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Impact of epigallocatechin-3-gallate (EGCG), a broad-spectrum anti-inflammatory, in controlling intestinal factors contributing to inflammatory bowel disease.

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IMPACT OF EPIGALLOCATECHIN-3-GALLATE (EGCG), A BROAD-SPECTRUM
ANTI-INFLAMMATORY, IN CONTROLLING INTESTINAL FACTORS
CONTRIBUTING TO INFLAMMATORY BOWEL DISEASE

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February 8, 2016

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DEDICATION

This dissertation is dedicated to my wife, Nan, for her love, support, and patience through this very long process. My children Anna, Sara and Emma also shouldered the burden of my time away from home working on this endeavor. Hopefully I have provided them with a model of tenacity and endurance that they can use to accomplish their lifelong goals.

I also dedicate this dissertation to Very Reverend Father Alexander Atty, PhD, who was my spiritual father, friend, and priest before his untimely death due to metastatic colon cancer. Memory Eternal! If only I could have perfected an immunologic treatment for colon cancer in time to help him. His enduring presence will continue to spur me on this quest.

ACKNOWLEDGMENTS

ABSTRACT

IMPACT OF EPIGALLOCATECHIN-3-GALLATE (EGCG), A BROAD-SPECTRUM
ANTI-INFLAMMATORY, IN CONTROLLING INTESTINAL FACTORS
CONTRIBUTING TO INFLAMMATORY BOWEL DISEASE

Gerald Wayne Dryden

February 8, 2016

This dissertation explores the role of epigallocatechin-3-gallate (EGCG), as a
potential treatment for patients with inflammatory bowel disease (IBD). IBD is a
common disorder that causes a great deal of suffering. Our understanding of the
etiologies, pathogenic mechanisms, and treatment targets continues to evolve. Many new
therapeutic targets are making their way through the pharmaceutical pipelines. However,
not all patients benefit from these therapies.

EGCG has long been studied as an anti-cancer agent. Most of our understanding
of this compound comes from the oncologic literature. As the pathways of oncology and
inflammation converge, new lessons can be taken from the cross discipline. EGCG's
effects on intracellular signaling bridges cancer to inflammation. Many of the same
cytokines, chemokines, and molecular signals influencing cancer cells to grow also
stimulate immune cells. Chapter 3 first explores the role of EGCG as both a preventative
as well as a therapeutic agent and its effect on the dextran sulfate sodium (DSS) mouse
model of colitis. The influence of EGCG on immune cell function is then explored in
chapter 4. One novel approach in chapter 4 has to do with a focus on intestinal epithelial
cells as agents of an immune response, and how EGCG impacts their function in that

role. Chapter 5 explores the impact of EGCG on bolstering barrier function, as this is an

important aspect of inflammatory bowel disease that is often neglected when considering

new approaches to treating IBD. Finally, chapter 6 ends this dissertation with the first

clinical trial in the world's literature to evaluate EGCG as a therapeutic for IBD.

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1.1.1   IBD EPIDEMIOLOGY AND SYMPTOMATOLGY

Inflammatory Bowel Disease (IBD) is a common chronic inflammatory process involving the digestive tract. IBD affects up to 1.5 million individuals in the US alone. The classification of IBD is generally divided into two main subgroups, Crohn's disease and ulcerative colitis (figure 1). A smaller category, indeterminate colitis, makes up about 10-15% of all IBD cases. The clinical presentations of each group vary greatly, and are, in part, determined by the pathophysiology of each disease state. Ulcerative colitis is limited to the colon, where it affects a variable portion of the colon from the rectum alone (proctitis) to disease involving the entire colon (pancolitis). Crohn's disease, on the other hand, may affect the digestive tract anywhere from the mouth to the anus. The most common site of involvement is the terminal ileum, which may be affected in up to 90% of cases. The colon is less commonly involved, and up to 40% may have both the colon
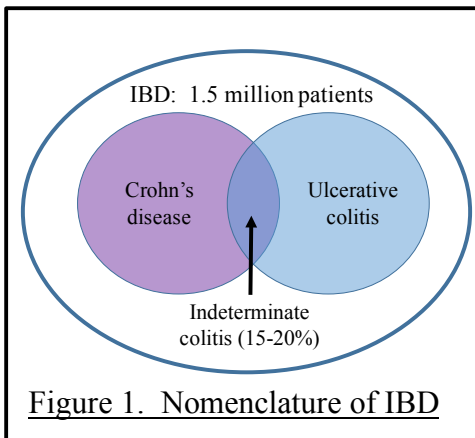
Figure 1. Nomenclature of IBD

IBD: 1.5 million patients

Crohn's
disease

Ulcerative
colitis

Indeterminate
colitis (15-20%)

and small bowel involved.

Diarrhea, abdominal pain, and rectal bleeding are hallmarks of IBD (Bernstein, Fried et al. 2010). Crohn's disease is usually accompanied by right lower quadrant abdominal pain, due to the high likelihood of ileal involvement (Silverberg, Satsangi et al. 2005). Ulcerative colitis more commonly presents with left lower abdominal cramps, urgency and tenesmus, as the rectum is universally involved. Both disease processes are often accompanied by diarrhea, but ulcerative colitis more commonly exhibits rectal bleeding due to the distal nature of its location and generalized involvement of the mucosa. Ulcerative colitis tends to be a superficial inflammatory process involving the epithelial lining (mucosa) and the adjacent submucosal tissue (Bernstein, Fried et al. 2010). In contrast, Crohn's disease begins at the mucosa, but the distinct ulcerations may penetrate deeply into the bowel wall, eventually leading to significant ulceration and even perforation of the bowel. The tendency of Crohn's disease to penetrate the bowel deeply predisposes patients to form abscesses, fistulas, and strictures (Bernstein, Fried et al. 2010).

1.1.2 PATHOGENESIS OF IBD

Both disease processes result from a convergence of three factors: 1. Genetic predisposition, 2. Environmental factors, and 3. Immune system abnormalities. Over 200 genes have been identified in genome-wide association studies (GWAS) of IBD patients and their relatives, with many of them encoding abnormalities of genes involved in innate and adaptive immunity (de Lange and Barrett 2015). The penetrance of

identified mutations varies greatly. The first described mutation associated with IBD, the NOD-2 mutation, dramatically increases the risk of IBD (Hugot, Chamaillard et al. 2001, Ogura, Bonen et al. 2001). While the presence of a mutation is not sufficient for the development of IBD, a double dose of the mutation elevates the risk of disease by 20-40 fold (Cuthbert, Fisher et al. 2002). Evidence from animal studies demonstrates that bacteria must be present in the lumen before inflammation can develop (Taurog, Richardson et al. 1994). Genetically susceptible, but germ-free, animals have under-developed immune systems (Taurog, Richardson et al. 1994). Upon exposure to different bacteria or bacterial products, inflammation develops in the GI tract with varying intensity (Rath, Herfarth et al. 1996). Of course, humans cannot be raised under germ-free conditions, but the composition of bacteria present in the microbiome may influence the development of IBD (Fabia, Ar'Rajab et al. 1993, Swidsinski, Ladhoff et al. 2002, Seksik, Rigottier-Gois et al. 2003).

Patients with IBD often experience a "trigger" that initiates their bowel inflammation. A trigger might consist of the use of non-steroidal anti-inflammatory medications, which may disrupt the epithelium and expose the underlying submucosal immune system to higher levels of bacterial exposure, or the development of an enteric infection, that initiates more than a self-limited inflammatory event.

1.1.3 INTERPLAY OF BACTERIA AND INTESTINAL BARRIER FUNCTION

A common estimate of the total number of human cells in a body approached 1 x 10^{12}, while the human intestine contains up to 3 x 10^{13} bacterial organisms plus an

undetermined number of discovered, as well as, uncharacterized viruses and fungi (Sender, Fuchs et al. 2016). It has been stated that intestinal bacteria outnumber human cells by a factor of 10:1, but this estimate has been recently revised to show that human cells equal the number of bacterial cells at steady state, and assume superiority temporarily after each defecation activity (Sender, Fuchs et al. 2016). Regardless of the ratio of human to bacteria, a huge number of organisms in a complex mixture resides in close proximity to the gastrointestinal immune system. The primary defense against bacterial invasion is a *physical barrier* that depends on the integrity of the epithelial border, consisting of a single layer of intestinal epithelial cells (IECs) buttressed by a variety of secreted substances that augment its barrier function that is spread over 200 m^2 of total surface area (Neish 2002). Specialized epithelial cells such as Paneth cells, enteroendocrine cells and goblet cells secrete anti-bacterial peptides, immunoglobulins, and mucins, respectively, that form a multi-functional coating that insulates the mucosa from direct contact with the luminal compartment (Peterson and Artis 2014). The secretion of mucins into the lumen forms a relatively impenetrable barricade separating the acquired microbial population from the host epithelial cells. Mucin-2 (Muc2) is the most abundant mucin secreted into the lumen (Johansson, Phillipson et al. 2008). The absence of mucin in experimental models is associated with inflammation and even colonic neoplasia in animal models deficient for Muc2 gene (Van der Sluis, De Koning et al. 2006, Dharmani, Leung et al. 2011). Additional contributors to mucin layer stabilization include IEC secretion of trefoil factor 3 and resistin-like molecule (RELM)-β that help maintain appropriate mucin content and structural integrity (Taupin and Podolsky 2003, Herbert, Yang et al. 2009). Underneath the mucin layer lies a unique

feature called the unstirred water layer. This thin aqueous environment is usually sterile due to the effects of secreted anti-microbial peptides such as cryptidins, cathelicidins, lysozyme, and REGIIIγ (Kim and Ho 2010, Gallo and Hooper 2012, Peterson and Artis 2014). Additionally, large amounts of immunoglobulins (Ig)G and secretory (s)IgA are transferred across the epithelial border into the unstirred water layer, eventually diffusing into the mucin layer. Genetic defects leading to impaired intracellular bacterial sensing, Paneth cell and enteroendocrine cell dysfunction, and mechanisms involved in autophagy and the unfolded protein response, are associated with an increased risk of IBD (Kaser and Blumberg 2011). A mutation in the gene encoding the intracellular protein responsible for sensing muramyl dipeptide (MDP) (Kanneganti, Lamkanfi et al. 2007), NOD2, was the first described genetic defect associated with Crohn's disease susceptibility (Cho 2001, Hugot, Chamaillard et al. 2001). This sensing protein has now been implicated in a number of mucosal defense and tolerance functions, including negative regulation of TLR stimulation (Watanabe, Kitani et al. 2004), upregulation of tolerance inducing IL-10 (Noguchi, Homma et al. 2009), and Paneth cell regulation of antimicrobial peptides such as α-defensin (Kobayashi, Chamaillard et al. 2005, Wehkamp, Salzman et al. 2005). NOD2 also functions as a key regulator for coordinating the activities involved in autophagy (Kaser, Lee et al. 2008). First, the binding of MDP to NOD2 actuates the formation of autophagic vesicles in enterocytes and dendritic cells. Reduced or aberrant expression of NOD2, on the other hand, results in impaired pathogen clearance (Cooney, Baker et al. 2010, Travassos, Carneiro et al. 2010). Second, the direct association of NOD2 with the normal autophagy protein ATG16L1 initiates the autophagy machinery through intracellular recruitment of

ATG16L1 to sites of bacterial invasion (Travassos, Carneiro et al. 2010). However, the presence of a human ATG16L1 variant, T300A, impairs activation of autophagy, as seen when the mutant gene was introduced into a murine model of autophagy (Lassen, Kuballa et al. 2014). With the similarities between the animal model and the human condition, the T300A mutation gains credibility as a mutation linked to Crohn's disease susceptibility, through its effect of blunting an adequate mucosal response in the face of appropriate inflammatory stimuli (Cooney, Baker et al. 2010). This crossover between intracellular pathogen sensing and activation of the autophagic pathway, which provides important input into the manner by which immune activation should proceed in response to commensals (immune tolerance), helps explain the seeming paradox whereby impaired bacterial sensing can lead to an inappropriately aggressive immune response to non-pathogenic resident microflora (Kaser and Blumberg 2011). Times of stress, such as during an intestinal infection, and other challenges to intracellular processing of bacterial products, also trigger other cellular protection processes, such as the unfolded protein response, which has also been linked to IBD (Kaser, Lee et al. 2008).

Intriguingly, optimal barrier function actually depends on the presence of bacteria. The total bacterial population, or microbiome, residing in the lumen of the gastrointestinal tract adds a considerable quantity of metabolic capacity to the human physiology. Most bacteria exist as non-virulent commensal organisms. Resident bacteria provide beneficial energy substrates obtained from complex carbohydrates that resist human digestion, in the form of short chain fatty acids. These compounds support important aspects of IEC function (Hamer, Jonkers et al. 2008). In addition, luminal

bacteria supply nutrients required for normal human function, such as vitamin K.
However, the close proximity of such a large number of potential pathogens requires the
presence of a ready defense in the form of the lamina propria immune system, which
creates an *immunological barrier* to augment the physical barrier of the IECs.

1.1.4 ROLE OF THE IEC IN ESTABLISHING AN INTESTINAL BARRIER AND ITS IMPACT ON INTESTINAL HOMEOSTASIS

IECs significantly contribute to the establishment of a functional, tolerant
immunological barrier that protects against mucosal invaders without inflicting
pathological damage to the host. The first line of defense against inadvertent activation
of the lamina propria immune system consists of a specialized network of protein-based
connections that bind IECs together into a variably permeable barrier. These connections
consist of a large collection of proteins called claudins and occludins arranged on a
scaffold protein called zonulin (McCarthy, Skare et al. 1996). Exchanges to the claudin
and occludin components can be made rapidly by IECs in order to regulate
transmembrane molecular flow (Shen, Weber et al. 2011). Manipulation of the tight
junction (TJ) proteins can either open the spaces between IECs or more tightly constrict
them. In addition to TJ pore size, specific changes in the protein content preferentially
dictate the charge of the ions allowed to flow across the epithelial barrier. Aggressive
changes in ion flow across the barrier can actually induce a protective "flushing" effect in
response to certain infections (Schulzke, Ploeger et al. 2009). In concert with ion flow,
changes to TJ composition also influence permeability of the epithelial barrier
permeability to the transmucosal flux of bacterial products. In a process closely tied to

IEC detection and identification of bacterial components, IECs continuously adjust the permeability of the barrier function (hence, the term variably permeable membrane) (Kirpich, Feng et al. 2012). Through the same mechanisms that microbial components elicit an immune response in formal immune cells, these microbial antigens can also induce IECs to secrete the same important effector cytokines, as well. IECs have been found to secrete a wide variety of cytokines polarized to the Th1 type of immune response (León, Sánchez et al. , Tiittanen, Keto et al. 2013), including IFN-γ, TNF-α, IL-1β, IL-6, IL-17, IL-12/23, IL-22, and IL-33 in our hands. Although many of these cytokines have demonstrated direct effects on barrier permeability, the first three (IFN-γ, TNF-α, IL-1β) have garnered the most attention in regard to their role in barrier dysfunction related to IBD. In the ultimate analysis, pro-inflammatory cytokine secretion serves as a tool for local communication between IECs. The signal sent out depends on the input detected through the interaction of bacterial products with the sensory machinery contained within IECs. In individuals susceptible to IBD, ineffectual signal detection or defective signal transduction leads to inappropriate secretion of inflammatory cytokines (Su, Shen et al. 2009, Ulluwishewa, Anderson et al. 2011). This unintended activation of a tightly controlled feedback loop, in turn, further degrades epithelial barrier integrity and sensitizes lamina propria immune components to bacterial antigens, ultimately resulting in an expansion of the local immune system activities.

1.1.5 HOW IECS MAINTAIN HOMEOSTASIS

Although IECs establish a robust barrier function, the intestinal epithelium is not invulnerable to bacterial invasion by bacterial pathogens. In order to facilitate

monitoring for the presence of pathogens, the immune system has developed a mechanism to survey the microbiome (Didierlaurent, Sirard Jc Fau - Kraehenbuhl et al. 2002). This is accomplished, in part, by non-specific mechanisms, such as epithelial transcytosis of bacteria and their antigens by standard IECs, as well as, uptake by specialized enterocytes called M-cells (figure 2). M-cells may also be able to selectively concentrate their uptake of pathogenic organisms through receptor-mediated transcellular transport mechanism, such as the glycoprotein GP2-FimH interaction (Ohno and Hase). Goblet cells have also been shown to transport soluble bacterial proteins to sub-epithelial dendritic cells (Peterson and Artis 2014). Finally, sub-epithelial dendritic cells extend dendrites across the epithelial barrier into the lumen to directly sample luminal contents, and then transport both viable bacteria and soluble antigens to mesenteric lymph nodes during their migration to the draining lymph nodes (Rescigno, Urbano et al. 2001). Dendritic cells generally acquire a tolerogenic tone as they mature during their transit to their eventual destination in the mesenteric lymph node in patients without IBD (Rescigno 2014). The sum total of these sampling techniques typically form the basis for setting the state of immune tolerance.
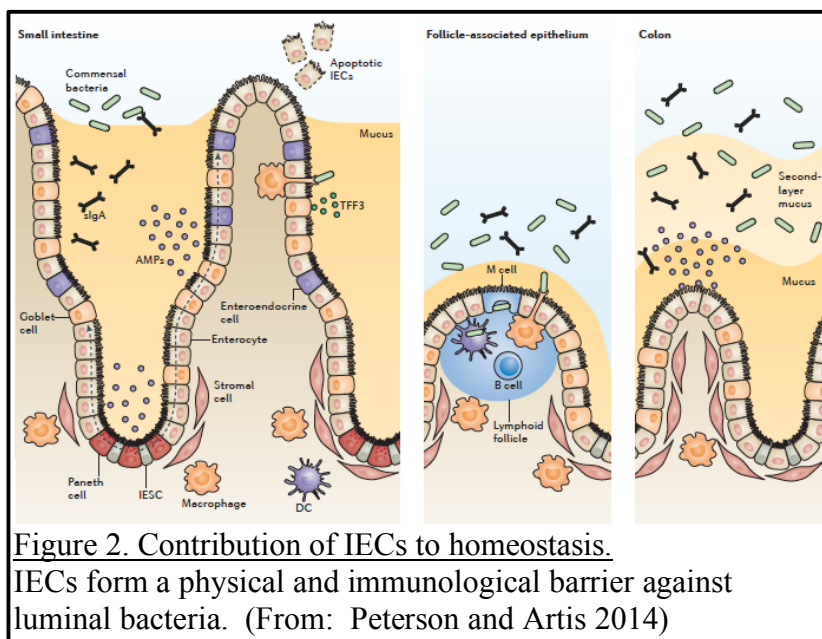
Figure 2. Contribution of IECs to homeostasis. IECs form a physical and immunological barrier against luminal bacteria. (From: Peterson and Artis 2014)

While these same events occur in

IBD patients, the perturbations within the maturation process that diminish mucosal tolerance in IBD patients still evade complete understanding.

One final contributor to the lamina propria immune compartment's state of tolerance and overall intestinal homeostasis comes from the IECs themselves. IECs act as frontline receptors for microbial organisms. They express a variety of pattern recognition receptors, including Toll-like receptors (TLR), NOD-like receptors (NLR), and RIG-I-like receptors (RLR) (Gallo and Hooper 2012). These receptors assist in maintaining tissue homeostasis. The initial indication that IECs contributed to the induction of tolerance came from studies of TLR deficient mice. TLR stimulation by bacterial ligands was required to maintain epithelial homeostasis and to repair injured epithelial barriers (Ostaff, Stange et al. 2013). From that work emerged the fact that several compensatory reactions emerge after TLR stimulation, including the upregulation of epidermal growth factors, intestinal trefoil factor 3, heat-shock proteins, and tight junction proteins responsible for enhancing epithelial barrier resistance to paracellular antigen flux (Putsep 2000). In concert with TLRs, additional receptor classes such as the Nod-like receptors and other inflammasome components have been found to be important contributors to the IEC regulation of GI homeostasis. NLRs and inflammasomes play a vital role in epithelial restitution in the aftermath of bacterial invasion.

Given the extensive array of bacterial sensors present on IECs, the propensity to secrete inflammatory cytokines, and the close proximity to trillions of bacteria, the interpretation of how homeostasis and immune tolerance can be maintained in this setting

is a complicated formula. When this fine balance is disrupted, inflammatory bowel disease ensues. IECs express specific negative regulators of pattern recognition receptor (PRR)-dependent pro-inflammatory responses to commensals (Didierlaurent, Sirard Jc Fau - Kraehenbuhl et al. 2002). Another mechanism for differentiating commensal organisms from pathogenic mechanisms has to do with the placement of the different bacterial sensors. Due to the polarized anatomy of IECs from apical to basolateral, with the tight junction complex providing a hard boundary between the two, differential responses to microbial exposure can occur. Apical exposure of TLR-9 to appropriate ligands results in a net inhibitory effect on IEC responsiveness due to stabilization of IκB, while basolateral exposure leads to canonical activation and subsequent nuclear translocation of NF-κB (de Kivit, van Hoffen et al. 2011). This provides a mechanism for generating tolerance when bacteria are appropriately located, while permitting activation of an immunological response in the setting of a mucosal breach. The intracellular sensor NOD2 provides an additional example of the importance of the site of localization to the impact on immune response. Upon stimulation with muramyl dipeptide (MDP), the leucine-rich repeats of NOD2 associate with the basolateral membrane based FERM and PDZ domain containing 2 (FRMPD2) scaffold protein, leading to induction of an inflammatory response (Boyle, Parkhouse et al. 2014). Location of pathogen associated molecular pattern (PAMP) stimulation may not be the only mechanism by which IECs determine whether an inflammatory response is needed or not. One additional mechanism may lie with the ability to recognize danger in relation to microbial viability, based on recognition of bacterial products by viability-associated PAMPs that can differentiate intact, alive microbes versus non-infectious debris

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(Mourao-Sa, Roy et al. 2013). This feat is accomplished by detecting virulence factors present on viable organisms, such as bacterial secretion systems or intact toxins (Keestra and Bäumler 2014). This level of differentiation provides another mechanism for IECs to discern between the need to activate an immune response against a pathogen versus maintaining tolerance to a commensal organism.

In their role as intestinal gatekeeper, IECs constituting the intestinal border self-regulate in response to bacterial signals. As detailed previously, mucin layer maintenance depends on the interaction of bacterial components with TLR sensors, anti-microbial peptide (AMP) production requires the presence of commensal organisms, and polymeric immunoglobulin receptor (pIgR)-mediated transport of IgA across the epithelial barrier is dependent upon the sensing of commensal bacteria by IECs. Completing the lineup of barrier functions dictated by the sensing of bacterial components, TJ protein redistribution by IECs to apical locations in the border structure occurs in part due to the effects of bacterial-dependent TLR-2 triggering (Ulluwishewa, Anderson et al. 2011).

1.1.6 IECs CONTRIBUTE TO IMMUNE REGULATION

IECs actively participate in the regulation of immune cells inhabiting the lamina propria located just beneath the epithelium through the use of immunoregulatory signals. Both the innate and adaptive immune compartments receive IEC mediated instruction through the production of cytokines such as thymic stromal lymphopoetin (TSLP), transforming growth factor-β (TGFβ) and IL-25 (Peterson and Artis 2014). Additional

IEC signals deliver information to B-cells including TNFSF13 (APRIL) and TNFSF13b (BAFF), which promote B-cell survival and plasma cell longevity. Resident mononuclear cells receive tolerogenic signals from IECs via production of TSLP, TGFβ and retinoic acid. These factors depend on commensal bacteria for their regulation. Two particular populations of mononuclear cells reside just below the epithelial layer: the pre-DC derived CD11c+CD103 dendritic cell (DC), and the monocyte derived CD11c^{low}F4/80CX3CR1^{hi} resident macrophages (Rogler, Hausmann et al. 1998, Farache, Koren et al. 2013). The intestinal epithelial dendritic cell performs a crucial step in inducing adaptive T cell responses, and therefore plays a pivotal role in determining the immune tone of the intestinal homing T cell population. Lamina propria (LP) residing DCs collect both luminal, as well as, LP derived bacterial components (Martín-Fontecha, Lanzavecchia et al. 2009). After activation, antigen-carrying DC migrate to regional lymph nodes to educate naïve T cells (Rescigno 2014). Using retinoic acid as a cue, DCs initiate antigen responsive naïve T cell imprinting to an intestinal phenotype by up-regulating the specific integrin profile combining the α4 integrin with the β4 integrin (Mora, Bono et al. 2003, Iwata, Hirakiyama et al. 2004, Johansson-Lindbom, Svensson et al. 2005, Jaensson, Uronen-Hansson et al. 2008, Mora and Von Andrian 2008). This particular combination interacts with MadCAM-1 to effect homing of activated T cells back to the intestinal mucosa during periods of inflammation (Arihiro, Ohtani et al. 2002). The presentation of additional co-stimulatory signal during T cell activation allow the migratory DC to condition the naïve T cell into an appropriate effector T cell, effectively providing a mechanism for the DC to communicate the signals that it received from the IECs prior to migration to the MNL (Reis e Sousa 2004). In contrast, non-

migratory CX3CR1[hi] lamina propria-based macrophages, located in close proximity to the IEC layer, have down-regulated CD14 and function primarily as phagocytes clearing out cellular and microbial debris from pathogenic or commensal organisms that traverse the epithelial barrier (Bain and Mowat 2014). In contrast to newly migrated monocytes that actively participate in active inflammatory processes (Zigmond, Varol et al. 2012), these resident macrophages mainly participate in the promotion of tolerance, by promoting expansion of regulatory T cells (Hadis, Wahl et al. 2011) and actively secreting IL-10, an anti-inflammatory cytokine that suppresses activated T cells. IECs promote this behavior via secretion of TSLP, TGFβ, retinoic acid, and semaphorin 7A signaling via αvβ1 integrin expressed on CX3CR1[hi] macrophages (Kang, Lee et al. 2007).

Another class of immune cells named innate lymphoid cells (ILCs) provide another critical contribution to intestinal homeostasis (Sonnenberg and Artis 2015). This class of cells lack adaptive effector cell properties, such as antigen specific receptors found in B and T cells, and are generally found at barrier surfaces such as the lamina propria of the intestine (Sonnenberg and Artis 2015). These cells function as regulators of tissue homeostasis, inflammation and innate early response to infection and consist of three subtypes (Artis and Spits 2015). Group 1 (ILC1s) includes classical natural killer (NK) cells which are characterized by secretion of Th1 cytokines IFN-γ and TNF-α. NK cells can kill target cells via cytotoxic activity, but other ILC1s can only secrete cytokines (Artis and Spits 2015). Non-cytotoxic ILC1s may play a role in IBD (Bernink, Peters et al. 2013, Fuchs, Vermi et al. 2013). Group 2 ILCs (ILC2s) secrete IL-5 and IL-13, the

Th2 type cytokines (Artis and Spits 2015). This class of ILCs may play a role in food allergy or wound repair. Their proliferation and activation depend on IEC production of IL-25, IL-33 and TSLP (Moro, Yamada et al. 2010, Neill, Wong et al. 2010, Price, Liang et al. 2010, Mjösberg, Bernink et al. 2012). Group 3 ILCs perform dichotomous roles depending on the environmental conditions communicated by IECs. They can secrete IL-17 in response to IEC secretion of IL-23 or IL-22 in response to commensal induced IEC secretion of IL-7 (Qiu, Guo et al.). This differential signaling pattern provides a mechanism for IEC to influence this lamina propria immune component to provide appropriate response based on actual conditions encountered by the epithelium. Their importance in human disease is not clear at this time. However, IL-22 supports containment of resident bacteria in gut-associated lymphoid tissue (Hanash, Dudakov et al. 2012, Sonnenberg, Monticelli et al. 2012, Kirchberger, Royston et al. 2013), in addition to directing γδ T cells in an immediate innate response to invading pathogens (Edelblum, Singh et al.). ILC3-derived IL-17A provides a predominantly pro-inflammatory effect in the setting of human IBD (Buonocore, Ahern et al. 2010, Geremia, Arancibia-Cárcamo et al. 2011, Coccia, Harrison et al. 2012). In addition, IECs play a direct role in regulating IEC3s. Under the influence of commensals, IEC produce IL-25, which suppresses IL-23 production by macrophages and decreases IL-22 secretion by ILC3s (Sawa, Lochner et al. 2011). Commensals also direct IECs to produce IL-7, which stabilizes the transcription factor retinoid-related orphan receptor γt (RORγt) (Satoh-Takayama, Vosshenrich et al. 2008, Vonarbourg, Mortha et al. 2010) and promotes ILC3 production of IL-22. Retinoic acid has also been shown to enhance IL-22

production, which aids in the suppression of experimental murine colitis (Mielke, Jones et al. 2013).

