HARNESSING WHAT LIES WITHIN:
PROGRAMMING IMMUNITY WITH BIOCOMPATIBLE DEVICES
TO TREAT HUMAN DISEASE

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ABSTRACT

REID AUSTIN ROBERTS: Harnessing What Lies Within – Programming Immunity with Biocompatible Devices to Treat Human Disease
(Under the direction of Jenny Ting, PhD and Joseph DeSimone, PhD)

Advances in our mechanistic insight of cellular function and how this relates to host physiology have revealed a world which is intimately connected at the macro and micro level. Our increasing understanding of biology exemplifies this, where cells respond to environmental cues through interconnected networks of proteins which function as receptors and adaptors to elicit gene expression changes that drive appropriate cellular programs for a given stimulus. Consequently, our deeper molecular appreciation of host homeostasis implicates aberrations of these pathways in nearly all major human disease categories, including those of infectious, metabolic, neurologic, oncogenic, and autoimmune etiology.

We have come to recognize the mammalian immune system as a common network hub among all these varied pathologies. As such, the major goal of this dissertation is to identify a platform to program immune responses in mammals so that we may enhance our ability to treat disease and improve health in the 21st century. Using advances in materials science, in particular a recently developed particle fabrication technology termed Particle Replication in Non-wetting Templates (PRINT), our studies systematically assess the murine and human immune response to precisely fabricated nano- and microscale particles composed of
biodegradable and biocompatible materials. We then build on these findings and present particle design parameters to program a number of clinically attractive immune responses by targeting endogenous cellular signaling pathways. These include control of particle uptake through surface modification, design parameters that modulate the magnitude and kinetics of biological signaling dynamics that can be used to exacerbate or dampen inflammatory responses, as well as particle designs which may be of use in treating allergies and autoimmune disorders. In total, this dissertation provides evidence that rational design of biocompatible nano- and microparticles is a viable means to instruct therapeutic immune responses that may fundamentally improve how we treat human disease.
DEDICATION

To the Big Bang, without which
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LIST OF ABBREVIATIONS

ATP – Adenosine triphosphate

BALF – Bronchoalveolar lavage fluid

BMDM – Bone marrow-derived macrophage

CLR – C-type lectin receptor

CNS – Central nervous system

CRD – Carbohydrate recognition domain

DC – Dendritic cell

DHA – Docosahexaenoic acid

DLS – Dynamic light scattering

EAE – Experimental autoimmune encephalomyelitis

EPA – Eicosapentaenoic acid

HP4A – Tetraethylene glycol monoacrylate

HPLC – High performance liquid chromatography

i.p. – Intraperitoneal

i.t. – Intratracheal

IL-18 – Interleukin 18
IL-1β – Interleukin 1β

iPS – Induced pluripotent stem cell

LDH – Lactate dehydrogenase

LPS – Lipopolysaccharide

M0 – Macrophage

MHC – Major histocompatibility complex

MP – Microparticle

MPL – Monophosphoryl Lipid A

MPS – Mononuclear phagocyte system

MSU – Monosodium urate

NCD – Non-communicable disease

NLR – Nod-like Receptor

NMP – Nanoparticles and Microparticles

NP – Nanoparticles

PBMC – Peripheral blood mononuclear cells

PEG – Polyethylene glycol

PET – Poly-(ethylene terephthalate)
PFA – Paraformaldehyde

PLGA – Poly(lactic-co-glycolic acid)

PLP – Proteolipid protein

PPS – Preparticle solution

PRINT – Particle Replication in Non-wetting Templates

PRR – Pattern Recognition Receptor

PS – Phosphatidylserine

PVOH – Polyvinyl alcohol

QD – Quantum Dot

RLR – RIG-like Receptor

SCM – Succinimidyl carboxy methyl ester

SEM – Scanning electron microscope

TEA – Triethanolamine

TFF – Tangential filtration flow

TGA – Thermogravimetric analysis

TLR – Toll-like Receptor

TPO – Diphenyl (2,4,6-trimethylbenzoyl)-phosphine oxide
CHAPTER 1
INTRODUCTION

This dissertation seeks to define a platform for programming immune responses in order to fundamentally improve how we treat disease in the 21st century. It is our tenet that therapeutically relevant instruction of the immune system will require extremely modular and biocompatible systems capable of providing a range of biological signals on the physical scale at which cellular networks communicate. These nano- and micro-scale devices will thus be capable of programming host biology in defined manners.

The research presented herein spans the fields of cell signaling biology, immunology, materials science and bioengineering. To this end, I first provide background of relevant information for each of these fields to frame this dissertation. Subsequent chapters will expound in more detail work performed and findings uncovered. While not exhaustive, this introductory overview of immune system function from a cellular network perspective provides the framework from which I pursued this dissertation. Immune cells precisely sense their environment in order to elicit appropriate responses. These responses are required to defend against infectious diseases but also drive pathology in the setting of non-communicable diseases (NCD) if uncontrolled. Because the vast majority of the world's population are susceptible to microbial infection and NCD, manipulating immune system
function in a targeted manner should become a key intervention in the clinician’s arsenal.

1.1 CELLULAR FUNCTION FROM A NETWORK PERSPECTIVE

In order to fully appreciate what is required for a human to maintain health over the course of an average modern lifespan of 67.2 years [3], it is worthwhile to first understand what constitutes a human body at the cellular level. There are trillions of human cells specialized for their functional purpose, be it detoxifying substances in the liver, perceiving light in the retina of the eye, forming memories in the brain, providing structural support as bone, or patrolling the body for signs of infection to name but a few examples [4]. Yet all these trillions of cells emerged from a single union between a sperm and an egg, and thus divided and specialized throughout development to fill all the niches required for a functioning human being.

While the details of development and niche specialization are complex, unique and beyond the scope of this dissertation, suffice to say that some simple repeating themes emerge that we can use to guide our understanding of human biology. Most critical of these themes is the overarching principle that a cell responds to environmental cues in order to ensure the survival of the organism it constitutes, be it unicellular or a multicellular organism. This response is dictated by signal transduction pathways that first emerged in unicellular organisms responding to intracellular signals picked up from the environment and then was expanded to extracellular detection in order to support the complexity of higher order multicellular species that are distant ancestors of humans [5,6].
At its most basic, signal transduction requires a cell to sense information encoded in its environment and then relay that information to immediate response networks such as G-protein coupled receptors as well as the nucleus of the cell, where expression of genes can be modulated in order to better adapt the cell to its current state of experience. A common example is the metabolic response of humans to glucose, a nutrient taken in from the environment in the form of dietary sugar consumption. Specialized cells in the pancreas - beta cells - bind glucose with glucose receptors and in response, upregulate expression of insulin and trigger its release into the bloodstream. Systemic insulin can then bind cell-surface bound insulin receptors throughout the body which coordinate uptake of glucose from the bloodstream for immediate or long-term energy use depending on the needs of the organism [7]. Using this simplified example, we can see all the hallmarks of signal transduction and network coordination of an appropriate response: 1) sensing of the environment; 2) relaying that information to the nucleus; 3) turning on appropriate gene programs; and 4) production of proteins that enable downstream functions required for the homeostasis of the organism at large, including secretion of host-derived environmental cues out of a cell.

We must also acknowledge that the human organism is not only integrating signals from its ~10 trillion human cells, but also must maintain order with the estimated 100 trillion bacterial cells which cohabit each of our bodies [8]. This latter insight has only come to the forefront of scientific knowledge in the past decade but has already transformed our understanding of the human body: we are not separate,
isolated entities in the world at large, but more of an ecosystem of multiple species that shifts depending on the geographic and dietary environment [8-14].

With this basic theme of cellular response to environmental cues in mind, let us now discuss how the human immune system maintains homeostasis of the organism as this information can enable more selective and integrated therapeutic interventions for the panoply of health ailments that afflict modern man. While we will delve into more explicit details from hereon, it is of utmost importance to recall the underlying theme that an organism’s survival depends explicitly on its ability to respond appropriately to its environment. As we will see, an appropriate response often requires involvement of numerous signaling pathways and biological networks within and between cells.

1.2 THE IMMUNE SYSTEM: PROVIDING CONTEXT, BRANDING RESPONSES

While there are thousands of signal transduction pathways in human cells, we will restrict ourselves to discussing some pathways which comprise major network hubs for function of the immune system. Abundant evidence suggests the immune system has profound effects on mammalian biology and is intimately involved in our maintenance of health through interactions with all major organ and tissue types [2,15-18]. Thus, clarifying our understanding of signal transduction principles in the immune system will frame our research directions in subsequent chapters.

Generally speaking, the main duty of the immune system is to differentiate self from non-self and mount appropriate cellular responses according to that
differentiation. This principle task is a hallmark of our evolutionary history which required cells to fend off attack by microbes such as fungi, bacteria and viruses in order to survive using the innate immune system. As multicellular organisms became entrenched on Earth and expanded in size and complexity, higher order immune systems became necessary, as is evidenced by the development of an adaptive immune system in jawed vertebrates over 500 million years ago [19]. However, as we have recently come to appreciate, the simple story of the immune system as defense against infection is only one aspect of the complex role this interconnected network of cells and soluble mediators plays in host homeostasis. To begin to appreciate this complexity, we will briefly outline major players of the mammalian immune system.

1.3 INNATE IMMUNITY: PROVIDING CONTEXT TO EXPERIENCE

In order to respond appropriately to an environmental encounter, innate immune cells much first identify whether the stimulus is self or non-self. This occurs through numerous classes of receptors located on the surface and within the cell. While beyond the scope of this dissertation, we briefly mention the critical role of T cell receptors (TCR) and B cell receptors (BCR) in immune system function as these receptor signaling networks constitute some exciting clinical advances for treatment of cancers and autoimmune disorders as will be highlighted later in this chapter. More germane to our studies are Pattern Recognition Receptors (PRRs) which have evolved to recognize conserved microbial structures, such as bacterial or fungal cell
wall components and viral nucleotides. This occurs through direct binding of these structures or through sensing intracellular responses to microbes, like the production of metabolites specific to infection with a virus [20,21]. Upon activation of PRRs within a cell, unique gene programs are turned on that result in expression and secretion of soluble mediators that alert other cells to both the presence and quality of the engaged stimulus [22,23]. These mediators, such as cytokines and chemokines, drive not only movement of cells into and out of tissues spaces, but also program stimuli-appropriate responses based on the combination of mediators present.

While many non-immune cells throughout the body have various PRRs, innate immune cells such as macrophages (M0) and dendritic cells (DC) are two of the primary effectors of innate defense. These innate cells have the capacity to eat microbes in a process termed phagocytosis that serves two purposes: 1) remove the offending stimuli from the body where it can do more harm, and 2) process the stimuli so that an adaptive immune response can be elicited against it [24-26]. A key point is that depending on the context of the stimuli, innate immune cells instruct other cells as to the quality of the stimuli encountered. Thus the innate immune response to a viral infection is qualitatively different than that to a bacterial infection or encounters with fungi [20,27]. This contextualization of environmental stimuli drives stimuli-appropriate tissue and adaptive immune responses as will be discussed in more detail shortly. There are a number of other cell types which also play critical roles in innate immunity but we will restrict ourselves to discussing M0 and DC as these are the cell types which are passively targeted by both
nanoparticles and microparticles and thus serve as the frontline for programming immunity in mammals.

Our understanding of innate immunity has come a long way since PRRs were first proposed to exist by Charles Janeway, Jr. in 1989 [28]. Since his prescient prediction of PRRs, we have identified not only multiple classes of PRRs but have increasingly refined our molecular understanding of these receptor families. There are currently four major classes of PRRs - Toll-like Receptors (TLRs), Nod-like Receptors (NLRs), C-type Lectin Receptors (CLRs) and RIG-like Helicases (RLHs) – though other individual PRR have recently been identified [29,30].

1.4 THE LESSONS OF TLRS AND NLRS

TLRs are the best studied PRR class and serve as a model for which we understand the function of other PRRs. Of the 10 TLRs identified in humans, 9 have known functions, with five being cell surface bound and four localized to intracellular compartments termed endosomes [31]. This distribution in various cellular compartments correlates with function; cell surface TLRs bind microbial motifs present on the surface of microbes whereas endosomal TLRs bind microbial nucleic acid motifs that are unmasked after successful degradation [32]. Upon binding cognate ligand, TLRs undergo post-translational modifications such as phosphorylation and relocalization in the cell, thus enabling downstream adaptors to interact with these receptors and trigger signaling pathways to drive gene expression changes commensurate with the microbe encountered [20,27,32-34]. For example,
triggering of TLR3 in the endosome by viral double stranded RNA results in activation of the TRIF adaptor and subsequent translocation of two transcription factors to the nucleus, NF-κB and IRF3, where they drive gene expression of pro-inflammatory cytokines and anti-viral interferons [27]. In summary, the location of the TLR corresponds to where its microbial ligand is perceived and the subsequent gene programs elicited are appropriate for the microbe encountered.

Whereas TLR signaling pathways have been well characterized, the NLR family of PRR are far less well known, in part due to their initial identification only in the early 2000s [35]. There are 22 human NLRs which are grouped as a family based on their structural homology, with C-terminal Leucine rich repeat (LRR) regions, a central nucleotide binding domain (NBD) and a N-terminal effector domain that is thought to confer unique properties among the NLR members [36,37]. As a beautiful example of convergent evolution, plants defend themselves with the NB-LRR receptor family using very similar protein domain architecture as human NLRs [38].

Most NLRs are believed to reside in the cellular cytosol, though some members have been found at the inner leaflet of the plasma membrane (NOD1 and NOD2) or localized to the outer membrane of the mitochondria (NLRX1) [39-41]. Since their initial discovery, NLRs have been shown to play a broad role in host immunity (Figure 1-1). NLR functions include transcriptional control of MHC class I and II expression, modulation of autophagy, inhibition and activation of pro-inflammatory pathways including NF-κB-mediated transcription, as well as a subclass of NLRs which form inflammasomes - multi-protein complexes which cause
activation of the cysteine protease caspase-1 and subsequent maturation of the pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) [42-45].

NLRs have been implicated in the innate immune responses to fungi, bacteria and viruses, with concomitant effects on ensuing adaptive immune responses [46-56]. Two NLRs, NOD1 and NOD2, have clear roles in the autophagic response to bacteria and subsequent loading of antigen in MHC class II complexes [40,57,58]. A separate means by which NLRs control adaptive immune responses is through upregulating expression of MHC I and MHC II genes via NLRC5 and CIITA, respectively [59-67]. As antigen-specific adaptive immune responses are critically dependent on MHC peptide presentation, these NLR members are thus placed at a central hub in initiating adaptive immunity.

As discussed shortly in more detail, the quality of an adaptive immune response is dependent not on just the antigen but also on the context in which that antigen is presented. Context is encoded in part through soluble messengers termed cytokines which greatly augment the quality of adaptive immune responses. One major type of adaptive immune response involved in defense against fungi and autoimmune pathology, termed Th17, is thought to be at least partially dependent on activation of inflammasome forming NLR proteins, such as NLRP3. Release of the potent pro-inflammatory cytokine IL-1β from innate immune cells with activated inflammasomes can initiate Th17 adaptive immune responses. Evidence supporting this comes from studies in mice deficient in inflammasome components which are more susceptible to fungal infection or protected from Th17-mediated pathology in settings of experimental autoimmune encephalitis [46,68-70]. Because of the potent
effects on Th17 immune responses elicited from NLRP3 inflammasome activation, designing vaccines which can trigger this PRR is an avid area of research in the nanomaterials field and one we will study in more detail in subsequent chapters [71-75].

**Figure 1-1. NLRs are sensors of the Intracellular Environment.**
While not exhaustive, this schematic overview presents cell locations and functions for NLR family members based on published literature. Of note, NLRs reside throughout the cell, from the inner leaflet of the plasma membrane (NOD1 and NOD2) to the mitochondria (NLRX1), the cytosol (inflammasome forming NLRs) and in the nucleus for those with known transcriptional functions (NLRC5 and CIITA). while some NLRs may be pro-inflammatory, others inhibit these same pathways.
Based on work we and others have published, NLRs mediate these wide-ranging immune effects in part through interactions with receptors involved in other innate immunity signaling pathways, such as the TNF receptor associated factor family (TRAF) [76-79]. Through the network nature of NLR effects on innate immunity, they have also been implicated in modifying the host microbiota [80-83]. Disease susceptibility to metabolic syndrome and cancer is modified by a number of NLR receptors based on studies using mice deficient in NLR genes [63,80,84-92]. Genome-wide association studies have also uncovered a powerful role for mutations in NLRs in a range of human disease, including bare lymphocyte syndrome, Crohn’s disease, auto-inflammatory disorders such as Muckle-wells and Blau syndrome, and autoimmune disorders such as vitiligo [37,45,93-104].

We have highlighted the NLR family in detail to serve as an example of how immune sensors can potently augment human physiology, with either beneficial or detrimental effects depending on the intact nature of these signaling networks. While this dissertation focuses on nanomaterial interactions with mammalian immunity, it is worthwhile to note that simultaneous research into the basic molecular functions of immune signaling networks will be crucial to broadening and enhancing the therapeutic potential of immunoengineering. To this end, we note our own research efforts to further molecular understanding of the NLR family via biochemical studies and bioinformatics approaches that are beyond the scope of this dissertation [76,88,105,106] (and unpublished data).
1.5 ADAPTIVE IMMUNITY: BRANDS OF RESPONSE

Perhaps one of the more astounding features of human biology is how unique immune responses are orchestrated for specific classes of microbes. We have already discussed the role of innate immune cells and PRRs such as TLRs and NLRs in contextualizing environmental encounters but how this context drives appropriate adaptive immune responses is equally fascinating. At root, an appropriate cellular response to its environment is elicited through networked orchestration of signal transduction pathways and the immune system is perhaps the clearest example of this concept. Here, we will attempt to synthesize a tremendous amount of immunology research into a few simple concepts reflecting this notion [2,15,16,18,20,23,24,32,33,56,107-115]:

1) innate immune cells, in particular dendritic cells (DCs), encounter microbes and process them according to the PRR that are triggered

2) the sum total of PRR signaling pathways triggered within the DC and surrounding tissues will elicit a unique milieu of cytokines and cell surface receptors that provide context to other cells and regional tissues in order to mount appropriate responses

3) within regional lymph nodes, DCs instruct T cells to mount adaptive immune responses to specific antigens presented by DC on MHC class I and II receptors to drive either CD8+ or CD4+ T cell effector responses appropriate to the microbe encountered (e.g., intracellular virus or extracellular bacteria)
4) the quality and quantity of T cell responses to an antigen depends on the context in which it is presented by a DC, which is encoded in the cytokine milieu initially triggered by PRRs within the DC and affected tissues.

5) these cytokines drive specific T cell gene programs that qualitatively shift the class of effector T cell response to tailor it for the task at hand, in part through inhibiting production of cytokines associated with inappropriate classes of T cell effector responses.

6) in a similar fashion, B cell antibody responses are appropriately tailored through B cell-intrinsic PRR signaling, sampling of antigens, and sensing of the cytokine milieu elicited by innate immune cells and effector T cells.

7) in the absence of PRR signaling, anergic or tolerogenic T cell responses are generated, such as would be the case to self-antigens.

Over the course of mammalian evolution, the end result of this immune symphony is the generation of general classes of microbe-specific adaptive immune responses as listed in Table 1.1 and visualized in Figure 1-2.

**Table 1.1 – General Description of Adaptive Immune Responses.**

<table>
<thead>
<tr>
<th>Effector Immune Class</th>
<th>Hallmark Cytokines</th>
<th>Triggers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IFN-γ, TNF-α, IL-1β, IL-2, IL-12</td>
<td>Virus, Bacteria, Intracellular parasites</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-4, IL-5, IL-13</td>
<td>Extracellular parasites, Helminths, Allergies</td>
</tr>
<tr>
<td>Th17</td>
<td>IL-1β, IL-6, TGF-β, IL-23, IL-17</td>
<td>Virus, Fungus, Autoimmune disorders</td>
</tr>
<tr>
<td>Treg</td>
<td>IL-10, TGF-β</td>
<td>Self-antigens, Tolerance induction</td>
</tr>
</tbody>
</table>
A) Microbes activate unique sets of PRR on Dendritic Cells which trigger production of cytokines and cell surface receptors appropriate to the microbe encountered. This contextualizes the threat at hand during antigen presentation to T cells, thus driving gene expression programs hallmarked by class-specific transcription factors. This, in turn, drives production of class-specific cytokines. Figure adapted from [2] with permission from Nature Publishing Group.

B) An immune hierarchy perspective enables appreciation for all levels of coordination, from signaling pathways activated by PRRs to cell-cell communication that modulates the tissue environment and vice-versa.
1.6 A CALL FOR IMMUNOTHERAPY

With this molecular understanding of innate immunity and the generation of adaptive immune responses, we have gained unheralded insight into the pathogenesis of both infectious diseases and those of non-microbial origin (termed non-communicable diseases, NCD). For infectious diseases, this can enable the production of more efficacious and microbe-specific vaccines. By designing vaccines that activate innate immune pathways normally elicited during the course of infection with a pathogen, we can generate adaptive immune responses appropriate to the pathogen we are trying to vaccinate against [18]. For example, we now know that a Th17 response is required to clear infection with the fungus Candida albicans so efforts are underway to design vaccines which elicit anti-fungal Th17 responses [116-120]. Similarly, using recent gains from immunology research a number of pathogens with high morbidity and mortality around the world for which there are currently no vaccines are being targeted. These include malaria, Dengue virus, HIV and cholera [121]. As we will discuss, many of these vaccines will rely on nanomaterials designed specifically to augment appropriate innate immune signaling networks for a given pathogen [74,122-124].

Non-Communicable Diseases – Our Modern Epidemics

A potentially transformative research direction made available through our understanding of immunological networks is the ability to entirely change how we treat non-communicable diseases (NCD). These illnesses – including heart disease, cancer, chronic obstructive pulmonary disorders and diabetes – have the highest morbidity and largest economic burden in the United States and other developed
societies [125-127]. It is estimated that 63% of deaths worldwide are due to NCD and the global economic output lost due to NCD over the next 20 years is predicted to reach over $30 trillion [127]. While NCD have behavioral risk factors that should be first line intervention strategies, specific aberrations in immune function correlated with these diseases are potential therapeutic targets that have yet to be fully addressed. Immunotherapies for NCD may show better efficacies at lower cost than currently used non-specific treatment approaches.

Cancer is one example of a NCD with immune therapeutic options. Cancer, at its core, is a cell which no longer recognizes its rightful place in the host organism and proliferates without concern for the body by co-opting its microenvironment [128,129]. It more recently has come to light that this co-opting of the microenvironment includes broad immune suppression in the tumor area as a means to keep the body’s own defenses at bay [17]. This occurs in part through modulating the cytokine milieu to promote suppressive polarization of innate immune cells while enhancing regulatory T cell responses to aid the cancer in hiding its true danger from the body [130-132]. The current standards of care to treat cancer include systemic chemotherapies that kill all rapidly dividing cells, radiation therapy, and systemic administration of antibodies or small molecules that target potent signaling pathways involved in both cancer and normal cellular function. These arduous and expensive treatment approaches can be debilitating to the patient and often non-curative [133-136]. Thus, alternative treatment approaches are desperately needed.
An avid area of current research is the concept of modulating the immune environment of cancers to reawaken the body’s own defenses and harness the inherent power of the immune system to then eradicate the cancer [137]. Multiple approaches are being pursued, with some involving antibody-based activation of cancer-dampened T cells already showing promise in the clinic [136]. Cancer cells also upregulate CD47, a cell surface receptor that serves as a ‘don’t eat me’ signal to macrophages [138]. Recent evidence suggests that blocking this receptor with antibodies is sufficient to enable macrophage clearance of cancers in mouse models [139]. The long-sought goal of generating cancer vaccines is also progressing, in part through immunoengineering approaches using nanomaterials as will be discussed in more detail below. With recent evidence indicating a Th1 immune response induces growth arrest in mouse and human cancers through the cytokines TNF-α and IFN-γ, targeted modulation of the immune environment could become a standard of care for cancer treatment [140].

Another NCD with immunotherapy potential that is now a world-wide epidemic is type 2 diabetes. Unlike type 1 diabetes which is of autoimmune origin (and amenable to immunotherapy), type II is caused by diet-induced metabolic and immune alterations which render patients insulin-resistant, thus mimicking the pathology of type 1 diabetes [7]. With increasing populations of the world eating high-fat and high-sugar diets, the levels of obesity are reaching staggering proportions and obesity is a primary risk factor for type 2 diabetes. With obesity prevalence rates at over 35% of US adults and 16% of US youth, the current and future economic costs to our health care system are recognized as unsustainable
While behavioral choices and policy changes to our food system are clearly important interventions that need to be pursued to stem these alarming obesity rates, augmenting the immune system is a potential therapeutic approach for those patients who have already become diabetic.

As an example of the intimate relationship with immune function and metabolic homeostasis, it is now clear that Th2 type immune responses - hallmarked by the cytokines, IL-4, IL-5 and IL-13 (Table 1.1) - are critical to the maintenance of metabolic function in adipose and hepatic tissues and are required for glucose homeostasis. Mice deficient in any of these cytokines are more susceptible to high-fat diet induced insulin resistance and hyperglycemia [144-146]. Conversely, pro-inflammatory Th1 cytokines like IL-1β are known to cause insulin resistance [89,147]. From a therapeutic approach, infection with intestinal helminths, which induce Th2 immune responses, have also been shown to restore normoglycemia and insulin sensitivity in otherwise diabetic mice [145,146].

As stated at the start of this chapter, immune cells precisely sense their environment in order to elicit appropriate responses. These responses are required to defend against infectious diseases but also drive pathology in the setting of non-communicable diseases if uncontrolled. Because the vast majority of the world's population are susceptible to microbial infection or NCD, programming immune system function in a targeted manner should become a key intervention in the clinician’s arsenal. To that end, we now turn to a discussion of our current state of knowledge regarding nanomaterial interaction with mammalian biology and efforts undertaken to use these materials to program immune responses.
1.7 NANOMATERIAL INTERACTIONS WITH BIOLOGICAL SYSTEMS

As the thrust of this dissertation is the analysis of the innate immune response to nano- and micro-scale particles, it is of use to first provide a background on the current state of our understanding as regards biocompatibility with materials in these size ranges. The overriding supposition is that by first understanding how size, shape and composition of particle parameters affect immune outcomes, we can then more appropriately design particles for a range of therapeutic interventions, in particular those which aim to modulate the host immune response in defined manners. Herein, we use the term nanoparticle (NP) to refer to particles that are less than 1 micron in all dimensions and microparticles (MP) for those that are greater than 1 micron in any single dimension. When discussing both nanoparticles and microparticles as a generalized group, we will refer to them as NMP.

