CRANBERRY MODIFIES INNATE IMMUNE CELL FUNCTION VIA PRIMING

By

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To my Heavenly Father who makes all things possible

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Ab	Antibody(ies)
ANOVA	Analysis of variance
APC	Antigen presenting cell
AP-1	Activator protein 1
APP	Apple peel proanthocyanidins
ARE	Adenylate/uridylate (AU)-rich element(s)
ARE-BP	Adenylate/uridylate (AU)-rich element- binding protein
C	Celsius
CAM	Cell adhesion molecule
CJC	Cranberry juice cocktail
CO_2	Carbon dioxide
DMEM	Dulbecco's Modification of Eagles Medium
DMSO	Dimethyl sulfoxide
EEN	Elemental enteral nutrition
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FL OZ	Fluid ounce(s)
GALT	Gut-associated lymphoid tissue
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
H	Hour(s)
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNRNA	Heterogeneous nuclear RNA

HPLC	High performance liquid chromatography
ICR	Institute of Cancer Research
IFNγ	Interferon γ
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12 (P40)	Interleukin 12 (p40)
IRF	Interferon regulatory factor
LBP	Lipid binding protein
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MD2	Myeloid differentiation 2
MFI	Median fluorescence intensity
MHC	Major histocompatability complex
MIN	Minute(s)
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1 beta
ML	Milliliter(s)
MRNA	Messenger RNA
MYD88	Myeloid differentiation factor 88
NDM	Nondialyzable material
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NM	Nanometer(s)
NTP	Nucleoside triphosphate
PAC	Proanthocyanidin(s)
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell(s)
PBS	Phosphate buffered saline
PCA	Procyanidin(s)
pIgR	Polymeric immunoglobulin receptor R
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
RANTES	Regulated on activation normal T cell expressed and secreted
RPL37A	Ribosomal protein L37A
RPMI	Roswell Park Memorial Institute medium
SEAP	Secreted embryonic alkaline phosphatase
sIgA	Secretory immunoglobulin A
T_H	T helper
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor alpha
TRIF	Toll/interleukin-1 receptor-domain-containing adaptor protein-inducing IFN-β
TTP	Tristetraprolin
µG	Microgram(s)
UTI	Urinary tract infection
UTP	Uridine 5’-triphosphate(s)
UTR	Untranslated region

VLA-1	Very late antigen-1
VUR	Vesico-ureteric reflux

Abstract of Dissertation Presented to the Graduate School
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CRANBERRY MODIFIES INNATE IMMUNE CELL FUNCTION VIA PRIMING

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Cranberries contain polyphenols, including proanthocyanidins (PAC). Recent research suggests that PAC may induce a priming effect in innate immune cells, resulting in a more robust response to secondary immune stimuli. The mechanisms underlying priming are not understood. Thus, our objective was to characterize the priming mechanism in THP-1 cells, a model of innate immune cells. In our previous *in vitro* study, cranberry bioactives containing PAC altered protein levels in the Toll-like receptor 4 (TLR4) signaling pathway. We hypothesized that treatment with cranberry would result in an enhanced response to secondary challenge via a TLR4-dependent mechanism.

Priming is postulated to be a subtle cellular response; therefore, a cranberry concentration that did not overtly activate cells was identified. THP-1 monocytes were treated with 5, 25, or 100 μg/mL of cranberry fraction rich in PAC (PAC-r) or a fraction containing moderate PAC levels (PAC-m) for 6 to 48 h. Activation status was determined by four measures: percentage of $CD14^+$, $CD49a^+$ cells; CD49a expression per cell; CD14 expression per cell; and cytokine and chemokine secretion. Three of four

measures suggested that optimal experimental conditions were 6 h incubation with 25 µg/mL cranberry fractions.

To investigate the priming mechanism, THP-1 macrophages were treated with 25 µg/mL PAC-r or PAC-m, followed by lipopolysaccharide (LPS) challenge. PAC-r treatment increased MIP-1α and TNFα secretion upon LPS stimulation. *TNFα* and *MIP-1α* steady-state transcript levels were upregulated by PAC-r in absence of LPS stimulation; however, transcript stability was not altered.

HEK-Blue™ hTLR4 cells and THP-1 macrophages with a neutralizing TLR4 antibody were used to delineate the role of TLR4 in mediating transcript increases. Although PAC-r increased TLR4 activation in HEK-Blue™ cells, use of the TLR4 neutralizing antibody in THP-1 macrophages treated with PAC-r did not depress steady-state *MIP-1α* and *TNFα* mRNA levels. TLR4 neutralization attenuated LPS-induced *MIP-1α* and *TNFα* mRNA increases. Therefore, TLR4 may play a partial but not obligatory role in priming.

Collectively, these data suggest that cranberry primes THP-1 cells for enhanced cytokine and chemokine synthesis and secretion upon subsequent stimulation that does not require TLR4 exclusively and may involve other pattern recognition receptors.

CHAPTER 1
LITERATURE REVIEW

Immune System

The immune system has evolved over time to protect the body from invasion and subsequent infection by bacterial, viral and parasitic organisms. A large variety of cells, proteins and other molecules work in unison to discriminate non-self from self-molecules and to generate an effector response to foreign molecules for their elimination or neutralization.

The human immune system is composed of an innate branch and an adaptive branch. The innate immune system responds rapidly and is the body's first line of defense against invading pathogens. Innate immune components include the epidermal layer, the epithelial lining of the digestive tract, digestive secretions, phagocytes (neutrophils and macrophages), dendritic cells, and natural killer cells.

In contrast to the rapid response of innate immune components, T lymphocytes (T cells) and B lymphocytes (B cells) of the adaptive immune system take days to respond to invading pathogens. The adaptive immune system, however, responds with a great degree of specificity and possesses "memory". After an initial exposure to an antigen, "memory" allows the adaptive immune system to initiate a heightened response to the antigen in subsequent encounters.

Upon penetration of the epithelium, pathogens encounter innate immune cells, such as neutrophils and macrophages. The pathogens are recognized by pattern recognition receptors (PRR) on innate immune cell surfaces. These receptors detect structural motifs from pathogens that are necessary for pathogen survival, known as pathogen associated molecular patterns (PAMP). The binding of a PAMP to its

respective PRR results in immune cell activation and release of cytokines, chemokines and complement factors. Chemokines attract additional immune cells to the area, while cytokines modify the behavior of target cells recruited to the area. The secreted complement factors can bind to the surface of pathogens and destroy them via cell membrane lysis. Alternatively, complement factors can coat the surface of the pathogen in a process called opsonization, which facilitates phagocytosis of the immune invader.

If the pathogen breaches the innate immune barriers and is taken up by dendritic cells or macrophages, inflammation and subsequent activation of the adaptive immune response may ensue. The adaptive immune response begins when antigen presenting cells (APC), which have taken up and processed pathogenic components, present an antigen to $CD4^+$ T helper (T_H) cells. The presentation of antigen along with inflammatory cytokines secreted by innate immune cells activates T cells and results in secretion of cytokines that can further activate B cells, macrophages and other immune cells to swiftly eliminate invaders.

B cells contain antigen-specific receptors on their cell surface capable of detecting minute differences in antigens. Naïve B cells that interact with antigens that match the antibodies attached to their cell surface will differentiate into antibody secreting plasma cells or into memory B cells that possess antigen-specific cell surface receptors for subsequent antigen encounters (1).

The immune system is very complex; however, it will not be covered in depth here as the focus of the research project in is on monocytes/macrophages in innate immunity.

Monocyte/Macrophage

Monocytes and macrophages are both myeloid lineage cells arising from a common bone marrow – derived precursor (2). These mononuclear cells can be identified from other immune cells by the relative abundance of CD14 antigen expressed on their cell surface. Monocytes are primarily found circulating in the peripheral blood and comprise 5-10% of human peripheral blood mononuclear cells (PBMC) (3). Chemoattractants, such as monocyte chemoattractant protein-1 (MCP-1), activate the binding of integrins on monocyte cell surfaces to cell adhesion molecules (CAM) on endothelial cell surfaces. Upon binding, monocytes migrate through the epithelial cell tight junctions and into the tissues where differentiation into specific tissue macrophages and dendritic cells occurs (4). The macrophage cells that reside in specific tissues have specialized functions influenced by their microenvironment and are named based on their location. These specialized macrophages include intestinal macrophages of the gut, alveolar macrophages of the lungs, histiocytes in connective tissues, Kupffer cells in the liver, mesangial cells in the kidney, microglial cells in the brain and osteoclasts in bone (1,3).

Although macrophages may develop into specialized cell-types, they possess some of the same indispensable anti-pathogenic mechanisms as monocytes during inflammatory responses. Both monocytes and macrophages express PRR on their cell surface and endosomal compartments, allowing them to respond to PAMP derived from pathogenic invaders. Recognition of PAMP by monocytes and macrophages activates anti-pathogen functions including release of inflammatory mediators, production of reactive oxygen and nitrogen species, cytokine secretion and phagocytic ability.

Although monocytes and macrophages possess these devices, macrophages, particularly when activated, exhibit enhanced potential for pathogen destruction (1,5).

Activated macrophages possess increased phagocytic ability and express higher levels of class II major histocompatibility complex (MHC) molecules, which are used to present antigen to $CD4^+$ T_H cells. In this respect, macrophages play a key role in bridging innate and adaptive immunity. The presentation of antigen via class II MHC molecules is essential to T_H cell activation and hence activation of the adaptive immune response. Upon macrophage secretion of inflammatory cytokines and presentation of antigens to T_H cells via class II MHC molecules, the T_H cells become activated and secrete cytokines, namely interferon gamma (IFNγ), which further enhance the antimicrobial functions of macrophages. Thus, macrophages and T_H cells function cooperatively to facilitate one another's activation for elimination of invading pathogens.

Toll-Like Receptors

The Toll-like receptor (TLR) family is one class of PRR that is responsible for recognition of a broad array of PAMPs including lipopolysaccharide (LPS), triacyl and diacyl lipopeptides, flagellin, peptidoglycan, single- and double-stranded viral RNA and bacterial DNA (6). Out of the ten known human TLRs, TLR4 was the first human TLR to be discovered (7,8). TLR4 is the cognate receptor for LPS, a cell wall component of gram negative bacteria (9). The process of LPS recognition by TLR4 begins when LPS in serum, is bound by LPS- binding protein (LBP). The soluble form of CD14 then delivers the LBP coated LPS to the TLR4- myeloid differentiation 2 (MD2) receptor complex located on the cell surface. Upon activation of the TLR4-MD2 receptor complex (Figure 1-1), signal transduction may proceed via either a myeloid differentiation factor 88 (MyD88)-dependent or TIR-domain-containing adaptor protein-inducing IFNβ (TRIF)-

dependent adaptor protein pathway (10). Signal transmission via MyD88 culminates in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1), which translocate into the cell nucleus to induce transcription of early-response inflammatory cytokine genes, such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) (10). MyD88 is an essential adaptor molecule in mediating the LPS triggered TLR4 signaling pathway. Mice lacking MyD88 are resistant to septic shock upon LPS challenge, and macrophages isolated from MyD88 -/- mice fail to produce proinflammatory cytokines upon LPS exposure (11).

The TRIF-dependent pathway is also required for effective TLR4 signaling but differs from MyD88 signal transmission in that TLR4 must first be transported to intracellular endosomes. Initiation of the TRIF branch of the TLR4 signaling pathway ends in activation of interferon regulatory factor (IRF) and NF-κB transcription factors, which are responsible for the activation of transcription in type I interferon and inflammatory cytokine genes, respectively (10). A full explanation of the TLR4 pathway will not be covered here as the aim of the research project focuses on the end products of the signaling cascade. A detailed explanation of the TLR4 pathway can be found in the reviews written by Akira and Takeda (6,10)

Cytokines and Chemokines

Chemokines form a superfamily of polypeptides 90 to 130 amino acids in length. They ensure a focused response to infection and inflammation via control of leukocyte activation, cellular adhesion and chemotaxis. The chemokines, such as interleukin 8 (IL-8), macrophage inflammatory protein 1 alpha (MIP-1α) and macrophage inflammatory protein 1 beta (MIP-1β), act on endothelial cells lining the blood vessels to increase expression of CAM. Additionally, chemokines stimulate the increased expression of

selectins and integrins on the surface of neutrophils, monocytes, macrophages and other immune cells. The increased expression of both CAMs and selectins helps to initiate the four step extravasation process. First, immune cells roll on the endothelial surface as selectins on immune cells bind loosely to CAM on the endothelium. Binding of chemokines to their receptors on immune cells activates the cells and induces a conformational change in cell surface integrins. The integrins on the immune cell then tightly adhere to the CAM on the endothelial surface. Following adhesion, immune cells traverse the tight junctions of epithelial cells and move into tissue during the diapedesis process. Once in the tissue, chemotaxis occurs as recruited immune cells follow the increasing chemokine gradient to the site of inflammation. Successful chemotaxis is essential to initiation and resolution of the immune response. Remarkably, immune cells can detect as little as a 1% difference in the chemokine gradient as they migrate (12). This sensitivity ensures rapid movement of immune cells to the site of inflammation for pathogen destruction and subsequent tissue repair.

Cytokines, like chemokines, are mediators of the immune response. These low molecular weight glycoproteins, typically less than 30 kDa, are secreted by cells of the immune system as well as other cell types in response to an immune stimulus (1). Cytokines bind to their respective receptors on target cell surfaces. The actions of cytokines are pleitropic and include induction of inflammation, control of cell proliferation and differentiation, regulation of hematopoiesis and promotion of wound healing. Interestingly, cytokines may alter the immune environment in an autocrine, paracrine or endocrine manner. Thus, the cell producing cytokines, cells in the nearby

microenvironment or cells in distant parts of the body can be impacted by cytokine activity.

T_H cells, macrophages and dendritic cells secrete the majority of cytokines during an immune response (1). The cytokines these cells produce are classified into groups based on their functional and structural characteristics. One type of functional grouping is classification based on the type of immune response that the cytokine tends to elicit within the cytokine milieu. Cytokines that are typically associated with inflammatory responses are classified as T_H1 type cytokines while cytokines that exert an anti-inflammatory effect in the host are referred to as T_H2 type cytokines.

Tight regulation of both T_H1 type and T_H2 type cytokine and chemokine production is necessary as perturbation of the cytokine balance can lead to physiologic abnormalities such as sepsis and rheumatoid arthritis. Thus, cytokine and chemokine expression are controlled at both the transcriptional and post-transcriptional levels. Transcriptional control is mediated by transcription factors that, when activated, bind to promoter regions of cytokine and chemokine DNA to initiate transcription. Transcription factors that play key roles in orchestrating inflammatory cytokine gene transcription in macrophages and other immune cells include NF-κB, IRF, and AP-1 (13,14).

Post-transcriptional control of cytokine and chemokine gene expression is controlled by the presence of adenylate/uridylate (AU) - rich elements (ARE) initially discovered in the 3´ untranslated region (UTR) of inflammatory cytokine gene and cancer oncogene mRNA (15). As reviewed by Zhang, three classes of AREs exist based on the abundance and distribution of AUUA pentamers or UUAUUUAUU nonamers in the 3´ UTR of the transcript (16). Each of the three ARE classes can confer

instability or stability to the mRNA based on the ARE binding protein (ARE-BP) that binds to the mRNA. The ARE-BP tristetraprolin (TTP), for example, binds to ARE in TNF transcripts facilitating their degradation, whereas the ARE-BP known as HuA stabilizes the TNF transcript (16). Thus, ARE and their respective binding proteins work in concert to control the degradation of gene transcripts for chemokines, cytokines and other proteins.

Post-transcriptional control of cytokine and chemokine expression via ARE may serve as an intrinsic coding of the temporal order of cytokine responses during inflammation. Hao and Baltimore examined the temporal gene induction patterns in both mouse fibroblasts and primary macrophages stimulated with TNFα over a 12 hour time period (17). Based on microarray analysis, the genes were classified into three groups based on the time of peak appearance in mRNA. Group I genes peaked at 0.5 hours, group II genes at 2 hours, and group III genes at 12 hours. Time course measurement of mRNA abundance, showed that half lives of group I mRNA were shortest and varied from 0.2 hours to 0.9 hours (17). Group II mRNA half lives were longer and varied from 1 hour to 8 hours. Group three half lives could not be measured due to a lack of consistent decrease. Interestingly, a database search revealed that mRNA of group I genes contained the highest number of 3´ARE sequences, followed by mRNA of group II and group III genes. Thus, the number of AREs in the 3´ UTR was hypothesized to be a determinant in the induction and kinetics of gene expression. In order to directly examine the contribution of the 3´ UTR to gene expression patterns, promoters from genes in group I, group II and group III were utilized in a set of transgenes that expressed green fluorescent protein (GFP) as the reporter. The GFP was linked to the

3’ UTR of a either a group I or group III gene. GFP mRNA from transgenes linked to the 3´ UTR of the group I gene were less stable than the GFP mRNA from transgenes containing the group III 3´ UTR. These differences in mRNA stability occurred regardless of the promoter in the transgene. This research strongly suggests that the induction and kinetics of cytokine and chemokine gene expression is at least partially dependent on mRNA stability as determined by the ARE content (17). Thus, an understanding of the molecular and genetic mechanisms controlling cytokine expression could provide essential information for analyzing and predicting immune response dynamics from initiation to resolution.

Cranberry

Cranberries (*Vaccinium macrocarpon*) belong to the plant family *Ericacea* and are one of the few berries of economic significance native to North America (18) . Over the centuries, cranberries have been exploited for a variety of purposes. The red berry served as a source of food, meat preservative, dye, and wound healing poultice for Native Americans and European settlers.

Cranberry Polyphenols

Beyond nutrients, such as ascorbic acid, cranberries contain phytochemicals, or secondary plant metabolites. Polyphenols are a type of phytochemical and contain multiple phenol rings in their structure. Cranberries have an extensive polyphenol profile with the most abundant classes of polyphenols in cranberry being anthocyanins, flavonols, flavan-3-ols, and proanthocyanidins (PAC) (19).

The anthocyanins found in cranberry are polyphenolic pigments also found in other red, purple and blue fruits. These compounds are responsible for the vivid red color of cranberry. The average concentration of anthocyanins in cranberry is 86 mg per

100 g edible portion of ripe fruit (wet weight basis) (20-22). These anthocyanins include cyanidin, delphinidin, malvidin, palargonidin and peonidin (20-22). Variability in environmental factors can influence plant production of anthocyanins; thus, concentrations of anthocyanins have been found to range from 65 mg per 100 g edible portion to 168 mg per 100 g edible portion in cranberries (20-22).

Cranberries also contain flavan-3-ols and flavonols. These two polyphenols can be distinguished from one another by the ketone group that is present at carbon 4 on flavonols but is absent at carbon 4 on flavan-3-ols (Figure 1-2). Flavan-3-ols are also known as catechins. According to Gu et al, cranberry content of flavan-3-ols in whole cranberry is approximately 7 mg per 100 g of fruit (23). Flavonol content of cranberries, however, is much higher and ranges from 7.70 mg to 48.27 mg per 100 g edible, ripe fruit (20). The most abundant flavonols present in cranberry are kaempferol, myricetin and quercetin with more than 70% of these compounds being quercetin glycosides (20).

PAC are perhaps the most well known of the cranberry polyphenols as they have been postulated to prevent urinary tract infections (UTI). PAC are polymers of flavan-3-ols and are thus referred to as condensed tannins. Cranberry PAC include a variety of structural forms distinguished by the linkages connecting their flavan-3-ol monomers (Figure 1-3). Interestingly, the rare Type-A PAC (Figure 1-3 panel C) is present in cranberry and constitutes a large proportion of the total 418.8 mg per 100g PAC content (24). During screening of 88 foods for presence of PAC, the only foods other than cranberry found to contain Type-A PAC were curry, cinnamon, peanut, avocado and plum (24).

Cranberry and Immune Function

Beyond imparting sensory qualities, cranberry polyphenols are bioactive compounds. Cranberry polyphenols have been studied for their potential to promote oral health, prevent urinary tract infections, inhibit cancer cell growth and to promote cardiovascular health (19,25).

