficiently phagocytized by neutrophils than 1 μm Poly-A, allowing us to investigate reduced dosages (Figure 4-7).

We find that the 4:1 particle to neutrophils ratio for 2 μm Poly-SA yielded 75.3% uptake which is close to the uptake obtained when using a 16:1 ratio of 1 μm particles (83.3%), as shown in Figure 4-7B and Figure 4-6B. Of the neutrophils treated with 2 μm Poly-A, all dosages resulted in the same rates of SYTOX+ staining, suggesting that particle phagocytosis is necessary for particle effects rather than dosage (Figure 4-7C). When we evaluate the particle +/- neutrophils within each particle-treated sample, we find that only the lowest ratio (1:1) of particles to neutrophils yields a significant reduction in SYTOX+ staining. The 2:1 ratio leads to a non-significant reduction in SYTOX+ staining and the 4:1 ratio result in a significant increase in SYTOX+ staining. Importantly, in wells not treated with particles SYTOX+ neutrophils ranged

from 11.55-29.19% and averaged at 20.74% (Figure 4-8). This increase from particle positive neutrophils was significant for all 2 μm particle dosing schemes; P-values for this significance was <0.0001, <0.0001, and 0.0024 for the 1:1, 2:1, and 4:1 ratio respectively.

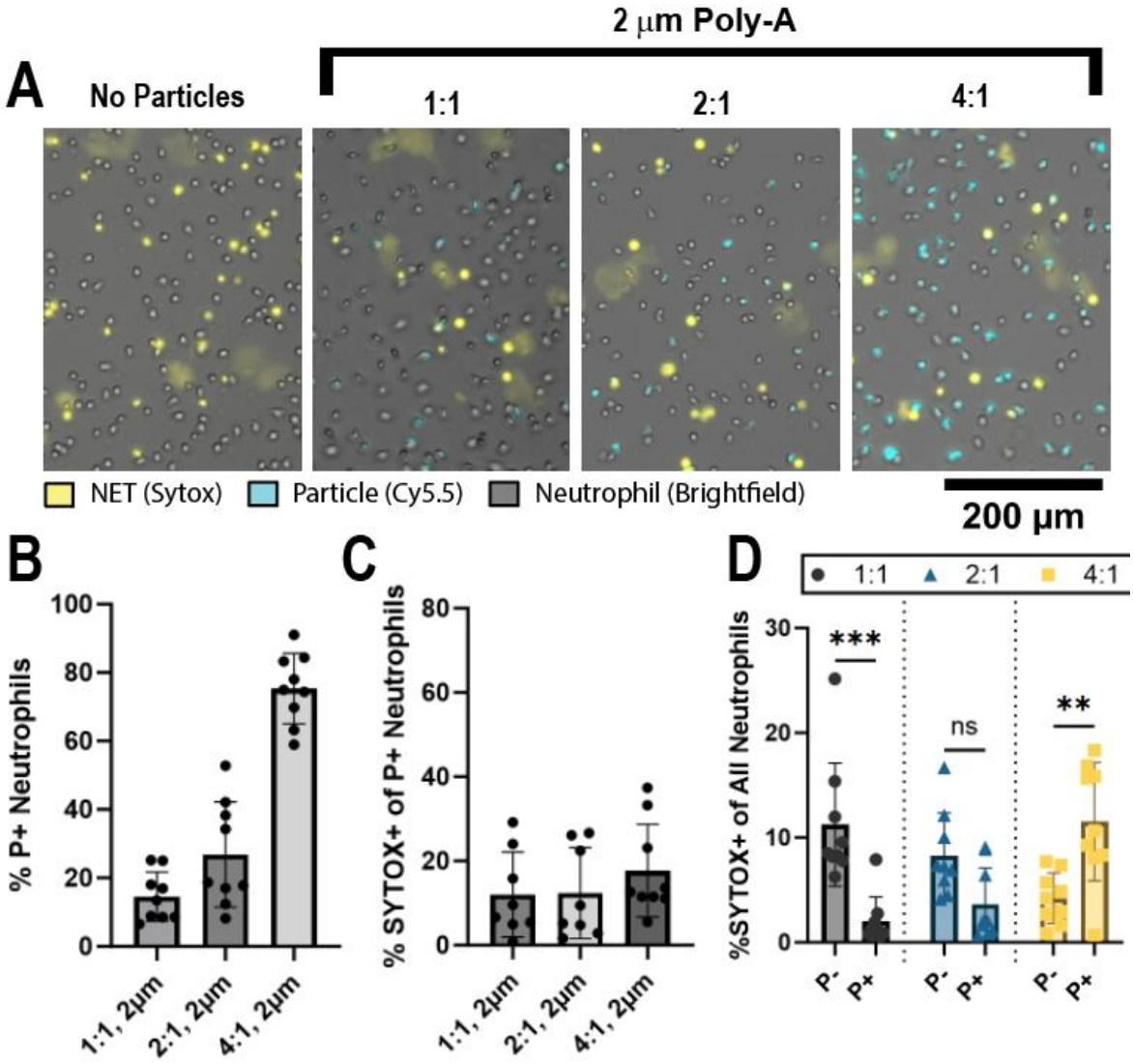


Figure 4-7 Evaluating effects on NETosis with larger particles (2 μm).

(A) Images taken at 20x magnification for cross-analysis of 2 μm Poly-A particles, SYTOX Green staining, and neutrophils. (B) Percent particle positive neutrophils with increasing particle dose. (C) Percent of particle-positive neutrophils that are stained with SYTOX Green. (D) Percentage of neutrophils that are stained with SYTOX Green of the entire neutrophil population broken down into either particle positive or particle negative. Statistical analysis was completed in Prism with ANOVA testing, following 2way ANOVA testing. Fisher's LSD test was used and the following symbols denote p-value not significant (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

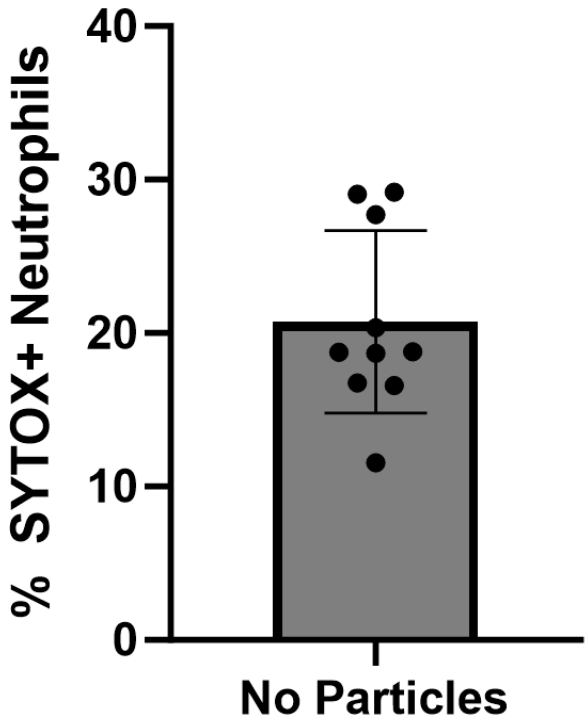


Figure 4-8 Percent SYTOX+ Neutrophils not treated with particles.

Statistical analysis was completed in Prism with ANOVA testing, following one-way ANOVA testing. Fisher's LSD test was used and the following symbols denote p-value not significant (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

We next investigated the full well images of wells treated with 2 µm Poly-A and found that only the 1:1 ratio 2 µm Poly-A lead to a reduction in percent well coverage (Figure 4-9). It could be that with larger particles excessive delivery of SA could be approaching toxic dosages. It is important to take into consideration that by increasing particle diameter 2-fold, particle volume and SA delivery is increased by 8-fold per particle, greatly enhancing SA delivery. This work further shows that preventing NETosis with Poly-A particles must be the perfect balance between particle size and dose to provide optimal therapeutic benefit.

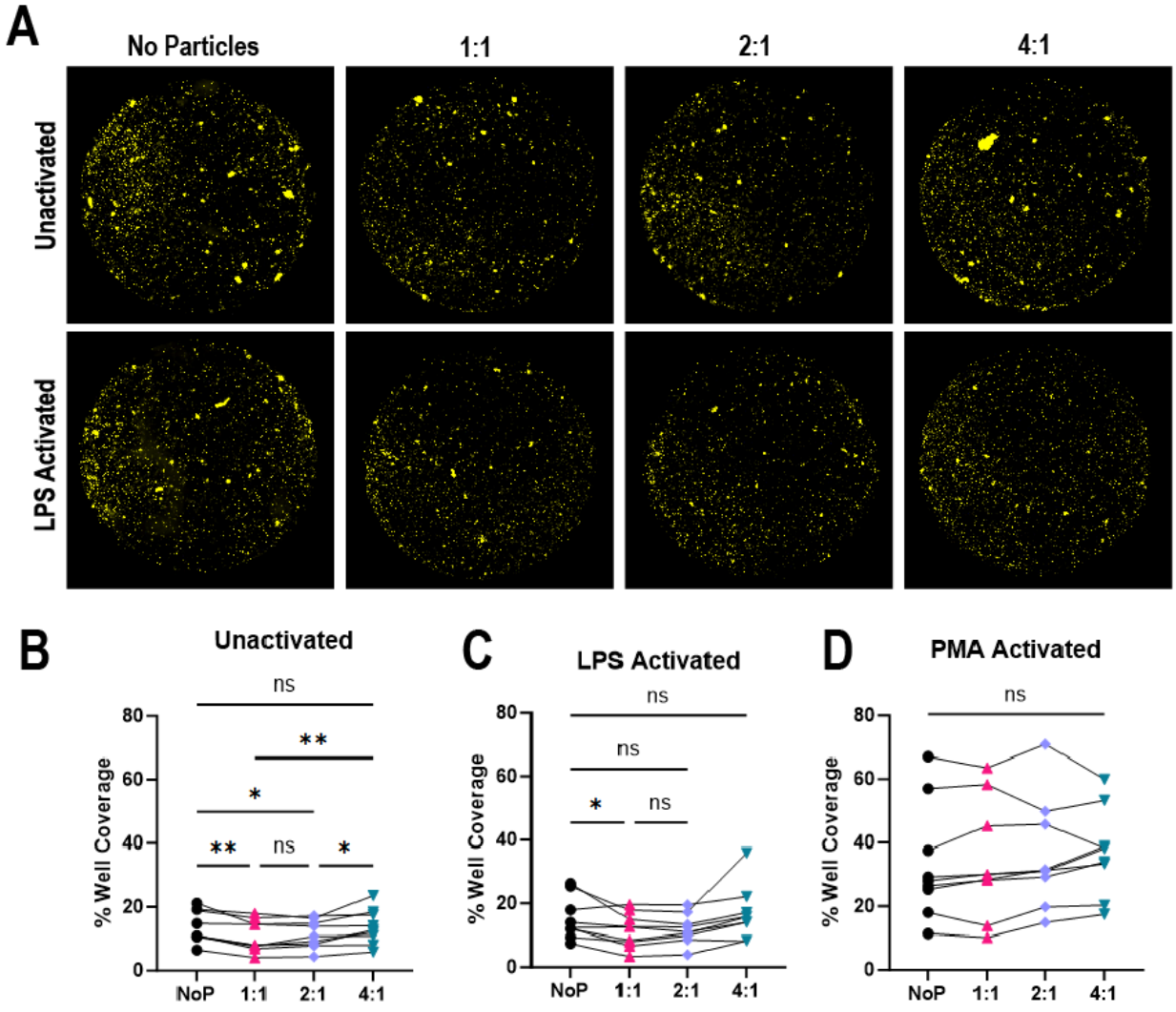


Figure 4-9 Full well analysis of 2 µm Poly-A Particles.

(A) Full well scans showing a much less obvious reduction in NETosis with particle treatment. Percent well coverage in (B) unactivated, (C) LPS activated, and (D) PMA activated conditions. Statistical analysis was completed in Prism with ANOVA testing, following 2way ANOVA testing with repeated measures for each donor. For both and Fisher's LSD test was used and the following symbols denote p-value not significant (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****)

4.5 Discussion

Particle-based therapeutics are a novel solution to inflammatory diseases that currently have minimal therapeutic solutions.[37] ARDS, DVT, and sepsis are a few diseases that can often be propagated by neutrophils and NETosis, thus it is imperative we understand particle impacts on NETosis in the development of particle-based therapeutics for inflammation. Particle properties such as material, shape, and size could be combined in so many iterations that finding the most optimized formulations would not be possible without high throughput methods. Plate reader assays are commonly used for measuring NETosis, however, plate readers assume the entirety of a sample will have a homogenous response rather than a response dependent on particle phagocytosis. Microscopy assays require intensive, time-consuming analysis procedures and results may be biased due to the user-dependent nature of image analysis and cell counting. Further, neutrophil isolation technique (anticoagulant, gradient, RBC lysis), NET inducing agent, and assay type are all compounding factors that can result in the wide variability observed in neutrophil response and resulting NETosis.[139-141] Thus, a sensitive yet high throughput assay would improve the current state of NETosis assays and enhance consistency from scientist to scientist. In this work, we first developed a high throughput method to quantify particle effects on NETosis, then investigated two particle materials, Poly-A or PLGA, in addition to multiple sizes of Poly-A particles on NETosis.

