Neutrophil Interactions with Particle Drug Carriers and Behavior in Inflammation

by

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Dedication

This dissertation is dedicated to:

Mom

Dad

Amanda

Grandma Kelley

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ACD	Acid citrate dextrose
aCL	Anticardiolipin
ALI	Acute lung injury
ANOVA	Analysis of variance
APS	Antiphospholipid syndrome
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic assay
BMM	Bone marrow macrophages
BSA	Bovine serum albumin
CAMP	Cyclic adenosine monophosphate
CAMs	Cellular adhesion molecules
CEACAM-1	Carcinoembryonic antigen-related cellular adhesion molecule 1
CFL	Cell-free layer
CFU	Colony forming units
CVD	Cardiovascular disease
CXCR4	C-X-C chemokine receptor type 4
DLS	Dynamic light scattering
EAE	Experimental autoimmune encephalitis
ECs	Endothelial cells
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food & Drug Administration

FITC	Fluorescein Isothiocyanate
fMLP	N-formylmethionine-leucyl-phenylalanine
GCSF	Gram colony stimulating factor
HSA	Human serum albumin
HUVEC	Human umbilical vein endothelial cells
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intercellular adhesion molecule 1
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukin 10
IL-17	Interleukin 17
IL-1β	Interleukin 1β
IL-23	Interleukin 23
IV	Intravenous
KLF	Krüppel-like factor
LAC	Lupus anticoagulant
LC/MS	Liquid chromatography/mass spectroscopy
LDL	Low-density lipoprotein
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MMP9	Matrix metallopeptidase 9
NAR	Normalized adhesion ratio
NETs	Neutrophil extracellular traps
NIR	Near-infrared

OCT	Optimal cutting temperature
OD	Optical density
PBS	Phosphate-buffered saline
PECAM-1	Platelet and endothelial cell adhesion molecule 1
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PLDA	Poly(DL-lactide)
PLGA	Poly(lactic-co-glycolic) acid
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear neutrophils
PPFC	Parallel plate flow chamber
PS	Polystyrene
PVA	Polyvinyl alcohol
RBCs	Red blood cells
ROS	Reactive oxygen species
RPMI	Rosell Park Memorial Institute medium
SDF-1	Stromal cell-derived factor 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFG	Sum frequency generation
SHG	Second harmonic generation
SLE	Systemic lupus erythematosus
sLeA	Sialyl Lewis-A
TEM	Transendothelial migration
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor α
VCAM-1	Vascular cell adhesion molecule 1
VTCs	Vascular targeted carriers

WBC	White blood cell
WSR	Wall shear rate

Abstract

Neutrophils are cells of the innate immune system which make up approximately 50-70% of all circulating white blood cells. Despite their abundance, the importance of neutrophils in many contexts has only recently begun to be explored in depth due to the difficulty in maintaining neutrophils in culture. The work in this dissertation aims to investigate the role of neutrophils in inflammatory diseases and how neutrophils interact with particulate drug carriers designed to mitigate such diseases.

We explored the impact of particle drug carriers on leukocyte adhesion in inflammation *in vitro*, finding that both targeted and non-targeted particles reduce leukocyte adhesion. This effect was linked to both particle size and concentration. Additionally, we found that under certain particle conditions, specific blocking of the endothelium via active targeting results in greater reduction in leukocyte adhesion. Intravenous particle administration resulted in a reduction in neutrophil adhesion in an *in vivo* model of acute mesentery inflammation and an *in vivo* model of acute lung inflammation, resulting primarily from particle uptake. Crucially, we found that after internalizing particles, neutrophils shed CD62L, resulting in faster rolling along the endothelium and reduced adhesion, making particle internalization the dominating mechanism for reducing neutrophil adhesion.

We investigated the impact of the use of a common non-fouling coating, polyethylene glycol (PEG), on neutrophil particle phagocytosis. In contrast to the behavior of other phagocytes, we found that human neutrophils preferentially internalize PEGylated particles compared to non-PEGylated particles. This effect was linked to factors in the plasma, specifically complement; we demonstrated that PEGylated particles adsorb more complement proteins than non-PEGylated particles, resulting in increased particle uptake.

We also explored whether drug-free particle effects can be used as therapeutics in inflammatory diseases, specifically in acute lung injury (ALI) and acute respiratory

distress syndrome (ARDS). For this purpose, we fabricated micron-sized salicylate-based particles, termed “PolyAspirin” or “PolyA”. These particles significantly reduced neutrophil infiltration into the lungs in an LPS-induced model of ALI and in a *P. Aeruginosa* lung infection model of ARDS. Further, the PolyA particles reduced the release of inflammatory cytokines in the lungs in ARDS, reduced the spread of the bacteria into the bloodstream, and dramatically improved survival. These effects appear to be driven by both the presence of particles in blood circulation as well as the degradation of PolyA particles into salicylic acid. We demonstrate a novel potential for drug-free particles to be used as a therapeutic for inflammatory disease.

Finally, we used *in vitro* and *ex vivo* tools to explore the role neutrophils play in antiphospholipid syndrome (APS). APS is an inflammatory autoimmune disease resulting in numerous complications including an increased risk of thrombus formation. We found that APS patient-derived neutrophils exhibit significantly increased adhesion to an unactivated endothelium, which may be in part responsible for the increased risk of thrombus. Additionally, we demonstrated that APS patient plasma can “condition” healthy donor neutrophils to exhibit enhanced adhesion. We found that this effect is driven by an increased expression of activated Mac1 on the surface of APS patient-derived neutrophils. Further, we ffound that activated Mac1 is responsible for enhanced NETosis of APS patient-derived neutrophils, which may also contribute to the increased risk of thrombosis in APS. These results suggest a novel role for neutrophils in APS pathophysiology and highlight a potential therapeutic target (activated Mac1) for the treatment of APS.

Chapter 1: Introduction

1.1 Background and Significance

The use of targeted particle drug delivery systems has remained an exciting potential method to improve the delivery of a wide array of therapeutics for diseases such as cancer, cardiovascular disease, and inflammatory diseases, among others.[1–6] Such platforms are attractive for several reasons, including reducing systemic side effects and increasing drug efficacy by delivery the drug directly to the site of disease rather than throughout the entire body, the potential to protect a drug from harsh physiological environments, the ability to modulate the release of the drug by tuning the degradation characteristics of the particle drug carriers, and the ability to selectively release therapeutics at the site of disease by designing drug carriers that degrade in response to specific enzymes or pH.[2,7] In particular, a great deal of research has focused on the use of vascular targeted drug delivery systems to improve the treatment of inflammatory diseases, due to the prevalence of convenient inflammatory markers such as cellular adhesion molecules (CAMs; including E-Selectin, P-Selectin, ICAM-1, VCAM-1, and L-Selectin) available for targeting using antibodies for those markers.[8–11]

However, despite the extensive research into and success in the use of targeted particulate drug delivery platforms, these platforms have seen very little success in clinical trials, with only 50 or so such platforms receiving FDA approval to date, of which only 17 are polymeric particle drug delivery systems.[12] Thus, there is a large gap between the success of polymeric particle drug delivery platforms in *in vitro* and *in vivo* experiments in a laboratory setting and their utility and efficacy in a clinical setting. There are several possible explanations for this gap, including limitations of *in vitro* models, differences between *in vivo* models of disease and human pathophysiology, unforeseen side effects of particle drug delivery systems, and potential interactions between particle drug carriers and immune cells in the human body. Therefore, it is crucial to the ultimate success of

the use of particle drug carriers for improving the efficacy of therapeutic drugs to explore the potential causes of this gap between success in the laboratory and in the clinic.

One area in which the current research is lacking is in understanding the role that neutrophils, a subset of white blood cell (WBC) or leukocyte, play in inflammation and their interactions with particle drug carriers in the bloodstream. In humans, neutrophils comprise up to 70% of circulating WBCs[13]; however, due to their short lifespan *ex vivo* and the lack of well-established *in vitro* models of neutrophils, their behavior in inflammation and interactions with particle drug carriers have proven difficult to evaluate, and thus have gone understudied. Here, we aim to study the interactions between particle drug carrier platforms and leukocytes, particularly neutrophils, *in vitro* and *in vivo*, as well as the role that neutrophils play antiphospholipid syndrome (APS), a chronic inflammatory disease. Specifically, we evaluate whether the introduction of particle drug carriers impacts neutrophils' response to inflammation in both *in vitro* and *in vivo* inflammation models, how commonly-used non-fouling coatings such as polyethylene glycol (PEG) impact human neutrophil phagocytosis of particle drug carriers, how neutrophils may contribute to complications related to APS such as thrombosis, and whether unloaded particle drug carriers can be used to modulate the immune response to inflammation as a therapeutic strategy.

1.2 Neutrophil Immunology

Circulating leukocytes are comprised of three primary subpopulations—neutrophils, monocytes, and lymphocytes. Neutrophils, also known as polymorphonuclear (PMN) leukocytes, are the most abundant subpopulation, comprising up to 70% of all circulating leukocytes.[13] They are among the "first responders" to inflammation, as they are rapidly recruited to the site of inflammation by the upregulation of CAMs (including E-Selectin, P-Selectin, ICAM-1, and VCAM-1) on the surface of endothelial cells (ECs).[11,14,15] These CAMs allow neutrophils first to roll along the endothelium before firmly adhering to the ECs. Then, the neutrophils respond to chemokines such as N-formylmethionine-leucyl-phenylalanine (fMLP) which induce the neutrophils to transmigrate into the tissue to address the cause of inflammation.[13] Under normal conditions, this response is desirable; neutrophils are able to rapidly

infiltrate the site of inflammation and remove or destroy the offending stimulus (often a bacterial infection or injury). However, when neutrophil recruitment becomes unregulated, they can cause damage to the tissue by creating gaps in the vasculature and releasing cytotoxic granules which carry reactive oxygen species (ROS).[16] Given the crucial role of neutrophils in responding to inflammation and their potential for causing damage if unregulated, it is important to develop a thorough understanding of neutrophil function when designing targeted particle drug carriers for the treatment of inflammatory diseases.

Neutrophil Life Cycle

Neutrophils, like other blood cells, are produced in the bone marrow, at a rate of approximately 0.85×10^9 cells/kg/day, or on the order of $10^{10} - 10^{11}$ neutrophils per day.[17] Inside the bone marrow, neutrophils develop and mature and are stored until their release into the bloodstream. The rate of neutrophil release is tightly controlled by numerous cytokines and chemokines, both inside and outside of the bone marrow. Inside the bone marrow, neutrophils are retained via interactions between C-X-C receptor 4 (CXCR4), expressed in low levels on neutrophils, and stromal cell-derived factor 1 (SDF-1), produced by stromal cells in the bone marrow.[18] This interaction is disrupted by granulocyte colony-stimulating factor (GCSF), resulting in the release of mature neutrophils into the bloodstream.[13,18,19] Additionally, macrophages outside of the bone marrow phagocytose apoptotic neutrophils, stimulating the secretion of interleukin-23 (IL-23), which enters a signaling pathway along with interleukin-17 (IL-17), ultimately resulting in the release of more mature neutrophils from the bone marrow into the bloodstream.[13,20,21]

After exiting the bone marrow, neutrophils enter the bloodstream. Because blood is a complex, multicomponent fluid, different blood cells localize to different regions of the flow.[22–24] The deformability of red blood cells (RBCs) and their unique discoidal shape give rise to collisions which result in the formation of an RBC core near the center of flow, while WBCs and platelets are excluded toward the vascular wall, creating a region known as the cell-free layer (CFL).[25,26] As neutrophils marginate to the vascular wall and occupy the CFL, they constantly sample the endothelial surface for the presence of

CAMs, which would cause adhesive interactions between neutrophils and endothelial cells, including rolling and firm adhesion.

In their role as first responders in inflammation, neutrophils are the primary leukocyte immediately recruited to the site of inflammation.[27] The leukocyte adhesion cascade consists of three phases: initial capture, slow rolling, and firm adhesion, each mediated by different ligand/receptor pairs on the surface of neutrophils and ECs. The capture and slow rolling phases are primarily mediated by the selectins (L-Selectin, P-Selectin, and E-Selectin), while the firm adhesion phase is primarily mediated by ICAM-1.[27–30]

Once firmly adherent, neutrophils undergo transendothelial migration (TEM), whereby they are able to migrate through the endothelial layer (either via the transcellular or the paracellular route), as mediated by a number of integrin proteins and immunoglobulins, including Mac-1, LFA-1, PECAM-1, ICAM-1, ICAM-2, VCAM-1, and others.[31–34] After TEM, the neutrophils are now in the tissue and able to address the cause of inflammation, through some combination of the release of granules containing cytotoxic molecules such as peroxidase to kill bacteria, phagocytosis of bacteria, and the release of neutrophil extracellular traps (NETs) to trap microbes.[35–39] Once the cause of inflammation is removed or destroyed, neutrophils undergo apoptosis and begin expressing phosphatidylserine as an “eat me” signal to tissue-resident macrophages, as well as releasing pro-resolution molecules such as Annexin A1.[40] After tissue-resident macrophages phagocytose apoptotic neutrophils, they also begin secreting pro-resolution molecules as well as signaling for more neutrophils to be released into the bloodstream, and the neutrophil life cycle begins anew.[40,41]

1.3 Role of Neutrophils in Inflammatory Disease

While the typical, well-regulated neutrophil response to inflammation is desired and helps to fight infections and other pathological conditions, there are cases where the inflammatory response becomes unregulated and neutrophils cause damage, potentially resulting in complications such as tissue and endothelial damage, thrombosis, sepsis, and even death. Thus, there are potentially opportunities for the deployment of therapeutic strategies to dampen or otherwise modulate the neutrophils response to

inflammation which could result in major improvements to the current state of therapies for inflammatory diseases. In order to leverage such strategies, it is crucial to develop an understanding of how neutrophils contribute to the pathophysiology of inflammatory diseases.

Role of Neutrophils in Propagating Acute Lung Injury and Acute Respiratory Distress Syndrome

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), terms often used interchangeably, describe severe sudden-onset lung disease which can arise due to any one of a number of triggering events including bacterial infection, pneumonia, pulmonary bruising, smoke inhalation, sepsis, shock, and others.[42] Due to the myriad of causes and the complexity and severity of the disease, ALI/ARDS is responsible for ~190,000 hospitalizations and ~75,000 deaths in the United States each year.[43] Thus, a critical need exists to develop more effective therapies for ALI/ARDS, and it is possible that a modulation of the neutrophil response to inflammation in ALI/ARDS may provide avenues for such therapies.

It is well-established that neutrophils play a major role in the pathogenesis and progression of ALI/ARDS.[44–46] Notably, the concentration of neutrophils in brochoalveolar lavage fluid (BALF) both in murine *in vivo* ALI/ARDS models and in human patients correlates strongly with the severity and mortality of the disease.[47,48] This is largely due to two major functions of neutrophils in ALI/ARDS: the recruitment of neutrophils to the site of injury and subsequent transmigration into the alveolar space results in damage to the lung epithelium; and, once present in the lungs, neutrophils release a number of cytotoxic chemicals including granule proteins and ROS which further cause damage and exacerbate the inflammatory response.[44–46]

The initial neutrophil response to ALI/ARDS proceeds similarly to the classical neutrophil response to inflammation outlined above: after injury, cytokines recruit neutrophils to the site of injury and upregulated CAMs cause neutrophils to roll along the surface of the lung endothelium and firmly adhere. However, as neutrophils transmigrate into the airspace, they increase the permeability of the alveolar space, resulting in an influx of fluid into the lungs, known as pulmonary edema, making it difficult for patients to

breathe. This pulmonary edema exacerbates the inflammatory response, creating a positive feedback loop which further damages the lung endothelial and epithelial tissue, resulting in the progression of ALI/ARDS.[44]

In addition to the lung tissue damage and resulting edema, neutrophils further progress ALI/ARDS through the release of cytotoxic materials into the lung space. Neutrophil elastase concentration, an important granule protein involved in the inherent antimicrobial activity of neutrophils, has been shown to correlate with the severity of ALI/ARDS.[49] Though the full mechanism of the contribution of neutrophil elastase to ALI/ARDS progression is not known, it has been shown that elastase exacerbates the increased lung epithelium permeability[50] and interfere with the function of surfactant proteins A and D, which are involved in the removal of both microbes and apoptotic neutrophils.[51,52]

Further, neutrophils also release ROS such as myeloperoxidase into the lung space after transmigration. Under normal conditions, these ROS are employed to assist in the degradation of phagocytosed microbes; however, when released into the alveolar space, ROS cause damage to the epithelium and increase lung permeability.[53,54] Additionally, the presence of ROS in the lungs can delay neutrophil apoptosis, resulting in the persistence of inflammation and further neutrophil influx into the lungs.[55]

Thus, it is clear that the recruitment, infiltration, and presence of neutrophils in the alveolar space in ALI/ARDS is a double-edged sword. Neutrophils are crucial in the killing and removal of pathogenic microbes, but excessive neutrophil recruitment and the release of cytotoxic chemicals such as elastase and ROS can damage the lung epithelium and disrupt normal antimicrobial processes. Therefore, a therapeutic strategy which modulates (but not necessarily totally inhibits) the neutrophil response in ALI/ARDS may provide new opportunities for successfully treating the disease.

Role of Neutrophils in Propagating Cardiovascular Disease

Neutrophils also play a role in propagating cardiovascular disease (CVD) including atherosclerosis, thrombosis, and others.[56,57] Neutrophils' contributions to such diseases follow a familiar pattern. They are initially recruited as first responders to the site of inflammation; however, in some circumstances their response becomes unregulated

and worsens the inflammation or causes other undesirable complications. In atherosclerosis, for example, neutrophils and neutrophil activity have been shown to exacerbate the disease: depletion of neutrophils in murine models of atherosclerosis reduces atherogenesis.[58] This is likely attributable to multiple mechanisms associated with neutrophil infiltration into the atherosclerotic lesion. Once present in the inflamed tissue, neutrophils release granule proteins such as cyclic adenosine monophosphate (CAMP) and azurocidin 1 which recruit inflammatory monocytes to the region and promote the upregulation of CAMs, resulting in increased inflammation and vascular permeability, which promotes the accumulation of low-density lipoproteins (LDL) that eventually give rise to the “foam cells” which make up atherosclerotic plaques.[56,57,59–61] Further, the release of ROS in atherosclerotic plaques damages the local endothelium and oxidizes LDL, contributing to plaque instability and thus the risk of blood clots, heart attack, and stroke.[62]

Neutrophils are also involved in another common form of CVD: thrombosis. Thrombosis is typically initiated by disturbed or static venous blood flow, resulting in vascular injury and the generation of a thrombotic occlusion (or blood clot) which can dislodge and lead to a pulmonary embolism.[63] Upon vascular injury, neutrophils are rapidly recruited to the site of thrombus formation where they contribute to the coagulation cascade by releasing tissue factor and NETs as well as by activating factor XII.[64,65] Darbousset and colleagues found that neutrophils express tissue factor *in vitro* and used a laser-induced vascular injury model to show that neutrophils rapidly localize and adhere to the site of vascular injury via LFA-1/ICAM-1 interactions *in vivo*, releasing tissue factor prior to the arrival of platelets.[64] Further, von Bruhl et al. demonstrated using a flow restriction model of deep vein thrombosis in mice that neutrophils and monocytes are rapidly recruited to the site of thrombus formation. Upon recruitment and interaction with activated platelets, neutrophils release NETs which bind and activate factor XII, resulting in further propagation of the thrombus formation.[65] Taken together, these results demonstrate that neutrophils are a crucial initiator of thrombus formation.

In addition to atherosclerosis and thrombosis, pathological neutrophil behavior has been implicated in ischemia-reperfusion injury[66–68] and sickle cell disease.[69] Clearly, almost any disease involving vascular injury will involve a neutrophil-driven process that

propagates and exacerbates the disease. Thus, there are again opportunities for the use of therapeutic strategies which modulate the neutrophil response. Preventing or reducing neutrophil recruitment to the site of injury may lead to improved disease outcomes and a reduction in lethal downstream consequences.

Role of Neutrophils in Chronic Inflammatory Disease: Antiphospholipid Syndrome

Given the role of neutrophils in the propagation of acute inflammatory diseases, it stands to reason that they might play a role in conditions characterized by chronic, low-level inflammation as well, and recent research is showing this to be the case. This section will focus on one such disease to be explored later in the dissertation: antiphospholipid syndrome (APS). APS is an autoimmune disease characterized by the presence of antiphospholipid antibodies including lupus anticoagulant, anticardiolipin (aCL), and anti-β_2 glycoprotein-I alongside vascular thrombosis or pregnancy morbidity.[70] In APS, the presence of antiphospholipid antibodies contributes to a prothrombotic state, wherein a secondary stimulus (such as vascular injury or infection) rapidly results in thrombus formation and associated downstream complications.[71]

Recent work has begun to explore the role of neutrophils in these complications. It has been shown that neutrophils in APS patients are primed for NET release, which contributes to thrombus development,[71–73] and that some APS patients exhibit a decreased ability to degrade NETs.[74] Further, it has been demonstrated that APS patient neutrophils exhibit a pro-inflammatory phenotype[75], and that the endothelium in APS patients is primed for leukocyte interactions via upregulated CAMs, further contributing to thrombus formation.[76–79]

Thus, neutrophils clearly play a major role in the pathogenesis of APS which has only recently begun to be appreciated. Again, we find that the unregulated recruitment and activity of neutrophils contributes to complications resulting from an inflammatory disease. Further understanding of the contributions of neutrophils to such diseases will inevitably provide numerous potential opportunities for therapeutic intervention by mitigating the neutrophil response.

1.4 Vascular Targeted Carriers as Leukocyte Mimetics—Design and Challenges

The upregulation of CAMs on the vascular wall in inflammatory diseases make such diseases an attractive target for vascular targeted carriers, or VTCs. Such carriers have been studied thoroughly for the treatment of conditions such as atherosclerosis, thrombosis, cancer, and others. The prevailing concept is simple; in order to protect therapeutics from harsh physiological environments and deliver them at the site of disease (increasing efficacy and decreasing systemic side effects), the drugs are packaged into biodegradable particles decorated with ligands targeted to specific disease markers. When the particles are administered, they will preferentially localize to the site of disease via their targeting schemes, and as they degrade their therapeutic cargo will be released, treating or curing the underlying condition. In this way, VTCs function analogously to leukocytes, taking advantage of the same binding sites and behaving as leukocyte mimetics.

Despite the simple concept, there are many complicating factors which make the design of successful VTCs extremely challenging. There are several types of particle platforms to choose from, including polymeric particles, liposomes, virus-like particles, micelles, and dendrimers; moreover, the most effective choice of carrier likely depends on the specific application at hand. There are also numerous potential targeting schemes possible, including dual-or-multivalent targeting schemes, and again the optimal choice depends on the phenotype presented in the condition of interest. Further, properties such as the particle size, shape, stiffness, and surface chemistry all influence the efficacy of any one particular particle platform. The difficulty in designing effective particle drug carriers is evident in the relative lack of success of such platforms in attaining FDA approval, as previously mentioned. Thus, it is crucial for any researcher interested in designing such drug carriers to develop a thorough understanding of how various particle design parameters impact the particles' behavior and efficacy *in vivo.* Given that the work in this dissertation focuses on polymeric particle drug carriers, the review in this section will focus on those platforms.

The Margination Problem: Particle Drug Carriers are Not Leukocytes

In order for any VTC system to be effective at targeting the vascular wall, it must efficiently localize and bind to the endothelium, in the same way that leukocytes do. At first thought, this seems simple; in laminar, single-component flow, particles would distribute evenly throughout the vessel and some fraction of those particles would localize near the vascular wall and bind. Blood, of course, is not so simple; it is a multicomponent mixture comprised of fluid (plasma), deformable discoidal particles (RBCs), and rigid particles (leukocytes and platelets). This results in a unique flow profile wherein, due to homogenous collisions between RBCs and heterogeneous collisions between RBCs and leukocytes or platelets, RBCs aggregate toward the center of blood flow (creating the RBC core), while leukocytes and platelets localize (or marginate) toward the vascular wall in an area known as the cell-free layer (CFL).[22–24,80] Given the behavior of VTCs as a kind of leukocyte mimetic, it stands to reason that these complex flow dynamics affect the ability of VTCs to marginate to the vascular wall as well, and indeed this is supported by many studies in the literature.

The ability of VTCs to efficiently marginate to the vascular wall is greatly impacted by both particle size and shape. Although nanoscale particles are generally preferred for their ability to navigate the microvasculature without risk of occlusion, microparticles have been shown to be much more efficient at marginating to the vascular wall than nanoparticles, with 1-3 μm particles marginating and binding most efficiently to the vascular wall.[81–88] This result is perhaps not surprising given that platelets and leukocytes, the blood cells which need to marginate, have an average size of 2 μm and 12-15 μm, respectively. This effect occurs because the collisions between RBCs and micron-sized particles push the particles to the vascular wall, while nano-sized particles simply become entrapped and distributed within the RBC core, which mirrors the dynamics observed with leukocytes. However, above a threshold particle size, the shear forces experienced by the particle at the wall are able to overcome the adhesive strength of the particle and rip the particle from the vascular wall. Importantly, this effect can be overcome by increasing the density of targeting ligands conjugated to the particle surface. Charoenphol et al. demonstrated that increasing the density of sLeA from 800 sites/μm^2 to 2000 sites/μm^2 significantly improved the adhesion of 10 μm VTCs, overcoming the enhanced adverse shear forces.[85]

Not surprisingly, VTC shape also influences the impact of particle-cell collisions and particle margination in blood flow. Although most VTC platforms are spherical, recent advances in particle fabrication methods and technology have allowed for the development of a variety of particle shapes, including rods, “worms”, hemispheres, cylinders, and numerous other shapes.[87,89–91] Importantly, differently-shaped particles undergo margination and adhesion differently; for example, rod-shaped particles tend to tumble in blood flow, resulting in more-efficient particle margination and adhesion (depending on the particle size).[92,93]

Thus, any researchers interested in designing VTCs for inflammatory diseases must choose a particle size and shape most appropriate for their application. If the therapy requires internalization by non-phagocytes (such as endothelial cells), less-efficient internalization considerations may outweigh margination considerations. However, if the application requires delivery of a therapeutic to the endothelial surface, the ability of micron-sized and/or rodlike particles to efficiently marginate to the vascular wall might take priority.

Particle Phagocytosis and Clearance: Effect of Particle Size, Shape, and Surface Chemistry

Another major obstacle to effective particle drug delivery is the phagocytosis and clearance of VTCs by professional phagocytes, including neutrophils, monocytes, and macrophages. When particles enter the blood stream, they rapidly develop a protein corona comprised of various plasma proteins.[94–98] Many of these proteins, including complement proteins and immunoglobulins, are known as “opsonins”, proteins which signal to phagocytic cells that a foreign body is present and should be phagocytosed and cleared.[99–102] Once a phagocyte recognizes an opsonin protein on the surface of a particle, the phagocyte will begin internalizing the particle for clearance; however, the size, shape, and surface chemistry of the particle all greatly impact the efficiency with which the particle is phagocytosed and cleared.

Unsurprisingly, particle size plays a major role in the efficiency of particle phagocytosis and clearance. Multiple studies have shown that macrophage phagocytosis increases with increasing particle size up to a maximum at around 2-3 μm in diameter

and then begins to decrease.[103–106] Similarly, neutrophils appear to most-efficiently phagocytose particles in the 1-2 μm size range.[107] Similarly, particle shape is a crucial parameter dictating the efficiency with which phagocytes internalize particles. Studies have shown that rodlike or wormlike particles with higher aspect ratios are difficult for phagocytes to engulf and internalize, and that the angle of attack by the phagocyte plays a major role in this effect.[90,108–110] Thus, it is crucial to carefully consider the optimal carrier size and shape for the given application.

Further, the surface chemistry of a particle drug carrier has a major impact on the composition of its protein corona, and thus its rate of phagocytic clearance by leukocytes. The composition of the protein corona is dictated by noncovalent interactions between the particle surface and various plasma proteins, including hydrogen bonding, electrostatic interactions, and hydrophobic interactions.[95–97] Because opsonin proteins are frequently found on the corona, much work has been dedicated to strategies for reducing or eliminating the protein corona. One commonly-employed strategy is to coat the particle surface with extremely hydrophilic PEG chains, which has been shown to reduce the total amount of plasma protein adsorption onto the particle surface by creating a hydration layer around the particle and increase the circulation time of particle drug carriers *in vivo*.[111–115] However, recent work has established the development of anti-PEG antibodies in humans due to the high prevalence of PEG in commercial cosmetics[116,117] which may reduce the effectiveness of PEG coatings in humans; additionally, other studies have called into question the efficacy of PEG coatings in improving VTC performance in human blood[118] and have shown that PEGylation may actually increase phagocytosis by human neutrophils.[119] (Note: this phenomenon is explored experimentally in this dissertation; see Chapter 4 for details.)

Another more-recent strategy for reducing, altering, or eliminating the protein corona is the use of zwitterions, which are molecules containing both positively and negatively charged functional groups such that the net charge of the molecule is zero, reducing the possible electrostatic interactions with plasma proteins and increasing circulation time *in vivo*.[120–122] Further, other groups have explored the use of biomimetic coatings which are designed to “trick” the immune system into recognizing the particle drug carrier as endogenous to the body, whether by using cell membrane

coatings or molecules such as CD47 which functions as a “don’t eat me” signal to phagocytes.[123–126] One alternative strategy recently explored by some groups is to alter the protein corona by selectively inducing the adhesion of beneficial plasma proteins known as “dysopsonins”, including serum albumin and apolipoproteins.[94,127] All of these strategies show promise for enhancing the performance of VTCs, but must be further scrutinized in human blood flows to determine the extent of their efficacy.

Again, the specific application at hand plays a critical role in the design choices made with regards to particle phagocytosis. For a drug carrier aimed at delivering an anti-inflammatory drug to the endothelial surface, phagocytosis by circulating leukocytes is undesirable and strategies should be employed to avoid it. However, some applications may require delivering therapeutics to leukocytes themselves to reduce their involvement in the inflammatory cascade; in these cases, it may be beneficial to enhance particle phagocytosis. Regardless, the exact choices of design parameters (especially particle size, shape, and surface chemistry) are critical in the success of any drug carrier for treating inflammatory disease.

1.5 Particle-Leukocyte Interactions in Blood: Current State of Understanding

Given the fact that both circulating leukocytes and VTCs both necessarily marginate to the CFL and potentially bind to the endothelium in inflammation, and that one role of leukocytes is to phagocytose and remove foreign materials from the body, it stands to reason that interactions between the two might occur which impact either leukocyte function, VTC efficacy, or both. As VTCs localize to the CFL near leukocytes, they will likely collide with one another in blood flow, and those collisions have the potential to impact either leukocyte or VTC adhesion. Additionally, VTCs are binding to and occupying the same sites on the endothelium as leukocytes, and may engage in competition for binding sites. Further, it is possible that particle phagocytosis has some impact on leukocyte function, the expression of various molecules on the surface, and how they behave in response to inflammation.

Despite these seemingly straightforward questions to consider, relatively little work has focused on the interactions between particles and circulating leukocytes in blood flow. Specifically, how neutrophils interact with targeted particle drug carriers has been largely

ignored, despite their comprising up to 70% of circulating leukocytes in humans and functioning as the first-responders to inflammation. However, some recent studies have begun to explore some of these questions, and the results of those works are important context for the work presented in this dissertation.

In 2014, Getts et al. explored the potential for intravenously-administered, drug-free particles to alter the immune response in a variety of inflammatory disease conditions, including myocardial infarction, experimental autoimmune encephalitis (EAE), colitis, peritonitis, and flavivirus (also known as West Nile Virus) associated encephalitis.[128] In these experiments, the researchers found that inflammatory monocytes rapidly scavenge negatively-charged microparticles [comprised of polystyrene, microdiamonds, or poly(lactic-co-glyolic) acid (PLGA)] via the macrophage receptor MARCO. After particle scavenging, the inflammatory monocytes were sequestered in the spleen, resulting in reduced monocyte accumulation at the site of inflammation and a reduction in disease symptoms. This was the first work demonstrating that the administration of drug-free particles can alter the immune response to inflammatory diseases and might provide a therapeutic benefit.

Following up on this work, in 2017 Jeong et al. investigated the potential for intravenously-administered PLGA nanoparticles (500 nm) to modulate the immune response in spinal cord injury.[129] From these experiments, the researchers concluded, again, that the nanoparticles were scavenged via the MARCO receptor, and prevented the infiltration of inflammatory monocytes to the site of injury. This resulted in a decrease in pro-inflammatory M1 macrophage polarization, reduced levels of inflammatory cytokines such as IL-10, reduced fibrosis, and ultimately improved clinical scores. Again, we see that the administration of drug-free particles alters the innate immune response to an inflammatory condition, potentially providing some therapeutic benefit.

Finally, in 2019, Saito et al. explored the impact of particle design on the level of immune modulation in murine EAE.[130] Specifically, the authors injected ~500 nm PLGA particles comprised of low and high molecular weight PLGA, as well as particles comprised of poly(DL-Lactide) (PLDA) into mice with EAE. The authors found that all particle types reduced the clinical scores of the EAE mice; however, the high-molecular weight PLGA particles were most effective in this, outperforming saline controls and both

other particle types. Notably, the authors also found that these high-molecular weight PLGA particles preferentially associate with neutrophils in the bloodstream *in vivo*, resulting in reduced neutrophil infiltration into the site of injury and a diversion of neutrophils into the spleen.

Though these works are of great interest and provide a basis from which to explore the interactions between leukocytes and particle drug carriers in inflammatory diseases, they leave many questions unanswered. They do not explore the effects of either nonspecific collisions in blood flow between leukocytes and particles or specific particle binding to an inflamed endothelium on the immune response to inflammation. They also largely focus on the impact of particle drug carriers on monocytes (though the lattermost work did explore the impact on neutrophils), while it is known that neutrophils comprise the overwhelming majority of circulating leukocytes and are the first responders to inflammation. In fact, we still know very little about how the use of particle drug carriers might impact neutrophils and even how neutrophils contribute to some inflammatory diseases; thus, this dissertation aims to shed light on some of these questions, exploring the mechanisms by which particle drug carriers might interfere with the immune response to inflammation using *in vitro* and *in vivo* tools, how non-fouling coatings such as PEG impact neutrophil phagocytosis of particle drug carriers, whether drug-free particle drug carriers can be developed for as a therapeutic strategy for the treatment of inflammatory diseases such as ALI/ARDS, and how neutrophils contribute to complications in chronic inflammatory diseases such as APS.

1.6 Organization of the Dissertation

The work presented in this dissertation aims to explore the interactions between leukocytes and particle drug carriers, with a specific focus on neutrophils, as well as the behavior of neutrophils in inflammatory diseases. We hypothesize that the presence of particle drug carriers will necessarily alter the leukocyte response to inflammation, that neutrophils will likely interact with particle drug carriers in ways that differ from other leukocytes, and that neutrophils play an important role in chronic inflammatory diseases such as APS. Further, we hypothesize that drug-free particles may be developed as a

therapeutic strategy by interfering with the neutrophil response to inflammation in acute inflammatory conditions such as ALI/ARDS.

Chapter 1 provides the background to the work presented in this dissertation, including a review of neutrophil immunology, the role of neutrophils in inflammatory diseases, the utility of particle drug carriers and the crucial design parameters for such drug carriers, and the limited recent work exploring the interactions between particles and leukocytes in inflammatory conditions.

Chapter 2 describes the materials and methods used in all the experimental work in this dissertation.

Chapter 3 is an *in vitro* and *in vivo* experimental exploration of the mechanisms behind the modulation of the leukocyte response to inflammation by particle drug carriers, including an examination of the impact of nonspecific collisions in blood flow, specific binding of particles to the inflamed endothelium, and particle phagocytosis.

Chapter 4 focuses on the impacts of PEGylation on phagocytosis by primary human neutrophils, given the importance of particle phagocytosis on immune modulation.

Chapter 5 aims to leverage the knowledge obtained in chapter 3 to develop biodegradable, salicylate-based microparticles as a therapeutic for inflammatory disease, specifically ALI/ARDS.

Chapter 6 is an *in vitro* investigation of the role of neutrophils in APS, a chronic inflammatory autoimmune disease, using *ex vivo* samples from APS patients to evaluate the adhesion of patient-derived neutrophils to a quiet endothelium. This chapter aims to shed light on the role of neutrophils in inflammatory disease, given the relative lack of understanding of their impact in a number of contexts.

Chapter 7 sums up the entirety of the work in this dissertation, providing overall conclusions and suggestions for future work following up on the work presented here.

Chapter 2: Materials and Methods

2.1 Introduction

This chapter describes the various materials and methods used to perform the experimental work in the remainder of the dissertation. Various particle drug carrier formulations are prepared for evaluation of their impact on the neutrophil response to inflammation in both *in vitro* and *in vivo* models of inflammation. Additionally, model particle drug carriers are introduced to human blood cells and other phagocytic cell lines to evaluate the impact of PEGylation on particle phagocytosis. Finally, parallel plate flow chamber (PPFC) assays are used to evaluate the adhesive potential of APS-derived neutrophils on a quiet endothelium.

2.2 Particle Functionalization and Preparation

Model Polystyrene Particulate Drug Carriers for PPFC Experiments

Fluorescent, carboxylated polystyrene (5 μm, 3 μm, 2 μm, and 500 nm, Polysciences, Inc.) were conjugated with IgG or sialyl Lewis A via avidin proteins covalently linked to the particle surface using carbodiimide chemistry.[131] Briefly, carboxylated particles were washed with 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, then resuspended in a 5 mg/mL NeutrAvidin® solution and rotated at room temperature for 15 minutes, at which time an equivalent volume of 75 mg/mL N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, Sigma) was added, and particles were rotated overnight. After overnight incubation, 7.5 mg/mL of glycine (Sigma) was added to the solution, and particles were rotated for another 15 minutes. Then, particles were washed with 50 mM PBS buffer, counted via fluorescence microscopy using a hemacytometer, and stored at 4° C.

After NeutrAvidin® conjugation, particles were then conjugated with either biotinylated rat IgG2b (Biolegend) or sialyl Lewis A (Glycotech). This was achieved by a 45-minute incubation with the desired ligand on a rotator, after which particles were stored

in a PBS buffer with calcium and magnesium ions and 1% BSA until use or site density characterization. Site densities were determined via flow cytometry using anticutaneous-lymphocite-associated-antigen-APC (for sLeA; Miltenyi Biotech) or anti-rat-IgG2b-PE (for IgG, eBioscience) as previously described.[84] Site densities for both sLeA and IgG were fixed at ~2000 sites/μm^2, consistent with previous experiments.

