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Nutraceutical efficacy in experimental animal models of inflammatory bowel disease: *Echinacea angustifolia*, *Prunella vulgaris* and *Hypericum gentianoides*

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Nutraceutical efficacy in experimental animal models of inflammatory bowel disease: *Echinacea angustifolia*, *Prunella vulgaris* and *Hypericum gentianoides*

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Major: Immunobiology

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2011

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DEDICATION AND ACKNOWLEDGEMENTS

This dissertation is dedicated to my family.

First and foremost to my daughter: Kiera Hailey DeHaven Haarberg. You are my life and a bright and shining light in this world. I do this and everything for you!

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ABSTRACT

Ulcerative colitis (UC) and Crohn’s disease (CD), collectively known as inflammatory bowel disease (IBD), are inflammatory diseases of the enteric mucosa whose etiology is currently undefined. While hypotheses for causation include genetic susceptibility, environmental risk factors, inappropriate and chronic immune responses to members of the intestinal microflora as well as failed immune regulatory mechanisms, many questions regarding IBD susceptibility, dysbiosis and immune dysfunction remain. Not surprisingly, current therapeutic targets are expensive, have questionable long term efficacy and are associated with risk of secondary bacterial and viral infections. As chronically ill patients substitute or supplement their treatments with alternative, nutraceutical remedies in ever greater numbers, more research is needed to ascertain the safety and efficacy of these products as therapeutics.

*Prunella vulgaris* is a common ‘cure all’ which is popular in traditional Chinese medicine. *P. vulgaris* can be steeped in water and drank as a tea or ground into a salve, and is known for its anti-viral, anti-inflammatory and antioxidant benefits. *P. vulgaris* has never, until now, been studied in the context of colitic therapy and has yet to gain a foothold as a commonly utilized nutraceutical in western medicine.

*Hypericum gentianoides*, akin to *Hypericum perforatum*/St. John’s Wort, is a unique and understudied species of *Hypericum*. *H. gentianoides* lacks the photoactive and cytotoxic compounds routinely found in other *Hypericum* species. While the bioactivity of this plant is not well characterized, it is known to have anti-inflammatory, immunomodulatory and antibacterial potential. This potential and the lack of cytotoxic, photoactive compounds make *H. gentianoides* a good candidate for study in colitis models.

*Echinacea angustifolia* is well studied, commonly utilized world-wide as a preventative or remedy for the common cold, and has great potential as a therapeutic agent in many disease modalities. The roots of this plant contain several types of compounds known to be bioactive. Like other nutraceutical, *E. angustifolia*
is documented anecdotally to cure or reduce symptoms related to countless maladies and diseases without sound scientific proof. *E. angustifolia* has never been studied as anti-colitic; however, pain suppressive and immunomodulatory capabilities of this plant lend credence to the potential for this type of study.

In this study, the anti-colitic potential of *P. vulgaris*, *H. gentianoides* and *E. angustifolia* were determined in the mdr1a deficient (-/-) mouse model of spontaneous colitis or the chemically induced dextran sodium sulphate (DSS) mouse model of acute colitis. Extracts of *E. angustifolia* root or above-ground sections of *P. vulgaris* or *H. gentianoides* were extracted using the Soxhlet method in 95% ethanol and were solubilized in a final concentration of 5% ethanol for use in studies. *P. vulgaris* and *H. gentianoides* were tested at daily doses of 2.4 mg (~ 100 mg/kg body weight) and 4.8 mg (~ 200 mg/kg body weight) respectively, while *E. angustifolia* was tested at daily doses of 100 mg/kg and 200 mg/kg body weight.

In the DSS model of acute colitis, *E. angustifolia* was not found to be effective during the period of DSS treatment (in low dose 1.75% to high dose 2.5% DSS in male and female C57BL/6 mice), or during restitution. *E. angustifolia* was not found to be cytotoxic in vivo. Conversely, *P. vulgaris* was able to significantly (p < 0.05) decrease weight loss and improve macroscopic indicators of severe colitic wasting. The disparity between *E. angustifolia* and *P. vulgaris* efficacies in this acute model of colitis could be due to the difference in the constituent types found in these two extracts. *E. angustifolia* is composed mostly of phenolics and alkylamides, while *P. vulgaris* contains phenolics, flavonoids and triterpinoids. Certain types of phenols have been shown to have efficacy in low dose DSS colitis; however, flavonoids have efficacy in models of colitis as well, and in combination with phenols may be synergistic. Since flavonoids have recently proven very effective in ameliorating spontaneous colitis, *P. vulgaris* was also evaluated in the mdr1a-/- model of spontaneous colitis.

In the mdr1a-/- mouse model of spontaneous colitis, *P. vulgaris* extract treatment was compared to mdr1a-/- mice treated with 5% ethanol vehicle, metronidazole (an antibiotic and anti-colitic control) and FVB<WT> mice. It was
discovered that *P. vulgaris* was able to delay onset of spontaneous colitis, significantly (p < 0.05) decrease macroscopic typhlocolitic and microscopic cecal disease scores, prevent cecal neutrophil influx, and downregulate nuclear factor-kappaB (NF-κB) related cytokines/chemokines and gene targets. The downregulation of innate immune signals and function is hypothesized to contribute to the observed decrease in cecal tonsil CD4+ helper and CD8+ cytotoxic T cells as well as germinal center B cells. Adaptive immune function was also altered. Loss of immune tolerance to the microflora is a characteristic of chronic colitis. Unlike mdr1a−/− vehicle treated mice, which displayed antibody production to microflora antigens, *P. vulgaris* treated mice displayed little to no Ab response to the same microflora antigens.

*H. gentianoides* contains flavonoids, phenolics and a unique antimicrobial compound called uliginosin A. As the mdr1a−/− mouse model of spontaneous colitis is driven by the host immune response to the microflora and *Hypericum* species are known to be immunomodulatory, this extract was of interest as a therapeutic anti-colitic in the mdr1a−/− model. Onset of spontaneous colitis was significantly delayed by *H. gentianoides*, but was not completely abolished. Macroscopic scores, microscopic scores, serum cytokine levels and myeloperoxidase production were all significantly (p < 0.05) reduced by *H. gentianoides* gavage. While *H. gentianoides* has immunosuppressant potential, a surprising increase in intestinal plasma cell infiltrate and an increase in serum IL-6 correlated with *H. gentianoides* gavage in mdr1a−/− mice. To date, anti-microbial functions have not been ruled out as a contributing factor in the efficacy of anti-colitic activity of this extract.

Together, these novel experiments have identified new candidates for anti-colitic therapy or therapeutic supplementation of current IBD treatment strategies. *P. vulgaris, H. gentianoides* and *E. angustifolia* merit further research as nutraceutical treatments of chronic inflammatory disorders, and these studies make it clear that synergy between phenolics, flavonoids and natural anti-bacterial compounds also warrant further research.
CHAPTER 1. GENERAL INTRODUCTION

General Introduction and Dissertation Organization

Inflammatory bowel disease (IBD) is a collection of idiopathic inflammatory enteric disorders which are growing in prevalence in adults and children every year (1, 2). With the growing cost of health care, increased instance of nosocomial infections in IBD patients, and the continued reliance on surgical intervention for patients suffering from Crohn’s disease and ulcerative colitis, the true impact of current IBD therapeutics are in question (1, 3, 4). New and safe therapeutic strategies are needed to combat the complex and multifactoral problem of IBD. Onset and chronicity of IBD is hypothesized to involve interactions among a compromised enteric mucosal epithelium, potential genetic susceptibilities, dysbiosis and aberrant and chronic immune reactivity (5). As the underlying interactions or causes of IBD are still poorly understood, physicians are forced to focus on symptomatic treatments for IBD. Successful IBD therapeutics will safely suppress chronic inflammation; encourage homeostatic immune reactivity and regulation; maintain a healthy microflora; and protect the patient from secondary infection through homeostatic maintenance or inherent antibacterial and antiviral activity. This list of activities might be unrealistic for pure compounds or individual biologic therapeutic modalities. Solutions to this problem turn patients and research to alternative nutraceutical remedies or supplements to current biologic therapies (6-9). The identification of nutraceutical plant extracts which harbor several bioactive compounds with synergistic efficacy in models of IBD will provide scientists, physicians and patients with new insights into the requirements for successful IBD therapeutics and supplemental treatments, not to mention new alternatives for safe treatment of IBD. This dissertation outlines the potential of *Prunella vulgaris*, *Hypericum gentianoides*, and *Echinacea angustifolia* as anti-colitic nutraceutical in multiple models of IBD, and uncovers potential mechanisms of anti-colitic and anti-inflammatory activity.
The dissertation is composed of 5 chapters. The current chapter, Chapter 1, encompasses the general introduction, organization of dissertation and literature review. Each of chapters 2, 3 and 4 consist of a journal paper. Chapter 2, entitled “Assessment of the in vivo anti-inflammatory activities of the ethanolic extracts of *Echinacea angustifolia* or *Prunella vulgaris* in the dextran sodium sulphate model of colitis”, is to be submitted for publication to the *Journal of Gastroenterology*. Chapter 3, entitled “*Prunella vulgaris* prophylaxis attenuates spontaneous colitis in mdr1a deficient mice”, was prepared for submission to the *American Journal of Physiology - Gastrointestinal and Liver Physiology*. Chapter 4, entitled “*Hypericum gentianoides* extract prophylaxis delays onset and severity of spontaneous colitis in mdr1a deficient mice”, is to be submitted for publication in the *Journal of Gastroenterology*. Chapter 5 includes general conclusions, encompassing a summary of results, discussion of significant findings and implications for future research. Formats of references for each research paper follow the requirements put forth by the appropriate, specific journal to which the paper will be submitted. References cited for Chapter 1, general introduction and literature review, and Chapter 5, general conclusions, are formatted in the style of *The Journal of Immunology*.

### Literature Review

**Enteric mucosal homeostasis and defense**

The intestinal milieu is constantly barraged by exogenous and endogenous antigens. Enteric mucosal homeostasis is a delicate balance maintained by pre-epithelial, epithelial and post-epithelial defense and regulatory components described in the following section (10).

**Enteric epithelium**

The intestinal epithelium is a dynamic single cell layer responsible for maintaining energy dependant absorption and secretion in the gut and preventing passive transport of compounds from the lumen into the submucosa and underlying tissues (11). These functions are responsible for blocking transport of chemicals and
xenobiotics, preventing undue water loss to the lumen, and compartmentalizing luminal contents while still allowing for nutrient absorption and exchange. Epithelial cells regulate transcellular transport through specific transmembrane proteins (12). These protein transporters include: the adenosine triphosphate (ATP)-dependant polyspecific efflux transporters of the ATP binding cassette (ABC) family; the polyspecific uptake transporter ATP independent H+/oligopeptide co-transporter family SLC15; the polyspecific ATP independent organic anion transporter family; the ATP independent organic cation/anion/zwitterion transporter family SLC22; and the ATP independent multidrug and toxin extrusion (MATE) H+/drug antiporters (13-17). The epithelium prevents passive paracellular transport through maintenance of tight junction or apical junction complexes (18). The paracellular pathway directs flow based on solute size, charge and hydrophobicity through two checkpoints, the apical junctional complex and subjunctional space (19).

The tight junction complex is built to guard passage of substances through the paracellular pathway, and at the same time create and sustain epithelial polarity by compartmentalizing the varied protein composition, lipid characters and physiologic roles of the apical and basolateral domains (20). Tight junction complexes (TJ) are divided into three vertical domains at the apical surface of the cell (21). Closest to the luminal surface of the cell, but below the brush border at the apical cell surface is the tight junction, below that is the adherens junction, and closest to the basolateral plasma membrane are the spot desmosomes. The tight junction domain is composed of the intramembrane proteins occludin, one of the 19 members of the claudins, and a junctional adhesion molecule (JAM). Studies have shown that occludens cannot confer tight junction functionality; only claudins can ensure efficient tight junction formation and function (22). Zonula occludens (ZO) are scaffolding cytoplasmic proteins that interact with occluden, claudins and JAM in the tight junction domain. ZO-1 interacts specifically with occluden, claudins and JAM, while ZO-2 and ZO-3 interact with ZO-1. ZO-1 has been molecularly associated with the cytoskeleton and could participate in tight junction function (23). Actin filaments, which extend from the inside of each microvillus in the brush border into the apical
cytosol, are bound with the peripheral actin-myosin ring/sphincter. This ring is bound to the adherens junction, in which E-cadherin associates with the ring and cytosolic signaling residues to control tight junction function (24). It is hypothesized that tight junction and adherens junction associations with the cytoskeleton and signaling molecules are two mechanisms by which TJ permeability is altered, and studies show that myosin light chain kinase (MLCK) is a key regulator of TJ permeability (25-27). Aside from apical TJ, the epithelium has luminal protections as well.

Compounds are further excluded from the epithelium and submucosa by pre-epithelial defenses. Goblet cells produce a thick mucus layer composed of mucins, trefoil peptides, glycoproteins, phospholipids and a glycocalyx (28-30). Trefoil peptides assist in the intracellular bundling of mucins and function to increase the viscosity of the mucus complex (31). Into the mucin layer a bicarbonate fluid and Immunoglobulin (Ig)A are secreted. The bicarbonate fluid maintains an almost neutral pH at the epithelium. IgA, known for its abundance relative to other isotypes, is secreted on the apical intestinal epithelial surface as a dimer bound to the polymeric Ig receptor (pIgR) (32). IgA assists antigen sampling by M cells and facilitates clearance of microbial antigens utilizing the Fc alpha receptor I (FcαRI) (33, 34).

If the epithelium is damaged, the cells have several mechanisms by which to heal and prevent excess water loss to the lumen. The epithelium naturally proliferates at a rapid rate. The lower third of crypts in the large intestine are comprised of proliferative stem cells, and as cells differentiate and mature they are pushed upward by the cells proliferating behind them at the base of the crypt (35-37). Thus, the most mature and differentiated cells are found at the surface epithelium at the shoulders of crypt and proliferation is balanced by cell shedding at the crypt shoulders. As crypt progenitors divide every 12 to 16 hours, the epithelium is completely replenished every 3 to 5 days. The process and speed of crypt proliferation can be observed by identifying crypt progenitors using tritium-thymidine injection or 5-bromo-2-deoxyuridine injection, which incorporates into the genetic material of the cell and is passed to daughter cells during mitosis (38, 39).
Proliferation is not, however, the first step in epithelial wound healing. Minutes to hours after superficial wounding, restitution begins with epithelial cells adjacent to the wound relocating into the wounded area by cytoskeleton rearrangement, and the formation of psuedopod-like processes called lamellae (40). Proliferation and differentiation of epithelial cells then replaces the diminished cell numbers. Extensive wounding activates a more complex healing process involving inflammation, extracellular matrix components and regulatory peptides. Acutely following deep colonic wounding, endothelin-1 binds endothelin A and B receptors, causing strong myofibroblast constriction and colonic shortening (41). This is a first line of barrier integrity defense. Transforming growth factor (TGF) \( \beta \) stimulates acute epithelial restitution while TGF\( \alpha \) encourages proliferation, migration, and differentiation of epithelial cells; while also upregulating the production of enzymes which aid in electrolyte and nutrient transport to the affected area (42). Fibroblast growth factors and insulin-like growth factors stimulate angiogenesis, epithelial restitution and matrix formation; and downregulate apoptosis while promoting chemotaxis of endothelial cells, respectively (43). Trefoil factors (TFF) protect the mucosa and discourage apoptosis, while colony stimulating factors (CSF) recruit inflammatory cells to clean up cell debris (44). The wound healing process underscores the need for epithelial interactions with nourishing enteric microbes and other cell types.

**Epithelial-neutrophil interactions**

Neutrophils are extravascular acute inflammatory cells which are chemotactically drawn into the tissues upon damage and distress (45). Neutrophil presence in the intestinal epithelium and submucosa is an indicator of active inflammation (46). Epithelial damage or recognition of pathogens through pattern recognition receptors (PRR) receptors or Toll-like receptors (TLR) on/in epithelial cells initiate a signaling cascade to attract neutrophils to the tissues. Activation of map kinase (MAPK) and nuclear factor-kappa B (NF-\( \kappa \)B) signaling cascades through TLR ligation have been implicated in the upregulation of epithelial basolateral expression of interleukin (IL)-8 and KC; the human and mouse chemotactic factors for neutrophils, respectively (47, 48). IL-8 directs neutrophil migration across the
endothelium utilizing $\beta_2$ integrins cluster of differentiation (CD) 11b/CD18, and through the lamina propria to the subepithelium, where the apical chemoattractant, pathogen-elicited chemoattractant (PEEC) mediates migration across the epithelium and into the lumen (49, 50). Recent studies have shown that basolateral epithelial contact of neutrophil produced serine proteases results in activation of epithelial protease-activated receptors (PARs) which increase epithelial permeability to allow neutrophil transmigration (51). Matrix metalloproteases (MMP) also participate in this process.

Upon arrival in the tissues, neutrophils stimulate chloride secretion which hydrates the mucosal surfaces and aids in washing away accumulated debris or microbes (52). Neutrophils also phagocytose debris or invading microbes, and degranulate to give off myeloperoxidase, lysozyme, elastase, cathepsins, and lactoferrin (53).

Under normal conditions, wound healing mechanisms, mentioned previously, restore transcellular integrity. Following complete transmigration of neutrophils, TJ reseal as a function of the aforementioned wound healing process. As wounds are closed by epithelial restitution, F-actin-myosin bands encircle the wound and restore TJ function by sphincter-like contraction (54). Anti-inflammatory epithelial trefoil factors and eicosanoids, such as lipoxin A4 (LXA4), downregulate inflammatory NF-$\kappa$B signaling and IL-8 production (55). This aspect of epithelial-innate immune cell interactions overlaps with microbial clearance of pathogens, however, the commensal microflora are bacterial species that are vital to epithelial maintenance and function.

**Epithelial-microflora interactions**

It is currently estimated that anywhere from 500 to 1000 species of bacteria are found in the gut (56). In fact, approximations place bacterial cell numbers within the human body 10-fold higher than human somatic and germ line cells (57). While the microflora benefit from host ingested nutrients and an intestinal niche, the host gains much from the presence of the microflora as well. The enteric microflora nourishes the intestinal epithelium by aiding in metabolism of plant polysaccharides
and producing short-chain fatty acids (SCFA) like butyrate (58). Butyrate is the primary nutrient source for colonocytes (59). Metabolized nutrients provided to the epithelium by the microflora play a vital role in wound healing and TJ function as well (60).

Much of what is now known regarding epithelial-microflora symbiosis comes from studies utilizing germ-free mice and rats. Mice lacking microflora are more susceptible to infection, exhibit reduced potential for angiogenesis, are deficient for digestive enzyme activity, display thickened muscularis tissues, are less capable of cytokine and immunoglobulin production, have underdeveloped peyer’s patches and fewer intraepithelial lymphocytes (57). Germ-free mice require an increased caloric intake to thrive comparably to conventionally colonized mice; emphasizing the importance of the microbial role in nutrient availability, uptake and storage (61). In 2003 it was shown that mono-association of germ-free mice with *Bacteroides thetaiotaomicron* restores angiogenic potential through paneth cell signaling, and similarly upregulates production of paneth cell proteins which are antibacterial for gram-positive gut pathogens like *Listeria* (62, 63). These experiments illustrate that even simple monoassociation of the gut is enough to restore more homeostatic epithelial function. It is also of importance that components of the microflora, such as LPS, play a role in homeostatic immune function and oral tolerance, which will be further described later in this review (64).

**Epithelial-immune cell interactions**

The epithelium is more than just a discrete barrier in the intestine. Epithelial cells possess antigen presentation capabilities which lead to the activation of adaptive immune responses through T cell interactions. Intestinal epithelial cells are a unique antigen presentation cell (APC) subset as they display classical type I presentation molecules and are the only non-professional APC to constitutively express MHC II molecules (65, 66). Intestinal epithelial cells are no different than any other nucleated cell in the body in that they express MHC I molecules that allow for presentation of antigen to CD8+ cytotoxic T cells. The class I presentation pathway involves antigens from infected or malignant cells whose proteins are
ubiquitinated and targeted to the proteasome complex. Proteasomal enzymatic processing of these proteins results in their transportation to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), where they are loaded into freshly synthesized MHC I molecules and shipped from the golgi to the cell surface (67). Thus far, it has only been shown that intestinal epithelial cells fail to insight cytotoxic T cell responses (68, 69).

Functionally, T cell interactions with epithelial MHC II molecules are polarized in similar fashion to the epithelium itself; apical antigens can only trigger a basolateral response (70). In 2005, Beers et al. showed that the lysosomal cysteine protease cathepsin S (Cat S) is responsible for invariant chain degradation and MHC class II maturation in intestinal epithelial cells (71). Moreover, this study illustrated for the first time that intestinal epithelial class II bound endogenous peptides are presented to naïve CD4⁺ T cells in vivo. Professional APCs secrete exosome-like membrane blebs containing classical restriction elements which can interact with T cells (72). Similarly, it has been shown that membrane blebs from intestinal epithelial cells also contain MHC II and could potentially cross into the lamina propria to interact with CD4⁺ T cells (73). Karlsson et al. went on to demonstrate that these epithelial membrane blebs could be isolated from peripheral blood and used to induce antigen-specific tolerance in vivo.

Intestinal epithelial cells have also been shown to express several non-classical class I-like molecules which are capable of interacting with many immune cell types (74). MHC class 1 chain-related protein A and B (MICA/B) are functionally indistinguishable molecules expressed on intestinal epithelial cells. These proteins interact with natural killer (NK) receptor NKG2D, which is expressed on NK cells, NK T cells, cytotoxic CD8⁺ T cells, γδ T cells, and macrophage (75). MICA/B activates NK cells and co-stimulates T cells (76). Tieng et al. described that upon *E. coli* adhesin to its cell receptor, MICA expression was upregulated on intestinal epithelial cells, implying that MICA expression and its functions are modulated by enteric microbes (77). UL16-binding proteins (ULBPs) on intestinal epithelial cells are genetically distinct from MICA/B, but functionally similar in that they are human
ligands for NK cell NKG2D receptors that activate cytokine secretion from NK cells (78). Human leukocyte antigen (HLA)-E (humans) and Qa-1 (mice) on intestinal epithelial cells can present peptides in similar fashion to MHC I (79). These molecules bind NKG2A, NKG2B and NKG2C on NK cells and cytotoxic CD8+ T cells; their expression can be IFNγ regulated (80). HLA-E and Qa-1 have been documented to stimulate CD8+ regulatory T cell subset expansion as well (81). The neonatal Fc receptor (FcRn) is a misnomer as it is expressed in adult intestinal epithelial cells as well as in neonates (82). FcRn does not present antigen, but binds the constant (Fc) region of IgG for the purpose of apical to basolateral transport and basolateral to apical transport (82). FcRn-IgG binding is optimal at acidic pH and bi-directional shuttling is regulated by sorting signals in the cytoplasmic tail of FcRn (83-85). Blumberg et al. were able to prove that antibodies capturing antigen in the lumen could be transported into the lamina propria by FcRn and delivered to CD11c+ dendritic cells (DC) cells which in turn activated antigen specific CD4+ T cell responses in vivo (86). These results solidify the role of intestinal epithelial cells as a signaling link between luminal antigens and immune cells in the lamina propria. Human CD1d, and murine homologs are expressed by professional APCs as well as intestinal epithelial cells and they present glycolipid and glycoprotein antigens to several T cell subsets (87, 88). CD1d is most associated in the literature with activation of NK T cells expressing the NK1.1 marker, which produce IL-4 and IFNγ upon activation (89). Interestingly, a regulatory subset of CD8+ T cells is restricted by CD1d expressed on intestinal epithelial cells and displays strong contact-dependant regulatory activity (90). CD1d expression is upregulated by IFNγ, cecal bacterial antigen exposure and in a positive feedback loop by regulatory T cells (91). While crosslinking of CD1d has been shown to upregulate IL-10 production, intestinal tolerance is not CD1d-restricted (74). Murine MHC T locus (TL) antigens, expressed on thymocytes and intestinal epithelial cells, do not present antigen, but bind CD8 and activate cytotoxic γδ and αβ T cells in a professional APC restricted manner (92-94). Together these observations clearly highlight the importance of
intestinal epithelial cell APC functions to immune cell activation and cytotoxic responses in the enteric mucosa.

Co-stimulation is a required aspect of specific T cell activation in addition to T cell receptor (TcR) engagement. Anergy is the consequence of TcR engagement in the absence of co-stimulation. Early on in this area of research it was found that intestinal epithelial cells do not express the typical CD80 or CD86 T cell co-stimulatory molecules; this observation raised questions about the APC functions of intestinal epithelial cells and causation of anergy (95). Since that time, expression of non-traditional co-stimulatory molecules has been characterized on intestinal epithelial cells. In vitro and ex vivo experiments identified the expression of B7h, positive signal ligand for inducible co-stimulator (ICOS) on T cells; and B7-H1, inhibitory ligand of PD-1 on T cells (96). These studies concluded that B7h and B7-H1 are only expressed on intestinal epithelial cells isolated from inflamed tissues. Additionally, only anti-B7h monoclonal antibody (mAb) inhibited proliferation of intestinal epithelial cell stimulated T cells, suggesting that B7h, but not B7-H1, is important for T cell activity in diseased intestinal tissues. Human intestinal epithelial cells also express basolateral CD58 (97). Studies by Framson et al. utilized anti-CD58 mAb to illustrate that blocking of CD58 co-stimulation could abolish intestinal epithelial-T cell HLA class II mediated antigen presentation (97). Intestinal epithelial cells hold potential as co-stimulatory mediators for T cell stimulation via B7h and CD58 molecules. Together, this evidence highlights the multiple niches the intestinal epithelial cell participates in with regard to T cell activity and regulation in homeostasis and disease states.

Cytokines, chemokines and inflammatory mediators

Cytokine, chemokines and other inflammatory mediators produced by multiple cell types in the gut, homeostatically and during inflammation or infection, are essential for intestinal protection and maintenance. Intestinal epithelial cells produce many neutrophil chemotactic molecules meant to basolaterally attract these cells for defense. CXCL chemokines 1, 2, 3, 5 and 8/IL-8 are all produced by intestinal epithelial cells for the purpose of attracting neutrophils upon infection or
inflammatory cytokine signals (98, 99). CXCL8/IL-8 is quickly released in large amounts by distressed intestinal epithelial cells and is short lived for expedient and substantial neutrophil chemotaxis (100). On the other hand, CXCL5 production in these cells is delayed, weakly potent and long lived for a mild but sustained chemotactic neutrophil response (101). Granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) are cytokine signals which are not chemotactic for neutrophils, but are responsible for local maintenance and survival of neutrophils once they have reached the intestinal tissues (102). These cytokines act to increase neutrophil proliferation and survival. CCL2/monocyte chemotactic protein (MCP)-1 and RANTES are produced as chemotactic factors for monocytes and macrophage (103). Intestinal signals for innate immune cells are not constitutively expressed, unlike chemotactic signals for T cells.

CXCL9/monokine induced by gamma interferon (MIG), CXCL10/interferon inducible protein 10 (IP-10) and CXCL11/interferon-inducible T cell α-chemoattractant (I-TAC) are all IFN inducible chemotactic factors for CXCR3 expressing CD4⁺ T cells (104). These chemokines are constitutively expressed in healthy intestinal tissues and can upregulate IFN production in CXCR3 expressing cells, including mononuclear cells and intraepithelial lymphocytes (104). These chemokines are usually associated with T helper 1 (Th1) type CD4⁺ lymphocytes, which are most common in the gut (105). Constitutive expression of CCL22/macrophage-derived chemokines (MDC) is chemotactic for T helper 2 type CD4⁺ T cells (106). Expression of this chemokine is upregulated by infection or increased production of TNF and IFN, but is downregulated by IL-4 and IL-13. This chemokines is one of many examples of NF-κB responsive chemical signals expressed in the enteric mucosa.

Dendritic cells (DC) are a critical linker of innate and adaptive immunity, serving as a professional antigen presenting cell to intraepithelial and more distal lymphocytes. CCL20 is produced by intestinal epithelial cells and most lymphocytes and is chemotactic for CCR6 expressing immature DC (107). Immature DC are
highly phagocytic, but upon antigen uptake will downregulate their phagocytic function and migrate to present the antigen they have collected and processed to T cells (108). Not surprisingly, CCL20 production is upregulated by epithelial infection, TNF production and IL-1 production. It is hypothesized that CCR6 expressing DCs that chemotax to the intestine participate in uptake of antigen across the epithelium (109). DCs have characteristic long dendrites which can form temporary TJ with intestinal epithelial cells as they insert their dendrites between epithelial cells to sample antigens in the lumen (110). After sampling, the dendrites retract and the dendrite-epithelial TJ are replaced immediately by epithelial-epithelial TJ.

Additional cytokine signals are upregulated in the intestine upon infection by pathogens. TNF and IL-1α serve as warning signals to initiate inflammatory cascades in the enteric mucosa. IL-1α is interesting in that it does not require cleavage, like IL-1β, for activity. IL-1α activity is abolished by IL-1 receptor agonist (IL-1Ra), which also downregulates IL-1 induced IL-8 production (111). It should be noted that mRNA expression of IFNγ, IL-2, IL-3, IL-4, IL-5 and IL-12p40 is not present in intestinal epithelial cells (112). This observation underscores the notion that while intestinal epithelial cells play an acute and vital part in the initiation of inflammation in the gut, these cells are not meant to sustain antigen specific immune responses.

One of the most important mediators of inflammation is the transcription factor NF-κB. In the absence of signaling leading to NF-κB activation, NF-κB dimers are sequestered in the cytoplasm by IκB inhibitors which mask the transcription factor's nuclear localization domains (113). Host cell detection of microbes or TNF or IL-1 production cause IκB phosphorylation, polyubiquitination, and proteasomal degradation, freeing NF-κB to translocate into the nucleus (114). Catalytic IKKβ mediated IκB degradation leads to activation of many innate defense mechanisms. Such mechanisms include CXCL1, CXCL8, CCL2, CCL20, CCL22, intercellular adhesion molecule 1 (I-CAM), TNF, nitric oxide synthase 2 (NOS-2) and cyclooxigenase 2 (COX-2) (115). The partnership of intestinal epithelial cells and local lymphocyte and monocyte populations in cooperatively initiating protective
mechanisms in the gut is a complex and highly regulated relationship in which homeostasis is balanced by inflammation and tolerance.

**Oral tolerance**

Oral tolerance could be described as the body’s way of extending self-tolerance to annexed host microflora and constant food antigen exposure. The traditional definition of oral tolerance relates the specific suppression of immune responses (cellular and/or humoral) to antigens previously dosed by the oral route. This tolerance functions to avert hypersensitivity to food antigens, immunologic depletion of the microflora, and the subsequent tissue damage that would result from these immune responses. This is a necessary immune mechanism as 130 to 190 g of food derived proteins are absorbed every day by the average intestine, and human stool contains $10^{12}$ bacteria/gram (60, 116). Additionally, the gut has a greater concentration of Ig-secreting cells than that found in all other lymphoid organs combined (117, 118). Indeed, dietary and microflora antigens usually induce tolerance without detrimental local or systemic immune reactivity. Experimentally, the role of food proteins in immune system development is valuable for normal maturation of gut-associated lymphoid tissues (GALT), IgA and IgG maintenance, normal lymphoid organ morphology and function, as well as balanced Th1/Th2 profiles and homeostatic IL-10, IL-4 and IFNγ ratios (119).

The two primary effector mechanisms of oral tolerance are in induced, in part, by antigen dose. One arm of oral tolerance is induced by clonal anergy or clonal deletion in response to high doses of oral antigen (120). T cell anergy was pinpointed as a tolerogenic mechanism when it was discovered that tolerance could be abolished by exogenous IL-2 (121). Now clonal anergy is defined as IL-2 responsiveness to high doses of oral antigen in the absence of transferable suppression (120, 122, 123). Anergic regulatory cells have also been hypothesized to function by producing IL-4 and IL-10 (122). Clonal deletion has also been observed in mice fed high antigen doses followed by systemic challenge with adjuvanted antigen (124). Additionally, long-term ovalbumin (OVA) feeding to T cell
receptor (TCR) transgenic mice increases caspase activity in Ag specific CD4+ T cells (125).

The second limb of oral tolerance is active suppression, induced by low doses of oral antigen (126). It has been documented in mice that autoimmune gastritis and ovarian/testicular inflammation associated with neonatal thymectomy is abrogated by the restoration of a CD4+CD25+ subset of T cells (127, 128). Anergic TNF receptor and FoxP3 transcription factor expressing T regulatory cells (Treg) mediate regulation through contact, while T helper 3 (Th3) and T regulatory cell type 1 (Tr1) Tregs regulate immune responses through the production of TGFβ and IL-10 respectively (129-132). CD8+ TGFβ secreting Treg cells have been identified in the maintenance of oral tolerance in several autoimmune models (133, 134). Specifically, IL-4 and IL-10 producing CD8+ regulatory cells are induced by feeding antigen, even after CD8+ T cell tolerization (135). It has also been shown that if an orally tolerized T cell and a naïve T cell of different specificities interact with the same DC, The tolerized T cell can influence the DC to induce differentiation of the naïve T cell to a regulatory phenotype (136). While it has been illustrated that CD8+ Tregs are not necessary for induction of systemic tolerance, it seems that these cells are able to fill certain regulatory niches in the mucosa (137). Ke et al. have also reported that γδT cells are vital to oral tolerance and enteric mucosal protection (138). In this study δTCR-deficient mice, which lack γδT cells, were incapable of eliciting tolerogenic responses to low doses of oral antigen. Conflicting reports exist regarding the role of NK T cells in oral tolerance. Several reports characterize the oral tolerance induced by NK T cells in the liver, however, other studies illustrate an intact oral tolerogenic response in mice lacking NK T cells (139-141). Perhaps this data implies that while not required for complete oral tolerance, NK T cells can serve as additional tolerogenic effectors in the liver and other organs. Most recently a subset of invariant FoxP3+ NK T cells has been described (142). This population was observed to arise in a transforming growth factor beta (TGFβ) dependant fashion in the cervical lymphnodes of mice with experimental autoimmune encephalitis as well as in the gut. Like FoxP3+ Tregs, these cells suppress in a
contact-mediated fashion, and like NK T cells, they preferentially migrate to the liver upon adoptive transfer into mice.