Other tissue resident T cells consist of specialized intraepithelial lymphocytes that reside in close contact with IECs, consisting of both activated conventional T cells as well as a smaller subset of T cells expressing a restricted repertoire of T cell receptors, which includes both γδ and NK T cells (Cheroutre, Lambolez et al. 2011). These T cells also receive bi-directional instructions from IECs to serve as an immediate mediator of cytotoxic responses to infectious organisms. Conventional T cells residing in intestinal tissue are primed to serve as rapid responders in the setting of active inflammation or infection and also provide the memory effect needed to mount a response to recurrent infections with a previously encountered infectious agent. CD8+ T-cells with an αβ T cell receptor constitute a significant proportion of the IEL population in humans (Gebhardt, Mueller et al. 2013). These tissue resident T cells interact with IECs via CD103 (αEβ7), which binds to E-cadherin expressed by IECs (Schön, Arya et al. 1999, Sathaliyawala, Kubota et al. 2013). In fact, this interaction with CD103 and E-cadherin has been shown to be important in acute infection with mucosal pathogens such as *S typhimurium*, as the CD103 directs γδ T cells into the lateral intercellular space between IECs (Edelblum, Singh et al. , Cerutti 2008), through an unknown mechanism dependent on the presence of IL-22. Finally, IECs appear to facilitate the positive selection and expansion of high affinity TCR $CD8^+$ T_{RM}cells (resident memory) expressing CD8α (also known as $CD8αα^+$ IELs) (Farber, Yudanin et al. 2014).

IgA secreting plasma cells play a significant role in maintaining defenses against pathogens and perhaps even regulating commensals. Maturation of naïve B cells into mature IgA-secreting plasma cells requires priming signals from mucosal DC plus live bacteria (Macpherson and Uhr 2004, Cerutti 2008). These mucosal DCs have been conditioned by IEC-derived signals to promote IgA class switching and a gut homing phenotype, via the effects of nitric oxide, IL-10 and retinoic acid combined with TGFβ signaling (Macpherson and Uhr 2004, Mora, Iwata et al. 2006, Mora and Von Andrian 2008). In the presence of a cognate CD4 T cell response, CD40L expression provides a necessary signal to activate class-switch recombination (CSR) within the B cell. In the absence of a cognate CD4 T cell response, CSR can occur under the influence of IEC expression of APRIL and BAFF signaling through transmembrane activator and CAML interactor (TACI) and BAFF receptor (Litinskiy, Nardelli et al. 2002, Salzer, Chapel et al. 2005, He, Xu et al. 2007, Xu, He et al. 2007). Commensals influence NF-κB activation which enhances IEC expression of TSLP signaling. This, in turn, enhances mucosal DC expression of APRIL and BAFF production and amplifies B cell stimulation.

Far from serving as just a mechanical barrier, the IECs of the intestinal mucosal interface function as a sensory organ for evaluating the luminal microbial environment, act as translators of the prevailing conditions to educate the underlying immune system, and provide both stimulatory and regulatory signals to evoke a graded response to invasive events when a counter response is necessary. Despite the central importance of the intestinal epithelium in coordinating and effecting immune responses, its role in inflammatory bowel disease has been relatively discounted, especially when considering

targets for IBD therapy. The majority of work performed to this date has targeted primary and secondary immune cells. Failure to consider the mucosal barrier function likely accounts, in part, for failures that have been seen with therapies directed at classical immune cells.

1.1.7 IBD: NOT JUST A DISORDER OF THE IMMUNE SYSTEM

The fundamental basis of IBD remains a hotly debated topic, with most theories taking the view that IBD results from a dysfunctional relationship between the microbiota residing in the intestinal lumen and the immune system lining the other side of the intestinal epithelial barrier. Many lines of evidence from the basic sciences have established the fact that IBD requires the presence of commensal organisms, but the idea that a loss of tolerance to commensals leads to IBD became entrenched in the collective thinking after Duchmann et al showed that immune cells isolated from the intestines of IBD patients responded vigorously to sonicates of both autologous and heterologous microflora, while immune cells from normal patients only responded to heterologous microflora (Duchmann, Kaiser et al. 1995). The general mechanism explaining the inappropriate activation assumes a loss of immune tolerance to the intestinal flora. One explanation assumes that the microflora maintains its baseline characteristics in both quantity and composition, while immune tolerance breaks down and a defective mucosal effector T cell arises and overreacts to commensal organisms. Collateral damage to the lining of the GI tract occurs as a consequence of the chronic inflammation. Another competing hypothesis contends that the primary problem arises due a shift in the composition of the intestinal microbiome, which initiates an immune response directed

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against the interlopers. The ensuing inflammation results in chronic, unremitting intestinal inflammation.

The role of bacteria in IBD pathogenesis has been well established. Host-microbe interactions are required for the normal development of the host immune system and effective responses to intestinal pathogens. The innate immune system stands up as the first responder in response to pathogen invasion. The innate immune system is also necessary and sufficient for the initiation of IBD, while the adaptive immune system is not. Animals without an adaptive immune system, such as the Rag-/- and severe combined immunodeficient (SCID) mice, do not spontaneously develop colitis, unless they are exposed to a trigger of innate immunity, such as the administration of dextran sodium sulphate (DSS), anti-CD40 antibody, or Helicobacter hepaticus (Dieleman, Ridwan et al. 1994, Buonocore, Ahern et al. 2010, Maloy and Powrie 2011). However, the absence of an adaptive immune system can protect against the development of colitis in certain genetically susceptible animal models. These data suggest that the control or prevention of an innate immune response could protect against the initiation of an IBD event. In chronic IBD, the role of the adaptive immune system takes over, as evidenced by the large numbers of activated T cells. The factors responsible for the induction of a pathologic adaptive T cell immunity against commensal organism have not been fully elucidated, but many clues point to dysfunction of the IECs as a significant contributor to the maladaptive response.

The main factors that appear to contribute to the breakdown in functional tolerance found in IBD include changes in the microbiome, defects in normal barrier function, dysfunction in the autophagy pathway, and abnormal responses to stress in the form of unfolded proteins. Normal compensatory regulatory functions responsible for reining in the active immune response fail to take hold, and the initial immune response gains steam and careens out of control.

1.1.8 CONTRIBUTION OF THE MICROBIOME TO IBD

Our understanding of the complexity of the intestinal microbiome continues to evolve. In fact, you just have to wait until your next meal for the composition of your microbiome to change (David, Maurice et al. 2014). Although IECs do not appear to directly impact the microbiome, dietary habits and other environmental factors implicated in the development of IBD have been shown to influence the relative composition of bacteria (Hansen, Gulati et al. 2010). Recent evidence suggests that some species play a larger role in promoting inflammation than others. Table 1 summarizes some of the classes felt to be important to the pathogenesis of IBD:

Table 1. Microbiota changes important to IBD susceptibility

Decreased	Increased
Bacteroides (SCFA)	Actinobacteria
Clostridium IXa and IV groups (SCFA)	Proteobacteria
Bifidobacterium populations	Bacteriodetes
Faecalbacterium prausnitzi (SCFA)	*B. fragilis*
Firmicutes	Mucosal adherent bacteria (AIEC)

Adapted from Frank et al. (Frank, Amand et al. 2007).

Many of the bacteria seen as protective against IBD produce short chain fatty acids (SCFA). *Clostridium* and *Bacteroides* species are the main producers of SCFAs in the human colon (Wong, de Souza R Fau - Kendall et al.). One particularly robust producer of SCFA, *F. prausnitzi*, has been shown to provide a strong anti-inflammatory effect in immune cells (Sokol, Pigneur et al. 2008) and to be deficient in patients with IBD (Sokol, Seksik et al. 2009). The sensing of other luminal inhabitants, such as fungal organisms may also play a role in immune tolerance. This has been demonstrated by the fact that dectin-1 knock out (KO) animals are more susceptible to induced colitis (Frank, Amand et al. 2007).

1.1.9 BARRIER FUNCTION DISTURBANCES AUGMENT MICROBIAL STIMULATION OF MUCOSAL IMMUNITY

Patients with both active and quiescent IBD have a reduced number of goblet cells within the intestinal mucosa (Xavier and Podolsky 2007). Genes encoding mucins are generally underexpressed, resulting in the presence of thin, ineffective mucin layers. Animal models with defects in mucin genes are highly susceptible to IBD.

Intestinal epithelial barrier permeability impacts many human disease states, especially IBD (McGuckin, Eri et al. 2009). Cytoplasmic-based proteins (zonnula occludens) and integral membrane-based proteins (claudins and occludins) knit the enterocytes forming the intestinal epithelial layer together in a manner that isolates the space below (lamina propria) from the microbial antigens present in the lumen of the intestines (Ivanov 2012). Defects of apical junctional complexes lead to permeable

epithelial barrier as evidenced by decreased epithelial electrical resistance and increased markers of flux such as fluorescein isothiocyanate (FITC) (Peterson, Costantini et al. 2010). These findings likely arise from alterations in the composition of tight junction proteins that precede any manifestation of disease. Changes in the ratio of pore forming claudins, such as claudin 2, claudin 4, and claudin 8 to pore tightening claudins, such as claudin 1, allow for increased ion flux across the epithelial border, resulting in osmotic diarrhea (Zeissig, Bürgel et al. 2007, Förster 2008). All components of the integral membrane-based TJ proteins, including occludin, cadherins, as well as, catenins have been found to be underexpressed in IBD patients (Weber and Turner 2007, Reuter and Pizarro 2009). Tight junction protein expression appears to be regulated through a variety of mechansims, including certain cytokines expressed by IECs and immune cells of the lamina propria (Nusrat, Turner et al. 2000, Weber and Turner 2007, Zeissig, Bürgel et al. 2007, Benjamin, Makharia et al. 2008)

1.1.10 IMPACT OF DEFECTS IN AUTOPAGHY ON HOMEOSTASIS

Defects in autophagy play a role in the pathogenesis of IBD (Tian, Kabi et al. 2001). Genome-wide association searches (GWAS) identified ATG16L1 as an important mutation in IBD patients (Fowler, Doecke et al. 2008). Animals transfected with defective ATG16L1 become more susceptible to both the DSS model and an infectious model of IBD involving *Y. enterocolitica* (Saitoh, Fujita et al. 2008, Murthy, Li et al. 2014). Paneth cells in terminal ileum of genetically altered animals resemble those seen in human Crohn's disease terminal ileum (Cadwell, Patel et al. 2010). While autophagy plays an important role in maintaining cellular energy stores during periods of stress or

starvation, it is also an integral process in handling bacterial products (Scharl and Rogler 2012). Defects in the proteins associated with autophagy can lead to ineffective degradation of phagocytosed bacteria and dysfunctional organelles. Disruption of the normal autophagy pathway leads to intracellular accumulation of secretory granules and subsequent decrease in antimicrobial peptide secretion (Kaser and Blumberg 2011). In fact, infection with norovirus in humans drives a pro-inflammatory phenotype similar to that seen in IBD (Kaser, Lee et al. 2008). Accumulation of antimicrobial peptides within the IECs leads to increased microbial contact with the epithelial barrier, activation of inflammation, and increased susceptibility to colitis.

1.1.11 STRESS RESPONSE ALTERED IN IBD PATIENTS

Circumstances that lead to accumulation of misfolded proteins in the endoplasmic reticulum (ER), such as shock, heat stress, metabolic stress, or bacterial infection typically induce a corrective cellular response called the misfolded protein response (Celli and Tsolis 2015). Misfolded proteins may occur in the setting of genetic mutations or environmental stimuli that are not conducive to normal protein, such as starvation (glucose/protein), hypoxia, or heat shock (Kaser and Blumberg 2010). Infection or inflammation also leads to stress within the endoplasmic reticulum, particularly in cells with a robust secretory function. The accumulation of misfolded proteins within the ER can result in disruption of normal cellular function. Affected cells can attempt to rescue themselves from death by activating the unfolded protein response (UPR) (Kaser and Blumberg 2009). Highly active secretory cells, such as Paneth cells located at the base of intestinal epithelial crypts, are highly sensitive to ER stress and require a robust UPR to

maintain homeostasis and normal function (Kaser and Blumberg 2009). A normal UPR to ER stress consists of upregulated synthesis of particular chaperone proteins and induction of autophagy to enhance breakdown of misfolded proteins (Kaser and Blumberg 2009). An irreversible accumulation of misfolded proteins initiates apoptosis of stressed cells. One method of recognizing misfolded proteins utilizes inositol requiring enzyme 1 (IRE1). Upon the recognition of misfolded proteins, IRE1 splices the mRNA of X box binding protein 1 (XBP1) into a transcriptionally active form that subsequently induces translation of a family of proteins responsible for managing the UPR (Kaser and Blumberg 2011). XBP1 has been identified as a risk factor for both UC and CD (McGovern, Gardet et al. 2010, Kaser and Blumberg 2011), and Paneth cells in the epithelium of XBP1 deficient animals die by apoptosis, leading to spontaneous colitis and enhanced susceptibility to enteric infections. Paneth cell loss leads to enhanced barrier permeability and inflammatory tone (Mumphrey, Changotra et al. 2007). Other mutations in UPR have been associated with IBD in GWAS searches, such as orsomucoid like gene 3 (ORMDL3) and anterior gradient 2 (AGR2) (Zheng, Rosenstiel et al. 2006, Kaser, Lee et al. 2008, Kaser, Flak et al. 2011). The intersection of defects in the inter- and intracellular bacterial sensing mechanisms in conjunction with the apparatus responsible for regulating apoptosis in the setting of overwhelming stress response or defective unfolded protein response provides a fascinating insight into how epithelial handling of bacterial products from commensal organisms could be involved with the incitement of the heightened state of inflammation found in IBD, without an overt infectious trigger accounting for the disease state.

1.1.12 LINKING ABNORMAL IEC FUNCTION TO DYSFUNCTION OF INNATE IMMUNE RESPONSE

Extra- and intracellular receptors help the intestinal epithelial cells and immune cells discern between friend or foe by discriminating between different bacterial components. As just discussed, intracytoplasmic bacterial sensors have been linked to the indirect development of inflammation and altered immune responses to commensal organisms through defects in intracellular sensors tied to inadequate autophagic responses. However, this process can be directly linked to the initiation of an inflammatory response, as well, through shared signaling components. Bacterial components can result in direct triggering of a parallel immune response via inflammasome activation (Hsu, Ali et al. 2008). The inflammasome represents a confluence of three individual components, including a NOD-like (NRL) receptor, an adapter protein called ASC, and caspase-1. Oligomerization of these three components into a single structure result in the formation of an inflammasome, responsible for cleaving certain immature cytokines (Dinarello 2002). Inflammasome activation by bacterial or viral components is required to initiate the cleavage of intracellular pro-cytokines (pro-IL-1β, pro-IL-18, and pro-IL-33), ultimately leading to the secretion of active cytokines and the subsequent induction of an immune response (Dinarello 2002, Martinon and Tschopp 2007). Inflammasome activation by PAMP/DAMP sensing also provides a basis for linking the mutated variants of NOD2 (Newton 2012), a genetic susceptibility factor found in some Crohn's disease patients,

Figure 3. Caspase-1 activation pathway. (From: Newton, 2012)

and the clinical manifestations of IBD (Cho 2001). After binding with MDP, activated NOD2 binds directly to caspase-1 via its N-terminal CARD domain and activates caspase-1 (figure 3), which subsequently cleaves immature, pro-cytokines such as IL-1β and IL-18 into their active forms, leading to the initiation of a robust innate immune response to bacterial penetration (Hsu, Ali et al. 2008). Deficiencies in this system lead to inadequate host immune responses in the Crohn's disease patient and the potential for overaggressive adaptive responses to uncontrolled bacterial contact with the intestinal epithelium. Further supporting this concept, multiple lines of evidence identify that inflammasome activation acts as a pivotal regulator of intestinal homeostasis, by coupling its ability to detect the presence of intracellular bacterial components to pathways for augmenting protective measures such as protective mucin and antimicrobial peptide (AMP) synthesis (Kaser and Blumberg 2011, Peterson and Artis 2014, Wlodarska, Thaiss et al. 2014). Therefore, the same germline mutations that are implicated in inappropriate immune responses to commensal organisms likely further compound the underlying inflammation of IBD by impairing the homeostatic processes that protect against inadvertent immune activation. Since innate lymphocyte recruitment and antimicrobial peptide secretion is frequently stimulated by the high levels of IL-1β and IL-18 secreted by IECs (Hornef and Fulde 2014, Bedi, McNair et al. 2015), intracellular processing of these pro-cytokines serve as yet another link between the microbial sensing function of NODs and NLRPs by IECs to activation of innate immune system responses. It appears that inappropriate sensing by defective intracellular sensors in some forms of IBD provides a perfect storm leading to enhanced bacterial proliferation and subsequent, over aggressive immune responses.

1.1.13 ROLE OF IECs IN MUCOSAL INFLAMMATION

Since the initial reports that IECs secrete various cytokines (Stadnyk 1994), evidence supporting the IEC as a source of a staggering number of immune mediating proteins has been steadily accumulated (Peterson and Artis 2014). The type of bacteria or immune cells interacting with IECs influence the type of cytokines and chemotactic factors secreted (Reinecker and Podolsky 1995, Haller, Bode et al. 2000, Borruel, Casellas et al. 2003, Watson and Galán 2005, Tiittanen, Keto et al. 2013). The complexity of cytokines known to be affiliated with the two most common forms of IBD has steadily grown, as has the catalog of cytokines identified from animal models and cell lines (Mitsuyama, Sata et al. , Sher, D'Angelo et al. 1995, Ruffolo, Scarpa et al. 2007, Seidelin, Bjerrum et al. 2010). However, several difficulties exist when correlating animal model cytokine production with human disease states. First, measuring systemic levels of cytokines may miss localized paracrine effects from low level IEC secretion. Second, cytokine profiles may vary greatly between diseased mucosa and adjacent normal tissue. This heterogeneity relates, in part, to the complex composition characterizing cells of the intestinal epithelium. Functional specialization of certain mucosal components, such as those associated with microfold (M) cells (specialized epithelial cells covering the follicle-associated epithelium responsible for augmenting antigen transcytosis) may enhance susceptibility to inflammation due to their close proximity to mucosal immune cells (Kucharzik, Lügering et al. 2000, Gullberg and SÖDerholm 2006). Lastly, established inflammation generates clinically apparent symptoms prompting endoscopic evaluation. Therefore, most biopsy specimens come

from chronically diseased patients. A true time course experiment, easily done with cell lines or cells from peripheral blood, becomes exceedingly difficult in humans, when the inconvenience or safety ramifications of serial colonoscopies at a close enough interval to make meaningful observations is considered. Therefore, most descriptions of clinical IBD represent a "snap shot", or moment in time. Accurate information distinguishing precursor environment from conditions during clinically apparent inflammation or even during the resolution phase are non-existent. These limitations have certainly impacted clinical research in dramatic fashion.

1.2 IMPACT OF DIETARY COMPONENTS ON INFLAMMATION

Diet plays a significant role in health and disease. The benefits of whole grains have been recognized since the time of Hippocrates (Slavin and Lloyd 2012), and fresh fruits and vegetables have been shown to protect against a number of metabolic diseases such as diabetes (Mursu, Virtanen et al. 2014), stroke and heart attack (He, Nowson et al. 2007), and even cancer (Mursu, Nurmi et al. 2008). Many of the benefits of a diet high in plant-based food items may have to do with the increased dietary fiber accompanying such diets. Dietary fiber serves as a metabolic substrate for certain bacteria and can preferentially shape a microbiome (Wu, Chen et al. 2011). This observation supports results seen in a study that analyzed the fecal microbiomes from 3rd world children growing up a rural African village compared to children from the 1st world city of Florence, Italy, that identified significant differences in fecal microbiota based on dietary intake (De Filippo, Cavalieri et al. 2010). In particular, children from Florence harbored high levels of bacteria from the phylum Firmicutes, while the stool from children from

Burkina Faso contained high levels of Bacteriodetes and low levels of Firmicutes (De Filippo, Cavalieri et al. 2010). The high levels of dietary fiber intake from the African traditional diet was considered to be a contributing factor to the population diversities seen based on location. Because of the close proximity of the nutrient containing lumen of the digestive tract to the intestinal epithelium, the effect of dietary components may be most strongly manifested at the mucosal interface. These data also support a connection between dietary intake, shifts in the microbiome, and increased potential for IBD.

1.2.1 ANTI-INFLAMMATORY COMPOUNDS FROM DIETARY SOURCES

A diet high in fruits and vegetables has aso been associated with protection against inflammation (Watzl 2008). Many of the beneficial effects have been attributed to the anti-inflammatory effects of phytochemicals. In fact, it has been estimated that a diet rich in plant food supplies over 25,000 phytochemicals that are missing from the highly refined diets typical of the Western diet (Rao 2003). Ingestion of tea has been associated with health for decades, including protection from chronic inflammatory disorders, diabetes, cardiovascular disease and even cancer (Higdon and Frei 2003, Lambert, Hong et al. 2005, Yang, Lambert et al. 2006). Green tea has especially been associated with benefits to health, likely due to the abundant polyphenols contained within the unfermented tea leaves. Massive amounts of tea are ingested throughout the world, second only to water in worldwide consumption (Costa, Gouveia et al. 2002, Rietveld and Wiseman 2003). Although total tea consumption exceeds 3 billion kilograms worldwide, the type of tea varies dramatically by region.

1.2.2 PROCESSING OF TEA FOR INGESTION

All tea begins as leaves collected from the tea bush, Camellia sinensis. The simplest preparations of tea, white and green teas, start with the steaming of freshly picked tea leaves (figure 4). Steaming stops polyphenol oxidation by phenol oxidase that activates spontaneously after picking (Cabrera, Artacho et al. 2006). After steam fixation, tea leaves are sun dried and processed for consumption. White tea is made with the youngest tea leaves, sometimes protected from light to limit the development of chlorophyll, and contains the highest polyphenol concentration by weight. Green tea is made from mature tea leaves, and incurs less manipulation during processing than black or oolong teas, leading to the highest retention of original polyphenol content (Graham 1992). Oolong and black teas alternate methods of preparation to induce different taste and appearance properties. Tea leaves bound for black and Oolong teas are crushed to activate polyphenol oxidase and expose internal contents to oxygen. The oxidation process is halted early for Oolong teas, but is allowed to continue until complete oxidation of the polyphenol content to theaflavins and thearugibins has occurred in the production of black teas (Roberts 1958). The oxidation process turns the tea leaves black. Black teas are naturally oxidized and occasionally bacterially fermented, while Oolong teas are always semi-fermented. Bacterial enzymatic digestion further alters the catechin profile even more dramatically. Health benefits appear to track with the type of tea consumed. Black tea consumption by Western countries and India accounts for 78% of worldwide consumption, while 20% is consumed as green tea in Asian countries and 2% is imbided as Oolong tea, mainly in Southern China (Balentine, Wiseman et al. 1997).

While the benefits of tea ingestion seen in epidemiologic studies are intriguing, the actual amount of tea required to receive a physiologically meaningful dose of EGCG would equal approximately 32 cups of green tea. Therefore, the work laid out in this dissertation is based on the concept of providing a concentrated dose of EGCG in a pharmacologically relevant oral dose of polyphenols extracted from green tea leaves.

Figure 4. Stepwise process for creating different forms of tea from freshly harvested tea leaves. The crucial step in maintaining a high polyphenol level in green tea has to do with the panfrying/steaming/firing step, as this heat inactivates the enzymes responsible for oxidizing polyphenolic compounds into theaflavins and thearugubins characteristic of black teas. (Source: Wikimedia Commons).

1.2.3 CHEMICAL COMPOSITION OF TEA LEAVES

Tea leaves contain a complex mixture of chemicals, including the methylxanthines (theobromine and theophylline) plus polyphenols (including the flavonols kaempferol, myricetin, and quercetin along with the flavanol catechins and flavanol gallocatechins) (Manach, Scalbert et al. 2004). Up to 30% of the dry weight is made up of flavanols plus flavonols (Balentine, Wiseman et al. 1997). Green tea contains the highest level of flavanols of any food source (Manach, Scalbert et al. 2004). Of the biologically active compounds contained in green tea, the most potent impact on

inflammatory pathways has been ascribed to the polyphenol content (Crouvezier, Powell et al. 2001). The main polyphenols found in green tea consist of a class of flavonoids called green tea catechins (Sano, Tabata et al. 2001). Four main catechins make up the bulk of all catechins (figure 5), including (-)-epigallocatechin gallate (EGCG-60%), (-)-epigallocatechin (EGC-19%), (-)-epicatechin-3-gallate (ECG- 13%) and (-)-epicatechin (EC-6%) (McKay and Blumberg 2002).

Figure 5. Structures of the predominant polyphenols found in green tea. (Kim, Tan et al. 2013).
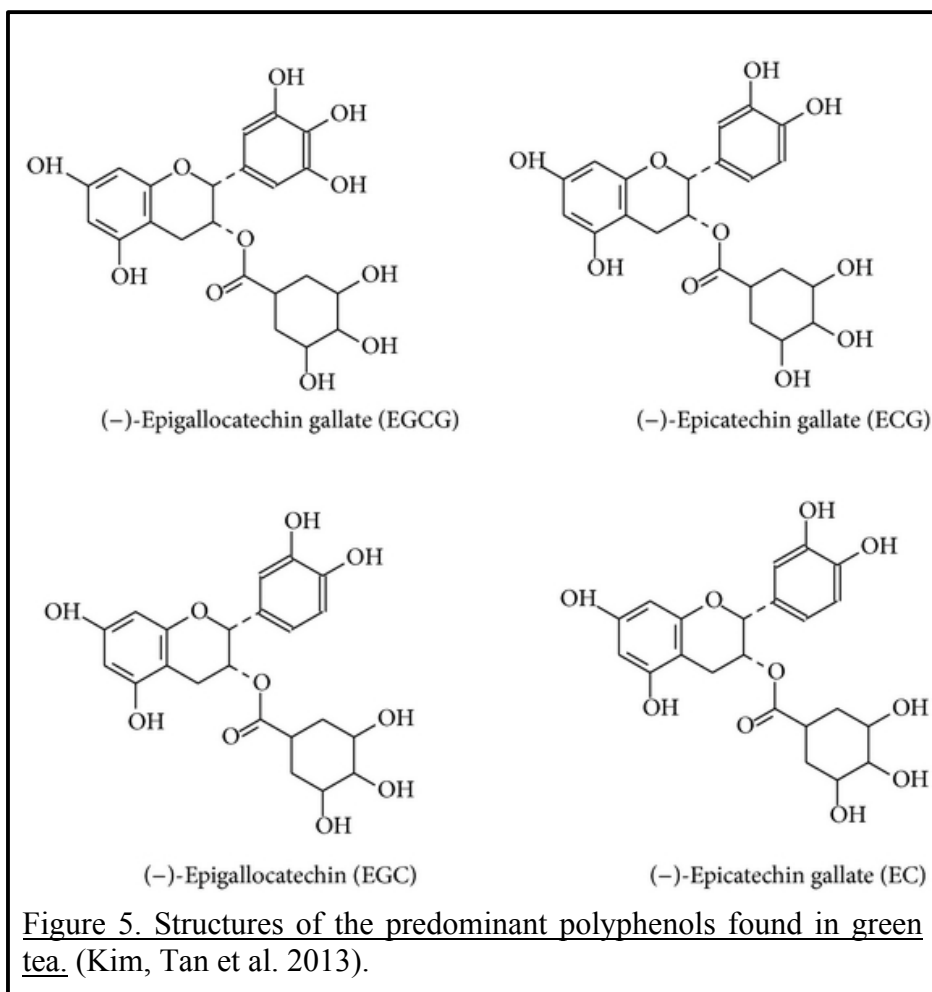
1.2.4 ATTRIBUTES OF GREEN TEA POLYPHENOLS THAT COULD BENEFIT IBD

Green tea polyphenols (GrTP) are potent anti-oxidants. The anti-oxidant properties of the GrTP EGCG are approximately 25-100 times more powerful than those

of vitamin C (Rice-Evans, Miller et al. 1996). The anti-oxidant properties of green tea polyphenols can be attributed to their donation of hydrogen radicals to alkoxyl (RO•) or peroxyl (ROO•) free radicals, converting them to more stable products (Wang, Kim et al. 2000). Many of the putative health benefits of tea are presumed due to the anti-oxidant effects of the polyphenols (Salah, Miller et al. 1995). One benefit may be cancer prevention, as epidemiological studies suggest that regular consumption of tea reduces the risk of cancer (Katiyar and Mukhtar 1996). In support of this, tea, or more specifically the polyphenol fraction, decreases the incidence of carcinogen-induced malignancies in animal models (Stoner and Mukhtar 1995). One proposed mechanism of action suggests that polyphenols induce apoptosis more readily in cancer cells than in their natural counterparts (Chen, Schell et al. 1998, Yang, Liao et al. 1998, Suganuma, Okabe et al. 1999, Ahmad, Gupta et al. 2000). Although epidemiological studies have evaluated the beneficial effects of green tea based on ingestion of cups of brewed tea, difficulty in changing dietary habits and in ensuring uniform concentrations of polyphenol intake have led most investigators to resort to the use of a standardized form of encapsulated polyphenol extract for clinical trials. Also, the amount of tea that would need to be consumed to obtain an adequate dose of polyphenols would exceed the threshold of tolerability for most people.