PEGylation of Model Polystyrene Particulate Drug Carriers for Phagocytosis Assays

Fluorescent carboxylated polystyrene (2 μm, 500 nm, and 200 nm, Polysciences, Inc.), PLGA (~2 μm, fabricated in-house), and silica (500 nm, Microspheres-Nanospheres, Inc.) were conjugated with aminated, methoxy-terminated polyethylene glycol (PEG, 5k molecular weight, Fisher) using N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, Sigma) chemistry. Particles (1% w/v) were suspended in 50mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer and incubated with PEG at varying concentrations for 15 minutes on a rotator. After 15 minutes, EDC (20mg) was added, and the particles were rotated for 20 hours. Glycine (4mg, Sigma) was added to quench the reaction. After conjugation, particles were washed 3x with PBS -/- (pH 7.4), and the particle concentration was determined using a hemocytometer on a fluorescent microscope.

Rhodamine-labeled, amine-terminated PEG (Nanocs) was conjugated to particles as described above. A rhodamine standard curve was used to determine the amount of PEG present on particles by taking fluorescent readings on BioTek Synergy H1 microplate reader. Then, the PEG density was calculated using the method described by Perry et al.[111]. For PEG chains with variable end groups, it was assumed that swapping out small functional groups on the end of the PEG chain will not alter the conjugation chemistry, and thus the same chemistry was used to conjugate PEGs of variable end groups.

Fabrication of PolyAspirin Particles for the Treatment of ALI/ARDS

PolyAspirin particles were prepared using a single emulsion solvent evaporation method. Briefly, 20 mg of PolyAspirin polymer was dissolved in 20 mL of dichloromethane. Then, 75 mL of 1% polyvinyl alcohol (PVA) solution was placed on a

mixer at 4250 rpm. PolyAspirin solution was slowly injected into the PVA solution, and allowed to mix for 2 hours. After mixing, the solution was allowed to settle for ~45 minutes, and then particles were removed and washed by centrifugation 3x with DI water. Particles were then lyophilized and stored at -40°C until use. Particle size was determined by SEM and DLS, and particle concentration was determined via counting on a hemocytometer.

2.3 Cell Culture and Preparation for Flow and Phagocytosis Assays

Human Umbilical Vein Endothelial Cell (HUVEC) Culture and Monolayer Preparation for PPFC Assays

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained from Mott Children's Hospital at the University of Michigan. Isolated HUVEC were cultured in T75 flasks at 37°C and 5% CO_2 until confluent, and then seeded onto glass coverslips coated with gelatin as previously described.[132] Monolayers were allowed to grow to confluence prior to use, typically over the course of 48 hours.

Bone Marrow-Derived Macrophage (BMM) Isolation and Culture:

BALB/c and C57BL/6 mice were euthanized via CO_2 and their hind legs were removed with surgical scissors. The bones were flushed with FACS buffer (PBS -/- with 2% FBS); fluid containing bone marrow progenitor cells was collected as described previously.[133] Cells were centrifuged at 500xG for 5 minutes and suspended in RBC lysis buffer (eBiosciences) for 1 minute. Cells were diluted in FACS buffer again, centrifuged at 500xG for 5 minutes, and suspended in DMEM medium + L929 conditioned media to promote differentiation into macrophages, and plated on T-75 cell culture flasks for 6 days prior to use.

Culture and Differentiation of HL-60 Derived Neutrophils:

HL-60 cells were differentiated into neutrophils by culturing the cells in RPMI with 10% FBS and 1.3% DMSO, as previously reported.[134] Cellular differentiation was monitored by shifting CD11b expression over time, as measured by flow cytometry. Neutrophil-like cells were used after the population exhibited a uniform CD11b expression peak, generally 4-5 days after culture in DMSO.

Alveolar Macrophage Assays

Murine alveolar macrophages were isolated according to an established protocol.

2.4 Blood Collection and Preparation for Flow and Phagocytosis Assays

Human Blood Collection

Venous blood was collected from healthy adults into a syringe using either acid-sodium citrate-dextrose (ACD, for non-phagocytic experiments) or heparin-sodium (for phagocytic experiments) as anticoagulant. Plasma was obtained by centrifugation of anticoagulated whole blood at 2250 x G for 20 minutes with no brake.

Mouse Blood Collection

Mouse blood was obtained via cardiac puncture per a protocol approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC) and in accordance with the National Institute of Health guidelines for the care and use of laboratory animals. Mice were obtained from Jackson Laboratories, and were either male BALB/c or male C57BL/6.

Neutrophil Isolation for PPFC and Phagocytosis Assays

Neutrophils were isolated from whole blood and then reconstituted as follows. Venous blood was collected from healthy adults into syringes using heparin-sodium as an anticoagulant. 20 mL of blood was then layered on top of 20 mL of lymphoprep (Fresenius Kabi Norge AS). The top plasma layer was then collected using a pasteur pipette and stored at room temperature. The middle layer, containing monocytes and lymphocytes, was discarded along with the lymphoprep layer. The RBC/neutrophil pellet was preserved, and plasma was added back to the solution. Then, PBS containing calcium and magnesium ions with 1% BSA was added to return the final volume to 20 mL to preserve a physiological concentration of neutrophils.

2.5 Parallel Plate Flow Chamber Assays

Venous blood was collected from healthy adults or APS patients into a syringe using either acid-sodium citrate-dextrose (ACD, for non-phagocytic experiments) or heparin-sodium (for phagocytic experiments) as anticoagulant. Confluent HUVEC monolayers were activated with 2 mL of 10ng/mL IL-1β (Fitzgerald) for 4 hours prior to flow experiments to induce CAM expression. After activation, blood with or without particles was perfused over the activated HUVEC monolayer using a parallel pate flow chamber (PPFC, Glycotech) and syringe pump.

For pulsatile flow experiments, blood was perfused forward for 14 seconds and backward for 7 seconds at a shear rate of $1000s^{-1}$, for a total of 15 minutes (chosen to replicate total blood volume for a 5-minute laminar experiment). The $1000s^{-1}$ shear rate and pulsatile flow profile were chosen to simulate the shear rate of arterial blood flow[135,136] and conditions relevant in vessels often associated with coronary artery disease, one condition where targeted particle drug carriers would be employed.[85,137] For laminar flow experiments, blood was perfused forward over the monolayer for a total of 5 minutes at a shear rate of $1000s^{-1}$. A 5-minute time was chosen for laminar flow experiments in order to establish a fully-developed laminar flow profile, while 15 minutes was chosen for pulsatile experiments to match the blood volume used in laminar flow experiments. For both types of experiments, after perfusion was completed, PBS with calcium and magnesium ions and 1% BSA was added and perfused at a shear rate of $500s^{-1}$ while images or videos were taken. For leukocyte counts, 10 images were taken along the length of the flow chamber using a Nikon TE-2000-S microscope with a digital camera (Photometrics CoolSNAP EZ with a Sony CCD sensor), and cells were counted using ImageJ software. For rolling velocity analysis, 10-second videos were taken for each sample, and rolling leukocytes were tracked using NIS-Elements® software.

Blood flow rates were determined based on the shear rate and channel size using Equation 2.1, below:

$$\gamma = \frac{6Q}{h^2 w} \tag{2.1}$$

where γ (s^{-1}) is the shear rate, Q is the volumetric flow rate (mL/s), h is the channel height (0.127 or 0.254 cm), and w is the channel width (0.25 cm).

For phagocytic flow experiments, particles at the desired concentration were added to blood 1 hour prior to flow, allowing for a substantial amount of particle uptake as previously characterized.[119]

2.6 Phagocytosis Assays

Various particle types (described above) were incubated with heparinized blood, primary neutrophils in plasma, primary neutrophils in RPMI, THP1 monocytes, bone marrow-derived macrophages, mouse blood, and HL-60 derived neutrophils at varying concentrations (1E7/mL for 2 μm particles, scaled concentrations to match total particle mass for smaller particles) and times at 37°C and 5% CO_2. For whole blood studies, samples were removed from the incubator and stained (CD45, CD11b, CD14, BioLegend) for 30 minutes on ice. The 1-step Lyse/Fix solution (eBiosciences) was then applied to lyse the RBCs and fix the WBCs. Fixed samples were washed with FACS buffer (PBS -/- + 2% FBS, pH 7.4) before flow cytometry analysis. For primary neutrophil/monocyte incubation studies, the incubation steps were performed as described above, after which cells were stained for 30 minutes on ice and then fixed using 2% paraformaldehyde (Sigma) solution.

For cultured cell studies, cells were seeded in 96-well plates at a concentration of 10^6 cells/well in medium and incubated with particles at 37°C and 5% CO_2. THP1 cells were removed from the wells and transferred to FACS tubes, centrifuged and resuspended in 2% paraformaldehyde solution for fixation. Bone marrow-derived macrophages were trypsinized for 5 minutes and then removed from the wells, centrifuged and resuspended in 2% paraformaldehyde solution. Cells were washed 3x with FACS buffer before analysis via flow cytometry. Fluorescent microscopy images of particle uptake were obtained with isolated neutrophils in plasma, where cells were exposed to particles at the same concentrations as all other uptake studies and imaged live.

2.7 Flow Cytometry

Particle Phagocytosis and Neutrophil Phenotype Assays

All flow cytometry was performed on an Attune Acoustic Focusing Cytometer and results analyzed using FlowJo software (Tree Star). Human whole blood samples were stained with CD45 and CD11b (BioLegend) on ice before addition of Lyse/Fix solution. For neutrophil phenotype assays, samples were also stained for CD62L. Cells identified as CD45+/CD11b+ were considered to be granulocyte neutrophils and monocytes, and the two populations were sorted by FSC/SSC. Mouse whole blood samples were stained with CD45, CD11b, and Ly6G (BioLegend) on ice before addition of Lyse/Fix solution. Cells identified as CD45+/CD11b+/Ly6G+ were considered to be neutrophils, while CD45+/CD11b+/Ly6G- cells were considered to be monocytes. Single-cell populations (Bone Marrow-Derived Macrophages, THP1 monocytes, and HL-60 derived neutrophils) were not stained before analysis via cytometry. Gating was applied to remove doublets. For all samples, particle-positive cells were identified by increased FITC MFI from untreated samples. Figure 2.1 below shows representative whole blood flow cytometry gating for these experiments.

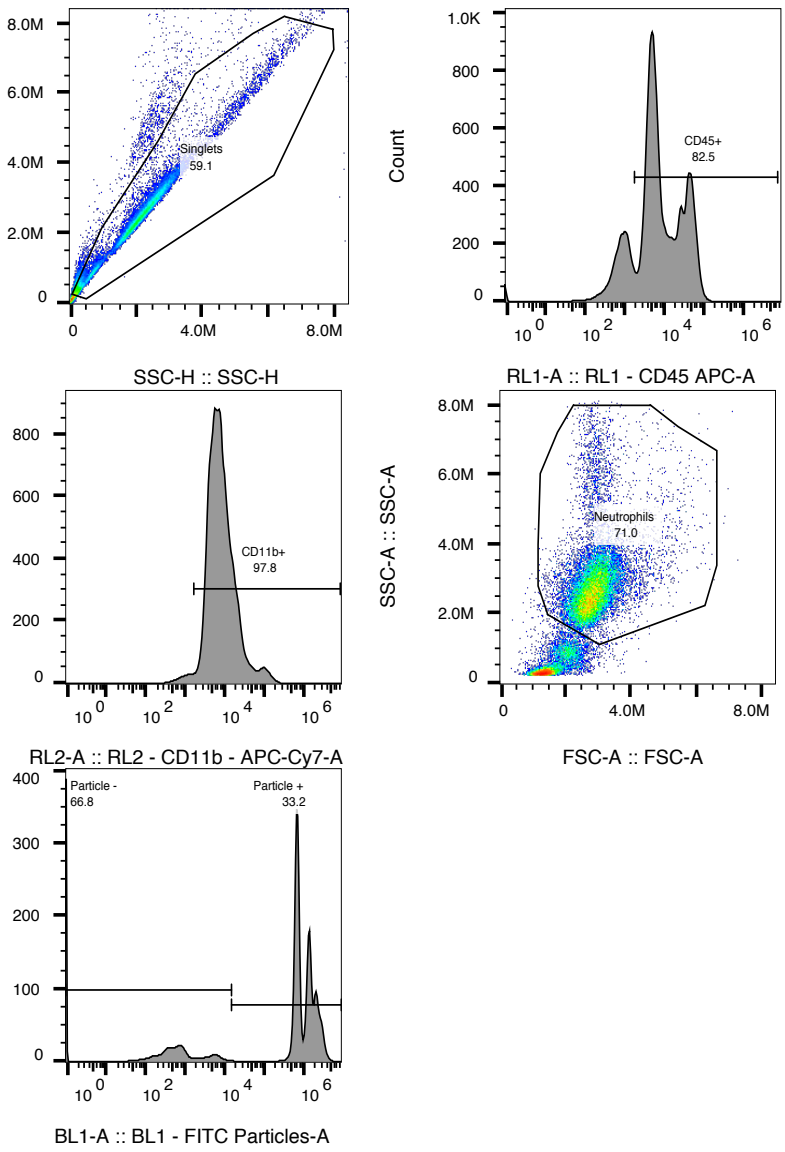


Figure 2.1—Representative whole blood flow cytometry gating for particle phagocytosis and neutrophil phenotype assays.

2.8 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gels

Particles were incubated in human plasma (1.83E7/mL) or buffer for 1 hour at 37°C, as done previously.[138] Then, particles were washed 3x in PBS -/- and resuspended in 50uL SDS stain (Pierce SDS-PAGE Sample Prep Kit). Particles were placed in a Bio-Rad T100 Thermal Cycler and boiled to strip proteins from the particle surface. Particles were then centrifuged and the supernatant solution was run on a Novex

WedgeWell 4-20% Tris-Glycine Gel alongside a protein ladder. Gels were then stained using EasyBlue Safe Stain, and destained by shaking overnight in deionized water. Images were taken using a Bio-Rad EZ Gel Imager. Specific bands were cut out, digested, and analyzed via LCMS at the University of Michigan proteomics core.

2.9 *In Vivo* Acute Mesentery Inflammation Model

Female C57BL/6 mice (Jackson) between 3 and 4 weeks in age were evaluated in a model of mesentery inflammation as described previously.[139] Neutrophil rolling and adhesion in mesenteric veins were visualized using a 25x oil objective on an inverted fluorescence microscope (Zeiss Axio Observer Z1Marianas microscope) using Slidebook 6 software. Mice were anesthetized, and a tail vein catheter was placed for delivery of reagents. Mice were placed on a custom-made microscopic heated stage at 37°C, and the mesentery was exteriorized to a glass coverslip through a midline incision. Rhodamine 6G (Rh6G, Sigma, 100 µL of 0.1 mg/mL in PBS) or antiLy6G (Biolegend) was injected IV, and a local injury was induced by topical application of TNF-α (Fitzgerald, 10 µL of 200 µg/mL in PBS). FITC-labeled particles suspended in PBS were injected via IV catheter 3 min following topical TNF-α application and imaged for another 7 min. Mice received 2 × 108 2 µm particles or 1.28 × 1010 0.5 µm particles in 200 µL injection volume, corresponding to ~0.6 mg/ mouse, ~30 mg/kg. Analysis was performed using Slidebook 6 and ImageJ by a blinded investigator. Images were recorded continuously in green, red, and bright field channels every 100 ms. Vessels were chosen in each mouse based on size and vessel exposure, with the average diameter of veins ranging from 90 to 190 µm, with an average of 130 µm. The total number of neutrophils and particles were counted per frame to obtain average neutrophil counts per 3 s of capture in 125 µm length of mesentery vessel, corresponding to 30 frames at 100 ms/frame.

2.10 *In Vivo* ALI Model

Male BALB/c or C57BL/6J mice were anesthetized briefly using isoflurane and given 50 uL of 0.4 mg/mL LPS orotracheally to induce inflammation. Particles were injected either 2 hours or 4 hours post-instillation via tail vein catheter. 2 hours after

particle injection, mice were euthanized via CO_2 asphyxiation and the lungs were lavaged 3x with PBS -/- to remove leukocytes present in the lungs for analysis.

BALF cells were counted via hemocytometer, stained on ice, fixed, and analyzed via flow cytometry to determine the percentage of neutrophils versus macrophages in the lungs. Following the lavage, organs were harvested and imaged using an Odyssey CLx infrared imaging system (LI-COR), and whole organ scans performed. Following NIR scan, lung, liver, and spleen were homogenized into single-cell suspensions, as previously described52 or frozen for histological examination. Frozen samples were embedded in OCT (Fisher) in disposable cassettes and flash frozen with an isopentane slurry; additional liver samples were fixed in 4% paraformaldehyde (PFA) or flash frozen in liquid nitrogen and stored for histology or protein analysis

2.11 *In Vivo* ARDS Bacterial Lung Infection Model

P. Aeruginosa was grown overnight in Difco nutrient broth at 37°C under constant shaking. The concentration of bacteria in the broth was determined by measuring the absorbance at 600 nm, then plotting the optical density (OD) on a standard curve generated by known colony-forming unit (CFU) values. Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine mixture and then given 30 uL of bacteria solution (15 uL in each nostril; 2E5 bacteria per mouse total) intranasally to induce lung infection.

At either 6 or 18 hours post-infection mice were placed in a restrainer and a catheter was inserted into the tail vein. Each mouse received 2E8 particles in 100 uL of injection volume, for a dose of approximately 30 mg/kg.

24 hours post-infection, mice were euthanized via CO_2 overdose. After euthanasia, the chest cavity was exposed and a cardiac puncture was used to collect blood from the mice. Additionally, the trachea was exposed and opened, and the lungs were lavaged with 3mL of PBS -/- to remove cells in the alveolar space. BALF was centrifuged and supernatants were saved at -80°C for enzyme-linked immunosorbent assay (ELISA) to quantify inflammatory cytokines. The cell pellets were resuspended in 500 uL of RPMI media, then aliquots were diluted 1:1 with Turk Blood Diluting Fluid and counted via

hemacytometer. Then, cytospin samples were prepared and cells were stained to differentiate neutrophils from mononuclear cells.

Blood and BALF were plated on auger plates and allowed to grow overnight at 37°C to determine CFUs. Then, the blood was centrifuged and plasma was collected and stored at -80°C for ELISA analysis.

2.12 APS Patient Recruitment, Cell Analysis, and Patient-Specific Assays

Patient Recruitment

Patients were recruited from rheumatology and hematology clinics at the University of Michigan. All 43 patients with APS fulfilled the clinical and laboratory criteria for APS established by the Sydney classification criteria.[70] None of the patients met American College of Rheumatology (ACR) criteria for systemic lupus erythematosus (SLE).[140] Of the patients with APS, some were classified as having “obstetric APS” if they had no prior history of vascular thrombosis, but did have APS-associated obstetric complications as defined by the Sydney criteria (≥3 unexplained, consecutive, spontaneous pregnancy losses; or ≥1 unexplained fetal deaths ≥10 weeks of gestation; or ≥1 preterm deliveries of a morphologically normal infant before 34 weeks of gestation due to severe preeclampsia, eclampsia, or features consistent with placental insufficiency).[70] Eleven patients with history of unprovoked venous thrombosis, but negative testing for antiphospholipid antibodies, were also recruited; many of these patients had genetic risk factors for venous thrombosis such as Factor V Leiden as detailed in. 38 healthy volunteers were recruited through a posted flyer; exclusion criteria included history of a systemic autoimmune disease, active infection, and pregnancy. All 38 controls were screened for IgG anti-β_2GPI and found to be negative. Blood was collected by phlebotomist venipuncture, and serum was prepared by standard methods and stored at -80ºC until ready for use. IgG, IgM, and IgA anti-β_2GPI, as well as IgG and IgM anticardiolipin, were determined by multiplex assay on a BioPlex 2200 System (BioRad). Lupus anticoagulant (LAC) was tested according to published guidelines.[141] This study was reviewed and approved by the University of Michigan Institutional Review Board. Written informed consent was received from all participants prior to inclusion. For patient information and demographics, see Table 2.1 below.

Table 2.1—Patients with APS (and controls)

	Primary APS		Controls	
Demographics				
Number	43		38	
Age (y)*	43 ± 27	(23-77)	39 ± 23	(20-71)
Disease duration (y)*	10 ± 14	(0-28)	N/A	
Since last thrombus (y)*	5 ± 8	(0-17)	N/A	
Female	27	(63%)	27	(71%)
White	42	(98%)	33	(87%)
Laboratory studies				
IgG anticardiolipin IgG	33	(77%)		
IgM anticardiolipin IgM	15	(35%)		
IgG anti-β_2GPI	33	(77%)		
IgM anti-β_2GPI	16	(37%)		
Lupus anticoagulant	26	(60%)		
"Triple-positive"	25	(58%)		
Clinical history				
Venous thrombosis	21	(49%)		
Arterial thrombosis	16	(37%)		
Microvascular	8	(19%)		
Pregnancy morbidity	15	(35%)		
Thrombocytopenia	17	(40%)		
Medications				
Aspirin	21	(49%)		
Warfarin	21	(49%)		
Enoxaparin	5	(12%)		
Direct oral anticoagulants	2	(5%)		
Hydroxychloroquine	24	(56%)		
Immunosuppressants‡	3	(7%)		

***** mean ± 95% CI (range)
‡ Why were patients receiving immunosuppressants?
2 patients: rituximab for CAPS
1 patient: mycophenolate mofetil for atopic dermatitis

APS Patient Neutrophil Characterization

Blood was collected into citrate tubes and immediately taken for further processing. Fc blocking of cells (in whole blood) was carried out using Human TruStain FcX

(BioLegend), according to the manufacturer's instructions. Subsequently, cells (still in whole blood) were stained with specific antibodies for 30 minutes on ice, followed by immediate lysis of RBCs and fixation of leukocytes using eBioscience 1-step Fix/Lyse Solution. Samples were analyzed on a LSRFortessa Cell Analyzer (BD Biosciences) and ZE5 Cell Analyzer (Bio-Rad). Further data were analyzed with FlowJo software (Tree Star). Specific primary antibodies were against: apolipoprotein H (ABS162, EMD Millipore), CD15 (W6D3, BioLegend), CD16 (3G8, BioLegend), CEACAM-1 (283340, R&D systems), CD64 (10.1, BioLegend), activated LFA-1 (m24, BioLegend), activated Mac-1 (CBRM1/5, BioLegend), and CD62L (DREG-56, BioLegend). Also used were eBioscience™ Fixable Viability Dye eFluor™ 506, and secondary antibody Alexa Fluor® 680 AffiniPure Donkey Anti-Rabbit IgG (H+L) (711-625-152, Jackson ImmunoResearch). For leukocyte conditioning experiments, the sample was either spiked with increasing concentrations of APS or control IgG (10 µg/mL or 100 µg/mL) or the citrated plasma of the sample was discarded and replaced with heterologous control or APS plasma, and incubated for 1 hour at 37°C before staining and flow analysis.

IgG Preparation

IgG was purified from human serum with Protein G Agarose according to the manufacturer's instructions (Pierce). Briefly, serum was diluted in IgG binding buffer and passed through a Protein G Agarose column at least five times. Elution of IgG was performed with 0.1 M glycine. The solution was neutralized with 1 M Tris, followed by overnight dialysis against PBS at 4ºC. After passing through a 0.2-micron filter, IgG purity was verified by SDS-PAGE. IgG was quantified by BCA protein assay (Pierce). IgG preparations were free of endotoxin contamination as determined by a chromogenic endotoxin quantification kit (Pierce).

APS Patient in vitro *PPFC Experiments*

HUVEC monolayers were prepared as described above in Section 2.5, and the PPFC experiments follow the same basic procedure described above, with the following specifics.

For some experiments, “leukocytes” were prepared by mixing together the buffy coat and RBCs (after discarding plasma). In other cases, “neutrophils” were prepared by retrieving the neutrophil-RBC pellet that remained after Ficoll gradient separation. For these leukocyte and neutrophil experiments, cells were always brought back to their original blood volume with “flow buffer” (PBS++ with 1% BSA). 2 mL of whole blood, leukocytes, or neutrophils were introduced into the chamber from an inlet reservoir via a programmable syringe pump (KD Scientific, Holliston, MA). For “low shear” experiments, samples were perfused across the HUVEC monolayer using a laminar flow profile. The wall shear rate (WSR, γw) was fixed by adjusting the volumetric flow rate (Q) through the channel according to Equation 2.1 above, where h is the channel height (127 μm) and w is the channel width (0.25 cm). The h of 127 μm and WSR of 200 s^{-1} were chosen to approximate the flow profile within veins and venules. Low-shear samples were perfused over HUVECs for 5 minutes. For “high shear” experiments, pulsatile flow was used in the horizontal PPFC as previously described.[85] Specifically, samples were perfused over HUVEC monolayers in pulsatile flow at a WSR of 1000 s^{-1} for 15 minutes (23-25). The flow time was chosen to ensure the same volume of blood passed through the chamber as for laminar/low-shear experiments.[85] At the end of the prescribed flow time, flow buffer was added to the PPFC to flush out nonadherent cells. Ten images per sample were collected along the length of the flow chamber using a Nikon TE-2000-S inverted microscope with a digital camera (Photometrics CoolSNAP EZ with a Sony CCD sensor). Results were imaged and analyzed via NIS-Elements® analysis software and ImageJ. The adherent cells were normalized to the controls run on the same day so as to minimize variation attributable to different batches of HUVECs. For experiments involving the pretreatment, or “conditioning,” of control leukocytes, the buffy coat/RBC sample was incubated at 37ºC for 1 hour with plasma, before washing again with flow buffer. For blocking experiments, anti-Mac-1 (20 μg/mL, clone CBRM1/5) antibody or isotype control were also included during the incubation.

TLR4 and Complement Inhibition

Anticoagulated control blood was preincubated with 20 μM toll-like receptor 4 (TLR4) inhibitor (TAK-242, Cayman Chemical) or 10 μM C5a receptor antagonist (W-

54011, Cayman Chemical) for 30 minutes. The sample was then spiked with IgG as above and incubated for 1 hour at 37°C.

Quantification of NETosis

Neutrophils were labeled with CytoTrace™ Red CMTPX (5 μM, AAT Bioquest) according to the manufacturer's instructions and resuspended in RPMI media (Gibco) supplemented with 0.5% BSA and 0.5% fetal bovine serum (Gibco). Neutrophils (1.5 x 10^5/well) were then incubated in 48-well plates with a pre-established monolayer of HUVECs at 37ºC. Samples were additionally treated with 100 μg/mL APS IgG or control IgG, in the presence of anti-Mac-1 (20 μg/mL, clone CBRM1/5) or isotype control. After three hours, SYTOX Green (Thermo Fisher Scientific) was added to a final concentration of 0.2 μM and incubated for an additional 10 minutes. Fluorescence was quantified at excitation and emission wavelengths of 485 nm and 520 nm, respectively, using a BioTek Cytation 5 Cell Imaging Multi-Mode Reader (BioTek). Representative images were captured by the BioTek Cytation 5 reader's 20x objective.

2.13 Statistical Analysis

For all experiments, all data points were included and no outlier tests were used for exclusion. Unless otherwise noted, data are plotted as averages with standard error bars. Data were analyzed using GraphPad Prism software using t tests (for comparing two averages) and ANOVA (for comparing multiple groups), unless otherwise noted. Statistical significance is generally denoted as follows: asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $P < 0.0001$, n.s. indicates "not significant".

For PPFC experiments, a N≥3 donors were included for every data point. For whole blood particle uptake studies, N≥3 donors performed in duplicate were included for every data point unless individual donors are denoted. *In vitro* cell culture particle uptake data points represent N=3 independent replicates averaged together. All *in vivo* experiments represent averages from N≥4 mice.

Chapter 3: Model Particulate Drug Carriers Modulate Leukocyte Adhesion and Response to Inflammation

3.1 Publication Information

The work in this chapter is largely published as:

William J. Kelley, Peter Onyskiw, Catherine Fromen, and Omolola Eniola-Adefeso. “Model Particulate Drug Carriers Modulate Leukocyte Adhesion in Human Blood Flows.” *ACS Biomaterials Science & Engineering.* October 22, 2019

Catherine A. Fromen, **William J. Kelley**, Margaret B. Fish, Reheman Adili, Jeffery Noble, Mark J. Hoenerhoff, Michael Holinstat, and Omolola Eniola-Adefeso. “Neutrophil-Particle Interactions in Blood Circulation Drive Particle Clearance and Alter Neutrophil Responses in Acute Inflammation.” ACS Nano 2017 11 (11), 10797-10807. October 13, 2017.

3.2 Abstract

Drug carriers have been widely explored as a method of improving the efficacy of therapeutic drugs for a variety of diseases, including those involving inflammation. However, few of these formulations have advanced past clinical trials. There are still major gaps in our understanding of how drug carriers impact leukocytes, particularly in inflammatory conditions. In this work, we investigated how targeted and non-targeted drug carriers affect the function of leukocytes in blood flow. We explored three primary mechanisms: (1) collisions in blood flow disrupting leukocyte adhesion, (2) specific binding to the endothelium competes with leukocytes for binding sites, and (3) particle phagocytosis alters leukocyte phenotype, resulting in reduced adhesion. We find that each of these mechanisms contributes to significantly reduced leukocyte adhesion to an inflamed endothelium, and that particle phagocytosis may be the most significant driver

of this effect. These results are crucial for understanding the totality of the impact of drug carriers on leukocyte behavior and response to inflammation and should inform the future design of any such drug carriers.

3.3 Background

To date, a great deal of research has focused on the use of particulate carriers for the delivery of drugs to increase their efficacy while decreasing systemic side effects.[2,142] Despite such extensive efforts, very few formulations have emerged from clinical trials and made it onto the market, likely due to inefficient targeting schemes, poor drug release kinetics, and possible mismatches between performance in artificial *in vitro* and *in vivo* experiments and clinical applications.[119,143,144] Indeed, despite the decades of research into polymeric particulate drug carriers for targeted drug delivery, all of the drug carriers for drug delivery approved to date are liposomal formulations except for Abraxane, an albumin-based particle formulation for the delivery of paclitaxel.[145] Notably, among the very few polymeric particulate drug carrier formulations to have reached Phase II/III clinical trials, none have convincingly demonstrated increased therapeutic benefit over free drug. For example, NK105, a polymeric micellar formulation of paclitaxel, showed no increased therapeutic benefit over paclitaxel alone in phase III clinical trial at a dose of 65 mg/m^2 (~1.8 mg/kg), and higher doses of NK105 (80 mg/m^2) resulted in complications due to neutropenia.[146] Additionally, relatively little work has explored the impacts of particle drug carriers on leukocyte function in response to inflammation in blood flow, which is crucial to fully understand the efficacy and potential side effects of vascular-targeted drug carriers.

Typically, in an inflammatory event (e.g., vascular injury), the endothelial cells (ECs) lining the lumen of the blood vessel begin releasing cytokines (such as IL-1β, TNF-α, and others), which recruit circulating leukocytes (particularly, neutrophils and monocytes) to the site of inflammation.[14,147] The ECs also begin expressing elevated levels of cellular adhesion molecules (CAMs; these include ICAM-1, E-Selectin, VCAM-1, PECAM-1, and others) which facilitate, via receptor-ligand binding, leukocyte rolling and firm adhesion onto the endothelium – a necessary precursor to leukocyte transmigration into the tissue space to remove the source of inflammation.[148]

Researchers have taken advantage of this overexpression of inflammation-associated CAMs for targeted therapy in several inflammatory diseases by designing particle drug carriers decorated with antibodies and ligands with binding specificity to these molecules.[149,150] To this end, several works have thoroughly investigated the impact of various particle parameters on the targeting efficacy of particulate drug carriers, including particle size[85,87,88,104,151], shape[87,92,108,152,153], and surface chemistry[106,112,154,155], resulting in a deep understanding of the interplay between these properties and ultimate drug carrier efficiency.

However, a major gap has emerged in prior works evaluating targeted drug carrier functionality. Specifically, it is well-established (and built into the conceptual design) that vascular-targeted drug carriers occupy the same physical space near the vascular wall and compete for the same binding sites as leukocytes in blood flow[22,80,81,83,92,144]. Additionally, it is well-understood that cellular collisions in blood flow are a major driver of hemodynamics[85,86,156]. Thus, the presence of vascular-targeted micro-or-nanoscale particles in the bloodstream is likely to impact leukocyte adhesion and response to inflammation. Further, neutrophils were recently reported to rapidly internalize particles in blood *in vivo* in mice, which prevents neutrophils from binding to the vascular wall, and it has been shown that the use of particle drug carriers can ameliorate the immune response in conditions such as West Nile Virus, EAE, and sepsis.[157–159] Indeed, a number of studies have shown that biomimetic drug carriers are effective at both modulating the inflammatory immune response and delivering anti-inflammatory therapeutics to the site of inflammation, suggesting the potential for competition between drug carriers and leukocytes for binding sites on the vasculature.[123,125,160–162] Despite this, no work has systematically explored the impacts of leukocyte and drug carrier interactions (including cell-particle collision, competitive binding, and phagocytosis) on the functionality of these cells in human blood.

In this work, we demonstrate that both non-targeted and targeted model drug carriers inhibit leukocyte adhesion in human blood, dependent on particle concentration and size. Additionally, we find that internalization of particles leads to phenotypic changes in neutrophils (increasing CD11b expression and decreasing CD62L expression; importantly, CD11b binds to ICAM-1 which mediates firm adhesion, while CD62L is

involved in the initial capture and slow rolling of neutrophils[14,163]) dependent on particle surface chemistry, which translate to faster leukocyte rolling velocity and reduced adhesion. Finally, we find that the magnitude of this effect varies with vessel size, likely due to changes in hemodynamics and cell-free layer (CFL) size in differently-sized vessels. These results have significant impacts for the design and efficacy of vascular-targeted drug carriers and shed new light onto previously-unexplored effects of vascular-targeted carriers on leukocytes.

3.4 Results

Targeted and non-targeted particles reduce white blood cell (WBC) adhesion in human blood flow

To investigate the impacts of the presence of particle drug carriers on WBC adhesion, we employed an *in vitro* parallel plate flow chamber (PPFC) assay as previously described.[85] For these experiments, human umbilical vein endothelial cells (HUVEC) were cultured on glass coverslips and activated for 4 hours with IL-1β prior to flow. Blood (with or without particles) was perfused over the monolayer in a pulsatile profile for 15 minutes as previously described.[85,88] For these experiments, sialyl Lewis A was chosen as a targeting ligand due to its high affinity for E-Selectin and ease of conjugation to our particle platform, and IgG was used as a non-targeted control to dampen non-specific interactions between particles and HUVEC.

When sialyl Lewis A-conjugated ("targeted") polystyrene spheres were introduced, we visibly observed a drastic reduction in WBC adhesion. Exploring this effect in-depth, we performed these experiments across a range of particle sizes (200 nm to 5 μm) and concentrations (5E5/mL through 1E9/mL; note: nanoparticles were tested at higher concentrations in order to achieve a "mass-based" dose on the same order of magnitude as the microparticles, and because little to no effect was seen for the nanoparticles at lower particle concentrations) and found some striking trends. First, we find minimal reduction in WBC adhesion for the 200 nm particles, even at high particle concentrations (Figure 3.1A). Moving forward, we see that the effect is concentration-dependent, with more significant inhibition of WBC adhesion generally occurring at higher particle concentrations. For example, focusing on the 500 nm spheres, we find that at a

concentration of 5E5/mL, minimal reduction in WBC adhesion occurs (~10%); however, at a concentration of 1E9/mL, we find a much greater reduction in adhesion (~70%). (Figure 3.1B). This effect holds across all particle sizes tested except for 200 nm particles, suggesting that a greater number of leukocyte-particle collisions and greater endothelium surface coverage results in a more significant reduction in WBC adhesion.

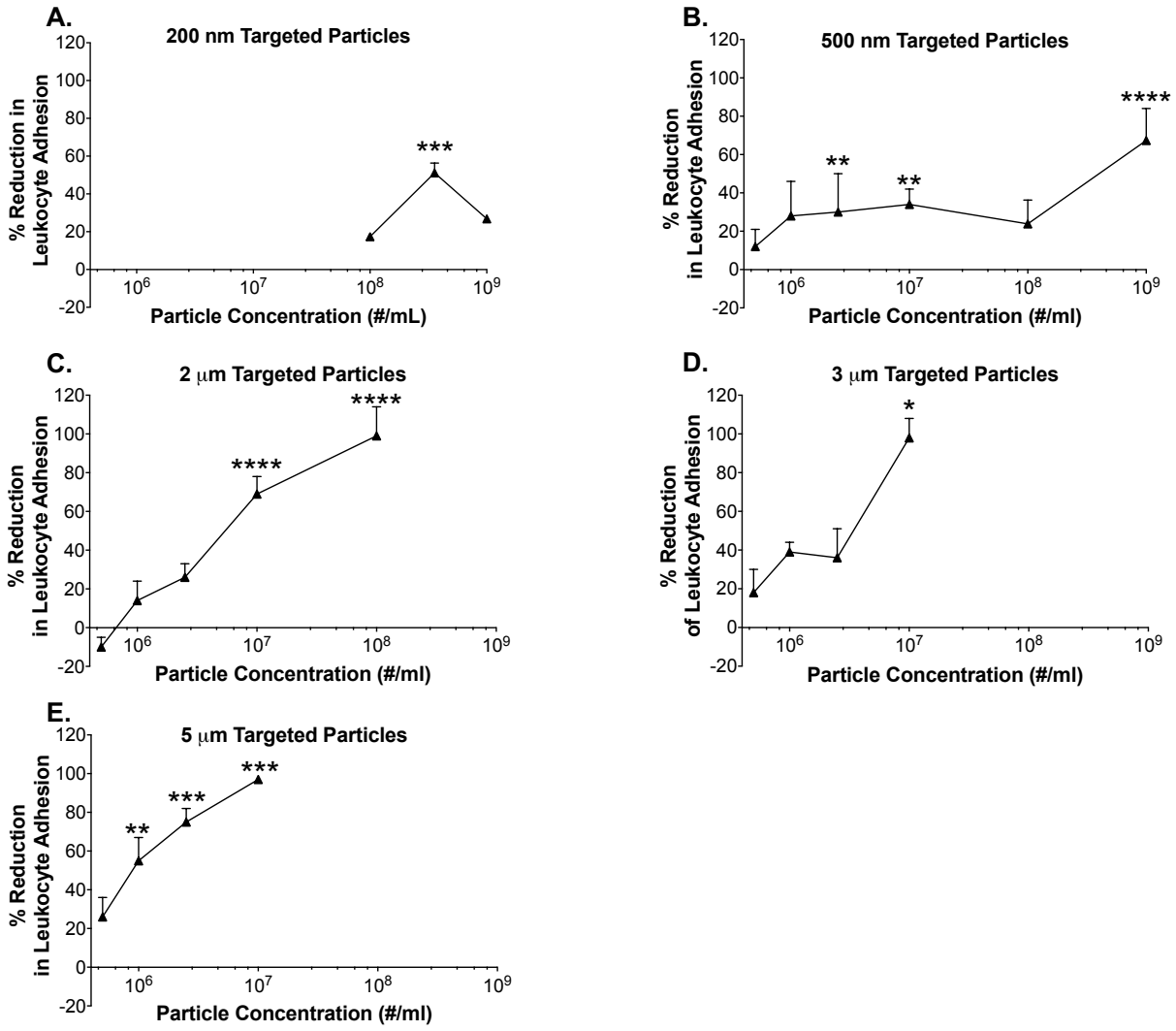


Figure 3.1 - Reduction of leukocyte adhesion observed following pulsatile flow with (a) 200 nm, (b) 500 nm, (c) 2 μm, (d) 3 μm, and (e) 5 μm targeted (sLeA) particles. (*) indicates significant difference in leukocyte adhesion of targeted (sLe^A) particles relative to particle-free blood, ($p<0.05$). Statistical analysis was performed Kruskal-Wallis with α

= 0.01. n ≥ 3 donors for each particle size and concentration. Error bars represent standard error.

