THE EFFECTS OF CRANBERRY SUPPLEMENTATION ON INFLAMMATION AND MACROPHAGE FUNCTION IN OLDER, OVERWEIGHT, OR OBESE INDIVIDUALS

A Thesis
by
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Abstract

THE EFFECTS OF CRANBERRY SUPPLEMENTATION ON INFLAMMATION AND MACROPHAGE FUNCTION IN OLDER, OVERWEIGHT, OR OBESE INDIVIDUALS

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Aging and obesity are two factors associated with chronic low-grade inflammation and increased risk for disease. Therefore, as the American population is experiencing a demographic shift to older age and heavier weight, investigating new anti-inflammatory therapies is becoming more important. Polyphenol compounds found in natural products have been shown to decrease inflammation in both human and animal subjects through the targeting of multiple inflammatory pathways and molecules. Cranberry, *Vaccinum macrocarpon,* is a fruit that is garnering increasing interest as a potential anti-inflammatory therapeutic due to its high levels of polyphenol compounds. However, much of the evidence on the anti-inflammatory effects of cranberry is derived from studies of polyphenol fractions at doses much higher than those circulating in human serum following supplementation. Additionally, studies have not investigated the effects of cranberry metabolites on the functions of macrophages, which serve an important role in both initiating and resolving inflammation. For our studies, THP-1 macrophages were pre-incubated with serum from cranberry or placebo supplemented individuals, followed by LPS stimulation to determine potential anti-inflammatory effects of serum metabolites. We first aimed to determine the levels of inflammation and functional ability of macrophages in this older, overweight population as compared to a younger cohort of athletic individuals. Results from our study

showed that serum from older overweight individuals was less effective at overcoming the cytotoxic effect of LPS stimulation on macrophages when compared to younger subjects. Additionally, macrophages exposed to serum from older subjects exhibited lower levels of phagocytosis and expression of molecules associated with activation of adaptive immunity (MHCII and CD80) than younger, athletic subjects. Because we observed evidence of immune dysfunction in older, overweight subjects, we aimed to determine whether cranberry supplementation of these individuals could alleviate LPS-induced inflammation or alter macrophage functioning. We hypothesized that human serum from cranberry supplemented individuals would downregulate inflammatory signaling pathways in THP-1 macrophages resulting in reduced levels of inflammatory cytokines, lower levels of antigen-presenting and costimulatory molecules, and reduced phagocytic abilities. When these older subjects were given an eight-week cranberry supplement, their serum did not lower levels of inflammation or significantly alter macrophage functioning in our LPS-induced model of inflammation as compared to serum from placebo supplemented controls. Therefore, we can conclude that the ingestion of 500 mg/day of cranberry polyphenols did not induce anti-inflammatory or immunomodulatory effects in our model. Because this dosage was already at a very high level, increasing the dosage of polyphenols may not be feasible. However, the employment of other anti-inflammatory strategies such as exercise and weight loss may be worth exploring in future studies.

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Dedication

I would like to dedicate this thesis to my husband Joseph Mel Rhoney Jr. who's love and support has carried me through the years and through this research. Without his encouragement this thesis would not exist. I also want to thank my mother, Eula M. Shook, who has instilled in me a strong work ethic and encouraged me all through my life to follow my interests. Lastly, I want to dedicate this thesis to the memory of my father, Allen E. Shook, whose creative, adventurous, and curious nature left a life-long impression on me.

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Chapter One

1. Introduction

Cranberry, *Vaccinium macrocarpon,* is a common fruit native to North America and has been used as a folk remedy for many ailments, especially bladder and kidney infections and cystitis. Because cranberries are polyphenol rich foods, which often exhibit anti-inflammatory properties, research into their use as a therapeutic has been growing (1). Remedies for inflammatory conditions are also gaining importance due to our society's aging population and unhealthy lifestyle habits, such as a modern sedentary lifestyles and caloric rich diets, all of which are associated with higher risk of chronic inflammation and inflammatory disease. Additionally, increased incidence of diseases related to long term inflammation, such as heart disease, diabetes, stroke, cancer, etc., now account for a large proportion of societal morbidity and mortality (2, 3). Given the overall prevalence of diseases related to chronic inflammation, identifying inexpensive dietary mitigation strategies, such as the ingestion of cranberries, is of growing importance to society. While numerous studies have revealed that cranberries exhibit anti-inflammatory properties, few studies have examined the effects of their metabolized polyphenols, found in serum and urine, on regulating the functions of essential innate immune cells involved in the inflammatory process, such as macrophages. Furthermore, obesity superimposed on aging, physical activity, diet, and sex drastically influence parameters of inflammation. Each of these cofactors will be examined or considered in this study as we investigate the anti-inflammatory effects of 8-week cranberry supplementation on a group of older, overweight, or obese adults.

1.1 Inflammation and Macrophages

Inflammation is an important defense mechanism to protect the body from pathogens, foreign objects, and injuries. The inflammatory process begins when a harmful or foreign stimulus, including pathogens, injuries, and foreign materials, activate resident sentinel immune cells such as macrophages, dendritic cells, and neutrophils. These cells can detect foreign pathogens and damaged cells via pattern recognition receptors (PRRs), which are molecules found on the surface or endosomal membrane organelles. PRRs can detect evolutionary conserved molecular structures on pathogens termed pathogen-associated molecular patterns (PAMPs) as well as endogenous danger molecules released from dead or dying cells called damage-associated molecular patterns (DAMPs). Engagement of PRRs with these conserved patterns leads to the stimulation of sentinel antigen presenting cells (APCs) and phagocytosis of damaged tissue or foreign substances and microbes (4). In addition, intracellular signaling cascades are stimulated, culminating in the release of inflammatory mediators such as prostaglandins involved in vasodilation, histamines to increase blood vessel permeability, and early cytokines and chemokines to promote activation and migration of additional APCs and T cells to the site of damage. APCs like macrophages promote inflammation but are also important players in the clearance of inflammatory stimuli through their role in initiating adaptive immunity and activating T cells. In a healthy inflammatory response, as the primary infection and debris are cleared, inflammatory signals decrease, and tissue repair is initiated (4).

As indicated previously, macrophages play a multifaceted role in the inflammatory immune response; they are important to pathogen recognition, phagocytosis, antigen presentation, and T-cell activation. Macrophages first recognize and respond to harmful or foreign material through activation of their cell surface and cytosolic PRRs. One of the most

widely studied of the PRRs involved in macrophage-mediated inflammation is toll like receptor 4 (TLR4). TLR4 specifically recognizes bacterial lipopolysaccharide (LPS) on the surface of gram-negative bacteria, as well as other components of pathogens and endogenous molecules produced during abnormal situations, such as tissue damage (5, 6). Activation of TLR4 mainly leads to the synthesis of pro-inflammatory cytokines and chemokines, as well as the expression of co-stimulatory molecules, leading to initiation of adaptive immunity.

LPS stimulation of the TLR4 pathway has been exstensively studied for numerous years leading to the identification of the molecules involved in TLR4-mediated signaling and players that are involved in the regulation of the pathway. Upon activation of TLR4 at the cell surface by LPS, the myeloid differentiation primary response protein (MyD88)-dependent and independent arms of the inflammatory signaling cascade are triggered. This cascade results in phosphorylation and activation of I kappa B (IκB) kinases which break down IκB proteins that are normally bound to and inhibit the transcription factor, NF-κB (nuclear factor kappa light chain enhancer of activated B cell). This allows the activation of NF-κB and movement to the nucleus where it induces the expression of inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1ß), and interferon gamma (IFNγ) in numerous cell types, including macrophages (6). Cytokines produced through this pathway, such as TNFα and IL-1ß, can also bind to surface receptors on macrophages to induce mitogen-activated protein kinase (MAPK) and the janus kinase/signal transducer and activator of transcription (Jak/STAT) pathways (7, 8). As cytokines accumulate in tissues, inflammation is enhanced due to the further expression and release of pro-inflammatory cytokines from macrophages and other innate immune cells. This leads to vasodilation and infiltration of additional macrophages and other adaptive immune cells, such as T cells, to the site of damage

(9, 10). Furthermore, macrophages exhibit plasticity in their behavior and phenotype depending on signals received from their environment; therefore, as inflammatory signals increase, resting state macrophages (M0), may be coerced to a more inflammatory phenotype (M1) (10). M1 macrophages express higher levels of inflammatory markers and present costimulatory molecules on their surface which are important to T-cell activation, an integral part of the adaptive immune response.

Phagocytosis of exogenous molecules and pathogens by macrophages is a critical component of antigen presentation and activation of T cells. After the material is endocytosed, phagosome acidity increases followed by fusion with lysosomes to form phagolysosomes, which degrade the microbes or particles they engulf (6). Following degradation, peptides are loaded onto major histocompatibility complex II (MHCII) in the late endosome and presented on the surface of cells. MHCII is a molecule found on professional APCs and its expression can be triggered via LPS activation of TLR4 on the surface of APCs like macrophages and enhanced by IFNγ following LPS-stimulated inflammatory gene transcription (11, 12). MHCII is crucial to T-cell activation, particularly $CD4^+$ (cluster of differentiation 4 positive) T cells. $CD4^+$ T cells, also called T helper cells, are adaptive immune cells that differentiate into different subsets to carry out various immune functions, such as further activation of cytotoxic T cells and B cells, as well as modulating the immune response through secretions of various cytokine and chemokines. MHCII antigen presentation at the cell surface is the first signal in a chain of signals required to activate adaptive $CD4^+$ T cells (13).

Following antigen presentation on MHCII molecules, a second signal is required to activate $CD4^+$ T cells. This second signal is the cell surface expression of the costimulatory molecule CD40 (cluster of differentiation 40). CD40 is found at low levels in unstimulated

macrophages but its expression is upregulated during inflammation (14, 15). Following an inflammatory signal from PRRs, and phagocytosis of stimulating molecules, NF-κB is upregulated as the molecule STAT-1α, part of the Jak/STAT signaling pathway, binds to part of the CD40 promoter in the nucleus of the cell resulting in an increase of CD40 expression at the surface of the cell (16). CD40 binds with cluster of differentiation 40 ligand (CD40L) on the surface of $CD4^+$ T cells which completes their second stage in activation. Once activated these T helper cells differentiate into different effector functions that aid in initiating cell mediated and humoral immune responses (13). Additionally, the interaction of CD40 with CD40L plays an important role in full macrophage activation, resulting in induction of interleukin 12 (IL-12), IFNγ, TNFα, and the production of nitric oxide (NO), which is important for pathogen killing (14). Therefore, the expression of CD40 on the surface of APCs and induction of its subsequent functions is critical for activating adaptive immunity (14).

Another molecule expressed on macrophages and other immune cells that is important for the activation of both $CD4^+$ and cytotoxic $CD8^+$ T cells is CD80. CD80 expression on the surface of macrophages is significantly upregulated by stimulation of TLR4 by LPS. CD80, which binds to cluster of differentiation 28 (CD28) on the T cell surface, allows T cells to proliferate and differentiate into different functional types in the adaptive arm of the immune response, which aid in antigen or pathogen clearance (13).

In a healthy acute inflammatory response, innate, antigen presenting, and costimulatory functions of macrophages function in synergy to clear foreign materials, dead cells, and pathogens from the body. Part of the resolution of inflammation incorporates an anti-inflammatory and repair phase of response once the stimulus is cleared from the body. In an acute inflammatory response, both innate and adaptive arms of the immune system work to

dampen the inflammatory process. Loss of a triggering or stimulating antigen normally results in the release of various endogenous molecules with immunosuppressive functions, including interleukin-10 and 13 (IL-10 and IL-13) and interleukin-1 receptor antagonist (IL-1Ra), from stimulated M1 macrophages and other APCs. These molecules suppress inflammation by blocking the activation of NF-κB and downstream expression of inflammatory genes in macrophages, leading to an anti-inflammatory and tissue repairing phenotype. These macrophages are said to be of a more M2 like phenotype in this phase (10, 17, 18). Additionally, increased levels of IL-10 also inhibits the expression of CD80 and MHCII, leading to a reduction in activated T cells (10). However, under various conditions, NF-κB expression and activation may be dysregulated and abnormally elevated resulting in chronic inflammation and potential damaging effects on tissue (10, 18). Several factors known to influence and increase the risk of chronic inflammation are increased age, obesity, low physical activity and poor diet.

1.2 Inflammation and Ageing

Aging is a large risk factor for chronic inflammatory diseases such as cardiovascular disease, obesity, chronic kidney disease, and dementia. Additionally, studies have shown that some inflammatory biomarkers such as IL-6, IL-1, TNFα and C-reactive protein (CRP) increase with age (19). The increase in many of these inflammatory markers are thought to be a result of several factors, including higher numbers of tissue resident macrophages. For example, one study showed that geriatric mice have higher numbers of macrophages in both spleen and bone marrow compared to young mice (20). Higher levels of tissue resident macrophages are just one of several factors contributing to higher levels of inflammation.

Another factor that may contribute to an increase in inflammatory markers is a change in function of circulating and tissue resident macrophages, associated with aging and senescence.

Senescence, or biological aging, is a slow decline in the function of an organism, its cells, and tissue, which also impacts the function of macrophages (20). Senescence in tissue and immune cells like macrophages occurs over a lifetime of DNA damage and oxidative stress to cells. The cumulative damage that occurs from this age-related damage results in functional changes of older macrophages when compared to younger ones. Senescent macrophages are functionally impaired in their phagocytic ability resulting in an inability to clear apoptotic cells and pathogens which may further drive inflammation in those tissues (20). Furthermore, changes in function of older senescent macrophages and monocytes are related to changes in inflammatory gene expression within these cells.

NF-κB activity and inflammatory gene transcription is implicated as a key player in age-related chronic inflammation (21). Inflammation, acute or chronic, is associated with an upregulation of NF-κB signaling pathways in many cell types, including macrophages, and has been implicated in progression of the aging process (21). NF-κB is activated via oxidative, genotoxic, and inflammatory stresses, all of which increase with age. Additionally, TNFα and IL-1, found to generally be upregulated in older persons, are activators and transcriptional targets of NF-κB. Consistent with these findings, studies have confirmed that NF-κB transcription is up-regulated in a larger percentage of cells within different tissues during natural aging (21).

In addition to the upregulation of NF-κB in cells, ageing is also associated with upregulation of the cell surface markers CD40 and CD80 on immune cells including monocytes and macrophages. Studies have reported higher expression of CD40 (22) and CD80 (23) on monocytes in studies with PBMCs (peripheral blood mononuclear cells) in older individuals as compared to younger individuals. In contrast, there is evidence of an age-related decline in other molecules expressed on the surface of immune cells. One of these is MHCII, whose levels have

been shown to be lower in peripheral macrophages of aged mice and humans compared to their younger counterparts (3, 23). Lower MHCII expression in macrophages from aging mice may be associated with a reduced ability to present antigens on their cell surface, potentially leading to poorer T-cell responses. Therefore, this evidence indicates that age alters macrophage function in several ways leading to reduced phagocytic ability and antigen presentation. With the addition of increased levels of circulatory and tissue-specific inflammatory molecules, ageing is associated with significant inflammatory disease risk.

1.3 Inflammation, Obesity and Diet

Another risk factor of chronic inflammation superimposed with age is obesity. Older individuals have higher visceral fat adiposity and rates of obesity tend to increase with age, peaking at 50-65 years (20, 24). Studies in macrophages have shown that obesity and high caloric, high fat diets are a contributor to chronic low-grade inflammatory responses (25). Individuals chosen to participate in the cranberry supplementation study presented in this thesis have body mass indices (BMIs) categorized as obese or overweight, which puts them at higher risk for inflammatory disease. There are several hypothesized mechanisms for the increase in inflammation associated with obesity, including the dysregulation of adipocytes. Adipocytes not only store lipids but also produce pro-inflammatory cytokines, and under conditions of obesity are shown to produce higher levels of IL-6, IL-1ß, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory proteins (MIPs), and TNFα. These inflammatory mediators recruit monocytes into the adipose tissue and polarize them to a pro-inflammatory M1 phenotype. Another hypothesized mechanism is through the upregulation of the NF-κB inflammatory transcription pathway (25). As previously explained, IκB kinases break down IκB proteins, leading to NF-κB activation. In a high fat and high calorie diet, IκB kinase expression is

dramatically increased, which leads to enhanced NF-κB-induced inflammatory gene expression. A mechanism theorized for enhancement inflammation associated with fatty diets, is through the binding of lipids on TLRs, which may enhance the activation of NF-κB (25). Overall, these data indicate high fat, high calorie diets and obesity promote NF-κB activation in immune cells like macrophages.

Alterations in the activation of TLRs and dysregulation of the NF-κB pathway in macrophages as a result of obesity and a high fat diet also suggests functional changes in these cells, including antigen presentation ability and costimulatory molecule expression. Generally, MHCII, CD40, and CD80 are found to be upregulated in obese individuals, mainly due to the over stimulation of these molecules from adipocytes and tissue resident macrophages in adipose tissue (25, 26, 27). An increase in expression of MHCII is associated with enhanced phagocytosis and antigen presentation. However, there is limited evidence indicating that phagocytic capacity is affected by obesity. A study investigating alveolar macrophage function reported no differences in the phagocytic ability in obese individuals versus normal weight individuals (28). While advanced age, obesity, and poor diet may increase overall inflammation and alter macrophage function, there are lifestyle choices that can ameliorate these factors. Regular routine exercise is one method for mitigating inflammation when appropriately balanced.