Historically, studies of nanomaterial interactions with mammalian biology have occurred with no real field standards but recent efforts have been made to establish general assays for toxicology and immunological properties of nanomaterials [148]. This is in part due to the complexity of carrying out controlled experiments to delineate differences between nanomaterials based on fine alterations of size, surface charge or composition, all of which clearly impact the mammalian immune response [148-150]. As an example, by altering the size, surface charge, surface hydrophobicity or surface-exposed functional groups, the quantity and quality of proteins adsorbed to NMP can be greatly affected [151-153]. Yet to give a sense of the disparity between in vitro nanoparticle studies and in vivo relevance, recent papers have shown that studies which fail to use serum surrogates
during *in vitro* analysis likely have little functional relevance to what occurs during *in vivo* experiments [154].

A relevant example for translational applications is the use of targeting NP to specific cell subsets by decorating antibodies for specific receptors on the surface of NP. One group recently found that NP targeting specificity to the transferrin receptor, which is upregulated in some cancers, was completely abrogated when switching *in vitro* assays from phosphate buffered saline media to one that was serum replete to more closely resemble host biology [155]. Remarkably, many NP studies do not use serum-containing media so this seemingly predictable result not only calls in to question numerous previously published findings but also serves as a wake-up call to the nanomaterials community at large as regards the translational relevance of experimental design. This example also serves to bolster the position we took during the NMP studies detailed in this dissertation: well-controlled, biologically relevant studies are needed to characterize nanomaterial interactions with mammalian immune system.

Admittedly, a difficulty in drawing broad conclusions regarding how particle design features such as size, shape, composition and surface charge affect host biology is due to major differences in NMP fabrication techniques, materials sourcing and experimental assays within and between labs [148,156]. Common NMP fabrication techniques include water-oil-water emulsion or spray-drying methods, yet particles fabricated in this manner can range in size by many hundreds of nanometers and have heterogenous shapes within the same batch [73,157,158].
Thus careful analysis of the role of size or shape on biological responses to nanomaterials using standard fabrication methods is difficult at best.

**Composition Considerations of NMP – The Inflammasome Example**

A remarkable example of the sensitivity of innate immunity to nanoscale details is how modifying the surface and composition of NMP can modulate activation of the NLRP3 inflammasome. As a reminder, the NLRP3 inflammasome is a multi-molecular protein complex that is formed in response to a variety of environmental triggers, ultimately resulting in the activation and secretion of pro-inflammatory cytokines, IL-1β and IL-18 [29,159]. Because particulate matter such as asbestos, silica and monosodium urate crystals are known to activate the NLRP3 inflammasome, it is expected that NMP may also trigger this innate immune response. There are many uses for NMP where it would be disadvantageous and clinically unacceptable to use particles that induced inflammation, such as diagnostic and drug-delivery applications. Conversely, activating the NLRP3 inflammasome by NMP is a potential vaccine adjuvant and thus the design principles controlling this response is an avid area of study. Through the work of a number of groups, we are starting to gain insight into how NMP can activate the NLRP3 inflammasome.

Work by the Tschopp group identified composition of metallic nanoparticles as the prime factor in triggering IL-1β release. Titanium dioxide and silica dioxide NP caused inflammasome activation while comparable sized zinc oxide particles did not [75]. Another group focused entirely on silica particles in the nano and micron range and found a size-dependent effect on IL-1β release. Fascinatingly, both
inflammasome activation and associated inflammatory signaling changes induced by these particles was inhibited simply through modifying the chemical groups exposed on the surface of particles [160-162].

While these findings have relevance to consumer products and environmental toxicology, the literature regarding inflammasome activation by NMP composed of polymers more relevant to biomedicine applications is inconsistent. Perhaps the two most commonly used polymers for biomedical applications are poly(lactide-co-glycolide (PLGA) and polyethylene glycol (PEG). These biocompatible polymers are approved by the Food and Drug Administration (FDA) and have a long, safe history of clinical use [163,164]. Yet when the biodegradable polymer PLGA is formulated into NMP, groups report differences in its inflammasome activating potential [71]. One report shows in vitro and in vivo evidence for inflammasome activation by PLGA NMP that is contradicted by another study [72,73]. However, recent evidence suggests these literature discrepancies could be due to fabrication differences among groups, as simply a non-smooth NMP surface is sufficient to induce inflammasome activation by particulate matter [165]. We have studied the issue of inflammasome activation by NMP at length, as will be discussed in subsequent chapters. Because composition is so clearly a factor in NMP interactions with human biology, we focused our studies on PLGA and PEG polymers as they have the most relevance for eventual clinical use given their safe history.
Physical Parameter Considerations of Particle Uptake

Though particle parameters such as size, shape and composition do impact host biology, field-wide conclusions regarding any one design feature is currently not possible. For example, while surface charge has been associated with cell uptake, if one modulates the size of the particle then conclusions regarding surface charge and uptake are not necessarily consistent [166-168]. Similarly, the mechanism by which cells uptake NMP is dependent on a variety of features, including size, shape and surface charge. Whereas mannose-coated NP are taken up by macrophages through mannose-receptor dependent phagocytosis [169-171], lipid-coated NP can be taken in via complement-receptor dependent pathways [172]. Iron oxide NP uptake is scavenger-receptor dependent [173]. From an immunological perspective, these are critical design parameters as the phagocytic uptake pathway can have downstream pro- or anti-inflammatory effects [26]. Multiple other examples of the inconsistent nature of how design parameters augment the biological fate of NMP abound, serving ultimately to highlight the absolute sensitivity of mammalian biology to just about every particle feature one chooses to test.

Separate from the specific phagocytic pathway used to ingest particles, the actual shape of the NMP plays a critical role in the initiation and successful completion of phagocytosis. A seminal study by the Mitragotri group identified the contact angle by which a macrophage encounters a particle as a critical parameter dictating successful phagocytosis of the NMP [174]. If the contact angle was greater than 45 degrees, then particle phagocytosis was unlikely to occur, likely due to physical difficulties of mobilizing the actin cytoskeleton to engulf particles in their
widest dimension (>500nm). The Mitragotri group has used this finding to design high aspect ratio NMP with worm like shapes to inhibit phagocytosis by restricting uptake to only those phagocytes which happen to encounter particles at the tip (where the contact angle < 45 degrees) [175]. Such shape design considerations may be of use for therapeutic modalities requiring enhanced circulation times and/or extracellular release of cargo.

More recently, these researchers have identified shape as critically augmenting the targeting specificity of antibody-coated NMP through harnessing this initial contact angle finding. By coating rod, spherous and disc shaped NMP with trastuzamab (a monoclonal antibody against HER2/neu receptors expressed by breast cancer tissue), the authors found rod-shaped NMP reduced non-specific cell uptake and reduced breast cancer cell line growth more efficiently than an equivalent dose of soluble trastuzamab [176]. These exciting findings point to an emerging theme in NMP studies whereby particularization of biological molecules (drugs, antibodies, ligands) confers unique properties not found with equivalent soluble forms of the same molecule, as will be discussed in greater experimental detail in Chapter 4.

One plausible explanation for why NMP are capable of eliciting emergent properties from cells when coated with bioactive molecules may be due to formation of a phagocytic synapse that macrophages and dendritic cells use to sense intact microbes [1]. This has formally been proven by the Underhill group using both soluble and particulate forms of the fungal cell wall component, β-glucan, which is recognized by Dectin-1, a cell surface receptor belonging to the C-type lectin (CLR)
family of PRR. Although both soluble and particulate forms of β-glucan are bound by Dectin-1, signaling is not initiated from the Dectin-1 receptor unless β-glucan is present in a particulate form (Figure 1-3). This causes receptor clustering of Dectin-1 and formation of a ‘phagocytic synapse’ that is able to physically exclude regulatory tyrosine phosphatases CD45 and CD148 from the site of phagocytosis [177]. In effect, immune signaling is not initiated from Dectin-1 unless an intact fungus (i.e., particulate form) is sensed. This clever biomechanical control enables innate immune cells to restrain initiation of potent anti-fungal responses to only those instances when an actual fungal microbe is encountered, thus sparing spurious innate responses that may otherwise be detrimental to the host. It is thus conceivable that coating of NMP with biologically active ligands, in particular antibodies or ligands which bind surface receptors, will result in emergent cellular responses through receptor clustering that soluble forms fail to elicit. We present evidence supporting this hypothesis in Chapter 4. germane to this issue, there is abundant literature reflecting the role of surface-ligand density in downstream cellular responses to NMP [178-181].
Figure 1-3. Formation of a Phagocytic Synapse in response to Particulate Ligand.

Consider Fungal β-glucan (orange) a bioactive ligand that can be either soluble or fabricated with nano- and micro-scale particles to appear as shown here (Russian doll outline). Receptor clustering afforded by particulation of ligand is one rationale for emergent properties seen with NMP. Figure is from [1] and used with permission from Nature Publishing Group.
The surface chemistry of nanomaterials used in NMP can also significantly impact the immune response to these particles. It has been shown that certain primary hydroxyl groups or other surface nucleophiles on nanomaterials can react with complement factor C3 to form the active C3b product, thus triggering potent complement-mediated effects on innate and adaptive immunity [182-185]. As discussed previously, inflammasome activation by metallic NP depends on the metal used, whereas silica nanoparticle activation of the inflammasome can be augmented through modification of surface chemical groups [75,186,187]. In total, these studies highlight the need to address nanomaterial interactions with the immune system for every given particle size and composition, as the only thing we can predict is that the body is clearly capable of discriminating among minute details.

Help Wanted: Control of Particle Design

With this nanomaterial background in mind, it should be evident that a process to reproducibly fabricate particles with complete control over all physical and chemical parameters would be a great boon to the NMP research community. In 2005, such a fabrication method was developed in the lab of Joseph DeSimone, Ph.D., by fusing the precision and engineering control offered by lithographic techniques from the microelectronic industry with state-of-the-art synthetic chemistry in a modular platform. The process – termed Particle Replication in Non-wetting Templates (PRINT) – is a top-down particle molding technology with unmatched control of the size, shape and composition of molded features. PRINT templates can be molded with an unprecedented variety of materials, including pharmaceutical
compounds, small molecules, proteins, siRNA, and bioabsorbable polymers containing therapeutics or diagnostic cargos [188-194].

An easy to grasp analogy is to consider PRINT molds as ice-trays with cubicles of defined size and shape that can be filled with solutions of one’s choosing. After PRINTing, the particles (ice cubes) can be popped out of the tray, resulting in a homogenous population of monodisperse particles of defined chemical composition (Figure 1-4). Using PRINT, well-controlled experiments isolating the effects of specific particle parameters such as size, shape and chemical composition on mammalian immune responses can be reproducibly explored.
Figure 1-4. Schematic of the PRINT fabrication process.

Master templates (grey) are fabricated using advanced lithographic techniques to defined size and shape parameters. A liquid fluoropolymer (green) is poured on the surface of the master template and photochemically cross linked to generate a precise mold having micro- or nanoscale cavities (green). A liquid solution comprised of user-specified components (red) fills the cavities in the mold through capillary forces. Once the liquid in the mold cavities is converted to a solid, the array of particles (red) are removed from the mold (green) through contact with a harvesting film (yellow). Free flowing particles or stable dispersions can be obtained by separating the harvesting film from the particles, often with water, thus enabling monodisperse particles to be used for downstream experiments. Figure adapted from http://www.desimone-group.chem.unc.edu.
To summarize this section, biological interactions with NMP fundamentally depend on the physical and chemical parameters of particles, though there are no overarching conclusions that can be drawn as regards all NMP for a given parameter. This is perhaps unsurprising given that the body does not process information in discreet groups but instead tailors an appropriate response to the individual stimulus, as discussed earlier in this chapter. Just as the body responds uniquely to various types of bacteria despite similarities in size, shape and genus, it follows that the response to NMP will also be context dependent. For these reasons, a bulk of the work presented in chapters 2 and 3 of this dissertation pertains to careful analysis of the mammalian innate immune response to NMP through controlled experiments using PRINT particles of strictly defined size, shape and composition. These fundamental studies are necessary in order to enable the rational design of NMP therapies for a range of health ailments that afflict 21st century society. As evidence of the potential for NMP to fundamentally change how diseases are treated through modulation of the immune system, an overview of immunoengineering approaches using NMP will now be discussed.

1.8 IMMUNOENGINEERING: WHERE WE ARE

The basic principle of immunoengineering is to design NMP that deliver biological signals in a targeted manner in order to program immune responses. This is commonly done through incorporating biological ligands and receptors into or on the surface of NMP that mimic endogenous immune cell interactions. The theoretical variety of immune signaling events that can be synthetically generated
through engineering approaches is rapidly approaching parity with the actual variety of immune interactions we know to occur in situ. This is in part due to the broad range of commercially-available immune active molecules, such as cytokines and antibodies to immune receptors, which enable modular approaches to programming immunity. We now present a survey of published immunoengineering studies which reflect the potential of this approach to treating human disease.

NMP have been designed to release cytokines in order to elicit specific immune outcomes. Modeling NMP after mast cell granules, the Abraham group complexed an immunostimulatory cytokine, TNF-α, into synthetic granules composed of heparin and chitosan in order to boost and direct the quality of immune responses [195]. These synthetic granules drained to the lymph node of mice and were able to enhance adaptive immune responses and survival to influenza more efficiently than soluble delivery of TNF-α. Furthermore, the group showed that NMP delivery of IL-12 was capable of skewing the adaptive immune responses towards the Th1 class. This simple yet powerful approach indicates artificial skewing of immune environments is feasible. Though the broad therapeutic potential of this approach remains largely unexplored, evidence suggests appropriately designed NMP can skew adaptive immune responses between classes (i.e., Th2 -> Th1, Th17->Treg) [196,197].

In an in vitro system, the Fahmy group generated artificial antigen presenting cells (aAPC) by anchoring peptide-MHC complexes and co-stimulatory ligands to the surface of MNP composed of the biodegradable polymer poly(lactide-co-glycolide) (PLGA) [198]. These aAPC MNP were loaded with the T cell stimulating cytokine IL-
2 and incubated in culture with T cells to assess their potential to act as dendritic cells (DC). The results show a size dependent activation of T cells, whereby microparticles 8µm in diameter were more efficient at stimulating proliferation of T cells than NP with 130nm diameter. This was not due to differences in total amount of ligands or receptors present. The study also found that particle encapsulated IL-2 was 10x more efficient than soluble IL-2 in terms of T cell proliferation, suggesting high localized IL-2 concentrations between the aAPC and T cell mediated more robust signaling akin to that which would occur between DC and T cells. This artificial system has relevance to ex vivo manipulation of T cells for clinical interventions, but also reveals how scaling biological interactions to the appropriate dimension (i.e., nano- and micrometer) confers broad enhancement of biological outcomes.

Next-Generation Vaccines

Perhaps the strongest illustration of the promise for immunoengineering with NMP is progress in vaccine development. This is ironic, given that vaccination is one of the most successful and oldest examples of engineering immunity in human society. Prior to the advent of vaccination in the late 18th century, the average lifespan was 35 years of age. It is now approaching 80 years, in large part due to the success of worldwide vaccination campaigns [121]. With recent elucidation of the molecular mechanisms behind the efficacy of vaccines, researchers are now avidly pursuing the rational design of vaccines to promote more tailored immune responses to a pathogen of interest [18,107,110,121,199,200]. This is made
possible in large part through our understanding of innate immune activation by microbes through specific pattern recognition receptors (PRR).

By coupling PRR activation to delivery of an antigen, antigen-specific adaptive immune responses can be generated with control over the quality of the adaptive response through judicious design of NMP [74,201]. As many ligands for PRRs are now known and commercially available, immunoengineers are rapidly incorporating these into NMP along with antigens of interest to improve the next generation of vaccines. PRR ligands published with NMP include those for the majority of TLRs, as well as those which activate CLR and the NLRs NOD1 and NOD2 [120,202-207]. The power of NMP from a vaccine perspective lies not only in the modularity afforded through selective or combinatorial PRR agonist inclusion, but also the ability to co-deliver innate immune agonists along with the antigen one desires an adaptive immune response towards. This mimics the spatiotemporal immune activation that occurs during natural infections and couples innate immune activation to a specific antigen of interest. In point of fact, NMP delivery of TLR agonists to mouse and human DC has been shown to be 100 times more effective than soluble delivery [206].

The Pulendran group recently used PLGA NP containing TLR4 and TLR7/8 agonists to show a profound synergistic enhancement of antibody and cell-mediated adaptive responses to co-delivered antigens in both mice and non-human primates. Combinatorial activation of these TLRs through NP delivery elicited antibody responses that were orders of magnitude greater than individual TLR ligand containing NP or the currently used vaccine adjuvant alum [204]. This finding is in
line with others who have seen a profound enhancement in adaptive immune responses to synergistic TLR agonism [200,205,207]. An as yet unexplored area is the use of PRR ligands from multiple classes of PRR families for use in vaccines, NMP or otherwise. It is our belief that combinatorial activation of multiple PRR classes, such as TLRs, NLRs and RIG-I-Lie receptors (RLRs), will likely result in even more profound enhancement of vaccine-induced immune responses as this can recapitulate the natural immune response to microbial infections which themselves trigger multiple PRR classes.

**Design Control of Nanomaterials**

A great strength of NMP approaches to vaccine delivery is the control over numerous biological parameters that can be designed into NMP and tailored to the clinical need at hand. For example, production of CD4+ T cell responses is required for potent humoral immunity while anti-viral responses require CD8+ T cell effector responses. These two responses depend on presentation of antigen through either MHC II (CD4+) or MHC I (CD8+), and the pathway to presentation is different between the two [208-210]. Class II peptides are acquired from the extracellular space as intact antigen and processed within the acidic environment of phagosomes into peptides that are then presented on MHC II receptors. Conversely, Class I peptides are generally acquired from the cytosol of cells. Thus, NMP vaccines with the goal of eliciting antigen-specific CD8+ T cell responses, such as those against viruses, must either avoid phagosomes entirely or be able to escape the phagosome once there.
Here, the materials science component of NMP design comes to play, with generation of polymers which are biologically responsive and thus can ensure cargoes within NMP are delivered to the cytosol. One approach is the use of multilamellar liposomal NP. These are highly sensitive to phagosomal phospholipases and thus rapidly degrade after endocytosis, releasing cargo (antigen) into the cytosol and potently enhancing CD8+ adaptive immune response [211]. An alternative approach for ensuring cytosolic delivery of NMP cargo makes use of the pH drop as phagosomes mature. In this case, NMP are made with pH-sensitive polymers such as polypropyl acrylic acid that display membranolytic activity during acidification of the phagosome. This disrupts the phagosome, releasing the NMP into the cytosol where MHC I presentation of antigenic cargo can occur to drive potent CD8+ T cell responses [212].

A key requirement for the success of immunoengineering approaches is the ability to target specific cells or regions of the body with NMP. Because regional lymph nodes are the location of adaptive immune response generation, ensuring NMP can get to these areas is crucial. NMP design affords a variety of approaches to this issue. Through size selection (500-2000nm diameter), one can passively target antigen presenting cells, such as macrophages and dendritic cells, in the periphery where they then can traffic to regional lymph nodes. Conversely, smaller particles (<100nm) will drain to regional lymph nodes on their own, where they can then be processed by resident immune cells [213]. For particles in the 200-500nm range, it is less clear whether they drain to lymph nodes or are passively trafficked by macrophages and dendritic cells [214].
NMP can also be directly targeted to certain immune cell subsets through the use of surface conjugated ligands and antibodies. DEC-205 is a cell surface receptor on a subclass of DC (CD8+) that are especially suited to priming CD8+ T cell responses. Thus, decorating MNP with antibodies to DEC-205 has been used to specifically target particles to this immune cell subset [215,216]. Interestingly, one study found that varying the surface density of DEC-205 antibody on NP could modulate the ensuing innate immune response from targeted DC, with increasing antibody density correlating with heightened production of the anti-inflammatory cytokine IL-10 due to enhanced cross-linking of the DEC-205 receptor [178].

Other studies targeting human DC through the DC-SIGN receptor showed that both the distance between the conjugated antibody and NMP surface and the size of the particle were critical parameters in ensuring targeted cell-specific uptake [217,218]. As evidence of how carefully coordinated the immune response is and how NMP design can both probe these basic mechanisms while offering therapeutically relevant tools, the Figdor group assessed uptake and antigen processing of NP targeted to either the carbohydrate recognition domain (CRD) or the neck region of the DC-SIGN receptor [219]. They found that NP which targeted the neck region of DC-SIGN were taken up through a clathrin-independent mechanism that led to prolonged retention in endosomes and enhanced MHC I cross-presentation of antigen as compared to the CRD-targeted NP. The cross-presentation of antigen using this strategy was 1000 times more effective than soluble antigen alone, again pointing to the power of biological scale imparted by NMP.
Whereas most current vaccines are delivered via intramuscular or subcutaneous injections, NMP afford a variety of alternative delivery routes, such as inhalation, oral and transdermal microneedle. Studies addressing route of NMP delivery have seen an impact on the quality of immune responses, with Th1 response in particular being more sensitive to delivery route than Th2 responses [220]. Importantly, it is increasingly appreciated that tissue-specific immune responses are critical to controlling local infections [16]. This is perhaps best seen as regards immunity of mucosal organs, such as the lungs and digestive tract. As the most common site of entry for infection, eliciting mucosal immunity via vaccination is a critical parameter to success [221-223].

A powerful example of mucosal targeting is the induction of antigen-specific CD8+ T cells and production of a Th17 immune environment in the lungs following intranasal vaccination with a NP designed to target DC and release antigen into the cytosol of these cells [224]. Such responses are crucial to protecting against respiratory infections with bacteria and viruses, such as influenza. The paradigm of inducing mucosal immunity also holds true for targeting mucosal tumors, whereby vaccination through the intranasal route is able to elicit anti-tumor CD8+ T cell responses while intramuscular vaccination fails to do so in orthotopic head, neck and lung cancers derived from the human papillomavirus 16 (HPV16) E7–expressing epithelial TC1 cell line [225].
Cancer Immunotherapy

In addition to the use of NMP for microbial vaccination, immunoengineering with NMP offers entirely new approaches to cancer treatment while avoiding the systemic toxicity associated with soluble delivery of cytokines or antibodies [136]. Similar to vaccines against microbes, a number of research groups are developing NMP-based cancer vaccines which incorporate PRR agonists and antigens associated with specific cancers [137,225-228]. While these approaches do show promise, a difficulty will be identifying cancer-specific antigens that don’t result in autoimmune pathology in healthy tissues. Thus, modulating the immune environment of cancer is a potentially more viable option.

As discussed earlier, cancers co-opt their microenvironment in order to hide from the immune system and divert nutrients towards their growth [130]. Therefore, some nascent anti-cancer NMP approaches involve blocking the mechanisms by which cancer quells immune responses in the tumor microenvironment. One example is through inhibiting the function of STAT3, a transcription factor upregulated in tumors that dampens production of Th1 cytokines while increasing secretion of immunosuppressive factors [229,230]. Designing NPs to carry siRNA against STAT3 has shown promise in shifting the immunosuppressive tumor environment towards one that enables immune surveillance and tumor clearance [231,232]. Another approach coupled NP-mediated delivery of IL-2, a T-cell activating cytokine, with a small molecule inhibitor of the immunosuppressive cytokine TGF-β. This multi-pathway approach inhibited tumor growth, provided 100% survival in mouse melanoma models and required NP delivery of both
molecules, as either compound alone or delivered in soluble fashion failed to protect mice [233]. This reflects a growing understanding in the cancer field that targeting multiple pathways at once will be required to avoid tumor resistance [234]. NMP are well suited to deliver multiple cargoes in this regard.

Due to clearance by innate immune cells as well as vasculature abnormalities in the tumor bed, it can be difficult to ensure NMP reach cancer regions at sufficient levels to exert therapeutic benefit \textit{in vivo} [235]. Thus, modification of immune cells \textit{ex vivo} prior to implantation back into the host is a ‘trojan-horse’ approach that relies on the intrinsic ability of immune cells to navigate to tumor sites \textit{in vivo}. A creative use of this concept was reported by the Irvine group where they conjugated NP to the surface of T cells \textit{ex vivo} using free thiol groups present on the surface of T cell membranes. By loading these NP with IL-15 and IL-21, potent T cell activating cytokines, these NP could in effect serve as paracrine signaling hitchhikers on T cells and greatly enhanced the T cell tumor killing capabilities \textit{in vivo} [236].

\textit{Hitchhiking Particles and Inflammation}

The ‘hitchhike’ tactic has been used by other groups in non-cancer applications to deliver NMP to sites usually occluded from particle transport. For example, by feeding macrophages NP with antiviral cargo, researchers have enabled delivery of anti-virals to the brain after these NP-laden macrophages navigated through the blood-brain barrier [237,238]. Access to the central nervous system (CNS) is notoriously challenging so NMP approaches which enable
therapeutic delivery of drugs or immune-modulators to this region have a tremendous amount of clinical potential [239,240].

Inflammation itself is a driver of numerous pathologies, wreaking havoc in the CNS after stroke for example, so approaches to limit inflammation are promising areas for NMP [34,96,241]. While encapsulating anti-inflammatory small molecules like cyclosporine or glucocorticoids in NP is an effective means of dampening inflammation, more recent efforts make use of immune signaling pathways to broaden the impact of NMP interventions [242,243]. One means of targeting inflammation is the development of reactive oxygen species-sensitive (ROS) polymers [244]. ROS production is upregulated by cells during inflammation associated with cancer and infection, thus NMP designed with ROS-sensitive polymers may selectively degrade in targeted areas of inflammation and release therapeutic payloads only in these sites.