Our high throughput model utilized the EVOS M7000 to collect automated fluorescent images with various activation strategies and across multiple timepoints and imaging magnifications (4x and 20x). Importantly, this work is not the first to propose a high throughput NETosis assay and analysis method. Gupta et al. proposed a similar, real-time fluorescent imaging method that utilized the IncuCyte ZOOM platform.[145] Both the EVOS M7000 and the IncuCyte

ZOOM platform have the capability to capture real-time images and reduce user bias by limiting human error. However, the IncuCyte is limited by two fluorescent channels and preprogrammed software that does not allow for assay flexibility necessary for evaluating novel particle-based therapeutics.[145] By developing a technique that utilizes a common piece of affordable lab equipment (EVOS M7000), and creating an analysis method in MATLAB, this work has generated an accessible and flexible technique for measuring and quantifying NETosis. Further, this method is modular in that many different fluorophores can be used at once, allowing for the analysis of multiple cell markers and particles.

Using our high throughput method, we first established similar results to the literature in that PMA induces a significant increase in NETosis compared to unactivated controls and LPS does not induce more NETosis compared to unactivated. Both LPS and PMA are reported to induce NETosis via activation of NADPHoxidase (NOX) and are (NOX-dependent).[141] NOX activation leads to downstream ROS production, inducing NETosis. Additionally, the literature has confirmed that LPS and PMA activation follow different pathways of ROS production, thus different pathways of NETosis induction.[141] While the exact pathway of LPS induced NETosis is known, the levels of NETosis induction vary drastically, some groups reporting that LPS does not induce more NETosis compared to unactivated groups similar to our results in the current study.[139-141] This discrepancy could be due to differences in neutrophil isolation techniques, anticoagulants, or even metric quantification. Additionally, we found that both PMA and LPS induced a well-characterized morphological change in neutrophils that is consistent with activation and NETosis.[145-147]

The impact of Poly-A particles on neutrophils in this study is consistent with the observation from prior work showing these salicylate-based polymeric particles functionally

reduce neutrophil activation and successfully redirect neutrophils from accumulation at sites of inflammation.[47] The literature has established salicylic acid as a NETosis inhibitor, but much of this work evaluates salicylic acid at a dose near or above the human toxicity dose (5 mM or 0.90 mg/mL).[145-147] In the blood, salicylic acid is therapeutic between 0.15-0.30 mg/mL, a factor of 3 lower than the dosage of SA that others report leads to significant reductions in NETosis.[148] In comparison, we investigated 1 μm Poly-A particles at a ratio of 16:1 particles to neutrophils (equivalent to 0.05 mg/mL SA if fully degraded) and found a significant reduction in the percent well coverage of NETs (Figure 1). By delivering the SA in a particle form directly into the neutrophil, we can reduce the concentration of SA necessary to interfere with NETosis pathways. For example, in particle form, Poly-A can deliver approximately 3 pg of SA per particle directly into the cell.

Our modified imaging protocol and imaging approach allowed us to explore the impact of Poly-A particles at a single cell level in a way that the traditional full well scan cannot. From our results we found that Poly-A particles had the biggest impact with LPS compared to unactivated and PMA. Based on the literature LPS does not induce more NETosis than unactivated samples, but LPS does induce NETosis via a different pathway. More specifically, LPS induces stress-activated protein kinase or c-Jun N-terminal Kinase (SAPK/JNK) which in turn leads to ROS production and NETosis.[141] It has been shown that reducing activation of JNK results in a reduction of resulting NETosis. Soluble SA in high doses is known to inhibit JNK.[149] Thus, it is logical that Poly-A particles would consistently reduce NETosis in LPS conditions.[149,150]

In our SYTOX Green region properties analysis we found that Poly-A particles significantly reduced NET progression from the permeabilization of the cellular membranes to DNA expulsion in both LPS and PMA activation conditions. This makes sense due to the early

timing of these images (1.5-hrs post activation) compared to the full well scan images (5-hrs post activation). The data collected from the 1.5-hr images shows a slowed progression of NET formation during this key time point, leading to the significant reduction in percent coverage that we see at the 5-hr time point.

In our work, PLGA was used as a non-therapeutically active control to Poly-A, thus we anticipated no impact on NETtosis. Instead, our assay showed that PLGA drove high rates of NETosis compared to non-particle treated neutrophils. This significant induction of NETosis was seen at long time points (5-hr post activation), but minimal changes at early time points (1.5-hrs post activation). This trend is opposite of the NETosis reduction we found with Poly-SA, suggesting the importance of material selection when designing particles for treating inflammatory diseases. This result needs to be investigated further since PLGA has been FDA approved for human clinical use and is typically proposed as a drug carrier for systemic delivery.[61] It has been shown in the literature that PLGA can be tuned to have anti-inflammatory properties in immune cells such as dendritic cells.[49] Thus, more research should be done to optimize PLGA formulation for targeting neutrophils in treating inflammatory diseases. NETosis assays, such as the high-throughput assay introduced in this work can be used to identify PLGA formulations that do not induce NETosis and could be used for systemic drug delivery.

When investigating Poly-A dosage, we found that Poly-A association with neutrophils is necessary for NETosis reduction and was not a dose-specific effect on the cellular level. It has been shown in the literature that once neutrophils begin to NET other surrounding neutrophils are prompted to NET as a chain reaction.[151] As we reduce NETosis to a larger proportion of the neutrophils in a sample, it follows that the surrounding neutrophils would also have reduced rates of NETosis as shown by our results (Figure 4-6D). Similarly, in samples that received lower

dosages of Poly-A (4:1, 2:1, 1:1, 1:2 particles to neutrophils) we saw a loss in systemic NETosis prevention by Poly-A particles.

The increase in the Poly-A particle size to 2 um effectively increased the A dose per particle 8-fold. By increasing particle size, particle uptake by neutrophils was enhanced and we were able to reduce the ratio of particles to neutrophils when using 2 μm particles. Salicylic acid, while anti-inflammatory, can exhibit toxicity at large dosages.[152] In neutrophils, high concentrations of salicylic acid has been shown to induce apoptosis.[153] In this assay, particle uptake can occur in a bulk manner allowing for several particles to accumulate in one neutrophil, potentially leading to excessive delivery in a 4:1 dose ratio for 2 um particles. From this, neutrophils that eat few particles in the 4:1 dose may have reduced rates of NETosis compared to those that eat many. In the lower ratios (2:1, 1:1), neutrophils are less likely to phagocytize many particles, and so may be receiving the optimized SA dose. While 2 μm particles are a more efficient drug delivery system, further dosing studies must be conducted to understand the optimal range of salicylic acid delivery.

4.6 Conclusion

NETosis is a well-known innate immune function to modulate and resolve inflammation. However, NETs are also known to contribute to the propagation of a wide range of inflammatory-related diseases. Particle-based therapeutics could be an optimized approach to controlling NETosis due to their ability to target, modulate, and redirect activated neutrophils as shown in previous work.[47] Furthermore, our research has shown the importance of polymeric material selection as certain materials can be used to reprogram neutrophils while others can have activating effects. For these reasons, we needed to develop a high throughput model that would allow us to investigate various particle properties and resulting impacts on NETosis.

In this work we developed a high throughput in vitro model for screening particle-based therapeutics effects on NETosis. Additionally, using this model we showed the drastically different effects degradable materials can have on NETosis thus demonstrating the necessity of screening particle-based therapeutics on both a systemic and cellular level.

Chapter 5 Investigating the Effects of Varying PLGA Composition Neutrophil Surface Protein Expression

5.1 Publication Information

The work in this chapter has been drafted for publication but has not been submitted. The manuscript is titled "Investigating PLGA composition effects on neutrophil surface protein expression and activation" with author list **Emma R. Brannon**, Xueqi Chu, and Omolola Eniola-Adefeso.

5.2 Abstract

Poly(lactic-co-glycolic acid) (PLGA) is an FDA approved, biodegradable polymeric material that is often used clinically in medical devices and drug delivery. Particle formulations of PLGA have been in the clinic for over 40 years yet have been limited in application to local delivery. PLGA is a versatile and tunable material that is an ideal candidate for systemic drug delivery—i.e. intravenous applications. Furthermore, PLGA has the potential to treat a wide range of diseases, including systemic inflammatory diseases that currently have minimal therapeutic options. The development of intravenous formulations of particle-based therapeutics has been hindered due to a lack of focus on the major circulating WBCs in humans, neutrophils. In this work we fabricated a range of PLGA particles of varying lactic and glycolic acid composition. We then developed a method to estimate PLGA degradation and lactic acid release within neutrophils. We next conducted phagocytosis experiments with each PLGA particle formulation and related the estimated lactic acid release to changes observed in neutrophil surface protein expression. The

work done in this thesis chapter informs the importance of material selection when designing particle-based therapeutics for treating inflammatory diseases.

5.3 Introduction

FDA-approved IV-delivered particle therapeutics all are either lipid- or colloidal-based, yet literature has shown time and time again that polymeric vehicles are more stable, reproducible, and tunable.[61] Particle formulations of PLGA have been used in the clinic since 1989 but have been limited to local delivery applications, such as drug eluting tissue deposits.[61,154] The hydrophobic nature of PLGA limits it's use intravenously due to the resulting opsonization and clearance post particle injection.[61,155,156] Particle formulations of PLGA, however, could act as an immunomodulatory therapeutic for inflammatory diseases in which enhanced interactions with immune cells would lead to improved function. To this end, it is important to investigate material effects on circulating white blood cells.

It is well established that immunotoxicity represents a major hurdle for particle-based therapeutics. However, it has been shown that we can use the aspect of targeting circulating white blood cells as an advantage.[37] Many works in literature have shown the utility of expanding particle-based therapeutics in treating inflammatory conditions.[42,45,47,157] Neutrophils can propagate inflammation by over-accumulating and initiate downstream inflammatory pathways preventing resolution.[6,158] Neutrophils are also responsible for clearance of foreign bodies in the bloodstream, including particle-based therapeutics, making neutrophils the optimal targets.[2,37] Much of the literature has failed to investigate neutrophil physiological response to various particle-based therapeutics due to the difficulty in accessing and handling primary neutrophils.[38,159] Neutrophils have short lifespans and activate easily, preventing the ability to culture neutrophils

and requiring access to fresh blood.[38,159] Immortalized cell lines, such as HL-60 cells, differentiated into neutrophils has shown to have different physiological responses to particle-based therapeutics compared to primary neutrophils.[38,118] For this reason, most researchers do not have the accessibility or skillset to study neutrophil behavior in response to particle-based therapeutics.

Early research has shown that neutrophil activation can be modulated by delivering anti-inflammatory materials in particle form.[47] PLGA is versatile in that tuning molecular weight and ratio of lactic to glycolic acid can result in a particle that has either inflammatory or anti-inflammatory properties.[49] In the case of designing particle-based therapeutics for treating inflammatory conditions we need to know what formulations of PLGA are the most anti-inflammatory in relation to neutrophil physiology. Lactic acid is known to have anti-inflammatory properties, thus tuning lactic acid release by selecting a low molecular weight (LMW) PLGA is crucial in designing an anti-inflammatory particle type. However, it is also essential to balance lactic acid release with polymer stability, thus it is important to select a high molecular weight (HMW) polymer to enhance shelf life. Furthermore, as lactic acid content increases, degradation rates decrease, thus a PLA polymer would theoretically have less lactic acid release compared to PLGA.

Previous work shown in chapter 4 revealed a negative neutrophil response to LMW 50:50 PLGA as measured by NETosis. In this chapter we investigate various formulations of PLGA to fully understand the impact of lactic acid release on neutrophil activation. More specifically, we designed experiments to examine the effects of altering PLGA composition on neutrophil surface protein expression. We found that PLGA particle uptake can exacerbate the inflammatory response, but when lactic acid is released intracellularly, we see levels of activation reduce to

baseline. From this work, we now know to select a low molecular weight, high lactic acid, and highly degradable polymer when designing particles to treat inflammatory diseases.

5.4 Results

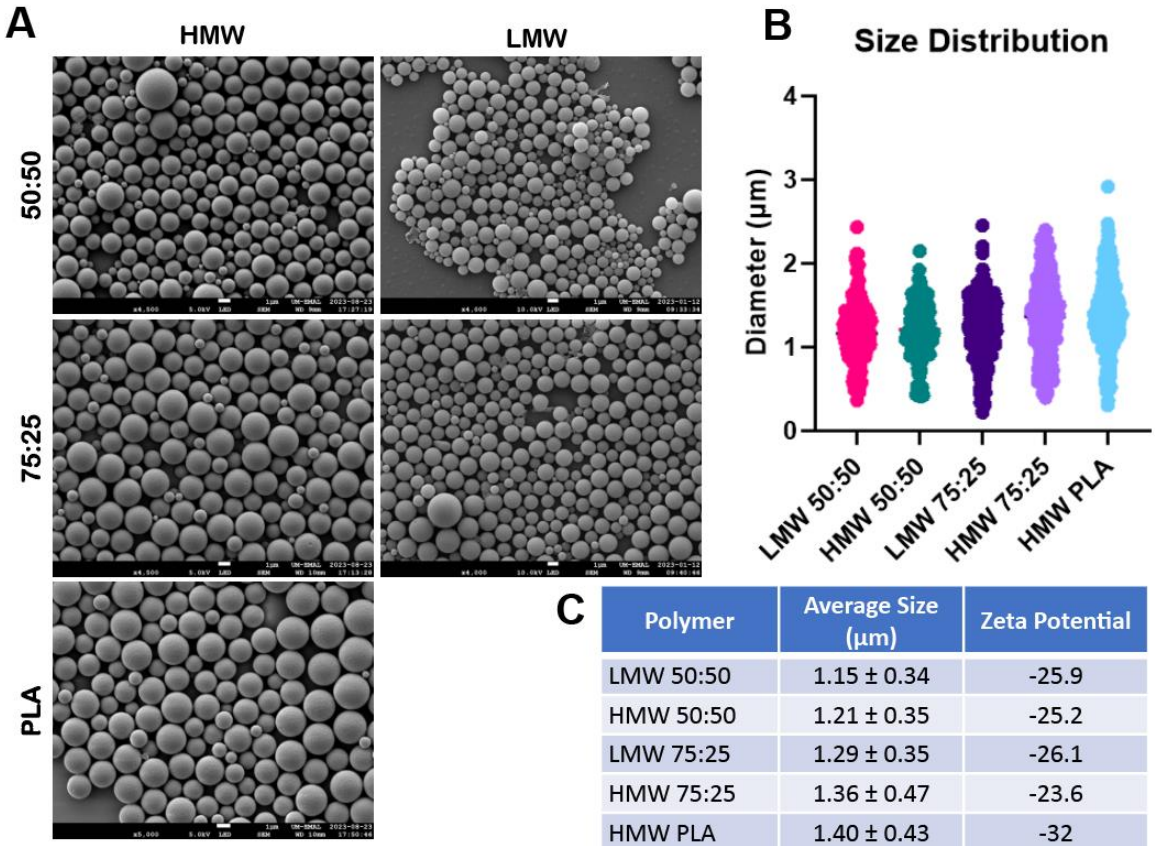
5.4.1 PLGA Particle Fabrication and Characterization

A variety of PLGA polymers ranging in molecular weight and lactic acid to glycolic acid ratios (LA:GA) were purchased for particle fabrication as listed in table 5-1. The polymers used for this study were either high or low molecular weight and lactic acid content was incrementally increased by 50% (50:50, 75:25, 100:0).

