# EXOGENOUS and ENDOGENOUS DANGER SIGNALS in INFLAMMATORY BOWEL DISEASE

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# EXOGENOUS and ENDOGENOUS DANGER SIGNALS in INFLAMMATORY BOWEL DISEASE

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The human chronic inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are ostensibly disorders of innate immunity with an exaggerated inflammatory response and loss of tolerance to the normal enteric microbial flora. In this project, we have extensively characterized innate immune responses driven by Pathogen Associated Molecular Pattern Molecules (PAMPs) and the more recently recognized Damage Associated Molecular Pattern molecules (DAMPs). The prototype DAMP, a chromatin-associated protein, high mobility group box 1 (HMGB1), is released during cellular necrosis and is secreted from activated macrophages. Extracellularly, it binds the receptor for advanced glycation end products (RAGE), as well as toll-like receptor (TLR) 2 and TLR4, important in the recognition of PAMPs. PAMPs and DAMPs trigger inflammatory signaling pathways in neighboring cells through activation of the transcription factor family, NF-κB.

Much attention has been given to the central role played by PAMPs in the form of the enteric bacterial flora in IBD pathogenesis. We hypothesize that DAMPs also play a pivotal role in this process. Accordingly, we have determined the significance of DAMPs and PAMPs in the mucosal inflammatory response in macrophages and *in vivo* in mouse models of IBD.

We first investigated expression of TLRs in the gut to determine cell types in the intestinal epithelium that may respond to danger signals. TLR expression was most prominent on intestinal epithelial enteroendocrine cells (EEC). Using a murine EEC line, multiple

functional consequences of TLR activation were demonstrated. Second, in IL-10 deficient (-/-) mice with chronic Th1-mediated enterocolitis, we demonstrate a role for HMGB1 in macrophage activation and IBD. Lastly, we examined an *in vivo* therapy targeted at inhibiting the prominent downstream effector of DAMP and PAMP signaling, NF-κB, in murine IBD. Inhibition of activated NF-κB with a short cell permeable peptide inhibited chronic enterocolitis in *IL-10*$^{-/-}$ mice.

In summary, this dissertation provides new insight into our understanding of intestinal innate mucosal inflammatory responses. We demonstrate the relevance of TLRs on EECs and the contribution of DAMP and PAMP signaling in disease. These results also provide proof of concept for new therapeutic approaches in IBD.

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# PREFACE

This dissertation would not have been possible without the assistance of many people over the past four years. First, I would like to thank my mentor, Dr. Scott E. Plevy. He has provided me with scientific guidance and intellectual freedom in the course of my experiments, taught me valuable skills for a career in academic medicine, and has been a role model as a physician-scientist. I appreciate the time and energy contributed towards my dissertation by the members of my thesis committee, Drs. Sid Morris, Tim Oury, Prabir Ray, and Paul Robbins. I also would like to acknowledge the assistance and the training I received from the members of the Stolz, Lotze, and Robbins labs. I thank my lab colleagues and friends for helpful discussions during the ups and downs of graduate school. Finally, I thank my parents Harish K. and Shaku Davé and my fiancée, Dr. Karen Au, who have been by my side through sickness and in health and have been a source of constant support throughout my training.

# 1.0 INTRODUCTION

The human chronic inflammatory bowel diseases (IBDs)[1], Crohn's disease (CD) and ulcerative colitis (UC), affect over one million Americans (1). The disease is characterized by chronic, intermittent inflammation of the gastrointestinal tract often associated with diarrhea, bloody stool, abdominal cramps, decrease in appetite, and weight loss. Distinguishing features between CD and UC are based on the anatomic distribution of inflammation, the depth of inflammation, and histological patterns of inflammation. UC is marked by a superficial inflammation in the lamina propria causing epithelial cell damage affecting only the innermost layers of the colonic wall. Inflammatory changes invariably start in the distal rectum and extend proximally through the colon in a symmetric, continuous manner. In contrast, inflammation in CD can affect any portion of the alimentary tract, from the mouth to the anus. Most frequently, the affected areas are the terminal ileum and ascending colon. CD has a patchy and discontinuous distribution throughout the gastrointestinal tract. It develops in a transmural fashion, affecting all layers of the bowel wall (2, 3). Transmural inflammatory changes in CD lead to the important clinical

[1]Abbreviations used in this chapter: BLP, bacterial lipoprotein; CARD, Caspase recruitment domain; CD, Crohn's disease; DAMP, damage associated molecular pattern; EEC, enteroendocrine cell; ELAM-1, endothelial-leukocyte adherence molecule 1; HMGB1, high mobility group box 1; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IKK, IκB kinase; *IL-10*$^{-/-}$, IL-10-deficient; IRAK, IL-1R-associated kinase; LPMC, lamina propria mononuclear cells; LPS, lipopolysaccharide; LRR, leucine rich repeat; MDP, muramyl dipeptide; NEMO, NF-κB essential modulator; NOD2, Nucleotide oligomerization domain 2; PAMP, pathogen associated molecular pattern; PGN, peptidoglycan; PRR, pattern recognition receptor; RA, receptor antagonist; RAGE, receptor of advanced glycation end products; Th, T-helper; TIR, Toll/IL-1R; TLR, toll-like receptor; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TRAF6, TNF-receptor associated factor 6; UC, ulcerative colitis.

consequences of intestinal perforation, abscess, and fistula formation. Finally, although only found in 10 to 25% of intestinal biopsies or surgical resections, the histological feature of granuloma formation is pathopneumonic for CD (4).

There is currently no cure for IBD and existing therapies such as corticosteroids, aminosalicylates, and immunomodulatory agents (e.g. azathioprine, 6-mercaptopurine, and methotrexate) are of limited effectiveness, have the potential for side effects, and/or are designed to non-specifically reduce intestinal inflammation. The frequent dosing schedules of these agents which are invariably prescribed in combination regimen also lead to poor patient compliance. As a result of the burden of their clinical symptoms and side effects of medications, most patients with IBD have a significantly impaired quality of life (5, 6). Due to complications of the disease (perforation, intestinal obstruction, colorectal cancer, failure of medical therapy), need for surgical intervention occurs in a significant proportion of patients over their lifetime. In fact, as many as 75% of people with CD and up to 40% of those with UC eventually require surgery (7).

Increased understanding of specific immune pathways that directly modulate inflammatory changes in IBD has led to the development of anti-TNF antibodies (infliximab) as a Food and Drug Administration approved therapy in CD and UC (8, 9). Although a significant therapeutic advance, these agents are effective in less than 40% of IBD patients. Moreover, there is the potential for long-term side effects, high costs, and the need to deliver the drug on a repeated maintenance basis by injection. Therefore, there are numerous unmet medical needs to identify new targets that may provide safer and more effective means of therapeutic intervention to successfully treat CD and UC.

## 1.1 PATHOGENESIS OF IBD

Although the etiologies of the human IBDs remain unknown, the pathogenesis of IBD is thought to result from interactions between genetic factors, the environment, and the immune system (10). Research has identified contributing factors that include a defect in the barrier mechanism of the lining of intestinal epithelial cells (IECs) and a poorly regulated immune response against the normal enteric microbial flora. These immunologic and barrier defects reflect a complex genetic susceptibility in the human IBDs. A prevailing unifying hypothesis is that persistent intestinal inflammation results from enhanced or aberrant immunologic responsiveness to the normal microbial constituents of the gut lumen. The intestinal tract is in direct contact with an external environment heavily inundated with bacteria. In fact, there are approximately ten-times the number of microbes that normally colonize the intestines than there are cells in the human body (11). An underlying and poorly understood feature of the mucosal immune system is the ability to co-exist with this biomass of commensal microbiota. In the event of invading intestinal pathogens, an appropriate immune response is generated to eradicate the foreign entities in part through the production of inflammatory cytokines and the recruitment of inflammatory cells. However, once a pathogen is eradicated, the inflammation is appropriately turned off, leading to a state of "controlled inflammation" (Figure 1.1). In IBD, the prevailing hypothesis is that intestinal inflammation results from an aberrant immune response in a genetically susceptible individual to the normal microbial constituents of the gut lumen. What is observed is a failure to downregulate the initial inflammatory response, leading to a chronic, "uncontrolled inflammation."

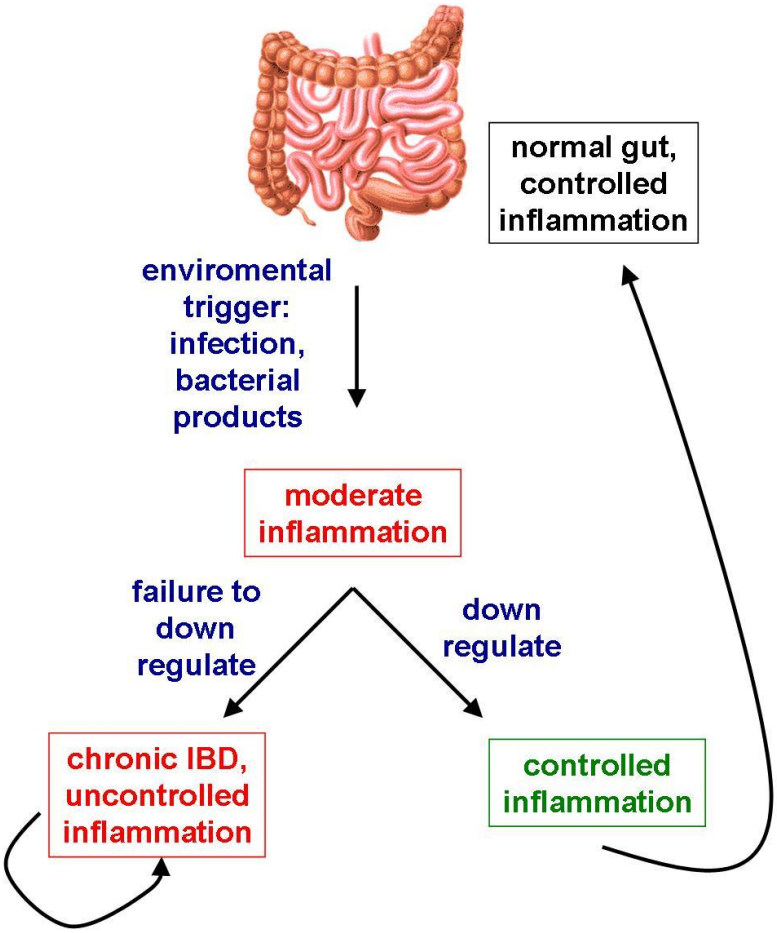


**Figure 1.1: Controlled and uncontrolled inflammation in the intestine.**

The intestines are normally in a controlled homeostatic state. The mucosal immune system is in a state of tolerance to the numerous environmental antigens and innate immune stimuli present in the GI tract. In the event of an environmental trigger, such as infection, the mucosal immune system responds with an appropriate inflammatory response to eradicate foreign pathogens. Once inflammation has served its purpose, it is appropriately downregulated restoring the "controlled inflammation" state. However, in a genetically susceptible individual with an aberrant immune response, there is a failure to downregulate the initial inflammatory response. This leads to a condition of chronic, "uncontrolled inflammation." In this setting, secondary signals are produced and released, perpetuating the inflammatory state.

### 1.1.1 Genetics of IBD

Epidemiological analyses demonstrating that different races and ethnicities have markedly varying prevalence of IBD were the first studies to suggest a genetic predisposition. For example, the incidence and prevalence of IBD is increased in North American and Northern European countries (12). Furthermore, in the United States the rate of IBD is lower amongst non-Caucasians. Compared to Caucasians, IBD is half as common in African Americans, while being about ten-times lower in Asians and Hispanics (13). Within ethnic groups, Jews (compared to non-Jewish Caucasians) in the United States have greater risk of developing IBD, with Ashkenazi Jews having the highest risk (14, 15). Some of the strongest evidence for genetic predisposition in IBD comes from twin studies where monozygotic twins have a far greater incidence of disease concordance than dizygotic twins (16-18). Importantly, the incidence of concordance amongst monozygotic twins for CD is approximately 50% and in UC it is approximately 15%. However, the lack of 100% concordance in monozygotic twins demonstrates that nongenetic factors, such as the environment, are involved in the pathogenesis of CD and UC.

In landmark studies published simultaneously out of North America and Europe, human genome wide scans identified the *Nucleotide oligomerization domain 2/Caspase recruitment domain 15* (*NOD2/CARD15*) gene as the first CD susceptibility gene (19, 20). NOD2 mutations are found in 27% to 39% of the CD population compared to 14% to 16% of non-IBD controls, and 12% to 14% of UC patients. Heterozygous carriers of NOD2 variants have a four-fold risk of developing CD; however, homozygous or compound heterozygous carriers run a 40-fold risk (12). Elucidation of the function of NOD2 has highlighted the importance of innate immunity in

the pathogenesis of CD. NOD2 is an intracellular pattern recognition receptor of the innate immune system. Its expression has been described exclusively in three types of cells: macrophages, dendritic cells, and the intestinal epithelial Paneth cell (further described in section 1.5.2). Other potential genetic loci for both CD and UC have been reported and are currently being verified. Elucidation of these specific genetic abnormalities will greatly contribute to our understanding of the pathogenesis of CD and UC, while better defining clinically important subsets of patients.

### 1.1.2 Immune System Dysregulation

Alterations in the intestinal mucosal barrier and amplification of inflammation through the activation of the innate and adaptive immune response are some of the inappropriate responses involved in the pathogenesis of IBD. In an aberrant response to microbial antigens, T cells are activated in CD and UC. As a result, there is increased expression of lymphocyte activation antigens on the cell surface of intestinal lymphocytes of IBD patients (21).

IBD is associated with T cell activation. CD and UC have been distinguished by distinct patterns of T-helper (Th) cell dysfunction. Lamina propria cells of patients with CD overproduce cytokines typically associated with a Th1 response, such as IL-12 and IFN-γ (22, 23). In contrast, a Th2 response has been observed in UC patients, with increased production of IL-5, although without the major Th2 cytokine IL-4 (23, 24). Recent data has demonstrated marked overexpression of the Th2 cytokine IL-13 in UC as compared to CD, which is produced by a novel NK-like T cell population (25). However, analyses on the production of immunoregulatory cytokines in human IBD have been inconsistent. There exists tremendous overlap between individual patients and the cytokines they are able to produce. Therefore,

distinguishing between CD and UC solely on the mere presence of Th1 and Th2 cytokine production is a vast oversimplification of a complicated disease. Additional research is needed to determine whether these T cell pathways differentiate the immunopathogenesis of CD and UC.

IL-12 has been identified to play an important role in Th1 immune responses. IL-12 is a heterodimeric cytokine, comprised of the IL-12 p35 and IL-12 p40 subunits. It is produced by macrophages in response to intracellular pathogens and bacterial products, such as lipopolysaccharide (LPS). IL-12 provides an obligatory signal for the differentiation of Th1 cells and for the secretion of the Th1 cytokine, IFN-γ (26). IL-12 production and Th1 cells are required for cell-mediated immunity and host defense against intracellular microbes (27). Notably, mice with a targeted disruption of the IL-12 p40 gene produce extremely low levels of IFN-γ. These deficient mice are unable to clear infections with intracellular organisms (28). The importance of IL-12 in the generation of a Th1 immune response during human infectious diseases, including leprosy (29), tuberculosis (30), and acquired immunodeficiency syndrome (31), has been well characterized. Although the induction of IL-12 by intracellular organisms is necessary for a protective Th1 immune response, overexpression of Th1 cytokines and IL-12 may contribute to the development and perpetuation of chronic inflammatory and autoimmune diseases (32), as observed in IBD.

Murine models provide strong evidence for the importance of Th1 cytokine regulation and IL-12 production in chronic intestinal inflammation. For example, IL-10-deficient (*IL-10*$^{-/-}$) mice develop weight loss, anemia, and a chronic enterocolitis (33). When these mice are housed under pathogen-free conditions, the enterocolitis is markedly attenuated. This observation suggests that the inflammatory response is triggered by enteric microbes and/or microbial

products. Enterocolitis in *IL-10*$^{-/-}$ mice is likely perpetuated by chronic overexpression of Th1 cytokines, which is reversible, as exogenous IL-10 or antibodies to IL-12 transiently cures the disease. As intracellular bacteria and bacterial products induce IL-12 production by macrophages and dendritic cells, mucosal expression of IL-12 perpetuates Th1 responses with resultant intestinal inflammation.