The bulk of research related to cranberry and immune health in recent years has concentrated on the anti-adhesive nature of the berry in relation to *E. coli, H. pylori and S. mutans* in the urinary tract, stomach and oral cavity, respectively (19,25). Despite the fact that the role of the immune system is to defend the body against pathogenic invaders and altered-self molecules, very few research studies have been conducted to date that explore the potential of cranberry in modulating immune function. Studies that have shown a potential role of cranberry in altering immune function present evidence that cranberry may act in a variety of ways. Han et al provided evidence of a potential anti-inflammatory role of cranberry in a rabbit model of vesico-ureteric reflux (VUR), a type of congenital defect of the urinary system (26). Male rabbits receiving VUR surgery were divided into five groups and received the following treatments: 1) sham control; 2) sterile urine instillation; 3) infection with *E. coli*; 4) infection with *E. coli* and dietary supplement of 1 g/kg body weight per day cranberry powder; or 5) infection with *E. coli* and intraperitoneal injection of melatonin, an antioxidant. Three weeks post surgery, rabbits given cranberry or melatonin and rabbits instilled with sterile urine showed only mild mononuclear cell infiltration in the absence of fibrosis. The rabbits infected with *E. coli* and receiving no supplemental treatment, however, had significant mononuclear cell infiltration and interstitial fibrosis in the infected kidney (26). Moreover, levels of malondialdehyde, an indicator of oxidative damage, was significantly lower in cranberry

fed, melatonin supplemented and sterile urine infused rabbits compared to animals infused with *E. coli*. The results of this study provide evidence that cranberry may act via an anti-inflammatory mechanism during infections of the urinary system to prevent excessive mononuclear cell infiltration and subsequent tissue death.

Pierre et al investigated the ability of cranberry to improve the function of gut-associated lymphoid tissue (GALT) in response to elemental enteral nutrition (EEN). GALT serves as a protective barrier against infection by resident and pathogenic microorganisms of the gut. EEN reduces the barrier function of GALT and results in decreased numbers of lymphocytes in the Peyer's patches (PP) and lamina propria, reduced secretory immunoglobulin A (sIgA) levels in the gut lumen and reduced expression of the sIgA transport protein, mucosal polymeric immunoglobulin receptor R (pIgR) (27,28). To test the hypothesis that cranberry could attenuate these decrements in GALT function, male Institute of Cancer Research (ICR) mice were randomized to one of three treatment groups: 1) chow via gastric catheter; 2) intragastric EEN via gastrostomy; or 3) EEN + cranberry PAC (100 mg/kg body weight) via gastrostomy (29). After receiving the specified treatment for five days, mice were sacrificed. The small intestine was removed from each animal for PP lymphocyte enumeration and determination of tissue interleukin 4 (IL-4), phosphorylated signal transducer and activator of transcription 6 (pSTAT-6) and pIgR. Luminal washes were collected from each animal for sIgA measurement. The number of PP lymphocytes and concentration of ileal tissue IL-4 were significantly increased in mice receiving EEN + PAC compared to EEN only mice, but no difference existed between chow fed and EEN + PAC mice (29). The ileal tissue sIgA and pIgR levels were also significantly elevated in EEN +

PAC mice versus animals receiving EEN feeding. No significant difference in sIgA and pIgR was noted between chow fed and EEN+PAC mice. Analysis of pSTAT-6 by an antibody microarray revealed that feeding PAC with EEN blunted the reduction in pSTAT-6 induced by EEN alone. As STAT-6 phosphorylation results in downstream activation of pIgR transcription, the authors suggested that increased ileal tissue levels of pIgR and lumen levels of sIgA in PAC+EEN mice were likely due to enhanced STAT-6 phosphorylation and signaling (29). This research study provides promising evidence of cranberry's capacity to modulate immune function through intracellular signaling pathways regardless of PAC absorption.

The mediators of potential immune modulation induced by cranberry were investigated in a randomized, double-blind, placebo controlled intervention involving frequency of asymptomatic bacteriuria in pregnant women (30). Twenty-seven females in their first or second trimester of pregnancy received 240 mL of cranberry juice cocktail (CJC) twice daily, CJC once daily and placebo once daily, or placebo twice daily. Each 240 mL bottle of CJC contained 80 mg PAC. After 3 days of beverage consumption, subjects provided 4 hour urine samples. Samples were analyzed for concentrations of anti-inflammatory cytokines IL-4, interleukin 10 (IL-10) and interleukin 5 (IL-5) and proinflammatory cytokines TNFα, interleukin 1 beta (IL-1β) and IL-6. Consumption of CJC twice each day resulted in two fold lower concentrations of urinary IL-6 as compared to urine of subjects receiving placebo beverage twice daily. Urinary concentrations of the other five cytokines tested were not significantly different between the three treatment groups. Absence of UTI and asymptomatic bacteriuria in all subjects, including the twice daily placebo group, precluded any evidence of a

uroprotective effect provided by CJC (30). Nonetheless, the reduction in urinary IL-6 concentrations following consumption of 480 mL CJC daily (160 mg PAC) does demonstrate the potential of cranberry to influence mediators of immunity in vivo.

Cranberry may also serve a role in boosting humoral immunity, or the aspect of immunity mediated by antibodies. Hochman investigated the impact of a cranberry nondialyzable material (NDM) with a molecular weight of 12,000-30,000 on lymphoma growth and antilymphoma antibody production in vivo (31). Syngeniec, immune competent Balb/C female mice were inoculated with Rev-2-T-6 murine lymphoma cells. Mice received either 4 mg cranberry NDM via intraperitoneal injection one day after inoculation followed by 2 mg cranberry every other day for 14 days or 2 mg cranberry NDM followed by 1 mg cranberry NDM every other day for 14 days. Four of five (80%) control mice injected with Rev-2-T-6 lymphoma cells developed tumors by three weeks post inoculation, while no mice consuming cranberry NDM developed tumors at study termination 60 days post inoculation. Western blot analysis of Rev-2-T-6 cells probed with sera from inoculated mice showed increased expression of anti-lymphoma antibodies in inoculated mice injected with cranberry NDM as compared to the low level of antibody production in tumor bearing control mice and the absence of antibody production in naïve mice (31). The absence of tumors in immune competent Balb/C mice inoculated with lymphoma cells combined with the increased expression of anti-lymphoma antibodies against Rev-2-T-6 cells does provide promising evidence of a potential role of cranberry in enhancing the immune response.

Jass and Reid investigated the potential of reconstituted cranberry extract to reduce pathogenic microbial adherence in the vaginal environment (32). During a

randomized, crossover study in 12 healthy females consuming reconstituted cranberry extract for one week, potential pathogens were lost from the vagina in five subjects (42%) consuming reconstituted cranberry drink twice daily (32). There were also no apparent harmful effects on the resident vaginal microbiota. The beneficial effects observed in the study strengthen the argument for research to fill the paucity of information that exists about cranberry's immune modulating potential.

Immune Cell Priming

The term "priming" in immunity often refers to pre-exposure of a cell or organism to a cytokine or antigen that modifies the ability of the cell or organism to respond to a subsequent immune challenge. However, priming with an antigen or cytokine takes immune cells out of a resting state and into a state of activation. Activated immune cells secrete inflammatory mediators that can lead to destruction of body tissues and chronic inflammation when the activation occurs in the absence of an immune challenge.

Interestingly, the work of Jutila et al in γδ T cells, which possess both myeloid and lymphoid characteristics, has suggested that priming may also be induced by plant polyphenols in innate immune cells. This antigen-independent priming, as termed by Jutila, invokes a much more subtle response in γδ T cells with a secondary stimulus being necessary to elicit overt activation and proliferation (33). The most potent γδ T cell dietary priming agent to date is PAC produced by certain plants (e.g. apples) (33,34). Treatment of human γδ T cells with unripe apple peel PAC resulted in increased expression of cytokine and chemokine transcripts of granulocyte macrophage – colony stimulating factor (GM-CSF) and IL-8 (35). IL-8 is a chemoattractant that functions to draw immune cells to sites of inflammation in the host, while GM-CSF controls the production and differentiation of macrophage and granulocyte cells. Interestingly,

transcripts for the prototypical lymphocyte inflammatory cytokine, IFNγ, were not induced in γδ T cells treated with unripe apple peel PAC (APP) (35).

A possible mechanism behind the increased expression of GM-CSF and IL-8 was explored in MOLT-14 cells, a proposed human γδ T cell line (36). Both IL-8 and GM-CSF contain ARE in the 3´ UTR of their mRNA (37). These ARE can be bound by ARE-BP to alter the stability of the transcript and hence control expression of cytokines and chemokines at a post transcriptional level. Thus, the authors hypothesized that the increase in cytokine and chemokine mRNA in γδ T cells treated with APP may be due to alterations in the transcript stability (36). Treatment of MOLT-14 cells with APP and subsequent addition of actinomycin D, to inhibit further *de novo* transcription, resulted in approximately 50% retention of the initial GM-CSF transcript level at 30 minutes (min) and 90 min post actinomycin D addition (36). In comparison, GM-CSF transcripts from MOLT-14 cells treated with TNFα, a negative control, were present at approximately 20% and 10% of initial levels 30 minutes and 90 minutes after actinomycin D treatment, respectively (36). IL-8 transcript levels also appeared to be stabilized by APP with greater than 80% of the initial transcript level remaining 90 min after actinomycin D treatment (36). Thus, one possible mechanism responsible for the priming effect seen in γδ T cells may be an increase in cytokine and chemokine mRNA stability. Increases in cytokine and chemokine mRNA stability and expression could offer a clear immunonological advantage to immune cells in that upon activation by an immune stimulus, the cells could have transcripts available for translation to protein and thus initiate a more rapid immune response. However, this hypothesis and the in vivo human relevance remain to be explored.

THP-1 Cells

THP-1 cells are a human leukemic cell line cultured from a young male with acute monocytic leukemia (38). These cells can be used as an appropriate model of a human innate immune cell in experimental research (39). Similar to primary human monocytes and macrophages, THP-1 cells express TLR4, among many other innate receptors, and express the monocytic cell marker CD14 on their surface (40). These cells can be used both as models of human monocytes and macrophages. Treatment of THP-1 cells with phorbol diester induces differentiation from a monocyte to macrophage cell (41). The differentiation process enhances the cells ability to respond to LPS and other mitogens, which is advantageous in experiments where responses of undifferentiated cells may be below detection limits (5).

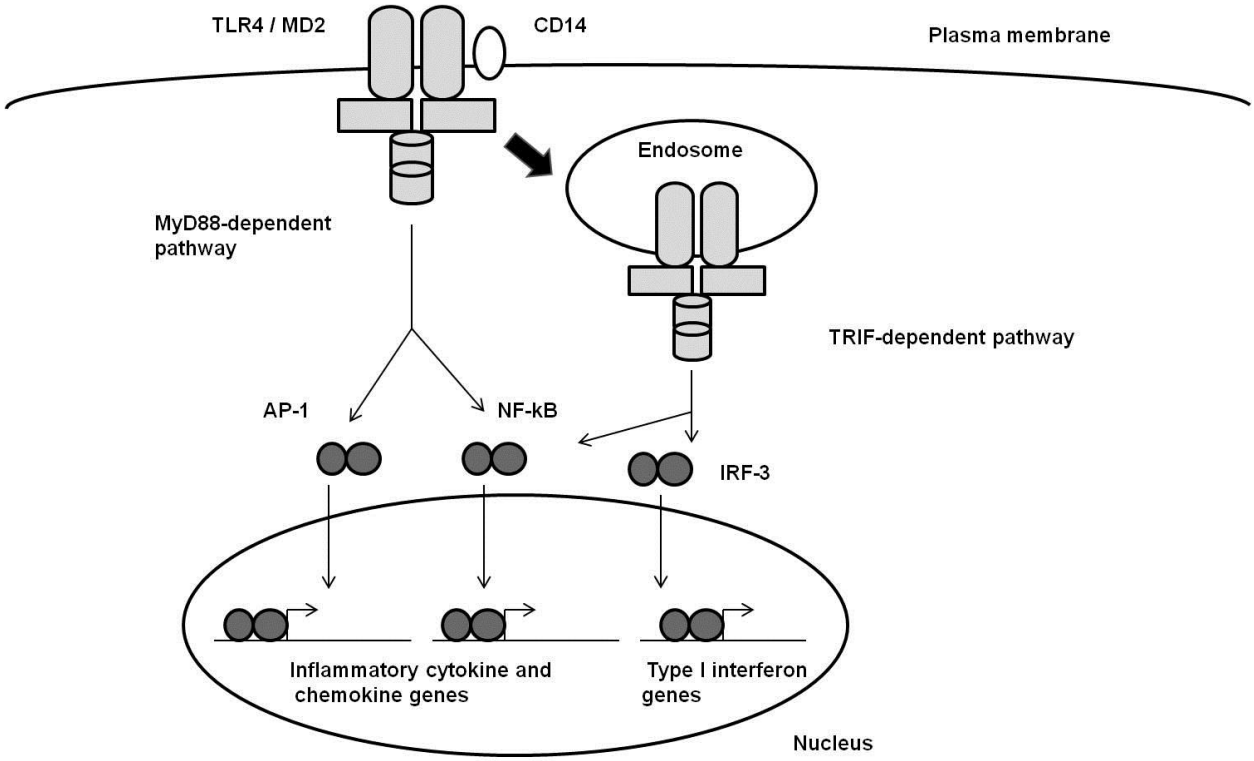


Figure 1-1. TLR4 pathway.

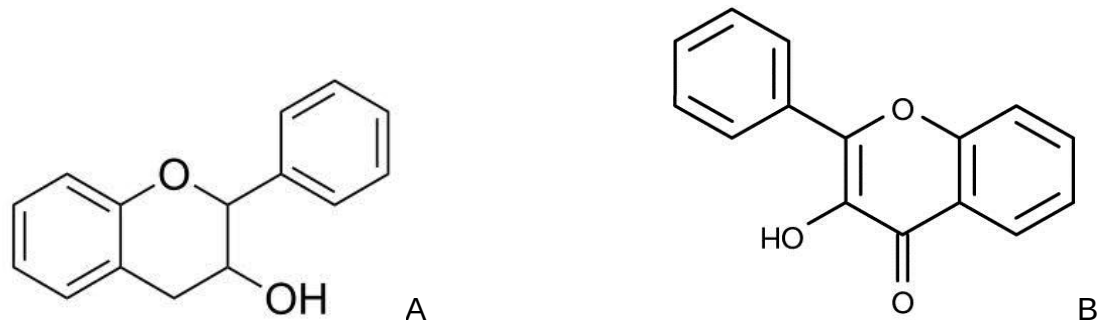


Figure 1-2. Basic structure of flavan-3-ols and flavonols. Subpanels represent: A) flavan-3-ol and B) flavonol.

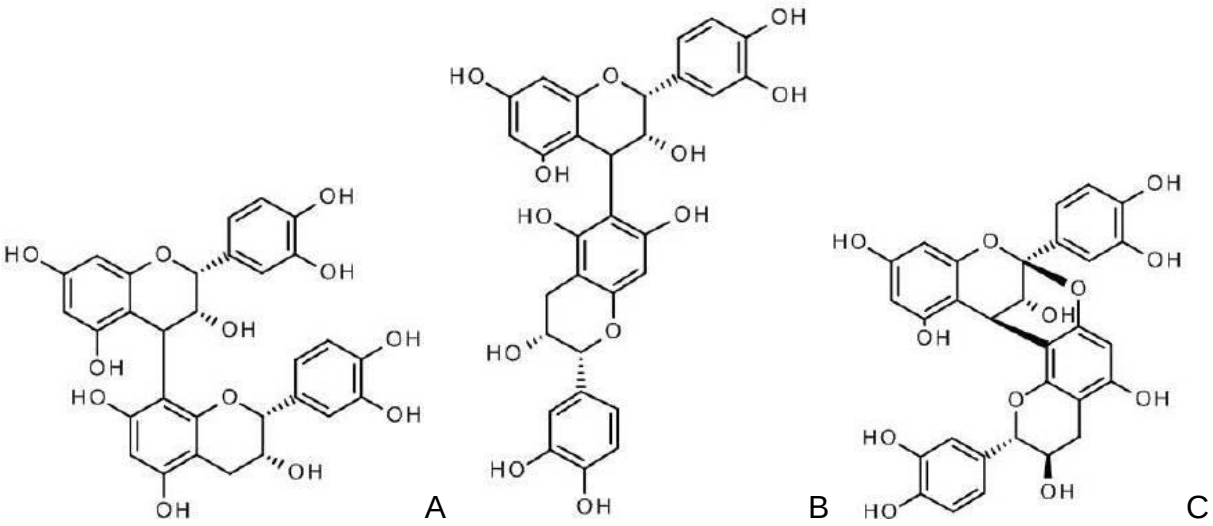


Figure 1-3. Proanthocyanidins found in cranberry. A) proanthocyanidin with a B-type linkage formed by a C4→C8 bond, B) proanthocyanidin with a B-type linkage formed by a C4→C6 bond and C) proanthocyanidin with an A-type linkage formed by a C2→O7 bond.

CHAPTER 2
INTRODUCTION

Significance and Rationale

Each year more than 62 million cases of the common cold occur in the United States alone and result in approximately $25 billion in lost work productivity (42,43). Additionally, an estimated 37.2 million food borne illnesses occur each year in the United States (44). These and other acute illnesses caused by bacterial and viral pathogens have a significant impact on quality of life and the economy. However, their impact could be lessened by decreasing the number of days of infection and/or reducing symptom severity. Consumption of dietary bioactives that alter innate immune cell function may facilitate these changes. The long term goal of our lab is to understand how food components modify the immune response to pathogen challenge and subsequent resolution of the inflammatory response. The goal of this research project was to characterize the priming mechanism in THP-1 monocytes/macrophages, a model of an innate immune cell. Priming refers to immune cell responses initiated by a compound prior to pathogen encounter.

The overall rationale for this research project was two-fold. First, the immune modulating potential of cranberry and responsible mechanisms has not been adequately studied. The paucity of research studies that have been conducted to date have focused on bacterial adherence in urinary tract infections and periodontal disease. An understanding of how cranberry modifies immunity may provide knowledge of prevention of these illnesses as well as evidence of how cranberry may enhance immune responsiveness to other infectious agents. Our research directly examined mechanisms by which cranberry bioactives alter innate immune cell function. Second,

having completed the research, we now have a better understanding of how cranberry bioactives interact with TLR4 on the innate immune cell surface to modify cell function, especially monocyte/macrophage function.

The monocyte and macrophage function in innate immunity is to phagocytize pathogens and to activate other immune cells via antigen presentation and secretion of inflammatory proteins. Thus, these cells are one of the first responders during pathogenic invasion, and are instrumental in initiating a rapid, effective immune response. Swift immune responses that eliminate pathogens and restore immune homeostasis provide potential for alleviation of infectious illnesses including the common cold, influenza and cholera.

Hypothesis

Our central hypothesis was that bioactives in cranberry fractions would enhance the responsiveness of THP-1 monocyte/macrophage cells to secondary challenge with a bacterial component in a Toll-like receptor 4 (TLR4) dependent manner.

Specific Aims

The primary aims of our research were to:

1. Characterize the activation state of THP-1 cells in response to increasing concentrations of cranberry to determine optimal experimental conditions.
2. Measure the chemokine and cytokine protein and mRNA levels of cranberry treated THP-1cells using the best determined cranberry concentration from Specific Aim 1.
3. Delineate the role of TLR4 in mediating cranberry-induced priming by measuring cytokine and chemokine mRNA levels under conditions in which TLR4 is inactivated.

CHAPTER 3
OPTIMAL EXPERIMENTAL CONDITIONS FOR CRANBERRY PRIMING ARE SIX HOURS AND 25 UG/ML CRANBERRY FRACTIONS

Background

In order to test the hypothesis that bioactives in cranberry fractions would prime THP-1 cells for an enhanced response to secondary challenge via TLR4, it was first necessary to identify a cranberry concentration that did not overtly activate the cells. Our proposed definition for innate immune cell priming involves a subtle response in the cell prior to a secondary challenge. An excessive cranberry concentration that activates the cell was postulated to prohibit detection of enhanced cytokine secretion upon secondary challenge with LPS, thus precluding our ability to use cytokine and chemokine secretion as a screening tool for which mRNA status to measure in future experiments.

Four approaches were used to analyze the activation pattern of THP-1 monocytes: percentage of $CD14^+$, $CD49a^+$ cells; CD49a expression per cell; CD14 expression per cell; and cytokine and chemokine secretion. CD14 is a cell surface marker for monocytic cell populations and is involved in cellular response to LPS via the TLR4/MD2 receptor complex (3,45). The marker is constitutively expressed on the cell surface; however, expression is upregulated during polarization of monocytic cells toward an inflammatory phenotype (46,47).

CD49a is the α-chain of the integrin, very late antigen 1 (VLA-1). When CD49a heterodimerizes with a β subunit, a receptor is formed that binds collagen and laminin on the extracellular matrix. This integrin is critical for migration of monocytic cells into the tissue during an inflammatory response.

As CD49a is expressed on activated T cells and monocytes, changes in CD49a expression per cell was used as the primary cell surface marker to classify the activation status of THP-1 cells (1).

Activated monocytic cells secrete cytokines and chemokines. Thus, the activation status of THP-1 monocytes in response to increasing concentrations of cranberry was also characterized by analysis of cytokines and chemokines secreted into culture medium using multiplex technology.

