EXPERIMENTAL MACROPHAGE POLARIZATION: A TWO-PRONGED APPROACH TO EVALUATE MODULATION OF MACROPHAGE FUNCTION

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by
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Abstract

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Monocytes and macrophages (MMs) are key effector cells of the innate immune system, and recent studies have implicated these cells in a wide array of additional homeostatic functions. Therefore, uncovering the stimuli that drive changes in MM function may yield important findings with far-reaching impact. **PURPOSE**: The purpose of this study was to create a procedure for testing the effects of human exercise and/or dietary interventions on macrophage function *in vitro*, after the incubation of macrophages with human subject serum. **METHODS**: This study involved three parts: 1. Protocol optimization for the differentiation of THP-1 monocytes to monocyte-derived macrophages (MDMs) using PMA; 2. Optimization of the LPS concentration for polarization of THP-1 MDMs to M1 macrophages; 3. Optimization for incubation of THP-1 MDMs with human serum prior to M1 polarization. For all optimizations, phagocytic capacity was determined relative to untreated controls using a commercially available kit. Additionally, prior to phagocytosis measurements, culture media was reserved and the concentration of cytokines IL-10, TNF-α, IL-1β, and IL-6 were analyzed for select samples. Finally, NucBlue staining immediately following phagocytosis measurements in the latter

two optimization experiments was used to qualitatively assess effect of treatment on cell numbers. Phagocytosis data were analyzed using a one-way ANOVA. Additionally, a two-way ANOVA was used to determine the effect of time x concentration in serum experiments. The level of significance for all analyses was set *a priori* to the level of 0.05 and, following a significant F-statistic, post-hoc analyses were conducted using Bonferroni Correction. Cytokine data was not subjected to statistical analysis due to insufficient sample numbers. **RESULTS**: There was a significant effect of PMA incubation time such that 48/24 hr incubation/rest led to greater phagocytosis than the longer incubations ($p < 0.01$), and a trend towards greater phagocytosis than 24/48 hr incubation/rest ($p = 0.065$). The 24/48 condition led to the highest levels, 48/24 condition led to intermediate levels, and 72 hr condition led to the lowest levels of all cytokines but IL-1β. There was no significant effect of LPS concentration on phagocytosis (p=0.386), though 0.5 μg/mL LPS led to the highest phagocytosis. Cytokine responses were similar for all cytokines but IL-1β in which a plateau in response was observed with 0.2, 0.5, 1.0 μg/mL LPS. There was a significant effect of serum concentration across time ($p < 0.01$) and incubation time across concentrations ($p < 0.001$), such that higher concentrations and longer incubation times were associated with higher and lower phagocytosis, respectively. Similar trends were observed in the cytokine data. **CONCLUSION**: The results of this study inform the following optimized procedure: 1. THP-1 monocyte differentiation to MDMs via 48 hr incubation with 25 nM PMA followed by 24 hr rest; 2. Incubation of MDMs with 35% human serum media for 3.0 hr before polarization. 3. THP-1 MDM polarization with 0.5 μg/mL LPS for 24 hr prior to the phagocytosis and cytokine measures.

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Dedication

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Chapter 1: Introduction and Literature review

Introduction

Macrophages are phagocytic cells of the innate immune system, most known for their role in pathogen clearance, tissue remodeling, and antigen presentation (28). However, specific populations of macrophages are also integrally involved in bone, spleen, and central nervous system homeostasis (24, 83). In order to fill these diverse roles, the functionality and phenotype of macrophages is uniquely plastic in nature (7). Macrophage function can vary widely depending on the specific suite of signals present in the tissue microenvironment (23). In addition to tissue specific subsets (e.g. osteoclasts in bone), soluble proteins and direct interaction with other cells can polarize (i.e. differentiate) macrophages toward either pro-inflammatory and microbicidal or anti-inflammatory and immunosuppressive phenotypes (64). These two divergent phenotypes have been classified as "M1" and "M2" macrophages, respectively, in analogous convention to Th1 and Th2 classifications of immune response (65). Though conceptually useful, macrophages *in vivo* likely manifest a continuum between these two extremes rather than binary M1 versus M2 (7, 65). Additionally, recent discoveries in developmental biology and epidemiology have shed light on the wider importance of these cells in tissue development, homeostasis, and disease (24, 28, 83). Consequently, understanding of the varied roles and significance of macrophages is expanding (64). Harnessing macrophage plasticity holds promise for increasing understanding of health, disease, and creating novel treatment options (64, 65).

Macrophage polarization both requires and manifests specific changes in cellular metabolism (45). Pro-inflammatory M1-type polarization includes upregulation of anaerobic

glycolysis, while M2-type macrophages rely more heavily on fatty acids to fuel oxidative phosphorylation (7, 64). These changes are an example of "form follows function" wherein the glycolytic metabolism showcased by M1-type macrophages supports rapid response and clearance of pathogens, while the consistent energy supply of oxidative phosphorylation fuels the often prolonged processes of tissue repair, remodeling, and the resolution of inflammation (64). However, aberrant appropriation of these phenotypes is associated with pathological states (65). For example, adipose tissue in obesity showcases excessive recruitment and polarization of adipose tissue macrophages (ATMs) towards the M1 state (45). This change in ATM population leads to pronounced inflammatory cytokine production fueling chronic systemic inflammation and initiating the path to insulin resistance (45). Conversely, virtually all known tumors actively recruit monocytes and macrophages causing them to assume pro-tumor, immunosuppressive, and metastatic functions (7). Beyond these two examples, macrophages are integrally involved in almost every known disease (83). The specific conditions may differ between disease states, but the unifying theme includes dysregulation of normal macrophage polarization and plasticity (40, 64, 65). Therefore, identifying means to differentially modulate or optimize macrophage polarization may reveal new therapeutic options. Of particular interest, diet and activity may have profound effects on monocytes and macrophage polarization.

Given the known associations of healthy diet and exercise with reduced morbidity and mortality, it is of interest to what extent these effects are mediated through the immune system (6, 59). So far, evidence in both mice and humans indicates that diet and exercise improve markers of disease and inflammation through changes in monocyte and macrophage polarization. In a mouse model of diet-induced obesity, exercise training was shown to

decrease macrophage recruitment into adipose tissue, pro-inflammatory signaling, and the inflammatory potential of ATMs (33). These changes were despite no differences in weight loss relative to the sedentary group, indicating the potential of exercise to specifically alter macrophage function and character (33). In elderly adult humans, 12 weeks of concurrent aerobic and resistance training reduced the proportion of $CD14^+/CD16^+$ (inflammatory) monocytes in circulation, as well as monocyte pro-inflammatory cytokine production (70). Again, the latter changes were despite no significant differences in body weight or % body fat over the course of the study (70).

As for diet, monocytes and macrophages are known to be sensitive to different macronutrients and accumulating evidence points towards the ability of plant chemicals (phytochemicals) to affect the phagocytes' metabolic functions. Both high glucose and free fatty-acids (FFAs) can initiate macrophage polarization towards an inflammatory M1 state (64, 65). However, perhaps paradoxically, consumption of carbohydrate during exercise is known to reduce both the stress-hormone and inflammatory cytokine response to the bout (5). Additionally, monocytes incubated with serum from athletes consuming bananas during extensive cycling exercise showed improved mitochondrial function compared to serum from athletes consuming a sports-drink or water only (53). Similar results were observed in a study by a different group, which found that polyphenolic extract from cocoa beans led to improved mitochondrial function as well as decreased inflammatory cytokine secretion in THP-1 monocyte-derived macrophages (MDMs) (22). In sum, research on the immunomodulatory effects of diet and exercise points towards the power of lifestyle practices to alter, support, and improve macrophage form and function. By more clearly delineating these relationships it may be possible to titrate diet and activity interventions to

specifically alter macrophage polarization on a case by case basis. Failing that, further insight could inform broad recommendations to support macrophage plasticity and prevent the inflexibility associated with many diseases (45, 65). It was the goal of this project to develop and optimize a two-part procedure for measuring monocyte-derived macrophage function and inflammatory character for future use evaluating exercise and dietary interventions.

In order to accomplish this goal, a multi-step procedure was developed to differentiate THP-1 monocytes to macrophages and then polarize the macrophages towards an M1-type phenotype. The first set of experiments involved comparing four differentiation protocols to optimize this process for later experiments. Differentiation of THP-1 monocytes to MDMs was initiated using the protein kinase C activator phorbol 12-myristate 13-acetate (PMA). PMA is the standard method for differentiating THP-1 monocytes and leads to MDMs that compare favorably to primary cells (15, 62). The next two stages of experiments involved optimization for the concentration of lipopolysaccharide (LPS), and for the incubation time and concentration of human serum leading to a robust and consistent increase in phagocytic function and cytokine secretion in THP-1 MDMs. Phagocytosis and increased inflammatory cytokine secretion are defining characteristics of M1 polarization, as well as key to M1 functions (11). Therefore, it was important for this and future projects to characterize both phagocytic function and the secreted cytokines following differentiation, serum incubation, and polarization. Phagocytic function was assessed using a commercially available kit (Vybrant Phagocytosis Assay Kit, ThermoFisher Scientific) and the concentration of secreted cytokines was measured using a multiplex, magnetic bead-based assay (Milliplex, MilliporeSigma). Finally, cell numbers were qualitatively assessed using NucBlue Staining (NucBlue Live ReadyProbes, ThermoFisher Scientific) during the LPS and

human serum optimizations to determine whether observed changes were due to alterations in cell behavior versus cell numbers.

Literature Review

Monocytes and Macrophages Overview: What they are, and What they do. Monocytes and macrophages are cells of the innate immune system that play integral roles in pathogen recognition, clearance, and tissue homeostasis (4). Specifically, monocytes and macrophages (MMs) are notable for their ability to phagocytose (engulf) debris and pathogens, leading to production of soluble signaling molecules and elimination of the target (64). As a part of the innate immune system, MMs arose approximately 500 million years ago shortly after the origin of single cell eukaryotes (24, 64). With the evolution of multi-cellular eukaryotes and adaptive immunity, MM function expanded to include activation of the adaptive immune system in addition to pathogen recognition and clearance (23, 64). MM functions continued to diversify as tissue specific populations emerged in more complex organisms (24, 83). MMs are highly plastic cells and the specific suite of signals within the tissue microenvironment directly and profoundly impacts their form and function (64). In response to both soluble mediators and physical interactions, MMs are differentiated and polarized towards a variety of functional subsets (7). This plasticity allows unique populations of macrophages to express features that support the specific needs of the tissue in which they reside. However, it also lends itself to perversion by pathogens or tissue dysfunction (83). Due to their widespread distribution, and their pivotal role in health and disease, MMs hold great potential for understanding the wider role of the immune system in organismal biology.

Furthermore, exploring the mechanisms of MM polarization offers a unique path to treat both acute and chronic disease.

Despite their primordial origins, monocytes were not recognized by science until the mid-19th century when they were characterized microscopically by Paul Ehrlich and functionally by Elie Metchnikoff (28). It was for this pioneering work on innate immunity that the two jointly received the 1908 Nobel Prize in Physiology or Medicine (28). However, it was not until 1910 that the term monocyte was first used to describe those cells and until 1924 that the term macrophage was introduced (23, 28). At this time MMs were classified as part of the reticuloendothelial system that also included fibroblasts, endothelial, and reticular cells (23). However, in the 1960's the mononuclear phagocyte system (MPS) was introduced to reclassify MMs ontogenetically (23, 28). This classification system, still used today, includes monocytes, macrophages, dendritic cells, and their bone marrow precursors (28, 83). In conjunction with the creation of the MPS classification, a hierarchical model of differentiation was introduced to describe the origins of each cell type (24). In this model, monocytes and dendritic cells differentiate from a common "monocyte-macrophage/dendritic cell precursor" (MDP), and macrophages mature from monocytes as the latter enters the tissue niche (27, 28). This paradigm had dominated until very recently despite early evidence of alternative developmental pathways (7, 24, 27, 28). However, recent work tracking the ontogeny of tissue resident macrophages has led to a broad revision of MM development and even suggestion that different tissue macrophages should be classified as distinctive cell types (64, 83). Conversely, rather than "immature" macrophages, monocytes may represent a dynamic "emergency squad" capable of patrolling and responding to disruptions in tissue homeostasis (28).

Monocytes. Due to the difficulty of isolating and maintain primary MM cells from human subjects, the majority of what is known about MM biology is derived from mouse studies (24, 83). Therefore, the majority of the following data applies specifically to mice, unless otherwise noted. However, despite some particular differences, it appears that murine and human MMs function vary similarly (83). So, conclusions drawn from animal studies are still broadly informative. Within the divisions of the MPS system multiple populations of both monocytes and macrophages have been observed. In bone marrow of adults, immature monocytes differentiate from MDPs (24, 27). These monocyte forerunners are positive for the cell surface marker Ly6c and have no phagocytic capacity (24). Following stimulation by CCL2/MCP-1, $Ly6c^+$ monocytes proliferate and egress from the bone marrow into the circulation where they constitute ~4% of circulating leukocytes in mice and ~10% in humans (28). Circulating monocytes are composed of two subsets, differentiated by their expression of the cell surface marker Ly6c (24, 28). $Ly6c^+$ cells, known as "classical monocytes," are the first to emerge from the bone marrow, and possess greater inflammatory potential (28, 65). Classical monocytes circulate for approximately 70 hours before either entering tissues and differentiating into macrophages, or differentiating into $Ly6c^-$ monocytes (23). $Ly6^-$ monocytes, known as "non-classical" or patrolling monocytes, have lower inflammatory potential but may persist in the circulation for up to 7 days (28). While both monocyte subsets patrol the circulation searching for problems, they perform divergent functions. $Ly6c^+$ monocytes form an "emergency squad" most responsive to CCL2/MCP-1, but sensitive to a variety of chemokines which cause the cells to migrate into inflamed tissue and differentiate into MDMs (24, 28, 64). Conversely, $Ly6c^-$ monocytes express higher levels of

adhesion molecules and, though capable of extravasation, primarily function to monitor and maintain the vascular endothelium (24).

Though pioneering work on monocytes was conducted in mice, analogous differentiation patterns and populations have been characterized in humans. Human classical and non-classical monocytes are identifiable as $CCR2^{+}/CD14^{+}/CD16^{-}$ and $CX3CR1^{+}/CD14^{-}/CD16^{+}$, respectively (28, 65). Humans also possess a third intermediate monocyte population that is $CD14^{+}/CD16^{+}$ and may represent a transitional phenotype with its own functionality (27). Similar to the $Ly6c^{+}$ to $Ly6c^{-}$ transition observed in mice, repopulation studies have shown that human monocytes progress from $CD14^{+}/CD16^{-}$ to $CD14^{-}/CD16^{+}$ with parallel changes in function (28). However, differences do exist between murine and human monocyte biology, particularly in the distribution of the subsets. While mouse monocytes are fairly evenly distributed between $Ly6c^{+}$ and $Ly6^{-}$, human classical monocytes compose 80-90% of the circulating population with intermediate and non-classical monocytes forming the remaining 10-20% (28). In addition, mouse monocytes do not and human monocytes do express major histocompatibility complex II (MHC-II) proteins, indicating functional differences in regard to antigen presentation (28).

Macrophages. For approximately 50 years since the introduction of the MPS system macrophages have been viewed as "mature" monocytes, the end result of monocyte differentiation following extravasation into the tissue (24). However, this was despite evidence to the contrary both in the 1960's and even as far back as Elie Metchnikov (24, 83). In the last 15 years, studies on macrophage population and repopulation have expanded conceptions on macrophage origin (83). The latter findings have not re-written the monocyte to macrophage pattern so much as diversified the definition of what macrophages are and

where they come from. Most notably, it is now accepted that the majority of tissue resident macrophages have embryonic origins and, rather than consistent repopulation by monocytes, self-renew *in situ* (24). The following section will cover an overview of these recent findings on macrophages during development, and then discuss the phenotypic and functional characteristics that discriminate different macrophage populations.