Further, we find a major impact of particle size on reduction in leukocyte adhesion, with larger particles generally resulting in a more significant decrease in leukocyte adhesion. For example, if we compare 500 nm (Figure 3.1B) and 2 μm particles (Figure 3.1C) at a concentration of 1E8/mL, we observe a reduction of ~20% in leukocyte adhesion for the 500 nm particles and ~100% for the 2 μm particles. Comparing 2 μm (Figure 3.1C) to 3 μm (Figure 3.1D) particles at a concentration of 1E7/mL, we find a percent reduction of ~70% for the 2 μm particles and a percent reduction of ~100% for the 3 μm particles. Similarly, if we compare 2 μm to 5 μm particles (Figure 3.1E) at a concentration of 1E7/mL, we find a percent reduction of ~70% for the 2 μm particles and a percent reduction of ~100% for the 5 μm particles, which is likely due more-efficient margination to the CFL[85,88].

Next, we sought to determine whether this effect was driven primarily by particle-leukocyte collisions or by specific blocking of binding sites on the endothelium by comparing the reduction in leukocyte adhesion for IgG-conjugated ("non-targeted") particles to targeted particles at the same size. For this comparison, we computed the 'Normalized Leukocyte Adhesion Ratio' using Equation 3.1 below:

$$Norm.\,Adhesion\ Ratio = \frac{\left(\frac{\#\ of\ adherent\ leukocytes\ for\ non-targeted\ particles}{\#\ of\ adherent\ leukocytes\ in\ particle\ free\ control}\right)}{\left(\frac{\#\ of\ adherent\ leukocytes\ for\ targeted\ particles}{\#\ of\ adherent\ leukocytes\ in\ particle\ free\ control}\right)} \quad (3.1)$$

Thus, a normalized leukocyte adhesion ratio (NAR) above 1 signifies a greater reduction in leukocyte adhesion for targeted particles versus non-targeted particles, with greater values signifying a greater effect. (Note: due to limitations in blood volume and cells, experiments were sometimes performed on different days and normalized to different controls, and thus the denominators in Equation 1 do not strictly cancel one another).

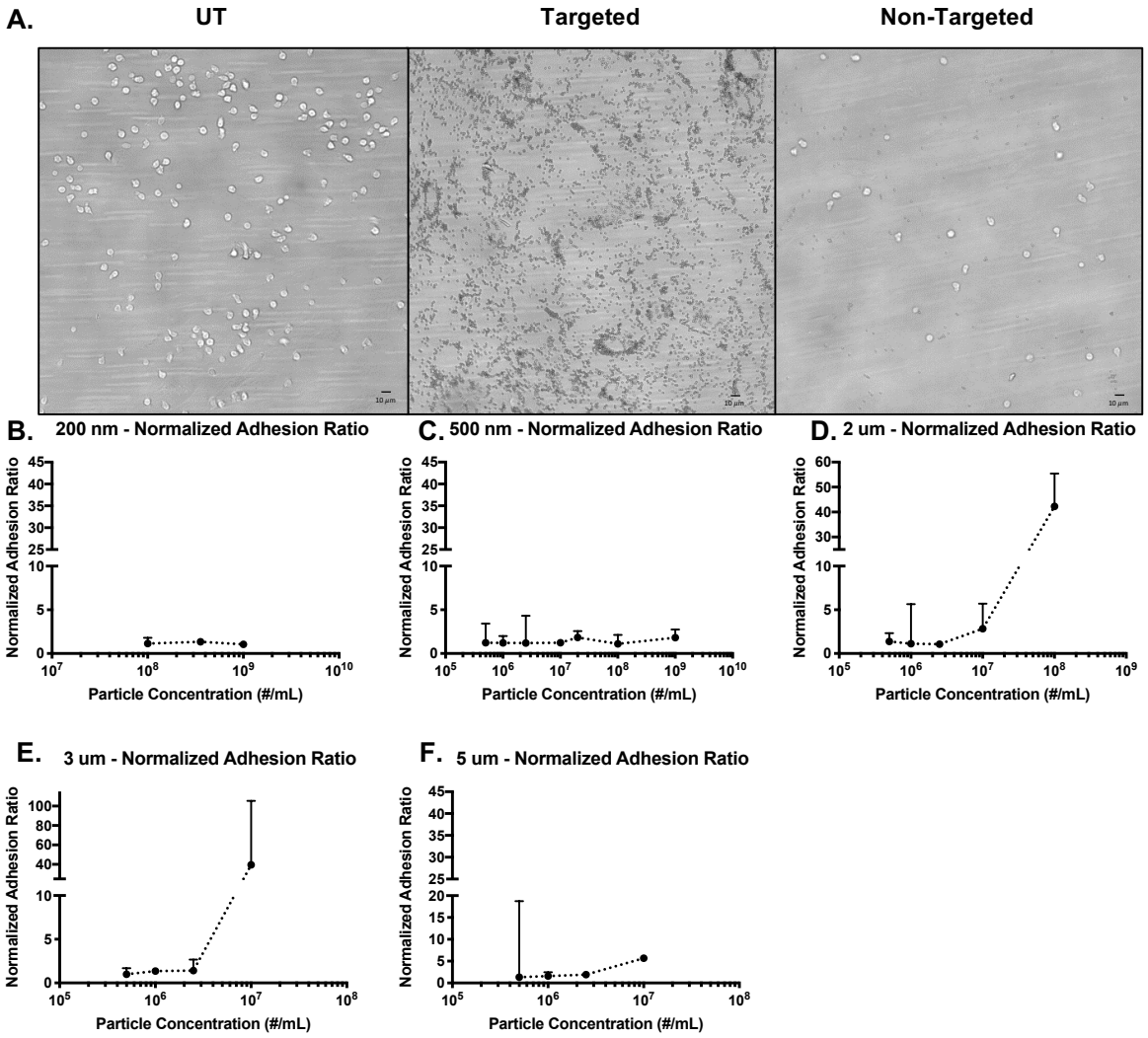


Figure 3.2 - Normalized Adhesion Ratio of Non-Targeted versus Targeted Particles. (a) Example images of leukocyte and particle (2 $\mu m, 1E8/mL$) adhesion on a HUVEC monolayer. Ratio of leukocyte adhesion (Non-targeted over Targeted Particles) relative to particle-free blood for (b) 200 nm, (c) 500 nm, (d) 2 μm, (e) 3 μm, and (f) 5 μm targeted (sLe^A) particles.

Figure 3.2A is a visual representation of the reduction in leukocyte adhesion for targeted particles versus non-targeted particles. Using the NAR analysis, we can determine conditions at which particle targeting confers an additional reduction in leukocyte adhesion (Figure 3.2B-F). A few findings stand out in this analysis. First, the added reduction in leukocyte adhesion for targeted particles primarily occurs with larger (micron-scale) particles, where the 2 μm, 3 μm, and 5 μm particles all exhibit NAR values

much greater than 1 at higher particle concentrations (Figure 3.2 D-F). The greater impact of micron-sized particles on cell adhesion is likely due to larger particles localizing more efficiently to the vascular wall and, once bound, occupying more physical space and receptor binding sites on the endothelium. Nano-sized particles are less efficient at marginating[85,88]; thus, they likely reduce leukocyte adhesion primarily through physical interactions in free stream. Hence, we observe low NAR values for nanospheres (Figure 3.2 B,C). Indeed, we observe nanoparticles associating with leukocytes in blood flow, which supports this hypothesis (Figure 3.3).

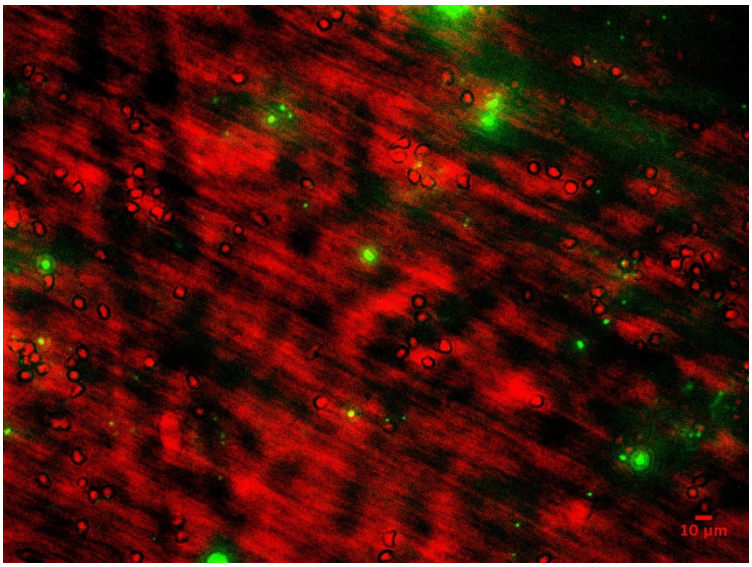


Figure 3.3—Colocalization of Nontargeted Nanoparticles with Leukocytes on HUVEC Monolayer. Representative fluorescent microscope image showing colocalization of nontargeted 500 nm nanoparticles (green) with leukocytes (red) on a HUVEC monolayer

Additionally, we primarily see high NAR values at high particle concentrations, suggesting that the specific targeting impact on leukocyte adhesion occurs primarily when bound particles occupy a large portion of the endothelium. Indeed, when plotting particle adhesion versus percent reduction in leukocyte adhesion, we find that as particle adhesion increases, the percent reduction in leukocyte adhesion also increases, particularly for larger particles (Figure 3.4).

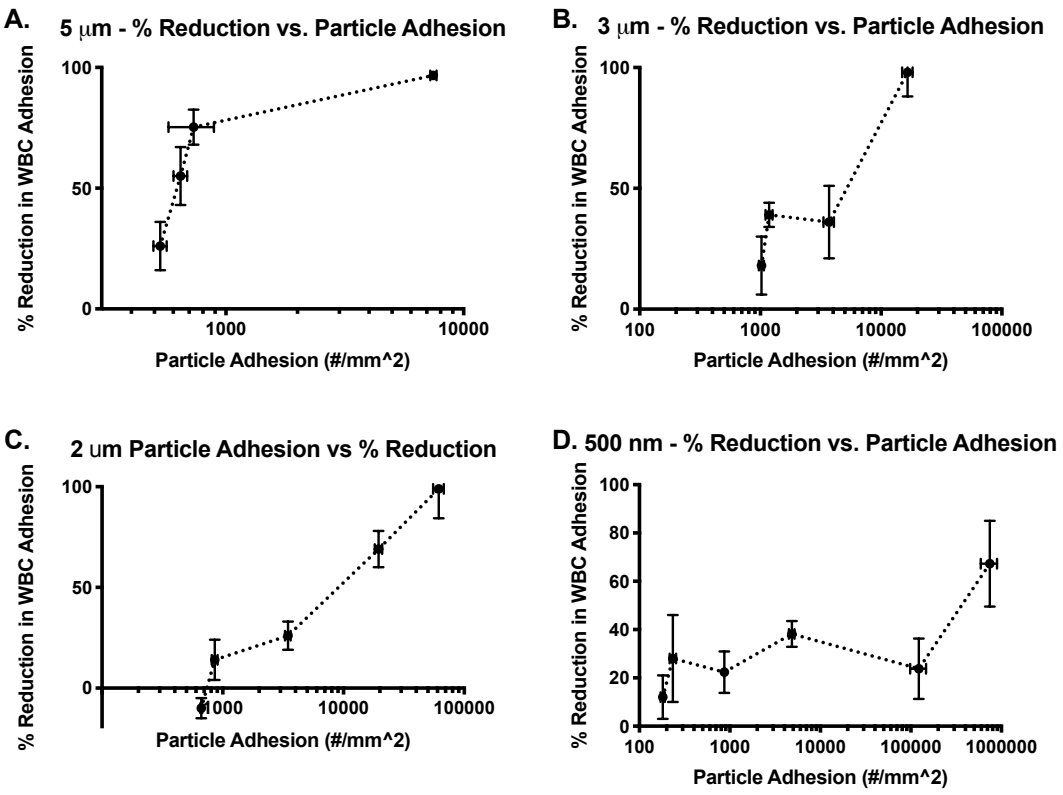


Figure 3.4 - Particle Adhesion versus Percent Reduction in Leukocyte Adhesion. (a) Particle Adhesion vs. % Reduction in Leukocyte Adhesion for 5 μm particles. (b) Particle Adhesion vs. % Reduction in Leukocyte Adhesion for 3 μm particles. (c) Particle Adhesion vs. % Reduction in Leukocyte Adhesion for 2 μm particles. (d) Particle Adhesion vs. % Reduction in Leukocyte Adhesion for 200 nm particles. Each data point represents at least 3 independent donors. Error bars are standard error.

This trend of adhesion versus %reduction in leukocyte adhesion supports the notion that, once a certain fraction of the available space for binding on the endothelium is occupied, targeted particles out-compete circulating leukocytes and prevent them from binding. Further, when plotting % reduction in leukocyte adhesion versus HUVEC surface coverage percentage for targeted particles, we find the greatest reduction in leukocyte adhesion (and the greatest NAR values) once surface coverage reaches above ~10%, suggesting that perhaps at lower particle concentrations, nonspecific collisions are responsible for the observed reduction in leukocyte adhesion, but once a certain level of

surface coverage is achieved via active targeting, the surface blocking effect begins to dominate (Figure 3.5).

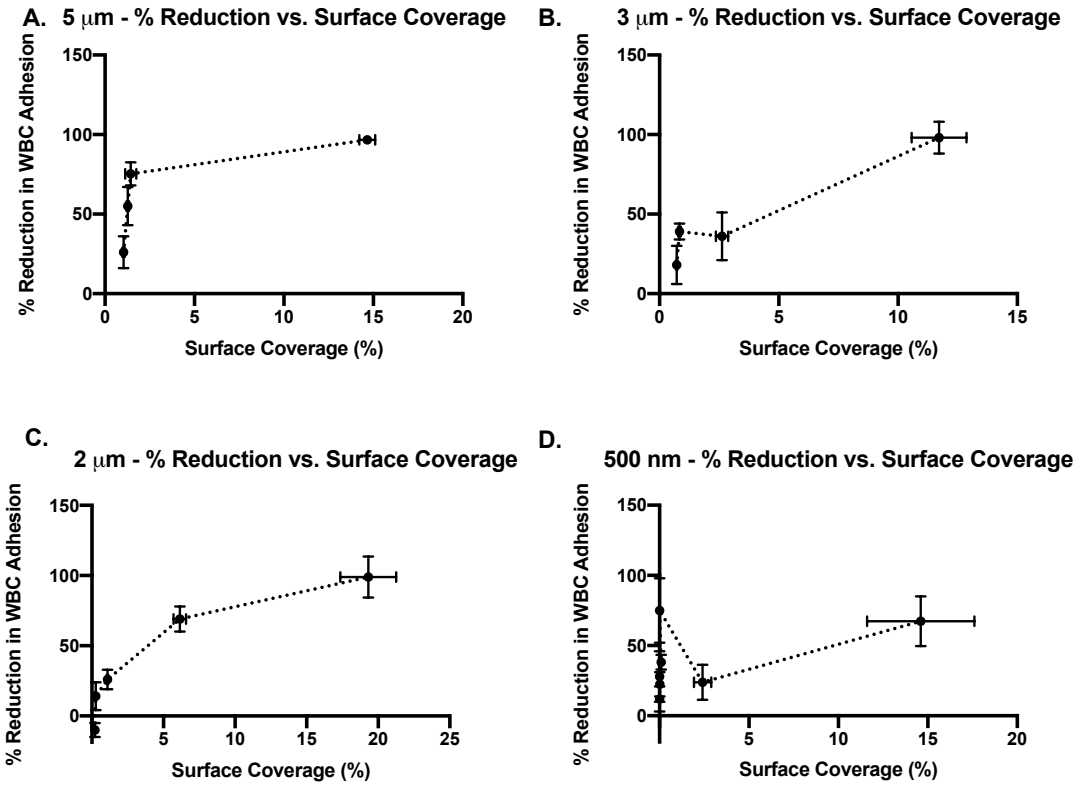


Figure 3.5 - Surface Coverage versus Percent Reduction in Leukocyte Adhesion. (a) Surface Coverage vs. % Reduction in Leukocyte Adhesion for 5 μm particles. (b) Surface Coverage vs. % Reduction in Leukocyte Adhesion for 3 μm particles. (c) Surface Coverage vs. % Reduction in Leukocyte Adhesion for 2 μm particles. (d) Surface Coverage vs. % Reduction in Leukocyte Adhesion for 500 nm particles. Each data point represents at least 3 independent donors. Error bars are standard error.

Inhibition of leukocyte adhesion in smaller blood vessels

Because hemodynamics can vary in blood vessels of varying sizes[25], we repeated a subset of the experiments from Figure 1 in a smaller channel (127 μm versus 254 μm channel height). These experiments are important for understanding if and how the observed reduction in leukocyte adhesion might manifest in blood vessels of varying sizes, informing the design of vascular-targeted drug carriers based on their intended destination. Thus, we evaluated leukocyte adhesion in the presence of 500 nm and 2 μm

particles across a range of particle concentrations in the 127 μm channel. These results are presented in Figure 3.6.

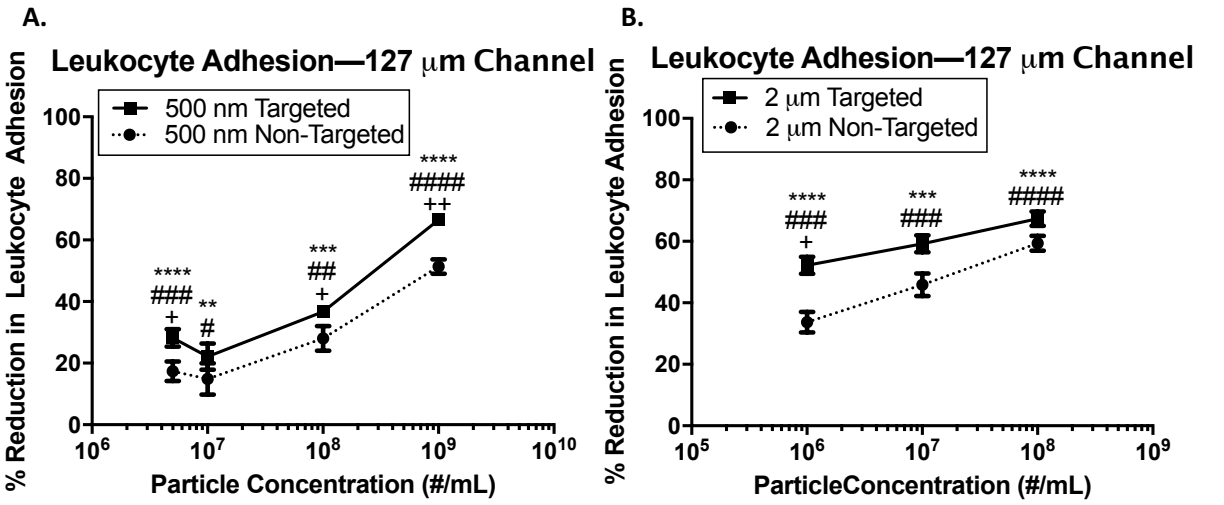


Figure 3.6 - Particle reduction of leukocyte adhesion in pulsatile blood flow in 127 μm channel. (a) Percent reduction in leukocyte adhesion to an inflamed endothelium in 127 μm channel following pulsatile blood flow with the addition of 500 nm targeted and non-targeted particles at varying concentrations. (b) Percent reduction in leukocyte adhesion to an inflamed endothelium in 127 μm channel following pulsatile blood flow with the addition 2 μm targeted and non-targeted particles at varying concentrations. (*) indicates significant difference in leukocyte adhesion of targeted (sLe^A) particles relative to particle-free blood, (#) indicates significant difference in leukocyte adhesion of non-targeted particles relative to particle-free blood, and (+) indicates significant difference between targeted and non-targeted groups ($p<0.05$). Statistical analysis was performed using one-way ANOVA using GraphPad Prism software. $n \geq 3$ donors for each particle size and concentration. Error bars represent standard error.

First, we find that some of the same trends observed in the 254 μm channel emerge here. In general, a higher particle concentration results in a more significant reduction in leukocyte adhesion. For example, the addition of non-targeted 500 nm spheres in blood at 1E7/mL results in a ~15% reduction in leukocyte adhesion, while a higher concentration of 1E9/mL leads to a 50% reduction in adhesion (Figure 3.6A). Similarly, for 2 μm non-targeted particles at 1E6/mL, we observe a ~30% reduction in

leukocyte adhesion versus a ~60% reduction for 2 μm non-targeted particles at a concentration of 1E8/mL (Figure 3.6B). Because we again observe greater reduction in leukocyte adhesion for higher concentrations of both targeted and non-targeted particles, this suggests that both greater numbers of collisions and higher specific blocking of the endothelial surface result in decreased leukocyte adhesion in the 127 μm channel, similar to the 254 μm channel. Additionally, similar to the results in Figure 3.2, we see that larger particles generally result in a more significant reduction in leukocyte adhesion.

However, we do observe some changes in the smaller channel versus the larger channel. Notably, the overall magnitude of the percent reduction in leukocyte adhesion is generally lower in the smaller channel versus the larger channel, particularly for the 2 μm particles. This could be due to the changes in the relative size of the CFL and RBC core in the different vessels; theoretical and experimental results have shown that, as vessel size increases, the relative size of the cell-free layer decreases.[164] Thus, in a larger vessel, leukocytes and particle drug carriers will be pushed closer to the wall, likely resulting in more collisions which in turn results in a greater inhibition in leukocyte adhesion. This hypothesis is also supported by experimental work from our lab showing that 2 μm particles marginate more efficiently in larger channels, while 500 nm particle margination is relatively unchanged.[85] However, calculations based on previous computational work show that, for a 127 μm channel versus a 254 μm channel, the CFL size will be approximately equivalent (3.25 μm versus 3.15 μm, respectively).[165] Thus, the differences observed could be simply due to a lower overall flow rate in the 127 μm channel, leading to a reduction in the magnitude of collision forces between particles and leukocytes. Of course, computational and many experimental works evaluate the CFL in actual blood vessels, which may differ slightly from PPFC experiments.

Intravenously-administered particles rapidly associate with neutrophils in vivo

Given the results of our *in vitro* PPFC experiments demonstrating that both targeted and non-targeted particles inhibit leukocyte adhesion to an inflamed endothelium, we sought to determine whether a similar phenomenon is observed in an *in vivo* setting. Thus, we injected our model particulate drug carriers into mice and investigated whether and in what ways they interacted with leukocytes in blood flow and

in inflammation. C57BL/6 mice were injected with a ~30 mg/kg dosage of either 2 or 0.5 μm polystyrene (PS) carboxylate-functionalized (COOH) particles (corresponding to 2 × 10^8 2 μm particles or 1.28 × 10^{10} 0.5 μm particles) and whole blood was obtained via cardiac puncture approximately 2 minutes following particle injection. Particles 2 μm in diameter were chosen due to their efficient margination (i.e., localization) in the RBC-FL, as well as a prior report of microparticle interference with the functionality of inflammatory monocytes by Getts et al.[88,128] Particles of 0.5 μm were chosen as representative nanoparticle therapeutics which do not localize to the CFL, but rather distribute uniformly in flow.[88]

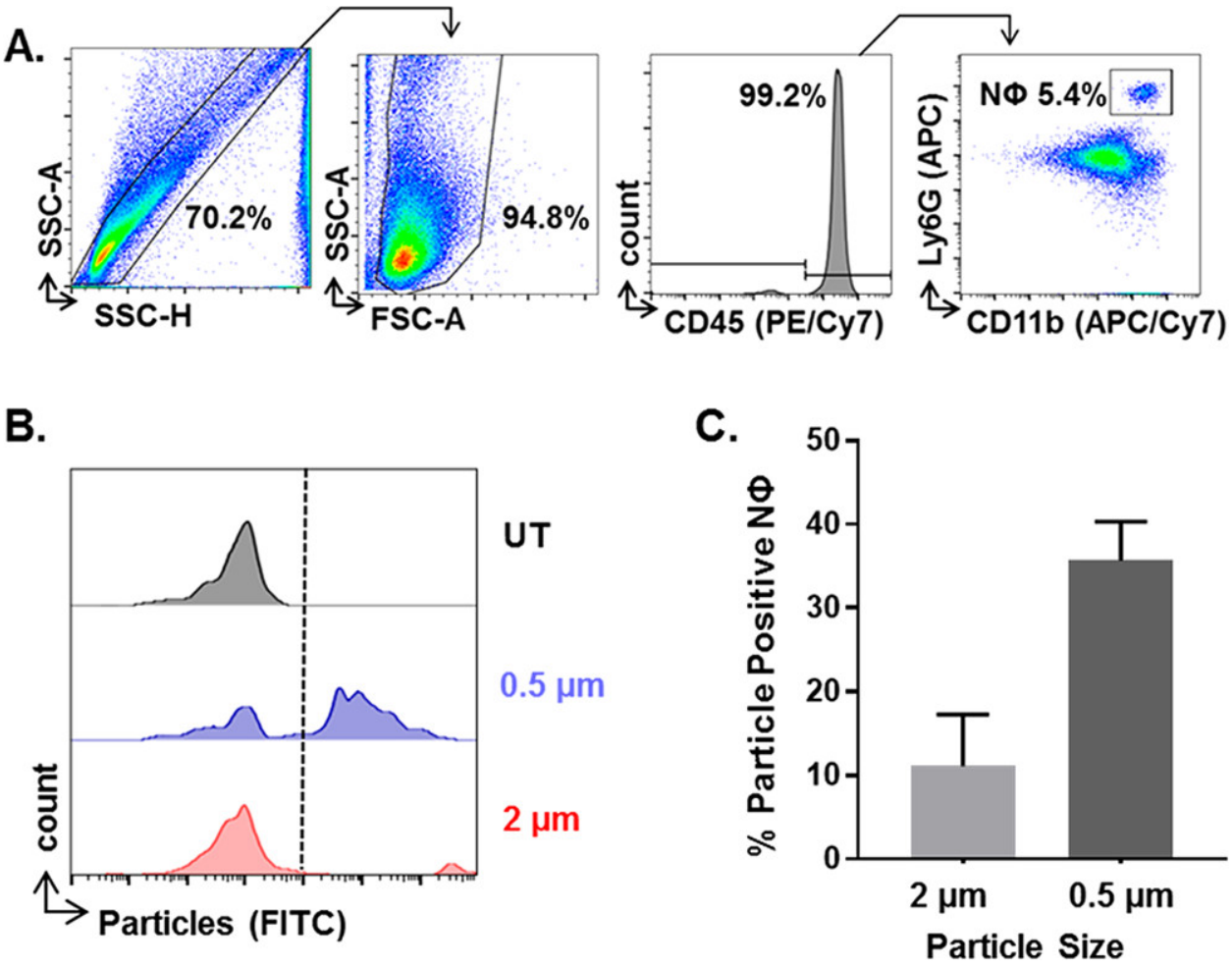


Figure 3.7 - Neutrophil association with particles immediately following IV injection. C57BL/6 mice were injected with equivalent mass dosages of either 2 or 0.5 μm COOH particles via tail vein, and blood was obtained within 2 min of particle injection via cardiac puncture. (a) Representative gating analysis of blood sample to identify neutrophil

population. (b) Representative gating analysis and (c) quantified results of particle positive neutrophils in mouse blood. Graphs are representative data from a single experiment (n = 3). Error bars represent standard error.

As shown in Figure 3.7A, mouse neutrophils were identified using flow cytometry first via FSC vs SSC and then as CD45+CD11b+Ly6G+. Ly6G is the most commonly used surface protein for mouse neutrophil identification.[166] A coexpression of CD11b, a leukocyte adhesion molecule and subunit of integrin αMβ2, with Ly6G differentiates neutrophils from monocytes.[167] From this population, particle positive neutrophils were identified as FITC+ cells (Figure 3.7B). When dosed at equivalent mass, 11.1 ± 1.0% of collected neutrophils from mice receiving 2 μm particles were particle positive, while 35.7 ± 2.9% of collected neutrophils from mice receiving 0.5 μm particles were particle positive (Figure 3.7C).

Intravenously-administered particles reduce neutrophil adhesion in acute mesentery inflammation

Given this measurable proportion of neutrophils in circulation found to be rapidly associated with particles of both sizes, we sought to visualize these interactions live within the bloodstream. Using intravital microscopy, we visualized vasculature in the mouse mesentery and monitored rolling neutrophils at the surface of an inflamed vascular wall. TNF-α was locally (topically) applied to the exposed vessel prior to particle injection to upregulate adhesive molecules and facilitate neutrophil rolling, enabling us to visualize a subset of neutrophils passing through the vasculature in real time

Circulating NΦs were prestained with anti-Ly6G and particles injected at the same equivalent mass dosage as before. As shown in Figure 3.8, both 2 and 0.5 μm particles were found associated with rolling neutrophils. Arrows indicate Ly6G+ neutrophils that were found with particles for at least three consecutive frames, indicating that particles are firmly associated with the cell. Interestingly from these observations, we can identify neutrophils that have bound both single or multiple particles. Both particle sizes were found associated specifically with neutrophil populations outside the RES organs,

indicating that neutrophils in the periphery are involved in binding particles and likely contribute to clearance of IV-administered particles.

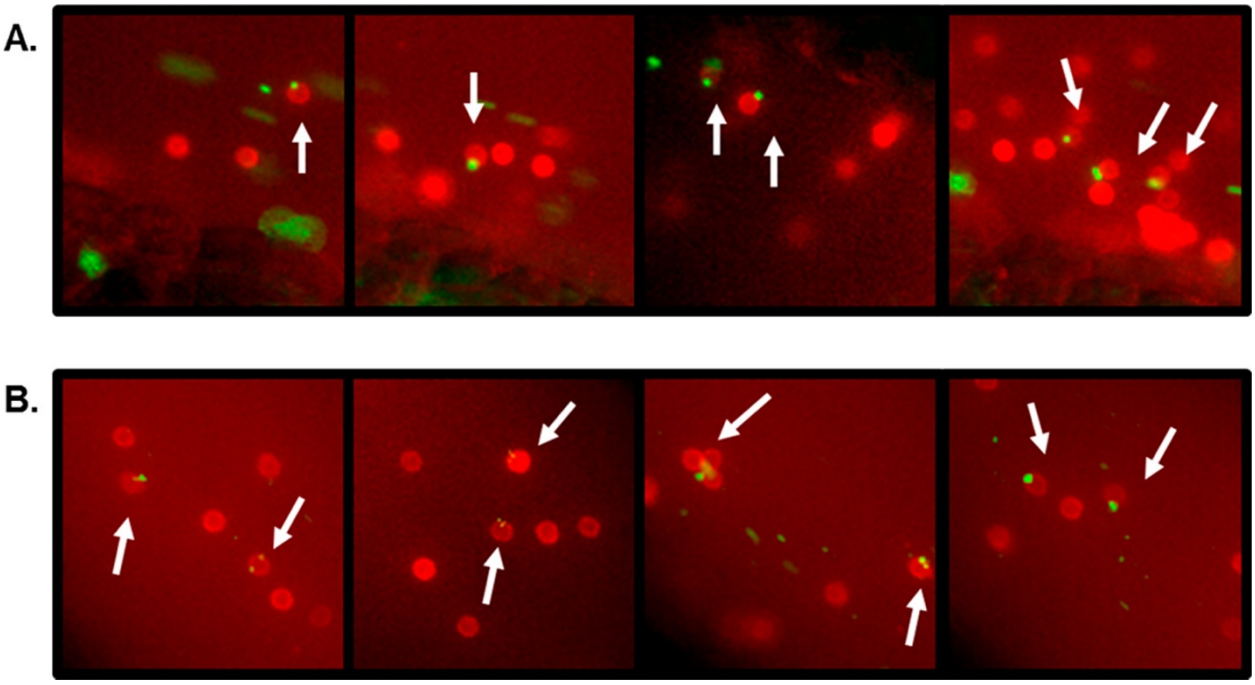


Figure 3.8 - Neutrophil association with particles immediately following IV injection. C57BL/6 mice were injected with equivalent mass dosages of either 2 or 0.5 μm COOH particles via tail vein, and blood was obtained within 2 min of particle injection via cardiac puncture. (a) Representative gating analysis of blood sample to identify neutrophil population. (b) Representative gating analysis and (c) quantified results of particle positive neutrophils in mouse blood. Graphs are representative data from a single experiment (n = 3). Error bars represent standard error.

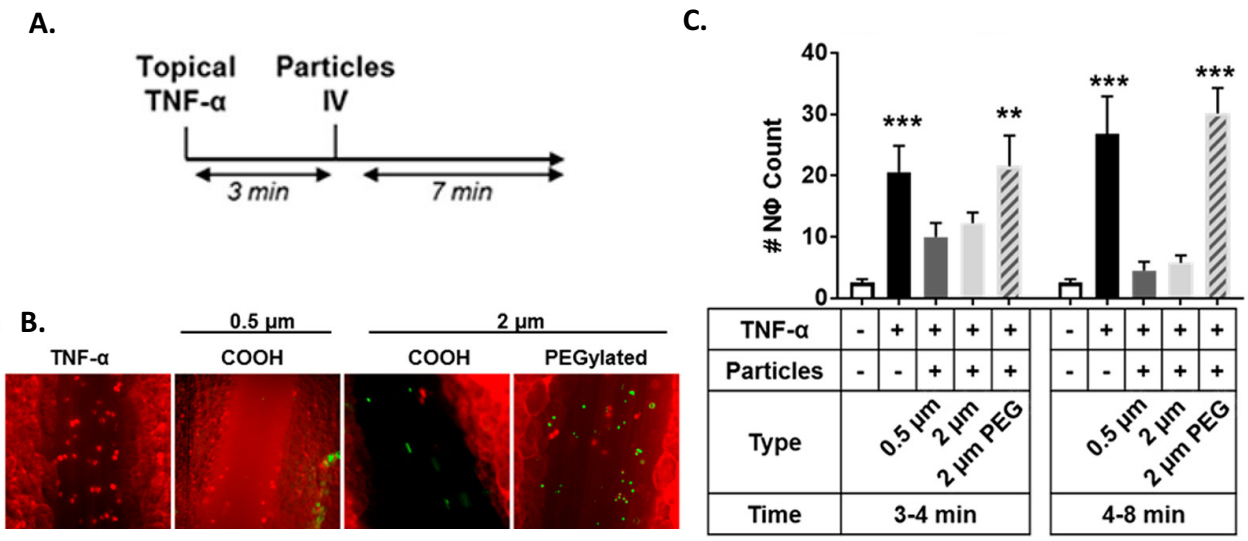


Figure 3.9 – Intravenously-administered particles reduce neutrophil adhesion in mesentery inflammation. (a) Dosing schedule of acute mesentery inflammation model in C57BL/6 mice. Mice received topical TNF-α at time 0 and a particle injection at 3 min. (B) Representative images shown at 8 min with NΦs labeled red and particles labeled green and (C) quantified results of NΦ frequency at the vascular wall following particle administration of 0.5 and 2 μm COOH particles or 2 μm PEGylated particles via IV injection. Results are averaged over two ranges of time points. The top image in B shows the merging of both red and green channels, with the individual colors split below. Average NΦ counts were quantified during the first minute following particle injection (3−4 min) and longer time points (4−7 min). (*) Indicates significant difference in cell counts relative to the corresponding TNF-α-only value. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparison test to the TNFα group with $\alpha = 0.01$. Bars represent averages from at least eight different vessels within groups, with $n \geq 4$ mice per group, and error bars represent standard error.

During our intravital investigation, we noted that the presence of particles in circulation appeared to reduce neutrophil adhesion at the vascular wall over time. To quantify this change, we assessed neutrophil adhesion over time in a previously established model of acute murine mesentery inflammation.[131,168] Following the dosing schedule shown in Figure 3.9A, neutrophil populations were quantified following 0.5 and 2 μm particle injection. In addition to the COOH 2 μm PS particles, we also evaluated 2 μm PS particles functionalized with a high-density poly(ethylene glycol)

(PEG) coating (brush conformation). As discussed elsewhere, high density PEG coatings mitigate cell uptake in mice, allowing us to confirm that any change in localization to the vessel wall would be an impact of particle internalization.[111,114,169] TNF-α was topically applied to mesenteric veins to induce local, rapid neutrophil accumulation on the vessel wall. We visualized an average of over 20 neutrophils on TNF-αtreated vessels after 3 min, more than double the number found after the first minute following TNF-α application. This increased number of neutrophils was maintained for the duration of the experiment in mice receiving only topical TNF-α, confirming a local inflammatory event in the vessel. Particles were injected following 3 min of inflammation; representative images of the vessel wall following particle injections are shown in Figure 3.9B. Average neutrophil numbers were quantified in the first minute following particle injection (3−4 min after TNF-α) and at subsequent (4−7 min after TNF-α) time points. In the first minute following injection, both COOH 0.5 and 2 μm particles produced an immediate and significant reduction in neutrophil localization versus particle-free TNF-α-only vessels and continued to do so for more than 4 min (Figure 3.9C). Neutrophil localization following COOH particle injections was not statistically different than untreated vessels at either time point evaluated and resulted in about 80% reduction versus the TNF-α group between 4 and 8 min (78% for 0.5 μm and 83% for 2 μm, nonsignificantly different from each other). In contrast, the 2 μm PEG particles failed to result in any reduction to the neutrophil localization to the vessel wall for the duration of the experiment. PEG particles were still found circulating in high quantities following 7 min, in comparison to both of the COOH particles, which had been largely cleared from circulation (Figure 3B bottom panel). The rapid decrease in neutrophil adhesion following COOH administration and minimal impact of PEG administration indicates that particle uptake by neutrophils may hinder normal neutrophil function during inflammation.