Materials and Methods

Cranberry Fractions

Lyophilized cranberry fractions were obtained from Ocean Spray Cranberries Inc. (Lakeville, MA). The cranberry fractions, designated PAC-m and PAC-r, were both derived from the juice portion of the berry. The primary difference between the two fractions, as shown in Table 3-1, is that PAC-r contained 35 - 40% more PAC than PAC-m (dry weight basis). PAC-m, however, contained 4.5 times more flavonols, 12 times more anthocyanins and 5 times more phenolic acids than PAC-r (dry weight basis). The high performance liquid chromatography (HPLC) chromatograms in Figure 3-1 and Figure 3-2 provide additional information on the composition of procyanidins (PCA), a type of proanthocyanidin, (Figure 3-1, A and B) and anthocyanins (Figure 3-2, A and B) in PAC-r and PAC-m.

The fractions were tested for the presence of endotoxin with a PyroGene® Recombinant Factor C Endotoxin Detection Kit (Cat # 50-658U, Lonza, Inc., Walkersville, MD) to ensure that cell activation was not due to LPS contamination. Both PAC-m and PAC-r contained less endotoxin than the minimum detectable limit (0.01 EU/mL) of the assay (data not shown).

In all cell culture experiments, cranberry fractions were dissolved in DMSO vehicle with a final concentration of less than 0.1% DMSO in culture medium.

THP-1 Monocyte Cell Culture

THP-1 human monocytes (American Type Culture Collection, TIB-202™) were maintained at 37°C, 5% CO_2 in RPMI 1640 complete medium (Cat# 17-105-CV, Corning Cellgro®, Manassas, VA) containing 10 % (v/v) FBS, 2 mM L-glutamine (25-005-CI, Corning Cellgro®, Manassas, VA), antibiotic-antimycotic supplement (100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin; Cat# 30-004-CI, Corning Cellgro®, Manassas, VA), 25 mM D-(+)-glucose (Cat# G8769 Sigma-Aldrich®, St. Louis, MO), 1 mM sodium pyruvate (Cat# SH30239.01, Thermo Scientific HyClone, Logan, UT), 10 mM HEPES (Cat# 25-060-CI ,Corning Cellgro®, Manassas, VA), 50 µM 2-mercaptoethanol (M7522, Sigma-Aldrich®, St. Louis, MO) and 50 µg/mL gentomycin (30-005-CR, Corning Cellgro®, Manassas, VA). The passage number of the cells was unknown upon receipt from American Type Culture Collection. After arrival, cells were not used beyond passage 50. Cell viability was 95% or greater for all experiments unless otherwise stated. Cells were maintained between 2 x 10^5 cells/mL to 8 x 10^5 cells/mL between experiments as recommended by ATCC (48).

Cell Viability

THP-1 cells were plated at 5 x 10^5 cells per well in a 24-well culture plate (Cat#07-200-84, Corning Costar®, Manassas, VA). Cells were treated with either 5, 25, 50 or 100 µg/mL of cranberry PAC-m or PAC-r, media or DMSO vehicle for up to 48 hours (h). At time points of 0, 24 and 48 h cells were harvested and viability was assessed using the trypan blue exclusion method and a Neubauer hemacytometer.

Flow Cytometry

THP-1 monocytes in RPMI complete medium were plated at 5 x 10^5 cells per well in a 24-well culture plate (Cat#07-200-84, Corning Costar®, Manassas, VA). Cells were incubated with either 5, 25 or 100 µg/mL PAC-m or PAC-r, medium or DMSO vehicle for 6 or 48 h.

Cells were harvested at aforementioned time points and placed into 5 mL polypropylene culture tubes (Cat#14-956, Thermo Fisher Scientific Inc., Pittsburgh, PA) containing 1 mL of flow wash buffer (0.01% sodium azide in PBS). Cell suspensions were centrifuged at 1500 rpm, 4˚C for 10 min. Supernatants were discarded and cell pellets were resuspended in 200 µL of flow stain buffer (2% FBS in flow wash buffer). 5 x 10^5 cells were stained with fluorophore-conjugated antibodies (Ab). The antibodies PE-conjugated anti-human CD14 (Cat#12-0149-73, eBioscience, San Diego, CA) and FITC-conjugated anti-human integrin 1α (CD49a) (Cat#sc-23942, Santa Cruz Biotechnology, Santa Cruz, CA) were used at the appropriate test size of 10 µL. Cells were stained in the dark for 30 min at 4˚C. After 30 minutes, cells were washed with 1 mL of flow wash buffer, centrifuged at 1500 rpm, 4˚C for 10 min. Supernatants were aspirated and cell pellets were fixed with 500 µL of 1% paraformaldehyde. A BD FACSort™ flow cytometer with BD CellQuest™ Pro software (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for data acquisition. Data were analyzed with FlowJo version 7.6.1 software (Tree Star Inc., Ashland, OR).

Multiplex Protein Analysis

THP-1 cells were plated at 5 x 10^5 cells per well in 24-well culture plates (Cat#07-200-84, Corning Costar®, Manassas, VA). Cells were treated with either 5, 25, or 100 µg/mL of PAC-m, PAC-r, or DMSO vehicle control for 6, 24 or 48 h. Cell suspensions

were harvested into 1.5 mL polypropylene microcentrifuge tubes and centrifuged for 1500 rpm, 4˚C for 10 min. Cell free supernatants were collected into pre-labeled 1.5 mL microcentrifuge tubes and stored at -80°C until analysis.

Cytokine and chemokine production in response to cranberry fractions was quantified with a Milliplex MAP Human Cytokine/Chemokine Bead Panel (Cat#MPXHCYTO-60K; EMD Millipore; Billerica, MA) according to the manufacturer's protocol. All washing steps were performed on a vacuum manifold apparatus (EMD Millipore; Billerica, MA). Specific cytokines analyzed included MIP-1α, MIP-1β, IL-8, IL-1β and TNFα. These proteins, incubation times of 6, 24 and 48 h and cranberry fraction concentrations were selected based on unpublished research from the Percival lab examining cytokine and chemokine production from human PBMC following treatment with cranberry fractions. A Luminex® 200™ multiplex system with xPONENT® 3.1 software (Luminex Corp., Austin, TX) was used for bead analysis.

Statistical Analysis

For all flow cytometry experiments, statistical analysis was carried out with a 2-way analysis of variance (ANOVA) and Tukey post-hoc all-pairwise test. All data is presented as mean ± SD with ($P<0.05$) considered significant.

Results

Cell Viability

The percentage of viable cells was greater than 90% for cells treated with PAC-m and PAC-r at each of the concentrations and time points with the exception of cells treated with 5 µg/mL PAC-r for 48 h (Figure 3-3). Eighty percent of cells were viable with this treatment and time combination.

Flow Cytometry

THP-1 monocytes were treated with increasing concentrations (5, 25 or 100 µg/mL) of either PAC-m or PAC-r, medium or vehicle control for 6 and 48 h. Initially, the percentage of $CD14^+$, $CD49a^+$ cells was examined. Using a two-way ANOVA to determine effects of time, treatment and interaction between time and treatment on percentage of $CD14^+$, $CD49a^+$ cells, time ($P=0.008$), treatment ($P<0.001$) and the interaction ($P=0.029$) between time and treatment were each found to have a significant effect on percentage of double positive cells (Figure 3-4).

For comparisons of time within each treatment, only 100 µg/mL PAC-m ($P=0.023$) and 100 µg/mL PAC-r ($P<0.001$) significantly increased the percentage of $CD14^+$, $CD49a^+$ cells from 6 h to 48 h. The shift in the $CD14^+$, $CD49a^+$ population with time can be seen in the representative dot plots in Figure 3-5.

Post-test analysis revealed that within the 6 h period, cells treated with 100 µg/mL PAC-r were activated as the percentage of $CD14^+$, $CD49a^+$ cells was significantly greater ($P<0.001$) than for cells receiving all other treatments (Figure 3-4).

Cell activation was also induced by 100 µg/mL of PAC-r and PAC-m treatments at 48 h. Following 48 h of 100 µg/mL incubation with PAC-r, the percentage of cells positive for both CD14 and CD49a was at least one fold greater ($P<0.001$) than for all other cell treatments within the same time point. The shift in the $CD14^+$, $CD49a^+$ population with increasing concentrations of PAC-r is shown in representative dot plots in Figure 3-6.

The trend for the highest fraction concentration to increase the percentage of $CD14^+$, $CD49a^+$ cells continued with PAC-m, as the percentage of double positive cells was greater ($P<0.01$) in 100 µg/mL PAC-m treated cells as compared to cells treated

with all other concentrations of PAC-m and 5 μg/mL PAC-r within the same time period (Figure 3-4).

Treatment with 5 or 25 μg/mL of either PAC-m or PAC-r at 6 h and 48 h had no effect ($p>0.05$) on the percentage of double positive cells when comparing treatments within time periods or time within treatments (Figure 3-4).

The median fluorescence intensity (MFI) was used to determine CD14 and CD49a expression per cell. For CD14, time ($P=0.007$), but not treatment ($P=0.301$) or interaction ($P=0.997$) between time and treatment, had a significant effect on expression of the monocytic cell marker based on a two-way ANOVA (Figure 3-7). A Tukey multiple comparison all-pairwise post-test, however, found no significant difference in CD14 expression with time in any of the treatment groups ($p>0.05$).

When two-way ANOVA was used to determine the effects of time, treatment and interaction between time and treatment, CD49a expression per cell was found to be significantly affected by treatment ($P<0.001$), but not time ($P=0.417$) or interaction ($P=0.996$) of treatment and time (Figure 3-8). One hundred μg/mL PAC-r treatment for 48 h resulted in significantly ($P<0.05$) greater CD49a expression per cell as compared to all other treatments at the same time point (Figure 3-8).

Multiplex Protein Analysis

Concentrations of MIP-1β (Figure 3-9, panel A), IL-8 (Figure 3-9, panel B), and MIP-1α (Figure 3-9, panel C) chemokines in culture medium from monocytes treated with 100 μg/mL PAC-r at 24 and 48 h were markedly higher than other treatments. The trend for greater secretion of chemokines from the 100 μg/mL PAC-r treated cells began at 6 h, and the magnitude relative to other treatments at the same time point increased at 24 and 48 h.

Although secretion of cytokines IL-1β (Figure 3-9, panel D) and TNFα (Figure 3-9, panel E) into culture medium was induced with 100 µg/mL PAC-r treatment, the magnitude of the secretion was minimal compared to the secretion of chemokines (Figure 3-9, panels A, B and C).

Discussion

The activation status of THP-1 monocytes was characterized using four approaches: percentage of $CD14^+$, $CD49a^+$ cells; CD49a expression per cell; CD14 expression per cell; and production of chemokines and cytokines. The primary goal of the experiments was to identify a cranberry concentration that did not overtly activate the cell. Although absence of a response is contrary to what many researchers desire, we were dependent on finding a cranberry concentration that elicited no or minimal changes in activation in order to examine the priming mechanism in later experiments.

The cell viability results showed no evidence that cranberry fractions were inducing cell death beyond basal levels. Thus, the 5 - 100 µg/mL concentration range was used in flow cytometry and cytokine/chemokine analysis experiments.

The concentration- and time-dependent increase in percentage of $CD14^+$, $CD49a^+$ cells suggested that cells were being activated at 6 and 48 h with 100 µg/mL PAC-r. A second measure of activation, CD49a expression per cell, confirmed that 100 µg/mL PAC-r was activating cells at 48 h in that each $CD14^+$ cell was expressing more of the CD49a activation marker compared with all other treatments except 100 µg/mL PAC-m. In comparison, 6 h treatment with 5 or 25 µg/mL PAC-r or PAC-m did not elicit cell activation as measured by percentage of $CD14^+$, $CD49a^+$ cells and CD49a expression per cell.

The expression per cell of CD14, an identification marker of monocytic cells, was not altered by time or increasing concentrations of PAC-m or PAC-r. CD14 is a constitutively expressed surface marker on monocytes but can be upregulated during monocyte polarization toward an inflammatory phenotype (47). This polarization occurs in the presence of potent mitogens and microbial components including peptidoglycan, CpG oligonucleotides, flagellin and LPS (46,47). As cranberry is not a microbial component or potent immune mitogen, it was expected that cranberry would elicit minimal or no changes in CD14 expression per cell.

Production of MIP-1α, MIP-1β and IL-8 chemokines in response to 100 μg/mL PAC-r supported the increase in expression of CD49a per cell and percentage of $CD14^+$, $CD49a^+$ cells induced with the same concentration of PAC-r. Although these chemokines increased in culture medium, it should be noted that the overall magnitude of the increase was lower than levels induced with LPS (Creasy unpublished observations).

The results of the four activation measures: percentage of $CD14^+$, $CD49a^+$ cells; CD14 expression per cell; CD49a expression per cell; and chemokine and cytokine production were all consistent in that incubation with 5 and 25 μg/mL cranberry fractions for 6 h did not activate THP-1 monocytes. Thus, the 6 h incubation period with cranberry fractions was selected for use in all future experiments with THP-1 cells. Between the two fraction concentrations (5 and 25 μg/mL), 25 μg/mL was selected as the concentration to use in later experiments as it was an intermediate concentration that was predicted to induce subtle, yet more measurable molecular responses in priming experiments.

Table 3-1. Composition of cranberry fractions

Component	Fraction	
	PAC-m	PAC-r
Sugars	1%	none detected
Organic Acids	0.5%	none detected
PACs	60-65%	99%
Flavonols	9%	0.5-2%
Anthocyanins	6%	0.5 or less%
Phenolic Acids	5%	0.1-1%

Composition data provided by Ocean Spray, Inc.

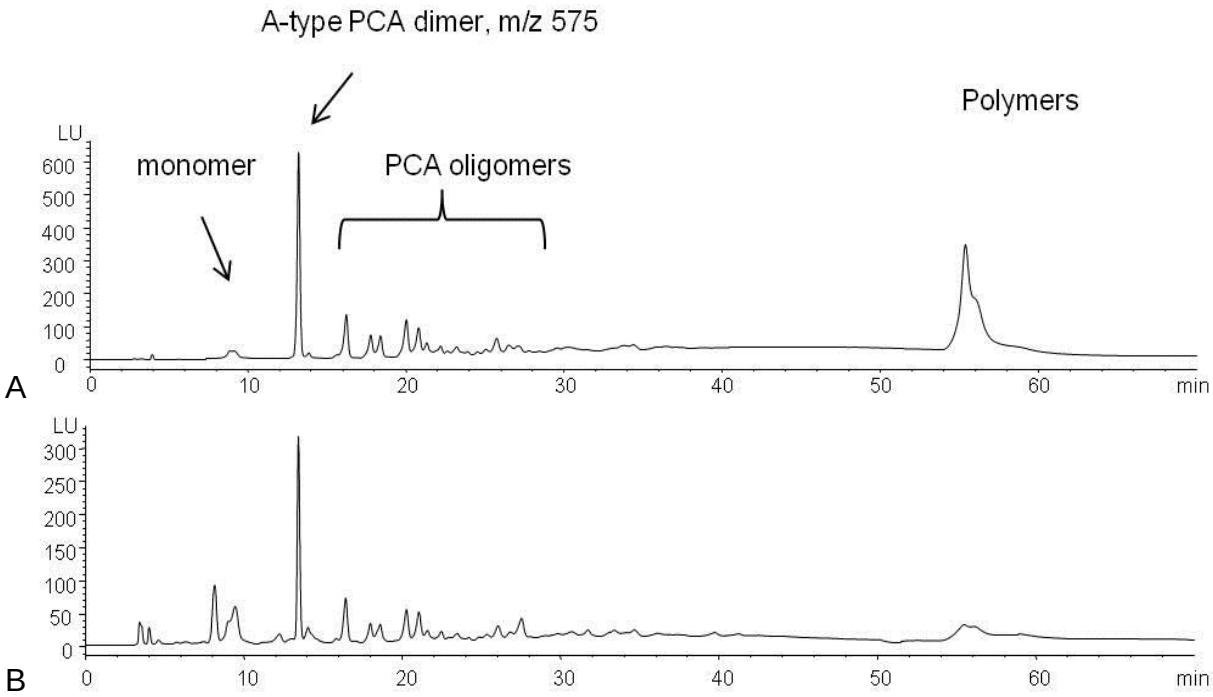


Figure 3-1. Normal phase HPLC chromatograms of procyanidins (PCA) recorded on a fluorescence detector (Ex=231 nm, Em=320 nm). Subpanels represent: A) PAC-r chromatogram and B) PAC-m chromatogram. Figure printed with permission (Sandhu et al unpublished).

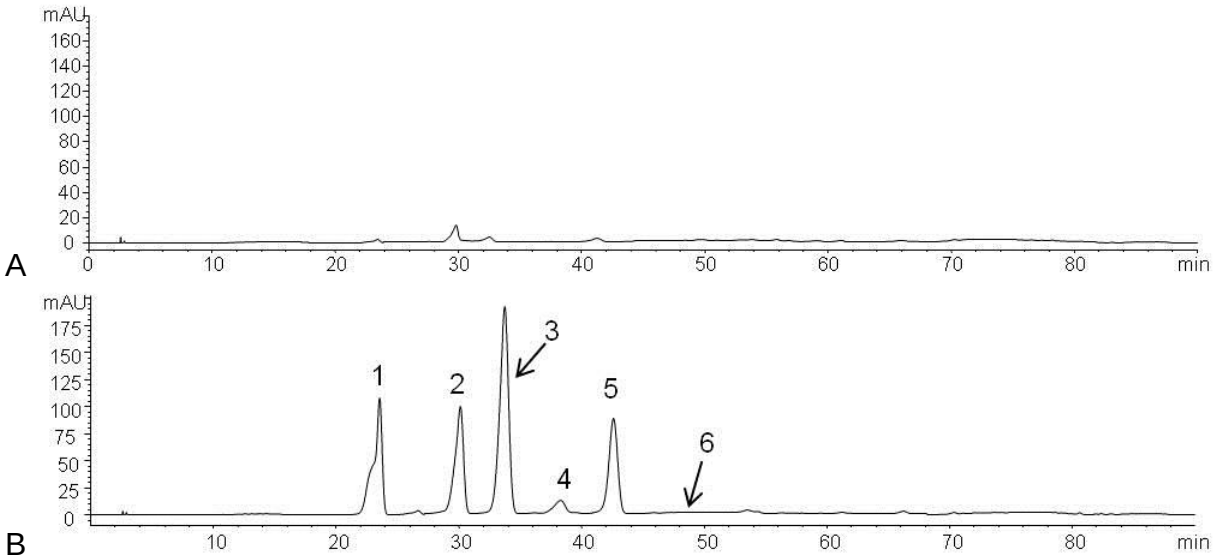


Figure 3-2. HPLC/diode array detector chromatograms of anthocyanins at 520 nm. Subpanels represent: A) PAC-r chromatogram and B) PAC-m chromatogram. Numbered peaks are: 1-cyanidin glucoside; 2- cyanidin xyloside; 3- peonidin glucoside; 4- peonidin xyloside and 5- peonidin xyloside. The numeral 6 and corresponding arrow on subpanel B refer to the retention time for delphinidin glucoside. Figure printed with permission (Sandhu et al unpublished).

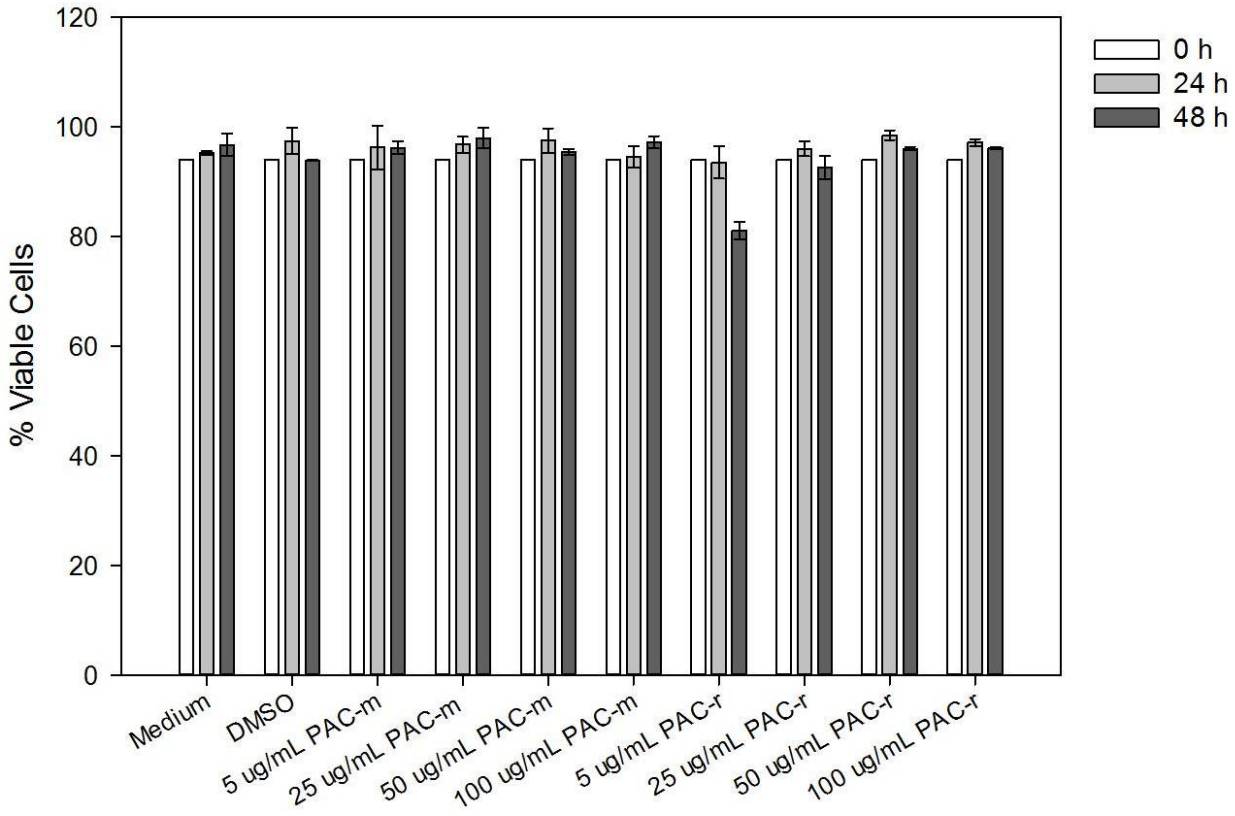


Figure 3-3. THP-1 monocytes maintained viability with cranberry treatment. THP-1 monocytes were treated with either 5, 25, 50 or 100 µg/mL PAC-m or PAC, media or DMSO. Cells were harvested at 0, 24 and 48 h. Viability was assessed using the trypan blue exclusion method.