Direct evidence for the role of IL-12 in chronic mucosal inflammation has emerged from murine models. Intrarectal administration of the haptenating reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS) induces a Th1-mediated colitis in BALB/c mice. Treatment with anti-IL-12 antibodies results in striking clinical and histopathologic improvement in the disease (34). Illustrating the importance of intestinal bacteria in the induction of colonic inflammation, mononuclear cells isolated from the intestines of mice with TNBS colitis proliferate when exposed to bacterial sonicates derived from autologous intestine. Tolerance was restored to autologous bacteria upon treatment with anti-IL-12 antibodies (35). Similarly, mice with targeted disruption of the IL-2 gene develop colitis and autoimmunity that is abrogated when they are raised in a germ-free environment. This colitis is Th1 mediated, and significant levels of IL-12 are detectable. Administration of anti-IL-12 antibody completely prevents the development of colitis (36). These Th1-mediated mouse models of chronic intestinal inflammation share several important histopathologic and immunologic features with the human chronic IBD, CD. Increased production of Th1 cytokines and increased IL-12 levels have been detected in lamina propria mononuclear cells (LPMC) isolated from CD patients (22, 37). Downregulation of mucosal Th1 responses may be an important therapeutic strategy in CD. In activated LPMC from CD patients treated with anti-TNF antibody, decreased IFN-γ production correlated with clinical and endoscopic improvement (38). Thus, an understanding of the

expression of IL-12 will provide insights into the pathogenesis of infectious and inflammatory diseases. Furthermore, this may elucidate new approaches for altering immune responses through manipulation of IL-12 production.

Other inflammatory cytokines have been shown to play integral roles in the pathogenesis of IBD. TNF is a proinflammatory mediator secreted by mucosal macrophages and T cells. Several studies have documented significant increases in TNF in the colonic mucosa of individuals with CD and UC compared with normal controls and stool samples of patients with active CD and UC (39-41). High concentrations of mucosal IL-2 and IL-2 receptors have also been observed in both CD and UC (42). Moreover, patients with CD and UC have demonstrated impaired activity of IL-1 receptor antagonist (RA). Decreased IL-1RA levels are associated with unopposed IL-1 secretion. One study has documented decreased ratios of intestinal IL-1RA to IL-1 to be characteristic of CD and UC, as the ratio in non-IBD inflamed mucosa was similar to that in normal controls (43).

Cytokines, such as TNF and IL-1, appear to induce expression of vascular adhesion molecules. Upregulation of these molecules can recruit leukocytes from the circulation to the site of intestinal mucosal inflammation in CD and UC. It has been reported that there are increased concentrations of endothelial-leukocyte adherence molecule 1 (ELAM-1) in the inflamed mucosa of patients with these disorders (44). ELAM-1 was associated with active inflammation and was found in similar concentrations in active CD and UC. This has lead to the proposal that ELAM-1 is integrally important in sustaining and amplifying inflammation in IBD (44).

Recent work has described important CD4+ regulatory T cell subsets in the intestinal mucosa. They are believed to have a primary role in inhibiting inflammatory responses. For

example, T-regulatory-1 cells make large amounts of IL-10 and some IFN-γ, while Th3 cells produce TGF-β (45). Both IL-10 and TGF-β are potent anti-inflammatory proteins. It has been shown that TGF-β producing T cells likely mediate the phenomena of oral tolerance. This is a state of immune unresponsiveness to intestinal luminal antigens. The significance of the T regulatory subset to IBD is perhaps best represented by experiments where lymphocytes from mice do not proliferate when stimulated with antigens from their own enteric flora, but proliferate when stimulated with flora from other mice. Furthermore, mice with Th1-mediated IBD lose this tolerance to their own enteric flora, demonstrating the importance of regulatory T cell subsets in maintaining intestinal homeostasis (46). This importance is further demonstrated in another mouse model of IBD, the C3H/HeJBir mice, where adoptive transfer of T regulatory cells can inhibit pathogenic antigen specific T cells (47). Regulatory T cell populations are identified by the expression of the transcription factor FoxP3, as well as their ability to directly inhibit inflammatory T cell activation, proliferation, and cytokine production.

A new inflammatory T cell subset known as Th17 cells appear to be important in the pathogenesis of numerous chronic inflammatory diseases including IBD (48, 49). Th17 cells are induced by the IL-12 family member IL-23, sharing a common p40 subunit with IL-12, and produced by macrophages and dendritic cells (48). They are characterized by expression of a recently identified inflammatory cytokine IL-17. Studies have demonstrated the importance of this cell type in mouse models of multiple sclerosis and rheumatoid arthritis. Additionally, this is an important T cell subset in murine IBD and may directly mediate intestinal inflammation that has been previously characterized as "Th1" (49). Therefore, cytokine profiles and elucidation of T cell subsets will likely be useful to determine subgroups within CD and UC rather than to distinguish one entity from the other.

### 1.1.3 Infectious and Environmental Triggers of Mucosal Inflammation

In genetically predisposed individuals, ill-defined initiating events appear to trigger an inappropriately sustained mucosal immune response. Research has suggested that environmental factors contribute to disease. Potential triggers that have attracted the most attention are commensal and pathogenic enteric microbes. Studies have revealed that the onset of disease has been associated with foreign travel, enteric infections, and perinatal viral infections (10, 50). It has been suggested that intestinal flora changes may be involved in the development or promotion of inflammation. There have been reports that during active IBD, there is a significant decrease in aerobic bacteria and *Lactobacillus* (1) and CD patients have been shown to have increased fecal concentrations of as well as elevated serum antibodies against *Eubacteria*, *Peptostreptococcus*, *Coprococcus*, and *Bacteroides* species (10). Though the triggering infection or antigen is only one step in the initiation of IBD, many of the implicated microbial triggers are not detectable even years after disease onset (10). This important observation suggests to the presence of a second source of signals. This second source may perhaps be responsible in perpetuating the inflammatory response and contributing to the chronic nature of this disease.

The study of mouse models of IBD has clearly demonstrated the importance of the bacterial flora in the initiation and perpetuation of IBD. For example, colitis prone mice do not develop IBD if they are maintained in a germ-free environment. However, when these mice are colonized with components of their normal enteric flora, they go on to develop IBD as before (51, 52). Through these investigations, single bacterial strains have been isolated and identified to induce colitis. However, the colitic strain of bacteria in a different animal host may have no effect on the development of colitis (53-55). For example, when the bacterium *Bacteroides*

*vulgatus* is added to a group of five bacteria isolated from CD patients, it induced colitis in HLA-B27 transgenic rats (53). However, this same bacteria mixture did not induce disease in germ-free derived *IL-10*$^{-/-}$ mice (56). This implicates an association between specific bacteria and disease location and severity (57). Furthermore, different commensal bacterial species can selectively initiate immune-mediated intestinal inflammation with different kinetics and anatomic distribution in the same host (58). These observations suggest that the loss of immunologic tolerance to the normal intestinal microflora appears to be confined to specific bacteria and to specific bacterial antigens.

Several observations have suggested a role for bacterial antigens in human IBD. Antibiotic therapies that alter enteric bacterial flora in CD patients and have resulted in clinical improvement (59). Administration of probiotic bacterial strains, which are normal constituents of the enteric flora, have been shown to have anti-inflammatory effects in IBD (60-62). A small study showed the importance of fecal stream and its components in the pathogenesis of CD. They showed by reintroducing a diverted fecal stream into a non-diseased segment of bowel, they can reactivate CD. This implicates the fecal stream and its contents as a source of antigens to cause disease (63). By characterizing serological B cell and mucosal T cell responses, it has been proposed that IBD patients specifically lose tolerance to their own bacterial populations. One analysis demonstrated that in human LPMC, tolerance selectively exists to intestinal flora from autologous but not heterologous intestines, and that this tolerance is broken in IBD patients (64). Though the exact mechanisms of action are unclear, a few hypotheses have been proposed. Several studies have investigated alteration of the bacterial flora with increased ratios of probiotic to aerobic adherent bacteria (65, 66), direct effects on IEC permeability preventing translocation of bacteria and bacterial products (66), and direct anti-inflammatory effects

demonstrated by alterations in cytokine and growth factor production (67, 68). A recent study has suggested that an enteric bacterial flagellin is a B cell and T cell antigen in murine IBD and human CD (69). All together, the genetic composition of the host as well as specific microbial determinants may influence the concomitant immune response and therefore the clinical phenotype of disease observed.

## 1.2 MURINE MODELS OF IBD

Animal models of intestinal inflammation provide a useful tool for investigating IBD. Animal models allow conduct of scientific investigations that could not be feasibly conducted in humans. For example, animal models can be used to dissect specific mechanisms of disease pathogenesis or to evaluate the ability of novel therapeutic approaches to prevent disease onset, or treat active disease. Although available animal models certainly do not always recapitulate human disease, preclinical data obtained from these models have justified the development of novel immunomodulatory therapies in humans (70).

To recapitulate observations from human IBD, various murine models of IBD have been developed and characterized (71). They have been broadly classified into four groups: chemically-induced, genetic knock-outs, immunologic, and spontaneous. Most of these models manifest Th1 mediated intestinal inflammation, sharing several immunologic features with CD, as increased production of Th1 cytokines and IL-12 have been detected.

A wide variety of genetically altered rodents that harbor mutations in immune response genes develop IBD. Most of the models described manifest Th1-mediated intestinal inflammation. For example, 60-80% of mice deficient in the potent anti-inflammatory cytokine

IL-10 develop weight loss, anemia, and a chronic enterocolitis at about 10 weeks of age (72). It has been shown that these mice are prone to colitis only in the presence of the intestinal bacterial flora. Mice that are housed germ-free or treated with antibiotics to eradicate their endogenous flora spares them from the development of colitis, implicating a role for bacteria in the pathogenesis of IBD (56, 73, 74). Enterocolitis in *IL-10*$^{-/-}$ mice is perpetuated by chronic overexpression of Th1 cytokines, which is reversible, as exogenous IL-10 transiently cures the disease.

In a widely used acute model of colitis induced intrarectally by the haptenating reagent TNBS, mice demonstrate a similar Th1 response (71). In this model, colitis develops with mucosal T cells losing tolerance to enteric bacterial antigen. This indicates colitis may be driven by the bacterial antigens provided by the luminal flora (35, 71).

Animal models are useful tools for assessing the efficacy of compounds that could not be feasibly carried out in humans. Furthermore, murine immunological reagents exist to further dissect specific pathways. However, animal models do not always recapitulate human disease. For example, drugs that have been proven to work in animal models, such as the potent anti-inflammatory cytokine IL-10, do not necessarily translate to clear efficacy in human IBD trials (75).

## 1.3 INTESTINAL EPITHELIAL CELLS

IECs make up a single cell layer of cells providing a physical and immunologic barrier against the intestinal microbial flora and the lamina propria, the mucosal immune compartment. The columnar epithelium is derived from stem cells in the basal crypts that differentiate into

absorptive enterocytes, goblet cells, enteroendocrine cells (EECs), and Paneth cells. The absorptive enterocytes makes up ≥95% of the entire epithelial population. Goblet cells secrete a mucus coat which becomes a physical barrier to pathogens, as organism become trapped and are passed in the stool. Paneth cells have been shown to have a role in inflammation by secreting preformed antimicrobial peptides and defensins (76, 77). EECs are activated by neuronal stimulation and discharge their secretions into the blood flowing through the capillary bed. This allows for their stored peptides to appear immediately at the tissue-blood interface. Some of these stored peptides have been shown to mediate leukocyte and monocyte recruitment (78, 79). Although bacteria interact with EECs and T cells are located in close proximity to EECs (80, 81), a direct immunologic function of EEC has not been described to date.

IECs form a physical barrier through tight junction formation. Tight junction proteins maintain the integrity of the epithelial layer and function as an exclusion barrier for pathogens. In IBD, the tight junctions may be altered resulting in a "leaky" epithelium, allowing for the luminal contents to gain access into the lamina propria (82, 83). Regulating tight junction protein function are many cytoprotective factors, including TGF-β, epidermal growth factor, and trefoil peptides (84, 85).

## 1.4 IECS AND BACTERIA

Amongst the immunologic functions ascribed to absorptive enterocytes are cytokine production. IECs are activated in response to a broad range of invasive pathogens such as *Salmonella*, *Shigella*, *Yersinia*, and *Listeria*. Studies have shown infection of human IECs with enteroinvasive bacteria increases the expression and secretion of a number of proinflammatory

cytokines and chemokines (86, 87). These include the chemokine family members IL-8 and MCP-1 and inflammatory cytokines TNF and IL-1β. Secretion of these mediators results in leukocyte and macrophage recruitment and activation. Therefore, activation of inflammatory gene expression in an IEC is an important component and initiator of the innate immune response against enteric pathogens.

However, not all interactions between IEC and bacteria result in an inflammatory response. In fact, interactions between resident bacteria and IEC may profoundly affect IEC gene expression, growth, and differentiation. For example, it has been shown that interaction of resident bacteria with IECs induces gene expression that is required for normal intestinal development and function (88). Also, interaction of certain bacteria with IECs inhibits specific immune responses. It has been shown that the enteric nonpathogenic *Salmonella* strain produce an effector molecule that inhibits inflammation in IECs (89, 90). This indicates transient and even resident bacterial species may contribute and/or alter the host's inflammatory gene response critical in chronic intestinal inflammation.

It has been hypothesized that specific microbes are directly involved in the pathogenesis of IBD. Many specific organisms, including *Mycobacteria*, *Listeria* and measles virus, have been proposed to directly contribute to the development of CD (10, 50). However, detecting immune responses to these organism's antigens have not been reproducible amongst groups. Furthermore, the commensal enteric microbial flora represents an enormous complex ecosystem, outnumbering our own body's total cell population by nearly ten-fold (11). Therefore, many of the microbes contained within this biomass have yet to be identified in IBD because some microorganisms are not culturable *in vitro* and/or there may not be a suitable animal model of infection to effectively study them. Perhaps large scale epidemiologic studies to detect particular

environmental and/or commensal organisms may allow for identification of specific species contributing to disease. If a single organism is to be found, this does not exclude the possibility, even the likelihood that an infectious agent would only explain the presence of IBD in a subset of genetically predisposed patients.

## 1.5 THE INNATE IMMUNE RESPONSE

In 1989, Dr. Charles Janeway put forward the concept of innate immunity, suggesting that host cells have the ability to recognize foreign pathogens through an innate family of receptors (91). Although the exact proteins were unknown at that time, a prediction was made that these potential receptors would be surveyors of the environment and be quick to initiate an immune response against specific repeating patterns found on foreign organisms. These pathogen associated molecular pattern (PAMP) "danger signals" would quickly activate macrophages and dendritic cells to alert the immune system. In 1994, Dr. Polly Matzinger proposed a new source of danger signals, suggesting endogenous factors released from tissues undergoing destruction, such as during necrotic cell death, can also alert the immune system (92). These endogenous damage associated molecular pattern (DAMP) "danger signals" could alert the immune system through direct activation of macrophages and dendritic cells, without the necessity of exposure to foreign substances. The innate immune system functions to deliver a "danger signal" to the adaptive immune system to alert it to the presence of bacteria, microbes, and even tissue damage during inflammatory diseases.

### 1.5.1 Toll-like Receptors

Receptors of the innate immune system that recognize conserved molecular patterns on microbes are known as pattern recognition receptors (PRRs). One evolutionary conserved family of PRRs, the toll-like receptors (TLRs), is a particularly important component of the innate immune system (93). This collection of receptors is unique in that it is hardwired to recognize a pathogen's specific molecular pattern and elicit a swift immune response. TLRs are proteins with an extracellular leucine rich repeat (LRR) domain, one transmembrane domain, and a cytoplasmic Toll domain. The Toll domain has homology to the IL-1R and is sometimes referred to as Toll/IL-1R (TIR) domain. The LRR domain affords the specificity for each TLR to recognize various ligands. PRRs recognize a conserved set of molecular structures expressed on diverse organisms. Hence, the innate immune system has evolved receptors that can recognize essential and unique features of microbial structure. A few PAMP-TLR recognition pairs include bacterial lipoprotein (BLP)-TLR1/2 (94), gram-positive peptidoglycan (PGN)-TLR2/6 (95, 96), gram-negative LPS-TLR4 (97), and flagellin-TLR5 (98). A critical co-receptor of the TLR4 signaling complex is MD2, a novel secreted protein which binds to the extracellular domain of TLR4 and is required for LPS recognition (99). TLRs are able to send a signal through an evolutionary conserved transduction pathway through its Toll domain (Figure 1.2). Signaling intermediates of the MyD88-dependpent pathway includes the adaptor molecule MyD88, IL-1R-associated kinase (IRAK), and TNF-receptor associated factor 6 (TRAF6) (93, 100). This culminates in the activation of the MAPK (ERK, p38, and JNK) and NF-κB (see section 1.5.4) pathways leading to the production of cytokines, chemokines, and adhesion molecules (101-103).