In mice, the first macrophages to develop derive from the embryonic yolk sac ectoderm around embryonic day 8 (83). At this point, these primitive macrophages are the only leukocytes present and this particular population goes on to colonize and perform trophic functions in the majority of the major organs (24, 32, 83). By embryonic day 10 hematopoietic stem cells (HSCs) become the main source of macrophages, proliferating and differentiating in the liver until shortly after birth when bone marrow becomes the main site of HSC differentiation (24). Liver-derived macrophages supplement the populations in all the previously mentioned tissue niches except the brain, whose population of macrophages is exclusively yolk sac-derived (24, 83). Following birth, the majority of tissue resident macrophages self-maintain with little supplementation by monocytes except in the case of injury or inflammation (24). The notable exceptions to this rule are macrophages in the digestive tract and osteoclasts in bone (27, 64). The digestive tract features a mixture and self-renewing resident macrophages supplemented by MDMs, while osteoclasts may be derived from HSCs or circulating monocytes (27, 64). Resident macrophages in each tissue niche vary widely in their phenotypes and functions, depending on the environment and the requirements of the organ (24). These differences have led to the development of nomenclature to differentiate resident macrophages from different tissues including: Microglia in the central nervous system (CNS), Kupffer Cells in the liver, Langerhans Cells

in the skin, Red-Pulp Macrophages of the Spleen, Osteoclasts in bone, and Histiocytes in both spleen and interstitial spaces (27, 64). This is not meant to be an exhaustive list of all tissue macrophages, rather, it is meant to give an appreciation for tissue macrophage diversity and recognition of their functional and phenotypic differences.

Regardless of their developmental origin, all macrophages in mice and humans express the cell surface receptor CSF1r (83). The ligand for this receptor, colony stimulating factor or macrophage colony stimulating factor (CSF/M-CSF), is involved in growth and proliferation of most macrophage populations though its specific effects vary between tissue niches (83). Additional markers of adult macrophages in mice include: CD11b, CD68, and F4/80 (83). CD68 and CD11b are also present in human macrophages, but the human equivalent of F4/80 is less diagnostic (27, 32). Additionally, macrophages may be distinguished by increased expression of the co-stimulatory proteins CD80 and CD86, though this too may vary by macrophage subset (36, 58). In comparison to monocytes, macrophages can be reliably identified by increased size and intracellular complexity (3, 23). The latter differences reflect changes in phagocytic capacity and are the result of a greater concentration of lysosomes and mitochondria (15). Beyond these few general characteristics, broad macrophage markers are difficult to confirm because of the diversity and plasticity in macrophage subsets. Macrophages in different tissues or functional states may be as transcriptionally different from one another as they are from monocytes (24).

As mentioned above, MDMs serve to supplement rather to replace tissue macrophages following fetal development (24). After entry into the tissue niche, MDMs are tuned towards different functional states based on contextual and tissue-specific conditions (64). This process is known as macrophage polarization and is described by the M1/M2 macrophage scheme (64).

M1 polarized macrophages, also known as classically activated macrophages, are the prototypical inflammatory macrophages tuned to fight viral and bacterial infection with cytotoxic functions (65). In contrast, M2 polarized macrophages, also known as alternatively activated macrophages, have a variety of functions including: anti-parasitic, anti-inflammatory/immunosuppressive, and tissue remodeling (23, 65). The wide variety of functions encompassed by M2 macrophages is indicative of the limitations of the M1/M2 scheme and dramatic plasticity of macrophages. At least four subsets have been identified within the M2 classification (M2a, b, c, d), all with variable expression patterns and divisions between them (23, 36). Furthermore, additional classes of macrophages have been identified that defy either M1 or M2 categorization (7, 9). Although the paradigm does have its limit, characterizing macrophages as M1 or M2-*like* is operationally useful and significant research has defined the changes associated with polarization towards either state.

M1 polarization is induced by bacterial lipopolysaccharide (LPS) alone or in combination with the cytokines IFNγ, TNFα, and/or GM-CSF (23, 64, 65). M1 macrophages mediate their inflammatory and anti-microbial functions through the release of reactive oxygen and nitrogen species (ROS/RNS), as well as the prolific production of inflammatory cytokines such as TNFα, IL-1b, IL-6, and IL-12 (65). M1 macrophages also possess potent phagocytic capacity, recruit additional immune cells to the site of infection, and promote a Th1-type adaptive immune response via the chemokines CXCL9 and CXCL10, as well as increased expression of CD80/86 and MHCII (23, 36, 65). In order to execute a rapid and potent inflammatory response, M1 macrophages undergo significant transcriptional changes during polarization, including metabolism (7, 9, 64). Besides increased expression of genes coding for inflammatory cytokines and chemokines, M1 polarization is associated with a dramatic

shift towards the anaerobic metabolism of glucose (64). This shift in energy metabolism supports M1 inflammatory function through the rapid production of ATP (64). Additionally, it allows citrate to be diverted from the Kreb's Cycle towards synthesis of ROS, RNS, and prostaglandins (64). Taken together, the features of M1 polarization support robust, rapid induction of inflammation, and recruitment of additional cytotoxic cells to contain and eliminate potential threats.

In contrast to M1-polarized macrophages, M2-polarized macrophages are tuned for resolution and repair following inflammatory events. Prototypical M2 polarization is induced by the cytokines IL-4, IL-13, IL-10, and TGF-β, and/or the phagocytosis of necrotic tissue (4, 36, 40). M2 macrophages promote a Th2-type immune response and the resolution of inflammation through the production of the cytokines IL-10, TGF-β, VEGF, and IGF-1 (23, 65). To support their role in tissue remodeling, M2 polarized macrophages have high phagocytic capacity, but are poor antigen presenting cells (APCs) due to lower expression of MHCII and CD80/86 (36, 65). Additionally, these cells express a variety of degradative enzymes, such as metalloproteinases, for breaking down extracellular matrix (23). In response to parasitic infections M2 macrophages recruit the help of eosinophils and basophils with the chemokines CCL17, CCL22, and CCL24 (65). Compared to M1 macrophages, M2 polarization involves relatively little transcriptional changes (9, 42). The majority of changes that occur during M2 polarization appear to involve genes associated with lipid metabolism, tissue growth and degradation, and extracellular pathogen response (42).

The features listed above are generally associated with M2 macrophages, but the degree to which they apply to the four defined M2 phenotypes varies between the subsets. Of the four, the M2a phenotype identifies most closely with the classic definition of alternatively-activated

macrophages and these macrophages are tuned to facilitate the clearance of cellular debris, as well as resolve inflammation and injury (64). Conversely, M2b macrophages appear to participate more actively in inflammatory immune responses as they are induced by some of the same stimuli as M1 macrophages (64). M2c polarized macrophages primed to specifically aid in clearance of cellular debris through the expression of scavenger receptors and anti-inflammatory cytokines (36, 64). Finally, M2d macrophages are the most competent at promoting vascular growth (64). For a more detailed list of M2 macrophage features please refer to Table 1 below. Despite the overarching designation as "M2 macrophages," the wide variety of stimuli and expression patterns listed above indicate that M2 macrophages may actually be more different than they are similar. The features that link these different populations may be less associated with similarity in expression patterns or functionality, and more related to the facility with which they transition from one phenotype to another. This phenomenon, known as macrophage re-programming or re-polarization, turns out to be a defining trait of macrophages that contributes to their diversity (2, 7, 64).

Class	Induced By	Express	Secrete
M2a	IL-4, IL-13	CD206, IL-1Ra, IL-1R, Arg-1 (Mice), FIZZ1 (Mice), Ym1/2 (Mice)	IL-10, TGF-β, CCL17, CCL18, CCL22, CCL24
M2b	Antigen-Antibody Complexes, Apoptotic Cells, LPS, IL-1Ra, IL-1β	CD86, MHCII	IL-1, IL-6, TNF-α, IL-10 (high)
M2c	IL-10, TGF-β, IL-1Ra, glucocorticoids	CD163, CD206, TLR-1, TLR-8, Arg-1 (mice)	IL-10, TGF-β, CXCL13, CCL16, CCL18
M2d	LIF, Il-6, Adenosine	IL-10 (high), VEGF (high), IL-12 (low), TNF-α (low)	IL-10 (high), VEGF (high), IL-12 (low), TNF-α (low)

Table 1: Features of M2 macrophage subsets. Compiled from (23, 36, 64).

Given the wide variety of characterized functional states, and the theoretically infinite mixtures of polarizing stimuli, macrophages *in vivo* likely reflect a continuum between the established M1 versus M2 (or M2a) extremes rather than discrete subsets (65, 83). The concept of macrophage polarization is further complicated by the lability of macrophage polarization states, and potential limitations set by tissue microenvironments. Following the absence of polarizing stimuli or prompting from opposite stimuli, polarized macrophages may revert to the original "M0" state or transition from one functional state to another (*i.e.* M1 ↔ M2) (7, 23). This remarkable plasticity appears essential to the progression of a healthy inflammatory response, and disruption of facile transitioning between polarization states is associated with a variety of diseases and poor healing (28, 64, 65). Nevertheless, the degree of plasticity does vary and may be purposefully governed between different tissue niches. For instance, regardless of their origin (*i.e.* resident or MDM) macrophages in the lung exhibit a more anti-inflammatory M2-like phenotype (65). This feature may limit inflammation-induced damage to the delicate tissue of the alveoli (64). In contrast, macrophages in the digestive tract exhibit a unique phenotype characterized by high M1-like bactericidal activity, but low inflammatory cytokine production (27). The latter traits appear to prevent dysbiosis by eliminating harmful bacteria, while concurrently fostering tolerance to beneficial bacterial populations (64). Thus, plasticity and diversity are essential features of macrophages leveraged for both immunologic functions and tissue homeostasis (7). In summary, more than simply effector cells of the innate immune system, macrophages are an endlessly diverse and dynamic population of cells employed as guardians of local and systemic homeostasis.

Monocytes and Macrophages: Why they matter. The facility with which macrophages re-polarize or re-program is more than a coincidence of their diversity. Rather, switching between phenotypes is integral to macrophage functions in promoting and resolving inflammation (4, 24, 28). However, the sensitivity of macrophages to their environment and their remarkable plasticity also leaves them susceptible to dysfunction in chronic disease, or exploitation by pathogens (64, 65). The following section will summarize a number of examples which showcase the utility of macrophage re-programming as well as how this process is disrupted during pathological states.

Analogous to an infectious insult, facilitating an effective and efficient healing response to physical trauma is central to macrophage function. Importantly, both M1 and M2 polarized macrophages serve distinctive roles in healing, and active transition between the two states appears essential for an optimal healing response (4, 28). In a seminal study by Arnold et al., MDM recruitment, phenotype, and tissue healing were observed following experimentally-induced skeletal muscle injury in mice (4). The investigators were most interested in examining whether the different MDM populations present at different stages of healing were representative of discrete invasions by different monocyte subsets, or one population with a transitioning phenotype (4). Over the course of multiple experiments, the authors concluded that the initial inflammatory response is induced following $Ly6c^+$ monocyte invasion, M1 polarization, and pro-inflammatory cytokine production (4). However, the decline in inflammatory MMs starting approximately 24 hours after injury occurred via re-programming of the initial invaders rather than new MDMs. Notably, phagocytosis of necrotic tissue and IL-4 production by muscle progenitor cells (MPCs) were shown to be essential to the transition of MMs from an inflammatory *(i.e.* $Ly6c^+$/M1) to an

anti-inflammatory ($Ly6c^-$/M2) phenotype (4). Both the inflammatory and anti-inflammatory/resolution stages of healing were essential to an effective healing response, with $Ly6c^+$/M1 MMs important to MPC growth and proliferation and $Ly6c^-$/M2 MMs responsible for MPC differentiation and fusion (4). The results of this study show the importance of both inflammatory and anti-inflammatory MMs, as well as effective macrophage reprogramming. They also suggest that overuse of anti-inflammatory interventions (*i.e.* NSAIDs, icing) in response to pain and injury may actually delay or impair the healing process.

Another example demonstrating the importance of macrophage polarization and plasticity is the systemic response to infection. During infection, the production of inflammatory cytokines (particularly TNF-α) induces a state of peripheral insulin resistance (65, 83). This response may save glucose for inflammatory cells, like M1 macrophages, that are primed for rapid glucose uptake and ATP production through anaerobic glycolysis (64, 83). Additionally, high glucose environments are capable of polarizing MMs towards the M1 state (64). This indicates glucose may serve as an immune signaling molecule to bolster the inflammatory response. However, while useful during brief infections, inflammation-induced insulin resistance and pro-inflammatory macrophage character become etiological in a number of diseases when prolonged (83). Chronic, sterile inflammation is symptomatic of obesity and linked to the progression of cardiovascular disease, non-alcoholic fatty liver disease, insulin resistance, and type-2 diabetes (7, 45). Understanding the role of inflammation and glucose on insulin resistance and macrophage polarization, it is logical to conclude that the ability of MMs to re-program towards an anti-inflammatory phenotype and reduce inflammation is essential to disease prevention. This hypothesis is supported by

studies show reductions in inflammatory cytokine production and re-programming MM following both exercise interventions and weight loss (25, 33, 70). Thus, similar to wound healing, rapid macrophage polarization and re-programming is essential for mounting and resolving an efficient response to infection.

Finally, perhaps the best example of the essentiality of macrophage polarization and plasticity is in the context of cancer. As a part of their inflammatory and cytotoxic phenotype M1 polarized macrophages are mostly tumoricidal (23). However, chronic activation and inflammation induced by M1 macrophages may lead to DNA damage that is causal in tumorigenesis (64, 65, 83). In contrast, most established tumors appear to "re-educate" macrophages toward an immunosuppressive, pro-angiogenic phenotype that most closely resembles M2d polarization or, perhaps, a developmental macrophage phenotype (7, 83). At the site of tumorigenesis, tumor associated macrophages (TAMs) are mostly derived from resident macrophages (28). These cells enable tumor growth by modifying existing extracellular matrix and specifically localization to hypoxic areas to promote angiogenesis (37). Additionally, TAMs are poor APCs and prevent tumor cytotoxicity through the production of anti-inflammatory cytokines and low production of IL-12 (64). Concurrently, metastasis is facilitated by monocytes recruited to colonize future tumor sites, perhaps by tumor released exosomes (28, 83). The importance of TAMs to cancer progression is evident from studies correlating the density of TAMs with poor prognosis and showing TAM depletion inhibits tumor vascularization and growth (7, 64). Therefore, identifying the signals that induce the TAM phenotype and targeting TAM polarization and plasticity is an attractive avenue for anti-cancer therapy. It is possible that TAM re-programming or supporting

macrophage plasticity could prove a powerful supplement in cancer treatment, if not cause spontaneous remission on its own.

The previous three examples showcase the importance of macrophage polarization and the dysfunction that can occur with the misappropriation of polarization states or disruptions to macrophage plasticity. Although inflammation is frequently designated "bad" and anti-inflammatory agents "good," hopefully the evidence detailed above illustrates that both inflammatory and anti-inflammatory potential are indispensable to effective macrophage function and systemic homeostasis. In thinking about future research avenues and potential therapeutic options, less emphasis should be placed on reinforcing a specific macrophage polarization state than on supporting appropriate sensitivity to stimuli and the ability to re-program following stimulation (65). The "right" polarization state is extremely context specific, therefore the benefit of inducing one functional state or another without adequate plasticity may lead to unforeseen complications. Fortunately, strategies to balance and maintain macrophage polarization and plasticity may already exist. In this vein, research into the beneficial effects of lifestyle interventions have shown promise in modulating macrophage phenotype.

Monocytes and Macrophages: Optimizing Response. With the understanding of MMs integral nature in the development and homeostasis of almost every tissue environment, it is clear that identifying means to modulate macrophage polarization and maintain plasticity offer distinct potential for improving and maintaining physiological function. Additionally, given the long lifespan of tissue macrophages, optimal versus impaired macrophage function may dictate long-term health (65). The question is, what are the best tactics to support MM function? Along these lines, a number of different strategies have started to be evaluated

including both targeted and more holistic approaches. Regarding the former, anti-CD14 antibodies have been evaluated for their ability to blunt excess macrophage inflammatory signaling in the etiology of sepsis (20). CD14 is a key cell surface receptor protein involved in the LPS signaling, and the development of sepsis and , given its role, anti-CD14 antibodies were hypothesized as a potential sepsis counter-measure. However CD14 also appears to participate in LPS clearance within the liver so, although anti-CD14 antibodies or oligonucleotides reduced inflammatory signaling *in vitro,* they did not lead to uniformly improved survival to sepsis challenge *in vivo* (20). Consequently, until understanding regarding the complexities of macrophage polarization and signaling are more refined, more judicious approaches may be warranted. Fortunately, the search for alternatives need not be exhaustive since interventions as mundane as exercise and diet may be remarkably potent.

It is well established that a healthy diet and remaining active are essential supporting physical and mental vitality. However, the means by which exercise and diet specifically support well-being have yet to be fully defined. Both exercise and the consumption of certain foods are known to have beneficial anti-inflammatory effects (25, 30, 69). Yet, *how* these effects are mediated and the correct dose to affect an optimal response remain to be elucidated. Given their atypical responsiveness, plasticity, and widespread distribution MMs are prime candidates. Furthermore, there is evidence to support MMs are one target for the effects of diet and exercise.