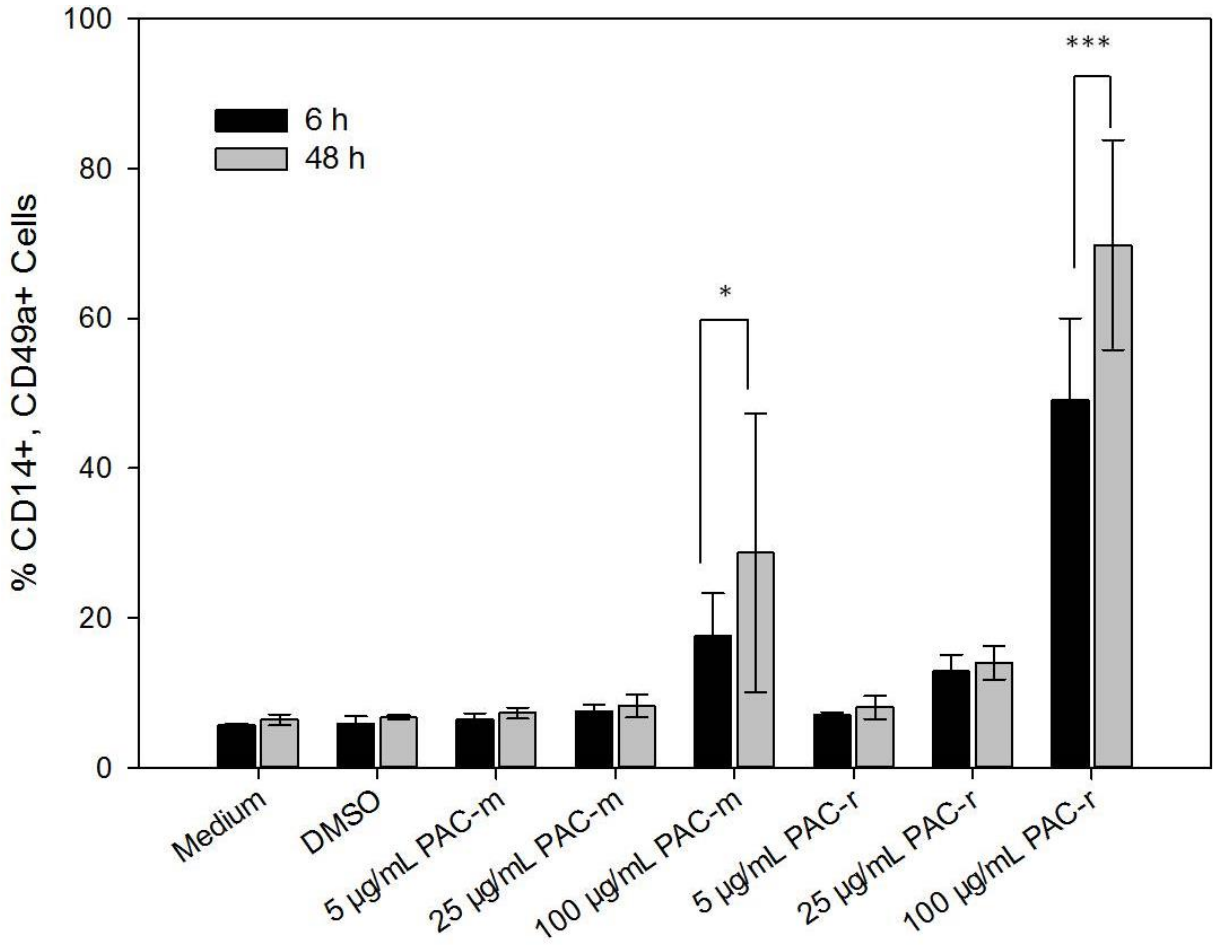


Figure 3-4. THP-1 monocytes were activated by 48 h treatment with 100 µg/mL PAC-r or PAC-m. Cells were incubated with increasing concentrations of PAC-m or PAC-r, medium or DMSO vehicle. Cells were harvested at 6 and 48 h. Flow cytometry was used to analyze the percentage of cells expressing CD14 and CD49a. Values represent means ± SD of two experiments each with duplicate treatments. Data were analyzed with a two-way ANOVA and Tukey post-test (*P<0.05, ***P<0.001).

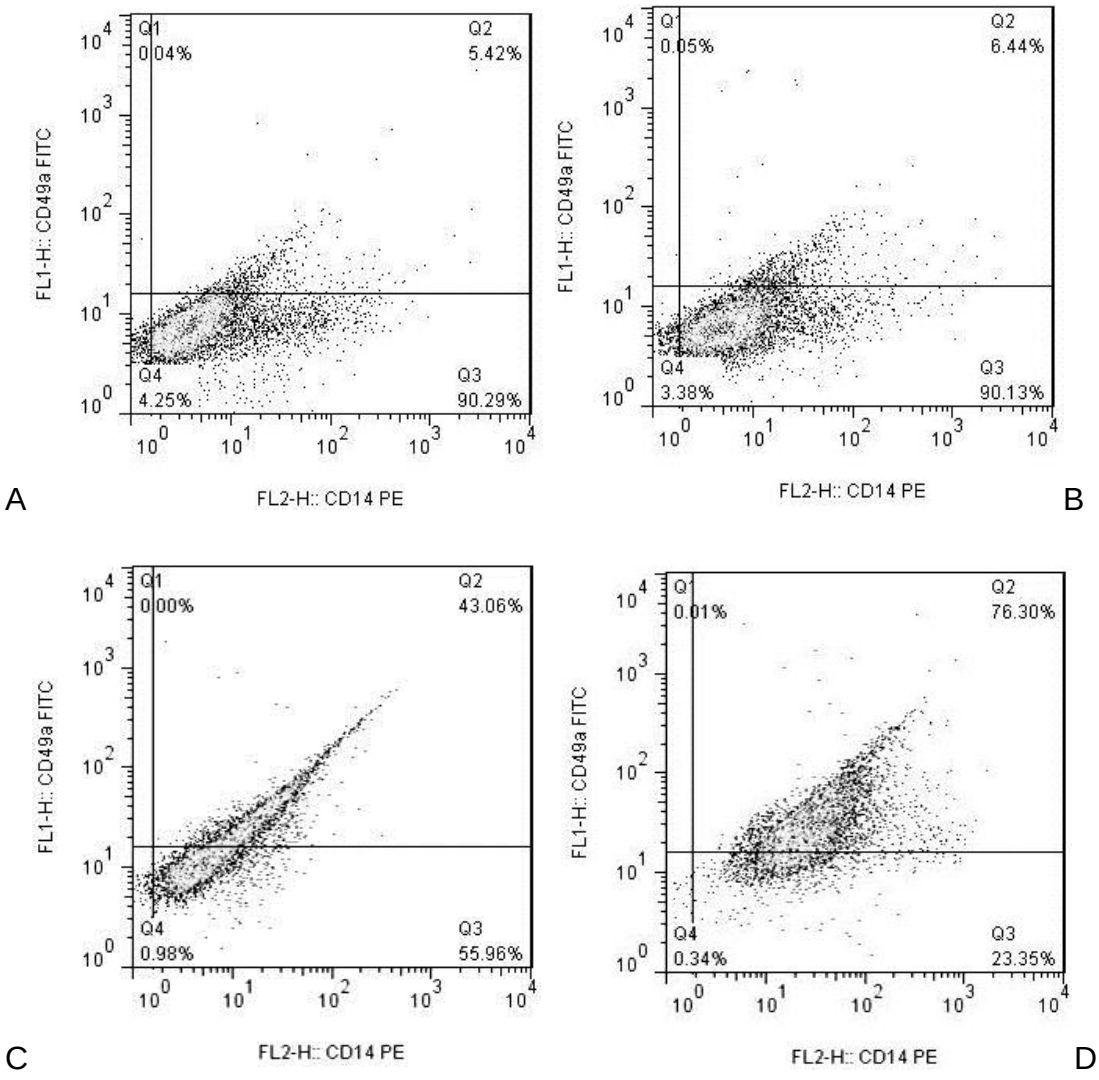


Figure 3-5. Representative dot plots showing the effect of time on percentage of THP-1 cells expressing CD14 and CD49a. Subpanels represent cells treated with: A) DMSO for 6 h, B) DMSO for 48 h, C) 100 µg/mL PAC-r for 6 h and D) 100 µg/mL PAC-r for 48 h.

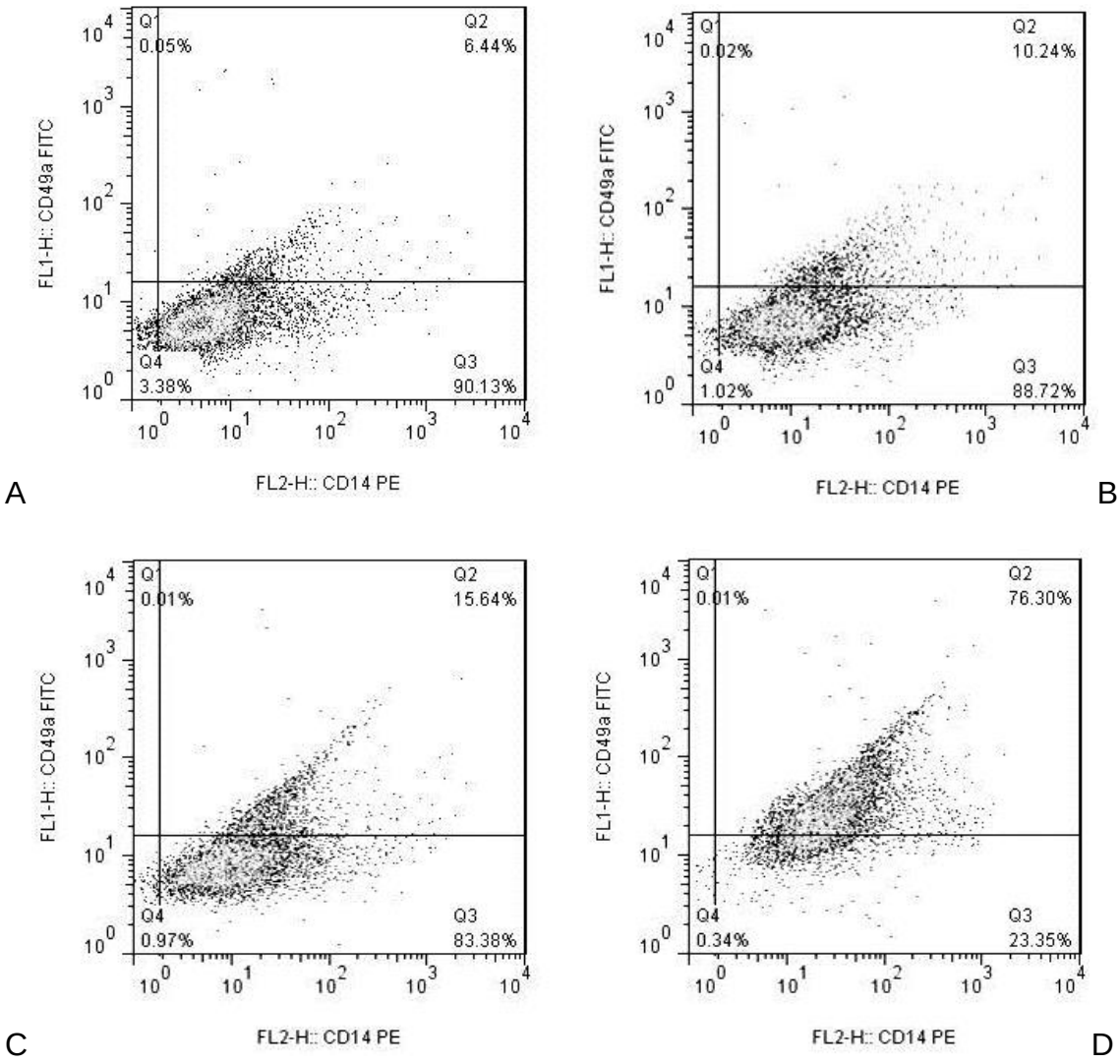


Figure 3-6. Representative dot plots showing the effect of concentration on percentage of THP-1 cells expressing CD14 and CD49a. Subpanels represent cells treated for 48 h with: A) DMSO, B) 5 µg/mL PAC-r, C) 25 µg/mL PAC-r and D) 100 µg/mL PAC-r.

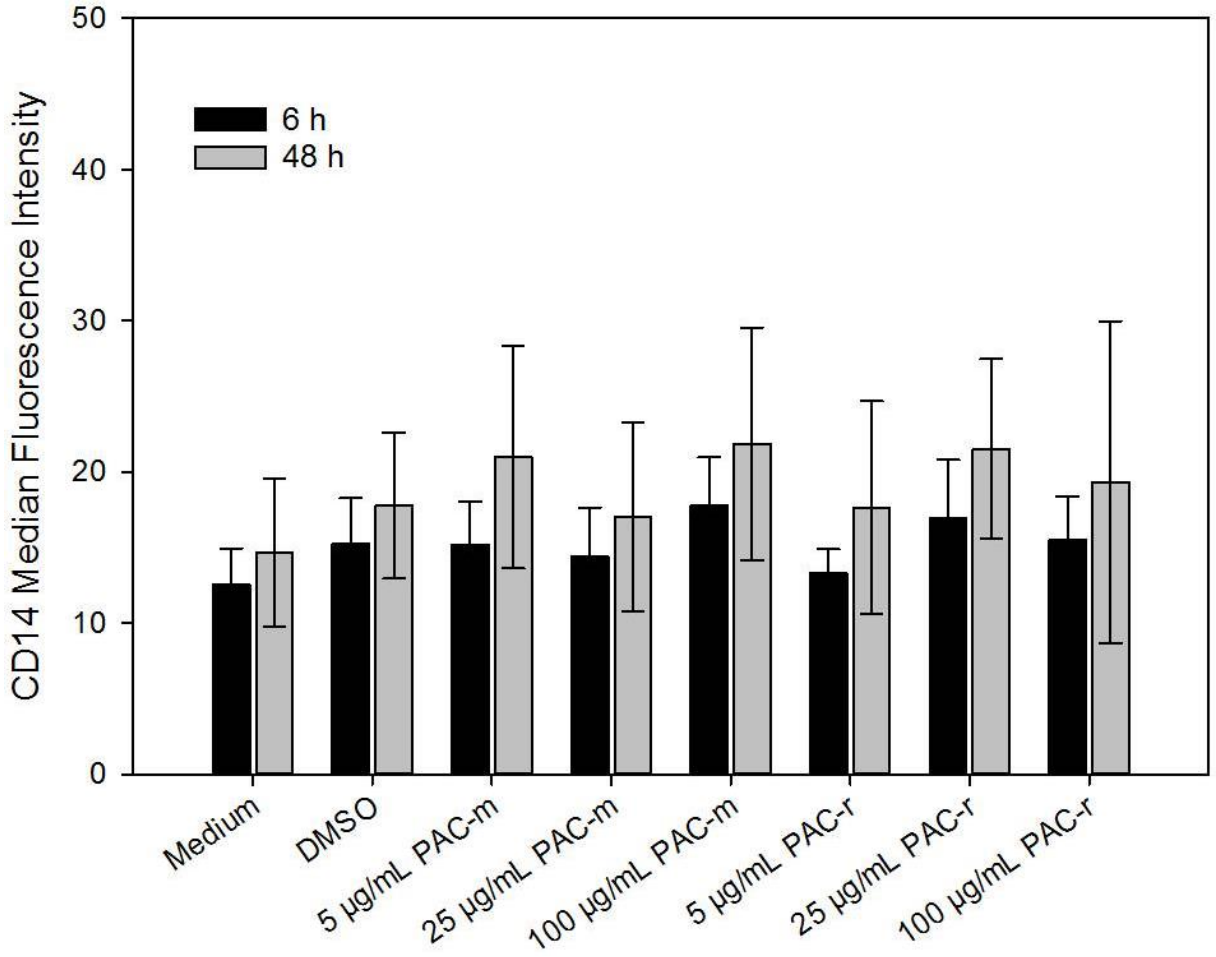


Figure 3-7. CD14 expression per cell did not differ on THP-1 cells from 6 to 48 h of cranberry treatment. Cells were incubated with increasing concentrations of PAC-m or PAC-r, medium or DMSO vehicle. Cells were harvested at 6 and 48 h. Flow cytometry was used to analyze CD14-PE MFI, which represents the CD14 expression per cell. Values represent means ± SD of two experiments each with duplicate treatments. Data were analyzed with a two-way ANOVA and Tukey all-pairwise post-test ($P<0.05$).

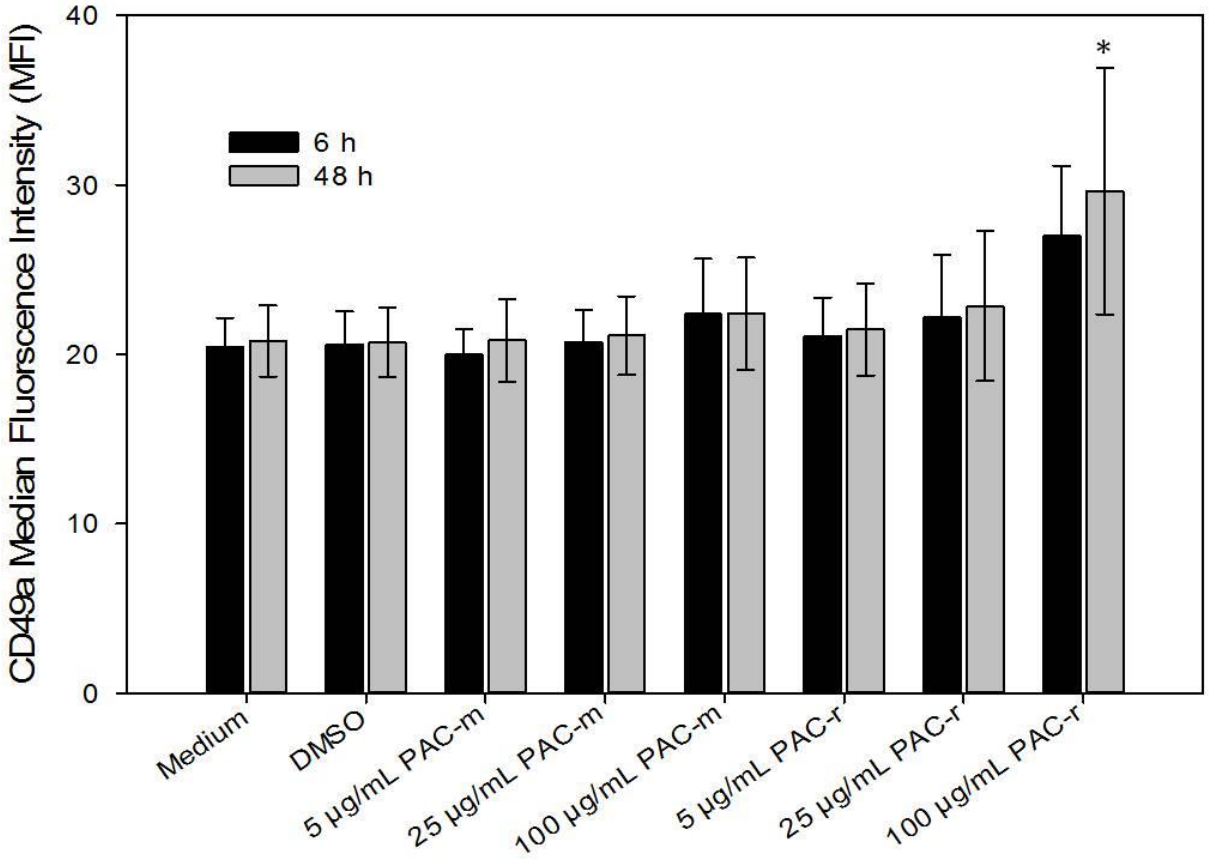


Figure 3-8. CD49a expression per cell was increased by 48 h treatment with100 µg/mL PAC-r. Cells were incubated with increasing concentrations of PAC-m or PAC-r, medium or DMSO vehicle. Cells were harvested at 6 and 48 h. Flow cytometry was used to analyze CD49a-FITC MFI, which represents the CD49a expression per cell. Values represent means ± SD of two experiments each with duplicate treatments. Data were analyzed with a two-way ANOVA and Tukey all-pairwise post-test. *Significant versus all other treatments within the same time point ($P<0.05$).