Intravenously-administered particles reduce neutrophil infiltration in acute lung injury (ALI)

Because the acute mesentery inflammation model is a simple model of inflammation which does not fully represent a physiological inflammatory condition, we sought to explore whether intravenously-administered particles alter the immune

response in a more complex model of inflammation. For this purpose, we explored the impact of intravenously-administered particles on neutrophil infiltration into the airways in a model of acute lung injury.[170] ALI was induced via lipopolysaccharide (LPS) instillation into mice lungs, causing recruitment of neutrophils to the airspace via increased expression of inflammatory cytokines and chemokines.[44,170,171] One hour after LPS instillation, we administered 2 and 0.5 μm particles at equivalent mass via IV tail vein injection to both C57BL/6 and BALB/c mice as in Figure 3.10A. To assess neutrophil migration, we evaluated the percentage of neutrophilss in the BALF (Figure 3.10B), and the total BALF neutrophil counts (Figure 3.10C-D). As shown in Figure 3.10B−D, mice receiving only LPS demonstrated increased BALF infiltrates, with neutrophil counts averaging $1.1 \times 10^6 \pm 1 \times 10^5$, which corresponded to over 46% of the collected cells in C57BL/6 mice; in BALB/c mice, BALF neutrophil counts averaged $3.3 \times 10^7 \pm 1 \times 10^7$, corresponding to over 67%. The BALF neutrophil counts following an LPS administration in both mouse strains indicate occurrence of an acute inflammatory event and correspond to previous findings that BALB/c mice are more sensitive to LPS instillation than C57BL/6. However, upon IV particle injections, the percentage of BALF neutrophils and the total number of neutrophil infiltrates decreased in both mouse strains. In C57BL/6 mice, ALI mice receiving 2 μm particles had total BALF neutrophil counts of $5.2 \times 10^5 \pm 2 \times 10^5$, representing a 52% decrease from the LPS-only group and corresponding to only 37% of collected cells. Similarly, ALI mice receiving 0.5 μm particles had total BALF neutrophil counts of $4.5 \times 10^5 \pm 1 \times 10^5$, representing a 59% decrease from the LPS-only group and corresponding to only 41% of collected cells. In BALB/c mice, the results were more pronounced. ALI BALB/c mice receiving 2 μm particles had total BALF neutrophil counts of $2.9 \times 10^6 \pm 2 \times 10^6$, representing a 93% decrease from the LPS-only group and corresponding to only 31% of collected cells. Similarly, ALI mice receiving 0.5 μm particles had total BALF neutrophil counts of $6.4 \times 10^5 \pm 5 \times 10^5$, representing a 98% decrease from the LPS-only group and corresponding to only 32% of collected cells. Both particle-treated groups were nonstatistically different than the untreated mice in both mouse strains.

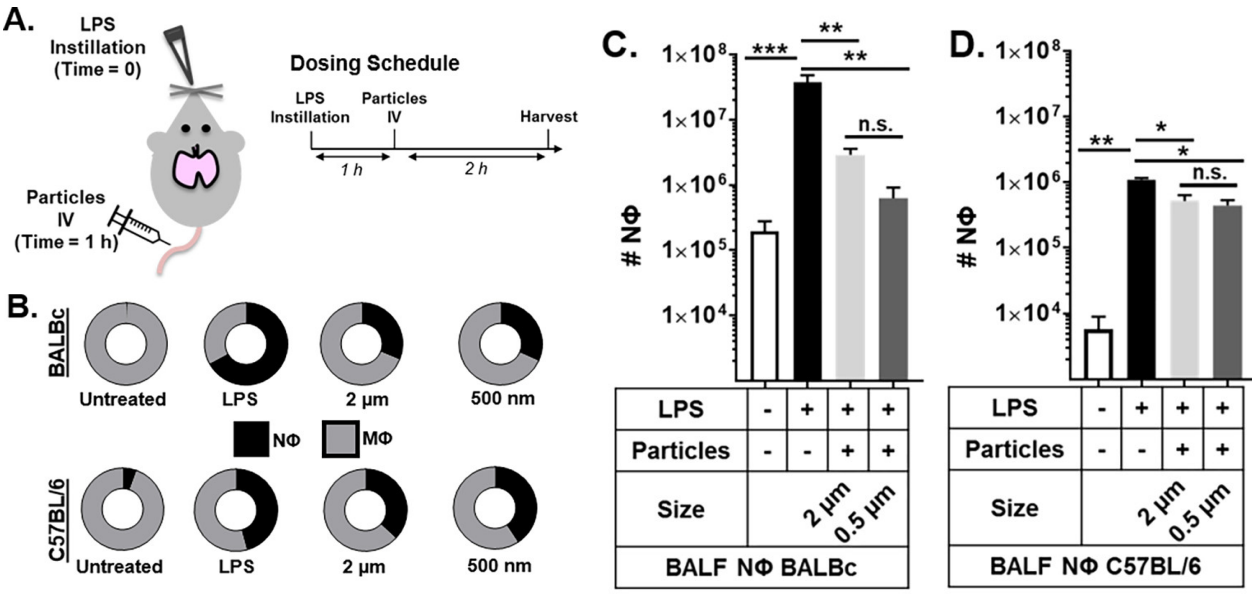


Figure 3.10 – Intravenously-administered particles reduce neutrophil infiltration into the lungs in ALI. (a) Diagram and dosing schedule of the ALI model performed in both BALB/c and C57BL/6 mice. (b) Average BALF distribution of neutrophil and macrophage populations (MΦ) following particle injection. Total BALF neutrophil counts in (c) BALB/c and (d) C57BL/6 mice.

Particle phagocytosis results in reduced leukocyte adhesion in blood flow in vitro

Given our experiments demonstrating that particle phagocytosis and clearance in mice *in vivo* results in a diversion of neutrophils to the liver[157], we sought to investigate whether particle internalization similarly influences adhesion for human leukocytes. Thus, we performed a series of experiments evaluating leukocyte adhesion in the presence of carboxylated and sialyl Lewis A-conjugated particles, where particles were either preincubated (1 hr) with cells prior to performing the flow experiments, or introduced immediately prior to the flow experiment (i.e., the "Non-incubated" condition). Thus, for the "Preincubated" condition, particle phagocytosis was allowed to occur prior to blood flow, while for the "Non-incubated" condition we expect particle phagocytosis to be minimal. 1 hour was chosen for incubation time based on our previous work showing that appreciable particle phagocytosis occurs after approximately 1 hour in human blood *ex vivo*.[119] We performed these experiments with both 2 μm (at 1E7/mL) and 500 nm particles (at 6.4E8/mL; chosen for equivalent particle mass to the 2 μm particles); the

results are shown in Figure 3.4. (Note: ACD was chosen for non-phagocytic experiments because it chelates Ca^{2+}, preventing phagocytosis, while heparin anticoagulates by activating antithrombin III, which in turn deactivates Thrombin, Factor IX, and Factor X, thus allowing for phagocytosis to occur.)

Notably, we see a striking difference between the "Non-incubated" and "Preincubated" conditions for both particle sizes. For 2 μm particles in the "Non-incubated" condition, we see little to no reduction in leukocyte adhesion for the non-targeted particle and ~30% reduction for the sialyl Lewis A-targeted particles (Figure 3.11A). However, for the "Preincubated" condition, we find that both particle types significantly reduce leukocyte adhesion at close to the same level (~30% reduction for non-targeted, ~50% reduction for sLe^A). This observation for the 2 μm size is replicated with the 500 nm particles; for the "Non-incubated" condition, we find ~15% reduction for the non-targeted particles and ~50% reduction for the sLe^A particles, and for the "Preincubated" condition we find a reduction of ~75% for both particle types (Figure 3.11B).

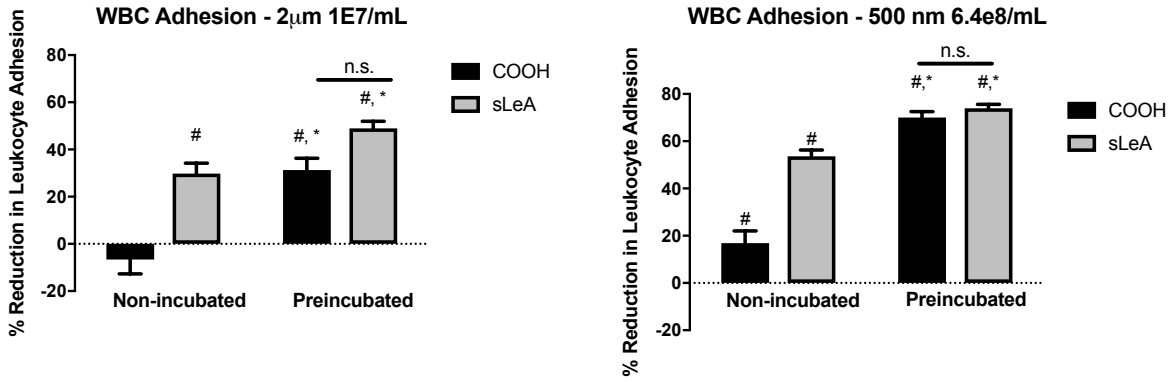


Figure 3.11 - Impact of Particle Internalization on Leukocyte Adhesion in Pulsatile Flow. (a) Leukocyte adhesion in whole blood following pulsatile flow at $1000s^{-1}$ shear over an activated HUVEC monolayer for 15 minutes for blood with carboxylated and sialyl Lewis A-conjugated 2 μm polystyrene particles at a concentration of 1E7/mL, normalized to the particle-free control. (b) Leukocyte adhesion in whole blood following pulsatile flow at $1000s^{-1}$ shear over an activated HUVEC monolayer for 15 minutes for blood with carboxylated and sialyl Lewis A-conjugated 500 nm polystyrene particles at a concentration of 6.4E8/mL, normalized to the particle-free control. (#) indicates a

significant reduction from the particle-free control; (*) indicates a significant reduction between the 'non-incubated' and 'preincubated' conditions for that particle type ($p<0.05$). $n \geq 3$ donors for each bar shown. Statistical analysis was performed using one-way ANOVA using GraphPad Prism software.

These results have a few major implications. First, we find that particle internalization can dramatically reduce the propensity for leukocytes to adhere to an activated endothelium. Additionally, we see that the added component of internalization eliminates much or all of the difference between targeted and non-targeted particles, suggesting that phagocytosis dominates in this effect over particle-leukocyte collisions and specific blocking of binding sites on the endothelium. This finding is critically important, as it more closely describes the situation found *in vivo*.

Particle phagocytosis results in altered neutrophil phenotype, changing ligand expression levels

The results in Figure 3.11 led us to ask whether particle phagocytosis results in specific changes in leukocyte phenotype which drive the reduction in leukocyte adhesion further. Because neutrophils comprise up to 70%[13] of circulating leukocytes and are the primary first-responders in inflammation, monocytes are responsible for relatively little particle uptake in *ex vivo* blood samples, and given recent work showing that neutrophil-particle interactions impact the immune response to inflammation *in vivo*[130,157], we focus this analysis on neutrophils. Additionally, the above experiments showing that particle surface chemistry greatly influences the impact of particles on neutrophil function *in vivo*, we investigated these changes resulting from phagocytosis of carboxylated polystyrene particles, PEGylated polystyrene particles, and sialyl Lewis A-conjugated particles (Figure 3.12).

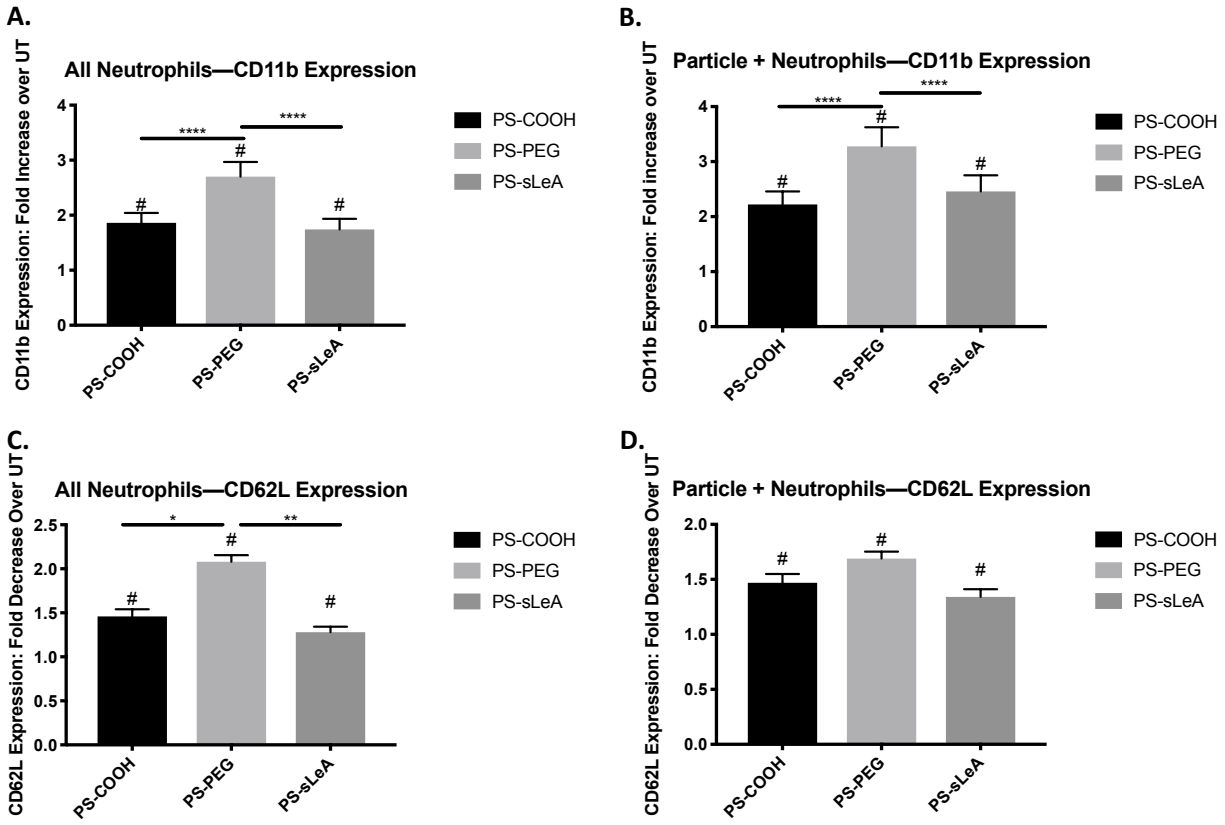


Figure 3.12 - Impact of Particle Internalization on Neutrophil Expression of CD11b and CD62L. (a) Fold change in CD11b expression over untreated for all neutrophils in whole blood exposed to carboxylated, PEGylated, and sialyl Lewis A-conjugated polystyrene particles. (b) Fold change in CD11b expression over untreated for particle-positive neutrophils in whole blood exposed to carboxylated, PEGylated, and sialyl Lewis A-conjugated polystyrene particles. (c) Fold decrease in CD62L expression over untreated for all neutrophils in whole blood exposed to carboxylated, PEGylated, and sialyl Lewis A-conjugated polystyrene particles. (d) Fold decrease in CD62L expression over untreated for particle-positive neutrophils in whole blood exposed to carboxylated, PEGylated, and sialyl Lewis A-conjugated polystyrene particles. (#) indicates significant changes in expression from the untreated samples for each donor; (*) indicates significant differences in expression levels between particle treatment groups ($p<0.05$). Statistical analysis was performed with one-way ANOVA using GraphPad Prism software. $n\geq3$ donors for each treatment, with duplicates for every donor.

For these experiments, we incubated heparinized human whole blood with 2 μm particles of the various surface chemistries for 2 hours, allowing for phagocytosis to occur. Then, we stained the samples for CD11b and CD62L and measured their expression levels via flow cytometry, comparing the expression levels to untreated samples. Notably, we found that neutrophils exposed to all particle types increased their expression of CD11b, by a factor of ~2 for PS-COOH and PS-sLe^A particles and by a factor of ~2.5 for PS-PEG particles (Figure 3.12A). Further, we found that this effect is more pronounced in particle-positive neutrophils, with fold increases of CD11b expression of ~2.5 for PS-COOH and PS-sLe^A and ~3 for PS-PEG (Figure 3.12B). As expected with an increase in CD11b expression, we find that particle internalization results in a reduction in CD62L expression by a factor of ~1.5 for PS-COOH and PS-sLe^A and ~2 for PS-PEG, with similar reductions in expression for particle-positive neutrophils (Figure 3.12-D). These results show that, in general, particle uptake significantly alters neutrophil phenotype, which can result in downstream effects on neutrophil (and, thus, leukocyte) functions. Further, we see that, because neutrophils more-readily phagocytose PS-PEG particles, this induces a greater change in CD11b and CD62L expression, resulting in weaker adhesion kinetics and thus faster neutrophil rolling on the endothelial surface (Figure 3.13).

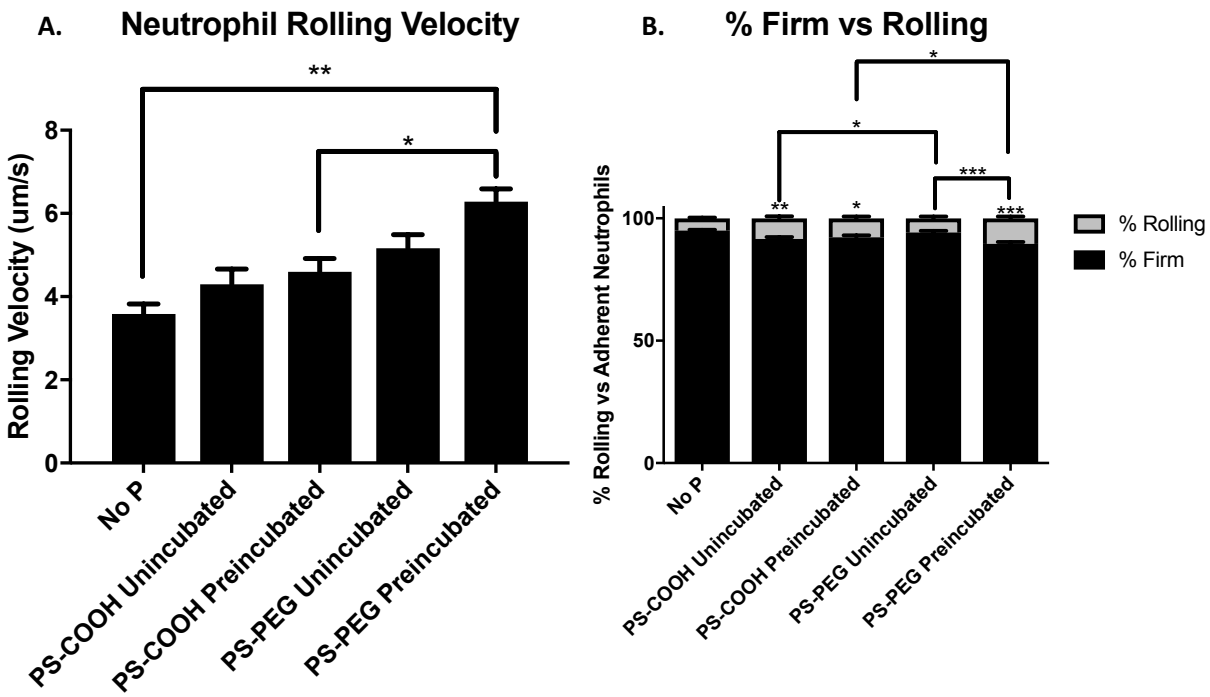


Figure 3.13 - Impact of Particle Internalization on Neutrophil Rolling Velocity and Firm Adherence in Laminar Flow. (a) Neutrophil rolling velocity on an inflamed endothelium with and without particle internalization for 2 μm PS-COOH and PS-PEG particles introduced at a concentration of 1E7/mL, measured by tracking leukocytes through 10-second videos. (b) Percentage of firmly adherent versus rolling neutrophils with and without particle internalization for 2 μm PS-COOH and PS-PEG particles introduced at a concentration of 1E7/mL, as measured by manually counting the number of rolling versus firmly adherent neutrophils in 10-second videos. (*) directly above a bar indicates significant difference from the untreated ("No P") condition, while other significance bars indicate significance between those two conditions ($p<0.05$). Statistical analysis was performed using one-way ANOVA using GraphPad Prism software. $n\geq3$ donors for each condition.

3.5 Discussion

While targeted drug carriers have been widely researched over the past several decades for their potential to protect therapeutic molecules from degradation and deliver drug directly to the site of disease, there is still much we do not understand about their

potential side effects, especially in humans. Additionally, very few of these formulations have successfully passed clinical trials, suggesting a need for more rigorous studies of the potential side effects of injectable particle drug carrier platforms. In this work, we aimed to investigate the impact of model drug carriers on the behavior and function of leukocytes broadly and neutrophils specifically in blood flow, as these impacts have not been thoroughly explored previously.

Importantly, we found that the introduction of both targeted and non-targeted polymeric particles results in significantly reduced leukocyte adhesion to an inflamed endothelium *in vitro* (Figures 3.1-5). This effect was highly dependent on both particle size and particle concentration, with larger particles and higher particle concentrations generally resulting in greater inhibition in leukocyte adhesion. Thus, it is possible that certain particle formulations/dosing schemes may result in a more pronounced impact, informing the design parameters for such formulations. Additionally, we find that, for certain particle sizes and concentrations, there is a significant increase in the reduction of leukocyte adhesion when particles are targeted to the site of inflammation (Figure 3.2), suggesting that both physical collisions and specific competition for binding sites on the endothelium are at play in this effect. Again, this finding reinforces the need to consider whether reducing leukocyte adhesion at the site of inflammation will result in unintended side effects.

Additionally, we found that vessel size is another important factor in the prominence of this effect. Because blood vessel size impacts the size of the CFL and particle margination efficacy[85,164], as well as the magnitude of collision forces in blood flow, it also affects how frequently particles and leukocytes will collide. Thus, we observe that the reduction in leukocyte adhesion is somewhat less prominent in a smaller chamber, particularly for the 2 μm particles, which carries important implications depending on the desired destination of a given particle drug carrier (Figure 3.5).

Given that neutrophils are by far the most abundant blood leukocyte, comprising up to 70% of all circulating white blood cells[13], we focused the remainder of our analysis on neutrophils specifically. To evaluate the impacts of particle drug carriers on neutrophil function in inflammation *in vivo*, we utilized two murine models of inflammation: acute mesentery inflammation and acute lung injury. In these experiments, we found that

intravenously-administered particles rapidly associate with neutrophils in inflammation *in vivo* (Figure 3.8), which results in a significant decrease in neutrophil adhesion in acute mesentery inflammation (Figure 3.9). Importantly, we find that this decrease is linked to the phagocytic clearance of particles by neutrophils, as PEGylated particles (which are not rapidly phagocytosed by murine neutrophils) do not reduce neutrophil adhesion in the acute mesentery inflammation model. These results carry over to a more complex model of inflammation—we find that intravenously-administered particles significantly reduce neutrophil infiltration into the airway in a murine model of acute lung injury (Figure 3.10)

Moving forward, we sought to investigate the phagocytosis-driven effects observed in mice *in vivo* with human blood in our *ex* vivo PPFC experiments. In these studies, we found that, in addition to particle-leukocyte collisions, particle phagocytosis is another major driver of altered leukocyte behavior in human blood (Figures 3.11-13), in agreement with the above work in mice. Based on previous work, and because the polystyrene spheres are not specifically targeted to any ligands on the neutrophil surface, we expect that the particle will rapidly undergo opsonization with plasma proteins, which facilitates interaction with the with FC receptors on the neutrophil surface. The engagement of Fc receptors is known to initiate cell eating.[102,172] We observe that particle internalization results in increased expression of CD11b and shedding of CD62L for neutrophils, and that this effect varies depending on the particle surface chemistry (Figure 3.12). These results are consistent with neutrophil activation. Further, we see that these changes are correlated with downstream changes in neutrophil function, resulting in decreased firm adhesion (Figure 3.11) and increased rolling velocity and proportion of rolling neutrophils versus firmly adherent (Figure 3.13). These results, upon first glance, may seem counterintuitive as reduced CD62L expression and increased CD11b expression are typical of neutrophil activation.[14] However, in the context of the normal inflammation and cell adhesion cascade, neutrophil activation occurs after the initial capture to the endothelium and rolling adhesion step. As L-selectin is a critical molecule for the initial capture, the pre-activation of these cells in circulation via particle internalization will lead to reduced adhesion.[14]

Importantly, we find that particle phagocytosis eliminates significant differences between the impact of targeted and non-targeted particles on leukocyte adhesion,

implying that phagocytosis is the dominating driver of this effect. Thus, if a drug carrier is modified with a coating that greatly reduces phagocytosis by circulating leukocytes, it is possible that this effect (or at least the phagocytosis-driven portion of it) will be sufficiently muted. However, the typical approach for achieving a non-fouling drug carrier surface, i.e., PEGylation, was recently reported to promote phagocytosis in human neutrophils, suggesting other approaches are needed.[119] Here, the use of zwitterionic coatings[120–122] or leukocyte mimetic surfaces[123,125] may be the key to reducing phagocytosis of particle drug carriers by circulating leukocytes in human blood and avoiding the reduction in leukocyte adhesion observed in this work.

Additionally, other strategies may be employed to alter particle margination, the magnitude of collision forces, particle phagocytosis, or some combination of these. It has been reported that changing particle shape can alter both particle margination dynamics[92] and phagocytosis[108,110], both of which would likely alter the magnitude of the reduction in leukocyte adhesion observed here. Further, manipulating the stiffness of particles can impact margination dynamics, collision forces, and particle phagocytosis[139,173,174], with softer particles resulting in lower collision forces and potentially decreased phagocytosis by circulating leukocytes. Thus, a thorough exploration of how these particle properties influence the reduction in leukocyte adhesion observed here could reveal strategies for mitigating this effect on the efficacy of drug delivery systems.

The results presented in this study have a wide range of implications for the field of particle drug delivery broadly, across a wide range of applications. First and foremost, the surface blocking mechanism of leukocyte adhesion reduction only applies to applications where particle drug carriers are targeted to inflammatory molecules (or potentially other surface molecules) on the vascular wall, and may not be a concern for passive targeting schemes or otherwise non-targeted particles. These effects may be undesirable in certain contexts (e.g., by preventing leukocytes from adhering/transmigrating to destroy a pathogen), but desirable in others (e.g., preventing an overactive immune response such as those seen in certain autoimmune diseases which result in downstream thrombus formation). Of course, the non-specific collision and phagocytosis mechanisms apply to all contexts, and again could be beneficial or

detrimental depending on the disease context. For example, one of the most prominent and widely-studied applications for targeted drug delivery is for the delivery of cancer therapeutics. Given that one of the major obstacles to cancer treatment is the immunosuppressive tumor microenvironment, preventing the innate immune system from recognizing and destroying cancer cells, the effects observed in these studies may result in decreased efficacy for particle drug carriers for cancer therapy.[175] On the other hand, tumor-associated neutrophils have recently emerged as a potential therapeutic target as neutrophils have been shown to promote tumorigenesis and metastasis.[176] Thus, the exact impact of these effects on drug carriers for cancer therapies is at question, and should be evaluated by groups involved in designing drug carriers for cancer applications.

It is important to highlight a few limitations to the work presented in this manuscript. First, all of the experiments described in this study utilized polystyrene spheres as model particulate drug carriers. While these are an excellent and convenient model for evaluating drug carrier properties, they do not necessarily always directly predict the behavior of actual drug carriers made from biocompatible materials, particularly because the efficiency and effect of neutrophil phagocytosis can vary greatly based on particle material.[177] Thus, employing particle drug carriers comprised of such materials (such as PLGA), may not result in the same effects seen here. Furthermore, while this study focuses specifically on the impact of leukocyte-particle interactions on neutrophil adhesion, we recognize that neutrophils also exercise other important functions in inflammation, including oxidative burst, degranulation, and NETosis. Thus, future work focusing on these other aspects of neutrophil behavior in inflammation may be necessary to shed more light on the totality of the impact of particle drug carriers on neutrophil function in inflammation. In particular, NETosis is an interesting phenomenon which often contributes to the damage caused by neutrophils in inflammatory diseases.[163,178] Typically, NETs are released after neutrophils transmigrate through the endothelium to destroy the cause of inflammation.[38,179] Thus, we expect that the prevention of neutrophil adhesion would reduce NETosis at the site of acute inflammation. However, there is evidence that in some chronic inflammatory conditions, e.g., autoimmune conditions, neutrophils are ‘preactivated’ and may release NETs into circulation.[73,74,180] Thus, further exploration into the impact on intravenously-

administered particle drug carriers on activated neutrophils and NETosis in circulation is warranted, though outside the scope of this paper.

3.6 Conclusions

Overall, this work demonstrates for the first time that polymeric drug carriers may have unintended effects on the ability of leukocytes to respond to an inflammatory event. Because drug carriers and leukocytes will necessarily be occupying the same physical space near and on the vascular wall, particularly in the case of vascular-targeted carriers which are designed to bind to inflammatory molecules, leukocytes and drug carriers (especially neutrophils) will interact with one another intimately in blood flow, potentially hindering the ability of leukocytes to bind to the endothelium. This could have unintended consequences for patients being treated with a drug carrier formulation, and might contribute to the general failure of drug carrier formulations advancing past clinical trials and onto the market. Additionally, we find that particle uptake dramatically inhibits the ability of human neutrophils to bind to an inflamed endothelium, in concurrence with our previous work.[157] However, that result suggests that this effect can perhaps be mitigated by sufficiently protecting drug carriers from phagocytosis, which is beneficial for drug carrier efficacy already. Additionally, these results suggest that there may be opportunities to effectively target circulating leukocytes simply by designing particles which will accumulate near the vascular wall, perhaps enhancing any such therapeutic strategy. In the grand scheme, this study provides crucial information as to how drug carriers interact with leukocytes in blood flow, and will help inform the design of particle drug carriers in the future.

Chapter 4: PEGylation of Model Drug Carriers Enhances Phagocytosis by Primary Human Neutrophils

4.1 Publication Information

The work in this chapter is largely published as:

William J. Kelley, Catherine A. Fromen, Genesis Lopez-Cazares, and Omolola Eniola-Adefeso. “PEGylation of Model Drug Carriers Enhances Phagocytosis by Primary Human Neutrophils.” *Acta Biomaterialia.* September 6, 2018.

4.2 Abstract

Targeted drug carriers are attractive for the delivery of therapeutics directly to the site of a disease, reducing systemic side effects and enhancing the efficacy of therapeutic molecules. However, the use of particulate carriers for drug delivery comes with its own set of challenges and barriers. Among these, a great deal of research effort has focused on protecting carriers from clearance by phagocytes via altering carrier surface chemistry, mostly with the use of polyethylene glycol (PEG) chain coatings. However, few papers have explored the effects of PEGylation on uptake by freshly obtained primary human phagocytes in physiological conditions. In this work, we investigate the effect of PEGylation on particle uptake by primary human neutrophils *in vitro* and compare these effects to several cell lines and other model phagocytic cells systems. We find that human neutrophils in whole blood preferentially phagocytose PEGylated particles (e.g., ~40% particle positive neutrophils for PEGylated versus ~20% for carboxylated polystyrene microspheres) and that this effect is linked to factors present in human plasma. Model phagocytes internalized PEGylated particles less efficiently or equivalently to carboxylated particles in culture medium but preferentially phagocytosed PEGylated particles in the human plasma (e.g., ~86% versus ~63% PEGylated versus carboxylated

particle positive cells, respectively). These findings have significant implications for the efficacy of PEGylation in designing long-circulating drug carriers, as well as the need for thorough characterization of drug carrier platforms in a wide array of *in vitro* and *in vivo* assays.

4.3 Introduction

Particulate drug delivery systems remain a relevant platform to improve the delivery of a variety of therapeutics for various diseases. The advantages of particulate drug carriers are numerous,[181] yet only a handful of such formulations have gained FDA approval.[182] Much of the perceived barriers to success include the inability to achieve targeted, localized delivery due to rapid clearance by professional phagocytes. Specifically, serum proteins rapidly adsorb onto the surface of particulate drug carriers as they enter the bloodstream in a process referred to as opsonization. These proteins can include any serum proteins but are most commonly complement factors and immunoglobulins.[99] This opsonin adsorption process quickly marks the particles as foreign, where receptors on phagocytes (monocytes, macrophages, and neutrophils) bind to the opsonins to initiate the process of phagocytosis or cellular eating.[183] Once internalized by cells, the particles are removed from the bloodstream, preventing them from achieving the goal of targeted drug delivery.[99] As a result, numerous studies have examined the effects of particle size,[104,110] shape,[90,108,109] and surface chemistry[106,155,184] on the phagocytic uptake of particulate carriers in an attempt to limit clearance via phagocytosis.

A typical approach to mitigating opsonization, and thus phagocytosis, involves the modification of particle surfaces with non-fouling, biocompatible polymers, which reduce opsin protein adsorption. Hydrophilic, high molecular weight polyethylene glycol (PEG) has been widely studied in this regard. Indeed, coupling PEG to the surface of a variety of particle drug carriers at high density has been shown to reduce macrophage uptake.[106,185] Due to this effect, many studies have demonstrated the long-term circulation of PEGylated particles in mice.[113,115] However, to date, no PEGylated polymeric formulations have been approved for clinical use, though there is a handful currently in clinical trials.[145] While some PEGylated particle formulations have gained

FDA approval by demonstrating increased circulation half-life,[3] these formulations were liposomes rather than polymeric particles. The lack of a widespread clinical translation of PEGylated polymeric carriers could be due to the bulk of the evidence regarding the success of these having primarily been gathered *via* immortalized cell lines with non-physiological media conditions and rodent *in vivo* models.[3,114,115,145] Only a few preclinical studies have explored particle uptake of PEGylated particles in freshly-obtained blood with primary human blood cells,[114,186] which is crucial to understanding the impact of PEGylation on particle phagocytosis *in vivo* in humans. For one, the exact cellular composition and serum proteins present can dramatically change the effectiveness of PEG in preventing phagocytosis, which the human blood environment is not recapitulated with mouse models and immortalized cell line assays. Of the limited studies using primary human blood cells, PEG modification has delivered mixed results. One study suggests that the reduction in uptake due to PEGylation for primary blood cells is heavily dependent on PEG grafting density, with the requirement that the Flory radius (R_F) is at least twice as large as the distance between PEG grafts[114]. Other studies have reported no reduction in uptake from PEGylation compared to other surface coatings such as pluronic and chitosan in primary human monocytes in culture medium (RPMI) with fetal bovine serum (FBS).[186] More importantly, the bulk of the prior studies investigating primary cell phagocytosis focus solely on phagocytosis by macrophages and ignore phagocytic contributions from neutrophils. While macrophages are critical professional phagocytes, neutrophils account for approximately 50-70% of cells in the human bloodstream[187]; and therefore are the first cells to be encountered by intravenously administered particulate drug carriers. Indeed, our recent work has demonstrated that these cells are significant contributors to the particle clearance process *in vivo*[157], which follows logically given their known role in pathogen removal. Furthermore, recent studies suggest that neutrophils may be activated and undergo degranulation in the presence of PEGylated surfaces.[188–190] Thus, it is crucial to investigate the impact of PEGylation on drug carrier phagocytosis by primary human neutrophils, as this remains a fundamental question in the literature.

In this study, we evaluate the impact of PEGylation on phagocytosis by primary human neutrophils across a range of PEG grafting densities and particle sizes (2 μm, 500

nm, and 200 nm) and compare obtained results to the uptake by model phagocytes. The 2 μm particles are included as micron-scale particles have been shown to exhibit better margination and vascular wall adhesion *in vitro* and *in vivo* [85,144], and a number of published drug carrier platforms feature particles sized between 100 and 500 nm (see [1,191–193]). We report a striking difference between the effect of PEGylation on phagocytosis by primary human neutrophils versus cultured phagocytic cells. In particular, we observe that the addition of PEG increases particle uptake specifically by primary neutrophils in whole blood and human plasma contrary to the observation with THP1 monocytes, mouse blood cells, and mouse bone marrow-derived macrophages. Furthermore, we note that the effect of PEGylation depends heavily on the cellular species of origin, as mouse neutrophils behave different from human neutrophils, and that factors in human plasma are at least partially responsible for this difference. These results have implications for researchers interested in designing PEGylated nanoparticles for drug delivery, highlighting the need for thorough characterization of particle coatings on uptake by phagocytic cells.

4.4 Results

Uptake of PEGylated Particles by Primary Human Cells

To determine how PEGylation affects the uptake of particulate drug carriers by primary human blood cells, we incubated model polystyrene drug carriers with whole blood samples taken from healthy donors in heparinized whole blood. In these studies, we compared the uptake of 2 μm carboxylated polystyrene particles with PEGylated particles of the same size. Particles were determined to have PEG grafted in the “dense brush” conformation (Table 4.1). [114]

Table 4.1—Characterization of PEG Density

Particle Size	PEG Concentration (mg/mL)	F Reading	F reading-background	Rhodamine Conc (mg/mL)	Rhodamine-PEG Molecules in well	PEG Density (#/um^2)	Area/Peg Chain (um^2)	Distance between Peg Grafts (D, um)	D, nm	Rf/D
2 µm	30	5.25	4.75	1.36E-04	1.50E+12	1.10E+06	9.09E-07	1.08E-03	1.08	5.54
	30	6.20	5.71	1.64E-04	1.80E+12	1.32E+06	7.56E-07	9.81E-04	0.98	6.07
	20	1.98	1.49	4.26E-05	4.68E+11	3.44E+05	2.90E-06	1.92E-03	1.92	3.10
	20	2.77	2.27	6.50E-05	7.14E+11	5.25E+05	1.90E-06	1.56E-03	1.56	3.83
	10	1.63	1.13	3.24E-05	3.56E+11	2.62E+05	3.82E-06	2.21E-03	2.21	2.70
	1	1.06	0.56	1.60E-05	1.76E+11	1.30E+05	7.70E-06	3.13E-03	3.13	1.90

Particle Size	PEG Concentration (mg/mL)	F Reading	F reading-background	Rhodamine Conc (mg/mL)	Rhodamine-PEG Molecules in well	PEG Density (#/um^2)	Area/Peg Chain (um^2)	Distance between Peg Grafts (D, um)	D, nm	Rf/D
500 nm	30	5.09	4.55	2.36E-04	2.60E+12	1.65E+05	6.04E-06	2.77E-03	2.77	2.15
	20	4.38	3.85	2.00E-04	2.20E+12	1.40E+05	7.16E-06	3.02E-03	3.02	1.97
	10	3.58	3.04	1.58E-04	1.74E+12	1.11E+05	9.05E-06	3.39E-03	3.39	1.76
	5	5.68	5.14	2.67E-04	2.93E+12	1.87E+05	5.35E-06	2.61E-03	2.61	2.28
	1	4.86	4.33	2.25E-04	2.47E+12	1.57E+05	6.36E-06	2.85E-03	2.85	2.09
	0.1	2.37	1.84	9.54E-05	1.05E+12	6.67E+04	1.50E-05	4.37E-03	4.37	1.36

Particle Size	PEG Concentration (mg/mL)	F Reading	F reading-background	Rhodamine Conc (mg/mL)	Rhodamine-PEG Molecules in well	PEG Density (#/um^2)	Area/Peg Chain (um^2)	Distance between Peg Grafts (D, um)	D, nm	Rf/D
200 nm	30	7.50	7.02	3.72E-05	4.08E+11	1.63E+05	6.15E-06	2.80E-03	2.80	2.13
	20	2.91	2.43	1.28E-05	1.41E+11	5.62E+04	1.78E-05	4.76E-03	4.76	1.25
	10	2.05	1.56	8.27E-06	9.09E+10	3.62E+04	2.76E-05	5.93E-03	5.93	1.00
	5	2.08	1.60	8.47E-06	9.30E+10	3.70E+04	2.70E-05	5.86E-03	5.86	1.02
	1	2.14	1.66	8.79E-06	9.66E+10	3.84E+04	2.60E-05	5.76E-03	5.76	1.04
	0.1	3.11	2.62	1.39E-05	1.53E+11	6.08E+04	1.65E-05	4.58E-03	4.58	1.30

Additionally, these studies were carried out across 16 donors, utilizing blood from both male and female donors (10 male, 6 female). Individual donor experiments for neutrophils are shown in Figure 4.1A. Except for Donor 1, there was a significant increase in the uptake of PEGylated particle by neutrophils over the carboxylated particles for all blood samples, Additionally, Fig 4.1B illustrates the impact of PEG on particle uptake by neutrophils over time, showing that PEGylated and carboxylated particles were initially taken up at a similar rate, but PEGylated particle uptake was eventually higher at longer timescales. No significant differences were observed between male and female donors.