1.2.5 ANTI-INFLAMMATORY PROPERTIES OF GrTP

Another potential benefit of green tea polyphenols stems from their anti-inflammatory properties. Studies in animal models show that GrTP decrease inflammation (Yang, de Villiers et al. 1998). Mice fed an extract of GrTP had decreased

tumor necrosis factor-alpha (TNF-α) production in response to an injection of lipopolysaccharide (LPS), and that ingestion of GrTP also prevented death following administration of a normally fatal dose of LPS (Yang, de Villiers et al. 1998). Haqqi et al (Haqqi, Anthony et al. 1999) reported that the ingestion of a green tea polyphenol extract reduced joint disease in mice with adjuvant-induced arthritis, which suggests that green tea may have a benefit in treating arthritic conditions, such as rheumatoid arthritis. Varilek reported that GrTP reduced disease activity in the autoimmune disease model of interleukin-2 deficient (IL-2-/-) mice (Varilek, Yang et al. 1999). These studies suggest that green tea polyphenols may be beneficial in treating chronic inflammatory diseases.

Many potential mechanisms responsible for the observed anti-inflammatory and anti-cancer properties of GrTP have been reported. One potential mechanism of action is the inhibition of NF-κB activation, an oxidative-stress sensitive transcription factor that regulates the expression of a wide variety of genes important in many cellular responses, including inflammation, innate immunity, apoptosis and growth. EGCG has been shown to decrease LPS-induced TNF-α production in the macrophage cell line RAW264.7 and in peritoneal macrophages by blocking the activation of NF-κB (Yang, de Villiers et al. 1998). Lin and Lin similarly reported that EGCG inhibited LPS-induced inducible nitric oxide synthase gene expression in mouse peritoneal macrophages by decreasing NF-κB activation (Lin and Lin 1997). Ahmad et al showed that GrTP modulate NF-κB in several cancer cell lines, rendering them susceptible to apoptosis (Ahmad, Gupta et al. 2000). These studies suggest that green tea modulates the activation of an important

transcription factor that is responsible for activating many cellular functions, including inflammation, immunity and programmed cell death.

1.2.6 PHARMACOKINETICS OF GrTP

The bioavailability of polyphenols from orally consumed tea has been difficult to determine from epidemiological studies, due to variations in types of tea consumed, differences in preparation methods, standardization of dosing based on cup size, etc. Suganuma and co-workers found that EGCG was widely distributed throughout the body of a mouse, with significantly elevated levels in the digestive tract, liver, lung, pancreas and skin (concentrations in the colon were 5x higher than the serum) (Suganuma, Okabe et al. 1998). Repeated dosing 6 hours later enhanced tissue levels in blood, brain, liver, pancreas, bladder, and bone. These levels were 4-6 fold greater than those found following the initial dose. These results suggest that frequent consumption of green tea enables the body to maintain higher tissue concentrations of polyphenols. Initial human studies returned highly variable absorption rates, ranging from 0.2-2.0% of ingested polyphenols (Unno, Kondo et al. 1996, Nakagawa, Okuda et al. 1997, Yang, Chen et al. 1998). The variation was likely due to non-standardized forms of tea ingested. Circulating EGCG existed mainly in the free form (>92%), while EGC and EC exist mainly in the conjugated form (Lee, Maliakal et al. 2002). More recent studies by Chow et al. evaluated the human pharmacokinetic parameters from consumption of a daily dose of a caffeine-free, well standardized, EGCG enriched capsule of green tea extracts (Chow, Cai et al. 2001, Chow, Cai et al. 2003, Chow, Hakim et al. 2005). However, the chronic consumption of 800mg EGCG as Polyphenon E over 4 weeks led to > 60% increase in the area under the curve

(AUC), when following a dosing schedule of 800mg of total EGCG once daily. Since the accumulation ratio was calculated at <1.05, the authors postulated that the observed increase of free EGCG was not related to drug accumulation after repeated dosing. Rather, they felt that the 800mg dose of EGCG/Polyphenon E resulted in significantly elevated EGCG levels in the gastrointestinal tract, subsequently inhibiting the pre-systemic elimination of EGCG (Chow, Cai et al. 2003). This is an important consideration, as it points to the fact that ample active constituent to induce the beneficial effects of EGCG on NF-κB inhibition and immune regulation will be available at the important interface between the microbiome and the epithelial cells lining the intestinal and colonic lumen. One would expect, based on the pharmacokinetic data from Chow and Suganuma, that the intestinal mucosal concentration of EGCG exceeds that of the serum.

1.2.7 SAFETY PROFILE OF GrTP

In human studies, single doses of up to 4.5 grams of green tea extract have been given without adverse reactions (Yang, Chen et al. 1998). In extended dosing studies, doses up to 1 g/m^2 of green tea polyphenols three times daily (equivalent to 21-24 cups of green tea/day) have been given for up to 6 months without evidence of toxicity (Pisters, Newman et al. 2001). Several animal studies have been performed using GrTP. Three grams/Kg in a single dose has been shown to be lethal for mice, while 500 mg/kg in a single dose was not toxic to mice or rats. In mice, 100 mg/Kg daily for 6 weeks was not associated with any evidence of toxicity. However, rats fed 500 mg/Kg daily for 3 weeks developed steatohepatitis with moderately elevated ALT levels. In a recent phase II study evaluating the safety and efficacy of green tea in cancer patients (given as an actual tea),

69% of subjects experienced mild (grade 1 or 2) toxicity, mainly consisting of nausea, insomnia, abdominal pain and diarrhea (Jatoi, Ellison et al. 2003). Most of these symptoms were attributed to the caffeine content of natural green tea. In a recent phase I study evaluating the caffeine free form of green tea extract known as polyphenon E, subjects experienced only mild side effects, such as excess gas, upset stomach, nausea, and diarrhea, at rates similar to those receiving placebo. In patients receiving active therapy, the symptom of nausea alone exceeded the placebo adverse event rate (Chow, Cai et al. 2003).

1.3 SUMMARY OF BENEFITS OF GrTP AND ROLE IN IBD

As detailed above, epidemiologic data suggests that the intake of bioactive compounds from fruits and vegetables positively impacts health and homeostasis, and in the case of green tea, its intake may protect against cancers and inflammatory disorders. In fact, one of the most important tea derivatives, EGCG, has been shown to possess many useful properties for treating inflammatory diseases. After putting this preliminary data into context with the expanding knowledge behind the pathogenesis of IBD, the potential impact from EGCG on IBD becomes increasingly apparent. Based on epidemiologic data described above, the most common focus of EGCG related research has concentrated on preventing or treating various forms of cancer. EGCG has been found to play a role in an expanding number of important processes related to cancer and cell signaling. Unfortunately, the promising *in vitro* findings in cancer research have not readily applied to *in vivo* evaluations of efficacy in human disease processes. While others have narrowly focused on these issues and their role in cancer, we have attempted to redirect these findings to the closely related discipline of IBD and translate key

findings from non-IBD related research to support the use of EGCG in IBD. Given the low systemic availability of EGCG but high exposure at the luminal interface of the intestinal mucosa, we hypothesized that while this phenomenon caused EGCG to fail in cancer research, we could harness all the beneficial properties in treating IBD.

We have taken a stepwise approach to evaluate the impact of EGCG on various aspects of IBD pathophysiology. First, in chapter 3 we questioned whether EGCG could be used as a preventative agent, as well as, a therapeutic agent in an animal model of DSS induced colitis. We next conducted experiments testing the anti-inflammatory effects of EGCG on activated immune cells taken from the systemic circulation, as well as, stimulated epithelial cells as a surrogate for intestinal epithelial cells as detailed in chapter 4. However, data pointing to the fact that orally administered EGCG concentrated at the epithelial barrier caused us to shift our focus in chapter 5 to measuring the effects of EGCG and its influence of barrier permeability, as this has been predicted to be an important front for controlling inflammation associated with IBD. Finally, chapter 6 finishes off our exploration into the effects of EGCG on IBD with a detailed report of our successful clinical trial using patients with active ulcerative colitis. In summary, the data presented in this dissertation lays out a convincing argument for the benefits of EGG in IBD and provides a springboard for exploring a whole new array of IBD-related clinical applications.

CHAPTER 2

MATERIALS AND EXPERIMENTAL METHODS

2.1 CELL CULTURE

   Human cell lines were purchased from the American Type Culture Collection (ATCC - Rockville, MD). Both Caco2 and HT-29 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10% fetal bovine serum (FBS), at 37°C in a 5% CO_2 environment. Culture medium was changed every 2-3 days. Caco2 cells ($1x10^6$ cells / well) were grown to 80% confluence prior to conducting experiments. Cells were subcultured after partial digestion with 0.25% trypsin-EDTA, and passages 12-15 were used. Caco2 and HT-29 cells grown on chamber slides (LabTek, Naperville, IL) were used for determination of tight junction proteins, whereas Caco-2 cells grown on six-well plates were used for immunoblotting analysis. For measurement of epithelial barrier function, Caco2 cells were cultured on 24-well inserts (pore size 0.4 μm; BD Biosciences, San Jose, CA). During co-culture experiments, EGCG was added to the culture medium at noncytotoxic doses of 2.5, 5, 7.5, and 10 μg/ml.

2.2 REAGENTS

The green tea catechin EGCG was purchased from Sigma Aldrich (St. Louis, MO) at 90% purity for administration to mice in drinking water, or at 99% purity for adding to cell culture medium as above. The source of LPS was from Sigma Aldrich as well. The LPS was derived from *E. coli* strain 0111:B4. Primary and secondary antibodies were all obtained from Santa Cruz Biotechnology (Dallas, TX). DSD-PAGE gels in various concentrations (6-12%) were obtained from Bio-Rad (Hercules, CA), as were blocking buffers, lysis buffers, TWEEN, and trypsin. A Luminata Forte Western HRP substrate chemilumiscent kit from Millipore (Billerica, MA) was used for imaging proteins by Western blot. Elisa kits were obtained from R&D Systems and Antibodies-on-Line.com. RNA isolation kits were obtained from Ambion (ThermoFischer – Grand Island, NY) and MACS (Miltenyi – San Diego, CA). Primers for reverse transcriptase polymerase chain reaction (RT-PCR) were obtained from Qiagen (Valencia, CA). Dextran sulfate sodium (molecular weight 36-50,000) was obtained from MP Biomedicals (Santa Ana, CA). All general lab chemicals were purchased from Sigma (St. Louis, MO).

2.3 CELL PROCESSING

Caco2 and HT-29 cells were used for cell culture studies due to the reproducibility of growth rates, reliability of cultures, and known cytokine output based on stimulation with LPS. A variety of analytical techniques were performed with the cultured cells as described in subsequent sections.

2.3.1 PERIPHERAL BLOOD *EX VIVO* IMMUNE ASSAY

Whole blood samples were collected in EDTA-containing vacuum tubes from IBD patients and healthy controls. Samples were thoroughly mixed and centrifuged, then divided and spiked with LPS (10 ng/ml) +/- EGCG. Samples were then incubated on a gentle shaker plate overnight under standard cell culture conditions. After 24 hours, plasma was collected by centrifugation and analyzed for cytokine levels.

2.3.2 CELL SEPARATION METHODS

Whole blood samples were collected in EDTA-containing vacuum tubes from IBD patients and healthy controls. Samples were thoroughly mixed and centrifuged. The isolated plasma was divided into separate culture tubes. Non-control aliquots were spiked with LPS (10 ng/ml) with or without EGCG. Samples were then incubated on a gentle shaker plate overnight under standard cell culture conditions. After 24 hours, cells were removed from the plasma by centrifugation and the supernatant was analyzed for cytokine levels.

2.3.3 CELL ISOLATION TECHNIQUE

PBMCs were isolated from the buffy coat of IBD patients (n=25) and healthy individuals (n=12) using Histopaque-1077 (Sigma Diagnostics, St. Louis, MO). The PMBC concentration was brought to 1×10^6 cells/ml in culture medium. The subpopulations of $CD14^+$ macrophages, $CD4^+CD45^+RO^+$ (memory) T cells and $CD4^+CD45^+RA^+$ (naïve) T cells were isolated with microbead technology (Macs, Invitrogen, Carlsbad CA) from the buffy coat aspirate.

2.3.4 IMMUNE CELL STIMULATION

CD14$^+$ macrophages were stimulated overnight (Sigma Aldrich, St. Louis, MO) at a concentration of 10 ng/ml, while T cells subpopulations (CD4$^+$CD45$^+$RO and CD4$^+$CD45$^+$RA) were stimulated by CD3/CD28 antibody coated plates. The stimulated immune cells were incubated in culture medium alone or in combination with varying concentrations of EGCG (2.5-10 μg/ml) at 37° C and 5% CO_2 for an additional 24 hours. All experiments were controlled with un-stimulated cells. At the end of the incubation period, cells were extracted by centrifugation. Supernatants were collected by pipette to measure pertinent secreted cytokine levels, while the pellets were used to isolate RNA for measuring the expression of the targeted cytokine messages.

2.4   METHODS FOR DETERMINING EFFECT OF EGCG ON MODEL OF INTESTINAL EPITHELIUM

Caco2 and HT-29 cells were grown to 80% confluence during experiments designed to evaluate the effects of EGCG on cytokine expression and TJ proteins, as this state of cell maturity most closely resembles intestinal epithelial cells during periods of inflammation. During experiments measuring transepithelial electrical resistance (TER), cells were allowed to grow to 100% confluence, which replicates epithelial cell behavior during periods of homeostasis.

2.4.1 CACO2 MONOLAYER CELL CULTURE

Caco2 cells from the American Type Culture Collection (Rockville, MD) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/ml

penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 10 mM HEPES, and 10% FBS, at 37°C in a 5% CO_2 environment. Culture medium was changed every 2-3 days. Caco2 cells ($1x10^6$ cells / well) were grown to 80% confluence prior to conducting experiments. Caco2 cells were subcultured after partial digestion with 0.25% trypsin-EDTA, and passages 12-15 were used. Caco2 cells grown on chamber slides (LabTek, Naperville, IL) were used for determination of tight junction proteins, whereas Caco-2 cells used for immunoblotting analysis were grown on six-well plates. For measurement of epithelial barrier function, Caco2 cells were cultured on 24-well inserts (pore size 0.4 μm; BD Biosciences, San Jose, CA). During co-culture experiments, LPS with or without EGCG was added overnight to the culture medium at noncytotoxic doses of 2.5, 5, 7.5, and 10 μg/ml.

2.4.2 IMMUNOBLOT ANALYSIS OF TIGHT JUNCTION PROTEINS

Protein was isolated by treating the monolayers with cell lysis buffer (20 mM TrisHCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM sodium vanadate, 1 ug/ml leupeptin, 1 mM PMSF, and a protease cocktail set (100X Protease Inhibitor Cocktail; Cell Signaling Technology, Danvers, MA) prior to mechanically separating the cells with a cell scraper. Cell lysates were centrifuged and the clear supernatants were removed. Protein measurements were determined using a BioRad Protein Assay kit (BioRad, Hercules, CA). Aliquots containing 10 μg proteins were loaded on to 6 or 12% SDS-polyacrylamide gel, depending on the protein being evaluated. Proteins were separated by electrophoresis at 120 V for 2 hours at room temperature. Proteins were

transferred onto membranes (Trans-Blot Transfer Medium, Nitrocellulose Membrane; Bio-Rad, Hercules, CA) for 1 hour at 120V at room temperature. The membranes were incubated for 60 min in blocking solution (5% dry milk in PBS-Tween 20 buffer). The membranes were incubated with primary Abs against claudins-1, -2, and -4, occludin, and ZO-1 (Life Technologies, Grand Island, NY) in blocking solution at dilutions recommended by the manufacturer for 16 hours at 4°C. The membrane was then washed thoroughly and processed with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (GE Healthcare, Piscataway, NJ) for each of the proteins of interest in blocking solution at a 1:2000 ratio for 1 hour at room temperature and then developed using Pierce ECL 2 Western Blotting Substrate (Thermo Scientific, Hudson, NH). The membranes were exposed from 0.5 seconds to 20 seconds and the protein bands were visualized by an enhanced chemiluminescence detection system (ChemiDoc MP Imaging System, Bio-Rad, Hercules CA) and quantified by densitometry analysis (Image Lab 5.0 for MAC, Bio-Rad, Hercules CA).

2.4.3 MEASUREMENT OF CYTOKINE LEVELS BY ELISA

Cell culture supernatants were used to assay and quantitatively analyze several IBD related cytokines (e.g., IL-1 β, INF-γ and TNF-α). An enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) was read by a BioTek Plate Reader with Gen5 1.08 software (BioTek Instruments, Winooski, VT) at 540 nm to perform testing and analyze experimental results.

2.4.4 ISOLATION OF RNA AND ESTIMATION OF CYTOKINE PRODUCTION BY RT-PCR

Cell pellets were used to study the RNA expression of targeted cytokine messages by real-time polymerase chain reaction (RT-PCR). The concentration and the quality of isolated RNA were determined by measuring the 260/280 ratios. cDNA was prepared using a Quantitect reverse transcription kit (Qiagen, Chatsworth, CA) after cleaning the sample of any genomic DNA contamination. The cytokine expression was measured via RT-PCR using TaqMan expression assays (Applied Biosystems, Branchburg, NJ) with β-actin used as a control for RNA integrity and load equality. The 96-well RT-PCR reaction plate was analyzed using a Step One plus RT-PCR system (Life Technologies, Grand Island, NY).

Table 2. Primers used to perform RT-PCR.

Primer	Human	Mouse
Claudin 1	5'-GGCGACATTAGTGGCCACAGCATG-3' 5'-CGCGGATCCTTTCCGGGGACAGGAGCA-3'	5'-CGATGAGGTGCAGAAGATGA-3' 5'-CCAGTGAAGAGAGCCTGACC-3'
Claudin 4	5'-AGCCTTCCAGGTCCTCAACT-3' 5'-AGCAGCGAGTCGTACACCTT-3'	5'-GGCGTCTATGGGACTACAGG-3' 5'-GAGCGCACAACTCAGGATG-3'
Claudin 5	5'-GACTCGGTGCTGGCTCTGAG-3' 5'-CGTAGTTCTTCTTGTCGTAG-3'	5'-TGGAACGCTCAGATTTCATC-3' 5'-AGGAAGGCAACCCCTCTAAG-3'
Claudin 8	5'-TCATCCCTGTGAGCTGGGTT-3' 5'-TGGAGTAGACGCTCGGTGAC-3'	5'-TGCAAGGGATTGTTCAAATG-3' 5'-AAGGCATAGAAAATGGGGAAA-3'
Mucin 1	5'-CCACCCATTTCACCAC-3' 5'-TACGCTGCTGGTCATAC-3'	
Mucin 2	5'-GCACTCACCATAGCACG-3' 5'-GGCCAGAGTCAATTGTAC-3'	
MMP9	5'-CGCAGACATCGTCATCCAGT-3' 5'-GGATTGGC CTTGGAAGATGA-3'	
TNF-α	5'-CTCTTCTCCTTCCTGATCGTGGCA-3' 5'-GAAAGCATGATCCGGGACGTGGA-3'	5'-AGATGGAGAAGGGCAGTTAG-3' 5'-GATTCGCAGCGCATCGCCTT-3'
IL-6		5'-TCCAGTTGCCTTCTTGGGAC-3' 5'-GTACTCCAGAAGACCAGAGG-3'
β-actin	5'-AACTGGGACGACATGGAGAA -3' 5'-ATACCCCTCGTAGATGGGCA -3'	5'-TGTGATGGTGGGAATGGGTCAG-3' 5'-TTTGATGTCACGCACGATTTCC-3'

2.4.5 MEASUREMENT OF TRANSEPITHELIAL ELECTRICAL RESISTANCE

Caco2 monolayer barrier function was evaluated by measuring the electrical resistance and paracellular permeability. The transepithelial electrical resistance (TER) of the filter-grown Caco2 monolayers was measured with an epithelial volt ohmmeter (Millicell ERS; Millipore Company, Bedford, MA, USA). Electrical resistance was recorded with three consecutive measurements after subtracting the resistance value of the filters alone.

2.5 ANIMAL STUDIES TO EVALUATE EFFECTS OF EGCG ON MUCOSAL INFLAMMATION

Animals used in the DSS colitis protocol consisted of C57/Bl6 mice ordered from Jackson Laboratory. All aspects of the study were carried out under protocols approved by the Institutional Animal Care and Use Committee (13.068M1). Mice were divided at random into groups of 5 mice per cage under standard conditions for light/dark cycle, temperature and humidity. Mice received *ad lib* standard mouse chow during the experiment. EGCG was added to the drinking water on a daily basis to avoid spillage and contamination, as well as, monitor daily intake to determine whether any discrepancies in dose occurred during the study.

2.5.1 DSS MODEL OF INTESTINAL INFLAMMATION

Groups of C57BL/6 mice (n=5) acclimatized for 5-7 days prior to initiating the experimental protocol. Animals were then exposed to 2.5% DSS (experimental) or plain drinking water (control) with or without EGCG at two doses (2 or 20 μg/mL) in their drinking water. DSS control groups were exposed to 2.5% DSS only from days 1-9,

while EGCG was given concurrently from days 1-9 as a preventative agent or after DSS administration on days 9-16 as a treatment agent. Weights were taken daily to assess colitis severity; in addition, endoscopies were completed on days 9 and 16. Mice were sacrificed on day 17, and blood samples were collected. Colon lengths were measured, and portions of intestinal tissue were collected and flash frozen in liquid nitrogen. All samples were stored at -80°C for further analysis.

2.5.1.1 INDICATORS OF HEALTH

Daily weights were measured during the experimental protocol. Mice experiencing greater than 10% weight loss were evaluated twice daily, to look for evidence of failure to thrive. If general appearance (including rough hair coat, hunched posture, lethargy, or persistent recumbent position) developed in addition to 10% weight loss, the animals were monitored on a daily basis by veterinary technicians. If an animal lost more than 15% body weight, it was removed from its cage and placed into an isolation cage with additional water sources, such as long sipper tubes or water gel. If weight loss continued to 20%, or additional indicators of moribundity developed (such as head tucked into abdomen, exudates around the eyes and/or nose, abnormal breathing, difficulty with ambulation, decreased food and/or water intake, or self-mutilation), then animals were euthanized.

2.5.1.2   MEASUREMENT OF COLONIC INFLAMMATION BY SERIAL ENDOSCOPY

Prior to colonoscopy, mice were anesthetized using intraperitoneal injection of ketamine/xylazine (100/10 mg/kg). The colonoscopy apparatus (Karl Storz, Tuttlingen, Germany) consisted of a miniature endoscope (scope 1.9 mm diameter), a xenon light source, a triple chip camera, and an air pump to achieve regulated inflation of the mouse colon. After mice were adequately sedated, the colonoscope was lubricated with sterile water then inserted under direct visualization into the rectum. The colonoscope was advanced after obtaining a view of the colon lumen until the bowel was obstructed by stool or the scope could not be advanced any further without impacting the wall of the colon. The colonoscopic procedure was viewed on a color monitor and digitally recorded for later viewing and archiving. Endoscopic scoring was performed in blinded fashion. A score was assigned immediately after the colonoscopy. Mice were placed in clean cages, which were then placed on a warming pad until movement was detected. Once mice began purposeful movements, cages were replaced in the housing racks.

2.6 STATISTICAL ANALYSIS

Results for cytokine expression were calculated using the $2^{-\Delta\Delta CT}$ method. Cytokine expression levels were represented as a percentage of the cytokine expression of the control at baseline conditions (no LPS, no EGCG). Each figure represents four individual experiments, each performed in triplicate, with results represented as mean ± standard deviation (SD). Statistical significance of these data was determined by two-way ANOVA with Tukey's Multiple Comparison test using Prism 6 for Mac software (Prism, La Jolla CA).

2.7 CLINICAL TRIAL EVALUATING EFFECT OF EGCG ON ULCERATIVE COLITIS

An investigator initiated clinical trial was designed and submitted to the Food and Drug Administration under Investigational New Drug application #74117. The clinical protocol was submitted to the University of Louisville Institutional Review Board (IRB) and approved for recruitment. All clinical trial activities were performed under a consent form approved by the University of Louisville IRB (protocol # 390.95). The trial was registered in clinicaltrials.gov under identifier NCT00718094. The trial was supported by NIH grant 5K23DK073750.

2.7.1 STUDY DESIGN AND DEFINITION OF CLINICAL ENDPOINTS

A randomized, double-blinded, placebo controlled phase IIa pilot study was used to evaluate EGCG in patients with ulcerative colitis. The primary objective of the study was to evaluate the safety and efficacy of (-)-epigallocatechin-3-gallate (EGCG) in patients with mildly to moderately active UC as determined by the UC Disease Activity Index (UCDAI), a standard method of measuring disease activity (Sandborn, Tremaine et al. 1997). Subjects were classified as responders if they experienced a drop in their baseline UCDAI score by three or more points at day 56, or if they recorded an exit score of < 2. Clinical remission was defined as a UCDAI score < 2 at day 56, with an endoscopic sub-score of 1 or less. Finally, an important secondary endpoint examined the impact of Polyphenon E® on quality of life (QOL), as determined by the Inflammatory Bowel Disease Questionnaire (IBDQ). The IBDQ is a validated, IBD-specific indicator

of QOL (Guyatt, Mitchell et al. 1989), and was used with permission from McMaster University (Hamilton, Ontario).