The suppressive or regulatory activities of these cell types are supported by cytokine signals. While suppression of interferon gamma (IFNγ) is a commonly recognized phenomenon in oral tolerance challenge experiments, it is also known that primed antigen-specific T cells produce large amounts of IFNγ during the inductive phase of tolerance (143). Preserved IFNγ production been linked to models of T cell anergy, while depletion or absence of this cytokine has also been shown to abrogate existing tolerance or prevent induction of tolerance (144-147). Confounding this issue are studies which report a lack of tolerance interference by in vivo IFNγ depletion or lack of the IFNγ receptor in vivo (148-150). Clearly, more research regarding the role of IFNγ in the development and maintenance of oral tolerance is needed. Perhaps the key cytokine modulator of adaptive suppression is TGFβ. TGFβ is a pleuripotent cytokine whose latent form relies on degradation of the coupled latency associated peptide (LAP) after αvβ integrin recognition for activity (151). This cytokine can be secreted in bioactive or latent forms. Miller et al. first suggested the link between TGFβ and oral tolerance by reporting the dependence of CD8+ T cell mediated suppression of experimental autoimmune encephalitis in mice orally tolerized to myelin basic protein on TGFβ (152). Since that time it has been found that TGFβ secreting cells can be isolated from peyer’s patches and mesenteric lymphnodes of orally tolerized mice, as well as from the blood of humans orally tolerized to myelin basic protein (153, 154). In addition to enhancing leukocyte recruitment and IgA secretion, TGFβ is known to aid in the differentiation of CD4+CD25- T cells into CD4+CD25+ Tregs (155-157). T cell clones from tolerized mice exhibit a CD4+CD25+ TGFβ producing Treg phenotype (153, 158). These cells have been found to express cytotoxic T-lymphocyte antigen 4 (CTLA4); an inhibitory molecule which binds B7 molecules on T cells; or FoxP3 and exhibit functional suppressive capabilities in vitro and in adoptive transfer models where tolerance is transferred by these cells to naïve cells of the recipient (159-161). TGFβ is also
released by apoptotic T cells in which mitochondrial membrane potential has been compromised and existing cytokine is released to the cytosol (162). Uptake of apoptotic cells by macrophage and dendritic cells induces TGFβ production and a decrease in pro-inflammatory cytokine production (163-165). In addition to secreted TGFβ, a membrane bound form of TGFβ has been characterized (166, 167). A 2010 study has shown that latent TGFβ is incorporated into the extracellular matrix by a complex, including latent TGFβ-binding protein 1, which is functionally dependant on pericellular assembly of fibrillin microfibrils (168), while a 2009 report related the importance of the leucine-rich repeat molecule GARP to tethering the complex to the cell membrane (169). The activity of TGFβ, and the subsequent generation of Tregs and tolerance is greatly influenced by the presence or production of the cytokines IL-10, IL-4 and IL-2. While IL-2 has been shown to support TGFβ induced Treg differentiation and expansion, IL-4 can also fill this role with respect to Th3 type Tregs from tolerized mice (153, 158). TGFβ induced Tregs are capable of producing IL-4 in combination with IL-10 or of producing IL-10 alone. In 2009, Zheng et al. observed the requirement for IL-4 in the production of highly suppressive CD8+ Tregs (170). IL-10 is produced by many cell types but is recognized as the key product of the Tr1 CD4+ Tregs (171). Studies involving IL-10 deficient mice illustrate the importance of IL-10 to immune tolerance, as these mice spontaneously develop severe colitis related to lack of sufficient immune regulation and subsequent chronic immune reactivity to antigens of the enteric microflora (172). Housing these mice in germ-free conditions prevents colitic onset (173). IL-10 is thought to act upon APCs by inhibiting MHC class II and co-stimulatory molecule expression in these cells (174). With regard to modulation of T cell effector functions, IL-10 serves to inhibit effector cells in a feedback control capacity (175). IL-10 therapy has been shown to restore enteric mucosal tolerance in mice with IBD (132).

In summary, the enteric mucosa relies on a complex and tightly regulated community of innate and adaptive immune cells, as well as cross talk with the resident microflora, to maintain homeostasis and immune tolerance. Antigen excluding epithelial barriers are maintained by tight junctions, nourished by microbial
metabolites and supported by innate immune surveillance. While effector T cells are readily called to action by APCs in the event of pathogen immune escape, regulatory cytokines and T cells are constantly acting to suppress inappropriate and chronic immune activation. The early induction of tolerogenic mechanisms by anergy, clonal deletion and active suppression are vital to enteric homeostasis. While the cellular interactions protecting the intestines on a daily basis are very thorough and not without several fail-safes, this system is not perfect and is subject to malfunction. The next section of this review will discuss the mechanisms and consequences of homeostatic breakdown in the enteric mucosa in the context of inflammatory bowel disease.

**Inflammatory Bowel Disease (IBD)**

Inflammatory bowel disease primarily encompasses the etiologically undefined and multifactorial disorders of Crohn’s disease and ulcerative colitis. These conditions involve an array of complications including, but not limited to, epithelial barrier dysfunction, microflora dysbiosis, chronic immune activation and ineffective immune regulation. The following section will discuss IBD from clinical and basic science perspectives, as well as current therapeutic strategies.

**Clinical and epidemiological perspectives**

Ulcerative colitis (UC) is a chronic inflammatory disorder which manifests in the lower intestine. Characteristically, lesions in UC patients are found in the rectum and progress proximally up the colon. The ileum is generally unaffected in UC unless ‘ileal backwash’ has occurred (176). UC can be broken into clinical categories based on the placement of colonic involvement: ulcerative proctitis is localized to the rectum, proctosigmoiditis refers to sigmoid colonic involvement, left-sided colitis encompasses the entirety of the descending colon to the intersection of the transverse colon and pancolitis describes UC lesions found beyond the splenic flexure and possibly into the cecum (177, 178). Physical examinations of patients suffering from mild to moderate UC are usually normal and common clinical signs of UC include increased frequency of bowel movement, blood in the stool, uncontrolled elimination, diarrhea, fecal incontinence, constipation, passage of hard stools,
abdominal pain, nausea, vomiting and weight loss (179). In severe cases malnourishment and abdominal distention can become externally apparent (180). There is as yet no standard UC disease activity index, although a combination of approaches including classical scoring developed by Truelove and Witts, Mayo index and Baron endoscopic scores are utilized in many clinical settings (181-183). The Truelove Witts index breaks disease phenotypes in to mild, moderate or severe based on bowel movement frequency, presence of blood in stool, heart rate, hemoglobin level and erythrocyte sedimentation rate (183). This index has been validated by reviews of retrospective data which found a correlation between Truelove Witts index score and severity of UC outcome (184, 185). Five primary outcomes are defined in UC patients: chronic relapsing disease, chronic unremitting disease, single episode without recurrence, colectomy and death (186). These outcomes are greatly affected by preceding disease activity and systemic symptoms associated with diagnosis. Flares can be aggravated by non-steroidal anti-inflammatory drugs, cessation of smoking, and mucosal infections (187). Endoscopic observations and scores, like that of the Baron endoscopic score, also tend to correlate well with clinical activity of UC and can lend insight to practitioners in outcome prediction (188). Several endoscopic features noted in UC affected intestines are common to other inflammatory disorders of the gut and include edema, loss of normal vascular pattern, mucosal friability and mucosal granularity (189). A somewhat unique endoscopic finding in UC patients is a continuous inflammatory distribution with severity peaking in the rectum and decreasing toward the transverse colon without healthy tissue between lesions. While patchy lesions and rectal sparing can occur in UC patients, most studies find that these are rare events and usually emerge in pediatric patients (189). Histologic lesions in UC patients are marked by depleted goblet cell numbers, common crypt abscesses and architecture distortion, uniformly heavy lymphocytic infiltrate, lack of granulomas, thickened muscularis mucosa, and scant submucosal inflammation (190). Endoscopic and histologic findings in UC patients are often indicators of the potential for UC patients to develop additional non-neoplastic or neoplastic intestinal
complications (191). In pancolitic and left-sided colitic UC patients, systemic toxicity accompanied by dilation of the colon more than 6 centimeters (cm) in the absence of obstruction is occasionally noted and termed toxic megacolon (192). This complication can be marked by the presence of *Clostridium difficile*, *Campylobacter*, *Shigella*, *Salmonella*, amebic infections or cytomegalovirus and is a risk factor for perforation. Other intestinal complicating factors for pancolitic UC patients, though rare, are hemorrhage and benign or malignant stricture. Patients suffering from pancolitis or left-sided UC also have a greatly increased risk of developing rectal cancer, colon cancer, or cholangiocarcinoma, with risk increasing even more in patients experiencing greater chronicity of UC or concurrent bouts with primary sclerosing cholangitis (193, 194). Studies show that in addition to intestinal complications of UC, patients will manifest at least one extraintestinal complication. Some recorded non-intestinal complications include osteoporosis, abnormal pulmonary functions, pyoderma gangrenosum, iritis, uveitis, venous thrombosis, arthralgias, and arthritis (195). It should be noted, however, that most extraintestinal complications are not uniquely linked to UC and are therefore not a distinguishing or diagnostic factor for practitioners attempting to differentiate between UC, CD or intermediate colitis. With advancing interest and funding for medical research and technology the life expectancy of UC patients is no different than that of the average member of the population (196). Mortality is slightly increased within the first few years of diagnosis, and chronicity and severity of UC is not surprisingly linked to higher mortality.

Crohn’s disease (CD) is a chronic, enteric inflammatory disorder which manifests as a patchy lesion anywhere from mouth to rectum (197). While CD lesions can be quite spread out, it seems that 90% of newly diagnosed patients present with gross small intestinal, colon restricted, or combined small and large intestinal lesions in a patchy pattern. Clinical categories or presentations for CD can be divided into terminal ileum, colonic, ileocolonic or upper gastrointestinal manifestations. The terminal ileum is by far the most consistently affected area of the intestine in CD patients. Lesions isolated to the mouth, esophagus, and stomach
are rare in the absence of primary intestinal lesions. It has been noted in several population-based retrospective studies that it is possible for the primary concentration of lesions or the location of CD involvement to change overtime in patients, but that is not the majority case (198). Common symptoms of CD include abdominal pain, diarrhea, weight loss, and occasional fever and bloody stools. Interestingly, there is a correlation between instance of abdominal pain and ileal lesion localization (199). There also seems to be a correlation between diarrhea, bloody bowel movements and colonic CD. Again, due to the variable and unpredictable nature of CD, no standard disease activity index exists for CD, however the Crohn’s Disease Activity Index (CDAI), Harvey-Bradshaw Index (HBI), Van Hees Index (VHI), Perianal Disease Activity Index (PDAI) and Pediatric Crohn’s Disease Activity Index (PCDAI) are all used in practice regularly, depending on the category of CD involvement, the patient type and some other CD-related complications (199-202). The CDAI is by far the most commonly utilized index (199). The CDAI takes into account 8 parameters over a seven day time period: instances of diarrhea, severity of abdominal pain, quality of life, extraintestinal complications, requirement of anti-diarrheal drugs for control, presence of abdominal masses, hematocrit and body weight. The HBI is a simplified version of the CDAI, while the VHI ignores subjective CDAI parameters (199, 200). It has been shown that CD patients suffering from perianal disease score deceptively low on the CDAI and subsequently require use of the PDAI (202). The past decade has been marked by a steady increase in severe CD development in pediatric patients, in whom the PCDAI is most appropriate (201). The two most common disease outcomes in CD patients are chronic intermittent disease and chronic continuous disease (203). Mild intermittent disease also occurs, but chronic intermittent CD is the most common outcome. External factors which affect CD outcome are smoking and use of the oral contraceptive pill; both of which encourage focal thrombosis and intensity of CD activity (204). Endoscopic scores, such as the Crohn’s Disease Endoscopic Index of Severity (CDEIS) or the Simple Endoscopic Score for Crohn’s Disease (SES-CD) in combination with various imaging methods are crucial for disease assessment due
to the highly variable and patchy manifestation of CD lesions (205). Again, several common endoscopic characteristics seen in CD patients are not limited to CD. Typical CD lesions are patchy with normal mucosa between lesions. Asymmetry and heterogeneity of lesions is also common. The presence of lesions interrupted by normal mucosa and more common rectal sparing are typical of CD. Small ileocecal valve or terminal ileal ulcerations are pathognomonic for CD. Histologically, CD patients exhibit thickened gut tissues interspersed with fistulas which can create a ‘cobblestone’ appearance, increased adipose wrapping around the gut tissue, normal goblet cell distribution, occasional to rare crypt abscesses, intact glandular architecture, patchy lymphocytic infiltrate ranging from mild to severe, granulomas, normal muscularis mucosa, and excessive submucosal inflammation (206). Related to these occurrences are the three main intestinal complications associated with CD: intestinal inflammation, stricture and penetration. Lesions in CD patients are transmural in nature and can involve fibrosis of the bowel wall leading to stricture. Bowel penetration and fistulae adjoining nearby organs and skin are not uncommon in CD. The development of these disease manifestations is not usually absolute. Most severe patients present with inflammation that leads to stricture and then penetration. The course of CD can be cyclical with any one or multiples of these manifestations occurring at any given time. Chronic inflammation can lead to penetration or fistula formation between the diseased colonic section and the urinary bladder, vagina or neighboring bowel segment (207). Like UC patients, those suffering with CD have an increased risk of colorectal cancer, however, as many of the most inflamed and severe cases of CD involve extensive or complete bowel resections, this lowers the risk of colonic adenocarcinoma in the most severely affected patients (208). In addition to intestinal complications of CD, extraintestinal CD complications can occur simultaneously with and be associated with a flare of disease or can occur in a fashion independent of intestinal CD activity (209). Joint inflammation is the most common extraintestinal disturbance associated with CD. Joint inflammation presents as acute peripheral arthritis involvement of the large joints associated with the duration of a CD flare or as acute peripheral arthritis
involvement of the small joints independent of a CD flare. Several inflammatory disorders of the skin develop in CD patients. While CD imparts a slightly increased risk for Sweet’s syndrome and pyoderma gangrenosum, erythema nodosum is the most frequently reported cutaneous disorder associated with CD. Erythema nodosum is characterized by painful red lumps on the skin caused by a hypersensitivity reaction in the panniculitis, or subcutaneous fat layer. This inflammatory reaction is self limiting and usually resolves within 3 to 6 weeks of onset. Cases of CD associated neutrophilic cutaneous disorders, such as Sweet’s syndrome are infrequent. Non-specific eczema and psoriasis seem to be genetically linked to CD. Although ocular inflammation and liver disease, such as iridocyclitis, episcleritis and primary sclerosing cholangitis respectively, are reported in CD patients, these are more commonly associated with UC. Osteoporosis and deep venous thrombosis are also reportedly linked to CD. Increased mortality in CD patients correlates with disease duration, with later stage cases at a greater risk.

Epidemiologic data collected and assessed in the context of IBD is complicated by many factors. Time to diagnosis, accuracy of diagnosis, patient access to medical care, and efficiency of patient tracking are only a few of the complicating factors to consider when reviewing epidemiologic data, however, much valuable epidemiologic data has been collected which sheds light on potential etiologic mechanisms in IBD. Recent estimates figure that 1.4 million Americans and 2.2 million Europeans suffer from CD or UC (210). While the worldwide instance, or relative number of newly diagnosed cases over a period of time, of UC has been stable for some years now, the instance of CD has only relatively recently plateaued since its more recent discovery in 1932. The more recent increase in instance of CD is likely attributed to its relative infancy compared to UC. There is a documented north-south gradient associated with risk of IBD (211). One hypothesis for this gradient is the ‘hygiene hypothesis’ (212). This hypothesis asserts that people living in southern geographic regions are more apt to spend time outdoors, come in contact with more beneficial microorganisms, acquire greater antigenic or immune stimulation from a less sterilized indoor environment, or come into contact with more
environmental pollutants or transmissible organisms. Theoretically, the hygiene hypothesis characterizes the environments in which IBD is most prevalent to have been made too sanitary. Thusly, people in those areas do not receive the right amount of immune stimulation. Supportive of the hygiene hypothesis is the observation that people with higher socioeconomic standing are more likely to develop IBD. Similarly, it has been shown that women whose occupation involves cleaning or maintenance and men whose occupations require them to work outdoors were less likely to develop IBD than those working indoors in office buildings or in jobs were they are not regularly exposed to greater levels of environmental antigen. Interestingly, instance of IBD has stabilized in geographic areas of high IBD frequency, but has recently begun to increase sharply in areas of lower IBD frequency. More specifically, cohorts in the United States and Northern Europe have seen a relatively steady instance of IBD over the past decade, while instances in eastern and southeastern Asia have increased greatly during that time. This increase in IBD instance specifically points to a rise in UC cases over CD. Some feel that this shift is due in part to “westernization” and cultural shifts in countries like Japan (213). Prevalence, or the number of recorded cases at a given time, is not a great epidemiologic measure of IBD (214). Prevalence is equivalent to incidence of disease multiplied by time. While prevalence should equal incidence for acute diseases, IBD is chronic in nature and manifests variably in terms of general and individual patient contexts such as cultural, geographical and microbial considerations. The patient to patient, region to region and culture to culture differences noted in IBD make prevalence a less accurate measure of chronic disease. Accordingly, most epidemiologic studies of IBD prevalence have resulted in variable numbers with little consensus within and among studies. While pediatric IBD totals have recently been estimated to encompass 33% of IBD cases in the United States, the average age of IBD onset is between late adolescence and early adulthood worldwide (215). In North America, the observed age of CD diagnosis is generally between 33 and 45 years of age, while UC diagnosis tends to occur 5 to 10 years later in life (210). Many epidemiologic studies have found a bimodal age
distribution in UC cohorts worldwide, in which a primary peak age of onset of UC occurs between the third and fourth decades of life; while a second peak age of onset occurs later in life beyond the fourth decade (216). This same bimodal observation is also noted in CD epidemiology, but not as reliably or extensively as that recorded for UC cohorts (216). While it is generally accepted that there is no significant sex bias in either CD or UC, many epidemiologic studies note that there is a slight skew toward increased female instance of CD while a similar skew shows an increased bias toward males with regard to UC instance (217). With regard to race and ethnicity, most studies conclude that both CD and UC are most prevalent in Caucasians but not that far from numbers in Black cohorts (218). Jewish populations are highly affected and this trend is most notable for CD (219). In the United States, the most infrequently represented groups are Hispanics and Asians, but as mentioned previously, these formerly low instance groups have seen increased instance of IBD in recent years in North American cohorts as well as in the native countries of Japan and Mexico (220). Diet is closely tied to health and culture around the world and epidemiologists have collected much data on the effect of certain items in the human diet on IBD. Several studies have asserted a link between sugar and carbohydrate intake and increased instance of IBD (221). More than a few IBD cohort studies have documented antibodies to wheat and corn in IBD patients (222, 223). Studies of Japanese cohorts explained that the increases in IBD instance seen in that country are significantly linked to an increase in sugar and fast food intake (224, 225). Another study also noted a lower intake of fruit and vegetable fiber and an increased intake of refined sugars by IBD patients (226). While studies such as these are not without some interest or validity, several opposing views have also been proposed. One such study concluded that sugar intake was not linked to increased instance of IBD (227). With regard to fruit and vegetable fiber intake by IBD patients it should be pointed out that those fibers are some of the most difficult to digest and might make these foods uncomfortable or unappealing for those with IBD to eat. Similarly, while some studies postulate a tie between milk protein sensitivity in childhood and adult onset of IBD, Sonnenberg has found that dairy
products and derivatives like margarine have no causative tie to IBD instance (228). It is also unclear if fat intake has any effect on IBD susceptibility. Some studies have suggested a lower instance of IBD in cohorts were large quantities of fish containing omega-3 fatty acids are regularly consumed, such as Asian cohorts (229). In general, it seems that data regarding the effects of foods and food additives on IBD risk is inconsistent. It remains unclear if food intake differences in IBD cohorts are causative or consequential factors of IBD. It cannot however be denied that dietary compounds have a great effect on individual host microbiota and it is well accepted that the microbiota play a key role in homeostatic and IBD related gut functions as will be discussed in later sections of this review. Likewise, genetic factors, which vary from population to population and with different cultural mating habits, have an effect on hormone production, immune function and homeostatic regulation in the human body (189). IBD epidemiology hints at the multiple complications involved in human susceptibility to UC and CD, and is a useful tool for pinpointing potential etiologic factors of IBD.

Etiopathogenesis of IBD

While many etiologic hypotheses exist for IBD, the causative nature of IBD remains undefined. Current theories of IBD etiology focus on four general instigating parameters: environmental interactions, genetic susceptibility, immune response, and luminal microbes (5, 230). In recent years it has become increasingly apparent that no single parameter listed above is likely to be individually causative (231). Multiple murine models of IBD, which will be discussed in more detail in following sections, have shown that while severe alteration of a single parameter is capable of inducing IBD, most models repeatedly highlight causes linked to multiple milder disturbances in homeostatic states. When considering the complexity of the gut environment, perhaps this finding is not surprising. Interactions between gut microbiota, microbial metabolites, epithelial cells, immune cells, and ingested compounds are a few of the exchanges which take place on a constant basis and greatly influence IBD (173, 230). When considering the four parameters mentioned previously, it is difficult to conceptualize them as autonomous. Most factors
hypothesized to contribute to IBD causality highlight the overlap between these four parameters. For example, much work has been done to characterize the contribution of persistent pathogenic infections to IBD onset and susceptibility.

Routes and mechanisms of persistent pathogenic infection could be considered environmental, persistent infection or ineffective clearance involve immune function and genetic susceptibility, while interactions between host and pathogen or host commensal and pathogen can alter the microflora balance in the gut as well as intestinal health and function (230). One of the first theories to link IBD etiology to bacterial pathogens was formed upon the observation of similarities between the granulomatous nature of *Mycobacterium paratuberculosis* infection and human idiopathic granulomatous enterocolitis (108). The similarities between CD and Johne’s disease extend beyond granulomatous lesions as several studies document the isolation of *M. paratuberculosis* DNA from CD tissue biopsies (232). Investigators such as Greenstein argue that *M. paratuberculosis* is the causative agent of CD; largely based on the satisfaction of Koch’s postulates and granulomatous lesions (233). Those in opposition point out that the success of anti-TNF therapy in CD management is contrary to the observation that anti-TNF seems to reactivate latent *M. paratuberculosis* infections (234). Similarities also exist between *E. coli* infections and IBD pathogenesis. Symptomatically, adherent-invasive *E. coli* (AIEC) infected macrophage release high levels of TNFα. The levels of TNFα released by these cells is above and beyond that released by lippopolysaccharide (LPS) exposed cells, and TNFα is known to be produced in large quantities and play a major role in CD (235). It is well documented that AIEC can be recovered from CD patient biopsies/samples of the ileum, ulcers, fistulae and lamina propria macrophages (236-238). *E. coli* is universally present in Boxer dog granulomatous tissues and initiates pathomomonic granulomas in the intestinal mucosa (239). Non-pylori species of *Helicobacter*, such as *H. hepaticus* and *H. bilis*, can contribute to initiation of colitic symptoms in animal models of IBD (240-243). These studies highlight the propensity of Helicobacter colonization to parallel dysbiosis. Several *Helicobacter* species have also been detected in colitic patients
and cotton-top tamarins (244, 245). In addition to the potential role of persistent pathogenic infections in IBD pathogenesis, defective pathogen clearance has also been suggested to contribute to IBD maintenance.

Defective pathogen clearance links genetic and immune response dysfunction to the pathogenesis of IBD. Caspase recruitment domain 15 (CARD15/NOD2) genetic mutations are well documented to predispose patients to CD (246). It has been shown that mutations in these pattern recognition receptors decrease NF-κB activity related to TLR ligands and consequently increase the severity of dextran sodium sulphate (DSS) induced colitis (247, 248). The specific truncation mutation addressed in this work causes defective bacterial killing. Likewise, some CD patients exhibit low copy numbers of human beta defensin 2 and reduced production of alpha defensin by paneth cells (249, 250). Defective bacterial clearance related to neutrophil dysfunction is thought to contribute to lesions in CD patients (251-253). CD patients present with abnormal neutrophil migration, lack of superoxide generation, decreased phagocytosis and lack of bacterial killing. All of these symptoms can arise from neutrophil abnormalities. Another related potential contributor to the pathogenesis of IBD is bacterial dysbiosis.

The gastrointestinal commensal microflora is usually quite stable. Alterations in the composition or functions of the host microflora can initiate and maintain chronic intestinal inflammation (254). Studies of germ-free mice and spontaneous colitic models support the importance of the microflora in the development of IBD as spontaneous colitic onset in these models is microflora dependant (255, 256). Dysbiosis has a major impact on gut homeostasis due to the critical involvement of the commensal microflora in maintaining a healthy gut environment, metabolism and immune homeostasis (257). It is difficult to effectively study the role of dysbiosis in IBD as primary and secondary effects are currently not clear (258). Adding to this difficulty are issues of microflora cultability and lack of fecal sample correlation relative to mucosal associated commensals. IBD is characterized by an increase in mucosally associated organisms and an increase in bacterial concentrations coupled with a decrease in diversity favoring gram-negative anaerobes and
Enterobacteriacea while curtailing Firmicutes (235, 238, 259, 260). The gut mucosal environment which supports the microflora can be altered by antibiotics, stress and diet, all of which can be linked as contributors to the predisposition for IBD (213, 261, 262). It has been hypothesized that hydrogen sulfide produced by anaerobic bacteria, the concentration of which is increased in CD patients, blocks beneficial short-chain fatty acid (SCFA) metabolism by colonic epithelial cells (263). The affects of this metabolic block are epithelial starvation and one less regulatory mechanism to dampen NF-κB activation. Furthermore, it has been shown that IL-1 production, common in inflammatory diseases like IBD, enhances proliferation of virulent *E. coli* (264). As previously mentioned, populations of mucosally associated bacteria are increased in IBD patients. This observation is linked to another potential contributor to IBD pathogenesis, mucosal barrier dysfunction.

It seems that intestinal barrier dysfunction is another prospective contributor which could play a primary or secondary role in onset and maintenance of IBD (265, 266). Lack of homeostatic epithelial integrity can come about genetically or through environmental disturbance. Deficiencies in the mucosal barrier can also be considered in the context of damage or impaired healing. Acquired permeability can be the result of corticosteroids, TNF secreted by lamina proprial cells, or zinc deficiency (267-269). Some postulate that barrier defects are a genetic predisposition due to the abundance of relatives of IBD patients who manifest leaky mucosa in the absence of colitic disease and the observation that IBD patients have mucosal dysfunction between flares (265). The environment plays a role in this deficit, as animal models of colitis develop severe intestinal inflammation mediated by host microflora following acute non-steroidal anti-inflammatory (NSAID) exposure (270, 271). The mucus layer enhances barrier function as proven by the 50-fold increased uptake of Formyl-Methionyl-Leucyl-Phenylalanine (FMLP), a product of protein degradation related to injury, in the absence of the mucus layer (272). The severity of spontaneous colitis observed in mouse models of IBD caused by genetic susceptibilities to barrier dysfunction, such as the mdr1a deficient mouse model, underscore the role of barrier integrity in IBD (273-276). Deficiencies in genes
related to epithelial healing or mucosal restitution have been shown to delay the healing process following colitic insult and enhance mortality (277). Overlapping the issue of barrier dysfunction is the instance of immune dysregulation and loss of tolerance to the microflora.

Human and experimental IBD is typified by an exaggerated immune response to the host microflora (278, 279). While the host immune system usually does not react adversely to the microflora, this tolerance is lost in the setting of IBD (135, 280). A Th1/Th17 cytokine profile has been observed in CD patients while those diagnosed with UC exhibit a mixed Th1/Th2 profile (281). Specifically, it has been shown that lamina proprial mononuclear cells produce elevated levels of IL-12, IL-17 and IFNγ, while UC patients produce more IL-5 and IL-13. Dendritic cells from IBD patients have been found to be hyperactive when stimulated with LPS (278). This is significant given the role of DC in pro-inflammatory cytokine production and promotion of effector T cell differentiation (109). Abnormalities in TLR and NOD receptor function are thought to contribute to the inappropriate activation of DC in the context of IBD (108). This DC hyperactivity leads to aberrant T cell activity and an imbalance between T effector and T regulatory cell populations (282, 283). This observation pairs with the fact that T effector apoptosis is greatly diminished in the context of IBD. Chronic intestinal inflammation in IBD is also typified by increased B cell activity in which plasma cells produce antibodies specific to the host microflora (223, 240, 241, 279). This observation underscores the loss of immunologic tolerance to the resident microflora in the setting of IBD and the role of immune dysregulation in the chronicity of disease.

In summary, current research has made an effective argument for the multifocal nature of IBD onset relating to genetic predisposition, environmental contributions, immune dysfunction and microfloral antigens. Several etiopathogenic hypotheses emphasize the overlap in these factors. Persistent infections, defective pathogen clearance, defective barrier function and restitution, dysbiosis and loss of immune tolerance to the microflora have all been implicated in the onset and
chronicity of IBD. The relevance of these implications are eloquently illustrated in the many animal models of experimental IBD.

**Animal models of IBD**

Research would be decades behind without the advent of animal models of gastrointestinal inflammation to uncover aspects of pathogenesis related to the idiopathic collection of disorders encompassing IBD. With respect to IBD, this area of research and model development began with the 1960s ovalbumin/formalin rabbit model of colonic hypersensitivity developed by Kraft and Kirsner. Since that time, mouse and rat models of colitis have become the most widely utilized tool in IBD research with regard to mechanistic, pathogenic, therapeutic and surgical investigations. Due to the multifocal and highly variable presentation among patients suffering from UC or CD, it is important to note that no “perfect” model of UC or CD exists. Most models highlight one or several aspects of a symptomatic/pathogenic mix of UC and CD. Table 1 below is adapted from Kirsner’s Inflammatory Bowel Diseases, sixth edition, chapter 9 (284). This table is meant to summarize the vast number and mechanistic depth of the most utilized and characterized models of IBD in use currently.
## Table 1. Summary of popular animal models of IBD

<table>
<thead>
<tr>
<th>Method of Induction</th>
<th>Animal Model Designation/Title</th>
<th>Immune Mediation of Inflammation</th>
<th>Pathogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemically Induced</strong></td>
<td>Acetic acid</td>
<td>Innate</td>
<td>Arachidonic acid pathway activation</td>
</tr>
<tr>
<td></td>
<td>DSS</td>
<td>Innate (acute)</td>
<td>Epithelial toxin</td>
</tr>
<tr>
<td></td>
<td>Indomethacin</td>
<td>Innate</td>
<td>Epithelial toxin</td>
</tr>
<tr>
<td></td>
<td>TNBS/alcohol</td>
<td>Innate (acute)</td>
<td>Overly aggressive effector cell response</td>
</tr>
<tr>
<td></td>
<td>PG-PS</td>
<td>Th1 (chronic)</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td></td>
<td>Oxazalone</td>
<td>Th2</td>
<td>IL-4 or IL-13 producing NK T cells</td>
</tr>
<tr>
<td><strong>Spontaneous</strong></td>
<td>Cotton-top tamarin</td>
<td>Mixed</td>
<td>Mucin and T cell abnormalities</td>
</tr>
<tr>
<td></td>
<td>C3H/HeJ Bir</td>
<td>Th1</td>
<td>Overly aggressive effector cell response</td>
</tr>
<tr>
<td></td>
<td>Samp- lYit</td>
<td>Th1</td>
<td>Overly aggressive effector cell response</td>
</tr>
<tr>
<td><strong>Genetically Engineered</strong></td>
<td>A20</td>
<td>Th1</td>
<td>Overly aggressive effector cell response</td>
</tr>
<tr>
<td></td>
<td>CD40 L TG</td>
<td>Th1</td>
<td>Overly aggressive effector cell response</td>
</tr>
<tr>
<td></td>
<td>HLA-B27/β2µ TG Rat</td>
<td>Th1</td>
<td>Regulatory cell activity</td>
</tr>
<tr>
<td></td>
<td>IL-2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Th1</td>
<td>Defective regulation/</td>
</tr>
<tr>
<td></td>
<td>IL-2R&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Th1</td>
<td>Regulatory cell activity</td>
</tr>
<tr>
<td></td>
<td>IL-10&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Th1</td>
<td>Defective regulation/</td>
</tr>
<tr>
<td></td>
<td>IL-10R&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Th1</td>
<td>Regulatory cell activity</td>
</tr>
<tr>
<td></td>
<td>Gio2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Th1</td>
<td>Overly aggressive effector cell response</td>
</tr>
<tr>
<td></td>
<td>Mdr1a&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Th1</td>
<td>Epithelial barrier dysfunction</td>
</tr>
<tr>
<td></td>
<td>N-cadherin DN</td>
<td>uncharacterized</td>
<td>Epithelial barrier dysfunction</td>
</tr>
<tr>
<td></td>
<td>STAT-3&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Th1</td>
<td>Defective regulation/</td>
</tr>
<tr>
<td></td>
<td>STAT-4 TG</td>
<td>Th1</td>
<td>Regulatory cell activity</td>
</tr>
<tr>
<td></td>
<td>TCRα&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Th2</td>
<td>Overly aggressive effector cell response</td>
</tr>
<tr>
<td></td>
<td>TGFβ RII DN TG</td>
<td>Th1</td>
<td>Defective regulation/</td>
</tr>
<tr>
<td></td>
<td>TGFβ&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Th1</td>
<td>Regulatory cell activity</td>
</tr>
<tr>
<td></td>
<td>TNF&lt;sup&gt;ΔARE&lt;/sup&gt;</td>
<td>Th1</td>
<td>Overly aggressive effector cell response</td>
</tr>
<tr>
<td><strong>T cell Transfer</strong></td>
<td>CD45RB&lt;sup&gt;hi&lt;/sup&gt; → SCID</td>
<td>Th1</td>
<td>Defective regulation/</td>
</tr>
<tr>
<td></td>
<td>BM → CD3ε TG</td>
<td>Th1</td>
<td>Regulatory cell activity</td>
</tr>
</tbody>
</table>

<sup>-/-</sup> = deficient or knockout, DN = dominant negative, TG = transgenic, BM = bone marrow
**Dextran sodium sulphate induced model of colitis**

This section will describe the DSS mouse model of colitis in some detail as it is relevant to later chapters of this dissertation. Dextran sodium sulphate is extremely chemically stable and used to induce colitic symptoms by dosing in drinking water. Generally used in rodent modalities, 2.5% to 10% dosed consecutively for 5 to 7 days results in pancolitis (285). Continuous dosing for 7 days is typical of a progressively severe DSS colitic model. A controlled lesion is achieved by dosing an initial high DSS dose for 2 days followed by a mild dose for up to 5 days (286). Repeated cycles of DSS dosing and DSS free restitution periods has been used to simulate chronic colitis (287). DSS susceptibility varies based on murine background, with Balb/c mice being very resistant and C57BL/6 mice being more susceptible.

Severity of DSS colitis and related inflammation is tunable in a dose response manner based on concentration and duration of dosing (285, 288). DSS colitis is variably characterized by weight loss, mild to severe intestinal ulceration, colonic shortening, lymphoid aggregation, as well as mononuclear and neutrophil infiltrate. Chronically, DSS colitis can lead to epithelial dysplasia and adenocarcinoma.

