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Mechanism Of Action Of American Ginseng And Its Components In The Treatment Of Ulcerative Colitis

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MECHANISM OF ACTION OF AMERICAN GINSENG AND ITS COMPONENTS IN THE TREATMENT OF ULCERATIVE COLITIS

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DEDICATION

I would like to dedicate this dissertation to my family for their unending and unwavering love and support.

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ABSTRACT

Inflammatory bowel diseases (IBD), mainly ulcerative colitis (UC), Crohn's disease (CD), are chronic, idiopathic, inflammatory diseases of the gastrointestinal tract affecting millions of people worldwide. The incidence of IBD is steadily increasing in the modern world due to changes in dietary habits and other environmental influences that originated from industrialization. The etiology is poorly understood but is believed to be a combination of genetic predisposition, environmental factors, and overactive immune system. UC is characterized by inflammation and ulceration of colonic mucosa and rectum and has a higher incidence than CD. Aside from severely affecting the quality of life of the patients, IBD also increases the risk of developing colorectal cancer (CRC). Hence, it is imperative to find a treatment that not only treats colitis but can also act as a chemopreventive agent. Current medications that include aminosalicylates, TNFα inhibitors, and corticosteroids help patients cope with the symptoms and induce temporary remission, but are paired with a risk of serious side effects and people become refractory. Many patients, therefore, turn to unconventional treatments for relief and plant-based products provide a safe, alternative option.

Many studies have shown that American Ginseng (AG), an herb native to North America is effective in the treatment of diabetes, cancer, cardiovascular diseases, and neurodegenerative diseases. Our lab has previously shown that AG treats colitis and prevents colon cancer in mice. This indicates the potential for AG to become part of mainstream medicine like other drugs that have natural antecedents, e.g., taxol, vincristine,

digoxin, etc. Drug discovery from plant products involves phytochemical and biological characterization of plants used in alternative medicine. This dissertation aims to address these issues by identifying the bioactive component of AG and elucidating the mechanism of action in the treatment of UC and prevention of CRC.

We used bioassay-guided fractionation to identify the most potent fraction of AG. A hexane fraction of AG (HAG) has shown remarkable anti-inflammatory and anti-cancer properties both *in vitro* and *in vivo*. Sub-fractionation of HAG revealed that Panaxynol (PA), a polyacetylene is the most abundant compound in this fraction and also showed anti-inflammatory potential *in vitro* as indicated by suppression of iNOS, an inflammation marker. PA effectively suppressed DSS induced colitis in mice and showed potential as a chemopreventive agent. PA targets macrophages (mΦ) for DNA damage and apoptosis while it requires >10X dosage to sustain cause similar effects in other cell types.

AG has been shown to decrease oxidative stress and Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2)-a transcription factor that is a master regulator of antioxidant response), we hypothesized that AG, HAG, and PA treat colitis by activating the Nrf2 pathway. AG, HAG, and PA decreased oxidative stress and activated the Nrf2 pathway *in vitro* and *in vivo*. Accordingly, *in vivo* experiments indicate that AG, HAG, and PA were not very effective in the treatment of DSS induced colitis in Nrf2 knockout mice.

We further delineated the mechanism of action of AG in the absence of inflammation using gene expression profiling of primary peritoneal mΦ by microarray. We found that AG and its compounds showed distinct immunomodulatory properties, as shown by the activation of both pro-inflammatory cytokines and anti-inflammatory molecules. These results will bring AG a step closer to being used as a conventional drug for the treatment

of colitis and pave the way for its use in the treatment of other inflammatory and autoimmune diseases with a similar genesis.

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LIST OF ABBREVIATIONS

AG..... American Ginseng

AOM .....Azoxymethane

CDI .....Clinical Disease Index

CRC .....Colorectal Cancer

CD..... Crohn's Disease

COX ..... .Cyclo-oxygenase

CRC .....Colorectal Cancer

DAB .....Diamino benzidine

DMEM ....Dulbecco's Modified Eagle Medium

DNA.....Deoxyribonucleic Acid

DSS .....Dextran Sulfate Sodium

HAG.....Hexane Fraction Of American Ginseng

HPLC .....High-Performance Liquid Chromatography

H&E .....Hematoxylin and Eosin

IBD.....Inflammatory Bowel Disease

IFNγ .....gamma Interferon

IHC.....ImmunoHistoChemistry

IL.....Interleukin

IP.....Intraperitoneal

IPA.....Ingenuity pathway analysis

KDa.....Kilo Dalton

LC-UV..... Liquid Chromatography-Ultra Violet

LPS..... Lipopolysaccharides

mΦ.....Macrophages

miR/miRNA.....microRNA

mRNA..... Messenger Ribonucleic Acid

MMPs.....Matrix Metalloproteinases

MS.....Mass Spectrometry

NF-κβ .....Nuclear Factor Kappa-light-chain-enhancer of activated B cells

NO..... Nitric Oxide

NOS .....Nitric Oxide Synthase

NRC .....National Research Council

NSAIDs..... Non Steroidal Anti Inflammatory Drugs

PA ....Panaxynol

PBS .....Phosphate Buffer Saline

PCR..... Polymerase Chain Reaction

PMM..... Primary Peritoneal Macrophages

qPCR..... Quantitative Polymerase Chain Reaction

ROS.....Reactive Oxygen Species

RONS.....Reactive Oxygen and Nitrogen Species

RT-qPCR.....Reverse Transcriptase – Quantitative Polymerase Chain Reaction

SFM .....Serum Free Medium

Th cells..... T-helper cells

TLR.....Toll-Like Receptor

TNFα..... Tumor Necrosis Factor-alpha

TREM.....Triggering Receptor Expressed on Myeloid cells

TUNEL .....Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling

UC..... Ulcerative Colitis

WT .....Wild Type

CHAPTER 1

INTRODUCTION

1.1 General overview

Inflammation is the immune system's first line of defense against foreign pathogens or, irritants. Inflammation protects the body against infection and injury by choreographing an immune response involving immune cells and cytokines, to eliminate the pathogens and/or repair the tissue damage (1,2). The important elements of this response include vasodilation to facilitate immune cell filtration and release of cytokines and chemokines that elicit an appropriate pro or anti-inflammatory response. The infiltration of inflammatory cells like macrophages (mΦ) and neutrophils can elicit physical symptoms of pain, redness, and swelling (1). These inflammatory cells release cytokines like nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and activate signaling cascades involving nuclear factor kappa-light-chain-enhancer of activated B cells (NFκb) and tumor necrosis factor (TNF) α, which can eliminate the invading pathogens and repair the tissue damage (2–4).

A genetic predisposition and environmental factors contribute to an overactive immune system and associated chronic inflammation. The chronic and cyclical inflammation associated with autoimmunity can drive tumorigenesis (5). More specifically, chronic inflammation in the affected tissue increases cancer risk in that tissue.

Patients with IBD (UC and CD) have an increased risk of CRC (6). Studying the molecular mechanisms has led to biological therapies including TNF-α inhibitors. However, overall treatment outcomes in IBD patients have been modest, people become refractory, and there are side effects. Therefore, approximately half of all patients turn to complementary and alternative medicines.

Complementary and Alternative Medicines (CAMs) that includes dietary and medicinal supplements like curcumin, grapes, garlic, ginseng, etc., are being explored as potential treatments with minimal side effects (7–9). A number of natural products have been assimilated into the mainstream medicine for the treatment of various diseases. However, CAMs such as plants, herbs, and spices are an amalgam of multiple compounds, and it is important to isolate a single, potent component that might be useful as a standardized stand-alone treatment, or in a standardized natural cocktail for the suppression of colitis. Mechanisms are also addressable, where this is more difficult with complicated mixtures making up natural mixtures. The general low toxicity of plant constituents make them candidates for long-term use and therefore the prevention of CRC. Over the past several years, our lab has shown that AG can treat UC and prevent CRC in mice. This project aims at identifying the bioactive component in AG and delineating the mechanism of action of AG and its derivatives.

1.2 Ulcerative Colitis

IBDs are chronic, inflammatory, idiopathic diseases that can affect the entire gastrointestinal (GI) tract (CD) or just the colon (UC) (10). IBD incidence has been increasing since the mid-20th century, indicating the effects of the changing lifestyle and dietary habits, including smoking, diets high in fats and sugar, medication, and stress (10).

UC has a higher incidence than CD with a rate of 10.2 to 20.3 cases per 100,000 per year and a prevalence of 7.6 to 246 cases per 100,000 per year. In the USA, this translates to 1.4 million people affected (10). The etiology of this disease is unclear but is believed to be multifactorial, with genetics, immunological, and environmental (alterations in the epithelial barrier, infection, microflora composition, nutrition, stress, etc.) factors playing an important role (11). The genetic predispositions can affect the interaction of the immune system with microbial and other environmental factors, leading to a disruption in the gut homeostasis and this causes an abnormally high proinflammatory response towards the commensal bacteria (12).

Colitis manifests mainly in patients between ages 15-30, but might have a secondary peak between the ages of 50-70 and is characterized by inflammation of the mucosal layer of the large intestine(13). The clinical symptoms include diarrhea, often with blood or pus, abdominal pain and cramping, rectal bleeding and pain, the urgency to defecate and inability to defecate despite the urgency, weight loss, dehydration, tachycardia, anemia, fatigue, and fever (14). Additionally, there are some extraintestinal manifestations of IBD in children and adolescents, which can lead to delayed growth and changes in cognitive and sexual development (14). Colitis also has a detrimental effect on the quality of life for the patients due to its chronic, recurring nature. However, the most important and extreme consequence of colitis is the increased risk of CRC. The chronic inflammatory state of the disease makes an ideal environment for tumorigenesis, and the risk of developing CRC increases with the length of disease, with long-term patients facing 20% risk (12). CRC is the second leading cause of cancer deaths with the prediction of up to 50,000 deaths per year (12). Scheduled colonoscopies in colitis patients act as good

preventative strategy, but it needs to be paired with potent treatments, which can act as chemopreventive agents.

1.2.1 IMMUNE CELLS IN THE INTESTINE

The organs with high interaction with the extraneous environment like lungs and intestines have additional layers of defenses from the immune system (15,16). The mucosal barrier in the intestines, along with the epithelial layer forms the first line of defense against the microorganisms and contains the immune cells (neutrophils, monocytes, mΦs, dendritic cells, natural killer cells, eosinophils, and basophils) responsible for the innate immunity (17)(18). These immune cells sample the antigens and elicit an appropriate response, which is often short-lived (18). Dendritic cells and mΦs in the intestines, along with the epithelial cells and myofibroblasts, sense conserved molecular patterns on microbes known as pathogen-associated molecular patterns (PAMPs), via pattern recognition receptors (PRRs) encoded in the germline (18). This engagement results in the initiation of an inflammatory response involving the release of inflammatory cytokines, chemokines, and antimicrobial peptides(19). This further leads to the phagocytosis of infected cells and pathogens, followed by antigen presentation by dendritic cells to the T-cells, thereby activating the adaptive immunity (20). The adaptive immune system comprises of B-cells and T-cells, which produce antibodies and cytokines, respectively (21).

In the intestine, which faces a constant exposure to a wide array of antigens in food, it is important to maintain tolerance to the symbiotic bacteria while also protecting from enteric pathogens by eliciting an immune response when needed. Any disturbance in this homeostasis can lead to pathogenic diseases like CD and UC (22).

1.2.2 MACROPHAGES

MΦs and dendritic cells belong to myeloid lineage along with neutrophils, differentiate from monocytes (23). Monocytes are produced in the bone marrow and are abundant in lymph nodes and spleen (24). In the case of an invading pathogen, monocytes migrate to the site of infection or damage through the bloodstream and mature into mΦs through a series of complex differentiation events that require the presence of interleukins (IL-1, IL-32, and IL-6) (25–27). Upon their discovery, the main function of mΦs was thought to be phagocytosis which is essential for wound healing and clearance of microorganisms (28). Later, the other main function of mΦs, pro-inflammatory cytokine production, was identified and is responsible for the initiation of adaptive immunogenic response and when left unchecked, leads to prolonged inflammation (29).

MΦ are a diverse group of cells and can be categorized based on their location and function into different types, M1 and M2 being the two main categories (30). M1 mΦ are pro-inflammatory and are considered classically activated or polarized mΦ. M1 mΦ mainly secrete cytokines and protect the host from invading microorganisms and have anti-tumorigenic properties (31). M1 mΦ can be activated by interferon-γ (IFN-γ) (secreted by helper T-cells (T_h1), cytotoxic T cells (T_c), NK cells and mΦ themselves) or lipopolysaccharides (LPS) (secreted by bacteria) (32). M1 mΦ also enhance the Th1 response and this response, in turn, enhances the activity of mΦs (33). M1 mΦ are characterized by increased glycolic rate and reduced mitochondrial oxidative phosphorylation when compared to the M2 mΦs (34). M1 mΦs secrete cytokines such as IL-1β, tumor necrosis factor (TNF) α, IL-12, IL-18, and IL-23 (31). MΦs can also increase

the production of nitric oxide from L-arginine, by activating nitric oxide synthase (iNOS), resulting in a cytotoxic activity towards various intracellular and extracellular intruders such as bacteria, fungi, helminths, viruses, and tumor cells (35). In case of inflammatory diseases, M1 mΦs are shown to be responsible for the initiation and sustenance of inflammation (36).

M2 mΦ (alternatively activated mΦ) and are considered anti-inflammatory with regenerative properties. M2 mΦ are activated as a part of the Th2 response, by IL-4, IL-13, IL-10, and transforming growth factor (TGF)-β (36). They are involved in tissue homeostasis and repair, and have a higher metabolism and are characterized by the secretion of high amounts of IL-10 and low levels of IL-12 (37). M2 mΦ also produce matrix metalloproteases and growth factors to aid in the wound healing (38).

The mΦ in the lamina propria (LP mΦs), also known as the resident mΦ, are largely anti-inflammatory. They have microbicidal activity and clear microbes and other stimuli that cross the epithelial barrier (39). However, they do not produce any pro-inflammatory cytokines (40). On the contrary, they support the transition from inflammatory to the anti-inflammatory environment (40). Inappropriate responses from mΦ to the gut microbiota has been shown to play an important role in the pathogenesis of colitis (41). Enhanced pro-inflammatory response from the mΦs releases nitric oxide (NO), and increases the oxidative stress that not only results in the disruption of the epithelial barrier, but also subcellular and molecular damage to macromolecules like DNA, RNA, protein, lipids, metabolites, etc., thereby contributing to the initiation and maintenance of the disease (42). Continued intracellular damage is one of the main causes of transition into colorectal cancer.

1.2.3 OXIDATIVE STRESS IN ULCERATIVE COLITIS

An imbalance between the production of free radicals and reactive metabolites, and their elimination results in oxidative stress, one of the hallmarks of inflammation (43). This imbalance could lead to the damage of important biomolecules and various signaling pathways, resulting in a negative impact on the organism (42). Inflammation can induce and exacerbate oxidative stress in multiple ways. During chronic inflammation, inflammatory cells that are recruited to the site of damage. This recruitment leads to a respiratory burst as a result of increased uptake of oxygen, thereby causing an increased accumulation of ROS at the site (44). Inflammatory cells also secrete cytokines like interleukins, tumor necrosis factor (TNF) α, and interferon (IFN) γ and chemokines like CX3-type chemokine receptor (CX3CR) 1, which can, in turn, stimulate the production of ROS and RNS (45). For example, TNF-α enhances the formation of ROS by neutrophils and other cells, while interleukin-1-β (IL-1-β), TNF-α and IFN-γ stimulate the expression of inducible nitric oxide synthase (iNOS) in inflammatory and epithelial cells.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) comprise the main reactive intermediates, and the antioxidants encompass the protective mechanisms responsible for their elimination of these metabolites. ROS are generated by the mitochondrial respiratory chain as a part of cellular metabolism and play an important role in the alteration of signaling pathways in cells in response to changes in intracellular and extracellular environmental conditions (46). When cells are subjected to prolonged environmental stress, and ROS are produced over a long period, it results in significant

damage to cell structure and also induces somatic mutations that can result in the neoplastic transformation of the cells (47). RNS are generated from NO, which is synthesized by iNOS, usually after a challenge by immunological or inflammatory stimuli (48). RNS increase the risk of mutagenesis by further generating other reactive species, e.g., reactive aldehydes-malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), by inducing excessive lipid peroxidation (49). Oxidative stress plays thus an important role in the initiation and progression of tumorigenesis by increasing DNA mutations or inducing DNA damage, genome instability, and cell proliferation. H_2O_2 is a less reactive but important type of ROS that is formed from superoxide anion (O_2^-) or spontaneously in peroxisomes from molecular oxygen. H_2O_2 can react with reduced transition metals like Fe^+ to produce hydroxyl radical (·OH), which in turn can cause DNA damage (50).

It is important to note that low or moderate levels of free radicals act as a defense mechanism against infectious agents by triggering the protective, antioxidant systems and signaling pathways. There are multiple endogenous antioxidant systems in place that are triggered and regulated by different metabolites. Glutathione reductase (GR), superoxide dismutases (SODs), and catalase are the important enzymatic antioxidants that can combat and scavenge the free radicals that can cause oxidative stress (51,52). On the other spectrum of antioxidant defense are the various transcription factors like AP1, CREB, HSF1, HIF-1, TP53, NF-κB, Notch, SP1, and CREB-1 (53). Of these, the Nrf2 (nuclear factor erythroid 2 [NF-E2]-related factor2)-Keap1 (Kelch-like ECH-associated protein-1) signaling pathway, involving the transcription factor, Nrf2, is one of the important regulators of cell's protective responses to oxidative stress (54).

1.2.4 NRF2

The transcription factor, Nrf2 attenuates inflammation by combating oxidative and xenobiotic stresses via the activation of multiple antioxidant genes like heme oxygenase 1 (HO-1) and NAD(P)H Quinone Dehydrogenase 1 (NQO1) (55). HO1 catalyzes the degradation of heme and NQO1 catalyzes the reduction of quinones to hydroquinones thereby causing detoxification of quinones (56). Nrf2 also controls the expression of enzymes involved in the production of glutathione (GSH), the most important antioxidant cofactor in cells (56,57).

Nrf2 has an extremely short half-life (~15 minutes) resulting in a rapid turnover and a slightly longer half-life of ~30 minutes when induced (58). The activity of Nrf2 is mainly regulated by Keap-1. Keap-1 is a substrate for a ubiquitin-ligase complex, and by sequestering Nrf2, it mediates the proteasomal degradation of Nrf2 (58). Conformational changes in the Keap-1 protein can free Nrf2, which can translocate into the nucleus and perform its function as a transcriptional factor (58). These conformational changes can occur in multiple ways, e.g., ROS can induce the oxidation of cysteine residues or some proteins like p62 and p21 can disrupt the binding of Nrf2 to Keap-1, thereby causing the activation of the Nrf2 pathway (58). Upon translocation, Nrf2 can bind to the cis-acting enhancer antioxidant response element (ARE; core sequence: TGAG/CNNNGC) on promoters of the target genes (59). The promoter region of Nrf2 also has two ARE regions which it can bind to and auto-regulate the activation (60).

The importance of Nrf2 in the alleviation of inflammatory diseases is demonstrated in various murine models. Deficiency of Nrf2 in mice resulted in exacerbation of inflammation in emphysema, pleurisy, and sepsis (61–64). Furthermore, activation of Nrf2 in myeloid cells resulted in an anti-inflammatory response. Recently, a Nrf2 inducer,

Tecfidera, has been approved for the treatment of multiple sclerosis, an autoimmune disorder (65). This suggests that Nrf2 activation can be used as a treatment strategy for various inflammatory diseases like colitis.

1.2.5 CURRENT TREATMENTS

Prolonged inflammation of colitis, when left untreated, can lead to hemorrhage, toxic dilation, perforation, and death (12). Various treatments are available for the treatment of colitis, depending on the severity of the disease and stage. NSAIDs like aspirin, sulindac, and ibuprofen are used to treat the symptoms of pain and inflammation for mild flares (12). NSAIDs act by interfering with cyclooxygenase (COX) pathways and are not selective towards either of the two isoforms, COX1 and COX2 (12). COX1 is a housekeeping gene that plays an important role in cytoprotection by producing prostaglandins in various tissues like stomach, duodenum, and kidneys (66). While COX1 is constitutively expressed in these tissues, COX2 is almost undetectable in normal conditions and is induced during inflammation to reach 200-300 times the basal levels (66). NSAIDs have adverse effects like mucosal damage and nephrotoxicity with prolonged use due to their lack of selectivity (67).

Moderate flares require steroids which suppress the hyperactive immune system or anti-inflammatory drugs like 5-aminosalicylic acid (5-ASA) (68). Severe attacks, especially in pediatric patients, require inpatient treatment with intravenous corticosteroids and two-thirds of colitis cases may require a colectomy (68). While steroids are effective in a short-term use, long-term intake is associated with multiple side effects, like a low rate of mucosal healing. 5-ASA is an anti-oxidant that is often ineffective and requires long-term use to achieve remission (14)(68).

More recently, biologics like adalimumab (Humira), certolizumab (Cimzia), infliximab (Remicade) and natalizumab (Tysabri) are being used to inhibit inflammation (68). The first three biologics mentioned target TNFα, and the fourth targets α4-integrin, a cell adhesion molecule (69). However, these biologics, especially the TNFα inhibitors have dangerous side effects like increased risk of infections (e.g., tuberculosis) due to suppressed immune function (69). Due to the ineffective nature of current treatments, paired with dangerous side effects, it is imperative to find a novel, safe, and effective treatment. Approximately 50% of patients turn to alternative treatments like acupressure, homeopathy, and Tai Chi for solace (70,71). There has also been an increased use of natural products like flax seeds, aloe vera, and garlic. Here, we have tested another natural product, American ginseng, for its use as an alternative therapy for colitis.