1.4 Inflammation and Physical Activity

A sedentary lifestyle is associated with multiple inflammatory and metabolic diseases. It is also associated with development of visceral fat which promotes the infiltration and overabundance of pro-inflammatory macrophages and the development of low-grade inflammation (29). In contrast, regular moderate exercise is associated with anti-inflammatory

effects on the body and immune system (30). Epidemiological studies also indicate that regular exercise is associated with lower frequency of chronic diseases, such as cardiovascular disease and diabetes, lower oxidative stress, as well as other inflammatory disease processes (31, 32). There are several possible mechanisms to explain the anti-inflammatory effects of regular exercise. One mechanism is through the overall reduction of visceral fat. As described previously, obese individuals have a larger proportion of tissue resident macrophages compared to lean individuals due to higher visceral fat. A second mechanism is the reduction of TLR expression on monocytes and macrophages seen during routine exercise, which leads to down-regulation of NF-κB gene expression. Lastly, during regular, moderate exercise, there may be additional recruitment of anti-inflammatory molecules from white blood cells in skeletal muscle (29). Each of these mechanisms lead to reduction of inflammation and inflammatory markers such as cytokines.

Routine exercise has been shown to affect both anti- and pro-inflammatory cytokine levels. Studies have shown that regular exercise elevates anti-inflammatory cytokine expression in humans and other animals (30, 31). In humans, a prolonged session of training is also capable of significantly decreasing IL-6, TNFα, CRP and increasing anti-inflammatory IL-10 (30). Studies investigating general levels of physical fitness also show that individuals who exercise more frequently and consider themselves highly fit also show reductions in CRP, TNFα and IL-6 in blood samples (31). This reduction of cytokine production supports that exercise downregulates the inflammatory signaling cascade, reducing NF-κB transcription. Lower levels of TNFα with exercise also elicits a suppressive effect on MHCII expression in macrophages (33). Furthermore, exercise has been shown to enhance IL-10 production to promote a more M2 like phenotype in macrophages, further reducing inflammatory responses (30, 31). Therefore, the

effects of routine moderate exercise on cytokine production indicate that inflammatory signaling pathways associated with cytokine production and antigen presentation are altered and result in an overall reduction of inflammation.

In addition to decreasing inflammation, moderate exercise has also been associated with changes in macrophage function (30, 31, 34). Studies have shown that the positive effect of exercise on macrophage function is dependent on intensity, frequency, and duration. These functional alterations are believed to be partially attributed to the release of stress hormones by the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) system. Additionally, changes in body temperature and cell metabolism also contribute to immune function alterations (32). In a study in humans, both macrophage chemotaxis and phagocytosis increased with acute exercise. This enhanced function is not thought to be attributed to increases in numbers of macrophages but due to the increase in TNFα and nitric oxide (NO) production (32). Despite these positive trends associated with exercise, the benefit to macrophage function and inflammatory responses is dependent on the duration and intensity of exercise.

Extreme exercise of high intensity and long duration may have a detrimental effect on the immune system. As previously mentioned, exercise as a form of stress increases the plasma levels of several immunomodulatory hormones, such as cortisol, adrenocorticotropic hormone (ACTH), adrenaline (epinephrine), and noradrenalin (norepinephrine) (35). A moderate amount of these hormones may be beneficial in decreasing some inflammatory conditions, but high levels lead to suboptimal immune function. Studies in exercise fatigued individuals have also reported reduced antiviral functions of macrophages (32). Additionally, if exercise is exhaustive, MHCII expression and antigen presentation by macrophages can be temporarily suppressed during the recovery period, which is in contrast to effects seen with moderate exercise. This

potential negative effect underscores the importance of moderate routine exercise for modulating immune function and inflammation, as exhaustive exercise can increase the risk of temporary immunosuppression (32).

1.5 Sex Differences in Inflammation

Other than stress hormones associated with exercise, sex hormones and steroids, along with other sex differences, can impact the inflammatory response. Studies have shown that both estrogen and progesterone, common female sex steroids, drive macrophages towards a more anti-inflammatory or alternative activation type, regulating wound healing, angiogenesis (blood vessel formation), and tissue remodeling (36). Estrogen has been associated with lower inflammatory molecule production in macrophages due to the inhibition of TLR4 expression. As a result, inflammation induced by LPS is also reduced through the downregulation of NF-κB activity, including the expression of pro-inflammatory cytokines such as TNFα, IL-1, and IL-6. Progesterone also influences macrophages by decreasing nitric oxide synthase (iNOS), NO, TNFα and IFNγ while increasing the more anti-inflammatory interleukin-4 (IL-4) cytokine. These alterations result in an overall decrease in inflammation in younger females (36, 37).

Functionally, sex steroids also influence differences in macrophage, as well as other immune cell, functions (36). Macrophages are more responsive to stimuli in females, and generally have greater phagocytic capacity as compared to those in males. This increase in sensitivity may be related to higher TLR expression in females and differences in NF-κB activity influencing antigen presentation and costimulatory molecule expression (37).

Studies have shown that the expression of macrophage surface molecules differs between males and females. As an example, mouse studies investigating macrophage functions showed that MHCII expression was slightly higher in younger female than younger male mice. However,

when age was considered, aged females had significantly lower levels of MHCII than their male counterparts as well as younger females (38). However, in the same study, CD40 levels did not differ significantly between male and female mice (38). In contrast, in a study of mice infected with *Taenia crassiceps* (a tapeworm in the family Taeniidae), female mice had higher expression of CD80, as well as MHCII, in their macrophage populations compared to male mice (39). These studies indicate that sex differences in macrophage costimulatory molecule expression may be dependent on several factors and should be investigated in more detail to also understand the impacts of anti-inflammatory treatments.

As described, numerous factors, including age, obesity, activity, diet, and sex, influence the risk of chronic inflammatory diseases. Therefore, with demographics shifts in this country favoring age related chronic inflammation, it is important to investigate strategies to mitigate disease, including dietary compounds.

1.6 Inflammation and Cranberry

Cranberries are a native plant to the northeastern United States and have been speculated to have numerous health benefits since before the 20th century (1). Research into the health effects of cranberries has revealed that they are composed of several bioactive chemicals, mostly polyphenols, which are compounds containing phenolic hydroxyl groups. The main bioactive polyphenol composition of cranberries includes flavan-3-ol monomers and dimers, proanthocyanidins (PACs), anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, terpenes, and flavanols (1). Expanding evidence indicates that cranberry polyphenols exhibit multiple anti-inflammatory effects through the alteration of inflammatory pathways in immune cells including macrophages.

The anti-inflammatory effect of cranberry polyphenols is attributed to their suppression of the MyD88-dependent inflammatory pathway which impacts NF-κB activity as well as the Jak/STAT inflammatory pathway (40). The downregulation of these inflammatory pathways in macrophages results in lower expression of inflammatory cytokines. For example, studies have shown that cranberry concentrates reduce inflammatory interleukin-8 (IL-8) and IL-6 expression in LPS-stimulated THP-1 macrophages. Conversely, an increase in the expression of anti-inflammatory cytokines such as IL-10 has also been observed in macrophages when exposed to potent cranberry concentrates (41).

The influence of cranberry on inflammatory pathways not only results in changes to inflammatory molecule expression but also to macrophage phenotype and function. In particular, the PAC fraction of cranberries specifically are shown to convert M1 inflammatory macrophages to a M2 anti-inflammatory phenotype in LPS-stimulated macrophages. Specifically, THP-1 macrophages exposed to cranberry PAC fractions of 50 and 100 μg/mL or PAC free controls, followed by LPS stimulation, resulted in an increase in the proportion of M2 macrophages. Along with this change in phenotype, these M2 macrophages expressed lower levels of pro-inflammatory cytokines such as IL-6 and IL-8, and higher anti-inflammatory cytokines like IL-10 (41). While it was unknown how these macrophages were coerced to a M2 phenotype, studies attributed it to the interaction of the PAC fraction to TLRs at the macrophages cell surface (42). Furthermore, the ability of macrophages in culture to endocytose LPS is inhibited after exposure to PACs. The mechanism behind the reduction in endocytosis is hypothesized to be due to the binding of the PAC fraction to LPS thus blocking its interaction with TLR4 on the cell surface (42). A downstream effect of limited binding of TLR4 to LPS is reduced expression of CD80 and MHCII whose expression and function are dependent on endocytosis and the development of

the late phagosome (23, 42). Consequently, reduced inflammation following cranberry polyphenol supplementation could theoretically lead to macrophage dysfunction.

1.7 Inflammation and Cranberry Metabolites

Much of the information on the anti-inflammatory effects of cranberries is gained from studies investigating the effects of raw cranberry fractions in *in vitro*. However, immunomodulatory effects of metabolized cranberry compounds found in blood, serum, and urine following consumption are less extensively reported. Absorption of polyphenols occurs in tandem with other reactions primarily within the small and large intestines and the liver. Metabolites of polyphenols are altered from their parent molecules through digestion and modified through sulfonation, methylation, or glucuronidation (43). Cranberry has been associated with approximately 60 compounds found in plasma. Most of these compounds fall under the categories of benzaldehydes, benzoic acids, catechols, cinnamic acids, dihydrocinnamic acids, flavanols, hippuric acids, phenylacetic acids, pyrogallol, and valeracetones (44). The most abundantly produced cranberry metabolites in human subjects, following ingestion of cranberry juice, are 3-(4-hydroxyphenyl) propionic acid, hippuric acid, and catechol-*O*-sulfate. However, proportions and amounts of cranberry metabolites are influenced by factors such as sex, genetic variability in metabolism, environmental factors, and the specific constituents of the individual's gut microbiome (44). Variability in these factors complicate studies investigating the anti-inflammatory effects of cranberry metabolites *in vivo*. However, studies have suggested that cranberry metabolites also play an important role in reducing inflammation.

Few studies have investigated the role of cranberry polyphenol metabolites on inflammation and immune function. One of the larger circulating metabolites after cranberry

consumption is 3-(3-hydroxyphenyl) propionic acid (3HPPA), a metabolite of quercetin. In studies on cultures of human aortic endothelial cells (HAECs) and THP-1 cell co-cultures stimulated with TNFα, 3HPPA was seen to inhibit the adhesion of THP-1 cells on HAECs, suggesting that macrophage function may be affected by 3HPPA. The mechanistic action behind this effect is thought to be through the inhibition of NF-κB activation (45). In addition to 3HPPA, ferulic acid (FA) and isoferulic acid (IFA), both hydroxycinnamic acids, influence immunity, including inflammatory immune response in macrophages. Both FA and IFA associated with *Cimicifuga sp*. (bugbane or cohosh) were shown to reduce the production of macrophage inflammatory protein-2 (MIP-2) in RAW264.7 cells infected with respiratory syncytial virus (RSV). FA and IFA associated with cohosh also decreased production of the pro-inflammatory cytokine IL-8 in mice (44, 46). In addition, FA and its derivatives have been shown to regulate inflammation by decreasing NF-κB activation (46). As hydroxycinnamic acids are one of many metabolites in human serum following cranberry ingestion, they are likely to be main compounds impacting macrophage functions in our study (44).

In addition to the metabolites mentioned previously, evidence suggests that metabolized flavanol glycosides also exert anti-inflammatory activity. Quercetin and kaempferol, both flavanol glycosides found in serum after cranberry supplementation, are known to inhibit nitric oxide, cyclooxygenase-2 (a prostaglandin), CRP expression, and NF-κB expression in Chang liver cells (47). Quercetin is also found to inhibit TNFα expression in LPS-stimulated THP-1 macrophages through inhibition of the JNK/SNPK (Jun amino-terminal kinases/stress-activated protein kinases) pathway, a member of the MAPK family (48). In THP-1 monocytes, kaempferol suppresses LPS-induced production of cytokines and chemokines. Kaempferol also suppresses type 1 T helper (Th1), type 2 T helper (Th2), and neutrophil-related chemokine production in

THP-1 cells. Kaempferol's mechanism of action is hypothesized to be via alteration of the MAPK inflammatory pathway (47). In addition to the compounds mentioned above, there are over 60 metabolized cranberry compounds which may also modulate inflammation (44).

1.8 THP-1 Macrophages as a Cell Model for Inflammation

While there is direct and indirect evidence that certain cranberry metabolites possess anti-inflammatory properties, we are unaware of any studies that have specifically examined the impact of these metabolites on macrophage cytokine production, cell surface molecule expression, and phagocytosis following cranberry supplementation. For our studies, we will utilize THP-1 cells, which are a human monocytic leukemic cell line with a long history of being used to study monocyte and macrophage functions and mechanisms of inflammation, such as inflammation triggered through activation of TLR cell receptors and through triggering the NF-κB inflammatory signaling cascade (49). This cell line has several advantages over peripheral blood mononuclear cell derived monocytes and macrophages. THP-1 cells are immortalized and actively proliferate and can be passaged up to 25 times without altering cell sensitivity. These cells are also genetically homogeneous, which improves reproducibility, and are not known to be virally contaminated or produce toxic byproducts. However, the power of this cell line lies in its ability to differentiate into a macrophage like phenotype (49).

When exposed to phorbol-12-myristate-13-acetate (PMA), THP-1 cells differentiate into resting adherent macrophages lacking the ability to proliferate. Additionally, MAPK and NF-κB pathways are activated following differentiation and stimulation of these cells with TLR agonists like LPS (50). As indicated previously, LPS induces the MyD88-dependent and independent signaling pathways which upregulates NF-κB gene expression and activation (49), thus serving as an effective cell model for inflammation.

1.9 Project Overview

We utilized an LPS-induced model of inflammation in THP-1 macrophages to investigate the effects of cranberry supplementation on inflammation in an older, overweight, or obese adult population. THP-1 macrophages were pre-incubated with serum from cranberry or placebo supplemented individuals, followed by LPS stimulation to determine the anti-inflammatory effects of serum metabolites on these cells. We first aimed to determine the background inflammation and cell function in this older, overweight population by comparing their levels of inflammation and macrophage functioning to a younger cohort of athletic individuals. Secondly, we aimed to determine whether cranberry supplementation of older, obese subjects alleviates LPS-induced inflammation or affects macrophage functioning. We hypothesized that the addition of human serum from cranberry supplemented individuals to THP-1 macrophages would downregulate inflammatory signaling resulting in reduced levels of inflammatory cytokines, lower levels of antigen presenting and costimulatory molecules, and reduced phagocytic abilities as a secondary consequence of the alteration of the inflammatory signaling cascade. This cell model investigating serum cranberry metabolites fills a gap in current understanding of the ant-inflammatory effects of metabolized cranberry as well as investigating secondary effects of these metabolites on macrophage function.

Chapter Two

2. Materials and Methods

2.1 Serum Samples Used in Study

Serum samples for this study were obtained from previous studies at the Appalachian State North Carolina Research Center (NCRC) Human Performance Lab in Kannapolis, NC. Both studies were designed, organized, and managed by NCRC Human Performance Lab under the principal investigator David C. Nieman with funding provided by Ocean Spray Cranberries, Inc. (for older group cranberry supplemented serum) and Dole Food Company, Inc. (for younger group serum). Blood samples were collected and processed into serum by NCRC Human Performance Lab (51). Details of the two studies are indicated below.

2.2 Cranberry Study Design: Influence of 8-Weeks Cranberry Supplementation on the Antibody Response to Influenza Vaccination and Ex-Vivo Viral Defense

The Cranberry Study design was focused on establishing the influence of an 8-week cranberry supplementation on antibody response to influenza vaccination. A cohort of 90 older (40-80 years of age), overweight or obese adults were chosen to participate and were chosen at random to receive either a 4 oz. cranberry beverage with breakfast and 2 cranberry gummies at lunch or a placebo beverage and gummies for a period of 8 weeks. The total phenolic content of the cranberry beverage was 170 mg total phenolics and each gummy had a content of about 160 mg total phenolics. Additionally, at 4 weeks into the study, participants received an influenza virus vaccine. Blood samples were collected by the lab at a pre-study visit, 4 weeks post-supplement immediately before vaccination, 1-week post-vaccination and 4 weeks post-vaccination. Serum samples were then extracted from blood samples taken from each visit for analysis and frozen for long term storage at -80°C.

2.3 Blueberry, Banana Study Design: The Combined Influence of Blueberry and Banana Consumption on Metabolic Recovery from 75-km Cycling Exercise Stress

The main aims of this study were to determine if consumption of blueberries, bananas or both could mitigate metabolic perturbation as well as inflammation and immune dysfunction following recovery from a 75 km cycling time trial. Supplements were sourced from Allen's Blueberries (Ellsworth, ME) and freeze-dried by Future Ceuticals (Momence, IL). Bananas were provided by Dole Foods (Westlake Village, CA, USA). Funding was provided by Dole Food Company Inc (51).

Participants in this study included healthy non-smoking males and females, 18-55 years of age, who regularly compete in road races and could cycle 75 km at a racing pace in a laboratory setting. They were randomized into four groups. Two weeks before the start of study, participants reported to the human performance lab for baseline fitness evaluation and blood samples collection. Subjects were then divided into supplement groups of blueberry-water, blueberry-water (mixed with banana), placebo-water, or placebo-water (mixed with banana), and supplemented for 2 weeks before trial with blueberry or placebo powder mixed in water. After a 2-week period of supplementation, on the day of time trial, participants provided a blood sample at time 0 hours, 1.5 hours, 3 hours, and 5 hours into trial and 24 and 48 hours after trial. Fifty serum samples taken from the time before beginning supplementation (2 weeks prior to trial) were randomly selected for our study samples (51).