2.7.2 SUBJECT SELECTION AND ETHICAL CONSIDERATIONS

Patients $\geq$ 18 years of age with mildly to moderately active disease (UCDAI score $\geq$ 4 and $\leq$ 10) were eligible for enrollment in the Institutional Review Board approved protocol. Informed consent was obtained prior to the conduct of any study related activity. Subjects had to have carried an endoscopically verified diagnosis of ulcerative colitis extending beyond the rectum for at least three months. Subjects already taking 5-ASA products were allowed to enter the study if they had been taking a stable dose for at least four weeks. Subjects previously treated with 5-ASA but who were intolerant of that therapy had to have been off 5-ASA therapies for at least two weeks prior to randomization. Subjects with persistent UC symptoms, despite taking a stable dose of immunomodulator therapy such as azathioprine (AZA) or 6-mercaptopurine (6-MP) for eight weeks, were allowed to enter the study on their baseline medication dose. Subjects currently taking corticosteroids, mycophenolate mofetil, methotrexate, cyclosporine, tacrolimus, or sirolimus were not permitted to enter the study. Those with recent use had to have undergone a four week washout period prior to randomization. Other exclusion criteria consisted of: (1) a positive stool sample for bacterial pathogens, ova and parasites or *Clostridium difficile* toxin; (2) pregnancy or lactation; (3) documented human immunodeficiency virus (HIV) infection; (4) signs or symptoms of severe progressive or uncontrolled renal, hepatic, hematologic, endocrine, pulmonary, cardiac, neurologic or cerebrovascular disease; (5) abnormal baseline lab studies including a serum creatinine

level $\geq$ 1.5x upper limits of normal (ULN); total bilirubin, aspartate transaminase (AST) or alanine aminotransferease (ALT) levels $\geq$ 2x ULN, or (6) concomitant, regular use of non-steroidal anti-inflammatory agents.

2.7.3 DETERMINATION OF ELIGIBILITY AND SUBJECT ENROLLMENT

Each subject was consented and evaluated for eligibility. Patients fulfilling all screening requirements were randomized in a double-blinded fashion according to a random number generator compiled by a statistician not involved in the study. Subjects were allocated to treatment or placebo in a 4:1 ratio (Figure 1A). Criteria for involuntary withdrawal from the study included the occurrence of a serious adverse event considered to be related to the study agent, or the presence of a UCDAI exceeding 10 at any visit. No subject required termination. Subjects withdrawing from the study were registered as non-responders.

2.7.4 MEASUREMENT OF CLINICAL ENDPOINTS OF EGCG SAFETY AND EFFICACY

All subjects were evaluated on days 1, 14, 28, 56, and 70. UCDAI was obtained on days 1 and 56 and the IBDQ was assessed on days 1, 14, 28, and 56. Safety labs consisted of: (a) complete blood count (CBC) with differential, (b) comprehensive metabolic panel, and (c) stool studies, including stool culture for enteric pathogens, *Clostridium difficile* toxin assay, and ova and parasite analysis. Compliance was evaluated by monthly pill counts $\geq$70%. Serial blood draws were assessed on day 1 and day 56 to evaluate the pharmacokinetics of EGCG in UC patients. Finally, the

endoscopic response was evaluated by flexible sigmoidoscopy on days 0 and 56, to complete UCDAI scoring.

Criteria for involuntary withdrawal from the study included the occurrence of a serious adverse event that were considered to be related to the study agent, or the presence of a UCDAI exceeding 10 at any visit.

2.7.5 SUBJECT RANDOMIZATION AND RATIONALE FOR CHOSEN DOSING REGIMEN

Twenty subjects were randomized to one of two groups: active therapy or placebo. Of the 10 subjects enrolled into cohort I, those subjects randomized to active therapy (low dose Polyphenon E[®]) ingested one Polyphenon E[®] capsule containing 200mg EGCG plus one identical appearing placebo, twice daily. Subjects randomized to active therapy in cohort II received high dose Polyphenon E[®] (400mg EGCG BID). The doses studied in this trial were determined during discussions with regulatory personnel while negotiating an Investigational New Drug (IND) application. The doses agreed upon were based on safety and pharmacokinetic data from the largest trial of green tea polyphenol components to date, which demonstrated that the chosen doses would result in an serum EGCG level that was immunologically active based on recent work on the immunoregulatory effects of EGCG (Chow, Cai et al. 2003, Dryden, Fernandez-Botran et al. 2011). Subjects randomized to placebo in either cohort received two capsules identical to Polyphenon E[®] twice daily. Subjects took the assigned study agent on days 1-56 (+/- 5 days), and underwent a physical exam and UCDAI calculation (including

endoscopy) prior to drug administration (day 0) and after 56 days of study agent exposure.

2.7.6 STATISTICAL ANALYSIS

Demographic information was compared between groups by χ^2 for categorical data and by unpaired t-test for continuous data. The percentage of subjects experiencing a response by the UCDAI (≥ 3 point decrease) or entering remission (≤ 2 point total) was compared between the aggregate subjects receiving Polyphenon E® or placebo by Fischer's exact test. All statistics were two-sided calculations with a significance level of 0.05 unless otherwise indicated. Calculations were performed with Prism 5 (GraphPad, La Jolla, CA).

CHAPTER 3

ROLE OF EGCG AS A PREVENTIVE AND THERAPEUTIC AGENT IN THE DSS

MODEL OF COLONIC INFLAMMATION

3.1 INTRODUCTION

Animal models of inflammation serve as useful tools for obtaining pre-clinical

information regarding the effects of a candidate therapy on inflammatory bowel disease.

A number of animal models of IBD exist, but one of the most commonly used is the

dextran sodium sulfate (DSS) model in mice. The DSS model of intestinal inflammation

most closely represents human ulcerative colitis (Okayasu, Hatakeyama S Fau - Yamada

et al.). This model depends on the oral administration of 40-50kDa DSS in drinking

water, but the exact mechanism is unknown. One recent investigation suggested that

colonic inflammation is mediated by dextran binding to dietary medium chain

triglycerides, with the resulting moiety fusing to colonocyte cell membranes (Laroui,

Ingersoll et al. 2012). Subsequent uptake into the cytoplasm triggers the release of

inflammatory cytokines. Unlike other models, the DSS model does not depend on the

adaptive immune system and can be used in RAG-/- and SCID mice to isolate the effect

on particular aspects of the immune response (Chassaing, Aitken et al. 2014). The model

is rapid and reproducible. We elected to use the DSS model to test orally administered

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EGCG to mimic the route of human administration, and to reproduce the mucosal uptake of this compound, which likely results in different distribution patterns of the compound in the body compared to other methods of administration, such as intraperitoneal injection. During our investigation, we also elected to evaluate two different treatment strategies, including early administration (mimicking a prophylactic approach) and late administration (mimicking a traditional treatment approach for suppressing already established colitis). In addition, two different doses were evaluated to determine whether a dose dependent response existed.

Figure 6. Schema for EGCG administration and endoscopy. Animals were divided into groups of 5. Control animals provided baseline data for comparison. DSS treated animals provided a positive control. Animals receiving EGCG had either low dose (2 µg/ml) or high dose (20 µg/ml) EGCG administered from day 0 through day 9 in the preventative group, or days 9-14 to animals in the treatment group.

3.2 RESULTS

3.2.1 EGCG REDUCES WEIGHT LOSS AND IMPROVES SURVIVAL IN THE SETTING OF DSS ADMINISTRATION

The initial manifestation of inflammation in the DSS model is weight loss, which begins around day 3 and bottoms out around day 7. DSS is typically administered at concentrations of 3-5%, with higher concentrations inducing greater weight loss and

severity of colitis. During dose optimization, we found that a concentration of 2.5% for 9 days provided the maximal differentiation of differences between treatment groups when using wild type C57/Bl6 animals. When using genetically modified animals that are susceptible to colitis or when another mucosal irritant such as ethanol is added to the diet, the concentration of DSS must be reduced to the range of 1-2%. In our investigation of EGCG in the DSS model, we tracked weights and viability on a daily basis. If an animal's weight dropped 15% from baseline, we housed the animal separately and provided additional sources of hydration. If an animal dropped more than 20% below baseline, it was euthanized to prevent unnecessary pain and suffering.

Figure 7. Mortality rates per treatment arm. All EGCG treatment arms experienced lower mortality rates than the DSS treated animals.
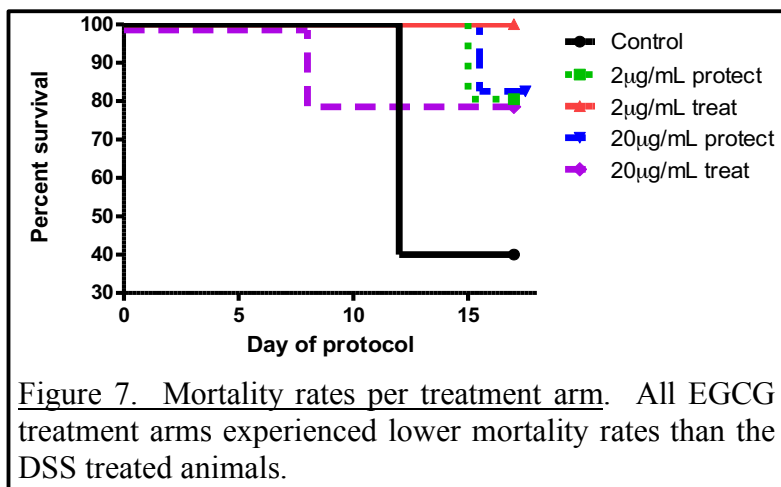
As seen in the adjacent figure (figure 7), mice treated with DSS alone had the worst survival rates, with up to 60% of animals dying or being euthanized due to excessive weight loss. The best survival rates occurred in mice pre-treated with the lowest dose of EGCG initiated as a preventative agent prior to the onset of inflammation. Other treatment groups all experienced some loss of animals, but in significantly fewer numbers than DSS alone. The addition of EGCG beginning at day 9 appears to have had a substantial effect of rescuing the mice exposed to the full course of DSS without a protective agent. Otherwise, we should have seen similar mortality rates compared to the DSS control animals.

In regard to weight loss, the animals receiving the lowest dose of EGCG in drinking water under the treatment protocol actually experienced the least amount of lost weight (figure 8). With the exception of the 20 μg/ml treatment group, EGCG treated groups of mice had intermediate levels of weight loss. The degree of weight lost by the highest dose treatment group tracked along with the DSS control group. The animals in the high dose treatment group, compared to the protective groups, did not rebound faster than the DSS control group. This observation could be related to changes in taste of the water caused by the higher dose of EGCG, leading to decreased water intake overall, or it could be related to an unknown impact on the inflammatory process, such as inducing increased apoptosis in intestinal epithelial cells. The latter explanation, however, is unlikely based on the fact that the endoscopic and histologic evaluations of colitis in the high dose treatment group were essentially no different from low dose treatment or either of the preventative groups (see below).
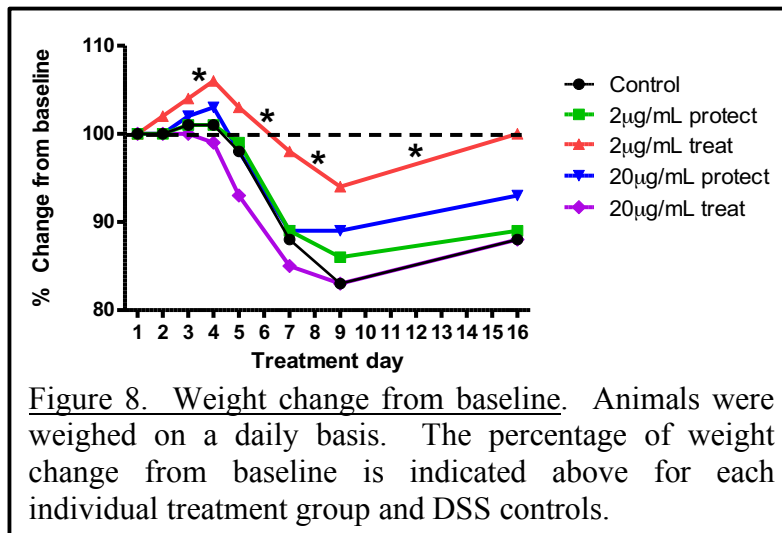
Figure 8. Weight change from baseline. Animals were weighed on a daily basis. The percentage of weight change from baseline is indicated above for each individual treatment group and DSS controls.

3.2.2 EGCG IMPROVES COLITIS SCORE AS DETERMINED BY SERIAL MURINE COLONOSCOPY

Murine colonoscopy has evolved into a useful tool for monitoring the evolution of mucosal inflammation. Initially described in 2002 (Huang, Carter et al. 2002), the technique has been rapidly developed to include objective measures of both inflammation and tumor burden (Becker, Fantini et al. 2005). These techniques apply particularly well to the DSS model of colitis or azoxymethane/DSS inflammation driven colon tumor model, due to the fact that the distal colon is most heavily involved with disease burden in both models. For the purposes our investigation, we performed serial endoscopies to monitor the maximal onset and subsequent resolution of colitis. This allowed us to better estimate the timing of onset and resolution of colitis to determine differences between the prophylactic administration compared to the reactionary treatment arms. The following scoring criteria were used to measure endoscopic disease severity in a blinded fashion by the endoscopist:

EIS Scoring Criteria

Colitis Score	0	1	2	3
Colon Thickening	Transparent	Blunting sharpness	Vague colors	Opaque
Vessel Changes	Normal	Small aneurysms	Loss of hierarchy	No vessels visible
Fibrin	Rare	White coats	Many clumps	Entire colon coated
Granularity	Normal	Patchy	More involved	Entire mucosa
Stool Consistency	Spicules	Barely deformable	Deep deformation	Coats mucosa

Table 3. Scoring criteria for colitis severity. Colitis scores are determined by endoscopy with each criterion being assigned a value between 0 - 3, with 3 being the most severe. Total severity scores are calculated by adding all five sub-scores, for a possible maximum score of 15 (Becker, Fantini et al. 2005).

As seen in figure 9, below, the groups receiving prophylactic therapy prior to the onset of inflammatory symptoms fared significantly better than "treatment" groups. The higher dose provided greater protection against inflammation. All groups receiving EGCG had significantly lower endoscopic scores of severity compared to animals

Figure 9. Effect of EGCG on mucosal findings and colitis severity scores. Animals were evaluated by endoscopy. (A) *top,* EIS scores on days 9 and 16 were determined via endoscopy, using Table 1 for analysis of findings. Data shown include mean and standard deviation (mean ± SD) of 3-5 mice. Statistical significance (p<0.01) was indicated by asterisks (**) and determined by ANOVA and Dunett's Multiple Comparison test. (B) *bottom left*, endoscopy of mouse with DSS-induced colitis. (C) *bottom right*, endoscopy of mouse treated with EGCG.

treated with DSS only by the second endoscopic evaluation. When considering the day 16 data, all EGCG treatment groups had significantly less inflammation, with no significant differences between the EGCG treated groups, although numerically, the anti-inflammatory effect appeared to impart a stronger treatment effect as evidenced by lower endoscopic scores in the animals receiving EGCG right up to two days before the second endoscopic assessment than the preventative treatment groups.

3.2.3 EGCG REDUCES INFLAMMATORY CYTOKINE PRODUCTION AS DETERMINED BY ELISA AND RT-PCR

Next, we evaluated the immunologic contributors to inflammation underpinning the endoscopic results seen in the previous section. Cytokines were evaluated in serum samples taken from the portal vein at the time of sacrifice and RT-PCR for various cytokines of interest was performed on homogenates of the distal colon from individual animals from each group. Each analysis consists of readings from 3-4 individual

samples. In all of the treatment groups, all five of the inflammatory cytokines evaluated

Figure 10. Cytokine analysis of portal vein plasma samples from animals in the prevention group. A panel of Th1 inflammatory cytokines were were analyzed by multi-bead ELISA (*left column)* or by PT-PCR (*right column*). Animals received either DSS alone or in combination with low dose or high dose EGCG. RT-PCR compared to DSS alone as baseline. Statistical significance of p<0.001 indicated by ****, as determined by ANOVA and Dunett's Multiple Comparison test. Data are expressed as mean ± SD of four independent experiments.

demonstrated a significant reduction from the levels seen in animals receiving DSS alone. The most prominently elevated cytokines in systemic circulation were IL-6 and IFN-γ. These two cytokines were essentially undetectable in portal vein serum samples. IL-17A, a more locally acting cytokine, was found at the lowest level. No significant differences were seen between treatment groups in the case of IL-17 or TNF-α. Gene expression levels also demonstrated a dose-dependent reduction in mRNA transcripts by RT-PCR analysis. In some cases, gene expression was reduced by up to 60%, as in the case of IFN-γ. RT-PCR analysis also demonstrated a dose-dependent, across the board reduction in inflammatory cytokine gene expression. As seen in the prevention groups, the treatment groups also exhibited dramatic

reductions of circulating cytokines. Again, IL-6 and IFN-γ levels were most aggressively
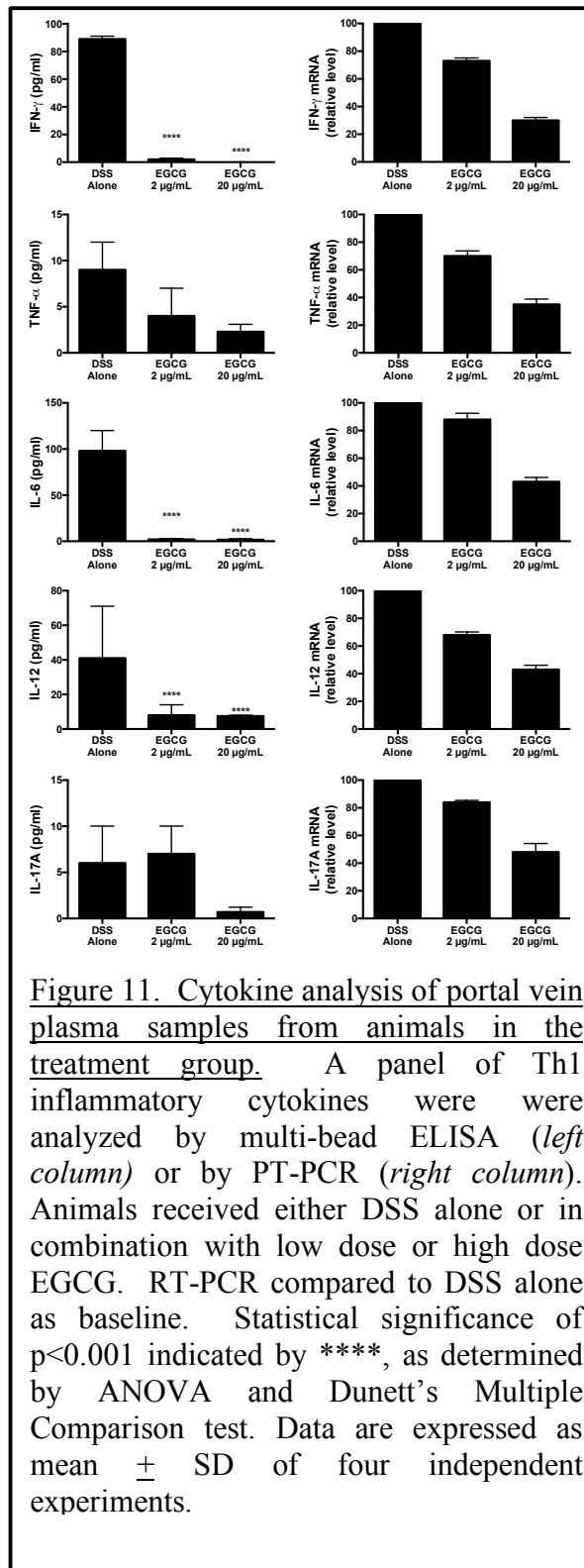
Figure 11. Cytokine analysis of portal vein plasma samples from animals in the treatment group. A panel of Th1 inflammatory cytokines were were analyzed by multi-bead ELISA (*left column*) or by PT-PCR (*right column*). Animals received either DSS alone or in combination with low dose or high dose EGCG. RT-PCR compared to DSS alone as baseline. Statistical significance of p<0.001 indicated by ****, as determined by ANOVA and Dunett's Multiple Comparison test. Data are expressed as mean ± SD of four independent experiments.

suppressed, but significant reductions were also seen in IL-12 concentrations. The gene expression measured by RT-PCR showed similar reductions in cytokine message on a per gene basis, but the appeared to be to a greater extent than the preventative group. This could be due to the fact that mice in this group were on treatment essentially up until the day of sacrifice. As before, the higher dose of EGG induced greater levels of suppression on cytokine message.

3.2.4 TRANSCRIPTION OF SELECT BARRIER RELATED PROTEINS AS DETERMINED BY WESTERN BLOT AND RT-PCR

Given the tight relationship of inflammation and enterocyte exposure to inflammatory cytokines and TJ protein composition, we next proceeded to evaluate the change of various TJ

proteins based on the relative levels of message and proteins in relation to the change in cytokine production.

Figure 12. Tight junction protein content from colonic epithelium. Colonic epithelial cells were isolated from mouse colons, lysed and harvested for protein analysis. Western blot analysis was carried for TJ proteins of interest. (A). Individual bands from Western blot of colonic cells from animals treated with EGCG 20 µg/ml. (B). Semi-quantitative densitometry analysis of WB bands using ImageJ analysis software. Statistically significant (p<0.001) increases are indicated by *** and were determined by ANOVA and Bonferroni's Multiple Comparison test.

3.3 DISCUSSION

The DSS model of colitis provides a useful model for evaluating novel therapies for IBD, with a particular relevance to UC. It provides an opportunity to evaluate the impact of a target therapy on several different aspects of the inflammation process. In our evaluation of EGCG as an anti-inflammatory therapy for DSS colitis, we focused on the effects of this topically active therapy on the mucosal interface. EGCG positively affected the general indicators of inflammation such as weight loss and mortality at both administered doses, without a clear-cut benefit of one over the other from those endpoints. Based on the fact that both active as well as preventative therapies are needed

in the clinical arena, we added a preventative arm to our evaluation, similar to how EGCG might be used to suppress the onset of inflammation to prevent post-operative recurrence of Crohn's disease. This is a particularly important unmet need in the field of IBD therapeutics, as the only form of medical therapy that has shown to be effective in preventing post-operative recurrence is the anti-TNF Ab class of therapeutics (Cottone, Orlando et al. 2003, Regueiro, Schraut et al. 2009, Carla-Moreau, Paul et al. 2015). The problem with surgical resection of Crohn's disease has always been the fact that Crohn's disease recurs rather quickly. Within 12 months after surgery, 70-90% of patients will have endoscopic recurrence of disease (Olaison, Smedh et al. 1992) and up to 30% will have recurrent clinical symptoms (Rutgeerts, Geboes et al. 1990). Because of the high cost ($777,732.00 per quality adjusted life year) and significant risks associated with long-term administration of anti-TNF therapy to prevent the possibility of a recurrence (Ananthakrishnan, Hur et al. 2011), it seems obvious that this indication should be a priority target for new drug development. The animals receiving preventative therapy were the only animals to have a statistically significantly lower degree of inflammation endoscopically. There appeared to be a dose dependent relationship to the endoscopic severity, such that the high dose of EGCG provided greater protection against DSS colitis than the low dose. We feel that the endoscopic evaluation of animals provides a clinically relevant evaluation of drug efficacy that observational scales such as the colitis severity index (based on a composite measurement of whether stool is loose or contains blood) cannot provide. The animals treated with preventative doses of EGCG also had essentially no colitis seen on microscopic evaluation of their colonic tissue taken at the end of the study. Additional animals euthanized at the mid-point of the study could have

provided a more definitive assessment of microscopic severity, but the mid-point endoscopic evaluation likely provides an equivalent surrogate without having to increase the number of mice studied.

Most recently approved therapies for IBD consist of parenterally administered immune modulating substances, mainly based on monoclonal antibody constructs targeting specific aspects of systemic immune cells. These therapies generally provide systemic anti-inflammatory effects, along with systemic immunosuppression. In fact, systemic infections constitute the largest number of serious side effects of most current therapies. One recently approved therapy, vedolizumab, targets the $\alpha4\beta7$ integrin to provide a relatively GI specific immunotherapy. This approach has limited the incidence of infectious complications to very low levels (Feagan, Rutgeerts et al. 2013). However, monoclonal antibody therapy incurs high costs and still puts patients at risk for complications such as infusion reactions or other infusion related complications. EGCG, on the other hand, has been implicated in reducing inflammation, but with few attendant "immunosuppressive" side effects such as those induced by interleukin-10 upregulation (Katiyar, Challa A Fau - McCormick et al.). This makes it an attractive therapy for IBD, as all of the current IBD therapies with the exception of 5-aminosalicylic acid (ASA) induce some degree of immune suppression and subsequently increase the risk of systemic infections.

Based on our analysis of the portal vein and colonic mucosal cytokine profiles from the DSS colitis study, we did see a significant anti-inflammatory effect from the

administration of EGCG. Although the systemic absorption of EGCG from the GI tract is poor, data from human pharmacokinetic studies suggest that circulating levels could reach the threshold for biologic activities. In order to understand the effects of systemic EGCG on circulating immune cells, we next performed a survey of the impact that EGCG could have on specific components of the immune system that play a role in IBD.

CHAPTER 4

EGCG IMPACTS PRODUCTION OF IMPORTANT CYTOKINE

INVOLVED IN INTESTINAL INFLAMMATION

4.1 INTRODUCTION: PERIPHERAL IMMUNE CELLS

Peripheral blood monocytes (PBMCs) provide a broad-based window into the immune status of a patient under evaluation. The PBMC population consists of a heterogenous collection of immune cells collected from the systemic circulation. This pool of immune cells reflects the general immune tone of the individual being examined as well as represents a population of cells that can readily be recruited to assist in the event of a immunonological crisis. These cells often exist in a precursor state that differs from the tissue resident state, such as the CD14+ bone marrow derived monocyte that is recruited into inflamed intestinal tissue under the guidance of the interaction of interferon gamma-induced protein (IP)10/chemokine receptor CXCR3 (Grimm, Pullman et al. 1995). These antigen presenting, cytokine producing monocytes rapidly evolve into purely phagocytic resident macrophages within days of migrating to the intestinal mucosa and cannot be stimulated to produce cytokines even with prolonged stimulation with bacterial antigens (Smith, Smythies et al. 2011).

Samples obtained from IBD patients participating in our studies contained monocytes and both effector and naïve T cells. Although we did not have before and after samples from patients receiving oral EGCG, we felt that it would be valuable to study the effects of pharmacologically relevant amounts of EGCG spiked into samples of immune cells harvested from IBD patients. Analysis of these cells would allow us to model the effect of systemically available EGCG on circulating immune cell cytokine production. Although many studies on patient samples inspect only a narrow range of cytokines, recent evidence has linked a suppressive effect on many of the regulatory pathways that control Th1-type effector cytokine protein production and secretion. We elected to begin our evaluation of EGCG as an IBD therapeutic useful for human disease by performing a survey of the effects of EGCG on the secretion of Th1 type cytokines from an assortment of the immune cells that play a significant role in IBD.

4.2 RESULTS

We found that EGCG produced a significant anti-inflammatory effect on all the immune cell populations that were studied. Interestingly, immune cells from IBD patients secreted much higher levels of cytokines in general when at rest in the growth medium compared to cells from normal volunteers. Upon stimulation with LPS or other appropriate immune cell activators, cells from IBD patients produced enormous amounts of cytokines compared to stimulated cells from normal volunteers. The effects from human plasma containing a mixture of immune cells, plus individual populations obtained by magnetic bead separation were probed more closely.

4.2.1 EFFECTS OF EGCG ON PRIMARY IMMUNE CELLS

Primary immune cells provide a more relevant platform for testing the effects of a potential anti-inflammatory therapy than cell lines. Cell lines are generally derived from either hematologic or solid organ malignancies, and may not represent a true physiological response to therapy. Cytokine responses or signaling pathways of interest may be disrupted by mutations that led to the development of the tumor in the first place. Primary immune cells reflect a more "normal" state of affairs, and generally tolerate short-term culture well. This makes time course evaluation possible. *In vitro* analysis also allows cells to be stimulated or inhibited under various conditions or tested against multiple concentrations of the potential therapeutic. We used primary immune cells to evaluate the impact of EGCG on cytokine production and gain insight into potential mechanism.