1.3 American Ginseng

Ginseng root has been used for centuries in traditional medicine for various ailments and general well-being. The botanical name of ginseng, *Panax*, means 'all-healing' and is closely related to the Greek word 'panacea.' Ginseng is a mixture of many components that include saponins, polyacetylenes, ginseng oils and phytosterol, carbohydrates and sugars, organic acids, nitrogenous substances, amino acids and peptides, vitamins and minerals, and certain enzymes (72). Among these, saponins (also known as ginsenosides) and polyacetylenes have shown to be the active ingredients and have been extensively studied for the treatment of multiple diseases including cancer, diabetes, and various inflammatory and cardiovascular diseases. Ginseng use is also intended for overall health with beneficial effects on immune, cardiovascular, nervous and endocrine systems.

Various species of ginseng are cultivated all over the world; Asian, Korean, or Chinese Ginseng (*Panax ginseng*), notoginseng (*Panax notoginseng*), and American ginseng (*Panax quinquefolius*) are the most commonly studied species of ginseng. These different species of Ginseng vary in their chemical composition, e.g., ginsenosides Rf and R1 are present in Korean ginseng and notoginseng but not American ginseng, which exclusively contains pseudoginsenoside F11 (73). Furthermore, the ratio of Rg_1/Rb_1 can be used for differentiation between the different species; ratios less than 0.4 indicate American ginseng while higher ratios indicate Korean or notoginseng (74). Korean ginseng has been used extensively in traditional medicine in Asia for thousands of years while American ginseng has been gaining popularity in the Western world in the recent decades for its wide range of pharmacological properties. Whole American ginseng root extract and its chemical components have been examined as potential treatments. More recently, different parts of this plant like fruit, leaves, and flowers are being studied not only for physical ailments but also, mental health, general well-being, and cosmetological benefits.

Like most medicinal plants, American ginseng (AG) has antioxidant properties due to which it is effective in the treatment of various diseases where free radicals, like reactive oxygen and nitrogen species, contribute to the pathogenesis of the disease. AG can protect cardiomyocytes from injury and prevent heart disease by scavenging hydrogen peroxide and hydroxyl radicals. AG also decreases oxidative stress in the central nervous system (CNS), thereby contributing to the prevention of memory loss. AG has been shown to have a hepatoprotective effect by increasing the activity of major antioxidant enzymes, GSH-P_x (glutathione peroxidase) and SOD (superoxide dismutase). AG has also been shown to

prevent myocardial injury and neurodegeneration by inhibiting various ion channels (Ca^+, K^+, Na^+, etc.) in a stereospecific manner.

1.3.1 American Ginseng for the treatment of colitis and colon cancer

In the past few years, our lab and Dr. Chong-Zhi Wang's lab at the University of Chicago have done separate and parallel studies to explore the potential of AG as a treatment for colon cancer (75–84). However, we have focused on investigating AG as a treatment for UC and specifically, colitis-induced colon cancer while the research in Dr. Wang's lab concentrated AG's effect on the different types of colon cancer. Our studies, however, have undeniably complemented one another. Here, I will summarize findings from both groups to highlight the significance of AG as a prospective treatment for colitis and colon cancer.

AG has significant anti-inflammatory effects *in vitro* and *in vivo*. Incubation of ANA-1 mΦ with AG, followed by activation with IFN-g (polarizes ANA1 cells into proinflammatory M1 type) results in decreased activation of iNOS, eNOS, NO release and I-kB, oxidative burst and oxidative stress. Preincubation of HT-29 colon cells with AG prevents DNA damage from cytokine-induced inflammatory stress when exposed to M1 type ANA1 cells. AG mimicked these effects *in vivo* – aside from suppressing the proinflammatory markers mentioned above, AG treatment of DSS treated mice also decreased the number of CD+ cells such as T helper cells, monocytes, mΦs, and dendritic cells in the inflamed colon. One of the mechanisms of action of AG is p53 dependent – AG does not cause apoptosis in p53-/- inflammatory cells (NH32) and is not effective in the prevention of colitis in p53-/- mice (79,84).

We have shown AG decreases the number of macroscopic lesions and the severity of microscopic adenomas in an AOM-DSS induced colon cancer model. Upon further investigation using protein arrays, it was shown that AG decreases the expression or activation of tumor-promoting genes like c-Jun, Pax 2 and ErbB2, while it increases the expression of tumor suppressors like PP6C, EphA1, and IRAK1 (80). Others have shown that AG attenuates colon cancer in different mouse models – AOM and western (high fat) diet-induced colon cancer in A/J mice, high-fat diet induced colon cancer in $Apc^{Min-/+}$ mice (85,86). Treatment of mice with AG in these models increased the phosphorylation of EGFR, ERK and AKT and also Bax, which is consistent with increased apoptosis (85). More recently, it has been shown that AG also altered the metabolomics and microbiota profiles in the AOM/DSS-induced colitis and colon carcinogenesis models. These profiles in the AG treatment groups are closer to the water control groups indicating that AG can alter the effects that inflammation can have on the microbiota (87).

1.3.2 Fractionation of American Ginseng

Our lab used bioassay-guided fractionation to obtain multiple fractions American Ginseng and tested their anti-inflammatory properties to identify the most potent ingredient in AG. AG was sequentially partitioned against various solvents (hexane, dichloromethane, ethyl acetate, water, and methanol) each containing a different composition of active and inactive chemical entities. The Hexane fraction of AG (HAG) appeared to be the most potent one as evidenced by its ability to suppress iNOS and COX-2 expression in proinflammatory (M1) ANA-1 mΦ and its cytotoxic effects on RAW264.7 cells (88,89). Furthermore, HAG also caused apoptosis of inflammatory cells in a manner comparable to the whole AG extract *in vitro* and *in vivo* (88). HAG also suppressed DSS-induced colitis,

and AOM-DSS induced colon cancer in mice further confirming that HAG contains the active ingredient/s responsible for similar effects by AG (88,90). However, the effects of HAG can only be partially attributed to p53. HAG caused apoptosis in p53-/- cells, even though the effect was significantly lower when compared to cells with wild-type p53. Similarly, HAG suppressed DSS-induced colitis in both p53 wild-type and p53 knockout mice, with better effects in the wild-type mice.

Global microRNA (miRNA) comparison of HAG treated HCT-116 colon cancer cells with untreated cells showed increased expression of Mir-29b by HAG. Mir29b suppresses matrix metalloproteinase-2 (MMP-2), thereby suppressing migration of colon cancer cells, indicating a potential for HAG and AG in the suppression of metastasis (81). Upon establishing the effectiveness of HAG as a potential fraction containing the bioactive ingredient of AG, we further fractionated HAG, and identified Panaxynol (PA) (also known as falcarinol and carotatoxin), to be the most abundant ingredient.

1.3.3 PANAXYNOL

PA is a polyacetylene found in common food and medicinal plants belonging to Apiaceae (e.g., carrots, celery, parsnip, etc.) and Araliaceae (e.g., AG, ivy, etc.) families(91). Polyacetylenes are a distinct group of molecules found in medicinal plants and have been recognized to have numerous pharmacological properties. PA was initially considered a dietary toxin (hence the name, carotatoxin) due to its pro-allergenic effects that are responsible for contact dermatitis from common ivy (92). However, the concentration of PA in vegetables and roots is lower, and when combined with desensitizing effect of oral intake, the risk of an allergic reaction is low (91). More recently,

PA is being studied as a potential medicinal drug due to its anti-cancer, anti-inflammatory, antimicrobial and neuroprotective properties.

The anti-cancer effects of PA are evidenced by its antiproliferative and cytotoxic effects towards various cancer cell lines such as; leukemia (L-1210), human gastric adenocarcinoma (MK-1), mouse melanoma (B-16), renal carcinoma cells (A498, Caki-1, and CURC I), and mouse fibroblast-derived tumor cells (L-929) (93–97). However, it is not clear how it compares with cytotoxicity towards normal cells as there is conflicting data in this regard and appears to depend on the cell lines tested rather than the cell type (91,95,98). It is interesting to note that the effect of PA is biphasic in intestinal cells (Caco-2), where it increased the cell proliferation at doses lower than 10 μM but apoptosis at doses higher than 20 μM (99). This biphasic effect conforms to the consensus that toxicity is dose-dependent and toxic compounds can have beneficial effects at lower concentrations. However, the anti-cancer effect of PA *in vivo* has not been extensively studied. PA from carrots decreased the number of azoxymethane (AOM)-induced pre-neoplastic lesions in rats and dehydrofalcarinol, a secondary metabolite of PA have been shown to have a modest effect on tumors induced in a LOX melanoma mouse xenograft model (100,101).

The mechanism of action of PA has not been elucidated but studies indicate the DNA alkylating potential of PA (99), and one of the signaling pathways that could be affected is MAP kinase pathway, where PA has been shown to inhibit the phosphorylation of ERK1/2 and CREB (102).

1.4 DSS MOUSE MODEL OF COLITIS

DSS induced colitis is one of the most widely used models to study UC in mice. The effect of DSS is limited to the mouse colon and is very similar to that of human colitis in etiology, pathology, and clinical outcome (103). The DSS treatment can be manipulated to achieve the different types of inflammation (103). For example, treatment with DSS for 1-2 weeks mimics acute inflammation while treatment with DSS in alternative weeks for multiple cycles represents chronic inflammation. We treated mice with DSS before or after treating the mice with PA to check the effectiveness of PA as a treatment and preventative for colitis respectively.

The precise mechanism of action of DSS is unknown but the sequence of events, where crypt loss precedes inflammation, suggest that DSS affects the epithelial layer and increases permeability (103). There are multiple theories proposed to explain the mode of insult to the epithelium by DSS. DSS affects the permeability by disrupting the tight junction protein (Zona occulens-1) (104) between the cells or the negatively charged sulfate groups in DSS can damage negatively-charged (anionic phospholipids) cell membranes by repulsive forces (105). This increased permeability increases the bacterial infiltration thereby causing an inflammatory response (105). It is also believed that the anticoagulant nature of DSS increases the intestinal bleeding further exacerbating the disease (105). However, the molecular weight of DSS plays an important role in determining the severity and extent of colitis, with 35000-50000 being the most effective range for inducing colitis (106).

1.5 OBJECTIVES OF THE RESEARCH

As discussed earlier, current medications are ineffective in the treatment of colitis and are accompanied by a horde of dangerous side effects. In order to find a natural and effective

treatment, we have previously tested AG and shown that it successfully suppresses colitis in mice. A natural next step is to identify the bioactive component of AG so that it can be used as a conventional treatment for colitis. The objective of this research is two-fold –

1. To identify the bioactive component of AG (Figure 1.1) and

2. To elucidate the mechanism of action of AG and its components in the treatment of colitis (Figure 1.2).

To this end, the following conclusions have been reached:

Conclusion 1: PA, a bioactive component isolated from AG, is effective in the treatment of DSS induced colitis in mice (Chapter 2)

Conclusion 2: PA specifically targets mΦ for DNA damage and apoptosis. (Chapter 2)

Conclusion 3: Activation of the Nrf2 pathway is essential for the treatment of colitis by AG, HAG, and PA. (Chapter 3)

To further elucidate the pathways influenced by the action of AG and its components, we performed gene expression profiling of primary mΦ treated by AG and identified the potential targets.

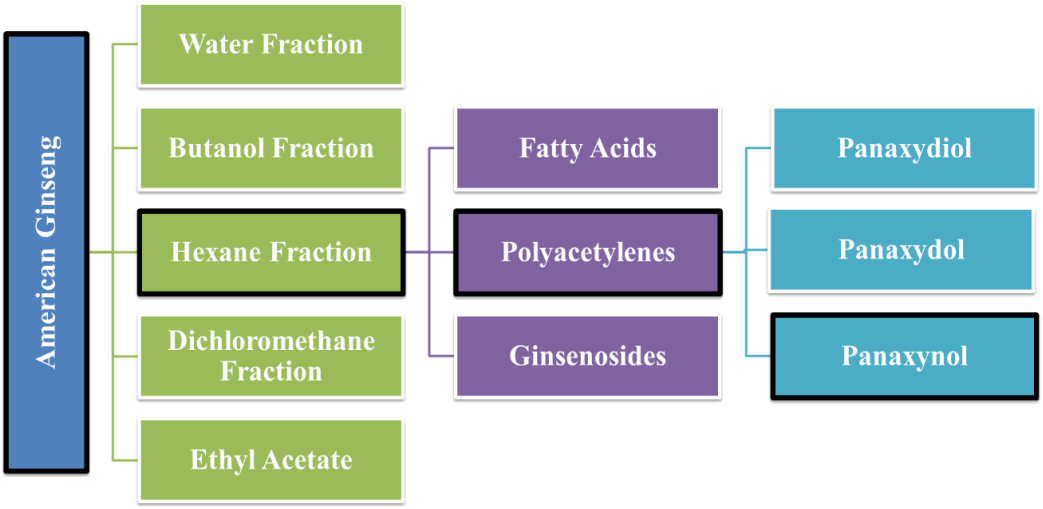

Figure 1.1. Schematic for the identification of a bioactive component of American Ginseng. Bioassay-guided fractionation determined that Hexane fraction of AG was found to be the most potent fraction of AG, of which Panaxynol was the most abundant molecule.

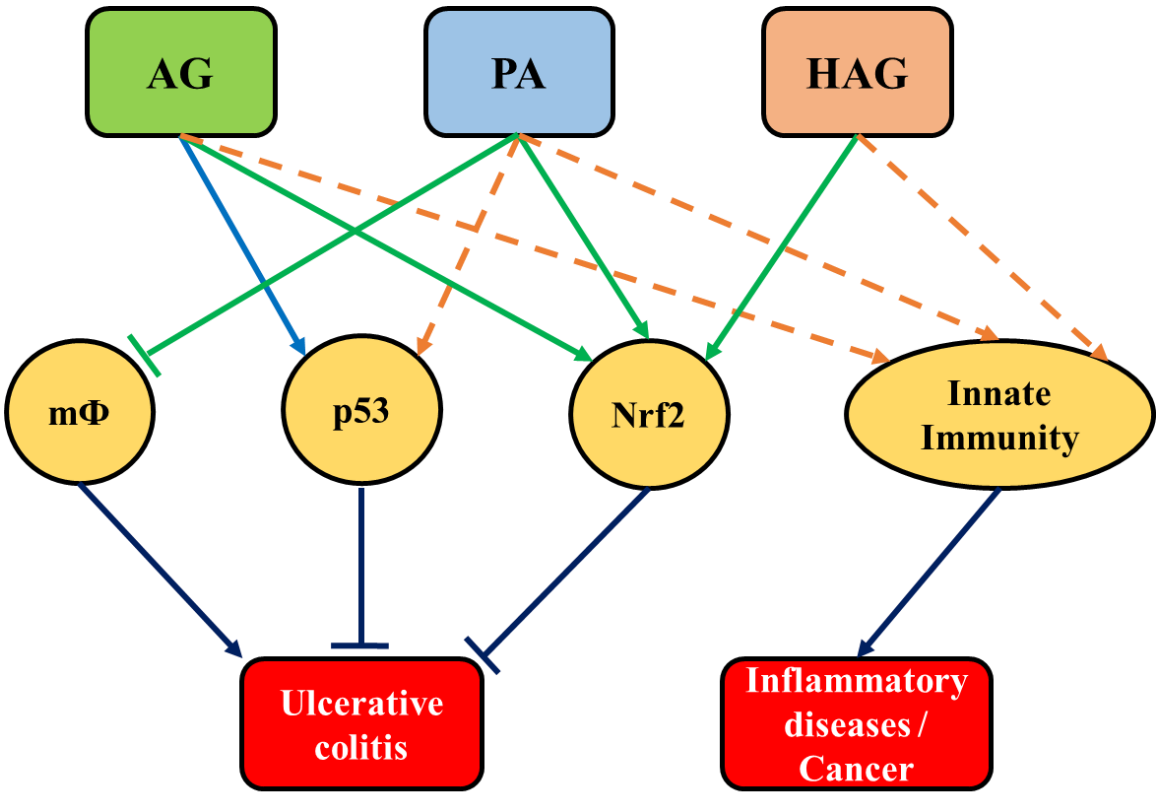

Figure 1.2 Schematic for mechanisms of action of AG and its components. Confirmed and potential mechanisms of action of AG, HAG, and PA in the treatment of UC, other inflammatory diseases, and cancer. The solid blue line represents previously confirmed mechanisms, solid green lines represent mechanisms confirmed in this dissertation, and dotted orange lines indicate potential mechanisms.

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CHAPTER 2

PANAXYNOL, A BIOACTIVE COMPONENT OF AMERICAN GINSENG, TARGETS MACROPHAGES AND SUPPRESSES COLITIS IN MICE

ABSTRACT

Ulcerative colitis (UC) has a significant impact on the quality of life for the patients, and can substantially increase the risk of colon cancer in patients suffering long-term. Conventional treatments provide only modest relief paired with a high risk of side effects, while complementary and alternative medicines can offer safe and effective options. Over the past decade, we have shown that American ginseng, particularly the hexane fraction of American ginseng (AG), has anti-oxidant and anti-inflammatory properties that can suppress mouse colitis and prevent colon cancer associated with colitis. With the goal of isolating a single active compound, we further fractionated the hexane fraction, and found the most abundant molecule in this fraction was the polyacetylene, Panaxynol (PA). After isolating and characterizing PA, we tested the efficacy of PA in the treatment and prevention of colitis in mice and studied the mechanism of action. We demonstrate here that PA effectively treats colitis in a Dextran Sulfate Sodium mouse model by targeting macrophages (mΦ) for DNA damage and apoptosis. Positive outcomes from this study will take American ginseng one-step further towards becoming a conventional drug for the treatment of colitis, possibly other autoimmune diseases associated with macrophage dysfunction, and for the prevention of inflammation-driven carcinogenesis.

2.1 INTRODUCTION

Inflammatory bowel diseases (IBDs), including UC and Crohn's disease (CD), are debilitating, significantly affect lifestyle, and carry a high colon cancer risk. IBD prevalence is particularly high in North America and Europe (affecting 3.8 million people), with an economic burden of $30 - $45 billion (1–4). Of note, incidence has been increasing for both males and females over the past 20 years (5), making this a health problem that needs to be addressed for both sexes. As a frustration to patients with IBD, conventional treatment outcomes are modest, e.g., 20% do not respond to anti-TNFα antagonists (6), and toxicity leads to dangerous side effects. As such, about half of all IBD patients (millions) turn to complementary and alternative medicines (CAMs). Although CAMs have been used for thousands of years, there is a gap in our knowledge of the mechanisms that support their effectiveness. Understanding these mechanisms will not only lead to standardized and more efficient treatment for IBD outside of toxic FDA-approved drugs but will also better our understanding of the potential applications of CAMs for other diseases with similar mechanisms.

The natural herb, American ginseng (*Panax quinquefolius*; AG), improves mental performance and detrimental endpoints associated with diseases, such as cardiovascular disease, diabetes, and influenza (7,8). Over the past decade, we have shown that AG has anti-oxidant and anti-inflammatory properties and can suppress mouse colitis and prevent colon cancer associated with colitis (9–11). Using bioassay-guided fractionation, more recently, we have shown that a hexane fraction of AG (HAG) was particularly potent in this capacity (12–14).

With a goal of isolating a single, bioactive compound from AG and HAG, we further fractionated HAG and found that the most abundant molecule in this fraction was the polyacetylene, PA. Polyacetylenes are a distinct group of naturally occurring products, whose numerous pharmacological properties have been recognized (15). PA ([3(R)-(9Z)-heptadeca-1, 9-dien-4, 6-diyn-3-ol]; falcarinol) is a bioactive member of this family. It has been identified in both traditional herbal medicines (such as AG), and in common dietary plants, e.g., carrots, celery, fennel, parsley, and parsnip (16). Interestingly, PA has been shown to have anti-cancer properties (16–19) and neuroprotective effects (20–22). However, there remains an unanswered question regarding PA's potential as an anti-inflammatory molecule and, therefore, its capacity to suppress chronic inflammatory diseases, such as UC. Intriguingly, PA (as compared to the hundreds of other potential CAMs currently used with success in animals) not only comes from a natural source, but is a single molecule, allowing the potential to be standardized on its own, or in a cocktail. What makes this molecule innovative is the putative mechanism we explore here by addressing the hypothesis that PA targets mΦ for apoptosis resulting in the suppression of colitis in mice.

2.2. Materials and Methods

2.2.1 Identification and isolation of Panaxynol

Characterization of HAG and extraction of PA were carried out by our collaborator, Dr. Anthony Windust at the National Research Council (Ottawa, ON, Canada). The method for characterization and analysis of HAG has been described in detail previously [12]. Briefly, for characterization of bioactive components of HAG, this fraction was sub-fractionated through preparative, reverse-phase HPLC, where the HAG was divided into

five sub-fractions based on elution time (4 minutes each). The fractions were collected over six repeat runs (6 x 50 mg injected) and evaporated to dryness. A comparative analysis by analytical scale LC-UV of both the whole and each sub-fraction was performed to confirm identities of constituents in each sub-fraction.

PA was isolated and purified from *Panax quinquefolius* grown on the Harper Ranch, Kamloops, BC, Canada. The method of extraction and purification of PA has been previously described (23). Briefly, dried root of 4-year-old AG was dissolved in ethanol, and the organic layer was concentrated using vacuum centrifuge to yield dark brown oil. This extract was further separated using flash chromatography, and the fractions containing PA were dried to yield crude PA. The crude PA was then subjected to multiple passes of chromatography, and the purity of the final extract was validated using liquid chromatography with UV diode array detection (LC-UV-DAD). Purified PA was dissolved in 95% ethanol for use in *in vitro* and *in vivo* experiments.