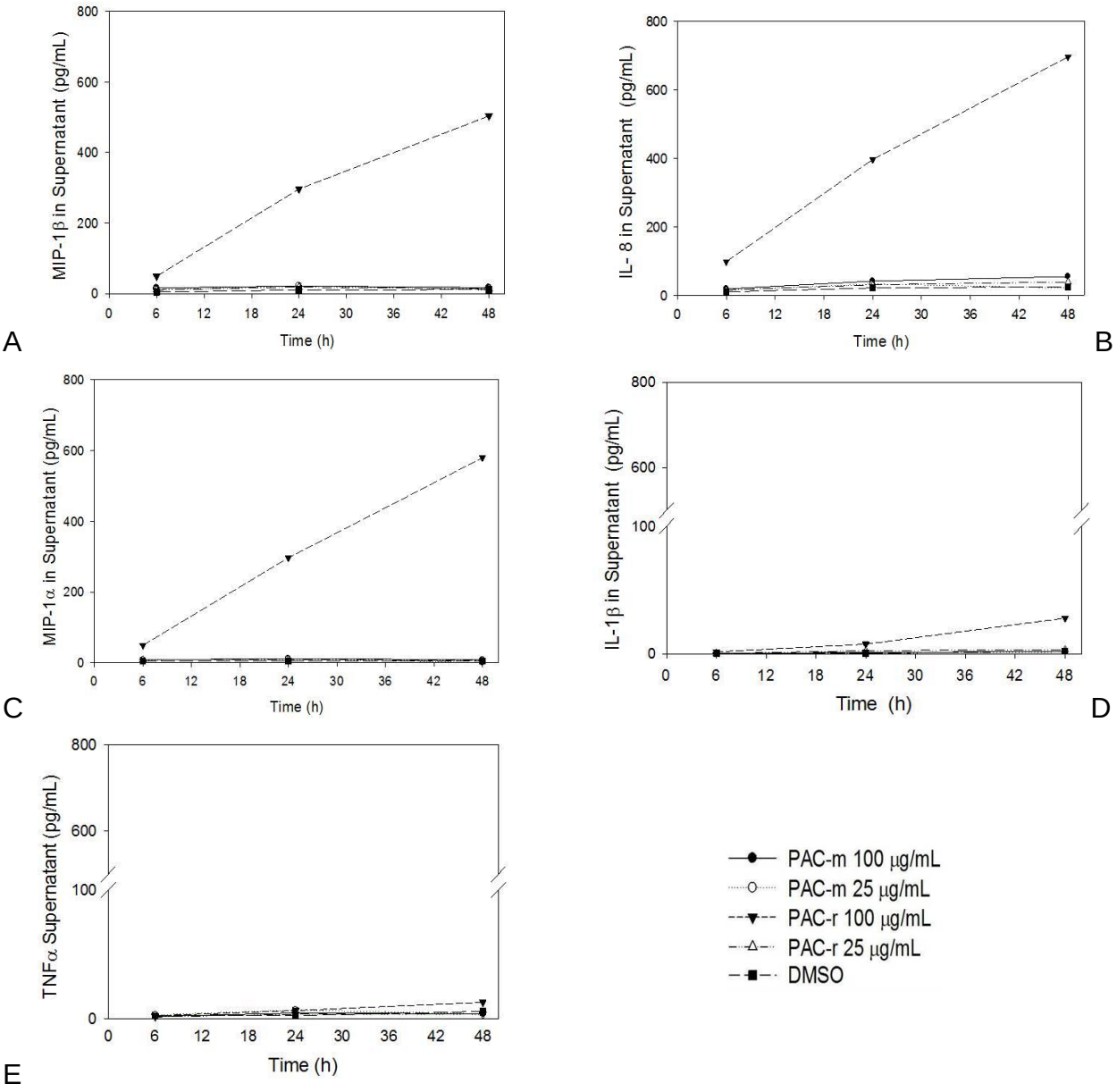


Figure 3-9. Chemokine secretion into supernatant was markedly increased by incubation with 100 µg/mL PAC-r. THP-1 monocyte chemokine and cytokine secretion in response to incubation with cranberry for 6, 24 and 48 h. Subpanels represent: A) MIP-1β concentration in supernatant, B) IL-8 concentration in supernatant, C) MIP-1α concentration in supernatant, D) IL-1β concentration in supernatant and E) TNFα concentration in supernatant.

CHAPTER 4
CRANBERRY BIOACTIVES INCREASE MIP-1α and TNFα PROTEIN SECRETION AND TRANSCRIPT ABUNDANCE IN THP-1 MACROPHAGES

Background

Having selected a cranberry concentration of 25 μg/mL and an incubation time of 6 h, the primary hypothesis that cranberry bioactives would enhance THP-1 response to secondary challenge with LPS was tested. The mechanism of enhanced response that was targeted was increased steady-state cytokine and chemokine transcript abundance and increased transcript stability.

Secretion of chemokines and cytokines during an immune response to pathogen challenge is of paramount importance to ensuring that leukocytes can migrate to the area of pathogen invasion and carry out functions required to swiftly eliminate the immune invader and prevent subsequent infection. Chemokines and cytokines produced by monocytes and macrophages in response to invading pathogens and other immune stimuli include GM-CSF, MIP-1α, TNFα IL-1β and IL-6 (49).

Production of these cytokines and chemokines are regulated via multiple mechanisms including gene transcription, mRNA stability and translation of mRNA to protein (50). The increased expression of cytokine and chemokine mRNA mediated by an increase in stability following treatment of MOLT-14 cells, a proposed γδ T cell line possessing innate and adaptive immune cell characteristics, with APP led us to hypothesize that the molecular actions taking place inside THP-1 cells exposed to low levels of cranberry may involve increased mRNA abundance and stability (36).

To identify the cytokine and chemokine mRNA whose expression might putatively be increased by cranberry fractions, we used Luminex® multiplex technology to analyze cytokine and chemokine proteins in cell supernatant following incubation with

25 µg/mL cranberry PAC-m and PAC-r with and without secondary LPS stimulation. Screening protein secretion for mRNA to analyze is supported by the work of Chanput et al in which cytokine and chemokine protein kinetics reflected mRNA kinetics (49).

Initially, THP-1 monocytes were utilized for screening experiments; however, the small magnitude of the cytokine and chemokine secretion in LPS challenged cells precluded our ability to test our hypothesis that cranberry could enhance innate immune cell response to secondary challenge. Differentiation of THP-1 monocytes into macrophage-like cells results in enhanced responses to immune stimuli; therefore, THP-1 monocytes were differentiated into macrophage-like cells using phorbol 12-myristate 13-acetate PMA for use in all further experiments (5).

Materials and Methods

THP-1 Differentiation

THP-1 monocytes were cultured in 60 mm x 15 mm tissue culture-treated plates at 5 x 10^5 cells/mL with a total of 3 x 10^6 cells per plate. Cell differentiation was achieved by incubating cells with 100 ng/mL PMA (Cat#P1585, Sigma-Aldrich®, St. Louis, MO) for 72 h in a 37°C, 5% CO_2 incubator as described by Schwende et al. (51)

THP-1 Macrophage Cell Culture

The cell culture methods for the cytokine and chemokine screening experiment and steady-state mRNA abundance experiment are explained separately to avoid confusion with LPS stimulation in the two experiments.

Cytokine and chemokine screening experiment

Differentiated THP-1 cells were washed with 2 mL of 37°C PBS. Fresh RPMI 1640 complete medium containing 25 µg/mL PAC-m or PAC-r or DMSO was added to culture dishes. Cells were returned to a 37°C, 5% CO_2 incubator for 6 h. Medium

containing treatments was removed, and cells were washed with 37°C PBS. THP-1 macrophages were then stimulated with 1 µg/mL LPS from *Escherichia coli* O55:B5 (Cat#L4524; Sigma-Aldrich®, St. Louis, MO) or PBS vehicle control for 6 h in a 37°C, 5% CO_2 incubator. After stimulation, cells were harvested and cell-free supernatants were stored at -80°C prior to multiplex analysis.

Steady-state mRNA abundance experiment

THP-1 macrophage-like cells were washed with 37°C PBS to remove residual PMA. Cranberry fractions, LPS and DMSO vehicle were diluted in 37°C RPMI 1640 complete medium and added to cells in culture dishes. Final concentrations of cranberry fractions and LPS in medium were 25 µg/mL and 1 µg/mL, respectively. The final percentage of DMSO in culture medium was less than 0.1%. Culture dishes containing cells were returned to a 37°C, 5% CO_2 incubator for 6 h.

Multiplex Analysis

Cytokine and chemokine production in response to cranberry fractions was quantified with a Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel (Cat#HCYTOMAG-60K; EMD Millipore; Billerica, MA) according to the manufacturer's protocol. All washing steps were performed on an AquaMax 4000 (Molecular Devices; Sunnyvale, CA) automatic plate washer. Specific cytokines analyzed included GM-CSF, IL-12 (p40), IL-1β, IL-6, MCP-1, MIP-1α, MIP-1β and TNFα. A Luminex® 200™ multiplex system with xPONENT® 3.1 software (Luminex Corp., Austin, TX) was used for bead analysis.

RNA Isolation, cDNA Synthesis and qRT-PCR

Culture medium was aspirated from tissue culture dishes and cells were harvested for RNA isolation without washing. RNA was purified from THP-1

macrophages using RNAzol® RT (Molecular Research Center, Inc., Cincinnati, OH). First-strand cDNA was synthesized from 1 µg RNA using a High-Capacity cDNA Reverse Transcription Kit (Cat#4368814, Applied Biosystems, Foster City, CA). Reverse transcription was performed on a MJ Research PTC-150 Thermal Cycler. Thermal cycling conditions consisted of 10 min at 25°C, 120 min at 37°C, 5 min at 85˚C and 1 min at 4˚C.

Quantitative RT-PCR was performed on an Applied Biosystems™ 7300 Real-Time PCR System using Power SYBR® Green PCR Master Mix (4367659, Applied Biosystems™, Foster City, CA) and four 10-fold serial dilutions. Conditions for quantitative RT-PCR were 2 min at 50°C for stage 1, 10 min at 95°C for stage 2 and 15 sec at 95°C, followed by 1 min at 60°C for 40 replicate cycles for stage 3.

Primers for *MIP-1α*, *TNFα, RPL37A* and *Cyclophilin B* (Table 4-1) were designed using Primer Express 3.0 software (Applied Biosystems™, Foster City, CA) and purchased from Eurofins MWG Operon (Huntsville, AL). Prior to use in experiments, a melting curve analysis was performed on all primers to confirm specificity of amplification (data not shown).

MIP-1α and *TNFα* values were normalized to the *average of RPL37A and Cyclophilin B* as the normalized values *of MIP-1α and TNFα* using each housekeeping gene independently were positively correlated (r^2= 0.99). Additionally, the use of *RPL37A* and *Cyclophilin B* as reliable reference genes in LPS-treated THP-1 cells and PMA differentiated THP-1 cells is supported by the work of Cao et al and Maess et al (52,53).

mRNA Stability

Cells were treated 6 h with 25 µg/mL PAC-m or PAC-r, 1 µg/mL LPS, or DMSO. Actinomycin D (Sigma-Aldrich®, St. Louis, MO) at a final concentration of 5 µg/mL was then spiked directly into culture medium to inhibit *de novo* transcription, following recommended mRNA decay measurement protocols (54). Cells were harvested for RNA isolation at 1, 30, 90 and 180 min post-actinomycin D addition. RNA isolation, cDNA synthesis and qRT-PCR were carried out as described above with the exception that *TNFα* and *MIP-1α* were normalized to the single housekeeping gene *Cyclophilin B*.

Statistical Analysis

All statistical analysis procedures were conducted with SigmaPlot version11.0 or 12.0 (Systat Software Inc., San Jose, CA). Separate statistical tests were performed for each cytokine/chemokine of interest in cells stimulated or not stimulated with LPS. A one-way ANOVA was used to test for significant differences among the means of treatment groups with the exceptions of IL-12 (p40) and MCP-1 from stimulated cells. For IL-12 (p40), a one-way ANOVA on $\log_{10}$ transformed data was used due to non-normality. Data is reported for IL-12 (p40) from stimulated cells as mean $\pm$ SD of untransformed data. Log_{10} transformation of MCP-1 values was attempted; however, normality could not be achieved. Thus, MCP-1 values were analyzed with Kruskal-Wallis one-way ANOVA on ranks. A Tukey multiple comparison procedure was used when necessary to determine what means differed between treatment groups. Data is reported as mean ± SD.

Differences in steady state mRNA abundance for *TNFα* were determined with a one-way ANOVA ($P<0.05$) on square root transformed data to correct for unequal

variance. For steady state *MIP-1α* mRNA abundance, a one-way ANOVA ($p<0.05$) on $\log_{10}$ transformed data was used to correct non-normal data. A Tukey multiple comparison all-pairwise test was used to determine which treatment means differed. Data are shown as mean ± SD of untransformed data.

For *TNFα* and *MIP-1α* transcript stability, relative mRNA abundance was plotted over time and generation of a trendline was used to derive the slope (relative *TNFα* abundance/min) for each experimental duplicate using Microsoft Excel 2007 (Microsoft, Redmond, WA). The slope values from two independent experiments were then used for statistical analysis.

For *TNFα* slopes, a one-way ANOVA ($P<0.05$) was used to test for significant differences among DMSO, PAC-m and PAC-r treatment group means. Data is shown as mean ± SD.

For *MIP-1α* slopes, statistical analysis was not conducted as there was little or no reduction of transcript abundance over the 3 h period following actinomycin D addition regardless of treatment. Data is shown as median with the range of values.

Results

Multiplex Analysis

In order to prevent confusion, THP-1 macrophages treated with cranberry or vehicle followed by subsequent LPS stimulation will be referred to as “stimulated” and macrophages treated with cranberry or vehicle followed by incubation with PBS will be referred to as “unstimulated” throughout the multiplex analysis portion of the results section.

The concentration of eight cytokines/chemokines in culture medium following treatment with cranberry followed by stimulation or no stimulation with LPS was

analyzed with multiplex technology. Detectable levels of all analytes were present in the medium of stimulated and unstimulated cells, except for GM-CSF in unstimulated culture medium (Figure 4-1).

For unstimulated cells, differences in mean values among PAC-r, PAC-m and DMSO treatment groups were significant only for the analytes MIP-1α ($P<0.001$), MIP-1β ($P<0.001$), TNFα ($P<0.001$) and IL-6 ($P<0.008$) (Figure 4-1). A Tukey all-pairwise multiple comparison test was used to determine which treatment group means differed. The MIP-1α concentration in culture medium of PAC-r and PAC-m unstimulated cells was significantly ($P<0.001$) less than the concentration present in the culture medium of vehicle-treated cells; however, no significant difference ($P<0.787$) existed between the mean MIP-1α concentrations in PAC-r-treated and PAC-m-treated cell culture medium. MIP-1β concentration in culture medium from DMSO-treated cells was greater than for PAC-m ($P<0.001$) and PAC-r ($P<0.001$) treated cells. PAC-r treated cells produced significantly less ($P=0.037$) MIP-1β compared to PAC-m treated cells. The concentration of TNFα in culture medium followed the same pattern as MIP-1β with TNFα in culture medium of cells treated with PAC-m and PAC-r being significantly ($P<0.001$) less than that in DMSO treated cell culture medium. PAC-r treated cells also produced significantly ($P=0.004$) less TNFα than PAC-m treated cells.

Stimulated THP-1 macrophages secreted concentrations of cytokines and chemokines that were of greater magnitude than unstimulated cells as noted by the concentration ranges of 0-120 pg/mL for the unstimulated cell graph (Figure 4-1) versus 0-5000 pg/mL for the stimulated cell graph (Figure 4-2). Out of the eight cytokines and chemokines analyzed, MIP-1α and TNFα were the only two analytes whose mean

concentration in culture medium was significantly (P=0.006 for MIP-1α and P<0.001 for TNFα) different among treatment groups based on one way ANOVA. Post-test analysis showed that treatment with PAC-r prior to stimulation resulted in a modest but significant (P=0.005) increase in MIP-1α secretion into medium compared with DMSO treatment (Figure 4-2). The concentration of TNFα in culture medium from all treatment groups was approximately 10 times lower than MIP-1α concentrations. PAC-r induced a subtle, but significant increase in TNFα secretion (P=0.013) compared to DMSO and (P<0.001) compared to PAC-m.

As MIP-1α and TNFα concentrations were both increased in cranberry-treated and stimulated cell culture medium as compared to vehicle-treated and stimulated cells, these two cytokines/chemokines were selected for analysis of steady-state mRNA and mRNA stability following cranberry treatment.

Steady-State mRNA Abundance

To determine if cranberry primes THP-1 macrophages via a mechanism involving increased cytokine/chemokine transcript abundance, qRT-PCR was used to measure the steady-state levels of *TNFα* and *MIP-1α* mRNA following 6 h treatment with cranberry fractions or vehicle control. Relative abundances of both *MIP-α* and *TNFα* were upregulated with PAC-r treatment in comparison to vehicle control and PAC-m treatment (Figure 4-3, panels A and B for *TNFα* and *MIP-1α*, respectively). The fold change in relative abundance was 1 and 2.5 for *TNFα* and *MIP-1α*, respectively for PAC-r treated cells vs. vehicle.

mRNA Stability

The possibility that the upregulation of steady-state *TNFα* and *MIP-1α* mRNA by PAC-r was due to enhanced mRNA stability was explored using actinomycin D, an inhibitor of *de novo* transcription, along with qRT-PCR.

Relative abundance of *TNFα* over time following actinomycin D addition declined from 1 to 180 min in all treatment groups (Figure 4-4). However, mean rate of change in *TNFα* transcript abundance did not differ significantly (P=0.057) among THP-1 macrophages treated with PAC-r, PAC-m and DMSO (Table 4-2).

Relative abundance of *MIP-1α* following actinomycin D addition remained stable over time (Table 4-3 and Figure 4-5).

Discussion

Work in this chapter focused on investigating the ability of cranberry fractions to heighten the THP-1 response to secondary challenge with a bacterial cell wall component via molecular changes in mRNA steady-state abundance and mRNA stability. We first used Luminex® technology to screen for cytokine and chemokine proteins that were secreted in greater quantities by cranberry-treated and LPS challenged versus vehicle-treated and challenged cells to identify potential cytokines and chemokines whose mRNA expression might be altered by cranberry bioactives. This reverse methodology was utilized because proteins are the active mediators of immune responses. THP-1 macrophages were treated with cranberry or vehicle and were unstimulated or stimulated with LPS to mimic aspects of a pathogen challenge. The unstimulated cells were used as a control to determine if cranberry alone was inducing cytokine and chemokine production, thus activating THP-1 macrophages. Although our previous experiments showed that 25 μg/mL did not activate THP-1

monocytes, it was imperative that we test the same concentration in the THP-1 macrophage model. Based on the absence of increase in cytokine and chemokine protein in unstimulated cells and the ten-fold lower concentrations of cytokines and chemokines in culture medium from unstimulated versus stimulated cells, we concluded that 25 µg/mL PAC-r or PAC-m did not activate THP-1 macrophages.

The enhanced production of TNFα and MIP-1α in cranberry-treated and stimulated cells suggests that cranberry does indeed prime THP-1 macrophages. Our findings both agree and disagree with other research involving modulation of cytokine and chemokine production by cranberry in immune cells.

Research that is in agreement with our findings that cranberry enhances cytokine and chemokine production includes an unpublished *in vitro* study of cranberry using the HL-60 neutrophil-like cell line and an unpublished human dietary intervention study both conducted in our lab. A 24 h incubation of HL-60 neutrophil-like cells with 50 µg/mL PAC-r and 250 ng/mL LPS resulted in TNFα secretion into medium that was five-fold greater than secretion induced by vehicle control or LPS alone (Muller unpublished). Although a different cell line was used in Muller's work, HL-60 cells were treated with the same PAC-r utilized in our research, suggesting that cranberry treatments utilized in experiments may have a greater effect on cellular response than differences in innate immune cell type.