Table 5-1 PLGA polymers purchased for particle fabrication.

Polymer (Lactic to Glycolic Acid)	***Molecular Weight***	***Supplier***
LMW 50:50	**5-10 kDa**	**LACTEL**
HMW 50:50	**24-38 kDa**	**Resomer RG 503H**
LMW75:25	**6-10 kDa**	**Sigma**
HMW 75:25	**15-34 kDa**	**Resomer RG 753H**
HMW PLA (100:0)	**18-24 kDa**	**Resomer RG 203H**

Polymers were then fabricated into 1 μm sized particles by an oil in water emulsification method. Particles were imaged on SEM to confirm smooth surface morphology and to measure particle size (Figure 5-1A). All of the polymers resulted in particles of similar size profiles (Figure 5-1B), all within standard deviations of each other (Figure 5-1C). Additionally, all of the PLGA particles had similar, negative surface charge (Zeta potential) as shown in Figure 5-1C.



C

Polymer	Average Size (µm)	Zeta Potential
LMW 50:50	1.15 ± 0.34	-25.9
HMW 50:50	1.21 ± 0.35	-25.2
LMW 75:25	1.29 ± 0.35	-26.1
HMW 75:25	1.36 ± 0.47	-23.6
HMW PLA	1.40 ± 0.43	-32

Figure 5-1 PLGA particle imaging and characterization.

(A) Particles were imaged on scanning electron microscopy (SEM). (B) Particle diameters were measured using ImageJ and n>100 were plotted to show distribution. (C) Particle size was tabulated with standard deviation and listed with the particle surface charge.

5.4.2 PLGA Degradation in "Phagosome-Like" Environment

PLGA composition only matters as much as the polymer can physically degrade. For this reason, we designed an experiment to estimate the potential lactic acid release from each particle type (Figure 5-2A). A neutrophil phagosome is basic—about pH 8.5—for 30 minutes post

phagocytosis, then returns to a neutral pH. To imitate this environment, particles were resuspended in a beaker on a stir plate fitted with a pH probe. Using concentrated NaOH, the pH was raised to 8.5 for 30 minutes with gentle agitation, then lowered to 7.4 with concentrated HCl for the remainder of the experiment. The degradation ran for 2 hours total to imitate the timeline of a phagocytosis assay. The particle degradation was quantified by calculating a percent decrease in volume (Figure 5-2B). Importantly, PLGA degrades by surface erosion in basic conditions, allowing for this type of measurement to be meaningful.[160] As expected, the 50:50 PLGA particles (high and low molecular weight) and the LMW 75:25 had the greatest reduction in particle volume—approximately 20% loss in volume. The HMW PLA and HMW 75:25 PLGA had the least amount of degradation, less than 10% volume loss.

We next wanted to convert the percent decrease in volume to an estimated lactic acid release per particle. To gain this estimation, we first obtained the mass per particle (Figure 5-2C) by weighing out and counting the particle mass suspended in solution. In this work, we found that the LMW 75:25 PLGA had the greatest mass per particle. The particle mass was then converted to a total lactic acid content per particle using the polymeric ratio of lactic to glycolic acid (Figure 5-2D). Finally, we multiplied the percent decrease in volume by the total content of lactic acid to obtain the estimated lactic acid release per particle (Figure 5-2E). Based on the ability for LMW 75:25 PLGA to degrade compared with its high availability of lactic acid and high particle mass, the LMW 75:25 PLGA particles have the highest potential (2.85 pg/particle) for lactic acid release. As follows, the LMW 75:25 particles also have the highest estimated lactic acid release (0.59 pg/particle) as estimated from the decrease in volume and the known lactic acid content.

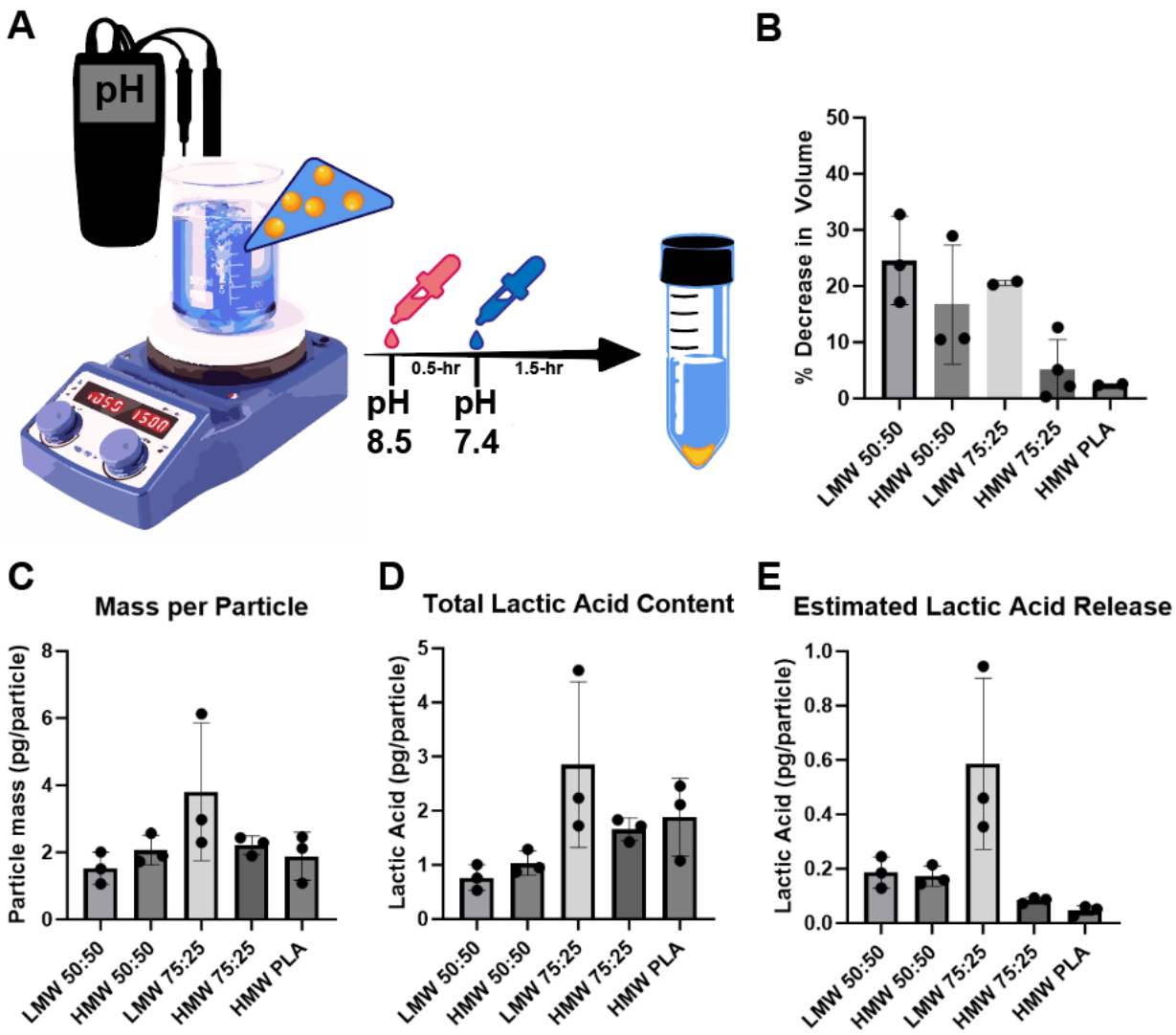


Figure 5-2 Particle Degradation in "Phagosome-like" environment.

(A) Experimental design for degrading particles. (B) Percent decrease in particle volume. (C) Measured mass per particle. (D) Total potential lactic acid per particle. (E) Estimated lactic acid release per particle.

5.4.3 PLGA Uptake and Effects on Surface Protein Expression

We next investigated the effects of lactic acid content on particle uptake and resulting changes in neutrophil surface protein expression. First, we confirmed that neutrophils phagocytosed particles at the same rate despite polymeric composition in both unactivated and LPS activated conditions (Figure 5-3). After confirming particle uptake, we measured the resulting effects of particle uptake on neutrophil surface protein expression using the experimental design shown in Figure 5-4.

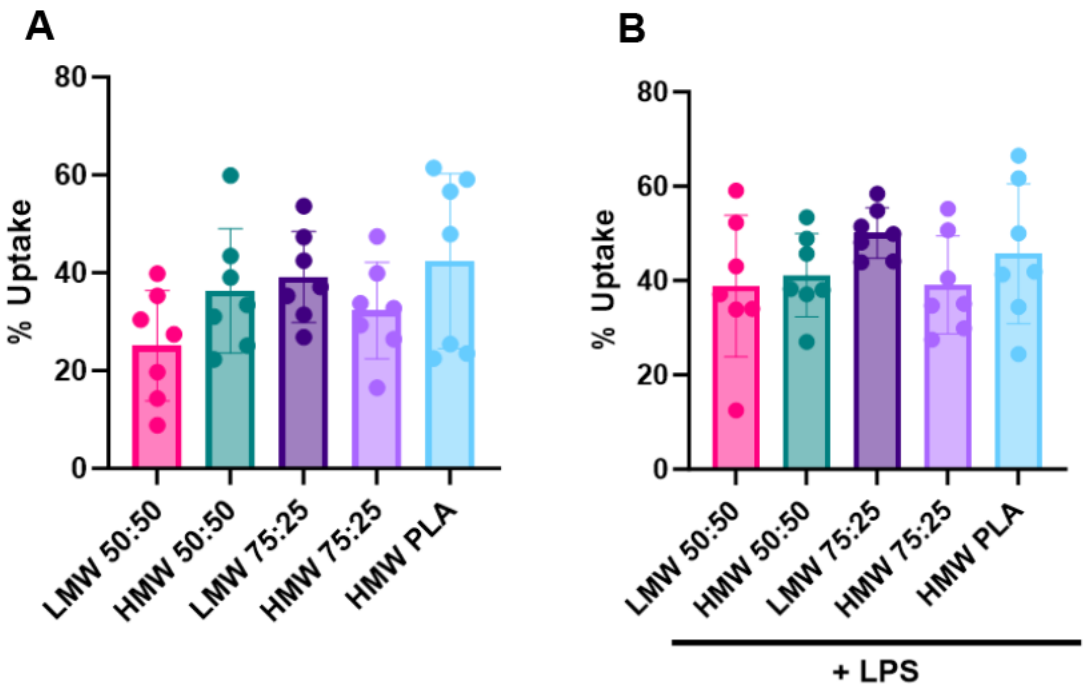


Figure 5-3 PLGA particle uptake by neutrophils.

Particle uptake in whole blood that is either (A) unactivated or (B) activated with LPS. Each sample received 2E8 particles/mL and each datta point represents a different donor.

In unactivated conditions we observed significant changes in the expression of PSGL1, MAC1, and LFA-1. Due to the high variance of CD62L surface protein expression we saw no significant changes in expression from particle type to particle type (Figure 5-5A). PSGL1 expression was significantly decreased in neutrophils positive for HMW 50:50 (1.40-fold), LMW 75:25 (1.30-fold), HMW 75:25 (1.36-fold), and HMW PLA (1.46-fofld) as compared to unactivated particle negative neutrophils (Figure 5-5B). Only 75:25 PLGA, both high and low molecular weights resulted in significant increase in the expression of Mac1 and LFA-1 as compared to the unactivated control (Figure 5-5C and D). Mac1 expression was increased by 2.68-fold and LFA-1 was increased by-1.15 fold on average, suggesting baseline activation.

While understanding baseline changes in surface protein expression in unactivated samples is important, it is also essential to investigate the effects of lactic acid on pathways of inflammation. In the following experiments we activated neutrophils in whole blood with LPS. As seen in previous work, LPS induced a 7-fold reduction in CD62L expression as compared to unactivated controls, thus we can use CD62L shedding as a marker for activation. The LWM 50:50, HMW 50:50, and HMW PLA induced a non-significant increase in CD62L shedding compared to the non-particle treated control. Interestingly, both the low and high molecular weight 75:25 PLGA lead to a significant preservation of CD62L compared to PLA positive neutrophils by 30.5% and 31.6% respectively (Figure 5-6A). These values were not different compared to the non-particle treated control, but importantly did not induce further CD62L shedding.