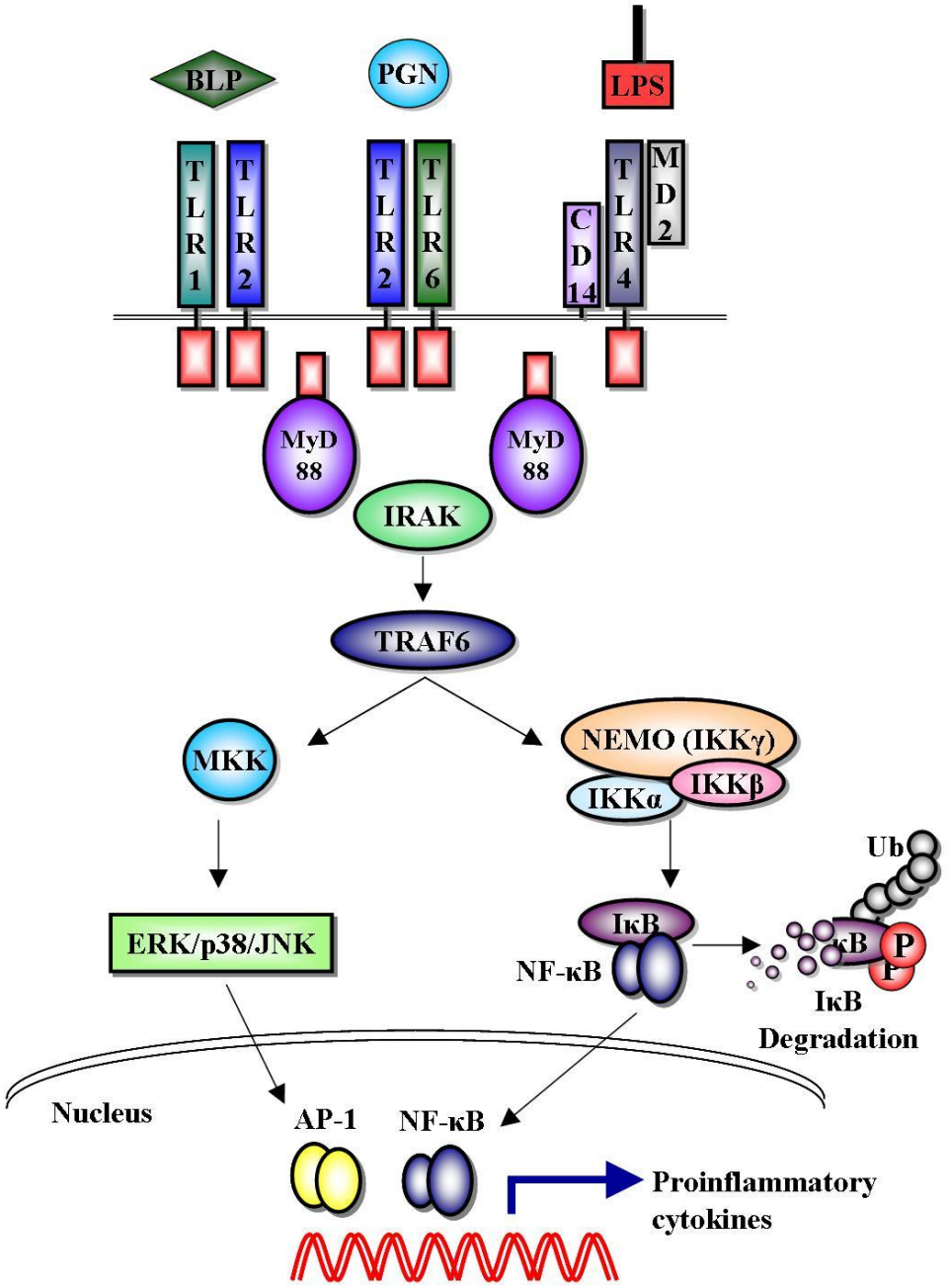


**Figure 1.2: The Toll-like Receptor and NF-κB pathways in the innate immune response.**

Bacterial recognition by IECs is mediated in part through TLRs. *In vitro* studies have shown IEC lines express TLRs which can be enhanced in the setting of inflammatory cues (104, 105). Nonetheless, the expression of TLRs on intestinal epithelia from normal or IBD subjects remains contentious and is not fully reconciled between groups. Previous studies have demonstrated TLR2 and TLR4 on IECs were "barely detectable" in human tissue sections (106). Upregulation of TLR4 in CD and UC, and downregulation of TLR3 in CD but not UC was also described (106). Conversely, another group reported that TLR2 and TLR4 were not expressed in IEC, specifically enterocytes, from normal or inflamed human intestine, and that TLR expression was exclusively confined to lamina propria macrophages from inflamed tissue sections (107). Likewise, there are controversial reports about TLR4 function in human absorptive enterocyte cell lines, with one group reporting LPS responsiveness (108), and another suggesting LPS resistance due to the absence of the co-receptor MD2 (104). Also, a functional role for TLR5 has been suggested from IEC line models (109). Therefore, it is of interest and potential clinical relevance to determine the role of TLRs in IEC innate immune responses. Furthermore, it has been suggested in TLR2-, TLR4-, and MyD88-deficient mice that the intestinal TLR pathway may function to recognize enteric commensals and orchestrate a protective response during intestinal injury and inflammation (110). However, no specific cell type was implicated in this study, leaving open the possibility of newly described functions to existing cell types.

### 1.5.2 NOD2/CARD15

Human genome wide scans have identified the *NOD2/CARD15* gene as the first CD susceptibility gene (19, 20). The *NOD2* gene encodes for an intracellular protein expressed in

the cytoplasm of monocytes, dendritic cells (111) and intestinal epithelial Paneth cells (112, 113). NOD2 is comprised of an N-terminal CARD, a central nucleotide binding domain, and a C-terminal LRR. The LRR is required for activation of an inflammatory process in response to the bacterial peptidoglycan component, muramyl dipeptide (MDP) (114). Following recognition of MDP, oligomerization of NOD2 leads to the recruitment of the CARD containing protein RICK/RIP2 (for RIP-like interacting CLARP kinase/receptor interacting protein-2) and subsequent activation of NF-κB (2, 111).

Initial characterization of CD-associated NOD2 mutations suggested that these mutations were associated with loss of function. In other words, cells containing mutant NOD2 demonstrated diminished MDP-induced NF-κB activation (20). This finding has recently been confirmed in human mononuclear cells from CD patients (115). However, studies from NOD2 deficient and mutant mice provide conflicting results. One study suggests that NOD2 deficient mice are defective in MDP-induced NF-κB activation (116). However, a second study suggests that NOD2 is a negative regulator of TLR2 mediated responses in macrophages (117). NOD2 signaling inhibited TLR2 activation of NF-κB. NOD2 mutations led to an exaggerated NF-κB response through TLR2, which resulted in the expression and secretion of IL-12. This may lead to defective killing of intracellular bacteria by macrophages and dendritic cells with persistent innate and adaptive immune responses stimulated through other pathways (117). In another study, transgenic mice expressing the most common NOD2 insertion mutation in CD were generated. These mutant mice demonstrated increased NF-κB activation in response to MDP (118). Although the functional effects of NOD2 mutations on NF-κB activation in CD is still controversial, the experimental evidence suggests that a hallmark of IBD is activated NF-κB, and specific targeting of this pathway is an important therapeutic approach.

### 1.5.3 Danger Signals: PAMPs and DAMPs

TLRs were originally described to detect constitutive and conserved products of microbial metabolism, termed PAMPs (93). Collectively, these are prototype "exogenous danger signals" for the immune system, as they originate from outside of the body. PAMPs have come to the forefront of attention as critical environmental factors in initiating and driving chronic inflammation in IBD. Examples of these molecules are the TLR ligands LPS, BLP, and flagellin. PAMPs make ideal targets for innate immune recognition and stimulation for three reasons (93). First, PAMPs are produced only by microbes and not by host cells. Second, they are invariant between microorganisms of a given class. Third, PAMPs are essential for microbial survival, where mutations or loss are either lethal or greatly reduces adaptive fitness for the microbe.

Research in IBD has long focused on the inflammatory response generated by the enteric flora, the so called "exogenous danger signals." However, IBD still can be present even in the absence of the initiating microbial source (10). This suggests that another signal may arise and be responsible for maintaining the inflammatory state. DAMPs have come to the forefront of attention as critical "endogenous danger signals" in perpetuating inflammation (92, 119). Works in other disease states mediated by cytokines are identifying signals secreted by our own cells after the presence of danger and/or tissue damage. Heat-shock proteins, high mobility group box 1 (HMGB1), S100 proteins, and uric acid, to name a few, are prototypic members of the DAMP family (119-121).

HMGB1, originally described as a non-histone, chromatin-associated nuclear protein, has emerged as a critical endogenous danger signal produced by host cells (122, 123). It is released during cellular necrosis and is secreted from activated cells (124-126). HMGB1 functions as a

diffusible signal of unprogrammed cell death, triggering inflammatory signaling pathways in neighboring cells (127). The first described receptor for HMGB1 was the receptor of advanced glycation end products (RAGE) (128). In addition, recent studies have demonstrated that HMGB1 also utilizes PRRs of the innate immune system, TLR2 and TLR4 (129, 130). These pathways can lead to activation of NF-κB and the production of potent proinflammatory signals, producing TNF, IL-1, IL-6, and IL-8 (127), and alter gut permeability, a defining feature of chronic IBD (131). Potential relevance of HMGB1 in IBD was suggested in studies performed by our colleagues in models of acute inflammation. These findings suggest HMGB1, and other DAMPs, may provide a second wave of inflammatory signals in the stressed intestinal epithelium and further perpetuate and sustain the inflammatory response through the release of more Th1 mediators. DAMPs, such as HMGB1, may play an important and virtually unexplored role in IBD.

### 1.5.4 NF-κB

Transcription factors are downstream targets of signal transduction pathways that bind to genomic DNA in the promoters of genes. Many families of transcription factors have been identified to play essential roles in acute and chronic inflammation through the regulation of expression of multiple inflammatory mediators. In the inflammatory response, the best characterized family of transcription factors is NF-κB. It is considered to be a "master switch" for inflammatory gene expression (101, 102).

In unstimulated cells, NF-κB proteins are localized in the cytoplasm through their association with inhibitory proteins known as IκB proteins. IκB proteins bind to NF-κB and mask their nuclear localization signals. Proinflammatory cytokines such as TNF and IL-1β,

PAMPs such as LPS, and DAMPs such as HMGB1 induce phosphorylation and ubiquitination of IκB proteins, targeting them for degradation by the proteosome. The phosphorylation of IκB proteins is a key step in the regulation of NF-κB. Phosphorylation is mediated by a specific IκB kinase (IKK) complex whose activity is induced by activators of NF-κB (102). IKK is made up of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit named "NF-κB essential modulator" (NEMO; also named IKKγ) (132). Activation of the IKK complex in response to proinflammatory mediators depends critically on the presence of the NEMO subunit of the IKK complex, as NEMO deficient cells lack detectable NF-κB binding activity in response to TNF, IL-1β, and LPS (133).

Activation of IKK leads to the release of bound NF-κB, which translocates to the nucleus. There, NF-κB can bind to NF-κB DNA binding sites on enhancer elements of target genes and induce transcription of inflammatory mediators. NF-κB plays an essential role in the inflammatory response through the regulation of genes encoding proinflammatory cytokines (IL-1β, TNF, IL-12), chemokines (IL-8, MIP-1α, MCP-1), and adhesion molecules (ICAM-1, VCAM-1, E-selectin) (101-103).

Activation of NF-κB has been shown to be important in many chronic inflammatory diseases such as asthma, rheumatoid arthritis, and IBD (103). The critical role of NF-κB in chronic intestinal inflammation is best illustrated by non-specific as well as selective blockade of NF-κB activation in animal models of IBD (134-137). The first studies demonstrating the activation of NF-κB in chronic intestinal inflammation were performed in the TNBS mouse model of induced colitis. Increased NF-κB DNA binding activity in nuclear extracts from lamina propria macrophages of mice with TNBS colitis have been described. The activated NF-κB complexes consisted of the p65 and p50 subunits. As NF-κB p65, but not p50, mediates

transcriptional activation of inflammatory genes, this study next tested the effects of a specific p65 antisense phosphorothioate oligonucleotide. A p65 antisense oligonucleotide specifically and dose-dependently downregulated p65 mRNA and protein, which was accompanied by reduced secretion of IL-1, IL-6, and TNF by LPS-stimulated lamina propria macrophages from mice with TNBS colitis. Strikingly, *in vivo* administration of p65 antisense abrogated established TNBS induced colitis following a single intravenous injection. Moreover, local administration of p65 antisense oligonucleotides into the colon of mice with TNBS colitis successfully treated established colitis (134, 135). Recently, local administration of antisense p65 oligonucleotides blocked intestinal inflammation as well as fibrosis in the TNBS model (137). In spontaneously occurring colitis in *IL-10*$^{-/-}$ mice, increased NF-κB DNA binding activity and increased p65 protein expression were found in lamina propria macrophages. Again, the essential role of p65 in maintaining chronic intestinal inflammation was demonstrated by successful treatment of established colitis in these mice with p65 antisense oligonucleotides. Treated mice showed a reduction in macroscopic and histological signs of colitis (135).

Based on animal models, it is not surprising that activated NF-κB is found in human intestinal inflammation and IBD. A significant increase in p65 protein in lamina propria macrophages and epithelial cells from CD patients was correlated with increased production of the inflammatory cytokines IL-1β, IL-6, and TNF (138). *In vitro* treatment of lamina propria macrophages from CD patients with p65 antisense oligonucleotides was more effective in down-regulating cytokine production than treatment with 5-aminosalicylates or glucocorticoids, suggesting a key role for NF-κB p65 in inflammatory cytokine expression in CD (138). Subsequently, higher levels of NF-κB p65 were demonstrated in nuclear extracts of lamina propria biopsy specimens from CD patients compared to normal controls or patients with UC. In

this study, increased nuclear p65 from CD patients was attributed to activation and nuclear translocation of NF-κB as total levels of NF-κB p65 from whole cell extracts did not differ between controls and IBD patients. Importantly, increased DNA binding activity of NF-κB was demonstrated in nuclear extracts from biopsy specimens as well as isolated LPMC from CD and UC patients which confirmed the activation of NF-κB in IBD. Furthermore, NF-κB p65 was identified as part of the DNA binding complex (139). Activated NF-κB has been reported in macrophages and epithelial cells from inflamed mucosa of patients with IBD *in situ* using a specific p65 antibody that exclusively detects the activated form of NF-κB (140). The number of cells positively stained for activated p65 correlated with the degree of mucosal inflammation. No significant differences were found between sections of inflamed mucosa from patients with CD, UC, or diverticulitis (140). This study indicated that activation of NF-κB is not specific for the pathophysiology of IBD: NF-κB activation could represent an important step in mucosal inflammation regardless of the etiology. Therefore, downregulation of NF-κB activity emerges as a potential key event in the control of chronic intestinal inflammation in humans and strategies to inhibit NF-κB activity more specifically are desirable.

## 1.6 STATEMENT OF THE PROBLEM

In this dissertation we utilized descriptive, functional, and genetic approaches to determine the significance of exogenous and endogenous danger signals in the pathogenesis of IBD. Under normal conditions, the IECs remain in a quiescent, suppressed state and are unable to mount an immune response to the intestinal microflora. However, upon the presence of invasive pathogenic bacteria, IECs upregulate TLRs, induce proinflammatory gene expression, secrete

cytokines, and recruit inflammatory cells to the site of injury. If these responses persist, the normal suppressive state of the IECs may be altered, resulting in the activation of innate responses to the normal intestinal microflora. It is in this chronic inflammatory setting that signals are released by activated and necrotic cells. This second source of signals may perpetuate the mucosal inflammatory response, leading to IBD.

We first attempted to better understand the TLR expression in the intestinal epithelium to determine cell types that may respond to danger signals. We found that the expression of TLRs was confined to epithelial cells expressing serotonin. This suggests a role for EECs and, perhaps their stored neuropeptides, in the innate immune response. We tested the hypothesis that an EEC line is capable of responding to PAMPs. This was assayed by looking at bacterial activation of the MAPK and NF-κB pathways and at release of neuropeptides in this specific cell type.

Although much attention has been given to the central role played by PAMPs in the form of the enteric bacterial flora in IBD pathogenesis, little is known about DAMPs in IBD. We looked at the role of HMGB1 as a putative endogenous danger signal in macrophage activation and IBD. We hypothesize that HMGB1 plays a pivotal and necessary role in the disease process, previously underappreciated in much of the pathobiology of the gut. Studies targeting HMGB1 have been shown to ameliorate end organ damage when used to treat acute inflammation. We investigated the use of ethyl pyruvate, which has been demonstrated to inhibit HMGB1. We predict that ethyl pyruvate will improve Th1-mediated murine enterocolitis through mechanisms that include inhibition of HMGB1 secretion and/or function.