In the context of increasing inflammation associated with age or chronic disease, exercise is a known to decrease or blunt such “inflammaging” and sterile inflammation (5, 25, 45). Despite this association, the mechanisms mediating the anti-inflammatory effects of exercise are just beginning to be discovered. So far, the evidence supports three main

strategies through which exercise modulates systemic inflammation: decreasing adiposity, anti-inflammatory signaling, and changing MM character.

In healthy adipose tissue, adipose tissue macrophages (ATMs) perform stromal functions, exhibit characteristics of M2 polarization, and maintain insulin sensitivity through the production of IL-10 (45, 83). However, adipose tissue stress induced by excess energy consumption or circulating free fatty acids (FFAs) leads to a cascade of maladaptive events including: increased pro-inflammatory signaling (*e.g.* MCP-1, LTB4, TNF-α), monocyte recruitment, MDM M1 polarization, and adipocyte apoptosis (45, 83). Consequently, adipose tissue in obesity or overnutrition becomes a site of prolific pro-inflammatory cytokine production concurrently inducing the release of additional FFAs and contributing to local and systemic insulin resistance (65). The central role of ATMs in adipose tissue inflammation and dysfunction is evident from experiments in which ATM depletion led to improved insulin sensitivity and glucose tolerance (45). Additionally, weight loss through the reduction of adipose tissue mass is associated with lower markers of systemic inflammation and a restoration of a healthy ATM population (25, 65). Given diet and exercise are the most common and effective weight-loss strategies, it is clear they can indirectly modify systemic inflammation through reductions in adipose tissue mass.

At a more acute level, there is also evidence to indicate that exercise can directly affect inflammatory vs. anti-inflammatory signaling. It has been observed that exercise induces a transient anti-inflammatory state following each bout (25, 74). This phenomenon may be contradictorily explained by inflammatory signaling *during* exercise, specifically the release of IL-6 (66, 68, 81). IL-6 infusion at levels similar to those observed during exercise has been shown to induce release of the anti-inflammatory cytokines IL-1ra, IL-10, and the

hormone cortisol (68). Additionally, IL-6 may blunt TNF-α and IL-1β signaling and promote a higher M2:M1 macrophage polarization balance (66, 74). Consequently, frequent doses of exercise-induced IL-6 signaling with habitual activity may summate to attenuate chronic low-grade inflammation (25). Evidence also exists to suggest consistent exercise may have immunological effects outside the context of each exercise bout.

As detailed above, weight loss is associated with a reduction in systemic inflammation and improvements in adipose tissue health whether with or without exercise (26, 64, 65). However, there is evidence to indicate that exercise may alter MM phenotype and decrease pro-inflammatory signaling even in the absence of weight loss. In a study by Kawanishi et al., introduction of a treadmill running program led to a reduction in adipose tissue MDM recruitment, MDM TNF-α production, and re-programming of ATMs towards a M2 phenotype in a mouse model of diet-induced obesity (DIO) (33). Notably, this was despite no differences in food intake or weight loss compared to the sedentary DIO group and, overall, exercise training caused a normalization of inflammatory cytokines and ATM character towards mice undergoing exercise training on a standard diet (33). Remarkably, similar results have also been shown in humans. As reported by Timmerman et al., twelve weeks of concurrent aerobic and resistance exercise training led to a reduction in circulating inflammatory monocytes, lower monocyte TLR4 expression, and a reduction in *ex vivo* basal and LPS stimulated TNF-α production (70). The latter changes were despite no significant changes in body mass, BMI, or percent body fat over the course of the study, indicating specific effects on monocytes themselves (70). Interestingly, when compared to those of physically active adults of the same age, the characteristics of the previously sedentary group undergoing training trended towards those of the physically active cohort (70). This suggests

that commencing physical training later in life may still offer similar benefits to life-long training in sedentary, but otherwise healthy elderly adults.

In the context of macrophage polarization and plasticity, the obvious caveat to the anti-inflammatory effects of exercise listed above is whether encouraging an anti-inflammatory environment is always warranted or wanted. MMs and other cells produce cytokines for the purpose of inflammatory signaling (23). Despite its negative connotations, both cytokine-dependent and independent inflammation has many essential functions including the mobilization of monocytes during homeostatic disturbances, containment and elimination of pathogens, as well as the previously detailed role in the initial stages of healing (4, 23, 28). Therefore, the anti-inflammatory effect of exercise may actually be detrimental in certain circumstances, and there is evidence showing this to be the case. It is well documented that heavy physical exertion can both acutely and chronically compromise immune function (54, 66, 84). For macrophages in particular, abundant evidence has shown that both acute exhaustive exercise and chronic heavy exertion can impair the cells' cytotoxic and antigen-presentation functions. Acute exhaustive, but not moderate, exercise is associated with reduced macrophage antigen presentation, inflammatory cytokine production, and intrinsic anti-viral defense (8, 16, 34, 80). Similarly, chronic heavy physical exertion is associated with increased risk for upper respiratory tract infections (URTI) likely mediated by impaired macrophage function, among other immune impairments (49, 52, 66, 84). However, in contrast to the effects of exhaustive exercise and chronic heavy exertion, regular moderate exercise is actually associated with improved immunity and reduced infection risk (5, 25). Notably, moderate exercise is associated with *reduced* risk for URTI (49). There is evidence to indicate this reduction is due to improved lung macrophage anti-viral resistance,

perhaps mediated by exercise induced increases in the inflammatory cytokine IL-6 (16, 48, 49). Additionally, both acute exercise and exercise training are associated with increases in macrophage phagocytosis, anti-tumor activity, ROS production, and improved sensitivity to the activating signals of LPS and IFN-γ (55, 73, 80). Therefore, exercise does not inherently suppress nor enhance immune function. Instead, the effect of exercise on immunity appears to be dose dependent with habitual moderate physical activity associate with improved immunity, yet reduced systemic inflammation (25, 49, 75). But, in heavy doses or in conjunction with additional stressors, exercise may lead to compromised immune function (25, 46). This biphasic relationship deserves further study as the stress responses of exercise mimic many of the same responses to psychological stress (18, 47). Investigations in this area may yield novel insight for treating both physiological and psychological ailments. In parallel, efforts devoted to bolstering or supporting the beneficial effects of exercise are also needed. Fortunately, work in the area of immunonutrition has shown promise both for supporting immune function in the face of exercise stress, and also for beneficially modulating macrophage polarization.

Like exercise, a healthy diet and the consumption of certain foods are known to support immune function and display anti-inflammatory effects, respectively (5, 19, 73). However, unlike exercise, the majority of work examining the anti-inflammatory properties of food products has been limited to *in vitro* work or in animal models (5). From this line of inquiry, plant-derived phytochemicals have demonstrated powerful anti-oxidant and anti-inflammatory properties (12, 37, 85–87). However, the degree to which these results translate *in vivo* or in humans is limited in two regards: the complexity of *in vivo* signaling dynamics and the bioavailability of phytochemicals (21, 39, 61). In regard to the latter point, the

activity of phytochemicals *in vivo* may differ considerably from the native due to extensive microbial and hepatic modification following ingestion, and prior to entering the systemic circulation (5). However, evidence does exist showing immunomodulatory effects of phytochemicals *in vivo* and demonstrating the transability of *in vitro* work, at least to a certain degree. Despite the potential for high glucose levels to polarize MMs towards the M1 (pro-inflammatory) state, consumption of carbohydrate during exercise is associated with a reduction in the stress hormones and inflammatory response to the bout (54, 64). Interestingly, in a recent study comparing the effect of banana versus sports drink or water-only consumption during an exhaustive cycling bout, banana consumption was associated with an increase in circulating banana-related metabolites, improved mitochondrial function and reduced COX-2 mRNA expression of THP-1 monocytes incubated with serum from banana provisioned cyclists (53). The latter changes were significantly different from the water-only condition and there was a trend toward lower COX-2 mRNA expression compared to sports-drink consumption (53). Similarly, incubation of THP-1 MDMs with a cocoa bean polyphenol extract reduced inflammatory cytokine secretion of M1-polarized macrophages and increased ATP production through oxidative phosphorylation (22). There is also evidence to indicate that specific nutritional practices may enhance immunity in the face of exercise stress, with the potential to directly bolster macrophage function.

Broadly, carbohydrate ingestion during exercise is a consensus counter-measure to exercise-induced immunosuppression by reducing the stress hormone and cytokine response to the bout, as well as attenuating some but not all adverse changes in immune cell function (5). On a more specific level, various plant-derived components have shown promise in boosting anti-viral defenses. Seventeen days consumption of a polyphenol-enriched protein

powder was associated with reduced *in vitro* cytotoxicity to the vesicular stomatitis virus when cells were incubated with post-exercise serum from supplemented vs. placebo-supplemented athletes (1). Additional evidence for the potential of phytochemicals to boost anti-viral responses comes from two studies which observed increased natural killer cell counts with six weeks consumption of either whole blueberries or a freeze-dried blueberry powder in athletes and non-athletes, respectively (43, 44). These studies also observed reductions in exercise-induced oxidative stress and increased anti-inflammatory cytokine levels in the athletes, and reduced blood pressure in the non-athletes (43, 44). Finally, added carbohydrate and/or oat β-glucan consumption has been shown to improve survival rates in mice infected with herpes simplex virus following exercise stress, as compared to non-supplemented mice (17, 50). The latter changes are likely mediated through enhanced lung macrophage function (47). Taken together, the results of the experiments listed above show the potential for transability of *in vitro* to *in vivo* models, as well as the potential for nutritional interventions to support immune function and modulate macrophage polarization both within and outside the context of exercise. However, more work remains to be done to reveal how the observed changes are occurring and uncover additional means to support macrophage and global immune function in different contexts.

In summary, MM polarization and plasticity are essential features to maintaining immune health, systemic homeostasis, and are an attractive target for supporting wellbeing in the face of infection or aging. To this end, both exercise and dietary interventions have shown promise in supporting immunity and modulating macrophage function. Although, identifying the most effective doses and compounds still remains to be determined. Therefore, strategies to consistently evaluate the effects of activity and/or nutritional

interventions are sorely needed. Ideally these strategies would allow for examination of conditions *in vivo*, without unduly invasive or tedious procedures. It was the goal of this project to optimize a procedure for evaluating macrophage function and polarization state *in vitro* following incubation with human subject serum to model *in vivo* conditions. In order to accomplish this goal, a multi-step process was devised to differentiate THP-1 monocytes to MDMs and then assess MDM phagocytic function and cytokine secretion in response to serum and/or polarizing compounds.

Monocytes and Macrophages: Modeling In Vivo conditions with Human Cell Lines. For optimal translation and applications, ideally, *in vivo* human studies would be used for exploring macrophage polarization. However, due to technical and ethical limitations, studying tissue macrophages *in vivo* or *ex vivo* is not feasible (80). To circumvent these problems both animal models and human monocytes isolated from peripheral blood mononuclear cells (PBMCs) have been used; although each alternative presents its own limitations either in applicability of results or availability and maintenance of the cells, respectively (11, 79). Consequently, human cell lines have been developed for use in experiments on MM biology with useful features not found in primary cells including: uniform genetic background, the ability for culture expansion, as well as long term culture and storage (3, 11). The most commonly used line for the study of human MM biology is the THP-1 monocyte line (3). This line of human monocytes was isolated from the circulation of a 1-year old male with acute monocytic leukemia in 1980 (71). Subsequent experiments revealed the cells behaved similarly to primary monocytes: showing lysozyme production, phagocytic activity to sheep red blood cells, and the ability to activate T-cells (71). Furthermore, supporting the practicality of the cell line, the cells could be cultured for

extended periods of time (months) without adverse genetic changes or diminished proliferative capacity (71). Since its establishment, the THP-1 cell line has been used extensively in experiments exploring the factors that influence monocytic and MDM behavior and function (11, 38). In the latter case, a variety of substances and protocols have been evaluated for their ability to differentiate THP-1 monocytes to MDMs, including Vitamin D_3 and phorbol 12-myristate 13-acetate (PMA) (9, 15, 57, 62). Of the two, PMA most reliably differentiates THP-1 monocytes to a phenotype that closely matches primary MDMs and, therefore, is the most common compound used in differentiation (9, 15, 38, 62). In THP-1 monocytes, PMA appears to mimic diacylglycerol signaling through protein kinase C (PKC), a family of enzymes involved in cell proliferation and differentiation (62). PMA treatment leads to arrest of proliferation, an increase in size and granularity of THP-1 cells, adhesion, and increases in surface markers of macrophage differentiation (15, 62). Despite (or perhaps because of) its popularity, numerous PMA differentiation protocols exist leading to variable phenotypes (3, 38, 57, 67). As a consequence of this non-agreement, one study aimed to critically compare the most popular differentiation protocols with the aim of introducing a standardized protocol for *in vitro* THP-1 differentiation (38). Following their experiments, the authors of the study recommended a differentiation protocol involving culture of THP-1's with 25 nM PMA for 48 hours, followed by 24 hours without PMA prior to subsequent experimental interventions (38). This protocol was shown to lead to adequate differentiation of the monocytes without excess activation of pro-inflammatory signaling, which has been shown to occur with overexposure to PMA (38, 57, 67).

Following differentiation, THP-1 MDMs can be polarized towards either the M1 or M2 polarizations states using similar substances to those in primary cells (IFN-γ + LPS and

IL-4, respectively) (9, 11). However, PMA differentiated THP-1 cells MDMs may be more amenable to M1 polarization as PMA differentiation does appear to bias the cells towards a more inflammatory phenotype (9, 15). Though standard M1 polarization involves incubation of differentiated THP-1 MDMs with a combination of IFN-γ and LPS, there is evidence to indicate that LPS alone may yield a similar polarization state (9). LPS, a pathogen associated molecular pattern (PAMP), is an integral component of the cell wall of gram-negative bacteria and consequently serves as a soluble indicator of bacterial infection *in vivo* (20). Antigen presenting cells, like monocytes and macrophages, express a wide array of pathogen recognition receptors for sensing PAMPs (76). In monocytes and macrophages, LPS signals primarily through the PRR TLR4 to activate NF-κB and upregulate inflammatory gene expression (20, 56). LPS-TLR4 interactions alone can induce NF-κB activation, however, optimal activation involves additional proteins participating in the LPS-TLR4 signaling complex (15, 38, 58). *In vivo*, LPS aggregates are bound by the LPS binding protein (LBP) which delivers them to soluble or membrane bound CD14 receptors (56). CD14 splits LPS aggregates into monomers and forms a complex with TLR4 and the adaptor protein MD-2, for initiation of the intracellular signaling cascade leading to NF-κB activation (20, 56). LPS-NF-κB activation leads to robust increases in inflammatory gene expression including those of inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 leading to the initiation of local inflammation (9, 13, 20). It is worthy of note that THP-1 cells appear more responsive to LPS treatment, as indicated by greater increases in phagocytosis and inflammatory cytokine secretion, following PMA differentiation (3, 11, 62). Therefore, PMA differentiation followed by LPS polarization may lead to consistent M1 polarization and provide a viable model of bacterial pathogen exposure prior to systemic immune activation (in the absence of

IFN-γ from previously activated immune cells) (23). Consequently, I proposed a 3-part differentiation and activation protocol in which THP-1 cells were differentiated with PMA and then incubated with human serum to simulate *in vivo* conditions, prior to activation/polarization with LPS. In order to optimize responses for utilization of this protocol with future human exercise and nutritional interventions, this project involved 3 stages of optimization: PMA incubation time, LPS incubation concentration, and human serum incubation and time.

Purpose of Study

Monocytes and Macrophages are multi-functional immune cells with a wide distribution and broad impact on the body. The cells are also remarkably plastic by nature and, therefore, the factors that influence their function are of great research interest and hold potential for profound discoveries. However, study of the dynamics influencing human macrophage function *in vivo* has been limited in the past by technical hurdles. Therefore, the purpose of this study was to develop and optimize and procedure for measuring macrophage phagocytic function and cytokine secretion *in vitro* following incubation with human subject serum to simulate *in vivo* conditions.

Hypothesis

I hypothesized that shorter PMA incubation times and higher LPS concentrations would be associated with greater inductions of phagocytosis and secretion of cytokines IL-10, TNF-α, IL-1β, and IL-6 from THP-1 monocyte-derived macrophages. I further hypothesized that shorter incubation times and higher serum concentrations would be associated with the greatest increases in phagocytosis and secreted cytokines, compared to longer incubations or lower serum concentrations.