Similarly, PSGL1 expression was equivalent for neutrophils treated with LMW 50:50 (1.96-fold), LMW 75:25 (1.99-fold), HMW 75:50 (2.08-fold), and particle negative neutrophils (1.84-fold). Only HMW 50:50 and HMW PLA induced further, significant, reduction of PSGL1 on neutrophils (Figure 5-6B). Unlike in the unactivated samples, MAC-1 expression did not significantly change with particle treatments in LPS activated conditions (Figure 5-6C). However, LFA-1 surface protein expression was found to be significantly increased in neutrophils positive for LMW 75:25 and HMW 75:25 compared to the non-particle treated control.

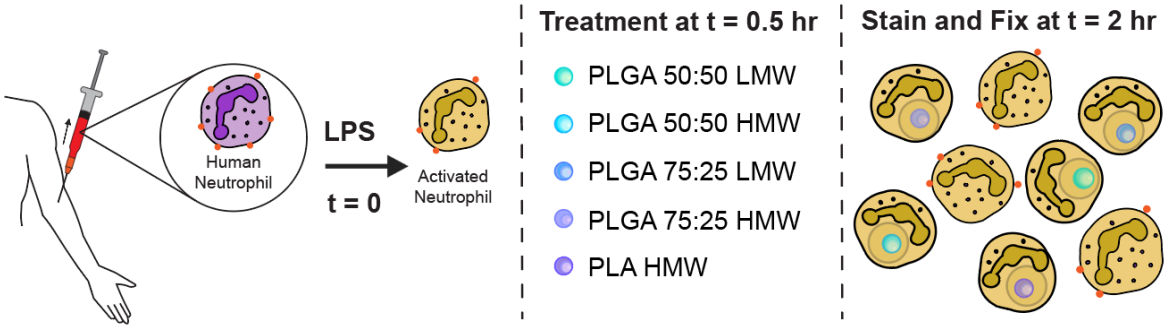


Figure 5-4 Experimental design for evaluating surface protein expression.

Human blood is taken from healthy donors and aliquoted for sample duplicates. The blood is activated with LPS at t = 0, treated with particles at t = 0.5 hr, and stained/fixed at t = 2 hrs to prepare samples for flow cytometry.

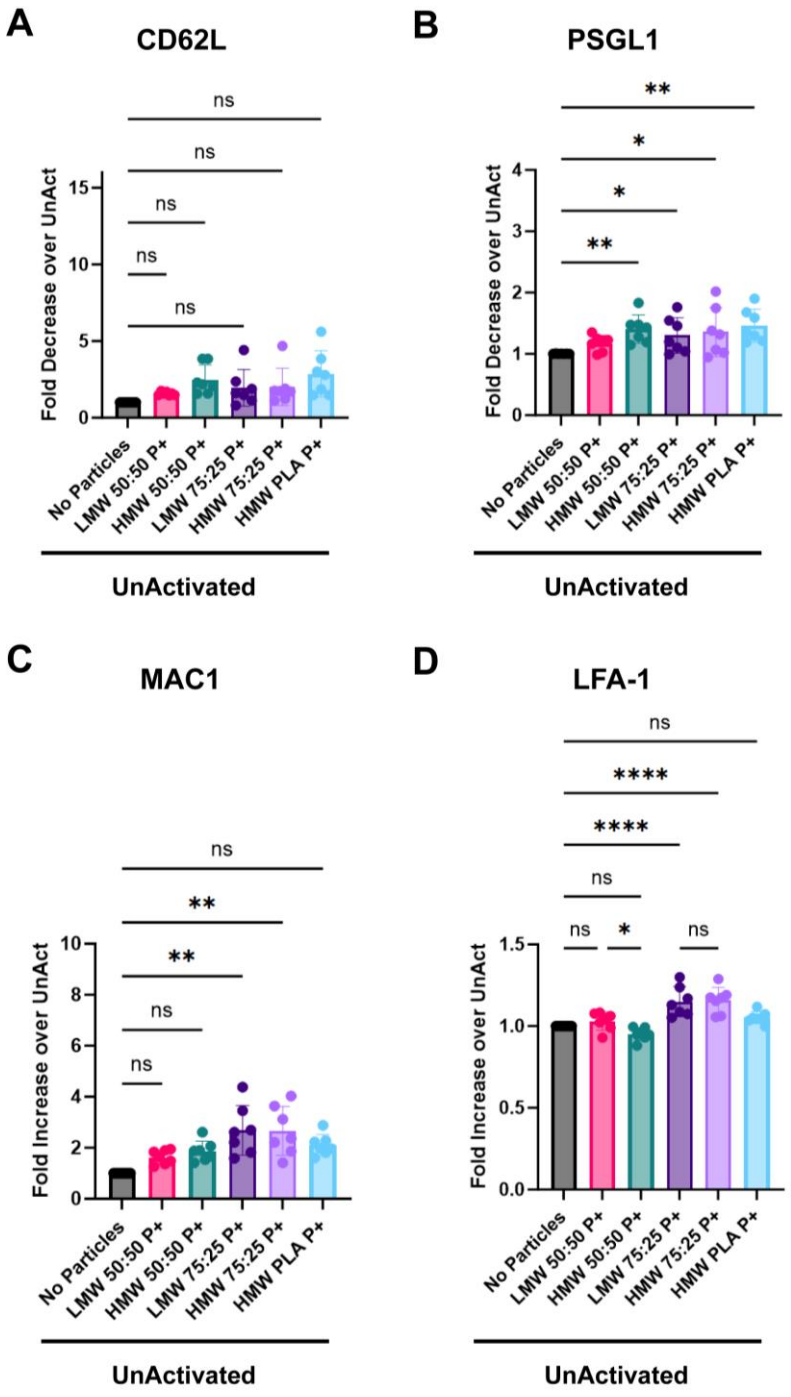


Figure 5-5 Neutrophil surface protein expression in unactivated conditions

Neutrophils in whole blood were treated with each particle type and evaluated on flow cytometry for surface expression of (A) CD62L, (B) PSGL1, (C) MAC1, and (D) LFA-1. Human donors were used for this experiment with n=7 for each condition. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $P < 0.0001$

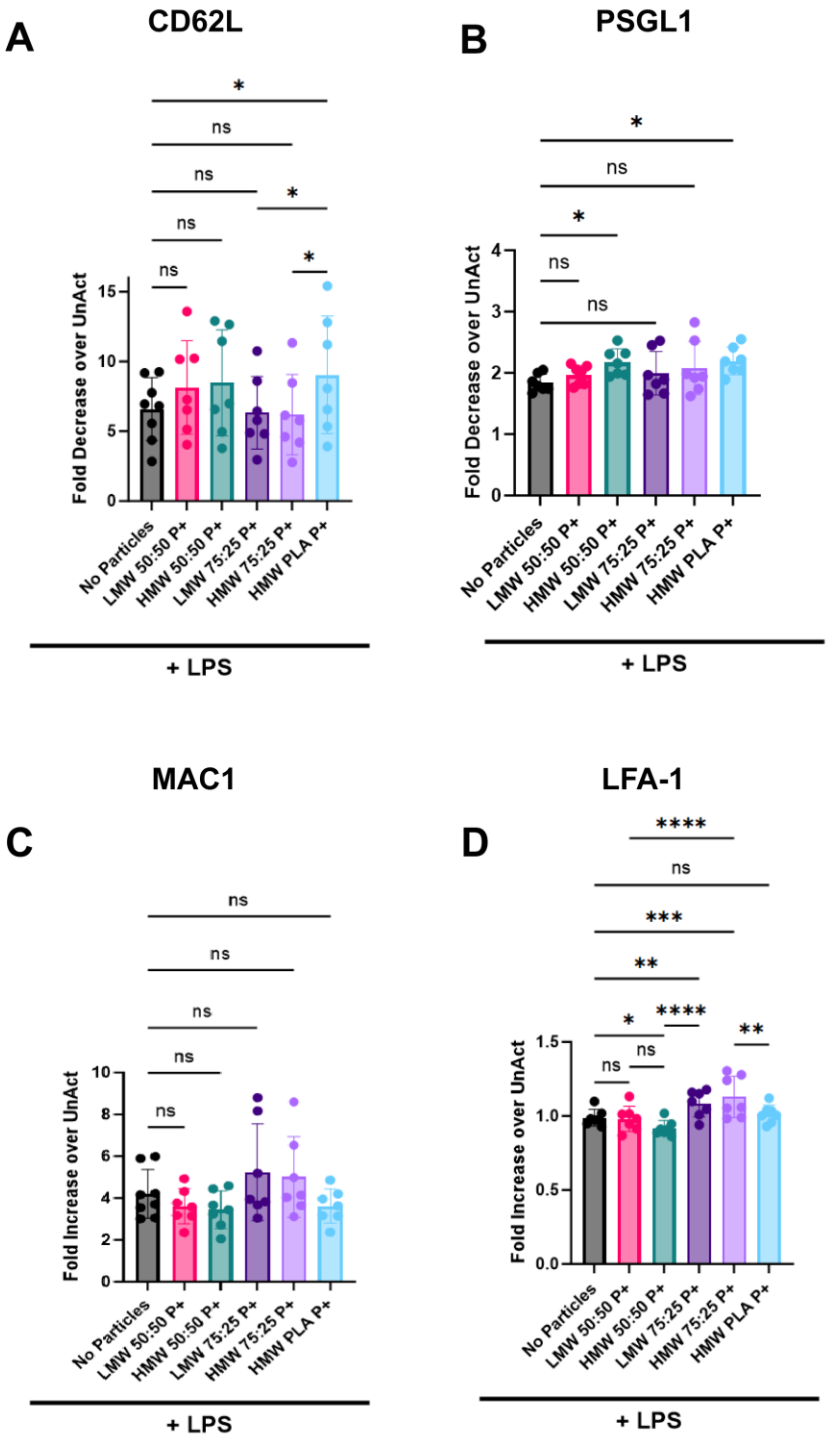


Figure 5-6 Neutrophil surface protein expression in LPS activation

Neutrophils in whole blood were activated with LPS and treated with each particle type and evaluated on flow cytometry for surface expression of (A) CD62L, (B) PSGL1, (C) MAC1, and (D) LFA-1. Human donors were used for this experiment with n=7 for each condition. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $P < 0.0001$

5.5 Discussion

Particle formulations of PLGA have been in the clinic since 1981, but have been limited to intramuscular, subcutaneous, periodontal, and intraarticular routes of injection.[61] Intravascular delivery of PLGA particles represents an untapped potential application for the treatment of inflammatory diseases, cancer, and gene therapy.[37,60] In order to develop intravascularly delivered PLGA particle therapeutics it is important to first understand the effects of PLGA on neutrophils, the major circulating WBC population in humans. PLGA is known to have pleiotropic effects on the immune system,[49,161,162] so it is necessary to understand the implications of various PLGA formulations when developing therapeutics for various applications, for example, inflammatory diseases would require immune suppressing formulations of PLGA, but gene therapeutics or cancer treatments may require an immunostimulatory formulation of PLGA.

In understanding the effects of PLGA on neutrophils, it is first necessary to understand the release of the active component of PLGA, lactic acid. From the literature we know that high contents of glycolic acid and low molecular weight increases the degradation rate of the polymer matrix.[163] Factors that influence degradation rate include temperature, pH, and the presence of enzymes, so knowing the exact rate of degradation intracellularly is a challenge.[160,164,165] In this work, we developed a method to estimate lactic acid release from each particle type in a "phagosome-like" environment by modulating pH. As expected, we found the most significant degradation in the 50:50 (low and high molecular weight) and in the LMW 75:25 as measured by decrease in volume. Further, while the 50:50 and LMW 75:25 degraded similarly, the greater proportion of lactic acid content in the 75:25 PLGA enhanced the available lactic acid significantly, yielding much more lactic acid release than the 50:50 PLGA particles.

PLGA degradation products and effects on inflammatory pathways have been well studied in the literature, but many results in the literature are conflicting. One reason for such a turbulent field is the intricate details that impact whether PLGA has inflammatory or anti-inflammatory properties. For example, PLGA used as a scaffolding material has been shown to cause inflammation at the sight of implantation due to its inherent acidic degradation products.[166] This acidity induces an inflammatory response and leads to the recruitment and differentiation of inflammatory cells (neutrophils, monocytes, macrophages). However, when delivered intracellularly via particle formulations, PLGA can induce either an inflammatory or anti-inflammatory response depending on degradation characteristics of the PLGA formulation.[49,162] In dendritic cells it has been shown that quick degrading PLGA (LMW PLGA) inhibits NF-κβ activation, preventing dendritic cell maturation.[49]

Throughout the literature it has been shown that immune cell phagocytosis of non-degrading (HMW) PLGA results in an inflammatory phenotype.[162,167] Despite this, PLGA is a versatile material that can be optimized to reduce inflammatory properties of slow degrading formulations. Chen. E., et al, for example, showed that by conjugating CD200, a self-associated regulatory protein, to the surface of PLGA, macrophage activation was inhibited.[167,168] PLGA can also be loaded with anti-inflammatory compounds (dexamethasone) and prevents the inherent inflammatory response seen in high molecular weight PLGA compared to low molecular weight.[169] Importantly, the literature has well characterized these responses to PLGA in animal models and cell lines, but has yet to investigate the neutrophil response to PLGA.