DSS is generally thought to be an epithelial toxin, but recent data allude to other mechanisms of pathogenesis as treatment of DSS exposed mice with muramyl-dipeptide (a NOD2 ligand) has been shown to ameliorate DSS induced colitis (289, 290). Blockade of IL-18 also abolishes DSS induced disease (291). DSS is concentrated in phagolysosomes of lamina proprial macrophage and has been shown to increase IL-1 production by macrophage in vitro (292). Data implicating bacterial involvement in the pathogenesis of acute DSS colitis is mixed. DSS insult causes an increase in *Bacteroides* species in the gut, can be attenuated by antibiotics in some instances and is variably effective in different germ-free mouse models (256, 293, 294). Pathogenesis of acute DSS colitis does not exhibit a T cell component but does result in typical Th1 cytokine production (288). Acute pathogenesis in this model is thought to involve epithelial injury and related activation of neutrophils and innate cytokine and chemokine cascades (288, 295-
Chronic DSS colitis does include T cell activity and is typified by a Th1 and Th2 cytokine profile mixture (298). Some hypothesize that repeated epithelial injury, and not T cell activity is the only true requirement for even chronic DSS colitis (299). The DSS model of colitis is cost-effective, easily adjustable with regard to severity, and convenient for simple screens of drug efficacy in acute colitis.

**Mdr1a deficient mouse model of spontaneous colitis**

This section will describe the mdr1a mouse model of colitis in some detail as it is relevant to later chapters of this dissertation. The genetic basis of this model involves the gene for one member, mdr1a, of the ATP binding cassette transporter family (300). This is the multiple drug resistance gene for P-glycoprotein 170. This transporter is expressed in many tissues including luminal intestinal epithelial cells, with a heavy concentration in colonic epithelial cells. The function of mdr1a pump is to transport xenobiotics and amphipathic cations across membranes in an ATP dependent manner. The mdr1a transporter has been identified in mouse and human tissues and is a vital means of drug efflux (301). As mdr1a is expressed in blood-brain capillaries, this knockout mouse model was originally developed to enhance the permeability of the blood-brain barrier for the purpose of studying compound uptake and efficacy in the brain in the absence of p-glycoprotein drug efflux (300). The surprise observation was noted that these mdr1a deficient (mdr1a^−/−) mice developed colitis between 8 and 30 weeks of age (274). As previously mentioned, mdr1a is heavily expressed on intestinal epithelial cells. It is therefore not surprising that deficiency of mdr1a predisposes these mice to intestinal epithelial barrier dysfunction (302). Despite this obvious contribution to colitic onset, the mechanism of colitic onset and maintenance is not fully understood for this model for several reasons. The mdr1a transporter is expressed on several immune cells, including CD4^+^ and CD8^+^ T cells, DCs, NK cells and PBMCs (276). While the functions of these cells seem intact in mdr1a^−/−^ mice, the implications of mdr1a deficiency for the individual functions of these cells have yet to be fully investigated. P-glycoproteins and transporters like them have been implicated in cholesterol metabolism and regulation of cell differentiation, proliferation and survival (276). This data would
indicate that mdr1a deficiency could have effects reaching beyond that of epithelial barrier integrity which could contribute to IBD pathogenesis. Additionally, the precise development and mechanism of barrier dysfunction in mdr1a^{-/-} mice is unclear. It has been hypothesized that lack of the mdr1a transport function leads to a build-up of bacterial byproducts within epithelial cells, leading to increased antigen presentation of bacterial products and a subsequent loss of tolerance due to a robust T cell response to those products (276). Early, pre-colitic cytokine changes have been discovered in mdr1a^{-/-} mice, which would support this hypothesis (302).

The average age of colitic onset in mdr1a^{-/-} mice occurs at 20 weeks of age (274). This colitis is non-transmissible and has been shown to be preventable by antibiotic therapy. Metronidazole dosed in the drinking water has specifically been shown to ameliorate colitis in this model (275). This evidence and the observation that mdr1a^{-/-} mice housed in germ free conditions do not develop colitis speaks to the vital contribution of the microflora to colitic onset in this model. Colitic lesions in mdr1a^{-/-} mice are transmural and generally continuous throughout the colon (303). Mucosal edema, thickening and lamina proprial inflammatory cell infiltrate are common to these lesions.

Colitic susceptibility of mdr1a^{-/-} mice is increased by DSS administration, but not by TNBS (275). No one bacterial species has been implicated as responsible for colitic susceptibility in mdr1a^{-/-} mice, but it has been shown that *H. bilis* accelerates colitic onset and severity in these mice while *H. hepaticus* delays onset of colitis in mdr1a^{-/-} mice (304).

This model is also relevant to human IBD. Enhanced expression of IFNγ, IL-6, IL-1β, TNFα, MCP-1, MIP-1α, RANTES and Eotaxin mRNA, along with increased expression of the chemokine receptors CCR2 and CCR5 is comparable to changes seen in patients with UC and CD (273). Furthermore, mdr1a has been directly implicated in human UC and CD (276, 300, 305).

The totality of this data indicates that the mdr1a^{-/-} mouse model of colitis is a relatively well characterized and relevant model for the study of human and animal IBD.
**Therapeutic and surgical perspectives on IBD**

This section will review relevant therapies commonly used for the treatment of IBD as well as the use of surgical interventions for UC and CD patients. Aminosalicylates, immunomodulators, biologic therapies and CAM use will be reviewed in the context of IBD therapeutic benefits, drawbacks to therapy, and patient and physician concerns associated with treatment.

Implications for surgery differ between patients suffering from CD and UC, and these implications also vary depending upon the severity and symptomatic manifestations and persistence of these forms of IBD (306-308). Current estimates emphasize that up to 75% of CD patients will require discussion with their physicians regarding surgical intervention (309). Absolute indications for surgical intervention in CD include perforation, severe hemorrhage, therapeutically non-responsive complete bowel obstruction, or high-grade dysplasia (203). Early laproscopic intervention is generally acceptable and feasible given the patchy nature of CD (309). It is still controversial as to whether early surgical intervention should immediately follow therapeutic non-responsiveness or if development of more severe symptoms should be allowed to manifest first (310). Bowel resection is unfortunately not rare in patients with CD, but is considered a last resort to therapeutic failure and minimally invasive procedures (311). Due to the continuous and concentrated colonic nature of UC, this form of IBD is viewed as a surgically ‘curable’ condition (312-314). This observation is leads to strong biases in opinions regarding medical practices of therapeutic vs. surgical intervention strategies (306). While it is true that immunomodulatory therapies are a newer arm of UC treatment, the idea of therapeutic first-line treatment is only now becoming more accepted in the treatment of UC. Absolute indications for surgical intervention in UC include perforation, colonic obstruction caused by stricture, high-grade dysplasia or uncontrolled hemorrhage (179, 312, 315, 316). Advances in therapeutic strategies have made surgical intervention less likely for patients with secondary toxic megacolon or fulminant colitis (317, 318). If colectomy is indicated, it usually occurs as two operations and up to 10% of patients must undergo further procedures to
repair ileal pouch failure (316). It is also estimated that 30 to 60% of patients will experience post-operative pouchitis. This information strengthens the need for development of new therapeutic strategies for UC and CD as well as continued optimization of current therapeutic modalities.

Aminosalicylates, or 5-amino salicylic acids (5-ASA)/mesalazine, are commonly used to prevent recurrent flares associated with mild to moderate UC but are not generally recommended for the treatment of CD as most clinical trial data in the context of mesalazine CD therapy is mixed (319, 320). One clinical trial noted a 90% positive response rate in UC patients receiving 5-ASA therapy who were non-responsive to prior treatments (321). 5-ASAs are generally not used as first line treatments but as maintenance therapies for UC (322). Mesalazine is generally safe and well tolerated. The mechanisms of 5-ASA efficacy are not well defined but mesalazine has been found to be inactive in 5-aminosalicylic acid is ineffective in peroxisome proliferator-activated receptor-gamma+/- mice (PPARγ+/−) (323). It is known that 5-ASA activity is related to topical, not absorptive effects (324, 325). Due to this observation, 5-ASA vehicles were being formulated to increase contact time with the mucosa and delay dissolution of the drug, but these have been found to be more toxic (323). 5-ASAs are most effective when dosed rectally when compared to oral 5-ASA efficacy or rectal corticosteroid treatment (326). Patient compliance related to 5-ASA dosing is very poor (327, 328). New oral dosing strategies and titration recommendations are being proposed to improve patient compliance (329). Side effects include headache, nausea, dyspepsia, and diarrhea. Less common are the side effects of pleuritis, pericarditis, myocarditis, pancreatitis, cholestatic hepatitis, nephritis, renal dysfunction and increased colitic symptoms related to osmotic 5-ASA effects (326, 330). The use of 5-ASA can also be very expensive, especially given the newly suggested daily oral treatment regimen.

Another common therapeutic modality for IBD is a group of immunomodulatory drugs. Thiopurines (azathioprine and 6-mercaptopurine), corticosteroids (budesonide, dexamethasone, hydrocortisone, methylprednisolone, prednisolone and prednisone) and methotrexate are indicated for maintenance of
remission in those diagnosed with mild CD or chronically active/relapsing UC which is refractory to 5-ASA drugs (331). Dosing these immunomodulators involves a slow, three month titration process for safety (331). Delicate titration underscores the toxicity caused by these modalities, which are also plagued with variable metabolism between individual patients (331-336). The greatest drawback to this treatment strategy is the broad-immunosuppression caused by these drugs. Secondary treatments, such as antibiotics, are often required to substitute for the lack of homeostatic immune function inhibited by these drugs. To address these issues, pharmaceutical companies are developing new formulations, dosing strategies, and delivery methods for immunomodulators. This is especially true for thiopurines and corticosteroids, which seem to have the most wide-spread side-effects (334, 337, 338). These therapies are generally more affordable to patients than several other therapeutic modalities (331).

Antibiotics have been investigated as therapies for the treatment and remission maintenance of UC and CD, but will not be discussed at length here as they are not commonly reliable for primary IBD treatment. Metronidazole, ciprofloxacin and other antibiotics are more commonly utilized to treat secondary complications of IBD (i.e. toxic megacolon, abscess, secondary infection, sepsis) and are not successfully utilized long term (339).

Perhaps the newest therapeutic options for IBD patients are biologic therapies, the most utilized and well studied being anti-TNFα antibodies. Other biologic therapies such as monoclonal antibodies to IL-12/23p40,(ustekiumab), IL-6 (tocilizumab), CD-25 (basiliximab), and chemokine/adhesion blockers (natalizumab) are also studied for efficacy in IBD therapy but will not be discussed at length in this review (340). As anti-TNF antibody therapies, infliximab, adalimumumab and certolizumab are specifically targeted in their immunologic suppression (340). It is generally accepted that anti-TNF infusions extend remission, shorten hospital stays and improve the quality of life of those IBD patients who do not respond to the more conventional therapies previously discussed in this section (341, 342). Biologic therapies intensify debates regarding ‘step-up’ vs. ‘top-down’ treatment strategies
and the combination of biologic and conventional therapies (343, 344). While antibody therapies exhibit great efficacy in the maintenance of remission of UC and CD, their use is not without drawbacks. TNFα is not simply a contributor to the detrimental inflammation associated with IBD, but is also necessary for homeostatic immune functions and host protection against illness and infection (338, 340). The most dangerous potential side effects of anti-TNF therapies are secondary infections or development of malignancy (345-348). Several studies have shown a correlation between the use of anti-TNF therapies and the increased instance of tuberculosis infections (349). An increased instance of T cell lymphomas also has been shown to correlate with biologic suppressive therapies (350). Furthermore, due to the relatively new use of this type of therapy, long term efficacy and safety studies are not complete (350). Patients can become refractory to anti-TNF therapies and these patients have been shown to respond poorly, or not at all, to alternate biologic therapies (351). IBD patient compliance is difficult to maintain with biologic therapies despite their efficacy, due to very high cost worldwide and general inaccessibility to these drugs in developing countries where the instance of IBD is on the rise (344). In summary, anti-TNF antibodies are the newest therapeutic option for IBD and have great therapeutic efficacy, but lack long term study and their use is not without safety concerns.

This section will conclude with a short discussion of probiotic and prebiotic/herbal CAM as primary and adjunct therapies for IBD, as the focus of the data in this dissertation relates to the use of herbal extracts as therapeutic and prophylactic modalities in models of IBD. In addition to the three forms of CAM listed above, exercise, meditation, chiropractic and osteopathy are also utilized but are not relevant to the work presented in this dissertation and will not be discussed at length. Using a very broad definition of CAM, Burgmann et al published that up to 60% of Canadian IBD patients surveyed used CAM as primary or supplementary treatments for CD or UC (352). As opposed to physician recommendation, CAM is usually sought out by patients for the purpose of direct or indirect disease related benefits (7). Probiotics are a relatively new CAM and are defined by the content of
viable microorganisms which at least transiently colonize the intestine. Examples of common probiotic organisms include lactic acid bacilli, Lactobacillus, Bifidobacterium, *E. coli Nissle 1917*, *Saccharomyces boulardii*, *Clostridium butyricum*, *Streptococcus salivarius thermophiles* and bacteria genetically engineered to secrete IL-10 (257). Broad probiotic bioactivity can encompass anti-pathogenic suppression of growth or suppression of binding and invasion, improved epithelial barrier function, and immunoregulatory activities (259). The greatest efficacy of probiotics is seen in studies involving pouchitis and UC therapy. One clinical study showed that daily dosing of VSL-3 (a combination of 4 *Lactobacillus* species, 3 *Bifidobacterium* species and an *S. salivarium*) prevented chronic pouchitis relapse for the 9 month trial following antibiotic induction of remission (353). With regard to UC therapy, *E. coli Nissle 1917* has been shown to be equally effective as low dose 5-ASA in maintenance of remission (354, 355). Prebiotic therapies for IBD can include dietary components that stimulate the growth or metabolism of the enteric host microflora or influence immune function (356-358). Nutraceuticals are considered by most to fall into this category. Examples of prebiotics documented in the literature include lactosucrose, fructo-oligosaccharides, inulin, bran, germinated barley and psyllium (359). These prebiotics/nutraceuticals can encourage the growth of *Lactobacillus* and *Bifidobacterium* species which produce the short chain fatty acid butyrate (360). Increases in butyrate production effectively nourish the intestinal epithelium. Prebiotics can also stimulate the growth of protective lactic acid bacilli to stave off pathogenic bacteria (361). Several prebiotics have also been implicated in immunomodulation and anti-inflammatory bioactivities (362-364). Antioxidant effects of berries have been shown to contribute to the amelioration of experimental colitis (365). Herbal therapies are underrepresented in the literature of most diseases, but surprisingly, are often documented as the most commonly utilized CAM by IBD patients (366-368). The following section will address the specific nutraceutical/herbal extracts investigated in the experimental chapters of this dissertation.
**Prunella vulgaris**

This section will briefly review the origins of medicinal use of *P. vulgaris*. Additionally, relevant chemical compounds identified in *P. vulgaris* related to potential bioactivity in vitro and in vivo will be described.

**Origin and preparation for medicinal use**

Termed ‘Self-heal’ or ‘Heal-all’, *P. vulgaris* (PV) is a perennial flowering plant found throughout Europe, Asia and North America. PV is well documented as a traditional medicinal Chinese herb (369). Traditional medicinal indications for PV include wound healing, indigestion, burns and anti-inflammatory therapy (370). In Asia, PV is generally steeped in hot or boiling water as a tea or occasionally ground into a save (369). Similar to traditional preparations, the experiments utilizing PV in later chapters of this dissertation present use of the extract of the above-ground portions of PV prepared by steeping and boiling in the soxhlet extraction method.

**Constituents**

*P. vulgaris* contains several bioactive phenolics, triterpenoids, and flavonoids (371). Dietary phenolics like rosmarinic acid, ursolic acid and caffeic acid, which are all found in *P. vulgaris*, have been shown to have antioxidant, anti-inflammatory and anti-cancer activities (372-377). Caffeic acid has also been shown to effectively attenuate chemically induced experimental colitis through upregulation of cychrome P450 (CYP4B1) (378). Flavonoids, like those found in *P. vulgaris*, have been implicated as potential therapeutics for IBD as well. The flavonoid luteolin was found to attenuate spontaneous colitis by downregulating NF-κB. Recently rosmarinic acid, the most plentiful phenolic compound found in *P. vulgaris*, was found to protect against sepsis by downregulating inflammatory genes in the NF-κB pathway and the related inflammatory cytokines TNFα and IL-6 (379).

**Bioavailability**

Bioavailability varies from compound to compound and can be considered in the context of a tissue/cell type or in the context of metabolic interactions with host microflora. Qiang et al recently described the permeability of rosmarinic acid and ursolic acid, two major components found in PV, across human intestinal epithelial
Caco-2 cell monolayers (380). As detected by HPLC, it was found that rosmarinic acid and ursolic acid contained in whole extracts of PV were able to permeabilize intestinal epithelial monolayers with equal efficiency as purified rosemarinic acid or ursolic acid. Additionally, UA is not subject to intestinal glucuronidation/sulfation which would potentially increase the bioavailability of this compound. It should be noted that this dissertation focuses on intestinal bioavailability, which can be uniquely altered by microflora metabolism and the condition of the intestinal, luminal epithelial barrier in the context of health and homeostasis as well as during onset and maintenance of colitis (381). The epithelial barrier changes which occur in the intestine in the context of colitis could dramatically alter the cell types and tissues to which target compounds could be exposed and bioavailable to (302). The diversity and quality of the microflora is also altered in the colitic intestinal milieu which could additionally alter bioavailability of these plant compounds (257).

**Aim of the use of *Prunella vulgaris* in this dissertation**

To date, PV has not been studied as a primary or adjunct therapy for IBD. The aim of use of ethanolic PV extracts in original studies described in the following chapters of this dissertation is to uncover potential efficacy of PV as a therapeutic or prophylactic modality for the treatment of experimental colitis relevant to human and animal medicine. Additional studies seek to address the mechanism of such potential efficacy in anti-colitic bioactivity.

**Echinacea angustifolia**

This section will briefly review the origins of medicinal use of *E. angustifolia*. Additionally, relevant chemical compounds identified in *E. angustifolia* related to potential bioactivity in vitro and in vivo will be described.

**Origin and preparation for medicinal use**

Termed ‘coneflower’ or ‘Kansas snakeroot’, *E. angustifolia* (EA) is a perennial flowering plant found throughout North America (382). Numerous traditional medicinal uses of EA are documented and most involve fresh or dried preparations of the roots. Past and present medicinal indications for EA include its use as an antibiotic, antiviral, antiseptic and anti-toxin for snake bites (382). EA is most
commonly considered as therapeutic for infections of the sinuses, respiratory tract, urinary tract, and skin, but also has been documented to speed wound healing/restitution (383). EA can be found in pill, capsule, powder, liquid and save form (384). The experiments utilizing EA in later chapters of this dissertation describe use of the root extracts of EA prepared by steeping and boiling in the soxhlet extraction method.

**Constituents**

Barnes et al. have hypothesized that no single compound in EA is responsible for its medicinal or bioactive efficacy (383). This hypothesis is supported by the idea of synergistic relationships between chemical constituents in whole or partially fractionated plant preparations or extracts, in which the mixture fosters chemical stability and biologic activity (385). Compounds in EA which are thought to contribute to bioactivity of EA include ketones, alkamides and caffeic acid derivatives (386-389). These constituents have been implicated in the anti-infective and anti-inflammatory activities of EA. Recent work has shown that EA extracts can cause reductions in prostaglandin E2 (PGE2) production and cyclooxygenase 2 (COX2) activity (387).

**Bioavailability**

Matthias et al. has characterized the ability of caffeic acid derivatives and alkamides from EA to cross monolayers of human intestinal epithelial Caco-2 cells (388). The caffeic acid conjugates caftaric acid, echinacoside and cichoric acid exhibited poor epithelial permeability, while cinnamic acid readily diffused across the monolayer. Alkamides readily permeated the intestinal monolayer. The authors concluded that alkamides and cinnamic acid, but not other caffeic acid conjugates, would be readily bioavailable in vivo. Permeability of these compounds was not altered by the presence of other chemicals in the whole extract as permeability was compared to, and not found to differ from, that of purified compounds. As mentioned previously, for the purpose of use in original studies described in the following chapters of this dissertation, this bioavailability data must be also be considered in the context of a compromised, colitic milieu.
Market availability and reliability

EA is commonly found as a nutraceutical supplement in commercial markets worldwide (384). Nutraceuticals and dietary supplements are not regulated by the Food and Drug Administration (FDA), in the United States, to a level greater than that of food safety. Several investigators have chemically analyzed commercial EA products sold to the public for content of EA constituents and quality. A 2003 study of 25 commercially available *Echinacea* extracts found that only 56% of the extracts tested were of claimed quality (390). Many of the 25 products did not state the type of *Echinacea* they contained or specify estimated *Echinacea* content at all. One of the 25 products was contaminated with 3 times the allowable concentration of microbes as defined by World Health Organization (WHO) guidelines. Another study found that only 10% of 59 commercially available *Echinacea* products contained any detectable constituents of *Echinacea* (384). *Echinacea* extracts and products containing *Echinacea* are readily available but rarely of dependable quality and are not FDA regulated for quality of claims.

Aim of the use of *Echinacea angustifolia* in this dissertation

To date, EA has not been studied as a primary or adjunct therapy for IBD. The aim of use of ethanolic EA extracts in original studies described in the following chapters of this dissertation is to uncover potential efficacy of EA as a prophylactic modality for the treatment of experimental colitis relevant to human and animal medicine.

*Hypericum gentianoides*

This section will briefly review the origins of medicinal use of *H. gentianoides*. Additionally, relevant chemical compounds identified in *H. gentianoides* related to potential bioactivity in vitro and in vivo will be described.

Origin and preparation for medicinal use

Very little reliable scientific data is available regarding *H. gentianoides* (HG). A current Pub med search yields only one reference. HG, also commonly called ‘orangepass’ is dispersed throughout the eastern United States. HG was used
medicinally by Native Americans to reduce gastrointestinal upset, fever and speed wound healing (391).

**Constituents**

This species of *Hypericum* is unique in that it contains the anti-bacterial compounds uliginosin A and B but lacks hypericin, pseudohypericin and hyperforin.(392, 393) This could indicate that HG could retain some anti-bacterial activity and have fewer potential toxic side effects when compared to other *Hypericum* species. Recently it was shown that HG reduces inflammatory prostaglandin E2 (PGE2) production in RAW 264.7 macrophage cells with no significant cytotoxicity.(393)

**Bioavailability**

Bioavailability of compounds in the context of HG extracts have not been evaluated scientifically.

**Aim of the use of *Hypericum gentianoides* in this dissertation**

To date, HG has never been studied in vivo or as a primary or adjunct therapy for IBD. The aim of use of ethanolic HG extracts in original studies described in the following chapters of this dissertation is to uncover potential efficacy of HG as a prophylactic modality for the treatment of experimental colitis relevant to human and animal medicine. Additional studies seek to address the mechanism of such potential efficacy in anti-colitic bioactivity.
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CHAPTER 2. Assessment of the in vivo anti-inflammatory activities of the ethanolic extracts of *Echinacea angustifolia* or *Prunella vulgaris* in the dextran sodium sulphate model of colitis

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Abstract

**Background.** Due to known side-effects associated with IBD therapies, complementary or alternative medicine (CAM) is being recognized as an adjunct therapeutic approach to treat or ameliorate chronic inflammatory diseases. In this context, and in light of the low toxicity and numerous bioactive compounds in these herbs, nutraceutical extracts derived from *Echinacea angustifolia* (EA) and *Prunella vulgaris* (PV) were investigated for their in vivo anti-inflammatory therapeutic potential in the dextran sodium sulphate (DSS) model of colitis. **Methods.** Vehicle (5% ethanol), EA (100 or 200 mg/kg) or PV (100 mg/kg) were orally administered daily to C57BL/6 mice (6-7 weeks old) prior to DSS treatment and during DSS insult. EA was also studied to evaluate its ability to enhance the restitution of epithelial lesions induced by DSS. Macroscopic disease assessment included measurement of daily weight, colon length and combined colonic/cecal score. **Results.** EA prophylaxis did not significantly attenuate the severity of weight loss, macroscopic
disease/inflammatory scores, or colon lengths directly following DSS treatment. Likewise, there was no observed benefit to lesion resolution for mice treated with EA. Mice receiving PV prophylactically had significantly ($P < 0.05$) less weight loss, lower macroscopic disease/inflammatory scores, and more normal colon lengths following exposure to DSS. In contrast, the use of PV as a therapeutic treatment did not attenuate the severity of colitis. **Conclusions.** The ethanolic extract of PV ameliorated the severity of DSS-induced colitis and warrants further examination as a therapeutic and immunomodulatory agent for the treatment of gastrointestinal inflammatory diseases.

**Key Words:** *Echinacea angustifolia, Prunella vulgaris*, colitis, IBD, DSS

**Introduction**

The addition of herbal forms of CAM to dietary and therapeutic regimens is growing in popularity worldwide. Low toxicity, high patient interest, improved compliance, low cost, and increased availability have resulted in acceptance of the general public, physicians and researchers as to the potential of botanically-derived materials to improve human and animal health. As therapeutic or prophylactic modalities, CAM is underrepresented in high-impact studies, clinical trials, and educational programs.¹ Contrary to this point, over half of the pharmaceuticals developed in the last two decades are derived from naturally occurring compounds.² Unlike purified compounds, whole herbal extracts in particular have the advantages of containing combinations of anti-inflammatory, anti-bacterial, anti-viral, and antioxidant compounds while inducing fewer toxic side effects due to synergy of the multiple components.³ ⁴ Two examples of herbal CAM which are used as dietary supplements or secondary therapeutic regimens are EA and PV.

Most thoroughly characterized for its use in the prevention or treatment of the common cold and other infections, EA is also gaining repute for anti-inflammatory and immunomodulatory activities.⁵ EA has been shown to reduce production of prostaglandin E2 (PGE2) and cyclooxygenase 2 (COX2) activity. ⁶ Recent studies point to the bioactivity of alkylamides, caffeic acid derivatives, and ketones as key
components in the anti-infective and anti-inflammatory activities of EA.\textsuperscript{6, 7} Due to its ease of cultivation and previous reputation as a cold remedy, it is readily available in the United States and worldwide.\textsuperscript{8}

As a medicinal plant, PV is infrequently utilized in the United States but has long been used in traditional Chinese medicine, primarily for the purpose of wound healing.\textsuperscript{9} The presence of polyphenols, triterpenes and flavonoids are well documented in PV extracts and these compounds have been implicated in anti-viral, anti-bacterial, anti-inflammatory, and immunomodulatory bioactivities.\textsuperscript{10-16} The variety of potentially bioactive compounds and growing amount of literature regarding this plant will certainly influence its medicinal use.

These nutraceuticals are prime candidates for therapeutic research involving idiopathic inflammatory diseases for several reasons. Many of these diseases are etiologically poorly understood. Problems with current therapeutic strategies arise with respect to the high cost, low specificity, symptomatic rather than causative treatment, poor patient compliance, and lack of reproducibility and predictable effect across patient populations. In this context, inflammatory bowel diseases (IBD) are no exception. The primary IBD presentations, Crohn’s disease (CD) and ulcerative colitis (UC) are highly complex and symptomatically variable among patients.\textsuperscript{17} Typical IBD manifestations include relapsing gut mucosal lesions of variable placement and severity throughout the digestive tract.\textsuperscript{18} With recent adult CD and UC instance in the USA estimated at 238/100,000 and 201/100,000 and pediatric instance estimated at 43/100,000 and 28/100,000, respectively, IBD is a growing medical and economic concern.\textsuperscript{19} Perhaps even more troubling is the knowledge that the most effective current biologic therapies, such as anti-TNF monoclonal antibody infusions, are as costly as to be scarce in countries where IBD instance is increasing at the greatest rate.\textsuperscript{20, 21} To compound this issue, 20 to 30 percent of patients with CD and 30 to 40 percent of patients with UC eventually become refractory to the continued use of anti-TNF therapy and fail to respond to a switch in anti-TNF agent.\textsuperscript{22-26} The difficulties in titration, toxicity, low tolerance, low patient compliance, and delayed activity related to use of the traditional corticosteroids,
mesalazines, and other immunosuppressants underscore the need for additional medications to combat side effects and secondary infections.\(^{27}\)

Because of the current drawbacks associated with IBD therapy to date, CAM is being recognized specifically in the context of IBD therapeutic regimens. An estimated 50 percent of IBD patients in North America utilize some form of CAM.\(^{28,29}\) IBD patients specifically utilizing herbal supplementation represent a large portion of CAM-related use worldwide.\(^{29}\) While the use of CAM is increasing worldwide, neither EA nor PV has been evaluated for biologic activity in acute colitis. In this study, we evaluated the efficacy of the ethanolic extracts of EA or PV to prevent or attenuate acute colitis induced by DSS. The results of these studies will increase the breadth of our understanding regarding the capabilities of these plants to be used as therapeutic modalities to combat the inflammatory complications associated with colitis.

**Materials and Methods**

**Reagents.** EA and PV dried plant materials were kindly provided by Dr. Mark Wiederlichner at the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS, Ames, IA). DSS (MW 36,000–50,000) was purchased from ICN Biomedicals (Aurora, OH) and 95% ethanol was purchased from Sigma (St. Louis, MO).

**Echinacea angustifolia or Prunella vulgaris extract preparation.** Information about the specific provenance of EA or PV accessions (PI 636395 or Ames 27748, respectively) obtained from the NCRPIS, is available on the Germplasm Resources Information Network database at http://www.ars-grin.gov/npgs/acc/acc_queries.html. Roots of EA (PI 636395) or arboreal portions of plants from PV (Ames 27748), harvested in 2008, were prepared for storage by drying for 8 days at 38°C in a forced-air dryer with constant humidity. The dried material was ground with a 40-mesh screen and stored at -20 °C until extraction. Extractions were made in 95% ethanol (EtOH) solvent by the Soxhlet method (6 hours).\(^{30}\) Upon complete drying of the extract by evaporation, the weight of the
extracted material was recorded; the residue was lyophilized and stored at -20 °C. Prior to administration to mice, the extract was solubilized in 5% EtOH (the lowest concentration of EtOH that would allow solubilization) at a final plant extract concentration of 100 or 200 mg/kg for an average 20 gram mouse (2.4 or 4.8 mg/mL respectively). The working solutions of each extract were divided into 2 mL aliquots and stored at -20°C until use. The ethanolic extracts were screened for endotoxin by using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD) according to manufacturers’ specifications for a microplate assay, and there was no detectable endotoxin present in either extract (data not shown).

**Animals.** All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University before any experiments were performed. Six to eight week old male and female C57BL/6 (B6) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed and maintained in the Laboratory Animal Resource facility at the College of Veterinary Medicine, Iowa State University. Established specific pathogen-free husbandry practices were followed and 12 hour light/dark cycles were applied. Upon arrival, mice were fed a defined Harlan Teklad AIN93 (M) rodent chow (Madison, WI) to control the amount of phytochemicals in their diet.

**Experimental Design.** This work describes two experimental designs used to assess 1) the ability of the extracts to prevent or attenuate colitis and 2) the impact of the extracts on mucosal restitution (i.e., mucosal healing) after the cessation of DSS treatment (Table 1).

For EA studies not evaluating lesion restitution, the following treatment groups were used and were comprised of both male and female B6 mice: 1) oral daily gavage with 100 mg/kg EA extract in a 200 μl volume (prepared as described above) in the absence of 2.5% DSS; 2) oral daily gavage with 100 mg/kg/day EA extract in a 200 μl volume in the presence of 2.5% DSS; 3) oral daily gavage with 200 mg/kg/day EA extract in a 200 μl volume in the absence of 2.5% DSS; 4) oral daily gavage with 200 mg/kg/day EA extract in a 200 μl volume in the presence of 2.5% DSS; 5) oral daily gavages with 200 μl 5% EtOH vehicle in the absence of
2.5% DSS; or 6) oral daily gavages with 200 µl 5% EtOH vehicle in the presence of 2.5% DSS. Gavages with the ethanolic extracts began on day -6 and continued through the day of necropsy (day 7), while the appropriate groups were given 2.5% DSS in their drinking water beginning on day 0 and ending on the day of necropsy (Table 1).

Additionally, the effect of EA on mucosal healing or restitution was observed. For all EA restitution studies the following treatment groups were included: 1) oral daily gavage with 200 mg/kg EA extract in the absence of DSS; 2) oral daily gavage with 200 mg/kg EA extract in the presence of low dose 1.75% DSS; 3) oral daily gavage with 200 mg/kg EA extract in the presence of high dose 2.5% DSS; 4) oral daily gavage with 5% EtOH vehicle in the presence of 1.75% DSS; 5) oral daily gavage with 15% EtOH vehicle in the presence of 2.5% DSS; or 6) oral daily gavages with 5% EtOH vehicle in absence of 2.5% DSS. All treatments were administered in a 200 µL volume and gavages began on day -6 and continued through the day of necropsy (day 14). The appropriate groups were given DSS in drinking water at 1.75% or 2.5% beginning on day 0 and ending on day 7. During the restitution period (day 8 to 14), the mice were provided drinking water free of DSS, but continued to receive the EA extract through day 14 (Table 1).

For all prophylactic PV studies, both male and female B6 mice were included in each treatment group. Separate groups of mice were treated as follows: 1) oral daily gavage with 100 mg/kg PV extract; or 2) oral daily gavage with 5% EtOH vehicle. The oral administrations (200 µL volumes) began on day -6 and continued daily through the end of the experiment (day 7). On day 0, half of the mice in each group continued to receive normal drinking water and the other half received drinking water containing 2.5% DSS. For the DSS treated mice, the concentration of DSS in the drinking water was adjusted to 1.5% DSS beginning on day 2 and continued through day 7 (Table 1). To evaluate the therapeutic potential of the PV extract, the treatment groups were the same as those mentioned above for the prophylactic studies. However, the treatment of the mice with the PV extract did not
begin until day 0 in conjunction with the DSS exposure (Table 1). The number of mice used for each experimental design is noted in the appropriate figure legend.

**Macroscopic typhlocolitis assessment.** All mice were euthanized at necropsy by CO$_2$ asphyxiation. Following euthanization, the colon and cecum were excised, photographed, measured and scored for severity of macroscopic lesions. Gross typhlocolitic lesions were scored using a nine point additive scale; a score of zero being assigned to a healthy colon and a score of nine being a maximally diseased colon. Score parameters evaluated included: 1) cecal atrophy, 2) enlarged cecal tonsil or other enlarged lymphoid aggregates, 3) cecal emptying, 4) abnormally watery or mucoid intraluminal cecal and/or colonic contents, 5) luminal blood in the cecum, 6) luminal blood in the colon, 7) visible thickening and rigidity of the cecum, 8) visible thickening and rigidity of the colon, and 9) absence of formed fecal pellets in the colon.

**Statistical analysis.** Statistical significance of parametric data was evaluated by student’s t-test. Non-parametric data (e.g., scores) were evaluated by the Mann-Whitney test. A P-value of ≤ 0.05 was considered statistically significant. Prism 5 software was used for all statistical calculations.