2.2.2 CELL LINES AND REAGENTS

All cells were maintained in appropriate media recommended by ATCC supplemented with 10% New Born Calf serum (NBCS) (Biofluids, Rockville, MD), penicillin (10 U/ml) and streptomycin (10 μg/ml, Biofluids) at 37°C in a humidified chamber with 5% CO2 atmosphere. Experiments with PA were carried out by treating the cells with indicated concentrations of PA dissolved in appropriate media with 0.1% NBCS. For polarization to M1 type mΦ, ANA-1 cells were exposed to 10 ng/ml interferon-γ (IFNγ) for 8 hours (R&D Systems, Minneapolis, MN) either before or after the treatment with PA. For differentiation of U-937 monocytes into mΦ, cells were treated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma; P1585) for 24 hours. After replacing with

fresh media containing no PMA, the cells were allowed to grow for 48 hours before treatment with PA. CD4+CD25- cells were isolated from the spleens of C57BL/6 mice as previously described [13]. Briefly, the mΦ and B cells were depleted before isolation of CD4+CD25- T cells using MACS separator along with CD4 and CD25 microbeads (Miltenyi Biotec, Auburn, CA). For co-culture experiments, HCT-116 cells were plated in 6-well dishes and ANA-1 mΦ activated with IFN-γ were placed in the inserts (2018-11, Grenier bio-one; Kremsmünster, Austria) to mimic an inflammatory environment.

2.2.3 WESTERN BLOT ANALYSIS AND ANTIBODIES

Phospho-Histone H2AX (Ser139) (cat # 9718S), phosphor-p53 (Ser15) (cat # 9284S), and GAPDH (cat # D16H11) (5174S) rabbit monoclonal primary antibodies (1:1000 dilution); and horseradish peroxidase conjugated anti-rabbit secondary antibody (7074S) (1:2000 dilution) were purchased from Cell Signaling Technology, Danvers, MA. Primary antibody incubations were carried out overnight at 4^0C Secondary antibody incubations were carried out at room temperature for 1 hour. The Western blot signal was detected by Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) and developed onto Hyperfilm (GE Healthcare Life Sciences, Pittsburgh, PA) or imaged using Bio-Rad ChemiDoc Imager.

2.2.4 FLOW-CYTOMETRIC TUNEL ANALYSIS

TUNEL labeling was performed using Fluorescein in situ cell death detection (cat # 11684795910, Roche Diagnostics, IN). Briefly, cells were incubated in 0.1% NBCS supplemented media containing appropriate concentrations of PA or vehicle. Cells were harvested after 12 hours of treatment, and TUNEL assay was performed as described by

the vendor. TUNEL positive cells were detected and quantified using Beckman Coulter F500 Flow Cytometer and CXP software.

2.2.5 IN VIVO EXPERIMENTS

DSS (MW 36000–50000) obtained from International Laboratories USA (San Francisco, CA) was used to induce colitis in mice. 8-10 week old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in a suitable environment according to the Institutional Animal Care and Use Committee (IUCUC) standards. The care and usage of the mice were monitored by Animal Resource Facility (ARF) at the University of South Carolina, Columbia. This study was approved by IACUC (Animal Use Protocol # 2178).

For the prevention model of colitis, mice were given PA, once daily, at different doses (0.01 mg/kg, 0.1 mg/kg, 0.5 mg/kg and 1 mg/kg diluted in ddH2O) by oral gavage for two weeks. The lowest dose was calculated based on our previous experiments with AG and HAG. The PA dose was equated to reflect the percentage composition of PA in HAG. Mice were given 2% DSS in drinking water to induce colitis starting on day 7. For the colitis treatment experiments, mice were given 2% DSS in their water for two weeks. Starting on day 7, mice were given PA at the same doses as the prevention experiments (0.01 mg/kg, 0.1 mg/kg, 0.5 mg/kg and 1mg/kg; Table 2.1) by oral gavage. Control mice were given ddH2O by oral gavage. The weight of mice was monitored over the duration of the experiment. The mice were sacrificed on day 14 and colons were harvested, length was measured and processed for further analysis.

For macrophage depletion, we administered 200 µl clodronate liposomes in PBS via intraperitoneal injection (i.p.) and empty liposomes in PBS were used as a vehicle

control. The first injection was on the day -1 with the subsequent injections on days 2, 6, 10 and 13. DSS was administered starting on day 0 through day 13, and PA treatment was given days 7-13. Mice were sacrificed on day 14.

Blood in stool was detected using Hemoccult (Beckman Coulter) fecal immunochemical test. Immediately before sacrifice, stool consistency (0-fully formed stool; 2-loose stool; 4-diarrhea) and blood in the stool (0-no blood; 2-detected using Hemoccult; 4-rectal bleeding) were scored, and these measurements were used along with the weight difference in mice from the beginning to the end of the experiment (0=no weight loss; 1= 0-5% weight loss; 2= 6-10% weight loss; 3=11-15% weight loss; 4=16-20% weight loss), to calculate the CDI.

2.2.6 INFLAMMATION SCORING

Paraffin-embedded colons were serially sectioned (5 μm), and one section from each mouse was stained with hematoxylin and eosin. The stained slides were blindly examined under a microscope by two investigators (A. Chaparala and A. Chumanevich) for histopathological changes and scored according to a system previously described and extensively used by our lab and many others (12,24,25). Briefly, the histology score for inflammation accounts for four parameters – 1) inflammation severity (0 (no inflammation), 1 (minimal), 2 (moderate), and 3 (severe)); 2) inflammation extent (0 (no inflammation), 1 (mucosa only), 2 (mucosa and submucosa), and 3 (transmural)); 3) crypt damage (0 (no crypt damage), 1 (one-third of crypt damaged), 2 (two-thirds damaged), 3 (crypts lost and surface epithelium intact), and 4 (crypts lost and surface epithelium lost)) and; 4) percentage area of involvement (0 (0% involvement), 1 (1-25%), 2 (26-50%), 3

(51-75%), and 4 (76-100%)). The scores for the first three parameters are added and the sum is multiplied by the fourth parameter, giving a range of scores between 0-40.

2.2.7 IMMUNOHISTOCHEMISTRY

Sections of paraffin-embedded colons were incubated with cyclooxygenase-2 (COX-2) (cat # 60126; Cayman Chemical Company, Ann Arbor, MI) CD11b (cat # ab133357; Abcam, Cambridge, UK) mouse polyclonal antibody, diluted 1:10,000 in Antibody Amplifier™ (ProHisto, LLC, Columbia, SC) overnight. The slides were then processed using EnVision+ System HRP kits (DAKO, Carpinteria, CA) according to the instructions provided by the kit, which uses the chromagen, diaminobenzidine to elicit dark brown reaction to the HRP-tagged secondary antibody provided in the kit. Methyl green was used as a secondary stain. Immunoreactivity score was obtained by multiplying scores from two criteria – 1) percentage of tissue stained (0-5: 0 (0% positive staining), 1 (< 10%), 2 (11-25%), 3 (26%-50%), 4 (51%-80%), or 5 (> 80%)), and 2) staining intensity (0-3: 0 (Negative staining), 1 (Weak), 2 (Moderate), or 3 (Strong)). The scores of two parameters are multiplied, giving a range of scores between 0-15.

2.2.8 STATISTICAL ANALYSIS

Data are expressed as a mean ± standard error of the mean. Mean differences between the groups were compared by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. A P-value of ≤ 0.05 was chosen for significance.

2.3 RESULTS

2.3.1 PANAXYNOL IS THE MOST ABUNDANT AND POTENT ANTI-INFLAMMATORY MOLECULE IN AG

We have previously shown that AG and HAG are effective in the treatment of colitis

and prevention of colon cancer (9–14). We have also demonstrated that fatty acids and polyacetylenes are both components in AG and HAG (12). In moving forward, to better understand the active components of HAG, we have sub-fractionated this fraction of AG (Fig 2.1A). Sub-fraction 1 (F1; <10% of the whole HAG) contains multiple minor components including two minor polyacetylenes tentatively identified based on UV spectra (Fig 2.1B). F2 (30% of HAG) contains two major polyacetylenes, Panaxadiol and Panaxydol, and four minor polyacetylenes tentatively identified based on UV spectra (Fig 2.1C). F3 (24% of HAG) contains a major polyacetylene, PA, and a fatty acid, linolenic acid (18:3n3) (Fig 2.1D). F4 (27% of HAG) contains linoleic acid and no detectable polyacetylenes (Fig 2.1E). F5 (10%) contains minor fatty acids including saturates, and no polyacetylenes (Fig 2.1F). F2 and F3, the only fractions containing major polyacetylenes, suppress iNOS induction in ANA-1 mΦ polarized to the M1 type with IFNγ (Fig 2.1G), which is predictive of colitis suppression (10,12). Of the three major polyacetylenes in F2 and F3 sub-fractions of HAG, PA was the most abundant (10.2%) (12); therefore, we proceeded to test its efficacy as an anti-inflammatory compound.

2.3.2 PANAXYNOL IS EFFECTIVE AS A TREATMENT FOR COLITIS IN DSS MOUSE MODEL

Following the isolation of PA from HAG, and an initial screening (iNOS suppression in vitro [23]), we tested the efficacy of this compound in the prevention and treatment of DSS-induced mouse colitis. In the prevention model, where mice were treated with PA for a week before the induction of colitis using DSS (Fig S1A), treatment with PA did not inhibit colitis in mice when compared to the control group. Moreover, there was a marginal increase in the inflammation score with the highest dose of PA (Fig 2.8 A, B) when compared to the vehicle group, indicating that pre-treatment with PA slightly

exacerbated DSS-induced colitis. Although we are currently exploring the possible mechanism of this finding, it appears caution has to be made when considering PA for any chemoprevention purposes.

Excitingly, PA was very effective in the treatment model of colitis (Fig 2.7 1B), where colitis was induced with DSS for a week followed by PA treatment. PA significantly decreased the Clinical Disease Index (CDI) (Fig 2.2E) and the inflammation score (Fig 2.2A, B) in a dose-dependent manner. Colonic inflammation from PA-treated mice was limited to the distal end of the colon, while in the vehicle group, inflammation involved a larger area. To examine a biomarker of inflammation, we tested each colon section for cyclooxygenase-2 (COX-2) immunoreactivity. There was a decreased expression of COX-2 with PA treatment (Fig 2.2C, D). When taken together, the results are consistent with the hypothesis that PA can be used to treat mouse colitis. To note, we monitored the weights of the mice over the course of the experiment and did not observe any unexpected weight loss even with the highest dose of PA, indicating the non-toxic nature of PA.

2.3.3 PANAXYNOL TARGETS MACROPHAGES FOR DNA DAMAGE IN VITRO

In an effort to identify the mechanism of action of PA, we studied the structure and observed that PA is a hydrophobic compound with several sites of potential modification that could convert it to a DNA reactive alkylating agent (Fig 2.9). The hydroxide at the 3-position (C3) can be converted to a α, ß unsaturated aldehyde, which is a Michael acceptor, while the double bond between the 9 and 10 position could potentially be converted to an epoxide. Furthermore, the hydroxide group on C3 can react with the amino group of nucleic acids and alkylate DNA. We, therefore, screened multiple cell types for PA-induced DNA damage. Strikingly, PA caused DNA damage, as identified by γ-H2AX expression.

However, γ-H2AX induction only occurred in mΦ. These included mouse mΦ (ANA-1, Fig 3A, B; RAW264.7, Fig 3C), primary peritoneal mouse mΦ (Fig 3D), and human mΦ (U-937, Fig 2.3E). For all non-macrophage cells (Fig 3F-K), γ-H2AX induction was not seen up to 10 μM PA treatment. As well, when U-937 human monocytes were not differentiated to mΦ, γ-H2AX induction was also not seen until 10 μM PA treatment (Fig 3K) when compared to induction at 1 μM in U-937 cells differentiated into mΦ (Fig 2.3E). This indicated the specificity of DNA damage to mature mΦ and not monocytes. PA also induced phosphorylation of p53 at Ser15 in ANA-1 cells, further confirming DNA damage (Fig 2.10).

2.3.4 PANAXYNOL SELECTIVELY TARGETS MACROPHAGES FOR APOPTOSIS *IN VITRO* AND *IN VIVO*.

Based on the understanding that DNA damage is associated with apoptosis, we hypothesized that PA could selectively cause apoptosis in mΦ. Results are consistent with this hypothesis in two macrophage cell lines (Fig2. 4A – C). Apoptosis was minimal in other non-macrophage cells, including HCT-116 cells (Fig 2.4D) and mouse embryonic fibroblasts (MEFs) (Fig 2.4E). To examine whether PA selectively causes apoptosis in mΦ in the presence of other cell types, we carried out a co-culture experiment with ANA-1 mΦ and colon cancer cells (HCT-116). Figure 4F shows that PA causes apoptosis in ANA-1 mΦ at significantly higher levels than in HCT-116 cells. This property of PA would distinguish it from broadly immunosuppressive drugs that are currently on the market for the treatment of UC.

2.3.5 PANAXYNOL TARGETS MACROPHAGES FOR APOPTOSIS *IN VIVO*

To confirm that PA targets mΦ *in vivo*, we used colons from the DSS-induced colitis experiment to perform IHC for mΦ. We used a CD11b antibody, which is a surface marker for mΦ and we saw that PA-treated colons have lower expression of CD11b when compared to the vehicle group, indicating that PA treatment decreased the number of mΦ *in vivo* (Fig 2.2A, B).

We also conducted a DSS-colitis mouse experiment with clodronate, a compound that can deplete mΦ. We administered clodronate before treatment with DSS to deplete mΦ and treated them with PA after inducing colitis with DSS. Macrophage depletion with clodronate decreased the intensity of colitis, but we did not see any synergistic effect with PA treatment. Interestingly, PA was more effective than clodronate treatment for the amelioration of colitis as evidenced by the changes in inflammation score (Fig 2.5 C, D).

2.4 DISCUSSION

Currently available treatments for UC have multiple side effects and affect major organs like kidneys, liver (hepatitis), and pancreas (pancreatitis) (26). Furthermore, immune targeting drugs, e.g., infliximab that targets the TNF pathway, are broadly immunosuppressive thereby weakening the immune system, and making the body more susceptible to other infections like tuberculosis. We have shown that AG treats colitis in mice; however, it is composed of multiple ingredients with diverse effects, making it unfit for use as a mainstream drug. Upon examining the different extracts of AG, we identified HAG to be the most effective fraction in the treatment of colitis. Further analysis examined the various components of HAG. PA, apart from being the most abundant molecule in HAG, is also more effective than the whole HAG in suppressing iNOS expression in mΦ

that are polarized to M1 (pro-inflammatory) type. Hence, testing PA for the treatment of colitis is a natural step towards the identification of the bioactive component to treat colitis and prevent colon cancer.

Consistent with our previous studies with AG and UC, we used DSS-induced mouse colitis model for studying the effect of PA on an inflammatory disease. We found that PA treats DSS-induced colitis in the mouse, as seen by decreased CDI, inflammation, COX-2 expression, and the halted weight loss in treated mice. There was no toxicity even at higher doses, as observed by the insignificant weight changes. In future experiments, we will examine the effect of PA on liver and kidneys to rule out possible toxicity.

One of the mechanisms by which AG and HAG treat colitis is by targeting immune cells for apoptosis (11,13). We also examined the structure of PA and identified it to be a hydrophobic compound, is a potential DNA-reactive alkylating agent. PA and its derivative, falcarindiol, have previously been shown to be protein-alkylating agents (27). Furthermore, it has been shown that PA causes DNA damage in CaCo2 cells (28). It can be reasoned that the mechanism of action of PA can be via the induction of DNA damage. Our preliminary results show that PA causes DNA damage in multiple cell lines and that mΦ are especially sensitive to DNA damage induced by PA. We predict that one possible anti-inflammatory mechanism of action of PA is targeting mΦ for DNA damage and apoptosis. This is an interesting finding with this property being unique to PA. Furthermore, we also show that mΦ are more sensitive to apoptosis by PA. We also saw in our preliminary experiments that treatment with Panaxynol increases the phosphorylation of p53 at Ser15 (Fig. S4). This indicates activation of p53 as a result of DNA damage and the activated p53 can then induce apoptosis or cause growth arrest. In

this case, p53 is possibly causing activation of apoptosis pathway. This further indicates that γ-H2AX was possibly induced because of DNA damage, and not the disintegration of DNA resulting from apoptosis.

PA, however, did not prevent colitis in mice. Furthermore, treatment with the highest dose of PA slightly increased the inflammation score when compared to the untreated mice. The resident mΦ in the lamina propria of the intestine are anti-inflammatory and important for the maintenance of homeostasis. They clear any microbes and other stimuli that cross the epithelial cell barrier, mainly by phagocytosis, but do not secrete any cytokines (29). Since PA targeted mΦ before induction of colitis in the prevention model, the disease was more severe, and PA was ineffective. This is consistent with previous studies that showed that depletion of mΦ before induction of colitis resulted in exacerbated DSS-induced colitis (30). However, upon initiation of UC, there is increased accumulation of proinflammatory mΦ that secrete cytokines to enhance the inflammatory response. An overactive response by the mΦ to the enteric microbiota at this stage greatly contributes to the pathogenesis of colitis (31). Treatment with PA to target mΦ at this stage was highly effective in suppressing colitis.

The reason for the mΦ being specifically targeted by PA is not completely understood. Future directions will explore the mechanisms of DNA damage and a possible defect in DNA repair. Investigating whether PA can prevent colon cancer is the next natural step, as macrophage depletion not only decreases inflammation but also suppresses tumorigenesis in AOM-DSS-induced model of colitis induced colon cancer in mice (32). PA is effective in the treatment of colitis and does so by targeting mΦ for DNA damage and apoptosis. We have tested a range of doses (0.01 mg/kg – 1 mg/kg), and demonstrate that PA is very

effective at 0.1 mg/kg, which would translate to 6 mg for an average patient weighing 60 kg. This is an extremely low dose when compared to the immunosuppressive drugs currently available, placing PA a step above the other treatments for UC.

CONTRIBUTIONS AND ACKNOWLEDGEMENTS

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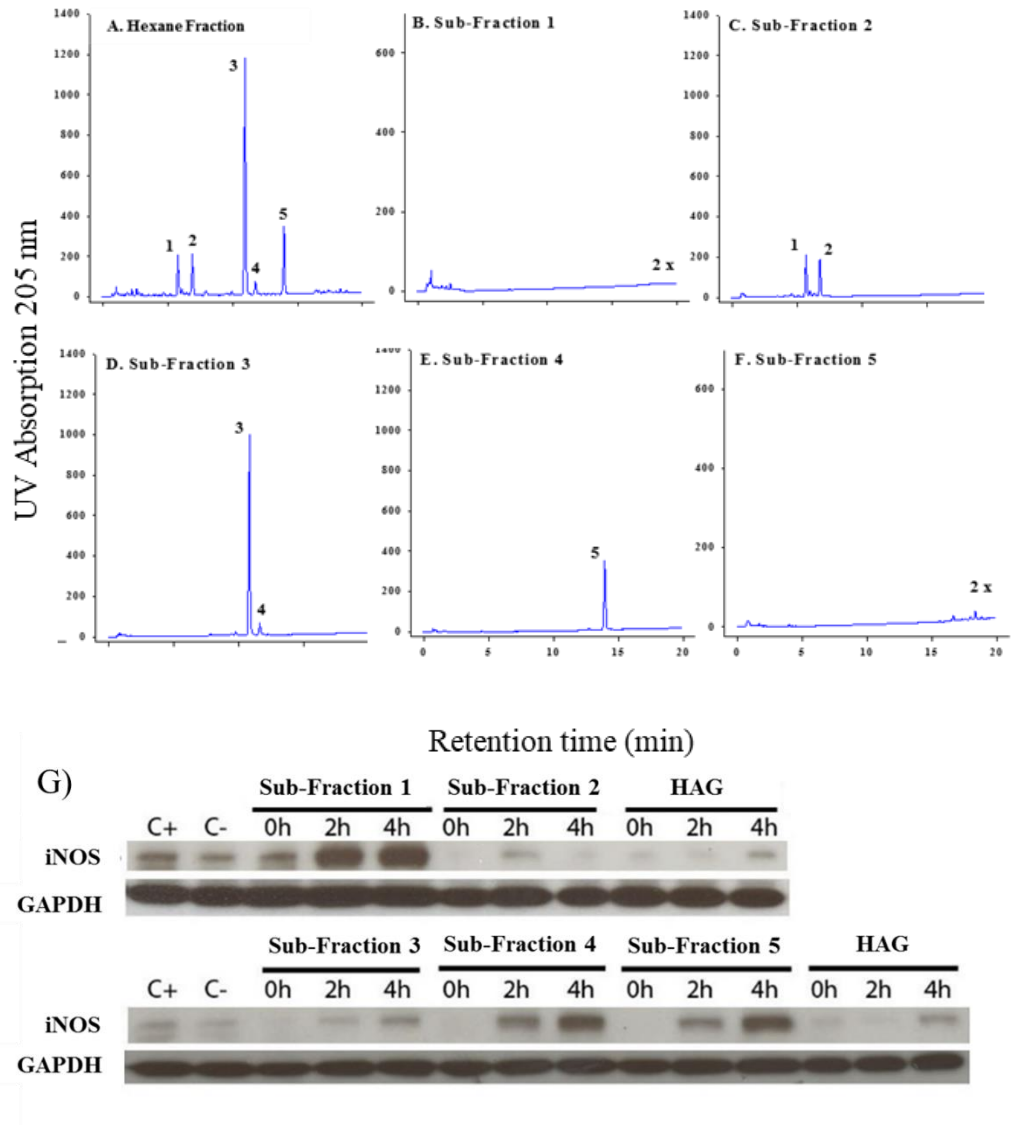

Figure 2.1. Isolation and characterization of various sub-fractions of HAG. A-F) LC-UV/DAD analysis of Hexane fraction and each sub-fraction. F1 to F5 represent the collected fractions, 4 minutes each. Peak identities: Panaxadiol (1), Panaxydol (2), PA (3), linolenic acid (4), linoleic acid (5) Column C-18 2.1 x 100 mm, 1 µl injection of a 5 mg mL^{-1} (whole) or equivalent fraction, gradient 55% to 90% acetonitrile/water in 15 minutes; hold 5 minutes; re-equilibration 10 minutes. Note: The scale magnification for sub-fractions 1 and 5 is 2X. **G)** Effect of HAG and different sub-fractions of HAG on IFNγ-induced iNOS expression. ANA-1 mouse mΦ were incubated for 12 hours with HAG or the indicated sub-fractions (10 µg/ml), washed, then exposed to IFNγ (10 ng/ml) for 0, 2, and 4 hours. C+ indicates the positive control, which is ANA-1 cells induced by IFNγ, and then incubated with media.