Lactic acid is produced naturally by anaerobic metabolism of mammalian cells and is known to modulate the immune response in inflammatory conditions.[170] More recently, lactic acid has been identified as a pleiotropic signaling molecule that can both contribute and interfere with

inflammatory pathways.[161] During a severe inflammatory response such as sepsis, lactic acid mediates a negative feedback loop to contain the inflammatory response.[171] This suppression of inflammatory cytokines by lactic acid has also been shown by Allen et al. in delivering fast-degrading PLGA to dendritic cells.[49] In the case of neutrophils, acidification of the cellular environment can greatly impact the neutrophil's response to an inflammatory stimulus, thus it is possible that lactic acid release from PLGA could influence the neutrophil's response to inflammation.[172] In this work we found that 75:25 PLGA induced baseline levels of inflammation as measured by PSGL1 shedding and increased MAC1/LFA-1 in unactivated conditions. However, in the case of LPS activation we see this trend reversed: the 75:25 PLGA had minimal impacts on surface protein expression as compared to the non-particle control, and significantly reduced CD62L shedding as compared to the non-degrading PLA.

We also see significant induction of LFA-1 expression by neutrophils that are positive for 75:25 PLGA particles. LFA-1 and MAC1 play an important role in modulating neutrophil slow rolling and transmigration. Neutrophil activation is well characterized and defined by an increase in MAC1 and LFA-1.[173] In this work we found that PLGA 75:25 induced the characteristic activation upregulation in unactivated samples, but in the activated samples we saw no additional MAC1 expression, yet a significant induction of LFA-1 expression. Similar to work shown in Chapter 3 of this thesis, LFA-1 upregulation by anti-inflammatory agents is an established mechanism that is not well understood in the literature. Ultimately, the conflicting results of 75:25 PLGA effects on neutrophils in unactivated and activated conditions further confirms the pleiotropic properties of lactic acid on neutrophils.

In our degradation study we found that the high molecular weight 75:25 PLGA did not release significant amounts of lactic acid, yet based on the modulation of surface protein

expression, we found that both the high and low molecular weight 75:25 PLGA had similar functionalities. It is likely that the degradation model used in this work did not fully capture the phagosome environment. The breakdown of high molecular weight PLGA is highly influenced by factors such as enzymatic degradation and temperature.[165] In short, the 50:50 PLGA polymers may not have enough available lactic acid to deliver intracellularly, whereas the high and low molecular weight 75:25 PLGA has the minimal amount of lactic acid release to influence surface protein changes. PLA is known to have the least amount of lactic acid release, due to the inability for degradation.[164,174-176] Future work should investigate a low molecular weight PLA, its potential to release lactic acid, and its ability to modulate neutrophil activation.

Importantly, the equivalence in neutrophil modulation between both high and low molecular weight 75:25 PLGA can help inform optimal particle design for treating inflammatory diseases. While the LMW 75:25 PLGA may have slightly more intracellular delivery of lactic acid, it is clear from these results that the HMW 75:25 meets the threshold of lactic acid release for neutrophil modulation. Thus, it would be important to move studies forward with the high molecular weight 75:25 PLGA, as a HMW polymer would be more stable in long term storage and throughout processing and sterilization practices.[177-179]

5.6 Conclusion

This work highlights the importance of investigating neutrophils to inform the design of particle-based therapeutics. Neutrophils are the first responder to inflammation and play a large role in modulating the inflammatory response. As shown by Kelley et al, ignoring primary human neutrophils in the development of PEGylated stealth coatings led to a major oversight in particle design and has ultimately led to major side effects in patients.[118] Specifically, Kelley et al.

identified the involvement of neutrophils in complement activation and particle clearance, illuminating the importance of such studies that investigate neutrophil-particle interactions. The information gained in this data chapter combined with findings from Kelley et al. show the major knowledge gaps in current understandings of neutrophil-particle interactions. Future work will investigate high lactic acid containing formulations of PLGA loaded with anti-inflammatory agents.

Chapter 6 Evaluating the Safety and Toxicity of Poly-A Particles as an Intravenous Therapeutic in Mice and Pigs

6.1 Publication Information

Much of the work in this chapter is unpublished. The toxicity study completed in mice was used as supplement in the publication titled “Cargo-free particles divert neutrophil-platelet aggregates to reduce thromboinflammation” with author list Alison L. Banka, M. Valentina Guevara, **Emma R. Brannon**, Nhien Q. Nguyen, Shuang Song, Gillian Cady, David J. Pinsky, Kathryn E. Uhrich, Reheman Adili, Michael Holinstat & Omolola Eniola-Adefeso published in *Nature Communications.*

Citation: Banka, A. L., Guevara, M. V., Brannon, E. R., et al. (2023). "Cargo-free particles divert neutrophil-platelet aggregates to reduce thromboinflammation." Nature Communications **14**(1): 2462.

6.2 Abstract

Poly-A has shown promise as a particle-based therapeutic for treating inflammatory diseases. To transition Poly-A to the clinic, safety and toxicity of Poly-A particle infusions must be examined. Any particle-based therapeutic is at risk for causing adverse infusion reactions, and as a salicylic acid releasing therapeutic there are additional toxicities to examine, i.e. liver toxicity from salicylic acid in the plasma. In this work we completed toxicity testing in mice and then further investigated

the safety of Poly-A infusions in pigs. Poly-A led to minimal long-term effects in a 5-day mouse study and was safely infused into a pig with no adverse reaction.

6.3 Introduction

Particle-based therapeutics represent an innovative strategy for intravascular, systemic drug delivery and are novel therapeutic strategies in modulating immune cells in inflammation. Since the development and utilization of PEGylated liposomes for chemotherapeutic drug delivery (i.e. DOXL/lipoDOX), the immune system, i.e. neutrophil and complement reactions, has represented a major hurdle that intravenously delivered nanomedicines have to face. Without the focus on neutrophils as a primary cellular component in poor translatability, intravenous formulations of particle-based therapeutics have not changed in 30 years and liposomal formulations are no longer thought of as novel or innovative.[180] Polymeric formulations of particle-based therapeutics show promise as a more stable, biocompatible, and novel inflammation modulators and thus work must be done to ensure translatability from benchtop to clinic.

Currently, all standardized assays developed by the Nanotechnology Characterization Lab (NCL), an organization within the FDA and National Cancer Institute (NCI), have excluded analysis on primary human neutrophils.[181,182] This oversight has led to an incredible knowledge gap in IV-delivered particle-based therapeutics, preventing the development of innovative solutions to decades-old problems. While *in vivo* models such as mouse models are helpful to initially evaluate therapeutics for inflammatory diseases, there is evidence suggesting inconsistencies with human diseases. For example, mice have a much more evolved immune responses to some pathogens, making it difficult to translate therapeutics from mouse models to the clinical setting.[183,184] Mice also lack similarities in immune cell composition—neutrophils

make up only 20-30% of circulating WBC as compared to 50-70%.[39,185] Additionally, mouse neutrophils lack functional similarities to human neutrophils.[39,185]

It is well known that poor tolerability of intravenously infused particles is due to a pseudo allergic response propagated by complement proteins, yet mouse models are incapable of replicating the exact symptoms and sensitivity seen in human patients.[186] This hypersensitivity reaction known as complement-activation related pseudoallergy (CARPA), is propagated by the cleavage of C3 to anaphylatoxins C3a and C5a.[187,188] Porcine models are better indicators of complement reactions due to their robust response to infused particles and closer similarity to human neutrophil composition and function.[185,189] Even so, porcine models are inherently more expensive and complex, posing additional challenges.

To translate novel, polymeric particle formulations, such as Poly-A, to the clinic, a wide range of safety/toxicity testing is required. The active degradation product of Poly-A, Salicylic acid, has been used to relieve pain and to treat inflammation since B.C.E. times, thus it is incredibly well characterized and understood.[190] With that said, it is well known that hepatotoxicity is a serious side effect of aspirin and is induced by high plasma concentrations of salicylic acid. Ideally, by polymerizing salicylic acid and delivering it directly to neutrophils, we can create a localized high concentration of salicylic acid to modulate neutrophils directly rather than modulating inflammation systemically. This will allow for therapeutic dosages to be drastically lower than that of orally prescribed aspirin, avoiding chances of hepatotoxicity all together. In this work we first evaluated Poly-A degradation in human plasma. We next utilized a mouse model to evaluate potential side effects of Poly-A. We then scaled the particle infusion to a pig model to evaluate the safety of Poly-A particle infusions.

6.4 Results

6.4.1 Investigating Poly-A Degradation in Human Plasma

Poly-A particles are known to degrade over a multi-day period as shown in chapter 3. To ensure particles remain intact in a biologically relevant environment we investigated the degradation of Poly-A particles in human plasma over the period of 30-hrs (Figure 6-1). The presence of particles was determined by SEM at 6-, 20-, 30-hrs post suspension in plasma. Some degradation occurred, but the detection of particles 30-hrs post resuspension shows that the particles maintain structure and do not simply disintegrate in human plasma.

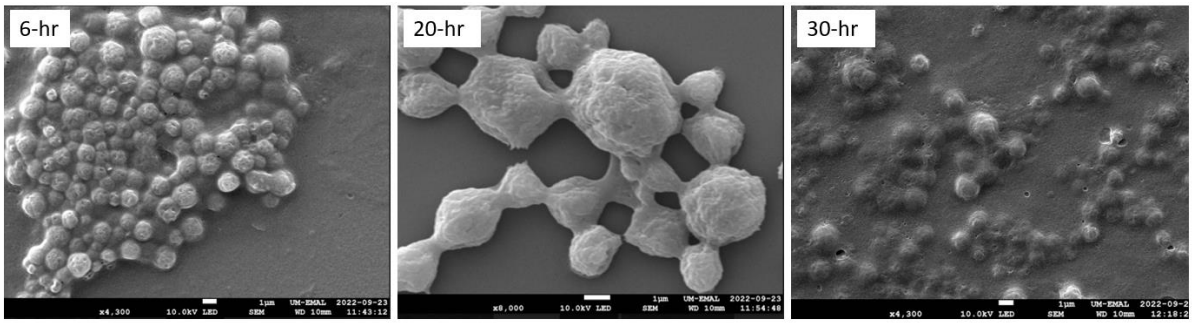


Figure 6-1 Degradation of Poly-A particles in human plasma.

Poly-A particles were suspended in human plasma and rotated at 37°C for 30 hours. SEM images were taken at various time points to establish the presence of particles.

6.4.2 Mouse 5-Day Toxicity Study

To understand the effects of Poly-A particles in a long-term study, we designed a mouse experiment to investigate the safety of a bolus Poly-A dosage in mice over a 5-day period. The mice were injected with 2E8 particles/mouse and monitored over a 5-day period. Five days post intravenous injection we evaluated the distribution of neutrophils in blood, liver, and spleen (Figure 6-2). In the blood, we found no significant changes in the percentage of monocytes or neutrophils across groups (control, soluble aspirin, or Poly-A injected) as shown in Figure 6-2A.

In terms of neutrophil count, we found that the soluble aspirin and Poly-A injected mice had a slightly higher, non-significant increase in total neutrophil count per mL of blood as compared to the control mice (Figure 6-2B). In the liver, we found that soluble aspirin injected mice had the greatest average count of neutrophils per gram of tissue, but not significantly greater compared to the control group (Figure 6-2C). Of the mice that received Poly-A particles, 2 had similar neutrophils/g liver tissue compared to the control group, but one Poly-A injected mouse had elevated neutrophils in the liver. There were no changes across groups in the neutrophil count in the spleen (Figure 6-2D).

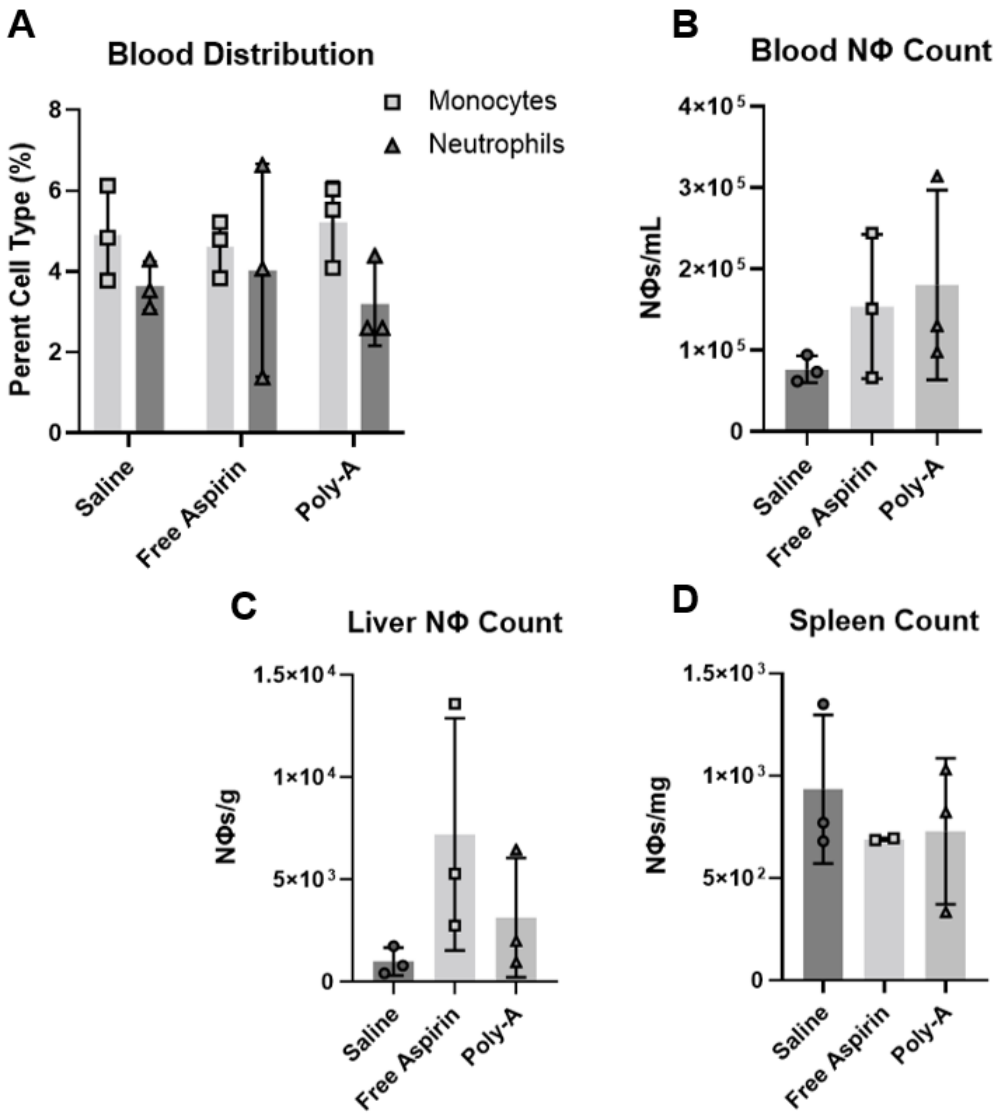


Figure 6-2 Distribution of neutrophils in a mouse toxicity study.