Lastly, we looked at the signaling pathway associated with DAMP and PAMP activation in the stressed gut. The two sources of danger signals are recognized by the common TLR family signaling pathway. This culminates in the activation of an important and prominent

downstream effector of DAMP and PAMP signaling, NF-κB. We hypothesized that inhibiting activated NF-κB with a short cell permeable peptide will inhibit chronic enterocolitis in *IL-10*$^{-/-}$ mice, leading to significant histologic improvement.

Through these experiments, we have gained new insights into the pathogenesis of IBD. We now understand that EECs express TLRs and have begun to look at the role of an endogenous danger signal, HMGB1. New therapeutic interventions uncovered during the course of this project provide a beginning to discern whether these intestinal inflammatory responses are relevant targets in human IBD.

# 2.0 ENTEROENDOCRINE CELLS EXPRESS FUNCTIONAL TOLL-LIKE RECEPTORS

## 2.1 ABSTRACT

Intestinal epithelial cells (IECs) provide a physical and immunologic barrier against the enteric microbial flora. Toll-like receptors (TLRs), through interaction with conserved microbial patterns, activate inflammatory gene expression in cells of the innate immune system. Previous studies of expression and function of TLRs in IECs report varying results. Therefore, TLR expression was characterized in human and murine intestinal sections, and TLR function tested in an IEC line. TLR1, TLR2, TLR4 are co-expressed on a subpopulation of human and murine IECs that reside predominantly in the intestinal crypt and belong to the enteroendocrine lineage. Enteroendocrine cell (EEC) lines demonstrate a similar expression pattern of TLRs as primary cells. A murine EEC line, STC-1, was activated with specific TLR ligands: lipopolysaccharide (LPS) or synthetic bacterial lipoprotein. In STC-1 cells stimulated with bacterial ligands, NF-κB and MAP kinase activation is demonstrated. Furthermore, expression of TNF and MIP-2 are induced. Additionally, bacterial ligands induce expression of the anti-inflammatory gene TGF-β. LPS triggers a calcium flux in STC-1 cells, resulting in a rapid increase in cholecystokinin secretion. Finally, conditioned media from STC-1 cells inhibit the production of NO and IL-12 p40 by activated macrophages. In conclusion, human and murine IECs that express TLRs

belong to the enteroendocrine lineage. Using a murine EEC model, a broad range of functional effects of TLR activation is demonstrated. This study suggests a potential role for EECs in innate immune responses.

## 2.2 INTRODUCTION

Host defense against microorganisms relies on both innate and adaptive immune responses. The molecular components of innate immunity are evolutionarily conserved over an extraordinary spectrum of life forms, from plants to mammals (141). Receptors of the innate immune system are known as pattern recognition receptors (PRRs)[2]. PRRs identify and bind to a conserved set of molecular structures expressed on diverse organisms. Hence, the innate immune system has evolved receptors that can recognize essential and unique features of microbial structure, such as lipopolysaccharide (LPS), teichoic acids, mannans, and double stranded RNA (93, 142). One evolutionarily conserved family of PRRs, the toll-like receptors (TLRs), is a particularly important component of the innate immune response. TLRs are transmembrane molecules composed of multiple extracellular leucine rich repeats, a single transmembrane domain, and an intracellular signaling domain. The signaling domain has been termed Toll/IL-1 receptor (TIR) related because conserved motifs are present in the IL-1 receptor family. Furthermore, TLRs and IL-1 receptors activate the transcription factor NF-κB and inflammatory gene expression through an evolutionarily conserved signal transduction pathway (141).

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[2] Abbreviations used in this chapter: BBS, bombesin; BM, bone marrow; CCK, cholecystokinin; CD, Crohn's disease; DAB, diamino-benzidine; DSS, dextran sodium sulfate; EEC, enteroendocrine cell; FITC, fluorescein isothiocyanate; HSP, heat shock protein; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; LPS, lipopolysaccharide; NO, nitric oxide; PRR, pattern recognition receptor; sBLP, synthetic bacterial lipoprotein; TIR, Toll/IL-1 receptor; TLR, toll-like receptor; UC, ulcerative colitis; VIP, vasointestinal protein.

Intestinal epithelial cells (IECs) serve as a physical and immunologic interface with the microbial flora that colonize and traverse the intestinal lumen. Amongst the immunologic functions ascribed to IECs are cytokine production upon intracellular infection with pathogenic intestinal bacteria (86, 87, 143). This implicates IECs as important participants in innate immune responses of the intestinal mucosa. However, the mechanisms involved in bacterial-epithelial interactions are unknown. Therefore, it is of great interest and potential clinical relevance to determine the role of TLRs in IEC innate immune responses. In human tissue sections TLR2 and TLR4 were "barely detectable" on IECs from human tissue sections (106). Upregulation of TLR4 in the inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis (UC), and downregulation of TLR3 in CD but not UC was also described (106). Conversely, another group reported that TLR2 and TLR4 were not expressed in IEC from normal or inflamed human intestine, and in fact TLR expression was confined to lamina propria macrophages from inflamed tissue sections (107). Likewise, there are controversial reports about TLR4 function in human IEC lines, with some reporting LPS responsiveness (108, 144), while others suggest LPS resistance due to the absence of TLR4 and/or the co-receptor MD2 (104, 105, 145), and a third report hypothesizing tolerance to TLR signaling in IECs due to induction of the inhibitory signaling molecule Tollip (144). A functional role for TLR5 has been suggested and one report describes hyporesponsiveness of TLR2 in IEC lines (108, 109, 146). Therefore, the nature of intestinal epithelial TLR expression and function remains unclear.

Recently, TLRs were demonstrated to have a counterintuitive role in regulating mucosal inflammation and maintaining homeostasis. In an acute inducible model of IBD, TLR2-, TLR4-, and MyD88-deficient mice challenged with dextran sodium sulfate (DSS) developed severe colitis and decreased survival compared to wild-type mice (110). Additionally, antibiotic-

treatment to deplete the intestinal flora decreased expression of cytoprotective proteins (110). Together, these findings suggested that intestinal TLRs may function to recognize enteric commensals and orchestrate a protective response during intestinal injury and inflammation. However, while demonstrating the biological significance of TLR in murine intestine, this study does not specify what cell type expresses TLRs that are responsible for such biological effects.

In this study, abundant expression of multiple TLRs is demonstrated on human and murine IECs that reside primarily in the crypt and belong to the enteroendocrine lineage. To study possible functional consequences of TLR expression and activation, the enteroendocrine cell (EEC) line STC-1 was utilized as a model system. STC-1 cells stimulated with bacterial ligands, LPS and synthetic bacterial lipoprotein (sBLP), demonstrate activation of NF-κB and MAPK signaling pathways, resulting in production of MIP-2 and TNF. Furthermore, bacterial ligands induce expression of the anti-inflammatory gene TGF-β. LPS triggers a calcium flux in STC-1 cells, leading to increased secretion of cholecystokinin (CCK). To implicate a role for EECs in mucosal immune responses, conditioned media from STC-1 cells inhibit the production of nitric oxide (NO) and IL-12 p40 by activated macrophages. This study suggests a potential role for EECs in innate immune responses, and that EEC-bacterial interactions help maintain mucosal immune homeostasis.

## 2.3 MATERIALS AND METHODS

**Cell lines and reagents.** The murine EEC line STC-1 (Mark Babayatsky, The Mount Sinai Medical School, NY) was maintained in DMEM with 4.5 g/l glucose and L-glutamine supplemented with 10% FBS (Gemini), 1% penicillin/streptomycin (Cellgro) and kept in a

humidified incubator at 37°C, 5% $CO_2$. Human embryonic kidney HEK293T, human intestinal epithelial Caco-2, and the human enteroendocrine Colo320DM and BON cell lines grown in subconfluent monolayers in DMEM with 4.5 g/l glucose and L-glutamine supplemented with 10% FBS were utilized to assess TLR expression as indicated in the Results. rTGF-β1 was purchased from R&D Systems. The synthetic bacterial lipoprotein (sBLP) $Pam_3Cys$-SKKKK was purchased from EMC Microcollections (Tuebingen, Germany). Lipopolysaccharide (LPS) from *Salmonella enteritidis* was purchased from Sigma and was repurified to eliminate TLR2 contaminating ligands by modified phenol extraction as previously described (147).

**Expression plasmids.** A multimerized NF-κB DNA binding element luciferase reporter was obtained from Dr. Adrian Ting (The Mount Sinai School of Medicine, NY). A heat shock protein (HSP) promoter-β-galactosidase reporter plasmid was used to normalize for transfection efficiency as previously reported (148).

**Murine macrophages.** Bone marrow (BM) from femurs of C57BL/6 mice were flushed with washing medium (RPMI1640 with 1% Pen/Strep), passed through a 70 μM nylon cell strainer into a 50 ml conical tube, and spun down at 1500 rpm for 5 minutes. RBCs were lysed using sterile-filtered 0.8% ammonium chloride, washed twice with washing medium, and resuspended in complete medium (washing medium with 10% FBS). BM cells were seeded in complete medium in a 150 mm dish and differentiated using recombinant murine GM-CSF (20 ng/ml) (R&D Systems). At day three, another 25 ml fresh culture medium containing GM-CSF was added to the culture plates. At day seven, the cells, representing the BM-derived macrophage population, were harvested. For peritoneal macrophages, 3 ml of 3% thioglycollate was injected

intraperitoneally into C57BL/6 mice. Five days later mice were euthanized and adherent cells isolated from peritoneal exudate fluid were utilized in experiments.

**Transient gene expression assays and cell activation.** STC-1 cells were plated in 6-well plates. Once 50-80% confluent, cells were transiently transfected using the Superfect (Qiagen) reagent following the manufacturer's protocol. Cells were transfected with the reporter genes NF-κB-luciferase (0.6 μg) and HSP-β-galactosidase (0.5 μg) in 1.1 ml. Following transfection, cells were activated with bacterial ligands. Eight hours after activation, cells were lysed in reporter lysis buffer and luciferase activity was measured with a luciferase assay system (Promega) using a Turner Designs Luminometer TD20/20. Transfection efficiency was assessed by β-galactosidase activity in the same cell lysates. The ratio of the two parameters (luciferase/β-galactosidase) was used for data presentation as previously reported (148).

**MAP kinase (MAPK) activation.** Cells were stimulated for times indicated in Figure 2.6. Cells were harvested and lysed as described previously (149). Whole cell lysates were immunoblotted for phosphorylated ERK1/2 MAPK and total ERK2 MAPK using specific polyclonal antibodies (Santa Cruz).

**Immunohistochemistry.** Paraffin-embedded tissue sections of histologically normal human colon and ileum derived from surgical resections of patients with colon cancer were deparaffinized and microwaved in antigen unmasking solution (Vector Laboratories) for 15 minutes. If cryosections were used, slides were fixed in 10% formalin. Intestinal sections were procured from the Department of Pathology, The Mount Sinai Medical Center, deidentified of

any patient data, as part of an IRB approved tissue bank. C57BL/6 murine colonic sections were fixed in 2% paraformaldehyde and incubated overnight in 30% sucrose prior to cryosectioning.

For diamino-benzidine (DAB) staining, slides were placed in 0.3% $H_2O_2$-methanol for 10 minutes to block endogenous peroxidase activity. Slides were preincubated with PBS containing 5% serum of the same allotype as the secondary antibody. Isotype-matched purified immunoglobulin or primary antibody was applied. Slides were washed in PBS and incubated with a secondary antibody against the Fc-fragment of the primary antibody. Depending on the immunohistochemistry technique, secondary antibodies were labeled with a fluorescent marker (Alexa-488, Cy3, fluorescein isothiocyanate or FITC, and Texas Red), biotin, or alkaline phosphatase. After incubation with biotin-labeled antibodies, horseradish peroxidase-streptavidin was applied to the slides followed by DAB solution (ImmunoCruz Staining System, Santa Cruz Biotechnologies). If alkaline phosphatase labeled antibody was used, slides were incubated with levamisole solution (Vector Laboratories) to block human alkaline phosphatase, and the slides were stained using the Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories).

Antibody binding to antigen was neutralized by pre-absorption with specific blocking peptides. Anti-TLR1 and anti-TLR2 antibodies were pre-incubated with a five-fold excess of corresponding blocking peptides (Santa Cruz Biotechnologies) overnight at $4^{o}$ C. Following, the antibody-peptide solution was applied to the slides.

Nuclei were counterstained with Harris Modified Hematoxylin (Fisher) or with Hoescht stain. Slides were dehydrated and mounted using Vectashield mounting medium with DAPI (Vector Laboratories), Gelvatol for fluorescent dyes, or Immu-mount (Shandon, Pittsburgh, PA) for DAB and Red dyes.

Slides were visualized with a microscope configured for fluorescence imaging (Olympus BX60 or Olympus Provis), fitted with a cooled CCD color camera and frame grabber (Optronix or Magnifier) or confocal laser scanning microscope (Leica TCP-SP), and analyzed using Metamorph (Molecular Devices, Corp., Sunnyvale, CA).

Antibodies and their working concentrations. Anti-human TLR1, TLR1-biotinylated, TLR2, TLR4, and TLR6 antibodies (goat polyclonal, Santa Cruz Biotechnologies) were used at concentrations between 2-5 μg/ml, anti-human TLR4 and anti-TLR4/MD2 antibody (rabbit polyclonal, eBioscience) were used at a concentration of 5 μg/ml. All anti-human TLR antibodies used (except TLR1) were cross-reactive against mouse TLRs. Anti-serotonin antibody (NCL-SEROTp, rabbit polyclonal, Novocastra, UK or rabbit polyclonal, Calbiochem) was diluted 1:200 or 1:500, respectively. Anti-multi-cytokeratin 4/5/6/8/10/13/18 antibody (NCL-C11, mouse monoclonal, Novocastra) antibody was diluted 1:10. Biotinylated secondary antibodies were obtained from Santa Cruz Biotechnologies.

**RT-PCR, nitrite determination, and ELISA.** Cells were harvested by TRIzol (Invitrogen) and total RNA was isolated as the manufacturer's protocol. For TLR mRNA detection, RNA samples were treated by DNaseI (Ambion). For reverse transcription, a SuperScript Preamplification System (Invitrogen) was used. PCR amplification was performed with Taq polymerase (Promega). Primer design and specific conditions are described in Table 1. All sequences were designed based on cDNA sequences obtained from GenBank database. For ELISA, STC-1 supernatants were removed and assayed for the presence of TNF (BD Pharmingen), MIP-2, and TGF-β (R & D Systems) following the manufacturer's protocol. The stable nitric oxide (NO) metabolite, nitrite, was measured using the Griess reagent as previously

described (150). Values were measured using a plate reader and SOFTMax Pro v4.0 software (Molecular Devices).

**Table 2-1.** Primers used for RT-PCR on murine genes.

| Gene | Forward Primer | Reverse Primer | Annealing T(°C) | Cycle Number | Product Size (bp) |
|---|---|---|---|---|---|
| TLR1 | ccgaatctcttcggcacgtt | taacttgggacgggcacagc | 58 | 38 | 501 |
| TLR2 | atggtgacctccgagcgtgt | gagcctcggaatgccagctt | 60 | 36 | 571 |
| TLR4 | ggcatggcatggcttacacc | attctcccaagatcaaccgatgg | 58 | 38 | 500 |
| TLR6 | tgctcccagttgctcacttgc | gggcacaccatgtggatgaa | 60 | 36 | 559 |
| MD2 | ttctgcaactcctccgatgcaa | tccctcgaaagagaatggtattgatg | 58 | 36 | 300 |
| TNF | ccaggcggtgcctatgtctc | agcaaatcggctgacggtgt | 60 | 32 | 407 |
| MIP-2 | gtgctgcactggtcctgctg | ggcttcagggtcaaggcaaa | 58 | 36 | 221 |
| TGF-β | ctcccactcccgtggcttct | gctctgcacgggacagcaat | 58 | 38 | 427 |
| β-actin | cctaaggccaaccgtgaaaag | tcttcatggtgctaggagcca | 56 | 21 | 646 |

**CCK Enzyme Immunoassay (EIA).** STC-1 cells were pretreated for 30 minutes in the presence or absence of 20 μM BAPTA-AM (Calbiochem) and then stimulated for 10, 30, 60, or 180 minutes with media, LPS, sBLP, or with bombesin (AnaSpec, San Jose, CA). Supernatants were centrifuged to remove cellular debris and analyzed for CCK by EIA following the manufacturer's protocol (Phoenix Pharmaceutical, Belmont, CA).