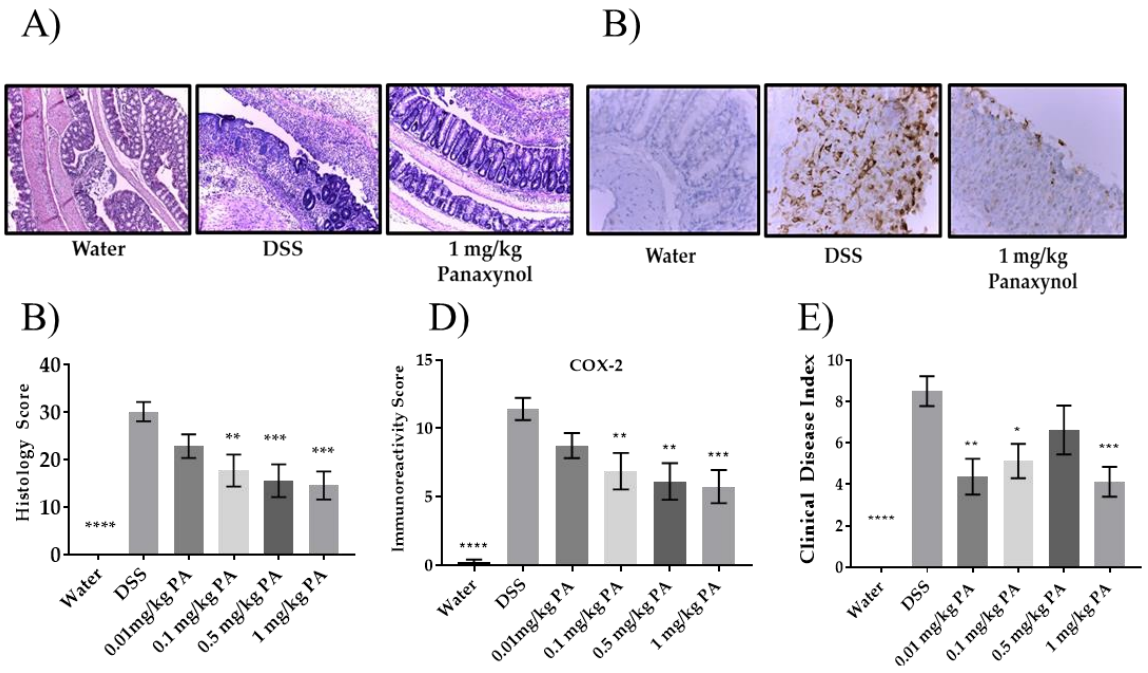

Figure 2.2. Panaxynol suppresses DSS-induced colitis in mice. A) Representative images (magnification – 100X) of histological sections from 3 groups; water, DSS only and highest dose of PA (1 mg/kg/day). **B)** Inflammation scores obtained from H & E slides of the colon cross-sections. **C)** Representative images of sections stained for COX-2 (magnification – 400X). **D)** Immunoreactivity score (IRS) of COX-2 from IHC staining. **E)** Clinical Disease Index (CDI) accounts for weight loss, blood in stool and stool consistency (n=8). Values represent mean ± SEM. One-way ANOVA followed by Dunnett's test was used for comparison between samples. p-value when compared to DSS group is indicated by: * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

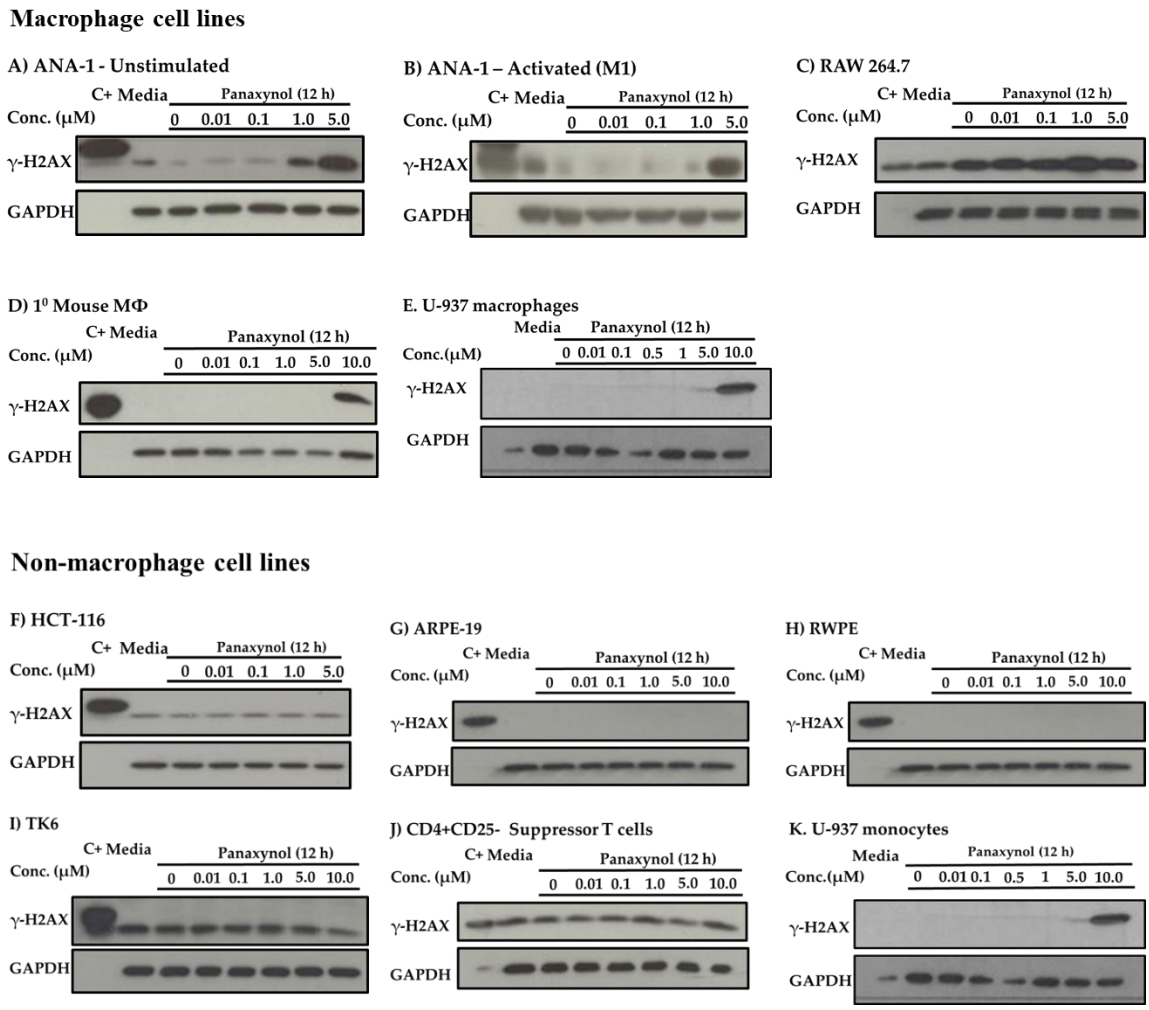

Figure 2.3. Panaxynol induces γ-H2AX in macrophages, but not in other cell types. All cell types were treated with PA at specified doses for 12 hours. Activated mΦ were generated by treating with IFNγ (10 ng/ml for 8 hours) before PA treatment. U-937 cells were treated with 10 ng/ml PMA for 24 hours for differentiation into mΦ. Nuclear protein extract from HCT116 cells overexpressing Flag-tagged γ-H2AX was used as a positive control for γ-H2AX. **A-E)** MΦ showed increased DNA damage with doses starting from 1 μM, as shown by the increase in the expression of γ-H2AX, a sensitive marker of DNA damage. **F-J)** Non-macrophage cell lines, including other immune cells (i.e., lymphoblasts and T cells) and epithelial cell lines, did not show any change in the protein expression of γ-H2AX and **K)** U-937 cells which are monocytes were more sensitive than U-937 mΦ.

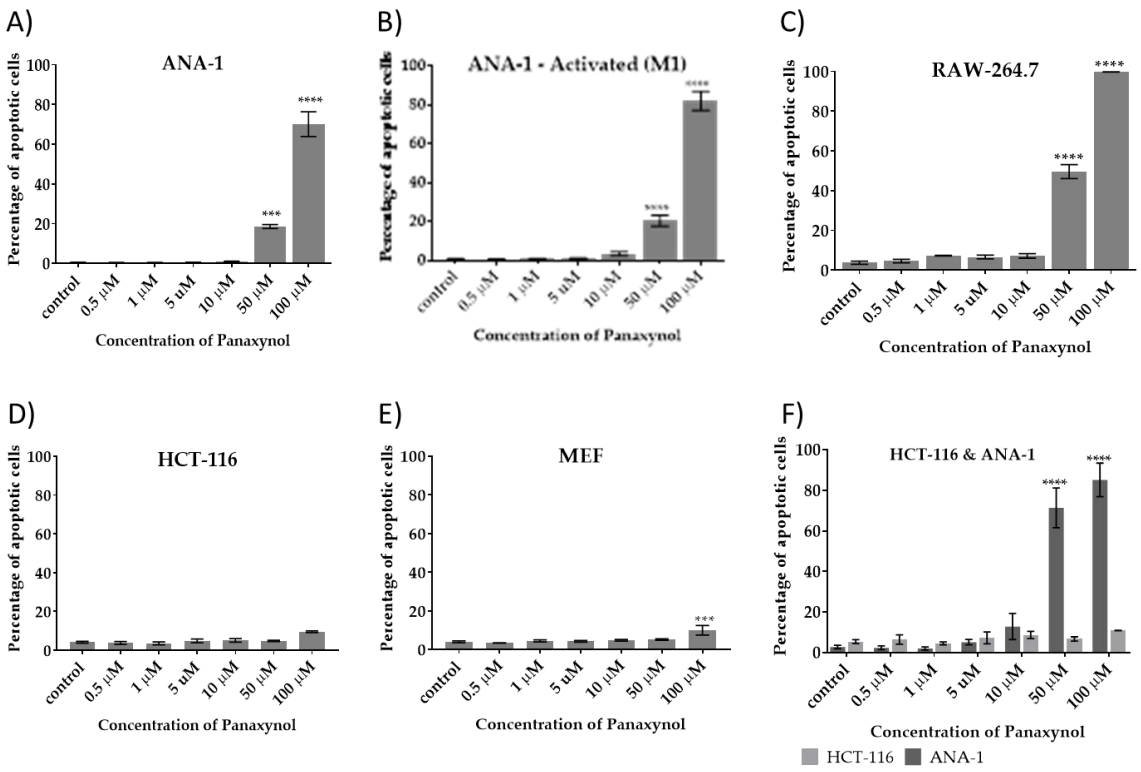

Figure 2.4 Panaxynol induces apoptosis in macrophages, but not in HCT-116 and MEF cells. Cells were treated with PA for 12 hours with indicated doses. PA significantly increased the percentage of apoptotic cells in **A**) unstimulated ANA-1 cells at 50 µM (18%) and 100 µM (70%), **B**) IFNγ stimulated ANA-1 cells at 10 µM (3.3%) and **C**) RAW264.7 cells at 50 µM (50%) and 100 µM (99%). **D**) PA had no significant apoptotic effect on HCT-116. **E**) PA induced apoptosis in MEFs only at a high dose of 100 µM (9.5%).**F)** In a co-culture experiment, PA caused apoptosis only in ANA-1 cells, but not HCT-116 cells. p-value indicated by; * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

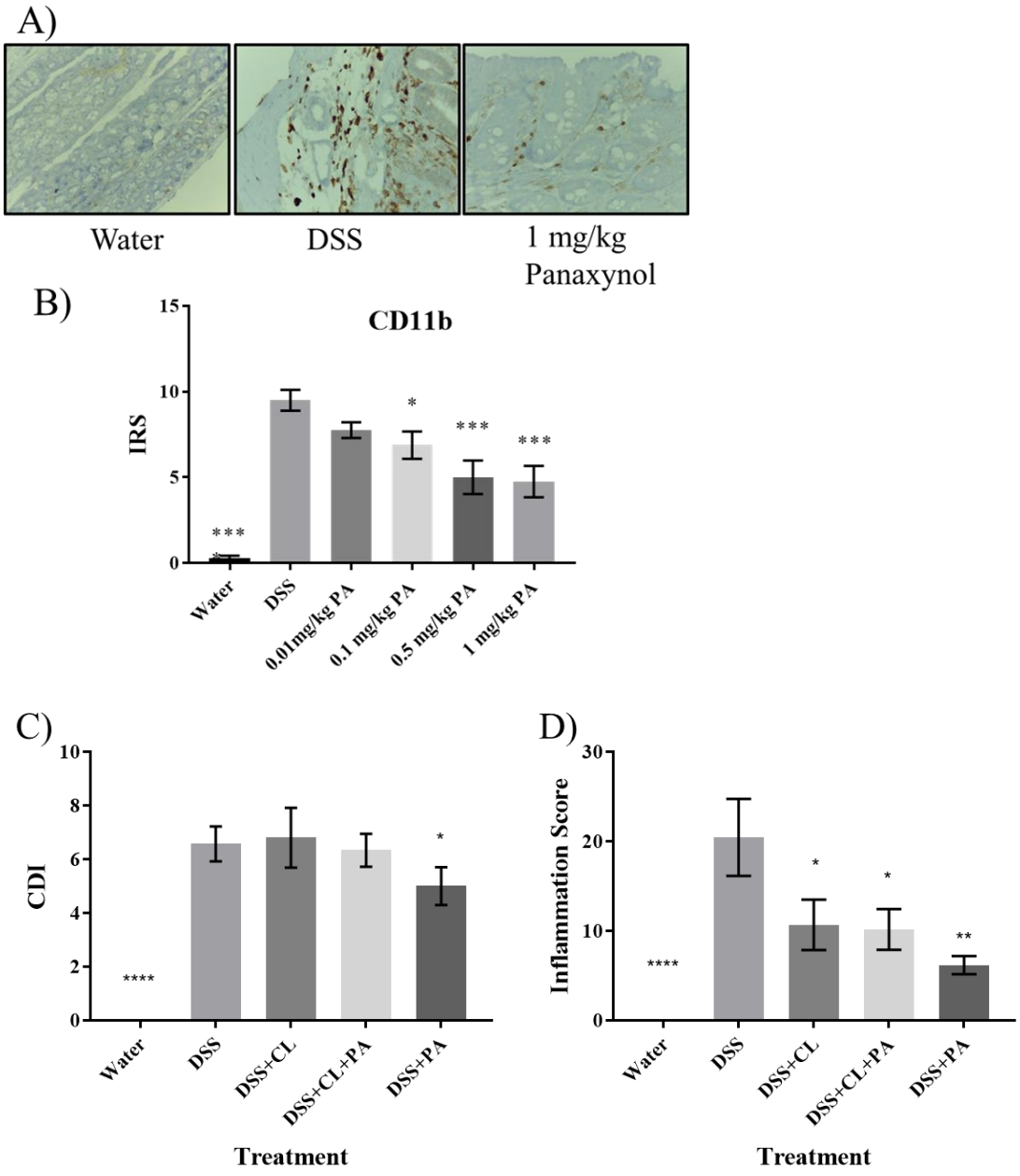

Figure 2.5 Panaxynol targets macrophages *in vivo*. Effect of PA on macrophages *in vivo*. **A)** Representative images of sections stained for CD11b (magnification – 400X) (N=8). **B)** Immunoreactivity score (IRS) of CD11b from IHC staining. **C, D)** DSS mice were treated in the presence and absence of clodronate (which depletes macrophages) to assess the involvement of macrophages in the treatment by PA. C) CDI). **C)** Clinical Disease Index (CDI) accounts for weight loss, blood in stool and stool consistency (n=6-8). **D)** Inflammation scores obtained from H & E slides of the colon cross-sections. Values represent mean ± SEM. One-way ANOVA followed by Dunnett's test was used for comparison between samples. p-value when compared to DSS group is indicated by: * = <0.05, ** = <0.01, *** = <0.001, **** = <0.0001.

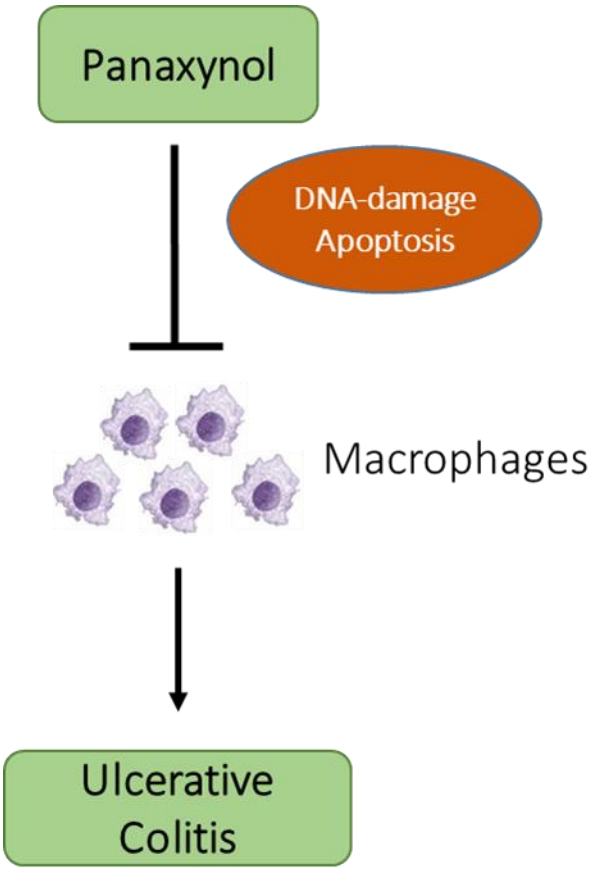

Figure 2.6 Schematic of the mechanism of action of Panaxynol. PA targets macrophages for DNA damage and apoptosis, thereby decreasing the inflammation and treating UC in mice.

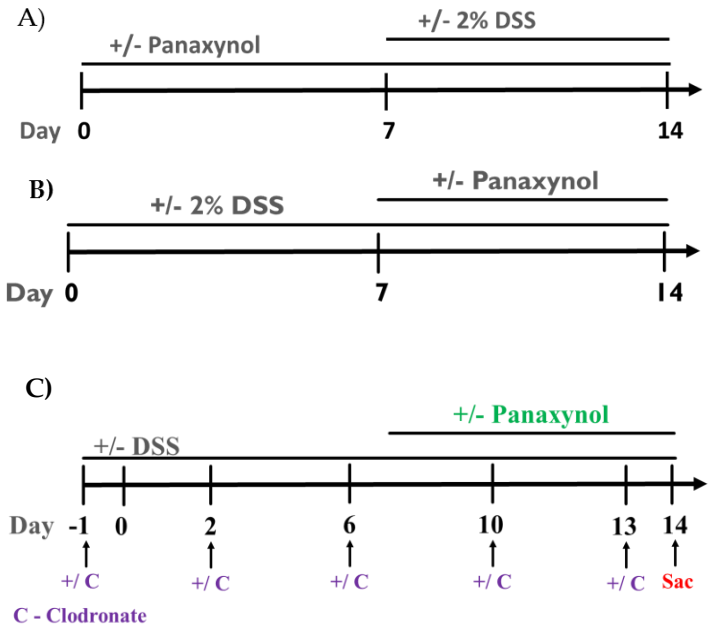

Figure 2.7: Schematics of *in vivo* experimental courses. A) Prevention model of colitis where the mice were treated with PA before induction of colitis using 2% DSS. **B)** Treatment model where mice were treated with PA after the manifestation of the disease due to DSS treatment. **C)** Clodronate experiment model where mice were treated with clodronate liposomes intermittently as indicated.

Table 2.1 Treatments and conditions for each group. n = 8

Group	DSS	Panaxynol
1	-	-
2	+	-
3	+	0.01 mg/kg
4	+	0.1 mg/kg
5	+	0.5 mg/kg
6	+	1 mg/kg

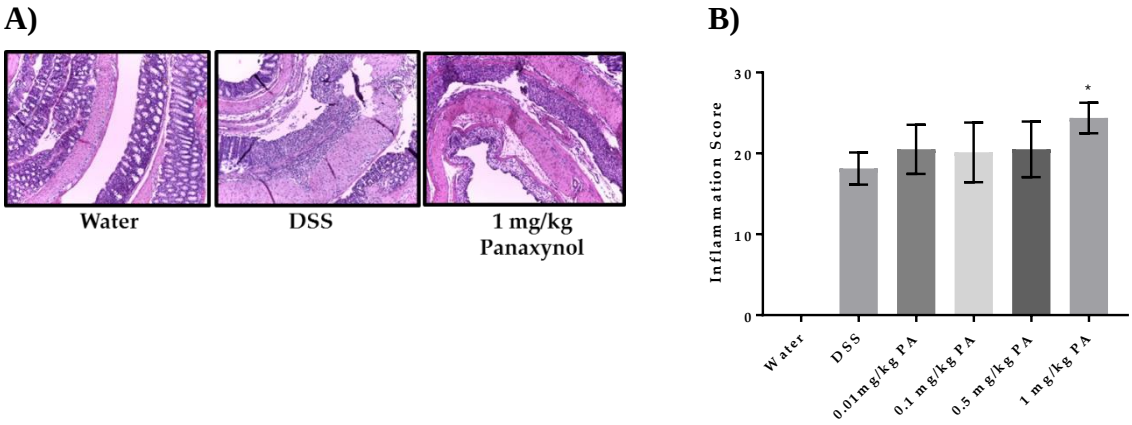

Figure 2.8: Panaxynol does not prevent colitis in mice. A) Representative images of H & E stained sections of colons (100X). **B)** Inflammation scores obtained from H & E slides of the colon cross-sections.