Chapter 2: Methods

Methods

To reiterate, the purpose of this project was to develop and optimize a procedure for evaluating the effects of human exercise and dietary interventions on macrophage function *in vitro*, through the incubation of THP-1 monocyte-derived macrophages (MDMs) with human serum. In pursuit of this purpose, the project had three aims: 1. Identify the optimal PMA differentiation protocol for differentiating THP-1 monocytes to monocyte-derived macrophages (MDMs) capable of M1 polarization using LPS. PMA protocols were compared based on functional characterization of M1 polarized macrophages using assessments of phagocytosis and secreted cytokines. It was the expectation that shorter PMA protocols would lead to greater levels of measured phagocytosis and cytokines upon LPS polarization. 2. Identify the concentration of LPS that most accurately polarizes THP-1 MDMs towards the M1 state, without excess stimulation or cytotoxicity. Similar to aim 1, LPS concentrations were compared based on measured phagocytosis and cytokine levels of polarized MDMs. Additionally, NucBlue staining and fluorescence was used to assess cell numbers following polarization. It is expected that both the lowest and highest concentrations of LPS will lead to lower levels of phagocytosis and cytokines due insufficient and overstimulation of the cells, respectively. However, no differences in cell numbers are expected between LPS concentrations as indicated by NucBlue fluorescence. 3. Distinguish the optimal human serum concentration and incubation time for incubating THP-1 MDMs prior to M1 polarization. Just as in aim 2 the outcome measures used to determine cellular responses were phagocytosis, secreted cytokines, and NucBlue fluorescence. Out of the tested conditions, optimal responses in this aim were considered intermediate levels of both

phagocytosis and cytokine secretions, without no difference or higher NucBlue fluorescence. This is due to the future goals for this procedure, in which both pro-inflammatory and anti-inflammatory effects the tested human intervention will need to be distinguished. It was expected that both higher concentrations, and longer incubation times would be associated with greater phagocytic and cytokine responses, and vice versa. The latter three aims formed the three stages of the overall study, the methods of which are detailed below.

THP-1 Culture. THP-1 monocytes were maintained at 37° C and 5% CO_2 in *THP-1 media* composed of RPMI-1640 media with 2mM glutamine, supplemented by 10% fetal bovine serum and 0.05 mM β-mercaptoethanol. Cell concentration was maintained between 400,000-1,000,000 cells/mL in a 30 mL culture flask by passaging every 3-5 days. Passaging included visual inspection and estimation of culture concentration. For visual inspection cells were observed by light microscopy within the culture flask for subjective detection of culture contamination or aberrant cell behavior. For estimation of culture concentration, two separate 200 μL aliquots were removed from the cell culture flask for counting with a hemocytometer. Ten microliters (10 μL) of each cell aliquot was mixed with trypan blue at a ratio 1:1, then added to the hemocytometer for counting. The cells counted alive and dead from each aliquot were averaged and used to calculate the culture concentration and cell viability.

Culture concentration estimated using the formula:

$$\left(\frac{(alive\ cells\ aliquot\ 1) + (alive\ cells\ aliquot\ 2)}{2}\right) \times 5000 = Cells/mL$$

Cell viability was estimated using the formula: $\left(\frac{Avg.Dead\ Cells}{Avg.Alive\ Cells}\right) \times 100 = \%\ Cell\ Viability$

When the culture concentration reached 800,000-1,000,000 cells/mL, a volume of the culture suspension was removed, and fresh THP-1 media was added to return the culture to ~400,000 cells/mL in 30 mL of THP-1 media.

Plating and Differentiation. To initiate differentiation of the THP-1 monocytes to M0 MDMs, cells were plated to 30 wells of a clear bottom 96-well plate at a total volume of 150 μL/well and a concentration sufficient to yield 100,000 cells/well. For all experiments but the PMA incubation optimization, cells were plated in a 6 x 6 format corresponding to four experimental rows and one positive control row with 6 replicates per row. THP-1 media only was plated to an additional row of 6 to serve as the negative (no cells) control condition. For the PMA incubation optimization experiments, cells were plated in a 6 x 3 format with each with row split between two of the incubation conditions, 3 replicates each. THP-1 media only was plated to an additional row of 3 to serve as the negative control condition. See Figures 1 and 2 for a diagram of the plate set-up. Differentiation was only commenced once the cell culture reached ≥ 800,000 cells/mL and no more than 1.1 million cells/mL as culture conditions have previously been shown to significantly impact differentiation in THP-1 monocytes (3).

PMA Differentiation. In order to determine the differentiation protocol which most clearly led to M1 polarization 4 different incubation protocols were compared: 96 hr PMA incubation (PMA included during 72 hr differentiation period and 24 hr LPS incubation; 96PMA); 72 hr PMA incubation (PMA removed during 24 hr LPS incubation; 72PMA); 48 hr PMA with 24 hr "rest" in culture media prior to addition of LPS (48PMA); and 24 hr PMA with 48 hr "rest" prior to addition of LPS (24PMA). In each incubation condition the same concentrations of 25 nM PMA and 0.5 μg/mL LPS were used. LPS treated cells in

each condition were compared to "positive control" cells unstimulated by LPS but differentiated in the same manner.

M1 polarization. In order to determine the effect of LPS concentration on THP-1 MDM function, four concentrations of LPS (0.1, 0.2, 0.5, 1.0 μg/mL) were tested for their effect on phagocytosis and cytokine secretion in M0 THP-1 MDMs. Specifically, following the 72-hour differentiation protocol outlined above, THP-1 media was removed and replaced with THP-1 media plus the addition of 0.1, 0.2, 0.5, or 1.0 μg/mL LPS, or control (no LPS). The cells were then incubated with LPS for 24 hours prior to assessment of phagocytosis. The media/supernatant following the 24-hour LPS incubation was saved for later assessment of secreted cytokines.

Serum Incubation. In order to determine the effect of concentration and incubation time with human serum (HS) on THP-1 MDM function, four concentrations (10%, 25%, 35%, 50%) of HS and five incubation times (0.5, 1.0, 2.0, 3.0, and 4.0 hours) were assessed for their effect on phagocytosis and cytokine secretion patterns in THP-1 MDMs subsequently activated with 0.5 μg/mL LPS (determined from LPS optimization experiments). Specifically, following differentiation to the M0 state (using the previously determined differentiation protocol and 25 nM PMA), THP-1 media was removed and replaced with RPMI-1640 media + X% HS or control (10% FBS). The cells were then incubated with HS for the allotted time followed by the addition of 0.5 μg/mL LPS to activate towards the M1 polarization state. Phagocytosis and cytokine secretion were assessed the same as detailed above (*M1 polarization*.)

Phagocytosis. Phagocytic function of differentiated and/or polarized THP-1 MDMs was evaluated using the Vybrant Phagocytosis Assay (Vybrant Phagocytosis Assay Kit,

ThermoFisher Scientific). In detail, fluorescein-labeled E. Coli K-12 BioParticles were suspended in Hank's balanced salt solution (HBSS) and diluted in sterile de-ionized water to a final concentration of 1 mg BioParticles/1 mL solution. As stated above, the media/supernatant following the 24-hour LPS incubation was saved for later assessment of secreted cytokines. Then, 100 μL of the Bioparticle solution was added to each of the wells and the plate was returned to the incubator for an additional 2-hour incubation to allow for cellular Bioparticle consumption (*i.e.* phagocytosis). At the end of 2 hours, the BioParticle solution was removed by vacuum aspiration and replaced with 100 uL of trypan blue to quench extracellular BioParticle fluorescence. The plate was incubated with trypan blue for one minute before removal by vacuum aspiration and reading on a fluorescent plate reader. Plates were read from the bottom with excitation wavelength set to 480 nm and emission wavelength set to 520 nm.

The % effect of the experimental intervention on phagocytosis was calculated using the formula below:

$$\frac{Net\ Experimental\ Reading}{Net\ Positve\ Reading} \times\ 100 = \%\ Phagocytosis\ Effect$$

To assess the qualitative effect of the LPS and HS treatments on cells numbers, cellular DNA content of each well was assessed using NucBlue (NucBlue Live ReadyProbes, ThermoFisher Scientific) immediately following the phagocytosis read. Specifically, 100 uL of NucBlue solution prepared in 1x PBS was added to each well and incubated at room temperature for 15 minutes. The plate was then read again, with an excitation wavelength of 360 nm and emission set to 460 nm.

Cytokines. Pro- and anti-inflammatory cytokines secreted by the cells from select experimental conditions were measured using a multiplex, magnetic bead-based assay panel (Milliplex, MilliporeSigma, Product #HCYTOMAG60) and fluorescent imaging platform (MagPix, Luminex). Specifically, cell culture media/supernatants collected following the 24-hour LPS incubation and prior to phagocytosis measurement, were screened for the concentrations of the anti-inflammatory cytokine IL-10, and the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α . These cytokines were assessed to screen for the relative polarization state of the macrophages towards an M1 phenotype, with greater secretion of pro-inflammatory and lesser secretion of anti-inflammatory cytokines indicating M1 polarization (36, 64). The results obtained from each intervention were compared to appropriate controls to distinguish basal or background cytokine secretion from that elicited by the interventions (*e.g*. LPS, Human serum concentration and incubation time.) Due to limited analysis materials, only one well from each experimental condition (*e.g.* 0.1 μg/mL LPS) was analyzed for cytokines. Wells selected for analysis were those closest to the mean phagocytosis reading for that condition, in order to determine the cytokine levels that approximated the mean response of that condition. In all, 10 samples were analyzed from the PMA differentiation experiments (4 conditions + 1 negative control per plate), 6 samples were analyzed from the LPS polarization experiments (5 conditions + 1 negative control), and 32 samples were analyzed from the serum incubation experiments (5 conditions + 1 negative control per time point, 1 sample each of control media and 50% HS media.)

Statistical Analysis. Phagocytosis data from the PMA (3 replicates each condition) and LPS (6 replicates each condition) optimizations were analyzed using a one-way analysis of variance (ANOVA) with an alpha level set to $p= 0.05$ to assess the effect of time and

concentration, respectively. Phagocytosis and NucBlue data from the Human serum optimization experiments (6 replicates each condition) were analyzed using a one-way ANOVA to assess the effect of serum concentration at each time-point (*e.g.* 1.0 hr incubation), the effect of concentration across incubation times, and the effect of incubation time across concentrations. Additionally, a two-way ANOVA was used address time x concentration effects. Following significant F-ratios, Bonferroni post-hoc analyses were performed to detect specific treatment condition effects. As with analysis of the PMA and LPS optimizations, the alpha value for both the human serum one-way and two-way ANOVA were both set $p = 0.05$. SPSS Statistics (IBM Corp.) was used for all statistical analyses. No statistical analyses were performed for the inflammatory cytokine assessments due to the selection of specific single wells for any given treatment condition per experiment.

Chapter 3: Results

PMA Differentiation. To determine the differentiation protocol which most clearly led to M1 polarization following differentiation and 24 hours incubation with 0.5 μg/mL LPS, 4 differentiation protocols (96PMA, 72PMA, 48PMA, 24PMA) were compared as detailed above in Methods. In each incubation condition the same concentration of 25 nM PMA was used. LPS treated cells in each condition were compared to "positive control" cells unstimulated by LPS but differentiated in the same manner.

Phagocytosis Data

48PMA led to a mean increase in phagocytosis of 774 ± 282% relative to positive controls. This is compared to 379 ± 79% with 24PMA, 42 ± 3% with 96PMA, and 13 ± 5% with 72PMA. Analysis of the data with a one-way ANOVA revealed there to be a significant effect of incubation time on phagocytosis ($p < 0.01$). Post-hoc tests using Bonferroni Correction showed the increase in phagocytosis with 48PMA to be significantly greater than either 96PMA or 72PMA ($p < 0.01$ for both), with a trend toward a significant difference between 48PMA and 24PMA ($p = 0.065$). The data are summarized in Figure 3.

Cytokine Data

Following differentiation, a 24-hour incubation with 0.5 μg/mL LPS increased levels of all measured cytokines (IL-10, IL-1β, TNF-α, IL-6), as compared to control (no LPS). Unfortunately, due to technical complications, cytokine data from 96PMA were lost during analysis. Of the three remaining conditions, 24PMA consistently led to the highest levels all cytokines. 48PMA led to the second highest concentrations of IL-10, TNF-α, and IL-6. While 72PMA led the second highest level of IL-1β and the lowest recorded levels of IL-10,

TNF-α, and IL-6. Cytokine data from one sample of each PMA condition are displayed in Figure 4.

LPS Concentration. Following the results of the preliminary PMA incubation studies, the 48PMA incubation protocol was chosen for the remainder of the optimizations of both LPS concentration and human serum incubation/concentration. This decision was made due to the high level of LPS-induced phagocytosis and pro-inflammatory cytokines, yet lower anti-inflammatory cytokines associated with the 48PMA condition. Next, to determine the optimal LPS concentration for polarizing PMA-differentiated THP-1 MDMs to the M1 polarization state, 4 different LPS concentrations (0.1, 0.2, 0.5, and 1.0 μg/mL) were compared for their effect on phagocytosis and cytokine secretion patterns.

Phagocytosis Data

The four LPS concentrations led to mean increase in phagocytosis of 350 ± 85% relative to unstimulated cells. By condition, 0.5 μg/mL led to an increase of 395 ± 102%, 0.2 μg/mL led to a 352 ± 43% increase, 0.1 μg/mL led to a 345 ± 116% increase, and 1.0 μg/mL led to a 307 ± 55% increase relative to cells unstimulated by LPS. Despite these absolute differences, analysis of the data with a one-way ANOVA showed there to be no significant effect of LPS concentration on phagocytosis ($p = 0.386$). The data are summarized in Figure 5.

Cytokine Data

Following differentiation to MDMs, 24 hr of incubation with all four concentrations of LPS led increased levels of each of the measured cytokines relative to unstimulated cells. Incubation with 0.5 μg/mL LPS led to the greatest increase in IL-10, while 1.0 μg/mL LPS led to the greatest increase in TNF-α and IL-6. The 0.1 μg/mL LPS treatment led to the

lowest levels of IL-1β with the other three concentrations showing similar responses. Cytokine data from one sample of each LPS concentration are displayed in Figure 6.

NucBlue Cellular DNA

To test for a differential effect of LPS concentration on cellular DNA, immediately following the assessment of phagocytosis all wells were treated with NucBlue and the plate was read again at an emission and excitation of 360 and 460 nm, respectively. The results indicated a significant effect of condition on NucBlue fluorescence ($p < 0.01$). Post-hoc analysis using a Bonferroni Correction revealed that the 0.1, 0.2, and 0.5 μg/mL LPS conditions led to significantly higher NucBlue fluorescence than the positive control, non-LPS treated condition ($p < 0.05$, $p < 0.05$, $p < 0.01$ respectively.) There was also a trend for higher NucBlue fluorescence in the 1.0 μg/mL LPS condition than the positive control condition ($p = 0.065$). There were no significant differences observed between concentrations ($p = 1.000$ for all). The latter data are summarized in Figure 7.

Summary

Although statistically significant differences were not observed between the four tested LPS concentrations, it was decided that 0.5 μg/mL LPS would be used in the remaining human serum (HS) optimizations. This decision was made as 0.5 μg/mL LPS led to the greatest absolute increase in phagocytosis, similar levels of cytokine secretion, and would lend consistency to the experiments, given that this concentration was used during the PMA optimization as well.

Human Serum Incubation and Concentration. To examine the effect of human serum (HS) concentration in the incubation media and incubation time on THP-1 MDM function, 4 different concentrations of HS were tested across 5 different incubation times as detailed in

the “Methods” section. Phagocytosis data are detailed by incubation time below, followed by cytokine data.

Phagocytosis Data - 0.5 hr HS Incubation

Following 0.5 hr of incubation, phagocytosis increased to 164 ± 60% with 10% HS media, 186 ± 126% with 25% HS media, 298 ± 153% with 35% HS media, and 290 ± 121% with 50% HS media relative to positive control cells incubated with 10% FBS. Analysis of the data (6 replicates per condition) with a one-way ANOVA indicated there was no significant effect of HS concentration on phagocytosis ($p = 0.143$). Phagocytosis data for 0.5 hr HS incubation are summarized in Figure 7.