A conceptually simple yet elegant approach to reduce inflammatory pathology is the passive targeting of monocytes with therapeutic NP that inhibit monocyte recruitment to sites of inflammation where they perpetuate and worsen disease. This can be done through encapsulating siRNA to CCR2 in NP, as CCR2 is a cell surface chemokine receptor required for recruitment of inflammatory monocytes. NP-delivery to these cells reduces upregulation of CCR2 and inhibits their ability to respond to inflammatory alarm signals because they are not able to sense it from their environment in the absence of CCR2. In mouse studies, this approach attenuates the number of inflammatory monocytes in atherosclerotic plaques, reduces infarct size after coronary artery occlusion, prolongs insulin sensitivity in
diabetic mice after pancreatic islet transplantation, and reduces tumor volumes and the number of tumor-associated macrophages [245].

*Autoimmune Disorders – No Need to Fight the Self*

A last area we will discuss regarding immunoengineering approaches relates to autoimmune disorders. Autoimmunity occurs when the immune system attacks the self, though the etiologies for these disorders still remain largely elusive [24,96,115]. Yet like so many aspects of human pathology, modulating the immune response can be a viable therapy in setting of autoimmunity. The current standard of care is the use of systemic immunosuppressive drugs to shut down the entire immune system non-specifically. Conversely, the immunoengineering approach uses NMP to deliver antigen-specific instruction to the immune system to either dampen the activity of self-targeted T cells or induce a regulatory T cell type of adaptive immune response [214,246,247]. These approaches rely on the same premise as microbial vaccines, but with the goal of inducing regulatory responses as opposed to effector T cells (Figure 1-2).

One example is decorating NP with MHC-peptide complexes specific for self-antigens in a NOD mouse model of diabetes. Diabetic mice treated with these NP induced auto-regulatory memory T cells that were able to suppress immune-mediated pathology and restore glucose homeostasis [248]. In analysis of the mechanism by which this regulatory T cell population was elicited, the authors believe the MHC-peptide conjugation to NP approach may work for any antigen that is targeted in autoimmune settings [249].
More recently, mice with experimental autoimmune encephalomyelitis (EAE), which models the autoimmune disorder multiple sclerosis, were effectively cured of disease through a NMP treatment approach. In this model, the autoimmune-inducing antigen is a proteolipid protein (PLP), one of the most abundant proteins in the CNS. Whereas delivery of soluble PLP exacerbated disease, simply conjugating PLP to the surface of PLGA NP was sufficient to dampen disease through shutting down the T cells responsible for mediating pathology [250]. While the mechanism is not fully clear, this finding suggests that antigen delivery by naked NP may mimic antigen presentation by dying self-cells, which results in tolerogenic immune responses. We will explore engineering tolerance in more detail in Chapter 4.

1.9 DISSERTATION OVERVIEW

Having presented a background on the role of signal transduction pathways in orchestrating immune responses as well as how NMP design parameters can be used to augment these functions for therapeutic aims, we will now turn to research performed during the course of this dissertation. Throughout this work, we make use of the PRINT fabrication process to precisely address how particle size, shape and composition affect the mammalian immune response. As has been detailed at length, the innate immune system is the prime driver of adaptive immune responses. Therefore, to help bring about the full potential of therapeutic immunoengineering pursuits, a baseline assessment of NMP interactions with the mammalian innate immune system is necessary. The ultimate goal is to identify immunologically inert
particles that then can be designed to skew immune responses towards any and all immune classes.

In Chapter 2, we focus on the murine innate immune system and address the potential for NMP particles to activate innate immunity through initiation of inflammatory responses in murine macrophages ex vivo, with a particular focus on particle-mediated inflammasome activation. We then address the use of particles across the nano and microscale as inert programming devices for pulmonary therapeutics using direct instillation into murine lungs as our model. Our results identify a cadre of particles across the nano- and micro-scale that can remain in the lungs for at least one week in an immunologically inert fashion.

In Chapter 3, we move our NMP studies into a human monocyte cell line and primary human immune cells from healthy donors. We assess the potential for these particles to activate inflammatory responses in human immune cells as well as carry out a broad cytokine screen to uncover other immune pathways that may be augmented by particle treatment. We identify human immune cell subsets which preferentially take up NP and a chemical modification that can augment this response. We also identify heterogeneity in the magnitude of human immune responses to NP among donors that may help inform future clinical studies. To lend further translational relevance to this work, we cross-validate these findings in a humanized mouse model to establish an in vivo system for future NMP studies with clinical applications.
In Chapter 4, we present a compilation of findings over the course of our studies that reflect not only the complexity of innate immune responses to NMP, but also highlight potential immunoengineering directions using rationally designed particles we have generated. Studies address: 1) the role of surface modification on augmenting nanoparticle uptake into human immune cells; 2) particle design parameters to control the kinetics and magnitude of inflammasome activation; 3) anti-inflammatory NMP designs; and 4) NMP designs which may enable programming of immune tolerance through two separate pathways. These findings have direct relevance to the development of microbial and cancer vaccines, reducing inflammatory damage in a range of disease settings, and offering new treatments for autoimmune disorders.

In Chapter 5, we conclude with an overview of the findings of this dissertation. Important lessons that may help guide future nanomaterials research in mammalian biology will also be highlighted. Lastly, we offer thoughts on future directions and implications of immunoengineering approaches to human disease.
CHAPTER 2

ANALYSIS OF THE MURINE IMMUNE RESPONSE TO PULMONARY DELIVERY OF PRECISELY FABRICATED NANO- AND MICROSCALE PARTICLES

Nanomedicine has the potential to transform clinical care in the 21st century. However, a precise understanding of how nanomaterial design parameters such as size, shape and composition affect the mammalian immune system is a prerequisite for the realization of nanomedicine’s translational promise. Herein, we make use of the recently developed Particle Replication in Non-wetting Template (PRINT) fabrication process to precisely fabricate particles across and the nano- and micro-scale with defined shapes and compositions to address the role of particle design parameters on the murine innate immune response in both in vitro and in vivo settings. We find that particles composed of either the biodegradable polymer poly(lactic-co-glycolic acid) (PLGA) or the biocompatible polymer polyethylene glycol (PEG) do not cause release of pro-inflammatory cytokines nor inflammasome activation in bone marrow-derived macrophages. When instilled into the lungs of mice, particle composition and size can augment the number and type of innate immune cells recruited to the lungs without triggering inflammatory responses as

assayed by cytokine release and histopathology. Smaller particles (80x320nm) are more readily taken up in vivo by monocytes and macrophages than larger particles (6µm diameter), yet particles of all tested sizes remained in the lungs for up to 7 days without clearance or triggering of host immunity. These results suggest rational design of nanoparticle physical parameters can be used for sustained and localized delivery of therapeutics to the lungs.

2.1 INTRODUCTION

The application of nanoparticles in medicine for disease treatment is a potentially transformative area of research. The possibility of potent instruction and modulation of host physiology through nanomaterials has been abundantly demonstrated. These efforts include modulation of cell-specific gene expression through delivery of antisense oligonucleotides, dose-sparing and targeted delivery of pharmacologics, as well as enhanced multi-functional imaging diagnostics through nanoformulations [245,251,252]. Another area where nanotechnology may revolutionize clinical care is the ability to direct immune responses in defined manners. The most obvious benefit is in the design of next-generation vaccines against microbial pathogens, whereby antigen specific immune responses can be elicited at levels far more potent than existing vaccines, commensurate with the immune response engendered by live organisms [123,204,253]. These latter advances owe much to our recent understanding of the critical role of innate immunity in contextualizing an appropriate adaptive immune response. This context is triggered through endogenous host receptor signaling pathways, most well
characterized by the TLR family of pattern recognition receptors (PRR), but hallmarked by a panoply of such PRRs including C-type lectin receptors, RIG-like helicases and the burgeoning understanding of Nod-like receptors (NLRs) as sentinels of the intracellular environment [18].

Researchers in the emerging field of immune-engineering are capitalizing on these exciting advances to open up the possibility to direct and instruct immunological outcomes to a variety of pathological conditions [246]. These include improving current pathogen vaccines, to the more nascent fields of cancer immunotherapy, tolerance induction in the setting of autoimmunity and organ transplantation, as well as general immunological rebalancing in diseased settings, such as the chronic inflammation associated with type 2 diabetes. The implications of such technology are profound and potentially represent a paradigm shift in clinical practice across a broad swath of medicine. However, efforts to use nanotechnology and material sciences engineering to modulate human biology in situ require a comprehensive understanding of the immune response, or lack thereof, engendered to introduced nanocarriers [148,150].

In order for the gamut of potential downstream therapeutic applications of nanomedicine to be realized, we must first understand how the physical properties of nanomaterials augment host immune responses. These principles will then enable the appropriate design of nano- and micro-scale interventions for specific purposes. For example, the use of nanoparticles to deliver potent biological molecules, such as oligonucleotides or small molecules augmenting intracellular signaling pathways, may squander the regulatory opportunity to reach the clinic if the nanocarriers for
such entities initiate off-target events that activate host immune responses.
Conversely, vaccines can be made more potent if nanocarriers are designed to
activate the appropriate innate immune response to tailor adaptive immune
responses to delivered antigens. As an example, the current state of the art is to
use Alum as a non-specific immunomodulatory adjuvant in vaccine formulations.
This could explain, in part, why some of the most pressing pathogens do not yet
have useful vaccines as Alum is known to engender a Th2-biased humoral immune
response [254]. It is conceivable that nano-carriers designed to elicit the appropriate
adaptive immune response to an antigen of interest - i.e., Th17 against fungal
pathogens - may enable the generation of vaccine-induced immune responses that
more closely mimic the natural immune response elicited by infection with a given
pathogen as opposed the non-tailored immunity induced by Alum.

A great issue in advancing nanotechnology from a laboratory pursuit into a
component of clinical care is a robust understanding of how physical particle
properties augment biological outcomes. There is a wealth of literature promoting
the use of nanotechnology in modern medicine, but much of this literature relies on
particle fabrication methods, such as oil-in-water emulsion, that generate
heterogeneous populations of particles that can vary widely between batches and
across labs [255,256]. In addition, most studies related to biomedical applications
of nanotechnology have not addressed the role of the immune system in the host
response to particulate delivery, a critical issue that threatens to diminish the utility of
such particles if these are rapidly cleared by innate immune cells and/or induce
localized or systemic immune responses that pose unintended complications for
clinical development. In cases where the immunological parameters of nanotechnology are being addressed, great variance is seen based on size, composition and even surface modification of particles, highlighting the tremendous complexity and exquisite sensitivity of the immune system to nanoscale events [148-150].

Most currently employed fabrication methods do not allow precise control over particle physical parameters, thus it is difficult to draw conclusions as to how size, shape and composition affect the innate immune response to particles in the nano and micron range. To address this lack of knowledge, we employed our recently developed top-down nanofabrication technique termed Particle Replication in Non-Wetting Templates (PRINT) [157,188,194,257]. Using soft-lithography techniques adopted from the semi-conductor industry, PRINT enables the production of monodisperse nano- and microparticles with well-defined control over particle size, shape, composition, modulus and surface chemistry. Therefore, the role of these physical parameters in augmenting biological responses can be reproducibly probed using PRINT technology.

To lend both clinical and field relevance to our findings, particles were designed with either the F.D.A approved polymer Poly-lactic co-gloycolic acid (PLGA) or derivatives of the commonly published polymer polyethylene glycol (PEG). While PLGA is an attractive polymer given its long history of clinical use, our study was aimed in part to clarify discrepancy in the literature as to whether particles made of PLGA trigger inflammation. As our main purpose was to define the ‘baseline’ status of whether particles of defined size, shape and composition
triggered inflammation, we did not augment particles in this study to include additional biologically active molecules, such as oligonucleotides, small molecules or adjuvants as previously published by our group and others [258-261]. To this end, we used in vitro assays with murine derived macrophages and in vivo delivery of particles to the lungs of mice to test the inflammatory potential of these particles. The lung is a highly desired site for therapeutic delivery of nanomedicine and we chose it for both clinical relevance and its sensitivity as an immunological organ [262]. Our findings imply that the delivery of PRINT nano- and micro-particles do not engender systemic or localized inflammatory responses and may not be impeded by host immune responses to the polymers used in this study. Future design strategies for the panoply of therapeutic opportunities made available by nanoengineering are likely available, as particles across broad size ranges can thus be rationally designed from an inert state.

2.2 MATERIALS AND METHODS

Particle Materials

Poly(ethylene glycol) diacrylate (Mn 700) (PEG700DA), 2-aminoethyl methacrylate hydrochloride (AEM), Diphenyl (2,4,6-trimethylbenzoyl)-phoshine oxide (TPO), and poly lactic co-glycolic acid (PLGA; 85:15 lactic acid/glycolic acid, MW=55 000 g/mol) were purchased from Sigma-Aldrich. Tetraethylene glycol monoacrylate (HP4A) was synthesized in-house as previously described [263]. Thermo Scientific Dylight 650 maleimide, PTFE syringe filters (13mm membrane,
0.220 µm pore size), dimethylformamide (DMF), triethanolamine (TEA), pyridine, sterile water, borate buffer (pH 8.6), Dulbecco’s phosphate buffered saline (DPBS) (pH 7.4), 1X phosphate buffered saline (PBS) (pH 7.4), acetic anhydride and methanol were obtained from Fisher Scientific. Conventional filters (2 µm) were purchased from Agilent and polyvinyl alcohol (Mw 2000) (PVOH) was purchased from Acros Organics. All PRINT molds used in these studies (80 nm x 320 nm, 1µm cylinder, 1.5µm and 6µm donuts) were kindly provided by Liquidia Technologies.

PRINT PLGA Particle Fabrication

The PRINT process for fabricating particles has been described previously [260,261]. Briefly, to fabricate PLGA particles, a preparticle solution containing PLGA was prepared in a DMSO (dimethylsulfoxide) / DMF (dimethylfluoride) / water solvent mixture (4:16:1) and cast on a poly-(ethylene teraphthalate) (PET) sheet (delivery sheet) using a #5 Mayer Rod (R.D. Specialties). The delivery sheet was placed in contact with a PRINT mold with desired features patterned (e.g., 80 × 320 nm). The delivery sheet and mold were passed through a heated laminator (150 °C, 5.5 × 105 Pa) and separated at the nip. This heating process enables the PLGA polymer solution to fill the molds, thereby forming nanoparticles of desired size and shape. Nanoparticles were then harvested from the PRINT mold by placing it in contact with a PET sheet coated with a layer (400 nm cast from water) of poly(vinyl alcohol) (PVA, MW = 2000 g/mol). This mold/PET-PVA ensemble was then passed through the laminator (150 °C, 5.5 × 105 Pa) to transfer the nanoparticles to the PVA sheet. Both laminator steps, the filling of the mold and transfer of particles onto the PVA-coated PET sheet, were performed at low humidity (~20–30%). Particles were
released from the PET/PVA sheet by delivering ~1ml of sterile water via a bead harvester to dissolve the PVA layer and remove the particles from the PET sheet. A typical yield of 80x320nm PLGA particles was ~0.4 mg particles/ft of PRINT mold, though this depended on the particle feature size of the mold. To remove excess PVA and concentrate the particles, tangential flow filtration (TFF; Spectrum Labs) was used to concentrate particles in sterile water (1-2mg/ml). For later use in particle characterization assays and experiments, particles were lyophilized by adding 10× mannitol and 8× sucrose (10× and 8× to mass of particles) using a tree lyophilizer. Mannitol and sucrose were used as cryoprotectants.

PRINT Hydrogel Fabrication

The process of fabricating 80x320nm hydrogel particles was conceptually similar to PLGA fabrication, but with important differences. The pre-particle solution (PPS) contained a composition of 67.5 wt% HP₄A, 20 wt% AEM (functional monomer), 10 wt% PEG₇₀₀₆ₐ (crosslinker), 1 wt% TPO (photo initiator) and 1.5 wt% Dylight 650 maleimide. This composition was then dissolved at 3.5 wt% in methanol and drawn as a thin film using a # 3 Mayer rod (R.D. Specialties) onto a roll of corona treated PET using an in-house custom-made roll-to-roll lab line (Liquidia Technologies) running at 12 ft/min. The solvent was evaporated from this delivery sheet by exposing the film to heat guns. The delivery sheet was laminated (80 PSI, 12 ft/min) to the patterned side of the mold, followed by delamination at the nip. Particles were cured by passing the filled mold through a UV-LED (Phoseon, 395 nm, 3 SCFM N₂, 12 ft/min). A PVOH harvesting sheet was hot laminated to the filled mold (140 °C, 80 PSI, 12 ft/min). Upon cooling to room temperature, particles were removed from
the mold by splitting the PVOH harvesting sheet from the mold. Particles were then harvested by dissolving the PVOH in a bead of water (1 mL of water per 5 ft of harvesting sheet). The particle suspension was passed through a 2 µm filter (Agilent) to remove any large particulates. To remove the excess PVOH, particles were centrifuged (Eppendorf Centrifuge 5417R) at 14000 rpm for 15 min, the supernatant was removed and the particles were re-suspended in sterile water. This purification process was repeated 4 times prior to lyophilization as detailed above. The 80x320nm particles were acetylated prior to experimental use to match negative charge of micron sized hydrogel particles.

The 1.5 and 6 µm donut shaped hydrogel particles were fabricated using a dropcast method. The pre-particle solution (PPS) was composed of 20% PEG$_{700-}$DA, 78% HP$_4$A, 1% TPO (photoinitiator), and 1% Dylight 650. The solution was spread onto a fluorocur mold and a poly(ethylene terephthalate) (PET) sheet was laminated on top of the mold and polymer mixture and run through a heated, pressurized laminator to fill the molds. The mold was then cured with a UV LED lamp for 30 seconds. Particles were transferred out of the mold onto a Luvitec harvesting layer by laminating the mold and Luvitec sheet together and running them through a heated laminator nip. The mold and harvesting sheet was separated, leaving free particles on the harvest layer. Particles were collected from the harvest sheet by bead harvesting with water and pelleted by centrifugation. The particles were re-suspended in tert-butanol and lyophilized overnight.
Particle Characterization

Thermogravimetric analysis (TGA) was used to determine stock particle concentrations (TA Instruments Q5000 TGA). Briefly, 20 µL of the stock nanoparticle solution was pipetted into a tared aluminum sample pan. The sample was heated at 30°C/min to 130°C and held at this temperature for 10 minutes. The sample was then cooled at 30°C/min down to 30°C and held for 2 minutes. A Hitachi S-4700 scanning electron microscope (SEM) was used to visualize particles. Prior to imaging, the SEM samples were coated with 1.5 nm of gold-palladium alloy using a Cressington 108 auto sputter coater. Particle size and zeta potential were measured by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Instruments, Ltd.).

Experimental animals

All studies were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill. All animals were maintained in pathogen-free facilities at the University of North Carolina at Chapel Hill.

In vitro inflammation assays

Bone marrow macrophages were isolated from the femurs of C57Bl/6 and BALB/c mice using standard procedures. Bone marrow-derived macrophages were cultured for six days in DMEM supplemented with 10% fetal bovine serum, L-
Glutamine, pen/strep and 20% L929-conditioned medium prior to use in particle experiments. Adherent cells were isolated and plated in complete Dulbecco’s Modified Eagle Medium (Gibco) with 10% fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine at 200,000 cells per well in a 96-well dish for 24 hours prior to treatment with particles. Some cells were primed with LPS (50ng/ml) for 24 hours prior to particle treatment to provide signal 1 for inflammasome activation. MSU (300μg/ml; Invivogen) or ATP (5mM) was used as a positive control for inflammasome activation. Particles were resuspended in PBS prior to dosing at various concentrations. After 24 hours of particle treatment in triplicate, supernatants were harvested and analyzed by murine IL-1β, TNF-α and IL-6 ELISA (BD Biosciences).

Assessment of airway inflammation

To assess whether PRINT particles induce airway inflammation, 10-12 week old female C57Bl/6 mice were anesthetized with isofluorane inhalation and particles were instilled via intratracheal (i.t.) administration. 50µg of particles were dosed in 50ul of PBS. Intratracheal administration of PBS (50ul) or LPS (20µg in 50ul PBS) served as negative and positive controls for airway inflammation, respectively, as previously described [264]. Mice were euthanized and airway inflammation was assessed 48 hours or 7 days post treatment.

Serum was collected from animals by cardiac puncture and centrifuged at 15,000 RPM for 10 minutes. The serum supernatant was collected and used for ELISA analysis of inflammatory markers. Bronchoalveolar lavage fluid (BALF) was
also collected to evaluate local leukocyte and cytokine levels in the lungs. For this purpose, lungs were lavaged three times with 1ml Hanks Balanced Salt Solution (HBSS; Gibco). After centrifugation at 1500 RPM for 5 minutes, cell-free supernatants were collected and used to assess cytokine levels of IL-1β, TNF-α and IL-6 via ELISA (BD Biosciences). RBC were lysed via brief hypotonic saline treatment and the cell pellet was resuspended in PBS. Total BALF cellularity was assessed with a hemacytometer. The cellular composition was determined by cytospin of BALF aliquots onto slides and staining with Diff-Quik (Dade Behring) for differential cell counts. Leukocytes were identified based on the morphology of ≥200 cells per sample. Following BALF harvest, the lungs were fixed by inflation (20-cm pressure) and immersed in 10% buffered formalin.

**Histopathological examination**

Inflammation was evaluated in 5µm sections of the left lung lobe after hematoxylin and eosin (H&E) staining. Serial paraffin-embedded sections were set and cut to reveal maximum longitudinal visualization of the intrapulmonary main axial airway and inflammation was scored by one of the authors (I.C.A.) who was blinded to genotype and treatment. As previously described, histology images were evaluated on each of the following inflammatory parameters and scored between 0 (absent) to 3 (severe): mononuclear cell infiltration, polymorphonuclear cell infiltration, airway epithelial cell hyperplasia/injury, extravasation, perivascular cuffing, and estimated percentage of the lung involved with inflammation [48,265]. Scores for each parameter were averaged for a total histology score.
Particle uptake in BALF

BALF aliquots from PEG treated mice were fixed in 2% paraformaldehyde and stained with DAPI (nuclei) and Phalloidin 488 (actin) and then viewed via epifluorescence microscopy for particle uptake (Dylight 650). Five distinct fields of view (FOV) were captured for each slide. The percentage of cell uptake was determined by dividing the number of cells showing particle internalization by the total number of cells in each field of view.

Statistical Analysis

GraphPad Prism 5 software was used to identify statistical significance. Single data point comparisons were evaluated by Student’s two-tailed t-test, whereas multiple comparisons were evaluated for statistical significance using Analysis of Variance (ANOVA) followed by Tukey-Kramer HSD post-test. All cytokine and cell count data are presented as mean +/- standard deviation (SD) or standard error of the mean (SEM), respectively, with a p-value less than 0.05 considered statistically significant.

2.3 RESULTS

PRINT enables the fabrication of monodisperse and homogenous particles

We employed Particle Replication in Non-Wetting Templates (PRINT) in an effort to address whether particles of defined size, shape and composition trigger an inflammatory response in mice. This fabrication platform enables production of
homogenous and monodisperse particles with user-defined physical parameters. As a large amount of literature shows crucial biological differences depending on size and shape, the PRINT technique enables reproducible probing of basic cell biology with nearly complete control of design parameters [122,150,266,267].

For the purposes of our studies, we fabricated particles across the nano and micron range to reflect biologically relevant sizes. These include 80x320nm particles (commensurate with the sizes of small bacteria and large viruses), 1 µm and 1.5µm particles (commensurate with bacteria and platelets), and 6µm particles (akin to a red blood cell in size) [266,268]. To characterize the fabricated particles, we performed dynamic light scattering (DLS) and zeta potential measurements as shown in Figure 2-1A. Note that DLS measurements are quantified based on particles with a perfect sphere shape, so that the size ranges detected are in line with non-spherical shapes of the molds used. The poly-dispersity index (PDI), a measure of heterogeneity in a particle population, indicates we were able to fabricate monodisperse particles of the same size and shape. This is further evidenced by scanning electron microscopy images (Figure 2-1B). As the surface charge of particles has been shown to play a role in biological outcomes, such as protein adsorption and cell uptake, we measured the Zeta potential of particles to quantify net surface charge [267,269,270]. For all PLGA particles, surface charge was negative and decreased with increasing particle size (Figure 2-1A).

While our initial studies used particles fabricated from the F.D.A-approved biocompatible and biodegradable polymer poly(lactic-co-glycolic acid) PLGA, we also incorporated studies using particles fabricated with derivatives of biocompatible
poly (ethylene glycol) (PEG). Chemical modification of PEG is more feasible than with PLGA and thus it is often used to add increased functionality to nanocarriers, such as decoration of cell-targeting ligands, imaging agents, and pharmacologic cargo incorporation. Characteristics of fabricated PEG particles were similar to that of the PLGA particles, with low PDI and negative surface charge (Figure 2-1C) and monodispersity as evidenced by SEM (Figure 2-1D). These PEG particles were fabricated with a dye (DyLight 650) to enable fluorescent imaging of particles in downstream assays.
Table 2-1. PRINT Particle Characterization.

<table>
<thead>
<tr>
<th>PLGA Particle Type</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Charge (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80x320nm</td>
<td>226</td>
<td>0.03</td>
<td>-4.21</td>
</tr>
<tr>
<td>1µm cylinder</td>
<td>1465</td>
<td>NA</td>
<td>-18</td>
</tr>
</tbody>
</table>

Figure 2-1. PRINT Particle Characterization.

A) Dynamic light scattering (DLS) and zeta potential measurements of PLGA particles used in studies. Particle charge decreases with increasing size for PLGA but this trend isn’t consistent across polymers. B) Scanning electron microscope (SEM) images of PLGA particles. C) PEG particle composition and characterization. D) SEM of PEG particles.
**PRINT particles do not induce inflammation in murine macrophages**

Much work has been done *in vitro* to assess the potential use of PLGA nanoparticles in a variety of therapeutic modalities, from delivery of chemotherapeutics and siRNA to imaging agents for improved diagnostics to name a few. However, less work has been done to characterize the innate immune response to such particles and whether physical parameters of particles can augment the immune response. As a primary sentinel of host homeostasis, the innate immune system is tasked with identifying foreign matter in the body and initiating an appropriate response. Subsequent activation of the innate immune response is hallmarked by release of soluble protein messengers like cytokines that serve to recruit other immune cells to the area to participate in defense and repair of the host [18]. This inflammatory response is initiated by release of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β, from innate immune cells, such as macrophages.