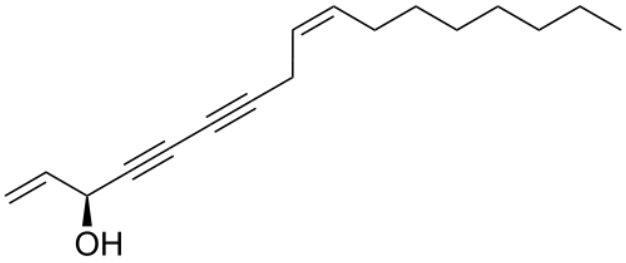

Figure 2.9: Structure of Panaxynol

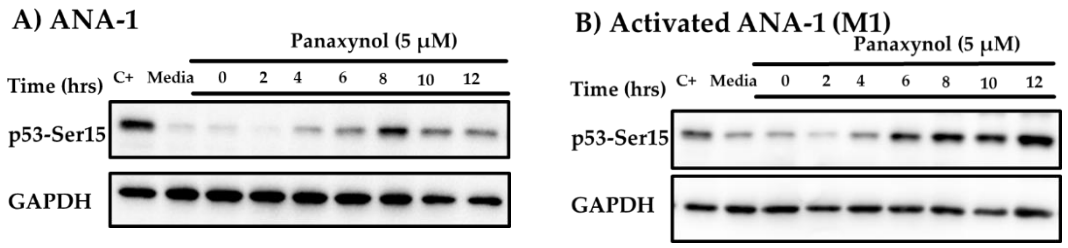

Figure 2.10: Treatment with Panaxynol increases the phosphorylation of p53 at Ser15 in ANA-1 cells. ANA-1 cells were treated with 10ng/ml IFNγ for 8 hours for activation of M1 type mΦ (B).

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CHAPTER 3

AMERICAN GINSENG AND ITS COMPONENTS TREAT COLITIS IN MICE BY INDUCING NRF2 DEPENDENT ANTIOXIDANT RESPONSE

ABSTRACT

Ulcerative colitis (UC) is a chronic, inflammatory, bowel disease that affects millions of people worldwide and increases the risk of development of colon cancer. We have previously shown that American Ginseng (AG) can treat colitis and prevent colon cancer in mice. We further fractionated AG and identified the most potent fraction, Hexane fraction (HAG), and the most potent compound in this fraction, Panaxynol (PA). Oxidative stress plays a significant role in the initiation and progression of colitis and contributes to tumorigenesis. Nrf2 is the master regulator of antioxidant response and hence plays an important role in the resolution of inflammation. To further the understanding of the mechanism of action of AG and its components, we examined the role of Nrf2 in the treatment of colitis. We determined the activation of the Nrf2 pathway and relieving oxidative stress *in vitro* and *in vivo*. We also used Nrf2 knockout mice to determine if Nrf2 is essential for AG and its components to operate. We found that AG and its components activate the Nrf2 pathway and decrease the oxidative stress in macrophages (mΦ) and colon epithelial cells. Consistent with these *in vitro* results, the Nrf2 pathway is activated combined with decreased oxidative stress in the DSS-induced colitis mice treated with AG, HAG or PA. Moreover, these compounds were not effective in the treatment of colitis in

the absence of Nrf2, when compared to the wild-type mice, further establishing Nrf2 as an important mediator of AG, HAG, and PA in the treatment of colitis.

3.1 INTRODUCTION

Inflammatory bowel disease (IBD), with rectal bleeding, severe diarrhea, and weight loss, debilitates approximately 2.6 million people in North America (1), with a health care cost of more than $3.9 billion annually. The worldwide health care cost is more than $46 billion annually. Treatment strategies for colitis (and the prevention of colon cancer) reduce periods of active disease and maintain remission, but outcomes are marginal. Patients become refractory, and there are dangerous side effects like cancer, infection, sepsis, hepatitis, and death (2–5). To this end, many colitis patients turn to CAMs to suppress the disease. 40- 50% of IBD patients use some form of CAM (6,7). Identifying new treatments that have minimal toxicity to treat the disease and prevent colon cancer, therefore, remains a high priority.

Previously, we demonstrated that American Ginseng (AG), may be ideally suited to treat IBD due to its ability to suppress colonic inflammation without apparent toxic side effects in mice (8–10). In further delineating the active components of AG, a fraction of AG (AG Fraction V), derived from extraction with n-Hexane as the solvent, was particularly potent in suppressing colitis and preventing colon cancer in mice (11–13). We have also seen that PA, an abundant molecule in this fraction suppresses colitis in mice. The next logical step is to explore the molecular mechanisms of the beneficial effects of AG and its components on the treatment of UC.

The etiology of UC remains to be fully elucidated, but involves interactions among genetic, environmental and immune factors, leading to uncontrolled, abnormal immune responses in the intestinal mucosa (14). Chronic intestinal inflammation is associated with enhanced RNOS production, and the consequent oxidative stress plays a critical role in the pathophysiology of IBD in both animals and humans. For example, elevated RNOS (reactive oxygen and nitrogen species) formation in colonic mucosa occurs at an early stage of IBD and correlates with the disease severity (15–17). Prevention of IBD has been shown by transgenic overexpression of endogenous antioxidant genes and administration of antioxidant compounds (18–21).

Nuclear factor E2-related factor 2 (NRF2), a master regulator of antioxidant response element, is a member of the cap'n collar family of basic region-leucine zipper transcription factor(22). As a transcription factor, NRF2 promotes the expression of phase II antioxidant enzymes that include, but not limited to, NAD(P)H quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), glutamate cysteine ligase (GCL), glutathione S-transferase (GST), and glutathione peroxidase (GPx), which all protect against reactive nitrogen and oxygen species (RNOS). The precise mechanisms of Nrf2 activation are not fully understood, but it is widely accepted that Keap1 is a key regulator of Nrf2 (23–25). Nrf2 is normally associated with Keap1 that serves as an adaptor for the interaction of the Cul3-based E3-ubiquitin ligase complex with Nrf2 leading to Nrf2 ubiquitination then proteasomal degradation (26). With various stresses or ARE inducers, the system is perturbed, and Nrf2 escapes from Keap1-mediated ubiquitination, accumulates in the nucleus, leading to increased Nrf2 target gene transcription(26). Several and probably overlapping mechanisms for Nrf2 nuclear accumulation in response to oxidative stress or

inducers like a direct modification of cysteine (Cys) residues of Keap1, Nrf2 phosphorylation of Nrf2, etc. (27–29).

With Nrf2 being an important initiator of antioxidant response required for the treatment of UC, it is reasonable to hypothesize Nrf2 plays an important role in the mechanism of action of AG, HAG, and PA. Here, we have shown that AG and its components (HAG and PA) suppress oxidative stress via activating Nrf2, a key endogenous regulator of antioxidant defenses, both *in vitro* and *in vivo*. We have also demonstrated that AG and its components suppress colitis by activation of the Nrf2 pathway. We used Nrf2 knockout mice to demonstrate that AG and its components are ineffective in treating colitis in the absence of Nrf2.

3.2 Materials and methods

3.2.1 Cell culture

All cell lines were maintained in cell culture media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and were kept at 37°C in a humidified incubator with 5% CO_2. ANA-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT) and HCT-116 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 media. ANA-1 cells were activated by treatment with 10 ng/ml interferon (IFN)-γ (R&D Systems, Minneapolis, MN) for 8 hours. AG/HAG/PA was dissolved in media and incubated with cells at specified concentrations for indicated times. For co-culture experiments, HCT-116 cells were plated in 6-well dishes and twice the number of activated ANA-1 cells were placed in the transwell inserts (cat # 2018-11, Grenier bio-one; Kremsmünster, Austria) to facilitate the exchange of cytokines and other cellular secretions.

3.2.2 TOS DETECTION:

ANA-1 mΦ were pre-treated with AG, HAG or PA for 12 hours in 6-well dishes. The media was replaced with media containing IFN-γ (10 ng/ml), and the cells were treated for 1 hour, 2 hours and 3 hours and processed according to the instructions of the Superoxide/TOS Chemiluminescent kit (lot # 101214A; Applied Bioanalytical labs. Briefly, the cells were washed with PBS and resuspended in Hanks's buffered saline solution (HBSS). Luminol and enhancer solutions were added before measuring the luminescence in a luminometer at 425nm. Luminometer quantifies an oxidative burst through the reaction of luminol with reactive oxygen species to produce a luminophore. Luminescence intensity is proportional to the number of reactive species in the sample and is quantified as relative light units.

3.2.3 WESTERN BLOT:

The primary antibodies, HO- 1 (ab13248; Abcam, Cambridge, UK) and GAPDH (5174S; Cell Signaling, Danvers, MA) were diluted to 1:1000 and incubated with the membrane overnight at 4^0C. Horseradish peroxidase-conjugated anti-rabbit secondary antibody (7074S; Cell Signaling, Danvers, MA) at 1:2000 dilution was incubated at room temperature for 1 hour. The Western blot signal was detected by Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) and imaged using Bio-Rad ChemiDoc Imager.

3.2.4 IMMUNOFLUORESCENCE:

Cells ($5x10^5$/well) were seeded into six well plates containing a coverslip. The next day, cells were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde for 10 min at room temperature, followed by permeabilization with 0.1% Sodium Citrate with 0.1%

Triton X-100. The cells were incubated with Nrf2 antibody at 1:1000 dilution (12721S; Cell Signaling, Danvers, MA) for one hour at room temperature. The cells were then washed with cold PBS three times (5 minutes each), and incubated with Alexa 514-labeled anti-rabbit secondary antibody (1:1000) (Invitrogen) at room temperature for 1 hour. The cells were then incubated with conjugated actin antibody for 15 minutes. After washing with PBS, the coverslips were mounted onto the slides with ProLong™ Diamond Antifade Mountant with DAPI (P36962; Thermo Fisher Scientific). The cells were examined by fluorescence microscopy (Olympus America Inc, Center Valley, PA). Fluorescence intensities from images of six randomly selected microscopic fields of cells were semi-quantitatively analyzed by densitometry (ImageJ software, NIH)

3.2.5 IMMUNOHISTOCHEMISTRY:

For immunohistochemical staining, serial sections of mouse colon tissues (processed as described above) were incubated with anti-HO-1 (ab13248; Rabbit Polyclonal, diluted 1:2000; Abcam, Cambridge,UK), 4-Hydroxynonenal (4-HNE) (ab46545; Rabbit Polyclonal, diluted 1:1000;Abcam, Cambridge, MA), and COX2 (Rabbit Polyclonal, diluted 1:1000cat # 60126; Cayman Chemical Company, Ann Arbor,MI). To ensure even staining and reproducibility, sections were incubated by slow rocking overnight in primary antibodies (4°C) using the Antibody Amplifier™ (ProHisto, LLC, Columbia, SC).Sections were processed with EnVision+ System-HRP kit according to kit protocols(K4011; Dako Cytomation, Carpinteria, CA). Immunohistochemistry was quantified as described previously (11).

3.2.6 RNA ISOLATION AND RT Q-PCR

The cells were lysed using Trizol and RNA was isolated using the RNeasy mini kit (Cat # 74104; Qiagen, Hilden, Germany) according to the instructions. RNA concentrations were measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). 1μg of RNA was used for cDNA synthesis using iScript cDNA synthesis kit (cat # 1708890; Bio-rad, Hercules, CA). The final product was diluted 1:10 for qPCR. qPCR was performed using iTaq™ Universal SYBR® Green Supermix (cat #1725121; Bio-rad, Hercules, CA). The list of primers used is as follows in the order forward then reverse in 5`-3` orientation. – HO-1: AAGCCGAGAATGCTGAGTTCA, GCCGTGTAGATATGGTACAAGGA; NQO1: AGGATGGGAGGTACTCGAATC, AGGCGTCCTTCCTTATATGCTA; Nrf2: TCTTGGAGTAAGTCGAGAAGTGT, GTTGAAACTGAGCGAAAAAGGC; GAPDH: AGGTCGGTGTGAACGGATTTG, TGTAGACCATGTAGTTGAGGTCA. Human primers – HO-1: CCACCTGTTAATGACCTTGCC, CACCGGACAAAGTTCATGGC; NQO1: GAGGCCAAATCAGGCTTACGG, CACTCCTGTCCACCAATCGC; GAPDH: AATCCCATCACCATCTTCCA, TGGACTCCACGACGTACTCA. qPCR was performed on CFX384 Touch™ Real-Time PCR Detection System (cat # 1855485; Bio-rad, Hercules, CA).

3.2.7 DSS-INDUCED COLITIS MOUSE MODEL

C57BL/6 and Nfe2l2tm1Ywk were obtained from The Jackson Laboratory. All mice were on an AIN93M diet as described previously. The mice were either given water ad libitum or 2% DSS for two weeks, starting on day 0. Treatments with AG (75 mg/kg/day), HAG (75 mg/kg/day) and PA (1mg/kg/day), dissolved in PBS, were carried

out by oral gavage from day 7-13. The control groups were treated with 1x PBS by oral gavage as well. All procedures performed were in accordance with the Guide for Care and Use of laboratory animals (National Research Council, Washington, DC) and by the approval of the Animal Resource Facility, University of South Carolina, Institutional Animal Care and Use Committee. On the day of the sacrifice, stool consistency (0-fully formed stool; 2-loose stool; 4-diarrhea) and blood in the stool (0-no blood; 2-detected using Hemoccult; 4-rectal bleeding) were scored, and these measurements were used along with the weight difference in mice from the beginning to the end of the experiment (0=no weight loss; 1= 0-5% weight loss; 2= 6-10% weight loss; 3=11-15% weight loss; 4=16-20% weight loss), to calculate the CDI. Blood in stool was detected using Hemoccult (Beckman Coulter) fecal immunochemical test. Mice were sacrificed on day 14 and colons were fixed overnight in formalin after they were cut longitudinally and their lengths were measured. The colons were then embedded in paraffin for sectioning.

3.2.8 INFLAMMATION SCORING

Paraffin-embedded colons were serially sectioned (5 μm), and one section from each mouse was stained with hematoxylin and eosin (H&E). The stained slides were blindly examined under a microscope by two investigators (A. Chaparala and A. Chumanevich) for histopathological changes and scored according to a system previously described (12,24,25). Briefly, the histology score for inflammation accounts for four parameters – 1) inflammation severity (0 (no inflammation), 1 (minimal), 2 (moderate), and 3 (severe)); 2) inflammation extent (0 (no inflammation), 1 (mucosa only), 2 (mucosa and submucosa), and 3 (transmural)); 3) crypt damage (0 (no crypt damage), 1 (one-third of crypt damaged), 2 (two-thirds damaged), 3 (crypts lost and surface epithelium intact), and 4 (crypts lost and

surface epithelium lost)) and; 4) percentage area of involvement (0 (0% involvement), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%)). The scores for the first three parameters are added, and the sum is multiplied by the fourth parameter, giving a range of scores between 0-40.

3.2.9 Statistical analysis

Data are expressed as a mean ± standard error of the mean. Mean differences between the groups were compared by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. A P-value of ≤ 0.05 was chosen for significance.

3.3 Results

3.3.1 American Ginseng and its derivatives activate Nrf2 pathway in macrophages

We have previously shown that American Ginseng decreases total oxygen species in mΦ activated to M1 type by IFN-γ. Here, we show that ANA-1 cells pre-treated with HAG and PA also show decreased total oxygen species when stimulated with IFN-γ (Figure 3.1 G). Antioxidant response mediated by Nrf2 is the most important phenomenon that is responsible for defense against inflammation. We hypothesize that American Ginseng and its components, HAG and PA decrease the reactive oxygen species by activating the antioxidant response via Nrf2. To test this hypothesis, we looked at the translocation of Nrf2 into the nucleus as well as activation of a key target gene activated by this transcription factor. Immunofluorescence data shows that Nrf2 is localized to the nucleus upon treatment with AG, HAG or PA, indicating its activation (Figure 3.3A). HO-1, a target of Nrf2, is upregulated by AG, HAG and PA treatment of ANA-1 mΦ, as shown by immunoblot and qPCR and immunoblot (Figure 3.3 B, D). Nrf2 has two ARE elements in its promoter region, and the elevated expression of Nrf2 gene indicates self-activation,

as seen in Figure 3.3C. To check the activation of Nrf2 in epithelial cells in an inflammatory environment, we co-cultured HCT-116 cells with activated ANA-1 cells for 3 hours. RT-qPCR data indicates that Nrf2 and HO-1 show increased expression when HCT-116 cells were pre-treated with AG, HAG, or PA (Figures 3.3 E, F).

3.3.2 American Ginseng and its derivatives suppress oxidative stress and activate Nrf2 pathway in vivo

To evaluate if Nrf2 is activated by the AG, HAG, and PA in vivo, we used DSS induced colitis mouse model. We used previously tested doses of AG (75 mg/kg/day), HAG (75 mg/kg/day) and PA (1 mg/kg/day) to treat mice by oral gavage. The colons were probed for 4-hydroxy-2-nonenal (4HNE) and HO-1. 4HNE is a product of lipid peroxidation and a biomarker for cellular oxidative stress; and HO-1, as indicated above, is a target of Nrf2. We see the decreased expression of 4HNE in the treatment groups indicating reduced oxidative stress (Figures 3.2A, B). We also see increased expression of the HO-1 protein, indicating the activation of the Nrf2 pathway (Figures 3.2C, D).

3.3.3 American Ginseng and its derivatives suppress colitis by activating Nrf2 pathway

We have previously shown that AG, HAG, and PA suppress DSS induced colitis in mice. Nrf2 activation is important for the antioxidant response, which in turn is important for defense against inflammation caused by ulcerative colitis. Since AG and its derivatives activate Nrf2 pathway in vitro and in vivo, we hypothesized that their mechanism of action requires activation of Nrf2 mediated anti-oxidant response. To test this hypothesis, we compared the effectiveness of AG, HAG, and PA in the treatment of DSS induced colitis in wild-type with that of their effectiveness in Nrf2 knockout mice. We used the previously

described 14-day DSS induced colitis mouse experiment (Figures 3.3A, B). AG, HAG, and PA lowered the CDI in wild-type mice but not in the Nrf2 knockout mice (Figure 3.3 C). We scored inflammation as indicated by ulceration, damage to crypts, and infiltration by inflammatory cells, using H and E slides of colon sections. Treated wild-type mice had significantly lower histology score than DSS-only mice, while the treatment was not as effective in case of Nrf2 knockout mice (Figures 3.4 A, B). We also tested immunoreactivity of COX2, a proinflammatory enzyme that has elevated protein expression during inflammation. We see that AG, HAG, and PA effectively decrease COX-2 expression in wild-type mice, but not the Nrf2-/- mice (Figures 3.4 C, D). Put together, this confirms that AG and its derivatives treat DSS induced colitis in mice by activating the Nrf2 pathway.

3.4 DISCUSSION

Deregulation of intestinal immune responses is considered a major contributor to IBD; the mechanisms of which are not well understood. A better understanding of this phenomenon would lead to better treatment strategies and would help millions of people, and reduce the tremendous cost of treatment with small synthetic molecules. Because IBD (aka colitis) is, by definition, chronic colon inflammation, it is reasonable that antioxidant-based approaches have shown efficacy in treating IBD (30). Current FDA-approved therapies involve treatment with 5-aminosalicylic acid (5-ASA) and its analogs, as well as glucocorticosteroids and anti-TNFα biologicals. Such treatment reduces periods of active disease and helps to maintain remission. Although these drugs have modest beneficial effects, patients become refractory, and there are significant side effects (31). This underscores the importance of developing more effective IBD therapies. To this effect, we

have explored the potency of a natural compound, AG in the treatment of colitis and identified the bioactive components that could be responsible for AG's efficacy. The identification of a single, active component would bring AG a step closer to being used as a mainstream medicine. In this context, it makes sense and would be significant to explore the molecular mechanisms of AG-mediated beneficial effects on the treatment of IBD.

An increased susceptibility also shows a key role of endogenous antioxidants in controlling intestinal inflammatory stress to chemically induced IBD and associated colon cancer in Nrf2-/- mice (32,33). As Nrf2 is a central regulator of tissue antioxidant defenses (23), these studies not only support a causative role of oxidative stress in IBD but also suggest that targeting Nrf2 provides a novel antioxidant-based intervention of IBD and the chemoprevention of colon cancer. AG and other plant products like resveratrol have been shown to combat cellular stress by activating the antioxidant mechanisms in the cell. Korean ginseng has been shown to activate Nrf2 pathway (34,35), and we have previously demonstrated with our collaborators that AG can activate Nrf2 in cardiomyocytes (36). Since AG and HAG are anti-inflammatory and suppress many of the molecular targets of Nrf2 (9–12,37,38), it is reasonable that Nrf2 is at the crossroads of the protective effects of AG/HAG/PA, and colitis-associated carcinogenesis. Nrf2 mediated antioxidant response plays an important role in resolving inflammatory diseases and could play an important role in AG mediated treatment of colitis.

In this study, we measured the antioxidant response and activation of Nrf2 signaling pathway upon treatment with AG and its components, macrophage cell lines (*in vitro*), and mouse colon (*in vivo*). Treatment with AG, HAG, and PA activated HO-1 in ANA-1 macrophage cell lines and mouse colons with DSS induced colitis. They also induced the

translocation of Nrf2 into the nucleus, indicating its activation. Furthermore, we saw that these treatments also decreased the production of TOS *in vitro*, and the expression of 4HNE *in vivo* indicated decreased oxidative stress. Upon confirming that AG and its components activate Nrf2 pathway, we used Nrf2 -/- mice to investigate if the Nrf2 pathway is essential for the treatment of colitis. As predicted, AG and its components were not very effective in the Nrf2-/- mice as evidenced by the inflammation scores and expression of COX2.