**Results**

*The ethanolic extract of EA has no significant macroscopic efficacy or toxicity in a DSS induced model of murine colitis.* To determine the prophylactic ability of the ethanolic EA extract to prevent or attenuate to DSS-induced colitis, separate groups of mice were gavaged daily with vehicle (5% EtOH) or 100 mg/kg or 200 mg/kg EA extract. EA treatment began 7 days prior to DSS treatment and continued for an additional 7 days while mice were dosed with 2.5% DSS in drinking water. As depicted in Figure 1a, all mice treated with DSS alone began to lose weight by day four of DSS treatment. Treatment of mice with either dose of EA failed to prevent the weight loss induced by exposure to 2.5 % DSS. Regardless of the dose of EA administered, there was no attenuation of the severity of typhlocolitis (Fig. 1b) or in the degree of colon shortening (Fig. 1c and Suppl. Fig. 1) observed.
Photographs of ceca and colons (Supp. Fig 1) show the extent of macroscopic lesions induced by 2.5% DSS. These observations held equally true for both male and female mice (data not shown).

**EA has no significant impact on restitution or healing following DSS induced colitis.** Recently, Zhai et al, demonstrated that the ethanolic extract of *E. pallida* had a positive effect on the healing of skin lesions in stressed mice. In this regard, we sought to evaluate the potential for the EA extract to accelerate mucosal healing following the exposure to DSS. As described above, EA treatment began 7 days prior to DSS treatment and continued daily for a total of 21 days including 7 days after the cessation of the DSS treatment. Two doses of DSS (1.75 and 2.5 %) were evaluated to discern the efficacy of EA in restitution following different levels of colitic severity. As described above, all DSS only recipients, regardless of the dose, lost an average of 10% of their baseline bodyweight (Fig. 2a). EA treatment did not significantly lessen weight loss during the DSS treatment period or accelerate weight recovery during restitution (Fig. 2a). Only one low DSS dose mouse presented with a score of 4 or greater at necropsy following restitution compared to 67% of high dose DSS recipients. DSS recipients that received EA treatment showed no significant improvement in macroscopic scores following restitution when compared to the corresponding DSS dose controls (Fig. 2b). Colon lengths were also not improved in either DSS dose treatment with the addition of 200 mg/kg EA (Fig. 2c and Supp. Fig). These observations were consistent in male and female mice (data not shown).

**The ethanolic extract of PV has significant prophylactic, macroscopic efficacy in a DSS induced model of murine colitis.** To determine the efficacy of PV extract in the treatment of DSS-induced colitis, mice were gavaged daily with vehicle (5% EtOH) or 100 mg/kg PV extract beginning seven days prior to treatment with DSS. To induce and maintain a consistent colitic lesion severity, DSS was dosed at 2.5% for two days and continued at 1.5% for another 5 days. This model was typified by weight loss in DSS treated mice (Fig. 3a). Prophylactic treatment with the PV extract significantly (**P < 0.01**) reduced weight loss during the last 3 days of DSS administration (Fig. 3a). Significant improvements
in macroscopic scores (Fig. 3b) and colon lengths (Fig. 3c) were also observed in mice treated with PV extract when compared to DSS only control mice (Fig. 3b and c, Supp. Fig. 5). There was no sex bias linked to these observations (data not shown) and administration of 100 mg/kg PV extract alone to mice for 14 consecutive days induced no clinical signs of disease or distress in the mice (Supp. Fig. 6). In contrast to the apparent prophylactic health benefits, the PV extract was not therapeutically efficacious when treatment began on the first day of exposure to DSS (Supp. Fig. 7).

**Discussion**

Botanical extracts are not a new line of research with regard to experimental colitis. A study by Araki et al, found that barley was able to reduce DSS-induced weight loss and mucosal damage in rats by preserving homeostatic balances among resident microfloral and increasing short-chain fatty acid production. Several studies have characterized the importance of certain chemical compounds or groups of constituents present in botanical extracts for bioactivity in DSS-induced colitic models. A recent study showed that a coca extract particularly rich in polyphenolic content was responsible for reduction of clinical signs induced by DSS due to modulation of STAT1 and STAT3 phosphorylation. Antioxidant potential is a major area of interest in nutraceutical research. Recently, the antioxidant activity of compounds in black raspberry powder were found to preserve mucosal health despite the colitic insult induced by DSS. In spite of a resurgence of interest, the potential of numerous botanicals with regard to anti-inflammatory and immunomodulatory bioactivity are still in question.

The present studies examined the in vivo efficacy of *Echinacea angustifolia* or *Prunella vulgaris* extracts to prevent or ameliorate the lesions induced by exposure to DSS. Because the DSS model offers the ability to tune severity of colitis by dosing more or less of the chemical in drinking water, the anti-inflammatory efficacy of EA or PV extracts was assessed in the development of moderate to severe and mild colitis and in the context of prophylaxis or onset therapy. Hypothetically, pre-
treatment/prophylaxis with an anti-inflammatory extract would allow for accumulation of bioactive compounds in the body which would have a greater chance to effectively influence the onset of inflammation caused by the exposure to DSS. As adsorption of some compounds into the deeper layers of the lamina propria or submucosa could be poor, there is a better chance that the mechanism of action for these bioactive compounds is to directly modulate the mucosal epithelium to exert beneficial effects. Because the colonic epithelium turns over or replenishes every 4 to 7 days, treatment with a medicinal extract for at least 7 days prior to the exposure to DSS would ensure that all intestinal epithelial cells would have matured in the presence of the anti-inflammatory compounds.

Previously, EA has been shown to inhibit inflammatory prostaglandin E2 (PGE2) production and modulate COX-2 signaling in cell culture experiments. Based on these in vitro observations, the anti-inflammatory bioactivity of EA as an anti-colitic preventative was evaluated. Exposure of mice to 2.5% DSS caused moderate to severe lesions based on the extent of macroscopic damage. Based on the progression of weight loss, macroscopic disease scores and observed colon lengths, prophylactic treatment of the mice with EA, regardless of dose, did not effect the severity of colitis induced by 2.5% DSS. If EA were toxic, it could potentially have caused exacerbated weight loss or additional adverse effects in mice treated with DSS; however this was not observed.

While EA had no macroscopic anti-colitic benefit during the epithelial injury phase of the study, the ability of the EA extract to accelerate epithelial healing or restitution was evaluated. The EA extract was tested in a DSS model that included the continuation of the treatment period for 7 days after the cessation of the exposure to DSS. Separate groups of mice were treated with either 1.75 % or 2.5 % DSS and the results demonstrated that treatment with EA was ineffective in attenuating the magnitude of inflammation. nor did the EA extract accelerate tissue restitution.

Previous studies have shown that PV is anti-inflammatory when tested in several models and contains several bioactive phenolics, triterpenoids, and
flavonoids. Because colitis leads to greater mucosal permeability, even poorly bioavailable components have an increased chance of exerting beneficial effects in colitic models. Prophylactic use of the ethanolic extract of PV was examined in the context of DSS-induced colitis. Analysis of the daily weight changes, macroscopic lesion scores, and colon lengths all indicated that PV significantly reduces the severity of colitis in DSS treated mice. It was also observed that PV was not efficacious if treatment began consequent to the exposure to DSS (Supp. Fig. 7). This would indicate that exposure of gut tissues to the PV extract is required prior to inflammatory insult. Again, this model also does not address inflammation related to dysbiosis or genetic polymorphisms that could also contribute to colitic development and maintenance.

In summary, the bioactivity of EA and PV in the DSS model of colitis is quite different. This could be explained in a number of ways. Previous studies highlight the importance of alkylamides and ketones in EA, while PV harbors many bioactive polyphenols and flavonoids making the totality of their chemical compositions are very different. Studies are ongoing to address the metabolic byproducts associated with these extracts, transport of extract components and their bioavailability in healthy, homeostatic gut tissues compared to leaky colitic tissues. This work indicates the need for further study of the PV extract, extract components, mechanism(s) of action, and ways to alter the extract to improve efficacy for therapeutic use in other models of inflammatory disease.
References


### Table 1. Experimental treatment groups.

<table>
<thead>
<tr>
<th>Ethanolic Extract</th>
<th>Efficacy Query</th>
<th>Treatment Groups</th>
<th>Extract Dose Timeline</th>
<th>DSS Dose and Timeline</th>
<th>Restitution Timeline</th>
</tr>
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<tbody>
<tr>
<td><strong>Echinacea angustifolia</strong></td>
<td>Prophylactic prevention of DSS colitis</td>
<td>1) 100 mg/kg EA 2) 200 mg/kg EA 3) 100 mg/kg EA + DSS 4) 200 mg/kg EA + DSS 5) 5% EtOH vehicle + DSS</td>
<td>Day -6 through day 7</td>
<td>2.5% DSS</td>
<td>Day 0 through day 7</td>
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<tr>
<td></td>
<td>Benefit to mucosal healing/ restitution</td>
<td>1) 200 mg/kg EA 2) 200 mg/kg EA + DSS 3) 200 mg/kg EA + DSS 4) 5% EtOH vehicle + DSS 5) 5% EtOH vehicle + DSS 6) 5% EtOH vehicle</td>
<td>Day -6 through day 7</td>
<td>1.75% or 2.5% DSS</td>
<td>Day 0 through Day 14</td>
</tr>
<tr>
<td><strong>Prunella vulgaris</strong></td>
<td>Prophylactic prevention of DSS colitis</td>
<td>1) 100 mg/kg PV 2) 5% EtOH vehicle 3) 100 mg/kg PV + DSS 4) 5% EtOH vehicle + DSS</td>
<td>Day -6 through day 7</td>
<td>2.5% DSS Day 0 through day 2</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Therapeutic prevention of DSS colitis</td>
<td>1) 100 mg/kg PV 2) 5% EtOH vehicle 3) 100 mg/kg PV + DSS 4) 5% EtOH vehicle + DSS</td>
<td>Day 0 through day 7</td>
<td>2.5% DSS Day 0 through day 2</td>
<td>None</td>
</tr>
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Figure 1. Efficacy of 200 or 100 mg/kg Echinacea angustifolia extract in a DSS chemically induced model of colitis.
Figure 2. Effect of 200 mg/kg *Echinacea angustifolia* extract on restitution following low or high dose DSS induced colitis.
Figure 3. Efficacy of 100 mg/kg *Prunella vulgaris* extract in a DSS chemically induced model of colitis.
Supplementary Figure 1. Photographs of colons and ceca from 2.5% DSS induced colitic or non-colitic mice treated with 5% EtOH vehicle, 100 mg/kg or 200 mg/kg *Echinacea angustifolia.*
Supplementary Figure 2. Neither 100 mg/kg or 200 mg/kg *Echinacea angustifolia* extract has negative impact on non-colitic mice.
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Supplementary Figure 5. Photographs of colons and ceca from DSS induced colitic (2.5% DSS for 2 days followed by 1.5% for 5 days) or non-colitic mice treated with 5% EtOH vehicle or 100 mg/kg *Prunella vulgaris*.
Supplementary Fig 6. 100 mg/kg Prunella vulgaris extract has no negative impact on non-colitic mice.
Supplementary Figure 7. 100 mg/kg *Prunella vulgaris* extract is not therapeutically efficacious in a DSS chemically induced model of colitis.
**Figure Legends**

**Figure 1.** Efficacy of 200 or 100 mg/kg *Echinacea angustifolia* extract in a DSS chemically induced model of colitis. Mice were prophylactically dosed *E. angustifolia* daily for 7 days prior to and 7 days during the administration of 2.5% DSS in drinking water. **a)** Body weights were recorded daily for the duration of the study, beginning on the first day of extract administration. Data is represented as a percent change from baseline. **b)** The additive macroscopic disease score was evaluated and recorded at necropsy based on 9 parameters as noted in the materials and methods. The maximally severe score is 9 while the minimal score for a completely healthy animal is 0. **c)** Colon lengths were measured at necropsy. Weight and colon length data were evaluated by non-parametric t-test, while macroscopic score was subject to non-parametric t-test. No significance (P < 0.05) in extract treatment groups was noted when compared to DSS treatment alone. 5% EtOH vehicle treatment n = 12, DSS only n = 14, 100 mg/kg *E. angustifolia* with DSS n = 6, 200 mg/kg *E. angustifolia* with DSS n = 6.

**Figure 2.** Effect of 200 mg/kg *Echinacea angustifolia* extract on restitution following low or high dose DSS induced colitis. Mice were prophylactically dosed *E. angustifolia* daily for 7 days prior to DSS, 7 days during the administration of 2.5% or 1.75% DSS in drinking water, and 7 days following discontinuation of DSS during restitution. **a)** Body weights, **b)** additive macroscopic disease scores and **c)** colon lengths were observed and statistically analyzed as noted in figure 1 and the materials and methods. No significance (P < 0.05) in extract treatment groups was noted when compared to appropriate DSS treatment alone. 5% EtOH vehicle treatment n = 18, 1.75% DSS only n = 7, 2.5% DSS only n = 18, 200 mg/kg *E. angustifolia* with 1.75% DSS n = 6, 200 mg/kg *E. angustifolia* with 2.5% DSS n = 11.

**Figure 3.** Efficacy of 100 mg/kg *Prunella vulgaris* extract in a DSS chemically induced model of colitis. Mice were prophylactically dosed *P. vulgaris* daily for 7 days prior to and 7 days during the administration of DSS (2.5% DSS for 2 days followed by 1.5% DSS for 5 days) in drinking water. **a)** Body weights, **b)** additive macroscopic disease scores and **c)** colon lengths were observed and statistically analyzed as noted in figure 1 and the materials and methods. Statistical significance (*P < 0.05, **P < 0.01) is noted for comparisons to DSS treatment alone. 5% EtOH vehicle treatment n = 21, DSS only n = 21, 100 mg/kg *P. vulgaris* with DSS n = 19.

**Supplementary Figure 1.** Photographs of colons and ceca from 2.5% DSS induced colitic or non-colitic mice treated with 5% EtOH vehicle, 100 mg/kg or 200 mg/kg *Echinacea angustifolia*. Photographs represent the average observed macroscopic score and colon lengths displayed in figure 1.

**Supplementary Figure 2.** Neither 100 mg/kg or 200 mg/kg *Echinacea angustifolia* extract has negative impact on non-colitic mice. Mice were prophylactically dosed *E. angustifolia* daily for 14 days. **a)** Body weights, **b)** additive macroscopic disease
scores and c) colon lengths were observed and as noted in figure 1 and the materials and methods. 5% EtOH vehicle treatment n = 12, 100 mg/kg *E. angustifolia* n = 6, 200 mg/kg *E. angustifolia* n = 6.

**Supplementary Figure 3.** Photographs of colons and ceca from low (1.75%) or high (2.5%) dose DSS induced colitic or non-colitic mice treated with 5% EtOH vehicle or 200 mg/kg *Echinacea angustifolia* following restitution from DSS. Photographs represent the average observed macroscopic score and colon lengths displayed in figure 2.

**Supplementary Figure 4.** 200 mg/kg *Echinacea angustifolia* extract has no negative impact on non-colitic mice when dosed for 21 consecutive days. Mice were prophylactically dosed *E. angustifolia* daily for 21 days. a) Body weights, b) additive macroscopic disease scores and c) colon lengths were observed and as noted in figure 1 and the materials and methods. 5% EtOH vehicle treatment n = 18, 200 mg/kg *E. angustifolia* n = 18.

**Supplementary Figure 5.** Photographs of colons and ceca from DSS induced (2.5% DSS for 2 days followed by 1.5% for 5 days) colitic or non-colitic mice treated with 5% EtOH vehicle or 100 mg/kg *Prunella vulgaris*. Photographs represent the average observed macroscopic score and colon lengths displayed in figure 3.

**Supplementary Figure 6.** 100 mg/kg *Prunella vulgaris* extract has no negative impact on non-colitic mice. Mice were prophylactically dosed *P. vulgaris* daily for 14 days. a) Body weights, b) additive macroscopic disease scores and c) colon lengths were observed and as noted in figure 1 and the materials and methods. 5% EtOH vehicle treatment n = 9, 200 mg/kg *P. vulgaris* n = 21.

**Supplementary Figure 7.** 100 mg/kg *Prunella vulgaris* extract is not therapeutically efficacious in a DSS chemically induced model of colitis. Mice were therapeutically dosed *P. vulgaris* daily beginning on the first day of DSS treatment and continuing for 7 days. DSS was dosed at 2.5% for 2 days followed by 1.5% for 5 days in drinking water. a) Body weights, b) additive macroscopic disease scores and c) colon lengths and d) cecal/colonic photographs were observed and as noted in previous figures and the materials and methods. Weight and colon length data were evaluated by non-parametric t-test, while macroscopic score was subject to non-parametric t-test. No significance (P < 0.05) in extract treatment groups was noted when compared to DSS treatment alone. 5% EtOH vehicle treatment n = 5, DSS only n = 5, 100 mg/kg *P. vulgaris* n = 5, 100 mg/kg *P. vulgaris* with DSS n = 8.
CHAPTER 3. *Prunella vulgaris* prophylaxis attenuates spontaneous colitis in mdr1a deficient mice

A paper to be submitted to the *American Journal of Physiology - Gastrointestinal and Liver Physiology*

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**Abstract**

The onset and extent of enteric injury associated with inflammatory bowel disease (IBD) is strongly influenced by epithelial barrier dysfunction and innate upregulation of inflammatory gene products, which enhance an aberrant, chronic adaptive inflammatory response. Previous studies have demonstrated the anti-inflammatory capabilities of *Prunella vulgaris*, but never in an *in vivo* model of colitis. We hypothesized that *P. vulgaris* extract prophylaxis would attenuate intestinal lesion severity and onset of disease associated with spontaneous typhlocolitis in mdr1a⁻/⁻ mice. Vehicle (5% ethanol), *P. vulgaris* (2.4 mg/day) or metronidazole (0.75 mg/mL drinking water, an antibiotic and anti-colitic control) were administered orally to separate groups of 6-7 week old mdr1a⁻/⁻ or FVBWT mice and continued daily until mice developed severe wasting or reached 20 weeks of age. Administration of *P. vulgaris extract* to mdr1a⁻/⁻ mice significantly (*p*<0.05) delayed onset of colitis, reduced macroscopic and microscopic cecal histologic scores when compared to vehicle-treated mdr1a⁻/⁻ mice. *P. vulgaris* extract prophylaxis resulted in significantly reduced (*p*<0.05) serum levels of G-CSF, GM-CSF, IL-9, IL-10, IP-10, KC, MIG, and TNFα as well as reduced gene expression of Ccl2, Ccl20, Cxcl1, Cxcl9, IL-1α,
Mmp10, VCAM-1, ICAM, IL-2, and TNFα in mdr1a<sup>−/−</sup> mice when compared to vehicle treated mdr1a<sup>−/−</sup> mice at necropsy. Histologically, cecal neutrophilic infiltrate was reduced in <i>P. vulgaris</i>-treated mdr1a<sup>−/−</sup> mice, as was myeloperoxidase (MPO) activity in the colon. CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and germinal center B cells observed in the cecal tonsils of <i>P. vulgaris</i>-treated mdr1a<sup>−/−</sup> were significantly reduced (p < 0.5) from vehicle treated mdr1a<sup>−/−</sup> mice. Vehicle-treated mdr1a<sup>−/−</sup> mice were found to produce serum antibodies to antigens derived from their resident microbiota, indicative of severe colitis and a loss of adaptive tolerance to the members of the microbiota. These serum antibodies were greatly reduced or absent in <i>P. vulgaris</i> and metronidazole treated mdr1a<sup>−/−</sup> and FVB<sup>WT</sup> mice. The anti-inflammatory activity of <i>Prunella vulgaris</i> ethanolic extract was shown to attenuate innate inflammatory responses and spontaneous colitis in mdr1a<sup>−/−</sup>.

**Introduction**

The intestinal epithelium and innate immune system cooperate to protect the host from damage induced nutrient and water loss to the lumen as well as colonization and invasion by pathogens. The constant low level of inflammation present in the gut is generally important for mucosal homeostatic balance. In the context of inflammatory bowel diseases (IBD), such as Crohn’s disease (CD) and ulcerative colitis (UC), regulation of mucosal inflammation and homeostasis fail and contribute to intestinal mucosal damage and chronic inflammation (61). While idiopathic in nature, current hypotheses regarding the etiology of IBD point to complex multifactorial causalities which could include a disrupted intestinal epithelial barrier, dysbiosis of the microbiota, genetic susceptibilities, chronically activated inflammatory immune cells, and failed immune regulatory responses (16, 44).

Much IBD research has focused on aberrant adaptive immune responses to antigens derived from the microbiota. More emphasis is now being placed on elucidating the role innate immune cells (i.e. neutrophils), cytokines, chemokines, and their related transcription factors play in the initiation and/or maintenance of a compromised epithelium as the initial step in the onset of IBD (3). Without a tight,
intact, mucin covered epithelial barrier, compartmentalization that is meant to separate immune cells in the lamina propria from the numerous bacterial and food antigens normally sequestered in the lumen is lost (70). A loss of epithelial barrier integrity is characteristic of UC and CD, and the consequential loss of immunologic tolerance to the microbiota initiates a cascade of immune signals and functions which lead to chronic, unproductive innate immune responses. These signals, in turn, exacerbate an aberrant and ineffectively regulated adaptive response (9). A key factor in the initial cascade of inflammatory signals is NF-κB.

Over a decade ago, the transcription factor NF-κB was characterized as a key regulator of inflammation in the intestine (48). NF-κB is known to regulate a host of genes participating in innate immune function and inflammation, including but not limited to G-CSF, GM-CSF, IFNγ, IL-6, IP-10, KC, MIP-1α/β, TNFα, VEGF, IL-1α, VCAM-1 and ICAM (51). In UC, CD, and experimental colitis, recruitment of neutrophils, exacerbated by NF-κB activation, is chronic and their production of myeloperoxidase (MPO), elastase, and nitric oxide damage the host tissues while their production of IL-1, IL-6, and TNFα contribute to activation of adaptive immune responses (17, 31, 37).

The most common therapies on the market for IBD are antibiotics or immune suppressive/anti-inflammatory agents (76, 77). Metronidazole and ciprofloxacin have been utilized in several clinical trials related to the treatment of UC, CD and pouchitis with underwhelming results, as evidenced by the fact that metronidazole is somewhat efficacious in the treatment of CD and pouchitis, but not UC (21). Immunosuppressive therapies include monoclonal antibodies against TNFα, 5-aminosalicylates (5-ASA), and steroids (59, 60, 65). In addition to the risks these therapies present with regard to secondary bacterial or viral infection, in the case of some therapies, the long term efficacy and safety is unknown for some therapies. Surgical intervention is still common for severe forms of IBD. A recent study showed that IBD related hospitalizations at high volume IBD treatment centers around the United States increased 6-fold from 1998 to 2004 (49). This data illustrates that despite advances in IBD research, current therapies are not decreasing the
frequency of IBD related hospitalizations. These notions and the burdens of difficult and expensive therapeutic regimens have lead patients to explore more unconventional means of coping with IBD.

A 1998 study showed that up to 51% of surveyed IBD patients had used alternative or complementary therapies, 16% of patients used the alternative therapies specifically for their IBD (18). For most nutraceuticals, many anecdotal claims of health benefits exist with very little scientific data to support or negate those claims. Given that increasing amounts of money are being spent every year on alternative medicines and supplements in the United States, more nutraceutical research is needed to identify potential biologic activity and safety of these products.

Prunella vulgaris is commonly used in traditional Chinese medicine for wound healing, indigestion, burns and anti-inflammatory therapy. P. vulgaris contains several bioactive phenolics, triterpenoids, and flavonoids (11). Dietary phenolics like rosmarinic acid, ursolic acid, and caffeic acid, which are all found in extracts of P. vulgaris, have antioxidant, anti-inflammatory and anti-cancer activities (19, 36, 45, 54, 72, 82). Caffeic acid has also been shown to effectively attenuate chemically induced experimental colitis through upregulation of cychrome P450 (CYP4B1) (83). Flavonoids, like those found in P. vulgaris, have been implicated as potential therapeutics for IBD as well. The flavonoid luteolin was found to attenuate spontaneous colitis by downregulating NF-κB. Despite this promising evidence, Prunella vulgaris extracts have never been studied as a treatment for IBD. In this context, we have designed this study to test the hypothesis that an ethanolic extract of P. vulgaris will decrease inflammatory signals that are mediated by NF-κB in the gut and thereby ameliorate the severity of spontaneous colitis.

**Materials and Methods**

**Reagents.** Prunella vulgaris dried plant materials were kindly provided by Dr. Mark Wiederlichner at the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS, Ames, IA). Ten percent buffered formalin for fixation of histologic samples; hydrogen peroxide and sulfuric acid for myeloperoxidase (MPO) assay
development; and all western blot reagents (tris, glycine, sodium lauryl sulfate, methanol) were purchased from Fisher Scientific (Fair Lawn, NJ). RNA later for tissue sample storage prior to RNA isolation was purchased from Ambion (Austin, TX). Phenylmethanesulfonyl fluoride (PMSF), a protease inhibitor utilized in the MPO assay; 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB), used in the development of the MPO assay; as well as Dimethylsulphoxide (DMSO) were all purchased from Sigma (St. Louis, MO). Heparin at 5,000 USP heparin units/0.5 mL was used as an anticoagulant in preparation of peripheral blood monocytes (PBMCs) and was purchased from Hospira, Inc. (Lake Forest, IL). Fetal bovine serum for cell culture medium was purchased from Atlanta Biologicals (Lawrenceville, GA) and heat inactivated prior to use. Cell wash/culture medium reagents (Dulbecco’s modified eagle’s medium (DMEM) 1X supplemented with 4.5 g/L glucose and sodium pyruvate, penicillin-streptomycin, glutamine and hepes were all purchased from Cellgro (Herndon, VA). All anti-mouse conjugated antibodies and anti-rat isotype controls used in flow cytometric cecal tonsil analysis were purchased from ebioscience (San Diego, CA): FITC-conjugated peanut agglutinin (PNA)+, PE-Cy7-conjugated anti-CD4+ monoclonal antibody (mAb), APC-conjugated anti-CD8β+ mAb, PE-conjugated anti-CD19+ mAb, Alexa 700-conjugated anti-B220+ mAb, PE-Cy7-conjugated anti-rat IgG2ak+ isotype, APC-conjugated anti-rat IgG2bk+ isotype, PE-conjugated anti-rat IgG2ak+ isotype, Alexa 700-conjugated anti-rat IgG2ak+ isotype. BD stabilizing fixative (for use with PE-Cy7 conjugates) purchased from BD biosciences (San Jose, CA) was used for cecal tonsil cells labeled for flow cytometric analysis. All reagents utilized for the Luminex platform multiplex flow cytometric analysis of serum cytokines and chemokines were purchased as part of the Milliplex MAP 32-plex mouse cytokine/chemokine kits from Millipore (Billerica, MA). TRIzol utilized for RNA isolation was purchased from Invitrogen (Carlsbad, CA). Materials utilized for RT2 profiler signal transduction pathway finder PCR array system including: RT2 profiler signal transduction pathway finder PCR array 96-well plates, RT2 qPCR-grade RNA isolation kit, RT2 first strand kit and RT2 SYBR green / ROX qPCR master mix were purchased from SABiosciences (Frederick, MD).
Forward and reverse primers (sequences provided upon request) for real-time PCR validation of Ccl2, Cxcl1, Cxcl9, IL-1α, Mmp10, VCAM-1, TNFα, ICAM and GAPDH were purchased from Integrated DNA Technologies (Coralville, IA). SYBR green / ROX master mix components for real-time PCR validation were purchased from Invitrogen (Carlsbad, CA).

**Prunella vulgaris extract preparation.** Information about the specific provenance of *P. vulgaris* accession Ames 27664, obtained from the NCRPIS, is available on the Germplasm Resources Information Network database at http://www.ars-grin.gov/npgs/acc/acc_queries.html. Arboreal portions of plants from *P. vulgaris* (Ames 27664), harvested in 2008, were prepared for storage by drying for 8 days at 38°C in a forced-air dryer with constant humidity. The dried material was ground with a 40-mesh screen and stored at -20°C until extraction. Extractions were made in 95% ethanol (EtOH) solvent by the Soxhlet method (6 hours). Upon complete drying of the extract by evaporation, the weight of the extracted material was recorded; the residue was lyophilized and stored at -20°C until solubilized in a final working solution of 5% EtOH (the lowest concentration of EtOH that would allow solubilization) at a final plant extract concentration of 12 mg/mL. The working *P. vulgaris* extract was divided into 2 mL aliquots and stored at -20°C until use.

*Prunella vulgaris* extracts from NCRPIS were screened for endotoxin by using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD) according to manufacturers’ specifications for a microplate assay, and there was no detectable endotoxin present in the extract (data not shown).

**Animals.** All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University. Four to five week old male Mdr1a -/- FVB.129P2-Abcb1a tm1BorN7 and wild type (WT) FVB.129P2 mice were obtained from Taconic Farms, Inc. (Germantown, NY). Animals were housed and maintained in the Laboratory Animal Resource facility at the college of veterinary medicine, Iowa State University. Established specific pathogen-free husbandry practices were followed and twelve hour light/dark cycles were applied.
Upon arrival, mice were fed a defined Harlan Teklad AIN93 (M) rodent chow (Madison, WI) to control the amount of phytochemicals in their diet.

**Experimental Design.** A total of six treatment groups of mice were utilized: groups 1 and 2) mdr1a<sup>-/-</sup> and FVB<sup>WT</sup> mice were orally gavaged with 2.4 mg/day *P. vulgaris* extract in a 200 µl volume (prepared as described above); groups 3 and 4) mdr1a<sup>-/-</sup> and FVB<sup>WT</sup> mice were orally gavaged with 200 µL 5% EtOH vehicle and given drinking water (refreshed weekly) containing 0.75 mg/mL metronidazole (Sigma, St. Louis, MO); groups 5 and 6) mdr1a<sup>-/-</sup> and FVB<sup>WT</sup> mice were orally gavaged with 5% EtOH vehicle alone. N = 4 to 10 mice per group per experiment. Gavage was performed using a 20 gauge feeding needle once a day beginning at 6 weeks of age until the mice reached twenty weeks of age or were removed from the study because of severe clinical wasting and/or weight loss exceeding 15% of their peak body weight. At necropsy, mice were euthanized by CO<sub>2</sub> asphyxiation. Following euthanasia, blood was collected by cardiac puncture and separate sections of ceca and proximal colon were excised, washed, and stored for further histological, MPO enzymatic, and real-time PCR analysis. Serum was analyzed by multiplex flow cytometric assay to measure cytokine and chemokine levels as well as western blot analysis for antibody reactivity to antigens derived from the microbiota. Cecal tonsils were also collected for flow cytometric analysis of T and B cell populations. All results are representative of two independent experiments.

**Macroscopic typhlocolitis assessment.** Following euthanization, the colon and cecum were excised, photographed, measured and scored for severity of macroscopic lesions. Gross typhlocolitic lesions were scored using a 9 point additive scale; a score of zero being a healthy animal and a score of 9 being a maximally diseased animal. Score parameters evaluated included: 1) cecal atrophy, 2) enlarged cecal tonsil or other enlarged lymphoid aggregates, 3) emptying of cecal contents, 4) abnormally watery or mucoid intraluminal cecal and/or colonic contents 5) bloody cecal contents, 6) bloody colonic contents, 7) visible thickening and rigidity of the cecum, 8) presence of visible thickening and rigidity of the colon, and 9) absence of formed fecal pellets in the colon.
Histopathological assessment. Sections of excised cecum and proximal colon were placed in 10% buffered formalin overnight, paraffin embedded, sectioned, and routinely stained with hematoxylin and eosin. Stained colonic and cecal sections were scored by a pathologist, Dr. Jesse Hostetter of Iowa State University (Ames, IA), blinded to the treatments as previously described (23, 24). Microscopic mucosal lesion scores were assessed by five parameters, with each parameter scored on a scale of 0-5 (5 = maximum severity). Score parameters include: 1) ulceration of the mucosa; extent of inflammatory cell infiltrate; 2) mucosal edema characterized by the extent of lymphatic and vascular distortion from the normal architecture; 3) stromal collapse and necrosis of the glands; 4) glandular hyperplasia characterized by the distribution of enterocytes along the base of the gland; and 5) cellular proliferation. In addition to score, mucosal heights were determined and recorded in micrometers (µm), and the specific inflammatory cell populations, if present, were recorded. Score parameters were considered individually and as an additive histopathological score with mucosal height included in the additive score.

Myeloperoxidase assay. Myeloperoxidase (MPO) activity was assessed as a measure of neutrophil/granulocyte accumulation in proximal colonic tissues. The MPO assay was performed as previously described with several modifications (83). Proximal colon sections collected at necropsy were gently flushed with PBS to remove luminal contents and stored in 1 mL of freshly prepared PBS supplemented with the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) at 0.1mM and 15% dimethylsulphoxide (DMSO) at -20°C for no more than 7 days prior to assay. Samples used as positive controls for MPO activity were prepared fresh the day the assay from peripheral blood. One FVBWT mouse, not on study, was euthanized by CO2 asphyxiation, and 500 µl to 1 mL of blood was immediately collected by cardiac puncture with a heparinized needle (heparin at 5,000 USP heparin units/0.5 mL is drawn into the needle and syringe and then expelled to coat the inside of the needle with heparin). The heparinized blood was centrifuged at 250 x g for 10 minutes, the supernatant discarded and the red blood cells (RBC) lysed. In brief, 1 mL of ACK
lysis buffer (8042.0 mg/L ammonium chloride, 1001.0 mg/L potassium bicarbonate, 3.722 mg/L ethylene diamine tetraacetic acid disodium, pH 7.2, Sigma chemicals, St. Louis, MO) is added to the pellet, vortexed gently for 1 minute, 1 mL of PBS is added, and the mixture is centrifuged for 10 minutes at 250 x g. The lysis was repeated until the pellet was white and the supernatant was clear. Following RBC lysis, the pellet was resuspended in 1 mL of PBS/PMSF (0.1mM), cell numbers were recorded (average yield of 3 x 10^6 cells/mL) (Z Series Coulter Counter, Beckman Coulter, Fullerton, CA) and the cells were sonicated at an amplitude of 5, pulse on for 4 seconds, pulse off for 1 second for 20 seconds total (Sonicator 3000, Misonix, Inc., Farmingdale, NY). The sonicate is then centrifuged at 250 x g for 15 minutes, the supernatant stored at 4 °C until the tissue samples were prepared. Frozen proximal colonic sections were thawed, blotted to remove as much excess fluid as possible, trimmed to roughly 35 mg and their weights recorded. Tissues were then homogenized for 1 minute at maximum power in 1 mL PBS/PMSF (0.1 mM) and the homogenizer probe was washed 5 times with PBS between tissue samples. Homogenate cell counts were recorded, and each sample was then sonicated as described above. The tissue sonicates were then centrifuged at 250 x g for 15 minutes, the supernatant collected and the pellet discarded. Each lysate prepared from tissue or PBMC was analyzed for total protein using aNanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Individual lysates were pipetted into 96-well, flat bottom microtiter plates. The PBMC lysates (150 µl per well) were serially diluted (10, two-fold dilutions) and analyzed in triplicate wells. For each tissue lysate, 150 µl was pipetted into separate wells and analyzed in triplicate. To each well, 50 µl of 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB) was added, followed immediately by the addition of 50 µl hydrogen peroxide (H_2O_2) (5 mM). The reaction was allowed to proceeded for roughly 2 minutes (wells turned bright blue), followed by the addition of 50 µl of sulfuric acid (1 M) to stop the reaction. The optical density (OD) was measured at 405 nm spectrophotometrically (V-Max, Molecular Devices, USA) using SOFTmax PRO 4.0 software. The MPO content was determined by comparison to the standard
curve and MPO activity was expressed as the relative units of enzyme activity per gram of wet weight of tissue.