The field of environmental toxicology has long studied the role of nanoparticulates in inducing inflammation, in particular in the lung [271]. Attempting to synthesize work by other groups using a range of particle compositions and sizes suggest that there is no clear correlation between the physical parameters of a particle and the ensuing inflammatory response to it. Generally speaking, the composition of a particle has greater bearing on the inflammatory response than its size or shape. As an example, titanium dioxide and silica dioxide nanoparticles trigger inflammation, whereas zinc oxide nanoparticles do not, even though all particles were of similar size (15-20nm) [75]. Others have identified size-dependent
inflammation and cell death that could be inhibited simply with surface modification of silica particles with common chemical groups such as aldehydes [160-162]. These findings highlight the sensitivity of the innate immune system as each particle may engender unique responses depending on its size, shape and composition.

We initially tested the inflammatory potential of PRINT particles in an *in vitro* cell culture system with bone marrow-derived macrophages from C57BL/6 mice. We used a panel of PRINT particles that differed in composition and size (Figure 2-1). After either a 5 hour or 24 hour incubation with a panel of PRINT particles comprised of either PLGA or PEG derivatives, no detectable levels of any tested pro-inflammatory cytokine (TNF-α, IL-6, IL-1β) across a range of doses (1-100μg/ml) were found (Figure 2-2A and data not shown). Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, was used as a positive control for inflammation induction. The lack of cytokine induction was in line with data from endotoxin assays indicating our fabrication process was endotoxin-free (Figure 2-2B). In addition, across all doses of particles tested, no particle-induce cytotoxicity was observed as measured by LDH assay (Figure 2-2C).

Given the recent discovery of the inflammasome as a mediator of the innate immune response to particulate challenge, we also sought to address whether PRINT-fabricated PLGA particles could cause inflammasome activation [72,75,272]. The inflammasome is a multi-protein complex that is formed in response to variety of environmental stimuli, including asbestos, silica and monosodium urate crystals, that results in the activation of caspase-1 and subsequent maturation and secretion of the pro-inflammatory cytokines IL-1β and IL-18 [43]. As our initial results did not
indicate any particle induction of IL-1β (Figure 2-2A), we next assessed whether
priming macrophages with LPS would cause particles to induce inflammasome
activation. LPS priming is thought to provide signal 1 to inflammasome formation by
upregulating the protein levels of pro-IL-1β and NLRP3, a main component of the
inflammasome complex [273,274]. As assessed by IL-1β release, PLGA particles
did not induce activation of the inflammasome in the presence or absence of LPS-
priming when tested in either BALB/c (Figure 2-2D) or C57BL/6 macrophages
(Figure 2-2E). Importantly, we tested particles across a range of doses (100ng-
3µg/ml) and sizes (80x320nm and 1µm cylinders). These results suggest that PLGA
particles across the nano and micron range do not synergize with TLR ligands (i.e.,
LPS) to induce inflammasome activation in vitro and lend further credence to the use
of PLGA particles for in vivo applications.
Figure 2-2. PRINT particles do not cause inflammation in bone marrow-derived macrophages from BALB/c or C57BL/6 mice.

A) Overnight stimulation with a panel of PRINT PLGA and hydrogel particles at 100μg/ml does not cause TNF-α, IL-6, or IL-1β release from bone marrow-derived macrophages from C57BL/6 mice as measured by ELISA. B) Both PLGA and hydrogel PRINT particles tested negative for endotoxin contamination using a Limulus amebocyte lysate assay. C) PRINT particles are not cytotoxic in bone-marrow derived macrophages as determined by lactate dehydrogenase (LDH) release. D) 80x320nm PLGA particles do not synergize with LPS to induce inflammasome activation as measured by IL-1β ELISA in BALB/c bone-marrow derived macrophages. E) Neither 80x320nm nor 1µm PLGA particles synergize with LPS to induce inflammasome activation as measured by IL-1β ELISA in C57BL/6 bone-marrow derived macrophages. MSU was dosed at 300μg/ml. *** = p<0.001. Experiments were performed in triplicate. Data shown are representative of at least three independent experiments.
**PLGA particles do not induce lung inflammation**

Bolstered by our *in vitro* findings, we next were interested in whether PLGA particles could be delivered to the lungs of mice without causing overt signs of immune activation as hallmarked by inflammation. The lung was chosen as a highly sensitive mucosal organ with clearly defined markers of inflammation that is the sight of numerous therapeutically relevant diseases, from allergies and asthma to chronic obstructive pulmonary disorder (COPD) and respiratory infections by microbial pathogens such as tuberculosis and influenza [262,275]. As such, therapeutic modulation of lung biology is a highly desired clinical goal with relevance to the vast majority of the human population. We used intra-tracheal (i.t.) delivery to determine whether 80x320nm PLGA particles (50µg) caused inflammation in the lungs, with PBS (50ul) and LPS (20µg) used as negative and positive controls, respectively. 48 hours after installation, mice (n=5 per group) were harvested and lung inflammation was assessed via field standards used in respiratory infection models [48,265]. Bronchoalveolar lavage fluid (BALF) cellularity indicated no recruitment of immune cells to the lungs after particle treatment, as cell numbers were no different than the PBS control (Figure 2-3A). LPS-treated mice revealed a robust accumulation of leukocytes as is expected during inflammatory responses. Assessing the composition of leukocyte populations in the BALF revealed no significant recruitment of immune cells to the lungs of particle-treated mice. Conversely, LPS-treated mice had high levels of both monocytes and neutrophils, key mediators of the innate immune system's inflammatory response (Figure 2-3B).
While BALF cellularity is widely used as a marker of lung inflammation, lung histopathology enables a deeper understanding of inflammatory effects on the lung parenchyma. Representative sections of histopathology slides of the main bronchi of the left lobe were examined to further delineate leukocyte infiltration around lung vasculature, parenchyma and the large and small airways (Figure 2-3C). Whereas LPS treatment caused a clear accumulation of leukocytes throughout the lung, treatment with 80x320nm PLGA particles showed no difference as compared to PBS controls (Figure 2-3D). To further verify the non-inflammatory nature of these particles, pro-inflammatory cytokine levels were assessed in the BALF and serum of treated mice. No significant release of IL-1β (Figure 2-3E) or IL-6 (Figure 2-3F) was seen in the BALF. Serum measurements for these same cytokines and TNF-α were undetectable (data not shown). In total, these results are in agreement with the in vitro findings and suggest that 80x320nm PLGA particles can be delivered to the lungs without causing innate immune activation and inflammation.
Figure 2-3. 80x320nm PLGA particles do not cause lung inflammation in mice. Mice were challenged with either 50µg of 80x320nm PLGA particles or 20µg LPS i.t. and airway inflammation was assessed 48 hours post-challenge. **A**) Total cellularity of bronchoalveolar lavage fluid (BALF) in treated C57BL/6 mice is no different after 48 hours than PBS-treated mice and is significantly less than the inflammatory cell recruitment seen in LPS-treated mice. **B**) PLGA particle treatment does not induce any appreciable immune cell recruitment to the lungs of mice, as opposed to the heightened levels of monocytes and neutrophils seen in the lungs of LPS-treated mice. **C**) Histopathology revealed no significant differences in lung architecture between PBS- and 80x320nm PLGA particle-treated mice. This is in stark contrast to the airway occlusion and significant innate immune cell recruitment seen in LPS-treated mice. **D**) Histopathology scoring confirmed that no significant differences were seen between the lungs of PBS and PLGA particle treated mice. **E-F**) The increased lung levels of pro-inflammatory IL-1β and IL-6 seen in LPS-treated mice is not found in PLGA-treated mice. PBS, n=3; 80x320nm PLGA particle-treated, n=5; LPS-treated, n=3. ND = Not Detected. * = p< 0.05, *** = p<0.001. Experiments were performed using 3-5 mice per group. Data shown are representative of at least two independent experiments.
**Sustained deposition of hydrogel particles in lungs without inflammation**

To broaden the implication of our *in vivo* findings, we fabricated a series of particles using PEG polymers and their derivatives (hydrogels) that incorporated fluorescent dyes which enabled us to track them *in vivo* over time after lung instillation. The hydrogel particles ranged in size from 80x320nm to 1.5µm and 6µm as characterized in Figure 2-1. *In vitro* experiments indicated they did not elicit inflammatory cytokines or cell death from bone marrow-derived macrophages (Figure 2-2). Using the same experimental approach as outlined above, 50µg of particles were instilled i.t. into C57BL/6 mice and lung inflammation was assessed at two time points, 48 hours and 7 days post-particle instillation. As shown in Figure 2-4A, total BALF cellularity does not increase in the presence of hydrogel particles as compared to PBS at 48 hours, which is in marked contrast to LPS-induced cell recruitment to the lungs. Breaking down the BALF cell types revealed a similar number of monocytes in the lungs PBS and particle-treated mice, whereas LPS-treatment induced a marked influx of both monocytes and neutrophils (Figure 2-4B). At 7 days post-particle treatment, there was no significant increase in the total BALF cellularity or composition in mice treated with any hydrogel particles (Figure 2-4C and 2-4D). Histopathology analyses indicated neither lung architecture disruption nor leukocyte infiltration into the lungs or airways of particle-treated mice as compared to PBS controls at either the 48 hour or 7 day time point (Figure 2-4E and 2-4F).
Figure 2-4. Hydrogel particles do not cause lung inflammation in mice.
Mice were challenged with 50µg of hydrogel particles (80x320nm, 1.5µm, or 6.0 µm donuts) i.t. and airway inflammation was assessed 48 hours and 7 days post-challenge. A) BALF analysis indicated no increased cellularity 48 hours after hydrogel particle treatment, whereas a significant cellular influx was seen in LPS-treated controls. B) At 48 hours, BALF cellular composition does not show any significant trend for immune cell recruitment in hydrogel particle-treated mice. C-D) BALF cellularity and composition was not significantly augmented seven days after hydrogel particle treatment. E) Histopathology analysis revealed no significant differences in lung architecture between PBS- and hydrogel particle-treated mice at either 2 or 7 days post-treatment. F) Histopathology scoring confirmed that no significant differences were seen between the lungs of PBS and hydrogel particle treated mice at any time points. ***=p<0.001. Experiments were performed using 2-5 mice per group. Data shown are representative of at least two independent experiments.
Despite the absence of overt signs of inflammation, 6µm particles with their hallmark donut appearance could be viewed within the lung spaces of multiple mice after H&E staining 7 days post-challenge (Figure 2-5A). The lack of immune cell recruitment or disruption of tissue architecture around these particles may suggest an immunologically inert deposition of particles within the alveolar spaces. Such a depot may provide sustained localized delivery of therapeutically attractive molecules. Because the 80x320nm and 1.5µm particles were too small to see in lung histology samples, immunofluorescence imaging on BALF samples was performed to determine whether lung-localized cells took up particles. As shown in Figure 2-5B, BALF cells contained hydrogel particles of all sizes at 48hrs (magnified view in Figure 2-5C). The percentage of cells with particles decreases as particle size increased (Figure 2-5D). Whether this is due to the quantitatively higher number of 80x320nm particles at the same dose weight of larger particles, the relatively easier ability for a cell to take up smaller particles as compared to larger ones, or an as yet unidentified size-dependent biological effect remains unanswered. Of note, BALF cells 7 days after particle instillation show particle uptake, albeit to a lesser extent than the 48 hour time point (Figure 2-5E). Finally, the levels of pro-inflammatory cytokines released into the BALF and serum of PEG particle-treated mice were quantified. At both the 48 hour and 7 day time points, IL-1β, IL-6, and TNF-α were undetectable for any particle treatment (data not shown). In total, these data highlight the ability of PRINT particles to remain localized to the lung for long periods of time in an immunologically inert manner.
Figure 2-5. Sustained deposition of hydrogel particles in murine lungs.

A) 6µm hydrogel particles (denoted by red arrows) are visible in the alveolar spaces of multiple mice 7 days after intratracheal installation. Lower insets are magnified views of particles. B) Two days after treatment with hydrogel particles, BALF cells were stained and visualized for particle uptake via epifluorescence microscopy. Particles (DyLight 650, red); nuclei (DAPI, blue); F-actin (Phalloidin 488, green). C) Magnified views of BALF cells taking up hydrogel particles from Figure B images. D) Quantification of particle uptake indicates smaller particles are more readily taken up in BALF cells than larger particles. E) All types of hydrogel particles can still be seen in BALF cells seven days after treatment, though there is a marked decrease in the number of particles present as compared to the 2 day time point. Scale bar is 20µm. Data shown are representative of at least two independent experiments.
2.4 DISCUSSION

Given the diverse therapeutic potential of nano- and microscale particles, this study sought to define whether particles composed of either PLGA or PEG-derivatives induced inflammatory responses in an in vitro and in vivo setting. By making use of the highly controlled PRINT fabrication method, we were also able to determine whether particle size affected any ensuing innate immune responses. Our findings reveal that PRINT particles do not cause any obvious activation of the innate immune response in murine macrophages or the murine lung and maintain long term (7 day) immunologic stability in the lungs of mice.

The wide array of polymers and particle fabrication techniques used in nanomedicine studies makes it difficult to reach definitive conclusions regarding particle effects on innate immune functions. We initially used particles fabricated from PLGA as this is a commonly used polymer with attractive clinical potential given its F.D.A approval. There is some discrepancy in the literature as to whether PLGA particles are inflammatory in situ. Some groups suggest PLGA particles are inflammatory in vitro and in vivo, whereas others have not found this to be the case [72,73,203,276,277]. Our study reveals that PLGA particles of nano and micron range fabricated by PRINT technology do not synergize with a TLR ligand to cause inflammasome activation nor inflammation in general, and that in vivo delivery does not trigger an inflammatory reaction, contrary to a previous report [72]. The discrepancy between these findings may be due to differences in particle fabrication or experimental settings. It has recently been shown that fabrication of particles with non-smooth surfaces can cause inflammasome activation, which may help explain
the discrepancy in the literature regarding PLGA nanoparticle-mediated IL-1β release [165]. However, given the long clinical history of PLGA and the broad literature reporting PLGA particle uses for biomedical applications, it seems unlikely that particles derived from PLGA would trigger potent inflammatory responses, yet this confusion is precisely why more research must be carried out to ensure such unwanted side-effects are avoided as fabrication methods or material sourcing may impact immune responses significantly [278-280].

In addition, studies using PEG particles enabled us to broaden our understanding of innate immune activation by particles comprised of a polymer composition that enables wide-ranging chemical modifications for enhanced functionality, such as cell targeting, pH-specific cargo release and siRNA incorporation as previously reported by our group and others [258,259,281,282]. Interestingly, although these PEG particles are not considered biodegradable, they did not induce lung inflammation as seen with other non-degradable particles such as those comprised of polystyrene [276]. This suggests our PEG polymer composition may also be an attractive alternative from an environmental toxicology perspective in applications currently employing polystyrene particles.

The issue of innate immune activation by particles is of central relevance to the translational application of nanotechnology. While particulate vaccines against some pathogens and cancers will likely be designed to trigger localized inflammation as part of the general innate immune activation required for robust adaptive immune responses, most other biomedical applications for nano- and microparticles will benefit by avoiding such responses. Additionally, a strong immune response might
lead to the undesirable outcome of rapid particle clearance as well as hypersensitivity responses. Drug delivery, diagnostic imaging and physiological biomimicry are examples of nanoengineering applications that may be impeded by innate immune activation. Importantly, many advances in immune modulation made available through rationally designed nano- and microscale particles such as tolerance induction in the setting of autoimmunity or organ transplantation, direct targeting of immune cell subsets and immune-skewing of pathological microenvironments such as tumors or sites of chronic inflammation, require that particles be designed initially from an inert immunological state [197,211,233,248,250,283,284]. To wit, if particles alone trigger inflammatory responses that skew towards any type of adaptive response (e.g., Th1, Th2), then many of these therapeutic goals will not be achieved. For these reasons, we feel it is of utmost importance that baseline innate immune responses to particles be assessed as part of field standards [148].

Our in vivo studies reveal that particle size augments uptake into innate immune cells of the lungs, with larger particles taken up less than smaller particles. This finding suggests a duality in design considerations depending on therapeutic application. For example, drug delivery to the lungs to ameliorate asthma would likely be best served by larger particles that can release their cargo to extracellular spaces. Conversely, if trying to deliver a respiratory vaccine, smaller particles that are more readily taken up by antigen presenting cells and traffic to lymph nodes would be more appropriate. The finding that particles of all tested sizes remain in the lungs up to 7 days post instillation also suggest the ability to provide sustained
localized delivery of therapeutically attractive molecules via particulate formulations. This is far different than the rapid clearance seen for smaller particles (<50nm diameter) and reflects the importance of particle design parameters when considering therapeutic interventions [285].

Having identified particles across the nano- and microscale that do not trigger inflammatory responses in mice while remaining in the lungs, we plan to next use these particles as delivery devices for a range of biologically relevant molecules, including siRNAs, anti-inflammatory agents, and immune-skewing compounds. These studies will test the hypothesis that targeted modulation of lung immunology via nanoengineering may enable a new class of therapeutics for lung disorders that avoid systemic side-effects while also reducing administration doses. Importantly, we will move studies into human cells to provide much needed data regarding the immunological response to nanomaterials in our own species. Using the design control inherent to PRINT technology, we will also be able to systematically address the role of particle size and shape during delivery of bioactive molecules. Such results may be crucial to advancing next-generation respiratory vaccines and treatments for asthma, allergies and chronic disorders of lung function.

2.5 CONTRIBUTIONS

RAR designed and performed all experiments. ICA helped design and perform in vivo mouse studies. TS and WH fabricated PRINT particles. Manuscript was written by RAR with edits from JPT. JMD and JPT supervised the overall research project.
Biocompatible particles at the nano- and micron scale are a promising platform for biological programming as they can be modularly designed to trigger specific cellular events in a variety of disease contexts. Because the immune system is a major mediator of host homeostasis and pathology, avoidance of immune responses as well as directed and specific programming of immune responses will likely constitute a major clinical advance in the near future. In order for this to occur, a clear understanding of the baseline human immune response to nano and microscale programming devices must be systematically characterized in order for the gamut of potential therapeutics to be achieved. To this end, we make use of Particle Replication in Non-wetting Templates (PRINT) technology to precisely fabricate biocompatible particles across the nano- and micro-scale in order to address the role of size, shape and composition on human immune response. We first characterize the inflammatory response to particles in a human monocyte cell line and then identify resident immune cell populations which are passively targeted by nanoparticles in \textit{ex vivo} human blood samples. We uncover a role for PEGylation in modulation of particle uptake into human immune cells as well as a role for lipid incorporation in mitigating chemokine induction by PLGA nanoparticles.
through global cytokine array screening. Finally, we recapitulate our *ex vivo* human PBMC findings in a humanized mouse model and validate this as a whole animal approach for future nanomedicine studies with translational applications. Our systematic methodology affirms the potential of biocompatible particles as biological programming devices for human use.

### 3.1 INTRODUCTION

Biocompatible materials when fabricated at the nano- and micro-scale enable interaction with the mammalian immune system where precise programming of host biology becomes feasible [74,148,201,214,246,247,283,286,287]. This has already been evidenced in mice for anti-microbial vaccines [73,120,123,124,178,195,196,203-205,212,224,253,288,289], cancer immunotherapy [137,231-233,236,245], induction of tolerance in autoimmune settings [248-250] and siRNA-mediated gene knockdown [231,232,245,260], with some promising pioneering examples also occurring in human studies [290]. Concomitant with advances in molecular immunology, there is a widespread appreciation for the role of the immune system in modulating host homeostasis in every major disease category, including those of metabolic, neurologic, and oncogenic origin [17,129,147,291,292].

While multiple efforts are underway to identify the molecular bases of these various diseases, a next generation of therapeutics will likely include the use of biocompatible materials fabricated at the nano- and micro-scale to deliver programming instruction to humans at the organ, tissue and cellular level [2,18,214]. Nano- and micro-scale particles (NMP) will be designed to precise physical and
chemical specifications, as size, shape and composition of particulate matter clearly impacts host biology (reviewed in Chapter 1 and [148,156,214,246,247]). However, our understanding of the impact of these particle design parameters on the human immune response is still in its infancy. This is due in part to a lack of reproducible and well-controlled particle fabrication techniques, as well as a dearth of studies in human cells.

In this report, we make use of the Particle Replication in Non-wetting Templates (PRINT) process to fabricate biocompatible, monodisperse nano- and micro-scale particles (NMP) with precise dimensions to probe the human immune response to such particulate matter [188,189,191,192,194,293]. NMP are comprised of polymers already used in clinical interventions, either poly(lactic-co-glycolic acid) (PLGA) or polyethylene glycol (PEG) hydrogel derivatives [235]. Particles can be fabricated across the nano (80x320nm) to micron scale (3μm) without inducing inflammatory responses in a human monocyte cell line. In addition, we identify a subset of primary human peripheral blood mononuclear cells (PBMC) passively targeted by nanoparticles in a manner that correlates with CD14 expression and identify PEGylation as a means to augment the specific quantity of particles taken up by these cells and ensuing biological responses. Global cytokine profiling reveals a general lack of immune induction by hydrogel nanoparticles in human PBMC cultures. This study also uncovered a role for lipid incorporation and/or surface charge in modulating induction of chemokine release in human immune cells by PLGA particles, while also revealing large differences in the magnitude of innate immune responses among donors. Finally, to translate our
findings into a whole animal model, we show humanized mice recapitulate our ex vivo human PBMC results and make the first report of nanoparticle targeting of human plasmacytoid dendritic cells and bone marrow-resident immune populations in a humanized mouse. These results may engender more appropriate models for nanomedicine studies aimed at eventual application in the clinic.

3.2 MATERIALS AND METHODS

Particle Materials

Poly lactic co-glycolic acid (PLGA; 85:15 lactic acid/glycolic acid, MW= 55 000 g/mol), poly(ethylene glycol) diacrylate (Mₙ 700) (PEG₇₀₀DA), 2-aminoethyl methacrylate hydrochloride (AEM), and Diphenyl (2,4,6-trimethylbenzoyl)-phosphine oxide (TPO) were all sourced from Sigma-Aldrich. Tetraethylene glycol monoacrylate (HP4A) was synthesized in-house as previously described [263]. Thermo Scientific maleimide-terminated Dylight 650 and Dylight 488, PTFE syringe filters (13mm membrane, 0.220 µm pore size), dimethylformamide (DMF), triethanolamine (TEA), pyridine, sterile water, borate buffer (pH 8.6), Dulbecco’s 1x phosphate buffered saline (DPBS) (pH 7.4), acetic anhydride and methanol were obtained from Fisher Scientific. Rhodamine PolyFluor 570 was sourced from Polysciences. Methoxy-PEG(5k)-succinimidyl carboxy methyl ester (mPEG₅₀⁻SCM) was purchased from Creative PEGWorks. Conventional filters (2 µm) were purchased from Agilent and polyvinyl alcohol (Mw 2000) (PVOH) was purchased from Acros Organics. Liquidia Technologies kindly provided all PRINT molds used
in these studies (80 nm x 320 nm, 200 nm x 200nm, 1µm cylinder, 1.5µm and 3µm donuts).

PRINT PLGA Particle Fabrication

Fabricating PLGA particles using the PRINT process has been previously described in detail elsewhere [260,261] so a brief description is included here. A preparticle solution (PPS) containing PLGA was prepared in a DMSO/DMF/water solvent mixture (4:16:1) and cast on a poly-(ethylene teraphthalate) (PET) sheet (delivery sheet) using a #5 Mayer Rod (R.D. Specialties). The delivery sheet was placed in contact with a PRINT mold with desired features patterned (e.g., 80 × 320 nm, 200x200nm, 1.5µm donut). The delivery sheet and mold were then passed through a heated laminator (150 °C, 5.5 × 105 Pa) and separated at the nip. Heating enables the PLGA polymer solution to fill the molds through capillary forces, thereby forming nanoparticles of desired size and shape. Nanoparticles were then harvested from the PRINT mold by placing it in contact with a PET sheet coated with a 400nm layer of poly(vinyl alcohol) (PVOH, MW = 2000 g/mol). This ensemble of mold/PET-PVOH was then passed through the laminator (150 °C, 5.5 × 105 Pa) to transfer the nanoparticles to the PVOH sheet. Both laminator steps - the filling of the mold and transfer of particles onto the PVOH-coated PET sheet - were performed at low humidity (∼20–30%). Particles were released from the PET/PVOH sheet by delivering ~1ml of sterile water via a bead harvester to dissolve the PVOH layer and remove particles from the PET sheet. A typical yield of 80x320nm PLGA particles was ~0.4 mg particles/ft of PRINT mold, though this depended on the particle feature
size of the mold. Tangential flow filtration (TFF; Spectrum Labs) was used to concentrate particles in sterile water (1-2mg/ml) and remove excess PVOH. For later use in particle characterization assays and experiments, mannitol and sucrose were used as cryoprotectants ((10× and 8× to mass of particles, respectively), particles were lyophilized, flash frozen and stored at -20 degrees.