Though the effects of AG and its components have been diminished considerably, there is still a decrease in inflammation with AG treatment of Nrf2 -/- mice. This could be due to the effect of p53, which also plays an important role in the activation of antioxidant mechanisms like the synthesis of GSH and NADPH generation (39,40). We have previously shown that the mechanism of action of AG in the treatment of colitis is p53 dependent, but the mechanism of HAG is only partially dependent on p53 (10,12). Apart from possessing antioxidant properties, Nrf2 has also been shown to negatively regulate the expression of pro-inflammatory genes that are induced in M1 mΦ (41). This is consistent with the decreased expression of ROS, a proinflammatory marker, and could be an additional mechanism by which Nrf2 activation helps in the treatment of colitis, that we would explore in the future.

AG, HAG, and PA caused a decrease in the expression of Histone Deacetylase 1 (HDAC-1) and DNMT-1 in ANA-1 mΦ (Figure A1). The increase in Nrf2 shown in Fig. 8B at the same time as a decrease in HDAC-1 and DNMT-1 is consistent with the hypothesis that these enzymes may regulate nrf2 expression. The AG fraction also down-regulates the expression of miR-142 (Fig. 9) (13), known to target Nrf2 (42), thereby suggesting that AG may increase Nrf2 through down-regulation of miR-142. Because Nrf2

is involved in colitis and associated colon cancer (32,33,43–45), we believe it would be of significance to examine the ability of AG, HAG, and PA to target Nrf2 as a mechanism for the protection of colitis and colon cancer. Such studies will not only better elucidate the mechanisms by which AG, HAG, and PA work in this disease, but also: (1) identify other diseases where Nrf2 is dysregulated that AG, AG-Fraction V, and individual constituents may be beneficial [e.g., obesity (46), neurodegenerative diseases (47), vascular diseases and aging (48), cardiovascular disease (23), and diabetes (49)]; (2) identify other CAMs or small molecules that target Nrf2 that may be beneficial to patients with colitis at a high colon cancer risk [e.g., triterpenoids that target Nrf2 also suppress experimental colitis (50,51)]; and (3) identify epigenetic biomarkers of Nrf2 regulation and attenuation of inflammation.

CONTRIBUTIONS AND ACKNOWLEDGEMENTS

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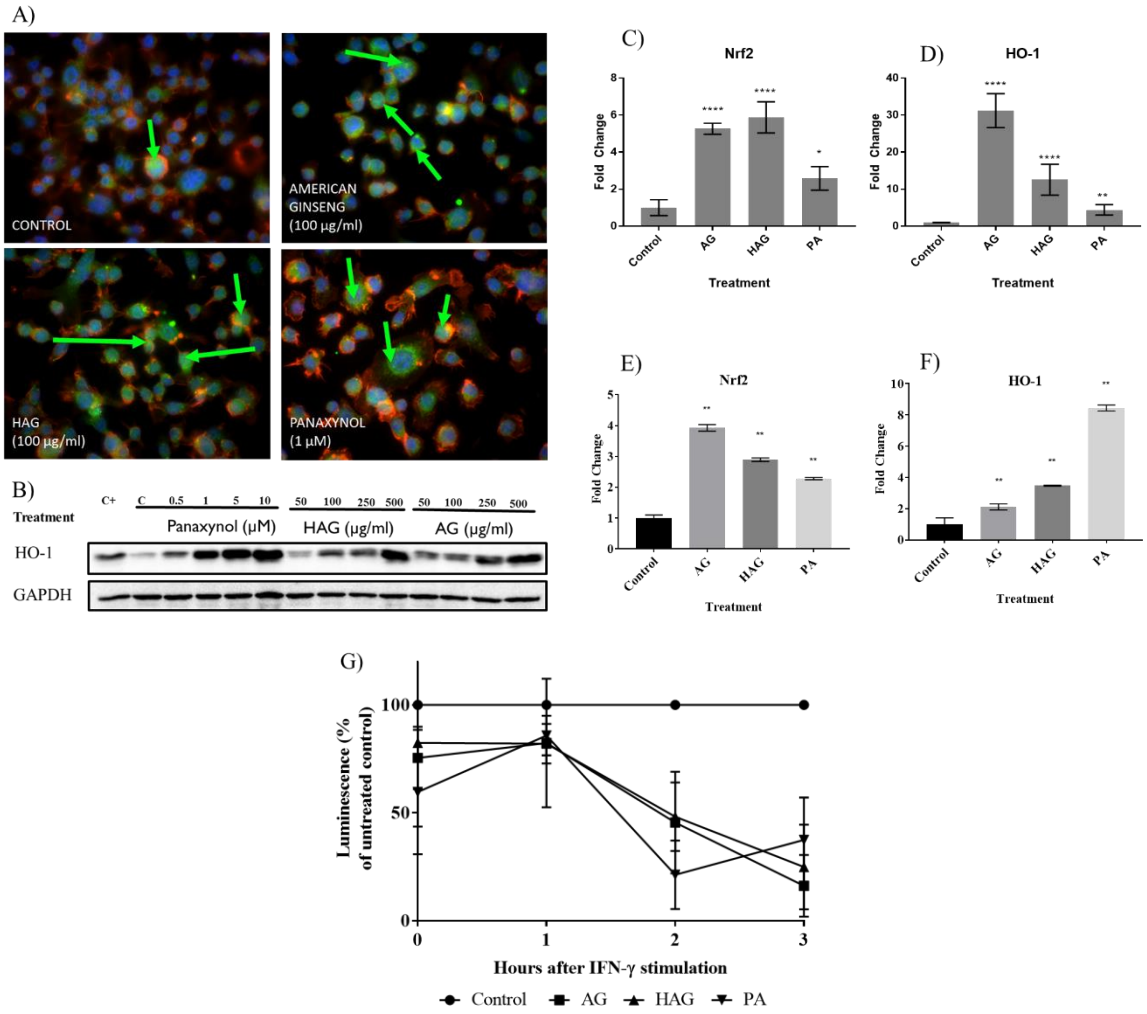


Figure 3.1: AG, HAG, and PA activate Nrf2 pathway and decrease ROS *in vitro*. A) AG, HAG, and PA induce translocation of Nrf2 into the nucleus. ANA-1 cells were treated with AG (100 µg/ml), HAG (100 µg/ml) and PA (1 µM) for 8 hours. Representative images of immunofluorescence (n=3). Green arrows indicate nuclei with Nrf2 expression. **B)** AG, HAG, and PA increase the expression of HO-1. Western blot image of ANA-1 cells treated with indicated doses of AG, HAG, and PA for 8 hours. **C, D)** AG (100 µg/ml), HAG (100 µg/ml) and PA (1 µM) increase the expression of HO-1 (C) and NQO1 (D) in ANA-1 cells. RT-qPCR data is cumulative of three separate experiments. **E, F)** AG, HAG and PA activate Nrf2 pathway in HCT-116 cells in the presence of activated mΦ. HCT-116 cells were pretreated with AG (100 µg/ml), HAG (100 µg/ml) and PA (1 µM) and co-cultured with activated ANA-1 (10 ng/ml IFNγ) for 3 hours and separated for qPCR. P-values - * - >0.05, ** - >0.005, *** - >0.001, **** - >0.0001. Values represent the mean ± S.D. The significance is compared with the DSS only group. **G)** An oxidative burst in ANA-1 mouse mΦ is suppressed by pretreatment with AG (100 µg/ml), HAG (100 µg/ml) and PA (1 µM). The protocol described in methods.

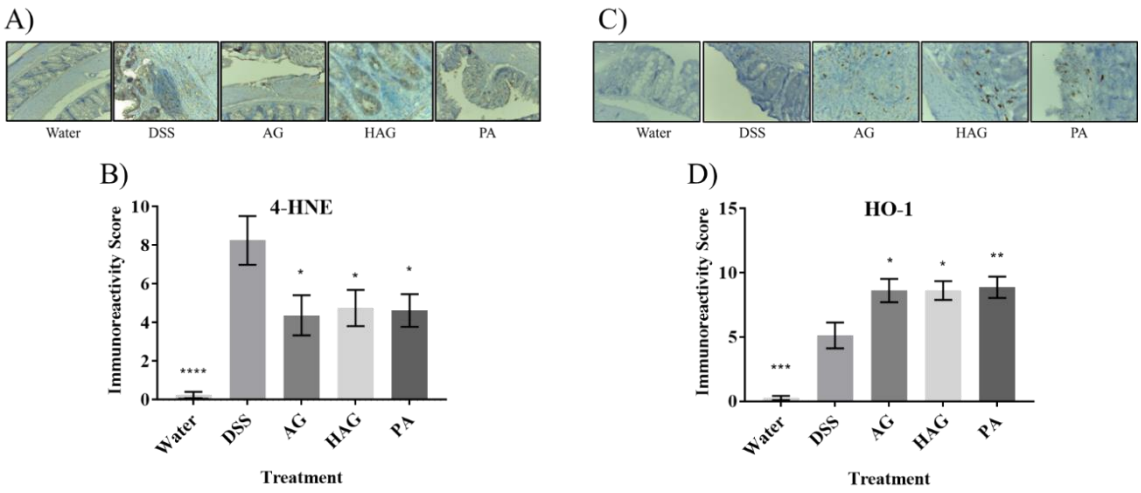
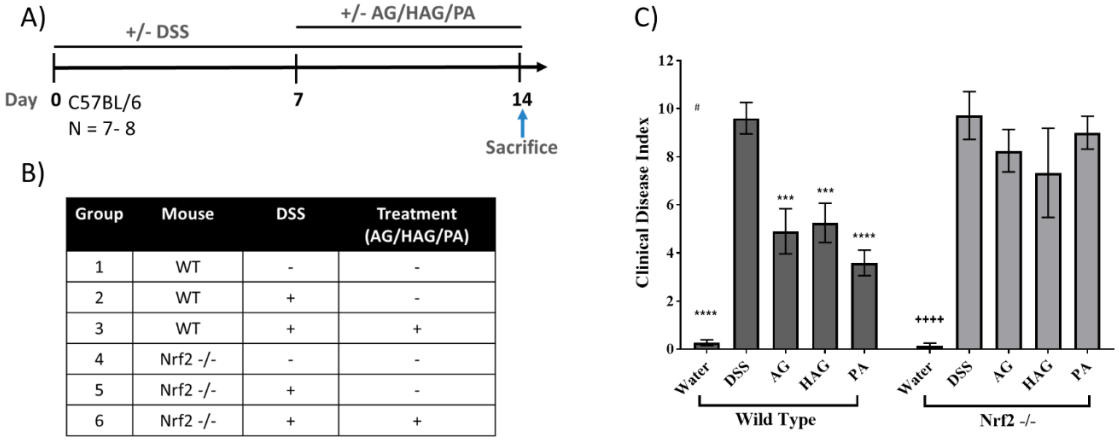


Figure 3.2: AG, HAG, and PA activate Nrf2 pathway and decrease ROS *in vivo*. Effect of AG, HAG, and PA on oxidative stress and Nrf2 pathway activation. DSS-induced colitis mice were treated with AG (75 mg/kg/day), HAG (75 mg/kg/day), or PA (1 mg/kg/day). Colons from these mice were probed for **A, B)** 4-HNE and **C, D)** HO-1 to indicate oxidative stress and Nrf2 pathway activation, respectively. Values represent the mean ± S.E. N= 8. The significance is compared with the DSS only group. P-values - * - >0.05, ** - >0.005. A and C are representative images (400X magnification)



Group	Mouse	DSS	Treatment (AG/HAG/PA)
1	WT	-	-
2	WT	+	-
3	WT	+	+
4	Nrf2 -/-	-	-
5	Nrf2 -/-	+	-
6	Nrf2 -/-	+	+

Figure 3.3 AG, HAG, and PA decrease the CDI in WT mice but not Nrf2 -/- mice. A, B) Schematic and groups of the experiment. **C)** Effect of AG (75 mg/kg/day), HAG (75 mg/kg/day) and PA (1 mg/kg/day) on the clinical disease index which accounts for weight loss, blood in stool and stool consistency. Values represent the mean ± S.E. Significance is compared with the DSS only sub-group within WT and Nrf2 -/- groups. P-values - * - >0.05, ** - >0.005, *** - >0.001, **** - >0.0001.

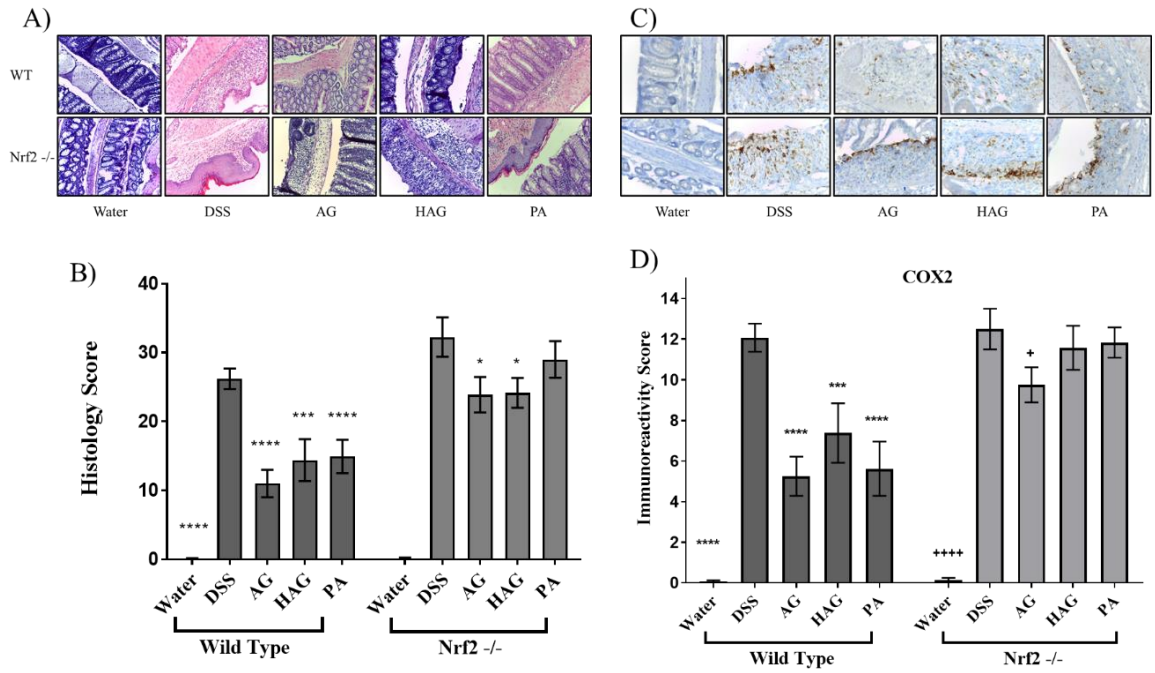


Figure 3.4 AG, HAG, and PA decrease the inflammation in WT mice but not Nrf2 -/- mice. Seven-ten mice from each group from Fig 3.3 were euthanized on day 14, and colons were harvested. **A, B)** Effects of AG, HAG, and PA on the colon histology score in the acute DSS colitis model. The histology score was determined as discussed in *Materials and methods*. Values represent the mean ± S.E. of the mean. Representative H&E-stained colons are shown for each group (A). Sections of the colon were probed for COX2. **C)** Representative images. **D)** Immunoreactivity score. *Significant difference from the DSS group (P values * - >0.05, ** - >0.005, *** - >0.001, **** - >0.0001.).

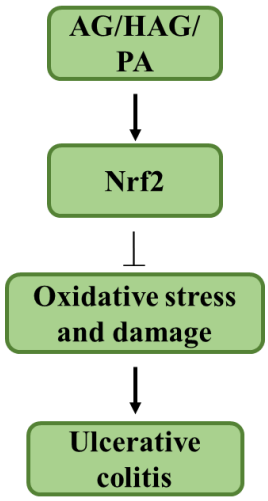


Figure 3.5 AG, HAG, and PA suppress colitis by activation of the Nrf2 pathway. Schematic representing the conclusions - oxidative stress is one of the factors that play a significant role in the progression of colitis. AG, HAG, and PA activate the transcription factor, Nrf2, which in turn activates antioxidant genes that decrease oxidative stress, thereby suppressing colitis.

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CHAPTER 4

GENE EXPRESSION PROFILING OF PRIMARY PERITONEAL MACROPHAGES TREATED BY AMERICAN GINSENG

ABSTRACT

Ginseng has been widely used for its beneficial effects on the general health as well as its medicinal properties in the treatment of various ailments. We have previously shown that American Ginseng (AG) and its components suppress ulcerative colitis (UC) in mouse, underscoring their anti-inflammatory properties, especially in the macrophages (mΦ). However, the immunostimulatory effects of AG on the innate immune system in the absence of inflammation are unclear. MΦ play an important role in the initiation of innate immune response and activation of the adaptive immune system. To elucidate the various key players in the mechanism of action of AG and understand the effects of AG on the immune system in a normal state without any inflammation, we conducted a microarray experiment using primary peritoneal mΦ isolated from mouse and treated with AG or one of its derivatives. Microarray analysis revealed that treatment with AG increases the expression of various proinflammatory pathways, as seen by the increased expression of proinflammatory cytokines like IL6, IL-1α, IL-1β, CXCL1, CXCL3, and CXCL5. This suggests that AG and its components stimulate the immune system to prime it against possible infection and any other environmental or endogenous insults to the body and this property of AG can establish it as an adjuvant therapy.

4.1 INTRODUCTION

Korean Ginseng is one of the most-used herbal medicines in the East. More recently, other types of ginseng, such as American Ginseng (AG) have been used as alternative medicines worldwide, due to their immunosuppressant, anti-tumorigenic, hypoglycemic, antioxidant, and anti-depressant effects (1–3). Furthermore, patients suffering from chronic inflammatory diseases use AG in the remission state between the flare-ups. Consistent with this, we have previously studied the immunosuppressive effects of AG in an inflammatory environment and found that AG and its components (a Hexane Fraction of AG, or HAG; and panaxynol, or PA) act as anti-inflammatory agents (4,5)(Chapters 2 and 3 of this dissertation). To better understand the underpinnings associated with the mechanisms by which AG, HAG, and PA suppress colitis and prevent colon cancer, we used gene expression profiling of primary peritoneal mΦ (PPMs) by microarray and validated the genes with significant increase in expression upon treatment, with qPCR.

We found that treatment with AG increases the expression of cytokines [interleukin (IL)-1α, IL-1β, IL-6, and COX-2] and chemokines [chemokine (C-X-C motif) ligand (CXCL)-1, CXCL3 and CXCL5. PA, the bioactive component of AG, also upregulated the expression of IL-1α, CXCL1, and CXCL5; while HAG activated IL-1α and CXCL5. These results indicate that AG and its components can act as stimulants of a resting immune system.

4.2 MATERIALS AND METHODS

4.2.1 ISOLATION AND TREATMENT OF PRIMARY PERITONEAL MACROPHAGES

Primary peritoneal mΦ (PPMs) were harvested from 8-10 week old C57Bl/6 mice. Mice were anesthetized and sacrificed by cervical dislocation. 8 ml of phosphate buffer

saline (PBS) with 5% fetal bovine serum (FBS) was injected into the peritoneal cavity using a 10-gauge syringe, and the peritoneal cavity was massaged to dislodge the immune cells in the cavity. The PBS is aspirated back and spun at 1000 RPM for 10 minutes, and the pellet is resuspended in RPMI media containing 5% FBS. Cells from one mouse were plated in one well in a 6-well dish to be used as a biological replicate. Cells were incubated for 2 hours at 37^0 C and 5% CO_2 and supernatant is aspirated, and the wells were washed with PBS. The attached cells are mostly mΦ and are incubated in whole RPMI media containing 50 μg/ml mouse macrophage colony stimulating (mM-CSF) factor overnight. The media was aspirated, and the cells were treated with 250 mg/ml AG in whole media with mM-CSF.

4.2.2 RNA ISOLATION AND QUANTIFICATION

Total RNA was isolated using the Qiagen RNeasy Plus Micro Kit according to the manufacturer's protocol. RNA concentration was measured using Nanodrop 2000. RNA quality was assessed using an Agilent 2100 Bioanalyzer, and RNA Integrity Numbers ranged from 9.8 to 10.0.

4.2.3 MICROARRAY ANALYSIS

Microarray experiments were performed using Affymetrix's platform. Total RNA samples were amplified and biotinylated using GeneChip WT PLUS Reagent Kit (Affymetrix, Santa Clara, CA). Briefly, 100 ng of total RNA per sample was reverse transcribed into ds-cDNA using NNN random primers containing a T7 RNA polymerase promoter sequence. T7 RNA polymerase was then added to cDNA samples to amplify RNA, and then RNA was copied to ss-cDNA and degraded using RNase H. ss-cDNA molecules were then fragmented and terminally labeled with biotin. Amplified and labeled

samples were hybridized to MoGene-2_0-st (Affymetrix, Santa Clara, CA) for 16 h at 45°C using a GeneChip Hybridization Oven 640 and a GeneChip Hybridization, Wash, and Stain Kit (Affymetrix, Santa Clara, CA). Hybridized arrays were washed and stained using GeneChip Fluidics Stations 450 (Affymetrix, Santa Clara, CA). Arrays (6 total) were then scanned using a GeneChip Scanner 3000 7G system and computer workstation equipped with GeneChip Command Console 4.0 software (Affymetrix, Santa Clara, CA).