**Serum cytokine/chemokine quantification.** Following euthanization of mdr1a^-/- and FVB^WT^ mice, blood was collected via cardiac puncture. The blood was allowed to clot for 24 hours at 4°C after which samples were centrifuged at 10,000 x g for 10 minutes. Serum was then removed and stored at -20°C until use. The day of assay, serum samples were thawed to room temperature. Concentrations of cytokines and chemokines of interest were measured using the Millipore (Billerica, MA) mouse cytokine-chemokine 32-plex multiplexed flow-cytometric assay kit. Analytes screened include: Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, VEGF. The assay was performed according to the manufacturer’s instructions. In brief, supplied analyte standards (range - 10,000 to 3.2 pg/mL), quality control standard, and buffer only control samples were analyzed in duplicate wells of the supplied 96 well plate. Mouse serum samples were diluted 1:1 in supplied assay buffer plated for each mouse. Supplied serum matrix and supplied assay buffer were added to all wells. Supplied pre-conjugated multiplex analyte beads were added to each well and the samples were incubated at 4°C overnight on a plate shaker (Barnstead International Titer Plate Shaker, setting #5, Model #4625). Supplied detection antibody was added to all wells and allowed to incubate at room temperature while shaking for 2 hours. Supplied streptavidine-phycoerithrin was incubated for 30 minutes at room temperature while shaking. The mean fluorescence intensity (MFI) was measured using Luminex platform technology (The FlowMetric System, Luminex, Austin, TX). MFIs were subsequently converted to concentrations using a 5-parameter logistic or line curve-fitting method in MasterPlex QT Software (MiraiBio Group, San Francisco, CA).

**Flow cytometric analysis of cecal tonsil cell populations.** Cecal tonsils from mdr1a^-/- and FVB^WT^ mice were excised, placed in complete cell medium (10 mL heat-inactivated FBS, 1 mL penicillin/streptomycin, 1 mL glutamine, 0.1 mL 50 mM
β-mercaptoethanol, 2.5 mL 1M hepes in 85.4 mL DMEM with 4.5 g/L glucose and sodium pyruvate), and homogenized mechanically on ice. Stainless steel wire strainers (60 mesh) were used to prepare a single cell suspensions and remove particulate matter. Cells (5 x 10⁵ cells/tube) were washed in FACs buffer, centrifuged at 250 x g and incubated in FACS buffer containing 1:100 rat IgG and fluorochrome labeled reagents for 15 minutes on ice. Following labeling, cells were washed with FACs buffer, centrifuged and fixed in 200 µl of BD stabilizing fixative. Cellular preparations from individual mice were labeled with the following fluorochrome-labeled reagents: germinal center B cells (PNA⁺B220⁺) were identified using FITC-conjugated PNA and Alexa 700-conjugated anti-B220⁺ mAb, CD4⁺ T cells were identified using PE-Cy7-conjugated anti-CD4⁺ mAb, CD8⁺ T cells were identified using APC-conjugated anti-CD8β⁺ mAb, B cells that have yet to mature to plasma cells (CD-19⁺) were identified by PE-conjugated anti-CD19⁺ mAb. The following isotype controls were utilized: Alexa 700-conjugated anti-rat IgG2ακ⁺, PE-Cy7-conjugated anti-rat IgG2ακ⁺, APC-conjugated anti-rat IgG2βκ⁺ and PE-conjugated anti-rat IgG2ακ⁺. PNA has no isotype control. Working dilutions of PNA, anti-B220 mAb, anti-CD4 mAb, anti-CD8β mAb, and anti-CD-19 mAb and their isotypes were 1:50, 1:100, 1:80, 1:80 and 1:50, respectively. Analysis was performed using a BD FACSCanto flow cytometer (BD, San Jose, CA) made available through the Flow Cytometry Core Facility at Iowa State University (Ames, IA). Data analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR).

**Western blot analysis.** Anti-sera from mdr1a⁻/⁻ and FVBWT mice was used to evaluate the presence of serum antibody against members of the resident microflora. Whole cell sonicates (WCS) of three members of the clostridial cluster group XIVa (ASF356, ASF500, and ASF502) were cultivated anaerobicly, cells were harvested by centrifugation, washed in PBS, lyophilized, and stored at -20°C until use. Cells were then weighed and suspended in PBS to 2 mg/mL. The resulting suspension was sonicated on ice for 3 minutes at the following settings: amplitude of 50 for 2, 30 second pulses with 5 seconds between each pulse; amplitude 75 for 2, 30 second pulses with 5 seconds between each pulse; amplitude 100 for 2, 30
second pulses with 5 seconds between each pulse. The sonicate was sterilized by UV light (six minute exposure) and sterility was confirmed bacteriologically. For each preparation, protein content was determined by bicinchoninic acid (BCA) analysis (Pierce Laboratories, New Haven, Connecticut, USA), aliquoted and stored at -20°C. Whole cell sonicates of ASF356, ASF500, and ASF502 (8 µg of each by protein content) were subjected to SDS-PAGE using 12% tris-glycine gels (BioRad, Hercules, CA) and transferred to PVDF membranes. Each individual antigen (ASF356, ASF500, and ASF502) was analyzed using pooled anti-sera (1:250) from separate treatment groups as described above. The membranes were then reacted with alkaline phosphatase (AP) conjugated anti-mouse IgG(H+L) (1:1000, Southern Biotech, Birmingham, AL) in a solution containing tris buffered saline (pH 7.6), 1% Tween 20 (TBST) and 2.5% non-fat, skim milk. Immunoreactive proteins were visualized using Sigma fast red tablets (Sigma, St. Louis, MO) according to manufacturers’ instructions.

**Pathway finder R2 profiler PCR array analysis and real-time RT-PCR validation.** To uncover signal transduction pathway gene targets modulated by *P. vulgaris*, cecal RNA was analyzed using the RT² profiler signal transduction pathway finder PCR array from SABiosciences (Frederick, MD) as per the Manufacturers’ instructions. In brief, RNA was isolated from cecal tissue collected at necropsy that had been stored at -20°C in RNAlater using the TRIzol method (26). RNA was further purified using the RT² qPCR-grade RNA isolation kit from SABiosciences (Frederick, MD) according to manufacturers’ instructions. RNA integrity was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA) made available by the GeneChip Core Facility at Iowa State University (Ames, IA). RNA integrity values ranged from 8.2 – 9.4 (data not shown). Prior to preparation of cDNA, RNA samples were tested by PCR using oligonucleotide primers for GAPDH to confirm the absence of genomic DNA contamination. Invitrogen SYBR Green / ROX, primers and 1 µg of isolated RNA from each mouse were subjected to the following PCR conditions and were run on an ABI 5700 (Applied Biosystems Inc., Carlsbad, CA): 95°C for 10 minutes, followed by 40 cycles of amplification (95°C for
10 seconds, $60^\circ\text{C}$ for 15 seconds). All cycle threshold (CT) values were greater than 30, and were acceptable for further use (data not shown). GAPDH oligonucleotide primers used were: 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-CCTGCTTCACCACCTTCTTGA-3'. RNA (1 µg) from each mouse was then converted to cDNA using SABiosciences RT$^2$ First Strand kit according to manufacturers’ instructions. Resulting cDNA from individual mice was pooled into diseased and healthy groups of mice for each treatment group and each experiment, mixed with the kit’s array master mix experimental cocktail preparation and subjected to the same PCR conditions and equipment noted above. PCR array data was analyzed using SABiosciences’ RT$^2$ PCR array analysis software and fold changes were calculated relative to house keeping genes by the software. Only 2-fold changes or greater were considered.

**Statistical analysis.** Statistical significance of parametric data was evaluated by student’s t-test. Non-parametric data (e.g., scores) were evaluated by the Mann-Whitney test. All survival curves were evaluated by the Log Rand (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test. A p-value of $<0.05$ was considered statistically significant. Prism 5 software was used for all statistical calculations.

**Results**

*The ethanolic extract of P. vulgaris decreases severity of macroscopic disease parameters and delays onset of severe colitis in mdr1a$^{-/-}$ mice.* To determine the efficacy of *P. vulgaris* extract in the treatment of spontaneous colitis, mdr1a$^{-/-}$ and FVB$^{WT}$ mice were gavaged daily with vehicle (5% EtOH) or 2.4 mg *P. vulgaris* extract. Previously published data shows that mdr1a$^{-/-}$ mice develop disease between 8 and 36 weeks of age, with the average age of disease onset occurring at 20 weeks (52). As expected, FVB$^{WT}$ mice treated with *P. vulgaris* were not adversely affected by the administration of the extract despite the long course of prophylaxis. Photographs (Fig. 1) show the extent of inflammation and damage caused in vehicle treated mdr1a$^{-/-}$ mice. In these mice, ceca are atrophied with visible, prominent cecal tonsils suggesting immune activation. The ceca are almost empty of contents and
both cecal and colonic tissues are notably thickened and rigid to the point they cannot be straightened. Occasional blood was noted in cecal and colonic contents, while no formed fecal pellets are noted. Conversely, the cecum and colon of the *P. vulgaris* treated mdr1a<sup>-/-</sup> mouse is markedly improved and more closely resembles the tissue appearance of healthy FVB<sup>WT</sup> mice with regard to improvements in all of the parameters noted above. Enlargement of the ceca from both mdr1a<sup>-/-</sup> and FVB<sup>WT</sup> mice treated with metronidazole was noted and would be a characteristic consequence of the metronidazole treatment by partially depleting the microbiota of the mice.

Grossly, mild to severe typhlocolitis (a score of 2 to 9 respectively) was observed in 100% of vehicle treated mdr1a<sup>-/-</sup> mice compared to only 53% of *P. vulgaris* treated mice which experienced only mild to moderate colitis (Fig. 2A). *P. vulgaris* prophylaxis significantly (*p* < 0.01) improved macroscopic parameters of disease when compared to vehicle treatment in mdr1a<sup>-/-</sup> mice. Despite these observations, *P. vulgaris* associated colon lengths displayed an improved trend when compared to vehicle treatment in mdr1a<sup>-/-</sup> mice, but not a significantly longer colon (Fig. 2B). As expected, no FVB<sup>WT</sup> mice, and only one metronidazole treated mdr1a<sup>-/-</sup> mouse, exhibited any parameters of disease (Fig. 2, A and B).

As anticipated, many mdr1a<sup>-/-</sup> mice developed severe colitic wasting and were removed from study prior to aging 20 weeks. The majority of vehicle treated mdr1a<sup>-/-</sup> mice which developed severe colitic disease prior to 20 weeks of age had to be removed from study (lost ≥ 15% of their maximal body weight) within 3 to 5 days of visible clinical signs (bloody stools, diarrhea, ruffled fur, hunched gate) (data not shown). Out of 16 mdr1a<sup>-/-</sup> mice treated with vehicle, 7 required removal from study prior to 20 weeks of age, compared to only 4 out of 13 *P. vulgaris* treated mdr1a<sup>-/-</sup> mice. While *P. vulgaris* extract was able to delay onset of severe colitis and reduce the number of mdr1a<sup>-/-</sup> mice which had to be removed from study, the difference was not significant when compared to vehicle treated mdr1a<sup>-/-</sup> mice (Fig. 3). When *P. vulgaris* treatment was delayed until clinical signs of colitis were visible in mdr1a<sup>-/-</sup> mice, only the macroscopic typhlocolitic scores were improved significantly, while
other gross indicators of disease were not significantly improved (Supplementary Fig. 1). These results indicate that *P. vulgaris* extract prophylaxis is able to significantly improve macroscopic disease and delay the onset of spontaneous colitis in mdr1a<sup>−/−</sup> mice.

**Impact of *P. vulgaris* treatment on the severity of histopathological lesions.** Histological inflammation of the cecum and colon was evaluated in the context of mucosal height, ulceration, extent and character of inflammatory cell infiltrate, edema, stromal collapse and glandular necrosis, and glandular hyperplasia (Table 1). The ceca of vehicle treated mdr1a<sup>−/−</sup> mice were typified by crypt hyperplasia, extensive transmural ulceration and inflammatory infiltrate, as well as submucosal edema and occasional stromal collapse (Table 1 and Fig. 4). The *P. vulgaris*-treated mdr1a<sup>−/−</sup> mice exhibited statistically significant (p < 0.01 – p < 0.05) improvement in every cecal microscopic parameter except edema (Table 1). In addition, there was no significant inflammation (p < 0.05 – p < 0.001 compared to vehicle mdr1a<sup>−/−</sup>) noted in the metronidazole-treated mdr1a<sup>−/−</sup> mice. While the FVB<sup>WT</sup> mice presented with no evidence of inflammation or adverse effects of the treatments, both *P. vulgaris* and metronidazole treated FVB<sup>WT</sup> mice did present with increased mucosal edema, which could indicate an effect on the vasculature. While 100% of vehicle treated mdr1a<sup>−/−</sup> mice exhibited extensive neutrophilic infiltration into the cecum, this population of inflammatory cells was absent in 62% of *P. vulgaris*-treated mdr1a<sup>−/−</sup> mice (Table 1 and Fig. 4). As a measure of the infiltration of granulocytes into the mucosal tissue, MPO activity was measured in tissue homogenates. In comparison to tissue samples from the vehicle-treated mdr1a<sup>−/−</sup> mice, the associated MPO enzymatic activity was significantly diminished in *P. vulgaris*- and metronidazole treated mdr1a<sup>−/−</sup> mice (p < 0.01) (Fig. 5). While colonic sections from the vehicle-treated mdr1a<sup>−/−</sup> mice were also histologically evaluated, the colons of these mice were not significantly improved in *P. vulgaris*-treated mice (data not shown) suggesting that the bioactive benefit of *P. vulgaris* localized in the cecum. When *P. vulgaris* treatment was delayed until clinical signs of colitis were visible in mdr1a<sup>−/−</sup> mice (i.e., therapeutically evaluated), there was no improvement in
microscopic typhlocolitic parameters relative to the vehicle-treated mdr1a⁻/⁻ mice (Supplementary Fig. 1 and Supplementary Table 1). Together, these data indicate that the protection *P. vulgaris* prophylaxis provides the cecal mucosa from severe inflammation and injury is associated with a reduced presence of inflammatory neutrophils and their enzymatic activity

**Impact of the ethanolic extract of *P. vulgaris* on the induction of innate chemotactic and pro-inflammatory cytokines.** To further investigate the mechanism related to improved cecal health and the associated reduction in neutrophil presence and enzymatic activity in the ceca of *P. vulgaris*-treated mdr1a⁻/⁻ mice, serum samples collected at necropsy were examined for the presence of chemokines and cytokines including: Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, VEGF. Several cytokines/chemokines (Eotaxin, IL-13, IL-15, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, LIF, LIX, M-CSF, MCP-1, MIG-1α, MIP-2, and RANTES) were not detectable in any treatment group (data not shown). Several analytes were significantly elevated in mdr1a⁻/⁻ mice compared to FVBWT mice: G-CSF, IP-10, and MIG (p < 0.001); IL-10 and TNFα (p < 0.01); GM-CSF, IL-7, IL-17, and KC (p < 0.05) (Table 2). Significantly reduced serum analytes detected in metronidazole treated versus vehicle treated mdr1a⁻/⁻ mice included: TNFα and IL-17 (p < 0.001); G-CSF, IL-10, IP-10, KC and MIG (p < 0.05) (Table 2). Numerous serum analyte concentrations were also lower in the *P. vulgaris*-treated mdr1a⁻/⁻ mice: IL-10 (p < 0.01), G-CSF, GM-CSF, IL-9, IP-10, KC, MIG, TNFα (p < 0.05) (Table 2). While not significant, there was a trend (p < 0.08) for reduced amounts of IFNγ, IL-6, IL-12(p70), MIP-1β, and VEGF in the serum of *P. vulgaris*-treated mdr1a⁻/⁻ mice when compared to vehicle-treated mdr1a⁻/⁻ mice (Table 2). When *P. vulgaris* treatment was delayed until clinical signs of colitis were visible in mdr1a⁻/⁻ mice, only IL-9 was significantly (p < 0.05) reduced (Supplementary Table 2). It is clear from these data that *P. vulgaris* is able to modulate production of many innate chemokines and inflammatory cytokines induced by the inflammatory response in mdr1a⁻/⁻ mice.
Differential regulation of gene expression pathways by in vivo treatment with the ethanolic extract of *P. vulgaris*. To further evaluate the mechanism(s) associated with the attenuation of mucosal inflammation, microarray analysis was performed in order to identify differential gene regulation between disease phenotypes (healthy mice and colitic mice) and treatment groups (mdr1a<sup>−/−</sup> and FVB<sup>WT</sup> mice treated with vehicle, metronidazole, and *P. vulgaris*). Disease phenotype was determined “healthy” if microscopic and macroscopic scores were within the standard deviation of vehicle-treated FVB<sup>WT</sup> mice (<2). At the extremes, it was observed that no vehicle treated mdr1a<sup>−/−</sup> mice were characterized as “healthy” and no metronidazole treated mdr1a<sup>−/−</sup> or FVB<sup>WT</sup> mice were characterized as “colitic” (data not shown). Genes modulating the production of Ccl2, Cxcl1, Cxcl9, IL-1α, Mmp10, TNFα, VCAM-1, Ccl20 and IL-2 were all downregulated more than 2-fold by *P. vulgaris* prophylaxis in mdr1a<sup>−/−</sup> mice that did not develop colitis (Table 3). In mdr1a<sup>−/−</sup> mice that remained healthy, the ethanolic extract of *P. vulgaris* downregulated the expression of TNFα-specific mRNA better than metronidazole. Out of 84 genes queried, 44 genes were differentially expressed in at least one comparison (Supplementary Table 3). *P. vulgaris*, but not metronidazole, effectively downregulates expression of several adaptive gene targets from NF-κB, nuclear factor of activated T cells (NFAT) and protein kinase C (PKC) pathways in mdr1a<sup>−/−</sup> mice.

Influence of *P. vulgaris* on local T cell and B cell populations. Innate signaling, inflammatory cytokines and chemokines as well as innate inflammatory cells can impact development of adaptive immune responses. Local mucosal T cell and B cell populations in the cecal tonsils of mdr1a<sup>−/−</sup> and FVB<sup>WT</sup> mice were recovered in order to evaluate the effects of *P. vulgaris* prophylaxis on local lymphocyte populations (Fig 6 and 7). Severe colitis in vehicle-treated mdr1a<sup>−/−</sup> mice resulted in a 3-fold increase in CD4<sup>+</sup> T cells (Fig. 6A) and a 6-fold increase in CD8β<sup>+</sup> T cells (Fig. 6B) in the cecal tonsil as compared to vehicle gavaged FVB<sup>WT</sup> mice. *P. vulgaris* prophylaxis significantly (p < 0.05) reduced numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the cecal tonsils of mdr1a<sup>−/−</sup> mice, while metronidazole treated mdr1a<sup>−/−</sup> mice
displayed baseline cecal tonsil T cell numbers (Fig. 6, A and B). Interestingly, both *P. vulgaris* and metronidazole reduced the percentage of CD4$^+$ and CD8$^+$ T cells in FVB$^{WT}$ mice by 47% and 64% (CD4$^+$) and 63% and 59% (CD8$^+$), respectively (Fig. 6C).

Vehicle-treated mdr1a$^{-/-}$ mice exhibited a 2-fold increase in CD19$^+$ B cells (Fig. 7A) and a 2.5-fold increase in PNA$^+$B220$^+$ germinal center B cells (Fig. 7B) in the cecal tonsil as compared to vehicle gavaged FVB$^{WT}$ mice. Remarkably, *P. vulgaris* treatment increased CD19$^+$ B cells in the cecal tonsils of mdr1a$^{-/-}$ and FVB$^{WT}$ mice (Fig. 7, A and C), but significantly (p < 0.05) decreased PNA$^+$B220$^+$ germinal center B cells in mdr1a$^{-/-}$ mice (Fig. 7B). Metronidazole significantly reduced CD19$^+$ and PNA$^+$B220$^+$ germinal center B cells in both mdr1a$^{-/-}$ (p < 0.05 and p < 0.01, respectively) and FVB$^{WT}$ (p < 0.001) mice (Fig 7). Therapeutically administered *P. vulgaris* did not affect the numbers of T cells or B cells in the cecal tonsil in comparison to those of vehicle-treated mdr1a$^{-/-}$ mice (Supplementary Fig. 2).

*P. vulgaris prophylaxis reduces CD4$^+$ T cell, CD8$^+$ T cell and PNA$^+$B220$^+$ germinal center B cell populations in the cecal tonsils of mdr1a$^{-/-}$ mice.*

**P. vulgaris prevents antigenic responses to some members of the resident microbiota.** Antigenic responses to the resident microbiota have been noted in IBD patients and in murine models of IBD (50, 79). These Ab responses are indicative of a loss of mucosal integrity and immune tolerance to the microbiota that do not occur in healthy humans or mice. Pooled serum samples from mdr1a$^{-/-}$ mice treated with *P. vulgaris* were evaluated by immunoblot analysis against WCS antigens from members of the microbiota (Fig. 8). As anticipated, sera from FVB$^{WT}$ mice did not display antibody reactivity against bacterial antigens suggesting that these mice maintained tolerance to the microbiota. PCR did previously confirm the presence of these members of the microbiota in FVB$^{WT}$ mice (data not shown). Conversely, sera from mdr1a$^{-/-}$ mice did contain reactive antibodies to each of the bacterial WCS, indicating a loss of immunologic tolerance to the microbiota. Sera from both *P. vulgaris* and metronidazole treated mdr1a$^{-/-}$ mice displayed little to no detectible antigenic response to the WCS.
Discussion

As the long term safety and efficacy of current parenteral therapeutics for IBD are in question and antibiotics are deemed more and more unreliable for consistent use in IBD patients, the need for new therapies and complementary treatments are ever growing (21, 76). Nutraceutical remedies hold realistic potential in treating or supplementing treatment of inflammatory disorders, as the anti-inflammatory and antioxidant benefits of plant-derived components like flavonoids, polyphenols, and triterpenoids are evidenced more extensively (30, 39, 67, 75, 88). *Prunella vulgaris*, already popular in Asian medicine, is a viable candidate for study as a therapeutic agent in the treatment of IBD as it contains several anti-inflammatory, immunomodulatory, and antioxidant flavonoids, polyphenols, and triterpenoids and has no documented toxic side-effects (8, 57, 67, 84-86). Recently rosmarinic acid, the most plentiful phenolic compound found in *P. vulgaris*, was found to protect mice against the deleterious effects associated with sepsis by downregulating inflammatory genes in the NF-κB pathway and the related inflammatory cytokines TNFα and IL-6 (25).

The mdr1a⁻/⁻ model of spontaneous colitis is ideal for study of potential IBD therapeutics relevant to human medicine as mdr1a⁻/⁻ mice are immunocompetent, develop spontaneous colitis in the context of a leaky intestinal epithelium exhibit cytokine profiles and immune responses similar to those documented in clinical IBD cases (4, 6, 43, 52, 58, 64, 80). In current studies, prophylactic treatment of mdr1a⁻/⁻ mice with an ethanolic extract of *P. vulgaris* was found to attenuate macroscopic lesions associated with the characteristic severe typhlocolitis observed in this model (Fig. 1 and 2). Ceca of mdr1a⁻/⁻ mice treated with a *P. vulgaris* extract retained normal mucosal architecture, lacked enlarged lymphoid aggregates, and retained luminal contents without the presence of blood or mucus, unlike vehicle-treated mdr1a⁻/⁻ mice. Colons of botanical-treated mdr1a⁻/⁻ mice were similarly improved in comparison to sham treatment with regard to presence of formed feces, and lack of gross tissue edema and rigidity (Fig. 1). *P. vulgaris* did not markedly improve colon
lengths in mdr1a<sup>−/−</sup> mice nor did the extract attenuate the microscopic lesions scored in the colon (Table 1). There was significantly less evidence of microscopic lesion formation observed in the cecum of mice treated with the extract compared to the vehicle-treated mdr1a<sup>−/−</sup> mice. This highlights the difference between colonic and cecal compartments. Perhaps this is due to the overwhelming concentration of microflora in this organ, which could more efficiently metabolize the extract, revealing active metabolic byproducts that might be more bioavailable to the host. Similar to previously published studies, onset of disease in vehicle-treated mdr1a<sup>−/−</sup> mice was observed, at roughly 10 weeks of age (Fig. 3) (52). Onset of colitis was delayed by treatment with the <i>P. vulgaris</i> extract as evidenced by the fact that 30% fewer mice developed severe colitis by 20 weeks of age. Limitations of the <i>P. vulgaris</i> extract could be due to the focused cecal benefit of extract therapy, or perhaps further dose titration or treatment schedule is required for more inclusive typhlocolitic benefit.

While the etiology of IBD is still ill-defined, it is recognized that the inductive phase of colitis involves a compromised intestinal epithelium and activation of innate immune responses that include neutrophil activation, transmigration, and enzymatic damage to host tissues (12, 16, 17, 34, 37). Flavonoids from licorice have been shown to inhibit neutrophil infiltration into lung tissue after lippopolysaccharide-induced inflammation, and reduce the severity of associated inflammatory damage to host airway tissues (81). Similarly, the <i>P. vulgaris</i> ethanolic extract greatly attenuated neutrophil infiltration into the cecal mucosa in 62% of extract treated mdr1a<sup>−/−</sup> mice (Table 1). Reduction of the neutrophil infiltrate in these mice was associated with significantly improved microscopic scores and a corresponding and significant decrease in MPO activity (Table 1, Fig. 4 and 5). MPO enzymatic activity is a known correlate to intestinal damage and is used as a marker of IBD severity in many models of colitis (2, 7, 15, 38, 41).

Since neutrophils are not resident in the tissues, cytokine and chemokine signals produced by epithelial cells and local macrophages are responsible for transmigration of neutrophils to the sight of tissue distress (20, 31). Homeostatic
production of these innate chemokines are central to mucosal health while over production contributes to the development of severe inflammation in colitis (12, 34, 53). Debate regarding the role of NF-κB and innate immune cells in acute intestinal inflammatory models continues, with some research pointing to a protective effect by these components and others revealing a deleterious effect (28, 32, 46, 55). Many, however, agree that dysregulation of NF-κB and its related innate cellular responses are causative factors in the inductive phase and maintenance of chronic models of colitis and in human CD and UC (29, 47, 63). Pro-inflammatory targets of NF-κB have been found to be abnormally upregulated in CD and UC patients (35). Nuclear translocation of NF-κB in epithelial cells and local monocytes upregulates production of pro-inflammatory cytokines and chemokines like TNFα, IL-1β, KC and MIG. These cytokines increase expression of adhesion molecules (i.e. VCAM and ICAM) on endothelial cells while chemokines create the chemical gradients to attract neutrophils and other innate inflammatory cells to the sight of injury (12, 20, 40, 53).

Regulation of cytokines (TNFα, IL-1α, IL-1β) which increase expression of adhesion molecules on endothelial cells, reduced production of cytokines (IFNγ) which upregulate chemokine production and downregulation of inflammatory monocyte-derived or neutrophil chemotactic factors (IL-8/KC and VEGF) have been shown to reduce inflammatory tissue damage in several disease models, including colitis (25, 46, 61, 74). Cytokine and chemokine production observed in aged-matched and sham-treated mdr1a−/− mice in the current study (Table 2) was consistent with that previously reported (43). Serum samples from mdr1−/− mice treated with the ethanolic extract of P. vulgaris had significantly lower levels of cytokines that would contribute to the production of granulocytes and monocytes (G-CSF and GM-CSF) as well as the neutrophil chemotactic factor KC (Table 2). Importantly, prophylaxis with the P. vulgaris extract reduced serum levels of TNFα (Table 2), a cytokine known to be a key regulator of inflammatory responses in colitis (78). This data implies that P. vulgaris extract reduced the chronic production of cytokines and chemokines which are critical in maintaining the chronicity of inflammatory cell infiltration. Maintenance of the delicate balance between effective and deleterious inflammation in the gut is
imperative, and current IBD therapies, like monoclonal antibodies to TNFα, have been shown to interfere with the beneficial functions of TNF, leaving patients vulnerable to secondary infections or inducing hypersensitivity (68).

Many studies have linked the activation of the transcription factor NF-κB and the regulation of gene expression to the chronicity of inflammation associated with IBD (26, 29, 47, 63). Recent studies have shown that flavonoids are capable of downregulating NF-κB, which is responsible for the gene regulation leading to production of innate chemotactic factors and pro-inflammatory cytokines (71, 73). One such study showed that the flavonoid luteolin decreased NF-κB expression in the ceca of IL-10−/− mice, and effectively ameliorated spontaneous cecitis (28).

Similarly, the ethanolic extract of *P. vulgaris*, which is known to contain several flavonoids (data not shown), downregulated expression of chemokine genes (Ccl2, Cxcl1/KC, Cxcl9/MIG, and Ccl20) and genes involved in increased expression of adhesion molecules (VCAM-1, ICAM, TNFα and IL-1α) and tissue remodeling to allow for inflammatory cell transmigration (MMP-10) (Table 3. All of these genes participate in activation of or are regulated by NF-κB (1, 27, 62, 69, 87, 89). Clearly, the ethanolic extract of *P. vulgaris* attenuates neutrophil recruitment in the cecal tissues of mdr1a−/− mice by downregulating gene expression of targets regulated by NF-κB signaling. The importance of regulating inflammation in mdr1a−/− mice, and in other colitic models, is underscored by recent data showing that regulation of inflammatory gene expression is altered in mdr1a−/− mice and in mice treated with dextran sodium sulfate (DSS) prior to any histologic signs of inflammation (13, 56). Since defects in gene expression precede inflammation, perhaps this suggests an advantage of prophylactic approaches to control IBD rather than therapy at flare onset. In the current study, it was demonstrated that initiation of the treatment with the ethanolic extract of *P. vulgaris* after the appearance of clinical signs of colitis was ineffective at reducing the severity of inflammation (Supplementary Tables 1 and 2, Supplementary Fig.1 and 2). This observation supports the current mechanistic hypothesis that the ethanolic extract of *P. vulgaris* is modulating innate inflammatory
gene expression in mdr1a\textsuperscript{-/-} mice, as treatment must begin at a time prior to onset of inflammation.

Adaptive immune responses play a part in the chronicity and severity of colitic disease in experimental models and in humans with IBD. Aberrant CD4\textsuperscript{+} T cell responses to antigens derived from the resident microbiota have been implicated in the pathogenesis of IBD (14, 50). It should, however, not be forgotten that innate cell signaling during the inductive phase of IBD has an impact on the chronicity of IBD and on the development and activity of adaptive immune responses. The ethanolic extract of \textit{P. vulgaris} prevented neutrophil transmigration into the cecal tissues of mdr1a\textsuperscript{-/-} mice, and serum cytokine and chemokine data indicates decreased production of IP-10, MIG, MIP-1\textbeta, IL-6, IL-9, IFN\gamma, and TNF\alpha (Table 2). IP-10, MIG and MIP-1\textbeta are interferon-inducible proteins (27, 33). IP-10 and MIG are chemotactic for T cells, while MIP-1\textbeta stimulates neutrophil and macrophage production of IL-6, TNF\alpha, and IL-1\beta. These cytokines and chemokines participate in inflammatory feedback loops that may be interrupted by treatment with the ethanolic extract of \textit{P. vulgaris}. The observation that there are reduced numbers of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the cecal tonsils of mdr1a\textsuperscript{-/-} mice (Fig. 6) are consistent with the reduction in the serum levels of IP-10 and MIG. Treatment with the ethanolic extract of \textit{P. vulgaris} reduced serum IL-6 in mdr1a\textsuperscript{-/-} mice (Table 2). IL-9 has been shown to enhance proliferation of CD4\textsuperscript{+} T cells (42) and the production of this cytokine is decreased by the \textit{P. vulgaris} extract in the current study (Table 2). Additional evidence for the secondary effect of treatment with the \textit{P. vulgaris} extract on T cell chemotaxis would be consistent with the decreased expression of genes regulating MIG, Ccl2, IL-1\alpha, TNF\alpha, and Ccl20 in extract treated mdr1a\textsuperscript{-/-} mice (Table 3 and 4). Ccl20 is strongly chemotactic for immature dendritic cells, which would mature upon collecting antigen in the tissues, present that antigen to T cells, and stimulate an adaptive immune response (22). Moreover, mice treated with the \textit{P. vulgaris} extract had decreased gene expression of IL-2 (Table 4), a cytokine responsible for CD8\textsuperscript{+} T cell differentiation and survival as well as memory T cells (66). The reduction of IL-2 gene expression is possibly due to a reduction in local CD4\textsuperscript{+} T cells and, therefore,
less IL-2 production. Perhaps this is the causative factor in the reduced presence of both CD4$^+$ and CD8$^+$ T cells in the cecal tonsils of mdr1a$^{-/-}$ mice treated with extract. This observation could contribute to the reduced serum antibody response to microfloral antigens in *P. vulgaris* treated mdr1a$^{-/-}$ mice (Fig. 8). While T regulatory cell populations were not assessed in this study, it is possible that *P. vulgaris* extracts could affect T effector cell function through modulation of T regulatory cell activity.