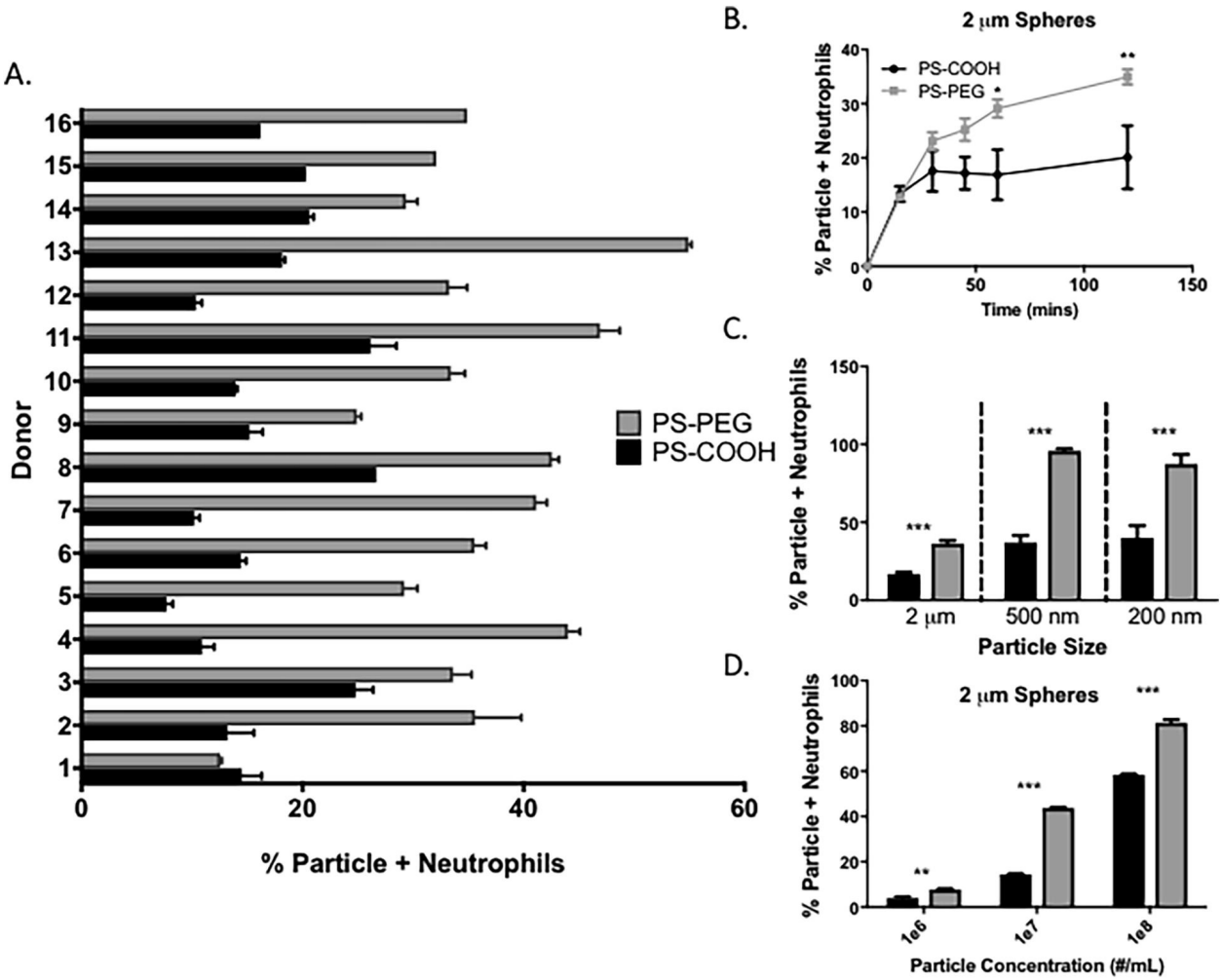


Figure 4.1 - Uptake of carboxylated and PEGylated 2 μm polystyrene particles by primary human neutrophils, as determined by flow cytometry. (a) Uptake by neutrophils from individual human donors. (b) Neutrophil uptake of 2 μm carboxylated and PEGylated polystyrene particles. (c) Aggregated neutrophil particle uptake for 2 μm, 500 nm, and 200 nm carboxylated and PEGylated polystyrene particles. (d) Neutrophil uptake of 2 μm carboxylated and PEGylated polystyrene particles at varying concentrations.

We observe that a brush PEG formation on the particle surface increases uptake by human neutrophils across a range of particle properties. Figure 4.1C demonstrates that this effect held true across a variety of particle sizes, and in fact, the impact of a brush PEG formation on particle uptake was more extensive for smaller particles. An average of ~37% of neutrophils internalized 2 μm PEGylated particles versus ~17% for carboxylated particles (aggregated results for all donors in Figure 1A). Meanwhile, ~98%

of human neutrophils internalized PEGylated 500 nm particles versus ~38% for carboxylated 500 nm particles, and ~90% of human neutrophils internalized PEGylated 200 nm particles versus ~40% for carboxylated. The increased uptake of PEGylated particles was maintained across a range of particle concentrations from 1E6/mL to 1E8/mL (Figure 4.1D). Notably, this effect was not observed in primary human monocytes, which demonstrated no difference in uptake between PEGylated and carboxylated polystyrene particles (Figure 4.2)

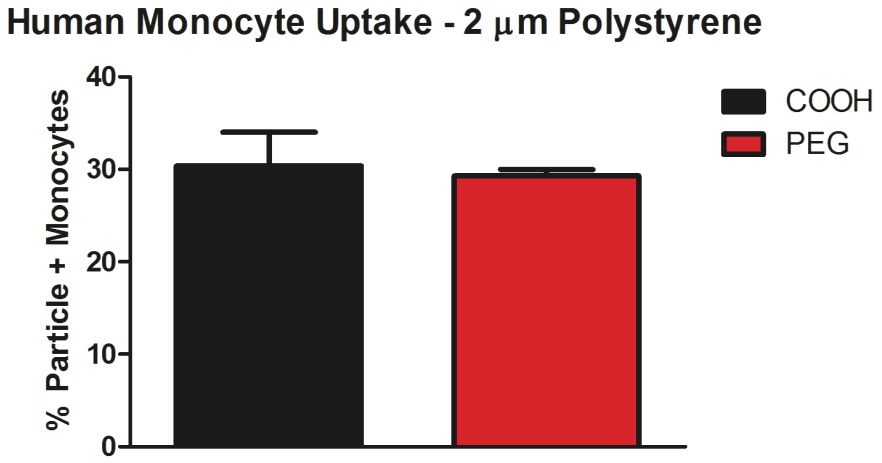


Figure 4.2 – Uptake of Carboxylated and PEGylated Particles by primary Human Monocytes.

Because PEG surface density is known to impact particle *in vivo* circulation time,[111,194] we sought to explore whether neutrophils' preferential phagocytosis of PEGylated particles remained consistent across an array of PEG grafting densities. Thus, we reacted 2 μm, 500 nm, and 200 nm polystyrene particles with a range of PEG concentrations, resulting in a range of PEG densities (characterized by R_F/D; the ratio of the Flory radius of the PEG polymer to the distance between PEG chains – a higher R_F/D value indicates a denser PEG grafting). These particles were then incubated in whole blood at concentrations matching those in above studies.

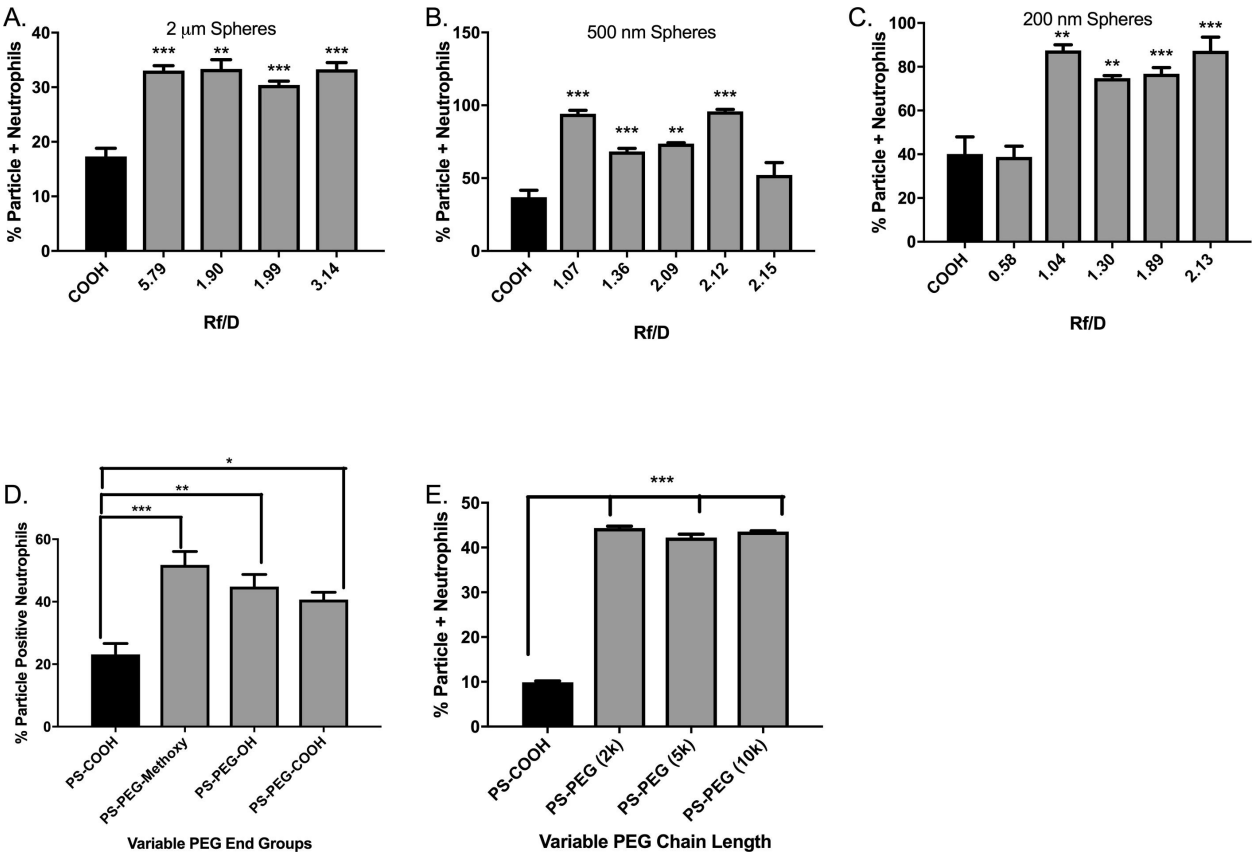


Figure 4.3 - Characterization of the Impact of PEG Density and Chain Length on PEGylated Particle Uptake in Whole Blood. Uptake of (a) 2 μm, (b) 500 nm, and (c) 200 nm carboxylated and PEGylated polystyrene particles of varying PEG densities. (d) Uptake of 2 μm PEGylated polystyrene particles with varying PEG chain endgroups. (e) Uptake of 2 μm PEGylated polystyrene particles with varying PEG chain lengths.

For 2 μm particles, all PEG densities from “dense brush” to “loose brush” were associated with a significant increase in particle uptake (Figure 4.3A). For 500 and 200 nm particles, all PEG densities except one were associated with a significant increase in particle uptake (Figure 4.3B-C). For the R_F/D of 2.15 for 500 nm particles, we observe a non-significant increase in PEGylated particle uptake relative to the carboxylated particles. For the R_F/D of 0.58 for the 200 nm particles, we see the same level of particle uptake between carboxylated and PEGylated particles. These results suggest that, across a range of particle sizes and PEG grafting densities, human neutrophils largely preferentially uptake PEGylated particles compared to carboxylated counterparts. Interestingly, for the 200 nm particles at the lowest PEG density, it appears that the uptake behavior approaches that of carboxylated particles, which may imply that there is a threshold amount of PEG, above which neutrophils will preferentially phagocytose

pegylated particles. The exception to this rule seems to be with 500 nm particles at the highest PEG density ($R_F/D = 2.15$). However, it is difficult to extrapolate based on this data point whether the nonsignificant increase in uptake for that particle type implies a change in behavior at extremely high PEG densities, although this was the highest PEGylation density we were able to achieve with our coupling chemistry for the 500 nm particles. Notably, 2 μm particles with the $R_F/D>2.15$ still exhibit increased uptake by primary neutrophils.

Because surface chemistry can dictate protein adsorption profiles and particle interactions with cellular membranes, it is possible that the PEG end group could play a role in modulating neutrophil phagocytosis. Thus, we examined the neutrophil uptake of PEGylated particles with methoxy, hydroxyl, and carboxyl end groups (Figure 4.3D). In agreement with the above experiments, neutrophils preferentially took up PEGylated particles, regardless of which end group was present. Additionally, we sought to explore the impact of PEG chain length on this phenomenon, as other groups have shown that PEG chain length can impact the performance of PEGylated particles *in vivo*.[112] As shown in Figure 4.3E, altering the PEG chain length between 2000 kDa and 10000 kDa, at R_F/D values approximately equal to those used for the 5000 kDa, had no impact on the increased uptake observed for the 2 μm particles with human neutrophils, i.e., all chain lengths resulted in an increased uptake over carboxylated surfaces.

Uptake of PEGylated Particles by Other Phagocytic Cells

The results in Figures 4.1 and 4.3 were initially quite surprising, as we had hypothesized that PEGylation would reduce particle uptake. Therefore, we next examined the uptake of PEGylated particles in a variety of other cell types frequently used for characterizing particle uptake, with the hypothesis that these studies should mostly match those presented in prior publications (that is, PEGylation would reduce particle uptake). For these studies, we used the same particle sizes and concentrations as used above for neutrophils. We incubated carboxylated and PEGylated particles with mouse whole blood and with THP1 monocytes and mouse bone marrow-derived macrophages (BMM; from both BALB/c and C57BL/6 mice) in RPMI with 10% FBS. For all the experimental conditions, PEGylation either reduced uptake by model phagocytes or showed no

significant difference from carboxylated particles, except for C57BL/6 mouse neutrophils taking up PEGylated 200 nm particles more than their carboxylated counterparts (Figure 4.4). Overall, these experiments matched prior reporting that PEGylation reduces particle uptake.

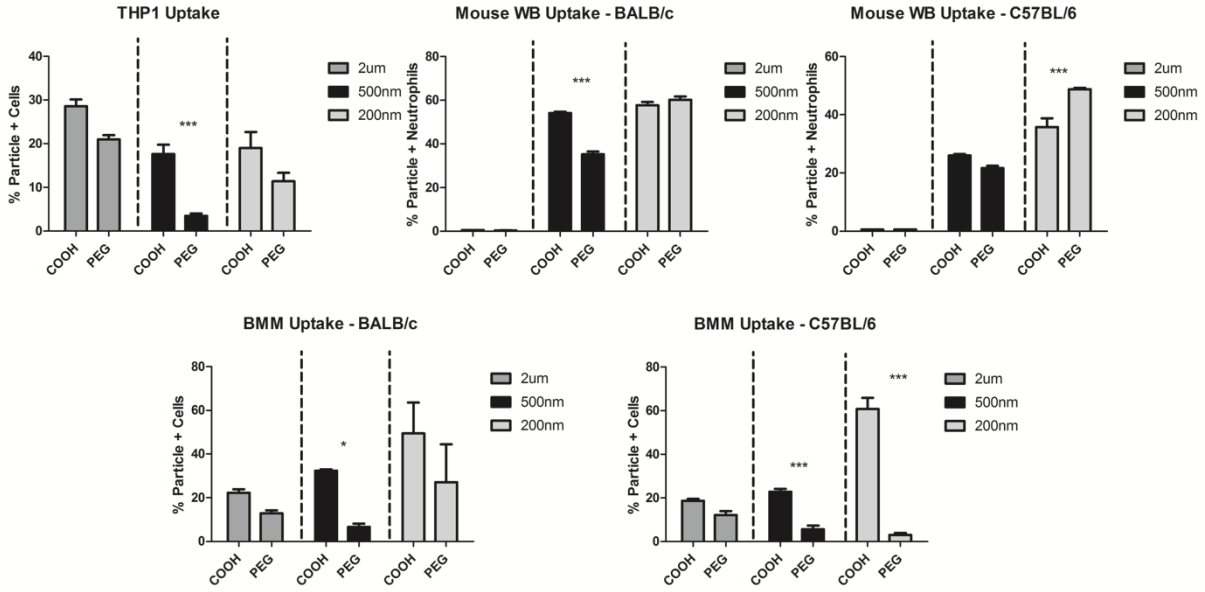


Figure 4.4 - Uptake of carboxylated and PEGylated polystyrene particles by a variety of cell types, as measured by flow cytometry. (a) Uptake by cultured THP1 monocytes. (b) Uptake by primary mouse (BALB/c) neutrophils in whole blood. (c) Uptake by primary mouse (C57BL/6) neutrophils in whole blood. (d) Uptake by bone marrow-derived macrophages isolated from BALB/c mice. (e) Uptake by bone marrow-derived macrophages isolated from C57BL/6 mice.

Given that one reason neutrophils are less widely-studied for phagocytosis is that there are few model neutrophil cell lines with which to work, we wanted to explore the uptake of PEGylated particles by one of the models that exist: HL-60 derived neutrophils. The HL-60 is a human promyelocytic leukemia cell line that is capable of differentiation to a neutrophil-like phenotype in the presence of DMSO or retinoic acid.[195] We hypothesized that HL-60 derived neutrophils in the presence of human plasma should behave similarly to primary human neutrophils with respect to PEGylated particle uptake.

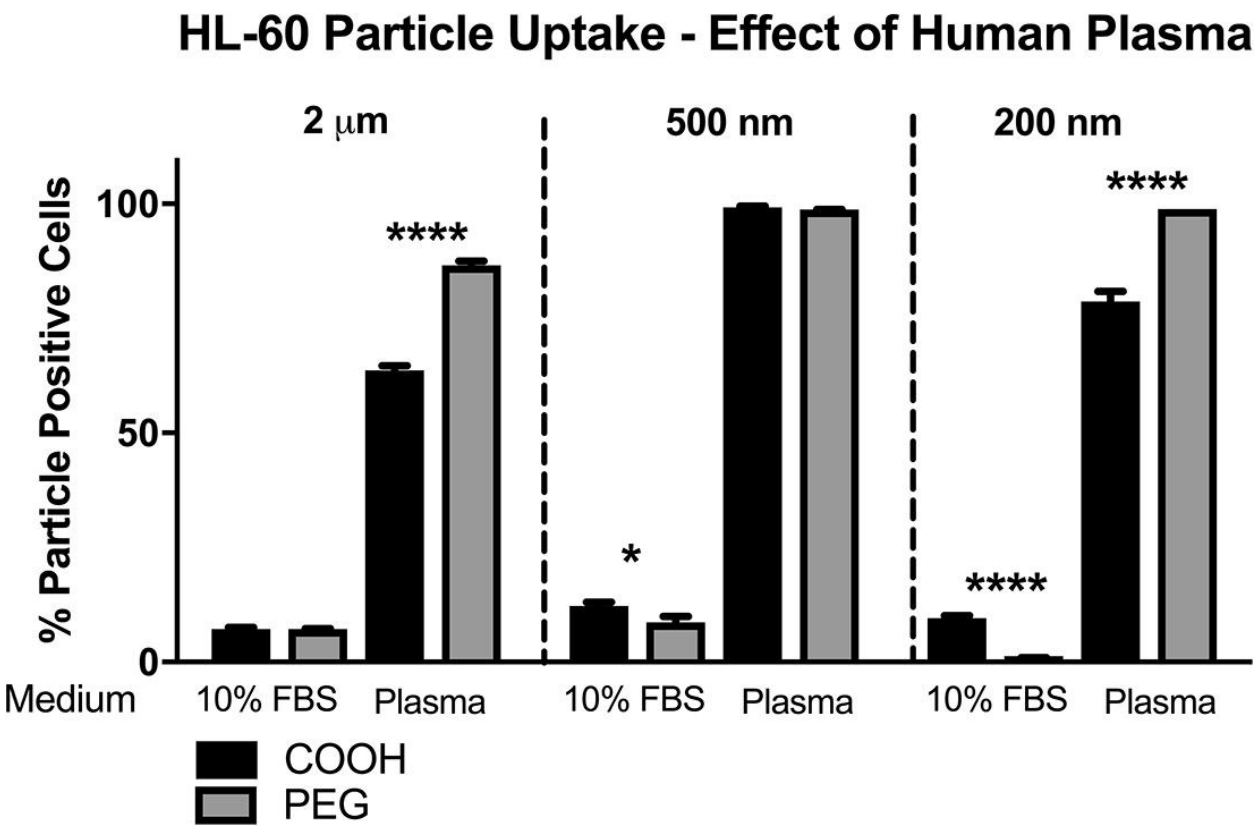


Figure 4.5 - Uptake of 2 μm, 500 nm, and 200 nm carboxylated and PEGylated polystyrene particles by differentiated HL-60 derived neutrophils in the presence of human plasma.

The HL-60 derived neutrophils were incubated with particles as above for primary neutrophils in either culture medium (RPMI + 10% FBS) or human plasma (Figure 4.5). Interestingly, HL-60 derived neutrophils seemed to preferentially take up carboxylated particles in the culture medium, in line with similar phagocytic studies of other secondary cells. However, in the presence of human plasma, HL-60 derived neutrophils behaved similarly to human neutrophils, with a significant increase in particle-positive cells for 2 μm and 200nm PEGylated particles versus their carboxylated counterparts. For 500 nm particles, both particle types resulted in approximately 100% particle uptake, suggesting that the combination of particle size and concentration dominated any effect PEG might have had on the uptake. These results, combined with the human whole blood uptake studies in Figures 4.1 and 4.3 strongly suggest that factors in human plasma drive an increased uptake of PEGylated particles by human neutrophils.

Impact of Human Plasma on PEGylated Particle Uptake

To further explore a potential human plasma effect as observed with the HL-60 cells, we incubated carboxylated and PEGylated particles with human plasma before incubation with BMMs from both BALB/c and C57BL/6 mice. The results of these studies are shown in Figure 4.6A and Figure 4.6B, respectively. For both mouse strains, the incubation of particles with human plasma prior to addition to cells increased PEGylated particle uptake. Specifically, in RPMI with 10% FBS, PEGylated particles were phagocytosed by approximately 20-40% fewer macrophages, while PEGylated particles were phagocytosed at the same level as carboxylated particles with the addition of human plasma. Additionally, we cultured THP1 monocytes in human plasma and evaluated the uptake of particles soaked in human plasma (Figure 4.6C). Similar to the BMMs, we saw an increase in uptake of PEGylated particles by THP1 monocytes, eliminating any significant differences between carboxylated and PEGylated particles. These cell-line results suggest that factors in human plasma help to drive PEG particle uptake, resulting in a mismatch between many cultured cell uptake assays and what can potentially occur in human *in vivo*.

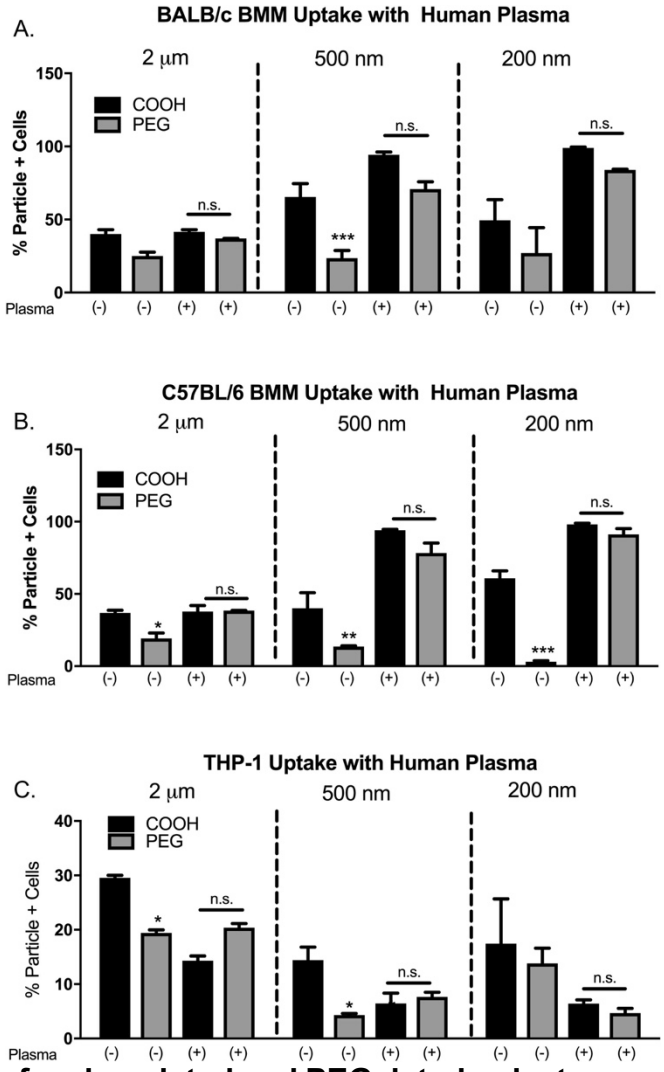
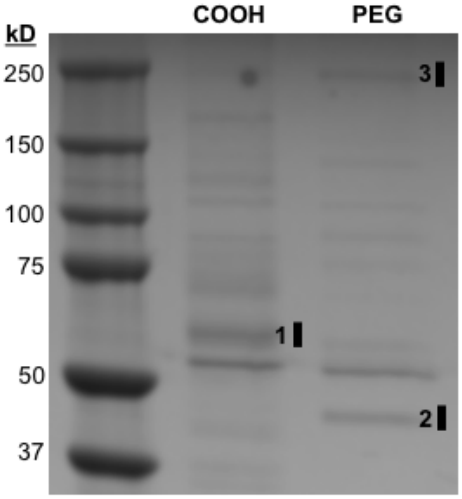


Figure 4.6 - Uptake of carboxylated and PEGylated polystyrene particles by various cell types in presence of human plasma. Uptake by (a) bone marrow-derived macrophages from BALB/c mice of particles soaked in human plasma; (b) bone marrow-derived macrophages from C57BL/6 mice of particles soaked in human plasma; and (c) THP1 monocytes cultured in human plasma; particles soaked in human plasma.

Given the appearance of a significant role played by human plasma in the increased uptake of PEGylated particles, we investigated potential differences in plasma proteins acquired by carboxylated and PEGylated particles. Accordingly, an SDS-PAGE analysis demonstrated that PEG was successful in reducing the total protein on the particle surface, repelling many of the low molecular weight plasma proteins (Figure 4.6). However, PEGylated particles demonstrated a clear band in the ~50 kDa and the ~250 kDa range that was not present for carboxylated particles. The presence of these bands indicates that there may be specific proteins in plasma that preferentially bind to PEG

particles and confer the preferential uptake of these particles. The higher molecular weight band corresponds to immunoglobulin (Ig) proteins.



Band #	Particle Type	Potential Proteins of Interest
1	COOH	Serum Albumin Complement Component (C9)
2	PEG	Apolipoprotein A-IV Properdin
3	PEG	Fibrinogen (α, β, γ chains) Apolipoprotein A-IV Complement Component (C9) Complement Component (C3) Gelsolin Alpha-2 macroglobulin

Figure 4.7 - SDS PAGE analysis of proteins adsorbed to carboxylated vs PEGylated 2 μm polystyrene particles and proteins of interest found in bands exclusive to each particle.

However, neutrophil uptake assays performed in Ig-depleted plasma still demonstrated increased uptake of PEGylated particles (Figure 4.8), suggesting that there may be other specific plasma proteins responsible for the PEGylation induced uptake effect. Additionally, inhibitor studies (using chlorpromazine to inhibit clathrin-mediated phagocytosis, sodium azide and 2-deoxyglucose to inhibit ATP-driven phagocytosis, and lovastatin to inhibit FC receptor-mediated phagocytosis) performed to probe the driving mechanism of PEGylated particle uptake by human neutrophils were inconclusive (Figure 4.8), but did point toward an ATP-driven process at play. Further, to explore whether PEG antibodies could be responsible for the increased PEGylated particle uptake, we spiked whole blood samples with free PEG before introducing particles (Figure 4.8). However, we did not observe any noticeable antibody-blocking effect, suggesting that PEG antibodies not be a primary driver of the observed increase in particle uptake.

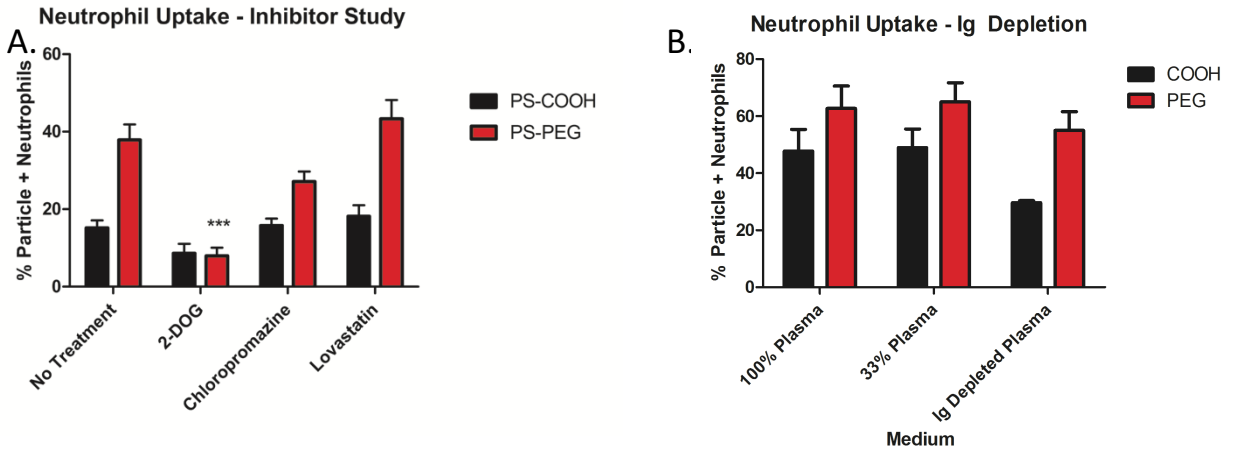


Figure 4.8 – Neutrophil particle uptake in the presence of phagocytosis inhibitors and in Ig-depleted plasma (a) Inhibitor studies to explore the mechanism for PEGylated particle uptake. Whole blood samples were treated with 0.05mM 2-deoxyglucose (2-DOG, an inhibitor of ATP synthesis), 10 ug/mL of chlorpromazine (an inhibitor of clathrin-mediated phagocytosis), or 10 μM of lovastatin (an inhibitor of Fc-mediated phagocytosis) for 1 hour prior to particle incubation. * = significant relative to untreated control. (b) Ig depletion studies to probe the impact of immunoglobulins on PEGylated particle uptake by neutrophils. Neutrophils were isolated from whole blood using a FICOLL separation and dextran separation to remove RBCs. Plasma was depleted of immunoglobulins using a Protein A Antibody Purification Kit (Sigma Aldrich) according to the product instructions. Neutrophils were then resuspended in 100% plasma, 33% plasma (diluted with PBS -/-), or the 33% Ig-depleted plasma generated by the column. Neutrophils were then incubated with 2 μm polystyrene-COOH or polystyrene-PEG particles for 2 hours, as in previous studies. No significant reduction was observed for PEGylated particles across plasma types.

Probing the Role of Complement and Serum Albumin in PEGylated Particle Uptake

Another possibility suggested by the SDS-PAGE analysis is that complement proteins and/or serum albumin might be responsible for the difference in uptake between carboxylated and PEGylated particles. Complement proteins were present in the bands that appeared in the corona for PEGylated particles but not carboxylated particles, and their role in particle phagocytosis has been well-documented.[101,154,196] Conversely,

human albumin was heavily present on the carboxylated particles, relative to PEGylated particles, and could be driving decreased particle uptake. Thus, we examined the uptake of carboxylated and PEGylated 2 μm polystyrene particles by isolated human neutrophils in RPMI with 10% FBS, RPMI with complete human complement sera (9.67 CH50 units/mL), RPMI with human complement C3 (119 μg/mL), and RPMI with human serum albumin (HSA, 35 g/L). We also examined particles that were incubated for 1 hour in serum albumin before introduction to the neutrophils. Complement C3 was chosen for its known role in phagocytosis of foreign bodies by neutrophils and its presence in the SDS-PAGE analysis.[197]

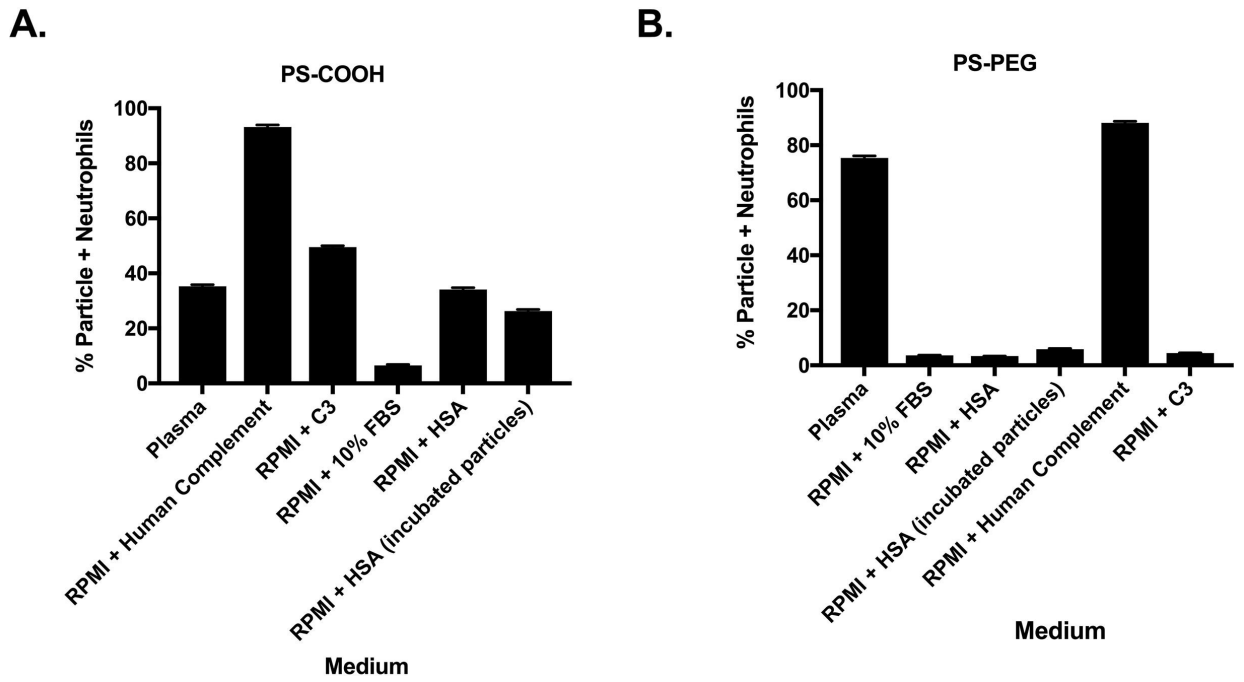


Figure 4.9 – Impact of Human Complement on the Uptake of Particles by Human Neutrophils_Uptake of (a) carboxylated and (b) PEGylated polystyrene particles by primary human neutrophils in plasma, RPMI + 10% FBS, RPMI + human complement sera, RPMI + human complement C3, RPMI + human serum albumin, and RPMI + human serum albumin with particles soaked in human serum albumin.

As shown in Figure 4.9a, when carboxylated particles were exposed to human complement only, they were phagocytosed much more readily by neutrophils than in plasma (~93% for complement-exposed particles, versus ~35% for particles in plasma).

Additionally, carboxylated particles exhibited an increase in uptake when exposed to C3 alone (~49%). Notably, carboxylated particles exposed to HSA only exhibited the same level of uptake as carboxylated particles in whole plasma and decreased uptake (~26%) when presoaked in HSA for 1 hr before adding to cells.

Similar to their carboxylated counterparts, when PEGylated particles were only exposed to HSA, i.e., no other protein in their corona, they exhibit drastically decreased uptake compared to the level seen in plasma (Figure 4.9b; ~3% in RPMI + HSA versus ~75% in plasma). Conversely, when PEGylated particles were exposed to complete human complement, they exhibit a slightly increased uptake (~88%) relative to whole plasma. However, PEGylated particles exhibit decreased uptake in C3 alone (~4%). Taken together, these results, along with the SDS-PAGE gel result, suggest that there is indeed an important role played by human complement in particle uptake by human neutrophils, and, more precisely, the increased affinity for complement by PEGylated particles is responsible for their increased uptake by human neutrophils relative to non-PEGylated particles, which may be mitigated by albumin.

4.5 Discussion

PEGylation is often employed as a strategy to reduce phagocytosis and clearance of particle drug carriers by circulating blood cells (i.e., neutrophils and monocytes), extending the circulation time of such particles. This strategy has been explored by numerous research groups in a variety of *in vitro* and *in vivo* assays,[111,112,114,115,184,185] demonstrating the potential of PEGylation to extend the circulation time of particles in the bloodstream. However, most of these studies are performed in artificial systems in the absence of human plasma, in model phagocytes such as cultured monocytes and macrophages (which are tissue-resident, rather than circulating), or in rodent models *in vivo*. Additionally, the phagocytic contributions of neutrophils are frequently overlooked due to the lack of model neutrophils systems and their difficulty in handling; yet, neutrophils typically make up 60% or more of leukocytes in human blood [198], making them the first WBCs to be encountered by IV-delivered drug carriers.

In this work, we thoroughly characterize the uptake by human neutrophils of model particle drug carriers with and without PEG coatings. Surprisingly, we find that PEG dramatically increases the uptake of particles by primary human neutrophils, regardless of particle size, particle material, PEG density, or end group (Figures 4.1 and 4.3). Notably, we do not observe a similar phenomenon in primary monocytes (Figure 4.2). Instead, we find that human plasma plays a role in the observed phenomenon, resulting in higher uptake of PEGylated particles when introduced to model phagocytes, including HL-60 derived neutrophils, mouse neutrophils, and BMMs from different mouse strain backgrounds (Figures 4.4-4.6).