Phagocytosis Data - 1.0 hr HS Incubation

Following 1.0 hr of incubation, phagocytosis increased to 150 ± 37% with 10% HS media, 209 ± 31% with 25% HS media, 236 ± 50% with 35% HS media, and 162 ± 20% with 50% HS media relative to positive control cells incubated with 10% FBS. Analysis of the data with a one-way ANOVA indicated there was a significant effect of HS concentration on phagocytosis ($p < 0.01$). Post-hoc tests using Bonferroni Correction showed the increase in phagocytosis observed with 35% HS was significantly greater than either 10% or 50% conditions ($p < 0.01$ and $p < 0.05$ respectively), and there was trend for 25% HS to be greater than the 10% HS condition ($p = 0.062$). There were no significant differences observed between the 25% and 35%, or 10% and 50% HS conditions ($p = 1.000$ for the latter pairwise comparisons.) Phagocytosis data for 1.0 hr HS incubation are summarized in Figure 8.

Phagocytosis Data - 2.0 hr HS Incubation

2.0 hr of HS incubation led to a phagocytosis effect of 98 ± 43% with 10% HS media, 133 ± 30% with 25% HS media, 155 ± 94% with 35% HS media, and 249 ± 108% with 50% HS media relative to positive control cells incubated with 10% FBS. Analysis of the data with a one-way ANOVA indicated there was a significant effect of HS concentration on phagocytosis ($p < 0.05$). Post-hoc tests using Bonferroni Correction showed the increase in phagocytosis observed with 50% HS was significantly greater than 10%, but not 25% or 35% ($p < 0.01$, $p = 0.092$, and $p = 0.267$, respectively). There were no significant differences observed between the 10%, 25%, or 35% conditions ($p = 1.000$). Phagocytosis data for 2.0 hr HS incubation are summarized in Figure 9.

Phagocytosis Data - 3.0 hr HS Incubation

3.0 hr of HS incubation led to a phagocytosis effect of 46 ± 16% with 10% HS media, 123 ± 25% with 25% HS media, 116 ± 20% with 35% HS media, and 121 ± 20% with 50% HS media relative to positive control cells incubated with 10% FBS. Analysis of the data with a one-way ANOVA indicated there was a significant effect of HS concentration on phagocytosis ($p < 0.001$). Post-hoc tests using Bonferroni Correction showed the increase in phagocytosis observed with 25%, 35%, and 50% HS was significantly greater than the 10% HS condition ($p < 0.001$ for all). There were no significant differences observed between 25%, 35%, or 50% conditions ($p = 1.000$). Phagocytosis data for 3.0 hr HS incubation are summarized in Figure 11.

Phagocytosis Data - 4.0 hr HS Incubation

4.0 hr of HS incubation led to a phagocytosis effect of 85 ±2 8% with 10% HS media, 86±29% with 25% HS media, 143 ± 34% with 35% HS media, and 105 ± 30% with 50% HS

media relative to positive control cells incubated with 10% FBS. Analysis of the data with a one-way ANOVA indicated there was a significant effect of HS concentration on phagocytosis ($p < 0.05$). Post-hoc tests using Bonferroni Correction showed the increase in phagocytosis observed with 35% HS was significantly greater than 10% HS or 25% HS, but not 50% HS ($p < 0.05$, $p = 0.342$ respectively). There were no significant differences observed between the 10%, 25%, or 50% conditions ($p = 1.000$). Phagocytosis data for 4.0 hr HS incubation are summarized in Figure 11.

Effect of Time by Concentration

As opposed to the effects of concentration at each time point (*e.g.* 25% HS at 2.0 hr) as detailed above, it was also of interest to evaluate the effect of incubation time across concentrations (*i.e.* overall effect of 1.0 hr vs. 2.0 hr*)*, concentration across incubation times (*i.e.* overall effect of 10% vs. 35% HS), as well as if there was a combined effect of concentration by time on phagocytosis (*i.e.* 25% at 2.0 hr vs 10% at 1.0 hr). A two-way ANOVA was used to analyze the latter question. The results indicated that while there was only a trend towards a significant interaction effect of human serum concentration by time ($p = 0.059$), there was a significant main effect of concentration ($p < 0.001$), as well as a significant main effect of time ($p < 0.001$). One-way ANOVA for the effect of concentration over time indicated a significant main effect of concentration ($p < 0.01$). Post-hoc tests using Bonferroni Correction showed the increase in phagocytosis with 35% and 50% HS across incubation times was significantly greater than that with 10% (190 ± 105% and 188 ± 102% vs 109 ± 57% respectively; $p < 0.01$ for both). There were no significant differences observed between 10% and 25% ($p = 0.513$), 25% and either 35% or 50% ($p = 0.363$ and $p = 0.421$, respectively), or 35% and 50% ($p = 1.000$). One-way ANOVA

examining the effect of incubation time across concentrations indicated a significant main effect of incubation time ($p < 0.001$). Post-hoc tests using Bonferroni Correction showed the increase in phagocytosis observed with 0.5 hr incubation across concentrations (234 ± 128%) was significantly greater than 2.0 hr (159 ± 91%; $p < 0.05$), 3.0 hr (101 ± 38%; $p < 0.001$), or 4.0 hr (105 ± 37%; $p < 0.001$). Additionally, phagocytosis with 1.0 hr incubation was significantly greater than either 3.0 hr or 4.0 hr ($p < 0.01$ for both). No significant differences were observed between 2.0, 3.0, or 4.0 hr incubation times ($p = 0.118$, $p = 0.183$, and $p = 1.000$, respectively). Data for the effect of time and concentration are summarized in Figures 12 and 13.

Cytokine Data

Two general trends emerged in analyzing the secreted cytokine levels from the human serum optimization experiments. For the cytokines IL-10, TNF-α, and IL-6, increasing incubation times were associated with lower cytokine levels. In contrast, IL-1β levels appeared to remain relatively consistent across incubation times, at a given concentration. Following the former trend, IL-10 levels were highest in the 50% HS conditions with 0.5 and 1.0 hr incubations. Similarly high levels of IL-10 were also observed in the positive control condition (10% FBS) with 3.0 hr incubation. The highest TNF-α levels were observed in the 10% HS condition with 0.5 hr incubation. In regard to IL-1β, the highest levels were observed in the 50% HS condition with peaks at both 1.0 and 3.0 hr incubation. With the exception of a low value at 1.0 hr incubation, the highest IL-6 levels were observed in the positive control (10% FBS) condition across incubation times. Although, following the larger trend, IL-6 levels still tended to fall as incubation time lengthened in the positive control condition. Negative control wells containing THP-1 media, but not cells, showed minimal

levels of all cytokines across all incubation times, compared to the HS or positive control conditions.

To account for background cytokine levels in the incubation media that might skew the latter results, both the THP-1 culture media and the 50% HS media (RPMI-1640 + 50% HS by volume) were analyzed and compared for their respective cytokine levels. Interestingly, the 50% HS media displayed markedly higher levels of all measured cytokines except IL-1β. Conversely, higher levels of IL-1β were observed in the THP-1 culture media, although the magnitude of difference was not as great (0.56 vs. 0.43 pg/mL). Nevertheless, these background levels were unlikely to meaningfully skew the results of the incubation experiments as the concentrations of the former were at least one order of magnitude lower than the latter. Cytokine data from both the HS incubation experiments and the incubation media are displayed below in Figures 15-19.

NucBlue Cellular DNA

To test for a differential effect of HS concentration and incubation time on cellular DNA, following assessment of phagocytosis all wells were treated with NucBlue and the plates were read again at an emission and excitation of 360 and 460 nm, respectively. The results indicated no significant effect of HS concentration or FBS at any single incubation time ($p = 0.239$ at 0.5 hr, $p = 0.525$ at 1.0 hr, $p = 0.354$ at 2.0 hr, $p = 0.399$ at 3.0 hr, $p = 0.202$ at 4.0 hr.) Additionally, there was no significant effect of concentration across incubation times or a concentration by time effect ($p = 0.658$, $p = 0.404$ respectively.) However, there was a significant effect of incubation time across concentrations ($p < 0.001$). Post-hoc analysis using a Bonferroni Correction revealed the 2.0 (56 5± 77MFI) and 3.0 hr conditions (537 ± 48 MFI) were not significantly different from one another ($p = 0.549$), but both incubation

times led to significantly higher readings than all other conditions (0.5 hr, 340.5 ± 54.8 MFI; 1.0 hr, 469 ± 48 MFI; 4.0 hr, 446 ± 50 MFI, $p < 0.001$ for all). Additionally, 1.0 and 4.0 hr conditions led to significantly higher readings than 0.5 hr ($p < 0.001$) but were not different from one another ($p = 1.000$). The latter data are summarized in Figure 20.

Chapter 4: Discussion and Conclusions

Discussion

The purpose of this project was to develop and optimize a procedure for evaluating the effects of human exercise and dietary interventions on macrophage function *in vitro*. It was necessary to create such a procedure because of the widespread and integral role monocytes and macrophages (MMs) play in physiological homeostasis (83). MM function and plasticity profoundly impact the balance of health and disease, however difficulties with isolation and maintenance of human primary cells restricts their broad application (11, 41). Previously, animal models and *in vitro* studies using both animal and human cell lines have been the primary means for exploring MM biology (11, 24, 79). Though these studies have produced important findings, differences do exist between murine and human MMs as well as signaling *in vitro* versus *in vivo* (5, 27). Therefore, it is important to explore changes in human MM in as close an approximation to *in vivo* conditions as possible. To serve this purpose, it would be advantageous to create a hybrid scheme for leveraging the utility of *in vitro* experiments while simulating *in vivo* conditions. To accomplish this goal, the current project had 3 aims: 1. Establish a PMA differentiation protocol to differentiate THP-1 cells to monocyte-derived macrophages (MDMs) capable of M1 polarization using LPS; 2. Identify the concentration of LPS that adequately polarized THP-1 MDMs towards the M1 state without excess stimulation or cytotoxicity; 3. Identify the human serum concentration and incubation time that induces an intermediate THP-1 MDM phenotype in which both pro-inflammatory and anti-inflammatory effects of future studies might be distinguished. A separate set of experiments was carried out to accomplish each aim, in the order listed.

PMA Optimization. The results of the PMA optimization showed that 48 hours of incubation with 25 nM PMA followed by 24 hours of "rest" in culture media (48PMA) led to the largest magnitude of increase in phagocytosis following 24 hours incubation with LPS, as compared to cells untreated with LPS (Figure 3). 24-hours of PMA incubation followed by 48 hours of rest (24PMA) prior to LPS led to an increase in phagocytosis about half that of the 48-hour incubation, while 96- and 72-hour PMA incubations (96PMA, 72PMA) actually led to a depression in phagocytosis relative to untreated cells. The reason for the latter results with 96PMA and 72PMA may have to do with similarities in PMA and LPS inflammatory signaling. PMA activates diacylglycerol signaling in monocytes, activating kinases in the protein kinase C (PKC) family and leading to cell differentiation (62). In conjunction with these effects, PMA also increases inflammatory gene expression including the cytokines IL-8 and TNF-α, and excess exposure to these cytokines may make cells less stress tolerant and prone to cell death (40, 50). Although we did not quantify cell viability in this portion of the study, it is possible that the prolonged incubation with PMA and/or the addition of LPS induced apoptosis of the cells, leading to lower measured levels of both phagocytosis and all cytokines.

In regard to cytokine levels, unfortunately, complications during sample analysis meant that the data on cytokine levels from 96PMA were lost. Concerning the other three conditions, 72PMA did display lower levels of all secreted cytokines except IL-1β (Figure 4). Interestingly, although 48PMA led to the greatest increase in phagocytosis, 24PMA displayed the highest levels of all analyzed cytokines. This was particularly true of IL-10 where the levels observed in 24PMA were more than 3- and 6-fold higher than the levels observed in 48PMA and 72PMA, respectively. Given the limited data set that was available

for analysis of cytokine levels, it is hard to speculate on the discrepancy between the trends observed in the phagocytosis and cytokine data. However, it's possible the robust secretion of cytokines, particularly IL-10, in 24PMA may have had an inhibitory effect on phagocytosis by the time it was measured following 24-hour LPS incubation. Cytokines have the capacity to signal in both a paracrine and autocrine manner, with the potential to create either positive of negative feedback loops for inflammation (23). It has been shown that Th-2 cytokines (e.g. IL-4) may actually have an inhibitory effect on phagocytosis yet may not affect the secretion of pro-inflammatory cytokines (60, 72). In contrast, it has been shown pro-inflammatory cytokines both potentiate and rely upon phagocytosis for their secretion (14, 29, 35, 51, 63) . The pro-inflammatory cytokines IL-6, IL-18, and TNF-α have been shown to increase phagocytic activity of macrophages (14, 35). Additionally, the act of phagocytosis of various target cells has been shown increase release IL-6, TNF-α, and IL-23; with TNF-α released directly from the phagocytic cup (29, 51, 63). Though IL-4 was not specifically quantified in the current study, the high levels of the anti-inflammatory IL-10 observed with 24PMA may reflect unmeasured levels of Th2 cytokines leading to the relative depression in phagocytosis. Alternatively, even in the absence of other anti-inflammatory cytokines, the high levels of IL-10 combined with the high levels of pro-inflammatory cytokines could have initiated deactivation or repolarization of the macrophages by the time phagocytosis was measured 24 hr later, through compensatory auto- and paracrine signaling (23, 64). In contrast, the marked induction of phagocytosis observed in 48PMA was associated with moderate levels of the measured cytokines, compared to the other conditions. This lends support towards the hypothesized relationship between phagocytosis and

cytokines in the 24PMA condition, as the levels of cytokines in 48PMA may have remained below the threshold leading to inhibition of phagocytosis.

In light of the significant increase in phagocytosis observed in 48PMA, the decreases observed with 96PMA and 72PMA, and the somewhat contradictory results in 24PMA, the 48PMA differentiation protocol was chosen for the subsequent aims. Again, the purpose of this aim was to identify the PMA differentiation protocol that most reliably led to M1 differentiation following exposure to LPS. M1 polarization by LPS is characterized by enhanced phagocytic capacity, robust secretion of pro-inflammatory cytokines, and lesser secretion of anti-inflammatory cytokines (36, 64, 82). It was concluded that the results in 48PMA best matched the latter criteria and supported the choice of 48PMA going forward.

LPS Optimization. Similar to the PMA optimization, it was the goal the second aim of the study to identify the concentration of LPS that best led to M1 polarization following differentiation of THP-1 monocytes to monocyte-derived macrophages with PMA. To accomplish this aim four different concentration of LPS were evaluated: 0.1, 0.2, 0.5 and 1.0 μg/mL. These concentrations were chosen based on a review of the literature for ranges of LPS used in macrophage activation (9, 10, 31, 77, 78). The results of this aim revealed that all four concentrations of LPS led to >3-fold increase in phagocytosis relative to cells untreated with LPS (Figure 5). Of the four, 0.5 μg/mL LPS led to the greatest increase in phagocytosis (394.5 ± 102.4%). Although, the latter result was not statistically different from the other conditions. Perplexingly, the latter increase in phagocytosis was less than that observed using the same LPS concentration in the PMA optimization experiments (395 ± 102% vs. 774 ± 282%). It is unclear why this difference occurred. Parallel to the phagocytosis results, all four LPS concentrations led to relatively similar cytokine responses

with the exception of IL-1β (Figure 6). 0.2, 0.5, and 1.0 μg/mL conditions all led to IL-1β concentrations of ~280 pg/mL while 0.1 μg/mL led to a concentration of 193 pg/mL. However, due to limited samples available for analysis, it was not possible to determine whether the latter difference was indicative of real response or sample variability.

In order to address the potential effect of LPS concentration on cell number, that might affect both phagocytosis and cytokine results, NucBlue was added to stain for cellular DNA following the phagocytosis readings and the plate was read again at the applicable excitation and emission wavelengths. The results of this experiment indicated that the application of LPS may have led to better maintenance of cell numbers, as all LPS conditions led to significantly higher readings than the positive control condition. Furthermore, there were no significant differences observed between LPS conditions. Though this result was surprising, it did mean that LPS was not differentially affecting cell numbers between concentration, and differences in the primary outcomes measures were due to responses of the cells specifically to LPS concentration rather than differences in cell numbers.

Considering the lack of significant differences observed in the phagocytosis data, and the relatively equivocal results between the three higher LPS conditions, it was decided that 0.5 μg/mL would be used as the M1 polarizing stimulus in aim 3. The rationale behind this decision was 3-fold: 1. Though not statistically significant, 0.5 μg/mL did lead to the greatest increase in phagocytosis of the four concentration; 2. Since it was the LPS concentration also used in aim 1, continuing to use 0.5 μg/mL in aim 3 would maintain consistency of the stimulus across the 3 aims; and 3. Previous work by another lab concluded that 0.7 μg/mL LPS led to similar inflammatory gene expression patterns as 1.0 μg/mL, but lower magnitude changes such that the effect of additional polarization modulating substances (*e.g.* serum)

might be more easily observed (10). Since it was the goal of the foregoing serum optimizations to be able to observe additional modulation of macrophage phenotype with human serum, it was decided that 0.5 μg/mL was the optimal concentration in which to proceed.