**Calcium Imaging.** STC-1 cells in 6-well plates were loaded in $Ca^{2+}$-/$Mg^{2+}$-free PBS (Invitrogen) containing 10% FBS and 5 μM fura-2AM (Molecular Probes) diluted from a 1

mM/DMSO stock for 15-20 min at 37°C. Following loading, cells were thoroughly rinsed with 10% FBS/PBS. For fluorescence recording, we used a BX61WI Olympus Optical (Tokyo, Japan) microscope, a CCD camera (Hamamatsu, Shizouka, Japan) and a Lambda-LS xenon arc lamp light source (Sutter, Novato, CA). Cells were imaged at room temperature using Compix Inc. imaging systems and SimplePCI software (Compix Inc., Cranberry, PA) at excitation wavelengths of 340/380 nm at a rate of 1 frame/3 sec before and during application of 10 μg/ml LPS.

**Statistical Analysis.** Statistical significance between groups was assessed by two-tailed Student's *t* test. A *p*-value equal or less than 0.05 was considered to be statistically significant.

## 2.4 RESULTS

**A human crypt intestinal epithelial cell co-expresses TLR1, TLR2 and TLR4.** Functional TLR2, TLR3, TLR4, and TLR5 gene products are expressed in IEC lines, but the distribution of TLRs in primary human IECs is more controversial. Therefore, the expression of TLR1 through TLR5 was determined in human intestinal epithelium by immunohistochemistry using intestinal specimens derived from surgical resections. Specific polyclonal antibodies to human TLR1, TLR2, TLR3, TLR4, and TLR5 were utilized in these studies. A crypt cell demonstrating strong immunoreactivity for the combination of TLR1 (Figure 2.1A), TLR2 (Figure 2.1B) and TLR4 (Figure 2.1C) was identified in uninflamed ileum (n = 5 of 5 specimens, Figure 2.1B) and colon (n = 9 of 9 specimens, Figure 2.1A, C). TLR expressing cells are almost exclusively located in

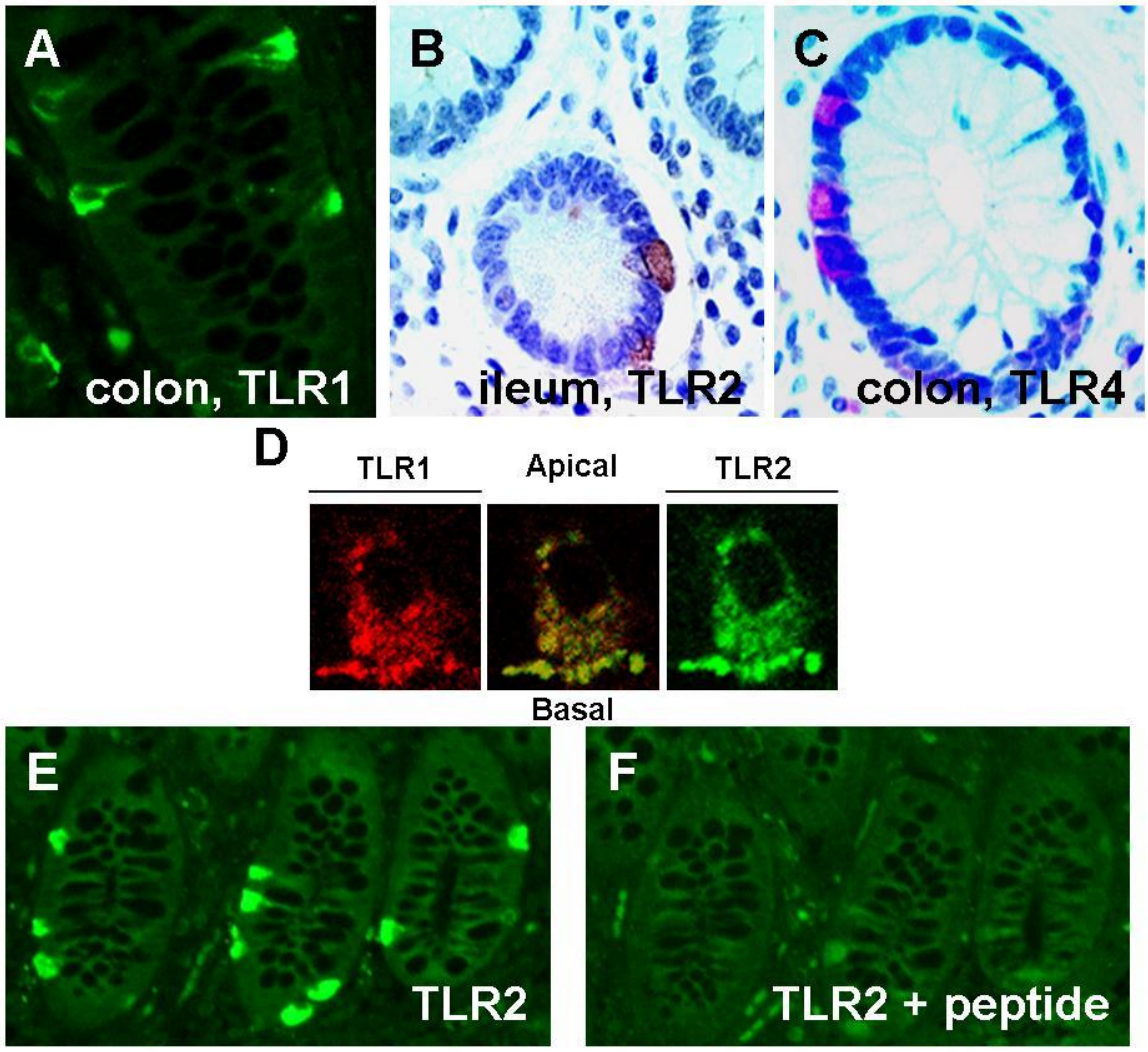


**Figure 2.1: TLR1, 2, and 4 are co-expressed by a human IEC that mainly resides in the crypts.**

Paraffin-embedded tissue sections of histologically normal human colon (n = 9 of 9 specimens) and ileum (n = 5 of 5 specimens) were immunostained with specific antibodies as detailed in the methods (A-C). (A) Colon; anti-TLR1 (FITC). (B) Ileum; anti-TLR2 (DAB). (C) Colon; anti-TLR4 (Red Alkaline Phosphatase Substrate). (D) Double staining of colon with anti-TLR1, Texas Red (left) and anti-TLR2, FITC (right) antibodies and a superimposed image of both (middle). A single epithelial cell located in a crypt is visualized by confocal microscopy. Preincubation of anti-TLR2 antibody with its corresponding blocking peptide abrogates immunoreactivity (E, F). Anti-TLR2 antibody was pre-incubated with a five-fold excess of corresponding blocking peptide. Following neutralization, the antibody/peptide solution was applied to the slides. (E) Colon stained with goat anti-TLR2 antibody (FITC). (F) A serial section of the same specimen stained with anti-TLR2 antibody preincubated with TLR2 blocking peptide (FITC).

the crypts (2-5 cells per crypt). Co-localization of TLR1 with TLR2 (Figure 2.1D) and TLR1 and TLR4 (data not shown) immunoreactivity is demonstrated by fluorescent double-labeling. No immunoreactivity is observed with an antibody to TLR3 or TLR5 in IEC or in the lamina propria (data not shown). TLR1-, TLR2-, and TLR4-staining IECs are observed in experiments performed on paraffin-embedded sections (Figure 2.1, 2.2) following antigen recovery and on frozen sections (data not shown). Immunoreactivity for TLR1, TLR2 and TLR4 is specific, as staining is not detected with species- and isotype-matched control primary antibodies (data not shown). Importantly, TLR1 (data not shown) and TLR2 (Figure 2.1E-F) immunoreactivity is abrogated by pre-incubating the respective antibody with specific blocking peptides. As a final specificity control, antibodies for TLR1, TLR2, and TLR4 detect their respective human receptor expressed in transfected HEK293T cells by immunohistochemistry, but do not cross-react with other TLR family members (data not shown).

In the lamina propria of intestinal tissue sections, TLR immunoreactivity of cells morphologically consistent with macrophages is present. However the intensity of staining is much lower than the crypt epithelial cell (Figure 2.1, 2.2). No other cells in the epithelium or lamina propria stain with anti-TLR1, 2, 3, 4 or 5 antibodies (Figure 2.1, 2.2, and data not shown).

**TLR expression in intestinal epithelial cells co-localizes with the enteroendocrine marker serotonin.** To further morphologically characterize IECs that express TLRs, co-staining experiments were performed with TLR antibodies and a monoclonal antibody to an epithelial cell marker, multi-cytokeratin. Co-localization of TLR2 immunoreactivity with NCL-C11 confirms that these cells are in fact IECs (Figure 2.2A-C). The IECs expressing TLRs are

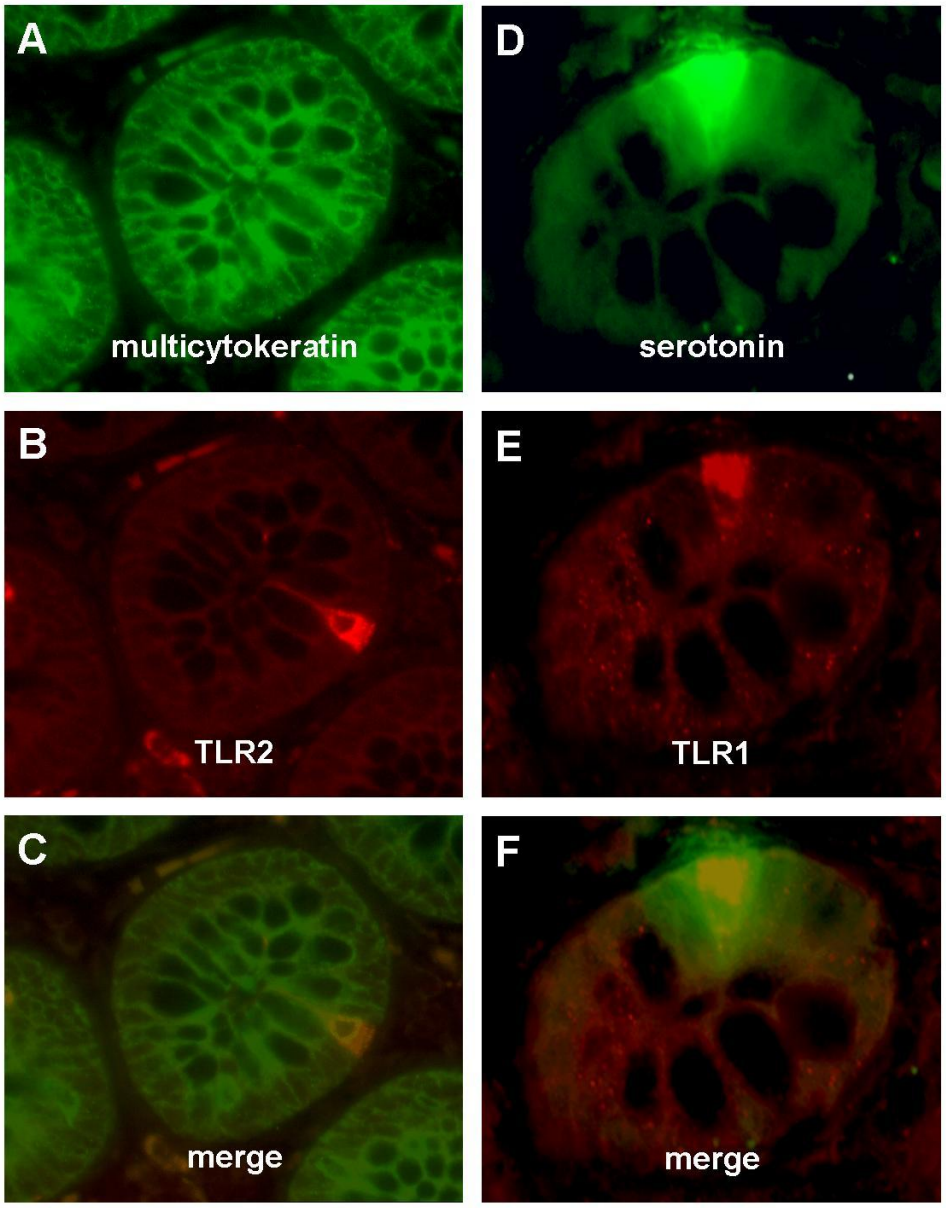


**Figure 2.2: TLR-immunoreactive cells in human intestinal specimens express an EEC marker, serotonin.**

(A-C) Double-staining of colon with (A) anti-multicytokeratin, FITC, an intestinal epithelial cell marker, and (B) anti-TLR2, Texas Red antibodies. (C) A superimposed image of (A) and (B). TLR-immunoreactive cells in human intestinal specimens produce serotonin. (D-F) Double staining of colon with (D) anti-serotonin, FITC, an enteroendocrine cell (EEC) marker and (E) anti-TLR1, Texas Red antibodies. (F) A superimposed image of (D) and (E). The IECs expressing TLRs have features reminiscent of the EEC lineage, including pyramidal shape with a large basolateral surface, an apically displaced nucleus, and a granular appearance. A representative image is shown.

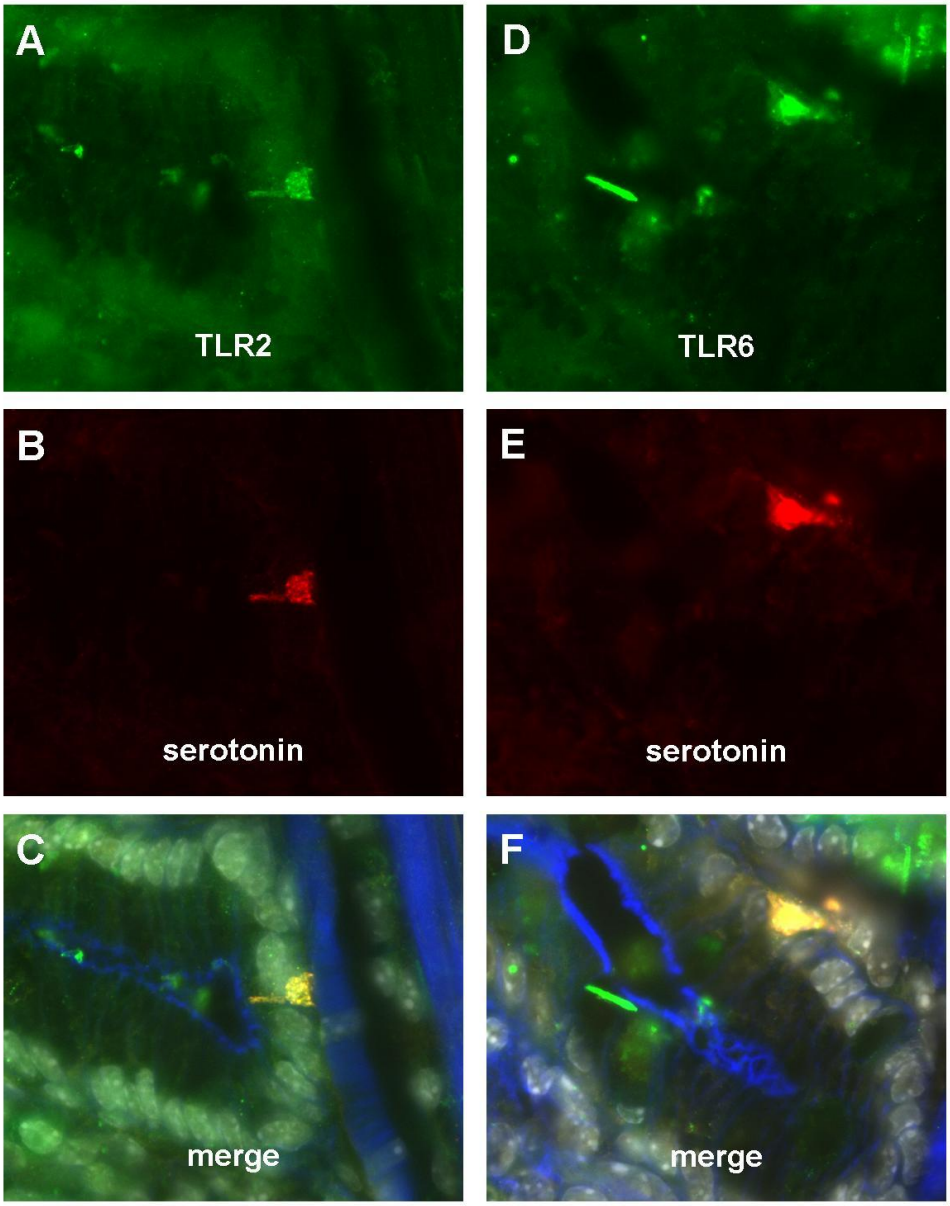


**Figure 2.3: TLR-immunoreactive cells in murine colonic specimens express an EEC marker serotonin.**

(A-C) Double-staining of murine colonic sections with (A) anti-TLR2 (Alexa-488 secondary) and (B) anti-serotonin (Cy3 secondary), an enteroendocrine cell (EEC) marker. (C) A superimposed image of (A) and (B) with nuclei depicted in white and actin in blue. (D-F) Double-staining of murine colonic section with (D) anti-TLR6 (Alexa-488 secondary) and (E) anti-serotonin (Cy3 secondary). (F) A superimposed image of (D) and (E) with nuclei depicted in white and actin in blue.

morphologically different from conventional absorptive epithelial, goblet, and Paneth cells. They have features reminiscent of the enteroendocrine lineage, including a pyramidal shape with a large basolateral surface, an apically displaced nucleus, and a granular appearance. To determine whether the IECs immunoreactive for TLR1, TLR2, and TLR4 are EECs, immunohistochemistry was performed with a polyclonal antibody to serotonin. Serotonin expression is detected in uninflamed human ileal and colonic sections, and serotonin immunoreactivity co-localizes with TLR1 staining (Figure 2.2D-F) and TLR2 (data not shown). Identical immunostaining patterns were observed in murine colonic sections using anti-TLR antibodies cross-reactive to murine TLR2 (Figure 2.3A-C), TLR6 (Figure 2.3D-F), and TLR4 (data not shown) that co-localize with serotonin immunoreactivity. Thus, an IEC that expresses a combination of TLRs represents a highly differentiated population and belongs to the enteroendocrine lineage. The characterization and distribution of these TLR-expressing epithelial cells suggests that they represent a novel population of IECs with a potential role in innate immune responses in the intestinal mucosa.