The results presented in this work were initially a surprise. Upon closer inspection of the literature, we found that, although PEG has been widely studied as a protective coating against protein adsorption and immune cell uptake, very few studies have explored the uptake of PEGylated particles in human neutrophils. We found one previous report demonstrating that very high-density PEG ($R_F/D > 2.8$) grafting results in reduced particle uptake in human neutrophils[114], but these cells were purchased as already-prepared buffy coats and incubated in culture medium with human serum added rather than in whole blood or the presence of complete human plasma. Additionally, the concept of anti-PEG antibodies has recently introduced another complication into the field of particle drug delivery. The application of PEG in a wide variety of personal care products (e.g., shampoos and cosmetics) can result in the development of anti-PEG antibodies which recognize and bind to PEG chains. These antibodies have been detected in humans by ELISA[117], and further research suggests that anti-PEG antibodies are not specific to the PEG head group, but to the PEG chain itself.[116] While our study spiking PEG into the media to saturate any anti-PEG antibodies does not support the contribution of anti-PEG antibodies to the effect reported here, taken together with the increased uptake of PEGylated particles by human neutrophils, these concerns surrounding PEGylated particles have dramatic implications for their effectiveness in humans *in vivo*. To ensure the surprising result obtained in our assays with primary neutrophils are not an artifact of our protocol, we characterized the uptake of our PEGylated particles in a variety of phagocytic cells typically used in assays to assess uptake. In these conventional *in vitro* tools, we find that PEGylation does, in fact, reduce uptake. Even using the most-

accurate model cell for human neutrophils, HL-60 cells, we do not observe uptake resembling the primary cell type unless these are cultured using human plasma. Thus, one conclusion from the presented results is that the conventional *in vitro* cell tools are incapable of fully capturing the behavior of primary human neutrophils. Often, groups choose a convenient phagocytic cell-line to model the uptake of their engineered particle systems *in vitro*, with the underlying assumption that the behavior of those cell lines will approximate the behavior of human phagocytes *in vivo*. Given time and financial constraints, this is understandable, but the results here highlight a need for comprehensive characterization of particle uptake properties in a variety of cell types and, more importantly, in physiological media such as complete plasma. Indeed, it has been reported that differences in plasma protein corona formation between species have an impact on the efficacy of targeted drug carriers,[199] as do differences in hemorheology.[200] Further, we know that mouse peripheral leukocyte subpopulations differ substantially from human subpopulations regarding both relative population abundance and receptor makeup and function.[201] Such differences ought to play a role in the characterization of the performance of particle drug carriers, and should be explored further before useful generalizations can be drawn between performance in murine *in vivo* models and humans. Here, we report evidence to suggest that not only do primary human leukocytes internalize PEGylated particles differently from many model phagocytic cells, but even cells isolated from two different mouse strains can behave differently from one another (Figures 4.4 and 4.6), in line with results obtained by at least one other group *in vivo*.[202] These results speak to some of the limits and caveats of experiments frequently used to characterize particle uptake and, ultimately, efficacy. To more effectively design particles capable of avoiding immune clearance and delivering their therapeutic payload, we need to thoroughly investigate the uptake of said particles in more relevant systems before concluding on their efficacy.

The results with HL-60 derived neutrophils, which only mimic results of human primary neutrophils when incubated with human plasma, highlight the critical role of plasma in particle phagocytosis, and suggests that there are factors in human plasma that drive PEGylated particle uptake by neutrophils and neutrophil-like cells. This finding was recapitulated with murine marrow-derived macrophages and THP-1 cells with said

particles in human plasma (Figure 4.6), removing any statistical difference between carboxylated and PEGylated particles and hinting that factors in the plasma are at least partially responsible for this result. SDS-PAGE analysis revealed that PEG was effective at reducing overall protein adsorption, particularly among low-molecular-weight proteins (Figure 4.7). However, PEGylated particles did show some prominent protein bands, identified to contain mostly immunoglobulins and complement proteins, not present on carboxylated particles, suggesting that this difference in protein adsorption could be responsible for the increased uptake of PEGylated particles by neutrophils. Indeed, the results from assays with particles with only human complement in the media highlight these proteins as a significant player in the increased neutrophil phagocytosis of PEGylated particles. In particular, the drastically increased uptake observed for carboxylated particles, on a similar level to the PEGylated particles, when exposed to human complement in isolation (Figure 4.9). Conversely, when PEGylated particles were exposed to serum albumin only, they exhibit drastically decreased particle uptake. Collectively, these results lead us to speculate that PEGylation of particle surface effectively filters out small proteins, most importantly albumin, from the particle corona, leaving room for the high adsorption of proteins that promote phagocytosis. Here, human serum albumin is shown to play a protective role in particle uptake, likely passively by its high-affinity adsorption preventing a significant amount of complement proteins from accumulating in the particle corona. Indeed, albumin has been reported to counteract IgG and complement adsorption on particle surfaces, reducing particle recognition by the mononuclear phagocytic system in some cases.[36,51-53] However, this study is by no means exhaustive, and it is possible that other proteins in plasma contribute to the differential uptake as well. Based on the SDS-PAGE analysis, proteins such as apolipoprotein A-IV, properdin, gelsolin, or α-2 macroglobulin could be contributing to PEGylation effect (Figure 4.7). Properdin, for example, is known to play a role in the alternative complement pathway activation and neutrophil phagocytosis of bacteria and apoptotic T-cells[54–56], but its role in the phagocytosis of drug carriers remains relatively unexplored. Gelsolin and α-2 macroglobulin have also been explored in their capacity to impact neutrophil function and phagocytosis[57,58], but also have gone unexplored in the context of drug carrier phagocytosis. A thorough study of the impact of these proteins on

human neutrophil phagocytosis of various particulate drug carriers could yield enlightening information as to how to best design a drug carrier to avoid phagocytic clearance.

One limitation of this study is the lack of demonstration of the preferential uptake of PEGylated particles by neutrophils *in vivo*. However, while *in vivo* studies are indeed important, in this work we notice a distinct difference between the behavior of mouse cells and human cells. As shown in Figure 4.4, in general, there is a decrease in the uptake of PEGylated particles by mouse blood cells, which is in line with historically published work, but we find the opposite occurs specifically for human neutrophils. Furthermore, we provide evidence that the increased phagocytosis of PEGylated particles is due to factors in human plasma. Therefore, an *in vivo* animal study, which would typically be conducted in mice, would likely not give any more information on that front. One method to further probe the positive impact of PEGylation on neutrophil phagocytosis would be to explore a spatial coupling strategy, in which PEG is coated only on one hemisphere of particles and employ live visualization to determine whether neutrophils preferentially orient the particles on the PEGylated side during the phagocytic process. This could be explored in future works to gain a more thorough understanding of neutrophils' apparent preference for PEGylated particles.

Importantly, these results may have implications for the effectiveness of PEGylation as a strategy for creating long-circulating particulate drug carriers in humans. Recent work has shown that circulating neutrophils phagocytose drug carriers within the bloodstream and play a significant role in the clearance of particle drug carriers.[157] While PEG has been widely used because it imparts long circulation times in mouse studies, it has not proven to be the "magic bullet" it was once expected to be in the particulate drug delivery field, and this effect likely plays some role in that result. Other non-fouling coatings, like zwitterionic coatings, may prove to be more effective in this regard. Indeed, zwitterionic coatings have shown some promise in significantly reducing protein adsorption on nanoparticle surfaces, but most uptake studies using such particles have neglected to characterize their uptake in primary human blood cells in the presence of complete plasma.[59] More thorough characterization of their uptake properties is required to determine whether zwitterionic coatings can have the impact PEG was once expected to

have, which we plan to explore in future studies. Overall, it is imperative to investigate the factors driving neutrophil phagocytosis of drug carriers more fully and to develop coatings or other strategies to combat the removal of drug carriers by circulating neutrophils.

4.6 Conclusions

Despite decades of research into the impacts of non-fouling PEG coatings on particle uptake by model phagocytes, there is a dearth of research into the impacts of these coatings on primary human phagocytes. In particular, neutrophils are understudied due to their inconvenience and a lack of immortalized cell lines to study them and their sensitivity *ex vivo*. In this work, we thoroughly characterize human neutrophil uptake of model particle drug carriers with and without PEG coatings, and the results are quite surprising. We find that PEG significantly increases the uptake of particles by primary human neutrophils, regardless of particle size, PEG density, or end group. We found that human plasma, particularly human complement proteins, plays a role in this phenomenon, resulting in higher uptake of PEGylated particles when introduced to model phagocytes. Finally, we see that HL-60 derived neutrophils behave similarly to primary human neutrophils in the presence of human plasma. These results suggest a need for the characterization of phagocytosis of drug carrier platforms in primary cells and human plasma to become standard practice. In the future, researchers should take care to characterize particle uptake in a wide variety of cell types and media conditions to develop a more robust understanding of the impact of particle properties on leukocyte behavior.

Chapter 5: Salicylate-based Microparticles Divert Neutrophils from Inflammation and Mitigate Acute Lung Injury and ARDS

5.1 Publication Information

The work in this chapter is not yet published. This chapter has been written up into a manuscript for submission to Science Advances, and will be submitted in the near future. The title will be as above, with the following author list:

William J. Kelley, Emma R. Brannon, Michael W. Newstead, Alison L. Banka, Kathryn E. Uhrich, Theodore J. Standiford, and Omolola Eniola-Adefeso.

5.2 Abstract

Acute, systemic inflammatory diseases such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are highly problematic due to their extremely high mortality rates and lack of effective therapeutic interventions. Neutrophils, in particular, are a major contributor to the lethality of ALI/ARDS by causing damage to the lung tissue as they transmigrate into the airspace and release reactive oxygen species and NETs in response to inflammation. Given recent work from our lab showing that drug-free, intravenously administered particles can interfere with the neutrophil response to inflammation, we sought to explore whether such neutrophil-particle interactions can be employed as a therapeutic for ALI/ARDS. For this purpose, we fabricated micron-sized salicylate-based particles, termed “PolyAspirin” or “PolyA” particles. In this work, we demonstrate for the first time that degradable, drug-free particles can potentially be used as a therapeutic for ALI/ARDS; we find that injecting PolyA particles reduces neutrophil infiltration into the lungs in ALI/ARDS, reduces the release of inflammatory cytokines in the lungs, reduces the systemic bacterial load in a *P. Aeruginosa* lung infection model of ARDS, and dramatically improves survival. This work represents an entirely novel

therapeutic strategy for ALI/ARDS, and potentially a paradigm shift in the treatment of systemic inflammatory diseases.

5.3 Introduction

Acute, systemic inflammatory diseases are difficult to treat and associated with extremely 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 post-operative deaths [210], while 2016 study revealed the worldwide mortality rate of ARDS alone to be as high as 40% [211]. Although methods of treating ARDS and ALI symptoms have improved, mortality rates remain high, establishing the need for new treatment methods and potentially therapeutics [212]. As systemic inflammatory conditions, ALI and ARDS are characterized by an unrestrained innate inflammatory response.

The role of neutrophils in the pathogenesis and severity of ALI/ARDS is well-established; importantly, the concentration of neutrophils in the bronchoalveolar lavage fluid (BALF) of ALI/ARDS patients strongly correlates with disease severity and mortality [44–48]. 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 (ECs). 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 transendothelial migration [11,14,15]. 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 [44]. Additionally, once present in the alveolar space, neutrophils release cytotoxic materials such as reactive oxygen species (ROS) and neutrophil elastase, both of which have been shown to contribute to the severity of

ALI/ARDS [49,50,53–55]. Thus, neutrophils are potentially a useful target for therapeutics designed to treat ALI/ARDS.

Despite the attractiveness of neutrophils as a target for ALI/ARDS therapies, no current therapies exist to mitigate the role neutrophils play in disease exacerbation. The vast majority of currently employed therapies are supportive in nature, intended to relieve downstream symptoms of ALI/ARDS, and include various methods of mechanical ventilation, neuromuscular blockade, β-2 agonists, and fluid management.[213] Corticosteroids, which are employed as an anti-inflammatory agent intended to manage the complications brought on by the innate immune system, but have shown limited to no efficacy in improving survival for ARDS patients, and in fact may be detrimental in some cases.[214] Recent work in animal models has shown stem cell-based therapies to be effective immunomodulatory therapeutics, reducing proinflammatory signaling while enhancing anti-inflammatory signaling, increasing bacterial clearance, and improving survival.[215] However, such therapeutics have still yet to gain FDA approval for ALI/ARDS, and face a number of challenges in terms of stem cell batch reproducibility and scale-up.

Recent work in the particle drug delivery field has demonstrated the potential to use particles to direct, train, or “piggy back” on immune cells for therapeutic benefit.[216–219] Additionally, 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, and spinal cord injury [128,130,157,220]. However, most of these studies focused primarily on interactions between particles and T-cells or monocytes, and many such formulations use antibodies such as anti-CD45 or anti-CD11b to bind to the surface of immune cells. Importantly, this approach has not been explored for the blocking of neutrophil function. We previously reported that vascular-targeted polystyrene particles interact with neutrophils in free stream and can interfere with their vascular wall adhesion.[157,221] 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.

Clearly, any particle-based therapeutics will require the use of a biocompatible, and preferably biodegradable, material. Poly(lactic-co-glycolic acid) (PLGA)-based polymeric drug delivery particles are frequently proposed for this purpose, have already achieved FDA approval and are used clinically for delivering active pharmaceutical agents in cancer applications [222]. Importantly, hydrolysis of PLGA-based polymeric particles produces biocompatible byproducts, lactic acid and glycolic acid [223,224], which can lead to an inflammatory response that would be harmful when treating an inflammatory disease [223,225]. For this reason, it is important to consider other polymers as the base of a polymeric particle treatment for inflammatory conditions.

In this work, we sought to investigate whether drug-free particles can be used as a therapeutic for the treatment of ALI/ARDS by modulating the neutrophil response to inflammation. For this purpose, salicylate-based poly(anhydride-ester) polymers ("PolyAspirin" or "Poly-A"), were fabricated into micron-sized spheres and evaluated as a potential therapeutic for systemic inflammation resolution. Poly-A degrades into the active component of aspirin, salicylic acid, which has been shown to be biocompatible and nontoxic, so it is hypothesized that drug-free Poly-A particles may confer therapeutic effects in ALI and ARDS [226,227]. 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.

5.4 Results

Fabrication and Characterization of Poly-A Microspheres

We used a single-emulsion solvent evaporation technique to fabricate ~ 2 μm Poly-A (comparable to polystyrene controls) microspheres and measured their size using SEM and DLS. Then, we suspended Poly-A particles in 10 mL of water and rotated them at 37°C for degradation studies, periodically removing and replacing the supernatant to determine salicylic acid content. Additionally, samples of degraded particles were taken and imaged via SEM to determine the type (bulk versus surface erosion) and timescale of particle breakdown. The results of these studies are presented in Figure 5.1.

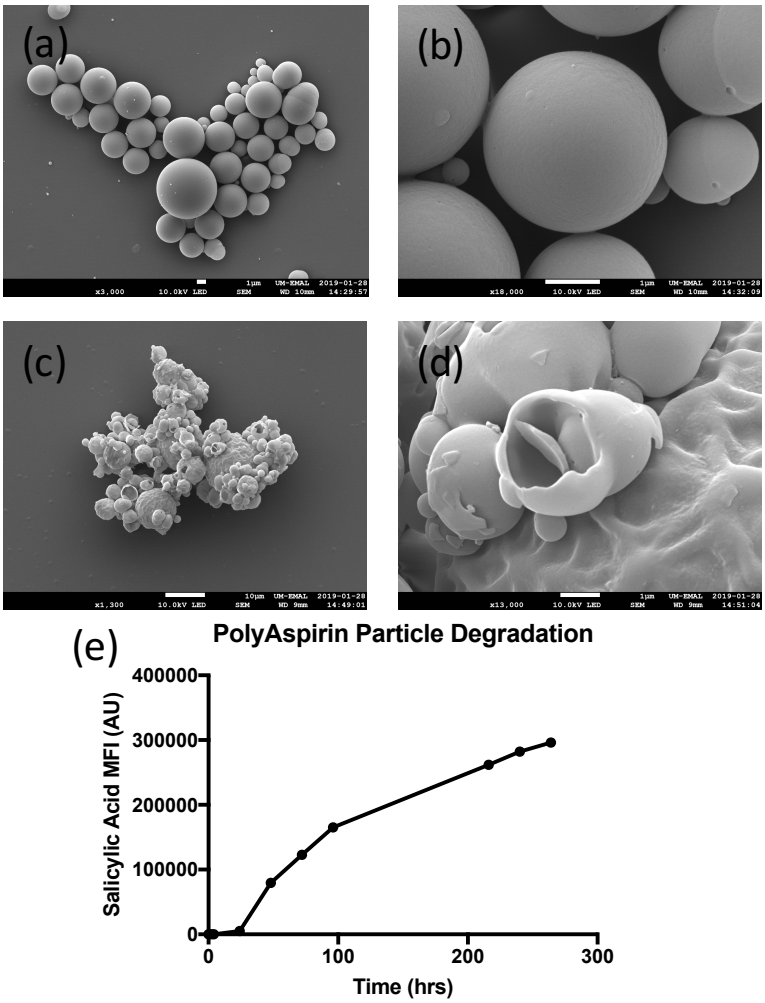


Figure 5.1 – SEM Characterization of PolyAspirin Particles and Degradation Profile **(a-b)** SEM images of freshly-prepared PolyAspirin particles. **(c-d)** SEM images of PolyAspirin particles after 13 days of degradation in water. **(e)** Hydrolytic degradation profile of PolyAspirin particles as measured by release of salicylic acid.

As shown in Figures 5.1a-b, we successfully fabricated smooth, micron-scale Poly-A spheres using our single-emulsion solvent evaporation technique, similar to those frequently fabricated using PLGA and other polymers for drug delivery applications [228]. Additionally, after 13 days of degradation in water we see that the Poly-A particles have undergone some degree of bulk degradation, and their morphology as dramatically changed (Figures 5.1c-d). Further, we find that these Poly-A particles undergo a prolonged period of degradation, continuing to degrade out to 2 weeks or more. Thus, we expect relatively little salicylic acid release to occur over the timescale of our ALI/ARDS

experiments by hydrolytic degradation. However, it is possible that, once phagocytosed by neutrophils or other leukocytes, Poly-A particles will be degraded more rapidly and provide some additional therapeutic benefit in the resolution of inflammation.

Impact of Poly-A Particles on an LPS Model of Acute Lung Injury

Given our previous study demonstrating the ability of drug-free polystyrene particles to reduce neutrophil influx in an LPS model of acute lung injury(cite ACS nano paper), we first sought to compare the impact of Poly-A particles to polystyrene and PLGA particles in this simplified model. In order to select optimal injection times and ensure a full understanding of the timeline of neutrophil influx in LPS-induced ALI in C57BL/6J mice, we first sought to fully characterize the model by inducing ALI and sacrificing mice at varying times, evaluating total BALF cells, total neutrophils in the BALF, and percentage of neutrophils versus macrophages in the BALF. The results of these experiments are presented in Figure 5.2.

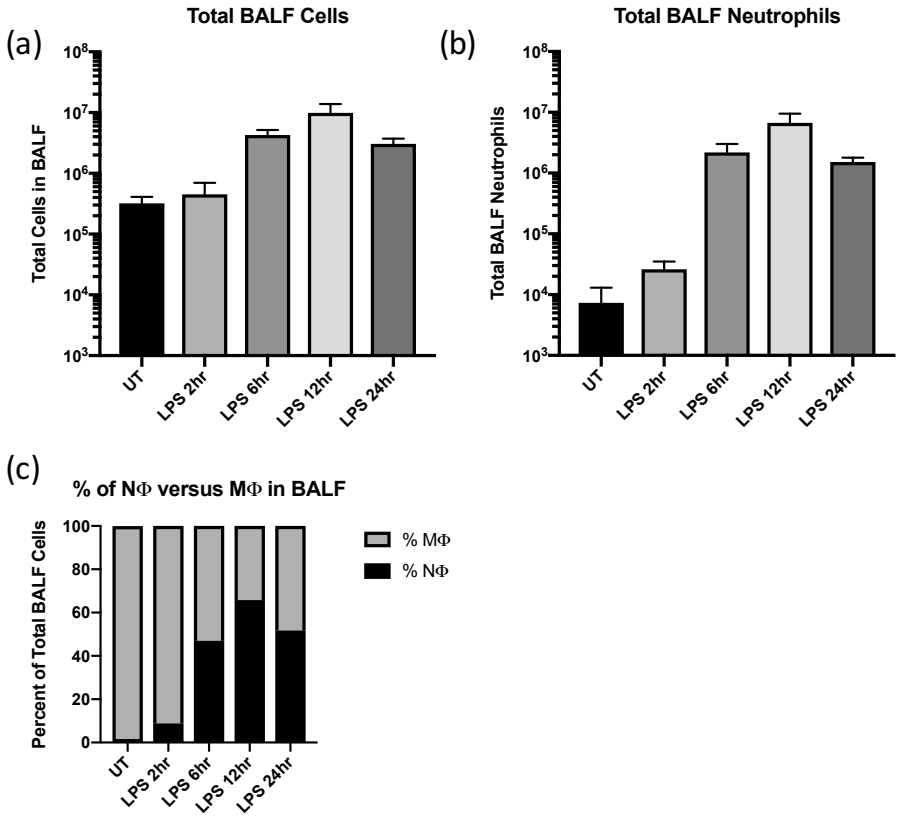


Figure 5.2—Characterization of LPS-induced ALI model in C57BL/6J mice. (a) Total cells in BALF 2, 6, 12, and 24 hours post-LPS instillation. **(b)** Total neutrophils in BALF 2,

6, 12, and 24 hours post-LPS instillation. **(c)** Relative percentage of neutrophils and macrophages in the BALF 2, 6, 12, and 24 hours post-LPS instillaton.

As shown in Figure 5.2, we see that the bulk of the neutrophil influx into the alveolar space occurs between 2 and 6 hours post-LPS instillation in C57BL/6J mice, and that the inflammation may begin to resolve after 24 hours.

We then evaluated the impact of intravenously-administered Poly-A, polystyrene, and PLGA particles on the inflammatory response in LPS-induced ALI. Because we observed the greatest influx of neutrophils into the lungs between 2 and 6 hours post-LPS instillation, we chose to investigate the impact of particle injections at 2 hours and 4 hours post-LPS instillation. In order to directly compare these two dosing schemes, we fixed the euthanasia time at 2 hours post-particle injection.

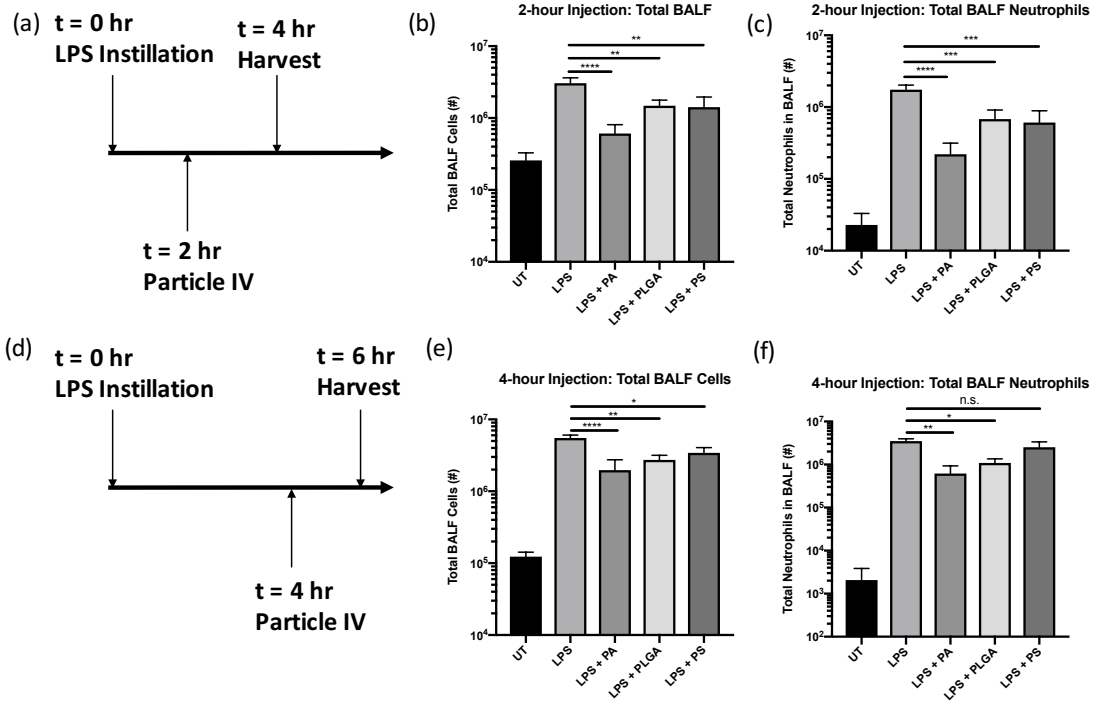


Figure 5.3—Total BALF cells and total BALF neutrophils in LPS ALI model in C57BL6J mice with 2 hour and 4 hour injection times. (a) Dosing/harvest schedule for 2-hour injections. **(b)** Total BALF cells in 2-hour injection time experiments for untreated (UT) mice, LPS-only, LPS + Poly-A (PA) particles, LPS + PLGA particles, and LPS + polystyrene (PS) particles. **(c)** Total BALF neutrophils in 2-hour injection time experiments. **(d)** Dosing/harvest schedule for 4-hour injections. **(e)** Total BALF cells in 4-

hour injection time experiments. (f) Total BALF neutrophils in 4-hour injection time experiments.

Figure 5.3 demonstrates the impact of injecting Poly-A, PLGA, and polystyrene particles at 2 and 4 hours post-LPS instillation. As shown in Figures 5.3b-c, 2-hour injection of all particle types significantly reduces the total cells in the BALF and total neutrophils in the BALF compared to LPS-only mice. Importantly, Poly-A particles exhibit the most significant reduction in both total BALF cells and BALF neutrophils compared to the other particle types, and was the only particle type to reduce both total BALF cells and BALF neutrophils to a level statistically insignificant versus the untreated controls. We hypothesize that the additional benefit of Poly-A particles relative to other particle types is due to an anti-inflammatory effect enacted upon circulating neutrophils, as direct treatment of mouse alveolar macrophages *in vitro* with Poly-A particles did not significantly reduce the production of inflammatory cytokines TNF, IL-6, IL-10, IP10, KC, MCP1, or MIP2. (Figures 5.4 and 5.5).

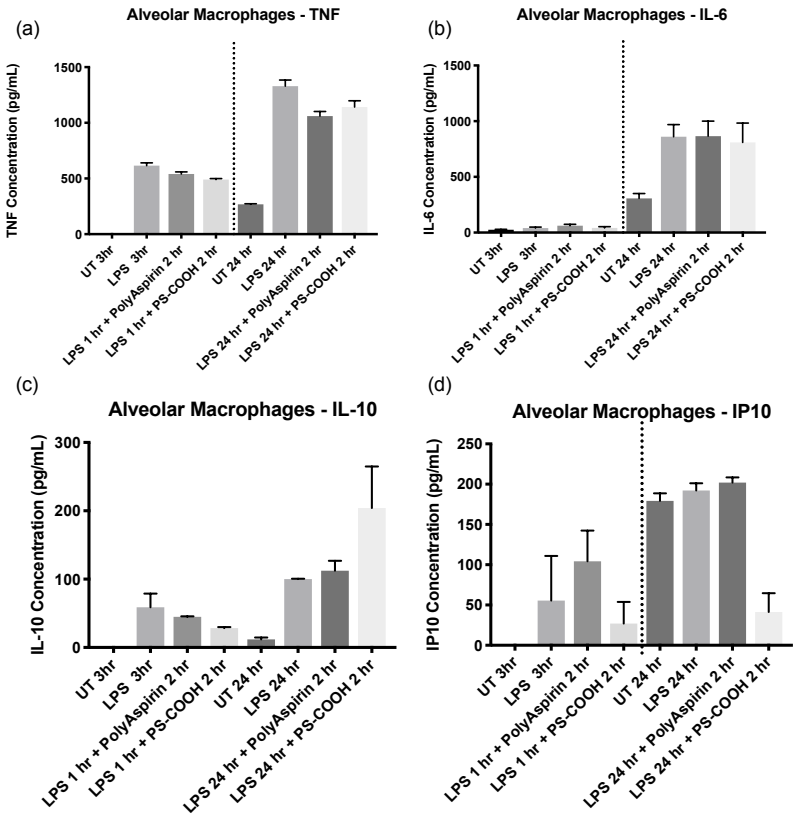


Figure 5.4—Alveolar macrophage production of (a) TNF, (b) IL-6, (c) IL-10, and (d) IP10 after treatment with LPS and either Poly-A particles or polystyrene particles.

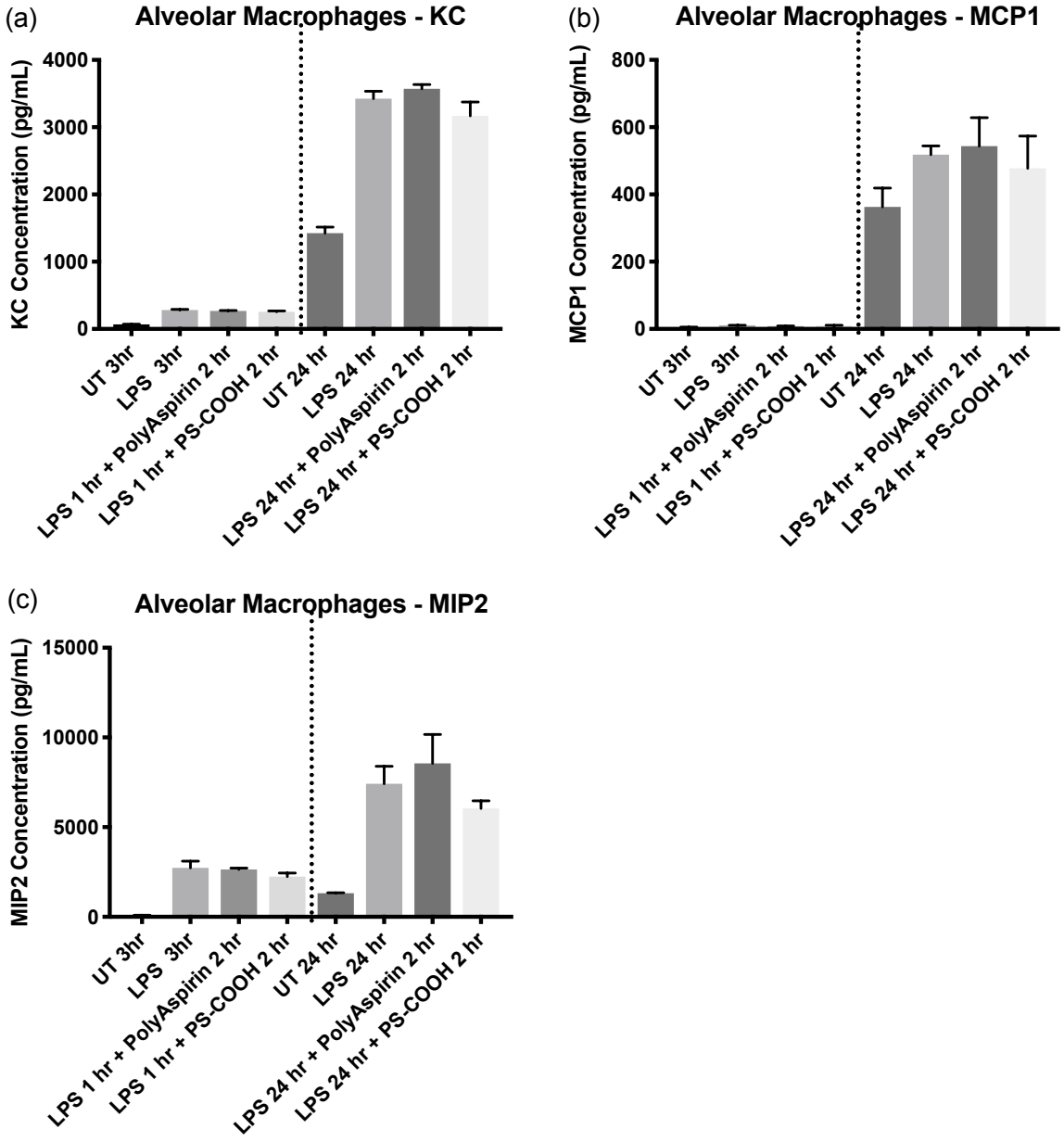


Figure 5.5—Alveolar macrophage production of (a) KC, (b) MCP1, and (c) MIP2 after treatment with LPS and either Poly-A particles or polystyrene particles.

Similarly, with the 4-hour injection time, we see that particle injections significantly reduce the total BALF cells and total BALF neutrophils, with the exception of polystyrene particles failing to significantly reduce total BALF neutrophils. Again, we see that Poly-A

particles confer a greater reduction in both BALF cells and BALF neutrophils than the other particle types. However, it is important to note that the overall reduction in BALF cells and BALF neutrophils is less significant at the 4-hour injection time than at the 2-hour injection time, emphasizing the importance of particle administration time relative to the influx of neutrophils into the lungs.

Impact of Poly-A Particles on Inflammation in P. Aeruginosa Lung Infection Model

Given the therapeutic effects of Poly-A particles in an LPS-induced model of lung inflammation, we sought to determine whether they could confer a similar benefit in a more complex lung injury model, potentially leading to the development of a therapeutic strategy. Thus, we explored the impact of IV-administered particles in a *P. Aeruginosa* lung infection model, as previously characterized [229]. Again, we first quantified the exact timeline of neutrophil influx into the lungs in *P. Aeruginosa* infection, as our results in the LPS-induced model of lung inflammation demonstrated that the timeline of particle administration relative to neutrophil influx plays a major role in the efficacy of treatment. The results of this characterization study are presented in Figure 5.6.

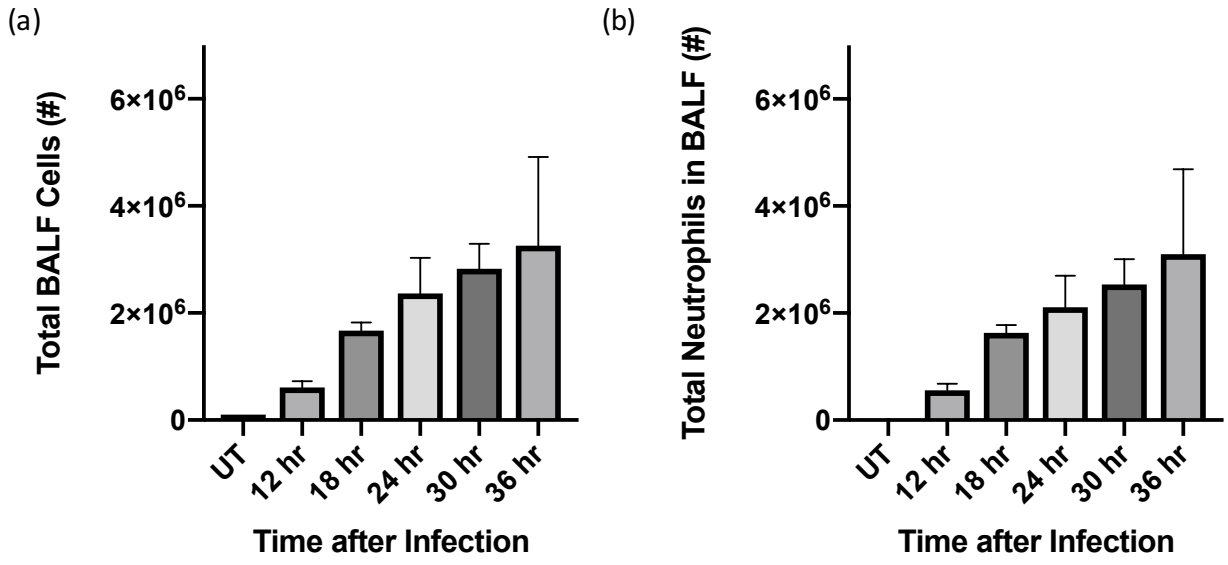


Figure 5.6—Characterization of P. Aeruginosa bacterial lung infection model in C57BL/6J mice. (a) Total cells in BALF 12, 18, 24, 30, and 36 hrs post-infection**. (b)** Total neutrophils in BALF 12, 18, 24, 30, and 36 hrs post-infection.

In Figure 5.6, we see a steady increase in total BALF cells and BALF neutrophils from 12 hours through 36 hours post-infection, with some plateauing seen at around 30-36 hours suggesting that the infection has ceased to progress after that point. Importantly, we see the greatest influx in neutrophils into the lungs between 0 and 18 hours. Thus, we again chose two different injection times to explore the impacts of Poly-A particles on the resolution of inflammation and infection: 6 hours and 18 hours post-infection, with a sacrifice time of 24 hours post-infection. Additionally, we sought to compare the impact of Poly-A particle injection with PLGA and polystyrene particles. The results of these experiments are shown in Figure 5.7.