2.4 Study Design for Evaluating Inflammation in Older, Obese Population as Compared to Younger, Active Population

Participants (49) from the group of cranberry and placebo subject samples (at the time prior to supplementation), were randomly chosen from this larger cohort to evaluate the immune

differences of the older, overweight group versus a younger group of active individuals (referenced in the "Blueberry Banana Study" above). Thawed serum samples from the younger, athletic group were used to compare parameters of macrophage function to the older, overweight group. Of this younger cohort, 50 total participants (from the time period before any supplementation began and before time trial) were randomly chosen as a subset of their larger size for cell viability and phagocytosis analyses. Due to limited serum availability of the younger cohort at the pre-supplement time series, MHCII and CD80 flow cytometry results from a previous analysis, by Dr. Maryam Ahmed in the Department of Biology at Appalachian State University, were used for our analysis. This data (from 13 subjects) was from the younger placebo subjects at the second time period (pre-time trial). Our investigations aimed to determine if our assumptions of higher inflammatory molecules and reduced macrophage function of the older population were supported when compared to this active younger cohort.

2.5 Study Design for Evaluating Inflammatory Differences in Older Individuals Treated with Either a Cranberry or Placebo Supplement for 8 Weeks.

To determine if cranberry supplementation alleviates macrophage induced inflammation in our older population, serum samples from participants in the cranberry study was subdivided into a smaller subset of 49 individuals: 23 from the placebo group and 26 from the cranberry supplemented group. Serum from the pre-supplement samples, the 4-week post-supplement samples and 4-week post-vaccine samples were used to evaluate differences between groups. For these studies, THP-1 macrophages in cell cultures were pre-incubated with 40% serum prior to LPS stimulation.

2.6 THP-1 Cell Culture and LPS Induced Model of Inflammation

THP-1 monocytes were cultured in THP-1 media at 37°C with 5% CO_2. THP-1 media consisted of 90% Roswell Park Memorial Institute (RPMI) media (Corning RPMI 1640 1X) and 10% fetal bovine serum (FBS) (R&D Systems Part no. S11150) with 0.05mM 2-mercaptoethanol. Cells were passaged when reaching a concentration of $8.0x10^5$-$1.0x10^6$ cells per ml or every 3-4 days.

Figure 1 shows a schematic of our standardized LPS induced model of inflammation for our experiments. Cells were differentiated using 25nM Phorbol 12-myristate 13-acetate (PMA) in a 96 well plate and incubated for 48 hours at 37°C and 5% CO_2. Following differentiation, supernatants were removed and replaced with fresh THP-1 media and incubated for 24 additional hours to allow cells to rest. This rest period ensures that the cells have time to recover from the effects of PMA and allows for better LPS stimulation of macrophages in our hands. Following the rest period, cells were pre-incubated with human serum (at 40% concentration by volume) in media containing Penicillin-Streptomycin (Pen strep) for a period of 3 hours. Cells were then stimulated with 500 ng/mL of LPS and allowed to incubate for 24 hours at 37°C and 5% CO_2.

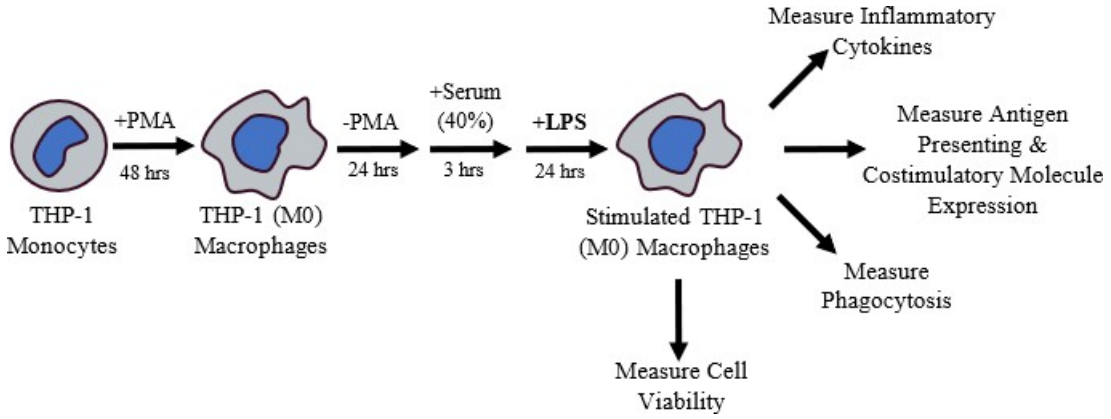

Figure 1: THP-1 macrophage LPS-induced inflammatory model for study. THP-1 monocytes were differentiated to a macrophage like state using PMA for 48 hours. Cells were then allowed to rest in the absence of PMA for 24 hours, pre-incubated with serum for 3 hours, and then stimulated with LPS for 24 hours prior to measuring cell viability, cytokine expression, antigen presenting and costimulatory molecule expression, and phagocytosis.

2.7 Phagocytosis by THP-1 Macrophages

The phagocytic ability of LPS-stimulated macrophages was measured using 2.0μm diameter red fluorescent latex beads (Sigma-Aldrich, Part# L3030). THP-1 monocytes were plated in a 96 well dish at $5.1x10^5$ cells/mL (100 mL per well volume) or $5.1x10^4$ cells/well, differentiated with PMA, and pre-incubated for 3 hours with 40% serum and 1% 100X Pen strep. Cells were then stimulated with LPS as described previously. Following 24-hour LPS stimulation, fluorescent latex beads were added at a 0.05% suspension (approximately $5.0x10^7$ beads/mL media) for 6 hours. Wells were washed two times with 100 μL sterile 1X PBS (Corning Part# 46-013-CM) solution followed by aspiration and replacement with fresh THP-1 media. Representative live cell images were immediately taken in triplicate at 20x magnification with an Olympus IX-81 inverted fluorescent microscope, DP80 camera and CellSens Software. Images were processed using Adobe Photoshop (2020). The percentage of cells undergoing phagocytosis and degree of phagocytosis was assessed using DotDotGoose (2022). The percentage of phagocytotic cells was calculated by measuring the number of cells that had at least one bead internalized relative to the total number of cells. The degree of phagocytosis was calculated as the percent of phagocytic cells with four or more beads internalized. The percentage and degree of phagocytosis in LPS-stimulated cells was normalized relative to the corresponding unstimulated values. Data was further normalized relative to pre-supplement levels in unstimulated cells in order to evaluate changes in phagocytosis over time.

2.8 Viability of THP-1 Macrophages

The viability of THP-1 macrophages in response to LPS and serum was assessed using an MTT Assay (Cell Proliferation Kit I, Millipore Sigma, Part#11465007001). This colorimetric assay measures cell viability through the conversion of MTT into formazan in active cell

metabolism and is measured using absorbance values in a microplate reader. THP-1 monocytes were polarized, plated in a 96 well dish at $1.2x10^6$ cells/mL (100 mL per well volume) or $1.2x10^5$ cells/well, followed by pre-incubation with 40% serum and 1% 100X Pen strep for 3 hours then stimulated as previously described. At 24h post-stimulation, 10 µL of 1X MTT labeling reagent was added to each well and incubated at 37°C/ 0.5% CO_2 for 4 hours. Following incubation, 100 µL of warmed solubilization buffer was added to each well and the plate was incubated overnight at 37°C/ 0.5 CO_2 for 8 hours. Absorbance was measured using a VersaMax Microplate reader with absorbance value set to 550nm and reference at 650nm. The viability of macrophages was determined by normalizing absorbance values relative to the cell control and further normalizing them relative to their unstimulated controls and pre-supplement levels (for cranberry study) to evaluate changes over time.

2.9 Expression of Surface Molecules on THP-1 Macrophages

The expression of antigen presenting and costimulatory factors on the surface of THP-1 macrophages was measured using fluorescently labeled antibodies to HLA-DR (for MHCII), CD40, and CD80, and analyzed using flow cytometry. THP-1 monocytes were plated in a 96 well dish at $5.1x10^5$ cells/mL (100 mL per well volume), or $5.1x10^4$ cells/well, polarized with PMA, followed by a 3-hour pre-incubation with 40% serum and 1% 100X Pen strep. Cells were then stimulated with LPS as described previously. Supernatants were collected following 24 hours of LPS stimulation and stored at -20°C for future sandwich enzyme-linked immunosorbent assay (ELISA) analysis. Cells were washed with 1X PBS, incubated with 50 µL of Accutase (Corning Part# 25-058-Cl) at 37°C/ 5.0 % CO_2 for 60 minutes, and collected with 100 µL of cold 1X PBS. Cells were transferred to a round bottom 96 well dish and centrifuged at 1200 rpm at 4°C for 4 minutes. Supernatant was removed and cells resuspended in 120 µL of wash/stain

buffer (5% FBS in 1X PBS). Samples were labeled with 100 µL of antibody at a 1:20 dilution in stain buffer (BioLegend - FITC anti-human HLA-DR item#307604, BioLegend - APC anti-human CD40 item#334310, BioLegend - PE anti-human CD80 item#305208). Cells were incubated on ice in the dark for 20 minutes, washed twice with 200 µL of wash/stain buffer, and resuspended in 250 µL of fresh wash/stain buffer for flow cytometry analysis using the Attune NxT Flow Cytometer. Data analysis was conducted using Microsoft Excel (Office 16) and FlowJo 9.9.6 software. Some of the existing data obtained from serum from younger subjects (Blueberry Banana Study) was carried out using the same methodology as above in the laboratory of Dr. Maryam Ahmed. The results from the serum from the older Cranberry study were compared to Dr. Ahmed's younger cohort results. Because results from younger subjects were obtained at a separate time period by a different individual, results from the older group and younger group were normalized to their internal controls.

2.10 IL-6 Production by THP-1 Macrophages

The secretion of IL-6 in supernatants (from the experiment described above) was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) (Invitrogen Human IL-6 Uncoated ELISA Part# 88-7066). Costar 9018 ELISA plates were coated with 100 µL of capture antibody per well (Invitrogen anti-human IL-6 100X) diluted to 1X in coating buffer (1X PBS). Plates were then sealed and placed in refrigeration at 4°C overnight. Wells were aspirated the following day and washed 3 times per well with 250 µL/well of wash buffer (1X PBS with 0.05% Tween -20) and blotted dry. Wells were then blocked with 200 µL of 1X ELISA/ELISPOT diluent and incubated at room temperature for 1 hour. IL-6 standard dilutions (0-200 pg/mL) were prepared using lyophilized human IL-6 reconstituted in 450 µL of ultrapure water and added (100 µL/well) in duplicate to the plate to generate a standard curve. Frozen

supernatants from samples were diluted in 1X PBS at a 1:5 ratio, and 100 µL of each diluted sample was added to the plates and sealed. Plates were incubated at room temperature for 2 hours. Wells were then aspirated and washed 4 times with wash buffer and blotted dry followed by the addition of 100 µL of diluted detection antibody (1X in ELISA/ELISPOT buffer). Plates were sealed and incubated at room temp for an hour. Wells were aspirated and washed 4 times with wash buffer solution and blotted dry. 100X Streptavidin-HRP Enzyme (diluted at 1:100 in ELISA/ELISPOT buffer) was then added to each well (100 µL/well) and incubated for 30 minutes at room temperature followed by incubation with 1X TMB solution (100 µL/well) for 15 minutes at room temperature and the addition of the stop solution (100 µL/well of 1M H_3PO_4). Plates were read at 450 nm with subtraction set at 570nm-450nm (Molecular Devices VERSAmax microplate reader). The concentration of IL-6 levels following LPS stimulation, was normalized to unstimulated and pre-supplement samples.

2.11 Statistical Analysis

Statistical analysis was carried out using Microsoft SPSS 28.0.0.0 software utilizing a general linear model with univariate analysis of variance for older versus younger (AIM 1) experimental comparisons. Cranberry study (AIM 2) analysis utilized a general linear model with repeated measure analysis of variance to determine variance over time. Interactions between time and treatment (cranberry versus placebo) groups were analyzed as well as group (older versus younger) and sex differences and interactions. Because much of the data was of unequal sizes, post hoc comparisons were analyzed using a Bonferroni multiple comparison of means for between subject effects. Microsoft Excel (Office 16) was also utilized for initial data collection, analysis, organization, and plots. P-values of significance were reported as * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Chapter Three

3. Results

Cranberries have a long history of use as a natural anti-inflammatory and anti-microbial, with multiple studies demonstrating these properties (1). Unfortunately, few studies have addressed the specific effects of cranberry metabolites on immune cells, such as macrophages, in blood or tissues. Studies that do test the effect of cranberries on macrophage functions often utilize polyphenol fractions from cranberries or cranberry concentrates (41). Unfortunately, these methods do not address the effects of metabolized cranberry polyphenols within the body on immune cells and their roles in inflammation. Therefore, investigating the effects of serum from subjects ingesting cranberries will more directly test the effects of metabolized substances on immune cells in tissue and blood, including macrophages. Our study aims to specifically determine how metabolites, as found in serum from older, overweight cranberry supplemented individuals, modulate THP-1 macrophages in an LPS-induced model of inflammation.

Before investigating whether cranberry supplementation alleviates LPS-induced inflammation in our older, overweight subjects, we wanted to compare the influence of non-supplemented serum on macrophage function from this group to that from a younger, active group (AIM 1). THP-1 monocytes were exposed to PMA to induce their differentiation into a naïve macrophage-like phenotype (M0 macrophages). Following a 48-hour incubation, the media was replaced with fresh THP-1 media and cells were allowed to rest for 24 hours. Cells were then pre-incubated with human serum (40% serum by volume) from the pre-supplement samples from our older, overweight group or the younger, athletic group. After a 3-hour pre-incubation, cells were either incubated with LPS for 24 hours to induce inflammation or left untreated. This procedure was carried out prior to assessing the effect of serum on the viability of THP-1 M0 macrophages, their ability to phagocytose fluorescent beads, and their surface expression of

MHCII and costimulatory molecule CD80. Due to availability of serum and time restraints, we did not investigate expression of CD40 or IL-6 molecules for this first aim.

3.1 Human Serum is Not Cytotoxic to THP-1 Macrophages.

To determine the effect of serum from the older, overweight group as compared to the younger, active group on the viability of M0 macrophages, an MTT assay was utilized following our standardized experimental procedure detailed above. Upon stimulation with LPS, we observed a 30% decrease in the viability of M0 macrophages compared to the unstimulated (Figures 2A and B). However, serum from both older and younger individuals exerted a protective effect on cell viability in LPS-stimulated cells (Figure 2B, diamonds). When results from LPS-stimulated cells were normalized to their unstimulated values (Figure 2C), the viability of M0 macrophages was significantly lower when exposed to serum from older subjects as compared to younger subjects. However, since cell viability in the presence of serum from both groups remained higher than that in the absence of human serum (NS), this suggests that serum exerts an overall protective effect against the cytotoxicity of LPS on THP-1 macrophages.

In addition to investigating the different effects of serum from the older group versus the younger group on cell viability, we also wanted to determine whether there were any sex differences. We did not observe any differences in cell viability of THP-1 macrophages exposed to serum from males versus females within the older group or younger group (data not shown). However, when the age groups were combined, we observed that serum from females elicited significantly higher cell viability than male serum following LPS stimulation (Figure 2E). No sex differences were seen with unstimulated cells (Figure 2D) or when values were normalized to unstimulated values (Figure 2F). In addition, serum from both groups did not lead to negative effects on the viability of M0 macrophages. With the knowledge that human serum is not

cytotoxic to these immune cells, we investigated the effect of serum from older and younger groups on phagocytosis by M0 macrophages.