Dendritic cell antigen presentation and T cell help is responsible for B cell proliferation, maturation, and antibody production. Germinal centers in lymphoid tissues, an important component of the B cell humoral response, develop upon B cell activation by T dependant antigen (5). Germinal center B cells bind peanut agglutinin (PNA) and are B220/CD45R$^+$ (10). This study illustrates that while CD19$^+$ B cells are slightly increased in number in the cecal tonsils of mdr1a$^{-/-}$ mice treated with the *P. vulgaris* extract, the number of PNA$^+$B220$^+$ B cells were significantly less than that detected in the vehicle-treated mdr1a$^{-/-}$ mice (Fig. 7). The impact of this arrest in B cell maturation is functionally relevant, as evidenced by the lack of antibody production to bacterial antigens in *P. vulgaris* treated mdr1a$^{-/-}$ mice (Fig. 8). Collectively, these data present evidence that the ethanolic extract of *P. vulgaris* acts to maintain immunologic tolerance to the host microbiota and preserve mucosal homeostasis in mdr1a$^{-/-}$ mice by regulating gene expression associated with innate immunity and subsequently attenuating the activation of the adaptive immune response. This work highlights the need for further study and consideration of *P. vulgaris* as a legitimate prophylactic or supplementary option for the treatment of IBD and other chronic inflammatory disorders.
Figure 1. Photographs of the cecum from mice treated prophylactically with *Prunella vulgaris*. Male mdr1a⁻/⁻ mice were treated with oral 5% EtOH vehicle (n = 16), 0.075 mg/mL metronidazole (n = 10) or 2.4mg *P. vulgaris* extract (n = 13) daily as described in methods. FVB<sup>WT</sup> mice were also represented in each treatment group (n = 6 per group). Images are representative of two independent experiments.
Figure 2. Macroscopic assessment of colitis in mdr1a⁻/⁻ mice treated with *Prunella vulgaris*. A) Macroscopic typhlocolitic scores of mdr1a⁻/⁻ and FVB<sup>WT</sup> mice treated with vehicle, metronidazole or *P. vulgaris* extract as described in Materials and Methods (Max/Severe = 9, Min/Healthy = 0.) B) Colon lengths of mdr1a⁻/⁻ and FVB<sup>WT</sup> mice treated with vehicle, metronidazole or *P. vulgaris* extract were measured at necropsy and the group range is represented. Whiskers indicate minimum and maximum values, while the horizontal line represents the group median. *p < 0.05, **p < 0.01, ***p < 0.001 compared to mdr1a⁻/⁻ vehicle. The n for each group is the same as noted in Figure 1 and data is representative of two independent experiments.
Figure 3. The percent of mdr1a⁻/⁻ mice remaining on study. The mdr1a⁻/⁻ mice were removed from the study as they developed severe colitis or at the termination of the experiment as described in Materials and Methods. *p < 0.05, **p < 0.01, ***p < 0.001 compared to mdr1a⁻/⁻ vehicle. This adapted survival curve is representative of two separate experiments.
<table>
<thead>
<tr>
<th>Microscopic Parameter</th>
<th>( V^{WT} ) (n = 6)</th>
<th>( M^{WT} ) (n = 6)</th>
<th>( PV^{WT} ) (n = 6)</th>
<th>( V^{-/-} ) (n = 16)</th>
<th>( M^{-/-} ) (n = 10)</th>
<th>( PV^{-/-} ) (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal Height (( \mu )m)</td>
<td>3.5 ± 0.2**</td>
<td>3.0 ± 0.0***</td>
<td>3.3 ± 0.2**</td>
<td>4.6 ± 0.2</td>
<td>3.5 ± 0.2**</td>
<td>3.9 ± 0.2*</td>
</tr>
<tr>
<td>Ulceration</td>
<td>0.2 ± 0.2**</td>
<td>0.0 ± 0.0***</td>
<td>0.0 ± 0.0***</td>
<td>1.9 ± 0.3</td>
<td>0.0 ± 0.0***</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Inflammation</td>
<td>1.3 ± 0.2**</td>
<td>1.0 ± 0.0**</td>
<td>1.0 ± 0.0**</td>
<td>3.6 ± 0.2</td>
<td>1.7 ± 0.3***</td>
<td>2.5 ± 0.4*</td>
</tr>
<tr>
<td>Edema</td>
<td>0.7 ± 0.5*</td>
<td>1.8 ± 0.2</td>
<td>3.0 ± 0.4</td>
<td>2.4 ± 0.3</td>
<td>0.8 ± 0.3**</td>
<td>0.9 ± 0.3**</td>
</tr>
<tr>
<td>Stromal Collapse (Necrosis)</td>
<td>0.0 ± 0.0*</td>
<td>0.0 ± 0.0*</td>
<td>0.0 ± 0.0*</td>
<td>1.7 ± 0.4</td>
<td>0.0 ± 0.0**</td>
<td>0.5 ± 0.4*</td>
</tr>
<tr>
<td>Gland Hyperplasia</td>
<td>1.2 ± 0.2**</td>
<td>1.7 ± 0.2**</td>
<td>1.0 ± 0.0**</td>
<td>2.8 ± 0.2</td>
<td>1.9 ± 0.3*</td>
<td>2.1 ± 0.3*</td>
</tr>
<tr>
<td>Additive Cecal Score</td>
<td>6.9 ± 0.9**</td>
<td>7.5 ± 0.3**</td>
<td>8.3 ± 0.5**</td>
<td>17.0 ± 1.2</td>
<td>7.9 ± 0.9***</td>
<td>11.1 ± 1.5**</td>
</tr>
<tr>
<td>% Mice Exhibiting Cecal Neutrophil Infiltrate</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
<td>10%</td>
<td>38%</td>
</tr>
</tbody>
</table>

\( V^{WT} \) = Vehicle (5% EtOH) treated FVB^{WT} mice, \( M^{WT} \) = Metronidazole treated FVB^{WT} mice, \( PV^{WT} \) = Prunella vulgaris treated FVB^{WT} mice, \( V^{-/-} \) = Vehicle (5% EtOH) treated mdr1a^{-/-} mice, \( M^{-/-} \) = Metronidazole treated mdr1a^{-/-} mice, \( PV^{-/-} \) = Prunella vulgaris treated mdr1a^{-/-} mice. Data are expressed as the mean ± SEM. Statistical significance is noted in three levels: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.

**Table 1.** *Prunella vulgaris* prophylaxis significantly decreases cecal histologic/microscopic damage associated with severe typhlocolitis in mdr1a^{-/-} mice, and decreases the occurrence of neutrophil influx associated with histologic inflammation. Cecal sections collected from mdr1a^{-/-} and FVB^{WT} mice treated with vehicle, metronidazole or *Prunella vulgaris* extract at the time of necropsy were formalin fixed, paraffin embedded and subject to routine hematoxylin and eosin staining. Stained histologic sections were scored for inflammation by a pathologist blinded to the treatments. The additive cecal score is based on the addition of mucosal height (\( \mu \)m) to the collective scores for ulceration, inflammation, edema, necrosis and gland hyperplasia. Scores for ulceration, Inflammation, edema, necrosis and gland hyperplasia were scored on a 0 (normal) to 5 (severe) scale. Average values are shown here ± standard error of the mean (SEM) except where noted. *p < 0.05, **p < 0.01, ***p < 0.001 compared to mdr1a^{-/-} vehicle. Mucosal heights were subject to parametric unpaired t-test, while microscopic scores were subject to non-parametric, Mann-Whitney t-test. Statistical analysis was performed using GraphPad Prism 5. Data are representative of two separate experiments.
Figure 4. *Prunella vulgaris* protects cecal microscopic architecture in mdr1a-/- mice and decreases inflammatory cell infiltrate. Representative photomicrographs (200x) of cecal sections collected from mdr1a-/- and FVBWT mice treated with vehicle, metronidazole or *Prunella vulgaris* extract at the time of necropsy were formalin fixed, paraffin embedded and subject to routine hematoxylin and eosin staining. FVBWT ceca of all treatments are characterized by normal mucosal architecture and height, intact glands, little to no mucosal or submucosal edema, and normal resident populations of mononuclear cells. In contrast, diseased mdr1a-/- ceca exhibit mucosal and glandular hyperplasia, transmural ulceration and inflammation, as well as stromal necrosis and an inflammatory population of neutrophils. *P. vulgaris* and metronidazole treated mdr1a-/- ceca are significantly improved toward the normal wild type condition.
Figure 5. Metronidazole and *P. vulgaris* reduce local myeloperoxidase (MPO) activity in the colon of *mdr1a\(^{-/-}\)* mice. Homogenates of colonic tissue were used to assay for MPO activity. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 compared to *mdr1a\(^{-/-}\)* vehicle.
<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>(V^\text{WT}) (n = 6)</th>
<th>(M^\text{WT}) (n = 6)</th>
<th>(PV^\text{WT}) (n = 6)</th>
<th>(V^-) (n = 16)</th>
<th>(M^-) (n = 10)</th>
<th>(PV^-) (n = 13)</th>
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<tbody>
<tr>
<td>G-CSF</td>
<td>140 ± 15***</td>
<td>132 ± 14***</td>
<td>99 ± 9***</td>
<td>9694 ± 2563</td>
<td>622 ± 199*</td>
<td>4597 ± 1931*</td>
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<tr>
<td>GM-CSF</td>
<td>0 ± 0*</td>
<td>0 ± 0*</td>
<td>0 ± 0*</td>
<td>21 ± 11</td>
<td>4 ± 3</td>
<td>2 ± 2*</td>
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<tr>
<td>IFNγ</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>8 ± 4</td>
<td>6 ± 3</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>IL-6</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>59 ± 28</td>
<td>9 ± 3</td>
<td>49 ± 40</td>
</tr>
<tr>
<td>IL-9</td>
<td>1 ± 1*</td>
<td>1 ± 1*</td>
<td>1 ± 1*</td>
<td>28 ± 11</td>
<td>3 ± 2</td>
<td>47 ± 21</td>
</tr>
<tr>
<td>IL-10</td>
<td>78 ± 27*</td>
<td>118 ± 21</td>
<td>129 ± 34</td>
<td>200 ± 38</td>
<td>130 ± 23</td>
<td>118 ± 14*</td>
</tr>
<tr>
<td>IL-12(p40)</td>
<td>0 ± 0**</td>
<td>0 ± 0**</td>
<td>0 ± 0**</td>
<td>19 ± 4</td>
<td>6 ± 2*</td>
<td>5 ± 2**</td>
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<tr>
<td>IL-12(p70)</td>
<td>4 ± 2*</td>
<td>3 ± 2*</td>
<td>0 ± 0***</td>
<td>16 ± 4</td>
<td>1 ± 1***</td>
<td>18 ± 13</td>
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<tr>
<td>IP-10</td>
<td>8 ± 6***</td>
<td>84 ± 43***</td>
<td>14 ± 10***</td>
<td>724 ± 136</td>
<td>280 ± 74*</td>
<td>397 ± 85*</td>
</tr>
<tr>
<td>KC</td>
<td>70 ± 24**</td>
<td>85 ± 10**</td>
<td>140 ± 61*</td>
<td>527 ± 119</td>
<td>232 ± 67*</td>
<td>253 ± 58*</td>
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<td>MIG</td>
<td>109 ± 37***</td>
<td>403 ± 136***</td>
<td>113 ± 35***</td>
<td>3901 ± 858</td>
<td>1499 ± 564*</td>
<td>1319 ± 277*</td>
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<tr>
<td>MIP-1β</td>
<td>48 ± 8</td>
<td>70 ± 5</td>
<td>72 ± 10</td>
<td>57 ± 8</td>
<td>57 ± 8</td>
<td>46 ± 9</td>
</tr>
<tr>
<td>TNFα</td>
<td>9 ± 0**</td>
<td>9 ± 0**</td>
<td>9 ± 0**</td>
<td>15 ± 2</td>
<td>7 ± 2***</td>
<td>10 ± 3*</td>
</tr>
<tr>
<td>VEGF</td>
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<td>0 ± 0</td>
<td>0 ± 0</td>
<td>13 ± 6</td>
<td>11 ± 4</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

\(V^\text{WT}\) = Vehicle (5% EtOH) treated FVB\(^\text{WT}\) mice, \(M^\text{WT}\) = Metronidazole treated FVB\(^\text{WT}\) mice, \(PV^\text{WT}\) = Prunella vulgaris treated FVB\(^\text{WT}\) mice, \(V^-\) = Vehicle (5% EtOH) treated mdr1a\(^-\) mice, \(M^-\) = Metronidazole treated mdr1a\(^-\) mice, \(PV^-\) = Prunella vulgaris treated mdr1a\(^-\) mice. Data are expressed as the mean serum cytokine/chemokines content (pg/mL) ± SEM. Statistical significance is noted in three levels: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.

**Table 2. Prunella vulgaris prophylaxis reduces serum content of several chemotactic and inflammatory cytokines associated with severe typhlocolitis in mdr1a\(^-\) mice.** Serum collected at necropsy from mdr1a\(^-\) and FVB\(^\text{WT}\) mice treated with vehicle, metronidazole or *P. vulgaris* subjected multiplex flow cytometric bead analysis for chemokine and cytokine concentrations using a 32-plex mouse chemokine/cytokine kit. Only cytokines and chemokines which were detectible in at least one treatment group are represented above. Average serum concentrations (pg/mL) are shown here ± standard error of the mean (SEM). *p < 0.05, **p < 0.01, ***p < 0.001 compared to mdr1a\(^-\) vehicle. Concentrations were subject to non-parametric, Mann-Whitney t-test. Statistical analysis was performed using GraphPad Prism 5. Data are representative of two separate experiments.
<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Pathway Affiliation</th>
<th>Fold change compared to diseased vehicle treated mdr1a⁻/⁻ mice (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl2</td>
<td>NF-κB, LDL</td>
<td>$V_{WT}^{Healthy}$ (n = 6): -7.3</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>NF-κB</td>
<td>-11.3</td>
</tr>
<tr>
<td>Cxcl9</td>
<td>NF-κB, Jak/Stat</td>
<td>-23.9</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>NF-κB, Phospholipase C</td>
<td>-3.2</td>
</tr>
<tr>
<td>IL-1α</td>
<td>NF-κB</td>
<td>-13.4</td>
</tr>
<tr>
<td>Mmp10</td>
<td>NF-κB, Jak/Stat</td>
<td>-6.6</td>
</tr>
<tr>
<td>TNFα</td>
<td>NF-κB</td>
<td>-10.2</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>NF-κB, Phospholipase C, LDL</td>
<td>-3.8</td>
</tr>
<tr>
<td>Ccl20</td>
<td>NF-κB</td>
<td>-3.6</td>
</tr>
<tr>
<td>IL-2</td>
<td>NF-κB, NFAT, Calcium, PKC</td>
<td>-3.7</td>
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</table>

$V_{WT}$ = Vehicle (5% EtOH) treated FVB WT mice, $M^{−/−}$ = Metronidazole treated mdr1a⁻/⁻ mice, $PV^{−/−}$ = Prunella vulgaris treated mdr1a⁻/⁻ mice. Healthy denotes mice with a macroscopic score of < 2. Colitic denotes mice with a macroscopic disease score of 2 or >. Data are expressed as Fold changes compared to colitic vehicle treated mdr1a⁻/⁻ mice.

Table 3. Fold change in gene expression of gene targets in healthy and colitic mice compared to colitic vehicle treated mdr1a⁻/⁻ mice.

Cecal tissue total RNA from mdr1a⁻/⁻ (healthy and colitic) and FVB WT mice (healthy) treated with vehicle, metronidazole or Prunella vulgaris was converted to cDNA and subjected to gene expression analysis using the Signal Transduction Pathway Finder RT² PCR array system. Gene target names, signaling pathway involvement and fold change compared to mdr1a⁻/⁻ vehicle are noted above. Fold changes were calculated relative to house keeping genes in SAbiosciences’ RT² PCR array analysis software for the Signal Transduction Pathway Finder 96-well format array. (-) indicates downregulation from control. Data are representative of two separate experiments.
Figure 6. Evaluation of CD4⁺ and CD8⁺ T cell subsets in the cecal tonsil lymphoid tissues of mdr1a⁻/⁻ mice treated with *P. vulgaris*. Cecal tonsils were excised at necropsy, single cell suspensions prepared and stained for flow cytometric analysis as described in materials and methods. **A** Numbers of CD4⁺ T cells in the cecal tonsil, **B** Numbers of CD8β⁺ T cells in the cecal tonsil, **C** Dot plots of percentages of CD4⁺ and CD8β⁺ T cells in the cecal tonsil. *p < 0.05, **p < 0.01, ***p < 0.001 compared to mdr1a⁻/⁻ vehicle. Flow cytometric data was analyzed using FlowJo software and statistical analysis was performed using GraphPad 5. The n for each group is equal to that noted in fig. 1 and data is representative of two independent experiments.
Figure 7. Modulated populations of CD19+ B cells and PNA+B220+ germinal center B cells in the cecal tonsil lymphoid tissues of mdr1a−/− mice treated with P. vulgaris extract compared to vehicle treated mdr1a−/− controls. Cecal tonsils were removed at necropsy, homogenized and stained for flow cytometric analysis as described in materials and methods with anti-CD19, peanut agglutinin (PNA) and anti-B220. A) Relative numbers of CD19+ B cells in the cecal tonsil. B) Relative numbers of PNA+B220+ germinal center B cells in the cecal tonsil C) Percentages of CD19+ B cells in the cecal tonsil. *p < 0.05, **p < 0.01, ***p < 0.001 compared to mdr1a−/− vehicle. Flow cytometric data was analyzed using FlowJo software and statistical analysis was performed using GraphPad 5. The n for each group is equal to that noted in fig. 1 and data is representative of two independent experiments.
Vehicle treated mdr1a<sup>−/−</sup> mice with severe colitic inflammation developed serum antibody responses to antigens derived from microflora members of the clostridial cluster XIVa, while those treated with metronidazole and <em>P. vulgaris</em> extract did not. Whole cell sonicates of each of three clostridial species found as part of the enteric microflora were subjected to SDS-PAGE. Western blot analysis was performed using anti-sera collected at necropsy. Antigens in lanes represented in each blot are as follows from left to right: ASF502, ASF500, ASF356.
Supplementary Figure 1. Mdr1a<sup>−/−</sup> mice treated therapeutically with the ethanolic extract of <i>P. vulgaris</i>, beginning at visible colitic onset, improves typhlocolitic score, but does not improve colon lengths or MPO activity associated with severe spontaneous colitis in mdr1a<sup>−/−</sup> mice.

A) Gross/macroscopic additive typhlocolitic score. Additive score parameters included cecal atrophy, enlarged lymphoid aggregate/s, cecal emptying, presence of watery/mucoid cecal or colon contents, cecal blood, colonic blood, cecal thickening, colonic thickening and lack of formed fecal pellets. Max/Severe = 9, Min/Healthy = 0.

B) Colon lengths of mdr1a<sup>−/−</sup> and FVB<sup>WT</sup> mice treated with vehicle, metronidazole or <i>P. vulgaris</i> extract were measured at necropsy and the group range is represented. Whiskers indicate minimum and maximum values, while the center bar represents the group median.

C) Colonic tissues collected at necropsy were subjected to colorimetric assay for MPO activity. *p < 0.05, **p < 0.01, ***p < 0.001 compared to mdr1a<sup>−/−</sup> vehicle. Colon lengths and MPO were subject to parametric unpaired t-test, while typhlocolitic scores were subject to non-parametric, Mann-Whitney t-test.

Statistical analysis was performed using GraphPad Prism 5. Data is representative of two separate experiments.
### Supplementary Table 1.

P. vulgaris dosed therapeutically does not improve cecal histologic/microscopic damage or neutrophil infiltrate associated with severe typhlocolitis in mdr1a\(^{-/-}\) mice.

Cecal sections collected from mdr1a\(^{-/-}\) and FVB\(^{WT}\) mice treated with vehicle, metronidazole or *Prunella vulgaris* extract at the time of necropsy were formalin fixed, paraffin embedded and subject to routine hematoxylin and eosin staining. Stained histologic sections were scored for inflammation by a pathologist blinded to the treatments. The additive cecal score is based on the addition of mucosal height (µm) to the collective scores for ulceration, inflammation, edema, necrosis and gland hyperplasia. Scores for ulceration, Inflammation, edema, necrosis and gland hyperplasia were scored on a 0 (normal) to 5 (severe) scale. Average values are shown here ± standard error of the mean (SEM) except where noted. *p < 0.05, **p < 0.01, ***p < 0.001 compared to mdr1a\(^{-/-}\) vehicle. Mucosal heights were subject to parametric unpaired t-test, while microscopic scores were subject to non-parametric, Mann-Whitney t-test. Statistical analysis was performed using GraphPad Prism 5. Data are representative of two separate experiments.
Cytokine/Chemokine | $\text{V}^\text{WT}$ (n = 6) | $\text{V}^-$(n = 16) | Prophylactic $\text{PV}^-$ (n = 10) | Therapeutic $\text{PV}^-$ (n = 7) |
<table>
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<tr>
<td>G-CSF</td>
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<td>0 ± 0**</td>
<td>19 ± 4</td>
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<tr>
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<td>724 ± 136</td>
<td>280 ± 74*</td>
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<td>70 ± 24**</td>
<td>527 ± 119</td>
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<td>1 ± 1</td>
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$\text{V}^\text{WT}$ = Vehicle (5% EtOH) treated FVB$^\text{WT}$ mice, $\text{M}^\text{WT}$ = Metronidazole treated FVB$^\text{WT}$ mice, $\text{PV}^\text{WT}$ = Prunella vulgaris treated FVB$^\text{WT}$ mice, $\text{V}^-$ = Vehicle (5% EtOH) treated mdr1a$^-$(n = 16) mice, $\text{M}^-$ = Metronidazole treated mdr1a$^-$(n = 16) mice, $\text{PV}^-$ = Prunella vulgaris treated mdr1a$^-$(n = 16) mice. Data are expressed as the mean serum cytokine/chemokines content (pg/mL) ± SEM. Statistical significance is noted in three levels: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.

**Supplementary Table 2.** *P. vulgaris* dosed therapeutically does not reduce serum content of chemotactic and inflammatory cytokines associated with severe typhlocolitis in mdr1a$^-$(n = 16) mice, with the exception of IL-9. Serum collected at necropsy from mdr1a$^-$(n = 16) and FVB$^\text{WT}$ mice treated with vehicle, metronidazole or *Prunella vulgaris* subjected multiplex flow cytometric bead analysis for chemokine and cytokine concentrations using a 32-plex mouse chemokine/cytokine kit. Only cytokines and chemokines which were detectible in at least one treatment group are represented above. Average serum concentrations (pg/mL) are shown here ± standard error of the mean (SEM). *p < 0.05, **p < 0.01, ***p < 0.001 compared to mdr1a$^-$(n = 16) vehicle. Concentrations were subject to non-parametric, Mann-Whitney t-test. Statistical analysis was performed using GraphPad Prism 5. Data are representative of two separate experiments.
Supplementary Figure 2. *P. vulgaris* dosed therapeutically does not decrease expression of CD4^+^ and CD8^+^ T cell markers or CD19^+^ and PNA^+^B220^+^ B cell numbers in the cecal tonsil lymphoid tissues of mdr1a^-/-^ mice compared to vehicle treated mdr1a^-/-^ controls. Cecal tonsils were removed at necropsy, homogenized and stained for flow cytometric analysis as described in materials and methods with anti-CD4 and anti-CD8. A) Relative numbers of CD4^+^ T cells in the cecal tonsil. B) Relative numbers of CD8^+^ T cells in the cecal tonsil. C) Relative numbers of CD19^+^ B cells in the cecal tonsil. D) Relative numbers of PNA^+^B220^+^ germinal center B cells in the cecal tonsil. *p < 0.05, **p < 0.01, ***p < 0.001 compared to mdr1a^-/-^ vehicle. Flow cytometric data was analyzed using FlowJo software and statistical analysis was performed using GraphPad 5. Data is representative of two independent experiments.
References


CHAPTER 4. *Hypericum gentianoides* extract prophylaxis delays onset and severity of spontaneous colitis in mdr1a deficient mice

A paper to be submitted to the *Journal of Gastroenterology*

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Abstract

**Background.** Nutraceuticals are gaining ground as therapeutic modalities for inflammatory and autoimmune disorders due to their low toxicity and high patient compliance. Several species of *Hypericum* possess immunomodulatory capabilities in many disease models; however the therapeutic potential of the chemically unique *Hypericum gentianoides* (HG) is largely untested. We investigated the efficacy of HG prophylaxis in the mdr1a deficient (mdr1a\(^{-/-}\)) murine model of spontaneous colitis.

**Methods.** Vehicle (5% ethanol), HG (4.8 mg/day) or metronidazole (0.75 mg/mL) were orally administered daily to mdr1a\(^{-/-}\) or FVB\(^{WT}\) mice (6-7 weeks old) until vehicle treated mdr1a\(^{-/-}\) mice developed severe wasting or reached 20 weeks of age. Macroscopic disease assessment included measurement of weight loss, colon shortening and combined colonic/cecal scores. Colonic/cecal inflammation was also scored histologically. Immune responses were assessed using myeloperoxidase (MPO) assay, analysis of serum cytokines/chemokines, and flow cytometry.

**Results.** HG prophylaxis significantly (p < 0.05) delayed colitic onset, reduced
mortality, macroscopic and microscopic disease/inflammatory scores, and
maintained colonic health. Histological evaluation of colonic tissue revealed a
decrease in neutrophil infiltrate in HG treated mice, substantiated by a significant
decrease in colonic MPO. HG prophylaxis inhibited induction of G-CSF, IL-7, KC,
LIX, MIP-1β, and TNFα while significantly increasing IL-6. Histologic and flow
cytometric analysis revealed B cell increases in gut tissues of HG treated mice.

**Conclusions.** HG exerts suppression of chemotactic signals which dampens colitic
damage. These observations provide novel evidence that HG has
immunomodulatory potential which can delay the onset and lessen the severity of
spontaneous IBD.

**Key Words:** *Hypericum gentianoides*, colitis, IBD, mdr1a deficient mice

**Introduction**

Inflammatory bowel disease (IBD) is complex and multifactoral, with a poorly
defined etiology and high variability in symptomatic presentation from patient to
patient. The two predominant forms of IBD, ulcerative colitis (UC) and Crohn’s
disease (CD), are characterized by relapsing inflammatory lesions. A 2007 study
found the instance of adult CD and UC in the United States to be 238/10^5 and
201/10^5, respectively, while the pediatric instance of these diseases was 43/10^5 and
28/10^5, respectively. The prevalence of IBD is increasing in developing countries
where funding and access to new biologic therapies, such as anti-tumor necrosis
factor alpha (anti-TNFα), are very scarce. Because the underlying etiology of IBD
is unknown, clinicians continue to focus on and treat the symptoms of IBD. The most
popular and effective therapeutics consist of mesalazines, corticosteroids and anti-
TNFα antibodies. More traditional treatments like corticosteroids, mesalazines and
the immunosuppressants azathioprine and methotrexate, are plagued by difficulties in
titration, toxicity, low tolerance, low patient compliance, delayed activity and the
need for additional medications to combat secondary infections. Additionally, the
long term effects of newer biologic therapies are still in question and patients can
become refractory to these treatments. In the case of anti-TNFα antibody therapies,
20 to 30 percent of patients with CD and 30 to 40 percent of patients with UC eventually fail to respond to treatment and fail to respond to a switch in anti-TNF agent.\textsuperscript{8-12}

In increasing numbers, physicians and their patients are adding natural products to therapeutic regimens to directly treat disease or to alleviate the side effects of primary therapies. It has been estimated that up to 50 percent of patients suffering with IBD in North America utilize some form of complementary or alternative medicine (CAM).\textsuperscript{13, 14} Herbal supplementation is one of the most popular complementary and alternative medications utilized by IBD patients worldwide.\textsuperscript{14} While the use of CAM is increasing worldwide, CAM is underrepresented in high-impact studies, clinical trials, and medical education programs.\textsuperscript{15} At the same time, over half of all pharmaceuticals developed in the last two decades arose from naturally occurring compounds.\textsuperscript{16} Complex plant extracts have the advantages of containing various combinations of anti-inflammatory, anti-bacterial, anti-viral and antioxidant compounds while being less costly and inducing fewer toxic side effects due to synergy between chemical compounds.\textsuperscript{17, 18} It has been recognized that consumption of selected species of Hypericum or their extracts could be therapeutically or supplementally (i.e., prophylactically) efficacious in the treatment of IBD due to their use in treating gastrointestinal upset as well as reported anti-inflammatory, analgesic, wound healing and free radical inhibitory effects.\textsuperscript{19-23} The chemical constituents of the most commonly known species of this genus, \textit{Hypericum perforatum} (common name, St. John’s Wort), are well defined and include several flavonoids: quercetin, hyperoside, isoquercitrin, quercitrin, rutin, and amentoflavone.\textsuperscript{24, 25} Of these compounds, rutin has been shown to ameliorate experimental colitis.\textsuperscript{26} \textit{H. perforatum} also consists of the phenols caffeic acid and chlorogenic acid, as well as tannins. Caffeic acid has been shown to modulate the IL-12/IL-23 pathway, while proanthocyanidins inhibit nuclear-factor kappa B (NF\textsubscript{κ}B) activity.\textsuperscript{27, 28} The bioactive compounds that have received the most attention in \textit{H. perforatum} are the photoactive compounds hypericin and pseudohypericin, as well as hyperphorin.\textsuperscript{29} While these three chemicals are reported to have a great deal of
biologic activity, they can also cause photosensitivity and toxicity in animals and humans.\textsuperscript{30-32}

While the species \textit{H. gentianoides} is dispersed throughout the eastern United States, their biochemical and medicinal properties are not well documented. However, it is known that HG has been used by Native Americans to reduce gastrointestinal upset and fever and to speed wound healing.\textsuperscript{33} Compared to other species of \textit{Hypericum}, the chemical composition of HG is unique in that it contains the anti-bacterial compounds uliginosin A and B but lacks hypericin, pseudohypericin and hyperforin.\textsuperscript{34, 35} This suggests that HG extracts would have fewer potential toxic side effects when compared to other \textit{Hypericum} species. Recently, it was shown that an extract of HG reduced inflammatory prostaglandin E2 (PGE2) production in RAW 264.7 macrophage cells with no significant cytotoxicity.\textsuperscript{35} Neither HG nor any extracts of HG have been evaluated for biologic activity in vivo. In this study, we evaluate the anti-inflammatory and immunomodulatory activities of HG in the \textit{mdr1a}\textsuperscript{-/-} mouse model of spontaneous colitis. Due to the recently reported chemical composition of HG, its ability to reduce inflammatory prostaglandin synthesis and its potential for anti-inflammatory biologic activity, we postulate that HG will reduce the severity of inflammatory complications associated with the onset of spontaneous colitis.

\section*{Materials and Methods}

\textbf{Reagents.} HG dried plant materials were kindly provided by Dr. Mark Wiederlichner at the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS, Ames, IA). 10\% buffered formalin; 30 \% hydrogen peroxide and concentrated sulfuric acid, were purchased from Fisher Scientific (Fair Lawn, NJ). Phenylmethanesulfonyl fluoride (PMSF), a protease inhibitor, 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB), and dimethylsulphoxide (DMSO) were purchased from Sigma (St. Louis, MO). Heparin (5,000 USP units/0.5 mL) was purchased from Hospira, Inc. (Lake Forest, IL). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Cell wash/culture medium
reagents (Dulbecco’s modified eagle’s medium (DMEM) supplemented with 4.5 g/L glucose and sodium pyruvate, penicillin-streptomycin, glutamine and hepes buffer) were all purchased from Cellgro (Herndon, VA). All anti-mouse conjugated antibodies and rat isotype control antibodies were purchased from eBioscience (San Diego, CA): PE-Cy7-conjugated anti-CD4+ monoclonal antibody (mAb), APC-conjugated anti-CD8β+ mAb, PE-conjugated anti-IgM+ mAb, Alexa 700-conjugated anti-B220+ mAb, PE-Cy7-conjugated rat IgG2ακ+isotype, APC-conjugated rat IgG2bκ+isotype, PE-conjugated rat IgG2ακ+isotype, Alexa 700-conjugated rat IgG2ακ+isotype. Stabilizing fixative (for use with PE-Cy7 conjugates) was purchased from BD biosciences (San Jose, CA). All reagents utilized for the cytokine and chemokine multiplex analysis were purchased as part of the Milliplex MAP 32-plex mouse cytokine/chemokine kit from Millipore (Billerica, MA).

Hypericum gentianoides extract preparation. Information about the specific provenance of HG accession Ames 28015, obtained from the NCRPIS, is available on the Germplasm Resources Information Network database at http://www.ars-grin.gov/npgs/acc/acc_queries.html. Arboreal portions of plants from HG (Ames 28015), harvested in 2009, were prepared for storage by drying for 8 days at 38°C in a forced-air dryer with constant humidity. The dried material was ground with a 40-mesh screen and stored at -20°C until extraction. Extractions were made in 95% ethanol (EtOH) solvent by the Soxhlet method. Upon complete drying of the extract by evaporation, the weight of the extracted material was recorded; the residue was lyophilized and stored at -20°C until solubilized in a final working solution of 5% EtOH (the lowest concentration of EtOH that would allow solubilization) at a final plant extract concentration of 24 mg/mL. The working HG extract was divided into 2 mL aliquots and stored at -20°C until use. HG extracts from NCRPIS were screened for endotoxin by using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD) according to manufacturers’ specifications, and there was no detectable endotoxin present in the extract (data not shown).
**Animals.** All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University. Four to five week old male mdr1a\(^{-/-}\) FVB.129P2-Abcb1a tm1BorN7 and wild type (WT) FVB.129P2 mice were obtained from Taconic Farms, Inc. (Germantown, NY). Animals were housed and maintained in the Laboratory Animal Resource facility at the College of Veterinary Medicine, Iowa State University. Established specific pathogen-free husbandry practices were followed and twelve-hour light/dark cycles were applied. Upon arrival, mice were fed a defined Harlan Teklad AIN93 (M) rodent chow (Madison, WI) to control the amount of phytochemicals in their diet. Food and water was provided *ad libitum.*

**Experimental Design.** A total of six treatment groups of mice where utilized: groups 1 and 2) mdr1a\(^{-/-}\) and FVB\(^{WT}\) mice were orally gavaged with 4.8 mg/day HG extract in a 200 µL volume (prepared as described above); groups 3 and 4) mdr1a\(^{-/-}\) and FVB\(^{WT}\) mice were orally gavaged with 200 µL 5% EtOH vehicle and given drinking water (refreshed weekly) containing 0.75 mg/mL metronidazole (Sigma, St. Louis, MO); groups 5 and 6) mdr1a\(^{-/-}\) and FVB\(^{WT}\) mice were orally gavaged with 5% EtOH vehicle alone. N = 4 to 10 mice per group per experiment. Gavage was performed using a 20 gauge feeding needle once a day beginning at 6 weeks of age until the mice reached twenty weeks of age or were removed from the study because of weight loss exceeding 15 % of their peak body weight. At necropsy, mice were euthanized by CO\(_2\) asphyxiation. Following euthanasia, blood was collected by cardiac puncture and sections of ceca and proximal colon were excised, washed, and stored for further histological and MPO enzymatic analysis. Serum was analyzed by multiplex flow cytometric assay to measure cytokine and chemokine levels. Cecal tonsils and mesenteric lymph nodes were also collected single cell suspensions prepared for flow cytometric analysis of T and B cell populations.

**Macroscopic typhlocolitis assessment.** Following euthanization, the colon and cecum were excised, photographed, measured for length, and scored for severity of macroscopic lesions. Gross typhlocolitic lesions were scored using a nine point additive scale; a score of zero being a healthy animal and a score of 9 being a
maximally diseased animal. Score parameters evaluated included: 1) cecal atrophy, 2) enlarged cecal tonsil or other enlarged lymphoid aggregates, 3) cecal emptying, 4) abnormally watery or mucoid intraluminal cecal and/or colonic contents, 5) bloody cecal contents, 6) bloody colonic contents, 7) visible thickening and rigidity of the cecum, 8) presence of visible thickening and rigidity of the colon, and 9) absence of formed fecal pellets in the colon.