PRINT Hydrogel Fabrication

The process of fabricating hydrogel particles was conceptually similar to PLGA fabrication, but with important differences as follows. For 80x320nm hydrogel particles, the pre-particle solution (PPS) contained a composition of 67.5 wt% HP4A, 20 wt% AEM (functional monomer), 10 wt% PEG700DA (crosslinker), 1 wt% TPO (photo initiator) and 1.5 wt% Dylight 650 or Dylight 488 maleimide. This composition was then dissolved at 3.5 wt% in methanol and drawn as a thin film using a # 3 Mayer rod (R.D. Specialties) onto a roll of corona treated PET using an in-house custom-made roll-to-roll lab line (Liquidia Technologies) running at 12 ft/min. The solvent was evaporated from this delivery sheet by exposing the film to heat guns. The delivery sheet was laminated (80 PSI, 12 ft/min) to the patterned side of the mold, followed by delamination at the nip. Particles were cured by passing the filled mold through a UV-LED (Phoseon, 395 nm, 3 SCFM N₂, 12 ft/min). A PVOH harvesting sheet was hot laminated to the filled mold (140 °C, 80 PSI, 12 ft/min). Upon cooling to room temperature, particles were removed from the mold by splitting the PVOH harvesting sheet from the mold. Particles were then harvested by dissolving the PVOH in a bead of water (1 mL of water per 5 ft of harvesting sheet). The particle suspension was passed through a 2 µm filter (Agilent) to remove any
large particulates. To remove the excess PVOH, particles were centrifuged (Eppendorf Centrifuge 5417R) at 14000 rpm for 15 min, the supernatant was removed and the particles were re-suspended in sterile water. This purification process was repeated 4 times prior to lyophilization as detailed above. The 80x320nm particles were acetylated prior to experimental use to match negative charge of micron sized hydrogel particles.

The 1.5 µm and 3 µm donut shaped hydrogel particles used in THP-1 studies were fabricated using a dropcast method. The pre-particle solution (PPS) was composed of 20% PEG\textsubscript{700}-DA, 78% HP\textsubscript{4}A, 1% TPO (photoinitiator), and 1% rhodamine. The solution was spread onto a fluorocur mold and a poly(ethylene terephthalate) (PET) sheet was laminated on top of the mold and polymer mixture and run through a heated, pressurized laminator to fill the molds. The mold was then cured with a UV LED lamp for 30 seconds. Particles were transferred out of the mold onto a Luvitec harvesting layer by laminating the mold and Luvitec sheet together and running them through a heated laminator nip. The mold and harvesting sheet was separated, leaving free particles on the harvest layer. Particles were collected from the harvest sheet by bead harvesting with water and pelleted by centrifugation. The particles were re-suspended in tert-butanol and lyophilized overnight as detailed above.

**Particle Characterization**

Stock particle concentrations were determined using thermogravimetric analysis (TGA; TA Instruments Q5000 TGA). Twenty µL of the stock nanoparticle solution was pipetted into a tared aluminum sample pan. The sample was heated at
30°C/min to 130°C and held at this temperature for 10 minutes. The sample was then cooled at 30°C/min down to 30°C and held for 2 minutes. To account for the mass of any stabilizer remaining in each sample, TGA was performed on a 20 µL aliquot of supernatant from a centrifuged sample of the stock nanoparticle solution. By subtracting the concentration of the stabilizer from the concentration of the stock particle solution, the actual particle concentration was determined. Particles were visualized with a Hitachi S-4700 scanning electron microscope (SEM). Prior to imaging, SEM samples were coated with 1.5 nm of gold-palladium alloy using a Cressington 108 auto sputter coater. A Zetasizer Nano ZS (Malvern Instruments, Ltd.) was used to measure particle size and zeta potential by dynamic light scattering (DLS).

**PEGylation of 80x320 nm hydrogel particles**

Purified 80x320 nm hydrogel particles were exchanged from water to DMF following the centrifugation. The fabricated particles contained free primary amine groups which were used as chemical handles to react with a methoxy-PEG$_{5k}$-SCM. The particles (1 mg/mL in DMF) were reacted with TEA (100 µL) for 10 min at room temperature on a shaker plate at 1400 rpm. 14 mg of methoxy-PEG$_{5k}$-SCM was dissolved in DMF and added to the reaction mixture, shaken overnight and then quenched with borate buffer (100 µL). The nanoparticle solution was then centrifuged (14000 rpm; 15 minutes) and washed 5 times with DMF.

To yield negatively charged nanoparticles following PEGylation, particles were acetylated with acetic anhydride to quench any unreacted amines. Nanoparticles (1 mg/mL in DMF) were reacted with an excess (10 µL) of pyridine
and acetic anhydride (7 µL) in a sonicator bath (Branson Ultrasonic Cleaner 1.4 A, 160 W) for 15 min. A second addition of acetic anhydride (7 µL) was added and the suspension was sonicated for another 15 min. Following acetylation, particles were washed by centrifugation one time in DMF, one time in borate buffer to neutralize any acetic acid that had formed, and then washed four times with sterile water. Particles were analyzed by TGA, DLS and SEM after acetylation.

In vitro Inflammation and Cell Death Assays

The THP-1 human monocyte cells (ATCC) were cultured in RPMI 1640 (Gibco) supplemented with 1% penicillin/streptomycin, 1% L-glutamine and 10% heat-inactivated fetal bovine serum. For experiments, THP-1 were plated at 200,000 cells per well in a 96-well dish in 200µl of media. Some cells were primed with LPS or MPLA (100ng/ml; Invivogen) for 4 hours prior to particle treatment to provide signal 1 for inflammasome activation. MSU (300μg/ml; Invivogen) or ATP (5mM) was used as a positive control for inflammasome activation. Particles were resuspended in PBS prior to dosing at various concentrations in triplicate. After denoted incubation times, supernatants were harvested and analyzed by human IL-1β ELISA (BD Biosciences) or a cytotoxicity detection kit (Roche) to determine cell death.

NF-κB / AP-1 Activation Assay

The THP1-XBlue reporter cells (Invivogen) were used to assay NF-κB and AP-1 activation by a panel of PRINT particles. These cells stably express an NF-κB/AP-1-inducible reporter secreted alkaline phosphatase system to facilitate the
colorimetric monitoring of NF-κB/AP-1 activation. THP1-XBlue cells were plated at 200,000 cells per well in a 96-well dish in 200μl of media and incubated with PRINT particles (10μg/ml) for 20 hours. Supernatants were harvested and assayed for secreted alkaline phosphatase per manufacturer’s protocol.

Particle Uptake in THP-1

THP-1 cells that had been incubated with rhodamine-loaded 3.0 μm hydrogel donuts were fixed on slides in 2% paraformaldehyde (PFA) followed by permeabilization for 10 minutes in 0.1% Triton-PBS. 1:500 solution of Biotinylated Ricinhus Communis Agglutinin (RCA, Vector Labs) was incubated with slides for 1 hour at 37 degrees followed by 3 washes for 5 minutes each in PBS. Avidin conjugated Alexa Fluor 594 (Invitrogen) was incubated 1:500 with slides for 45 minutes at room temperature. Slides were washed 3 times for 5 minutes each in PBS. Slides were mounted in Vectashield with DAPI (Vector Labs). Slides were viewed via immunofluorescence microscopy to assess particle uptake.

Human PBMC Isolation and Culture

Blood was donated by healthy donors in accordance with the University of North Carolina’s Office of Human Research Ethics (IRB #12-1858). Peripheral blood mononuclear cells were prepared from buffy coats by density gradient centrifugation using Lymphoprep (Cosmo Bio USA). PBMC were cultured in AIMV medium (Gibco) supplemented with 1% penicillin/streptomycin, 1% L-glutamine and 10% heat-inactivated AB-human serum (Invitrogen). PBMC were plated at 300,000 cells per well in a 96-well dish in 200μl of media and allowed to rest for 4 hours prior to
incubation with particles (10µg/ml) for 8 hours. After experimental time point, plates were spun at 1500RPM for 5 minutes and supernatants were stored at -80 degrees until further cytokine human 30-plex analysis (Life Technologies) using the Luminex platform. Cells were then stained and fixed for flow cytometry.

*Flow Cytometry of Human PBMC*

To differentiate immune cell subsets, cells were stained with fluorescent human antibodies as follows CD3-FITC, CD19-PacBlue, CD56-PerCP-Cy5, CD14-PE, CD11b-APC-Cy7, and CD11c –PE-Cy7 (Becton Dickinson and eBiosciences). Live/Dead Fixable Dead Stain Kit was from Invitrogen. Flow cytometry was performed on a LSR II using FacsCalibur software (BD Biosciences). Flow data was then analyzed via FlowJo (Tree Star, Inc.).

*Humanized Mice Experiments*

A detailed protocol for preparation of NRG-hu humanized mice has previously been published [294-297]. In brief, human CD34+ cells were isolated from 15- to 18-week-old fetal liver tissues. The cell suspension released from the liver was filtered through a 70-µm cell strainer (BD Falcon) centrifuged at 150g for 5 minutes to remove hepatocytes. The mononuclear cells were purified through a Ficoll gradient (GE Healthcare Bio-science AB). Cells were labeled with the CD34 MicroBead Kit from Miltenyi Biotec, and then CD34+cells were positive selected with autoMACS by following the manufacturer’s instructions (Miltenyi Biotec). After irradiating NRG mice at 200rad, a total of 0.5-1×10^6 CD34+ HSC cells were injected into the liver of each NRG mouse at 1-3 days of age. Mice that received a transplant were bled
through tail vein at 3-4 months after transplantation to check human cell reconstitution by flow cytometry and gating on human CD45+ cells. All animal experiences were reviewed and approved by the University of North Carolina-Chapel Hill Institutional Animal Care and Use Committee (IACUC).

Humanized mice were injected with 100µg of 80x320 nm hydrogel particles via tail vein injection in 100µl of sterile water. Acetylated particles (HP4A Hydrogels) contained Dylight 650, while PEGylated versions of the same particle (PEG-Hydrogel) contained Dylight 488. Twenty-four hours after particle injections, mice were euthanized for downstream analysis. To assess particle distribution via flow cytometry, blood was drawn via retro-orbital bleed, spleens were homogenized and femurs were harvested using standard methods to access bone marrow. Roughly 1/3 of the spleen was processed via a tissue homogenizer and RNeasy Plus Minikit (Qiagen) for RNA analysis.

Human cytokine expression in the spleen was assayed using RT-PCR and KiCqStart SYBR Green primers for Interleukin-1β, Interleukin-8, Interleukin-6, and TNF-α (Sigma Aldrich). Primer sequences (5’-3’) are as follows: IL-1β Forward - ACAGTGGCAATGAGGATGAC, IL-1β Reverse – CCATGGCCACAACAACCTGA; IL-6 Forward - GTGCCTCTTTTGCTGCTTTCAC, IL-6 Reverse – GGTACATCCTCGACGGCATCT; TNFα Forward – CTCTTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
Flow Cytometry of Humanized Mice

Cells were stained with the following human antibodies: CD11c-FITC, CD56-FITC, CD14-PE, CD4-PE-Cy5, CD3-PE-Cy7, CD123-PE-Cy5, CD19-PB, CD11c-APC, CD56-APC, CD45-APC-Cy7 (all from Biolegend); CD8-PE-TR and CD4-PE-TR (Invitrogen). Mouse CD45-Pacific Orange and Live/Dead Fixable Dead Stain Kit were from Invitrogen. All data were collected on CyAn™ (Beckman Coulter) and analyzed using FlowJo (Treestar, Inc.)

Statistical Analysis

Statistical significance was analyzed using GraphPad Prism 5 software. Single data point comparisons were evaluated by Student’s two-tailed t-test, whereas multiple comparisons were evaluated for statistical significance using Analysis of Variance (ANOVA) followed by Tukey-Kramer HSD or Bonferroni post-test. All data are presented as mean +/- standard deviation (SD) or standard error of the mean (SEM) as noted, with a p-value less than 0.05 considered statistically significant.

3.3 RESULTS

Precise fabrication of monodisperse and homogenous particles with PRINT

To address whether PRINT nano- and micro-particles are immunologically inert in human cells, we generated a panel of particles made of either PLGA or PEG, both biocompatible polymers which have a safe history in clinical use. We used the
PRINT fabrication process to generate particles on the nano (80x320nm and 200x200nm) and micron scale (1.5μm and 3.0 μm donuts). In some cases, we incorporated fluorescent dyes to enable tracking of particles in downstream assays. Characterization via dynamic light scattering (DLS, measures size), zeta potential analysis (measures surface charge) and scanning electron microscopy (SEM) indicated monodisperse populations of particles that could be used to reproducibly probe the role of size and shape and composition on human immune responses to nano and microscale particles (Figure 3-1). The polydispersity index (PDI), a measure of how similar in size particles within a batch are to one another, was below 0.1 for all particles fabricated, reflecting the homogeneity of these preparations and the precision of PRINT fabrication. Note that DLS analysis cannot be used on larger particles with nonspherous shapes, which explains why donut particles have no size values. Size was further verified for all particles via SEM. All particles used in studies had a negative surface charge.
Figure 3-1. PRINT particle characterization.

A-C) Size, polydispersity index (PDI) and surface charge data for PRINT particles used in these studies. D-E) SEM images show homogenous particle populations in the appropriate size range (scale bars at lower right of each image).
PRINT particles do not induce inflammation in THP-1 cells

We next carried out in vitro assays using the THP-1 human monocyte cell line as a surrogate for human innate immune cells of the monocyte and macrophage lineage. Monocytes and macrophages, as part of the mononuclear phagocyte system (MPS), are the cell types primarily responsible for clearing NMP from circulation when delivered in vivo to mammals, including humans [235,245,293,298,299]. Previous studies have shown NMP made of PLGA and other materials can induce inflammatory responses [72,186]. As drug delivery, diagnostic and many immunoengineering applications would be precluded in humans if NMP trigger innate immune activation of inflammation, we deemed it important to address this issue using PRINT particles.

Initial time course studies with the PRINT particles indicated an absence of inflammatory mediator release, as evidenced by the complete lack of IL-1β in culture media for all particles tested at the 10μg/ml dose (Figure 3-2A). PRINT particles that ranged from 1-100μg/ml also failed to induce IL-1β secretion (Figure 3-2B). As IL-1β secretion is largely dependent on activation of an inflammasome - typically requiring two signals to induce IL-1β secretion in THP-1 cells - we asked whether PRINT particles could synergize with a TLR4 ligand to cause IL-1β release [29,37,43,300]. The TLR4 ligand LPS is used to provide signal 1 for inflammasome formation, which induces transcriptional upregulation of both IL-1β and NLRP3 [273]. For all particles tested, particles did not synergize with LPS to induce IL-1β secretion (Figure 3-2C). We also asked whether TRIF-mediated signaling events could synergize with PRINT particles to induce IL-1β secretion by using the TLR4 ligand monophosphoryl lipid A
MPL), which preferentially engages the TRIF adapter as opposed to MyD88 [301]. In concordance with the literature regarding soluble monophosphoryl lipid A (MPL) alone, there was no indication of inflammasome-mediated IL-1β release when particles were delivered in the presence of MPL (Figure 3-2C). In these experiments, positive controls monosodium urate (MSU) and ATP+LPS induced IL-1β secretion as expected. These findings are different than published literature on the inflammatory potential of PLGA particles in mouse cells as well as literature showing inflammasome activation in THP-1 cells by silica nano- and microparticles [72,161]. This indicates that both particle fabrication and composition can augment inflammatory responses to NMP.

PRINT particles do not activate pro-inflammatory transcription factors

To gain a broader understanding of the potential for inflammation induction by particles, we made use of the THP1-XBlue reporter cell line (Invivogen) to enable colorimetric analysis of NF-κB and AP-1 transcriptional activation. These cells encode the alkaline phosphatase gene downstream of NF-κB and AP-1 promoter sites, so activation of either of these transcription factors results in the secretion of alkaline phosphatase into the supernatant where it can be detected using colorimetric analysis. For all particles tested, there was no indication of inflammatory transcriptional activation as measured by secreted alkaline phosphatase. As a control, heat killed *Listeria monocytogenes* induced pro-inflammatory transcription (Figure 3-2D). This suggests PLGA and PEG particles do not activate the NF-κB or AP-1 transcriptional programs, a key finding given the desired use of these polymers in a broad array of nanomedicine efforts where NMP
carriers should preferably be immunologically inert. In line with these results, incubation with these particles for 24 hours did not induce cell death as measured by LDH assay (Figure 3-2E). The lack of a measurable immunologic effect is not due to a failure of uptake, as these particles were rapidly taken up by THP-1 cells (Figure 3-2F).
Figure 3-2. PRINT particles are non-inflammatory in THP-1 human monocytes.

A) PRINT particles (10μg/ml) comprised of PLGA or PEG do not cause IL-1β release at any time point tested when dosed on THP-1 cells. B) PRINT particles dosed from 1-100μg/ml to not cause IL-1β release at 6 hours post treatment with THP-1 cells. C) PRINT particles (10μg/ml) at the nano and micron scale do not synergize with either LPS or MPL to induce IL-1β release in THP-1 cells. D) PRINT particles (10μg/ml) do not activate NF-κB or AP-1 transcription in THP1-XBlue reporter cells as measured by alkaline phosphatase secretion. E) PRINT particles (10μg/ml) do not induce cell death after 24 hours as measured by LDH assay. F) Fluorescence microscopy images of THP-1 cells containing rhodamine-loaded 3.0μm PEG particles (red), with nuclei (DAPI-stained blue) and cell surface lectins (RCA-stained green) at two and four hours post treatment. All data are presented as mean values +/- SD. *** = p<0.001. Experiments were performed in triplicate. Data shown are representative of at least three independent experiments.
Assessment of Nanoparticle uptake in Human PBMC

With the ultimate goal of using nano and microscale particles in a broad range of clinical interventions, we first sought to identify which cells of the human immune system are targeted by particles, followed by global profiling of soluble immune mediators induced by particle delivery. To our knowledge, this information is not readily available in the literature and thus we made use of the reproducibility of PRINT to address this gap in knowledge. Peripheral blood mononuclear cells (PBMC) were prepared from two healthy females and two healthy males using standard procedures. PBMC were treated with a panel of particles for 4 and 8 hours at 10µg/ml dose. Supernatants were analyzed in a multiplex cytokine assay and cells were stained for flow analysis when fluorescent particles were used (hydrogel +/- PEGylation).

We used particles of the same size, 80x320nm, but varied the composition between biocompatible polymers. DC-Cholesterol is a cationic lipid commonly used in transfection reagents and was incorporated into PLGA particles to address whether surface charge affected ensuing immune responses (kindly provided by Liquidia Technologies). For these studies, an improved hydrogel formulation published by our group using tetraethylene glycol monoacrylate (HP4A), a PEG derivative, as the base monomer afforded enhanced chemical conjugation opportunities. We conjugated PEG chains (5K) to the surface of HP4A particles (termed PEGylation), which improves circulation times in vivo in mice [293]. PEGylation has long been reported in mouse studies to augment uptake of nanoparticles, but little work has been done in primary human cells to address the
effects of this chemical modification. Here, fluorescent dyes (Dylight 650) were incorporated into the HP4A series to identify which immune cells take up particles and whether PEGylation could modulate this to any extent.

Forward scatter and side scatter were used to identify PBMC of the lymphocyte and myeloid lineage from all donors. Lymphocytes (T, B and NK cells) did not undergo cell death in any donor nor could nanoparticles be detected in any of these cell populations (Figure 3-3A). Conversely, roughly 14% myeloid cells were identified as dead and three discreet populations of myeloid cells based on CD14 expression levels were detected (Figure 3-3B). We termed these populations CD14-High (Figure 3-3C), CD-14 Intermediate (Figure 3-3D), and CD-14 Low (Figure 3-3E). There was a clear correlation between particle uptake and CD14 expression levels. This indicates 80x320nm hydrogel nanoparticles are selectively taken up by myeloid monocyctic cells and not the lymphocytic subsets, a key finding that may enable more effective nanoparticle design strategies for a range of human diseases.
A. Lymphocytes

% Cell Death

% with Particles

B. Myeloid Gate

% Cell Death

CD14 expression

Discreet CD14+ Populations

CD14 Lo  CD14 Int  CD14 Hi

39.3  24.2  36.6
Figure 3-3. 80x320nm PRINT hydrogel particles target myeloid and not lymphocytic cells from freshly isolated human peripheral blood mononuclear cells. A) Hydrogel particles do not cause cell death or target human lymphocytes (T cells, B cells, and NK cells). B) Hydrogel particle treatment induces a moderate cell death in myeloid cells (~14%). Myeloid populations form three discreet populations based on CD14 expression. C-E) Hydrogel particle uptake positively correlates with CD14 expression. Flow plots from one donor are representative of all four donors.
By pooling data from all four donors, a number of interesting results regarding nanoparticle uptake were uncovered. First, CD14 expression distinctly correlates with particle uptake (Figure 3-4A). Of the cells expressing the highest amount of CD14 (CD14 Hi), roughly 70% had taken up particles by the 8 hour time point, whereas those expressing the lowest amount of CD14 (CD14 Lo) took up particles to the same extent (~10%) as the phagocytosis control (cells kept at 4 degrees). Particle uptake is an active process, as keeping cells at 4 degrees greatly reduced the percentage of cells with particles. In addition, the correlation between CD14 expression levels and hydrogel particle uptake in human cells has not been reported to our knowledge and suggests a passive targeting of an innate immune cell population that is critical in mediating a variety of host functions. There are reports noting CD14 receptor correlations with uptake of silver or iron-oxide nanoparticles in human PBMC, but those particles are potentially toxic and thus have limited therapeutic use [302-304]. As CD14 expression is a marker of monocytes, macrophages (M0) and dendritic cells (DC), CD11b (M0) and CD11c (DC) staining were used to further verify these innate immune subsets take up particles (data not shown). Passive targeting to these three CD14+ subsets may afford a wealth of therapeutic programming opportunities.

Of note, while PEGylation of the particle surface does seem to impede the uptake of hydrogel particles at the 4 hour time point, an equivalent percentage of CD14 Hi cells take up particles regardless of PEGylation by 8 hours (compare HP4A to PEG group). This is not the case for the intermediate CD14 expressing cells (CD14 Int) and may reflect phagocytosis differences intrinsic to these two cell
populations. The CD14 Lo population did not take up an appreciable number of particles regardless of PEGylation status.

While the percentage of cells taking up HP4A and PEG particles appears to be equivalent at 8 hours among CD14 Hi cells, PEGylation greatly impedes the quantity of particles taken up by a given cell as measured by mean fluorescence intensity (MFI) (Figure 3-4B). Because the MFI of PEG treated groups at both 37 degrees and 4 degrees are similar, this may suggest that PEGylated particles decorate the surface of CD14 cells as opposed to becoming internalized, though imaging studies are needed to validate this interpretation. These trends are consistent regardless of CD14 expression levels and indicate a simple chemical modification, PEGylation, may quantitatively restrict particle internalization but not particle interactions with the cell surface. This may be an important design consideration when developing nanoparticles for either targeted or systemic drug delivery. Effective dosing concentrations may be adjusted via surface modification with PEG chains to enable autocrine and paracrine delivery of immunomodulators or other therapeutics if these particles remain bound to the cell surface.

There was a clear correlation with uptake of HP4A particles and cell death in the CD14 population (Figure 3-4C). Those cells which took up the most HP4A particles (CD14 Hi) had higher levels of cell death than the CD14 Int and CD14 Lo populations. This was phagocytosis dependent as cells kept at 4 degrees did not have appreciable levels of cell death. PEGylation of HP4A particles entirely abrogated the cell death seen with HP4A particles alone. This could be due to the larger quantity of HP4A particles taken up by a given cell as compared to the
PEGylated form (Figure 3-4B) or the possibility that PEGylated particles remain surface bound. Further analysis of the mechanism by which HP4A particles induce cell death is necessary though preliminary data suggest particle surface charge may be a critical driver of cell death in response to particle uptake.
Figure 3-4. Human PBMC differentially process PRINT particles depending on CD14 expression and PEGylation.

A) Particle uptake correlates with CD14 expression levels in human PBMC.  
B) PEGylation of HP4A particles (PEG) decreases the absolute number of particles taken up by CD14+ human PBMC as measured by MFI.  
C) PEGylation abrogates HP4A particle-induced cell death in CD14+ human PBMC as measured by Live/Dead staining.  All data are presented as mean values +/- SEM from four blood donors. * = p<0.05, ** = p<0.01, *** = p<0.001.
Global Cytokine Profiling in NP-treated Primary Human Immune Cells

Having addressed which human PBMC take up particles, we next assessed the immune environment of PBMC after 8 hours of incubation with our particle panel (Table 3.1). We used a 30-plex human cytokine array (Invivogen) in an attempt to capture the soluble immune profile from all donors. Immune changes induced by particles - such as cytokine, chemokine and growth factors alterations - could be revealed through this method. For all particles tested, there was no induction of a number of immune mediators at the 8 hour time point, including growth factors, interferons and cytokines associated with Th1, Th2, and Th17 immune responses (Figure 3-5A). The lack of induction of numerous cytokines from multiple classes of immune response may reflect a general inactivation of innate immunity by the particles tested herein. Given that HP4A particles were able to induce considerable cell death in CD14 expressing cells (Figure 3-4C), we were surprised to see no differences in any of the 30 cytokines assayed between the untreated, HP4A and PEGylated-HP4A treatment groups (Figure 3-5B). This suggests that HP4A particle-induced cell death may be immunologically inert and thus may lend itself to potential therapeutic interventions where transient depletion of circulating phagocytes is of use.
Figure 3-5. Hydrogel nanoparticles do not induce a broad spectrum of cytokines in human PBMC.

A) Table listing soluble mediators not detectable by multiplex array analysis from human PBMC after 8 hours of treatment with 80x320nm PLGA particles (+/- DC-Cholesterol) or 80x320nm hydrogel particles (+/- PEGylation). B) HP4A particle treatment and subsequent cell death (Figure 3-4C) does not induce cytokine release from human PBMC. All data are presented as mean values +/- SD from all four blood donors.
**Heterogeneity in Human Immune Responses to Nanoparticles**

Among the different particles tested, 80x320nm PLGA particles were the only one to induce any cytokines over untreated controls. In this experiment, PLGA particles induced a subset of inflammatory mediators, namely TNF-α and the chemokines CCL3 and CCL4 (Figure 3-6). Interestingly, complexation of PLGA with DC-cholesterol was sufficient to abrogate this modest inflammatory response through an as yet undetermined mechanism that may depend on surface charge. However, subsequent analysis of 80x320nm PLGA particles did not show induction of TNF-α, suggesting this may have been a single batch fabrication issue with unidentified contaminant(s). Nonetheless, we think these data are noteworthy and include it here because there was considerable inter-donor variation in the magnitude of cytokines detected by the same particle treatment. This was recapitulated in separate work we recently published where the immune status of primary human macrophages augmented uptake of nanoparticles [305]. Polarizing CD14+ monocytes from human donors towards the M2 phenotype significantly enhanced particle uptake, whereas polarizing towards the M1 phenotype reduced particle phagocytosis. In line with our findings, another group recently found tremendous donor variability in the innate immune response of humans to carbon-based nanoparticles [306]. We suggest that future ex vivo studies addressing nanoparticle use in humans should employ multiple blood donors so that individual immune variation can be revealed. In total, these findings highlight the need for a systematic analysis of the human immune response to biocompatible particles as simple modifications may augment the immune response in profound ways.
Figure 3-6. Heterogeneity in the Human Immune Response to Nanoparticles.