**Enteroendocrine cell lines express TLRs.** To begin to determine functional consequences of TLR expression by EECs, the presence of TLRs in intestinal derived EEC lines was determined. The murine EEC line STC-1 (151) expresses mRNA transcripts for TLR1, 2, 4, 6, and the TLR4 co-receptor MD2 (Figure 2.4A). The RAW264.7 murine macrophage cell line was used as a positive control for TLR expression. Compared to RAW264.7, STC-1 cells express qualitatively lower levels of TLR mRNA. Two human EEC lines, Colo320DM and BON, demonstrate identical expression patterns (data not shown). Furthermore, STC-1 cells express TLR2 (Figure

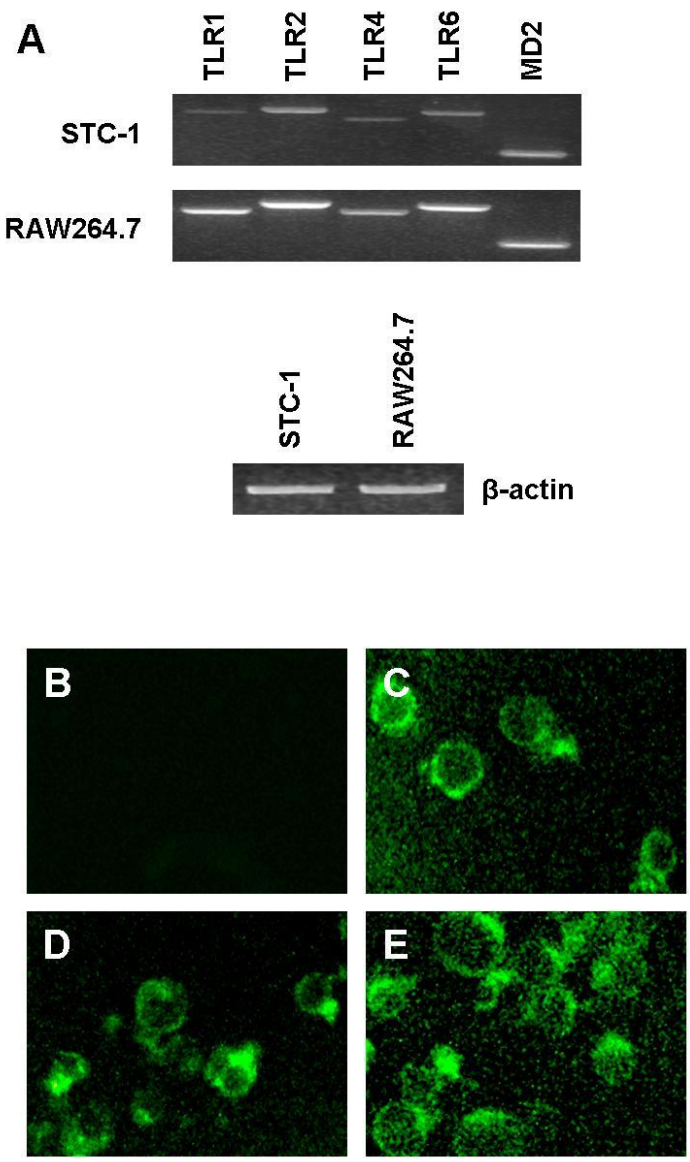


**Figure 2.4: The murine enteroendocrine cell line STC-1 expresses TLRs.**

(A) Expression of TLR and MD2 mRNA were analyzed by PCR of reverse transcribed total RNA isolated from STC-1 cells. RNA isolated from RAW264.7 macrophage cells were used as a positive control. STC-1 cells were stained (FITC-secondary) with (B) control IgG, (C) anti-TLR2, (D) anti-TLR4/MD2 complex, and (E) anti-TLR6 antibodies. Experiments were repeated three times and a representative result is displayed.

2.4C), TLR4/MD2 complex (Figure 2.4D), and TLR6 (Figure 2.4E) protein. Thus, the STC-1 EEC line expresses the same repertoire of TLRs observed in primary human and murine EECs.

**TLR ligands activate NF-κB and MAPK pathways in STC-1 cells.** Activation of the NF-κB family of transcription factors is a central downstream signaling event in TLR-mediated cellular activation, and regulates gene expression in many cell types. To determine whether TLR expression on EECs is functional, STC-1 cells were transiently transfected with a NF-κB luciferase reporter plasmid and stimulated with the TLR4 ligand LPS or the TLR1/TLR2 ligand sBLP. STC-1 cells demonstrate a dose-dependent transcriptional activation of the NF-κB reporter to TLR ligands (Figure 2.5). The activation of MAPK is another important signaling event in TLR-mediated responses. Accordingly, STC-1 cells were stimulated with LPS or sBLP to assess MAPK activation. Phosphorylation of ERK1/2 MAPK was detected as early as 2 minutes (Figure 2.6). These experimental results support the presence of an intact intracellular signal transduction pathway downstream of TLRs, leading to NF-κB and MAPK activation in EECs.

**TLR ligands induce cytokine and chemokine expression in STC-1 cells.** Absorptive enterocytes participate in innate immune responses through the elaboration of cytokines such as TNF and chemokines like IL-8 when activated by pathogenic bacteria through TLRs. Whether EECs are programmed for similar responses is unknown. STC-1 cells were stimulated with LPS or sBLP. MIP-2 (the murine homologue of human IL-8) and TNF expression was evaluated by RT-PCR (Figure 2.7). The ligands induced rapid accumulation of MIP-2 mRNA, detected after

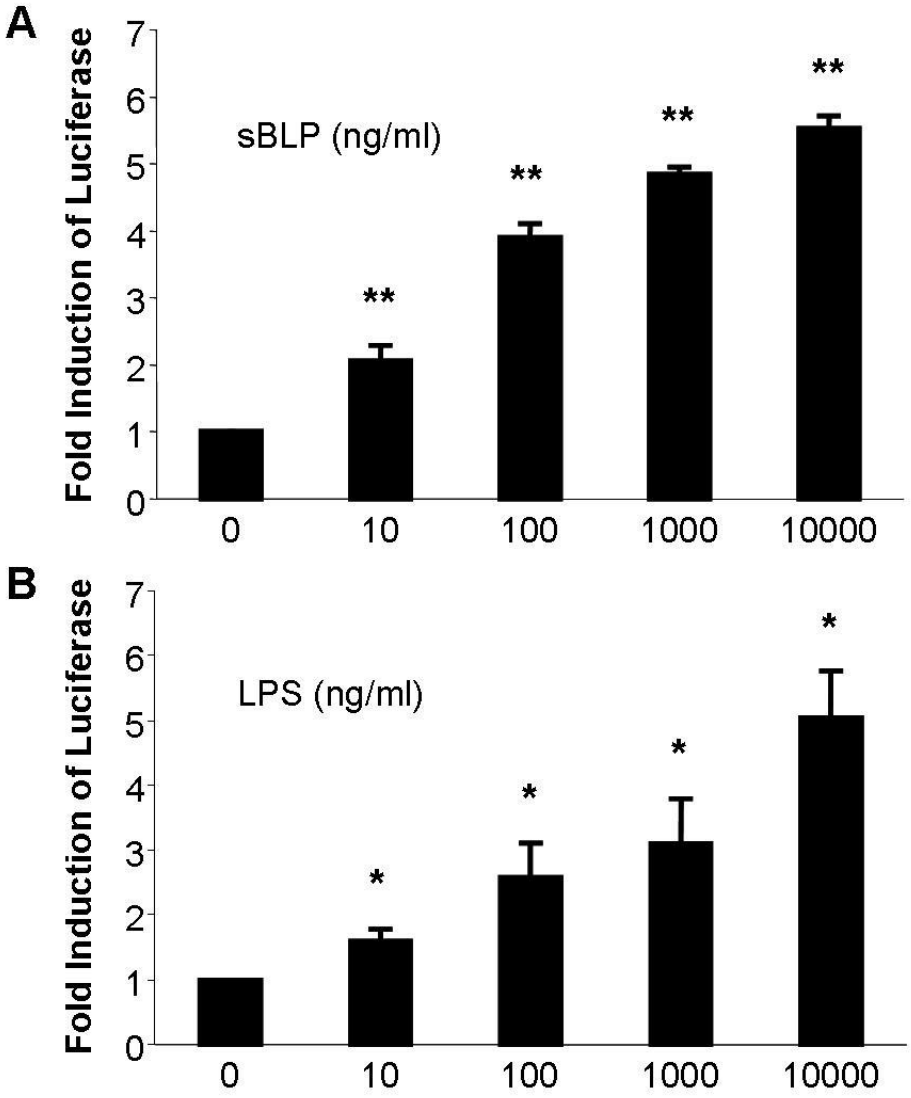


**Figure 2.5: The TLR1/2 (sBLP) and TLR4 (LPS) ligands activate NF-κB in the murine STC-1 EEC line.**

NF-κB activation was assessed by transient transfection of a plasmid with a multimerized NF-κB DNA binding element luciferase reporter. STC-1 cells were stimulated for eight hours with a dose titration of (A) sBLP or (B) LPS. Results are expressed as relative light units normalized to β-galactosidase activity from a co-transfected HSP promoter-β-galactosidase plasmid to correct for transfection efficiency. Fold induction of NF-κB luciferase is compared to values in unstimulated cells (=1) for each group. Each result represents the mean ± standard deviation of three to five experiments. *, $p<0.05$; **, $p<0.005$ compared to unstimulated cells for each group.

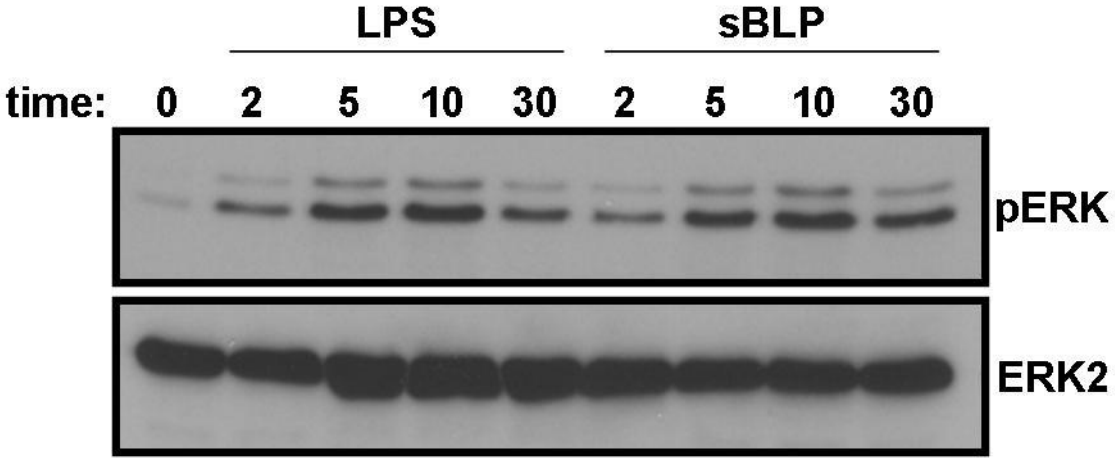


**Figure 2.6: sBLP and LPS activate the ERK MAPK pathway in STC-1 cells.**

MAPK activation was assessed in cells stimulated with LPS or sBLP (10 μg/ml) for 0, 2, 5, 10, or 30 minutes. Whole cell extracts were isolated as described in the materials and methods section. Western blots were probed for MAPK activation via phospho-ERK1/2 and total ERK2 to determine equal loading. Experiments were repeated three times and a representative result is displayed.

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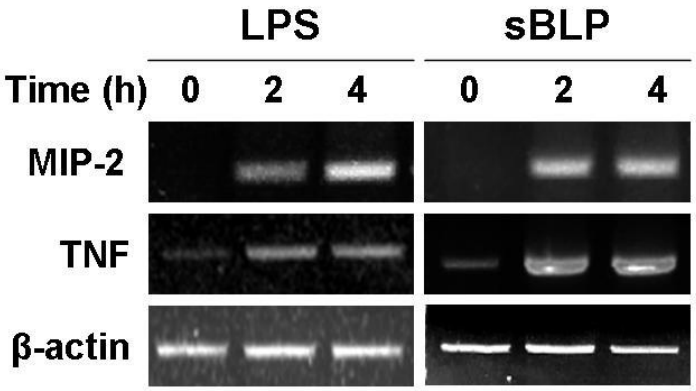


**Figure 2.7: TLR ligands induce TNF and MIP-2 mRNA expression in STC-1 cells.**

STC-1 cells were stimulated with 10 μg/ml of LPS or sBLP for 0-4 hours. Cells were harvested via TRIzol. Total RNA was isolated, digested with DNaseI, and subsequently reverse-transcribed into cDNA. TNF, MIP-2, and β-actin PCR primer sequences are described in the methods section. Experiments were repeated three times and a representative result is displayed.

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2 hours with maximal expression after 4 hours of stimulation. Kinetics for TNF mRNA induction show maximal levels between 2 and 4 hours and slowly returning to baseline levels at the end of 8 hours for each TLR ligand (data not shown). Likewise, LPS and sBLP induce concentration dependent secretion of MIP-2 (Figure 2.8A-B) and TNF (Figure 2.8C-D) protein that remain elevated after 96 hours (data not shown).

**LPS induces TGF-β in STC-1.** IECs also secrete factors that inhibit immune responses and may serve to maintain mucosal homeostasis *in vivo*. TGF-β is a regulatory cytokine constitutively expressed in freshly isolated IECs, with increased mRNA expression detected towards the base of the crypts (152). The relevant source among primary IECs remains to be determined, although TGF-β protein is produced in both the small intestine and colon (153). In addition to inhibiting T and B cell proliferation and macrophage activation (154), TGF-β is involved in oral tolerance (155) and is pertinent in the pathogenesis of IBD (156). In unstimulated STC-1 cells, basal expression of TGF-β mRNA is detected (Figure 2.9A). Upon stimulation with LPS, maximal expression is induced at 4 hours. Supernatants were also analyzed for TGF-β protein over 72 hours. An increase in spontaneous secretion of TGF-β in unstimulated samples is observed from 24 to 72 hours. Additionally, a dose-dependent increase of TGF-β protein over the baseline amounts is detected in LPS-activated STC-1 cells (Figure 2.9B). Expression of pro- and anti-inflammatory molecules by an EEC line suggests that TLRs expressed on EECs may contribute to innate immune responses and mucosal homeostasis.