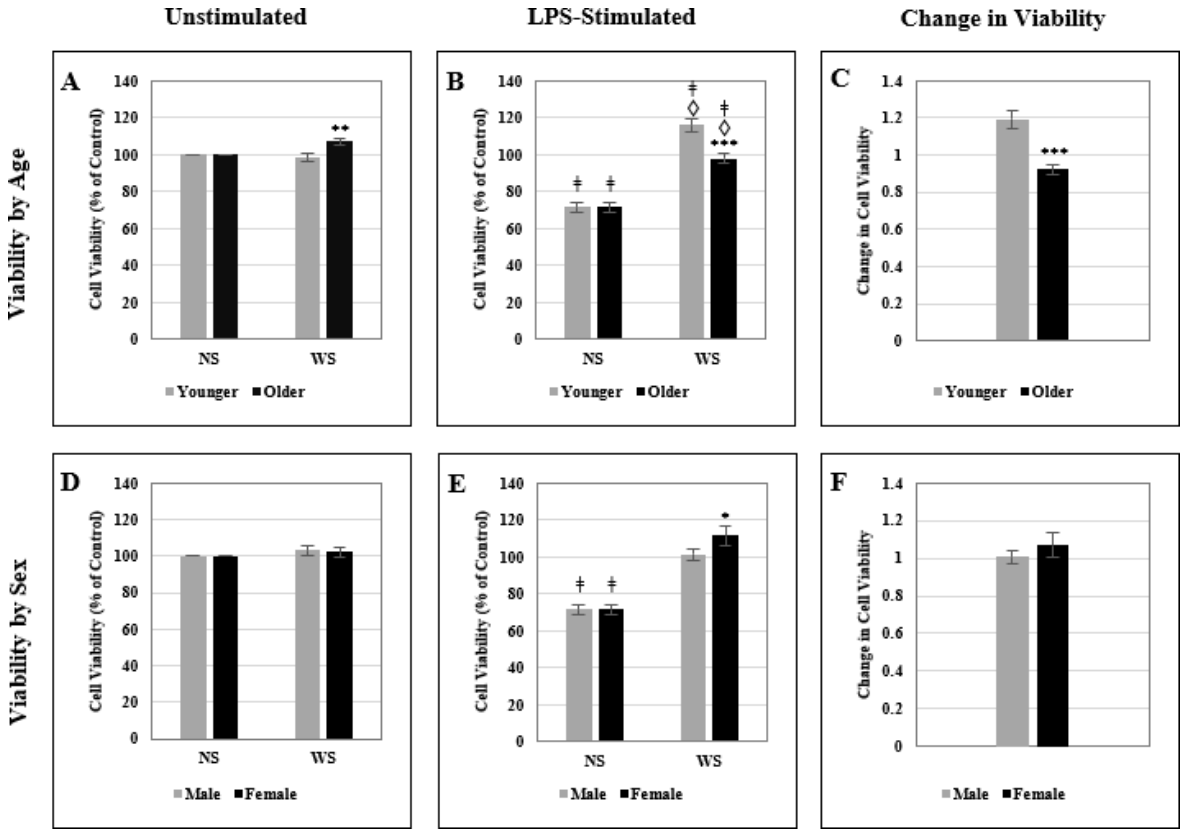

Figure 2. Cell viability of THP-1 macrophages pre-incubated with serum from younger active, or older overweight, or obese adults. THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum (WS) from un-supplemented younger or older individuals. Cells were either left unstimulated (A, D) or stimulated (B, E) with LPS for 24 hours and both subjected to MTT assay. Results were normalized to unstimulated controls (NS). (C) Represents fold change in viability following LPS stimulation, relative to unstimulated values. (D) Represents sex differences in viability of unstimulated and (E) LPS-stimulated M0s. (F) Represents fold change in viability following LPS stimulation, relative to unstimulated values, with serum from both sexes. Asterisks indicate significant differences in cell viability between age and sex groups (*, $P \leq 0.05$), (**, $P \leq 0.01$), (***, $P \leq 0.001$). Diamonds represent significant differences between M0s (NS) and serum (WS) groups. (◇, $P \leq 0.05$) Crosses represent significant differences of viability of LPS-stimulated cells relative to respective unstimulated values in A and D (ǂ, $P \leq 0.05$).

3.2 Serum from Older Subjects Reduces the Phagocytic Capacity of THP-1 Macrophages Compared to Younger Subjects

Previous studies have shown that age influences macrophage function by reducing phagocytic ability in older individuals (20). Conversely, exercise has the opposite effect of

increasing phagocytic ability of macrophages (32). Therefore, we wanted to determine the baseline level of phagocytosis when cells were exposed to serum from older, overweight individuals as compared to our younger, active group. We hypothesized that we would observe a reduction in phagocytic ability of our THP-1 macrophages when incubated with serum from older subjects as compared to that from younger individuals.

To test this hypothesis, we employed our standardized experimental procedure and pre-incubated our THP-1 macrophages with serum (40% by volume) from the older or the younger groups for 3 hours followed by LPS stimulation for 24 hours. Fluorescent latex beads were added (0.05% by volume) for 6 hours followed by two washes. Triplicate images of each sample were taken at 20x magnification. The percentage of cells with at least one bead internalized was calculated to determine the phagocytic ability of M0 macrophages. We also determined the percentage of cells with 4 or more internalized beads to measure the degree of phagocytosis.

Figure 3A shows representative images of both LPS-stimulated and unstimulated cells with (WS) and without (NS) human serum. We observe a greater number of cells with at least one bead internalized under conditions of LPS stimulation versus without stimulation. This observation is supported in our analysis of Figure 3C versus 3B, where we note that there is a significant increase in phagocytosis in stimulated versus unstimulated cells (crosses). Images also show that there are generally a greater number of internalized beads in THP-1 macrophages following LPS stimulation (Figure 3A). Figure 3E and F support that observation, as we see an increase in the percent of cells that have internalized 4 or more beads following LPS stimulation.

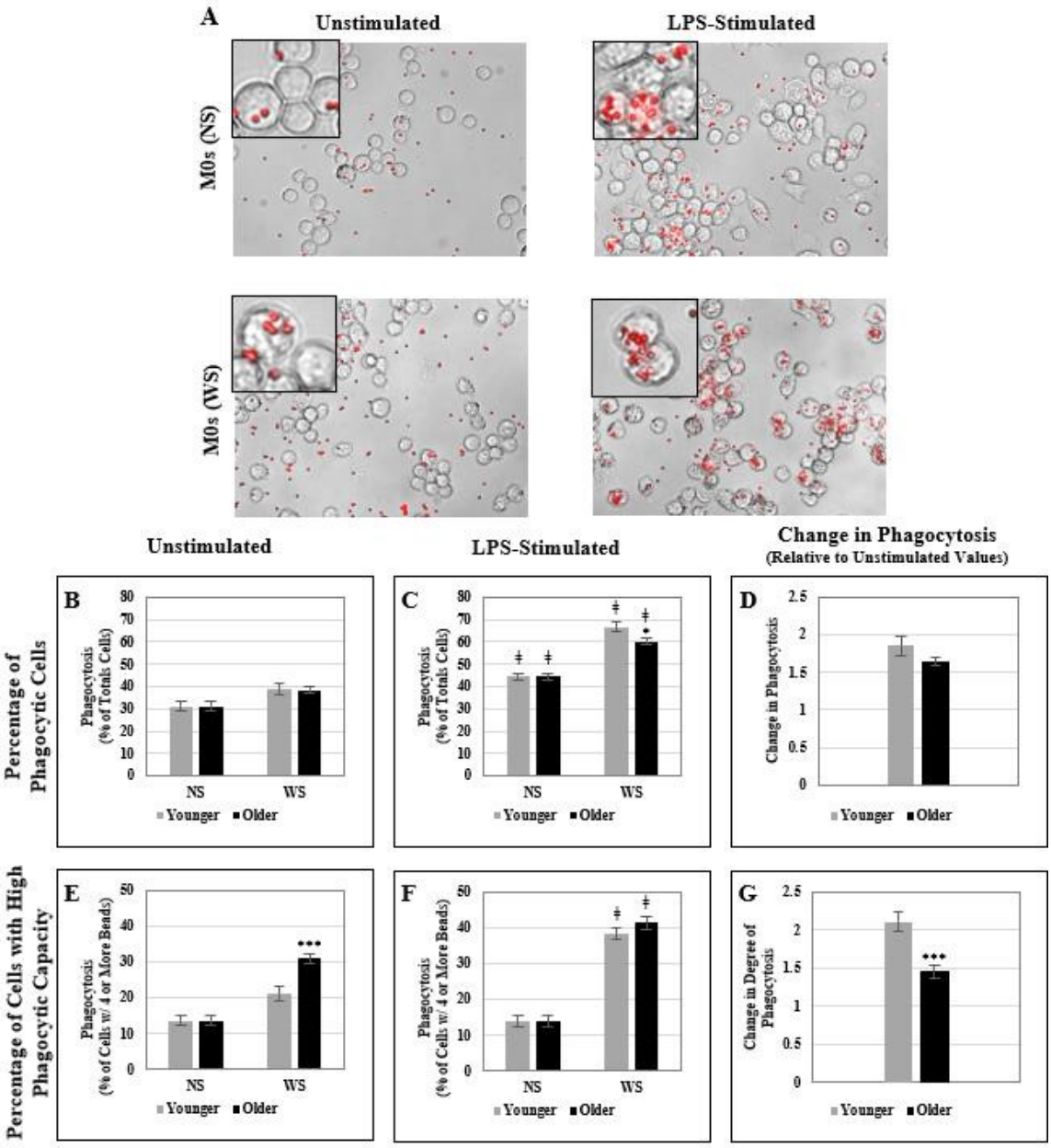

Figure 3. Phagocytic ability of THP-1 macrophages (pre-incubated with serum from younger active, and older overweight, or obese adults). THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum (WS) from un-supplemented younger or older individuals. Cells were either left unstimulated (B, E) or stimulated with LPS (C, F). Fluorescent latex beads were added at a 0.05% concentration by volume and incubated for 6 hours followed by washing and imaging at 20x magnification. Triplicate representative live cell images were taken for each sample to obtain a sample average. (A) Shows representative images of phagocytic THP-1 macrophages. The percentage of cells with internalized beads are shown (B, C). (E, F) represent the percent of cells with 4 or more internalized beads. Phagocytosis was normalized to unstimulated values and is shown as the fold change in phagocytosis in LPS-stimulated cells (D) and fold change in phagocytic degree (G). Asterisks indicate significant differences between older and younger groups in in phagocytic ability (*, $P \leq 0.05$), (***, $P \leq 0.001$). Crosses represent significant difference in phagocytic ability of LPS-stimulated cells relative to respective unstimulated values in B and E (‡, $P \leq 0.05$).

Consistent with our hypothesis, we observed a lower level of phagocytosis when LPS-stimulated cells were incubated with serum from older individuals as compared to the younger group (Figure 3C). While this did not translate to significant differences when data from LPS-stimulated cells were normalized to unstimulated levels (Figure 3D), the degree of phagocytosis was significantly lower upon exposure to serum from the older group (Figure 3G). We also analyzed sex differences in phagocytosis by THP-1 macrophages but did not observe any differences between males and females (data not shown).

3.3 Serum from Older Subjects Reduces the Expression of MHCII on THP-1 Macrophages Compared to Younger Subjects.

As phagocytosis is a necessary step in exogenous antigen presentation, we wanted to determine if these functional differences extend to MHCII expression on THP-1 macrophages. Studies have shown that aging is associated with lower levels of MHCII expression in macrophage populations in both mice and humans (3, 23). Because MHCII expression is required for antigen presentation and the first step in T-cell activation, lower expression suggests that macrophages have reduced ability to present antigens as aging occurs (11). To explore how MHCII expression may be altered with age, we incubated unstimulated and LPS-stimulated M0 macrophages with FITC conjugated anti-human HLA-DR (MHCII) antibody followed by flow cytometry analysis. We measured the percent of live cells expressing MHCII as well as the geometric mean fluorescence to determine the degree of MHCII expression on the surface of these cells. It is important to note that results from the younger subjects were measured previously in the Ahmed lab in a related study, but the experimental procedure, antibodies, and analysis were the same as those for the older group. Because of this time difference, results from

both the older and younger groups were normalized to their internal control values (M0 macrophages with no serum - NS) to correct for any differences in experimental techniques.

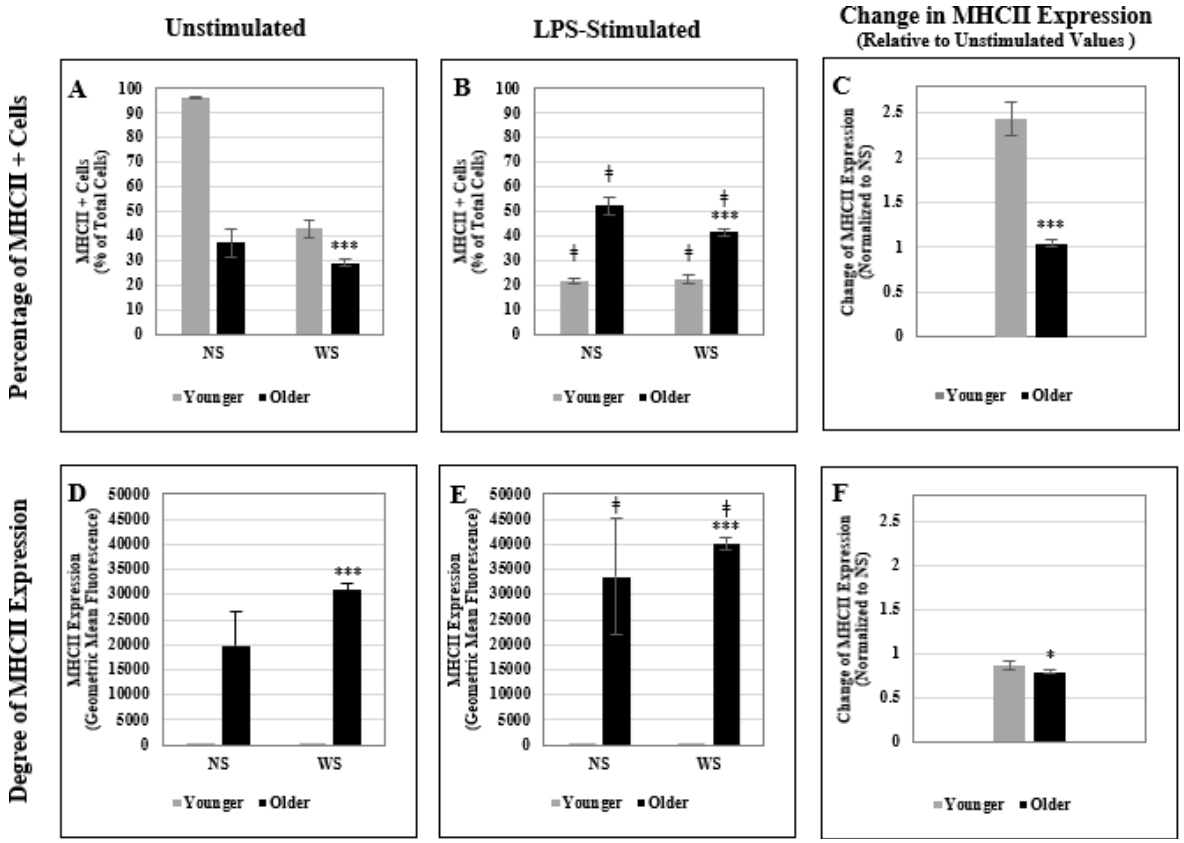

Figure 4. MHCII expression on THP-1 macrophages (pre-incubated with serum from younger active, and older overweight, or obese adults). THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum (WS) from un-supplemented younger or older individuals. Cells were either left unstimulated (A, D) or LPS-stimulated (B, E) and incubated for 24 hours. Samples were labeled with fluorescent antibodies (FITC anti-human HLA-DR) and analyzed via flow cytometry. The percentage of MHCII positive cells are shown (A, B). (D, E) represent degree of MHCII expression. MHCII expression was normalized to unstimulated values and serum free (NS) values and is shown as the fold change of MHCII expression on LPS-stimulated cells (C) or fold change in degree of MHCII expression (F). Asterisks indicate significant differences between older and younger groups in MHCII expression (*, $P \leq 0.05$), (***, $P \leq 0.001$). Crosses represent significant differences of MHCII expression on LPS-stimulated cells relative to respective unstimulated values in A and D (‡, $P \leq 0.05$).

In unstimulated cells, we observed a decrease in MHCII expression in the presence of serum (WS) as compared to cells that were left untouched (NS) (Figure 4A). In addition, a lower percentage of unstimulated cells expressed MHCII when incubated with serum from older subjects compared to serum from younger subjects. However, the degree of expression in

unstimulated cells was higher with serum from older subjects versus younger (Figure 4D). Upon stimulation with LPS, we saw an increase in levels of MHCII positive cells (Figure 4B) as well as a higher degree of expression (Figure 4E) when cells were incubated with serum from older subjects. While these results suggest the opposite of our hypothesis, when we normalized these values to their corresponding values in the absence of serum (NS) and relative to unstimulated cells, we observed that serum from older subjects resulted in a lower percentage of cells that expressed MHCII (Figure 4C), as well as a reduced degree of MHCII expression relative to serum from younger subjects (Figure 4F). These results agree with our expectations that MHCII expression is reduced with age, suggesting that older individuals have some level of dysfunction in antigen presentation driven by Immunosenescence.

3.4 Serum from Older Subjects Reduces the Degree of CD80 Expression on THP-1 Macrophages Compared to Younger Subjects

For a more complete picture of the effect of age on macrophage function, we investigated the expression of the costimulatory molecule, CD80, which is critical to the activation of T cells. Experimental evidence suggests that with age, CD80 expression increases on PBMCs (23). To determine if serum from our older cohort exerted similar effects on the THP-1 macrophages, we stained the M0 polarized cells with PE-conjugated anti-human CD80 antibodies following our standard experimental procedure and analyzed the expression of CD80 on the surface of cells via flow cytometry. We measured the percent of cells expressing CD80 as well as geometric mean fluorescence, to determine the degree of CD80 expression on these cells. As before, because results from the younger individuals were obtained from a previous study carried out in the Ahmed lab, results from both the older and younger groups were normalized to their respective serum free control values (NS) to correct for any differences in time or technique.

Results in unstimulated cells showed that a lower percent of macrophages expressed CD80 (Figure 5A) when incubated with serum from older subjects as compared to serum from younger individuals. However, we observed an opposite result in the degree of CD80 expression (Figure 5D) in unstimulated cells. As expected, LPS stimulation increased the percentage of cells expressing CD80 (Figure 5B versus 5A) as well as the degree of CD80 expression (Figure 5B vs E) (13). We also observe a dampening effect of LPS stimulation when serum from older subjects was added to cells ($P \leq 0.001$) as compared to serum free controls (NS).