A-C) There are large differences in the magnitude of nanoparticle-induced production of pro-inflammatory cytokines and chemokines among healthy human blood donors. Coating PLGA particles with DC-Cholesterol (DC-Chol) may dampen these inflammatory responses. All data are presented as mean values +/- SD.
Cross-Validation of ex vivo PBMC findings in a Humanized Mouse Model

Having characterized the resident immune cells which take up hydrogel particles in human PBMC and captured the global immune profile via cytokine array, we next sought to determine whether our findings were translatable to emerging models of human disease that employ humanized mice, such as infection with human immunodeficiency virus (HIV) or hepatitis C virus (HCV) [294-297]. As nanomedicine is increasingly sought out as a means for therapeutic improvement, we aimed to recapitulate aspects of human immune interactions with nanoparticles in humanized mouse models in order to help translate basic research to the clinic.

To this end, humanized mice were generated using established protocols that repopulate immune-deficient mice with a human immune system derived from fetal liver cells [294-297]. After reconstitution, mice were injected with 100µg of dye-loaded 80x320 HP4A particles or PEGylated-HP4A particles via the tail vein. Twenty four hours after injection, flow cytometric studies were performed on the spleen, blood and bone marrow of mice to identify resident human immune cells which took up particles. Anti-human CD45 antibodies distinguished human and murine immune cells. All major human immune cell subsets were identified in humanized mice. These include CD4+ T cells, CD8+ T cells, CD19+ B cells, CD56+ NK cells, CD14+ monocytes and macrophages, CD11c+ myeloid dendritic cells (mDC) and CD123+ plasmacytoid dendritic cells (pDC) (Figure 3-7).
Figure 3-7. Humanized mice reconstitute major types of human immune cells.

Gating example used to characterize human immune cells in humanized mice. Human CD45 was used to gate on human immune cells only, followed by specific gating schemes for individual immune cell subsets. These include CD3+CD4+ T cells, CD3+CD8+ T cells, CD19+ B cells, CD56+ NK cells, CD14+ monocytes and macrophages, CD11c+ myeloid dendritic cells (mDC) and CD123+ plasmacytoid dendritic cells (pDC). Representative flow plot shown from one of 14 humanized mice used in studies.
In the spleens of humanized mice the cell types which took up the most particles, regardless of PEGylation status, were the CD14+ monocytes and macrophages as well as CD11c+ myeloid dendritic cells (Figure 3-8A). This is similar to the human PBMC findings which showed uptake primarily in CD14+ cells. PEGylation of particles reduced uptake into CD14+ human immune cells of the spleen by about half, while modestly decreasing uptake in CD11c+, which also recapitulates the ex vivo human PBMC findings. Like the human PBMC studies, CD14 expression levels in humanized mice correlated with particle uptake regardless of PEGylation status (Figure 3-8B). Though more thorough analysis is needed, PEGylation may also enhance targeting of nanoparticles to pDC (Figure 3-8A). As pDC are a primary interferon producing cell of the immune system, this could be a useful nanoparticle design strategy for therapeutic targeting of this cell population during viral infection in humanized mouse models [110,296,307-312].

A low level of hydrogel particles were seen in lymphocyte subsets of the spleen, such as CD4+ T cells. As lymphocytes did not take up particles in the ex vivo PBMC studies, this difference may be due the complexity of in vivo physiology and/or the longer time course of the in vivo studies. Alternatively, the possibility of particle drainage to the spleen and lymphocyte sampling of particles directly or through passive transfer by myeloid cells is a potential explanation. If true, then lymphocyte targeting with nanoparticles in vivo may also be feasible.

Hydrogel particles failed to elicit inflammatory responses during the human PBMC as measured by cytokine production. Because the number of human immune cells in humanized mice is relatively small, the levels of human cytokine
production are often too low to be detectable using ELISA. Therefore, we assayed human mRNA levels of four pro-inflammatory cytokines form the spleens of humanized mice. As seen in Figure 3-8C, the expression levels of human \textit{IL-8}, \textit{TNF-\alpha}, \textit{IL-6} and \textit{IL-1\beta} were indistinguishable between mock treatment groups and mice treated with hydrogel nanoparticles +/- PEGylation, which recapitulates the human PBMC data.

We were somewhat surprised to see that PEGylation of hydrogels did not abrogate uptake into immune cells of the blood because this chemical modification has been published as reducing particle uptake. In both blood and spleen, CD14+ and CD11c+ cells are the primary targets of particles yet no difference in uptake is seen between the two particle types in the blood population (Figure 3-8D). This may reflect the fact that PEGylation enhances circulation times of these same nanoparticles in normal mouse strains [293]. Over the course of 24 hours, circulating immune cells have more time to encounter and process PEGylated particles and uptake differences seen between hydrogels and PEGylated hydrogels may be diminished. The \textit{ex vivo} PBMC data reflects this, where the only difference in particle uptake for CD14 Hi expressing cells occurred at 4 hours but not at 8 hours (Figure 3-4A).

When assessing nanoparticle uptake in human immune cells resident in the bone marrow of humanized mice, we found a small but measurable amount of hydrogel particles in innate immune cells (CD14+ and CD11c+) as well as T cells (Figure 3-8E). PEGylation abrogated this effect entirely, revealing nanoparticle design principles that enable selective targeting of the bone marrow niche. To our
knowledge, this is the first report of nanoparticle targeting of human cells in the bone marrow compartment of humanized mice and may enable therapeutic modulation of hematopoietic niches.
Figure 3-8. Humanized mice recapitulate human PBMC nanoparticle studies.

A) Particle uptake in the spleens of humanized mice 24 hours after tail vein injection is most pronounced in CD14+ human monocyte/macrophage population and CD11c+ dendritic cells (mDC). PEGylation of particles reduces uptake into these populations while potentially enhancing targeting to pDC, an attractive cell population for antiviral therapeutics.  

B) As seen with human PBMC, CD14 expression levels in humanized mice correlates with particle uptake regardless of PEGylation status.  

C) Expression levels of human pro-inflammatory cytokines are unchanged over mock in the spleens of nanoparticle-treated humanized mice.  

D) CD14+ and CD11c+ cells are the primary targets of particles in the blood. PEGylation does not alter uptake in these cells.  

E) Hydrogels show enhanced targeting to immune cell subsets in the bone marrow as compared to PEGylated hydrogels.  

Mock (n=2), Hydrogel (n=3), PEG-Hydrogel (n=3). All data are presented as mean values +/- SEM.  * = p< 0.05, ** = p<0.01, *** = p<0.001. Data shown are representative of at least two independent experiments.
3.4 DISCUSSION

The goal of these studies is to identify baseline human immune responses to nano and microparticles (NMP). This information is critical to eventual translational applications of nanomedicines in humans yet is grossly understudied. As the immune system is supremely sensitive to the host environment but also a central integrator of host physiology, clinical application and efficacy of NMP will ultimately depend on our ability to understand and control immune responses to NMP.

Using the PRINT system to fabricate particles comprised of biodegradable and biocompatible polymers across the nano and micron scale, we observed no activation of inflammatory pathways in a human monocyte cell line as evidenced by cytokine release and inflammatory transcriptional activation. To provide physiologic relevance to these studies, primary human immune cells isolated from four healthy individuals were studied. Using these cells, we found that 80x320nm particles were not targeted to lymphocyte subsets but instead preferentially targeted cells that express CD14. Particle uptake correlated with the level of CD14 on the surface of cells and this was impeded by PEGylating particles. PEGylation also inhibited the modest cell death seen after uptake of hydrogel particles. Neither the mechanisms of hydrogel-induced cell death nor how PEGylation abrogates cell death and uptake into human immune cells are clear at this time.

Next, we employed a 30-plex cytokine array to generate a global picture of the inflammatory state induced by nanoparticle treatment of human PBMC. These results indicated that 80x320nm hydrogel particles +/-PEGylation are immunologically inert because none of the 30 assayed cytokines were different in
any donors as compared to untreated controls. Therefore, the hydrogel cell death identified by flow cytometry may thus be considered ‘silent’ in the sense that no indication of immune activation was found.

We were surprised to find an increase of the inflammatory mediators TNF-α, CCL3 and CCL4 by 80x320nm PLGA particles. In all our extensive in vitro and in vivo experiments using mouse and human cells with PLGA particles (Chapter 2, 3, 4 and data not shown), this was the only instance an inflammatory response to PLGA particles was observed. One explanation is that the single particle batch used in the cytokine array may have been inadvertently contaminated during fabrication as other particle batches do not show activation of inflammation in human cells. Regardless, we include the data here because of the insight it reveals about the heterogeneity of human immune response to nanoparticles. Though all four donors received the same dose of PLGA particles, the magnitude of cytokine production among them varies considerably. One donor consistently had the highest amount, one donor consistently had the lowest amount, and two donors had comparable amounts of detected cytokines. Coupled with our findings in a recently published paper that macrophage polarization differentially affects nanoparticle uptake in primary human CD14+ cells (M2>M1) [305], we believe this might reflect heterogeneity in human immune responses to nanoparticles that may suggest a one-size-fits-all approach to NMP clinical interventions is too simplistic a model. A recent paper showing tremendous human variability in innate immune responses to carbon-based nanoparticles validates our conclusions [306].
As a means to further the translational relevance of these studies, we generated humanized mice to assess the human immune response to hydrogel nanoparticles in a more dynamic in vivo system. Many of our ex vivo human PBMC findings were recapitulated in this mouse model, including preferential targeting of nanoparticles to CD14+ monocyte/macrophages and CD11c+ dendritic cells (DC), and CD14 expression levels correlating with particle uptake. PEGylation was able to reduce particle uptake in vivo, though this effect was greater in the spleen than in the blood. Splenic mRNA analysis of the human cytokines IL-8, TNF-α, IL-6 and IL-1β may indicate hydrogel particles do not cause inflammation, which replicates results from the human PBMC global cytokine array data. However, studies using more humanized mice are necessary to control for variation in reconstituted human immune systems between animals.

Interestingly, we see evidence of particle-specific uptake into immune cell subsets, with PEGylated hydrogels preferentially targeting plasmacytoid DC (pDC) in the spleen and the blood while hydrogels access bone marrow niches including T cells. As humanized mice are primarily used to model viral infection, targeting interferon-producing pDC with NP may be a means to enhance our basic understanding of these human cells types during an in vivo infection. Because pDC are a primary interferon producing cell of the immune system and interferon is a key anti-viral and anti-cancer defense, this could be a useful nanoparticle design strategy for therapeutic targeting of this cell population during viral infection and cancer [110,140,296,307-312]. Accessing the bone marrow niche via nanoparticles may also have relevance for studying and treating hematopoietic abnormalities. To
our knowledge, this is the first report of nanoparticle targeting of human pDC and bone-marrow resident immune cells in a humanized mouse model.

Ideally, NMP will be fabricated from polymers that are immunologically inert at baseline. This would enable diagnostic and drug delivery applications without concern for unwanted immune activation. In addition, the ultimate goal of immunoengineering is the ability to design NMP that can program all types of immune responses. Therefore, using a NMP platform that is perceived as a blank canvas by the immune system would enable the full spectrum on immunological ‘colors’ to be painted in any combination. We believe these studies indicate NMP composed of PLGA or hydrogel polymers may be perceived by the human immune system as relatively inert, thus bolstering their use as a blank canvas for therapeutic immune painting.

3.5 CONTRIBUTIONS

This work was made possible through the good-will and collaborative nature of many talented scientists. PRINT particles used in THP-1 studies were fabricated with the help of Dr. Warefta Hasan and Tammy Shen. Kevin Reuter generated all hydrogel particles used in the PBMC and humanized mice studies. We thank the four healthy human donors who agreed to participate in these studies as well as the assistance of Dr. Karen McKinnon while drawing their blood. Dr. Greg Robbins was instrumental in human PBMC isolation, experimental setup and design, and flow analysis. Through SERCEB funding, Dr. Doug Widman and the Sempowski lab at
the Duke Human Vaccine Institute made the 30-plex cytokine array study a reality. Our sincere thanks to Dr. Lishan Su and his laboratory for generating and sharing precious humanized mice. Dr. Haitao Guo deserves a special thank you for his tremendous assistance with all aspects of the humanized mouse studies. Lastly, this work would not have been possible without the generous support and overall experimental guidance of Dr. Jenny Ting and Dr. Joseph DeSimone.
CHAPTER 4

LESSONS IN IMMUNE PROGRAMMING WITH PRECISELY FABRICATED NANO- AND MICROSCALE PARTICLES

Programming biological responses with nanomaterials has the potential to transform clinical care in the 21st century, in particular through control of immune responses in situ. In order to help make this a reality, we reported in Chapters 2 and 3 on the generation of precisely fabricated biocompatible and biodegradable particles using the PRINT process across the nano and micron scale (NMP) that are immunologically inert in two mammalian species, mouse and human. Here, we probe the programming landscape for engineering immune responses in mammals by rational design of PRINT NMP. We identify NMP design features that enable control of uptake into immune cells, activation and silencing of inflammatory responses, and the induction of immune tolerance. These findings may have bearing on the clinical treatment of many pressing diseases, including types 1 and 2 diabetes, allergies and asthma, organ transplantation and autoimmune disorders.
4.1 INTRODUCTION

As detailed extensively in Chapter 1, the immune system is exquisitely sensitive to the environment. Thus, if one is trying to program immune responses through rational design of NMP, it is absolutely imperative that the starting material be as immunologically inert as possible so as not to inadvertently trigger unwanted signaling pathways. In effect, we desire a blank canvas on which to paint (i.e., program).

As presented in Chapters 2 and 3, our own careful analysis of the murine and human immune response to PRINT nano and microparticles (NMP) composed of PLGA and hydrogels established these particles are inert from an immunological perspective. This is precisely the desired finding, as the ultimate goal of these particles is to design them into programming devices that can modulate immune functions in mammals.

In this Chapter, we present a compilation of findings over the course of our studies that reflect not only the complexity of innate immune responses to NMP, but also highlight potential immunoengineering directions using rationally designed particles we have generated. Studies discussed herein address: 1) the role of surface modification on augmenting nanoparticle uptake into human cells; 2) particle design parameters to control the kinetics and magnitude of inflammasome activation; 3) anti-inflammatory NMP designs; and 4) NMP designs which may enable programming of immune tolerance through two separate pathways. These findings have direct relevance to the development of microbial and cancer vaccines,
reducing inflammatory damage in a range of disease settings, and offering new treatments for autoimmune disorders.

4.2 MATERIALS AND METHODS

Particle Materials

Poly(D,L-lactide-co-glycolide) (PLGA) (lactide:glycolide 85:15, 0.65 dL/g Inherent Viscosity at 30 °C) was purchased from Sigma-Aldrich. Chloroform and solvents (acetonitrile and water) for high performance liquid chromatography (HPLC) were purchased from Fisher Scientific. Poly(ethylene terephthalate) (PET) sheets (6” width) were purchased from KRS plastics. Fluorocur®, d = 80 nm; h = 320 nm; all prefabricated molds and 2000 g/mol polyvinyl alcohol (PVOH) coated PET sheets were kindly provided by Liquidia Technologies.

PRINT PLGA Particle Fabrication

Fabricating PLGA particles using the PRINT process has been previously described in detail elsewhere [260,261] so a brief description is included here. A preparticle solution (PPS) containing PLGA was prepared in a DMSO/DMF/water solvent mixture (4:16:1) and cast on a poly-(ethylene teraphthalate) (PET) sheet (delivery sheet) using a #5 Mayer Rod (R.D. Specialties). The delivery sheet was placed in contact with a PRINT mold with desired features patterned (e.g., 80 × 320 nm, 200x200nm, 1.5µm donut). The delivery sheet and mold were then passed through a heated laminator (150 °C, 5.5 × 105 Pa) and separated at the nip. Heating enables the PLGA polymer solution to fill the molds through capillary forces, thereby forming nanoparticles of desired size and shape. Nanoparticles were then harvested
from the PRINT mold by placing it in contact with a PET sheet coated with a 400nm layer of poly( vinyl alcohol) (PVOH, MW = 2000 g/mol). This ensemble of mold/PET-PVOH was then passed through the laminator (150 °C, 5.5 × 105 Pa) to transfer the nanoparticles to the PVOH sheet. Both laminator steps - the filling of the mold and transfer of particles onto the PVOH-coated PET sheet - were performed at low humidity (∼20−30%). Particles were released from the PET/PVOH sheet by delivering ~1ml of sterile water via a bead harvester to dissolve the PVOH layer and remove particles from the PET sheet. A typical yield of 80x320nm PLGA particles was ~0.4 mg particles/ft of PRINT mold, though this depended on the particle feature size of the mold. Tangential flow filtration (TFF; Spectrum Labs) was used to concentrate particles in sterile water (1-2mg/ml) and remove excess PVOH. For later use in particle characterization assays and experiments, mannitol and sucrose were used as cryoprotectants ((10× and 8× to mass of particles, respectively), particles were lyophilized, flash frozen and stored at -20 degrees.

Quantum Dot, Docosahexaenoic acid and Phosphatidylserine Particle Fabrication

Particles were fabricated using the same techniques as described in detail in Chapters 2 and 3. Details unique to the studies reported in this chapter are included here. Q-Dot (655 ITK Organic, Invitrogen) were incorporated into the pre-particle PLGA solution at 3% by weight. Docosahexaenoic Acid (DHA; 90310, Cayman Chemicals) and phosphatidylserine (PS; 840032, Avanti Polar Lipids) and PLGA were dissolved separately in chloroform. The solutions of DHA or PS and PLGA were mixed at ratios of 30:70 (DHA:PLGA) or 10:90 (PS:PLGA), and the sample was diluted to 2 wt% (mass/mass) solution with chloroform. A thin film of DHA or PS and
PLGA was deposited on a 6”x12” sheet of PET by spreading 200 μL of solution using a #5 Mayer Rod (R.D. Specialties). The solvent was evaporated with heat. The PET sheet with the film was then placed in contact with the patterned side of a mold and passed through heated nips (Chem Instruments Hot Roll Laminator) at 130 °C and 80 psi. The mold was split from the PET sheet as they both passed through the hot laminator. The patterned side of the mold was then placed in contact with a sheet of PET sheet coated with 2000 g/mol PVOH. This was then passed through the hot laminator to transfer the particles from the mold to the PET sheet. The mold was then peeled from the PET sheet. The particles were removed by passing the PVOH coated PET sheet through motorized rollers and applying water to dissolve the PVOH to release the particles. To remove excess PVOH, the particles were purified and then concentrated by tangential flow filtration (Spectrum Labs). Particles were lyophilized, flash frozen and stored at -20 prior to use in experiments.

**HPLC Analysis of Ligand Incorporation into Particles**

Ligand encapsulation (DHA, and PS) was quantified via HPLC in a similar fashion. DHA or PS incorporation into PLGA particles was measured using an Agilent Technologies Series 1200 HPLC with a C18 reverse phase column (Zorbax Eclipse XDB-C18, 4.6×100 mm, 3.5 micron). A linear gradient from 85:15 of methanol with 0.1% trifluoroacetic acid (TFA): water with 0.1% TFA to 100% methanol with 0.1% TFA was run over 25 minutes. The flow rate was 1 mL/min and an ELSD detector was used for quantification. Particle samples were prepared by diluting the sample with a 50:50 acetonitrile:water solution and mixing the sample to
break down the particle and dissolve the PVOH. Standards of PS and DHA were prepared in 50:50 acetonitrile:water.

PRINT Hydrogel Fabrication

The process of fabricating hydrogel particles was conceptually similar to PLGA fabrication, but with important differences as follows. For 80x320nm hydrogel particles, the pre-particle solution (PPS) contained a composition of 67.5 wt% HP₄A, 20 wt% AEM (functional monomer), 10 wt% PEG₇₀₀DA (crosslinker), 1 wt% TPO (photo initiator) and 1.5 wt% Dylight 650 or Dylight 488 maleimide. This composition was then dissolved at 3.5 wt% in methanol and drawn as a thin film using a #3 Mayer rod (R.D. Specialties) onto a roll of corona treated PET using an in-house custom-made roll-to-roll lab line (Liquidia Technologies) running at 12 ft/min. The solvent was evaporated from this delivery sheet by exposing the film to heat guns. The delivery sheet was laminated (80 PSI, 12 ft/min) to the patterned side of the mold, followed by delamination at the nip. Particles were cured by passing the filled mold through a UV-LED (Phoseon, 395 nm, 3 SCFM N₂, 12 ft/min). A PVOH harvesting sheet was hot laminated to the filled mold (140 ºC, 80 PSI, 12 ft/min). Upon cooling to room temperature, particles were removed from the mold by splitting the PVOH harvesting sheet from the mold. Particles were then harvested by dissolving the PVOH in a bead of water (1 mL of water per 5 ft of harvesting sheet). The particle suspension was passed through a 2 µm filter (Agilent) to remove any large particulates. To remove the excess PVOH, particles were centrifuged (Eppendorf Centrifuge 5417R) at 14000 rpm for 15 min, the supernatant was removed and the particles were re-suspended in sterile water. This purification
process was repeated 4 times prior to lyophilization as detailed above. The
80x320nm particles were acetylated prior to experimental use to match negative
charge of micron sized hydrogel particles.

**OVA-Conjugated Hydrogel Fabrication**

Fabrication of the base hydrogel particle is the same as reported above.
Conjugation of ovalbumin is as follows. Ovalbumin (OVA) is attached to the surface
of hydrogel particles using a bifunctional PEG linker, which contains a maleimide
group that reacts with thiols present in cysteines in the protein. HP$_4$A AEM-
containing particles are first reacted with maleimide-PEG$_{5k}$-succinimidy carbocxy
methyl ester (MAL-PEG$_{5k}$-SCM, Creative PEGWorks). Amine groups in the
nanoparticle are activated with triethylamine in DMF for 10 minutes prior to the
addition of MAL-PEG$_{5k}$-SCM. For a high surface PEG density maintaining a brush
conformation, 14mgs of MAL-PEG$_{5k}$-SCM is added per 1 mg of NP as previously
reported [293]. This reaction is left overnight (~12 hr) on a shaker at room
temperature. Particles are then centrifuged at 14000 rpm for 15 minutes three times
to remove excess reagents. This results in the reaction of surface amine groups
PEG$_{5k}$ with a functional terminal maleimide handle. Unreacted amine groups are
then quenched through the addition of acetic anhydride in the presence of pyridine
for 30 minutes. Centrifuge wash steps are carried out in DMF and 1x borate buffer
pH 9.5, with particles ultimately resuspended in 2x Borate Buffer: 1x PBS + 0.1%
PVOH. OVA is added to the particles at a ratio 1:1 by mass and left on a shaker
overnight at room temperature. Conjugated particles are then washed three times in
water to remove excess OVA. HP$_4$A-PEG$_{5k}$-OVA particles are confirmed by DLS,
TGA and SEM, with OVA loadings determined through a colorimetric BCA Protein Assay (Thermo Scientific Pierce Protein Assay Kit). The difference in absorbance between a known amount of conjugated HP₄A-PEG₅k-OVA particles from unconjugated PEG₅k-OVA control particles is attributed to bound OVA, quantified through the use of an OVA standard curve.

**Particle Characterization**

Thermogravimetric analysis (TGA) was used to determine stock particle concentrations (TA Instruments Q5000 TGA). Briefly, 20 µL of the stock nanoparticle solution was pipetted into a tared aluminum sample pan. The sample was heated at 30°C/min to 130°C and held at this temperature for 10 minutes. The sample was then cooled at 30°C/min down to 30°C and held for 2 minutes. A Hitachi S-4700 scanning electron microscope (SEM) was used to visualize particles. Prior to imaging, the SEM samples were coated with 1.5 nm of gold-palladium alloy using a Cressington 108 auto sputter coater. Particle size and zeta potential were measured by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Instruments, Ltd.).

**Particle uptake in THP-1 cells**

THP-1 cells were seeded at 500K/ml media and particles were dosed in triplicate at 100µg/ml over the time course (1, 4, 24 hours). Analysis of PLGA-QDot and hydrogel particle uptake in THP-1 cells was performed on an LSR II (BD Biosciences) using scatter gating and analyzed via Flow Jo (Treestar, Inc.)
**THP-1 inflammasome activation assays**

THP-1 human monocyte cells (ATCC) were cultured in RPMI 1640 (Gibco) supplemented with 1% penicillin/streptomycin, 1% L-glutamine and 10% heat-inactivated fetal bovine serum. For experiments, THP-1 were plated at 200,000 cells per well in a 96-well dish in 200µl of media. Some cells were primed with 0.5µM PMA for 3 hours prior to particle treatment to provide signal 1 for inflammasome activation. MSU (300μg/ml; Invivogen) or ATP (5mM) was used as a positive control for inflammasome activation. Particles were resuspended in PBS prior to dosing at various concentrations in triplicate. For adjuvant coated particle studies, particles were harvested in water with LPS or MPL at 1mg/ml concentration and filtered by TFF (50nm pore, Spectrum labs) at least 3x prior to use in experiments. After denoted incubation times, supernatants were harvested and analyzed by human IL-1β ELISA (BD Biosciences).