*Human Serum Optimization***.** With the ultimate goal of utilizing these optimized procedures in future studies in which the imposed human intervention would be the main variable of interest, this aim set out to identify the ideal HS concentration and incubation time that would yield a macrophage phenotype in which both depressions and enhancements in the inflammatory response might be observable. Previous research has shown that acute exercise can increase, while chronic physical exertion and stress can decrease macrophage function (18, 66). Similarly, dietary compounds have the potential to both increase or decrease the inflammatory responses of macrophages (9, 17, 67). Considering the human serum used for this study was from a resting subject not currently undergoing any specific training program or undertaking a special diet, maximal or minimal responses to LPS following serum incubation would limit future applications of this procedure. It was important to keep the latter purpose in mind when contextualizing the results of this aim.

In comparing the mean effect on phagocytosis of the four concentrations of human serum across incubation times, the 35% and 50% human serum concentrations both led to more significant inductions of phagocytosis with LPS than the 10% human serum condition (Figure 12) . Furthermore, 35% and 50% human serum conditions also led to, on average, greater induction of phagocytosis than 25% human serum, though this was not statistically significant. These results were supported by the observation that 35% HS led to the highest phagocytosis induction following 0.5, 1.0, and 4.0 hr incubation while 50% HS led to the

highest phagocytosis induction following 2.0 hr HS (Figures 7, 8, 9, 11). Technically, 25% HS led to the highest induction of phagocytosis following 3.0 hr incubation (Figure 10). Although, the difference between 25% and 35% or 50% at 3.0 hr was minor (123 ± 25% with 25% HS vs. 116 ± 20% with 35% HS, and 121 ± 20% with 50% HS.)

In contrast, the longer incubation times were associated with smaller mean inductions of phagocytosis with LPS. Phagocytosis induced following 3.0 and 4.0 hr HS incubation was significantly less than after 0.5 hr incubation, but not significantly different from 2.0 hr incubation (Figure 13). Further, there was no significant difference observed between the two longer incubations. This suggests that the initial exposure to new media may make the cells more prone to LPS induced phagocytosis, and that longer exposure to HS leads to an attenuation of the response to LPS. Similar trends were observed in the cytokine data.

Unfortunately, due to limited resources, only a restricted sample of the HS data was able to be analyzed for cytokine levels. As a result of this limited sample, it is difficult to make broad conclusions about the cytokine responses of the different treatments and whether outlying values are in fact that, or if they reflect a real trend. Nevertheless, some interesting relationships were observed in the data. Though less dramatic than the reduction in induced phagocytosis, in general, longer HS incubation times were associated with lower secreted cytokines following 24 hr incubation with LPS (Figures 14-17). This trend held true across HS concentrations and the positive control condition, although was most notable in regard to the levels of IL-10 and TNF-α. The latter results may point towards the addition of HS as a potentially stressful event that potentiates both cytokine release and phagocytosis upon the addition of LPS. Conversely, increasing HS concentration was associated with greater IL-10 and IL-1β secretion but reduced TNF-α and IL-6 secretion. It is also noteworthy that the

highest levels of IL-6 across incubation times were observed in the positive control condition. The conflicting trends of increasing IL-10 and IL-1β but decreasing TNF-α and IL-6 levels with higher serum concentration are difficult to reconcile. However, they may point towards either reciprocal relationships between IL-10/IL-1β and TNF-α/IL-6 (*i.e.* increasing IL-10 decreases TNF-α) or mirror relationships between IL-10 and IL-1β, and TNF-α and IL-6. Previously, it has been shown that IL-6 infusion at levels similar to those observed during exercise potentiates the release of IL-10, IL-1ra, and cortisol in humans (68). If a similar relationship were observed here, it would have been expected that the peak levels of IL-6 observed with the positive control treatment would have led to commensurately high levels of IL-10. However, this was not the case. The differences between the IL-6:IL-10 relationship in the current study and the latter one may be explained by the source of the cytokines. Cytokines in the latter study were isolated from the systemic circulation, meaning that they could be derived from multiple cell populations and tissue compartments. In contrast, the cytokines in the current study were solely MDM derived. It would be interesting to explore the relationships in cytokine signaling between macrophages themselves, as well as macrophages and different tissue/cell types in the future. This is worthy of additional investigation.

In order to address the potential effect of LPS concentration on cell numbers, that might affect both phagocytosis and cytokine results, NucBlue was added to stain for cellular DNA following the phagocytosis readings and the plate was read again at the applicable excitation and emission wavelengths. The result of this portion of the experiments revealed no significant effect of HS concentration or the positive control condition on NucBlue fluorescence at any specific incubation time or across incubation times. Additionally, there

was no significant effect of concentration by time on NucBlue fluorescence. However, there was a significant effect of time across concentrations such that all higher incubation times led to significantly higher NucBlue fluorescence than at 0.5 hr Additionally, 2.0 and 3.0 hr led to higher readings than 1.0 and 4.0 hr. Comparing this information to the phagocytosis and cytokine data, it would appear that higher NucBlue readings were associated with lower phagocytosis and cytokine levels. This relationship lends support to the hypothesis that the addition of HS is stressful to the cells and may concurrently lead to heightened inflammatory responses and decreased cell numbers. Contradicting this hypothesis is the fact the positive control condition containing FBS, not HS, led to consistently lower NucBlue readings, though none of these differences were statistically significant. If the HS was negatively affecting cell numbers, it would be expected that the FBS condition would have led to higher readings, particularly at the shorter incubation times. However, this was not the case.

Taken together, the results of the HS optimization experiments (aim 3) point towards an attenuation of LPS response with increasing incubation time and heightened responses with increasing serum concentration. Given the goals of this aim was to define conditions that lead to an intermediate response in THP-1 MDMs, it was concluded that 3 hr incubation and 35% HS are the optimal conditions for HS exposure prior to LPS polarization. This decision was made given the plateau in phagocytosis responses starting at 3 hr. incubation and 35% HS in the context of time and concentration, as well as the intermediate cytokine responses observed with the same time/concentrations. Although both 25% and 50% HS led to similar phagocytosis responses as 35% at 3.0 hr incubation, the cytokine responses were either higher or lower than 35% HS. Therefore, the choice of 35% HS gives a better chance at observing higher or lower responses to LPS in future investigations. In regard to time, 4.0

hr incubation led to similar phagocytosis responses as 3.0 hr overall. However, the cytokine responses were reduced relative to 3.0 hr incubation and may diminish the ability to observe decreases in cytokine response to LPS with additional variables. Hence, 3.0 hr incubation was deemed the more prudent option.

Conclusions and Future Directions

To reiterate, the overall goal of this project was to develop an experimental procedure for the measurement of *in vitro* THP-1 MDM function following incubation with human serum to simulate *in vivo* conditions. In regard to the three specific aims of the project, it was determined that: 1. Of the tested protocols, the optimal PMA-differentiation scheme involved 48 hr incubation with 25 nM PMA followed by 24 hr "rest" prior to LPS exposure; 2. Though not significantly different from the other conditions, 0.5 μg/mL LPS may provide a "happy medium" of M1 polarization without overstimulating the cells; 3. 35% human serum concentration for 3.0 hr incubation provides the best chance at capturing increased or decreased MDM responses to LPS in future investigations. In summary, based on the current results it is recommended that THP-1 monocytes be differentiated to M0 macrophages with 25 nM PMA for 48 hr, followed by 24 hr "rest" in culture media. It is then recommended that differentiated THP-1 MDMs be incubated with human subject serum at a concentration of 35% for 3.0 hours, prior to exposure to 0.5 μg/mL LPS for a further 24 hr incubation. This procedure should reliably afford adequate stimulus to polarize THP-1 MDMs towards the M1 state and allow for examination of the modulating effect of soluble factors in human subject serum upon the latter phenotype. To build on this scheme, future investigations should evaluate whether the same differentiation and serum incubation conditions are amenable to M2 polarization with IL-4 or IL-13. Furthermore, LPS alone was chosen as the M1

polarizing stimulus in this study for simplicity, and to model primary responses to bacterial infection. However, IFN-γ is also a potent M1 polarizing compound and frequently used in conjunction with LPS to polarize THP-1 cells towards the M1 state (9, 23, 65). It would be interesting to examine to what degree treatment with a combination of IFN-γ + LPS might alter cell responses in the current procedure. Additionally, it is possible that M1 polarization using LPS or IFN-γ + LPS may not be applicable to all research questions. The LPS stimulus was chosen for this experiment as it was the goal to expand on the commonly used steps of THP-1 differentiation and polarization with the addition of serum incubation, and model macrophage responses to initial bacterial stimulus (23, 56). By modifying an existing paradigm, it was the aim to facilitate comparisons between previous studies and the changes observed with serum incubation. However, for studies evaluating changes in the inflammatory response to acute *exercise* LPS polarization is not applicable. In the latter case the current procedure would likely need to be modified by the measurement of phagocytosis and cytokines immediately following serum incubation, with no polarization step. Then, comparisons could be made between the effects of serum at different timepoints around exercise (pre-, intra-, post-) and due to the variable of interest. Along the same lines, within the current procedure, analysis of phagocytosis and cytokine secretion following each step of the procedure (*e.g.* PMA differentiation) would allow for the determination of changes in macrophage function with each step, and more accurate identification of when (and perhaps why) changes are occurring. Finally, in light of the important role that macrophage plasticity appears to play in health vs. disease, it is of interest whether serum incubation following MDM polarization (rather than before it) might modify or re-polarize the cells. Both exercise training and food derived compounds have shown evidence of modulating MM polarization

and phenotype, and it is possible re-polarization of activated macrophages could be the mechanism (9, 22, 70). The latter additions would expand the applications of the current procedure and the translation of its results. Consequently, though the results of this study outline a useable framework for assessing *in vitro* MDM function following human serum incubation, more work is needed to enhance the procedure's usefulness and impact.

With the recognition that additional experiments may be necessary to fully optimize the recommended procedure, some limitation of the current study also need to be acknowledged. First of all, due to time and resource constraints, replicate experiments under each aim were limited. This means that the long-term repeatability of the procedure is unknown and in addition, limited data points may have hampered the statistical significance of some of the results. In particular there were trends towards a significant effect of HS concentration x incubation time ($p = 0.059$), as well as a significant difference between 48PMA and 24PMA ($p = 0.065$). It is possible that additional data points would push the latter relationships towards statistical significance, although they might not change the conclusions of the project. Furthermore, due to the same constraints, only a small selection of samples from each condition was used for cytokine analysis. This meant that statistical analysis of trends in the data was not possible and restricts the conclusions that can be drawn. So, in addition to the added experiments outlined in the previous paragraph, it is recommended that each aim be repeated to confirm the results reported here. Finally, the lack of significant differences between concentrations in the LPS optimization (aim 2) could have been due to an inappropriate incubation time rather than, or in conjunction with, inadequate data points. The 24-hour incubation time was chosen as this was previously shown to be when cytokine secretion peaked or plateaued in LPS stimulated THP-1 MDMs (10).

However, it may have been advisable to use a shorter incubation time to examine the cytokines while they were ascending to their peak, and the rate of change was highest. Furthermore, cytokine kinetics and phagocytosis may operate on different time scales and the ideal time to measure phagocytosis post-LPS exposure could be different than that of cytokine secretion. It is recommended that future experiments explore this question.

Summary. Monocytes and macrophages are omnipresent throughout the body, serving a plethora of auxiliary functions in pathogen detection, metabolism, and organ homeostasis (83). Consequently, the diversity, character, and plasticity of monocytes and macrophages has a profound impact on organismal development, disease risk, and aging (65). Despite their importance, relatively little is known about the factors that influence transitions in monocyte and macrophage phenotype, particularly in humans (24, 28). Therefore, increasing knowledge on the monocyte and macrophage biology and signaling hold immense potential for enhancing understanding of the etiology of numerous diseases, the dynamics of aging, and the impact of lifestyle habits such as diet and physical activity. Unfortunately, technical hurdles limit examinations of monocyte and macrophage function *in vivo* or using primary cells (11, 80). Hence, devising a means to reliably measure macrophage function *in vitro* using simulated *in vivo* conditions may provide valuable insights for later confirmation in primary cells. This was the goal of the current project. Upon completion of the 3 project aims the following procedure is recommended: 1. THP-1 monocytes cultured to between 800,000-1,000,000 cells/mL should be differentiated in culture media supplemented with 25 nM PMA for 48 hours, followed by 24 hours “rest” without PMA; 2. PMA differentiated THP-1 MDMs should be incubated in 35% human serum for 3 hours prior to the administration of 0.5 μg/mL LPS; 3. To measure THP-1 MDM function, following 24 hours LPS incubation

culture supernatant should be reserved for analysis of secreted cytokines at a later time, and phagocytosis should be assessed immediately. This 3-step procedure provides a novel approach to studying macrophage function through the use of human serum incubation to simulate *in* vivo conditions *in vitro*, providing a means to tackle previously untested research questions. With this procedure, future studies will provide new insight on the modulating effects of soluble factors in human serum, such as dietary compounds or exercise induced changes in hormones, on monocyte-derived macrophage behavior and provide valuable information on monocyte and macrophage signaling dynamics *in vivo*.

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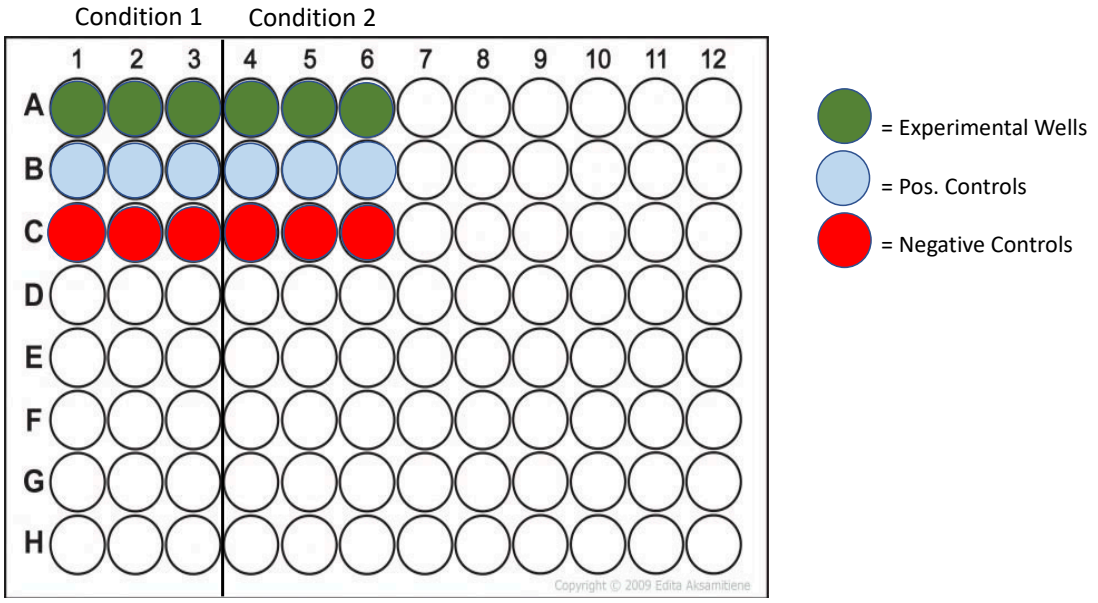

Figure 1: Diagram of 6x3 plate set-up for PMA optimization experiments. Cells were plated in rows A and B. Cells in row A were treated with both PMA and LPS, while cells in row B were served as the positive controls and were treated with PMA but no LPS. Columns 1-3 and 4-6 corresponded to one incubation condition each as detailed below. Only media was added to row C, as these wells served as the negative control/background reading.