4.2.4 REVERSE TRANSCRIPTASE-QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR)

The cells were lysed using Trizol and RNA was isolated using the RNeasy mini kit (Cat # 74104; Qiagen, Hilden, Germany) according to the instructions. RNA concentrations were measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). 1μg of RNA was used for cDNA synthesis using iScript cDNA synthesis kit (cat # 1708890; Bio-rad, Hercules, CA). The final product was diluted 1:10 for qPCR. qPCR was performed using iTaq™ Universal SYBR® Green Supermix (cat #1725121; Bio-rad, Hercules, CA). The list of primers used is as follows in the order forward then reverse in 5`-3` orientation. – HO-1: AAGCCGAGAATGCTGAGTTCA, GCCGTGTAGATATGGTACAAGGA; NQO1: AGGATGGGAGGTACTCGAATC, AGGCGTCCTTCCTTATATGCTA; GAPDH: AGGTCGGTGTGAACGGATTTG, TGTAGACCATGTAGTTGAGGTCA., IL6: CCAAGAGGTGAGTGCTTCCC, CTGTTGTTCAGACTCTCTCCCT; CXCL3: TAACCCTCATCAGAGTACTGGC, GAAAGAGGCTCCAGGTAGGG; CXCL1: TGAGCTGCGCTGTCAGTGCCT, AGAAGCCAGCGTTCACCAGA; CXCL5: GCATTTCTGTTGCTGTTCACGCTG, CCTCCTTCTGGTTTTTCAGTTTAGC; IL-1b, CCTTCCAGGATGAGGACATGA, TGAGTCACAGAGGATGGGCTC; IL-1a: CGAAGACTACAGTTCTGCCATT,

GACGTTTCAGAGGTTCTCAGAG; Ptgs2: TGCCTGGTCTGATGATGTATG, GCCCTTCACGTTATTGCAGATG. qPCR was performed on CFX384 Touch™ Real-Time PCR Detection System (cat # 1855485; Bio-rad, Hercules, CA).

4.2.5 Statistical analysis

Following completion of array scans, probe cell intensity (CEL) files were imported into Expression Console Software (Affymetrix, Santa Clara, CA) and processed at the gene-level using the Robust Multichip Analysis (RMA) algorithm to generate CHP files. After confirming data quality within Expression Console, CHP files containing log2 expression signals for each probe were then imported into Transcriptome Analysis Console Software version 4.0 (Affymetrix, Santa Clara, CA) to analyze cell type-specific transcriptional responses using one-way between-subject analysis of variance (ANOVA). A p-value of 0.05 and a fold change of 2.0 was used as cutoff parameters. Subsequently, pathway analysis of the differentially expressed genes was performed using Ingenuity Pathway Analysis software (IPA) (Qiagen, Hilden, Germany).

qPCR data is expressed as a mean ± standard error of the mean. Mean differences between the groups were compared by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. A p-value of ≤ 0.05 was chosen for significance.

4.3 Results

4.3.1 Gene expression profile of primary peritoneal macrophages treated with American Ginseng

PPM isolated from C57B/6 mice were cultured for a day and treated with 250 mg/ml of AG for 8 hours in quadruplicates. RNA collected from these samples was analyzed for concentration, purity, and integrity, upon which three biological replicates for

each condition were selected for microarray analysis. We determined the treatment doses prior to microarray to ensure that these concentrations would cause a change in gene expression. We accomplished this by probing PPMs treated with various doses of AG, HAG, and PA, for HO-1, a known target of all three compounds. We compared the treated and untreated samples using Kruskal-Wallis statistics with Benjamini-Hochberg multiple testing corrections at a fold-change cut-off of 2 and a *p*-value of 0.05. The number of differentially expressed genes with at least 2-fold change were identified. 32 genes were upregulated, and 20 genes were downregulated upon treatment with AG when compared to the vehicle group (Figure 4.1A). However, HAG only had one gene that showed more than 2-fold change in expression and PA did not have any viable target genes that had any change in expression profile (Figure 4.1A). There was only one common target for AG and HAG which was NQO1 (Figure 4.1B). Surprisingly, a number of pro-inflammatory genes were activated by AG. Of these, the expression of IL-6 was elevated the most with the AG treatment with a fold change of 5.87 and a *p*-value of 0.0005. Scatter plot of differentially expressed genes and hierarchal clustering of intensities of mRNA expression are shown in Figures - 4.2 and 4.3 respectively. The list of genes that are differentially expressed with AG treatment is presented in Table 4.1.

4.3.2 INGENUITY PATHWAY ANALYSIS (IPA) OF MICROARRAY RESULTS

IPA shows distinct activation of triggering receptor expressed on myeloid cells (TREM1) signaling pathway, with the target genes CXCL3, IL1B, IL6, NLRP3, and CSF2, with the first three genes showing more than 2.5 fold increase in gene expression (Figure 4.4). Activation of the TREM1 receptor that is expressed on mΦ and neutrophils plays an important role in systemic infection by causing a cascade of events that result in cytokine

production. The top- hits like CXCL2, CXCL3, PTGS2, IL1A, IL1B, and IL6 are also involved in toll-like receptor (TLR) signaling, especially TLR2, TLR3, TLR4, TLR5, and TLR9. The target genes identified here are upstream regulators of various proinflammatory genes and play an important role in the recruitment, activation, and migration of phagocytes, in this case, mΦ.

4.3.3 Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analysis of selected target genes

To confirm the differential expression of the targets identified in the microarray analysis, we conducted the RT-qPCR analysis. Since HAG or PA did not yield multiple results, we validated the targets of AG using qPCR and compared the gene expression in the samples from HAG and PA. This analysis confirmed the activation of proinflammatory cytokines by AG. We saw that, while some of AG's targets only showed increased expression upon AG treatment, e.g., IL6, IL-1β, and CXCL3 (Figure 4.4A, B, C), others showed changed gene expression profiles in HAG and/or PA. IL-1α and CXCL1 showed increased expression in both HAG and PA-treated cells (Figures 4.4 D, E), while CXCL5 expression was increased in PA but not HAG (Figure 4.4F).

PTGS2 or COX2, one of the potential targets was not validated by qPCR (Figure 4.4 G). We also examined the expression of NQO1 and HO-1 as they have previously been shown to be upregulated in AG treatments. NQO1 was also one of the targets of both AG and HAG that manifested in the microarray analysis. However, there was no change in the expression with any of the treatments when validated with qPCR. HO-1, on the other hand, showed increased expression with AG, HAG, or PA treatments, even though it was not one of the genes with changed expression profile in the microarray (Figure 4.4 I).

4.4 DISCUSSION

AG has been used as an anti-inflammatory agent for the treatment of UC in our previous studies (6,7), and we found that PA, a polyacetylene in AG specifically targets mΦ for DNA damage and apoptosis. PA was the most abundant compound in the hexane fraction of AG (HAG), the most potent of the various fractions of AG (5). To further understand the mechanisms of action of AG, HAG, and PA in the treatment of colitis and prevention of colon cancer, we profiled the gene expression patterns in PPMs using microarray analysis.

Curiously, treatment with AG resulted in the upregulation of a number of cytokines and chemokines, which is in contrast with their anti-inflammatory properties. Unfortunately, microarray analysis of treatment with HAG only yielded one gene with an at least 2-fold change in expression and treatment with PA did not produce any valid targets, even though we standardized the dose and treatment times prior to the microarray. However, when we followed up on the top-hits with AG treatment using qPCR in the samples treated with all three compounds to identify any common targets and targets unique to AG treatment, we found that HAG and PA also increased the expression of some of the cytokines and chemokines. This discrepancy in results could be due to the fact that qPCR is more sensitive and quantitative than microarray.

This immunostimulatory effect of AG could indicate that AG can boost the innate immune system to prime it against infections and other environmental insults. This property of AG can also be exploited in cancer immunotherapy for use as an adjuvant. The immune system is comprised of two separate and interacting components – innate and adaptive immunity. The innate immune system forms the first barrier of defense against

the infections and includes physical barriers like skin and mucous membranes; phagocytic immune cells like mΦ and natural killer cells; inflammatory mediators like eosinophil granules; and cytokines (8,9). These components recognize infection and initiate primary reactions to prevent and eliminate an infection. Toll-like receptors (TLRs) are pattern recognition receptors (PPRs) that are present on the myeloid cells like mΦ and dendritic cells, recognize pathogen-associated molecular patterns (PAMPs) on the invading pathogens and danger associated molecular patterns (DAMPs) from tissue injury, and initiate an appropriate immune response (10–12). This immunological signaling is finely balanced. Lack of appropriate regulation of TLRs can result in chronic inflammation (13,14). Conversely, the decreased response in early stages of infection or neoplastic transformation can have adverse effects like worsened infection or tumor formation (15). MΦ form an important part of the innate immune system and are activated by binding of ligands to surface TLRs like TLR4 (16–18). In the presence of an impending infection, mΦ are activated to phagocytose the pathogens and produce proinflammatory cytokines like TNFα, and inflammatory mediators like nitric oxide (NO) and H_2O_2 (19). Plant extracts have been previously shown to modulate the TLR activation and function (20–23).

Among the predicted upstream regulators of the top genes, TLR4 appears to be of importance. It has been previously shown that plant extracts can initiate an innate immune response by activating TLRs, particularly TLR4 (20–22). This can result in the activation of transcription factors like NF-κB and AP-1, which in turn increase the production of proinflammatory mediators like TNF-α, IL-6, IL-1, and COX2 (24). The activation of proinflammatory cytokines by AG could be a result of TLR4 activation. TLR agonists are currently being investigated in the development of immunotherapy strategies for the

treatment of cancer. TLR ligands have been previously used as adjuvants in the development of vaccines in cancer immunotherapy (25–27). For example, Monophosphoryl lipid A (MPL), a TLR4 ligand is being tested as an adjuvant to vaccines against colorectal carcinoma, melanoma, glioma, etc., to initiate tumor-specific immunity in response to vaccination (28). Adjuvants can decrease the immunotolerance in the tumor microenvironment and aid in the initiation of anti-tumor response(25,27). If AG is established as a TLR ligand, it can be used as an adjuvant with vaccines for cancer and infectious diseases.

We used PPMs, which have not been polarized to either M1 or M2 type in our study. For future studies, we will confirm the findings above, then compare the effects of AG and its components between the different types of mΦ, *in vivo* and *in vitro* by examining the expression patterns of proinflammatory cytokines. We will also examine the effects of AG on dendritic cells which along with mΦ are the sentinels of innate immune system and function via TLR signaling. These studies will provide an insight into the divergent effects of AG, especially in the treatment of UC. UC is a chronic disease with periods of remission alternated with a period of flare-ups. It is important to have active immune system in the remission periods to enable the body to fight the infections. The property of AG to modulate the immune system will be especially appreciated in these periods of remission to ensure the patient is not susceptible to infections.

CONTRIBUTIONS AND ACKNOWLEDGEMENTS

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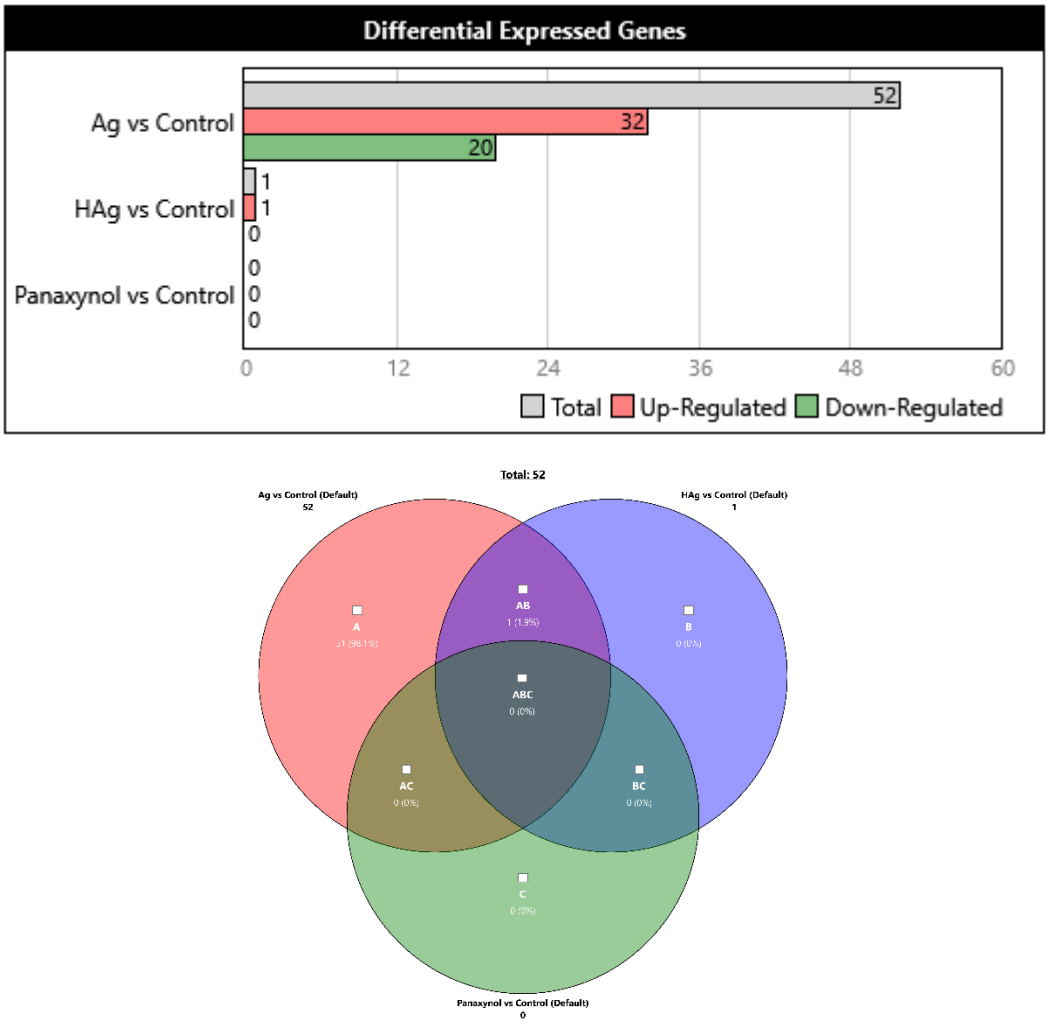

Figure 4.1 Gene expression profiling of primary peritoneal macrophages treated with AG, HAG, and PA. A) Summary of the microarray analysis of PPMs treated with AG (100µg/ml), HAG (100µg/ml) and PA (1µM) to address the number of genes that have been differentially regulated. **B)** Venn diagram representing common targets that manifested in microarray analysis.

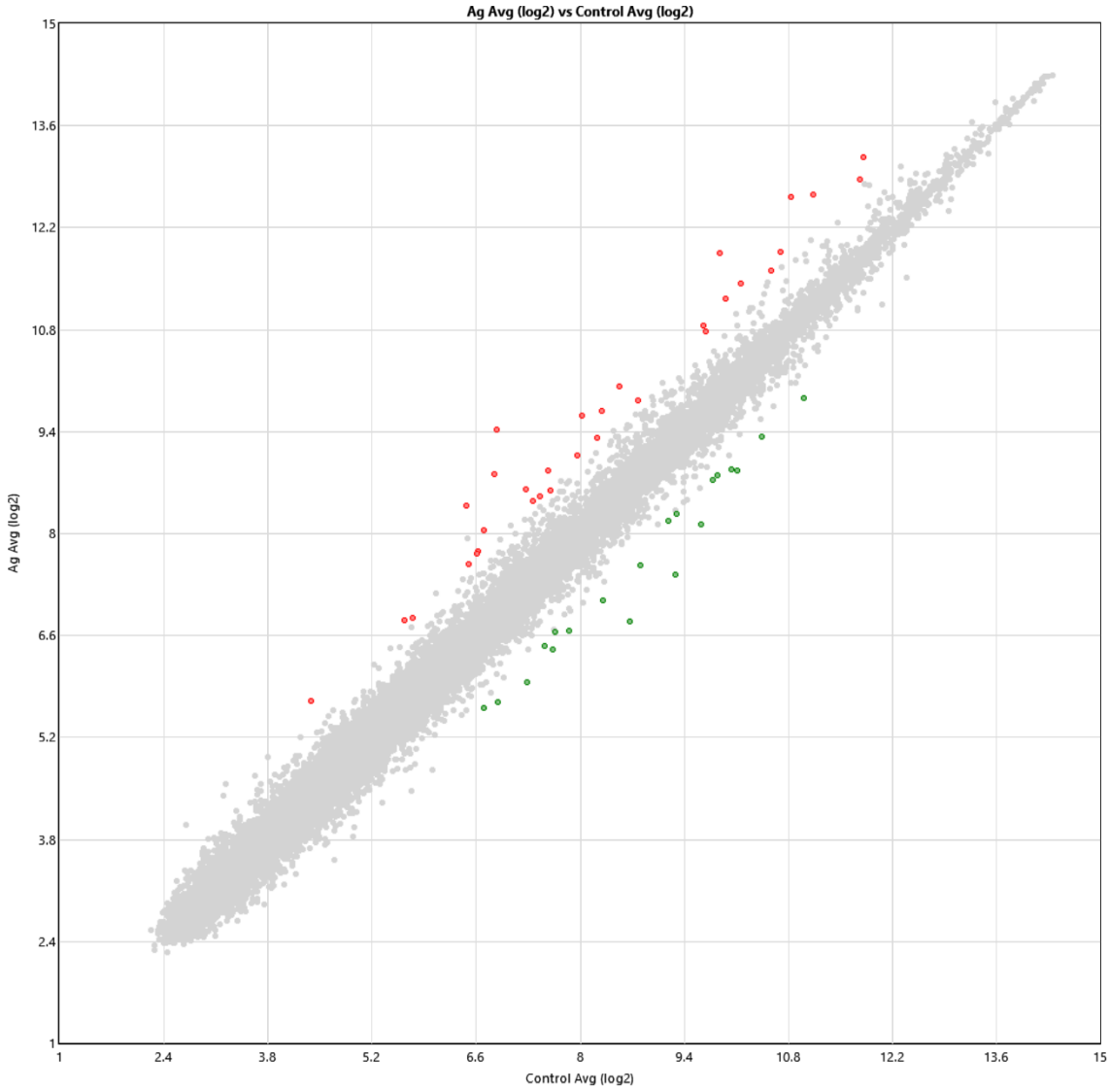

Figure 4.2 Representation of differential gene expression with AG treatment when compared to the control group. Scatter plot of mRNA expression determined by mRNA microarray analysis. The expression profile of ~35,000 mRNAs on a log2 scale with AG treatment and control group is plotted. Red and green dots represent the mRNAs that were significantly ($P < 0.05$ and 1.5 fold-change cut-off) up-regulated and down-regulated, respectively, in AG group. Grey dots represent a lack of differential expression.