Following LPS stimulation, a lower percentage of cells expressed CD80 in the presence of serum from older subjects versus younger subjects (Figure 5B). However, the degree of CD80 expression was higher when exposed to serum from older subjects compared to younger subjects (Figure 5E). Given these results, it appears that CD80 expression is variable, where serum from younger subjects tends to result in a higher percentage of cells expressing CD80 but the degree to which each cells expresses this molecule is dependent on stimulation status. However, as mentioned previously CD80 expression in serum from younger versus older subjects was measured by different researchers, at different times, which could result in experimental differences. Consequently, these values were normalized to results under serum-free conditions (NS) and we observed no differences in the percent of cells expressing CD80 in older versus younger subjects (Figure 5C). However, serum from older subjects resulted in a significant decrease in the degree of CD80 expression as compared to that from younger subjects (Figure 5F).

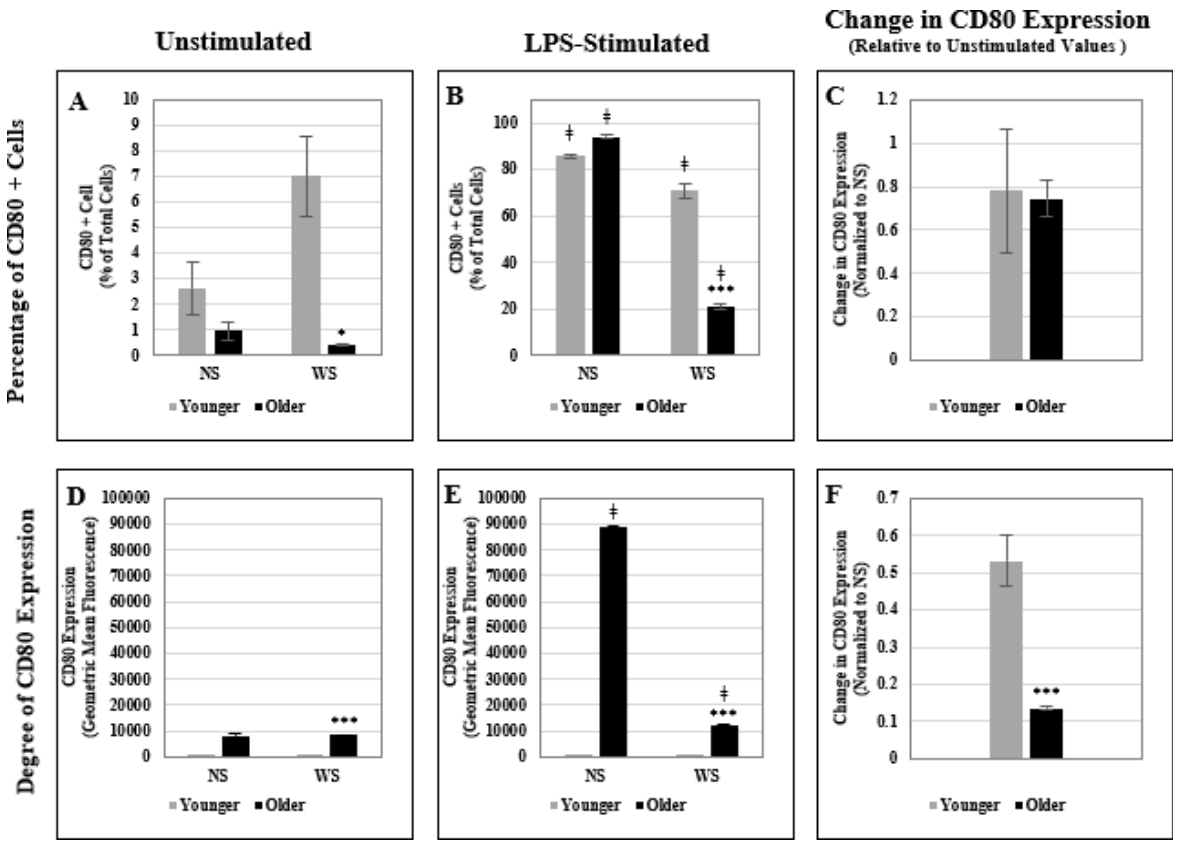

Figure 5. CD80 expression on THP-1 macrophages (pre-incubated with serum from younger active, and older overweight, or obese adults). THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum (WS) from un-supplemented younger or older individuals. Cells were either left unstimulated (A, D) or LPS-stimulated (B, E) and incubated for 24 hours. Samples were labeled with fluorescent antibodies (PE anti-human CD80) and analyzed via flow cytometry. The percentage of CD80 positive cells are shown (A, B). (D, E) represents the degree of CD80 expression. CD80 expression was normalized to unstimulated values and serum free (NS) values and is shown as the fold change of CD80 expression on LPS-stimulated cells (C) or fold change in degree of CD80 expression (F). Asterisks indicate significant differences between older and younger groups in CD80 expression (*, $P \leq 0.05$), (***, $P \leq 0.001$). Crosses represent significant differences of CD80 on LPS-stimulated cells relative to respective unstimulated values in A and D (‡, $P \leq 0.05$).

In addition to investigating the differences in serum from the older group versus the younger group on MHCII and CD80 expression, we also wanted to determine whether there were any differences in the expression of these molecules when exposed to serum from different sexes. We evaluated the degree of MHCII expression of THP-1 macrophages when exposed to serum from males versus females, upon normalization to unstimulated controls. We detected a sex-linked effect, where THP-1 macrophages exposed to serum from females all groups,

expressed higher levels of MHCII than when exposed to male serum (Figure 6A). With regards to the percentage of cells expressing MHCII, there were no significant differences between serum from different sexes (data not shown). As before, there was no significant interaction effect of serum from different sexes within each age group.

In contrast to the trends observed with MHCII expression, we found that cells exposed to serum from females expressed significantly less CD80 on their surface as compared to serum from males (Figure 6B). In this case, we also detected a significant interaction effect of sex within the younger age group where serum from younger females exerted lower levels of CD80 expression on THP-1 macrophages than serum from young males (Figure 6C).

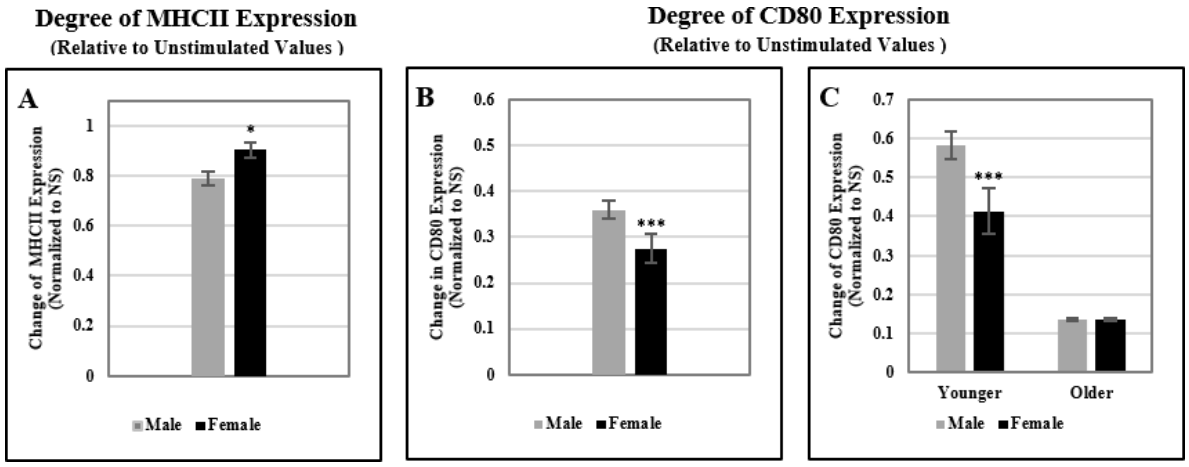

Figure 6. Sex differences in MHCII and CD80 expression of THP-1 macrophages (pre-incubated with serum from younger active, versus older overweight, or obese adults). THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum (WS) from un-supplemented younger or older individuals. The fold change of degree of MHCII expression (A) and fold change in degree of CD80 expression (B) are shown by sex. The interaction effect of age and sex on CD80 expression are shown in (C). MHCII and CD80 expression were normalized to unstimulated values and serum free values (NS). (*, $P \leq 0.05$), (***, $P \leq 0.001$).

The data above indicates that age is a factor in reducing the phagocytic ability of macrophages and the expression of key molecules to stimulate adaptive immunity; MHCII and CD80. These results further highlight the need for interventions to alleviate the immune dysfunction seen in older individuals. Therefore, the second aim of our study is to determine if cranberry supplementation in these older individuals exerts anti-inflammatory or

immunomodulatory effects on these dysregulated macrophages by investigating the effect of cranberry supplemented serum on cell viability, phagocytic ability, MHCII expression, costimulatory molecule expression, and IL-6 expression.

3.5 Serum from Cranberry Supplemented Subjects is Not Cytotoxic to Macrophages Compared to Serum Free Controls.

To determine the effect of serum from older, overweight subjects supplemented with cranberry, as compared to serum from placebo subjects, we utilized an MTT assay following our standardized experimental protocol. Results show that in the absence of serum (NS), LPS decreased the viability of THP-1 macrophages as compared to unstimulated cells. However, serum from both cranberry and placebo supplemented subjects exerted a protective effect on cell viability in LPS-stimulated cells (Figure 7B, diamonds). When results from LPS-stimulated cells were normalized to their unstimulated values and pre-supplement levels (Figure 7C), the viability of M0 macrophages was significantly lower when exposed to serum from cranberry supplemented subjects as compared to serum from placebo supplemented subjects. However, in the presence of serum from both groups, cell viability remained higher than in the absence of human serum (NS). This suggests that human serum from both groups exerts an overall protective effect against the cytotoxicity of LPS on THP-1 macrophages. We also analyzed sex differences in viability and did not observe any significant differences between males and females (data not shown).

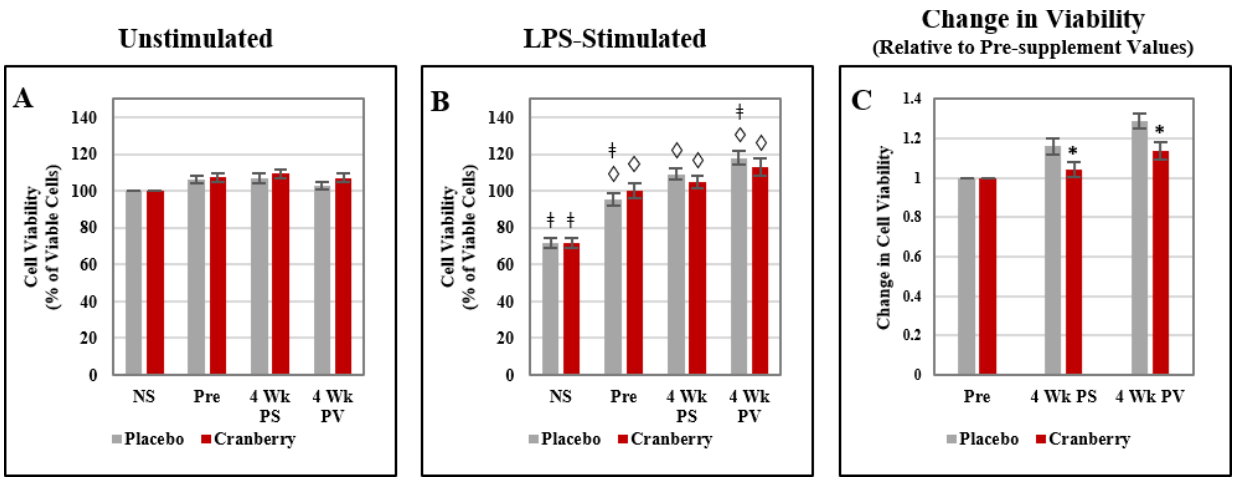

Figure 7. Cell viability of THP-1 macrophages pre-incubated with serum from placebo or cranberry supplemented older adults. THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum from either cranberry or placebo supplemented individuals. Cells were either left unstimulated (A) or stimulated with LPS for 24 hours (B) and both subjected to MTT assay. Results were normalized to unstimulated values (NS). Time periods evaluated were pre-supplement (Pre), 4 weeks post-supplement (4 Wk PS) and 4 weeks post-vaccine (4 Wk PV). (C) Represents fold change in viability following LPS stimulation. Asterisks indicate significant differences in cell viability of older versus younger individuals. (*, $P \leq 0.05$). Diamonds represent significant differences between M0s (NS) and with serum (Pre, 4 Wk PS, 4 Wk PV) groups. (◇, $P \leq 0.05$) Crosses represent significant differences of viability of LPS-stimulated cells relative to respective unstimulated values in A (‡, $P \leq 0.05$).

3.6 Serum From Cranberry Supplemented Subjects does Not Alter Phagocytic Ability of THP-1 Macrophages Compared to Placebos.

Previous studies have shown that cranberry polyphenols affect macrophage function by reducing endocytosis. However, we are not aware of any research on the effects of metabolized cranberry on endocytic or phagocytic abilities of macrophages (42). Therefore, we aimed to determine the impact of serum from cranberry supplemented subjects on the phagocytic ability of THP-1 macrophages as compared to serum from placebo supplemented subjects. We hypothesized that we would observe lower phagocytic ability in THP-1 macrophages exposed to serum from cranberry supplemented subjects compared to placebo supplemented subjects. We expect this outcome due to the known effects of the PAC polyphenol fraction to bind to LPS and interfere with endocytosis (42) As described in Figure 3, following our standardized

experimental protocol, cells were incubated with fluorescent latex beads (0.05% by volume) for 6 hours and images of each sample taken at 20x magnification. The percentage of cells with at least one bead internalized was calculated to determine the phagocytic ability of M0 macrophages. We also determined the percentage of cells with 4 or more internalized beads to measure the degree of phagocytosis.

We observed a significant increase in phagocytosis in LPS-stimulated versus unstimulated cells as indicated by an increase in the percentage of phagocytic cells (Figure 8B versus 8A). This increase was more apparent when THP-1 macrophages were incubated with serum from all subjects (Figure 8B versus 8A, and 8E versus 8D). However, we observed no significant differences in the percentage of phagocytic cells or the degree of phagocytosis in unstimulated or LPS-stimulated cells incubated with serum from cranberry supplemented subjects versus placebo supplemented subjects. We also saw no significant differences between serum groups in LPS-stimulated cells after data was normalized to unstimulated levels and relative to pre-supplement levels (Figure 8C and 8F). We analyzed sex differences in phagocytosis by THP-1 macrophages but observed no significant differences between males and females (data not shown). These data indicate that cranberry supplementation does not impact phagocytosis of THP-1 macrophages in our assay.

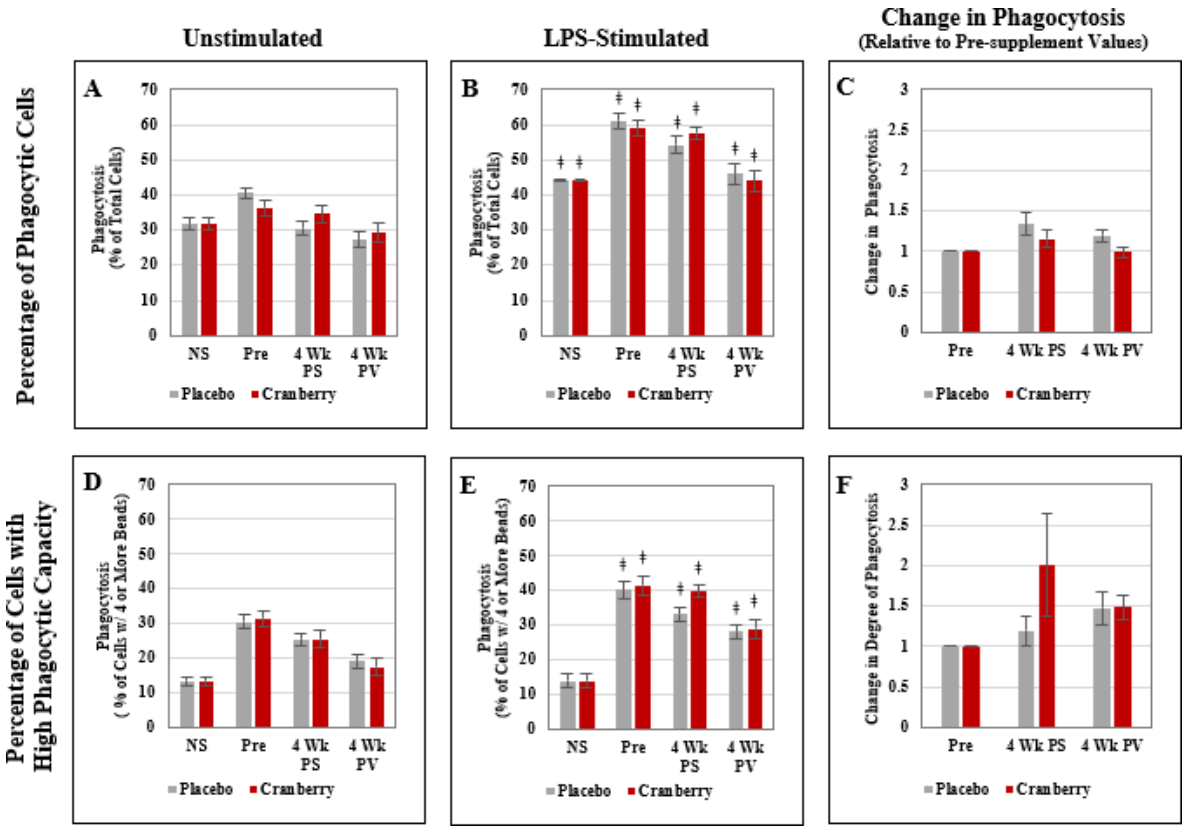

Figure 8. Phagocytic ability of THP-1 macrophages pre-incubated with serum from placebo or cranberry supplemented older adults. THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum from either cranberry or placebo supplemented individuals. Cells were either left unstimulated (A, D) or stimulated with LPS for 24 hours (B, E). Fluorescent latex beads were added at a 0.05% concentration by volume and incubated for 6 hours followed by wash and imaging at 20x. Triplicate representative live cell images were taken for each sample to obtain a sample average. The percentage of cells with internalized beads are shown (A, B). (D, E) represent the percent of cells with 4 or more internalized beads. Phagocytosis was normalized to unstimulated values and is shown as the fold change in phagocytosis in LPS-stimulated cells (C) and fold change in phagocytic degree (F). Time periods evaluated were pre-supplement (Pre), 4 weeks post-supplement (4 Wk PS) and 4 weeks post-vaccine (4 Wk PV). Crosses represent significant differences in phagocytic capacity of LPS-stimulated cells relative to respective unstimulated values in A and D (‡, $P \leq 0.05$).