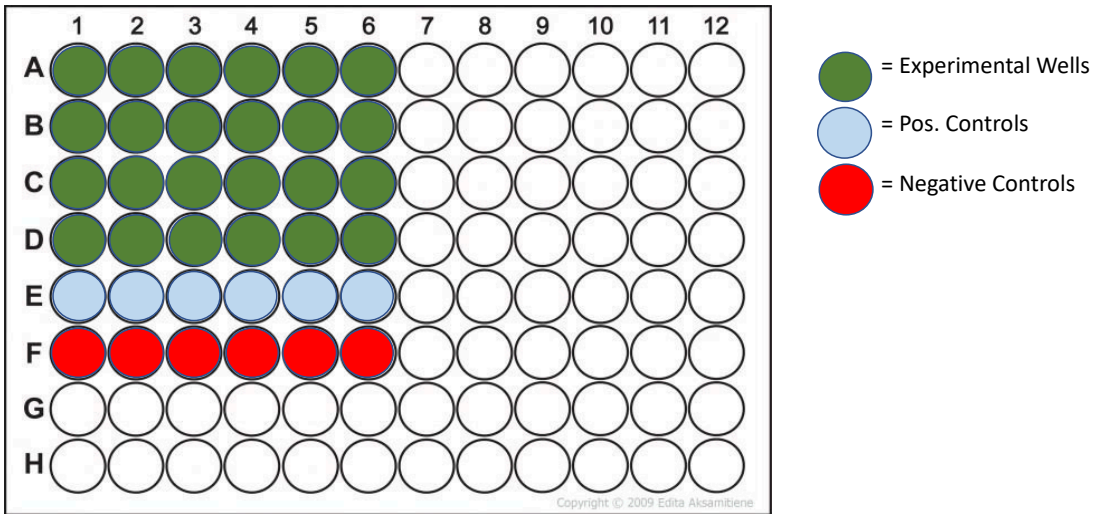

Figure 2: Diagram of 6x6 plate set-up for LPS and human serum optimizations. Cells were plated to rows A-E. Rows A-D corresponded to one experimental condition each (*i.e.* 0.1 µg/mL LPS) and row E was used as the positive control condition in which cells experienced all treatments except for the independent variable *(i.e.* LPS for LPS optimizations, humans serum for the human serum optimizations.) Only media was added to row F, as these wells served as the negative control/background reading.

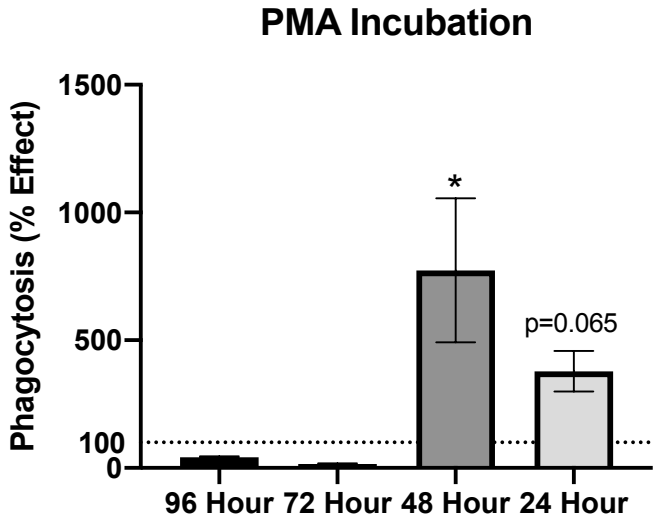

Figure 3: Effect of THP-1 monocyte PMA differentiation protocol on phagocytosis following differentiation and 24 hr incubation with 0.5 μg/mL LPS. Phagocytosis is expressed as % effect relative to cells untreated with LPS but differentiated in the same manner as indicated by the 100% dotted line. " * " indicates $p<0.01$ compared to 96- or 72-hour incubations. Data are presented as mean±SD.

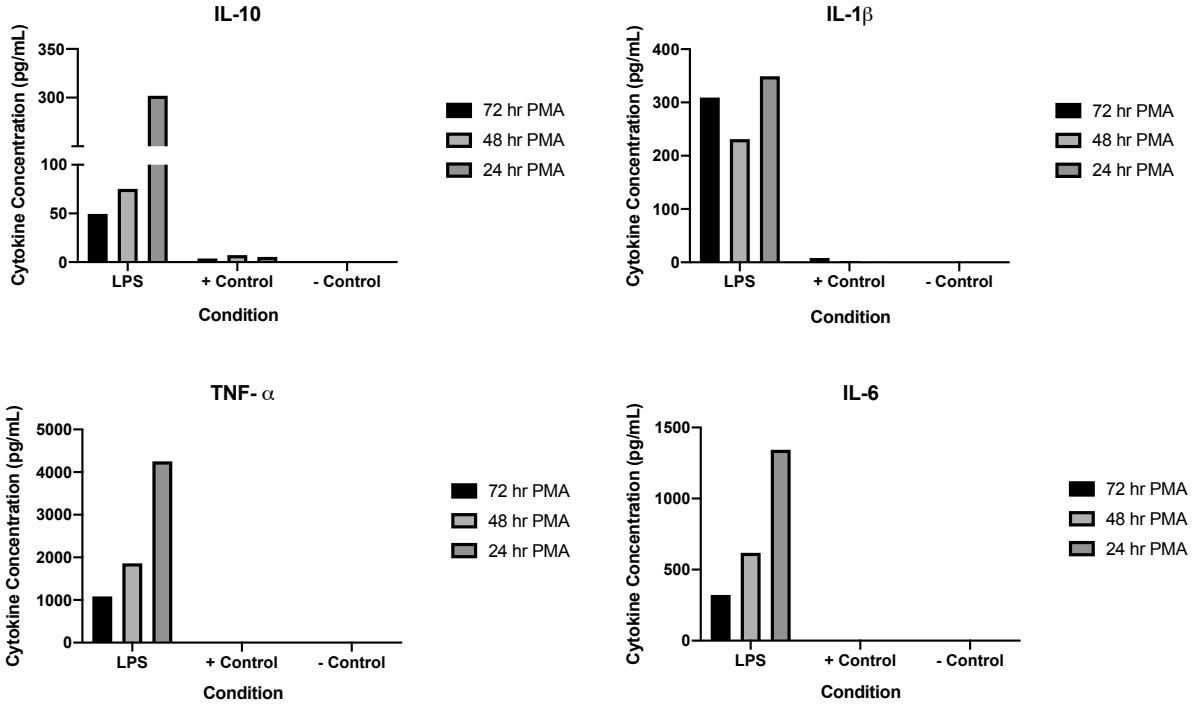

Figure 4: Effect of THP-1 monocyte PMA differentiation protocol on the secretion of cytokines IL-10, IL-1β, TNF-α, and IL-6 following differentiation and 24 hr incubation with 0.5 μg/mL LPS. "LPS" refers to LPS stimulated cells, "+ Control" refers to cells differentiated using the same PMA protocol but unstimulated by LPS, and "- Control" refers to background cytokine levels of the culture media. Data represent one sample analyzed from each condition.

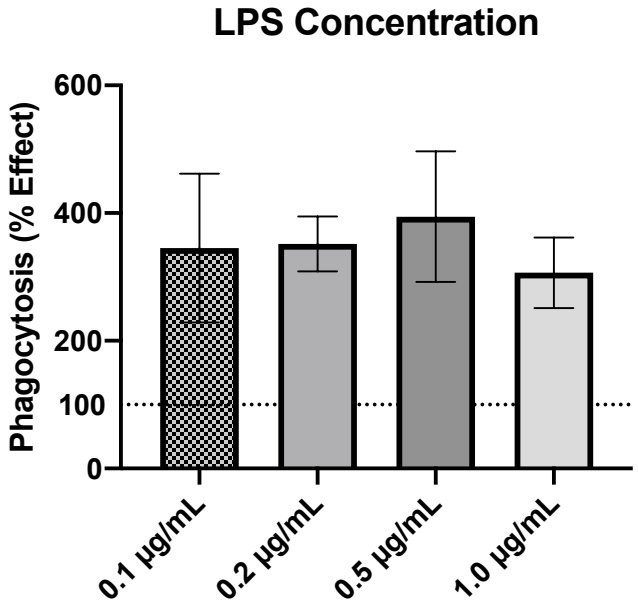

Figure 5: Effect of LPS concentration on phagocytosis in THP-1 MDMs following 24 hr of LPS incubation. Phagocytosis is expressed as % effect relative to cells untreated with LPS as indicated by the 100% dotted line. Data are presented as mean±SD

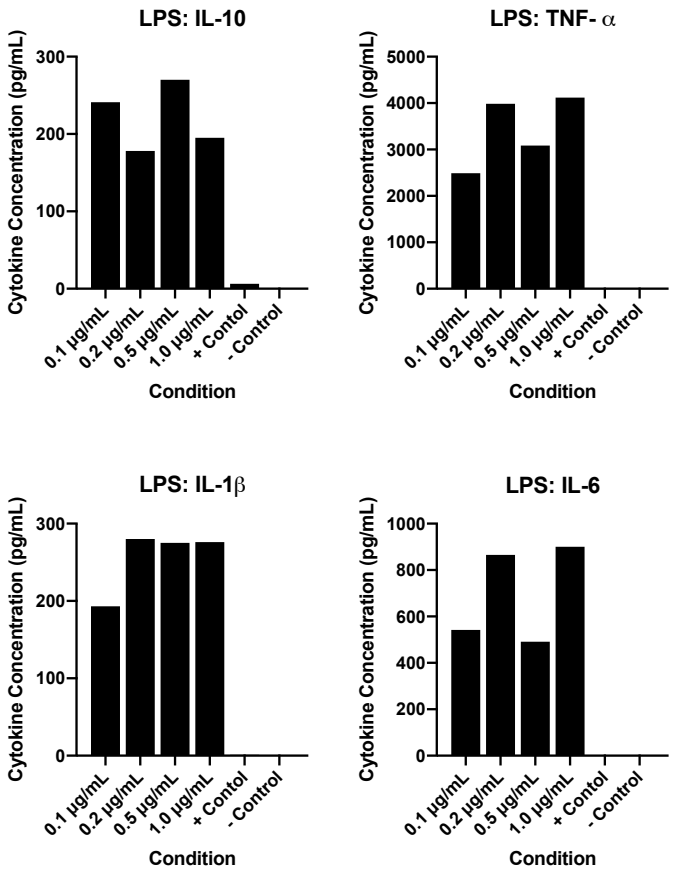

Figure 6: Effect of LPS concentration on the secretion of cytokines IL-10, IL-1β, TNF-α, and IL-6 following a 24 hr incubation with THP-1 MDMs. "Pos" refers to positive control wells in which cells were differentiated using the same PMA protocol but unstimulated by LPS, and "Neg" refers to negative control wells with only THP-1 media (no cells) to account for background cytokine levels of the culture media. Data represent one sample analyzed from each condition.

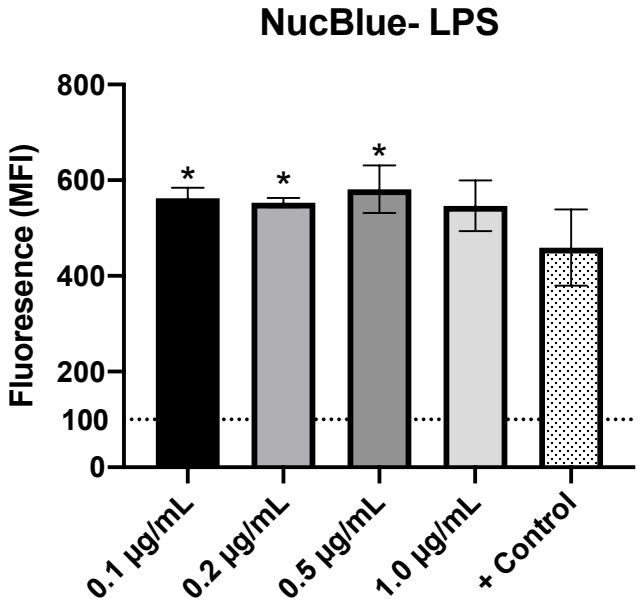

Figure 7: Effect of LPS concentration on NucBlue fluorescence in THP-1 monocyte-derived macrophages following 24 hr of LPS incubation. NucBlue is expressed in mean fluorescence intensity (MFI). " * " indicates $p<0.05$ compared to + Control. Data are presented as mean±SD.

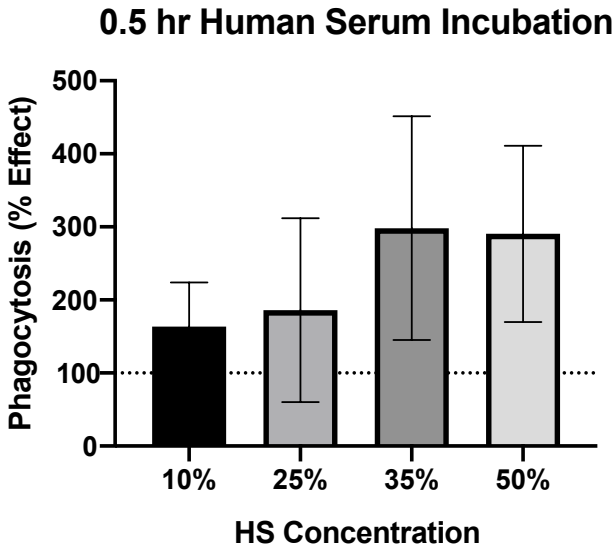

Figure 8: Effect of human serum (HS) concentration in culture media on phagocytosis in THP-1 MDMs following 0.5 hr HS incubation and 24 hr incubation with 0.5 µg/mL LPS. Phagocytosis is expressed as % effect relative to cells incubated for the same time in normal culture media (10% FBS) and similarly incubated with LPS, as indicated by the 100% dotted line. Data are presented as mean±SD.

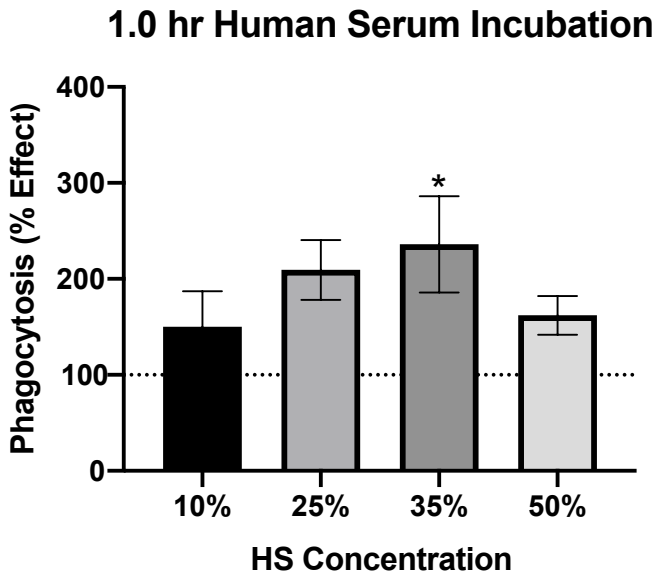

Figure 9: Effect of human serum (HS) concentration in culture media on phagocytosis following 1.0 hr HS incubation and 24 hr incubation with 0.5 μg/mL LPS. Phagocytosis is expressed as % effect relative to cells incubated for the same time in normal culture media (10% FBS) and similarly incubated with LPS, as indicated by the 100% dotted line. " * " indicates $p<0.01$, $p<0.05$ between 35% and 10%, 50% conditions respectively. Data are presented as mean±SD.

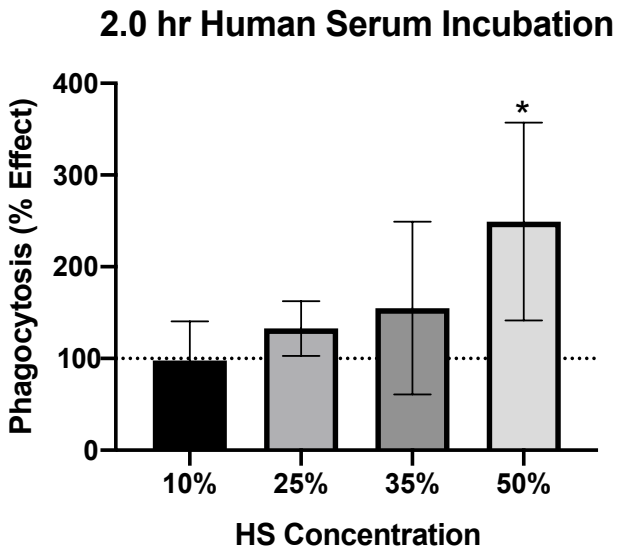

Figure 10: Effect of human serum (HS) concentration in culture media on phagocytosis following 2.0 hr HS incubation and 24 hr incubation with 0.5 μg/mL LPS. Phagocytosis is expressed as % effect relative to cells incubated for the same time in normal culture media (10% FBS) and similarly incubated with LPS, as indicated by the 100% dotted line. " * " indicates $p<0.05$ between 50% and 10% conditions. Data are presented as mean±SD.