The physiological relevance of cranberry in boosting immune cell response to secondary challenge was demonstrated in a human dietary intervention previously conducted in our lab. Healthy subjects consumed 12 fl oz of cranberry beverage or placebo each day for 10 weeks. At baseline and after 10 weeks, the concentrations of

cytokines and chemokines were measured in culture medium of PBMC stimulated *ex vivo* with phytohemagglutinin (PHA). For the cranberry group, IFNγ secretion increased by 148 pg/mL from baseline to week 10. In the placebo group, IFNγ decreased by 24 pg/mL after 10 weeks of beverage consumption. Although five other cytokines were analyzed, including IL-1β, TNF-α, IL-17, IL-1α and MIP-1β, no significant difference in secretion from baseline to ten weeks between the cranberry and placebo groups was observed (Percival unpublished).

In contrast to the increases in cytokine and chemokine production seen with LPS challenge in our research, other studies suggest that cranberry attenuates LPS induced cytokine production. Bodet et al investigated the ability of cranberry juice non-dialyzable material (NDM) with a molecular weight greater than 14,000 to inhibit LPS induced inflammatory cytokine production in the U937 leukemia monoblastic cell line (55). Following a 2 h stimulation with cranberry and subsequent 24 h incubation with LPS derived from five separate sources (*F. nucleatum, P. gingivalis, T. Denticola and T. forsythia*), the concentrations of TNFα, IL-1β, IL-6 and RANTES (regulated upon activation normal T-cell expressed and presumably secreted) in cell supernatants were measured with enzyme linked immunosorbent assay (ELISA). Although reductions in cytokine secretion occurred in cells treated with cranberry and stimulated with LPS, the change was dependent on the type of LPS used as well as the concentration of cranberry NDM. When LPS derived from *F. nucleatum* was used as the immune activator, 50 μg/mL cranberry NDM suppressed IL-1β, TNFα and IL-6 production. The 50 μg/mL concentration of NDM was also capable of reducing TNFα induced by *E. coli*

O55:B5 LPS. The 10 μg/mL cranberry NDM treatment did not prevent LPS induced cytokine production for IL-1β, TNFα, IL-6 or IL-8 regardless of the LPS source (55).

Although the anti-inflammatory effect of cranberry on LPS stimulated U937 cells contrasts with the increase in TNFα and MIP-1α secretion in our research, the differences in the results can be explained by a combination of differences in experimental design (55). Primary differences include the removal of medium containing cranberry in our study prior to LPS challenge, utilization of a cranberry concentration one half the concentration used by Bodet et al, variations in cranberry and LPS treatment times and use of different cell lines. In order to determine the effects initiated by cranberry interaction with the THP-1 cell, we treated cells with cranberry for 6 h, followed by a wash and addition of fresh medium containing LPS. Bodet et al incubated cells simultaneously with cranberry NDM and LPS (55). Cranberry NDM with a molecular weight >6000 reduces LPS binding to the TLR4/MD2 receptor complex on the surface of HEK293 cells as detected by immunofluorescence (56). Thus, the anti-inflammatory effect reported by Bodet et al may be due to an interaction of cranberry with LPS rather than by a molecular effect induced by cranberry interaction with receptors on the immune cell surface.

The composition differences between the cranberry NDM used by Bodet et al and the PAC-m and PAC-r fractions used in our research could also account for dissimilar cytokine production between the two studies. The NDM used by Bodet et al contained 0.35% anthocyanins and 65.1% PAC and was void of sugars and organic acids (55). The NDM PAC, sugar and organic acid levels were approximately equivalent to those in PAC-m; however, the anthocyanin level was closer to the <0.5% found in

PAC-r. The fact that cranberry NDM induced different cellular responses than PAC-r and PAC-m and fully reflected neither fraction's chemical makeup emphasizes the dependence of cranberry's immune modulating effects on treatment composition.

In order to elucidate the molecular mechanism responsible for the priming effect seen in PAC-r treated cells, we conducted qRT-PCR on RNA isolated from THP-1 macrophages treated for 6 h with PAC-r, PAC-m, or vehicle control. PAC-r upregulated *TNFα* and *MIP-1α* mRNA by 1 and 2.5 fold, respectively, compared with vehicle control and PAC-m.

When actinomycin D was added to cranberry-treated THP-1 macrophages, *MIP-1α* transcripts did not appear to decay over the 3 h time period. In contrast, *TNFα* mRNA decayed with PAC-r, PAC-m, LPS and DMSO treatment; however, there was no difference in the rate of degradation between vehicle control and PAC-r or vehicle control and PAC-m treatments. The decay rate of *TNFα* is well studied, and the *TNFα* half-life in our study of approximately 30 min is within the range of half- lives published in the literature (17,57,58).

The absence of decay in *MIP-1α* mRNA in the 3 h post-actinomycin D time period, may be explained by the number of ARE present in the 3'UTR. Using the ARED Organism database which offers a quantitative compilation of AREs in the human, mouse and rat transcriptomes combined with the AREsite database, we found that *MIP-1α* contains four ATTTA pentamers, while *TNFα* contains nine ATTTA pentamers (59,60). The lower abundance of *cis*-regulatory elements in the *MIP-1α* 3'UTR means that less *trans*-acting regulatory proteins, such as TTP, can bind to the mRNA to promote deadenylation of the poly(A) tail and subsequent mRNA decay (61,62).

The increase in steady-state *TNFα* and *MIP-1α* mRNA abundance following cranberry treatment in the absence of alterations in transcript stabilization compared to other treatments could be due to an increase in the rate of transcription. *MIP-1α* and *TNFα* share response elements in their gene promoters for transcription factors including NF-κB, IRF3, AML1a and C/EBPβ (14). These transcription factors are activated in macrophages and other immune cells by immune stimuli and are capable of initiating the transcriptional activation of *TNFα* and *MIP-1α* during both early and late phases of an immune cell response (14).

In summary, PAC-r but not PAC-m enhances THP-1 macrophages' ability to respond to LPS challenge as PAC-r treated cells displayed enhanced production of TNFα and MIP-1α protein secretion upon LPS stimulation. Moreover, this increased cytokine and chemokine production is mediated by a priming mechanism involving upregulated steady-state mRNA abundance that is independent of *TNFα* and *MIP-1α* mRNA stability based on the time points used in our experiments.

Table 4-1. qPCR primer sequences

Primer Name		Primer Sequence
MIP-1α	Forward	5’ – CCATGGCTCTCTGCAACCA - 3’
	Reverse	5’ – GCGGTCGGCGTGTCA - 3’
TNFα	Forward	5’- GCAGGTCTACTTTGGGATCATTG - 3’
	Reverse	5’ – GCGTTTGGGAAGGTTGGA - 3’
RPL37A	Forward	5’ - GGCCTCCCTCCGGAAA - 3’
	Reverse	5’ – TTGGCGTGCTGGCTGAT - 3’
Cyclophilin B	Forward	5’ – TGCCATCGCCAAGGAGTAG - 3’
	Reverse	5’ - CTGCACAGACGGTCACTCAAA - 3’

Table 4-2. *TNFα* mRNA relative abundance rate of change

Treatment	Relative *TNFα* mRNA / min
DMSO	-0.0382 ± 0.0093
PAC-r	-0.0384 ± 0.0047
PAC-m	-0.0540 ± 0.0117

TNFα mRNA levels were normalized to levels of the housekeeping gene *Cyclophilin B*. Rate of change values were ascertained by plotting relative *TNFα* abundance over time, followed by generation of a trendline to derive the slope (relative *TNFα* abundance/min) for each experimental duplicate. Values represent mean ± SD of two independent experiments. Data were analyzed with a one-way ANOVA ($P<0.05$).

Table 4-3. *MIP-1α* mRNA relative abundance rate of change

Treatment	Relative *MIP-1α* mRNA / min
DMSO	0.0021 (0.0003 - 0.0027)
PAC-r	0.0000 (0.0076 - 0.0339)
PAC-m	0.0103 (-0.0052 - 0.0023)

MIP-1α mRNA levels were normalized to levels of the housekeeping gene *Cyclophilin B*. Rate of change values were ascertained by plotting relative *MIP-1α* abundance over time, followed by generation of a trendline to derive the slope (relative *MIP-1α* abundance/min) for each experimental duplicate. Values represent median with the range of values from two independent experiments.

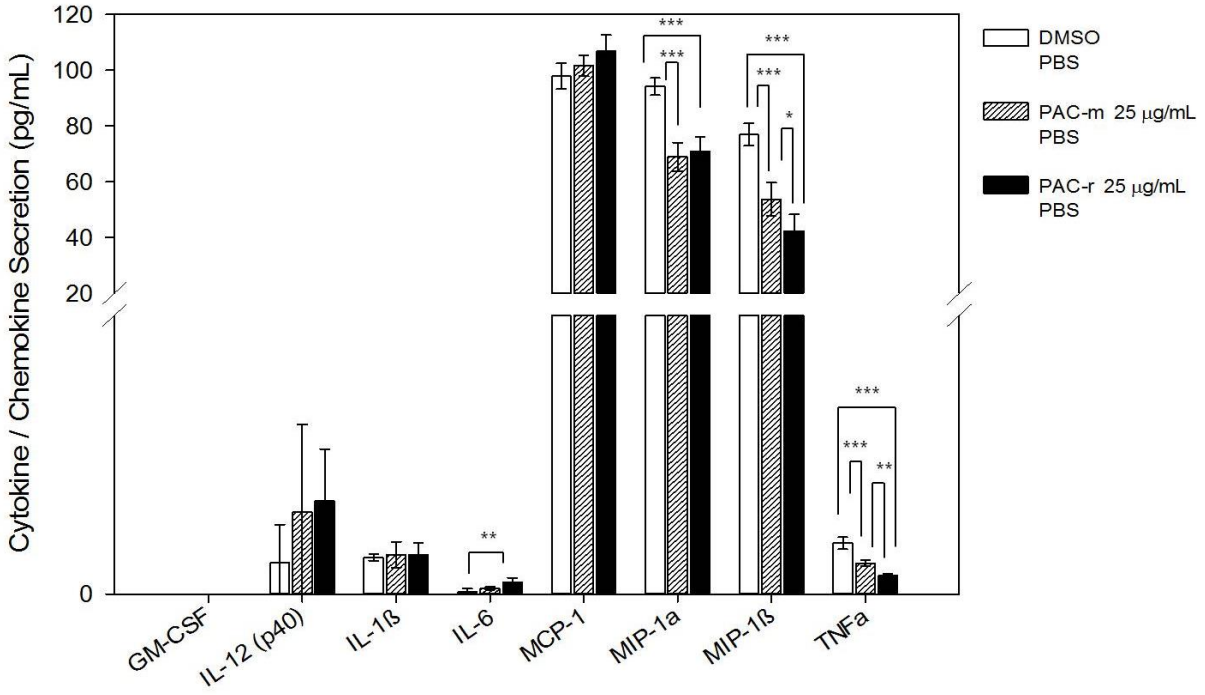


Figure 4-1. Cranberry did not activate unstimulated THP-1 macrophages. THP-1 macrophages were treated with 25 µg/mL PAC-r, PAC-m, or DMSO for 6 h, followed by 6 h incubation with PBS vehicle control. Cell supernatants were collected and analyzed for cytokines and chemokines using multiplex technology. Values represent mean ± SD from two independent experiments. *P<0.05, **P<0.01, ***P<0.001

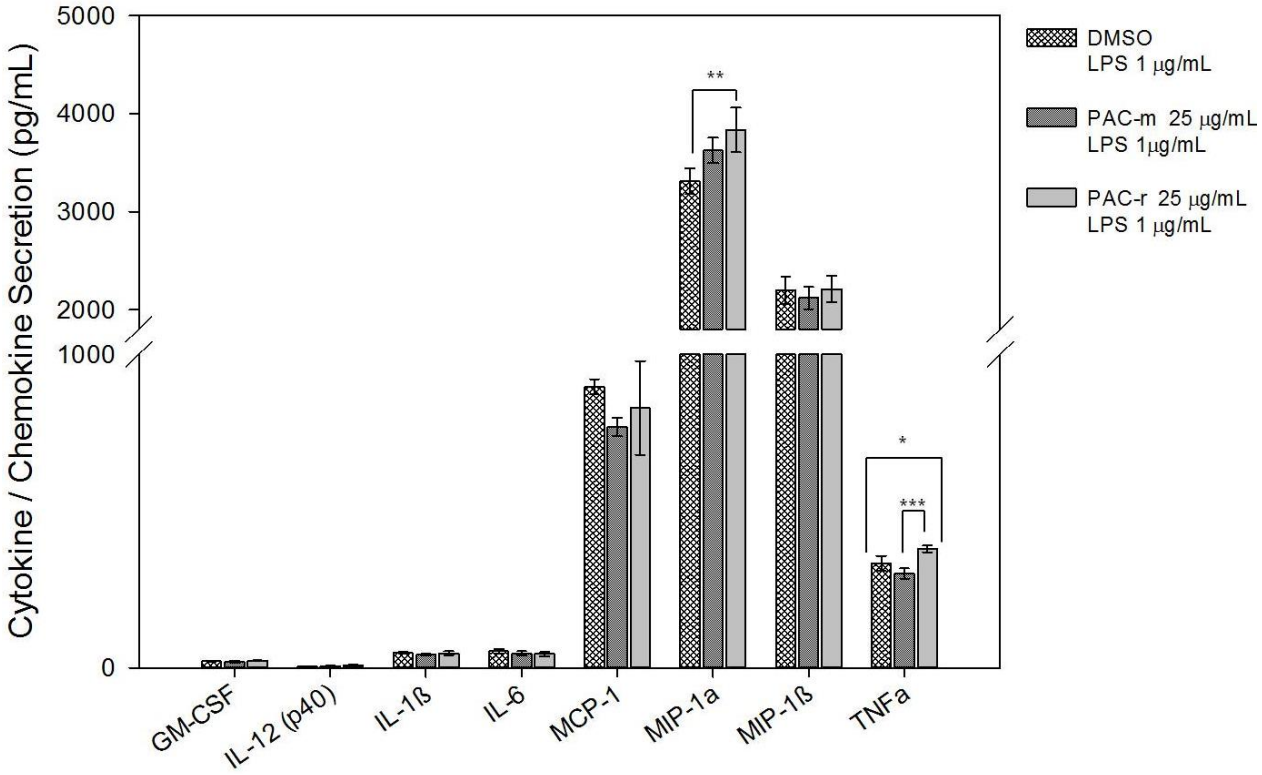
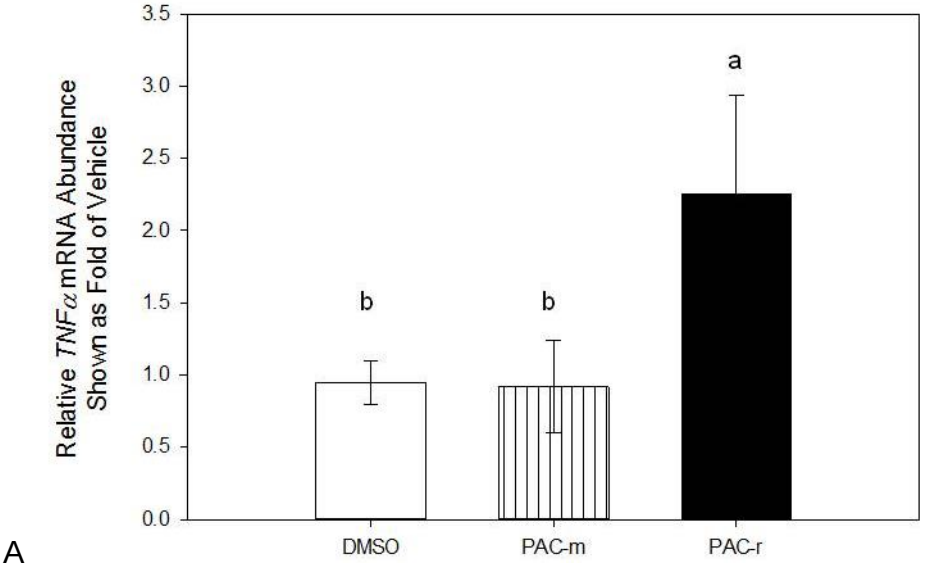
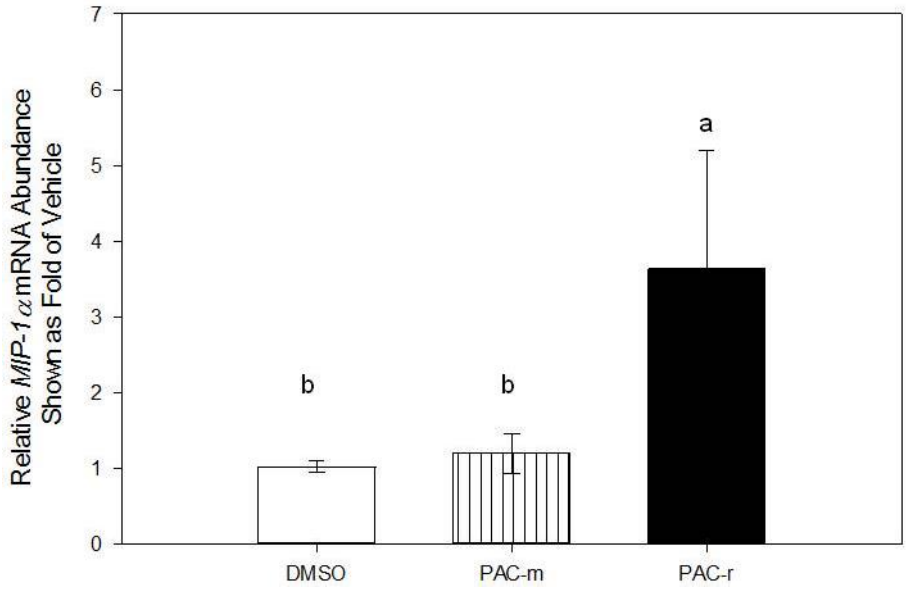


Figure 4-2. PAC-r enhanced secretion of MIP-1α and TNFα from stimulated THP-1 macrophages. THP-1 macrophages were treated with 25 μg/mL PAC-r, PAC-m, or DMSO for 6 h, followed by 6 h incubation with LPS. Cell supernatants were collected and analyzed for cytokines and chemokines using multiplex technology. Values represent mean ± SD from two independent experiments. *P<0.05, **P<0.01, ***P<0.001



A



B

Figure 4-3. Cranberry upregulated expression of *TNFα* and *MIP-1α* mRNA. A) *TNFα* mRNA relative abundance. B) *MIP-1α* mRNA relative abundance. THP-1 macrophages were treated for 6 h with 25 µg/mL PAC-m or PAC-r or vehicle control. Cellular RNA was isolated and reverse transcribed to cDNA. Quantitative RT-PCR was performed on an Applied Biosystems® 7300 RT-PCR system. *MIP-1α* and *TNFα* levels were normalized to the average level of two housekeeping genes (*RPL37A* and *Cyclophilin B*). Data were analyzed with a one-way ANOVA and Tukey post-hoc analysis. Bars without common letters are significantly different from one another ($P<0.05$).