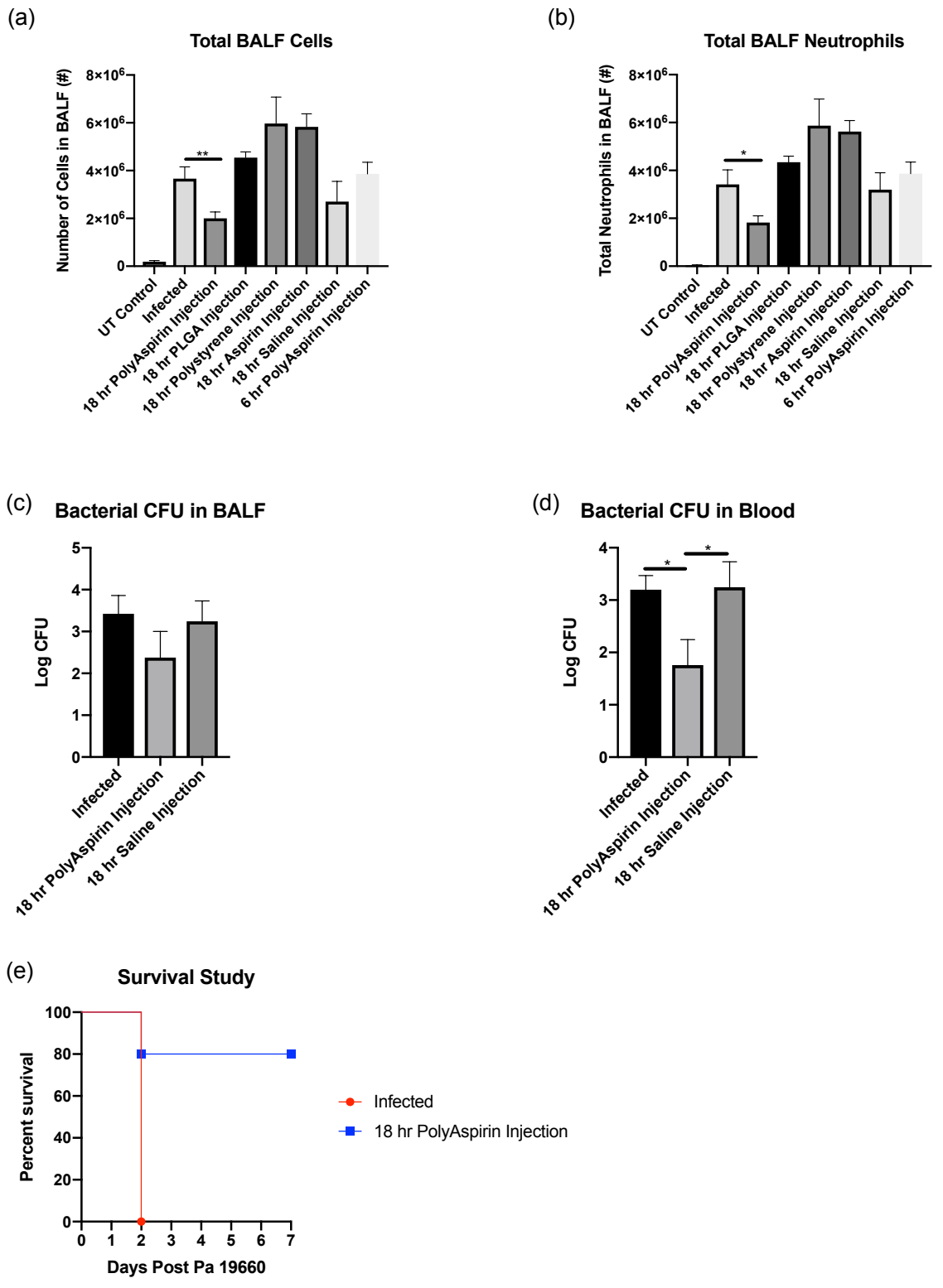


Figure 5.7—Impact of PolyAspirin Particle Injection on P. Aeruginosa lung infection in C57BL/6J mice. (a) Total cells in BALF 24 hrs post-infection after 6-hr or 18-hr

PolyAspirin injection, 18-hr PLGA injection, 18-hr polystyrene injection, or 18-hr aspirin injection compared to infected mice and saline controls. **(b)** Total neutrophils in BALF 24 hrs post-infection after 6-hr or 18-hr PolyAspirin injection, 18-hr PLGA injection, 18-hr polystyrene, or 18 hour aspirin injection, compared to infected mice and saline controls. **(c)** Bacterial CFU in BALF 24 hrs post-infection after 18-hr PolyAspirin injection. **(d)** Bacterial CFU in blood 24 hrs post-infection after 18-hr PolyAspirin injection. **(e)** Post-infection survival for *P. Aeruginosa* infected mice with and without 18-hour PolyAspirin Injection.

As seen in Figure 5.7a-b, we again find that the timing of particle injection is crucial; the 6-hour Poly-A injection failed to reduce total BALF cells and total BALF neutrophils, while the 18-hour Poly-A injection significantly reduced both total BALF cells and total BALF neutrophils. Additionally, the 18-hour PLGA injection insignificantly reduced both total BALF cells and total BALF neutrophils, while the 18-hr polystyrene injection had no effect on either the total BALF cells or total BALF neutrophils. Further, an injection of free aspirin had no effect on either the total BALF cells or total BALF neutrophils. All together, these results suggest that there is some therapeutic effect resulting from injecting a degradable particle, with additional therapeutic effect provided by the degradation of Poly-A particles into salicylic acid. Importantly, the mere injection of an anti-inflammatory agent alone is not enough to create a therapeutic effect.

Further, in order to evaluate the potential therapeutic benefit of Poly-A particles in lung infection, we evaluated the bacterial CFU in both the BALF and blood following an 18-hour Poly-A particle injection. In Figure 5.7c-d, we see that the injection of Poly-A particles does not significantly reduce bacterial CFU in the BALF; however, the injection did significantly reduce the bacterial CFU in the bloodstream. This result suggests that, while Poly-A particle injection does not directly assist in the clearance of the infection in the lungs, the diversion of neutrophils from the site of inflammation prevents neutrophils from damaging the lung endothelium and epithelium to the extent that the bacteria cannot escape the alveolar space and enter the bloodstream, thus alleviating the risk of a systemic infection. Additionally, we performed a survival study comparing the survival time of mice receiving only *P. Aeruginosa* inoculation versus infected mice which also received an 18-hour Poly-A injection (Figure 5.7e). Crucially, we find that an 18-hour Poly-

A particle injection results in a significant improvement in survival. All of the infected-only mice died within 48 hours post-infection, while 80% of the mice receiving a Poly-A injection lived out to one week, at which point the mice were sacrificed. Additionally, blood and BALF samples were taken from the surviving mice after sacrifice and plated, with no bacterial CFU found (data not shown).

Finally, we sought to investigate whether the injection of Poly-A particles had an impact on inflammatory markers in the lungs, either by diversion of neutrophils or by the direct anti-inflammatory effects of salicylic acid as the particles degrade. Thus, we used ELISAs to measure the levels of IL-10, KC (CXCL1), MCP1 (CCL2), MIP2 (CXCL2), TNF, and IL-6 in the BALF. Additionally, we measured levels of IgM and albumin in the lungs as markers of lung endothelial and epithelial damage. The results of these experiments are shown in Figure 5.8.

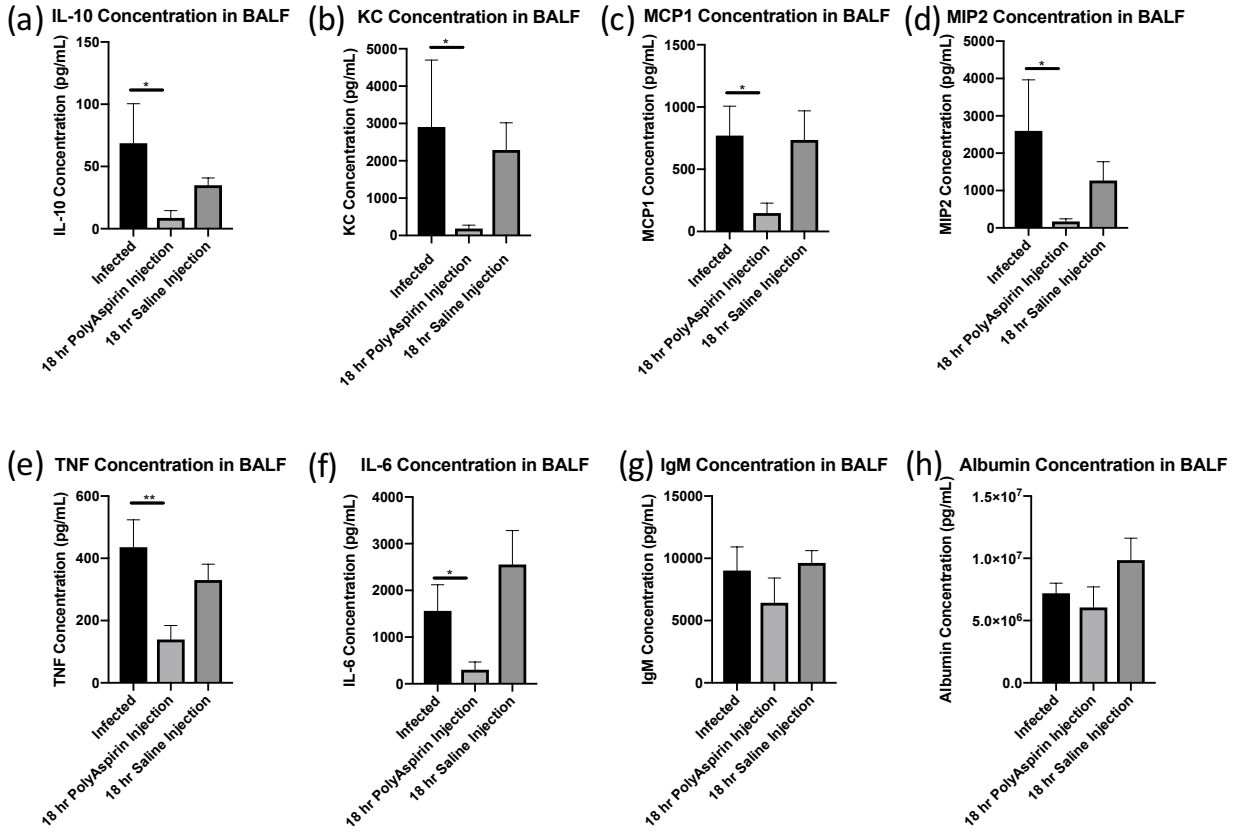


Figure 5.8—Cytokine and Protein Content in BALF of P. Aeruginosa infected mice after 18-hour PolyAspirin injection, as measured by ELISA. Concentration of **(a)** IL-

10, **(b)** KC, **(c)** MCP1, **(d)** MIP2, **(e)** TNF, **(f)** IL-6, **(g)** IgM, and **(e)** albumin in BALF of *P. Aeruginosa* infected mice, 24 hours post-infection with an 18-hour PolyAspirin particle or saline injection.

As shown in Figure 5.8a-f, Poly-A particle injection significantly reduced the levels of all the inflammatory cytokines and chemokines in the BALF measured as compared to infected controls. This result suggests that the administration of Poly-A particles alleviates the inflammatory response, again supporting the hypothesis that Poly-A particle injection may serve as an effective therapeutic alone or in combination with other therapies such as antibiotics in the treatment of ALI/ARDS. However, the Poly-A particle treatment did not significantly reduce IgM or albumin levels in the lungs (despite an insignificant trend toward a decrease), as shown in Figure 5.86g-h, suggesting that some level of lung tissue damage still occurs even with particle administration. It is possible that further experimentation with injection timing or multiple injections would result in significant reduction in IgM or albumin concentration; thus, further study is required.

5.5 Discussion

In this study, we investigated the impact of intravenously-administered, drug-free Poly-A particles on inflammation in ALI/ARDS, hypothesizing that Poly-A particles would both divert neutrophils from the site of inflammation and potentially provide additional anti-inflammatory benefit as they are degraded into salicylic acid. To this end, we fabricated micron-sized Poly-A particles using a single-emulsion solvent evaporation technique, measured their size using SEM, and characterized their degradation profile in buffer in terms of salicylic acid release (Figure 5.1). Importantly, we find that the Poly-A particles undergo degradation in buffer over an extended period of time (on the order of weeks), suggesting that passive degradation of the particles may provide very little salicylic acid for anti-inflammatory therapy. However, it remains unclear whether the Poly-A particles are more rapidly degraded by neutrophils and other leukocytes once internalized, thereby releasing more salicylic acid *in vivo*.

In order to determine the feasibility of using Poly-A particles as a therapeutic strategy for alleviating ALI/ARDS, we first explored the impact of intravenously-administered Poly-A particles in an LPS-induced model of ALI. Based on our previous work, we hypothesized that the timing of particle injection relative to LPS instillation might

be important to the efficacy of the particle treatment [157]. Thus, we investigated two injection times: two hours and four hours post-LPS instillation. Additionally, we compared the impact of Poly-A particle injection to PLGA and polystyrene particles in order to determine whether Poly-A particle injection conferred any additional therapeutic benefit.

To this end, we first characterized the LPS model of ALI in C57BL/6J mice to determine the timeline of neutrophil influx into the alveolar space (Figure 5.2). Notably, we found that the greatest influx of neutrophils occurred between 2 and 6 hours, hence the choice of 2-hour and 4-hour injection times, in order to maximize the impact of particle injection on neutrophil influx. Additionally, we found that all particle types significantly reduced total BALF cells and total BALF neutrophils at the 2-hour injection, with Poly-A particles causing the most significant reduction. (Figure 5.3b-c). Further, we found that all particle types except polystyrene significantly reduced total BALF cells and total BALF neutrophils at the 4-hour injection, again with Poly-A particles performing the best (Figure 5.3e-f) However, we found that the 2-hour injections generally caused a greater reduction in BALF cells and BALF neutrophils, highlighting the importance of injection time. If particles are injected before excess neutrophils are recruited and released from the bone marrow, they will likely be cleared before they can divert neutrophils from the inflammation. Conversely, if they are injected long after neutrophils have been recruited and infiltrated into the lungs, the window of opportunity for intervention will have passed. This result suggests that any therapeutic strategy designed to alter the innate immune response to inflammation ought to take into account the timeline of inflammation, in alignment with other studies investigating the impact of innate immunity modulation on lung infection [230], and that diagnostics to determine when and how neutrophils in particular are responding to inflammation may be useful in this regard. Additionally, the increased benefit of Poly-A particle injection versus PLGA and polystyrene suggests that the degradation of Poly-A into salicylic acid may be conferring some additional therapeutic effects, despite the extended timeline of Poly-A particle degradation; this could be due to degradation of the Poly-A particles by neutrophils or other circulating leukocytes, resulting in the release of salicylic acid and an anti-inflammatory effect. However, more work is required to confirm this hypothesis.

Moving forward, we sought to determine the impact of intravenous particle administration on a more complex model of ALI/ARDS: a *P. Aeruginosa*-induced model of lung infection. Again, given our prior work and our results in Figures 5.2-3, we first sought to characterize the timeline of neutrophil influx into the lungs in this model (Figure 5.6). We found that the greatest neutrophil influx occurs between 0 and 18 hours post-infection; thus, we evaluated the impact of Poly-A particle injection at both 6 and 18 hours post-infection. Notably, this longer timeline for *P. Aeruginosa*-induced lung inflammation provides a much wider potential therapeutic window for eventual application in the clinic, which may be even wider in human cases of ARDS versus mice.[231,232] Thus, it is possible that our therapeutic strategy may be even more applicable in clinical settings.

Importantly, we found that the 18-hour Poly-A particle injection was most impactful at reducing total BALF cells and total BALF neutrophils (Figure 5.7a-b), while the 6-hour particle injection had no impact on the number of total BALF cells or total BALF neutrophils. Based on the neutrophil influx curve in Figure 5.6, it is likely that the 6-hour injection time results in particles being cleared before significant neutrophil recruitment occurs, blunting the potential therapeutic impact. We can infer from these results that injecting particles around the time of greatest neutrophil influx (i.e., the point on a curve of neutrophils versus time with the greatest slope) is the most effective at potentially providing a therapeutic effect in ARDS, which should be taken to account in any clinical application.

Comparing 18-hour Poly-A particle injection to PLGA and polystyrene, we see only Poly-A significantly reduces total BALF cells and total BALF neutrophils, although PLGA particles do result in a nonsignificant reduction in total BALF cells and total BALF neutrophils (Figure 5.7a-b). These results suggest that the mere presence of degradable particles is enough to confer some therapeutic benefit in terms of reducing neutrophil influx into the lungs; however, Poly-A particles may be conferring additional therapeutic benefit via the release of salicylic acid. Further, injecting soluble aspirin alone confers no reduction in total cells or neutrophils in the BALF, in alignment with clinical results demonstrating little to no therapeutic benefit from the use of anti-inflammatory agents alone.[213] This result shows that a solid particle (or perhaps some other strategy) used

to divert neutrophils from the inflammation in the lungs in addition to some anti-inflammatory therapeutics may be most effective at treating ARDS.

Additionally, we find that 18-hour Poly-A particle injection results in a significant decrease in bacterial CFU in the bloodstream but not in the BALF (Figure 5.7c-d), suggesting that the prevention of neutrophil influx into the lungs also prevents some degree of lung damage from occurring, lessening the ability of bacteria to extravasate and create a systemic infection. However, we do not find a significant reduction in either IgM or albumin content in the BALF (though we do find a trend toward a decrease), suggesting that some degree of lung tissue damage is still occurring even with Poly-A particle injection. Critically, we found that an 18-hour Poly-A particle injection also dramatically improves survival for infected mice, with 80% of mice receiving Poly-A living for a week post-infection, while nontreated mice died within 48 hours post-infection. Further, we found that 18-hour Poly-A particle injection significant reduced the amount of a number of inflammatory cytokines in the BALF, including IL-10, KC, MCP1, MIP2, TNF, and IL-6, again suggesting that Poly-A particle injection is conferring some anti-inflammatory effect in ARDS (Figure 5.8a-f). These results, taken together, suggest that Poly-A particle injection may function effectively as part of a comprehensive therapeutic strategy in combination with antibiotics or other anti-inflammatory agents. Importantly, compared to current therapeutic strategies for ALI/ARDS employed in the clinic, our particle platform addresses a major underlying cause of the mortality of ALI/ARDS (namely, neutrophil-induced damage to the lungs and subsequent systemic infection), rather than aiming to alleviate the symptoms long enough for the condition to subside.

As with any study, this work is not without limitations. Because we were interested in developing the therapeutic strategy and not the therapeutic formulation itself, we did not fully optimize our Poly-A particle system to result in maximum therapeutic benefit. Future work iterating over various particle parameters (such as concentration, size, shape, polymer chemistry, etc.) may shed more light on both the therapeutic mechanism and the optimal formulation for maximum therapeutic effect, as evidence suggests that these parameters all influence the degree to which particles interact with immune cells.[119,150,157,221] More critically, these results are based entirely on murine models of ALI/ARDS. While such models are useful tools for developing potential therapies, more

complex and physiologically-relevant models such as large-animal models or *ex vivo* human lung models should be explored to further probe the potential use of Poly-A particles as a therapeutic for ALI/ARDS.[215] Further still, human cases of ALI/ARDS are extremely heterogeneous in their presentation, making translation of therapeutics from the bench to the clinic difficult and hard to predict.[42,43,213,233]

5.6 Conclusions

All in all, our findings show for the first time that drug-free Poly-A based particles can be used as a therapeutic intervention in ALI/ARDS, potentially creating a new therapeutic paradigm for the treatment of ALI/ARDS and other similar acute inflammatory diseases. We find that Poly-A based particles potentially confer additional therapeutic benefit above other particle types, including PLGA and polystyrene particles. Additionally, we find that the timing of particle injection relative to neutrophil recruitment and infiltration is crucial, highlighting the importance of the transient nature of such inflammatory conditions and shedding light on how to best design future therapeutic interventions. As with any study, there are limitations to our findings. First and foremost, the makeup of murine immune cells differs drastically from that of humans, which could alter the impact of this therapeutic strategy in human ALI/ARDS [201]. Additionally, fine-grained exploration of the dosing scheme (i.e., injection times, multiple particle injections, combination therapies, etc.) and particle properties (e.g., particle size, shape, concentration, polymer composition) are outside the scope of this paper; however, further investigation into these parameters may shed further light onto the potential of Poly-A particles as a therapeutic for ALI/ARDS.

Chapter 6: Increased Adhesive Potential of Antiphospholipid Syndrome Neutrophils Mediated by β_2 Integrin Mac-1.

6.1 Publication Information

The work in this chapter is largely published as:

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Note: * indicates co-authorship.

6.2 Abstract

While the role of antiphospholipid antibodies in activating endothelial cells has been extensively studied, the impact of these antibodies on the adhesive potential of leukocytes has received less attention. Here, we endeavored to determine the extent to which APS neutrophils adhered to resting endothelial cells under physiologic flow conditions, and to elucidate the molecules required for that adhesion. Patients with primary APS (n=45) and healthy controls (n=38) were included in the study. Cells were introduced into a flow channel and perfused across a resting human umbilical vein endothelial cell (HUVEC) monolayer. Adhesion molecules on APS neutrophils were identified by flow cytometry. Neutrophil extracellular trap (NET) release was assessed in neutrophil-HUVEC co-cultures.

Perfusion of anticoagulated blood through the flow channel resulted in increased adhesion of APS patient neutrophils as compared with controls under conditions

representative of either venous or arterial flow. At the same time, APS patient neutrophils demonstrated upregulation of CD64, CEACAM-1, beta-2 glycoprotein I, and activated Mac1 on their surface. Similarly, treating control neutrophils with APS plasma or APS IgG resulted in increased neutrophil adhesion and surface marker upregulation as compared with controls. A monoclonal antibody specific for activated Mac1 reduced the adhesion of APS neutrophils to HUVECs in the flow-channel assay. Interestingly, the same monoclonal antibody reduced NETosis in neutrophil-HUVEC co-cultures. Thus, APS neutrophils have an increased adhesive potential, which is dependent upon the activated form of Mac1. In patients, this may lower the threshold for neutrophil-endothelium engagement, NETosis, and possibly thrombotic events.

6.3 Background

Antiphospholipid syndrome (APS) is an autoimmune condition of unknown cause defined by the presence of circulating “antiphospholipid” antibodies (anticardiolipin, anti-beta-2-glycoprotein I/β2GPI, or lupus anticoagulant).[70] The morbidity and even mortality of APS are significant, as patients carry a markedly increased risk of thrombotic events (especially stroke and deep vein thrombosis) and pregnancy loss.[234] Beyond these disease-defining events, patients with APS may also develop cytopenias, heart valve damage, nephropathy, and cognitive decline, among other complications.[235] While it has long been recognized that circulating leukocytes play a vital role in the pathophysiology of APS, the impact of neutrophils has only come to light in the past few years.[72] Studies have revealed that APS neutrophils are prone to the exaggerated release of neutrophil extracellular traps (NETs), prothrombotic tangles of DNA and protein released from dying neutrophils.[236] At the same time, at least some APS patient blood does not degrade NETs normally.[74] Indeed, dismantling NETs with deoxyribonuclease has proven effective in venous models of APS.[73] Even more recently, work has demonstrated that the activated neutrophil transcriptome in APS is highlighted by the upregulation of a number of meta-groups, including a cellular defense node that includes L-selectin and P-selectin glycoprotein I, amongst other adhesion molecules.[75]

Beyond neutrophils, APS animal models and descriptive studies of APS patients have demonstrated signs of smoldering endothelial activation. For example, tissue factor

activity is increased in carotid homogenates from antiphospholipid antibody-treated mice, which correlates with increased leukocyte-endothelium interplay.[76,237] In keeping with the latter concept, antagonizing either E-selectin or P-selectin (the key selectins expressed on endothelium) is protective against thrombosis in mice; the same is true for strategies blocking the endothelial integrin ligands VCAM-1 and ICAM-1.[77,78] One study has suggested that downregulation of endothelial nitric oxide synthase by antiphospholipid antibodies may be another important factor in increased leukocyte-endothelium interplay.[79] Beyond these *in vivo* data, there is robust evidence *in vitro* that antiphospholipid antibodies can activate endothelial cells to express tissue factor and adhesion molecules.[238,239] Mechanistically, NF-κB, p38 MAPK, and Krüppel-like factors (KLFs) have all been implicated in antiphospholipid antibody-mediated activation of endothelial cells, demonstrating how antiphospholipid antibodies may co-opt pathways normally associated with more “authentic” activating stimuli.[240–242]

Mac-1 is a heterodimeric beta-2 integrin primarily expressed by myeloid-lineage cells. In its activated state, Mac-1 mediates cell-cell interactions by engaging a variety of surface molecules, including the endothelium-expressed glycoprotein ICAM-1. In this study, we focused not on the endothelium, but rather leukocytes (and especially neutrophils), in terms of what they bring to the heterotypic adhesive interactions relevant to APS. We interrogated both fresh APS patient blood and APS plasma-conditioned leukocytes for leukocyte adhesion to resting endothelial cells under flow. We also characterized key adhesion molecules, including Mac-1, on the surface of APS neutrophils, exploring their role in not just adhesion, but also NETosis.

6.4 Results

APS patient neutrophils demonstrate increased adhesion under flow.

Utilizing anticoagulated whole blood collected from primary APS patients or matched healthy volunteers (Table 2.1), we tested leukocyte adhesion to early-passage human umbilical vein endothelial cells (HUVECs) in a parallel-plate flow chamber (PPFC) assay. Representative images of leukocyte adhesion in the PPFC assay are shown in Figure 6.1A. As compared with control blood, we observed increased adhesion of APS leukocytes under high-shear (1000 s-1) pulsatile flow conditions, as might be found in arteries or the arterioles (Figure 6.1B). Similar results were observed when blood was

passed through the chamber under lower-shear (200 s-1) laminar flow, as would be found in the venous system (Figure 6.1B). If the adhesion were being driven by factors inherent to the leukocytes themselves, we reasoned that a similar phenotype would be observed when plasma (along with cytokines and autoantibodies that might activate the HUVECs) were discarded. Indeed, isolated APS patient leukocytes, in the absence of plasma, still adhered in exaggerated fashion to HUVECs (under both high- and low-shear flow conditions) (Figure 6.1C). Finally, we removed not just plasma, but also peripheral blood mononuclear cells by spinning the blood through a density gradient. Again, we observed increased adhesion of neutrophils to HUVECs as compared with controls (Figure 6.1D). Insummary, these data reveal that leukocytes, and specifically neutrophils, demonstrate increased adhesion to unstimulated HUVECs under various flow conditions. The phenotype remained even after plasma was discarded, consistent with an inherent role for neutrophils in the adhesive interaction.

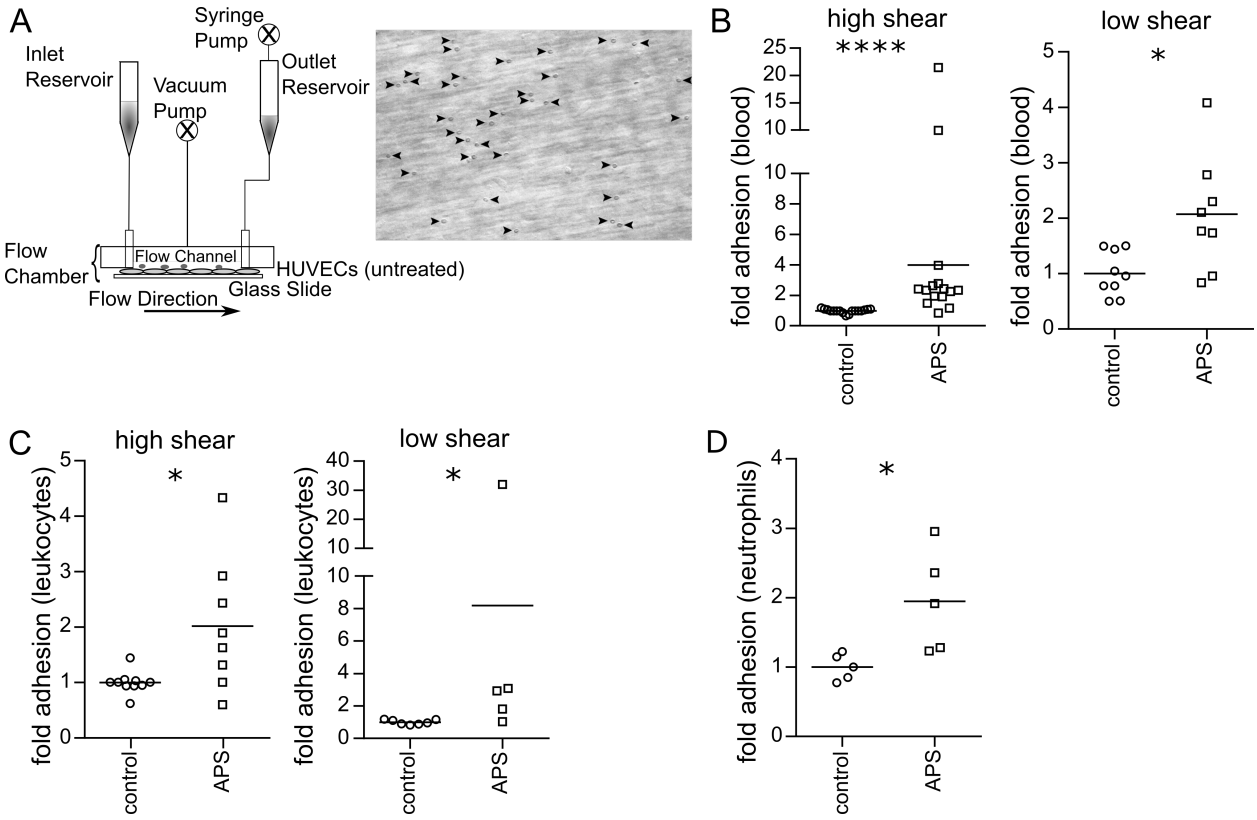

Figure 6.1 - APS neutrophils demonstrate increased adhesion. Adhesion was measured under either pulsatile, high-shear (1000 s-1) conditions or laminar, low-shear (200 s-1) conditions. (a) Schematic of the parallel plate flow chamber, and a representative image from the adhesion assay. (b) Anticoagulated whole blood from healthy controls or APS patients were perfusedthrough the flow chamber. At the end of

the run, adherent cells were quantified; ****p<0.0001 (Mann Whitney test) and *p<0.05 (t test). (c) Control or APS leukocytes were isolated, resuspended in flow buffer (plasma discarded), and perfused through the flow chamber. At the end of the run, adherent cells were quantified; *p<0.05 (Mann Whitney test). (d) Control or APS neutrophils were isolated, resuspended in flow buffer, and perfused through the flow chamber. At the end of the run, adherent cells were quantified; *p<0.05 (t test).

Adhesion molecules are upregulated on the surface of APS patient neutrophils.

In an effort to understand what seemed to be an inherent increase in APS neutrophil adhesion, we evaluated the surface expression of various adhesion molecules on the neutrophil surface (Figure 6.2A). As ICAM-1 is known to be expressed even by resting HUVECs, we reasoned that beta-2 integrin family members (which are well known to interact with ICAM-1) might be upregulated on APS neutrophils, thus mediating the increased adhesion. While we observed no difference in the activated form of beta-2 integrin LFA1 (Figure 6.2B), the activated form of another beta-2 integrin, Mac1, was robustly upregulated on the surface of APS neutrophils (Figure 6.2C). In support of these data, an evaluation of other potential markers of neutrophil activation revealed no significant difference in CD62L (L-selectin), but did reveal upregulation of both CD64 and CEACAM1 (Figure 6.2D-F). Interestingly, autoantigen β2GPI was also present at increased levels on the surface of APS neutrophils (Figure 6.2G). In summary, these data demonstrate increased expression of activated Mac1, but not activated LFA-1, on the neutrophil surface, which correlates with the upregulation of other known neutrophil activation markers such as CD64 and CEACAM1.

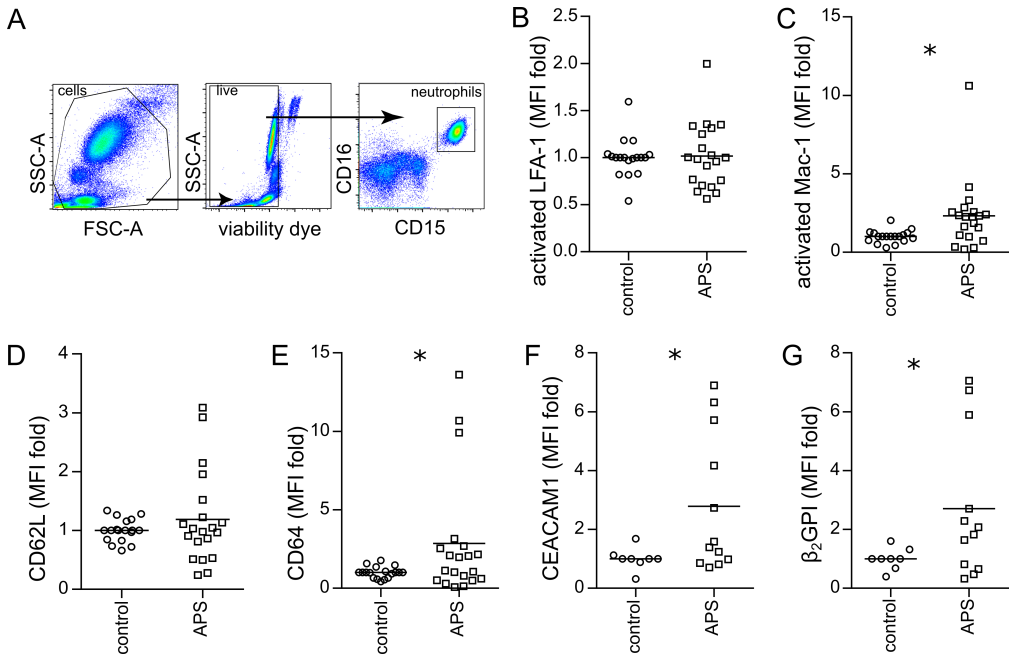

Figure 6.2 - Increased expression of activated Mac-1 and other adhesion molecules on APS neutrophils. Flow cytometry was performed after treating anticoagulated whole blood with fluorescently-labeled antibodies. Mean fluorescence intensity (MFI) was normalized to controls run in the same batch. (a) Schematic of gating strategy for identification of neutrophils in whole blood. (b) Activated LFA1 (not significant by t test). (c) Activated Mac1 (*p<0.05 by t test). (d) CD62L (not significant by t test). (e) CD64 (*p<0.05 by t test). (f) CEACAM1 (*p<0.05 by t test). (g) Beta-2 glycoprotein I (*p<0.05 by t test).

APS IgG mediated upregulation of Mac1 on neutrophils is dependent on TLR4 and complement anaphylatoxins receptors.

Previous work has demonstrated that NETosis can be triggered from control neutrophils by incubation with either APS serum or APS IgG. Here, we explored whether adhesion molecules were also upregulated by similar treatment (Figure 6.3A). When we "conditioned" control blood cells with APS plasma, we did not find increased expression of activated LFA on the surface of neutrophils (Figure 6.3B). In contrast, there was a striking increase in surface expression of activated Mac1 (Figure 6.3C). We also found evidence of shedding of CD62L from neutrophils, and upregulation of both CD64 and CEACAM1 (Figure 6.3C-E). β2GPI was measured, but was not significantly upregulated (Figure 6.3F).

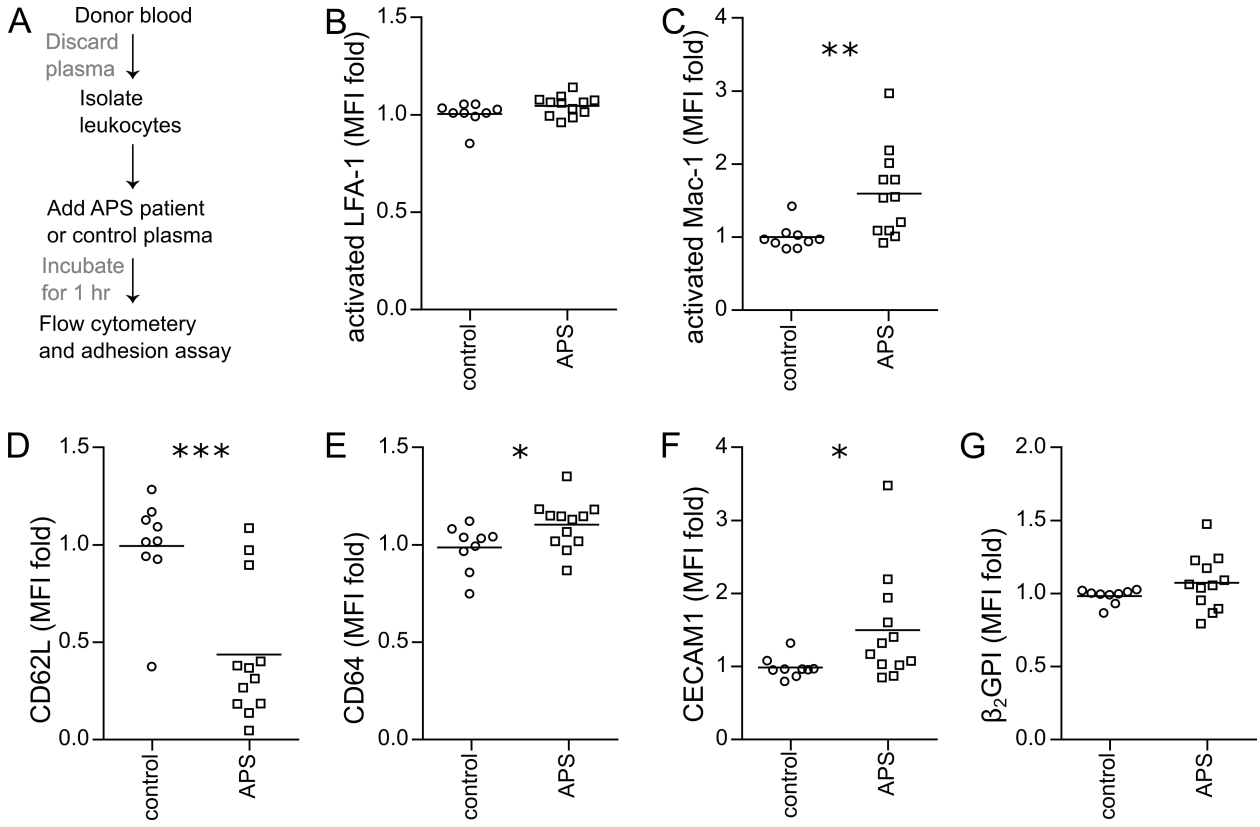

Figure 6.3—Increased expression of activated Mac-1 and other adhesion molecules when control neutrophils are conditioned with APS plasma. Control leukocytes were conditioned with heterologous control plasma or APS plasma (a), and then incubated with fluorescently labeled antibodies. Mean fluorescence intensity (MFI) was normalized to controls run in the same batch. (b) Activated LFA1 (not significant by t test). (c) Activated Mac1 (**p<0.01 by t test). (d) CD62L (***p<0.001 by t test). (e) CD64 (*p<0.05 by t test). (f) CEACAM1

We then turned our attention to conditioning control blood with purified IgG from primary APS patients. Under these conditions, we observed upregulation of activated Mac1 on neutrophils (Figure 6.4A), along with shedding of CD62L (Figure 6.4B). Having previously observed that APS IgG-mediated NETosis is dependent upon Toll-like receptor 4 (TLR4), we assessed that same pathway here—now in the context of Mac1 activation. Indeed, the TLR4- signaling inhibitor TAK-242 prevented APS IgG from upregulating activated Mac1 on neutrophils (Figure 6.4C). We reasoned that we might also find a role for the complement cascade in neutrophil activation. When blood was treated with a C5a receptor-inhibitory antibody, upregulation of activation Mac-1 on neutrophils was blunted (Figure 6.4D). In summary, these data together indicate that control neutrophils

upregulate activated Mac1 in response to conditioning with either APS patient plasma or IgG, and that this upregulation requires TLR4 and the C5a receptor.