3.7. Serum From Cranberry Supplemented Subjects Does Not Alter MHCII Expression on THP-1 Macrophages Compared to Serum from Placebo Supplemented Subjects

To determine if macrophage function is altered by cranberry metabolites, we wanted to ascertain if there were any differences in MHCII expression on the surface of THP-1 macrophages. Due to the overall lowered activation of TLR4 and NF-κB observed by other groups following cranberry supplementation, we hypothesized that serum from cranberry supplemented subjects would elicit lower levels of MHCII expression compared to serum from placebo supplemented subjects (23, 42). To test this hypothesis, we incubated unstimulated and LPS-stimulated M0 macrophages with FITC conjugated anti-human HLA-DR (MHCII) antibody followed by flow cytometry analysis. We measured the percent of live cells expressing MHCII as well as the geometric mean fluorescence to determine the degree of MHCII expression on the surface of these cells.

As expected, LPS increased the percent of cells expressing MHCII (Figure 9B versus 9A) and the degree of MHCII expression (Figure 9E versus 9D) as compared to unstimulated cells. We observed no significant differences in MHCII expression in unstimulated cells incubated with serum from cranberry subjects versus placebo subjects (Figure 9A and 9D). In contrast to our expectations, we also observed no significant differences in MHCII expression when LPS-stimulated cells were incubated with serum from cranberry supplemented subjects versus placebo supplemented subjects (Figure 9B and 9E). These results were consistent with those obtained when data was normalized to unstimulated levels and relative to pre-supplement levels (Figure 9C and 9F). As before, there were no significant sex differences in MHCII expression by THP-1 macrophages (data not shown).

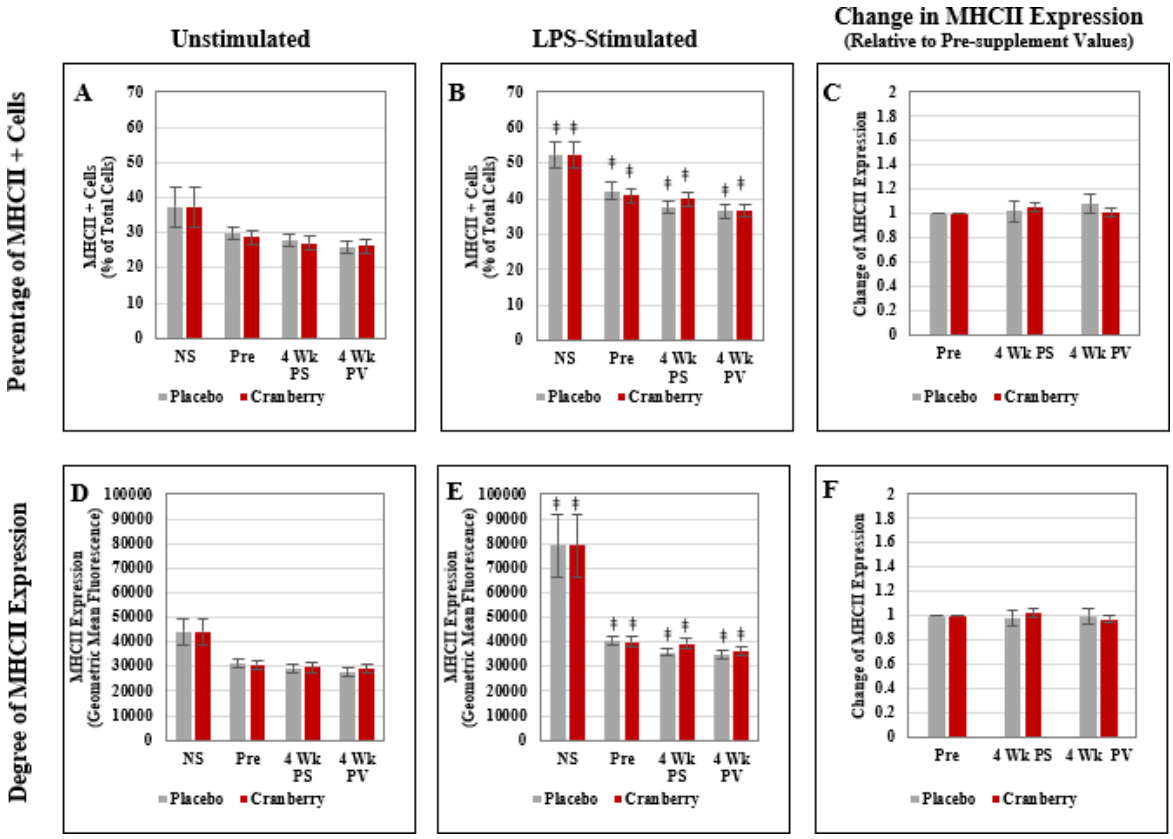

Figure 9. MHCII expression on THP-1 macrophages pre-incubated with serum from placebo or cranberry supplemented older adults THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum from either cranberry or placebo supplemented individuals. Cells were either left unstimulated (A, D) or stimulated with LPS for 24 hours (B, E). Samples were labeled with fluorescent antibodies (FITC anti-human HLA-DR) and analyzed via flow cytometry. The percentage of MHCII positive cells are shown (A, B). (D, E) represent degree of MHCII expression. MHCII expression was normalized to unstimulated values and pre-supplement (Pre) values and is shown as the fold change of MHCII expression on LPS-stimulated cells (C) or fold change in degree of MHCII expression (F). Time periods evaluated were pre-supplement (Pre), 4 weeks post-supplement (4 Wk PS) and 4 weeks post-vaccine (4 Wk PV). Crosses represent significant differences of MHCII expression on LPS-stimulated cells relative to respective unstimulated values in A and D (ǂ, $P \leq 0.05$).

3.8 Serum from Cranberry Supplemented Subjects does Not Alter Costimulatory Molecule Expression on THP-1 Macrophages following LPS Stimulation.

We are not aware of studies that directly investigate the effects of cranberry polyphenols or their metabolites on expression of the costimulatory molecule, CD40. Cranberry polyphenols and their metabolites are known to downregulate NF-κB gene transcription (40). As NF-κB is an inducer of CD40 expression, we hypothesized a reduction of CD40 expression of THP-1

macrophages when incubated with serum from cranberry supplemented subjects versus placebo supplemented subjects (16, 40). To determine if serum from the cranberry supplemented group lead to a difference in CD40 expression on our THP-1 macrophages, we incubated our cells with APC-conjugated anti-human CD40 antibodies following our standard experimental protocol and analyzed the expression of CD40 on the surface of cells via flow cytometry. We measured the percent of cells expressing CD40 as well as geometric mean fluorescence to determine the degree of CD40 expression on these cells.

Similar to results of MHCII expression (Figure 9), LPS stimulation increased the percent (Figure 10B versus 10A) and degree (Figure 10E versus 10D) of CD40 expression compared to unstimulated cells. Serum from cranberry supplemented subjects did not alter CD40 expression in unstimulated THP-I macrophages (Figure 10A). However, serum from cranberry supplemented subjects did lead to an increase in the degree of CD40 expression in unstimulated THP-1 macrophages at 4 weeks post supplementation and 4 weeks post vaccination (Figure 10D). We also observed that exposure to serum dampened the effect of LPS stimulation (Figure 10E), as CD40 expression is generally much lower ($P \leq 0.001$) when incubated with serum from both groups as compared to serum free controls (NS). Again, consistent with results of MHCII expression in Figure 9, there were no significant differences between cranberry and placebo groups in LPS-stimulated cells after normalization to unstimulated levels and relative to pre-supplement values (Figure 10C and 10F). We also analyzed sex differences in CD40 expression by THP-1 macrophages but observed no significant differences between males and females (data not shown).

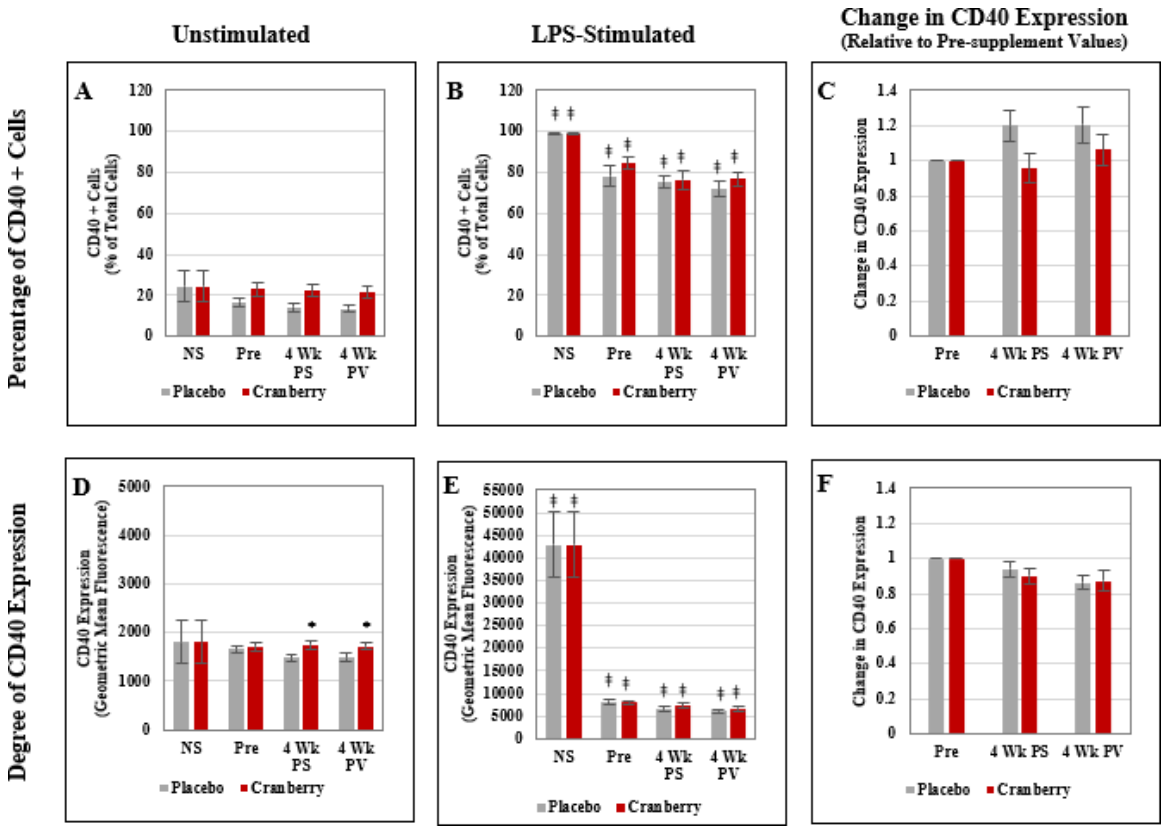

Figure 10. CD40 expression on THP-1 macrophages pre-incubated with serum from placebo or cranberry supplemented older adults. THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum from either cranberry or placebo supplemented individuals. Cells were either left unstimulated (A, D) or stimulated with LPS for 24 hours (B, E). Samples were labeled with fluorescent antibodies (APC anti-human CD40) and analyzed via flow cytometry. The percentage of CD40 positive cells are shown (A, B). (D, E) represent degree of CD40 expression. CD40 expression was normalized to unstimulated values and pre-supplement (Pre) values and is shown as the fold change of CD40 expression on LPS-stimulated cells (C) or fold change in degree of CD40 expression (F). Time periods evaluated were pre-supplement (Pre), 4 weeks post-supplement (4 Wk PS) and 4 weeks post-vaccine (4 Wk PV). Asterisks indicate significant differences between placebo and cranberry groups (within time series) (*, $P \leq 0.05$) Crosses represent significant differences of CD40 expression on LPS-stimulated cells relative to respective unstimulated values in A and D (ǂ, $P \leq 0.05$).

We also hypothesized that serum from cranberry supplemented subjects would decrease CD80 expression due to the effect of unmetabolized cranberry polyphenols on inhibition of TLR4 pathways (23, 42, 46). Similar to the protocol for evaluating CD40 expression, we determined whether serum from cranberry supplemented group alters CD80 expression in our THP-1 macrophages.

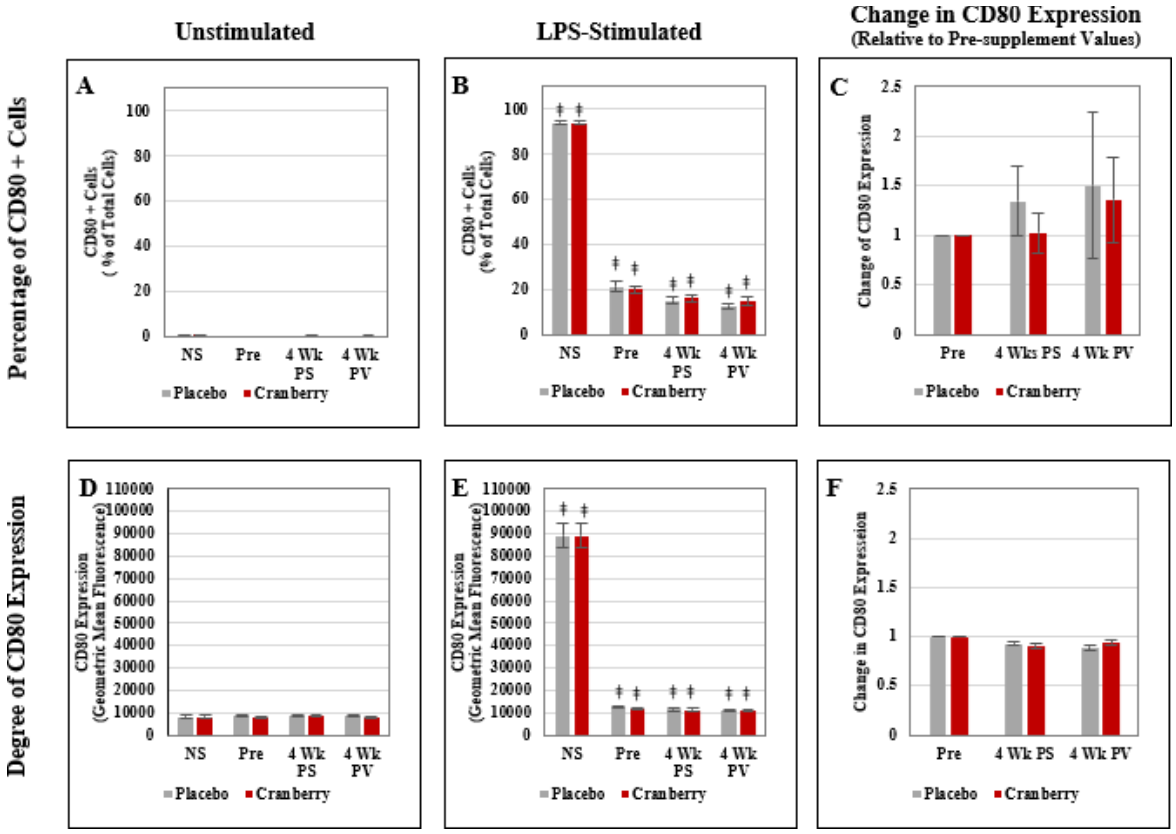

Figure 11. CD80 expression on THP-1 macrophages pre-incubated with serum from placebo or cranberry supplemented older adults. THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum from either cranberry or placebo supplemented individuals. Cells were either left unstimulated (A,D) or stimulated with LPS for 24 hours (B,E). Samples were labeled with fluorescent antibodies (PE anti-human CD80) and analyzed via flow cytometry. The percentage of CD80 positive cells are shown (A, B). (D, E) represents the degree of CD80 expression. CD80 expression was normalized to unstimulated values and pre-supplement (Pre) values and is shown as the fold change of CD80 expression on LPS-stimulated cells (C) or fold change in degree of CD80 expression (F). Time periods evaluated were pre-supplement (Pre), 4 weeks post-supplement (4 Wk PS) and 4 weeks post-vaccine (4 Wk PV). Crosses represent significant differences of CD80 expression on LPS-stimulated cells relative to respective unstimulated values in A and D (ǂ, $P \leq 0.05$).