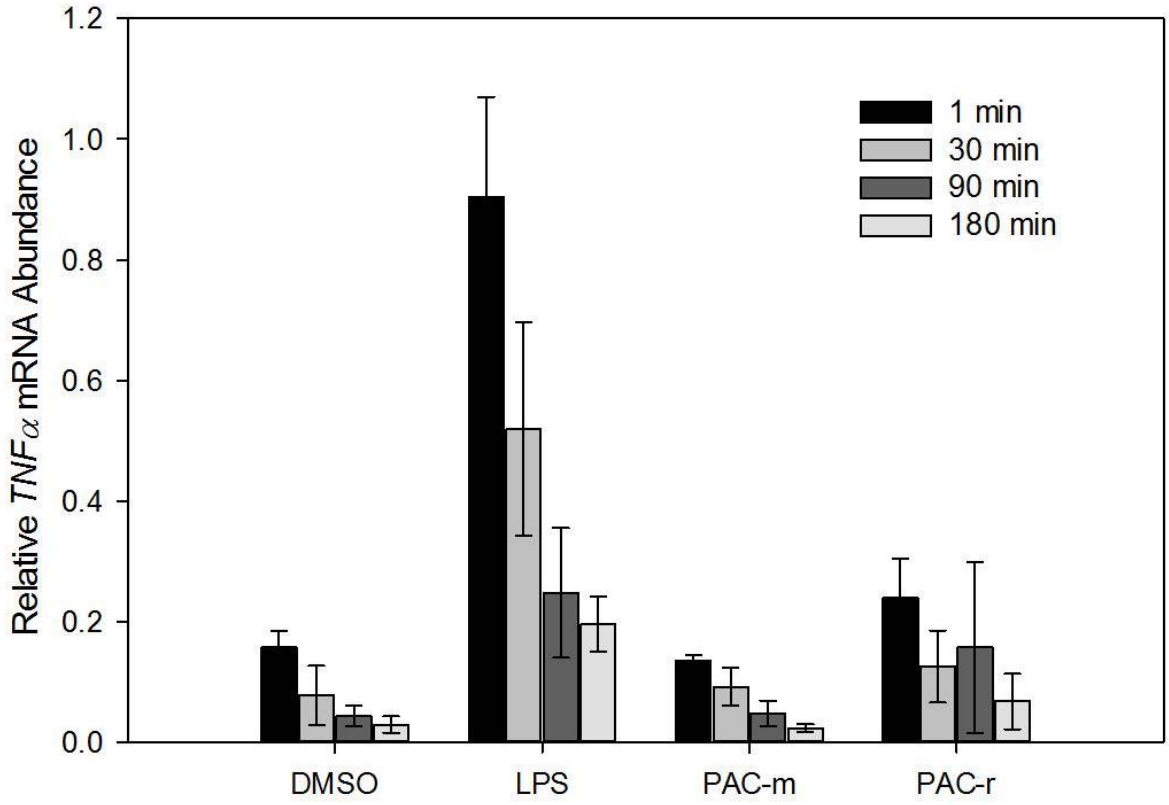


Figure 4-4. Cranberry did not stabilize *TNFα* mRNA. THP-1 macrophages were treated for 6 h with 25 µg/mL PAC-m or PAC-r or vehicle control followed by addition of 5 µg/mL actinomycin D. Cellular mRNA was isolated at 1, 30, 90 and 180 min post-actinomycin D addition and was reverse transcribed to cDNA. Quantitative RT-PCR was performed on an Applied Biosystems® 7300 RT-PCR system. *TNFα* transcript levels are shown relative to housekeeping gene *Cyclophilin B*. Values represent mean ± SD of two independent experiments.

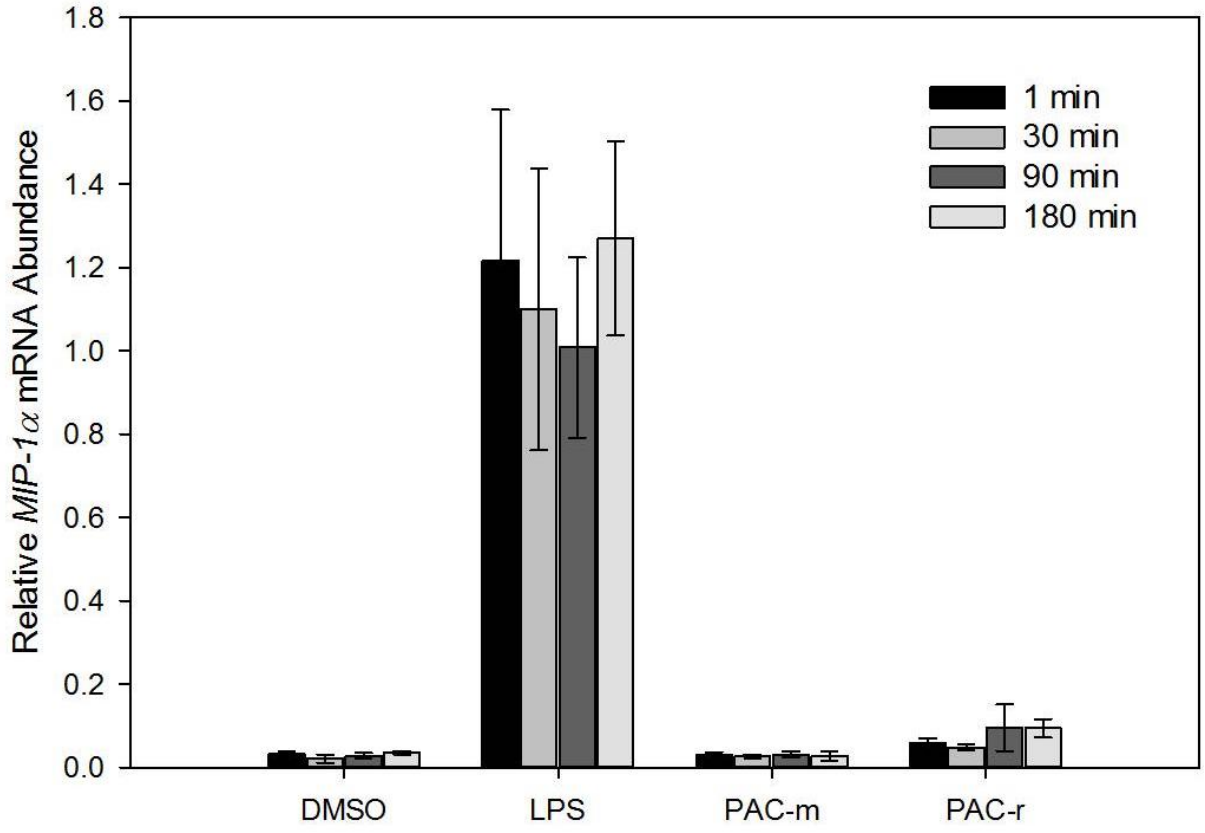


Figure 4-5. *MIP-1α* mRNA stability was not altered by cranberry. THP-1 macrophages were treated for 6 h with 25 µg/mL PAC-m or PAC-r, 1 µg/mL LPS, or vehicle control followed by addition of 5 µg/mL actinomycin D. Cellular mRNA was isolated at 1, 30, 90 and 180 min post-actinomycin D addition and was reverse transcribed to cDNA. Quantitative RT-PCR was performed on an Applied Biosystems® 7300 RT-PCR system. *MIP-1α* transcript levels are shown relative to housekeeping gene *Cyclophilin B*. Values represent mean ± SD of two independent experiments.

CHAPTER 5
COMPLETE TLR4 ACTIVATION IS NOT REQUIRED FOR CRANBERRY-INDUCED INCREASES IN *MIP-1α* AND *TNFα* mRNA

Background

TLR4 is found on a variety of cell types including monocytes, macrophages, δγ T-cells and dendritic cells (8,63). Although TLR4 is the cognate receptor for LPS, it is also somewhat promiscuous in comparison to other PRR as it recognizes fibronectin, fatty acids and fusion protein from respiratory syncytial virus (9,64,65)

Previous research conducted in our lab using the HL-60 human neutrophil-like cell line provided evidence that cranberry may also be recognized by TLR4. Treatment of HL-60 cells with cranberry fractions altered levels of the TLR4 signaling protein, IRAK4 (Muller unpublished). As both HL-60 and THP-1 cell lines express TLR4, this PRR was the most logical cell surface receptor for us to investigate.

Our primary objective for this set of experiments was to determine if TLR4 mediated the cranberry-induced increases in cytokine and chemokine mRNA expression. The activation of TLR4 was first measured in the HEK-Blue™ hTLR4 cell line. The ability of cranberry to modulate cytokine and chemokine mRNA levels during priming was then explored with an hTLR4 neutralizing antibody in THP-1 macrophages.

HEK-Blue™ hTLR4 Cells are a reporter cell line designed to detect human TLR4 activation via the transcription factor NF-Kb (66). The HEK 293 cells are co-transfected with TLR4 and co-receptors CD14/ MD2, along with a secreted embryonic alkaline phosphatase gene. The SEAP gene is fused to the promoter of the pro-inflammatory cytokine gene IL-12 (p40). This promoter contains five binding sites for NF-κB and AP-1. When TLR4 is activated, the resulting signaling cascade ends in the activation of NF-κB and AP-1 which then bind to the IL-12 (p40) promoter and induce SEAP

production. SEAP production is measured with QUANTI-Blue™ detection medium. The medium turns blue in the presence of SEAP (66).

Materials and Methods

HEK- Blue™ hTLR4 Cells

HEK-Blue™ hTLR4 Cells (Cat# hkb-htlr4, Invivogen, San Diego, CA) were cultured in DMEM (Cat# 10-017-CV, Corning Cellgro®, Manassas, VA) supplemented with 50 U/ml penicillin and 50 mg/ml streptomycin (Cat# 1670049, MP Biomedicals, Santa Ana, CA), 1X HEK-Blue Selection (Cat# hb-sel, Invivogen, San Diego, CA) antibiotics and 10% FBS. The hTLR4 assay was conducted with 70% confluent cells. Cells were detached from the culture flask using PBS warmed to 37°C and resuspended at a concentration of 1.4 x 10^5 cells/mL. One-hundred eighty µL of the cell suspension was added to a 96-well, flat bottom, tissue culture plate (Cat# 07-200-90 Corning Costar®, Manassas, VA). Twenty uL of PAC-r, PAC-m, LPS from *Escherichia coli* O55:B5 (Cat#L4524; Sigma-Aldrich®, St. Louis, MO) or DMSO vehicle diluted in medium were added to the 180 uL cell suspension. The final concentration of PAC-r, PAC-m and LPS in wells was 25, 25 and 1 µg/mL, respectively. The final percentage of DMSO in the culture medium was less than 0.1%. The HEK-Blue™ hTLR4 cells were incubated at 37°C for 20 h as described by Invivogen (66). After 20 h, 20 µL of cell supernatant was mixed with 180 µL of QUANTI-Blue™ detection medium (Cat# rep-qb1, Invivogen, San Diego, CA) in a 96-well, flat bottom, tissue culture plate (Cat# 07-200-90 Corning Costar®, Manassas, VA). The plate was incubated for 1 h at 37°C. Absorbance at 655 nm was read on a SpectraMax 340PC 384 plate reader (Molecular Devices, Sunnyvale, CA).

THP-1 Macrophage and hTLR4 Neutralizing Antibody

THP-1 monocytes at 5 x 10^5 cells/mL were plated in 60 mm x 15 mm tissue culture dishes with a total of 3 x 10^6 cells per plate. Cell differentiation was achieved by incubating cells with 100 ng/mL PMA (Cat#P1585, Sigma-Aldrich®, St. Louis, MO) for 72 h in a 37°C, 5% CO_2 incubator as described by Schwende et al. (51)

Differentiated cells were washed with 2 mL of 37°C PBS. Two mL of RPMI 1640 Complete Medium containing either 1µg/mL anti-hTLR4 antibody (Cat# mabg-htlr4, Invivogen, San Diego, CA) or an equal volume of sterile water was added in duplicate to cell culture dishes. Cells were incubated for 1 h in a 37°C, 5% CO_2, humidified environment.

After the 1 h incubation, 1 mL of RPMI 1640 complete medium containing either PAC-r, DMSO vehicle or LPS was added to duplicate cell culture dishes with or without anti-hTLR4 antibody to achieve final concentrations of 25 µg/mL PAC-r and 100 ng/mL LPS. The final percentage of DMSO in culture medium was < 0.1%.

THP-1 macrophages were incubated with PAC-r, DMSO vehicle or LPS treatments for 6 h in a 37°C, 5% CO_2 incubator. After incubation, culture medium was aspirated from cells and RNAzol® RT (Cat# RN 190, Molecular Research Center, Inc., Cincinnati, OH) was used to isolate RNA. Synthesis of cDNA and qRT-PCR were carried out as described in Chapter 4. *TNFα* and *MIP-1α* were normalized to the single housekeeping gene *Cyclophilin B*.

The PAC-m cranberry fraction was not used in hTLR4 neutralization experiments as it did not upregulate *TNFα* or *MIP-1α* steady-state mRNA levels in previous experiments. The primary purpose of neutralizing hTLR4 was to determine the role of the receptor in mediating cranberry-induced increases in cytokine and chemokine

mRNA. Thus, we focused specifically on PAC-r, which displayed the most bioactivity in initial experiments. The concentration of anti-hTLR4 antibody and LPS used in the neutralization experiments were determined in a preliminary optimization experiment (see Appendix).

Statistical Analysis

SigmaPlot versions 11.0 and 12.0 (Systat Software Inc., San Jose, CA) were used for statistical analysis. Absorbance values from HEK-Blue™ hTLR4 cells are reported as mean ± SD. Differences between mean absorbance values among treatment groups were tested with a one-way ANOVA and a Tukey multiple comparison all-pairwise post-test.

For the hTLR4 neutralization experiments, data were analyzed by comparing the mRNA relative abundance from cells incubated with or without anti-hTLR4 antibody in each treatment group (PAC-r, DMSO and LPS) using a one-way ANOVA and a Tukey post-test. Data are reported as mean relative mRNA abundance $\pm$ SD with ($P<0.05$) considered significant.

Results

HEK-Blue™ hTLR4 Cells

Analysis of absorbance values from HEK-Blue™ hTLR4 cells with a one-way ANOVA revealed that a significant difference ($P<0.001$) existed between treatment group means (Figure 5-1). The mean absorbance value of supernatants from cells treated with LPS was greater ($P<0.001$) than for all other treatments based on a Tukey multiple comparison post-test, indicating that more SEAP was present in the supernatant of these cells (Figure 5-1). Treatment of cells with PAC-r resulted in more

blue color and higher absorbance values of supernatant as compared to medium ($P<0.001$) and PAC-m ($P=0.002$) treatments. Absorbance values for supernatant from HEK-Blue™ hTLR4 cells treated with PAC-m did not differ ($P=0.454$) from supernatants from medium-treated cells (Figure 5-1).

Neutralization of TLR4 in THP-1 Macrophages

The involvement of TLR4 in cranberry-induced increases in steady-state *TNFα* and *MIP-1α* mRNA abundances was investigated using an anti-hTLR4 neutralizing antibody. For cells treated with 25 μg/mL PAC-r, neutralization of TLR4 activity with an anti-hTLR4 antibody did not attenuate the upregulation of *TNFα* and *MIP-1α* mRNA (Figure 5-2, panels A and B for *TNFα* and *MIP-1α*, respectively). The ability of the positive control, LPS, to increase *TNFα* and *MIP-1α* mRNA, however, was significantly reduced ($P<0.001$ and $P<0.05$ for *TNFα* and *MIP-1α*, respectively) by hTLR4 neutralization (Figure 5-2, panels A and B for *TNFα* and *MIP-1α*, respectively).

Discussion

The objective of our work in this chapter was to determine if TLR4 plays a role in mediating the upregulated steady-state levels of *TNFα* and *MIP-1α* mRNA induced by cranberry. TLR4 is a pattern recognition receptor that is critical for initiating host responses to LPS, a conserved component of gram negative bacterial cell walls (67,68). When TLR4 is activated, signaling may proceed via either a MyD88-dependent pathway or TRIF-dependent pathway (69,70). Signaling via the TRIF pathway ends in translocation of NF-κB and IRF-3 into the nucleus, where these proteins induce transcription of type I IFN and proinflammatory cytokine genes (71). Signaling through the MyD88 pathway leads to the translocation of the transcription factors NF-κB, AP-1

and IRF-5 into the nucleus with subsequent initiation of transcription of proinflammatory cytokine and chemokine genes, including *TNFα* and *MIP-1α* (70,72).

We used two models, HEK-Blue™ hTLR4 cells and THP-1 macrophages treated with an anti-hTLR4 neutralizing antibody, to determine if cranberry fractions could indeed activate the TLR4 pathway and induce upregulation of steady-state *TNFα* and *MIP-1α* mRNA levels.

In HEK-Blue™ hTLR4 cells, the increased secretion of SEAP from PAC-r treated cells compared to cells treated with PAC-m and medium indicated that PAC-r bioactives activated the TLR4 receptor pathway. The degree of activation in the PAC-r treated cells, however, was less pronounced than in LPS treated cells. These results support our proposed definition of priming being a subtle response in the cell as compared to an inflammatory state induced by PAMP such as LPS.

The absence of a difference in SEAP production in PAC-m versus medium treated cells concomitant with greater SEAP production in PAC-r treated cells may be due to the approximately 35% lower level of PAC in PAC-m compared to PAC-r. However, the presence or absence of a single bioactive component or synergy of components in cranberry fractions being responsible for the immune modulating effects in our experiments remains to be investigated.

Surprisingly, the use of a TLR4 neutralizing antibody in THP-1 macrophages treated with PAC-r did not result in reduced steady-state levels of *MIP-1α* and *TNFα* mRNA. In contrast, the ability of LPS to increase *MIP-1α* and *TNFα* transcript levels was reduced by approximately 34% and 67%, respectively, in the presence of the neutralizing antibody. This magnitude of *TNFα* reduction was consistent with preliminary

optimization experiments and demonstrated that the concentration of antibody used was sufficient to reduce TLR4 activation.

The seemingly incongruous results from the HEK-Blue™ hTLR4 cell experiment and TLR4 neutralization experiment could be due to activation of other PRR pathways that result in increased levels of cytokine and chemokine genes. In addition to TLR4, macrophages and other APC express a host of PRR on their surface, including TLR2, TLR1, TLR6, TLR5 and Dectin-1 (7,70,73). Interestingly, the MyD88 pathway, which is responsible for transcriptional activation of early pro-inflammatory cytokine genes, including *TNFα* and *MIP-1α*, is shared by all of these receptors (70). PRR work both independently and synergistically via dimerization to modulate immune responses to both microbial components and plant-derived substances (74-76). Thus, it is highly probable that cranberry could be activating TLR4 in cells with increases in *MIP-1α* and *TNFα* mRNA being mediated via activation of an alternate PRR pathway

In summary, PAC-r did react with the TLR4 receptor in HEK-Blue™ hTLR4 cells. However, when TLR4 was neutralized in THP-1 macrophages, *MIP-1α* and *TNFα* transcript levels should have gone down but did not. Transcript levels did decrease with the LPS positive control, so the assay was working properly. PAC-r must be interacting with other receptors on the THP-1 cell surface to induce signaling for chemokine and cytokine gene transcription without specifically needing TLR4 involvement.

Our findings are novel in that to date, no other research has examined the ability of cranberry bioactives to modulate TLR4 receptor signaling and subsequent effects on priming. Although PAC have been widely studied for their ability to promote an anti-inflammatory state or bind pathogen components (56), their potential to modulate

immune cell function prior to pathogen encounter in a manner that boosts the cell's ability to respond remains a novel field of exploration.