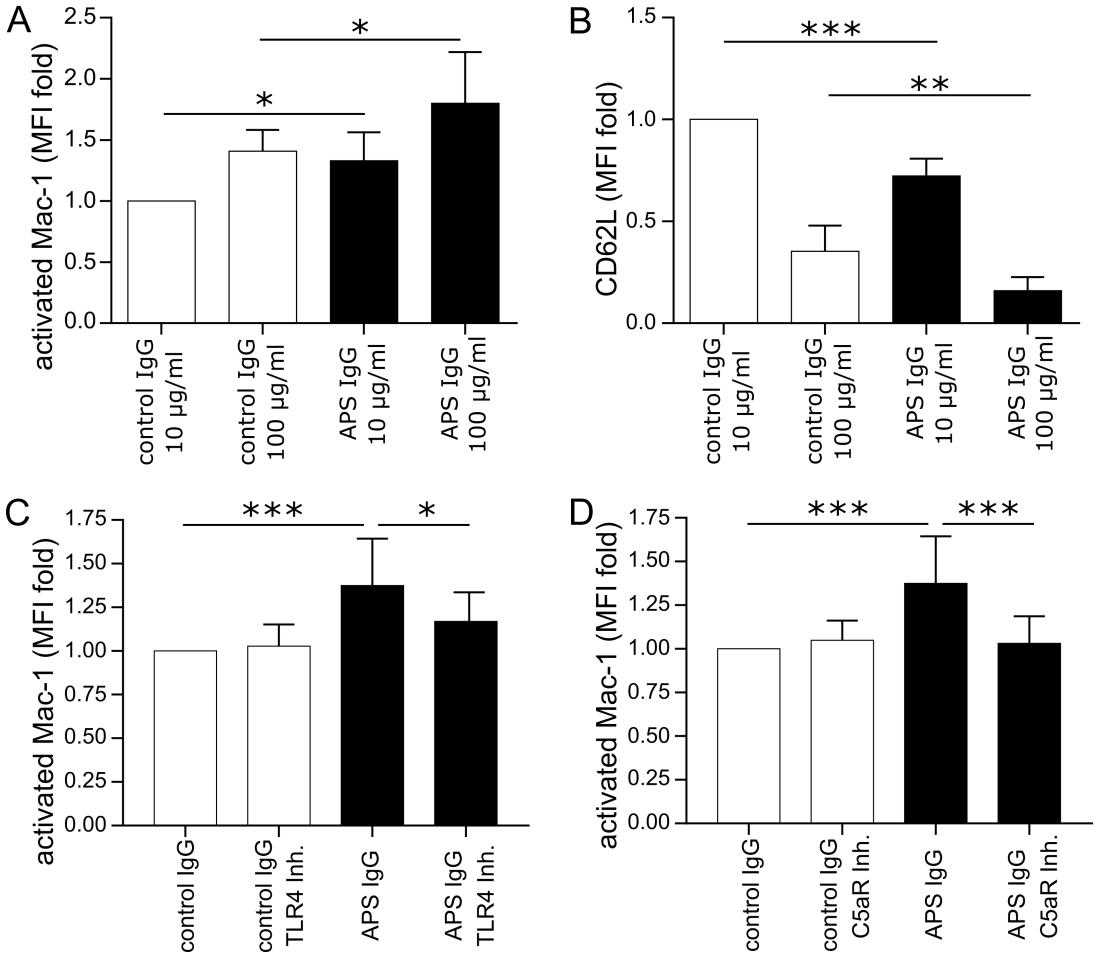

Figure 6.4 - Exposure to purified APS IgG increases the expression of activated Mac1 on control neutrophils in TLR4- and complement-dependent fashion. Control leukocytes were treated with control or APS IgG as indicated. Activated Mac1 and CD62L were quantified by flow cytometry. (a) Activated Mac1 (*$p<0.05$ by one-way ANOVA with correction for multiple comparisons by Holm-Sidak method; n=4 independent experiments). (b) Shedding of CD62L (**$p<0.01$ and ****$p<0.0001$ by one-way ANOVA with correction for multiple comparisons by Holm-Sidak method; n=4 independent experiments). (c) Control leukocytes were treated with control or APS IgG (100 µg/ml) in the presence or absence of TLR4 inhibitor. Activated Mac1 was quantified by flow cytometry (*$p<0.05$ and ***$p<0.001$ by one-way ANOVA with correction for multiple comparisons by Holm-Sidak method; n=8 independent experiments). (d) Control leukocytes were treated with control or APS IgG (100 µg/ml) in the presence or absence of C5a receptor (C5aR) inhibitor. Activated Mac1 was quantified by flow cytometry (***$p<0.001$ by one way ANOVA with correction for multiple comparisons by Holm-Sidak method; n=7 independent experiments).

Activated Mac1 is required for increased adhesion of APS neutrophils.

Having found that APS plasma up-regulates Mac-1 on the surface of control neutrophils, we reasoned that this up-regulation might be directly responsible for increased neutrophil adhesion. Indeed, APS plasma–treated cells, but not control plasma–treated cells, demonstrated increased adhesion under both high-shear and low-shear flow conditions (Figure 6.5A). Furthermore, a monoclonal antibody specific for the activated form of Mac-1 effectively neutralized adhesion in the context of conditioning with APS plasma, but had no effect in the setting of control plasma (Figure 6.5B). To determine whether the ability of plasma to stimulate cell adhesion was unique to patients with APS or whether the phenotype might extend to any patient a with history of thrombosis, we recruited 11 patients with a history of unprovoked VT but with test results negative for aPLs. When compared to plasma samples from healthy controls, plasma samples from the VT cohort triggered no increase in cell adhesion (Figure 6.5C). Similar to this finding, conditioning neutrophils with plasma from the VT cohort did not alter levels of activated Mac-1, CD62L, or CD64 (Figures 6.5D–F) on the neutrophil surface. Finally, we asked whether the increased cell adhesion triggered by APS plasma might be limited to patients with a history of “thrombotic APS” (i.e., at least 1 documented arterial, venous, or small vessel thrombotic event). In summary, these data demonstrate that antagonizing the activated form of Mac-1 is sufficient to reduce APS-relevant adhesion to levels seen in controls.

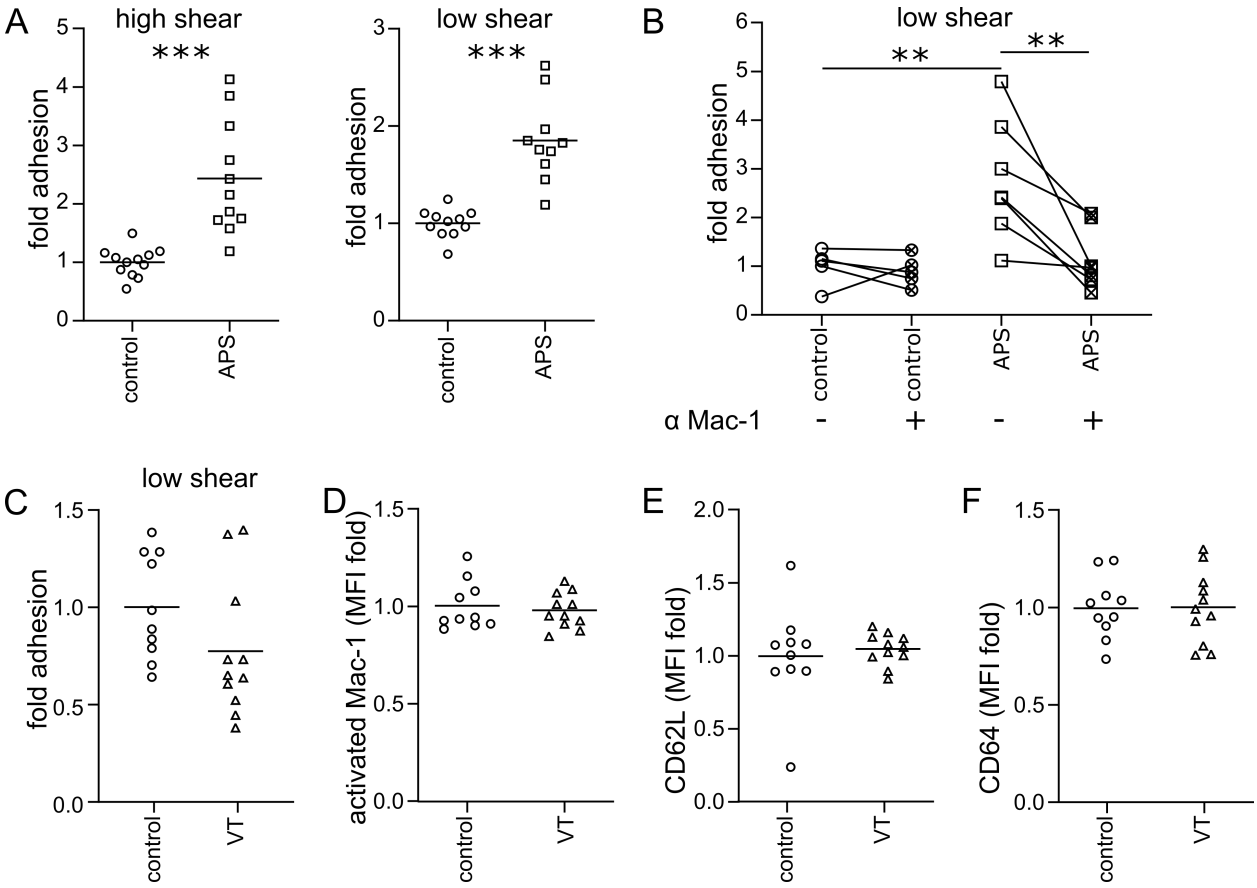

Figure 6.5 - Increased adhesion of APS leukocytes mediated by activated Mac-1. Control leukocytes were incubated with heterologous control or APS plasma, resuspended in flow buffer, and perfused through the flow chamber. Adherent cells were quantified. A, Increased adhesion of APS plasma–treated cells. *** = $P < 0.001$ by t-test versus controls. B, Conditions similar to A, except with the addition of a blocking antibody for activated Mac-1 to some samples. ** = $P < 0.01$ by one-way analysis of variance (ANOVA) with correction for multiple comparisons by the Holm-Sidak method. C, Conditions similar to A, control leukocytes incubated with heterologous control plasma or plasma from patients with history of unprovoked venous thrombosis (VT) and negative test results for antiphospholipid antibody. The leukocytes were then resuspended in flow buffer and perfused through the flow chamber. Adherent cells were quantified (not significant by t-test). D–F, Similar to Figure 3, control leukocytes conditioned with heterologous control plasma or VT plasma and incubated with the fluorescently-labeled antibodies activated Mac-1 (not significant by t-test) (D), CD62L (not significant by t-test) (E), and CD64 (not significant by t-test) (F). MFI was normalized to controls run in the same batch. Symbols represent individual samples. See Figure 6.2 for other definitions.

Activated Mac1 is required for NETosis by APS neutrophils bound to endothelial cells.

Given recent evidence that NETosis proceeds most efficiently upon cell adhesion, we asked whether the aforementioned antibody targeting activated Mac1 might mitigate NETosis. To test this, we adhered vital-dye-labeled neutrophils to resting HUVECs, and

then tracked NETosis in real time via the loss of vital dye and the local release of decondensed DNA (Figure 6A). As compared with isotype treatment, the Mac1 monoclonal antibody significantly neutralized NETosis in response to APS IgG, but not the phorbol ester PMA (Figure 6B). In summary, these data demonstrate that inhibition of the activated form on Mac1 can neutralize NETosis, at least in the context of APS.

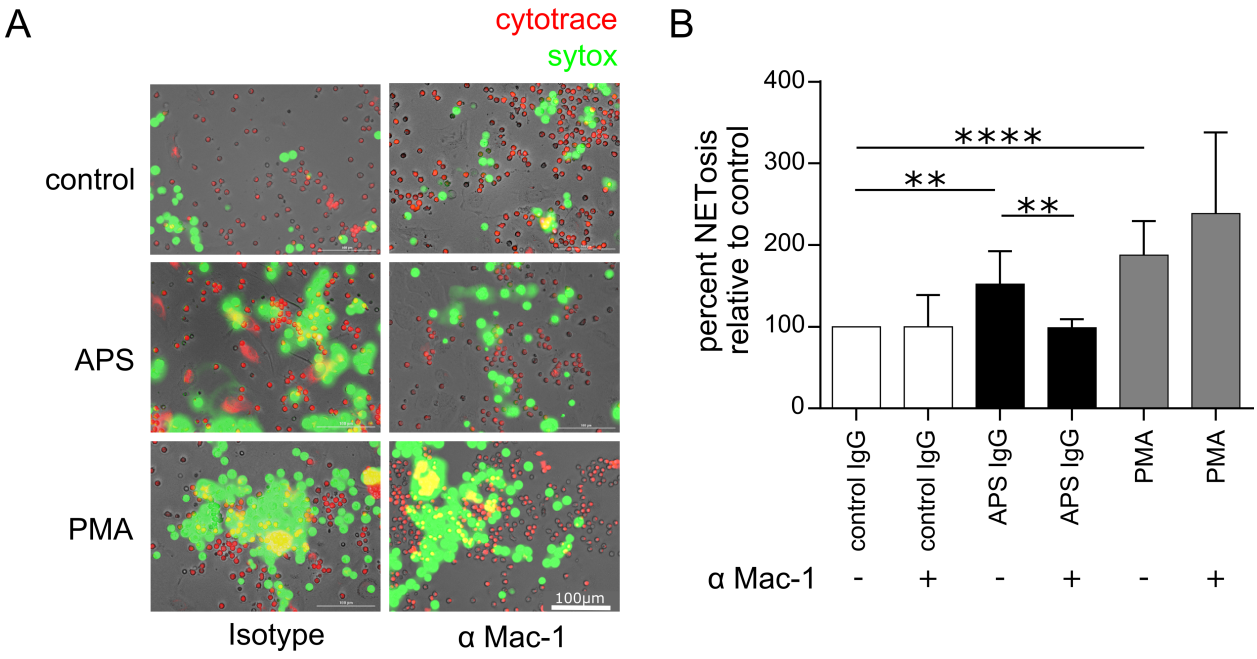

Figure 6.6 - Activated Mac-1 regulates APS IgG-mediated NETosis. Control neutrophils were plated over a monolayer of HUVECs in the presence of either activated Mac1 blocking antibody or isotype control. Cultures were then stimulated with control IgG (100 µg/ml), APS IgG (100 µg/ml), or phorbol myristate acetate (PMA, positive control). After 3 hours, SYTOX Green was added to the culture, and fluorescence intensity was quantified. A, In these representative images, live cells are labeled by CytoTrace™ Red and extracellular DNA (NETs) by SYTOX Green. Scale bar=100 µm. B, Quantification of NETosis by fluorescence intensity of SYTOX Green (**p<0.01 and ****p<0.0001 by one-way ANOVA with correction for multiple comparisons by Holm-Sidak method; n=4 independent experiments).

6.5 Discussion

While there are many studies characterizing activated endothelium in APS (10, 14), comparatively little is known about the adhesive nature of circulating cells (26).[76,238,243] In this study we examined the adhesive potential of APS leukocytes, and in particular, neutrophils. We found augmented adhesion of APS neutrophils to resting HUVECs irrespective of the flow conditions. Notably, this functional increase in adhesion was in the context of upregulated adhesion molecules on the neutrophil surface including CD64, CEACAM1, and the activated form of Mac1. Of note, all flow experiments were performed in the presence of red blood cells, which are known to stabilize leukocyte adhesion, more closely modeling conditions observed in vivo.[244]

In previous work, it was shown that inhibition of TLR4 signaling could mitigate APS IgG-mediated NETosis.[236] This is in addition to the work of others demonstrating that TLR4 deficiency protects mice form APS *in vivo*, and that neutrophil TLR4 supports phagocytosis and reactive oxygen species production by APS neutrophils.[237,245] We now show that the TLR4 inhibitor TAK-242 prevents APS IgG from upregulating activated Mac1 on neutrophils. These data would seem to support further investigation of TLR4 signaling as a potential therapeutic target in APS. Along with TLR4 pathway, complement contributes to neutrophil activation in many contexts. Here, we show that inhibition of C5a receptor attenuates the upregulation of activated Mac1 on APS IgG-stimulated neutrophils. These data are in line with previous studies demonstrating that C5a receptor contributes to upregulation of Mac1.[246,247]

Despite extensive CD62L shedding in control neutrophils conditioned with APS plasma, CD62L shedding was not detected at a significant level in neutrophils freshly isolated from APS patients. One possibility is that the patient neutrophils have had time to upregulate CD62L expression, thereby effectively compensating for shedding. In support of this hypothesis, CD62L was upregulated at the gene level in a recent transcriptomic profiling of APS neutrophils.[75] Alternatively, it is possible that neutrophils that have shed CD62L in vivo are strongly activated to the point that they have preferentially left circulation, thereby being unavailable for our expression analysis.

Blocking experiments demonstrate that the adhesion of APS neutrophils is at least partially mediated by activated Mac1. Interestingly, we also found that APS IgG-mediated

NETosis was Mac1 dependent. This latter finding is reminiscent of work by Neeli and colleagues, who found Mac1 to be required for both hypercitrullination of histones and NETosis in response to lipopolysaccharide stimulation.[248] Interestingly, both lipopolysaccharide and APS IgG activate neutrophils via TLR4.[236] Collectively, the data presented here reveal a previously unknown role for activated Mac1 in the adhesion and NETosis of APS neutrophils.

Beyond activated Mac1, we also observed consistent upregulation of CD64 on the surface of APS neutrophils. This is somewhat reminiscent of work in patients with sickle cell disease. Sickle cell neutrophils demonstrate increased levels of CD64 and increased adhesion to endothelial cells, with some evidence that CD64 directly contributes to the adhesion.[249,250] Future studies should ask whether this surface molecule, typically thought of as an IgG receptor, might also play a role in APS neutrophil adhesion. CEACAM1 (CD66a) expression was also consistently upregulated on APS neutrophils. Interestingly, there are studies to suggest that signaling through CEACAM1 results in activation of Mac1 and increased adhesion to endothelial cells.[251,252] CEACAM family members are involved in neutrophil adhesion by activation of neutrophils and via regulation of the activity of other adhesion molecules.[253,254] At the same time, recent reports have suggested that CEACAM1 may have inhibitory functions, protecting against neutrophil hyperactivation and neutrophil-mediated tissue damage. For example, studies in mice have demonstrated that CEACAM1 protects against ischemic stroke by inhibiting MMP9.[255,256] CEACAM1-deficient mice also form larger carotid thrombi in a ferric chloride injury model, suggesting that CEACAM1 may inhibit arterial thrombus.[257] Whether these studies are simply indicating a difference between humans and mice, or whether they are indicating that CEACAM1 activation might be pursued as a therapeutic target in APS, remains to be determined.

In the general population, numerous reports have suggested a link between Mac1, neutrophils, and endothelium in thrombotic vascular diseases.[258–260] For example, significant upregulation of neutrophil Mac1 has been detected at the time of myocardial infarction and for up to one week after the event.[261] In another study, neutrophils from myocardial infarction patients displayed enhanced adhesion to endothelial cells ex vivo, which could be reduced by blocking Mac1.[262] In patients with acute ischemic stroke,

there was significant upregulation of neutrophil Mac1 immediately after the event, and persisting into the subacute phase of the stroke.[263] In patients with venous thromboembolism, increased adhesive potential of neutrophils was associated with a higher rate of recurrence.[264] The average time from last thrombotic event to blood collection for subjects included in this study was some 4.5 years. One might hypothesize that upregulation of activated Mac1 is detected only acutely (i.e., at the time of thrombosis) for persons in the general population, but continues to smolder in APS patients, potentially consistent with the life-long anticoagulation that such patients require. To further explore this question, it will be necessary to build longitudinal APS cohorts and study them alongside cohorts from the general population.

6.6 Conclusions

In summary, our study has uncovered a novel role for activated Mac1 in regulating APS neutrophils and NETosis, and hints at a role for Mac1 in APS pathophysiology. While Mac1 can be considered as a therapeutic target in APS, mutations in CD11b are a well-recognized risk factor for lupus, and many, but not all, mouse studies have suggested that CD11b deficiency has the potential to exacerbate autoimmunity.[265–268] Having said that, since Mac1 binds to a variety of ligands, selective inhibition of specific Mac1 interactions could emerge as a potential therapeutic strategy. For example, one proof-of-concept study has demonstrated that targeted inhibition of the Mac1-CD40L interaction improved bacterial clearance and survival in a polymicrobial model of sepsis.[269] Another innovative approach has involved the utilization of small-molecule Mac1 agonists. These agonists tend to induce an intermediate-affinity conformation in Mac1, which may permit neutrophil adhesion, with less potential for endothelial damage.[270] Indeed, a partial Mac1 agonist not only protected MRL/lpr mice from organ injury and but also enhanced endothelium-dependent vasorelaxation, demonstrating a vasprotective effect.[271] Taken together, these studies indicate targeting Mac1 might indeed be feasible, and emphasize the need for future research in APS patients.

Chapter 7: Conclusions

7.1 Overall Conclusions

This dissertation aims to illuminate the impact of particle drug carriers on the behavior of leukocytes, particularly neutrophils, in inflammatory diseases, as well as how neutrophils may contribute to certain inflammatory conditions. Despite the many decades of research into inflammatory diseases and various therapeutic formulations for the treatment of said diseases, the role of neutrophils in propagating inflammation and in phagocytosis has only recently begun to be fully appreciated. Thus, a great deal of work remains to be done investigating both how particle drug carrier formulations interact with neutrophils and how neutrophils contribute to inflammatory disease. Although particle drug carriers for the delivery of anti-inflammatory therapeutics remain a promising avenue for improving outcomes, it is imperative that we first establish the effects of such drug carriers on cells of the innate immune system involved in the inflammatory response in order to fully evaluate their efficacy. It is likely that many different particle properties, including particle size, shape, surface chemistry, material, and ligand density (among others) have an impact on the interactions between particle drug carriers and neutrophils, and all of these effects should be investigated fully. In this work, we aimed to begin an investigation into some of these effects, exploring how particle drug carriers modulate neutrophil adhesion in inflammation both *in vitro* and *in vivo*, how PEGylation of drug carriers impacts phagocytosis by neutrophils, whether salicylic acid-based particles can be used as a therapeutic for the treatment of inflammatory disease such as ARDS, and how neutrophils contribute to some of the inflammatory pathophysiology of antiphospholipid syndrome.

In Chapter 3, we evaluated the impacts of the presence of particle drug carriers on the neutrophil response to inflammation. To this end, we employed an *in vitro* PPFC model of blood flow to evaluate how free-stream collisions, specific targeting to the endothelium, and particle uptake affect neutrophil adhesion in inflammation. Here, we

found that all of these mechanisms result in decreased neutrophil adhesion, with particle uptake dominating the other two effects. These results were validated in two *in vivo* models of inflammation, wherein intravenous particle injections decreased neutrophil adhesion in an acute mesentery inflammation model and an acute lung injury model. Importantly, the magnitude of these effects are greatly dependent upon both particle size and concentration, with larger (particularly micron-sized) particles and particles at higher concentrations resulting in a greater decrease in neutrophil adhesion.

In Chapter 4, we investigated whether a commonly-employed nonfouling coating, PEG, was effective at reducing phagocytosis by primary human neutrophils. Surprisingly, and contrary to the literature consensus, we found that PEGylation actually increases phagocytosis of particle drug carriers by human neutrophils while reducing phagocytosis by other major phagocytes. This enhanced phagocytosis was observed regardless of PEG chain length and end group. We were able to specifically link this effect to factors in human plasma, and specifically human complement proteins.

In Chapter 5, we sought to leverage the results in Chapter 3 to develop a therapeutic strategy for reducing the pathophysiological effects of neutrophils in *in vivo* models of inflammatory disease. Thus, we developed salicylate-based microparticles formulated from PolyAspirin or PolyA polymer graciously supplied by Dr. Kathryn Uhrich's group at UC-Riverside. Here, we found that these PolyA particles significantly reduce neutrophil infiltration into the lungs in a model of acute lung injury, resulting in a greater impact than either PLGA or polystyrene. Further, we see that PolyA particles significantly reduce neutrophil infiltration and inflammatory cytokine production in the lungs of mice infected with *P. Aeruginosa* bacteria and, most importantly, prevent systemic infection and mortality.

In Chapter 6, we partnered with Dr. Jason Knight's group to explore the behavior of neutrophils in a chronic inflammatory autoimmune disease, antiphospholipid syndrome. Here, we used our *in vitro* PPFC model to compare the adhesion of APS-derived neutrophils to healthy donor neutrophils on a quiet endothelium, finding that APS

neutrophils display greatly-enhanced adhesion. We find that this effect is driven by an upregulation of Mac-1 on APS neutrophil surface, which itself results from a TLR4-dependent pathway. Further, we find that APS neutrophils display an enhanced propensity to undergo NETosis, dependent upon increased Mac-1 expression. These novel results show that neutrophils may play a significant role in the pathophysiology of APS and highlight potential therapeutic targets for treating the disease.

In conclusion, we found that particle drug carriers impact neutrophil function and behavior in many contexts, some of which are unexpected. Particle drug carriers reduce neutrophil adhesion in inflammation through free-stream collisions, specific blocking of endothelial surface, and particle uptake. Further, particle surface chemistry does not necessarily impact phagocytosis in the same way as it does with other phagocytes. We explored the possibility of using particles to modulate the neutrophil response in inflammatory conditions as a therapeutic strategy, finding that this strategy holds great promise and should be further explored. Finally, we found that neutrophils in APS display enhanced adhesion to a quiet endothelium *via* Mac-1, which may be a therapeutic target for the treatment of APS.

7.2 Impact and Future Directions

The work in this dissertation highlights the importance of interactions between particle drug carriers and neutrophils, as well as the heretofore underappreciated role of neutrophils in inflammatory diseases, setting the stage for significant further exploration into these interactions. While much progress has been made on his front within this dissertation and elsewhere, much work remains to be done both to improve the state of particulate drug delivery and to understand the many ways in which neutrophils contribute to inflammatory disease.

The work in Chapter 3 is the first systematic exploration of the impact of targeted and nontargeted particulate drug carriers on leukocyte (particularly neutrophil) adhesion in blood flow. Given the prevalence of literature on the subject of targeted particle drug carriers, with 24,153 research articles published on nanoparticle technology in 2018

alone, this work is crucial to understanding how such particle drug carriers interact with the immune system and their ultimate efficacy.[1] Further, the work in Chapter 3 provides the first *in vivo* evidence of rapid association of particle drug carriers with neutrophils in blood circulation, resulting in reduced neutrophil adhesion and transmigration in acute inflammation. This result reorients the conversation around particle-immune cell interactions to focus on neutrophils, highlights the dominant role neutrophils play in particle clearance, and suggests that particle drug carriers may provide a platform by which we are able to modulate neutrophil function in inflammation.

Moving forward, there is a great deal of future work that logically flows to follow from the work in Chapter 3. In the *in vitro* PPFC experiments, we primarily explored the impact of two particle parameters, particle size and concentration, on the reduction in leukocyte adhesion. However, given the evidence that other particle properties, such as shape, density, choice of targeting ligand, ligand density, and particle surface chemistry, have a major impact on particle performance, the effects of these properties on leukocyte adhesion should be further explored.[87,92,110,118,131,272] Further, we find that particle internalization plays a dominating role in reducing neutrophil adhesion, likely by inducing CD62L shedding; however, elucidating the cellular mechanism by which this occurs is well outside the scope of this work, and should be done in a follow-up study. Defining this mechanism is crucial for understanding how to effectively design particle drug carriers to either avoid or leverage this effect. Digging further, the impacts of particle internalization on other crucial neutrophil functions (e.g., NETosis, and granule release) have yet to be evaluated. As all of these functions play a major role in the neutrophil response to inflammation, understanding how particle internalization modulates each of them will provide a more complete picture of the ultimate outcome of the interactions between particle drug carriers and neutrophils.[16,38,273] Finally, the *in vivo* work in Chapter 3 is limited to murine models of inflammation. While these models are convenient due to their simplicity and reproducibility, they often do not fully replicate the effects observed in humans. Importantly, the proportions of immune cell populations in mice differ

drastically from those in humans, potentially creating entirely different effects.[201] Thus, further work should explore these effects in a system which more-closely replicates the human immune system, either through a “humanization” approach to murine models or through other animal models with immune systems which more closely resemble that of humans, such as pigs or nonhuman primates.[274–276]

The work in Chapter 4 provides the first evidence that PEGylation enhances particle phagocytosis by human neutrophils in blood, cutting against the conventional wisdom that PEGylation will necessarily improve particle circulation time in humans and, thus, the efficacy of particle drug carriers. This work represents a major shift in the particle drug carrier field, both in terms of the efficacy and applicability of PEG as a nonfouling coating and in terms of the ways in which we characterize particle uptake in the field. Because PEGylation clearly reduces phagocytosis in many *in vitro* cellular uptake assay conditions and enhances circulation time in mice, a conventional wisdom developed that PEGylation improves the performance of particle drug carriers. This work, and other work in our lab, contradict that wisdom, emphasizing the need to thoroughly characterize particle drug carrier platforms in a wide range of conditions.[118]

While the work presented in Chapter 4 makes major strides in advancing the understanding of how a commonly-employed nonfouling coating impacts particle drug carrier phagocytosis by neutrophils, much work remains to be done. Most obviously, the inefficacy of PEG at reducing phagocytosis by human neutrophils highlights the need for the development and characterization of other nonfouling coatings to create truly long-circulating particle drug carrier platforms. These may come in the form of zwitterionic coatings or biomimetic coatings which mimic the surface of immune cells.[277] For example, recent work suggests that the use of RBC-derived cell membranes or leukocyte cell membranes may effectively reduce particle opsonization and clearance by phagocytes, thus improving drug carrier performance.[125,278–281] However, further characterization of these effects on neutrophils, specifically, must be performed. Additionally, while we were able to link the enhanced phagocytosis of PEGylated particles

to complement proteins, the exact mechanism by which neutrophils preferentially phagocytose PEGylated particles is not fully understood. Fully fleshing out this mechanism, as well as the various other factors which drive neutrophil phagocytosis, would provide a more well-informed basis by which to design effective particle drug carriers to either evade or target neutrophils. Finally, the work in Chapter 4 reveals that the bulk of the literature regarding how various particle parameters, such as particle shape and surface chemistry, impact phagocytosis and clearance, may not hold when it comes to neutrophils. Thus, further characterization of how these parameters modulate neutrophil particle phagocytosis is required.

The work presented in Chapter 5 demonstrates, for the first time, that the use of intravenously-administered, drug-free particles to reduce neutrophil recruitment in inflammation has potential to be used as a therapeutic strategy. In this work, we fabricated micron-sized particles made from PolyA, a polymer comprised of repeating units of salicylic acid. We then injected these particles into mice in both an LPS-induced ALI model and a *P. Aeruginosa* infection model of ARDS. In both models, the PolyA particles reduced neutrophil infiltration into the lungs and reduced the concentration of various inflammatory cytokines. Crucially, in the ARDS model, PolyA particles were able to prevent the infection from spreading systemically, and ultimately resulted in greatly improved survival. These results, especially viewed in light of other recent work using drug-free particles to improve outcomes in EAE, spinal cord injury, West Nile virus, and sepsis, prove a novel basis for the development of therapeutic strategies using particles themselves to treat inflammatory diseases.[130,159]

While the work in Chapter 5 provides exciting new potential avenues for the treatment of inflammatory diseases, much work remains to be done. First and foremost, the exact mechanism by which PolyA particles alleviate ALI/ARDS symptoms and improve survival remains unsettled. We hypothesize that PolyA particles are directing neutrophils away from the site of inflammation, allowing some neutrophils to infiltrate the lungs and destroy the bacteria without doing serious damage to the lung tissue which

tends to result in systemic inflammation. Additionally, we hypothesize that the degradation of PolyA particles to salicylic acid confers some additional anti-inflammatory effects. However, this has yet to be fully confirmed, and further studies should be undertaken to flesh out this mechanism; *ex vivo* experiments examining the degradation of PolyA particles in the presence of neutrophils, as well as their response to inflammatory molecules such as LPS, would provide a great deal of insight into this outstanding question. After fully elucidating the mechanism by which PolyA particles provide a therapeutic effect in ALI/ARDS, the formulation of PolyA particles should be optimized to provide maximum benefit. This work utilizes a single particle formulation as a proof of concept study; however, many properties of both the polymer and the particles can be altered to change the ultimate impact. Altering the particle size, polymer molecular weight, choice of linker, or dosing scheme could all change the efficacy of PolyA particles in treating ALI/ARDS. Further, the addition of ligands for targeting inflammation may confer an additional therapeutic benefit. Finally, given the wide range of potential applications for the use of PolyA particles in treating inflammatory diseases, these particles should be explored as a therapeutic in other conditions such as sepsis.

The work in Chapter 6 demonstrates a novel role for neutrophils in the pathophysiology of a chronic inflammatory autoimmune disease, APS. Specifically, we demonstrated that APS neutrophils exhibit enhanced adhesion to a quiet endothelium, mediated by an increased expression of Mac-1 on the surface of APS neutrophils. This increased Mac-1 expression was linked to a TLR4-dependent pathway. Further, the increased expression of Mac-1 was found to promote increased NETosis in APS neutrophils. These results provide an evidentiary basis for the role that neutrophils play in APS and highlight potential new therapeutic targets for the treatment of APS.

Moving forward, there is yet more work to be done to build on the results obtained in Chapter 6. In addition to the finding that upregulated Mac-1 mediates increased adhesion of APS neutrophils on a quiet endothelium, we also found upregulated CD64, an FC receptor which binds IgG, on the surface of APS neutrophils. While our study did

not focus on the role of CD64 in APS, future work should explore whether CD64 is partly responsible for the role of neutrophils in APS. Similarly, we found upregulated CEACAM1 on the surface of APS neutrophils, the effects of which should also be explored. More broadly, the results of our work in Chapter 6 provide a potential new therapeutic target, activated Mac-1, for the treatment of APS. Future work should explore the efficacy of activated Mac-1 in the treatment of APS, beginning with the use of anti-Mac-1 antibodies in murine models of antiphospholipid syndrome.[282] Additionally, given our work in Chapters 3 and 5 demonstrating the ability of drug-free particles to divert neutrophils from inflammation, it may be fruitful to explore the impact of such particles on chronic inflammatory diseases such as APS.

7.3 Overall Outlook

The work presented in this dissertation falls into two main "thrusts": developing a better understanding of how particle drug carriers interact with the innate immune system, particularly neutrophils, and illuminating the role of neutrophils in inflammatory diseases and potentially identifying therapies to mitigate their negative impacts. Here, I detail my own insights into the outlook in each of these areas, as well as the field of particle drug delivery holistically. Additionally, I propose some new and emerging areas of study for the improvement of targeted drug delivery.

For many years, the discussion of interactions between particle drug carriers was primarily limited to phagocytic interactions between carriers and macrophages or monocytes. Recent results from our lab and others have shifted this conversation substantially to examine how particle drug carriers interact with neutrophils, and much of the conventional wisdom in the field is being challenged as a result.[119,157,166,283] Given the recent revelations as to the importance of neutrophils in the efficacy and impact of particle drug carriers, we now have an opportunity to take a step back and consider aspects of particle drug carrier-leukocyte interactions which may have been previously elided over. Based on work in this dissertation, we now know that incubation medium has a major impact as to how leukocytes interact with particle drug carriers. This finding

necessitates a great deal of future work fully characterizing how various particle parameters (size, shape, surface chemistry, surface charge, etc.) affect the protein corona in human plasma and serum, as well as the impacts of individual proteins on downstream interactions between particle drug carriers and leukocytes. For this purpose, a number of techniques to characterize the protein corona composition may be employed, including conventional techniques such as SDS-PAGE, liquid chromatography-mass spectroscopy (LC/MS), and dynamic light scattering (DLS), as well as emerging techniques such as sum frequency generation (SFG), second harmonic generation (SHG), and computational simulations of protein folding on particle surfaces.[284] SFG and SHG are techniques in which visible and infrared lasers are overlapped both spatially and temporally at the protein-surface interface, resulting in a summed vibrational frequency signal which can provide information about the secondary structure of proteins adherent to a surface.[285,286] These techniques, combined with SDS-PAGE and LC/MS, can be used to develop a mechanistic understanding as to which proteins, and which active sites, are crucial in driving particle-leukocyte interactions such as phagocytosis. Additionally, new tools for measuring leukocyte activity may be employed to determine the fully impact of particle drug carriers on leukocyte function. For example, Salminen et al. recently developed a multiscale *in vitro* model for evaluating neutrophil transmigration across an endothelial monolayer which provides a more physiologically-relevant platform for evaluating neutrophil function, as compared to previous models such as transwells.[287] Finally, our work has revealed some major differences between the response of primary human leukocytes to particle drug carriers and the response of some commonly-employed models, such as immortalized cells cultured *in vitro* and murine models. Given that access to primary human cells is limited, more work should be done to develop cultured cell lines and culture conditions which more-accurately replicate the behavior of primary human leukocytes. Given recent work highlighting the impacts of leukocyte-particle drug carrier interactions, as well as the emergence of new tools for evaluating these interactions, I fully believe we are moving toward a space in which we

can begin to effectively design particle drug carriers which can evade (or modulate) interactions with leukocytes, opening the doors to a great deal of unexplored space in the field.

In addition to the unexplored contributions of neutrophils in particle phagocytosis and clearance, neutrophil contributions to inflammatory diseases have also gone largely understudied until recently. The reasons for this are manifold and include the fact that there is a lack of reliable *in vitro* cultured cell models for neutrophil behavior, as well as the fact that neutrophils are difficult to manipulate and do not live long *ex vivo*. However, recent work has highlighted the myriad roles played by neutrophils in inflammatory diseases, including APS, influenza, cancer, cardiovascular disease, and others.[56,57,72,74,75,176,230,236] Further, recent advances in cellular manufacturing technology could alleviate many of the challenges with working with neutrophils *ex vivo*, creating new opportunities to probe important neutrophil functions such as adhesion, phagocytosis, transmigration, and NETosis in inflammatory diseases.[288] In my view, we will soon begin to fully appreciate both the positive and negative aspects of neutrophil contributions to inflammation and use this information to develop more-effective therapeutics for tackling inflammatory disease.

Overall, I believe we are entering an exciting time in the field of particle drug delivery. Over the course of my doctoral work, I have developed a philosophy of learning to take what nature has given us when thinking about designing particle drug carriers, rather than working to fight against nature within the human body. This concept is illustrated most clearly in Chapter 5, where, rather than working so hard to design elaborate particles to evade immune clearance, we seek to leverage these interactions to develop a potential new therapeutic strategy for the treatment of ARDS. Other groups have recently begun employing similar strategies, as previously discussed, in the context of various diseases, from spinal cord injury to West Nile Virus to sepsis.[158,159,220] Additionally, other groups have begun leaning on nature for design inspiration, developing particles which mimic the functions of leukocytes in order to create more-effective particle

drug delivery platforms.[123–126,279,280,289] As we begin to understand more about how particle drug carriers interact with and influence the innate immune system, as well as the positive and negative impacts of immune cells on inflammatory disease, we can begin to draw more inspiration from nature to design simple, elegant particle drug carriers which can leverage the intrinsic interactions with the immune system to enact effective therapies. It is my view that this is the future of the particle drug delivery field, and that the coming decades will see a dramatic increase in effective particle-based therapeutics based on these principles.

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