Mice were injected with 2E8 particles/mouse and monitored for 5 days. On day 5 the mice were euthanized to evaluate the percentage of neutrophils in the (A) blood along with neutrophil counts in the (B) blood, (C) liver, and (D) spleen.

We next investigated inflammatory markers in the plasma (Figure 6-3). We first measured two inflammatory cytokines, Il-6 and CXCL1/KC. IL-6 was significantly reduced in the plasma of mice that received either soluble aspirin or Poly-A particles. Soluble aspirin lead to a 67% reduction and Poly-A particles lead to an 87.8% reduction in IL-6 (Figure 6-3A). CXCL1 was unchanged across groups (Figure 6-3B). Aspartate Aminotransferase (AST) was measured as a marker against liver damage. In this work we found no change in AST content in plasma across treatment groups, suggesting there was no effect on liver function.

While we did not detect liver damage, we wanted to better understand the release profile of Poly-A particles compared to soluble aspirin post injection. We isolated plasma collected from mice after either a soluble aspirin or Poly-A injection and ran the samples on liquid

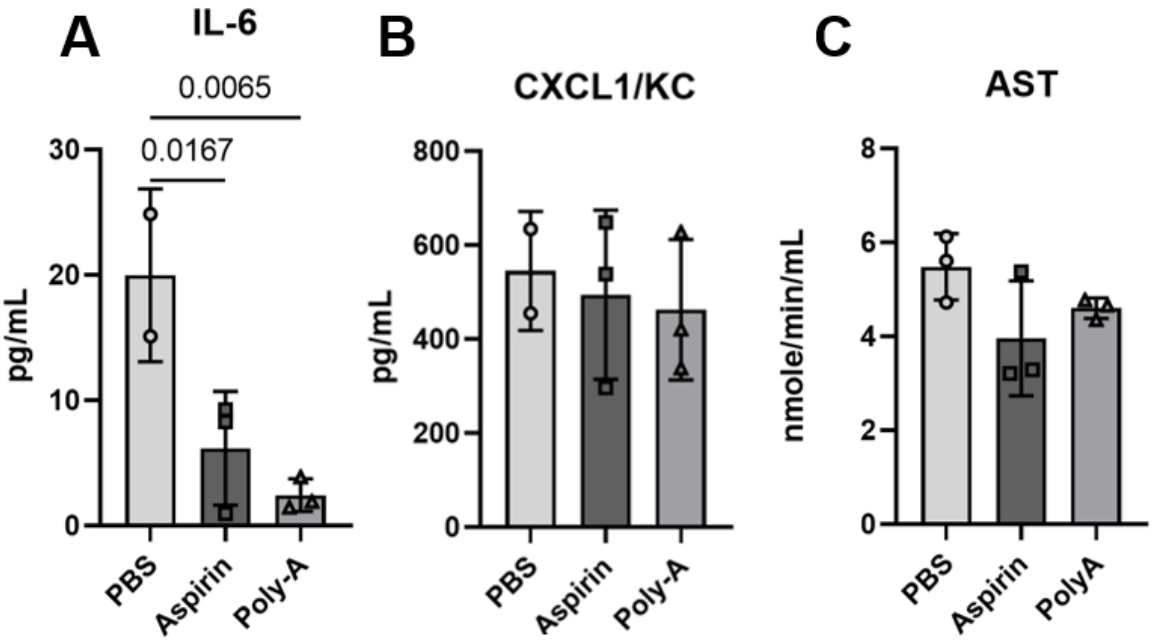


Figure 6-3 Quantifying inflammatory markers in the plasma.

The plasma was isolated from blood collected on day 5 of the toxicity study. Inflammatory cytokines (A) IL-6 and (B) CXCL1/KC were measured using ELISAs. (C) Aspartate Aminotransferase (AST) was measured to investigate liver damage. Statistical analysis was performed using GraphPad Prism Software using One-Way ANOVA with Fisher's LSD test with a 95% confidence interval. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $P < 0.0001$

chromatography-tandem mass spectrometry (LS-MS/MS) (Figure 6-4). From this experiment we

found that soluble aspirin immediately leads to a spike in salicylic acid content in the plasma, approximately 4000 ng/mL, but Poly-A particles have minimal release of salicylic acid.

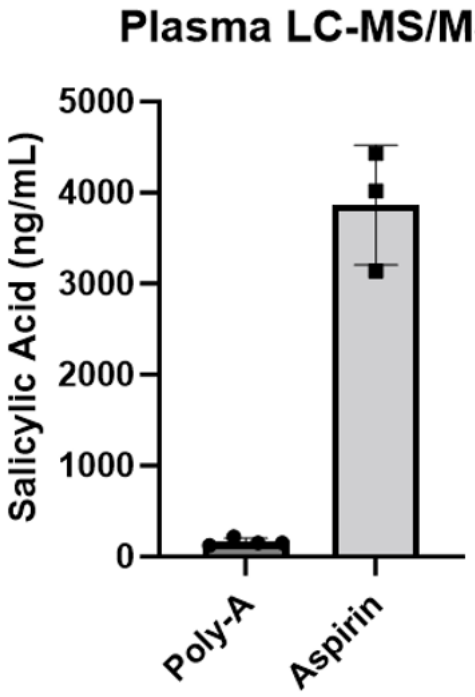


Figure 6-4 Salicylic acid detection in plasma.

Mice were injected via tail vein with either Poly-A particles or soluble aspirin. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) we quantified the salicylic acid concentration in the plasma.

6.4.3 Poly-A Uptake Across Species

After confirming the long-term safety of Poly-A particle infusion, we began scaling Poly-A to a porcine model. Prior to investigating an *in vivo* porcine model, we had to first develop an understanding of Poly-A particle interactions with pig neutrophils. Using flow cytometry, we developed a staining protocol to identify neutrophils in whole blood samples (Figure 6-5). Forward and side scatter were used to isolate pig neutrophils by size, then cells stained positively for SWC1 and CD172a were identified as neutrophils.

After developing a flow cytometry protocol, we moved on to particle uptake studies. In this work we spiked increasing particle concentrations into whole pig blood, then evaluated the resulting particle uptake on flow cytometry (Figure 6-6). We found that as particle concentration was increased, a greater proportion of neutrophils were particle positive (Figure 6-6A). In

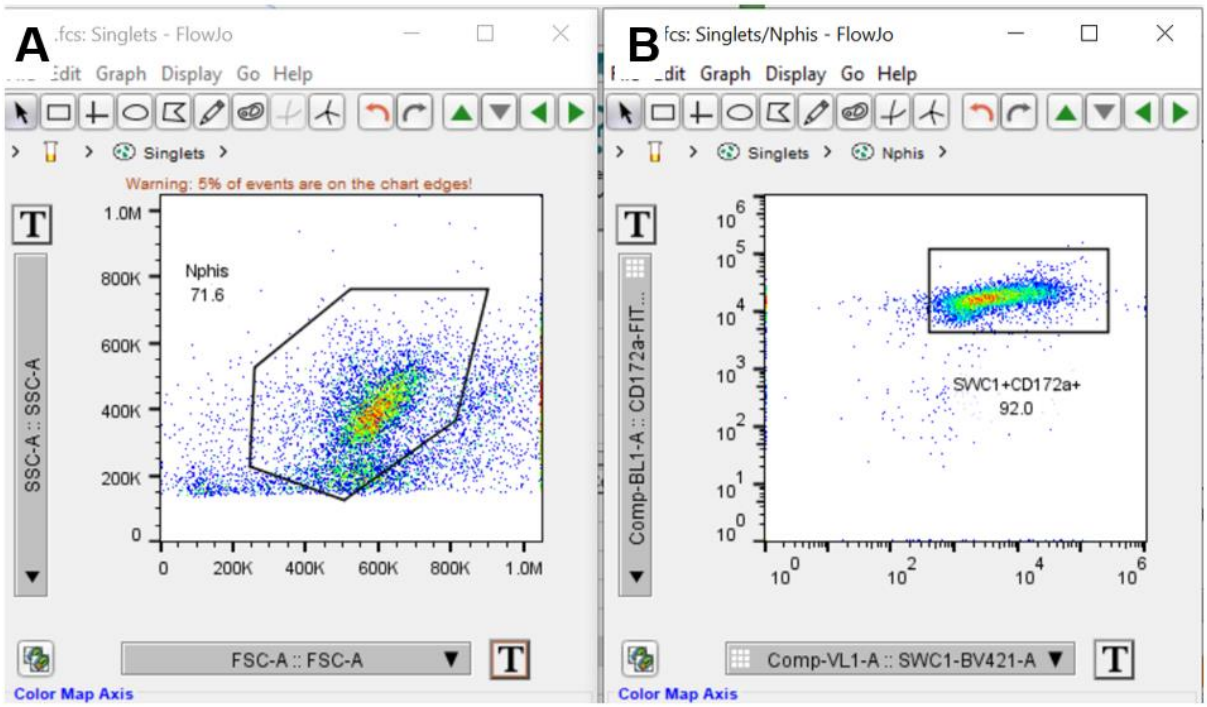


Figure 6-5 Flow cytometry of pig neutrophils stained in whole blood.

Pig neutrophils were stained with SWC1 and CD172a to identify the neutrophil population. (A) Forward and side scatter (FSC/SSC) was used to isolate neutrophils by size differentiation. (B) Surface protein expression of SWC1 and CD172a was then used to confirm neutrophil identification.

comparison to human and mouse blood, we found that pig neutrophils phagocytosed Poly-A particles at similar rates to mouse neutrophils (about 20%) particle positive neutrophils, and less than human neutrophils (Figure 6-6B). The data collected here informed the rate and dose of Poly-A infusion for our *in vivo* pig experiments.

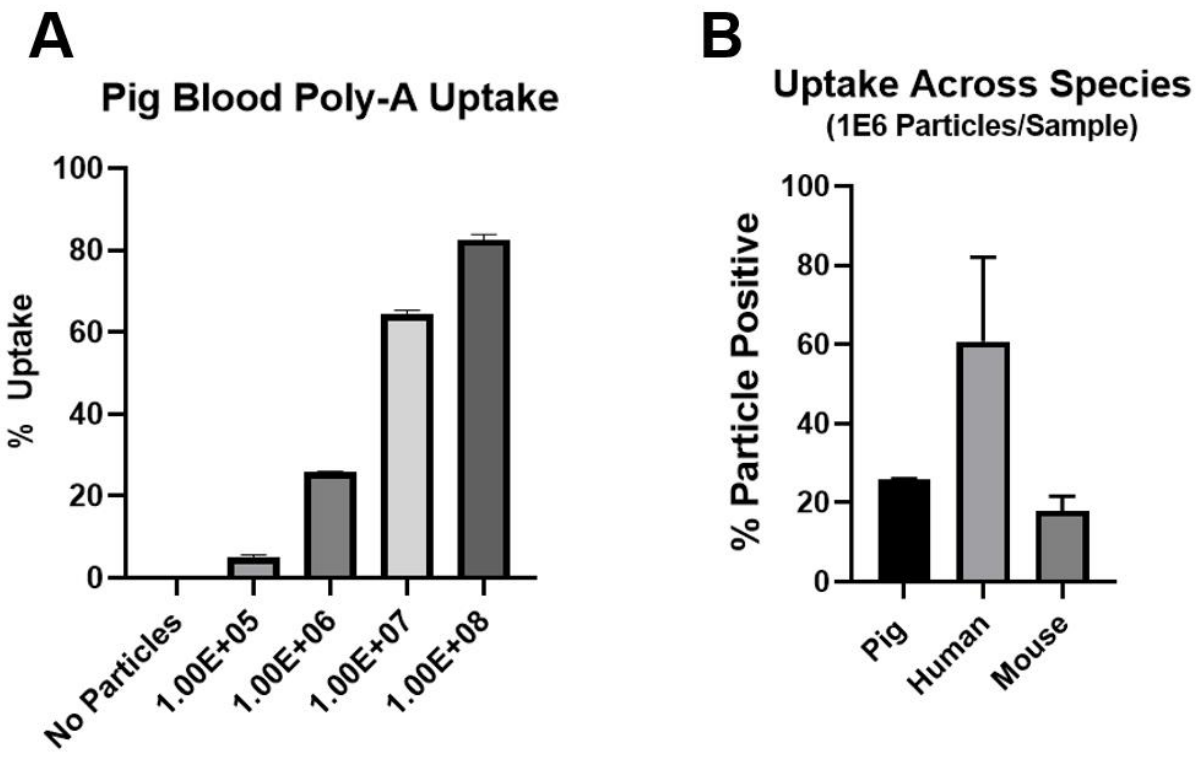


Figure 6-6 Poly-A particle uptake by pig neutrophils and compared to mouse and human neutrophil uptake.