**LPS induces calcium flux in STC-1 cells.** One of the identifying characteristics of EECs is the

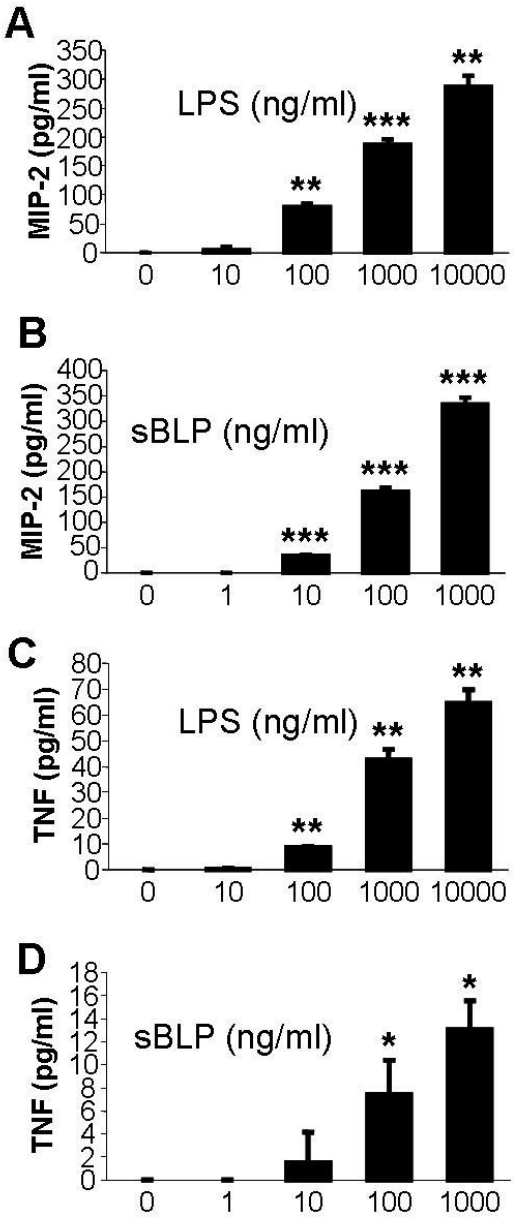


**Figure 2.8: TLR ligands induce TNF and MIP-2 protein expression in STC-1 cells.**

STC-1 cells were stimulated with increasing amounts of (A, C) LPS or (B, D) sBLP for 48 hours. ELISA was performed on cell free supernatants for (A, B) TNF and (C, D) MIP-2. Experiments were performed in triplicate and results represent the mean ± standard deviation. *, $p<0.05$; **, $p<0.01$, and ***, $p<0.001$ compared to unstimulated cells for each group.

presence of preformed storage vesicles that contain numerous bioactive compounds with hormonal and neurotransmitter properties. Calcium fluxes are associated with fusion events of storage vesicles (157, 158) and have been previously described in EECs stimulated with fatty acids (159, 160). To determine whether bacterial ligands cause calcium flux in STC-1 cells, cells were loaded with Fura-2 and challenged with LPS (Figure 2.10). STC-1 cells were rested for baseline readings. After introducing LPS, there was an increase in the 340:380 ratio indicating an increase in intracellular calcium. Levels remained elevated out to 15 minutes. As controls, RAW264.7 macrophages and Caco-2 cells, which express functional TLR4, did not demonstrate calcium flux (data not shown) in the presence of LPS. We speculate that increased intracellular calcium following TLR ligation leads to degranulation of EEC vesicles, suggesting a specialized function for TLR signaling in this cell type.

**LPS induces rapid secretion of CCK from STC-1 cells.** We next addressed whether TLR activation may induce degranulation of EEC vesicles and secretion of neurohormones. STC-1 cells were stimulated with LPS, sBLP, and bombesin (BBS), as a positive control, and supernatants assayed for serotonin and CCK (Figure 2.11). Serotonin levels were relatively unchanged during stimulation of up to 24 hours (data not shown). However, CCK levels increased with LPS, with maximal release after 30 minutes (Figure 2.11A, white bars). CCK release was dependent on calcium as preincubation with a calcium chelator, BAPTA-AM, significantly reduced CCK release (Figure 2.11A, black bars).

**STC-1 conditioned media inhibits LPS-activated macrophages.** Neurohormones produced by

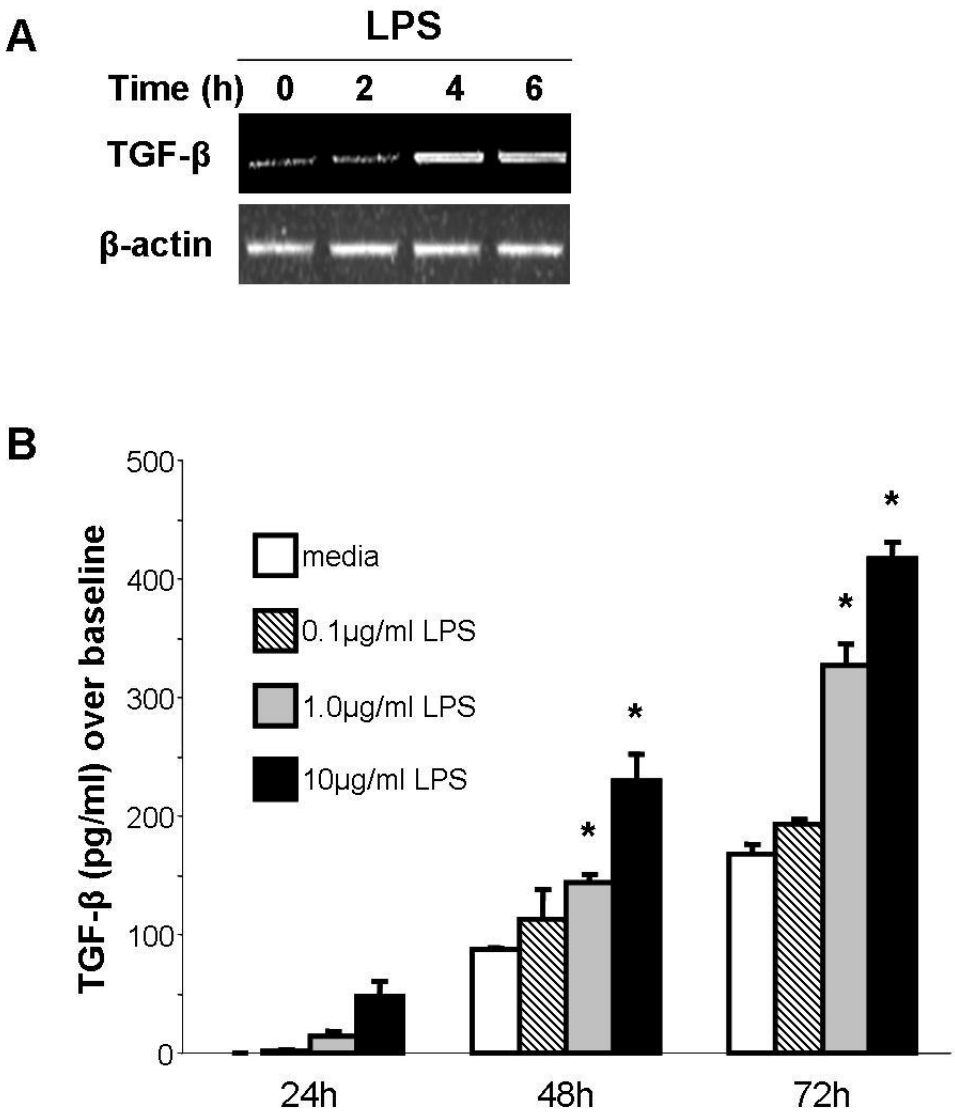


**Figure 2.9: LPS induces TGF-β expression in STC-1 cells.**

(A) STC-1 cells were grown in 1% FBS and stimulated with 10 μg/ml of LPS for 0-6 hours. Total RNA was isolated and reverse-transcribed. TGF-β and β-actin primer sequences were described in the methods section. Experiments were repeated three times and a representative result is displayed. (B) STC-1 cells were stimulated with 0.1-10 μg/ml of LPS for 24, 48, or 72 hours. ELISA was performed on the supernatants for total TGF-β according to the manufacturer's protocols. Experiments were performed in triplicate and results represent the mean ± standard deviation. *, $p<0.05$ compared to unstimulated cells for each time group.

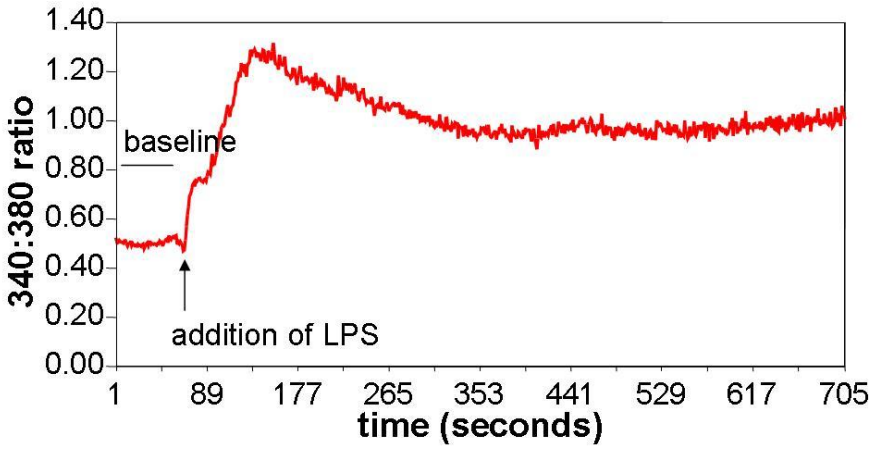


**Figure 2.10: LPS induces a rapid calcium flux in STC-1 cells.**

STC-1 cells were loaded with Fura-2 and incubated in DMEM with 10% FBS. Cells were initially stabilized ("baseline"). Cells were then subsequently treated with 10 μg/ml LPS ("addition of LPS") causing an increase in the internal calcium concentration. Experiments were performed in triplicate, and a representative result is shown.

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EECs and other cells, such as somatostatin, vasointestinal peptide (VIP), and CCK have been demonstrated to inhibit immune responses (78, 79, 161-163). Resident lamina propria macrophages are notably deficient in the expression of innate immune receptors and therefore do not produce proinflammatory cytokines when stimulated by bacterial ligands (164). Therefore, we explored the possibility that EEC may be involved in the downregulation of innate immune responses in macrophages. Conditioned media obtained from 4 day STC-1 cultures was transferred on to RAW264.7 cells which were then stimulated with LPS for 24 hours. EEC supernatants inhibit NO production in LPS-stimulated RAW264.7 cells (Figure 2.12A). This inhibitory activity is concentration dependent, as incremental inhibition of NO is demonstrated with 20% to 100% conditioned media (data not shown). In contrast, 4 day conditioned media from the IEC line Caco-2 and HEK293T cells did not inhibit NO production in LPS activated

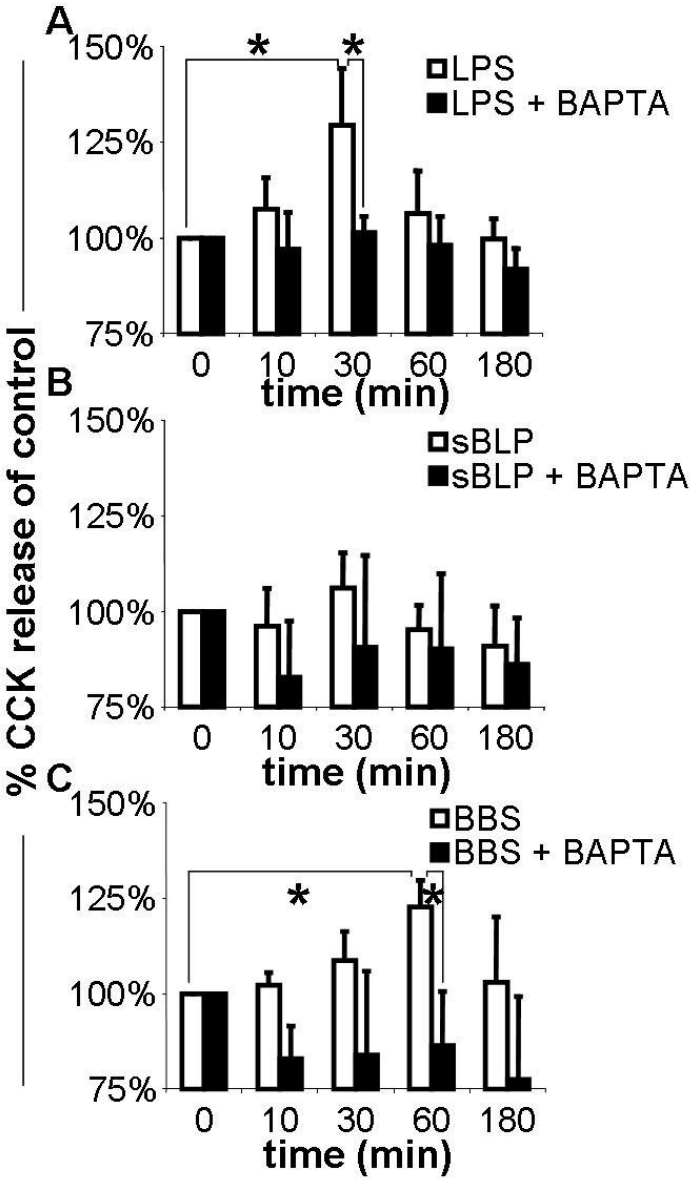


**Figure 2.11: LPS induces secretion of CCK in STC-1 cells.**

Cells were pretreated in the presence (black bars) or absence (white bars) of the calcium chelator BAPTA-AM (20 μM) 30 minutes prior to being stimulated for 10, 30, 60, and 180 minutes with (A) 10 μg/ml LPS, (B) 10 μg/ml sBLP, or (C) 10 nM Bombesin (BBS). EIA was performed on cell free supernatants for CCK. Experiments were performed in duplicate. Due to the variation of the baseline production of CCK, results were normalized to controls (unstimulated) and presented as percentage of control values. The average baseline production was 1.8±0.6 ng/ml CCK. *, $p<0.05$.

RAW264.7 cells. Cell viability was assayed by trypan blue exclusion, which was ≥90% in each condition. Furthermore, STC-1 conditioned media inhibits NO production and IL-12 p40 protein expression in LPS-activated murine peritoneal (Figure 2.12B-C) and BM-derived macrophages (Figure 2.12D-E).

## 2.5 DISCUSSION

In this study, we show that a subset of human and murine IECs co-expresses TLRs and belongs to the enteroendocrine lineage. Using an EEC model, a broad range of functional effects of TLR activation is demonstrated, including NF-κB and MAPK activation, calcium fluxes and CCK secretion, and pro- and anti-inflammatory gene expression. Taken together, this study proposes a novel role for EECs as participants in mucosal innate immune responses and raises the hypothesis that EEC-bacterial interactions help maintain intestinal immune homeostasis.