4.2.1.1 EFFECTS OF EGCG ON IMMUNE CELLS FROM IBD PATIENTS BY *EX VIVO* PLASMA CYTOKINE ASSAY

Perpetuation of the immune response in IBD comes from continuous recruitment of immune cells from the systemic circulation to replenish aging or damaged resident immune cells. In most disorders, immune modulating therapies must target circulating immune cells, as it might be difficult to get drug to the affected tissue, especially in situations like central nervous problems (due to the blood brain barrier), bone, or active infection. Other tissues are quite amenable to drug delivery by oral ingestion, such as the intestinal mucosa, due to the fact that the drug must cross the gastrointestinal tract/luminal interface prior to absorption. In order to evaluate the potential systemic

effects of EGCG on circulating immune cells, we utilized a whole blood *ex vivo* model established to assess potential effects of anti-cytokine therapies. We and others have used this method to effectively screen candidate compounds for anti-inflammatory activity (McClain, Barve et al. 1998, Stadlbauer, Mookerjee et al. 2008).

Figure 13. *Ex vivo* cytokine testing. EGCG suppresses the ability of LPS to elicit inflammatory cytokine production by peripheral blood leukocytes in an *ex vivo* model developed to screen for anti-inflammatory activity of potential therapeutic agents. Plasma was obtained by centrifugation of 5 ml of the whole blood immediately after being drawn from IBD patients and normal healthy individuals. All plasma samples were individually incubated in the presence of varying concentrations of EGCG (0-10 µg/ml) at 37^0C and 5% CO_2 for 24 hours. Samples were stimulated overnight with lipopolysaccharide 10 ng/ml. All experiments were controlled with unstimulated cells.

As seen in figure 13, EGCG induced a significant reduction in *ex vivo* cytokine production in LPS-stimulated samples from IBD patients. We initially chose a small number of type 1, IBD-related cytokines for this *ex vivo* testing (upper row). Samples spiked with LPS demonstrated a sharp increase in all tested cytokines. Samples that were initially stimulated with LPS then co-incubated with EGCG saw levels of TNF-α and IFN-γ suppressed, nearly to their pre-stimulated baselines. In the case of IL-1β, EGCG

suppressed cytokine production to a level below unstimulated baseline. In this model, the maximal suppressive effect occurred at a concentration of 10 μg/ml, which is clinically feasible based on available pharmacologic data (Chow, Cai et al. 2003). Given the positive results from the whole blood *ex-vivo* assay, we opted to evaluate the effect on other important Th1 effector cytokines that play a significant role in IBD. In all instances, there appeared to be a suppressive effect from EGCG on the production of these inflammatory cytokines. IL-12 seemed to be least affected, but reductions in IL-23 and IL-17A were profound in the plasma samples from IBD patients. The results of this experiment suggest that peripheral exposure to pharmacologically feasible levels of circulating EGCG might significantly dampen LPS-mediated activation pathways of immune cells while in the intravascular compartment, validating the potential for EGCG as a systemic therapy.

4.2.1.2 CD14$^+$ MONOCYTES

Since CD14$^+$ monocytes play such a large role in the innate immune response to inflammation and infection, we desired to know whether EGCG could suppress cytokine secretion in their pre-migratory state. This is pertinent to stemming the inflammation from IBD, as newly recruited CD14$^+$ monocytes can act as antigen presenting cells during acute inflammation, and serve as chief sources of Th1 type cytokines in the early phase of an immune response (Smith, Smythies et al. 2011). They are continuously replenished through a variety of mechanisms, including chemokine receptor (CXC3R)1/fractalkine binding or interaction the gut-specific α4β7 integrin and the cell adhesion molecule mucosal addressin cell-adhesion molecule-1 (MadCAM-1) (Waddell,

Ahrens R Fau - Steinbrecher et al. , Zigmond and Jung , Schippers, Muschaweck et al. 2015). Under homeostatic conditions of the non-inflamed bowel, these cells evolve under the influence of IL-10 into tissue resident macrophages which no longer contribute to the cytokine storm (Zigmond, Bernshtein et al.). However, EGCG could provide a suppressive influence on IBD by dampening the production of cytokine responses from newly arriving monocytes within the lamina propria.

CD14^{+} macrophages from both IBD patients and normal volunteers were isolated from the buffy coat of peripheral blood samples by magnetic bead technology. LPS was added to the culture medium in designated arms of the experiment to stimulate cells during overnight incubation. Next, cells were incubated in the presence of EGCG (0-10 μg/ml) at 37^oC and 5% CO_2 for 24 hours. Read outs from stimulated cells +/- EGCG were compared to basal cytokine secretion from un-stimulated cells. Additional testing to determine the impact of EGCG on unstimulated cells demonstrated similar, but lesser, impacts of EGCG (data not shown).

Figure 14. CD14^{+} monocytes exposed to multiple concentrations of EGCG. A panel of Th1 cytokines were analyzed from supernatant taken from CD14^{+} monocyte culture.
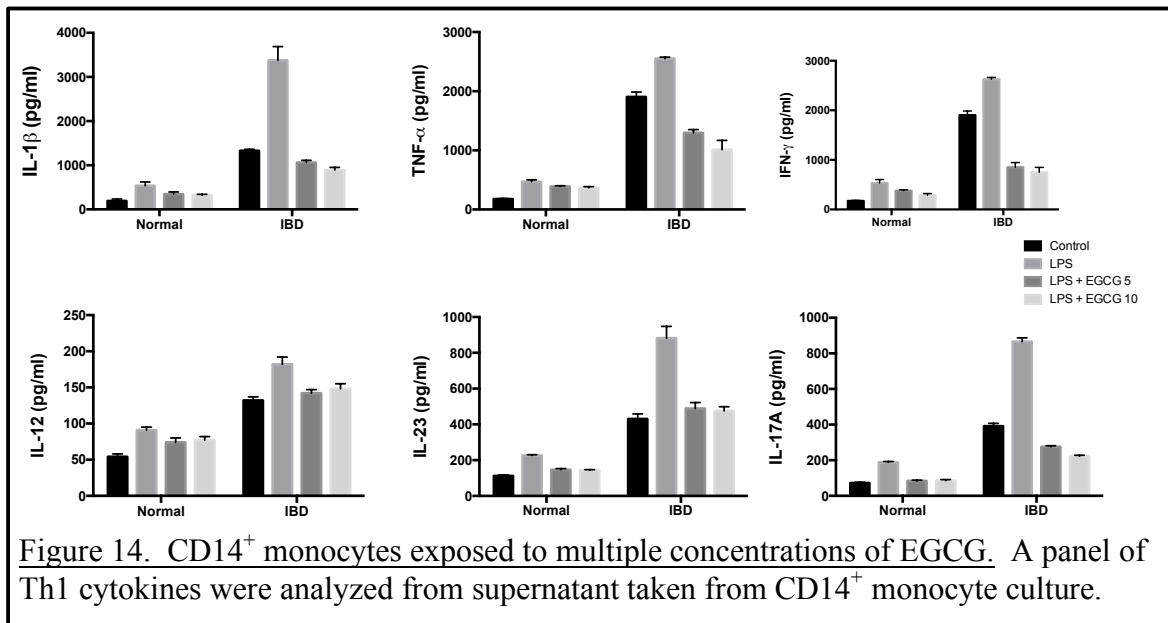
As in the case of the cell-containing serum samples from the previous section, individual cultures of $CD14^+$ monocytes also demonstrated significant reductions in inflammatory cytokine secretion. Again, the most vigorous response to LPS was seen in IBD patient monocytes, as baseline cytokine secretion in unstimulated cells occurred at the least an order of magnitude higher than in normal cells, especially for the type 1 cytokines such as interferon-γ, tumor necrosis factor-α, and IL-1β. IBD cells also expressed higher levels of the effector cytokines IL-12, IL-23 and IL-17 compared to normal cells, but not to the same extent. LPS-induced rises in cytokine production/ secretion followed similar patterns, although the response to LPS produced less variance than in the plasma samples. This experiment demonstrated that EGCG effectively specifically dampens the robust monocyte secretory response found at baseline in circulating monocytes from IBD patients, while the effect on LPS-stimulated cells predicts a suppressive effect once the monocytes migrate into inflamed mucosa and encounter antigenic stimulation.

4.2.1.3 $CD3^+CD45^+RO$ T CELLS

Although monocytes enter the inflamed tissue in a less mature state, effector T cells homing to the intestinal tract have generally matured through an antigen-specific activation process in the mesenteric lymph node. Circulating activated effector T cells enter the intestinal tissue in a controlled manner under the guidance of intestinal specific cell adhesion molecules that interact with the intestine homing specific integrin combination α4β7 (Erle, Briskin et al. 1994). Although the T cell population expressing this integrin heterodimer makes up only about 1-3% of circulating lymphocytes, its contribution to the

dysregulated adaptive immune response in the inflamed GI tract is responsible for causing a

considerable amount of the injury in IBD (Arihiro, Ohtani et al. 2002). In order to evaluate

the ability of EGCG to impact the Th1 effector T cell population's contribution to intestinal

inflammation, we isolated $CD3^+CD45^+RO$ T cells and activated them by CD3/CD28

antibodies as detailed in chapter 2: methods section.

Figure 15. Effect of EGCG on activated effector T cells. $CD3^+CD45^+RO$ T cells were
separated by magnetic bead technology from peripheral blood monocytes obtained
from the buffy coat of normal volunteers vs IBD patients. The rate of baseline un-
stimulated cytokines production ranges from 2-4 x higher in IBD patients than in
samples from volunteers.
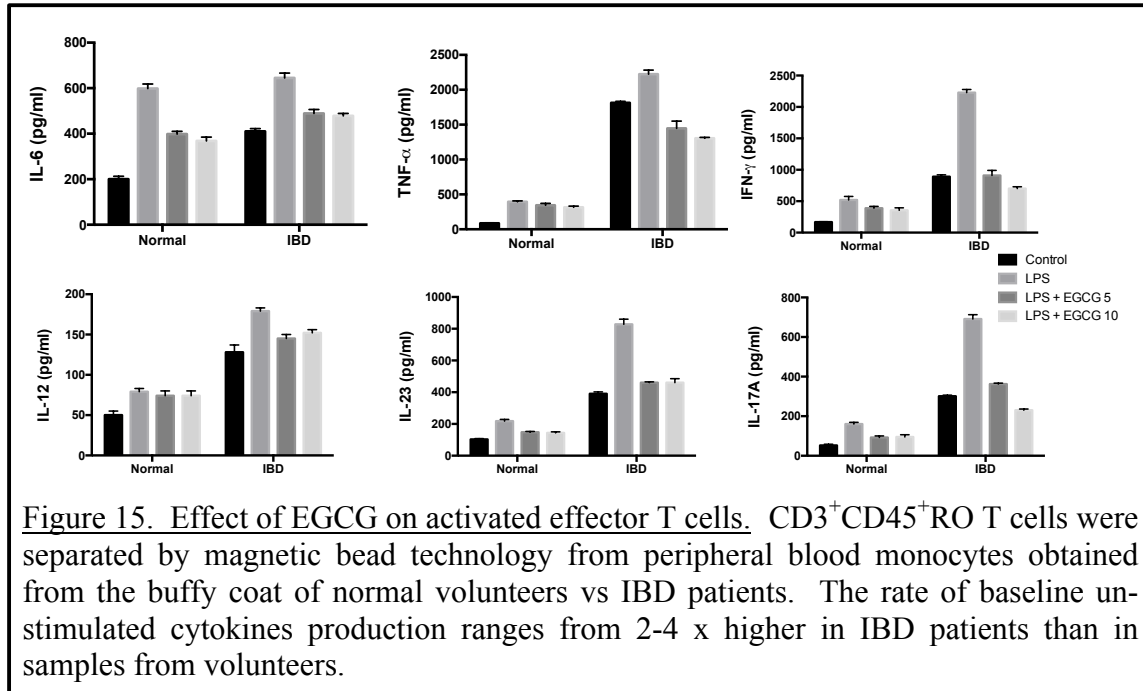
The resting pattern of cytokine secretion from normal volunteer and IBD patient

effector T lymphocytes exhibited a pattern similar to that of serum and isolated $CD14^+$

monocytes. Interestingly, IL-6 secretion from normal samples were 50% of the baseline

from IBD patients. This is the lowest ratio of normal to IBD resting cytokine secretion seen

among the tested cytokines. As in the other examples, stimulation of the cells produced a

much greater rise when comparing IBD patients to normal patients. The only exception was

IL-6 secretion, where stimulated RO T cells from normal samples secreted as much IL-6 as

those from IBD samples. This suggests that T cells do not acquire a "primed" state for IL-6

as they do for other inflammatory cytokines. These data also demonstrate that EGCG produces a dose dependent reduction for a variety of inflammatory cytokines from activated effector T cells. This should prove to be beneficial for control of inflammatory bowel disease, as infiltrating T cells provide such a large contribution to the chronic inflammation of IBD.

4.2.2.3 SUPPRESSION OF MONOCYTE INFLAMMATORY CYTOKINE TRANSCRIPTS BY EGCG AS DETERMINED BY RT-PCR

In order to ensure that loss of cytokine production was not simply due to inactivity of cells or loss of cells due to apoptosis under culture conditions, we evaluated the production of mRNA transcripts by RT-PCR. Although mRNA was performed for all analyzed cytokines, the results for the prototypical type 1 cytokines have been presented below in figure 16.

Figure 16. Effects of EGCG on selected cytokine message from stimulated CD14$^+$ monocytes. Stimulated CD14$^+$ monocytes were tested for the expression of (A) IL-1β, (B) TNF-α, and (C) IFN-γ pro-inflammatory cytokine messages after exposure to EGCG (0-10 μg/ml). Data are expressed as mean $\pm$ SD of four independent experiments. ****$P<0.0001$; ***$P<0.001$; **$P<0.01$; NS = not significant. No change was seen in RNA expression of these three TJ cytokines when CD4$^+$CD45$^+$RA T cells were evaluated (data not shown).

Cytokine suppression by EGCG was confirmed by RT-PCR on cDNA from cell pellets isolated after culture of isolated immune cells (figure 16). In the case of all three cytokines examined, the strongest suppression on mRNA message was seen in immune cells from stimulated IBD subjects. In most cases, there were no significant differences between EGCG 5 or 10 µg/ml doses, while the mRNA transcripts were sometimes numerically greater at higher doses of EGCG. This did not correspond to cytokine production in supernatant, suggesting that some cytokine production in response to LPS may have been release of preformed intracellular sources. This would especially apply to IL-1β, a cytokine that is made as a pro-cytokine and relies on caspase-1 cleavage to achieve its secretory form (figure 3).

4.3 EFFECT OF EGCG ON TRANSCRIPTION FACTORS IMPORTANT TO THE GENERATION OF EPITHELIAL INITIATION OF INNATE IMMUNE RESPONSE

The LPS induced upregulation of cytokine production is generally related to the activation of various signaling pathways associated with TLR-2 or TLR-4 activation in the case of Th1 cytokines. LPS activated CD14^{+} monocytes can also stimulate T cell activation and even Th17 differentiation within inflamed intestinal tissues (Bogunovic, Ginhoux et al. 2009, Shaw, Kamada et al. 2012). Intestinal epithelial cells also possess the ability to differentiate between pathogenic and commensal bacterial antigens and tailor cytokine secretion based on environmental cues (Campeau, Salim et al. 2012). Furthermore, many sources from the oncology literature have documented the ability of EGCG to influence a large number of signaling and cell differentiation pathways. Since the intestinal epithelial cell is really the first cell line in the body to interact with EGCG,

we decided to evaluate the impact of EGCG on intestinal epithelial cytokine secretion and cell signaling pathways that are important for promoting tolerance in the intestinal immune response. IECs secrete a variety of cytokines (Li, Seth et al. 1998, Lyu and Park 2009). Given reports that EGCG suppresses STAT3 and enhances suppressor of cytokine signaling (SOCS) 3 (Sharma, Shankar et al. 2014, Yang, Lee et al. 2014), we chose to evaluate its effects this important cytokine regulatory pathway in Caco2 cells.

Table 4. Effects of EGCG on selected signaling pathways.
Cytoplasmic Extract of Caco-2

Transcription Factor	STAT3	ROR-γt	SOCS3
No LPS	100.00	100.00	100.00
(1) Hour Incubation			
+ LPS only (10ng/mL)	247.17±6.5	175.06±5.3	102.34±3.4
+ LPS & EGCG (5μg/mL)	106.30±8.2	95.69±6.2	115.34±6.7
+ LPS & EGCG (10μg/mL)	104.56±1.5	102.78±1.8	121.56±11.6
(2) Hour Incubation			
+ LPS only (10ng/mL)	325.33±1.7	183.84±5.7	119.45±6.9
+ LPS & EGCG (5μg/mL)	294.49±5.9	146.89±6.3	132.67±7.0
+ LPS & EGCG (10μg/mL)	282.56±4.4	138.29±6.6	140.48±4.71
(4) Hour Incubation			
+ LPS only (10ng/mL)	237.94±5.4	188.45±3.6	125.89±5.6
+ LPS & EGCG (5μg/mL)	135.37±6.7	138.67±4.6	144.47±4.9
+ LPS & EGCG (10μg/mL)	133.06± 4.8	142.78±6.3	148.56±73.5

Nuclear Extract of Caco-2

Transcriptional Factor	STAT3	ROR-γt	SOCS3
No LPS	100.00%	100.00%	100.00%
(1) Hour Incubation			
+ LPS only (10ng/mL)	123.53±3.4	111.26±4.7	140.87±5.2
+ LPS & EGCG (5μg/mL)	127.75±5.6	39.13±5.1	138.98±4.3
+ LPS & EGCG (10μg/mL)	125.89±6.2	38.78±2.3	136.56±2.4
(2) Hour Incubation			
+ LPS only (10ng/mL)	129.09±5.4	116.77±5.7	151.56±6.8
+ LPS & EGCG (5μg/mL)	116.80±3.7	37.34±6.3	155.45±6.9
+ LPS & EGCG (10μg/mL)	112.67±5.6	36.89±6.6	158.89±4.6
(4) Hour Incubation			
+ LPS only (10ng/mL)	139.45±5.4	125.89±3.6	148.45±5.5
+ LPS & EGCG (5μg/mL)	109.89±6.7	34.67±4.6	152.56±4.3
+ LPS & EGCG (10μg/mL)	103.00± 8.2	35.78±6.3	151.78±7.3

It has been well documented that IL-6 and IL-23 are common activators of STAT3 (Takeda, Kaisho et al. 1998, Cho, Kang et al. 2006). STAT3 then activates RORγt, an integral step in Th17 polarization of naïve T cells undergoing immune activation (Yang, Panopoulos et al. 2007). As seen in the table above, during our investigation into the signaling processes in Caco2 cells, EGCG substantially affected STAT3. Though STAT3 signaling is typically ascribed to IL-6, the administration of LPS to our Caco2 cultures at 80% confluence initiated cytokine secretion and STAT3 activation, but at 100% confluence, no STAT3 activation occurred in response to LPS stimulation (data not shown). STAT3 signaling was attenuated moderately in the cytoplasm, both suppressing as well as prolonging the time to maximal activation. EGCG had a mild attenuating effect on STAT3 translocation to the nucleus. The largest impact, however, occurred on RORγt. The cytoplasmic levels of RORγt decreased by approximately 50%, while the nuclear translocation of RORγt decreased by over 70% compared to non-treated cells. Our findings also correlated with others that EGCG administration modestly enhanced the upregulation of SOCS3.

4.3.1 CYTOKINE SECRETION BY IECs, AN IMPORTANT ORGAN OF THE INTESTINAL IMMUNE RESPONSE

The intestinal epithelium cell is a multifunctional workhorse capable of maintaining a barrier protecting the sensitive immune cells located below the barrier in the lamina propria as well as influencing the immune system to promote pathogen destruction while avoiding uncontrolled inflammation. IECs have been shown to secrete a variety of cytokines, chemokines and immune regulatory proteins such as thymic

stromal lymphopoietin (TSLP) (Rescigno 2014). IL-17, IL-23 secretion induced by LPS in human colon cancer cell lines HT29 and T84 (Paolillo, Carratelli et al. 2009, Ghadimi, Helwig et al. 2012), while IL-1α, IL-6, MCP-1, IL-23 secreted by Caco-2 cell line (Mascia, Maina et al. 2010). In all instances, LPS provides a strong trigger for signaling cytokine release, initiating secretion of greater amounts of cytokines than co-culture model, with basolateral exposure leading to higher levels than apical (Ghadimi, Helwig et al. 2012). We embarked on a survey of the type 1 and type 2 cytokines that could be produced by Caco-2 cells, since primary human epithelial cells pose several difficulties for long-term culture that we used to investigate certain aspects of cell signaling during time course experiments.

Figure 17. Survey of cytokines produced by Caco2 cells after LPS exposure The effects of two concentrations of EGCG were evaluated in this study. Cells were exposed to 10 ng/ml LPS or cultured in medium alone for three hours. EGCG was added at various concentrations for an overnight exposure.
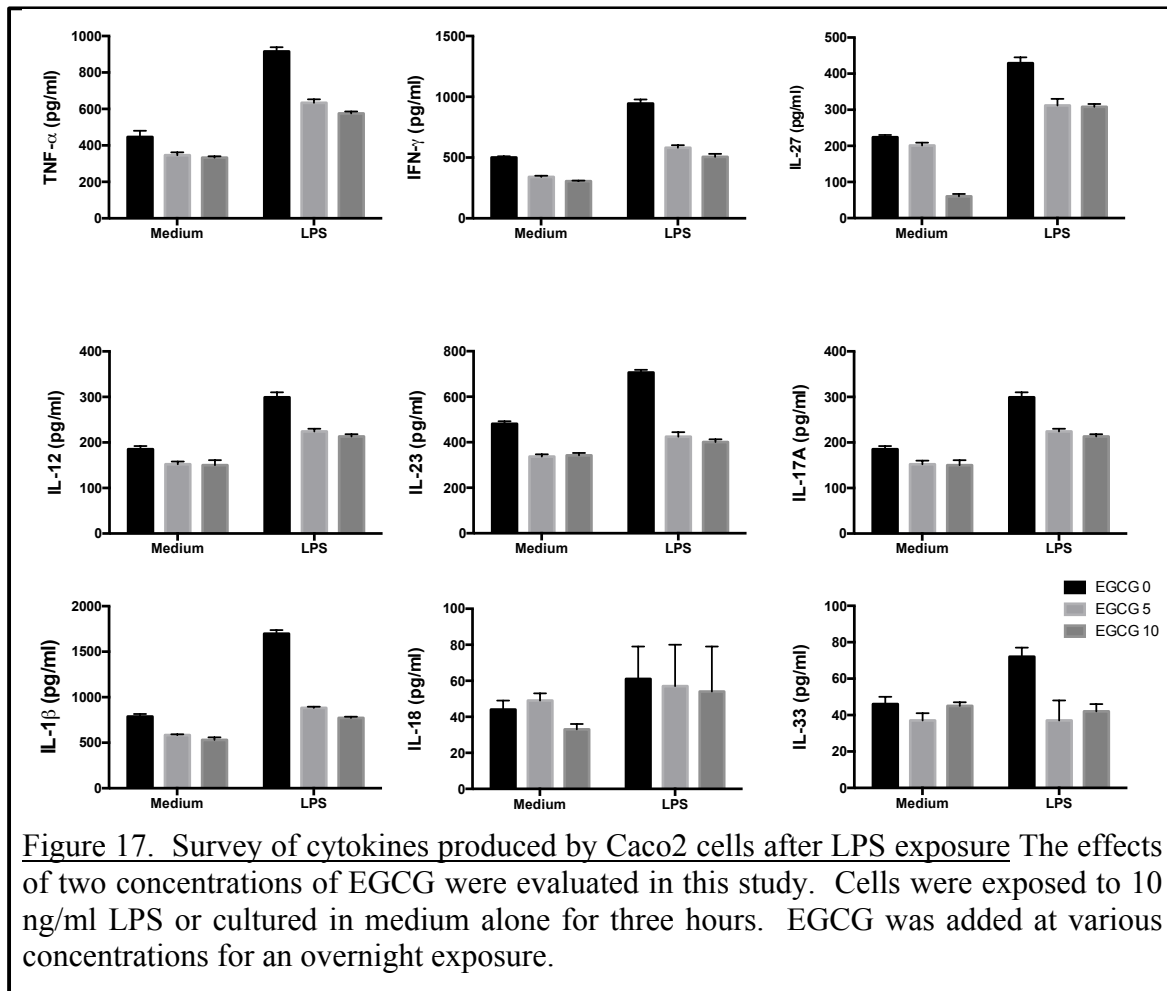
As seen in the figure above (figure 17), a variety of cytokines are produced by Caco2 cells at baseline. Significant upregulation of several cytokines occurred after exposure to LPS. EGCG typically suppressed the cytokine secretion down to basal rates, or even below unstimulated levels in the case of IL-33. These findings suggest that EGCG provides a broad-based suppressive effect on intestinal epithelial cell cytokine secretion. In most cases, the mRNA transcripts mirrored cytokine protein production (data not shown).

4.3.3 EGCG SUPPRESSION OF PRE-FORMED, PRO-CYTOKINE SECRETION REQUIRES EXPOSURE TO LPS

Data from our current work and others suggest that EGCG can suppress NF-κB induced cytokine transcripts dramatically, supporting the theory that EGCG is exerting an effect on inflammation by inhibiting the nuclear signaling effects of NF-κB. A suppressive effect of EGCG also occurs in the case of IL-1β, but the degree of suppression cannot be accounted for only by NF-κB inhibition. Interestingly, the cells from colitis subjects experienced significantly higher rates of reduction from baseline than unstimulated cells. IL-1β contained in the cytoplasm is not readily secreted into the surrounding environment, due to the fact that it is translated into a pro-form of IL-1β that requires cleavage to its active form prior to secretion (Lopez-Castejon and Brough 2011). So, relatively little IL-1β is secreted unless stimulatory signals are received through the binding of IL-1β to its cognate IL-1receptor with or without ATP, or through TLR signaling. Downstream signaling molecules lead to inflammasome activation and subsequent cleavage of pro-IL1-β to its active form (Lamkanfi, Kanneganti et al. 2007).

The inflammasome is a multiunit construct that comes together when an appropriate intracellular signaling mechanism is activated (Hsu, Ali et al. 2008). This leads to Caspase-1 activation. Caspase-1 cleaves pro-IL-1β, pro-IL-18, and pro-IL-33. Based on that fact, we decided to evaluate the effects of EGCG on the three pro-cytokines to see if there was a class effect against them.

Figure 18. Three pro-cytokines need exposure to LPS prior to secretion. In order to activate caspase-1, LPS must be added to the medium. LPS activates inflammasome dependent cleavage of the pro-cytokines into an active form for secretion. Cells were incubated with EGCG overnight, then exposed to LPS for three hours prior to collecting supernatants.
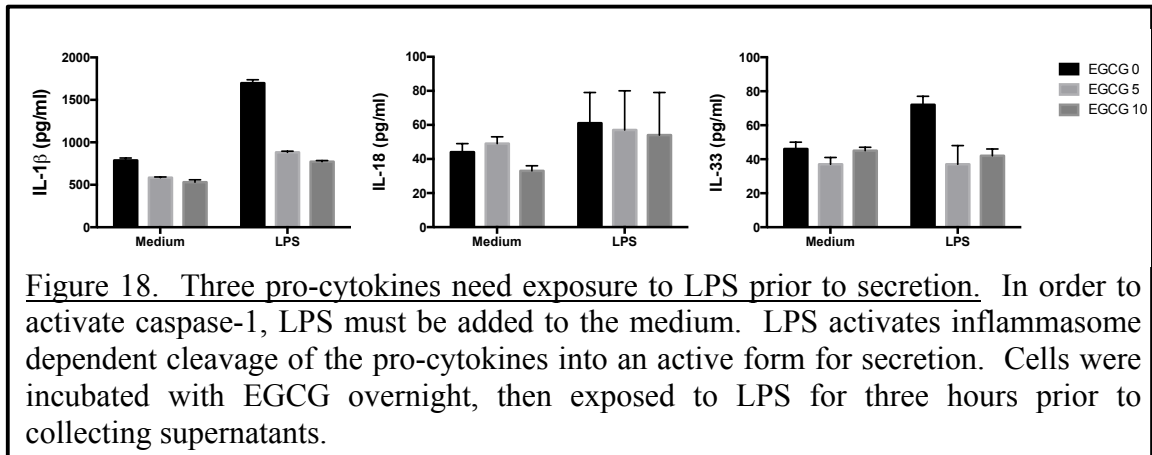
The data detailed in figure 18 demonstrates that EGCG likely provides a dual mechanism for suppressing inflammatory cytokine secretion. Based on the effects on IFN-γ, TNF-α, IL-12 and -23, EGCG is conferring a suppressive effect on NF-κB mediated cytokine modulation. This could also be providing an effect on pro-IL-1β production as well, but the suppression seen in figure 18 likely represents a direct suppression of caspase-1 mediated pro-cytokine cleavage under the effects of the inflammasome. The culmination of these effects should play well with desire to treat IBD patients with an effective anti-inflammatory agent that works well at the microbial/intestinal interface.