**Murine inflammasome activation assays**

Bone marrow macrophages were isolated from the femurs of C57Bl/6 and BALB/c mice using standard procedures. Bone marrow-derived macrophages were cultured for six days in DMEM supplemented with 10% fetal bovine serum, L-Glutamine, pen/strep and 20% L929-conditioned medium prior to use in particle experiments. Adherent cells were isolated and plated in complete Dulbecco’s Modified Eagle Medium (Gibco) with 10% fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine at 200,000 cells per well in a 96-well dish for 24 hours prior to treatment with particles. Some cells were primed with LPS (50ng/ml) for 24 prior to
particle treatment to provide signal 1 for inflammasome activation. MSU (300μg/ml; Invivogen) or ATP (5mM) was used as a positive control for inflammasome activation. Particles were resuspended in PBS prior to dosing at various concentrations. After 24 hours of particle treatment in triplicate, supernatants were harvested and analyzed by murine IL-1β ELISA (BD Biosciences). 50μM doses were used for cells pre-treated with particle-delivered ligand or soluble ligand (DHA and PS). Blank particle controls were dosed at the same concentration of particle required to deliver a 50μM dose of ligand. Coated particles were generated by resuspending lyophilized PLGA particles in a water solution containing 50μM of the coated ligand DHA, PS, or DOTAP/DOPE (Avanti Polar Lipids).

Mouse Model of Asthma

The OVA-alum murine model of asthma has been used extensively by multiple laboratories and details have been previously reported by us [105]. Briefly, C57/Bl6 mice were immunized with interperitoneal (i.p.) injections of a 200μl emulsion of OVA and alum (20μg OVA per dose) on day 1 and day 7. Starting on Day 13, mice were challenged with intratracheal instillations of 1μg/50μl dose OVA in either soluble form or conjugated to hydrogel particles. PBS and PEGylated hydrogels were used as controls. After 5 days of challenge, mice were euthanized and harvested for analysis. Serum was collected from animals by cardiac puncture and centrifuged at 15,000 RPM for 10 minutes. The serum supernatant was collected and used for ELISA analysis of antibody production. OVA-specific antibody ELISAs were sourced from Biolegend, BD and Invitrogen.
Bronchoalveolar lavage fluid (BALF) was also collected to evaluate local leukocyte levels in the lungs. For this purpose, lungs were lavaged three times with 1ml Hanks Balanced Salt Solution (HBSS; Gibco). After centrifugation at 1500 RPM for 5 minutes, RBC were lysed via brief hypotonic saline treatment and the cell pellet was resuspended in PBS. Total BALF cellularity was assessed with a hemacytometer. The cellular composition was determined by cytospin of BALF aliquots onto slides and staining with Diff-Quik (Dade Behring) for differential cell counts. Leukocytes were identified based on the morphology of ≥200 cells per sample. Following BALF harvest, the lungs were fixed by inflation (20-cm pressure) and immersed in 10% buffered formalin.

Histopathological examination

Inflammation was evaluated in 5μm sections of the left lung lobe after hematoxylin and eosin (H&E) staining. Serial paraffin-embedded sections were set and cut to reveal maximum longitudinal visualization of the intrapulmonary main axial airway and inflammation was scored blinded to genotype and treatment. As previously described, histology images were evaluated on each of the following inflammatory parameters and scored between 0 (absent) to 3 (severe): mononuclear cell infiltration, polymorphonuclear cell infiltration, airway epithelial cell hyperplasia/injury, extravasation, perivascular cuffing, and estimated percentage of the lung involved with inflammation [48,265]. Scores for each parameter were averaged for a total histology score.
DC-2D2 Co-culture assays

Bone-marrow dendritic cells (DC) were isolated from murine femurs and differentiated for 6 days in 20ug/mL GM-CSF. Anti-CD11c beads (Miltenyi Biotec) were used to isolate DC, which were plated in 96 well plates at 40,000 cells per well. T cells were isolated from 2D2 mouse spleens using anti-CD4 beads (Miltenyi Biotec) and were plated at 200,000 cells per well in co-culture with DC. 2-4 hours prior to adding T cells, DC were incubated with soluble phosphatidylinerine (PS) or PRINT particles fabricated with or without PS. DC-T cell cultures were then incubated for 2 days at 37 degrees prior to supernatant harvesting for cytokine analysis by ELISA or cell fixing and staining for flow cytometry analysis using antibodies to IFN-γ, IL-10 and FOXP3+ (eBiosciences).

Experimental animals

All studies were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill. All animals were maintained in pathogen-free facilities at the University of North Carolina at Chapel Hill.

Statistical Analysis

GraphPad Prism 5 software was used to identify statistical significance. Single data point comparisons were evaluated by Student’s two-tailed t-test, whereas multiple comparisons were evaluated for statistical significance using Analysis of Variance (ANOVA) followed by Tukey-Kramer HSD post-test. Data are
presented as mean +/- standard deviation (SD) or standard error of the mean (SEM), with a p-value less than 0.05 considered statistically significant.

4.3 RESULTS

*Inside or Out? – Design Considerations for Particle Uptake*

There are a multitude of therapeutic applications for NMP that require control over the location of NMP deposition depending on the desired outcome. For example, drug delivery may yield better results if NMP remain in extracellular spaces to target more tissues and cells. Conversely, targeting NMP to specific cells is desirable in vaccine settings or when targeting specific tumors. Thus, underlying principles which can modulate uptake into cells is an important area of basic research in the nanomedicine field [74,163,251,256,313-315]. By making use of fluorescent 80x320nm PRINT particles comprised of either PLGA or hydrogels, we addressed how composition affects cell uptake of NP in the THP-1 human monocyte cell line in a quantitative manner using flow cytometry. We chose THP-1 as a surrogate for phagocytic cells responsible for clearance of NMP *in vivo*. It has been difficult in the past to ensure fluorescent dyes remain within PLGA NMP. To remedy this, quantum dots (QD) - ~5-15nm diameter metallic alloys which auto-fluoresce at the 650nm wavelength – were incorporated into the pre-particle solution in order to fabricate PLGA NP that could be tracked as recently reported (Figure 4-1A) [316]. Hydrogel particles were fabricated with Dylight 488 to enable fluorescent monitoring. To further the impact of our studies, we looked at the role of a lipid coating on
modulating PLGA particle uptake, as well as the role of PEGylation and conjugation with a model antigen (ovalbumin, OVA) on the uptake of hydrogel particles.

For 80x320nm PLGA particles, there was very little to no uptake into THP-1 cells. Even after 24 hours, only 12% of cells were positive for particle uptake (Figure 4-1B). As these particles have a slightly negative surface charge (-5 mV), we proposed coating these NP with a cationic lipid after fabrication would alter particle uptake. As coating PLGA particles with the common transfection reagent DOTAP/DOPE is known to make the nanoparticle surface positively charged and enhance siRNA delivery, DOTAP/DOPE was selected for these studies [260]. Within 1 hour after particle dosing, nearly 100% of THP-1 cells had taken up particles and this held constant through 24 hours of treatment (Figure 4-1C).

It is not clear whether this robust enhancement is strictly due to a change from negative to positive surface charge or whether the lipid coating itself aids in masking other physical or chemical PLGA surface properties that impede THP-1 uptake. Studies are underway to tease this apart. However, preliminary in vivo studies suggest uncoated 80x320nm PLGA-QDot particles may not be readily taken up by immune BALF resident cells in the lungs of mice as they cannot be found in these cells by flow analysis after intratracheal instillation, whereas hydrogel particles are (Figure 2-5 and data not shown). From a translational perspective, design features which can impede NP uptake into phagocytic cells may enhance in vivo circulation times of the NP as well as promote extracellular delivery of cargo. A lipid coating would thus be used for applications where uptake into phagocytes is more desirable, such as immunoengineering approaches targeting macrophage function.
Figure 4-1. Design control of PLGA particle uptake in THP-1 cells.

A) Particle characterization details.  
B) Uncoated 80x320nm PLGA particles are not taken up by THP-1 cells to any significant extent across a 24 hour time course.  
C) Coating 80x320nm PLGA particles with the cationic lipid DOTAP/DOPE results in rapid and complete uptake by all THP-1 cells.  

Data are representative of two independent experiments.
We next carried out similar uptake assays using the 80x320nm hydrogel system. THP-1 cells were incubated with three types of 80x320nm hydrogels: acetylated, PEGylated and acetylated, and PEGylated and conjugated to the model antigen OVA to mimic a vaccine application (see Figure 4-7 for characterization). Acetylation confers a negative surface charge to the hydrogels and ensured all three particle types had similar charges. As seen in Figure 4-2A, acetylated hydrogels were taken up by nearly all THP-1 cells as soon as 1 hour after treatment. By modifying the surface of these particles with either OVA or PEG chains, uptake was entirely blocked through 4 hours of treatment. Even after 24 hours, OVA-particles were present in only half the cells, while PEGylated particles were found in only 25% of cells (Figure 4-2B and C).

The PEGylation finding is perhaps not surprising given the published literature regarding surface decoration with PEG impeding uptake of NMP [293]. However, the 24 hour result in THP-1 cells is somewhat at odds with our own findings in human PBMC where PEGylation does not seem to confer great masking of uptake by cells per se, but does impede the quantity of particles per cell (Figure 3-4). This may also reflect differences between primary human cells and those derived from immortalized cell lines, a further testament to the importance of validating in vitro findings in primary cells. In addition, simply conjugating OVA to the surface of hydrogels impeded uptake. Because of the intricacies of antigen processing through either MHC I or MHC II pathways discussed in the introduction chapter, more detailed experiments are underway to address how conjugation strategies can modify the processing of antigen from hydrogel particles.
Figure 4-2. Design control of hydrogel particle uptake in THP-1 cells.

A) Within 1 hour, nearly all THP-1 cells are positive for 80x320nm hydrogel particles.

B) Conjugating the model antigen OVA to the surface of these particles impedes their uptake entirely at early time points and to roughly half that seen with blank hydrogels at 24 hours. C) PEGylating the surface of hydrogels greatly reduces uptake into THP-1 cells at all time points tested. Data are representative of two independent experiments.
**Design control over the kinetics and magnitude of immune signaling**

As discussed at length in the introduction, triggering immune signaling pathways via NMP is a potent means for programming biological responses. There is a large field of literature which indicates the magnitude and duration of biological signaling events are interpreted by dynamic cellular networks to fundamentally alter the cell response to environmental stimuli [317]. Thus, as NMP technology advances and our knowledge of immune principles increases, it likely will be desirable to impart control over the kinetics and magnitude of signaling events programmed by NMP devices. It is already clear that particle parameters such as size, shape and density of surface-displayed ligands can affect the biological output induced by NMP [168,178,206,217-219,246]. Because of the importance of controlled inflammatory responses to host physiology [34], we sought to address whether the kinetics and magnitude of inflammasome activation by NMP could be controlled through varying the size, shape and composition of PRINT particles. Such findings may have relevance to vaccine design and Th17 immune skewing applications [47,48,54,68,69,84,94].

As our previous studies indicated PRINT particles alone or in the presence of the soluble TLR4 ligands LPS and MPL did not activate the inflammasome to release IL-1β (Figures 2-2 and 3-2), these TLR4 ligands were coated onto PRINT particles to adjuvant them. Initial studies focused on the TLR4 ligand lipopolysaccharide (LPS), a bacterial cell wall component known to synergize with other signals to activate the NLRP3 inflammasome [29,49,50,159]. The same PRINT particles fabricated in Chapter 3 studies - a series of PRINT particles across
the nano and micron range composed of either PLGA or PEG - were coated with LPS during the harvesting phase. Using the established THP-1 inflammasome activation model, we were able to screen a number of parameters as regards their impact on inflammatory activation.

As shown in Figure 4-3A, particles alone did not cause release of IL-1β across a range of doses. Unlike what was seen when particles were delivered with soluble LPS (Figure 3-2C), simply coating these particles with LPS was sufficient to generate an inflammatory response (Figure 4-3A). In addition, the magnitude and kinetics of the response depended not only on the size of the particle (1.5 µm more efficient than 3.0µm), but also the composition. PLGA particles induced a comparable level of IL-1β at 1/10 the dose of PEG particles. In all cases, LPS-coated particles were more inflammatory than MSU crystals.

When carrying out kinetic studies, it was clear that the inflammatory response was hastened when LPS-coated particles were composed of PLGA as opposed to PEG (Figure 4-3B), with 80x320nm particles leading to more rapid inflammatory induction than those in the micron range. This could be due to the fact that at the same dose weight (10µg/ml) there will be far more 80x320nm particles than micron size particles. Thus, more cells may be exposed to particles or take them up compared to larger particles. Regardless, these findings point to the possibility that altering the size, shape or composition of adjuvanted-NMP can enable control over the kinetics and magnitude of inflammatory immune responses.
To verify these particles were causing IL-1β release through inflammasome activation, we made use of previously reported THP-1 cell lines that stably knockdown the inflammasome components, *NLRP3* and *ASC* [106,318,319]. As shown in Figure 4-3C, IL-1β release induced by 80x320nm PLGA particles coated with either LPS or MPL is mediated by the NLRP3 inflammasome, as knockdown of either *NLRP3* or *ASC* completely abrogates IL-1β production. Similar results were seen for 200x200nm PLGA, 1.5µm PLGA, 1.5µm PEG and 3.0µm PEG particles coated with LPS (data not shown).

It is interesting to note that coating particles with MPL also induces a modest inflammasome-dependent IL-1β release. This is somewhat surprising as MPL has a more favorable clinical profile than LPS and is not thought to activate the inflammasome. The reduced toxicity of MPL is thought to occur because it signals primarily through the TRIF adaptor of TLR4 as opposed to LPS, which uses the MyD88-adaptor [301,320]. It has been reported that TRIF activation is less potent at priming inflammasome events than MyD88, but these studies were done using soluble forms of these ligands [301]. To our knowledge, the effect of particulate MPL on inflammatory responses has not been studied, thus these studies are clinically relevant. Along these lines, the data revealed a size- and shape-dependent sensitivity to the inflammatory response induced by particulate MPL (Figure 4-3D). At the same dose of MPL-coated particles, 200x200nm PLGA cylinders induce a significantly greater amount of IL-1β as compared to 80x320nm particles. This effect is not seen when these same particles are coated with LPS.
One could hypothesize that the receptor clustering events that occur upon TLR4 activation are differentially sensitive to shape and size depending on whether TRIF or MyD88 is enlisted as the adaptor. In order to carry out well-controlled experiments to address this issue, absolute quantification of the amount of biological ligands complexed with particles is necessary. This is a difficult task that many groups fail to carry out in their published reports as it requires technically challenging analytical chemistry assays. However, given our own findings, we strongly believe that these types of exhaustive particle characterizations are absolutely required in order to move the field forward. As soon as these techniques became available to us, we made use of analytical chemistry assays during the remainder of our dissertation studies.

While the mechanism of particle shape differences in MPL-induced inflammatory responses is not clear, this finding exemplifies the molecular details of immune signaling that can be uncovered by NMP studies, in particular when using the PRINT platform’s inherent control over particle size and shape. Design control of inflammasome activation may have therapeutic relevance, such as enhancing the efficacy of microbial vaccines against fungal and respiratory infections through generation of Th17 adaptive immunity [46,48,54,94]. In addition, these studies reflect the concept that emergent biological properties can manifest when a soluble ligand is particularized. This is a common theme we see in the studies detailed below.
Figure 4-3. Design control over the kinetics and magnitude of inflammasome activation.

A) Dose studies using a panel of PRINT particles reveals coating NMP with LPS induces IL-1β release in a manner that depends on both particle size and composition. B) Kinetic studies using a panel of PRINT particles reveals coating NMP with LPS induces IL-1β release in a manner that depends on both particle size and composition. C) IL-1β release induced by LPS- and MPL-coated 80x320nm PLGA particles depends on the inflammasome components NLRP3 and ASC. D) MPL-dependent signaling events show sensitivity to particle size and shape at the nanoscale not seen for LPS signaling. Data are representative of at least three independent experiments.
Turning off Inflammation using Endogenous Pathways in a Targeted Manner

Having addressed design features which can activate inflammatory pathways, we next sought to develop a series of particles that could be used to dampen or inhibit inflammatory signaling cascades. Inflammation is a major cause of disease pathology in a multitude of clinical settings, from driving cancer to exacerbating the detrimental effects of heart attacks and strokes, and acting as a prime instigator in type 2 diabetes [17,34,127,137,241,321-323]. Therefore, therapeutics which inhibit inflammatory signaling pathways have tremendous clinical merit. By coupling anti-inflammatories with NMP, we may also gain translational advantages through the modular freedoms inherent to this delivery platform.

The body has numerous endogenous signaling networks to resolve inflammation, as tight control over this physiologic response is critical to ensure the long term health of the host [34]. These include down regulation of PRRs and the production of anti-inflammatory mediators such as resolvins, protectins and lipoxins [324-327]. The latter classes of molecules are metabolic by-products of polyunsaturated fatty acids, such as the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Long known for their beneficial effects on human health, omega-3 fatty acids are derived entirely from the diet, with seafood providing the lion share for most people. Recently, the report of GPR120 as the cell surface receptor for omega-3 fatty acids has helped unmask the molecular mechanisms explaining the anti-inflammatory effects of these molecules [328]. We thus sought to address whether incorporating DHA into PLGA NMP would generate an anti-inflammatory programming device.
As part of these studies, we also generated a separate panel of PLGA NMP containing one of two lipids. One set were coated with DOTAP/DOPE, as this enhanced uptake into cells (Figure 4-1) and could control for the role of lipids on any ensuing cell response. The second lipid we fabricated PLGA particles with was phosphatidylserine (PS). PS is an endogenous lipid known to have unique immune signaling properties [329,330]. It is located on the inner surface of the plasma membrane and is exposed on the surface of cells which are undergoing apoptosis. This informs phagocytes that they are engulfing ‘self’, so they are tempered from activating inflammatory responses or presenting antigen from the apoptotic cell in a manner that would cause an autoimmune response. This occurs in part through PS triggering release of TGF-β and IL-10, potent immunoregulatory cytokines involved in tempering immune activation and inducing tolerance [26,329-332]. We reasoned that PS NMP could dampen inflammation and/or potentially skew the immune environment towards immunoregulatory conditions, which would be of great use in autoimmune disorders. In support of our reasoning, recent papers reveal PS NMP preparations can dampen pro-inflammatory cytokine release and promote production of the anti-inflammatory cytokines IL-10 and TGF-β [333-335].

An inflammasome activation model in bone-marrow derived mouse macrophages (BMDM) was used to address the anti-inflammatory potential of this panel of particles. Given our previous studies with TLR agonists (Figure 4-3), high performance liquid chromatography (HPLC) analysis of all particle types was used to determine the explicit amount of encapsulated ligand to carry out well-controlled experiments (details in Figure 4-4). Cells were pre-treated overnight with soluble
DHA, soluble PS or PLGA NMP containing ligands encapsulated or coated on the surface (all doses were 50µM of ligand). After treating cells with the inflammasome-activating signals LPS+ATP, IL-1β release was assessed.

As seen in Figure 4-5, nearly all particle types were able to significantly dampen IL-1β release as compared to the untreated, blank particle or soluble ligand controls. Coating or encapsulating particles with DHA resulted in significant inhibition of IL-1β, with the 80x320nm DHA particles having comparable effects as the 1µm particles. Similarly, all PS particles significantly inhibited IL-1β release regardless of particle size. In addition, neither soluble form of DHA or PS inhibited inflammation. It is also worth noting that coating a 1µm particle with DOTAP/DOPE was able to significantly inhibit IL-1β release whereas DOTAP/DOPE-coated 80x320nm particles did not, though the mechanism of how this occurs is unclear. These findings support a previous comment regarding the emergent properties that occur when biological ligands are delivered in particulate fashion and reflect how control over size and shape afforded by the PRINT system unmasks emergent biological details that may otherwise be missed. A final point is the attractive clinical feature of using anti-inflammatory programming devices that are both biodegradable and composed of natural compounds (PS or DHA). Studies are underway to tease out molecular mechanisms and test the in vivo efficacy of these devices in mice.
A. **80x320nm Blank PLGA PRINT Particles**

- Pre-Particle Formulation: 100% PLGA
- Mass of Particles per Tube: 2.029mg
- Size = 223.0 ± 1.997 nm
- PDI = 0.057
- Zeta Potential = -8.82 ± 1.00 mV

B. **80x320nm PLGA:PS (90:10) PRINT Particles**

- Pre-Particle Formulation: 90% PLGA and 10% PS
- Encapsulation Efficiency: 67.9%
- Mass of Particles per Tube: 1.109mg
- Mass of PS per Tube: 0.234mg
- Size = 223.5 ± 4.336 nm
- PDI = 0.307
- Zeta Potential = -68.7 ± 1.38 mV

C. **80x320nm PLGA:DHA (70:30) PRINT Particles**

- Pre-Particle Formulation: 70% PLGA and 30% DHA
- Encapsulation Efficiency: 5.85%
- Mass of Particles per Tube: 2.395mg
- Mass of DHA per Tube: 0.043mg
- Size = 226.2 ± 2.931 nm
- PDI = 0.081
- Zeta Potential = -7.74 ± 0.336 mV

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**Figure 4-4. Characterization Details for Anti-Inflammatory PRINT particles.**

A) Blank 80x320nm PLGA particles.  B) 80x320nm PLGA and phosphatidylserine (PS) particles.  C) 80x320nm PLGA and docosahexaenoic acid (DHA) particles.
Figure 4-5. Biodegradable PRINT Particles Complexed with Natural Ligands are Anti-inflammatory Programming Devices.

A) Inflammasome activation in bone marrow derived mouse macrophages is significantly inhibited by PLGA nano- and microparticles containing the natural ligands phosphatidylserine (PS) and docosahexanoic acid (DHA) (50µM doses). Inhibition by DOTAP/DOPE coating is only effective at the 1µm particle size. Experiments were performed in triplicate. Data are representative of three independent experiments.
Inducing Immune Tolerance through Particle Design

An immunoengineering area that has the potential to entirely change the clinical outcomes for those with allergies, autoimmune disorders or organ transplantations is the ability to induce tolerance to antigens which trigger undesired or overactive immune responses. This is based on the principle that immune responses have inherent plasticity, meaning responses to an antigen can be shifted among the various classes of adaptive immunity depending on the context in which the antigen is presented [111]. This principle has already shown efficacy in treating food allergies to either peanuts or eggs. By delivering escalating doses of allergen over multiple months, tolerance to peanut and egg allergen is induced in children [336-338].

In a similar fashion, rationally designed NMP that deliver antigens and/or immunoregulatory signals have the potential to switch pathological immune responses off and replace them with tolerance [197]. This has recently been demonstrated in a mouse model of multiple sclerosis, where simply coupling the triggering antigen, PLP, to a PLGA particle induced anergy of effector T cells and promoted a tolerizing immune response that reduced clinical severity [250]. Soluble delivery of PLP exacerbated disease, which suggests the particulate delivery of antigen on an inert particle is able to access and induce immunoregulatory pathways.

To test this principle, we made use of an established mouse model of asthma we have previously published [105]. Mice are immunized against the model antigen
OVA through co-delivery into the intraperitoneal (i.p.) space with the vaccine adjuvant alum and then challenged intratracheally (i.t.) with soluble OVA for 5 days to induce an allergic response in the lungs. Because we were interested in how the immune response to an allergen is affected by particulate delivery of that antigen, we challenged the lungs of immunized mice with equivalent doses of either soluble OVA (n=5) or OVA conjugated to the surface of 80x320nm hydrogel particles (PEG-OVA, n=3), as well as PBS controls (n=3). As OVA was conjugated to PEG chains on the surface of PEGylated 80x320nm particles, PEGylated 80x320nm particles alone (PEG) were used as controls for particle delivery. Particle characterization and an asthma model schematic are show in Figure 4-6. OVA-conjugation to hydrogel particles was quantified using BCA Protein assay to ensure equivalent dosing with soluble OVA.

To assess the immune response of challenged mice, we carried out a number of assays including assessment of cell recruitment to bronchoalveolar lavage fluid (BALF), OVA-specific antibody ELISAs and histopathology analysis of mouse lungs to visualize the extent of allergic responses. As compared to PBS treated mice, there was a small increase in the number of cells recruited to the lungs regardless of how OVA was delivered, as to be expected when encountering an allergen (Figure 4-7A). For total IgG as well as OVA-specific IgG1 and OVA-specific IgG2a, there were no differences among any treatment groups (data not shown).

We next assessed the level of IgE production in the serum of mice, as IgE is an antibody class associated with allergic responses [339]. For total IgE, there was no statistically significant difference between OVA-sensitized groups, though there
was a trend for soluble OVA to increase the total amount of IgE (Figure 4-7B). The high level of total IgE seen even in PBS treated mice reflects the role of the alum-OVA immunization in boosting production of this antibody class, as the unsensitized mice treated only with PEGylated particles (PEG- No OVA IP) show no IgE in the serum. When assaying for OVA-specific IgE production, a statistically significant increase was seen only in the groups treated with soluble OVA (Figure 4-7C). The relatively lower OVA-specific IgE seen in PEG-OVA treated groups may reflect the fact that particulate OVA would not necessarily encounter the same number of cells or tissue regions as soluble OVA. The sequestration of the antigen may thus restrict the amount of immune activation otherwise seen in soluble delivery.

Perhaps most telling from these studies is histopathology analysis of the lungs of asthma-induced mice (Figure 4-7D). A robust allergic response induced by soluble OVA can be seen, with numerous infiltrating immune cells and general widespread damage to the air spaces as seen at both 1.25x and 10x magnification. Surprisingly, mice treated with PEG-OVA do not show lung pathology and in fact have histopathology scores no different than mice treated with either PBS or PEG particles alone (Figure 4-7E). This finding suggests particulate delivery of an allergen (here, OVA) significantly reduces an inflammatory allergic response to that allergen. Clearly, more work needs to be done to further characterize the immune response to OVA in these mice, such as cytokines present as well as T cell responses to OVA.