Table 4.1 List of differentially expressing genes with AG treatment

Transcript Cluster ID	Fold Change	ANOVA p-value (Ag vs. Control)	FDR p-value (Ag vs. Control)	Gene Symbol	Description
17435725	5.87	0.0005	0.072601	Il6	interleukin 6
17438955	3.97	0.000114	0.055876	Cxcl5	chemokine (C-X-C motif) ligand 5
17218060	3.96	0.001347	0.096285	Ptgs2	prostaglandin-endoperoxide synthase 2
17517097	3.78	0.000204	0.059353	Plet1	placenta expressed transcript 1
17391565	3.47	0.002125	0.115594	Il1b	interleukin 1 beta
17490160	3.03	0.000048	0.053384	Siglece	sialic acid binding Ig-like lectin E
17391554	2.88	0.001147	0.093706	Il1a	interleukin 1 alpha
17438987	2.83	0.000847	0.084791	Cxcl1	chemokine (C-X-C motif) ligand 1
17438993	2.75	0.017462	0.282706	Gm22816	predicted gene, 22816
17417699	2.63	0.00019	0.059353	Gm12840	predicted gene 12840
17438975	2.57	0.002232	0.118429	Cxcl3	chemokine (C-X-C motif) ligand 3
17479849	2.53	0.000029	0.053384	Tmc3	transmembrane channel-like gene family 3
17356427	2.53	0.001331	0.095517	Fosl1	fos-like antigen 1
17261942	2.52	0.000071	0.053906	Mir146	microRNA 146
17237280	2.45	0.00099	0.090302	Phlda1	pleckstrin homology-like domain, family A, member 1
17333731	2.43	0.001221	0.094862	Fpr2	formyl peptide receptor 2
17402181	2.43	0.001308	0.095517	F3	coagulation factor III
17344873	2.32	0.000051	0.053384	H2-M2	histocompatibility 2, M region locus 2
17302475	2.27	0.000197	0.059353	Irg1	immunoresponsive gene 1
17337750	2.26	0.00811	0.201988	Gpr110	G protein-coupled receptor 110
17439021	2.21	0.0124	0.240546	Ereg	epiregulin
17280729	2.18	0.000313	0.065014	Ahr	aryl-hydrocarbon receptor
17521300	2.17	0.03818	0.391805	Cish	cytokine inducible SH2-containing protein
17469455	2.16	0.001723	0.10704	Prok2	prokineticin 2
17327580	2.16	0.002959	0.130882	Mefv	Mediterranean fever
17405082	2.14	0.000651	0.076346	Slc7a11	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11
17485510	2.13	0.000051	0.053384	Tarm1	T cell-interacting, activating receptor on myeloid cells 1
17512732	2.13	0.000431	0.069242	Nqo1	NAD(P)H dehydrogenase, quinone 1
17265386	2.12	0.001296	0.095517	Scimp	SLP adaptor and CSK interacting membrane protein
17284839	2.1	0.00306	0.131533	Itgb8	integrin beta 8
17272619	2.08	0.009459	0.212684	Socs3	suppressor of cytokine signaling 3
17378440	2.07	0.000516	0.072601	Procr	protein C receptor, endothelial
17366726	2.07	0.011793	0.233441	Gm23578	predicted gene, 23578
17351330	2.06	0.000042	0.053384	Malt1	mucosa associated lymphoid tissue lymphoma translocation gene 1
17294352	2.01	0.000487	0.072601	Ahrr	aryl-hydrocarbon receptor repressor
17218845	-2.01	0.000733	0.080987	Selp	selectin, platelet
17287066	-2.02	0.000122	0.055876	Rnf144b	ring finger protein 144B
17433283	-2.02	0.000424	0.069242	Gm13091	predicted gene 13091
17219382	-2.05	0.000038	0.053384	Cd244	CD244 natural killer cell receptor 2B4
17227764	-2.08	0.001138	0.093381	Rgs2	regulator of G-protein signaling 2
17507161	-2.09	0.000438	0.069872	Cd209a	CD209a antigen
17289388	-2.12	0.000834	0.084588	Gm5086	predicted gene 5086
17216469	-2.16	0.00052	0.072601	Serpinb10	serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 10
17432014	-2.21	0.000313	0.065014	Padi4	peptidyl arginine deiminase, type IV
17427051	-2.21	0.002536	0.122953	Ptplad2	protein tyrosine phosphatase-like A domain containing 2
17236552	-2.29	0.001709	0.106523	Hal	histidine ammonia lyase
17266851	-2.29	0.004552	0.154841	Slfn9	schlafen 9
17224211	-2.32	0.000053	0.053384	Tns1	tensin 1
17324420	-2.34	0.000494	0.072601	St6gal1	beta galactoside alpha 2,6 sialyltransferase 1
17450142	-2.37	0.000509	0.072601	Hpse	heparanase
17520396	-2.37	0.00301	0.13152	Slc9a9	solute carrier family 9 (sodium/hydrogen exchanger), member 9
17500089	-2.38	0.007568	0.196308	Gm26714	predicted gene, 26714
17450414	-2.51	0.000253	0.062256	Abcg3	ATP-binding cassette, sub-family G (WHITE), member 3
17339772	-2.84	0.000099	0.053906	Rasgrp3	RAS, guanyl releasing protein 3
17507605	-3.59	0.000042	0.053384	Gas6	growth arrest specific 6
17455386	-3.7	0.000102	0.054403	Slc46a3	solute carrier family 46, member 3

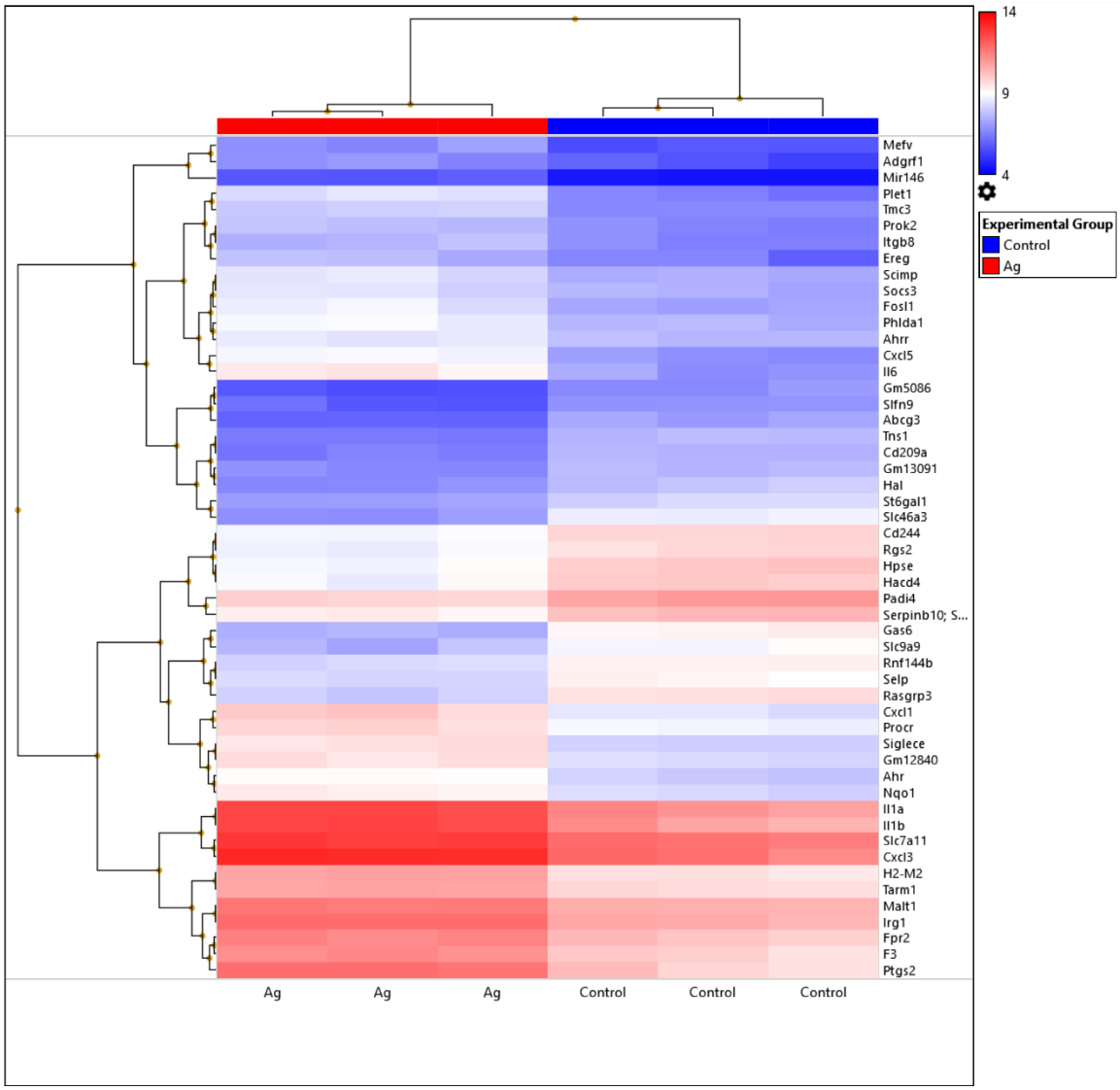

Figure 4.3 Hierarchal clustering of replicates. Hierarchical clustering of samples based on differentially expressed genes with at least 2-fold change and controlled by false discovery rate of 0.1, from AG treated vs. control groups. In the clustering heat map, red indicates upregulation while green indicates downregulation. In the sample clustering dendrogram, red indicates AG samples while blue indicates control samples. The intensity of expression is indicated by blue (low) and red (high), with white being medium.

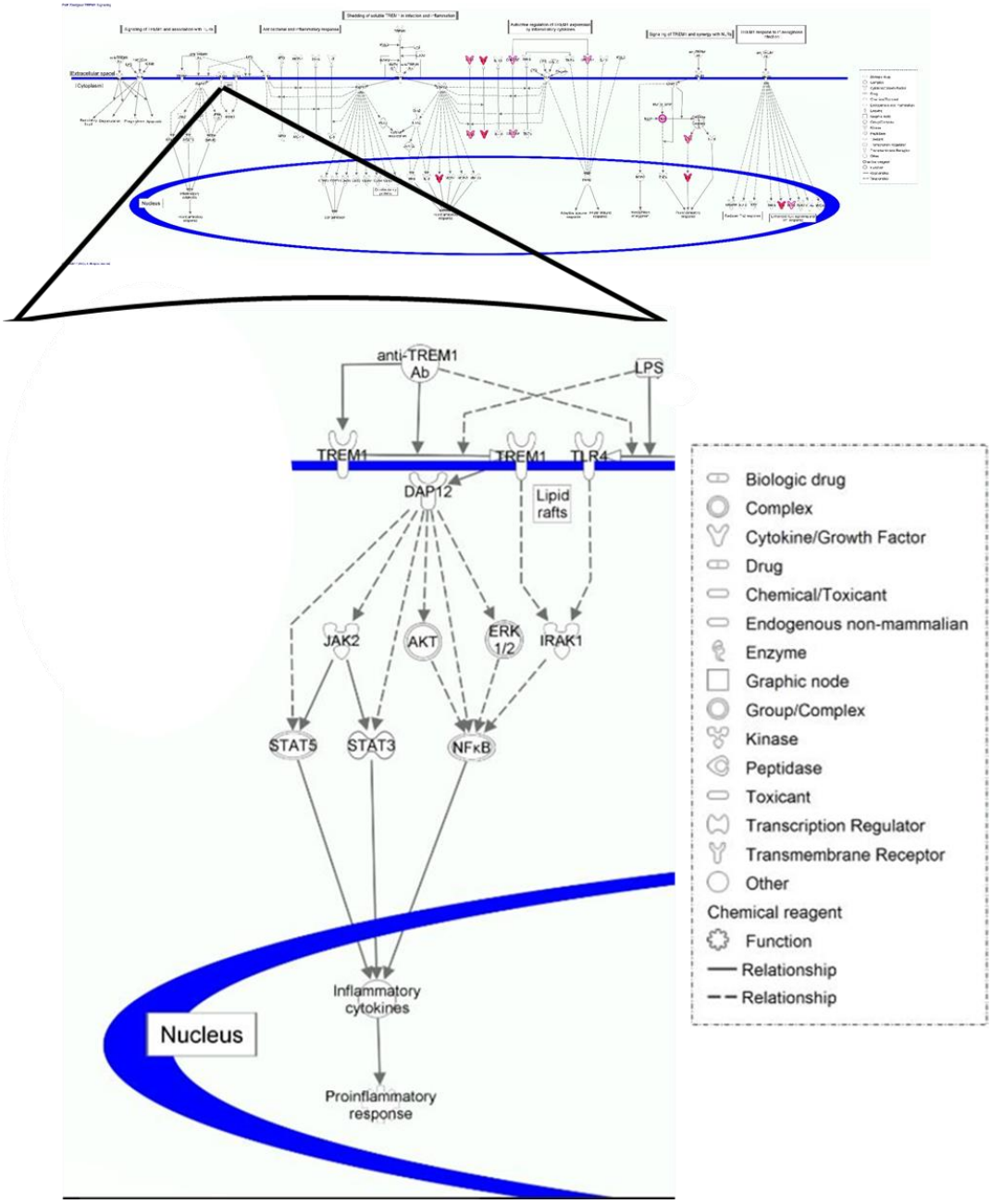

Figure 4.4 Activation of TREM pathway from IPA. TREM pathway showed activation in IPA. TREM pathway involving TLR4 is upstream of the cytokines and chemokines that were upregulated by AG.One of the TREM pathways, involving proinflammatory response is highlighted here.

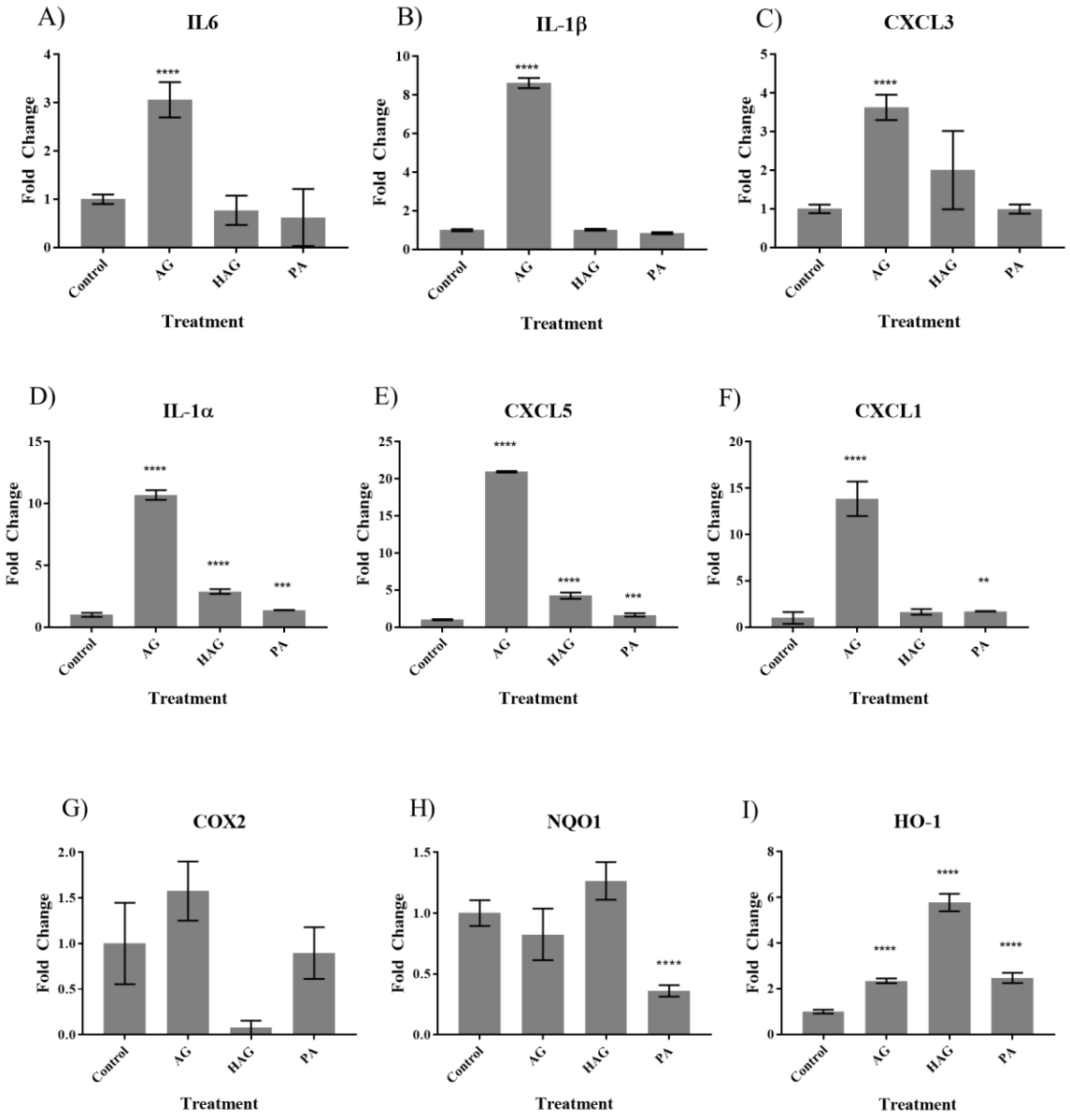

Figure 4.5 Validation of differentially expressed genes by qPCR. Targets identified by microarray analysis of AG treated mΦ was validated in all three treatments. A, B, C) Genes that were validated only in AG. D, E, F) Genes also verified in HAG and or PA. G, H) Identified targets of AG that were not validated by qPCR. Fold changes presented are cumulative mean of three separate experiments.

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CHAPTER 5
CONCLUSION AND FUTURE DIRECTIONS

5.1 SUMMARY AND CONCLUSIONS

American Ginseng (AG), and one of its fractions, HAG, have been shown to be effective in the treatment of colitis and colon cancer in mice. In chapter 2, we aimed to identify the bioactive component of AG. We used bioassay-guided fractionation to isolate various sub-fractions of HAG and test their ability to suppress the expression of iNOS, an inflammatory marker. PA was the most abundant compound in the fraction with the anti-inflammatory potential and was further studied as a potential treatment for colitis. PA successfully suppressed DSS-induced colitis in the mouse. We also found that PA selectively targets macrophages (mΦ) for apoptosis *in vivo* and *in vitro*. Additionally, PA was more effective than clodronate, a compound that specifically targets mΦ and did not enhance the effect of clodronate in the treatment of colitis. However, PA was not effective in the prevention of colitis.

In chapter 3, we explore the mechanism of action of AG, HAG, and PA. We have previously seen that AG decreases oxidative stress in proinflammatory mΦ. Here we see that HAG and PA decrease the production of ROS and thereby oxidative stress in the mΦ. AG, HAG, and PA also activate the Nrf2 dependent antioxidant pathway in both ANA-1 and HCT-116 cells that were co-cultured with ANA-1 cells. These components also activate Nrf2 pathway and decrease oxidative stress *in vivo* in DSS-induced colitis colons. In the absence of Nrf2, AG and its components are not very effective in the treatment of

colitis as seen by the comparison between wild-type and Nrf2 knockout mice. However, even in the absence of Nrf2, AG slightly decreased the extent of colitis. This could be due to the role that p53 plays in the treatment of colitis by AG.

In chapter 5, we wanted to further explore the effect of AG on the innate immune system. We conducted a gene expression profiling of primary peritoneal mΦ treated with AG using microarray. Surprisingly, the gene expression profile indicated that AG increased the expression of proinflammatory cytokines like IL-1α, IL-1β, and IL-6 and chemokines like CXCL2, CXCL3, and CXCL5. We confirmed the top hits of microarray using RT-qPCR in AG samples, as well as HAG and PA samples. The pathway analysis of these targets and other genes with increased expression indicated that proinflammatory pathways like NFκb and TNF-α pathways could be activated. This contradicts with the anti-inflammatory activity of AG in the treatment of inflammatory diseases. However, this indicates that AG can act as an immune boost to prime the immune system to fight potential infections

From the findings in this dissertation, we have identified the bioactive component of AG and gained insights into the mechanism of action of AG and its components. PA treats colitis in mice by the selective targeting of mΦ for DNA damage and apoptosis. Activation of the Nrf2 antioxidant mechanism is the key to the mechanism of action of AG, HAG, and PA. Activation of proinflammatory pathways by AG in a normal state provides insight into the immunomodulatory effects of AG and provides a segue for future investigations.

5.2 FUTURE DIRECTIONS

To further the outcomes from chapter 4, we will compare the expression of proinflammatory cytokines and activation of proinflammatory pathways between activated and non-activated mΦ *in vitro* using primary cells and established cell lines. *In vivo*, we will study the effect of treatment with AG on the innate immunity in the absence of an infection. This will help us understand its effectiveness in the prevention of infections.

We would like to explore further, the mechanism by which PA targets mΦ. Since PA causes DNA damage, we will concentrate on DNA repair pathways that could be impaired in the mΦ, leading to apoptosis. Identifying the DNA repair mechanism and its status in non-macrophage cell lines would provide understanding into the specificity of PA in targeting the mΦ.

We will test the toxicity and side effects of PA in the organs collected during our *in vivo* experiments (e.g., Liver: Serum Bilirubin, Serum Albumin, ALT [Alanine amino transferase], and AST [Aspartate amino transferase] test; Kidney: Urinalysis, Blood Urea nitrogen). Upon validation of PA as a safe and effective treatment for colitis in classic models of IBD, we hope to explore its potential as a treatment for other inflammatory diseases like rheumatoid arthritis, lupus, and psoriasis, improving the quality of life for millions of people.

Since colitis increases the risk of colitis-associated colon cancer, we conducted preliminary studies to determine the effectiveness of PA in the prevention of colon cancer. These studies conducted with AOM-DSS mouse model showed that PA-treated mice had smaller tumors (> 2mm). Preliminary data also indicates that PA decreases the expression of DNMT1 and HDAC in activated ANA-1 cells, indicating its role in epigenetic regulation

(Figure A.1). We will explore the possibility of PA as a chemopreventive agent for colon cancer using various colon cancer mouse models with an emphasis on epigenetics.

We will also study other components of AG in the hexane fraction, mainly polyacetylenes like Panaxydol and Panaxadiol.

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APPENDIX A.

EPIGENETIC EFFECTS OF AMERICAN GINSENG AND ITS COMPONENTS

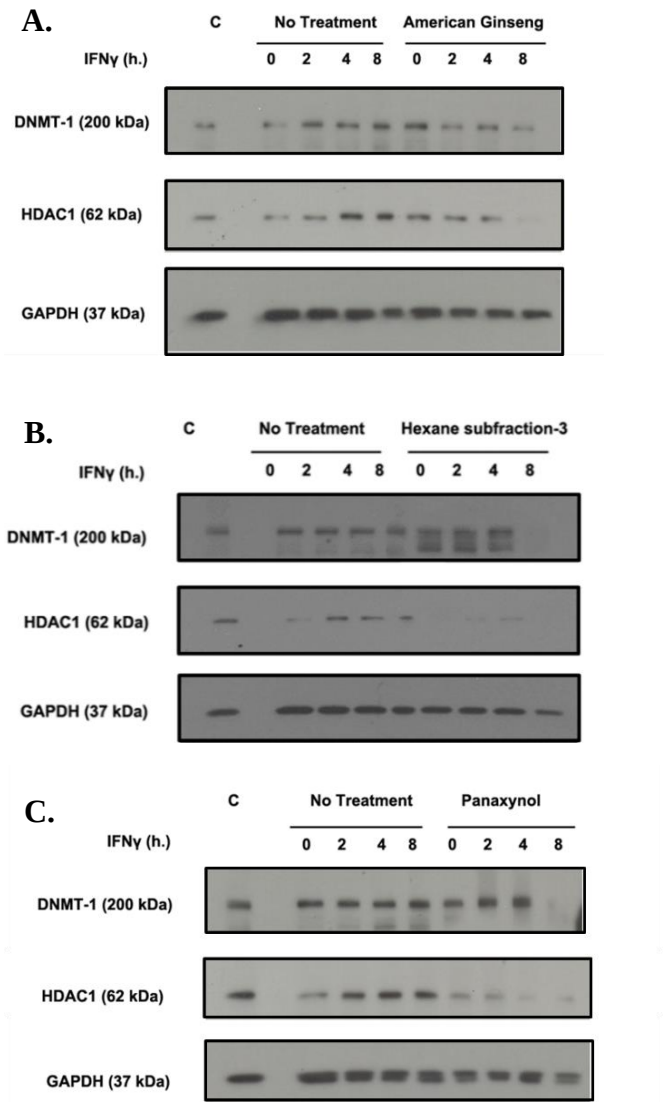

Figure A.1 AG, HAG, and Panaxynol decrease the expression of DNMT-1 and HDAC1 in stimulated ANA-1 cells. ANA-1 cells were pretreated with AG (250 μg/ml), HAG (10 μg/ml), or Panaxynol (5 μM), followed by stimulation with IFN-γ (10 ng/ml) for indicated times.