**Histopathological assessment.** Sections of excised cecum and proximal colon were placed in 10% buffered formalin overnight, paraffin embedded, sectioned, and routinely stained with hematoxylin and eosin. Stained colonic and cecal sections were scored by a pathologist, Dr. Jesse Hostetter of Iowa State University (Ames, IA), blinded to the treatments as previously described \(^{36, 37}\). Microscopic mucosal lesion scores were assessed by five parameters, with each parameter scored on a scale of 0 to 5 (5 = maximum severity). Score parameters include: 1) ulceration of the mucosa; extent of inflammatory cell infiltrate; 2) mucosal edema characterized by the extent of lymphatic and vascular distortion from the normal architecture; 3) stromal collapse and necrosis of the glands; 4) glandular hyperplasia characterized by the distribution of enterocytes along the base of the gland; and 5) cellular proliferation. In addition to score, mucosal heights were determined using an ocular micrometer and recorded in micrometers (µm), and the presence of inflammatory cells was recorded.

**Myeloperoxidase assay.** Myeloperoxidase (MPO) activity was assessed as a measure of neutrophil/granulocyte accumulation in proximal colonic and cecal tissues. The MPO assay was performed as previously described with several modifications \(^{38}\). Proximal colon and cecal sections collected at necropsy were gently flushed with PBS to remove luminal contents and stored in 1 mL of freshly prepared PBS supplemented with the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) at 0.1 mM and 15% dimethylsulphoxide (DMSO) at -20°C for no more than 7 days prior to assay. Samples used as positive controls for MPO activity were prepared fresh the day the assay from peripheral blood. One FVB\(^{WT}\) mouse, not on study, was euthanized by CO\(_2\) asphyxiation, and 500 µl to 1 mL of blood was
immediately collected by cardiac puncture with a heparinized needle (heparin at 5,000 USP heparin units/0.5 mL is drawn into the needle and syringe and then expelled to coat the inside of the needle with heparin). The heparinized blood was centrifuged at 250 x g for 10 minutes, the supernatant discarded and the red blood cells (RBC) lysed. In brief, 1 mL of ACK lysis buffer (8042.0 mg/L ammonium chloride, 1001.0 mg/L potassium bicarbonate, 3.722 mg/L ethylene diamine tetraacetic acid disodium, pH 7.2, Sigma chemicals, St. Louis, MO) is added to the pellet, vortexed gently for 1 minute, 1 mL of PBS is added, and the mixture is centrifuged for 10 minutes at 250 x g. The lysis was repeated until the pellet was white and the supernatant was clear. Following RBC lysis, the pellet was resuspended in 1 mL of PBS/PMSF (0.1mM), cell numbers were recorded (average yield of 3 x 10^6 cells/mL) (Z Series Coulter Counter, Beckman Coulter, Fullerton, CA) and the cells were sonicated at an amplitude of 5, pulse on for 4 seconds, pulse off for 1 second for 20 seconds total (Sonicator 3000, Misonix, Inc., Farmingdale, NY). The sonicate is then centrifuged at 250 x g for 15 minutes, the supernatant stored at 4°C until the tissue samples were prepared. Frozen proximal colonic sections were thawed, blotted to remove as much excess fluid as possible, trimmed to roughly 35 mg and their weights recorded. Tissues were then homogenized for 1 minute at maximum power in 1 mL PBS/PMSF (0.1 mM) and the homogenizer probe was washed 5 times with PBS between tissue samples. Homogenate cell counts were recorded, and each sample was then sonicated as described above. The tissue sonicates were then centrifuged at 250 x g for 15 minutes, the supernatant collected and the pellet discarded. Each lysate prepared from tissue or PBMC was analyzed for total protein using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Individual lysates were pipetted into 96-well, flat bottom microtiter plates. The PBMC lysates (150 µL per well) were serially diluted (10, two-fold dilutions) and analyzed in triplicate wells. For each tissue lysate, 150 µL was pipetted into separate wells and analyzed in triplicate. To each well, 50 µL of 3,3′,5,5′-tetramethylbenzidine dihydrochloride hydrate (TMB) was added, followed immediately by the addition of 50 µL hydrogen peroxide (H_2O_2) (5 mM). The
reaction was allowed to proceed for roughly two minutes (wells turned bright blue), followed by the addition of 50 µL of sulfuric acid (1 M) to stop the reaction. The optical density (OD) was measured spectrophotometrically at 405 nm (V-Max, Molecular Devices, USA) using SOFTmax PRO 4.0. The MPO content was determined by comparison to the standard curve and MPO activity was expressed as the relative units of enzyme activity per gram of wet weight of tissue.

**Serum cytokine/chemokine quantification.** Following euthanization of mdr1a⁻/⁻ and FVBWT mice, blood was collected via cardiac puncture. The blood was allowed to clot for 24 hours at 4°C after which samples were centrifuged at 10,000 x g for 10 minutes. Serum was then removed and stored at -20°C until use. The day of assay, serum samples were thawed to room temperature. Concentrations of cytokines and chemokines of interest were measured using the Millipore (Billerica, MA) mouse cytokine-chemokine 32-plex multiplexed flow-cytometric assay kit. Analytes screened include: Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, VEGF. The assay was performed according to the manufacturer's instructions. In brief, supplied analyte standards (range - 10,000 to 3.2 pg/mL), quality control standard, and buffer only control samples were analyzed in duplicate wells of the supplied 96 well plate. Individual serum samples were diluted 1:1 in supplied assay buffer. The manufacturer’s supplied serum matrix and supplied assay buffer were added to all wells. Supplied pre-conjugated multiplex analyte beads were added to each well and the samples were incubated at 4°C overnight on a plate shaker (Barnstead International Titer Plate Shaker, setting #5, Model #4625). Following vacuum filtration and washing, supplied detection antibody was added to all wells and allowed to incubate at room temperature while shaking for two hours. Supplied streptavidine-phycocerithrin was then added to each well and incubated for 30 minutes at room temperature while shaking. After a final vacuum filtration and rinse, the beads/samples were resuspended in supplied sheath fluid and the mean fluorescence intensity (MFI) was measured using Luminex platform technology (The
FlowMetric System, Luminex, Austin, TX). MFI values were subsequently converted to concentrations using a 5-parameter logistic or line curve-fitting method in MasterPlex QT Software (MiraiBio Group, San Francisco, CA).

**Flow cytometric analysis of cecal tonsil cell populations.** Mesenteric lymph nodes and cecal tonsils from mdr1a\(^{-/-}\) and FVB\(^{WT}\) mice were excised, placed in complete cell medium (10 mL heat-inactivated FBS, 1 mL penicillin/streptomycin, 1 mL glutamine, 0.1 mL 50 mM β-mercaptoethanol, 2.5 mL 1M hepes in 86 mL DMEM with 4.5 g/L glucose and sodium pyruvate), and homogenized mechanically on ice. Stainless steel wire strainers (60 mesh) were used to prepare a single cell suspensions and remove particulate matter. Cells (5 x 10\(^5\) cells/tube) were washed in FACs buffer, centrifuged at 250 x g and incubated in FACS buffer containing 1:100 rat IgG and fluorochrome labeled reagents for 15 minutes on ice. Following labeling, cells were washed with FACs buffer, centrifuged and fixed in 200 µL of BD stabilizing fixative. Cellular preparations from individual mice were labeled with the following fluorochrome-labeled reagents: B cells (B220\(^+\)) were identified using Alexa 700-conjugated anti-B220 mAb, CD4\(^+\) T cells were identified using PE-Cy7-conjugated anti-CD4 mAb, CD8\(^+\) T cells were identified using APC-conjugated anti-CD8β mAb, Plasma cells (IgM\(^+\)) were identified by PE-conjugated anti-IgM mAb. The following isotype controls were utilized: Alexa 700-conjugated anti-rat IgG2aκ, PE-Cy7-conjugated anti-rat IgG2aκ, APC-conjugated anti-rat IgG2bκ and PE-conjugated anti-rat IgG2aκ. Working dilutions of anti-B220 mAb, anti-CD4 mAb, anti-CD8β mAb, and anti-IgM mAb and their isotypes were 1:50, 1:100, 1:80 and 1:50, respectively. Analysis was performed using a BD FACSCanto flow cytometer (BD, San Jose, CA) made available through the Flow Cytometry Core Facility at Iowa State University (Ames, IA). Data analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR).

**Statistical analysis.** Statistical significance of parametric data was evaluated by student’s t-test. Non-parametric data (e.g., scores) were evaluated by the Mann-Whitney test. All survival curves were evaluated by the Log Rank (Mantel-Cox) test.
and the Gehan-Breslow-Wilcoxon test. A P-value of < 0.05 was considered statistically significant. Prism 5 software was used for all statistical calculations.

Results

The ethanolic extract of HG delays the onset of severe colitis and decreases the severity of macroscopic disease parameters in mdr1a<sup>−/−</sup> mice. To determine the efficacy of HG extract in the treatment of spontaneous colitis, mdr1a<sup>−/−</sup> and FVB<sup>WT</sup> mice were gavaged daily with vehicle (5% EtOH) or 4.8 mg HG extract. Previously published data shows that mdr1a<sup>−/−</sup> mice spontaneously develop colitis between 8 and 36 weeks of age, with the average age of disease onset being 20 weeks<sup>39</sup>. As anticipated, the majority of vehicle treated mdr1a<sup>−/−</sup> mice (11 out of 16) developed severe colitic disease prior to 20 weeks of age. Only 6 of the 16 HG treated mice developed severe colitis prior to reaching 20 weeks of age (Fig. 1). The delay in colitic onset in HG treated mdr1a<sup>−/−</sup> was significant (P < 0.05) when compared to vehicle treated mdr1a<sup>−/−</sup> mice.

Representative photographs of ceca and colons (Fig. 2) show the extent of macroscopic tissue changes that developed in vehicle treated mdr1a<sup>−/−</sup> mice. In these mice, ceca are atrophied with visible, prominent cecal tonsils suggestive of local immune activity. Decreased presence of cecal and colonic contents was common while both cecal and colonic tissues were notably thickened. Blood was observed in some cecal and colonic contents and formed fecal pellets were absent. Conversely, the ceca and colons of HG treated mdr1a<sup>−/−</sup> mice were markedly improved and more similar to those from healthy FVB<sup>WT</sup> mice. The cecal and colonic tissues of mdr1a<sup>−/−</sup> mice treated with metronidazole were also markedly improved and healthy and all of these mice survived to the end of the experiment (Fig. 1).

Grossly, severe typhlocolitis (a score of 5 or greater) was observed in 75% of vehicle treated mdr1a<sup>−/−</sup> mice compared to only 25% of HG treated mdr1a<sup>−/−</sup> mice and none of the metronidazole treated mdr1a<sup>−/−</sup> mice. (Fig. 3A). All vehicle treated mdr1a<sup>−/−</sup> mice displayed at least mild macroscopic disease, while all of the macroscopic scores for HG treated mdr1a<sup>−/−</sup> mice are below the median score of the
vehicle treated mdr1a<sup>−/−</sup> mice (Fig. 3A). HG prophylaxis significantly (P < 0.01) prevented macroscopic parameters of disease when compared to vehicle treatment in mdr1a<sup>−/−</sup> mice. Similar to metronidazole treated mdr1a<sup>−/−</sup> mice, colon lengths in HG treated mdr1a<sup>−/−</sup> mice displayed significant (P < 0.001) improvement compared to vehicle treatment (Fig. 3B). As expected, no FVB<sup>WT</sup> mice exhibited any signs of disease (Fig. 3, A and B).

**Impact of HG treatment on the severity of histopathological lesions.**

Histological inflammation of cecal and colonic tissues was scored based on changes in mucosal height, ulceration, extent and character of inflammatory cell infiltrate, edema, stromal collapse and glandular necrosis, and glandular hyperplasia (Tables 1 and 2). Both the colons and ceca of vehicle treated mdr1a<sup>−/−</sup> mice were typified by crypt hyperplasia, extensive transmural ulceration and inflammatory infiltrate, as well as occasional submucosal edema and stromal collapse (Table 1 and Fig. 4). HG-treated mdr1a<sup>−/−</sup> mice exhibited statistically significant (p < 0.05) improvement in colonic edema (Table 1), cecal ulceration, edema, and stromal collapse or necrosis (Table 2). As expected, tissues from metronidazole-treated mdr1a<sup>−/−</sup> mice did not exhibit significant inflammation (p < 0.05 – p < 0.001 compared to vehicle mdr1a<sup>−/−</sup>), nor did FVB<sup>WT</sup> mice. Importantly, HG prophylaxis in WT mice did not induce any adverse toxic or inflammatory responses. While 100 % of vehicle treated mdr1a<sup>−/−</sup> mice exhibited extensive neutrophil infiltration into colonic and cecal tissues, inflammatory cells were absent in 31% and 44% colons and ceca of HG-treated mdr1a<sup>−/−</sup> mice, respectively (Tables 1, 2 and Fig. 4).

As a measure of inflammation and infiltration of granulocytes into the mucosal tissue, MPO activity was measured in colonic and cecal homogenates (Fig. 5). In comparison to tissue samples from the vehicle-treated mdr1a<sup>−/−</sup> mice, the associated MPO enzymatic activity was significantly diminished in colons of HG and metronidazole treated mdr1a<sup>−/−</sup> mice (P < 0.05 and P < 0.01, respectively) (Fig. 5a). Cecal tissue of HG-treated mdr1a<sup>−/−</sup> mice exhibited a decreasing trend (P < 0.08) in MPO activity that was not statistically significant (Fig. 5b)
Unexpectedly, HG prophylaxis corresponded with a notable increase in plasma cell infiltrate into both colonic and cecal tissues of mdr1a$^{-/-}$ mice (Tables 1, 2 and Fig. 4). Together, these data indicate that reduced neutrophil and increased plasma cell populations present in the ceca and colons of HG-treated mdr1a$^{-/-}$ mice provide some protection from severe inflammation and injury associated with spontaneous colitis.

**Impact of the ethanolic extract of HG on the induction of chemotactic and pro-inflammatory cytokines.** To further investigate inflammatory modulation related to improved macroscopic and microscopic cecal and colonic health, serum samples collected at necropsy were examined for the presence of cytokines and chemokines. Many analytes (G-CSF, IL-6, IL-7, IL-17, IP-10, KC, MIG, TNF$\alpha$ and VEGF) were significantly ($P < 0.5$) elevated in vehicle-treated mdr1a$^{-/-}$ mice compared to FVB$^{WT}$ controls (Table 3). Significantly reduced ($P < 0.5$) serum cytokines/chemokines (G-CSF, IL-6, IL-7, IL-17, KC and TNF$\alpha$) were detected in metronidazole treated verses vehicle treated mdr1a$^{-/-}$ mice included: (Table 3). Other serum analytes (G-CSF, IL-7, KC, LIX, MIP-1$\beta$, TNF$\alpha$) were significantly ($P < 0.5$) lower in HG-treated mdr1a$^{-/-}$ mice (Table 2). Of note, IL-6 serum concentrations were significantly ($P < 0.5$) increased in HG-treated mdr1a$^{-/-}$ mice when compared to vehicle-treated mdr1a$^{-/-}$ mice. It is clear from these data that HG is able to modulate production of many chemokines and inflammatory cytokines involved in the immune response in mdr1a$^{-/-}$ mice.

**Influence of H. gentianoides on local T cell and B cell populations.** The extent of inflammation and magnitude of the accompanying cytokine and chemokine responses can impact the course of adaptive immune responses. T cell and B cell populations in the mesenteric lymph nodes and cecal tonsils of mdr1a$^{-/-}$ and FVB$^{WT}$ mice were examined in order to evaluate the effects of HG prophylaxis on lymphocyte populations in these tissues (Fig. 6 and 7). Even in the presence of severe colitis, there was no significant difference in CD4$^+$, CD8$^+$, B220$^+$, or IgM$^+$ cell populations present in the mesenteric lymph nodes of vehicle treated mdr1a$^{-/-}$ mice (Fig. 6a-d). Even though no cell population was elevated above the FVB$^{WT}$ baseline
in vehicle-treated mdr1a<sup>-/-</sup> mice, HG and metronidazole prophylaxis reduced the percentage of IgM<sup>+</sup> cells below baseline (Fig. 6d).

In contrast to the mesenteric lymph node, vehicle-treated mdr1a<sup>-/-</sup> mice did display an approximate 2-fold (P < 0.5) elevation in CD4<sup>+</sup> T cells and B220<sup>+</sup> B cells in cecal tonsils when compared to FVB<sup>WT</sup> mice (Fig. 7a and c). CD8<sup>+</sup> and IgM<sup>+</sup> cell percentages were not significantly altered in the ceca of these mice (Fig. 7b and d). Though not statistically significant, HG treatment did cause an increase in the percentage of CD4<sup>+</sup> T cells detected in the cecal tonsils of FVB<sup>WT</sup> and mdr1a<sup>-/-</sup> mice when compared to vehicle controls (Fig. 7a). In comparison to metronidazole therapy, HG prophylaxis in mdr1a<sup>-/-</sup> mice significantly (P < 0.5) increased the percentage of detectable B220<sup>+</sup> B cells in cecal tonsil tissues when compared to FVB<sup>WT</sup> and mdr1a<sup>-/-</sup> vehicle-treated mice (Fig. 7c).

HG prophylaxis consistently reduced the percent of IgM<sup>+</sup> B cells in both cecal tonsil and mesenteric lymphoid tissues, while simultaneously increasing cecal tonsil percentages of B220<sup>+</sup> B cells. Additionally, HG increases CD4<sup>+</sup> T cell presence in cecal tonsils regardless of health status.

**Discussion**

Mice deficient in mdr1a are genetically susceptible to barrier disruption and increased antigen presentation to the lamina propria. This model is characterized by an increase in colonic inflammation which is grossly observable by 8 weeks of age. This colitis becomes transmural, multifocal, and is characterized by the production of multiple inflammatory cytokines and chemokines. The mechanism of colitic onset associated with this model as well as the genetic basis for the onset are consistent with some forms of human IBD, adding to the relevance of this model in screening for potential therapeutic or prophylactic modalities for IBD.

This study is novel in evaluation of the therapeutic potential and biologic activity of *Hypericum gentianoides* in vivo in a spontaneous model of murine colitis. Using the mdr1a<sup>-/-</sup> model of spontaneous murine colitis, we evaluated the anti-colitic
efficacy of oral HG in comparison to vehicle (5% EtOH) alone and metronidazole. The timeframe and severity of colitic onset in vehicle-treated mdr1a<sup>−/−</sup> mice was consistent with that reported in previous studies. As expected, FVB<sup>WT</sup> mice remained free of colitis. Only two metronidazole-treated mdr1a<sup>−/−</sup> mice developed mild clinical signs, which underscores the importance of the role of the resident microflora in the process of colitic onset. HG prophylaxis significantly delayed mortality by 33 days when compared to vehicle treated mdr1a<sup>−/−</sup> controls. Despite this delay by HG treatment of mdr1a<sup>−/−</sup> mice, the majority of these mice did develop clinical signs of colitis as evidenced by weight loss around 20 weeks of age. The HG treatment regimen used in these studies was unable to completely prevent the onset of disease in the mdr1a<sup>−/−</sup> mice.

Despite the inability to prevent the onset of colitis, HG significantly reduced macroscopic disease parameters in mdr1a<sup>−/−</sup> mice when compared to controls. While longer studies would be required, this could indicate that HG could further reduce long-term mortality, while allowing mice to survive with only mild to moderate clinical disease.

The improvements in macroscopic data in HG-treated mdr1a<sup>−/−</sup> mice correlated with significantly decreased microscopic colonic edema and significantly reduced cecal ulceration, edema and stromal necrosis in these mice. The improvements in microscopic and subsequent macroscopic disease morphology in HG treated mdr1a<sup>−/−</sup> mice could be attributed to the significant reduction in cecal and colonic neutrophil infiltrate in these mice (a 25% and 31% improvement, respectively). MPO activity, a known contributor to and marker of inflammation, was similarly reduced in the colons and ceca of HG treated mdr1a<sup>−/−</sup> mice. Neutrophil damage is known to contribute to disease severity in IBD. A greater number of microscopic parameters were improved in the ceca of HG-treated mdr1a<sup>−/−</sup> mice, yet the reduction in neutrophil infiltrate and subsequent MPO activity was less dramatic in the cecal tissues of these mice than in the colon. This would indicate that neutrophils are not the only inflammatory cell population affected by HG or contributing to colitic pathogenesis in these tissues.
The increase in the presentation of plasma cells seen histologically in the colons and ceca of HG-treated mdr1a−/− mice was unexpected. It has been previously documented that increasing numbers of plasma cells are found in the tissues of diseased mdr1a−/− mice. Given that HG treated mdr1a−/− mice exhibited significantly reduced colitic disease in most parameters in this study, it is unclear what the cause and function of the increased plasma cell migration into the cecal and colonic tissues of these mice is. HG prophylaxis did not cause any plasma cell infiltrate in FVBWT mice, perhaps indicative of the fact that there is no direct cause or effect related to the HG treatment and the increase in plasma cells.

Serum cytokine data from HG-treated mdr1a−/− mice illustrates another aspect of the anti-inflammatory potential of the extract in this model. HG prophylaxis in mdr1a−/− mice resulted in a significant reduction in the production of cytokines and chemokines G-CSF, KC, LIX, MIP-1β, IL-7 and TNFα. G-CSF and KC are both stimulatory and chemoattractant for neutrophils. Their downregulation by HG prophylaxis is consistent with the decrease in colonic and cecal neutrophil infiltrate seen in HG-treated mdr1a−/− mice. LIX is also a chemoattractant for neutrophils and is the murine homologue of human epithelial neutrophil-activating peptide-78 (ENA-78). The decrease in LIX production associated with HG treatment further supports histologic reductions in neutrophil infiltrate. This could be a significant interaction as the inhibition of LIX by anti-sense oligonucleotides has been shown to ameliorate experimental colitis. MIP-1β, or CCL4, is a macrophage inflammatory protein which has been shown to have high activity during the acute phase of experimental colitis while sharply decreasing in concentration during the recovery phase of colitis. IL-7 is known to be an important growth and maturation factor for colitogenic memory T cells and B cells, but also contributes to non-T or B cell colitic related inflammatory processes. HG reduction of TNFα serum concentrations is a significant finding, as TNFα has been directly implicated in the pathogenesis and chronicity of IBD. HG reduction of TNFα serum concentrations is a significant finding, as TNFα has been directly implicated in the pathogenesis and chronicity of IBD.42
prophylaxis in mdr1a−/− mice did result in the increase of IL-6 in the serum. IL-6 can be pro-inflammatory or anti-inflammatory by enhancing Th17 cell development or Treg cell development dependant on the cytokine milieu of the microenvironment. H.G prophylaxis results in a reduction in macroscopic and microscopic disease parameters and an increase in serum concentrations of IL-6. This can perhaps be explained by a recent study which has shown that IL-6 levels are elevated in the tissues of human patients with active IBD in the absence of microscopic and macroscopic inflammation. The authors concluded this to be evidence of immune activity even in the absence of histological inflammation. Much of the cytokine/chemokine data presented here could relate to the reduction in microscopic and macroscopic disease associated with reduced granulocyte and neutrophil activity in HG-treated mdr1a−/− mice. The increase in IL-6 serum concentrations in these mice could be one facet of the limitation of long-term anticolitic maintenance of HG. The increase in IL-6 could also explain the unusual increase in plasma cell and CD4+ T cell concentrations in the tissues of these mice noted histologically and by flow cytometry. It has been shown that IL-6 can enhance or facilitate B cell differentiation and production of IgG in a ‘feed-forward’ mechanism. Perhaps the increased production of IL-6 and increase in B cell presence in the tissues of HG treated mdr1a−/− are functionally linked.

In summary, the ethanolic extract of Hypericum gentianoides has anti-inflammatory and immunomodulatory potential based on its ability to attenuate inflammation in the mdr1a−/− mouse model of spontaneous colitis. This activity could be related to the flavonoid and polyphenolic compounds in the extract. This work highlights the need for further study of HG, the potential synergy of its components, and its efficacy in other disease models.

References


[27] Danesi F, Philpott M, Huebner C, Bordoni A, Ferguson LR. Food-derived bioactives as potential regulators of the IL-12/IL-23 pathway implicated in inflammatory bowel diseases. *Mutat Res*. 2010; 690: 139-44.


Figure 1. The percent of mdr1a<sup>-/-</sup> mice remaining on study.
Figure 2. Photographs of colons and ceca from WT or mdr1a⁻/⁻ mice treated with vehicle, metronidazole or *Hypericum gentianoides*.
**Figure 3.** Macroscopic assessment of colitis in WT or mdr1a<sup>−/−</sup> mice treated with vehicle, metronidazole or *Hypericum gentianoides*. 
Table 1. *Hypericum gentianoides* prophylaxis significantly decreases colonic edema associated with severe typhlocolitis in mdr1a<sup>−/−</sup> mice, decreases the occurrence of neutrophil influx associated with histologic inflammation and increases colonic plasma cell infiltrate.

<table>
<thead>
<tr>
<th>Microscopic Parameter</th>
<th>V&lt;sub&gt;WT&lt;/sub&gt; (n = 6)</th>
<th>M&lt;sub&gt;WT&lt;/sub&gt; (n = 4)</th>
<th>HG&lt;sub&gt;WT&lt;/sub&gt; (n = 6)</th>
<th>V&lt;sup&gt;−/−&lt;/sup&gt; (n = 16)</th>
<th>M&lt;sup&gt;−/−&lt;/sup&gt; (n = 10)</th>
<th>HG&lt;sup&gt;−/−&lt;/sup&gt; (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal Height (µM)</td>
<td>3.2 ± 0.2***</td>
<td>3.0 ± 0.0***</td>
<td>3.7 ± 0.7**</td>
<td>5.2 ± 0.2</td>
<td>4.4 ± 0.3*</td>
<td>4.9 ± 0.2</td>
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<tr>
<td>Ulceration</td>
<td>0.0 ± 0.0***</td>
<td>0.0 ± 0.0***</td>
<td>0.5 ± 0.5**</td>
<td>1.9 ± 0.3</td>
<td>0.4 ± 0.3**</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Inflammation</td>
<td>1.0 ± 0.0***</td>
<td>1.0 ± 0.0***</td>
<td>1.3 ± 0.3***</td>
<td>3.5 ± 0.2</td>
<td>2.1 ± 0.2***</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Edema</td>
<td>0.2 ± 0.2*</td>
<td>0.3 ± 0.3*</td>
<td>0.3 ± 0.2*</td>
<td>1.2 ± 0.3</td>
<td>0.4 ± 0.2*</td>
<td>0.4 ± 0.2*</td>
</tr>
<tr>
<td>Stromal Collapse (Necrosis)</td>
<td>0.0 ± 0.0**</td>
<td>0.0 ± 0.0*</td>
<td>0.0 ± 0.0**</td>
<td>1.1 ± 0.3</td>
<td>0.1 ± 0.1*</td>
<td>1.0 ± 0.2</td>
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<tr>
<td>Gland Hyperplasia</td>
<td>0.3 ± 0.2***</td>
<td>0.0 ± 0.0***</td>
<td>0.8 ± 0.5**</td>
<td>3.0 ± 0.2</td>
<td>1.4 ± 0.3***</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>

% Mice Exhibiting Colonic Neutrophil Infiltrate

- 0% 0% 0%
- 100% 20% 69%

% Mice Exhibiting Colonic Plasma Cell Infiltrate

- 0% 0% 0%
- 12% 0% 31%

V<sub>WT</sub> = Vehicle (5% EtOH) treated FVB<sub>WT</sub> mice, M<sub>WT</sub> = Metronidazole treated FVB<sub>WT</sub> mice, HG<sub>WT</sub> = *Hypericum gentianoides* treated FVB<sub>WT</sub> mice, V<sup>−/−</sup> = Vehicle (5% EtOH) treated mdr1a<sup>−/−</sup> mice, M<sup>−/−</sup> = Metronidazole treated mdr1a<sup>−/−</sup> mice, HG<sup>−/−</sup> = *Hypericum gentianoides* treated mdr1a<sup>−/−</sup> mice. Data are expressed as the mean ± SEM. Statistical significance is noted in three levels: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.
Table 2. *Hypericum gentianoides* prophylaxis significantly decreases cecal edema associated with severe typhlocolitis in mdr1a<sup>−/−</sup> mice, decreases the occurrence of neutrophil influx associated with histologic inflammation and increases colonic plasma cell infiltrate.

<table>
<thead>
<tr>
<th>Microscopic Parameter</th>
<th>V&lt;sup&gt;WT&lt;/sup&gt; (n = 6)</th>
<th>M&lt;sup&gt;WT&lt;/sup&gt; (n = 4)</th>
<th>HG&lt;sup&gt;WT&lt;/sup&gt; (n = 6)</th>
<th>V&lt;sup&gt;−/−&lt;/sup&gt; (n = 16)</th>
<th>M&lt;sup&gt;−/−&lt;/sup&gt; (n = 10)</th>
<th>HG&lt;sup&gt;−/−&lt;/sup&gt; (n = 16)</th>
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<tbody>
<tr>
<td>Mucosal Height (µM)</td>
<td>3.7 ± 0.2***</td>
<td>3.0 ± 0.6***</td>
<td>3.5 ± 0.2**</td>
<td>4.5 ± 0.2</td>
<td>3.8 ± 0.2*</td>
<td>4.6 ± 0.2</td>
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<tr>
<td>Ulceration</td>
<td>0.0 ± 0.0***</td>
<td>0.0 ± 0.0***</td>
<td>0.0 ± 0.0***</td>
<td>2.3 ± 0.3</td>
<td>0.1 ± 0.1***</td>
<td>0.7 ± 0.3**</td>
</tr>
<tr>
<td>Inflammation</td>
<td>1.5 ± 0.3***</td>
<td>1.0 ± 0.0***</td>
<td>1.5 ± 0.2***</td>
<td>3.2 ± 0.2</td>
<td>2.1 ± 0.3**</td>
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<tr>
<td>Edema</td>
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<td>0.8 ± 0.5</td>
<td>0.8 ± 0.3**</td>
<td>2.1 ± 0.2</td>
<td>0.9 ± 0.3**</td>
<td>1.6 ± 0.3*</td>
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<tr>
<td>Stromal Collapse (Necrosis)</td>
<td>0.0 ± 0.0***</td>
<td>0.0 ± 0.0**</td>
<td>0.0 ± 0.0***</td>
<td>1.7 ± 0.3</td>
<td>0.1 ± 0.1**</td>
<td>0.6 ± 0.3*</td>
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<tr>
<td>Gland Hyperplasia</td>
<td>0.7 ± 0.3***</td>
<td>0.8 ± 0.5**</td>
<td>0.3 ± 0.2***</td>
<td>2.8 ± 0.3</td>
<td>1.7 ± 0.3*</td>
<td>2.6 ± 0.2</td>
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<td>% Mice Exhibiting Cecal Neutrophil Infiltrate</td>
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<td>0%</td>
<td>0%</td>
<td>81%</td>
<td>20%</td>
<td>56%</td>
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<tr>
<td>% Mice Exhibiting Cecal Plasma Cell Infiltrate</td>
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<td>0%</td>
<td>0%</td>
<td>12%</td>
<td>10%</td>
<td>31%</td>
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V<sup>WT</sup> = Vehicle (5% EtOH) treated FVB<sup>WT</sup> mice, M<sup>WT</sup> = Metronidazole treated FVB<sup>WT</sup> mice, HG<sup>WT</sup> = *Hypericum gentianoides* treated FVB<sup>WT</sup> mice, V<sup>−/−</sup> = Vehicle (5% EtOH) treated mdr1a<sup>−/−</sup> mice, M<sup>−/−</sup> = Metronidazole treated mdr1a<sup>−/−</sup> mice, HG<sup>−/−</sup> = *Hypericum gentianoides* treated mdr1a<sup>−/−</sup> mice. Data are expressed as the mean ± SEM. Statistical significance is noted in three levels: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.
Figure 4. Hypericum gentianoides protects microscopic architecture in mdr1a<sup>-/-</sup> mice, decreases inflammatory cell infiltrate, and increases plasma cell infiltrate.
Figure 5. Metronidazole and *Hypericum gentianoides* reduce local myeloperoxidase (MPO) activity in the colons and ceca of mdr1a<sup>-/-</sup> mice.
Table 3. *Hypericum gentianoides* prophylaxis reduces serum content of several chemotactic and inflammatory cytokines associated with severe typhlocolitis in mdr1a<sup>+/−</sup> mice.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>V&lt;sup&gt;WT&lt;/sup&gt; (n = 6)</th>
<th>M&lt;sup&gt;WT&lt;/sup&gt; (n = 4)</th>
<th>HG&lt;sup&gt;WT&lt;/sup&gt; (n = 6)</th>
<th>V&lt;sup&gt;−&lt;/sup&gt; (n = 16)</th>
<th>M&lt;sup&gt;−&lt;/sup&gt; (n = 10)</th>
<th>HG&lt;sup&gt;−&lt;/sup&gt; (n = 16)</th>
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<tr>
<td>Eotaxin</td>
<td>443 ± 42</td>
<td>468 ± 28</td>
<td>559 ± 102</td>
<td>742 ± 126</td>
<td>956 ± 160</td>
<td>901 ± 198</td>
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<tr>
<td>G-CSF</td>
<td>156 ± 43***</td>
<td>1223 ± 1073*</td>
<td>175 ± 41***</td>
<td>7944 ± 2635</td>
<td>443 ± 124***</td>
<td>2009 ± 625**</td>
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<tr>
<td>GM-CSF</td>
<td>17 ± 2</td>
<td>20 ± 5</td>
<td>18 ± 2</td>
<td>18 ± 3</td>
<td>13 ± 4</td>
<td>15 ± 3</td>
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<td>IFNγ</td>
<td>2 ± 0</td>
<td>6 ± 2</td>
<td>4 ± 1</td>
<td>19 ± 4</td>
<td>9 ± 3</td>
<td>11 ± 3</td>
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<tr>
<td>IL-1α</td>
<td>104 ± 17</td>
<td>140 ± 7</td>
<td>132 ± 13</td>
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<td>125 ± 13</td>
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<td>IL-1β</td>
<td>10 ± 2</td>
<td>16 ± 4</td>
<td>9 ± 2</td>
<td>14 ± 3</td>
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<td>IL-6</td>
<td>9 ± 3*</td>
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<td>2 ± 1**</td>
<td>16 ± 12*</td>
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<td>336 ± 68</td>
<td>340 ± 54</td>
<td>265 ± 28</td>
<td>217 ± 41</td>
<td>269 ± 31</td>
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<td>18 ± 4</td>
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<td>18 ± 4</td>
<td>20 ± 2</td>
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<td>IL-12(p40)</td>
<td>29 ± 4</td>
<td>43 ± 7</td>
<td>33 ± 6</td>
<td>85 ± 35</td>
<td>176 ± 56</td>
<td>100 ± 36</td>
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<td>IL-12(p70)</td>
<td>49 ± 8</td>
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<td>28 ± 3</td>
<td>39 ± 7</td>
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<td>IL-13</td>
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<td>834 ± 214</td>
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</tr>
<tr>
<td>IP-10</td>
<td>25 ± 5***</td>
<td>56 ± 8**</td>
<td>25 ± 4***</td>
<td>612 ± 151</td>
<td>300 ± 69</td>
<td>361 ± 80</td>
</tr>
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<td>KC</td>
<td>125 ± 31***</td>
<td>182 ± 546***</td>
<td>92 ± 26***</td>
<td>801 ± 122</td>
<td>251 ± 83***</td>
<td>251 ± 30***</td>
</tr>
<tr>
<td>LIF</td>
<td>3 ± 0</td>
<td>3 ± 0</td>
<td>3 ± 0</td>
<td>4 ± 2</td>
<td>1 ± 0</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>LIX</td>
<td>14000 ± 1</td>
<td>14000 ± 1</td>
<td>130310 ± 617</td>
<td>137510 ± 1264</td>
<td>169750 ± 1922</td>
<td>8088 ± 1415**</td>
</tr>
<tr>
<td>MIG</td>
<td>73 ± 19***</td>
<td>230 ± 73*</td>
<td>90 ± 25**</td>
<td>2119 ± 469</td>
<td>1734 ± 561</td>
<td>1461 ± 248</td>
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<tr>
<td>MIP-1β</td>
<td>40 ± 5</td>
<td>40 ± 4</td>
<td>43 ± 9</td>
<td>42 ± 5</td>
<td>40 ± 8</td>
<td>28 ± 6*</td>
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<tr>
<td>TNFα</td>
<td>2 ± 0**</td>
<td>3 ± 0**</td>
<td>3 ± 1**</td>
<td>12 ± 2</td>
<td>5 ± 1**</td>
<td>7 ± 1*</td>
</tr>
<tr>
<td>VEGF</td>
<td>2 ± 1*</td>
<td>1 ± 0*</td>
<td>2 ± 1*</td>
<td>13 ± 6</td>
<td>12 ± 4</td>
<td>9 ± 4</td>
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V<sup>WT</sup> = Vehicle (5% EtOH) treated FVB<sup>WT</sup> mice, M<sup>WT</sup> = Metronidazole treated FVB<sup>WT</sup> mice, HG<sup>WT</sup> = *Hypericum gentianoides* treated FVB<sup>WT</sup> mice, V<sup>−</sup> = Vehicle (5% EtOH) treated mdr1a<sup>−/−</sup> mice, M<sup>−</sup> = Metronidazole treated mdr1a<sup>−/−</sup> mice, HG<sup>−</sup> = *Hypericum gentianoides* treated mdr1a<sup>−/−</sup> mice. Data are expressed as the mean serum cytokine/chemokines content (pg/mL) ± SEM. Statistical significance is noted in three levels: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.
Figure 6. Evaluation of CD4⁺, CD8⁺, B220⁺ and IgM⁺ cell subsets in the mesenteric lymph nodes of mice treated with vehicle, metronidazole or Hypericum gentianoides.
Figure 7. Evaluation of CD4⁺, CD8⁺, B220⁺ and IgM⁺ cell subsets in the cecal tonsils of mice treated with vehicle, metronidazole or *Hypericum gentianoides*. 
Figure Legends

**Figure 1.** The percent of mdr1a<sup>−/−</sup> mice remaining on study. Mdr1a<sup>−/−</sup> mice were removed from the study as they developed severe colitis or at the termination of the experiment as described in Materials and Methods.*P < 0.05, **P < 0.01, ***P < 0.001 compared to mdr1a<sup>−/−</sup> vehicle. This adapted survival curve is representative of two separate experiments (vehicle n = 16, 0.075 mg/mL metronidazole n = 10, 4.8 mg/kg *Hypericum gentianoides* n = 16).