It is known that many allergens are particulate in nature, pollen being a prime example, so that cannot explain the lack of an inflammatory response to PEG-OVA
entirely. However, the answer may be due to the uptake pathway which PEG-OVA accesses in comparison to soluble OVA, a pathway that may promote less robust allergic responses through as yet undefined mechanisms. As an example, preliminary work in our lab indicates that OVA conjugated to hydrogels is presented by DC to T cells, though the kinetics and dosing are different than soluble OVA (data not shown). Given literature in the NMP field where modulating the duration or uptake pathway of an antigen can augment ensuing adaptive responses, we believe that this may help explain our findings in this asthma model [219]. It may also be useful to address the binding affinity of OVA-specific antibodies when OVA is conjugated to a particle, as this may also affect immune responses to an antigen through reduced Fc receptor activation on mast cells and eosinophils for example. Future studies employing NMP-delivery of known allergens, such as food products and environmental substances that trigger asthma, may enable low-cost immunomodulatory therapeutics to help treat the increasing number of people with allergies and asthma [12,340,341]. This approach also has clear relevance for autoimmune disorders, as published recently [250].
Figure 4-6. OVA Asthma Study Details.

A) Characterization of OVA-conjugated and PEGylated 80x320nm hydrogel particles. B) Asthma model schematic where mice are immunized by i.p. injection with prime and boost doses of 20µg of OVA in an alum emulsion. For the challenge phase, mice were given 1µg doses of OVA in either soluble form or OVA-conjugated particles via intratracheal instillation.
Figure 4-7. Particulate delivery of OVA reduces allergic responses to an allergen.

A) Cell recruitment in the bronchoalveolar lavage fluid (BALF) of mice reveals a modest increase when OVA is delivered in either soluble or particulate form. B) No significant differences were seen in total IgE production among immunized mice, though soluble OVA trends higher than other groups. C) OVA-specific IgE levels are significantly higher than PBS-treated mice when OVA is delivered in soluble form, a finding not seen with PEG-OVA delivery (* = p < 0.05). D) Histopathology analysis reveals soluble OVA induces widespread lung damage through immune cell recruitment and inflammation. Similar to PBS and PEG treated mice, PEG-OVA treatment does not cause histopathology. E) Histopathology scores indicate only soluble OVA significantly increases lung pathology, whereas particulate delivery does not. PBS, n=3; Soluble OVA, n=5; PEG-OVA, n=3; PEG, n=3; PEG (No OVA-IP), n=1. Data represent a single experiment.
Inducing Immune Tolerance through Particle Design – Part 2

It is known that there are many different types of immune tolerance and that the pathways eliciting each are unique and appropriate to certain circumstances \[24,115,342\]. For example, aggressive T cells may be directly turned off or dampened through anergic mechanisms, or a population of regulatory T cells can be induced to feedback on other immune cells and ‘change the message’ towards tolerance. Many of these pathways depend explicitly on the context by which dendritic cells encounter and present antigen \[24\]. As discussed in Chapter 1, other pathways to tolerance also exist, so identifying means of programming various qualities of tolerance with NMP may be a boon to human health.

As mentioned previously, one group recently demonstrated an approach where coupling the PLP antigen to PLGA particles induced a tolerizing immune response and protected mice against experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. The mechanism did not depend on production of regulatory T cells (FOXP3+) or IL-10 production, but instead occurred through anergizing effector T cell responses \[250\]. As shown in Figure 4-7, we have preliminary in vivo data suggesting a diminution of an allergic immune response to an antigen by conjugating OVA to a NP which supports the published finding that coupling antigen to inert NP may promote tolerance. However, we sought to design NMP that may program alternative tolerance pathways, in particular the production of regulatory T cells.
To achieve these goals, we hypothesized that phosphatidylserine (PS) could program tolerizing signals when delivered via nano- and microparticles. PS is a natural lipid exposed on the surface of apoptotic cells of the host that signals phagocytes not to mount an immune response against the contents of the engulfed apoptotic cell in order to reduce autoimmune pathology that would otherwise ensue [26,329-331,343]. PS is also known to trigger TGF-β and IL-10 release, critical immunoregulatory cytokines that drive production of FOXP3+ regulatory T cells [329,332,335,342]. In order to assess the potential for PS-NMP to program regulatory immune responses, a panel of PLGA-encapsulating PS particles was produced as described earlier (Figure 4-4).

We used a co-culture system with bone marrow-derived murine dendritic cells (BMDC), the myelin oligodendrocyte glycoprotein (MOG) and 2D2 T cells, which are transgenically engineered to encode T cell receptors (TCR) specific for the MOG peptide (amino acids 35-55) [344,345]. 2D2 T cells are specific for MOG antigen and are skewed towards Th1 responses (low IL-10, low FOXP3 expression, and high IFN-γ). Therefore, this experimental system addressed whether PS-NMP could modulate the functions of 2D2 T cells in the presence and absence of MOG antigen presentation. As shown in Figure 4-8A, delivery of PS (50µM) via 80x320nm PLGA particles (PS PAR) is able to significantly (p < 0.05) increase the production of FOXP3+ regulatory T cells (~20% of total T cells) in the presence or absence of MOG. Neither blank 80x320nm PLGA particles (BLK PAR) nor soluble PS (PS SOL, 50µM) could induce such high levels of FOXP3+ T cells. In addition, IFN-γ production by T cells, a hallmark of Th1 effector immune responses, was
significantly (p < 0.05) impeded by both soluble and NP-delivery of PS during antigen stimulation with MOG. This latter finding is interesting because it shows a bifurcation in programming requirements for PS signaling events: PS in either soluble or particulate form can inhibit IFN-γ production, whereas FOXP3+ induction requires that PS is presented on a particle.

This bifurcation of PS signaling also holds true for production of the anti-inflammatory cytokine IL-10 by FOXP3+ T cells (Figure 4-8B). Only NP-delivered PS significantly (p < 0.05) increased the percentage of IL-10 and FOXP3 double positive T cells (>20% of T cells). Blank particles and soluble PS were equally ineffective at generating this population of regulatory T cells. These preliminary findings show induction of robust amounts of regulatory T cells that inhibit pro-inflammatory cytokines while increasing anti-inflammatory cytokines in a nanoparticle-dependent fashion. Current studies to delineate the role of particle size and ligand density on these effects indicate 1µm PLGA-PS particles also induce regulatory T cell populations, though 80x320nm particles are more potent. In vivo studies in an EAE mouse model with PS particles will determine whether these regulatory responses are antigen-specific and sustainable in vivo.

Through NP-mediated programming, we show evidence of skewing Th1 adaptive immune responses towards regulatory T responses, a finding that has clear translational implications for autoimmune disorders, allergies and the field of organ transplantation. A recent paper identified phosphatidylserine and other natural fatty acid-derivatives as therapeutic targets in humans with multiple sclerosis, giving further credence to the translational relevance of data presented herein [291].
Figure 4-8. Programming Regulatory T cells with Phosphatidylserine Nanoparticles.

**A)** Flow plots of 2D2 T cells after co-stimulation with bone-marrow derived dendritic cells +/- the MOG peptide. Incubation with 50µM of soluble phosphatidylserine (PS SOL) or 50µM of phosphatidylserine encapsulated in 80x320nm PLGA particles (PS PAR) is able to inhibit IFN-γ production by T cells in the presence of MOG, whereas blank 80x320nm PLGA particles (BLK PAR) have no effect. PS PAR induce a significant increase in FOXP3+ T cells, not seen in the BLK PAR or PS SOL groups.

**B)** Only particulate delivery of PS (PS PAR) is capable of inducing significant percentages of IL-10 and FOXP3 double positive T cells. * = p<0.05. Data are representative of at least three independent experiments.
4.4 DISCUSSION

In the studies detailed in this chapter, we have explored potential particle parameters that may enable broad programming of immune responses through modular and rational design of NMP. While this is by no means an exhaustive list of the potential signaling events that can be designed into NMP, we believe it reflects the merit in pursuing basic science studies addressing the potential of biological programming through NMP. As evidenced by our findings, there is a tremendous space for discovery through basic probing using size and shape specific delivery of biological ligands. Given the potential to control the kinetics and magnitude of biological responses through size, shape and composition of NMP, there are multiple levels of program control afforded by this approach (Figure 4-3). It should also be noted how well suited the PRINT platform is for these studies.

These studies not only enable more thoughtful interpretation of experimental data, but also open up the possibility of uncovering unique signaling events that have translational merit. For example, our findings uncovered a bifurcation between the signaling events downstream of phosphatidylserine signaling (Figure 4-8). Inhibition of IFN-γ production is insensitive to how PS is delivered, whereas generation of IL-10 and FOXP3 regulatory T cells requires particulate delivery of PS. Whether this is because particulate PS enables receptor clustering on immune cells to drive this regulatory switch is currently unclear, but as discussed throughout this dissertation, emergent properties occur when biological ligands are presented in particulate fashion [1].
It is an added benefit that the majority of these studies were performed using PLGA as the base composition of our particles, as this polymer is biodegradable, biocompatible and F.D.A approved for use in humans. In addition, we have purposefully designed NMP with biological ligands that are endogenously produced in the body to generate entirely biodegradable and natural programming devices that modulate mammalian biology in clinically relevant fashion.

A last note regards the potential for modular perturbation of signaling networks through NMP design. There is much literature showing the potential of NMP as vaccines through incorporation of TLR agonists [73,74,123,124,201,203,204,211,214,246,247]. Yet, TLRs are but one class of pattern recognition receptor (PRR) and there are multiple other network families beyond PRRs that have yet to be explored. As cells integrate cues from all aspects of their environment in every moment, we deem it judicious to consider combinatorial approaches to network perturbation.

Coupled with our studies using natural lipids and fatty acids, one could envisage a combinatorial programming device at the nano and microscale that triggers multiple biological networks simultaneously to generate unique and, ideally, clinically useful outcomes. Given the modularity of NMP, this combinatorial signaling need not necessarily occur on a single device. One could envisage targeting NMP to certain tissues or cell types that program responses which promote or enhance the efficacy of the ultimate payload delivered by a separate NMP. The feasibility of this general concept has already been proven using nanoparticles which communicate in vivo to amplify tumor targeting of drugs [346].
4.5 CONTRIBUTIONS

Much of the work presented in this chapter would not have been possible without the generous time and effort of a number of colleagues. Matthew Detter fabricated and characterized PLGA particles containing Quantum Dots. Dr. Warefta Hasan and Tammy Shen helped fabricate PLGA and hydrogel particles used in the adjuvanted (LPS/MPL) particle studies. Cathy Fromen generated and characterized OVA-conjugated and PEG-hydrogels. Both Cathy and Tammy, along with Dr. Coy Allen, were instrumental in the asthma studies. Dr. Greg Robbins assisted in flow experiments and was always willing to lend an extra hand. James Byrne and Patrick Short fabricated and characterized the PLGA particles containing either DHA or PS. Dr. Haitao Wen helped perform the inflammasome inhibition assays with these particles. We are also indebted to Dr. Tim Eitas technical expertise during the co-culture experiments with 2D2 T cells and PS particles. Finally, the constant support, collaboration and overall guidance of Dr. Joseph DeSimone and Dr. Jenny Ting made these studies a reality.
CHAPTER 5
GENERAL CONCLUSIONS

Given that cellular function is both sensitive and malleable in response to environmental input, we sought to demonstrate the utility of a platform capable of programming cellular function for therapeutic benefit as a paradigm shift in how we treat disease. This requires the use of biocompatible devices that can be modularly designed at the physical scale which cellular networks communicate: the nano ($10^{-9}$) and micron ($10^{-6}$) scale. These devices are termed nano- and microparticles (NMP).

As discussed at length throughout this dissertation, the immune system constitutes a vast communication network that integrates cues from within and without the body in a coordinated fashion with all organ and tissue systems to ensure the maintenance of health for the entire organism. Given its direct and commanding role in host survival, it is not surprising that immune network signaling is implicated in all major disease categories. This spans microbial infections, neurodegenerative and autoimmune disorders, cancer, organ transplantation rejection, metabolic abnormalities such as type 2 diabetes, and other non-communicable diseases (NCD). In addition, it is the immune system that naturally recognizes and responds to NMP devices. Therefore, we have direct access to one of the most important and powerful communication networks in the body and the
potential to program the immune system in a targeted manner to evoke cellular functions that may have benefit in all major disease categories.

A new and promising scientific field, termed immunoengineering, merges the disciplines of materials science, chemistry, immunology, and bioengineering to pursue the ultimate goal of harnessing the full potential of the human immune system to treat disease [214,246,247]. Early results from this nascent field suggest rationally designed NMP programming devices can make this goal a reality in the foreseeable future. Some major highlights include: generation of highly potent microbial and cancer vaccines [73,123,124,195,203,204,207,211,227,253,289]; inhibition of inflammatory immune cell recruitment in vivo with associated improvement in multiple mouse models of disease [245]; inhibition of cancer-mediated immune suppression and activation of T cell mediated tumor killing [231-233,236,284]; induction of potent mucosal immunity [224,225,347]; skewing of adaptive immune classes [196,197]; and tolerance induction in mouse models of autoimmunity [248-250].

While these results are very promising, they all used different methods of particle fabrication and design with no overall consideration of a platform approach to immunoengineering. In particular, programming the full spectrum of immune responses will be greatly abetted by a NMP platform that offers precise control over particle fabrication, as even slight differences in size, shape or chemistry can have profound biological implications (reviewed in Chapter 1 and [148-150,246,247]). Recent breakthroughs in materials science have resulted in development of a precise fabrication technique, termed Particle Replication in Non-wetting Templates.
(PRINT), which offers complete control over the size, shape, composition and chemistries of nano- and microscale particles (NMP) [188,189,191,192,194,257,348]. Using PRINT technology, this dissertation sought to systematically and reproducibly address the feasibility and potential of programming mammalian biology through rational design of NMP.

As our ultimate goal is the ability to program all types of immune response in vivo, it is critical that we use NMP that are immunologically inert at baseline. To use a metaphor, we want to use NMP that are a blank canvas to the immune system so that we can paint with all colors. Thus, the initial work we performed was systematic analysis of the mammalian immune response to PRINT-fabricated NMP. For all studies, we fabricated NMP with biodegradable and biocompatible polymers with a safe history of use in humans to lend the most translational relevance to our studies (PLGA, PEG or PEG derivatives).

**PRINT Particles are Immunologically Inert in Mice**

In chapter 2, we presented evidence that PRINT NMP do not cause inflammatory responses in vitro or in vivo in mice. As the mouse is the most common mammal used in biomedical research, we initiated our studies in this species so that we could help clarify confusion in the field as to the inflammatory potential of PLGA NMP [72,73]. In addition, future murine studies employing PRINT NMP for therapeutic purposes now have a baseline of data to guide design strategies. We were able to show sustained deposition (7 days) of NMP in the lungs of mice without immune cell recruitment, inflammation or evidence of
pathology. This suggests that PRINT NMP are immunologically inert in mice, and can reside in the lung for extended periods of time, a desirable attribute for drug delivery efforts. In addition, modulating particle size corresponded with differential uptake into lung cells. This particle design parameter may enable differential targeting to the intracellular and extracellular space, a feature that could be exploited if one wanted to deliver cytokines locally to the lung while also delivering an antigen into innate immune cells so that the ensuing immune response could be skewed in a targeted manner. Unexpectedly, particle deposition in the lungs may have direct relevance to treatments for autoimmune disorders such as multiple sclerosis and type 1 diabetes or other CNS and intestinal abnormalities. This is because the lung serves as a network hub where circulating T cells are licensed to enter the CNS or the intestines depending on the cues in the lung environment [349,350].

*PRINT Particles are Immunologically Inert in Humans*

In chapter 3, we performed a systematic analysis of the human immune response to PRINT NMP. We did this to address the gap in literature regarding human immune responses to nanomaterials, as most studies are performed in mice. Of mice and men, there are large immunological differences [351,352]. In the human monocyte cell line, THP-1, we found no signs of inflammatory responses to PRINT NMP, nor an ability to synergize with soluble TLR ligands to activate the inflammasome, as assessed by cytokine production. More broadly, we also found PRINT NMP do not activate the pro-inflammatory transcription networks NF-κB or AP-1. This is a critical finding as it suggests a large part of the canvas we so desire to paint is blank.
For direct analysis of human immune responses to a series of 80x320nm PRINT NP, we acquired blood from four healthy donors and identified immune cell subsets which are targeted by particles. Using dye-loaded hydrogels, we found no uptake into lymphocyte subsets (T, B and NK) and targeting to CD14+ innate immune cells. Particle uptake correlated with the level of CD14+ expression and could be abrogated by PEGylation to the particle surface. In addition, the cells which took up the most particles stained positive for a marker of cell death, a response that was entirely diminished by PEGylation.

When we performed a global cytokine screen to capture a portrait of the human immune response to PRINT 80x320nm hydrogels, we found no differences between treated and untreated donor samples for any of the 30 cytokines tested. This reflects the broad immunological inertness of these particles and suggests the cell death we identified via flow cytometry for hydrogels is itself non-immunostimulatory. While the mechanism needs more analysis, broad depletion of innate immune populations for transient periods of time may be of clinical merit in certain settings. For example, many NMP applications are limited by their rapid clearance from the circulatory system by innate immune cells [235,287,353]. By first treating patients with particles that deplete this same population transiently, subsequent delivery of a therapeutic or diagnostic NMP may show prolonged circulation times and enhanced efficacy, as published in mouse studies [354].

In conjunction with our published data on the role of human macrophage polarization on particle uptake [305], our uncovering of heterogeneity in the human innate immune response to nanoparticles points to the complexity of
future clinical nanomedicine interventions and suggests humans may have
differential responses to NMP programming depending, in part, on their immune
status. Some evidence exists for this already [299,306]. In future, pre-screening of
patients for known immune parameters (e.g., allergies, asthma, autoimmune
disorders, recent sickness, age, gender) may be an important aspect of ensuring
appropriate dosing and delivery strategies of NMP therapeutics.

We translated our *ex vivo* findings into a humanized mouse model to gain a
better appreciation of the complexities of human immune cell targeting in a whole
animal. Our findings were largely recapitulated, with hydrogel particles taken up
primarily by human CD14+ myeloid cells and CD11c+ DC, with PEGylation impeding
this process. CD14 expression levels also correlated with particle uptake in this *in
vivo* setting. We did not find any indication of an inflammatory response to NP
treatment as assessed by mRNA analysis for human cytokines. Interestingly, we
noted enhanced targeting of PEGylated particles to human plasmacytoid dendritic
cells (pDC), which may be an attractive cell population to program in applications
related to viral infection, such as vaccines.

In the absence of PEGylation, hydrogels showed a modest uptake into
human CD4 and CD8 T cells in the bone marrow. This is also an exciting finding, as
access to T cells by NP may enable direct programming of these very potent
adaptive immune cells. As the bone marrow contains T cell precursors as well as
memory and regulatory T cell subsets, further characterization of which classes are
targeted by particles is necessary [355]. However, targeting any, or all, of these
populations could confer unique therapeutic opportunities in a variety of disease
spaces. Clearly, more work needs to be done to show the low level of in vivo targeting we see is sufficient for therapeutic instruction. Alternative targeting strategies, such as T cell specific antibody conjugation on the particle surface, may enable more robust efficacy in downstream applications. The general cross-validation of our ex vivo PBMC studies and humanized mouse work further validates each study and suggests the humanized mice may be a more clinically relevant model for NMP translational applications.

**Programming Immune Responses with PRINT Particles**

Having systematically characterized the immune response to PRINT NMP in both mouse and humans, we felt confident in the general immunologic inertness of our particle platform. Thus, in Chapter 4, we turned to early sketches on this blank canvas to reveal some of the potential programming opportunities available through rationally designed NMP. Through the fabrication control inherent to PRINT, we were able to uncover biological details relevant to programming immunity that would have been masked using other fabrication systems. They are summarized here as testament to the power of the PRINT platform for probing basic biology.

We first identified simple surface modifications to particles that could modulate uptake in a model human immune cell (THP-1). 80x320nm PLGA particles could be programmed to remain outside or enter these cells simply by adding a cationic lipid to the surface. This may be a powerful yet low-tech means of delivering cargos to extracellular or intracellular spaces, depending on the therapeutic need.
In a similar fashion, PEGylation of the surface of hydrogels or conjugation of a model antigen (OVA) reduced uptake. Control of the kinetics of uptake and duration of antigen presentation are key parameters in modulating adaptive immune responses, in part through differential targeting of MHC I or MHC II pathways [24,115,178,246]. As has been shown in other particle systems, this is a potentially simple yet powerful approach to skewing vaccine responses towards CD4+ or CD8+ effector cells [246,356].

By coating the TLR4 ligand LPS onto PRINT NMP, we identified differences in the timing and magnitude of inflammasome activation based on particle size, shape and composition. This provides evidence for the ability to control both the kinetics and intensity of biological signaling pathways with NMP. These are two critical components of cell network communication and will likely be a boon to programming efforts in the immunoengineering space [317]. In addition, design control over inflammasome activation may itself enhance vaccine efficacy, such as for those targeting fungal infections or induction of Th17 responses.

As inflammation drives pathology in numerous diseases including cancer, metabolic syndrome, atherosclerosis, stroke and neurodegenerative disorders [17,34,127,137,241,321-323], we sought design features that would enable NMP to program anti-inflammatory responses. To this end, we fabricated PLGA particles with the omega-3 fatty acid docosahexaenoic acid (DHA), as literature supports its potent anti-inflammatory properties [326,328]. In addition, we incorporated the lipid phosphatidylserine (PS) into particles as it is known to have strong immunoregulatory functions, in part through production of anti-inflammatory and
immunoregulatory cytokines [329,333,335]. Both particles compositions were able to significantly inhibit inflammasome activation \textit{in vitro}, whereas soluble controls were ineffective at the same dose. Size-dependent differences in the magnitude of inflammation inhibition may reflect the power of PRINT to tease out small biological details and the complex role of ligand presentation on ensuing biological responses.

This theme of emergent properties manifesting when bioactive ligands are delivered via NMP is one we cannot stress enough, as any small molecule may have its own unique properties when made particulate. These NMPs are entirely natural and biodegradable anti-inflammatory devices that we are now testing in \textit{in vivo} models with inflammatory sequelae. We also note the modularity and fabrication ease of incorporating fatty acids and lipids into PLGA particles with the PRINT process. This is a research space worth pursuing given the potent biological properties of commercially available endogenous fatty acids and lipids.

Work we would like to see undertaken involves combinatorial delivery of ligands for multiple receptor families. A prime example is the design of vaccine carriers which would contain adjuvants for multiple classes of pattern recognition receptors. For example, this could include TLR, NLR and/or RLR ligands to potently augment innate immune activation. It is known that multiple TLR ligands when delivered by NMP show synergistic effects and enhance adaptive immune response to co-delivered antigens [204,207], thus it is likely that combinatorial signaling between PRRs will be even more advantageous as this more closely mimics the cellular response to natural infection. To our knowledge, this is a design space that has not been studied to date.
Our final studies in Chapter 4 focused on programming immune tolerance, as this has immediate relevance to a number of poorly treated diseases that afflict millions of people, including allergies, asthma, organ transplantation and autoimmune disorders. We believe we have identified NMP design parameters that may enable the programming of two immune tolerance pathways. In the first, we simply conjugated the model antigen OVA onto the surface of our previously identified inert 80x320nm hydrogel particles (Chapters 2 and 3). In a mouse model of asthma where mice are sensitized to OVA as an allergen, particle delivery of OVA to the lungs completely abrogates the allergy response. The mechanism for this has not been elucidated as of yet. However, we are bolstered in our findings by a paper published after our studies showing induction of tolerance in a mouse model of multiple sclerosis by simply conjugating the antigen at issue to a PLGA particle [250]. The study authors identified anergy of effector T cells as the primary mechanism of tolerance induction by antigen-conjugated PLGA particles with no evidence for a role of regulatory T cells (Treg).

Because there are different pathways to generate tolerance and Treg are critical to maintaining immune tolerance throughout the body, we deemed it useful to identify NMP design parameters that could program expansion of the Treg population. We hypothesized that particles fabricated with PLGA and phosphatidylserine (PS) would be processed similarly to apoptotic cells (which have PS exposed on their surface), namely in an immunosuppressive manner characterized by production of IL-10 and TGF-β [329,330,332,335]. In support of our reasoning and during the course of our dissertation work, two groups published
that delivery of liposomal PS could indeed produce these immunoregulatory cytokines [333,334]. However, they did not address the potential of their PS particles to modulate tolerance, which is our main interest.

Using a co-culture system with mouse DC and Th1-skewed MOG-specific 2D2 T cells, 80x320nm PLGA-PS NP dampened production of IFN-γ from T cells while significantly increasing production of IL-10 and FOXP3 expression. At the same dose, soluble PS was unable to induce IL-10 or FOXP3 expression in T cells, exemplifying the theme of emergent properties when using particulate ligands. These exciting findings may suggest that an entirely natural and biodegradable PLGA-PS NP can reprogram T cells with effector-skewed responses to an antigen into Treg cells with tolerizing responses to that same antigen. While much work remains, this may be a powerful and clinically unheralded means of treating autoimmune disorders and improving clinical outcomes after organ transplantation. In addition, it epitomizes our belief that rationally designed NMP have the potential to fundamentally change treatment and clinical outcomes for the panoply of modern diseases while also serving as immensely detailed probes of biology.

We have glimpsed only a small spectrum of the potential of NMP for immunoengineering approaches. Given their modularity, these devices can be designed to include small molecules, siRNAs, expression vectors, proteins, antibodies, in short anything one could possibly want to paint with. The possibilities for biological programming are truly awe-inspiring.
Final Thoughts

The prevailing realization one has when considering the complexity of biological systems is how impossibly simple it all is: input<->output. Of course, there’s a lot that goes on in the in between space! What is absolutely clear is the tremendous amount of integration and collaboration that must occur within and between cells in order for the higher organism to exist. This integration and collaboration goes beyond the cellular level, as collaboration between organisms is a prerequisite for life; without peaceful coexistence with the 100 plus trillion bacteria that cohabit our body, we quickly fall into disrepair [8,10,12,80,147,357,358].

Through collaboration, higher functions manifest. We take this to heart, as the work presented in this dissertation required collaborations with at least 16 scientists across multiple disciplines. We also benefited from knowledge collaborations with thousands of scientists, referenced throughout this dissertation, whose research laid the ground work and intellectual framework for our studies.

Only through massive collaboration across disparate disciplines will scientists and non-scientists alike solve the world’s most pressing problems. By learning about biology, we must also learn from it. We, and all the other living things among us, will be better for it.

“There’s a natural mystic blowing through the air…”

-Bob Marley
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