As expected, LPS stimulation significantly increased the percent (Figure 11B versus 11A) and degree (Figure 11E versus 11D) of CD80 expression compared to unstimulated cells. We observed no significant differences in CD80 expression in unstimulated cells incubated with serum from cranberry supplemented subjects versus placebo supplemented subjects (Figure 11A and 11D). We also observed no significant differences in CD80 expression in LPS-stimulated

cells between groups (Figure 11B and 11E). However, we observed a dampening of the effect of LPS stimulation with serum in Figures 11B and 11E, as CD80 expression with serum is generally much lower ($P \leq 0.001$) as compared to serum free controls (NS). Importantly, when results from LPS-stimulated cells were normalized to their unstimulated values and relative to pre-supplement values (Figure 11C and 11F), we also saw no significant differences between groups in CD80 expression of THP-1 macrophages, nor did we observe any sex differences between groups (data not shown). Overall, these results indicate that cranberry metabolites in serum from supplemented subjects did not impact the expression of costimulatory molecules.

3.9 Serum from Cranberry Supplemented Subjects does not Reduce the Expression of IL-6

Cranberry polyphenols and metabolites are known to exert an anti-inflammatory effect on macrophages by downregulating expression of several inflammatory cytokines, including IL-6 (41). To explore how IL-6 expression of THP-1 macrophages may be altered by serum from cranberry supplemented subjects versus placebo supplemented subjects, we followed our standardized protocol and utilized a sandwich ELISA to determine IL-6 expression. We hypothesized that we would observe a reduction in IL-6 expression of our THP-1 macrophages when incubated with serum from cranberry supplemented subjects as compared to serum from placebo supplemented subjects.

Our results show LPS stimulation significantly increased IL-6 expression by THP-1 macrophages compared to unstimulated cells (Figure 12B versus 12A). We also saw a dampening effect on LPS stimulation with serum in Figures 12B, as IL-6 expression with serum is generally much lower ($P \leq 0.001$) as compared to serum free controls (NS). We did not see any significant differences between groups in IL-6 expression before or following LPS stimulation of THP-1 macrophages (Figure 12A and 12B). When results from LPS-stimulated

cells were normalized to their unstimulated values, relative to pre-supplement values (Figure 12C), we unexpectedly also saw no significant differences between cranberry or placebo groups. We also analyzed sex differences in IL-6 expression by THP-1 macrophages but observed no significant differences between males and females (data not shown).

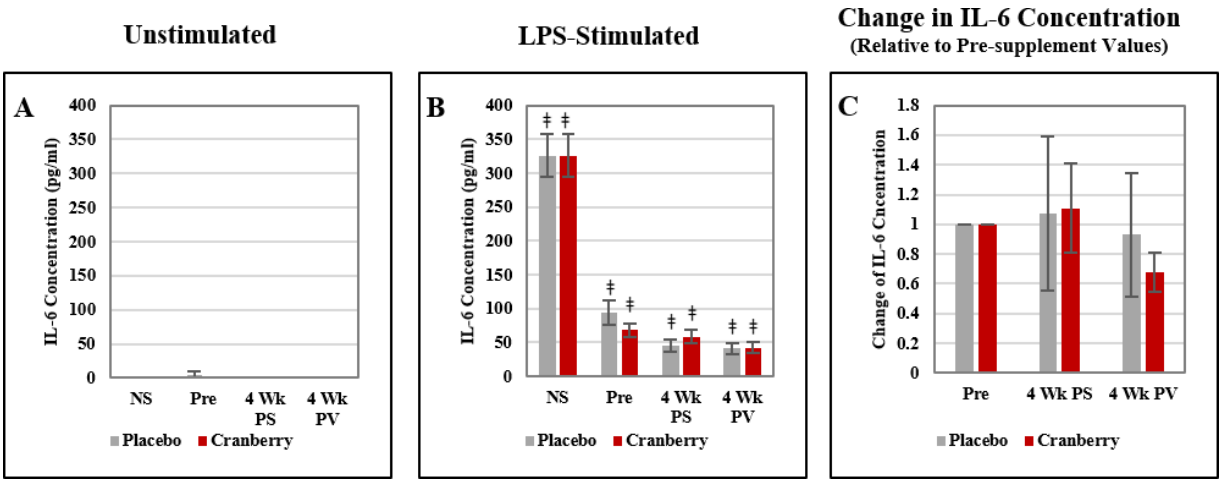

Figure 12. IL-6 expression of THP-1 macrophages pre-incubated with serum from placebo or cranberry supplemented older adults. THP-1 macrophages (M0s) were pre-incubated with no added human serum (NS) or with 40% human serum from either cranberry or placebo supplemented individuals. Cells were either left unstimulated (A) or stimulated with LPS for 24 hours (B). Supernatants were collected and frozen (20°C) and later thawed and diluted in 1X PBS at a 1:5 concentration. IL-6 concentrations of diluted samples were measured using a sandwich ELISA assay. IL-6 concentration is shown (A, B). IL-6 concentration was normalized to unstimulated values and pre-supplement (Pre) values and is shown as the fold change of IL-6 concentration in LPS-stimulated cells (C). Time periods evaluated were pre-supplement (Pre), 4 weeks post-supplement (4 Wk PS) and 4 weeks post-vaccine (4 Wk PV). Crosses represent significant differences of IL-6 expression on LPS-stimulated cells relative to respective unstimulated values in A. (ǂ, $P \leq 0.05$)

Overall, these data indicate that serum from older individuals promotes macrophage dysfunction by decreasing their phagocytic ability and lowering expression of antigen presenting molecules and costimulatory molecules. Furthermore, cranberry polyphenols or metabolites in serum from supplemented older subjects do not impact macrophage functions and are key factors to promote adaptive immunity.

Chapter 4

4. Discussion

4.1 Serum from Older Subjects Imparts Dysfunction on THP-1 Macrophages

The goal of this study was to determine the ability of cranberry supplementation to promote anti-inflammatory activity in a group of older, overweight individuals using an LPS-stimulated model of inflammation in THP-1 cells. Numerous studies have reported that this is a high-risk group for inflammatory diseases and does not respond efficiently to vaccines. Therefore, our intent was to test the effects of serum from cranberry or placebo groups to alleviate LPS-induced inflammation of THP-1 macrophages or alter immune function following influenza vaccination. However, we first wanted to investigate the baseline levels of inflammation in this older group as compared to a younger subject group to confirm whether age impacts inflammatory responses in our model system. Our results agreed with numerous studies demonstrating that age and obesity significantly affect the function of macrophages (20, 25). We observed specific differences in inflammatory and functional markers of THP-1 macrophages when incubated with serum from older, overweight or obese subjects versus serum from younger, more active subjects. Many of these differences support our hypotheses that age and obesity lead to macrophage dysfunction. Our results are summarized in Table 1.

Our results indicated that exposing THP-1 macrophages to serum from both groups exerted a protective effect on cells following exposure to LPS (Figure 2A and 2B). Because human serum contains growth hormones and vitamins, this may explain the positive effect on viability of THP-1 cells. Unexpectedly, we observed that unstimulated THP-1 macrophages exposed to serum from older subjects had significantly higher viability as compared to when they

Function	Measurement	Older vs. Younger (No stimulation)	Older vs. Younger (LPS Stimulation)	Older vs. Younger (Fold Change)
Cell Viability		Higher in older subjects*	Lower in older subjects Higher in females	No differences*
Phagocytic Ability	Percentage of phagocytic cells	No differences*	Lower in older subjects*	No differences*
	Degree of phagocytosis	Higher in older subjects*	Higher in older subjects*	Lower in older subjects
Surface Molecule Expression	MHCII (% to total cells)	Lower in older subjects*	Higher in older subjects*	Lower in older subjects
	MHCII (degree of expression)	Higher in older subjects*	Higher in older subjects*	Lower in older subjects Higher in females
	CD40 (% to total cells)	Not assessed	Not assessed	Not assessed
	CD40 (degree of expression)	Not assessed	Not assessed	Not assessed
	CD80 (% to total cells)	Lower in older subjects*	Lower in older subject*	No differences*
	CD80 (degree of expression)	Higher in older subjects*	Higher in older subjects*	Lower in older subjects Lower in females
Cytokine Production (IL-6)		Not assessed	Not assessed	Not assessed

*No significant sex differences observed

Table 1: Data comparing the effects of serum from older versus younger subjects on THP-1 macrophages. Blue indicates an increase in function in serum from older subjects and **red** indicates a reduction in function. Main sex differences are included.

were exposed to serum from younger subjects. However, this effect changed with LPS stimulation, where we observed that the viability of macrophages was higher when incubated with serum from younger subjects. This result indicates that serum from older subjects does not offer as much protection to THP-1 cells from the cell killing effects of LPS, as compared to serum from younger individuals.

Studies have shown that LPS has two mechanisms for inducing cell death in THP-1 macrophages. One mechanism is through the increase in autocrine secretion of TNFα by macrophages following stimulation and the other is through increased oxidative stresses on cells due to an increase in nitric oxide production (52). TNFα expression is triggered in LPS-stimulated macrophages via activation of the Type I p55 TNFα receptor mediated pathway. The TNFα expression pathway triggers apoptosis via inhibiting protein synthesis, inducing the release of ceramides (triggering autophagy), increasing reactive oxygen intermediaries, activating caspase 3 (an enzyme that breaks down protein), and inducing DNA fragmentation

(53, 54, 55). Nitric oxide production is associated with the expression of p53 and Bax, which are genes that are upregulated in apoptosis. Bax expression alone is sufficient to activate killer proteases (54). The release of NO also initiates cell death through oxidative stress as well as disrupted energy metabolism and DNA damage (56). Studies have shown that older individuals have higher levels of inflammatory molecules such as TNFα, CRP and IL-6 (19). Therefore, a possible explanation for the differences in cell viability we observed between older versus younger groups may be due to the higher expression of TNFα which triggers cell apoptosis and may be exerting an additive effect to LPS-induced oxidative stresses. Regardless of these differences, we still saw a strong protective effect of serum from both groups against LPS-induced cell death. This suggests that the negative impact of LPS on the viability of THP-1 macrophages is alleviated by human serum.

Functional differences in THP-1 macrophages were also observed when THP-1 cells were exposed to serum from different age groups. Results of our study showed that serum from older subjects decreased the degree of phagocytosis in LPS-stimulated THP-1 cells when compared to serum from younger subjects (Figure 3G and Table 1). This result is consistent with our hypothesis and agrees with studies that show that macrophages from older individuals have diminished capacity to undergo phagocytosis as compared to macrophages from younger individuals (20). The underlying mechanism of this effect is not fully understood but one factor may be the differences in anti-inflammatory cytokine levels in the environment that these cells reside in. A study showed that when macrophages from younger mice were transferred into the peritoneum of older mice, they experienced a decline in phagocytosis compared to controls in younger mice. Furthermore, when macrophages from older mice were transferred into the peritoneum of younger mice, the macrophages did not undergo a reduction in phagocytosis. The

authors of this study hypothesized that the mechanism of this effect was higher expression of IL-10 in the older microenvironment versus the younger microenvironment, but this response may differ dependent on tissue or serum type (20, 57). Additionally, the drop in cell viability seen in THP-1 cells incubated with serum from older subjects versus younger subjects may also play a role in our results, perhaps due to greater oxidative stress contributing to LPS-induced cytotoxicity. (52, 54). All of these mechanisms may play some role in the reduction we see in phagocytic ability of THP- 1 cells exposed to serum from older subjects.

In addition to dysfunctions in cell viability and phagocytosis, we also observed that serum from older individuals decreased the expression of surface molecules associated with antigen presentation and T-cell functions (MHCII, CD80) on the surface of macrophages, following LPS stimulation. This further highlights an age-related effect on immune function. While this result is consistent with our hypothesis, we did consider that since data from younger subjects was collected prior to the data for older subjects, there may be some experimental inconsistencies. Therefore, we place a heavier emphasis on the results we observed following normalization to unstimulated values and serum free controls.

We observed that LPS-stimulated macrophages exposed to serum from older subjects showed a lower percentage of cells expressing MHCII, as well as a lower degree of MHCII expression, relative to serum from younger subjects (Figure 4C and 4F, and Table 1). These results agree with our hypothesis and other studies that show declines in macrophage surface expression of MHCII in older mice and humans (3, 23, 58). Following LPS stimulation, inflammatory gene transcription increases as does the production of endogenous IFNγ, which binds to the interferon gamma protein receptor (IFNGR) at the cell surface to produce a secondary signaling cascade which results in increased expression of MHCII (59). A study of

IFNγ-induced MHCII gene expression revealed that MHCII IAß gene transcription in aged mice is lower than in younger mice (12). Our study suggests that the reduced ability to express antigens when macrophages were exposed to serum from older individuals when compared to younger individuals may lead to a degree of immune suppression.

A study in healthy aged individuals showed there was a tendency toward an increase in the percent of cells expressing CD80 on peripheral blood monocyte cells (PBMCs) (23). Based on the results of this study we hypothesized that THP-1 macrophages would express higher levels of CD80 when exposed to serum from older subjects versus serum from younger subjects. However, this hypothesis was not supported in our results as we observed a lower degree of CD80 expression of cells when exposed to serum from older subjects (Figure 5F and Table 1) (23). A factor that may be contributing to this result may be the higher exercise levels of the younger individuals. Studies have shown that acute bouts of aerobic exercise can enhance immune surveillance through the temporary increase in TNFα and NO production (19, 32). However, we did not measure the serum levels of TNFα or NO in either of our subject groups and our younger subjects may or may not be reaching high enough intensity or duration of exercise to see this effect. Regardless, a moderate 12-week exercise program can specifically increase CD80 expression in monocytes in humans (60). While it is thought that upregulation of NF-κB may be causing an increase in CD80 levels following exercise, the authors of this study point out that exercise should lead to a decrease in NF-κB activity. Therefore, more research is needed to fully understand the mechanisms behind these observations (60).

An additional question we wanted to investigate was the effects of serum from females versus males on macrophage functional markers in our older subjects versus younger subjects. We observed that serum from female subjects exerted a stronger protective effect against the

cytotoxic effects of LPS on THP-1 macrophages, compared to male serum (Figure 2E). Literature review suggests that females may have lower levels of oxidative stress than males, with lower levels of stress biomarkers, lower ROS production, and greater antioxidant potential than males (61). While these differences may be responsible for the protective effect of serum seen in our study, more research is needed to fully investigate this effect. Regardless, because this outcome was seen when data from our older and younger subject groups were combined, this effect could be influencing results of our cranberry supplementation study, but a deeper investigation on this effect would be required to determine if this sex-linked difference imparts a significant effect on our results.

We also had expected to see some sex-linked effects on macrophage functional markers, including increased levels of phagocytosis and CD80 expression in THP-1 macrophages exposed to serum from females as opposed to males in both older and younger subject groups. Phagocytic ability has been observed in studies to be higher in females compared to males as they are more responsive to stimuli which is thought to be related to higher TLR expression in females and differences in NF-κB activity influencing antigen presentation and costimulatory molecule expression (37). Additionally, other studies have shown that CD80 expression in females is also higher than that of males, as seen in one study of peritoneal macrophages from mice infected with tapeworms (39). Again, this is thought to be due to higher TLR expression in females as well as sex-steroid differences. Unexpectedly, we saw that sex differences did not influence results when evaluating phagocytic ability of THP-1 cells using serum from either older or younger subjects. As our sample size is small, 10 or less individuals per group, it may be that our study did not have the statistical power to detect an effect. However, we did observe that CD80 expression was higher on macrophages exposed to male serum compared to female serum in our

younger, active cohort. An explanation as to why we observed this unexpected outcome may be related to the overall high levels of exercise that the younger group is exerting and possible differential effects of sex and exercise on CD80 expression.

Additionally, we had anticipated that THP-1 cells exposed to serum from older females would express lower levels of MHCII expression than older males, with the reverse observation seen when using serum from younger subjects. Studies of peritoneal mouse macrophages show a similar situation, with a mixed effect of age and sex on MHCII expression (38). The effect of 17ß Estradiol (E2) is thought to be a strong factor in the results of the above referenced study, as E2 is produced in the ovaries and known to increase levels of IFNγ concentrations in mouse splenocytes (62). IFNγ gamma may be sufficient to tip the scales toward higher MHCII expression in young females, as IFNγ is also known to increase MHCII expression (62, 63). While we did not see sex-linked differences in our study, we did however see higher MHCII expression in THP-1 macrophages when using serum from females versus males when both age groups were combined. Again, the small sample size of our study may be limiting the statistical power of our study to detect an effect within each age group, but larger sample sizes would need to be tested to explore this idea farther. Because there were not sex-linked differences within our older age group, this also implies that sex is not a confounding factor in our cranberry study results investigating MHCII expression.

As we observed some unexpected results when investigating sex-linked differences on functional markers associated with age, this also highlights the possibility that there may be more complex interactions influencing these sex-linked differences, including interactions of exercise superimposed on sex and age differences. It also suggests that a larger sample size investigating sex differences may be of value to future studies, as some anticipated sex-linked effects weren't

seen. Regardless, differences we observed associated with age and sex are consistent with previous results indicating there is macrophage dysfunction in older individuals when comparing against younger individuals.

4.2 Serum from Cranberry Supplemented Subjects does Not Alter Macrophage Function or Decrease Inflammation

An additional objective of this study was to determine if cranberry supplementation could alleviate immune dysfunction in older subjects. We hypothesized that serum from cranberry supplemented subjects would reduce inflammatory and functional markers in LPS-stimulated THP-1 macrophages as compared to that from placebo individuals. However, we did not observe any differences in macrophage cell viability, phagocytic capacity, and expression of MCHII, CD40, CD80, and IL-6 when exposed to serum from cranberry versus placebo supplemented older adults. A summary of these results is shown in Table 2.