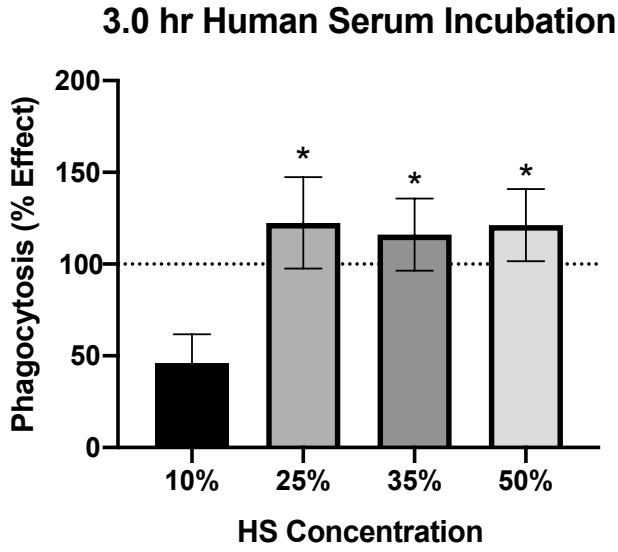

Figure 11: Effect of human serum (HS) concentration in culture media on phagocytosis following 3.0 hr HS incubation and 24 hr incubation with 0.5 μg/mL LPS. Phagocytosis is expressed as % effect relative to cells incubated for the same time in normal culture media (10% FBS) and similarly incubated with LPS, as indicated by the 100% dotted line. " * " indicates $p<0.001$ between 25%, 35%, 50% and the 10% condition. Data are presented as mean±SD.

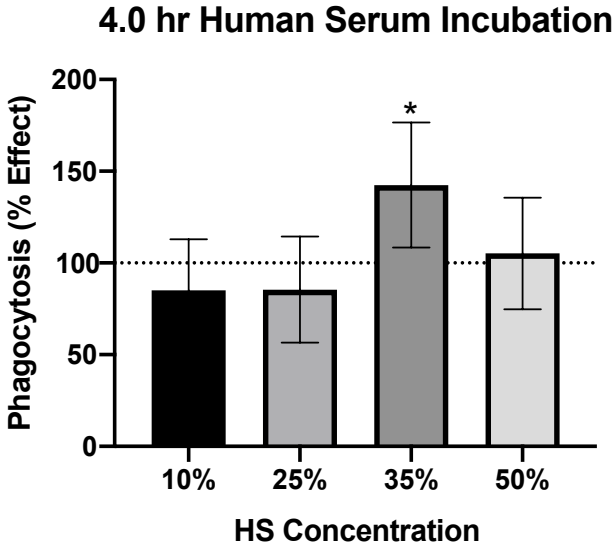

Figure 12: Effect of human serum (HS) concentration in culture media on phagocytosis following 4.0 hr HS incubation and 24 hr incubation with 0.5 μg/mL LPS. Phagocytosis is expressed as % effect relative to cells incubated for the same time in normal culture media (10% FBS) and similarly incubated with LPS, as indicated by the 100% dotted line. " * " indicates $p<0.05$ between 35% and 10%, 25% conditions. Data are presented as mean±SD

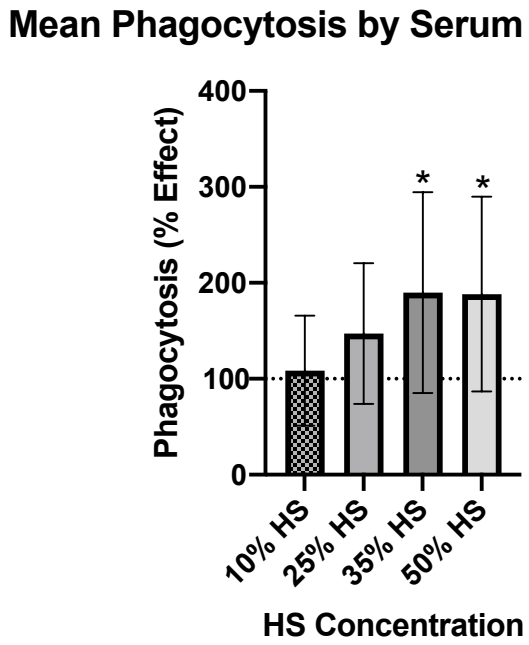

Figure 13: Effect of human serum (HS) concentration on phagocytosis in THP-1 MDMs following 0.5-4.0 hr serum incubation and 24 hr incubation with 0.5 μg/mL LPS. Phagocytosis is expressed as % effect relative to cells incubated for the same time in normal culture media (10% FBS) and similarly incubated with LPS, as indicated by the 100% dotted line. " * " indicates $p<0.01$ between 35%, 50% and the 10% HS condition. Data are presented as mean±SD.

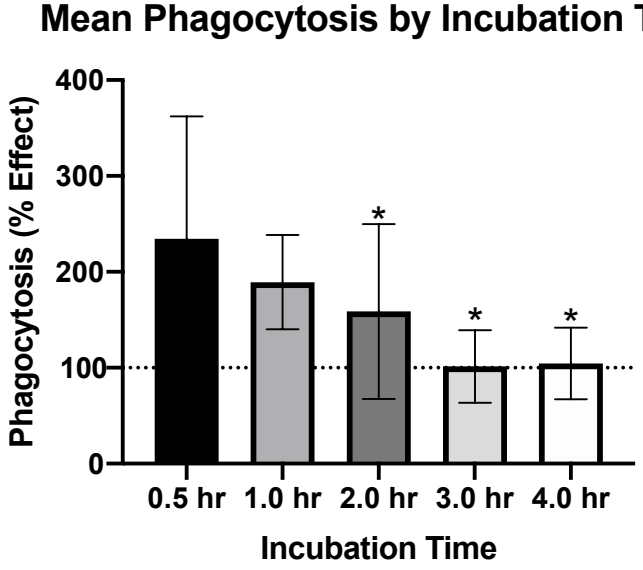

Figure 14: Effect of human serum (HS) incubation time prior to 24 hr incubation with 0.5 μg/mL LPS on phagocytosis in THP-1 MDMs. Phagocytosis is expressed as % effect relative to cells incubated for the same time in normal culture media (10% FBS) and similarly incubated with LPS, as indicated by the 100% dotted line. " * " indicates $p<0.05$ between 0.5 hr and 2.0 hr, 3.0 hr, 4.0 hr conditions. Data are presented as mean±SD.

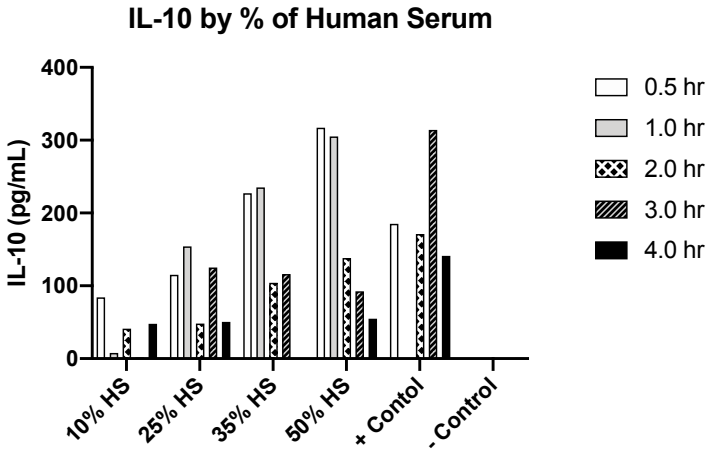

Figure 15: Effect of human serum (HS) concentration and incubation time on the secretion of IL-10 by THP-1 monocyte-derived macrophages following serum incubation and 24 hr incubation with 0.05 μg/mL LPS. "Pos. Control" refers to cells incubated for the same time and manner in THP-1 culture media (RPMI-1640 + 10% FBS) rather than HS. "Neg. Control" refers to cytokine levels in the THP-1 culture media, no cells.

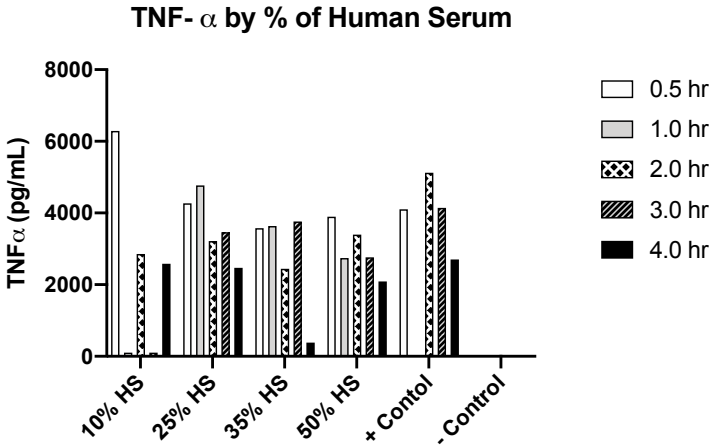

Figure 16: Effect of human serum (HS) concentration and incubation time on the secretion of TNF-α by THP-1 MDMs following serum incubation and 24 hr incubation with 0.05 μg/mL LPS. "Pos. Control" refers to cells incubated for the same time and manner in THP-1 culture media (RPMI-1640 + 10% FBS) rather than HS. "Neg. Control" refers to cytokine levels in the THP-1 culture media and no cells.

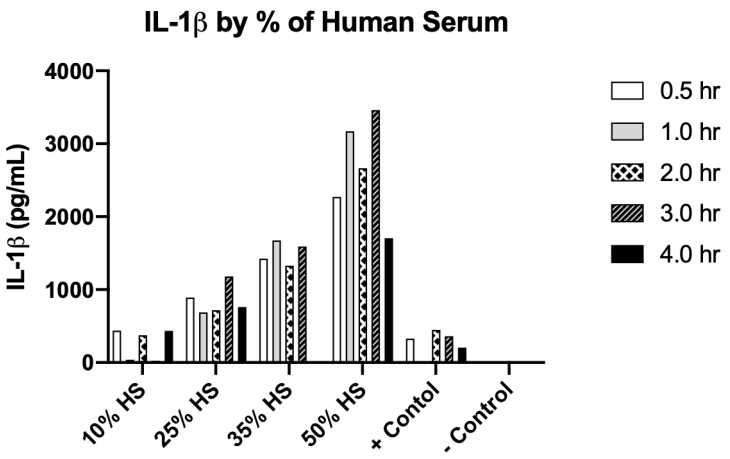

Figure 17: Effect of human serum (HS) concentration and incubation time on the secretion of IL-1β by THP-1 MDMs following serum incubation and 24 hr incubation with 0.05 μg/mL LPS. "Pos. Control" refers to cells incubated for the same time and manner in THP-1 culture media (RPMI-1640 + 10% FBS) rather than HS. "Neg. Control" refers to cytokine levels in the THP-1 culture media and no cells.

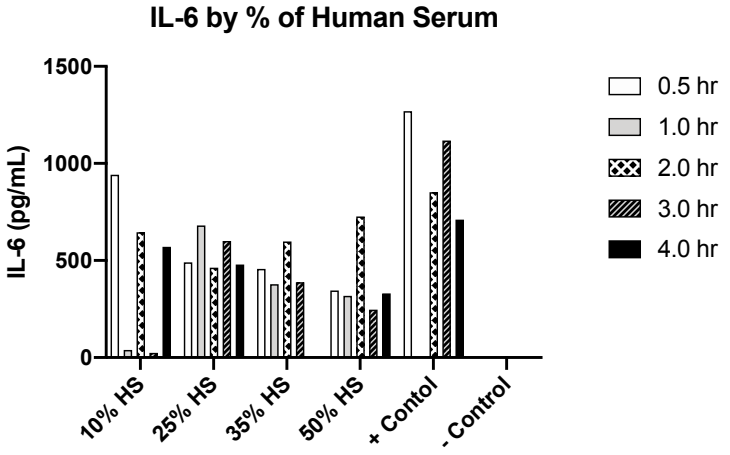

Figure 18: Effect of human serum (HS) concentration and incubation time on the secretion of IL-6 by THP-1 MDMs following serum incubation and 24 hr incubation with 0.05 μg/mL LPS. "Pos. Control" refers to cells incubated for the same time and manner in THP-1 culture media (RPMI-1640 + 10% FBS) rather than HS. "Neg. Control" refers to cytokine levels in the THP-1 culture media and no cells.

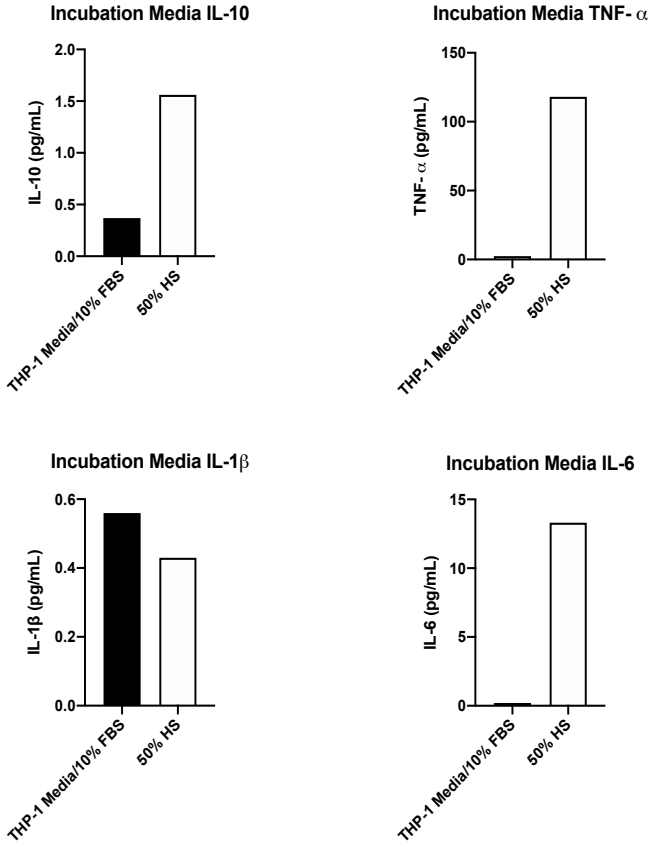

Figure 19: Concentration of cytokines IL-10, TNF-α, IL-1β, and IL-6 within the media used for human serum (HS) incubation experiments. 50% HS media was a mixture of RPMI-1640 with 50% HS by volume. THP-1 media was mixed as detailed in "Methods" and contained 10% FBS.

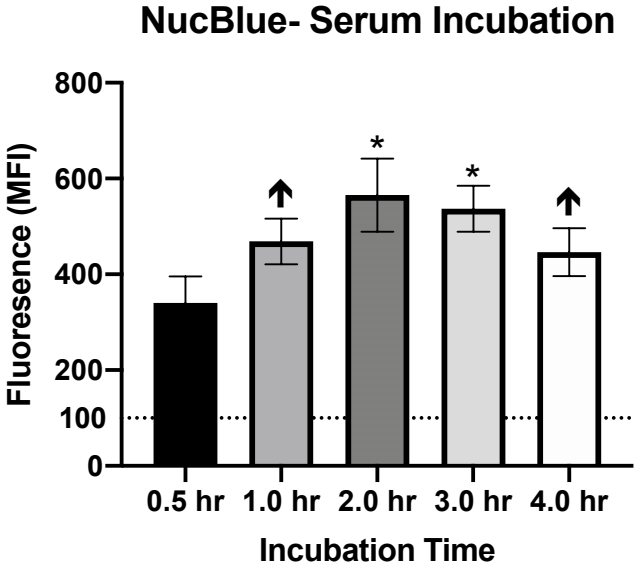

Figure 20: Effect of HS incubation time on NucBlue fluorescence in THP-1 MDMs following 24 hr of LPS incubation. NucBlue is expressed in mean fluorescence intensity (MFI). " * " indicates $p<0.001$ compared to 0.5, 1.0, 4.0 hr. "↑" indicates $p<0.001$ compared to 0.5 hr. Data are presented as mean±SD.

Vita

Charles Fee Hodgman was born in Seattle, WA, to Will and Carol Hodgman. He spent the majority of his childhood in Seattle, where he graduated from Lakeside High School in 2010. He then attended Colorado College between fall 2010 and fall 2012 before transferring to the University of Oregon. Charles attended the University of Oregon from the fall of 2013 to the spring of 2016, where he majored in human physiology and volunteered in the Lovering Cardiopulmonary Laboratory before graduating summa cum laude with a Bachelor of Science Degree. Following a year spent taking post-baccalaureate classes and volunteering in a lab at the University of Utah, Charles began a master's degree in exercise science at Appalachian State University in the fall of 2017. He was awarded a Master of Science degree from Appalachian State in the Fall of 2019 and began work towards a Ph.D. in exercise immunology at the University of Houston the same term.