(A) Whole blood collected from pigs was incubated with a range of Poly-A particles to investigate rates of phagocytosis. (B) Particle uptake was compared across species (mouse/pig/human).

6.4.4 Infusing Poly-A Particles into a Healthy Pig

The preliminary challenge in moving to a large animal model is determining the injection dosage and infusion rates. Specifically, particle-based therapeutics can induce a pseudo allergic reaction upon infusion leading to life threatening complications.[191,192] This reaction, also known as Complement Activation Related Pseudo Allergy (CARPA), is initiated by complement involvement with intravenously injected particles.[192] There is no literature that investigates a dosage regimen for a bolus injection of 1-micron sized particles for large animal models. Due to the similarities between mouse and pig uptake of Poly-A particles, we chose to scale the Poly-A infusion based on particles per blood volume as established in our mouse acute lung injury model (1E8 particles/mL blood). We chose to infuse the particles through the left external jugular due to ease of access based on the Tiba lab's experience in using the left external jugular for infusions. BALF and blood samples were collected throughout the 12-hr experimental period. Lung and liver samples were harvested for histology analysis. The overall experimental layout for this first experiment is shown below in Figure 6-7.

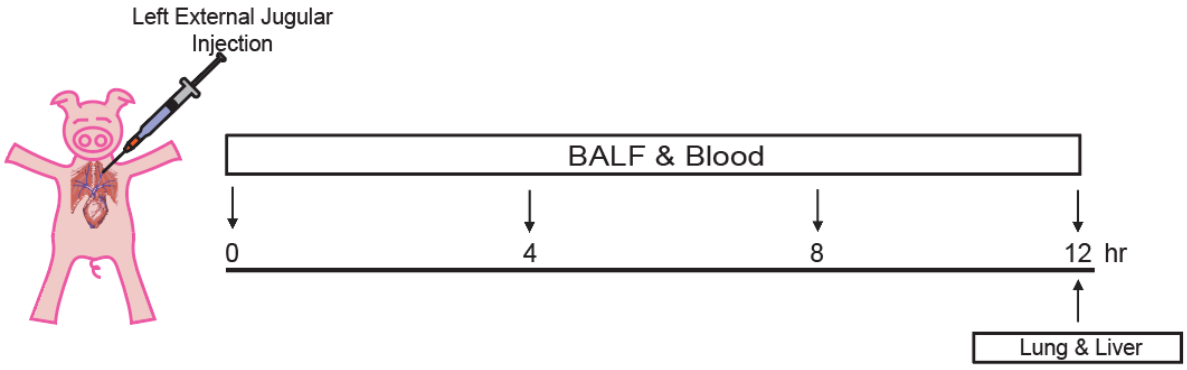


Figure 6-7 Experimental layout for the first particle infusion into a pig.

Poly-A particles were infused through the left external jugular. Blood and BALF samples were collected throughout a 12-hr period.

Particles were infused at rate of 10 mL/min, inducing a hypotensive event. We hypothesized that the particles induced sudden vasodilation that led to a drop in blood pressure. The pig was resuscitated with 1 mg of epinephrine and crystalloids, stabilizing the pig's blood pressure. The Poly-A infusion was then continued at a reduced rate of 2 mL/min for 10 minutes, then the rate was increased to 4 mL/min and then 6 mL/min. The pig experienced a second hypotensive event at the 6 mL/min infusion rate that was controlled by reducing the infusion rate to 4 mL/min. The pig experienced residual hypotension and bradycardia (MAP < 60mmHg, HR < 55 BPM) for another hour and was treated with a crystalloid infusion. The pig then remained stable for the remainder of the experiment.

In the blood we monitored circulating monocytes and neutrophils (Figure 6-8). Monocytes remained stable through the infusion period, but throughout the day the monocyte population incrementally reduced over the first 8-hrs post infusion, then increased between the 8- and 12-hr time points. Neutrophils spiked at the completion of the infusion and remained irregular throughout the experiment.

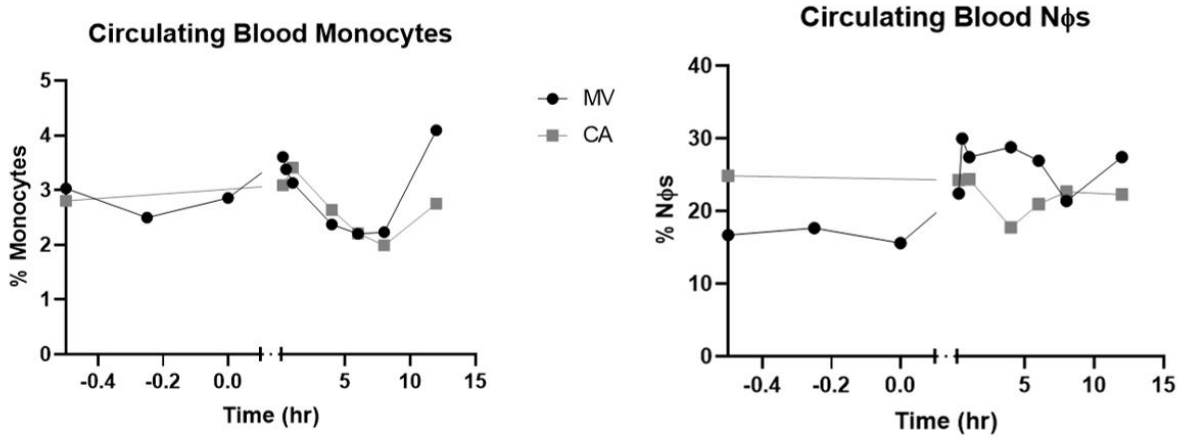


Figure 6-8 Circulating WBC populations post infusion, pig #1.

Blood monocytes were found to decrease throughout the day post-infusion. The neutrophil population spiked post-infusion and we found erratic differences in neutrophil percentage between mixed venous and carotid arterial blood samples.

The symptoms experienced by the pig were similar to symptoms from a CARPA response. Thus, we next sought to measure the complement protein C5a—an anaphylatoxin that propagates a CARPA response (Figure 6-9). We found that C5a was slightly increased at the infusion completion, but not significantly raised from baseline samples and within the normal range of C5a for pigs.[193] Throughout the experiment, C5a remained stable with no significant changes.

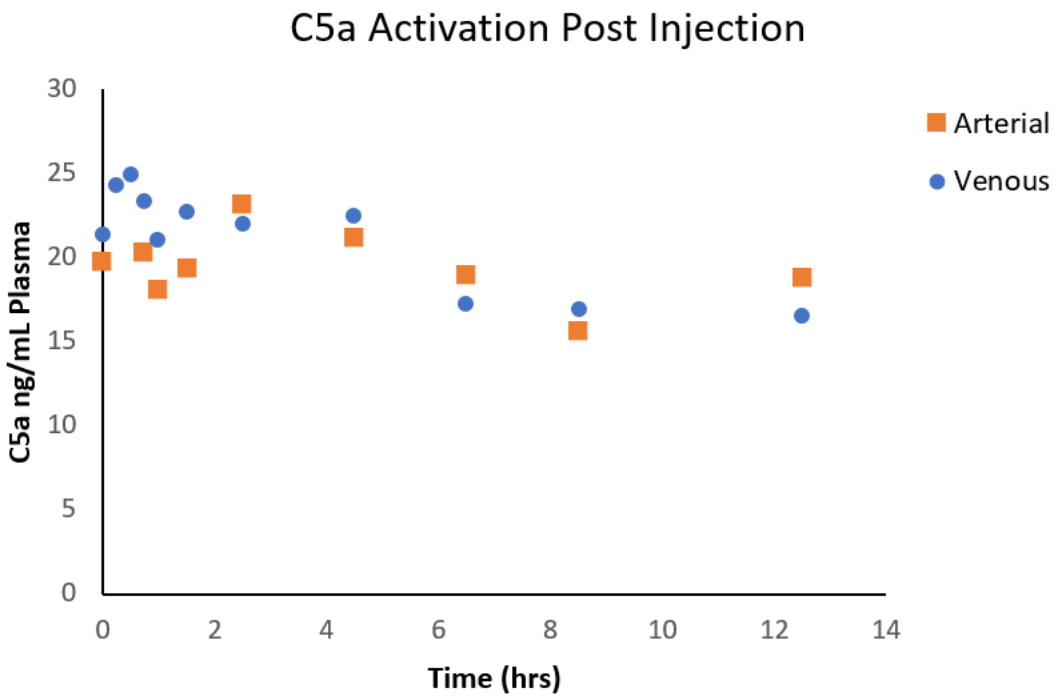


Figure 6-9 Complement activation in plasma as measured by anaphylatoxin C5a.

C5a was measured in the plasma via an ELISA.

Due to the minor complications experienced in the first Poly-A pig infusion, we chose to repeat the experiment with slight modifications to reduce infusion side effects. In this experiment, we moved the infusion location to the lateral saphenous vein to allow for maximum particle circulation prior to particles reaching the heart. Similar to the first experiment, blood and BALF samples were collected throughout a 12-hr timeline and lung and liver samples were harvested for histology at the end of the experiment (Figure 6-10).

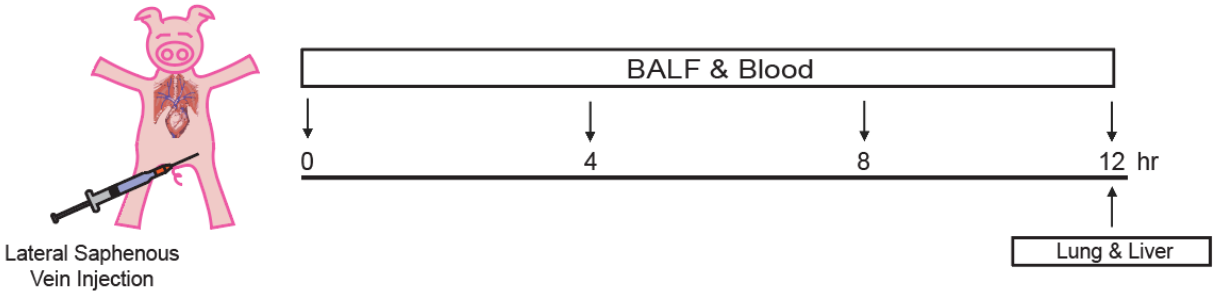


Figure 6-10 Experimental layout for pig #2.

The particles were infused into the lateral saphenous vein as shown above. Blood and BALF was collected over a 12 -hr period.

Poly-A particles were infused at 4 mL/min and the pig remained stable throughout the infusion and experiment. Neutrophil counts remained stable for the first 6 hours of the experiment but increased at 8- and 12-hr post infusion. Similarly, the BALF neutrophil content was found to increase throughout the day after a minor drop in neutrophils at 4-hr post-infusion (Figure 6-11). Histology reports from the second report showed no remarkable signs of inflammation in the lungs or liver (see Chapter 6 appendix). This was a major improvement from the first experiment in which inflammation in the lungs was detected. More specifically, the first pig had mild multifocal lobar edema accompanied by moderate to marked numbers of neutrophils and fewer macrophages in alveoli. Higher neutrophils in the lung tissue is a well-known marker for inflammation. The second pig, however, had no significant findings in the lung tissue as reported by blind analysis.

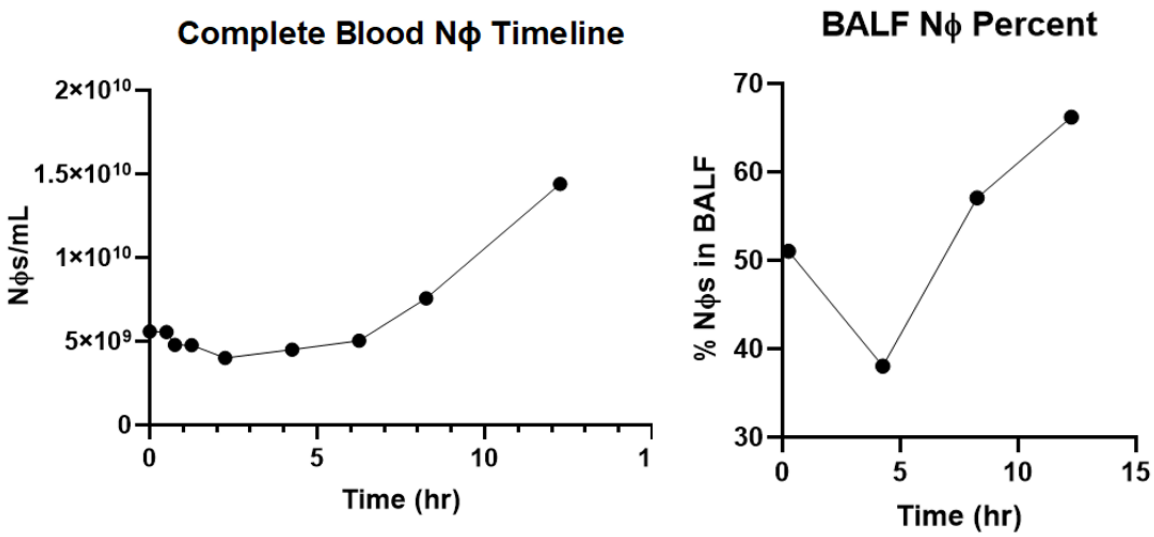


Figure 6-11 Neutrophil profile in the blood and BALF post-infusion.

6.5 Discussion

IV-delivered particle-based therapeutics have great potential in treating inflammatory diseases but have been limited in clinical use to cancer and diagnostics. This limitation is partially due to regulatory shortcomings, i.e. minimal flexibility in defining particle-based therapeutic safety, but also due to limitations in translatability.[60,181,194,195] Many particle-based therapeutics fail in translation from the difficulty in testing therapeutics in multiple small and large animal models.[60] Rodent models often poorly represent human diseases and reactions since our immune systems evolved so differently.[184] Thus, in this work we evaluated Poly-A particles, a novel therapeutic for treating inflammatory diseases, in two animal models for safety and toxicity. The work presented here shows the value in further investigations of Poly-A as it is both safe and has great potential.