4.4 DISCUSSION

Experimental evidence demonstrates that EGCG effectively suppresses intestinal epithelial cell secretion of cytokines. Likely happens due to inhibition of signaling factors as in table 4, but also appears to inhibit cleavage of pro-form into active form via caspase-1 activation as seen in figure 18. We continue to explore the direct activity on caspase-1 as part of our ongoing efforts to characterize the immune effects of EGCG on IBD. Again, the potential impact of a luminally active agent is relatively uncharacterized. While most developments in the field of IBD therapeutics deal with systemic treatments to suppress the systemic immune system, with hopes of creating the desired anti-inflammatory effect on the intestinal inflammation associated with IBD, the promise of effective luminal therapy is great.

Clinical trial endpoints generally examine the change in clinical parameters over time based on the response to treatment or placebo. Now, many new trials have begun to embrace the concept of mucosal healing to evaluate the effect of newer, more focused therapies for IBD by including a measurement of endoscopic disease severity before and after treatment. Again, this attempts to answer the question of whether bystander healing of the mucosa occurs as the systemic immune effect of the medication takes effect, but unfortunately, methods for addressing the impact of therapy on the barrier function are essentially non-existent. This is problematic in many ways, as the adaptive immune response has become almost the sole focus for explaining the pathophysiology of IBD, but not all patient symptoms can be explained by infiltrations of adaptive T cells and loss of regulatory T cells (Seiderer, Elben et al. 2008). Infiltrative reactions by the adaptive immune compartment generally take days to weeks to initiate and resolve, which does not

exactly mirror the pattern of complaints voiced by IBD patients. Rather, patients often complain of short-lived "flares" that are most often attributed to irritable bowel syndrome, but this gives the patient two diagnoses rather than just one. While this may sometimes be justified, if we look beyond the common definition of a traditional IBD "flare" consisting of a full-blown adaptive immune response, we should consider the possibility that the intermittent symptoms may be occurring due to less catastrophic changes occurring in the intestinal epithelial cells. Evidence for this concept can be found, to a small extent, in the analysis of tight junction proteins from tissue taken from the distal colon of mice from the DSS colitis experiment. By Western blot analysis of those proteins, it appears that EGCG significantly alters the constituent components of the tight junctions in a manner that has important ramifications for ion and water transport across the epithelial barrier. The main clinical impact of changing ion transport across the intestinal epithelium into the lumen would be to alter diarrheal symptoms. Diarrhea is one of the most prominent symptoms in both ulcerative colitis and Crohn's disease. Current anti-diarrheal therapies do not target the source of diarrhea (ion flux and subsequent water movement into the lumen), they only mask the symptoms by slowing motility through their effects on intestinal opioid receptors (Holzer 2014). Indeed, anti-diarrheal therapies often result in uncomfortable constipation and can occasionally induce more serious complications such as toxic megacolon (Strong 2010). A more holistic approach would involve the administration of substances that actually manage immune responses of the intestinal barrier and address the root cause of the diarrhea problem: that is, manage the rapidly inducible fluctuations in tight junction proteins that cause short-term changes in ion and water movement across a "semi"-permeable barrier while

suppressing inflammation from contact with bacterial antigens. Based on the findings from our evaluation of the DSS model of colitis, we next turned our attention to how EGCG affected the barrier function of the intestinal epithelium by performing a number of illuminating experiments using intestinal epithelial cell lines.

CHAPTER 5

EFFECT OF EGCG ON TIGHT JUNCTION COMPOSITION AND FUNCTION

5.1 INTRODUCTION

Certain clinical data suggest that a primary barrier defect may be present in IBD patients and their first-degree relatives (Hollander, Vadheim et al. 1986), while other reports conclude differently (Teahon, Smethurst et al. 1992, Munkholm, Langholz et al. 1994). In either event, the origin of that potential barrier defect or the actual barrier problems observed in active IBD remains imprecisely defined. The inciting event could be caused by a primary barrier etiology (such as those seen in pathogenic infections or non-steroidal anti-inflammatory induced injury), or it could be due to low-grade inflammation from infiltrating immune cells. When it comes to evaluating the status of TJ protein contribution to IBD pathophysiology, the story becomes even more confusing. Insight into the various roles that tight junctions play in intestinal barrier function continues to evolve. Two methods exist for transporting material across the epithelial barrier: either around or through the cells making up the border of the intestine (Anderson and Van Itallie 2009). The intestinal border consists of a variety of specialized cells, but the vast majority of the surface is covered by enterocytes bound tightly together by a multitude of interwoven strands of TJ proteins (Schneeberger and Lynch 2004). Originally felt to be inert solute barriers, our understanding now points to

the fact that the TJ composition is controlled by a highly complex regulatory system that causes rapid changes in TJ elements based on environmental conditions. IECs take cues from both the basolateral interface with the luminal environment, as well as, the apical interface with the lamina propria.

As the largest absorptive surface area of the body, the chief role of the intestinal epithelium involves moving nutrients across the barrier from the lumen to the inner layer. This process is most important in the proximal gut, which also carries the lowest burden of intestinal bacteria. Here, the absorptive process often relies on carrier proteins for bulk movement of specific nutrients (Rao, Sarathy et al. 2015). The lower digestive tract carries out relatively little absorption compared to the upper tract, but must cope with the largest exposure to commensal bacteria. Two separate pathways exist for the transport of materials across the epithelial barrier. The first consisting of a charge selective system of pores up to 4 Å in diameter, the second involving larger discontinuities in the membrane that are indifferent to charge status (Anderson and Van Itallie 2009). Paracellular ion movement is determined by the claudin composition of the epithelial cells, which varies over the length of the digestive tract (Anderson and Van Itallie 2009). Transcellular transport generally requires carrier proteins, although specialized epithelial cells such as M cells, permit wholesale transport of large antigen particles via tunnels that facilitate transcellular extension of dendrites from follicular-associated epithelial dendritic cells (Lelouard, Fallet et al.). The relative movement of ions across an epithelial barrier depends on the resistance of the barrier, as diagramed in figure 19. Changes in barrier permeability are dictated by resistors (which represent claudins in the actual barrier).

Figure 19. Model of Transepithelial Resistance. Representation of the epithelial barrier function modeled after an electrical circuit. Author unknown.
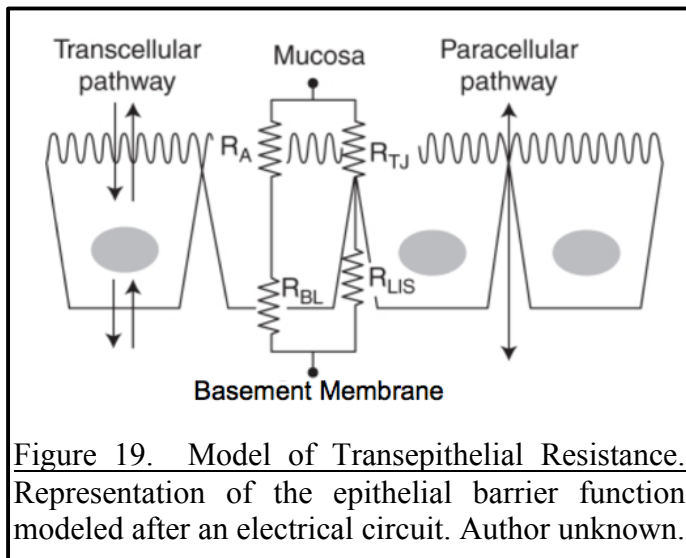
Several cell lines exist for investigating how TJ respond to various stimuli, such as cytokines or bacterial/food antigens. One of the most frequently studied is the Caco-2 cell line. Given the fact that the Caco-2 cell line is derived from a human colon cancer, it is not surprising to learn that the electrical resistance resembles that of the distal colon (Van Itallie and Anderson). This resistive property provides a firm foundation for developing a robust model for evaluating the effects of therapeutics on the TJ's contribution to intestinal physiology.

The relative contribution of individual claudins to barrier resistance (and permeability, to a certain extent) has been established by knocking out or establishing a constitutively active form of the various claudin genes. For instance, the importance of claudin 1 as a contributor to maintaining barrier integrity can be inferred based on a hypothesis developed by Furuse after observing that claudin 1 deficient mice die of dehydration after birth (Furuse, Hata et al. 2002) and confirmed when experiments overexpressing claudin 1 led to increased TER (Inai, Kobayashi J Fau - Shibata et al. ,

McCarthy, Francis et al. 2000). Furthermore, expression of claudin 2 in cultured cell lines selectively alters permeability for solutes smaller than 4 Å, while the pores formed by claudins 4 and 18 pores allow for passage of larger molecules without sensitivity for charge (Van Itallie, Holmes et al. 2008). These contrasting discriminant features create incongruencies when interpreting results, as similar pores in different membrane settings may discriminate differently on similar electrical charges, or monomeric expression of certain "tightening" claudin blocks ion passage based on charge selectivity, while co-expression of that same claudin with a different claudin leads to diametrically opposite permeability to solutes (causing a diarrheal state in the intestine) while the ion permeability remains low (still possesses a high TER).

The juxtaposition of size versus charge may be readily sorted out in the lab, where precise manipulation of cell lines allows for accurate measurement of physiologic outcomes, but physical realities can promote confusion in the clinical setting when interpreting the role of TJ proteins in health or inflammatory bowel disease. For instance, some investigators found evidence for the upregulation of claudin 1 (a pore sealing claudin) in some patients with IBD, while others showed no change or even down-regulation in their patients with IBD (Kucharzik, Walsh et al. 2001, Zeissig, Bürgel et al. 2007, Weber, Nalle et al. 2008). On the other hand, claudin 2 is regularly associated with paracellular leakiness and has frequently been found to be upregulated in clinically active Crohn's disease but not in normal controls (Zeissig, Bürgel et al. 2007). However, claudins 3 and 8 have shown variably decreases in Crohn's disease compared to controls, while claudin 4 did not change (Zeissig, Bürgel et al. 2007). Others have confirmed

elevated expression of claudin 2 in inflamed colonic epithelium, but diminished levels or topographical redistribution of claudin 4 (Prasad, Mingrino et al. 2005). One reason for the confusion lies in the fact that a consistency in baseline conditions can be difficult to establish. Due to the risk, cost and inconvenience of endoscopy in humans, the basis for the permeability changes shared between IBD patients and their first degree relatives mentioned previously (Hollander, Vadheim et al. 1986)has never been identified. Second, the claudin composition of normal intestine versus inflamed intestine differs within the same individual, depending on the proximity of the biopsy to the inflamed mucosa, relative location in the bowel, or even chronological stage of the disease, making precise determinations difficult and generalizations more common. Third, the alterations in claudin composition may vary depending on the etiology of the inflammation (Heller, Florian et al. 2005, Prasad, Mingrino et al. 2005).

Little is known regarding the effects of EGCG on claudin physiology. EGCG has been shown to not change claudin 1 expression in a liver cell line (Ciesek, von Hahn et al. 2011), while it reduced inflammatory cytokine-based changes to claudin 5 and occludin in cultured vascular endothelial cells (Li, Ye et al. 2012). Based on the fact that EGCG is readily taken up by IECs when administered orally, and that it has been shown to reduce secretion of inflammatory cytokines associated with barrier disruption, we sought to evaluate the effects of EGCG on epithelial integrity and key tight junctions associated with the pathophysiology of IBD.

5.2 RESULTS

5.2.1 EFFECT OF EGCG ON EPITHELIAL BARRIER FUNCTION AFTER LPS CHALLENGE

In order to determine whether EGCG could effectively restore TJ composition after LPS exposure, Caco2 cells were co-cultured with two concentrations of EGCG, both in the presence or absence of LPS exposure. TJ composition plays a role in maintaining epithelial barrier function. As TJ relax or open in response to changes in TJ claudin composition, electrical resistance goes down due to increased ion flux across the epithelial barrier. After TJ homeostasis is restored, electrical resistance rises.

Figure 20. Effect of LPS and EGCG on Caco2 model of epithelial barrier. Caco2 cells were cultured for TER testing with two doses of EGCG). Data are expressed as a ratio of TER experimental condition to TER control values for each time point. The results shown are representative of data obtained from three independent experiments. **$P<0.01$; *$P<0.05$.
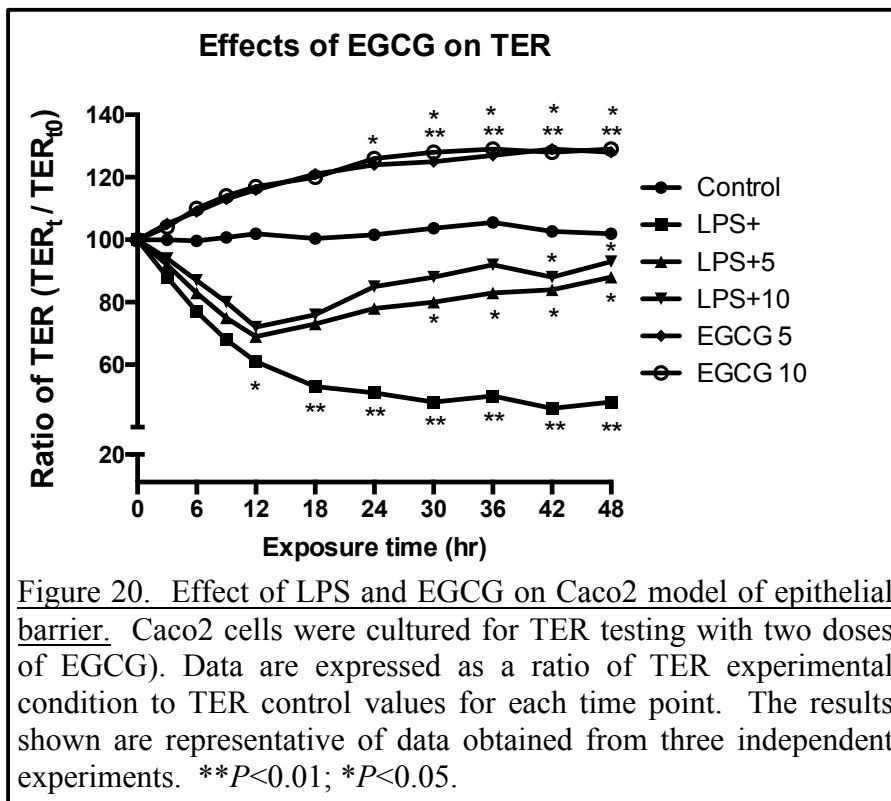
Figure 20 demonstrates that trans-epithelial resistance dropped significantly from baseline within 12 hours of exposure to LPS, with no evidence of improvement by 24 hours. However, the reduction in TER was abrogated by the addition of EGCG. The highest dose of EGCG (10 μg/ml) provided the most robust protection against LPS-

induced degradation of TER, returning levels to near baseline within 18-24 hours after exposing Caco2 cell monolayers to LPS and EGCG. Interestingly, the presence of EGCG in culture medium in cells not exposed to LPS actually caused a rise in TER above the baseline of control Caco2 cells.

5.2.3 EFFECT OF EGCG ON TJ PROTEIN PRODUCTION AT BASELINE AND AFTER STIMULATION WITH LPS

Based on the dramatic changes in TER that were seen in Figure 20, an understanding of the relative composition of TJ proteins expressed in those cells was sought. In order to accomplish this task, semi-quantitative analysis of the individual TJ of interest was performed by Western blot analysis.

LPS (10ng/ml)	Pre	-	-	Pre	Pre	Post	Post
EGCG (µg/ml)	0	5	10	5	10	5	10

Figure 21. EGCG affects TJ protein expression in Caco2 cells. (A, B) 10 mcg protein/lane exposed from 0.5-20 sec. Densitometry analyzed with Image Lab 5.0. Data expressed as a ratio of TER exp. to TER ctrl for each time point. Represents 3 experiments. **P<0.01 *P<0.05. (C) RT-PCR for selected claudins.
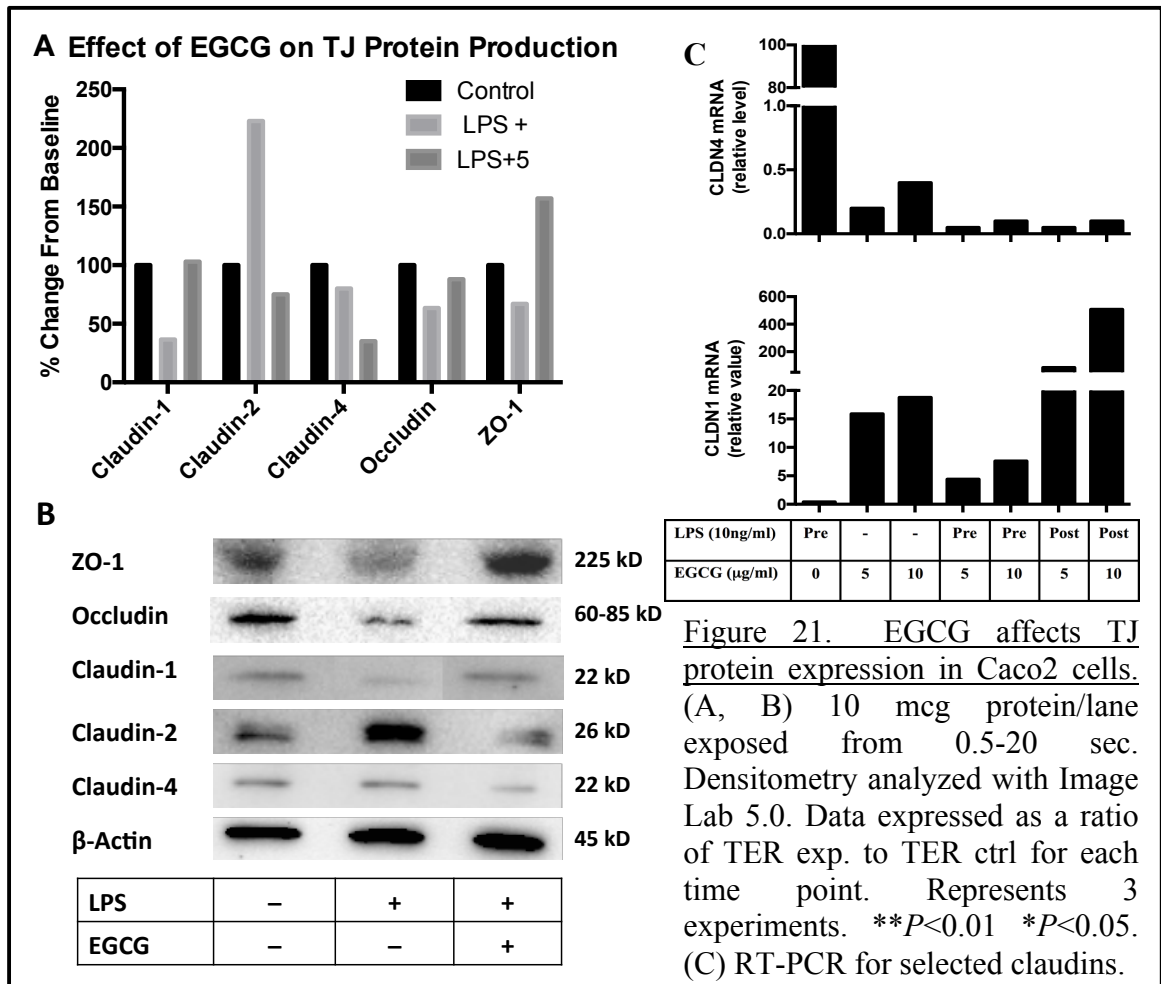
Caco2 cultures exhibited significant differences in the relative production of specific TJ proteins after LPS exposure (figure 21). The most significant change was noted with claudin 2, a claudin found to be upregulated during acute colitis (Lu, Ding et al. 2013). Claudin 2 protein levels increased in our model by more than 200% after exposure to LPS. Co-incubation with EGCG 5 μg/ml actually reduced claudin 2 levels by 25% below the basal production of un-stimulated cells. Claudin 4, a pore-tightening TJ protein associated with inflammation did not appear to be affected by LPS on a protein level, but message expression increased 130 fold by RT-PCR. Upon exposure to EGCG, claudin 4 message was completely suppressed and could not be rescued by post-exposure to LPS after an initial exposure to EGCG, and protein levels fell to 35% of basal production when cells were incubated with both LPS and EGCG. Levels of claudin 1, a pore tightening claudin, dropped by over 60% from basal levels in response to LPS exposure, but returned to baseline upon adding EGCG to culture medium. By RT-PCR, message for claudin 1 increased by 16-18 fold from EGCG exposure alone, but most intriguing was what happened after pre-incubating Caco-2 cells with EGCG <u>then</u> LPS. Claudin 1 levels skyrocketed by 508 fold according to RT-PCR. Finally, occludin, a barrier enhancing TJ protein, also decreased in response to LPS but was rescued by EGCG. LPS impacted the TJ protein ZO-1 the least, with approximately a 33% reduction from baseline protein levels in the control samples. However, incubation with EGCG increased ZO-1 protein levels by over 50% from baseline. This variable impact in subsets of TJ proteins makes sense in light of the unique role each component plays in the function of a tight junction. ZO-1 forms the foundation for TJ construction, acting as a scaffold protein upon which the tight junction is built, while the accessory components

(claudins and occludins) fluctuate in response to the local environmental or physiologic needs of the barrier function. Although the relative protein levels fluctuate dramatically in some cases, the importance of these changes to physiological function can only be measured by functional assays. Combined with data from the TER experiment, it appears that the "tightening" protein claudin 1 plays a large role in augmenting barrier resistance after EGCG exposure, while claudin 2, a "leaky" protein, is suppressed by the administration of EGCG. EGCG's suppression of claudin 4, according to our Western blot and RT-PCR data, bring into question its role as a tightening claudin. How can rising levels of a "tightening" claudin in the setting of an increasingly "leaky" barrier in response to LPS be interpreted, especially when the "tightening" of that barrier in the setting of EGCG occurs without a rise in claudin 4? The answer lies in part with the concept of relative contributions from TJ proteins, as supported by additional experiments showing changes in relative abundance and claudin distribution. In this set of experiments, Caco-2 cells were exposed to LPS for 3 hours, washed, then incubated with or without EGCG at clinically relevant doses of 5 and 10 μg/ml. Cells were then washed with fresh medium and prepared for analysis, as reported in Chapter 2.

5.2.6 BARRIER PERMEABILITY CHANGES LIKELY RELATED TO RELATIVE CLAUDIN EXPRESSION AND DISTRIBUTION

The expression of claudin 2 itself, which rose 225 fold after LPS exposure, sufficiently alters epithelial monolayer porosity to allow net flux of positive sodium ions, while prohibiting passage of larger molecules such as lactulose or mannitol (Amasheh, Meiri et al. 2002). Simultaneous with the rise in claudin 2, LPS increased claudin 8

levels by 6 fold. This change in relative concentration of claudins 4 and 8 change helps explain how a "tightening" claudin (that is, claudin 4) can become more plentiful while the barrier resistance drops. Others have shown that when claudin 4 co-expresses with claudin 8, the combination of claudins forms a pore for negatively charged chloride ions (Hou, Renigunta et al. 2010). The movement of chloride ions into the lumen promotes diarrhea, such as that occurring after cholera toxin induced calcium activated (CLCA) chloride channel cyclic AMP mediated opening of the cystic fibrosis transmembrane conductance regulator (CFTR) channel, which have been harnessed pharmacologically to treat chronic idiopathic constipation (Hodges and Gill 2010). The pattern of claudin 4/8 co-expression found in our experiment with Caco-2 cells helps explain the paradoxical drop in electrical resistance seen after LPS exposure. Furthermore, the dramatic rise in claudin 4 mRNA transcripts after administration of LPS was fully suppressed by EGCG, and LPS could not elevate claudin 4 after exposure to <u>either</u> concentration of EGCG. Claudin 1 protein levels, on the other hand, were initially supressed by LPS, but either pre- or post-LPS exposure to EGCG enhanced claudin 1 message levels enormously.

On the other hand, LPS appeared to have no effect on claudin 1 expression, while exposure to EGCG alone increased claudin 1 transcripts significantly in a dose dependent fashion. When Caco-2 cells were pretreated with LPS, claudin 1 transcripts were suppressed relative to EGCG exposure alone, but when cells were pretreated with EGCG followed by LPS, a synergistic, dose dependent surge of claudin 1 transcripts followed. This finding is likely explained by effects of EGCG on HIF1a stabilization. Increased levels of HIF1α appear to be related to HIF1α protein stabilization in response to mir201,

an important microRNA induced by EGCG. The CLDN1 promoter has been shown to be a prominent target for HIF1α binding by ChIP-on-chip analysis (Saeedi, Kao et al. 2015), has been shown to bind to the promoter for claudin 1 with subsequent rises in claudin 1 protein production. In order to gain a better understanding of this relationship, we have initiated an active line of investigation to determine possible clinical implications.