Function	Measurement	Placebo vs. Cranberry (No stimulation)	Placebo vs. Cranberry (LPS Stimulation)	Placebo vs. Cranberry (Fold Change)
Cell Viability		No differences*	No differences*	Lower with cranberry
Phagocytic Ability	Percentage of phagocytic cell	No differences*	No differences*	No differences*
	Degree of phagocytosis	No differences*	No differences*	No differences*
Surface Molecule Expression	MHCII (% to total cells)	No differences*	No differences*	No differences*
	MHCII (degree of expression)	No differences*	No differences*	No differences*
	CD40 (% to total cells)	No differences*	No differences*	No differences*
	CD40 (degree of expression)	Higher with cranberry	No differences*	No differences*
	CD80 (% to total cells)	No differences*	No differences*	No differences*
	CD80 (degree of expression)	No differences*	No differences*	No differences*
Cytokine Production (IL-6)		No differences*	No differences*	No differences*

*No significant sex differences observed

Table 2: Data comparing the effects of serum from cranberry supplemented subjects versus from placebo supplemented subjects on THP-1 macrophages. Blue indicates an increase in function in serum from cranberry subjects and **red** indicates a reduction in function. Main sex differences are included.

Exposing THP-1 macrophages to serum from both groups exerted a protective effect on THP-1 macrophages following exposure to LPS, both before and after vaccination (Figure 7B). Again, this is likely because of the growth hormones and vitamins found in human serum. Unexpectedly, we observed that LPS-stimulated THP-1 macrophages exposed to serum from cranberry subjects had significantly lower viability, following normalization to unstimulated values, as compared to exposure to serum from placebo subjects (Figure 7C and Table 2). Previous studies that exposed stimulated THP-1 macrophages to cranberry A-type PACs (up to 100 μg/ml) saw no cytotoxic effects within 24 hours (41). A hypothesized reason for the differences between our results and the previous study is that our cells were exposed to a variety of metabolized compounds in the serum which may have resulted in an additive effect to reduce cell viability. Additionally, when the macrophages in the previous study were exposed to concentrations of 25 and 50 μg/ml of the A-type PAC fraction for longer time points, cell viability decreased (41). Sufficiently high levels of these polyphenolic compounds in the serum of our subjects may explain this enhanced cell-killing effect, but this information is currently unknown.

We also examined the effects of serum from cranberry supplemented subjects on the phagocytic ability of THP-1 macrophages. We are unaware of any studies that have investigated the effects of cranberry metabolites on phagocytosis but expected to observe a decrease in phagocytosis based on previous studies indicating that the PAC fraction of cranberry polyphenols interferes with TLR4 signaling, which is required to initiate phagocytosis (42). However, we observed that there were no differences in phagocytic ability between groups either before or after vaccination (Figure 8), suggesting that cranberry supplementation does not affect this function of macrophages, even after an inflammatory stimulus like vaccination.

The expression of MHCII on THP-1 macrophages in response to serum from cranberry supplemented subject was examined to determine impacts on antigen presentation. We expected that MHCII expression would be reduced on macrophages exposed to serum from cranberry supplemented subjects versus serum from placebo subjects due to the disruption in TLR4 signaling by the PAC cranberry fraction. The recognition of LPS by TLR4 and subsequent signaling cascade is a necessary step for antigen presentation (23, 42). The mechanism behind this disruption is thought to be due to the binding of the PAC fraction to LPS thereby blocking its interaction with TLR4 on the cell surface (42). Additionally, a reduction of endogenous produced inflammatory cytokines, such as IFNγ, which is associated with enhancing MHCII expression following cranberry supplementation, may also contribute to this effect (11,12). However, our results showed no differences between serum from either group either before or after vaccination (Figure 9 and Table 2) perhaps due to inadequate dosage of cranberry polyphenols and variability in individual metabolism of polyphenols.

Similar to results obtained from the analysis of MHCII, we did not observe significant impacts of cranberry supplementation on expression of the costimulatory molecules CD40 and CD80. We expected to see a reduction in the expression of these molecules on THP-1 macrophages due the ability of cranberry polyphenols and metabolites, like ferulic acid, to downregulate expression of NF-κB and STAT-1α signaling pathways necessary for expression of CD40 (16, 46). Additionally, we expected to see a reduction in CD80 expression on THP-1 macrophages due to the ability of quercetin and kaempferol metabolites, associated with cranberry supplementation, to downregulate the MAPK inflammatory pathway, which is thought to be necessary for the expression of CD80 (47, 48, 64). Furthermore, the cranberry PAC fraction is known to disrupt the recognition of LPS by TLR4 at the cell surface, leading to

suppression of CD80 expression (42). Interestingly, we did observe a modest increase in expression of CD40 on unstimulated THP-1 cells suggesting that cranberry metabolites could be triggering an increase in baseline STAT-1α expression to upregulate transcription of CD40 (Figure 10D) (16). However, because this effect is not seen following LPS stimulation, this may not be an effect that serves to strongly enhance the function of macrophages following vaccination or when fighting infections (14).

One of our main goals was to determine whether cranberry metabolites promoted anti-inflammatory effects following LPS-stimulation by measuring levels of the inflammatory cytokine IL-6. IL-6 expression is known to increase following exposure of THP-1 macrophages to LPS and circulating levels of this cytokine are elevated during the aging process (6,19). In addition, numerous studies that demonstrated cranberry polyphenols and their metabolites lead to a reduction in IL-6 and NF-κB expression (40, 41, 46). Contrary to our hypothesis, we did not observe any differences in IL-6 expression when macrophages were exposed to serum from cranberry versus placebo supplemented subjects prior to and after vaccination (Figure 12 and Table 2). This result is consistent with our previous findings indicating that serum from cranberry supplemented older, overweight individuals did not exert anti-inflammatory or immunomodulatory effects on THP-1 macrophages.

One of our goals was to determine if there are sex linked interactions during cranberry supplementation that are influencing our study results both after initial supplementation but also post-vaccination. Previous studies have shown that macrophages from females typically have higher TLR expression, which may increase phagocytic function (37), as well as lower expression of MHCII versus male mice (38). However, we did not observe any sex-related differences in our analyses of serum from cranberry versus placebo subjects. The sample sizes in

male and female groups were approximately 10 individuals in each group, which may be too limited to detect slight differences. However, similar to our studies in younger and older subjects, these results indicate males and females are not differentially susceptible to the effects of cranberry supplementation on THP-1 macrophages.

4.3 Factors Influencing Results from the Cranberry Study

Overall, our results indicate that cranberry supplementation does not alleviate LPS-induced inflammation or affect macrophage functionality in our model system. These results are consistent with those carried out in the Human Performance Laboratory showing that cranberry supplementation of these subjects does not impact immunity even with doses at levels that should elicit a response. Early unpublished results of metabolite content in urine samples taken from cranberry supplemented subjects shows that levels of cranberry metabolites were consistently over twice the levels found in urine of placebos subjects. Hippuric acid, 4-hydroxycinnamic acid, and 3,4-dihydroxybenzoic acid are three metabolites that showed statistically higher levels after supplementation with cranberry versus placebo. Additionally, currently unpublished results of cytokine levels in subjects treated with cranberry versus placebo supplements also show no statistically significant differences in IL-6, IL-10, IL-1ß, IL-17, and IL-18 concentrations between groups, including after vaccination. As vaccination is an inflammatory stimulus, if any anti-inflammatory effects of cranberry supplementation existed, then we would likely see significant differences between groups in some or all these cytokine concentrations. Furthermore, early unpublished results of a viral replication assay using serum from cranberry versus placebo supplemented subjects, showed no significant differences between treatment groups on immunoglobulin levels. Combined with these results, our study

reinforces the conclusion that cranberry supplementation does not impact immune responses in this cohort of subjects.

While we were confident our results in this inflammation model system show no anti-inflammatory or immunomodulatory effects of serum from cranberry supplementation, we did have several possible study limitations to consider: bioavailability of cranberry polyphenols and metabolites in serum samples, individual variability in metabolite production in serum, sample sizes limiting our statistical power, and the possibility that this model was not sensitive enough to respond to cranberry metabolites.

First, depending on the cranberry polyphenol and its metabolism, inadequate levels of metabolites may be bioavailable after a 24-hour period to exert any systemic anti-inflammatory or immunomodulatory effects. Many cranberry metabolites such as cinnamic acid aglycones, benzoic acids, dihydrocinnamic acids, cinnamic acids, flavanols, hippuric acids, catehols, and phenyacetic acids reach their maximum concentration at 5 to 10 hours after ingestion (44). Timing of the blood draw related to the last ingestion of cranberry supplement could therefore be a contributing factor to our results. However, this is an unlikely scenario in our study because each subject was instructed to take a split daily dose, and blood draws were overnight fasted, which should still result in high levels of circulating metabolites.

The dose of the cranberry supplement also affects overall plasma and urine concentrations of polyphenols and their metabolites. For example, in a similar cell model study of inflammation, cranberry fractions whose total PAC A2 content was 183-317 μg/ml were preincubated (at concentrations of 0.1, 1.0, 10, and 100 μg/ml) with PMA-differentiated THP-1 macrophages for 6 hours, followed by LPS stimulation for 24 hours. Unlike our study, they did find a significant reduction in IL-6 expression of THP-1 macrophages using the 10 and 100

μg/ml samples, but this effect was not seen at the 1.0 μg/ml or lower concentrations of these fractions (48). Moreover, when evaluating the actual content of the PAC A2 fraction found in serum after a cranberry juice supplementation, polyphenol concentrations can be much lower than necessary for activity. For example, another study that assessed the levels of the PAC A2 fraction of cranberry polyphenols, following 237 ml double strength cranberry juice consumption, found that maximum PAC A2 levels peaked in urine at an average of 24 ng/ml at 11 hours post consumption and had less than 2.5 ng/ml of the PAC A2 fraction in plasma post consumption. The concentration found in urine is approximately 416 times lower than what the previously mentioned study indicated as the lowest effective dose, with the amount of PAC A2 in plasma being even smaller (65). To complicate matters, most of the PAC A2 fraction is metabolized to several secondary compounds. Therefore, studies that have shown reductions in inflammatory parameters in cell models following cranberry supplementation may not necessarily be measuring the impacts of bioavailable and metabolized compounds found in serum following supplementation as our study does.

Connected to the issues mentioned above, both the dosage and timing of blood draw affect the concentrations of cranberry polyphenols and their metabolites in serum. The estimated daily polyphenol content consumed in our study was approximately 500 mg which is a very high dose. However, cell culture studies that have demonstrated anti-inflammatory effects are utilizing levels of parent polyphenols (not metabolites) that are not produced at such high levels in serum following ingestion. This may be the most likely reason we see different results in our study compared to other cell models.

It is known that an individual's personal metabolism also impacts the amounts of metabolites present in the serum after ingestion. For example, studies have shown even when

subjects consumed a cranberry beverage of a standardized concentration of polyphenols, there was a high degree of variability between metabolite concentrations found in plasma. Some individuals had a coefficient of variance (ratio of the standard deviation to the mean) of up to 216%. Such variability is related to an individual's ability to generate each metabolite, which is dependent on sex, gut microbiome, genetics, and other environmental influences (44). However, unpublished results of metabolite content in urine samples taken from these cranberry supplemented subjects, shows that levels of cranberry metabolites were already significantly higher than the levels found in urine of placebos subjects. This suggests that variability in metabolism of polyphenols may not be a strong factor in our study, and that we are seeing a true effect with our results.

Lastly, using THP-1 macrophages as a model for inflammation may also be a limitation to understanding the full anti-inflammatory effects of cranberry polyphenols and their metabolites. Using a single cell line like THP-1 macrophages or PBMCs may simplify experiments, but they have limitations, as they only show effects of these compounds on a small subset of cells. In addition, by the time that cranberry polyphenols and their metabolites are disseminated in serum, levels may be too low to elicit any effects. Additionally, diet, exercise, weight, and other environmental factors that affect inflammation can only be controlled loosely in human subjects. However, mouse models may be an alternative to consider in future studies as we can more closely control for diet, weight, age, activity, and amount of outside dietary polyphenol ingestion. Additionally, in mouse models, we can investigate specific effects of cranberries on tissue resident immune cells like macrophages via dissection and histology and or disaggregation of tissue into single cell suspensions for analysis via flow cytometry. This could yield tissue specific immune cell effects, beyond macrophages, in areas like the GI tract which

would presumably also have more direct contact with higher concentrations of polyphenols and their metabolites after ingestion. Additionally, aspects of the interaction of the gut microbiome and cranberry polyphenol metabolism could also be investigated concurrently with a mouse model system.

Overall, we see that there are many factors that could be influencing our results. However, our randomized experimental setup as well as careful study design should control for many of these potential confounding factors. Regardless, future studies related to ours should consider our study limitations and successes when designing experiments investigating the effects of serum from cranberry supplemented subjects.

4.3 Additional Intervention Strategies to Reduce Inflammation

Although we did not detect anti-inflammatory or immunomodulator effects in our cranberry supplementation study, there may be additional intervention strategies that, in synergy with cranberry supplementation, may exert benefits. For example, numerous studies have shown that moderate routine exercise and better general fitness can lead to a downregulation of inflammatory signaling pathways and the promotion of a more anti-inflammatory state in macrophages (30, 31). Also, the effects of moderate exercise in short bursts results in increases in the phagocytic capacity of macrophages (32). This lifestyle change is just one of several that can be utilized to promote anti-inflammatory effects in older populations.

In addition to the potential effect of exercise, weight loss also exerts a positive effect on immune and macrophage function. As adipocytes in obese individuals express higher levels of inflammatory markers such as IL-6 and TNFα, it is reasonable to expect that weight loss may lead to a reduction of these inflammatory markers in adipocyte resident macrophages (25). This may produce a stronger effect when combined with exercise and cranberry supplementation.

Weight loss has been associated with an alteration in the number of tissue resident macrophages in adipose tissues, as demonstrated in a study of mice that showed that weight loss and a calorie restricted diet leads to lower adipose tissue macrophage numbers (66). Weight loss alone has also been shown in studies to lead to a reduction in blood circulating inflammatory markers such as CRP, IL-6 and TNFα (67).

One important point that our study demonstrates is that dietary modifications beyond calorie restriction and weight loss are also another intervention strategy that on its own or in combination with the previous strategies, is likely to reduce levels of systemic inflammation. Dietary modifications in older subjects can be of benefit to reduce systemic inflammation. Diets that are high in saturated and trans fats are associated with higher production of inflammatory markers in blood such as high sensitivity CRP (HS-CRP) and TNFα. Additionally, high glycemic diets are also associated with an increase in HS-CRP levels, whereas a high fiber low glycemic diet is associated with low levels of HS-CRP, IL-6 and TNFα (68). An increase in the overall consumption of fruits and vegetables, flavonoids, and carotenoids, are also associated with lower levels of HS-CRP and IL-6 levels (68). Dietary alterations alone are a positive influence on reducing inflammatory markers but the combined effects of diet, weight loss and moderate exercise may provide additive or synergistic effects that contribute to the reduction of chronic inflammation in high-risk individuals.

4.4 Future Studies

Because the amounts of bioavailable polyphenols and metabolites in serum may be a factor in our study results, quantification of common cranberry metabolites in these serum samples would help determine the polyphenol and metabolite levels that our cells were exposed to. We already have unpublished results showing that urine metabolites are significantly higher

in the cranberry supplemented group versus the placebo group, but quantification of levels in serum would further support that the dose of polyphenols was sufficiently large to see differences in serum levels as well. Regardless, future studies may add these measurements to their design to aid in validation of results.

Future THP-1 cell model studies could also investigate the anti-inflammatory effects of specific polyphenol fractions and metabolites at concentrations found in serum. Many of these molecules are available for purchase commercially. Designing additional studies in subjects based on molecules found at highest concentrations in serum would focus future research on the most likely molecules to succeed in producing anti-inflammatory effects.

Future studies in animal models and humans may also consider evaluating the effects of lifestyle changes on chronic inflammation such as the addition of dietary modification, exercise and weight loss. This may produce anti-inflammatory and immunomodulatory results without medications and lead to results like those in studies showing anti-inflammatory effects of a healthy low saturated fat diet, maintaining a healthy weight, and routine moderate exercise.

In conclusion, while this study did not determine that serum metabolites from cranberries were immunomodulatory or anti-inflammatory to THP-1 macrophages at our current dosage, we have suggested that other lifestyle factors may produce stronger immunomodulatory effects. Studies that further explore these possible effects may lead to new avenues of treatment for chronic inflammatory disorders that rely less on medication and more on lifestyle factors.

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Vita

Gloria Jean Rhoney, nee Shook, was born in Hickory, NC to Allen E. Shook and Eula M. Shook. She grew up in Morganton, NC and graduated from Freedom High School in 2002. She then attended Western Piedmont Community College and graduated with an A.S. in 2004. Mrs. Rhoney received her B.S. in Geology from Appalachian State University in 2006. She spent several years working in private industry while also continuing career related coursework through NC State University in Soil Science, before she began working in Public Health in 2013. In 2015 she married her husband Joseph Mel Rhoney Jr. and received her Registered Environmental Health Specialist licensure for the state of North Carolina the same year. Mrs. Rhoney worked in public health as an Environmental Health Specialist for 7 years, until 2021 when she began studying for her Master of Science degree in Cell and Molecular Biology. Her degree was awarded in 2023.