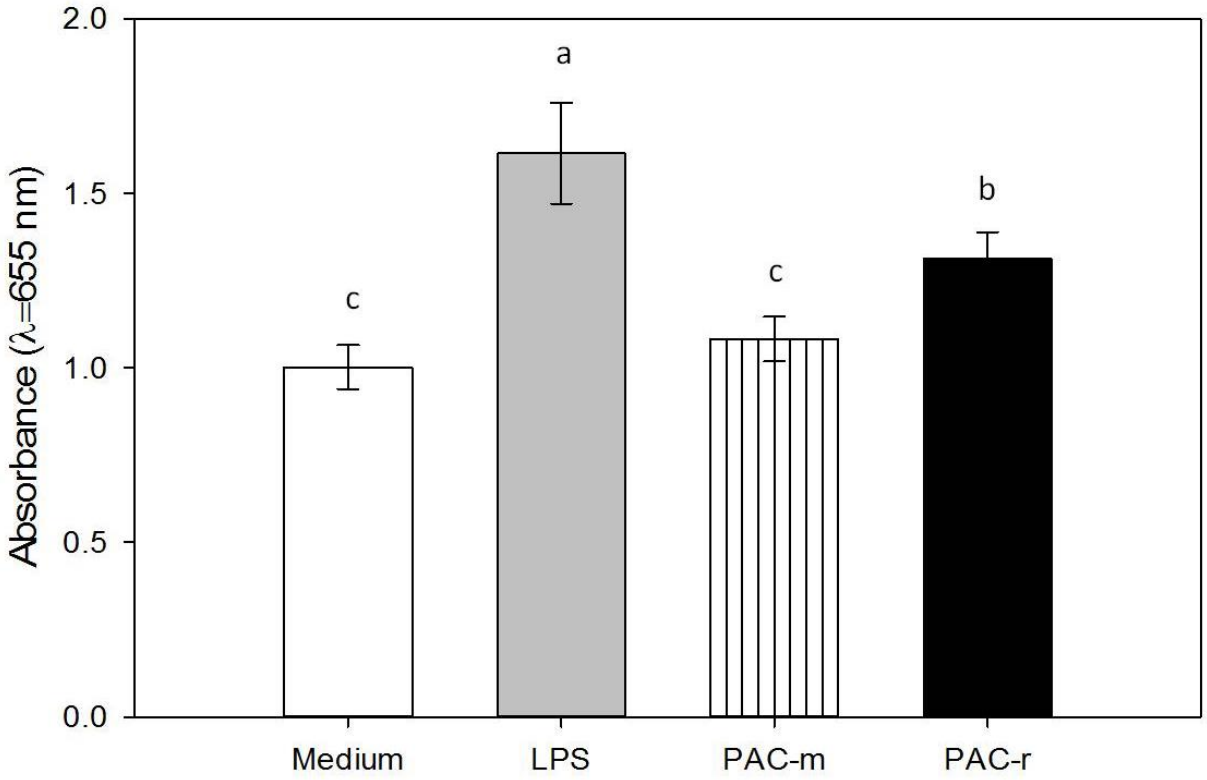
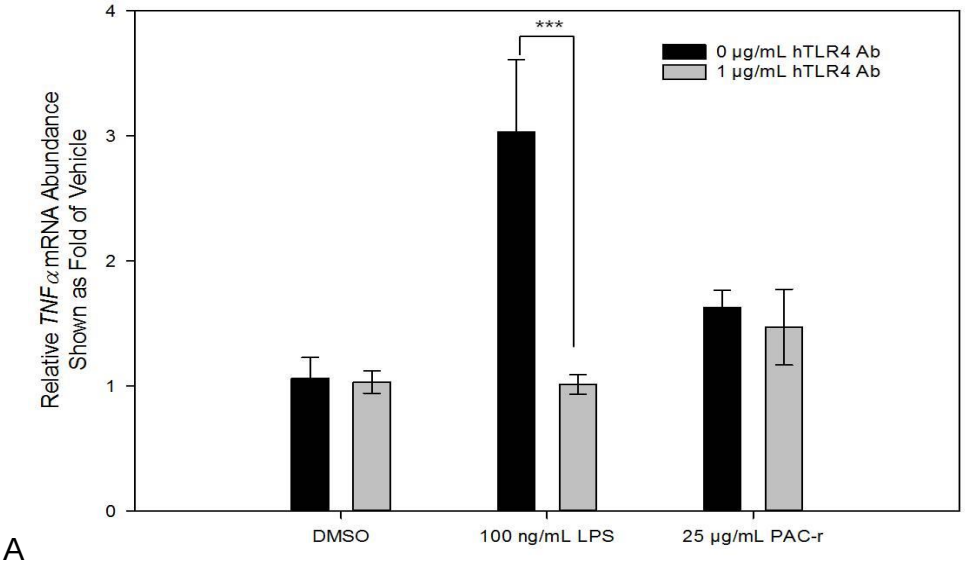
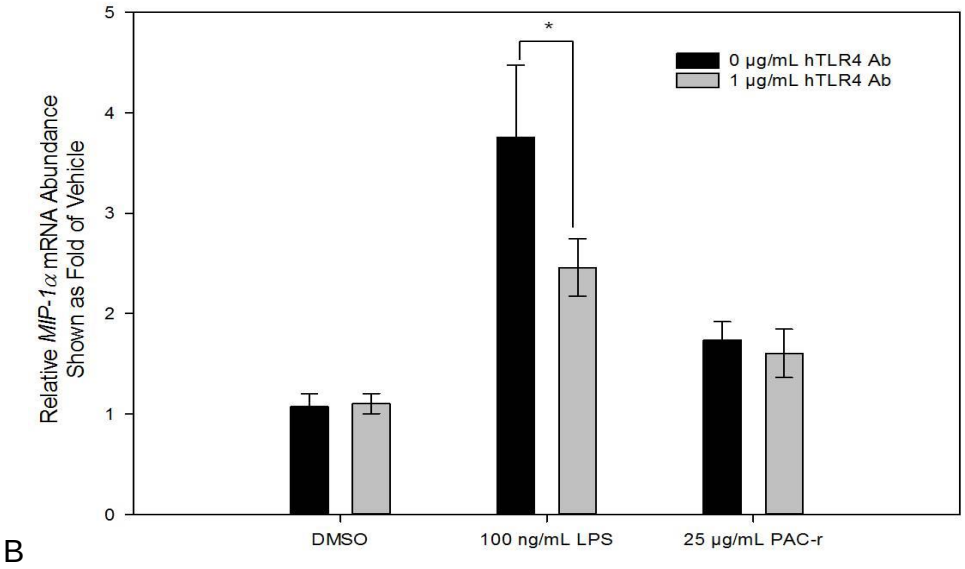


Figure 5-1. PAC-r, but not PAC-m, activated hTLR4 signaling pathway. Absorbance values represent SEAP production in HEK-Blue™ hTLR4 cells. Data were analyzed with a one-way ANOVA and Tukey post-hoc analysis. Values are the mean absorbance ± SD of two independent experiments. Bars without common letters are significantly different from one another ($P<0.05$).



A



B

Figure 5-2. TLR4 neutralization did not prevent cranberry-induced increases in *TNFα* and *MIP-1α* mRNA. The subpanels represent: A) TNFα mRNA relative abundance and B) MIP-1α mRNA relative abundance. THP-1 macrophages were pre-treated for 1 h with or without 1 µg/mL anti-hTLR4 neutralizing antibody, followed by 6 h incubation with PAC-r, vehicle control, or LPS. Cellular mRNA was isolated and reverse transcribed to cDNA. Quantitative RT-PCR was performed on an Applied Biosystems® 7300 RT-PCR system. *MIP-1α* and *TNFα* levels were normalized to the housekeeping gene Cyclophilin B. Data were analyzed with a one-way ANOVA and Tukey post-hoc analysis. Values represent mean ± SD of two independent experiments. *P<0.05, ***P<0.001

CHAPTER 6
SUMMARY, FUTURE DIRECTIONS AND CONCLUSION

The primary objective of our research was to characterize the priming mechanism in THP-1 cells, a model of innate immune cells. Three specific aims were designed to meet the objective. The Specific Aims were:

1. Characterize the activation state of THP-1 cells in response to increasing concentrations of cranberry to determine optimal experimental conditions.
2. Measure the chemokine and cytokine protein and mRNA levels of cranberry treated THP-1cells using the best determined cranberry concentration from Specific Aim 1.
3. Delineate the role of TLR4 in mediating cranberry-induced priming by measuring cytokine and chemokine mRNA levels under conditions in which TLR4 is inactivated.

For the first aim, the activation status of THP-1 monocytes in response to 5, 25 and 100 µg/mL of PAC-r and PAC-m was characterized using four measures of activation: percentage of $CD14^+$, $CD49a^+$ cells; CD49a expression per cell; CD14 expression per cell; and cytokine and chemokine secretion. Three out of four of the activation experiments suggested that 48 h incubation with 100 µg/mL cranberry fraction overtly activated cells. A 6 h incubation with 5 or 25 µg/mL PAC-r and PAC-m were the only time/concentration combinations that did not result in increased cell activation. Thus, a 6 h incubation time along with the 25 µg/mL concentration was selected as the treatment conditions for all subsequent experiments.

For the second specific aim, the molecular changes taking place inside THP-1 cells exposed to cranberry fractions were explored at the mRNA level. The upregulation of steady-state levels of *MIP-1a* and *TNFa* in PAC-r treated cells versus vehicle control treated cells, along with the corresponding increase in protein secretion into culture medium, led us to conclude that cranberry primes THP-1 macrophages for a heightened

response to secondary challenge via a mechanism involving increased steady-state levels of cytokine and chemokine mRNA.

For the third specific aim, the final portion of our central hypothesis that cranberry bioactives enhance responsiveness of THP-1 macrophages challenged with LPS via TLR4 was tested. Two models, HEK-Blue™ hTLR4 cells and THP-1 macrophages with a neutralizing anti-TLR4 antibody, were used to determine if TLR4 plays a role in priming. Although PAC-r increased TLR4 activation in HEK-Blue™ cells, use of the TLR4 neutralizing antibody in THP-1 macrophages treated with PAC-r did not depress steady-state levels of *MIP-1α* and *TNFα* mRNA. Thus, TLR4 is not required for cranberry-induced THP-1 cell priming and subsequent increases in *MIP-1a* and *TNFa* mRNA. TLR 2, TLR1, TLR6, TLR5 and Dectin-1 are other cell surface receptors that could potentially be responsible for cranberry-induced priming. Activation of these PRR can lead to transcriptional activation of *MIP-1a*, *TNFa* and other pro-inflammatory cytokine and chemokine genes (7,70,73).

It is of interest to note that in experiments measuring steady-state mRNA and SEAP levels, the magnitude of the increase in these parameters were significantly less in PAC-r-treated cells than in LPS treated cells. The lower level of increase in mRNA and SEAP suggests that cranberry not only primes cells, but that this priming is a subtle response from the cell. Thus, our definition of priming in innate immune cells being a subtle response is consistent with the novel concept of priming proposed by Jutila et al (33,36,77).

In synthesizing experimental results from chapters 3-5, it must be noted that PAC-r had more efficacy as a priming agent in THP-1 macrophages than PAC-m.

Although the specific component in the cranberry fractions was not investigated, the fact that PAC-r contained ~35% more PAC than PAC-m suggests that the greater efficacy of PAC-r may be due to higher levels of PAC. Other differences between PAC-r and PAC-m compositions were present. PAC-m had 4.5 times more flavonols, 12 times more anthocyanins and 5 times more phenolic acids than PAC-r. Although the possibility exists that the difference in bioactivity between PAC-m and PAC-r was due to lower levels of flavonols, anthocyanins and phenolic acids in PAC-r, the minimal level of these compounds is not likely to stimulate increases in cytokine and chemokine mRNA and protein.

Future research should be targeted primarily toward investigating the cranberry fraction component(s) responsible for the priming of THP-1 macrophages and identifying the mechanism responsible for increased steady-state transcript abundance. Delineation of the bioactive component(s) in the cranberry fractions could be investigated by treating THP-1 cells with proanthocyanidin, flavonol and anthocyanin standards separately and in combination at levels present in PAC-r and PAC-m. Cellular RNA could then be harvested, reverse transcribed to cDNA, and *MIP-1α* and *TNFα* transcripts analyzed by qRT-PCR.

Increases in steady-state mRNA abundance can be due to either enhanced mRNA stability or an increased rate of transcription. As PAC-r and PAC-m did not alter *TNFa* and *MIP-1a* transcript stability, future experiments could incorporate one of two different methods for measuring the transcription rate. These two methods are nuclear run-on assays and qRT-PCR utilizing primers specific for heterogeneous nuclear RNA (hnRNA). Nuclear run-on assays measure frequency of transcription initiation via a

series of steps including cell nuclei isolation, RNA polymerase pausing, and incubation of cell nuclei with nucleoside triphosphates (NTP) and radiolabeled uridine 5′-triphosphates (UTP) (78). No new transcripts are synthesized during cell nuclei incubation with NTP and radiolabeled UTP. The NTP and radiolabeled UTP, instead, are incorporated into transcripts being synthesized at the time of polymerase pausing. The nascent transcripts containing radiolabeled UTP are conjectured to be proportionate to the frequency of transcription initiation (78).

Quantitative RT-PCR analysis of hnRNA is a surrogate method for nuclear run-on assays and is advantageous in that it does not require use of radioactive reagents (79). Heterogeneous nuclear RNA is a short-lived, transcriptional intermediate that can be further processed to mRNA (80). By using primers that span the intron and extron regions, analysis of hnRNA with qRT-PCR has been used successfully in both cell culture and animal studies as a reliable, sensitive index of transcription rate (79,81).

No research study is without limitations. The primary limitation of our research is that it is an *in vitro* study utilizing a single cell type as a model. Although cell culture studies are valuable in that they allow for greater control over experimental conditions, which facilitate the discovery of mechanisms responsible for results seen in human and animal studies, they do not fully recapitulate and reflect what occurs in higher organisms. We are, however, in the process of completing a human dietary intervention study in which the role of cranberry in modulating subsets of monocytic cell populations and MIP-1α production will be explored. The study also includes analysis of steady-state cytokine and chemokine mRNA abundance and protein secretion from PBMC.

A second limitation of our research is that it utilizes only a single cranberry fraction concentration and time point. Although this time and concentration were carefully selected based on our experimental results, the literature and unpublished research previously conducted in our lab, modification of the cranberry fraction concentration and time may yield different results.

Although limitations exist in our research, our study also possesses strengths. Use of the bacterial cell wall component, LPS, is one strength as LPS more closely mimics a pathogen challenge as compared to plant-derived mitogens. Another strength is that 25 μg/mL is a physiologically relevant cranberry fraction concentration. In a dietary intervention study in pregnant women, 240 mL of cranberry beverage, containing 80 mg PAC was consumed twice daily for three days and bioactive effects were noted (30). Moreover, the absorption of PAC following cranberry consumption has been suggested in humans in two different studies utilizing differentiated CaCo-2 cells (82,83). Ou et al found that procyanidin dimer A2 and A-type trimers and tetramers traversed the CaCo-2 cell monolayer with ratios of 0.6%, 0.4%, and 0.2%, respectively (82). In a second study, a small percentage of B-type PAC dimers and trimers were also capable of crossing the Caco-2 cell monolayer (83).

Although the absorption of PAC and other polyphenols may be minimal, there are other routes whereby these compounds may alter immune cell function. Bioactive components in cranberry could be sampled from the luminal milieu via transcytosis in M cells. The M cells deliver antigens to B cells, γδ T cells and macrophages and dendritic cells, which are innate immune cells (84).

In summary, PAC-r, but not PAC-m, primes THP-1 macrophages for an enhanced response to secondary challenge with LPS via a mechanism involving increased steady-state levels of *MIP-1a* and *TNFa* transcripts. This increase in steady-state transcripts appears to be independent of transcript stability. The significance of our findings is that we have discovered one mechanism whereby cranberry bioactives can modulate innate immune cell function in preparation for an immune challenge. By having increased levels of cytokine and chemokine transcripts already present in the cell when a pathogen breaches the immune barrier, innate immune cells would be able to produce a greater abundance of cytokine and chemokine proteins within a set time period to attract and activate other immune cells. Thus, the immune system could react more rapidly than if lower levels of transcripts were present at the time of immune challenge.

We have indeed demonstrated the ability of cranberry to boost the THP-1 cell response to a bacterial component. The subsequent effects that cranberry may have on the overall immune system response time and reduction in symptoms and duration of illnesses, such as cold, flu and food borne illnesses, remains to be fully elucidated.

APPENDIX
OPTIMIZATION OF ANTI-TLR4 ANTIBODY CONCENTRATION

To determine what concentration of anti-TLR4 neutralizing antibody to use in experiments investigating the role of TLR4 in cranberry-induced priming, a preliminary optimization experiment was performed.

THP-1 macrophages were treated for 1 h in a 37°C, 5% CO2 incubator with 2 mL of RPMI 1640 complete medium containing either 1µg/mL anti-hTLR4 antibody (Cat# mabg-htlr4, Invivogen, San Diego, CA) or an equivalent volume of sterile water. After 1 h, 1 mL of RPMI 1640 complete medium containing either 1 µg/mL, 100 ng/mL, or 10 ng/mL LPS or vehicle was added to cells. Culture dishes were returned to a 37°C, 5% CO_2 incubator for 6 h. Culture medium was then removed and RNAzol® RT (Cat# RN 190, Molecular Research Center, Inc., Cincinnati, OH) was used to isolate RNA from cells. Synthesis of cDNA and qRT-PCR were carried out as described in Chapter 4. *TNFα* was normalized to the single housekeeping gene *Cyclophilin B*.

Neutralization of TLR4 with 1 µg/mL hTLR4 Ab depressed LPS-stimulated *TNFα* relative abundance to a greater extent than did 0.5 µg/mL hTLR4 Ab in THP-1 macrophages stimulated with 10 and 100 ng/mL LPS (Table A-1 and Figure A-1). Therefore, 1 µg/mL hTLR4 Ab was used in all TLR4 neutralization experiments.

Table A-1. Percentage of change in relative *TNFα* mRNA abundance

LPS (ng/mL)	Anti-hTLR4 Antibody (µg/mL)	% Change in Relative *TNFα* mRNA Abundance
1000	1	31.4
1000	0.5	134.8
1000	0	0.0
100	1	-28.4
100	0.5	-20.8
100	0	0.0
10	1	-35.6
10	0.5	-26.0
10	0	0.0

TNFα mRNA levels were normalized to levels of the housekeeping gene *Cyclophilin B*. The percent change in *TNFα* mRNA abundance was calculated relative to the abundance in THP-1 macrophages stimulated with an equivalent concentration of LPS but without TLR4 neutralization. Values represent mean of treatment duplicates.

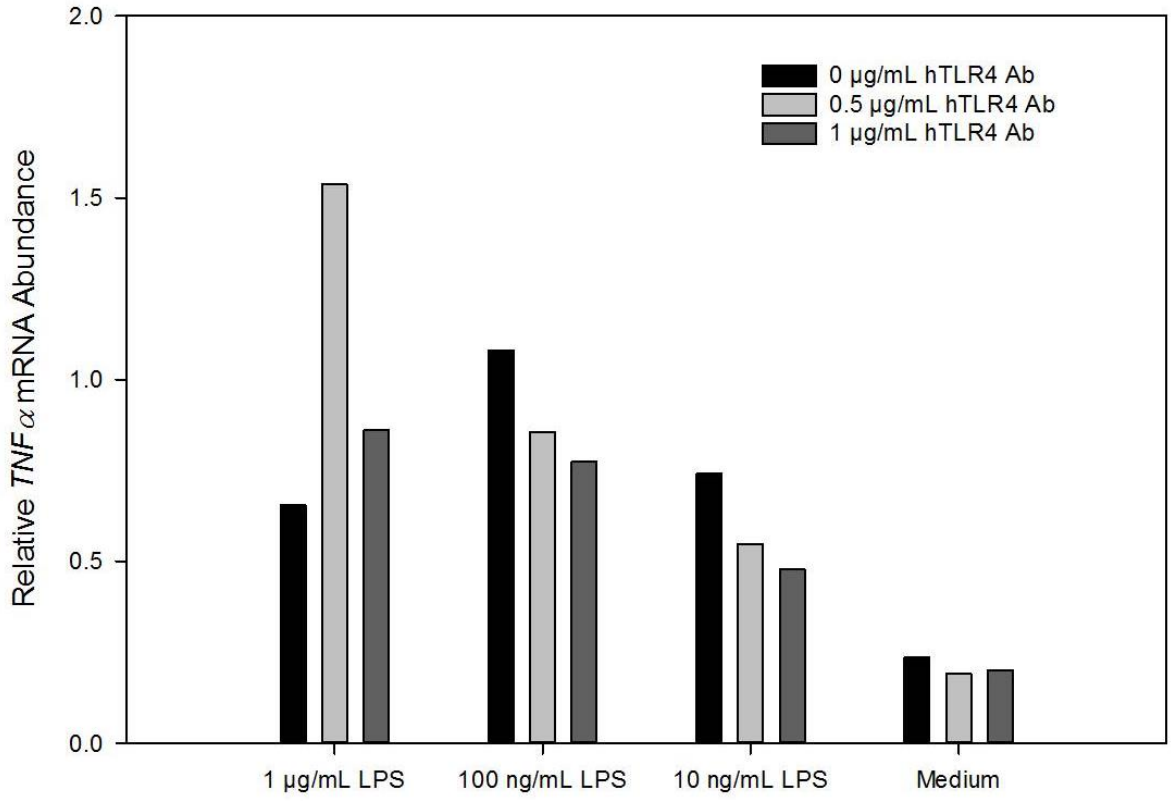


Figure A-1. *TNFα* mRNA abundance following TLR4 neutralization and LPS stimulation. THP-1 macrophages were treated for 1 h with 1 µg/mL anti-hTLR4 antibody or sterile water followed by 6 h incubation with 1 µg/mL, 100 ng/mL or 10 ng/mL LPS or vehicle. Cellular RNA was isolated and reverse transcribed to cDNA. Quantitative RT-PCR was performed on an Applied Biosystems® 7300 RT-PCR system. *TNFα* transcript levels are shown relative to housekeeping gene *Cyclophilin B*. Values represent mean of treatment duplicates.

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BIOGRAPHICAL SKETCH

Rebecca Anne Creasy was born in 1985 and raised in Denmark, Georgia. She grew up on a small farm and developed a passion for agriculture. Rebecca attended Brooklet Elementary School and Southeast Bulloch High School, both in Brooklet, Georgia. Her love of learning, agriculture, and innate curiosity led her to pursue a Bachelor of Science in Agriculture (B.S.A) degree with a major in Food Science and a minor in Nutrition Science at The University of Georgia beginning in the fall of 2003. Upon receiving her B.S.A. in December 2007, Rebecca spent six months working as a researcher on nutrient stability and sensory properties of foods packaged in a nitrogen atmosphere at The University of Georgia. She also delved into the fitness field working at a fitness center while also earning her personal trainer certification from the National Strength and Conditioning Association.

In July of 2008, Rebecca began her pursuit of a Ph.D. in Food Science and Human Nutrition at the University of Florida so that she could further her understanding of the intimate relationship between food and nutrition sciences. After her first year of graduate studies, Rebecca was accepted into the lab of Dr. Susan S. Percival. Her research project focused on the role of cranberry in boosting human immune function. Rebecca graduated with her Doctor of Philosophy in Food Science and Human Nutrition and a minor in Agricultural Education and Communication from the College of Agricultural and Life Sciences in the spring of 2013. Her future plans are to utilize the gifts and knowledge God has given her to improve the lives of others.