5.2.6 EGCG IMPARTS A DISPARATE EFFECT ON TJ PROTEINS AS DEMONSTRATED BY IMMUNOFLUORESCENCE OF HT-29 CELLS

Immunofluorescence can provide additional information about protein function, especially in situations where the physical location of proteins within the cell provide meaningful context regarding their physiological role. In the case of claudin proteins, impact on barrier resistance to ion passage requires insertion of the protein into the cell membrane, so that the extracellular loops of the protein can interact with claudin loops from neighboring cells for the creation of ion channels. In order to correlate the data derived from Western blot and RT-PCR of important pore forming claudins with the provocative TER data, immunofluorescence imaging of the most conspicuous proteins, claudins 1 and 4, was undertaken. HT-29 cells were grown under standard conditions and exposed to LPS with or without EGCG, depending on the parameters dictated by the arm of the experiment. In order to investigate the concept that EGCG could be used as a preventative agent on non-inflamed mucosa to prevent effects from bacterial antigens, cells in one experimental arm were exposed to EGCG prior to LPS, while the response to a "treatment effect" was examined by pre-conditioning cells with LPS for three hours

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prior to exposing them to EGCG. Supernatants were taken for cytokine analysis and cells

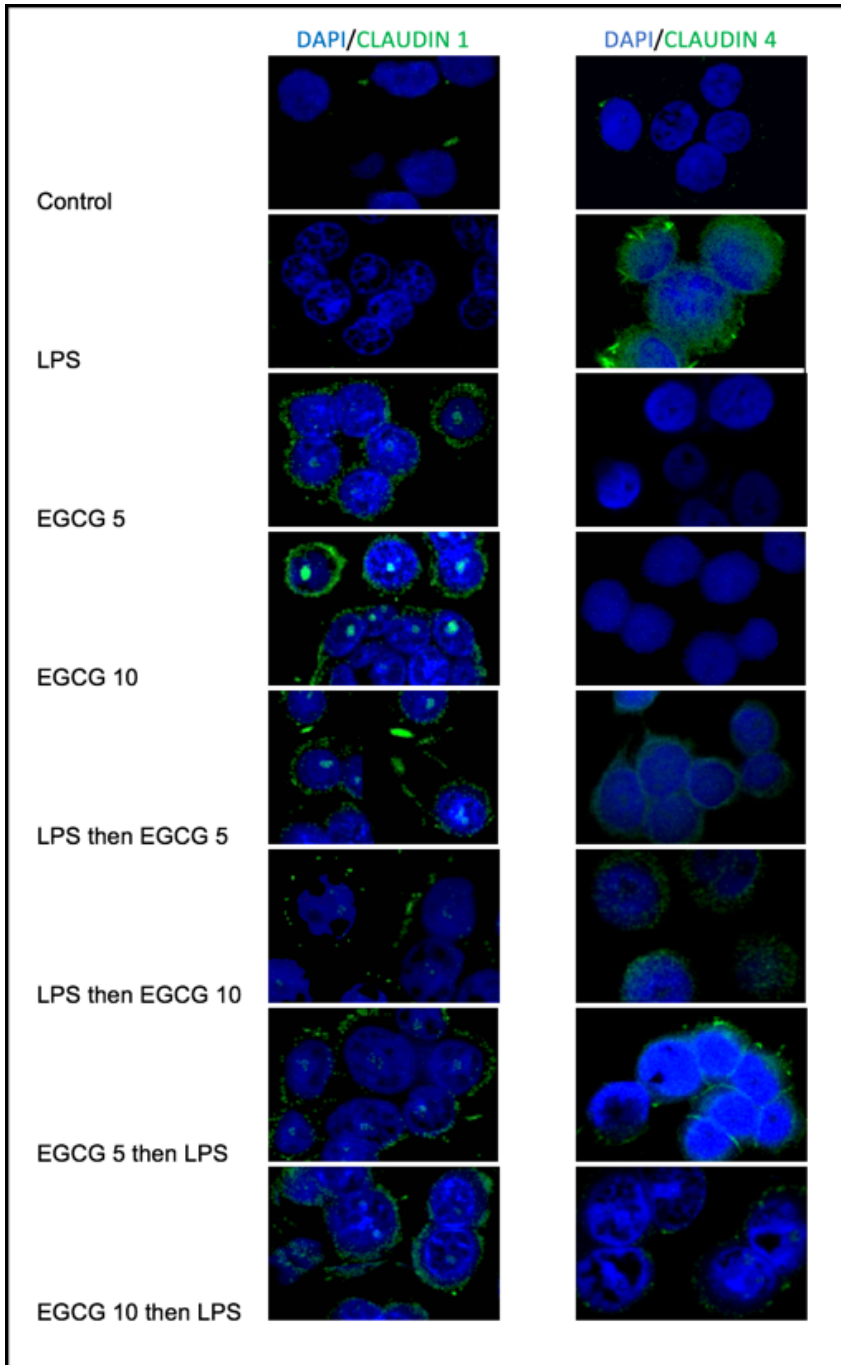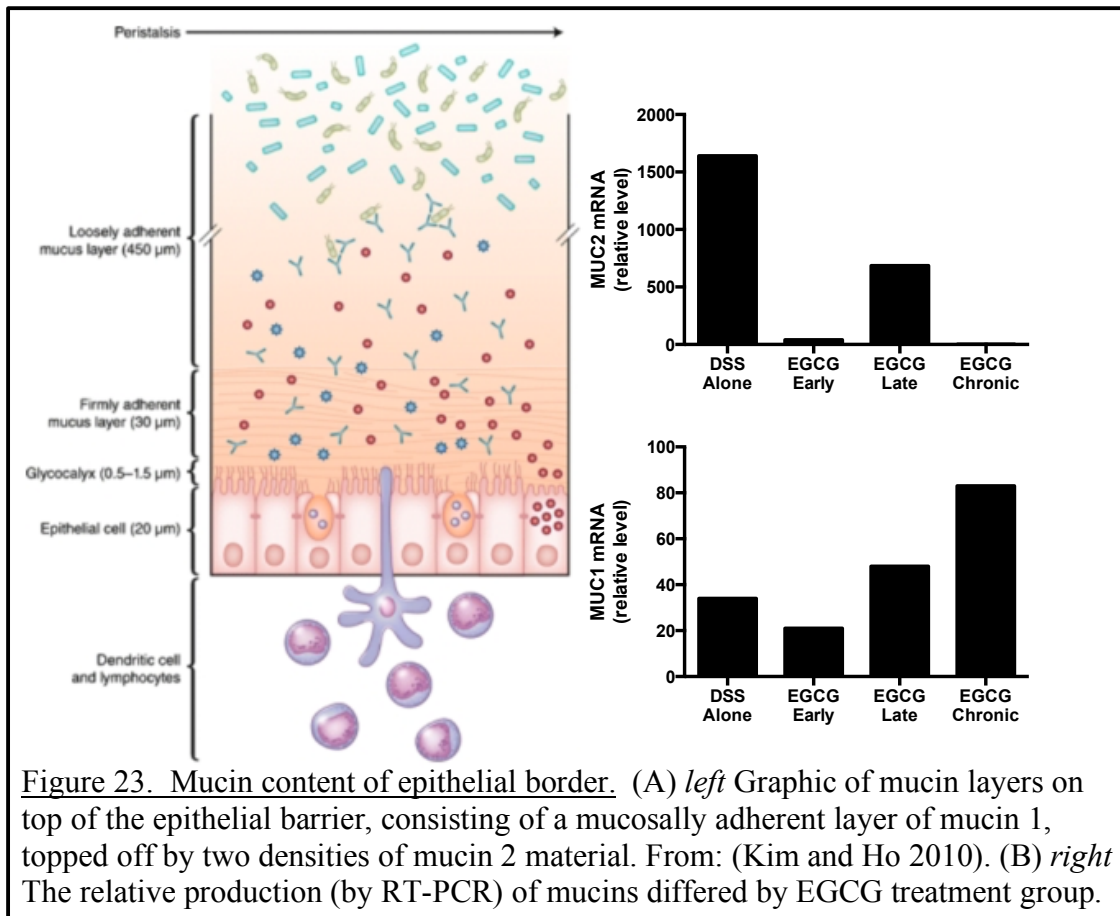were harvested for mRNA and protein isolation according to standard protocols.

Figure 22. Immunofluorescence of TJ proteins under experimental conditions. HT-29 cells were cultured with or without LPS, and with or without two doses of EGCG. Immunofluorescence for either claudin 1 or claudin 4 showed striking differences in both quantity of protein expression as well as location of protein.

Although technological considerations make quantification of fluorescence difficult and subsequent comparisons between conditions inaccurate, gross differences in appearance of the relative fluorescence intensity based on experimental conditions provide insight into the functional status of claudins 1 and 4 (figure 22). EGCG clearly has an antithetical effect on claudin production and distribution within the cellular architecture. In the presence of LPS, claudin 1 expression did not change, but in the presence of EGCG, claudin 1 expression increased and the resulting protein was distributed to the inner aspect of the cell membrane, where it can materially participate in sealing the gap between cells. On the other hand, claudin 4 mRNA and protein levels noticeably rose in response to LPS. Interestingly, claudin 4 staining revealed an even distribution of protein throughout the cytoplasm. The addition of EGCG by itself had no effect on claudin 4 message or protein, but the administration of EGCG after LPS appeared to not only diminish the amount of claudin 4 protein, but it also appeared to cause the claudin 4 to redistribute and concentrate around the nucleus. The functional ramifications of this rearrangement are not clear, but it speaks to the fact that claudin 4 can no longer participating in a sealing capacity at the cell membrane.

5.2.7 EGCG EFFECTS EXPRESSION OF BOTH Muc1 and Muc2 GENES IN THE DSS COLITIS MODEL

Mucins are ubiquitous proteins produced mainly by goblet cells contained in the epithelial layers lining the internal interfaces between the body and the environment. The most abundant mucin proteins produced in the gastrointestinal tract are encoded by MUC1 and MUC2. The protein products of these genes differ in structure and function. The protein encoded by MUC1 contains a transmembrane component that localizes the

protein to the apical membrane of the IEC, and may be involved in cell signaling, detection of cell-cell contact, or docking of signaling proteins for intracellular communication (Kim and Ho 2010). Mice deficient in Muc1 have increased susceptibility to certain infections and increased susceptibility to autoimmune disorders such as EAE (Kim and Ho 2010). Along those lines, MUC1 mucin has also been shown to negatively regulate the NLRP3 inflammasome through reduced interleukin-1 receptor-associated kinase (IRAK4)-dependent NF-κB activation, with the resulting suppression of inflammation providing a protective mechanism against lethality from *Helicobacter pylori* infection (Ng, Menheniott et al. 2015). On the other hand, the protein encoded by MUC2 is a gel-forming protein that constitutes the bulk of the intestinal mucin layer that creates the physical barrier between the IECs and the microbiome (Kim and Ho 2010). MUC2 mucin polymerizes to form a dense hydrogel that separates into two layers as seen in figure 23: a thinner, firmly adherent sterile layer adjacent to the IECs and a much thicker, loosely connected layer that acts as a trap for secreted antimicrobial peptides, antibodies and bacterial products (Kim and Ho 2010).

Figure 23. Mucin content of epithelial border. (A) *left* Graphic of mucin layers on top of the epithelial barrier, consisting of a mucosally adherent layer of mucin 1, topped off by two densities of mucin 2 material. From: (Kim and Ho 2010). (B) *right* The relative production (by RT-PCR) of mucins differed by EGCG treatment group.

We saw no changes in Muc1 expression with LPS exposure in Caco2 or HT-29 cell lines (data not shown), but we wanted to determine whether chronic administration of EGCG in the DSS model of colitis altered mucin gene expression over time. Although increased mucin levels from elevated Muc1 expression have been implicated in causing increased Th17 activity, instead of exacerbating epithelial injury, the downstream effect of enhanced mucin production ultimately acts to promote barrier restitution through the intensification of the restorative effects of IL-22 (Nishida, Lau et al. 2012). On the other hand, the intestinal epithelial cells of mice deficient in Muc2 undergo excessive exposure to antigens from both gram positive and gram negative bacteria leading to the development of massive inflammation and eventually colon cancer (Dohrman, Miyata et

al. 1998). In the setting of Muc2 deficiency, bacteria directly interface with the epithelial surface of the intestine of Muc2$^{-/-}$ animals (Johansson, Phillipson et al. 2008). Upregulation of Muc2 gene expression occurs diametrically in response to LPS, a TLR-4 agonist, as opposed to MDP, a TLR-2 agonist (Rodrigues, Slobbe et al. 2015). Indeed, mice treated with DSS alone demonstrated a massive upregulation of Muc2, and to a lesser extent, Muc1 (figure 23), which was blocked in animals treated with early EGCG (prior to the onset of inflammation). Instead of the robust DSS-triggered TLR stimulation driving increases in Muc2 expression, we actually detected an initial fall in both Muc1 and Muc2 readouts. Animals treated with late EGCG had attenuated levels mRNA production, suggesting a suppressive effect that occurred after LPS initiated an upregulation of Muc2 expression. This finding is plausible, given the fact that EGCG inhibits signaling pathways emanating from activation of both TLR-2 and TLR-4 (which is activated by LPS) (Lee, Yeo et al. 2004, Hirao, Yumoto et al. 2010). Others have shown that over time, DSS decreases Muc2 expression by 15-18 fold around days 3-6, which is accompanied by goblet cell depletion (Dharmani, Leung et al. 2011). The loss of goblet cells and disappearing mucin layer heralds the onset of inflammatory immune cell infiltrates and deteriorating clinical conditions. However, in our experience, while the massive upregulation of Muc2 mRNA seen in animals treated with DSS was suppressed with EGCG, relative expression of Muc1 increased in a dose dependent fashion to EGCG. In our experience, this correlated with a well maintained goblet cell population in animals treated with EGCG compared to controls. In the context of a reactive treatment paradigm, the DSS-dependent expression of Muc2 was strongly down-regulated by EGCG, causing a 3-fold reduction in mRNA expression in the EGCG

treated animals, while preventative treatment almost completely subdues Muc2 transcripts to water-fed baseline levels. The reduction of Muc2 gene expression tracks with a lower endoscopic score in animals treated pre-emptively with EGCG, and more rapid endoscopic recovery after administration of EGCG in a treatment protocol (see data in chapter 5). Gene expression of Muc1, however, increased with longer periods of exposure to EGCG, and higher levels inversely corresponded lower inflammatory measures. In line with this finding, Muc1 appears to extend a protective effect to DSS treated animals by converting Th17 inflammatory signals from pathogenic T cells into a healing effect by enhancing mucosal responses to IL-22 (Nishida, Lau et al. 2012). The comprehensive benefits from orally administered EGCG in DSS exposed mice demonstrated a parallel dose-dependent response, as greater protective effects from colitis were seen with the higher dose of EGCG (20μg/ml) than with the lower dose (EGCG 2μg/ml).

5.2.8   MMP9, A MAJOR DETERMINANT OF COLITIS, IS STRONGLY DOWNREGULATED BY EGCG ADMINSTRATION

Matrix metalloproteinase (MMP)-9 has demonstrated a role in initiating and determining the severity of colitis in both animal models of inflammation and human IBD (Medina, Santana et al. 2006, Garg, Vijay-Kumar et al. 2009, Annaházi, Molnár et al. 2012). Mice with deleted or inhibited MMP-9 are protected against colitis, and treatments directed at inhibiting MMP-9 effects on extracellular matrix can improve colitis (Shimshoni, Yablecovitch et al. 2015) .

Figure 24. MMP9 transcripts. MMP9 mRNA message level was evaluated by RT-PCR for 3 treatment conditions.
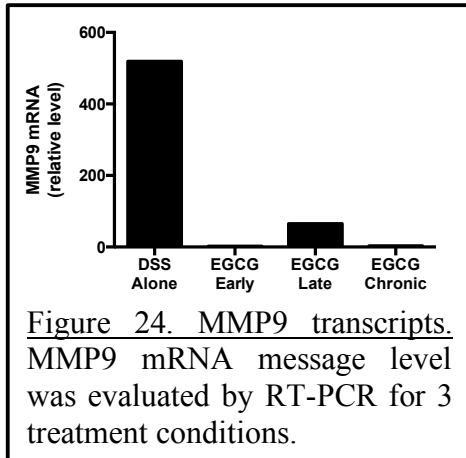
In our animal trial evaluating the early (preventative) versus late (treatment) effects of EGCG administration, as seen in figure 24, colonic tissue from DSS only treated animals exhibited a dramatic rise in MMP9 transcripts, while both treatment regimens profoundly inhibited the DSS-induced stimulatory effects on MMP9. It appears that early administration of EGCG completely prevents the induction of MMP9 mRNA production. From a treatment consideration, even when waiting for the inflammation to become established, EGCG still retains the ability to suppress MMP9 mRNA transcription to a fraction of the untreated animals. To put these findings into context, elevated MMP9 mRNA and protein levels in infiltrating immune cells correlate directly with severity of colitis (Medina and Radomski 2006, Medina, Santana et al. 2006, Santana, Medina et al. 2006). Elevated stool levels of MMP9 in humans with IBD correlate with disease activity (Annaházi, Molnár et al. 2012), and this protein is now the active target of pharmaceutical manipulation by monoclonal antibody therapy for treating the human disease state (Adamkewicz, Smith et al. 2013).

5.3 DISCUSSION

Barrier function may be one of the most important, yet under appreciated, components of the IBD susceptibility triad. Barrier disruption by medications such as nonsteroidal anti-inflammatory drugs, pathogenic bacteria, or even shifts in the microbiome can induce the onset of IBD. First degree relatives of IBD patients, who

have increased barrier permeability (Hollander, Vadheim et al. 1986), also have higher rates of developing IBD as well (Kevans, Silverberg et al. , Gaya, Russell et al. 2006, Van Limbergen, Russell et al. 2007). One IBD susceptibility mutation in particular, encoded by the XBP1 mutation (Kaser, Flak et al. 2011), directly impacts autophagy-related epithelial homeostasis and points to a genetic factor that could tie abnormal barrier permeability to first degree relatives of IBD patients (Fowler, Doecke et al. 2008).

One persisting unanswered chicken and egg question remains: that is, which problem comes first? Does the barrier defect exist first, then leads to an abnormal immune response, or does the activation of intestinal inflammation leave intestinal permeability in a shambles as an unintended consequence of the inflammatory cytokine storm? The answer could have relevance to future work developing IBD therapies. With our ever expanding knowledge of the intricacies of the mechanisms of inflammation guiding drug development, new therapies generally target the newest pathway discovered. Drug targets with broad effects on the immune system, such as TNF-α, have provided significantly better relief than non-specific immunomodulators such as azathioprine, 6-mercaptopurine or methotrexate. However, targeting other pervasive cytokines such as IL-6 or IL-1β have not provided relief, while drugs targeting very specific downstream cytokines, such as antibodies directed against IL-17, have actually worsened inflammation in treated patients compared to controls (Hueber, Sands et al. 2012). In retrospect, it turns out that interrupting IL-17 effects prevented compensatory increases in IL-22, which resulted in a cascading worsening of barrier function followed by heightened inflammation (Eyerich, Eyerich et al. 2010). Clinical trials, by design,

generally implement one primary intervention, though multiple outcomes may be measured. As clinical trial acumen improves, our so does our ability to interpret the results. We have now to come to a consensus in IBD trials that clinical do not provide reliable results for evaluating the success of a treatment. Therefore, newer clinical trials have started targeting mucosal healing as the primary or co-primary outcome to measure success of the trial for both Crohn's disease (ClinicalTrials.gov identifier NCT02596893) and ulcerative colitis (Dave and Loftus 2012). However, the recognition of mucosal healing as a worthwhile outcome has not yet translated into a focus for drug development. Based on our observations, agents that provide both a healing stimulus as well as an anti-inflammatory effect on the intestinal epithelial barrier provide a synergistic effect that translate into excellent outcomes. By the work detailed in this chapter, we have laid a substantial foundation for using EGCG as a therapeutic agent in IBD patients, especially for ulcerative colitis. First, EGCG exerts a beneficial effect on the composition of tight junction proteins in the colonic epithelium of mice treated with DSS and on human epithelial cells treated with LPS by promoting the production of tight junction "tightening" claudins while decreasing "loosening" claudins. Further support comes from measurements of Caco2 monolayer resistance to the passage of current (TER), showing that EGCG attenuates the loss of resistance induced by administrating LPS. In the absence of LPS, the administration of EGCG elevates TER above that of control Caco-2 cells, suggesting that cells do not require an inflammatory stimulus to respond to the effects of EGCG. This provides us a great deal of confidence as we plan clinical trials to evaluate the effects of EGCG on post-operative Crohn's disease patients. We feel that EGCG will prevent the return of inflammation that normally occurs in 70-

90% within one year of intestinal resection by bolstering epithelial resistance to antigen penetration, reduce diarrheal symptoms by augmenting expression of "tightening" claudins, and impart an anti-inflammatory effect on intestinal epithelial cells, circulating immune cells, and especially lamina propria immune cells. Other potential benefits from EGCG stem from its ability to reduce MMP9 production (an early manifestation of and possible precipitator of inflammation) and improve the quality of the mucin layer by enhancing mucin 1 production to assist with mucosal healing through effects of lamina propria produced IL-22 (Nishida, Lau et al. 2012). These data, plus additional data described previously, provide a sound basis for the evaluation of EGCG in further clinical trials for IBD.

CHAPTER 6

CLINICAL EFFECT OF EGCG ON HUMAN UCLERATIVE COLITIS[1]

6.1 INTRODUCTION

The treatment of patients with active UC generally follows a step-wise approach,

beginning with the administration of 5-aminosalicylic acid (ASA) agents.   While a

majority of UC patients respond to topically active 5-aminosalicyclic acid (5-ASA)

preparations currently available, up to 35% of patients fail to respond initially and 20% of

responders lose efficacy over time (Howell 2008).   Refractory cases of UC require an

escalation in therapy, usually involving steroids, immunomodulators, biologic agents, or

various combinations of all three to attain remission.   The fact that multiple forms of

susceptibility (genetic, immunologic) culminate in one common clinical manifestation

that is recognized as ulcerative colitis, likely explains why no single therapy works for all

affected individuals.   Although all 5-ASA agents are considered quite safe, the therapies

used during escalation for those with partial or no response to 5-ASA exhibit a relatively

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[1] The results discussed in this chapter have been published in *Inflammatory Bowel Diseases*, Volume 19, No. 9, pp 1904-1992 (2013) titled "A pilot study to evaluate the safety and efficacy of an oral dose of (-)-epigallocatechin-3-gallate-rich Polyphenon E in patients with mild to moderate ulcerative colitis".
Authors: Dryden G, Lam A, Beatty K, Qazzaz H, and McClain C.

high propensity for inducing significant side effects. For this reason and others, a large number of patients have turned to complementary and alternative medicine (CAM), either as an adjunctive therapy (complementary) to their current regimen or as a substitute for conventional therapy (alternative) (Hilsden, Scott et al. 1998, Ganguli, Cawdron et al. 2004).

6.1.1 ROLE OF COMPLEMENTARY AND ALTERNATIVE MEDICINE IN IBD

Patients managing chronic disease states, such as diabetes or hypertension (which are generally well controlled with conventional therapy) are less likely to use CAM than patients with disease processes that exhibit a variable response to commercially available medications (Eisenberg, Davis et al. 1988).   In light of this fact, patients with gastrointestinal diseases (particularly inflammatory bowel diseases) find dissatisfaction with conventional medicine (Hilsden, Scott et al. 1998).   Several CAM agents under investigation for IBD have demonstrated an ability to modulate inflammation, alter gut flora, or reduce psychosocial stress (Krueger, Wright et al. 2004, McClain, Dryden et al. 2008).   Unfortunately, clinical evidence for the therapeutic claims of CAM agents is often limited, and safety issues have often been inadequately addressed.

6.2 CLINICAL TRIAL CONSIDERATIONS: SUBJECT DEMOGRAPHICS, RANDOMIZATION SCHEME, AND DISPOSITION

A total of 30 patients were screened for inclusion. Twenty patients met all inclusion and exclusion criteria and were enrolled in the study. The demographics of each group are summarized in Table 5. There were no statistically significant differences

between treatment groups according to height, weight, UCDAI or baseline laboratory values such as white blood cell count (WBC), hemoglobin (Hgb), hematocrit (Hct), AST, ALT, and c-reactive protein (CRP) (Table 5). However, subjects randomized to the placebo arm did have a significantly lower baseline IBDQ score (Table 5). All randomized subjects were taking 5-ASA medications at time of enrollment. Almost half (7 of 16) of subjects randomized to active therapy were also taking AZA/6-MP. No subjects randomized to placebo were taking an immunomodulator at enrollment. Four subjects had previously received steroid therapy.

	Polyphenon E	Placebo	p value
Age	44.9 +/- 15	30.7 +/- 14.4	p=0.26
Weight	84.8 +/- 14.2 (61-77)	76.7 +/-8.1 (70-88)	p=0.29
Male gender	46.7	0%	p=0.5
Tobacco use	7%	50%	p=0.11
Azathioprine use	5	0	p=0.64
Day 0 DAI	6.5 +/- 1.9 (4-10)	7.3 +/- 1.7 (5-9)	p =0.5
Day 0 IBDQ	152.3 +/- 26 (108-206)	101.3 +/- 21.4 (77-129)	p = 0.02
Day 0 Endo score	2 +/- 0.68 (1-3)	2 +/- 0.5 (1-2)	p>0.99
Day 0 CRP	0.702 +/- 0.89 (0.03-2.8)	1.5 +/- 2.1 (0.13-3.9)	p=0.27

Table 5. Baseline characteristics for randomized subjects. Both the low-dose and high-dose cohorts were combined for analysis. No statistically significant differences were noted in any category, with the exception of IBDQ score in the placebo group. A lower IBDQ score suggests more severe disease. The treatment group contained all of the patients refractory to azathioprine.

A total of sixteen subjects were randomized to receive study drug (low vs. high dose Polyphenon E®), while four subjects were randomized to receive placebo. One subject assigned to treatment with Polyphenon E®, who withdrew just after randomization but before receiving any therapy, was excluded from final analysis. Three subjects assigned to active therapy voluntarily withdrew during the study period due to lack of efficacy (two in the low-dose cohort, one in the high-dose cohort). One subject

randomized to placebo withdrew prior to completion of the study. All drop-out subjects were coded as non-responders. (See Figure 25 for Subject Allocation).

*One withdrew prior to dosing with EGCG.

Figure 25. Subject allocation. Subjects were assigned to treatment at random.
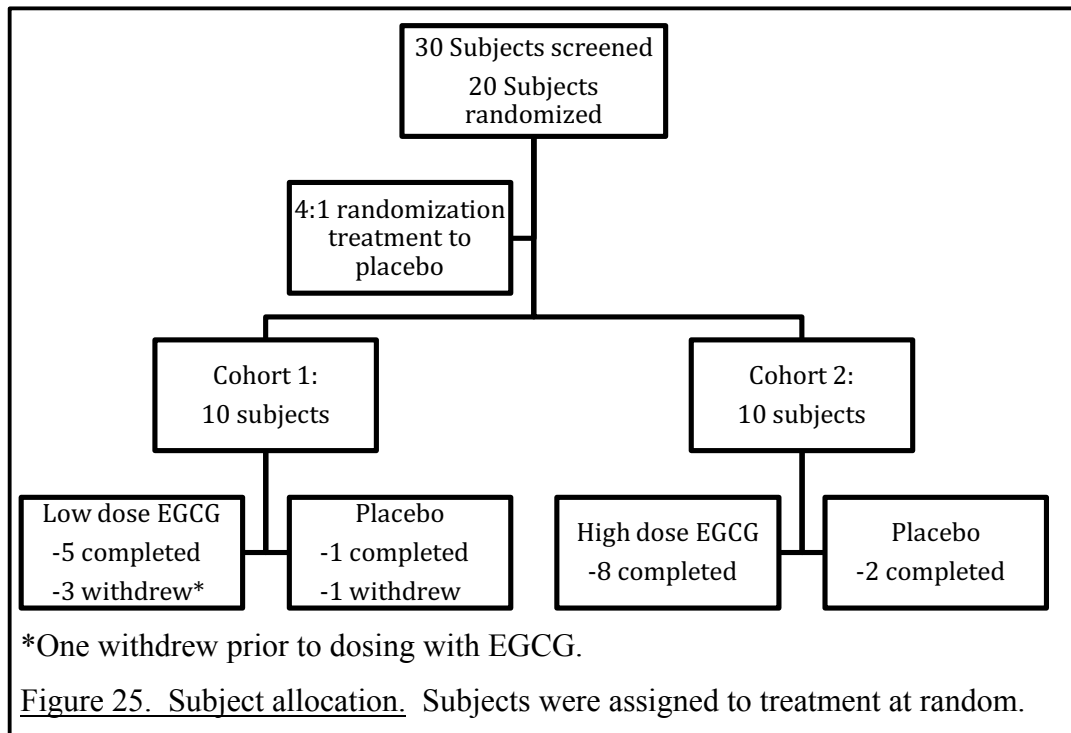
6.3 RESULTS

6.3.1 EFFECT OF EGCG ON OVERALL TREATMENT OUTCOMES OF CLINICAL RESPONSE

Subjects were evaluated for response after 56 days of therapy by UCDAI. As demonstrated in Figure 26, a total of 10 of 15 (66.7%) subjects randomized to Polyphenon E® (aggregates of cohorts I and II) responded to therapy, as evidenced by a decrease in their UCDAI score by at least three points ($p = 0.03$), (OR 17.2, 95% CI 0.77-381.1). When response rates were evaluated by individual treatment group (low or high dose Polyphenon E® or placebo), overall response rates for the active therapies appeared similar irrespective of dose (Figure 27). Both high and low dose groups contained a

majority of responders. No formal comparisons were made for the individual treatment groups, due to the small number of subjects in each group.