**Figure 2.** Photographs of colons and ceca from WT or mdr1a<sup>−/−</sup> mice treated with vehicle, metronidazole or *Hypericum gentianoides*. Mdr1a<sup>−/−</sup> mice were gavaged with 5% EtOH vehicle (n = 16), 0.075 mg/mL metronidazole dosed in drinking water (n = 10) or gavaged with 4.8 mg *Hypericum gentianoides* extract (n = 16) daily as described in Materials and Methods. FVB<sup>WT</sup> mice were also represented in each treatment group (n = 6, FVB<sup>WT</sup> mice per treatment). Images are representative of two independent experiments.

**Figure 3.** Macroscopic assessment of colitic severity. a) Macroscopic typhlocolitic scores of mdr1a<sup>−/−</sup> and FVB<sup>WT</sup> mice treated with vehicle, metronidazole or *Hypericum gentianoides* extract as described in Materials and Methods (Max/Severe = 9, Min/Healthy = 0.) b) Colon lengths of mdr1a<sup>−/−</sup> and FVB<sup>WT</sup> mice treated with vehicle, metronidazole or *Hypericum gentianoides* extract were measured at necropsy and the group range is represented. Whiskers indicate minimum and maximum values, while the horizontal line represents the group median. *P < 0.05, ** P < 0.01, *** P < 0.001 compared to mdr1a<sup>−/−</sup> vehicle. Data is representative of two independent experiments with n = those listed in Figure 1.

**Figure 4.** *Hypericum gentianoides* protects microscopic architecture in mdr1a<sup>−/−</sup> mice, decreases inflammatory cell infiltrate, and increases plasma cell infiltrate. Representative photomicrographs (200x) of cecal and colonic sections collected from mdr1a<sup>−/−</sup> and FVB<sup>WT</sup> mice treated with vehicle, metronidazole or *Hypericum gentianoides* extract at the time of necropsy were formalin fixed, paraffin embedded and subject to routine hematoxylin and eosin staining. FVB<sup>WT</sup> ceca of all treatments are characterized by normal mucosal architecture and height, intact glands, little to no mucosal or submucosal edema, and normal resident populations of mononuclear cells and lack of plasma cells. In contrast, diseased mdr1a<sup>−/−</sup> ceca exhibit mucosal and glandular hyperplasia, transmural ulceration and inflammation, as well as stromal necrosis and an inflammatory population of neutrophils. *Hypericum gentianoides* and metronidazole treated mdr1a<sup>−/−</sup> ceca and colons are improved toward the normal wild type condition. A significant increase in the tissue presence of plasma cells was noted in mdr1a<sup>−/−</sup> mice treated with *Hypericum gentianoides*. Photographs were chosen based on the average macroscopic and microscopic score data for each group and are representative of two independent experiments with n = those listed in Figure 1.
Figure 5. Metronidazole and Hypericum gentianoides reduce local myeloperoxidase (MPO) activity in the colons and ceca of mdr1a⁻/⁻ mice. Homogenates of colonic or cecal tissue were used to assay for MPO activity. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 compared to mdr1a⁻/⁻ vehicle. Data is representative of: FVB<sup>WT</sup> vehicle n = 6, metronidazole n= 4, Hypericum gentianoides n = 6; mdr1a⁻/⁻ vehicle n = 10, metronidazole n= 4, Hypericum gentianoides n = 10.

Figure 6. Evaluation of CD4<sup>+</sup>, CD8<sup>+</sup>, B220<sup>+</sup> and IgM<sup>+</sup> cell subsets in the mesenteric lymph nodes of mice treated with vehicle, metronidazole or Hypericum gentianoides. Mesenteric lymph nodes were excised at necropsy, single cell suspensions prepared and stained for flow cytometric analysis as described in materials and methods. a) Percent of CD4<sup>+</sup> T cells in the mesenteric lymph nodes, b) Percent of CD8<sup>+</sup> T cells in the mesenteric lymph nodes, c) Percent of B220<sup>+</sup> B cells in the mesenteric lymph nodes. d) Percent of IgM<sup>+</sup> B cells in the mesenteric lymph nodes. *P < 0.05, ** P < 0.01 compared to mdr1a⁻/⁻ vehicle. Flow cytometric data was analyzed using FlowJo software and statistical analysis was performed using GraphPad 5. Data is representative of: FVB<sup>WT</sup> vehicle n = 6, metronidazole n= 4, Hypericum gentianoides n = 6; mdr1a⁻/⁻ vehicle n = 10, metronidazole n= 4, Hypericum gentianoides n = 10.

Figure 7. Evaluation of CD4<sup>+</sup>, CD8<sup>+</sup>, B220<sup>+</sup> and IgM<sup>+</sup> cell subsets in the cecal tonsils of mice treated with vehicle, metronidazole or Hypericum gentianoides. Cecal tonsils were excised at necropsy, single cell suspensions prepared and stained for flow cytometric analysis as described in materials and methods. a) Percent of CD4<sup>+</sup> T cells in the cecal tonsils, b) Percent of CD8<sup>+</sup> T cells in the cecal tonsils, c) Percent of B220<sup>+</sup> B cells in the cecal tonsils. d) Percent of IgM<sup>+</sup> B cells in the cecal tonsils. *P < 0.05, ** P < 0.01, *** P < 0.001 compared to mdr1a⁻/⁻ vehicle. Flow cytometric data was analyzed using FlowJo software and statistical analysis was performed using GraphPad 5. Data is representative of: FVB<sup>WT</sup> vehicle n = 6, metronidazole n= 4, Hypericum gentianoides n = 6; mdr1a⁻/⁻ vehicle n = 10, metronidazole n= 4, Hypericum gentianoides n = 10.
Table Legends

Table 1. Hypericum gentianoides prophylaxis significantly decreases colonic edema associated with severe typhlocolitis in mdr1a<sup>-/-</sup> mice, decreases the occurrence of neutrophil influx associated with histologic inflammation and increases colonic plasma cell infiltrate. Colonic sections collected at the time of necropsy were formalin fixed; paraffin embedded and subject to routine hematoxylin and eosin staining. Stained histologic sections were scored for inflammation by a pathologist blinded to the treatments. Scores for ulceration, Inflammation, edema, necrosis and gland hyperplasia were scored on a 0 (normal) to 5 (severe) scale. Data is represented as mean ± SEM (except where noted). *P < 0.05, ** P < 0.01, *** P < 0.001 compared to mdr1a<sup>-/-</sup> vehicle. Mucosal heights were subject to parametric unpaired t-test, while microscopic scores were subject to non-parametric, Mann-Whitney t-test. Statistical analysis was performed using GraphPad Prism 5. Data are representative of two separate experiments with n = those listed in Figure 1.

Table 2. Hypericum gentianoides prophylaxis significantly decreases cecal edema associated with severe typhlocolitis in mdr1a<sup>-/-</sup> mice, decreases the occurrence of neutrophil influx associated with histologic inflammation and increases colonic plasma cell infiltrate. Cecal sections were prepared and assessed as noted in Table 1. Data is represented as mean ± SEM (except where noted). *P < 0.05, ** P < 0.01, *** P < 0.001 compared to mdr1a<sup>-/-</sup> vehicle. Mucosal heights were subject to parametric unpaired t-test, while microscopic scores were subject to non-parametric, Mann-Whitney t-test. Statistical analysis was performed using GraphPad Prism 5. Data are representative of two separate experiments with n = those listed in Figure 1.

Table 3. Hypericum gentianoides prophylaxis reduces serum content of several chemotactic and inflammatory cytokines associated with severe typhlocolitis in mdr1a<sup>-/-</sup> mice. Serum collected from individual mice at was subjected to multiplex flow cytometric bead analysis for chemokine and cytokine concentrations using a 32-plex mouse chemokine/cytokine kit. Only cytokines and chemokines which were detectable in at least one treatment group are represented. Data is represented as average serum concentrations (pg/mL) ± SEM. *P < 0.05, ** P < 0.01, *** P < 0.001 compared to mdr1a<sup>-/-</sup> vehicle. Concentrations were subject to non-parametric, Mann-Whitney t-test. Statistical analysis was performed using GraphPad Prism 5. Data are representative of two separate experiments with n = those listed in Figure 1.
CHAPTER 5. GENERAL CONCLUSIONS

Summary

The experiments presented in this dissertation illustrate that mice exposed to 2.5% DSS, an epithelial toxin and colitic instigator, develop worsening colitic lesions which do not respond to daily oral prophylaxis with 100 or 200 mg/kg of the ethanolic extract of *Echinacea angustifolia* (EA). Restitution from a mild (1.75%) or severe (2.5%) DSS colitic insult is likewise not significantly enhanced by 200 mg/kg EA prophylaxis. In a model of moderate, controlled DSS colitis (titration from 2.5% to 1.5%) daily oral prophylaxis with an ethanolic extract of *Prunella vulgaris* (PV) (100 mg/kg) significantly reduced weight loss and intestinal lesion formation. This effect was not maintained if PV ethanolic extract was dosed therapeutically in the same DSS model.

Additional analysis of the prophylactic anti-colitic and anti-inflammatory efficacy of PV was examined in the human relevant mdr1a/-/- model of spontaneous colitis. PV ethanolic extract (2.4 mg/day = 100 mg/kg/day) significantly delayed onset of colitic clinical signs and mortality related to onset of severe spontaneous colitis. Colitic injury was macroscopically and microscopically improved in PV treated mdr1a/-/- mice. Anti-colitic effects of PV in this model were associated with reduced innate neutrophil function and presence in the intestinal tissues. These observations in PV treated mdr1a/-/- mice were accompanied by suppression of serum inflammatory chemokines and cytokines which function as growth factors and chemoattractants for inflammatory cells. PV prophylaxis in mdr1a/-/- mice lead to reduced expression of genes related to NF-κB activity and subsequently reduced the presence of cecal CD4+ and CD8+ inflammatory T cells. As noted in the DSS induced model of colitis, all of these effects of PV ethanolic extracts were abolished when PV was dosed in a therapeutic fashion at the onset of colitic clinical signs in mdr1a/-/- mice. These combined experiments underscore the immunosuppressive and related anti-colitic activities of PV ethanolic extracts in vivo. PV is a prophylactic medicinal option for further study in colitic therapy.
Similar to PV, ethanolic extract of *Hypericum gentianoides* (HG) was examined as a prophylactic anti-colitic in the mdr1a<sup>−/−</sup> model of spontaneous colitis. A 4.8 mg/kg dose of HG ethanolic extract dosed to mdr1a<sup>−/−</sup> mice significantly delayed colitic onset and significantly reduces colitic mortality and macroscopic as well as microscopic inflammatory intestinal injury. Microscopically and enzymatically, colonic and cecal neutrophil related inflammation was reduced by HG prophylaxis in mdr1a<sup>−/−</sup> mice. HG prophylaxis was also responsible for increased plasma cell infiltrate into the intestines of mdr1a<sup>−/−</sup> mice but had no effect on intestinal T cell populations in these mice. While several inflammatory cytokines and chemokines were reduced by HG prophylaxis, IL-6 levels in the serum were increased in mdr1a<sup>−/−</sup> mice. These novel in vivo studies of HG ethanolic extracts uncover the potential of a brand new treatment modality for IBD.

**Discussion**

Inflammatory bowel disease (IBD) is a collection of idiopathic inflammatory enteric disorders which continue to take a heavy toll on the financial, economic and quality of life status of patients suffering from these diseases (1). It is effective and important to optimize old therapeutic strategies related to IBD treatment. However, the continued cost inflation and growing inaccessibility to evermore complex IBD remedies and the absence of confidence in long-term safety and efficacy of current anti-colitics underscore the need for concentration of scientific research to effectively and quickly screen new, safe, and broadly efficacious anti-IBD treatment modalities (2-8). Herbal or nutraceutical CAM is an attractive medicinal option for IBD primary or adjunct therapy due to its documented safety, low cost, public and commercial accessibility, and generally high patient compliance (9-15). In addition to these positive aspects of CAM, evidence supporting the bioavailability and anti-inflammatory bioactivity of herbal compounds is growing (13, 16-28).

Therapeutic and prophylactic medicinal targets must be studied in animal models of disease to assess safety and efficacy. The DSS induced model of colitis offers an inexpensive, highly flexible and disease relevant method of quickly titrating...
and assessing the activity of potentially anti-colitic and anti-inflammatory compounds for further examination in even more accurate and complex models of disease (29). In this context, ethanolic extracts of EA, PV and HG were screened for anti-colitic and anti-inflammatory activities in the DSS induced mouse model of colitis.

The mdr1a−/− mouse model of spontaneous colitis is a well described, repeatable and relevant model for the study of human and animal IBD (30). This model encompasses the vital contributions of the intestinal epithelial barrier dysfunction and the host microflora to colitic onset. Similar to human and animal IBD, colitic lesions in mdr1a−/− mice are transmural and generally continuous throughout the colon (31). Mucosal edema, thickening and lamina proprial inflammatory cell infiltrate are common to these lesions. Furthermore, enhanced expression of IFNγ, IL-6, IL-1β, TNFα, MCP-1, MIP-1α, RANTES and Eotaxin mRNA, along with increased expression of the chemokine receptors CCR2 and CCR5 in mdr1a deficient mice is comparable to changes seen in patients with UC and CD (32). Mdr1a has been directly implicated in human UC and CD (30, 33, 34). In this context, ethanolic extracts of PV and HG were screened for anti-colitic and anti-inflammatory activities in the mdr1a−/− mouse model of spontaneous colitis.

**Echinacea angustifolia**

Utilizing the DSS model of chemically induced colitis, the macroscopic anti-colitic activity of oral EA in comparison to 5% EtOH vehicle alone as a sham treatment was assessed. With regard to experiments evaluating EA anti-colitic activity, extract prophylaxis was chosen due to the low concentration of individual bioactive compounds in whole extracts and the knowledge that the colonic mucosa turns over or replenishes every 4 to 7 days (35, 36). Pre-treatment would allow for accumulation of potentially bioactive compounds and would ensure thorough exposure of the gut epithelium to these compounds prior to inflammatory DSS insult, increasing the likelihood of immediate and effective constituent influence on the rapid onset of inflammation caused by the initial exposure to DSS. As penetrance of some compounds into the deeper layers of the lamina propria or submucosa could
be poor, there is a better chance that bioactive compounds could interact directly with the mucosal epithelium to exert effects (37).

EA effectively inhibits inflammatory prostaglandin E2 (PGE2) production and modulates COX-2 signaling (23). Potential anti-inflammatory bioactivity combined with the inherent feature of increased colonic permeability and potential for increased chemical exposure in the colitic gut, make EA an interesting potential anti-colitic therapy (38). The progression of weight loss, severity of macroscopic disease scores reduced colon lengths seen in DSS treated mice alone were unchanged in EA treated mice exposed to DSS. EA did not positively or negatively statistically effect the severity of colitis induced by 2.5% DSS. It is clear that EA is non-toxic macroscopically. Because colitic intestinal epithelia are more permeable or ‘leaky’, if EA were toxic, it could potentially have caused excess weight loss or additional adverse effects in mice treated with DSS. Additionally, EA was not toxic in healthy control mice.

It was considered that the potent inflammatory insult of 2.5% DSS might mask significant bioactivity of EA. For this reason, EA was tested in a DSS model which included an additional 7 days of DSS free restitution and a lower dose of DSS (1.75%) was also examined. While 1.75% DSS clearly caused lesser colitic damage than 2.5% DSS; as evidenced by weight loss, restitution weight recovery, disease score and colon lengths; EA was still ineffective in treating this milder colitic insult and was also inefficient at enhancing mucosal restitution.

While the totality of this data would indicate that EA is not an ideal option for anti-colitic therapy, it is possible that further extract or DSS dose adjustments could be required to uncover the bioactivity of EA in this model. Moreover, it is possible that the efficacy of EA might be directed more towards an aspect of colitis not addressed in this model, such as a colitic model driven more by dysbiosis or a different inflammatory insult than an epithelial toxin like DSS. These experiments did not address metabolic end products or their concentrations in feces or serum. While these studies are ongoing, the serum concentrations, penetrance and metabolic
byproducts related to EA extract treatment in this model could underscore the lack of activity seen here or point to need for adjustments to treatment or the model.

**Prunella vulgaris**

Dosing strategies for PV in the DSS induced model of colitis were influenced by the same considerations as those previously mentioned for EA. The macroscopic anti-colitic activity of oral PV ethanolic extract in comparison to 5% EtOH vehicle alone was assessed in this model. PV anti-colitic activity was identified in DSS studies presented in this dissertation. Previous studies show that PV is capable of anti-inflammatory effects in several models and contains several bioactive phenolics, triterpenoids, and flavonoids (27, 39-42). These studies supported the hypothesis that PV could reduce colitic inflammation. PV prophylaxis was examined in the context of a ‘controlled lesion’ DSS dosing model in which a moderate to severe colitic insult was initiated with 2.5% DSS followed by an extended period of mild DSS dosing (1.5%) to maintain a constant, non-progressive lesion (43). Daily mouse weight maintenance, decreased macroscopic score, and increased colon lengths all indicate that prophylactic PV significantly reduces the severity of DSS colitis. Prophylaxis is required for this activity as therapeutic dosing in this model abolished anti-colitic efficacy of PV ethanolic extract. This would indicate that prior exposure of gut tissues to PV is required in the absence inflammatory insult. This might not be true at a lower dose of DSS not investigated here.

The anti-DSS colitic efficacy of ethanolic PV extracts required further testing in a more complex, human relevant model of IBD. The mdr1a<sup>−/−</sup> model of spontaneous colitis is ideal for study of potential IBD therapeutics relevant to human and animal medicine (30). PV extract prophylaxis was found to improve the macroscopic damage associated with severe typhlocolitic lesion as ceca of mdr1a<sup>−/−</sup> mice gavaged with PV retained normal architecture, lacked grossly developed lymphoid aggregates, and retained luminal contents without the presence of blood or mucus, unlike vehicle treated mdr1a<sup>−/−</sup> mice. Presence of formed feces and lack of gross tissue edema and rigidity in colons of PV treated mdr1a<sup>−/−</sup> mice underscored improved function and homeostasis in these mice. Significant microscopic benefit of
extract treatment was targeted to the cecum, perhaps not surprising as the concentration of microflora in this organ could more efficiently metabolize PV, revealing active metabolic byproducts (44). Colitic onset was slightly delayed by *P. vulgaris* and 30% fewer mice developed severe colitis by 20 weeks of age when gavaged with PV extract. Limitations of *P. vulgaris* could be due to the focused cecal benefit of extract therapy, or perhaps further dose titration is required for more inclusive typhlocolitic benefit.

The inductive phase of colitis involves a compromised intestinal epithelium and innate neutrophil activation, transmigration and enzymatic damage to host tissues (45-49). Flavonoids from licorice have been shown to inhibit neutrophil infiltration into lung tissue after lippopolysaccharide-induced inflammation, and reduce the severity of associated inflammatory damage to host lungs (50). Similarly, PV prevented cecal neutrophil infiltrate in 62% of mdr1a<sup>-/-</sup> mice. Related to this effect, microscopic scores and MPO activity were improved by PV.

Since neutrophils are not resident in the tissues, cytokine and chemokine signals produced by epithelial cells and local macrophage are responsible for transmigration of neutrophils to the sight of tissue distress (51, 52). Innate immune chemokines are central to the development of severe inflammation in colitis (45, 48, 53). Downregulation of cytokines which increase expression of adhesions on endothelial cells (TNFα, IL-1α, IL-1β), reduced production of cytokines which upregulate neutrophil chemokine production (IFNγ) and downregulation of inflammatory monocyte derived or neutrophil chemotactic factors (IL-8/KC and VEGF) have been shown to reduce inflammatory tissue damage in several disease models, including colitis (54-57). PV treatment of mdr1a<sup>-/-</sup> mice lead to reductions in innate growth factors (G-CSF and GM-CSF), the neutrophil chemokine KC, and TNFα; a key potentiator of colitis (58). This data illustrates that PV reduces the chronic production of cytokines and chemokines critical in replenishing the supply of inflammatory immune cells and their damaging products in the intestine but does not completely block their production. In other words, PV is not completely shutting down cytokine and chemokine production, perhaps allowing for homeostatic or
maintenance levels of inflammatory signaling. Trying to maintain the delicate balance of minimal but constant homeostatic inflammation in the gut is imperative, and current IBD therapies like monoclonal antibodies to TNFα disrupt that balance by abolishing the necessary homeostatic functions of TNF, leaving patients vulnerable to secondary infections or inducing hypersensitivity (59).

NF-κB and its gene targets are inflammatory targets in IBD (60-63). Flavonoids are capable of downregulating NF-κB and ameliorating spontaneous cecitis (26, 43, 64). Perhaps the flavonoids present in PV contributed to the downregulation of gene targets controlling neutrophil and monocyte chemotaxis (Ccl2, Cxcl1/KC and Cxcl9/MIG), adhesion expression (VCAM-1, ICAM, TNFα and IL-1α), tissue remodeling and inflammatory cell transmigration (MMP-10) mdr1a⁻/⁻ mouse ceca. All of these genes participate in activation of, or are regulated by NF-κB (65-70). Clearly, PV is decreasing neutrophil chemotaxis and activity downregulating gene expression of targets regulated by or participating in NF-κB signaling. Prophylaxis might be key in this point due to the observation activation of inflammatory gene regulation is altered in mdr1a⁻/⁻ mice and DSS colitic mice prior to any histologic signs of inflammation (71, 72). Supporting this hypothesis is the evidence that therapeutic PV ethanolic extract gavage had no anti-colitic efficacy in mdr1a⁻/⁻ mice. PV might be modulating early innate inflammatory gene expression in mdr1a⁻/⁻ mice, as treatment must begin at a time prior to inflammatory protein activity.

PV also inhibited serum presence of IP-10 and MIG (chemotactic for T cells), MIP-1β (enhances innate cell production of IL-6), TNFα and IL-1 in mdr1a⁻/⁻ mice. These cytokines and chemokines participate in inflammatory feedback loops and could support the evidence of reduced numbers of CD4⁺ and CD8⁺ T cells in the cecal tonsils of mdr1a⁻/⁻ mice treated with PV. PV related reduction in serum IL-9 in mdr1a⁻/⁻ mice, shown to enhance proliferation of CD4⁺ T cells, is additional evidence for the secondary decrease in T cell chemotaxis caused by PV (73). PV related reduction of IL-2 gene expression is possibly due to a reduction in local CD4⁺ T cell presence. Perhaps PV related reduction of gene expression and production of
inflammatory and chemotactic signals for dendritic cells and T cells is the causative factor in the reduced presence of both CD4$^+$ and CD8$^+$ T cells in the cecal tonsils of mdr1a$^{-/-}$ mice treated with extract.

Changes in DC and T cell function and location would B cell proliferation, maturation and eventual antibody production. Germinal centers in lymphoid tissues, an important component of the B cell humoral response, develop upon B cell activation by T dependant antigen (74). Germinal center B cell populations in the cecal tonsils of PV treated mdr1a$^{-/-}$ mice are significantly reduced. The functional relevance of this data could be tied to the lack of antibody production to microflora WCS in PV treated mdr1a$^{-/-}$ mice. Collectively these data present evidence that PV acts to maintain immunologic tolerance to the host microflora and preserve cecal homeostasis in mdr1a$^{-/-}$ mice by arresting NF-$\kappa$B regulated innate cell chemotaxis, and starving the adaptive immune response of inflammatory cytokine and chemokine signals necessary for complete activation and chronicity. This work highlights the need for further study and consideration of PV as a legitimate primary prophylactic or adjunct option for the treatment of IBD and other chronic inflammatory disorders.

**Hypericum gentianoides**

To date, work presented in this dissertation is a novel evaluation of the bioactive potential of *Hypericum gentianoides* in vivo. Mdr1a$^{-/-}$ mouse studies presented here are also the first evaluate the effects of any species of *Hypericum* in a spontaneous colitic model. Using the mdr1a$^{-/-}$ model of spontaneous murine colitis, we evaluated the anti-colitic efficacy of oral HG in comparison to 5% EtOH vehicle. HG prophylaxis significantly delayed colitic. Notably, roughly one third of HG-treated mdr1a$^{-/-}$ mice were removed from study during the final 20 days of the experiment, and another third of these mice expressed 2 or greater scorable macroscopic disease parameters by the time the study ended and the mice reached 20 weeks of age. These observations highlight a limitation in HG efficacy. While HG is capable of significantly staving off severe colitic onset, this potential diminishes overtime. HG is not therefore capable of totally preventing colitic onset.
HG was potent in significantly reducing macroscopic disease parameters in mdr1a\(^{-/-}\) mice and significantly increasing colon lengths in these. While longer studies would be required, this could indicate that HG could further reduce long-term mortality, while allowing mice to survive with only mild to moderate symptoms of colitis.

Macroscopic and microscopic improvements in HG-treated mdr1a\(^{-/-}\) mice were correlative. HG prophylaxis resulted in significantly decreased microscopic colonic edema and reduced cecal ulceration, edema and stromal necrosis. These observations could be attributed to the significant reduction in cecal and colonic neutrophil infiltrate in these mice. As previously described, neutrophil damage is known to contribute to disease severity in IBD (48, 55, 75-77). A greater number of microscopic parameters were improved in the ceca of HG-treated mdr1a\(^{-/-}\) mice, yet the reduction in neutrophil infiltrate and subsequent MPO activity was less dramatic in the cecal tissues of these mice than in the colonic tissues. This would indicate that neutrophils are not the only inflammatory cell population acted upon by HG and contributing to colitic pathogenesis in these tissues. An interaction between HG and another inflammatory moiety must be responsible for the excess microscopic improvement in the ceca of HG-treated mdr1a\(^{-/-}\) mice compared to colonic improvements.

The unexpected increase in plasma cells seen histologically in the colons and ceca of HG-treated mdr1a\(^{-/-}\) mice could involve the increase in IL-6 serum levels also noted in these mice. The function of these plasma cells was not investigated in these experiments, so the mechanism of their presence and activity is unknown. HG prophylaxis did not cause any plasma cell infiltrate in FVB\(^{WT}\) mice, perhaps indicative of increased bioavailability of HG beyond the mucosa in the “leaky” gut tissues of the mdr1a\(^{-/-}\) mice. This idea would be supported by the general lack of biologic effects observed in HG-treated FVB\(^{WT}\) mice.

HG prophylaxis in mdr1a\(^{-/-}\) mice resulted in a significant down regulation of the cytokines and chemokines G-CSF, KC, LIX, MIP-1\(\beta\), IL-7 and TNF\(\alpha\). G-CSF and KC are both stimulatory and chemoattractant for neutrophils (78, 79). Their
downregulation by HG prophylaxis supports the decrease in colonic and cecal neutrophil infiltrate seen in HG-treated mdr1a\textsuperscript{-/-} mice. LIX is also chemoattractant for neutrophils and is the murine homologue of human epithelial neutrophil-activating peptide-78 (ENA-78). The downregulation of LIX by HG further validates histologic reductions in neutrophil infiltrate, and inhibition of this chemokine by anti-sense oligonucleotides has been shown to ameliorate experimental colitis (80). MIP-1\textbeta, or CCL4, is a macrophage inflammatory protein which has been shown to have high activity during the acute phase of experimental colitis while sharply decreasing in concentration during the recovery phase of colitis (81). Increased levels of related isoforms of MIP-1\textbeta have also been shown to increase the severity of colitis (82). IL-7 is known to be an important growth and maturation factor for colitogenic memory T cells and B cells, but also contributes to non-T or B cell colitic related inflammatory processes. (83, 84). HG reduction of TNF\textalpha serum concentrations is a significant finding, as TNF\textalpha has been thoroughly implicated in the pathogenesis and chronicity of IBD (85). HG prophylaxis in mdr1a\textsuperscript{-/-} mice did result in the upregulation of IL-6 serum concentrations. This is an interesting observation as IL-6 is pro-inflammatory, and generally perpetuates adaptive T cell and B cell responses (86). IL-6 levels are elevated in the tissues of human patients with active IBD in the absence of microscopic and macroscopic inflammation (87). HG modulation of serum cytokines/chemokines explains the reduction in microscopic and macroscopic disease which could be associated with reduced granulocyte and neutrophil activity in HG-treated mdr1a\textsuperscript{-/-} mice. The increase in IL-6 serum concentrations in these mice could be one facet of the limitation of long-term anticolitic maintenance of HG or could contribute to the unusual increase in plasma cell and CD4\textsuperscript{+} T cell concentrations in the tissues of these mice noted histologically and by flow cytometry. The mechanism behind this increase in IL-6 will require more investigation.

In summary, Hypericum gentianoides has immunomodulatory potency in the mdr1a\textsuperscript{-/-} mouse model of spontaneous colitis. This activity could be related to the flavonoid and polyphenolic compounds in the extract. Additionally, this work
does not address the potential impacts of HG and its antibiotic compound, uliginosin, on the microflora or colitic associated dysbiosis in this model. This work highlights the need for further study of HG, the potential synergy of its components, and its efficacy in other disease models.

**Recommendations for Future Studies**

Dosing of EA, PV and HG were primarily chosen due to previous studies and lack of host toxicity. In all models presented in this dissertation, it is possible that further extract or DSS dose adjustments could be required to uncover the true range of bioactivities of EA, PV or HG. With regard to DSS studies, it is possible that further efficacy of PV or EA might be directed more towards an aspect of colitis not addressed in this model, such as a colitic model driven more by dysbiosis or a different inflammatory insult than an epithelial toxin like DSS. EA should be evaluated in the mdr1a⁻/⁻ mouse model of spontaneous colitis, as a different colitic mechanism addressed by this model could highlight bioactivity of EA not seen in the presence of DSS insult. While the microflora is a vital part of spontaneous colitic development in the mdr1a⁻/⁻ mouse model of spontaneous colitis, the microflora and potential effects of EA, PV or HG were not specifically addressed to a great extent in these experiments. Additionally, these experiments did not address metabolic end products or their concentrations in feces or serum. While these studies are ongoing, the serum concentrations, penetrance and metabolic byproducts specifically related to EA extract treatment are worth consideration as they could underscore the lack of activity seen in DSS experiments or point to the need for adjustments to treatment or the model.

The bioactivity of EA and PV in the DSS model of colitis is quite different. This could be explained in a number of ways. Previous studies highlight the importance of alkylamides and ketones in EA, while PV harbors many bioactive polyphenols and flavonoids making the totality of their chemical compositions are very different (23, 27, 42). These differences in chemical constituents could lead to different compound stabilities or synergies impacting bioactivity. This furthers the need for studies to address the metabolic byproducts associated with these extracts, transport of extract
components and their bioavailability in healthy, homeostatic gut tissues compared to leaky colitic tissues. Studies of extract component synergy and related synergistic bioactivity of target compounds would clarify ways to alter the extracts to make them more efficacious for therapeutic use.

Because of the lack of literature and study of HG, and the results summarized above, the specific cellular responses related to HG extract prophylaxis could be of great importance to study further. Specifically, the propensity of HG to encourage IL-6 serum increases and plasma cell activity in gut tissues of mice with barrier dysfunction and genetic susceptibility to colitis. Additionally, this work presented here does not address the potential impacts of HG and its antibiotic compound, uliginosin, on the microflora or colitic associated dysbiosis in the mdr1a⁻/⁻ mouse model of spontaneous colitis.

The totality of this dissertation identifies PV and HG as nutraceutical modalities with legitimate evidence for continued scientific pursuit as primary or adjunct therapies for IBD. EA will require more in depth investigation for potential anti-colitic activity in IBD model systems. Clearly, herbal extracts are not simply “snake-oil” and warrant greater scientific exposure and investigation as reasonable medicinal therapies and supplements.

References