Salicylic acid is a thoroughly researched anti-inflammatory agent with well understood toxicities and side effects. As Poly-A is polymerized salicylic acid, it's important to investigate its

potential side effects such as liver toxicity. In our 5-day mouse study we found no signs of liver toxicity as measured by AST, and further neutrophil distributions returned to normal in blood, liver, and spleen by day 5. However, we did find that both the soluble aspirin and Poly-A lead to significant reductions in plasma IL-6 5-days post injection. IL-6 is a well characterized cytokine that promotes the acute inflammatory response. As a therapeutic for inflammation, this side effect of reduced IL-6 can act as a benefit in controlling an overzealous inflammatory response. IL-6 is known to be a major contributor to cytokine storms,[196] thus a therapeutic that reduces IL-6 could significantly improve outcome. Further, salicylic acid is a known inhibitor of NF-κβ, a protein that promotes IL-6 production, so it makes since that both soluble aspirin and Poly-A particles would reduce IL-6. [196] Importantly, CXCL1/KC was not significantly reduced across treatment groups, suggesting that Poly-A particles may only interfere with parts of the inflammatory response and not inhibit all inflammatory pathways. CXCL1/KC plays a role in the initial phases of acute inflammation by recruiting neutrophils. While Poly-A may dampen the inflammatory response for 5-days via IL-6 reduction, some inflammatory pathways remain viable.[197]

One major concern for Poly-A is the potential bolus release of salicylic acid once injected into the blood stream and the related toxicity of salicylic acid.[198] To investigate this, we ran plasma samples taken from mice receiving either soluble aspirin or Poly-A particles on mass spectrometry to determine the salicylic acid content. From this experiment, we found only mice injected with soluble aspirin had significant amounts of salicylic acid in the plasma. Thus, we confirmed that Poly-A particles remain intact in the blood stream and instead release salicylic acid once internalized or slowly over time as determined by degradation characterization.[47,157]

When translating Poly-A particles across species it is important to understand the differences in particle-neutrophils interactions from mouse to pig and eventually to human. In this

work we investigated these differences by evaluating neutrophil uptake across species. We found that pig neutrophils internalize Poly-A similarly to mouse neutrophils, thus justifying our scaling of particles to blood volume in the pig infusion studies. Both mouse and pig neutrophils phagocytose Poly-A at reduced rates as compared to human neutrophils. Thus, if we were to scale Poly-A particle infusions to a human model, fewer particles would be needed to see similar results as observed in mouse and pig studies.

While the inflammatory pathways have been researched for decades, there is still much for us to learn in terms of particle design and the resulting inflammatory response, i.e. CARPA.[62,199] Despite the known relationship between complement and particle-based therapeutics, *in vitro* assays are limited and complement reactions vary heavily across species, making it difficult to develop *in vivo* assays.[62] Further, complement activation is not the only known pathway for adverse reactions to particle-based therapeutics.[200] CARPA is classically characterized by systemic vasoconstriction rather than vasodilation.[201] In this work, the first pig experienced an adverse reaction characterized by hypotension and low blood pressure due to vasodilation. In an early study investigating the negative reaction to particle-based therapeutics in dogs, it was found that histamine rapidly released in the blood stream propagated a hypotensive event similar to what was observed in this pig study.[202] By slowing down the infusion, the hypotensive event was not observed, similar to findings by Garavilla et al.[202] We confirmed through plasma quantification of C5a that the adverse reaction seen in the pig was not propagated by CARPA. Pigs are known for having intense CARPA reactions to particle-based therapeutics, but reliable model to test particles.[62,203] Thus, it is incredibly promising that Poly-A particles did not induce a CARPA reaction.

6.6 Conclusion

Particle-based therapeutics have been in clinical use since the approval of Doxil in 1995.[60] As a pioneering therapeutic, liposomal doxorubicin opened the door to a new realm of therapeutics enhancing drug delivery by concentrating potentially systemically toxic drugs into vesicles to be delivered to tumors.[60,204] Despite this, only 25 additional IV-delivered particle-based therapeutics have been introduced to the clinic since.[60] Particle-based therapeutics have untapped potential for treating inflammatory diseases. Poly-A represents an innovative therapeutic that has great potential in treating inflammation with minimal side effects and safe infusibility.

6.7 Appendix

6.7.1 Histology Report from Pig 1

Request Date: 11/3/2020
Pathologist: MJH
Returned Date: 11/6/2020

University of Michigan Medical School

ULAM In Vivo Animal Core
North Campus Research Complex
2800 Plymouth Road B36/G157
Ann Arbor, MI 48109-0614
Lab: (734) 647-0654
Email: ULAM-IVAC@umich.edu

Fax: (734) 936-3395
Web Site: http://med.umich.edu/ulam/services/pathology.html

Case number: 20M064-20M066
Date of necropsy: NA
Species: SUS
PI: Tiba
Contact: Colmenero

History: Three submissions of tissues from a pig were made (20H304, 20H310, 20H312) comprised of lung (20H304), spleen, liver, kidney, small intestine (20H310), and trachea (20H312) for gross examination and histopathology. These tissues will be reported in a single report for clarity.

RESULTS:
Gross examination: There were no gross lesions noted in any tissues.

Histopathology:
Lungs (20H304):
In the RLL, RML, 20201016-1, and 20201016-2, there was mild multifocal lobar edema accompanied by moderate to marked numbers of neutrophils and fewer macrophages in alveoli, and multiple medium to large bronchioles and bronchi contained neutrophils and macrophages. There was mild to moderate interlobular expansion by edema. In other lung samples, there was multifocal mixed interstitial infiltrates mostly arranged around bronchioles and arterioles.

Liver, spleen, kidney, small intestine (20H310):
Sections of liver and small intestine (duodenum) were unremarkable. In sections of kidney, there were multifocal minimal to mild mononuclear interstitial infiltrates in the medulla and cortex, with focal and minimal renal tubular epithelial hyperplasia.

Trachea (20H312):
In sections of trachea, there were minimal to mild mixed inflammatory infilrates within the submucosa, with focal and minimal epithelial blunting and loss of cilia. There was atrophy of epithelial cells with the presence of few neutrophils along the epithelial surface.

DISCUSSION:
The significant findings on histopathologic examination were the inflammatory infiltrates in the lung, which were consistent with a mild bacterial bronchopneumonia; there were no infectious agents noted histologically, and other findings (interstitial infiltrates) may suggest past viral infection. This may have been a subclinical inflammatory process in an asymptomatic animal. The majority of the inflammatory reaction was present in the right lung, which may be related to lateral recumbency during anesthesia, and it is also possible the changes in the trachea could be secondary to endotracheal intubation. Findings in other organs are considered background findings.

Pathologist: Mark J. Hoenerhoff, DVM, PhD, DACVP
November 6th, 2020

Figure 6-12 Histology report from pig 1.

6.7.2 Histology Report from Pig 2

University of Michigan
Medical School

Request Date: 1/21/2021
Pathologist: MJH
Returned Date: 1/22/2021

HISTOPATHOLOGY REPORT

ULAM In Vivo Animal Core
North Campus Research Complex
2800 Plymouth Road B36/G157
Ann Arbor, MI 48109-0614
Lab: (734) 647-0654
Email: ULAM-IVAC@umich.edu

Case number: 21M003
Date of necropsy: NA
Species: SUS
PI: Tiba
Contact: Colmenero

History
Samples of small intestine (duodenum), lung, liver, kidney, spleen, and trachea were submitted for gross and histopathologic examination.

RESULTS (descriptive)

On gross examination, submitted tissues were within normal limits.
Histologically, there were no significant findings observed.

DISCUSSION:
The purpose of this evaluation was to evaluate the submitted kidney samples grossly and histologically for evidence of inflammation and other pathologic alterations. There were no significant changes noted in sections examined above typical background alterations observed in conventionally raised pigs.

Pathologist: Mark J. Hoenerhoff, DVM, PhD, DACVP

January 22nd, 2021

This report is intended for rapid communication of histopathology results to the submitting researcher. If portions of this report are subsequently utilized in a publication or presentation, please communicate this to the pathologist so that the draft may be reviewed to ensure a narrative appropriate to the particular forum.

Figure 6-13 Histology report from pig 2.

Chapter 7 Conclusion

7.1 Publication Information

The text in this chapter was adapted from "*Polymeric particle-based therapies for acute inflammatory diseases"* in Nature Reviews Materials with author list **Emma R. Brannon**, M. Valentina Guevara, Noah J. Pacifici, Jonathan K. Lee, Jamal S. Lewis & Omolola Eniola-Adefeso.

Citation: Brannon, E. R., et al. (2022). "Polymeric particle-based therapies for acute inflammatory diseases." Nature Reviews Materials **7**(10): 796-813.

7.2 Outlook and Final Remarks

The introduction of particle-based therapeutics to clinical medicine has significantly improved efficacy and convenience for treating a range of diseases, including endometriosis, cancer, growth failure, gum disease, and even mood disorders. Despite the wide range of polymeric particle-based therapeutics used clinically and the plethora of literature on the topic, only 12 PLGA (polymeric) particle-based formulations have been approved by the FDA in the past 30 years.[59] This stark contrast between research and clinically approved therapeutics lies in the challenges at hand in creating a safe and effective polymeric particle-based treatment. Many challenges result from the complexity of biology and thus translation from animal models to clinical trials.

Severe acute inflammatory diseases currently have no curative treatments, yet particle-based therapeutics have shown incredible promise as immune-modulators. In chapter 3 I showed

the capability of Poly-A particles to redirect neutrophils in acute inflammation more efficiently than other particle types due to its inherent ability to reprogram neutrophils from the inside out. I next showed in chapter 4 that Poly-A can inhibit an inflammatory pathway, NETosis, further exemplifying its value as an anti-inflammatory therapeutic for both long term (5-hrs) and short term (2-hr) *in vitro* studies. Importantly, a more clinically relevant polymeric material, PLGA, induced NETosis after 5-hrs of incubation with neutrophils. PLGA, however, shows promise as an immune modulating material in shorter time frames (2-hrs) as work presented in chapter 5. By tuning the lactic to glycolic acid ratio, we can control the resulting inflammatory properties. Future work should investigate faster degrading PLGA polymers that deliver therapeutic doses of lactic acid intracellularly. Additional studies could investigate the incorporation of anti-inflammatory agents into the polymer matrix of PLGA to enhance anti-inflammatory properties. Polymeric materials represent an untapped potential in the field of IV-delivered particle-based therapeutics. Poly-A can be safely transfused intravenously into pigs as shown in chapter 6. Future studies investigating optimal dosing schemes are necessary to properly translate this work into the clinic. The work presented in this thesis not only shows the utility of particle-based therapeutics but also shows the safety and translatability of polymeric particles. Ultimately, using methods developed in this dissertation along with better translation of *in vitro* assays to *in vivo* applications will be necessary to transition particles in the clinic for the treatment of inflammatory diseases.

Novel particle-based therapeutics pose a challenge inherent to innovative design. Unlike traditional bulk therapeutics, particle-based medicines are typically constructed of multiple components: polymeric vehicle, therapeutic agent, and surface modifications.[181,194,195] Slight changes in any of these components can significantly change particle function, biodistribution, and toxicity, making it difficult for the FDA to efficiently evaluate these new platforms. Thus, the

National Cancer Institute (NCI) instigated the establishment of the Nanotechnology Characterization Laboratory (NCL) to develop standardized assays to characterize particle-based therapeutics and related toxicities.[181] While the NCL was built to streamline the clinical trial and FDA approval process, the streamlined processes are designed for cancer therapeutics rather than generalized therapies.[181,194,195] Thus, certain guidelines such as prolonged evasion of the mononuclear phagocyte system would inherently exclude particle-based therapeutics designed to target circulating phagocytes.[195] It will be crucial to update current methodologies for testing the safety of particle-based therapeutics to ensure translatability. PEGylated liposomes, while effective, are now considered "old technology" due to the lack of innovation since the approval of DOXIL. This lack of innovation can be attributed to the poor translatability across models and ultimately the lack of consideration of neutrophils.[180,194,195] To this day, neutrophils have been ignored in all immunological assays designed and used by the NCL.[205] This oversite has limited safe translation of current uses of particle-based therapeutics (cancer/diagnostics) in addition to the application of particle-based therapeutics to immunomodulation (inflammatory diseases).

Neutrophils are inherently difficult to study as there are no good cell lines and major differences in neutrophil composition across animal models. To successfully develop neutrophil modulating therapeutics, it will be necessary to fully develop *in vitro* assays that replicate the *in vivo,* human environment. Otherwise fully characterized models with known limitations could be used for therapeutic development. The work in this dissertation is just the beginning in terms of characterizing particles for the treatment of inflammatory diseases. Particles-based therapeutics can be optimized for targeting neutrophils by changing particle shape, including targeting ligands, and loading effective therapeutics. Due to the current prevalence of PLGA in the clinic, PLGA is a good candidate for material optimization to quickly translate this work into a human ready

therapeutic. However, it will be important to first optimize the anti-inflammatory properties of PLGA prior to use as a neutrophil modulation tactic. Thus, it is imperative that more neutrophil assays are developed to thoroughly test the safety and efficacy of particle-based therapeutics for modulating the inflammatory response. Ultimately, the consistent progress made by the scientific community and the eventual streamlined approval for use in patients can revolutionize particle-based therapeutics and the treatment of inflammatory diseases.

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