6.3.2 EFFECT OF EGCG ON OVERALL TREATMENT OUTCOMES OF CLINICAL REMISSION

Eight of 15 (53.3%) subjects (p=0.07), (OR 10.2, 95% CI 0.47-222.6) who received active therapy went into remission, as evidenced by a UCDAI < 2. Some subjects qualified as both responders and remitters, depending on their UCDAI score on day 0. No subjects randomized to placebo demonstrated either a response or remission to study medication. When evaluated separately, both active therapy dosing regimens of Polyphenon E® appeared to have similar patterns of response (Figure 27).

Figure 26. Combined response and remission rates. Subjects treated with EGCG had a statistically significant response to therapy.
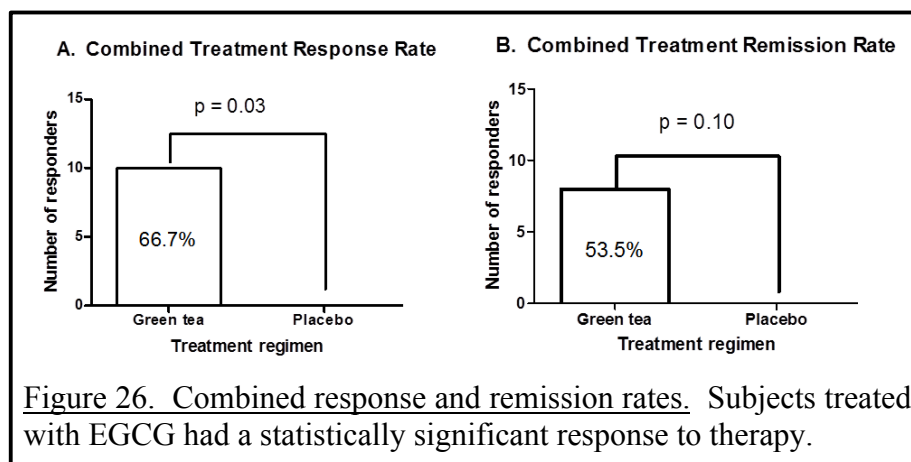
6.3.2.1 ANALYSIS OF INDIVIDUAL RESPONSES BASED ON TREATMENT ASSIGNMENT

The following graphs represent the individual treatment responses, based on either high dose EGCG, low dose EGCG, or placebo. The only patient exhibiting a worsening of symptoms during the clinical trial actually developed infection with *C. difficile* after a course of antibiotics for an unrelated infection.

Figure 27. Individual responses to EGCG based on dose of EGCG or placebo. The majority of individuals improved while taking EGCG, whereas, only one subject on placebo demonstrated any improvement.
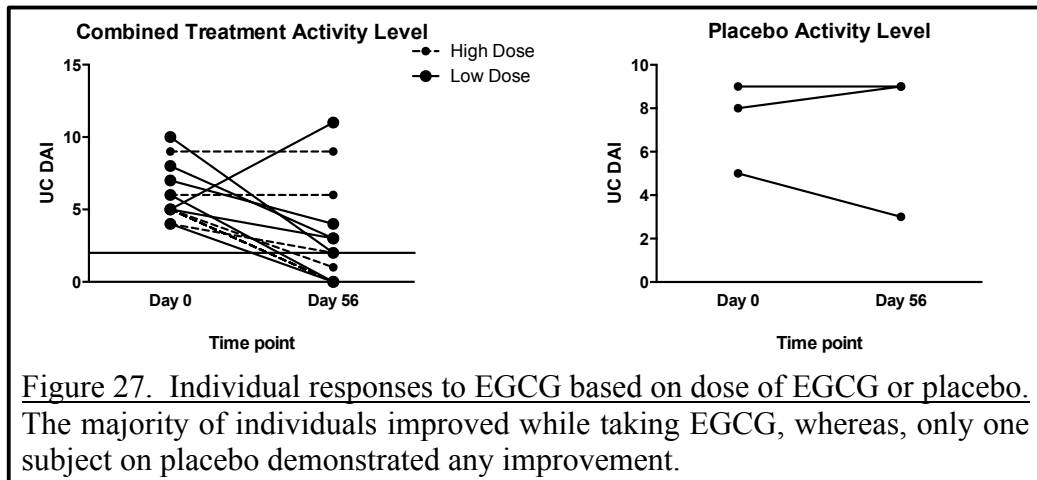
6.3.3 EFFECT OF EGCG ON SUBJECT QUALITY OF LIFE

Quality of life (QoL) was evaluated by the Inflammatory Bowel Disease Questionnaire (IBDQ). The IBDQ was administered on a weekly basis between days 0 and 56. The IBDQ is a validated, IBD-specific tool for measuring QoL in patients with UC or CD. Although not a direct measure of clinical effect from an intervention, the IBDQ generally correlates well with measures of clinical disease (Sandborn, Tremaine et al. 1997). In general, the IBDQ paralleled subject's responses to therapy by UCDAI. Individual treatment responses based on cohort allocation have been broken out and are demonstrated in Figure 3.

Figure 28. Impact on QoL by different doses of EGCG or placebo. A rising IBDQ indicates improving quality of life. A majority of subjects treated with EGCG experienced a rise in their IBD, while most randomized to placebo did not.
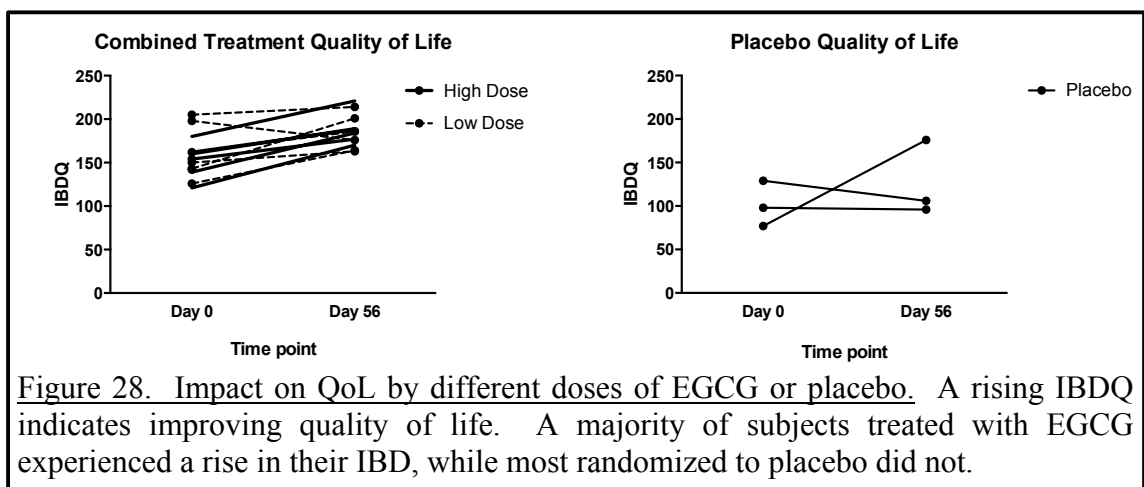
6.3.4 IMPACT OF EGCG ON MUCOSAL INFLAMMATION AS DETERMINED BY SERIAL ENDOSCOPY

All subjects underwent flexible sigmoidoscopy on day 0. All but one subject completing the full treatment course underwent a follow up procedure. Only one subject randomized to active therapy demonstrated a worsening in the endoscopic score after therapy. All subjects who clinically responded to treatment with Polyphenon E® (10/15) also exhibited at least a single point drop in the endoscopic score. Two subjects receiving active therapy demonstrated a dramatic 3-point drop in their endoscopic score, and their images are highlighted in Figure 4. A total of 5 subjects completed the study with an endoscopic score of 0. There were no clinical non-responders who received active therapy who also exhibited a drop in their endoscopic score. Only one subject randomized to placebo demonstrated an improvement in the endoscopic component of the UCDAI, changing from a score of 2 to 1 at day 56.

Day 0 - Screening
Diffuse, continuous erythema, edema and friability in rectum and distal sigmoid colon.

Day 56 – Post-treatment
Minimal edema or erythema remain, compatible with resolving colitis.

Figure 29. Endoscopic response to orally administered EGCG. Figure 29 demonstrates clinical effect of EGCG administered over a 56-day clinical trial. The initial picture depicts a grade 3 colitis as evidenced by the presence of spontaneous hemorrhage and diffuse erythema, edema and granularity of the mucosa. A follow up picture from the same patient on day 56 demonstrates complete endoscopic remission occurred due to the administration of EGCG. The bottom row depicts the effects of continuous treatment over a 7-year period in a responder from the original clinical trial.
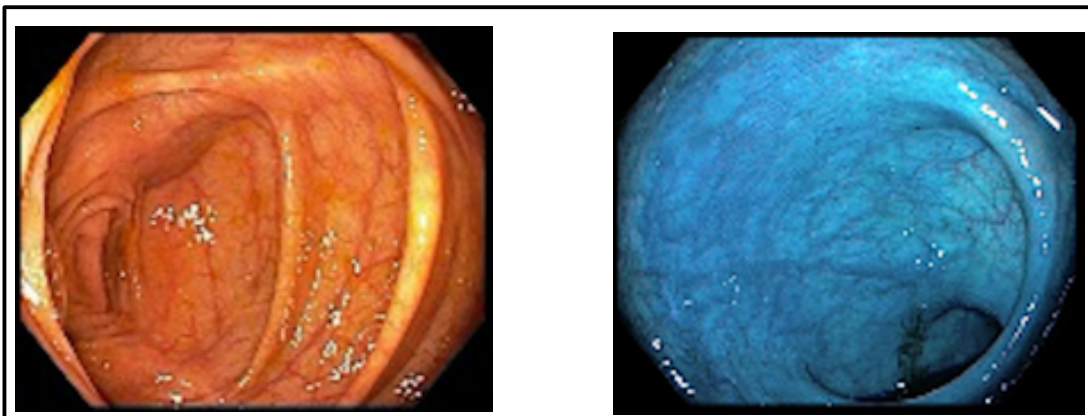
Figure 30. Seven year followup. (A) demonstrates the white light appearance of a normal colon. (B) depicts the appearance of the mucosa imaged by narrow band imaging (NBI), a technique that highlights red to visualize highly vascular structures. In this picture, NBI shows that the entire rectal mucosa is normal. This patient had been failed 5-ASA, azathioprine and prednisone. He has remained in remission, taking EGCG alone.

6.3.5 SUBJECT SAFETY

There were no serious adverse events or greater than grade 2 toxicities related to treatment. One subject randomized to treatment required hospitalization due to disease progression, which appeared to be related to antibiotics administered for a non-UC indication and subsequent *Clostridium difficile* infection. No significant differences were noted between pre- and post-treatment laboratory studies, including ALT and AST levels. The following table summarized the most common study related adverse events.

Symptom	Polyphenon E	Placebo
Heartburn	27%	0%
Bloating, flatulence	27%	33%
Nausea	20%	33%
Headache	13%	33%
Increased thirst	7%	0%
Increased diarrhea	7%	0%

Table 6. Common adverse events. The common adverse events attributed as possible or likely related to therapy with Polyphenon E® have been listed above. There were no adverse events exceeding grade 2 severity, according to Common Terminology Criteria for Adverse Events version 3.0. The small number of subjects enrolled in the placebo group prevents any meaningful comparison between groups.

6.4 DISCUSSION

This first ever study of Polyphenon E® in patients with ulcerative colitis provides convincing preliminary evidence for a significant treatment effect in patients with inflammatory bowel disease. The observed clinical effect, coupled with a paucity of acute side-effects, lends strong support for more thorough evaluation of Polyphenon E® as a therapeutic agent for UC. The numbers of patients enrolled in this study were small, but with the 4:1 randomization scheme, a large number of subjects were exposed to the active therapy. There were two main reasons for randomizing a larger number of subjects to Polyphenon E® than placebo. First, we wanted to enhance the statistical confidence regarding response rates in the active therapy group for planning subsequent clinical trials. Secondly, with a previously untried therapy, we felt that recruitment would be enhanced if potential subjects felt that they had a better chance of receiving therapy. In later phase clinical trials, the risk of randomization to placebo is counterbalanced by the possibility of rolling over into an open-label arm, but regulatory constraints prevented this option for our early phase trial. During the final analysis, we noted that subjects randomized to placebo had a significantly lower IBDQ score, which could indicate a more severe state in this group. However, the UCDAI did not significantly differ between the active and placebo groups.

Considering our positive results from this pioneering clinical trial of Polyphenon E® in UC, a lack of significant side effects from the therapy, and the mass of pre-clinical evidence of anti-inflammatory effects of EGCG, we are currently in the process of planning a subsequent clinical trial to validate the current results. If the improvements

seen in both clinical and QoL parameters hold up in this difficult to treat demographic (5-ASA and/or azathioprine refractory) and Polyphenon E® retains its favorable side-effect profile, this treatment option would be well placed to serve as an alternate therapy for first-line therapy of UC, or as an alternative therapy for 5-ASA intolerant patients. The possibility that Polyphenon E® could also serve as a "step-up" treatment for those patients who do not respond to either 5-ASA or immunomodulators needs to be further investigated. Given the serious side-effect profile of the immunomodulator agents, Polyphenon E® could potentially become a preferred alternative to this class of medications.

In summary, we anticipate that the synergy created by a favorable side effect profile, a commendable efficacy rate against a formidable treatment challenge, and a convenient once daily dosing schedule will cement the appeal of Polyphenon E® as a treatment for UC. Taking these results into account, we are planning a continuation of this line of research in the form of an adequately powered, controlled trial to definitively confirm the benefits of Polyphenon E® in active UC.

CHAPTER 7

CONCLUSION: EGCG IS AN EFFECTIVE ANTI-INFLAMMATORY TREATMENT

FOR PATIENTS WITH INFLAMMATORY BOWEL DISEASE

While the underlying etiology of IBD remains a mystery, the discovery of new immunological mechanisms provides a basis for new therapies. Ample evidence supports a role for the activation and nuclear translocation of NF-κB in the development and maintenance of IBD (Abboud, Hake et al. 2008). Although an exact mechanism of action for EGCG has not been established in IBD, the results of our work detailed in this dissertation point to a multitude of potential roles for EGCG. EGCG exerts a strong inhibitory effect on NF-κB nuclear translocation through inactivation of IκB kinase (IKK), which subsequently liberates NF-κB from its inactive cytosolic sequestration by IκB (Yang, Oz et al. 2001). Liberated NF-κB traverses the nuclear envelope, and engages with the promoters of multiple genes, promoting or suppressing gene transcription. Nuclear translocation of NF-κB within cells of the innate immune system (such as macrophages and dendritic cells) results in a coordinated increase in the expression of genes whose products mediate inflammation and immune responses, including cytokines (e.g., IL-1, IL-6, IL-12, TNF-α), chemokines (e.g., IL-8), enzymes involved in metabolism (e.g., inducible nitric oxide synthase, inducible cyclooxygenase-2), and cell adhesion molecules (e.g., E-selectin, intracellular adhesion molecule-1, and

vascular adhesion molecule-1). Protein products of these genes are expressed at high levels in patients with chronic inflammatory bowel disease. During the process of disentangling the effects of EGCG on nuclear signaling, it has become evident that EGCG exerts a much more profound effect on immune regulation of inflammation and activation of immune cells than can be explained by NF-κB inhibition alone (Wu, Wang et al. 2012). Insight into the interplay of EGCG with cell signaling events in cancer cells has greatly expanded our knowledge of the mechanisms of action of EGCG in inflammation (Khan, Afaq et al. 2006). EGCG exerts potent inhibitory effects on signaling intermediaries involved in both generation of inflammation as well as control of cell growth and proliferation, such as MAPKs, JAKs, and AP-1. A thorough synopsis of the numerous interactions has been published by Singh *et al.* (Singh, Akhtar et al. 2010). We have also demonstrated an effect on RORγt signaling and its nuclear translocation. In addition to its dampening effects on active inflammation, EGCG has been shown to actively up-regulate important compensatory, anti-inflammatory pathways simultaneously. Up-regulation of the anti-inflammatory cytokine TGF-β, as well as its membrane receptors, has been reported in chondrocytes cultured with EGCG (Andriamanalijaona, Kypriotou et al. 2005). Additionally, EGCG administered to endothelial cells downregulated the production of monocyte chemotactic protein (MCP)-1 by activating the heme oxygenase-1 pathway, an important factor regulating cellular responses to oxidative stress (Zheng, Toborek et al. 2010). Cellular proliferation and survival signals evoked by the binding of IL-2 and IL-6 to their membrane receptors are abrogated by EGCG, which simultaneously enhances compensatory pathways for counterbalancing their pro-inflammatory effects. This compensation mechanism takes

place by way of activation of suppressor of cytokine signaling (SOCS) proteins, particularly SOCS1 (Ripley, Fujimoto et al. 2010). SOCS1 protein dampens inflammatory responses elicited by multiple activators of the immune system, such as the binding of pro-inflammatory cytokines to their cognate receptors or bacteria-derived peptides to the Toll-like membrane-bound receptor (TLR)-4. This action of SOCS1 is attributable to direct interference with JAK kinase activation, as well as the enhancement of JAK kinase degradation through the proteasome system (Yoshimura, Naka et al. 2007). Finally, SOCS1 also binds directly to an important co-factor in TLR-4 signaling, MyD88-adaptor-like (MAL) protein. The direct binding of SOCS1 to activated MAL accelerates proteasome degradation of this important factor linking TLR-4 signaling to NF-κB activation (Mansell, Smith et al. 2006). In light of the important role these intermediaries play in the immune activation cascade, which are central to the initiation and maintenance of inflammation, it is not surprising that EGCG was able to effectively subdue and control human ulcerative colitis.

The clinical manifestations of IBD are largely related to the indiscriminant effects of cytokine release from activated immune cells. We have demonstrated that EGCG effectively down-regulates the production of cytokines that are important in the development of intestinal permeability. Mucosal immune activation results in part from a loss of protection of the immune system from bacterial antigens, due to a breakdown of the intestinal epithelial barrier (Sandle, Higgs et al. 1990, Braegger and MacDonald 1994). Although the initial instigator of barrier dysfunction remains a topic of debate, clinical evidence demonstrates that prolonged exposure of the intestinal mucosa to high

levels of Th1-type cytokines produces mucosal ulcerations in CD (Braegger and MacDonald 1994). Therapies that reduce mucosal exposure to inflammatory cytokines assist in healing the damage associated with IBD, ultimately re-establishing a protective barrier that isolates the mucosal immune cell populations from exposure to antigenic stimulation (Baert, D'Haens et al. 1999). In this study, we established a general protective role for EGCG in IBD through the reduction of cytokine production by the major initiators of intestinal inflammation: the immune system and the epithelium. An extensive body of data supports the notion that IL-1β, IFN-γ and TNF-α signaling, through independent pathways, ultimately converge at the point of myosin light chain kinase (MLCK) phosphorylation to enhance TJ permeability (Stow and Murray 2013). However, the relative composition of TJ proteins occupying the TJ scaffold also determines the functional state of the epithelial barrier. The pore-forming claudins (2, 7, 10, 15, and 16) and pore-sealing claudins (1, 4, 5, 8, 11, 14 and 19) (Krause, Winkler et al. 2008) exist as preformed proteins which can be shuttled in or out of the TJ complex, as influenced by external stimuli. Although the exact role played by many of these claudins in IBD remains to be investigated, a recent study by Zeissig *et al.* found downregulated expression of occludin as well as claudins 5 and 8 globally throughout the mucosa of Crohn's patients with active disease, while claudin 2 was upregulated (Zeissig, Bürgel et al. 2007). These changes in claudin expression were not exhibited during periods of remission, suggesting that TJ remodeling and changes in claudin expression occurred under the influence of cytokine mediators (Weber and Turner 2007). Along these lines, Laukoetter *et al.* demonstrated that the increase in susceptibility to experimentally induced colitis seen in JAM-A knockout animals correlated with

increased expression of pore-forming claudins (10 and 15) that were present prior to induction of the experimental injury (Laukoetter, Nava et al. 2007). Cytokine related permeability changes have also been tied to cytokine signaling through TNFR2 (Shen, Weber et al. 2011, Noda, Tanabe et al. 2012, Su, Nalle et al. 2013).

Based on the results from our experiments, it is evident that EGCG improves barrier function. This is likely through two complementary pathways: by reducing cytokine production from activated immune cells and their downstream effects on TJ composition, as well as by directly influencing TJ protein composition. It is known that epithelial cells regularly rotate claudins during normal junction maintenance and homeostasis (Matsuda, Kubo et al. 2004). During periods of inflammation and NF-κB activation, cytokine-dependent alterations in TJ constituent protein cycling patterns can degrade barrier function by the substitution of pore-forming components integrated into the TJ complex (Matsuda, Kubo et al. 2004). Elevated levels of claudin 2 have also been documented in patients with acute colitis, and its presence correlates with increased barrier permeability (Lu, Ding et al. 2013). This finding was corroborated in our results with the increase in both claudins 2 and 4 when Caco2 cells were exposed to LPS. Enhanced levels of these claudins correlated with decreases in the TER. However, EGCG appears to have a direct effect on TJ permeability independent of cytokine inhibition, as the addition of EGCG in the absence of LPS actually increased TER by over 20%, while the level of TJ proteins remained essentially at baseline. This observation prompted additional experiments to determine the mechanism of this observation. This effect of EGCG is likely tied to increased claudin 1 production, a claudin that appears to exert tremendous influence on TJ resistance to ion flux. The data

from WB and RT-PCR analysis of mRNA for claudin 1 supports the notion that EGCG upregulates this important tightening claudin. We are currently pursuing a novel mechanism exploring the effects of HIF1α on CLDN1 promoter.

The data presented here demonstrate that the potent anti-inflammatory effects of EGCG modulate the secretion of three cytokines (IL-1β, TNF- α and IFN-γ) by circulating immune cells and intestinal epithelial cells. These three cytokines have been shown to be integrally involved with inflammation-induced changes in epithelial tight junction permeability. Examination of TER data presented in this paper clearly demonstrates that EGCG increases epithelial electrical resistance and TJ protein expression levels after 18-24 hours of incubation with Caco-2 cell monolayers. These data suggest an additional mechanism underlying EGCG-mediated protection against colitis (Azuma, Shigeshiro et al. 2013). Future studies on the anti-inflammatory effects of EGCG should evaluate its impact on the regulation, expression, and function of barrier related components in clinical subjects with IBD. These insights will provide critical information for better understanding how EGCG impacts the molecular mechanisms involved in inflammatory bowel disease, and guide the development of this potentially beneficial therapeutic agent. Data binding EGCG's anti-inflammatory and TJ regulatory effects to the preservation of barrier function provides an explanation for the successful treatment of ulcerative colitis documented in our clinical trial (Dryden, Lam et al. 2013). These data and others provide justification for larger clinical trials to further evaluate the impact of EGCG on the inflammatory pathways related to the IBDs, as well as confirm its efficacy on the clinical manifestations of those disorders.

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EDUCATION

12/2008-present	University of Louisville, School of Medicine PhD Candidate
8/2006-12/2008	University of Louisville, School of Medicine Master of Science in Microbiology and Immunology
8/2001-5/2003	University of Louisville, School of Public Health and Information Sciences Master of Science in Public Health
8/1988-5/1992	University of Kentucky, College of Medicine Doctor of Medicine
8/1984-5/1988	University of Kentucky, College of Agriculture Bachelor of Science in Agriculture

POSTGRADUATE TRAINING

7/1995-7/1997	Naval Medical Center San Diego, Department of Internal Medicine
	    Gastroenterology Fellowship
	    - Credentialed in diagnostic/therapeutic upper endoscopy, colonoscopy, ERCP including sphincterotomy, biliary dilation, stent placement and esophageal/rectal manometry

| 7/1993-7/1995 | Naval Medical Center Portsmouth, Department of Internal Medicine |
| | Internal Medicine Residency |

| 6/1992-7/1993 | Naval Medical Center Portsmouth, Department of Internal Medicine |
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ACADEMIC APPOINTMENTS

| 5/2014-present | University of Louisville School of Medicine
Professor of Medicine
Medical Director, Clinical Trials Unit |

| 11/2010-5/2014 | University of Louisville Speed School of Engineering
Associate Professor, Department of Bioengineering |

| 5/2009-present | University of Louisville Division of Gastroenterology
Director, Luminal GI Research |

| 7/2006-present | University of Louisville Division of Gastroenterology
Associate Professor of Medicine |

| 7/2005- present | University of Louisville Division of Gastroenterology
Director, Inflammatory Bowel Disease Program |

| 7/ 2000-7/2006 | University of Louisville Division of Gastroenterology
Assistant Professor of Medicine |

| 1/2000-7/2000 | Naval Hospital Jacksonville, Department of Internal Medicine
Department Head, Command Combined Endoscopy Suite |

| 1/1998-7/2000 | Naval Hospital Jacksonville, Department of Internal Medicine
Head, Gastroenterology Division |

| 7/1997-1/1998 | Naval Medical Center San Diego, Department of Internal Medicine
Teaching staff, Gastroenterology Division |

CERTIFICATION AND LICENSURE

Medical License: State of Kentucky   #33791
                  State of Virginia (Inactive)   #0101049542

2013   BCLS, ACLS certification current
2008   Diplomate, American Board of Internal Medicine (Gastroenterology)

#160615
1995   Diplomate, American Board of Internal Medicine (General Internal Medicine)
        #160615
1992   Diplomate, National Board of Medical Examiners
        #34119438

PROFESSIONAL MEMBERSHIPS AND ACTIVITIES

1995   Member, American College of Gastroenterology
1995   Member, American Gastroenterological Association
                        Elected **Fellow**, AGA December 2010
1995   Member, American Society of Gastrointestinal Endoscopy
                        Elected **Fellow**, ASGE July 2010
2007-2008   **President**, Kentucky Society of Gastrointestinal Endoscopy

HONORS AND AWARDS

2012   1st Place Faculty Award – "Potential for Major Clinical Application"
                Research!Louisville 2012
2007   Golden Key International Honor Society
2006   University of Louisville Outstanding Community Service Award
2003   Dean's Scholarship Citation
2003   Phi Kappa Phi National Honor Society
2003   Outstanding Achievement Award in Clinical
                Research and Academic Medicine

MEDICAL SCHOOL:
1991-1992   A.J. Beale Scholarship for Primary Care
1991-1992   Elected Senior Class representative,
1991-1992   Medical Student Government Association
                        Appointed as student member,
1991, 1992   Internal Medicine Liaison Committee
1989, 1990, 1992   Lampoons Committee Chairman
1989-1992   Naval Health Professions Scholarship

UNDERGRADUATE:
1988   Oswald Research and Creativity Competition
                    Second place for paper on soybean tissue culture   and   molecular
genetics
                    research
1988   Gamma Sigma Delta Agriculture Honor Society
1987   Alpha Zeta Agriculture Honor Society
1987, 1988   Dean's List
1987   Kappa Alpha Scholarship Citation
1984-1985   Keenland Scholarship

MILITARY SERVICE

2000-present   Active reserve member, United States Navy
Current rank: Captain, Medical Corps
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1992-2000   Active duty member, United States Navy

ABSTRACTS AND MEETING PRESENTATIONS

ORAL PRESENTATIONS

Eugene D. Boland, Carlos C. Chang, **Gerald W. Dryden**. Fibrinogen based scaffold for chronic wounds. *Regenerative Medicine: Technologies Enabling Novel Therapies*. Hilton Head, SC. March 20-23, 2013.

POSTER PRESENTATIONS

Andrew N Stillman, Michael A Connors, Matthew E Miller, Hassan M Qazzaz**, Gerald W Dryden.** Oral Administration of EGCG, a Green Tea Polyphenol, Both Suppresses and Rescues Mice from DSS-Induced Colitis. #P-145. Advances in Inflammatory Bowel Disease: Crohn's and Colitis Foundation's Clinical and Research Conference, Orlando, FL. December 10-12, 2015.

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