Reports of TLR expression on normal healthy IECs have been controversial (104-109, 144-146). Previous immunohistochemical analyses of TLR expression in human intestinal mucosa revealed different results from our study. These discrepant results may have several explanations. First, different antibodies were used in these studies. Therefore, we performed numerous controls to demonstrate the specificity of our immunohistochemical analysis (Figures 2.1, 2.2, and Results). Secondly, our staining for TLR2 and TLR4 may have been more sensitive, enabling detection of TLR2 and TLR4 expressing IECs that were not noted in the previous studies. Another report first described the immunohistochemical expression of TLR4 on a murine IEC line and on isolated murine intestinal crypt epithelium (165). In this well

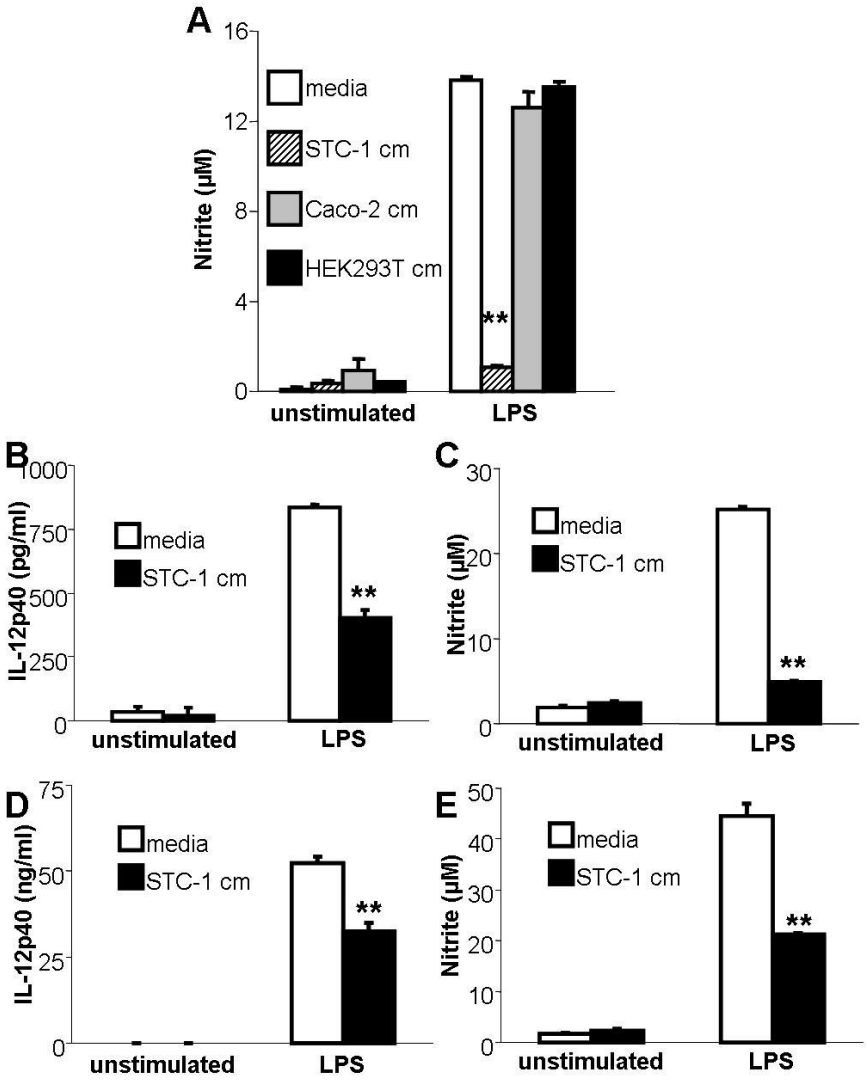


**Figure 2.12: STC-1 conditioned media inhibits LPS-stimulated IL-12 p40 and NO production in macrophages.**

Four day conditioned media from STC-1 cells was added to (A) RAW264.7 or (B, C) murine peritoneal macrophages or (D, E) murine BM-derived macrophages. Four day conditioned media (cm) from the IEC line Caco-2 and epithelial cell line HEK293T were used as controls (A). Macrophages were subsequently stimulated with 1 μg/ml of LPS for 24 hours (A, B, D) or 48 hours (C, E). Experiments were performed in triplicate and results represent the mean ± standard deviation. **, $p<0.01$, compared to media control for each group.

controlled analysis, staining of the crypt appeared to be diffuse, suggesting that cells of the enteroendocrine lineage as well as other IEC types in the mouse may express TLR4 (166). Our study does not exclude the possibility that TLRs are expressed on other IEC type in lower quantities. However, there may also be species specific differences in TLR expression. Furthermore, this analysis was limited to the first five members of the TLR family. It is certainly possible that EECs and other IECs express other TLR family members.

In the current study, a specific crypt epithelial cell morphologically different from conventional absorptive epithelium co-expresses TLRs and is identified as a serotonin-positive EEC (Figure 2.2 and 2.3). EECs represent less than 1% of terminally differentiated cells in the intestinal mucosa. The intestinal epithelium harbors at least 15 types of EECs that have been characterized based on morphological criteria, the nature of their principal secretory product, specific marker molecules, and their ability to express certain transgenes (167-169). Serotonin expressing cells represent the largest population, can be detected throughout the whole intestine, and can be located in the crypt as well as along the villus (167, 170, 171). Therefore, TLR-expressing IECs likely represent a distinct subpopulation of EECs.

Immune function associated with EECs is largely unknown. However, bacterial interactions with EECs have been described: Cholera toxin - a secretory enterotoxin produced by *Vibrio cholerae* - evokes intestinal fluid secretion via binding to its receptor GM1 ganglioside on EECs, followed by serotonin release and activation of the enteric nervous system (81, 172). Furthermore, the EEC product CCK has been described to be elevated in an acute upper gastrointestinal infection with *Giardia* and prevents bacterial translocation (173, 174). There have been few reports directly implicating the EEC in immune responses. One study described a close proximity between EECs and T lymphocytes in non-human primate gut mucosa (80),

indirectly suggesting a functional interaction. Additionally, colitis prone T cell receptor-α deficient mice were demonstrated to have a decrease in number of colonic EECs compared to age and strain matched controls (175), suggesting that EECs may possess homeostatic function.

EECs are activated by nervous stimulation and discharge their secretions into the blood flowing through the villus capillary bed. This allows for stored peptides and secreted proteins to appear immediately at the tissue-blood interface, where it may exert an effect on leukocyte and monocyte recruitment. If this is the case, it suggests a role for EECs as highly specialized "life-guards" of the intestinal mucosa with the capacity for early recognition of bacterial invasion with consequent recruitment of protective mechanisms mediated by the enteric immune and nervous systems. Here, we report that TLR activation in an EEC line is associated with a rapid calcium flux. It has been shown that exocytosis of secretory granules requires membrane fusion, a calcium dependent process (157, 158). We further show that CCK, an EEC product, was released in response to TLR signaling, perhaps through activation of the MAPK signaling pathway (176). Although mobilization of intracellular calcium is not a well described downstream event of TLR activation, degranulation of secretory products in response to TLR signaling has been previously described in mast cells (177). We speculate that this may be a specific adaptation of EECs with consequent release of enteric neurohormones from secretory vesicles in response to TLR activation.

Various neuropeptides expressed and released by EECs have been shown to inhibit immune responses. For example, somatostatin inhibits bacteria-induced IL-8 in IECs (78), CCK inhibits LPS-mediated responses (162, 163), and VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) also inhibit macrophage activation (79, 161). To date, we have not identified the bioactive component that inhibits macrophage activation in STC-1 conditioned

media. In preliminary analyses, we have tested several described EEC products to determine whether they may mediate this inhibitory activity. Incubation of RAW264.7 cells and BM-derived macrophages with serotonin, somatostatin, or CCK does not inhibit NO or IL-12 p40 expression (data not shown). We were interested in the observation that TGF-β is upregulated in LPS-activated STC-1 cells given its well-described immunoregulatory functions (154, 155) and its role in wound healing (178). TGF-β inhibits NO in bone-marrow derived macrophages and IL-12 p40 expression in peritoneal macrophages (Figure 2.13). Therefore, TGF-β may partially but not fully mediate the inhibitory effect of STC-1 conditioned media.

A recent pivotal report further examines the importance of TLRs in the intestines. Mice deficient in TLR2, TLR4, or the signaling molecule MyD88 demonstrated increased severity of DSS induced colitis and decreased survival as compared to control mice (110), demonstrating that engagement of TLRs through recognition of ligands on commensal bacteria plays a beneficial role in intestinal homeostasis. This study is consistent with hypotheses raised by our findings: EECs express TLRs and actively produce factors, such as TGF-β, which may contribute to intestinal homeostasis and protection from epithelial damage. Constitutive expression of TLRs suggests that EECs may actively recognize bacteria even under normal physiologic circumstances and contribute to the normal immunosuppressive state in the intestine.

Another possible consequence of TLR ligation in EECs is the production of the neutrophil attractant human IL-8 or murine MIP-2, used in our study as a read-out in an EEC line model system. The intestinal epithelium, through production of chemotactic factors, initiates the recruitment of immune cells to the lamina propria and sites of infection. Consequently, reduced numbers of neutrophils are observed in the lamina propria of TLR4- and MyD88-deficient mice treated with DSS (179).

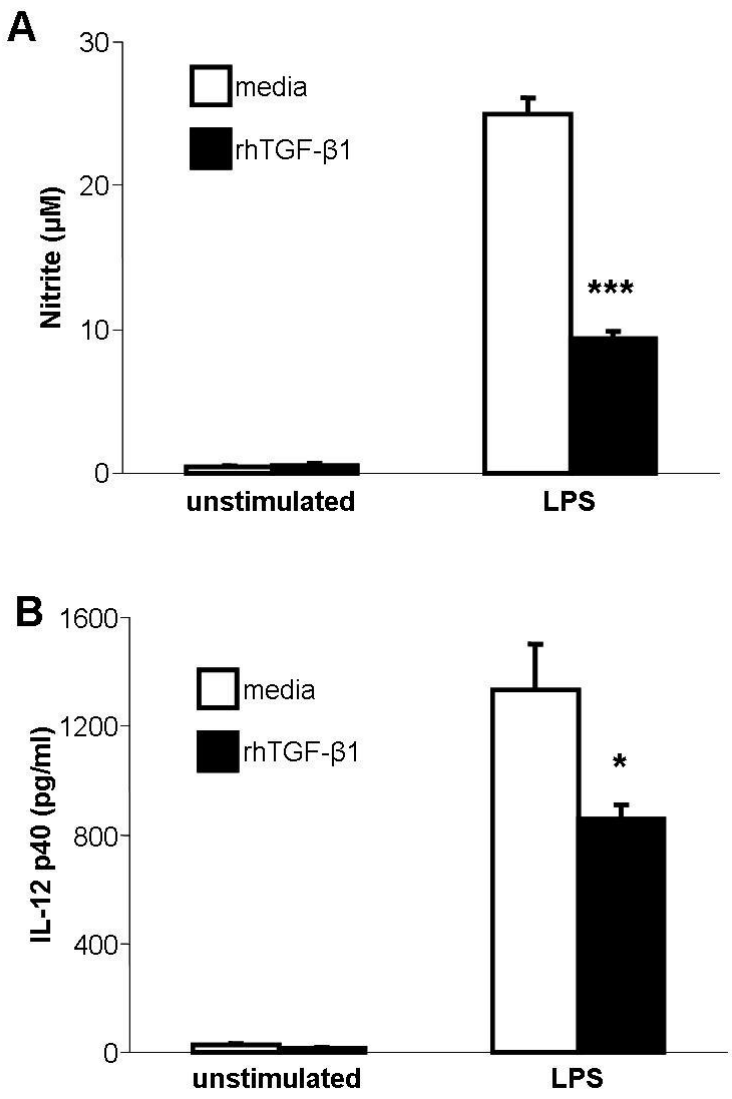


**Figure 2.13: TGF-β inhibits LPS-stimulated IL-12 p40 and NO production in macrophages.**

(A) BM-derived or (B) peritoneal macrophages were incubated with (black bars) or without (white bars) 5 ng/ml rhTGF-β1 prior to stimulation with 1 μg/ml LPS. Supernatants were assayed for (A) NO and (B) IL-12 p40. Experiments were performed in triplicate and results represent the mean ± standard deviation. *, $p<0.05$; ***, $p<0.001$, compared to media control for each group.

That TLR-expressing IECs belong to the enteroendocrine lineage raises intriguing hypotheses about how bacterial interactions regulate physiologic and pathologic inflammation. Multiple effects of TLR activation were demonstrated in a murine EEC line, but another caveat to this study is that there may be functional differences in TLR activation between mice and humans. However, the striking similarity in immunostaining patterns in EEC observed between species may suggest a conserved functional role. To fully understand the functional consequences of TLR expression on EECs will necessitate much more investigation, combining cell line and descriptive data with mouse models of mucosal immunity and inflammation.

# 3.0 ETHYL PYRUVATE AMELIORATES ACUTE AND CHRONIC MURINE COLITIS

## 3.1 ABSTRACT

Ethyl pyruvate has been shown to inhibit secretion of high mobility group box 1 (HMGB1) and other proinflammatory cytokines by macrophages and improve survival in models of endotoxemia and hemorrhagic shock. We reasoned that ethyl pyruvate may be protective in colitis, where pathogenesis also depends on activation of similar inflammatory pathways. In this study, we investigated the immunomodulatory effects of systemically and locally delivered ethyl pyruvate in murine models of colitis. In IL-10-deficient (*IL-10*$^{-/-}$) mice with chronic colitis, ethyl pyruvate administered intraperitoneally resulted in weight gain, amelioration of histologic colitis, and reduced spontaneous intestinal inflammatory cytokine production compared to vehicle-treated controls. Fecal HMGB1 levels were decreased in ethyl pyruvate-treated mice. Furthermore, ethyl pyruvate induced the anti-inflammatory enzyme heme oxygenase-1 (HO-1) in intestinal tissue. In TNBS-induced acute colitis, mice administered ethyl pyruvate intrarectally demonstrated weight gain, amelioration of histologic colitis, and a reduction in spontaneous intestinal inflammatory cytokine production. Mechanistically, anti-inflammatory effects of ethyl pyruvate on innate immune responses were studied in murine macrophages. In macrophages, ethyl pyruvate decreased expression of IL-12 p40 and nitric oxide (NO) production, but did not

affect IL-10 and TNF levels. Ethyl pyruvate incubation did not inhibit nuclear translocation of NF-κB family members, but resulted in attenuated NF-κB binding. Additionally, ethyl pyruvate induced HO-1 mRNA and protein expression, and HO-1 promoter activation in macrophages. In conclusion, ethyl pyruvate demonstrates pleiotropic anti-inflammatory effects and ameliorates murine colitis following systemic and local delivery. Therefore, ethyl pyruvate may have therapeutic potential in the treatment of inflammatory bowel disease.

## 3.2 INTRODUCTION

Pyruvate, the end product of glycolysis and a key intermediary of aerobic and anaerobic metabolism, has been demonstrated to function in cells as an endogenous antioxidant and free radical scavenger (180). It is protective in numerous *in vitro* and *in vivo* models of oxidant-mediated cellular or organ system injury. Pyruvate, which normally is present in cells at millimolar concentrations, can reduce hydrogen peroxide nonenzymatically, and specifically reduce levels of hydroxyl radicals. However, because of the poor stability of pyruvate in solution (181), ethyl pyruvate, a simple aliphatic ester derivative (180), was formulated in a calcium- and potassium-containing balanced salt solution as a stable antioxidant for therapeutic interventions.

Ethyl pyruvate administration has been shown to be therapeutic in ameliorating intestinal, renal, or hepatic injury in animal models of mesenteric ischemia and reperfusion, hemorrhagic shock, endotoxemia, and polymicrobial bacterial sepsis (182-185). In many of these models of acute inflammation and in cell culture systems, ethyl pyruvate demonstrated pleiotropic anti-inflammatory effects, including downregulation of key proinflammatory genes such as inducible

nitric oxide synthase (iNOS)[3], TNF, cyclooxygenase-2, and IL-6; and protection against inflammation-induced intestinal epithelial barrier dysfunction (183, 185-187). Importantly, many of the anti-inflammatory effects of ethyl pyruvate in models of acute inflammation may be related to inhibition of a soluble signal of cell stress and damage, high mobility group box 1 protein (HMGB1) (184).

Collectively, these results support the concept that ethyl pyruvate may have a role as a therapeutic agent in acute and chronic inflammatory disease. In particular, an agent that inhibits proinflammatory cytokines and augments intestinal epithelial barrier function may be an attractive therapeutic candidate in the human inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis (UC). Although the etiologies of the IBDs are unclear, chronic gut inflammation may result from inappropriate and excessive inflammatory responses (1, 2, 10, 188, 189), including an overabundance of proinflammatory cytokines such as IL-1β, IL-6, IL-12, TNF, and IFN-γ (22, 51, 134, 135, 138, 139), and the overproduction of reactive oxygen species (ROS) (190-193). Furthermore, abnormal innate immune responses to enteric microbes are involved in the pathogenesis of IBD. For example, IL-10-deficient (*IL-10*$^{-/-}$) mice develop spontaneous chronic colitis and T-helper-1 (Th1) polarized immunity that is dependent on the presence of the gut flora (56, 72-74, 194). Finally, defects in intestinal epithelial barrier function may predispose to innate immune activation in IBD (52). Therefore, therapeutic strategies that dampen excessive innate immune activation and cytokine production, and restore intestinal barrier integrity may ameliorate IBD.

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[3] Abbreviations used in this chapter: ARE, antioxidant response element; BM, bone marrow; CD, Crohn's disease; DAMP, damage associated molecular pattern; EMSA, electrophoretic mobility shift assay; HMGB1, high mobility group box 1; HO, heme oxygenase; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; *IL-10*$^{-/-}$, IL-10-deficient; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; PPAR-γ, peroxisome proliferator-activated receptor γ; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; Th1, T-helper-1; TNBS, 2,4,6-trinitrobenzene sulfonic acid; UC, ulcerative colitis.

Accordingly, we reasoned that ethyl pyruvate may be protective in IBD, because the pathogenesis of sepsis, shock, and ischemia reperfusion, like IBD, depends on activation of inflammatory mediators (both cytokines and ROS) and subsequent release of endogenous danger signals, such as HMGB1. Here we show that local and systemic ethyl pyruvate administration in acute and chronic murine colitis models ameliorated disease. Ethyl pyruvate inhibited IL-12 p40 and nitric oxide (NO) levels in lipopolysaccharide (LPS)-stimulated macrophages. Furthermore, the anti-inflammatory effects of ethyl pyruvate are pleiotropic, and we also demonstrate that ethyl pyruvate decreased NF-κB DNA binding, inhibited HMGB1 secretion, and induced the anti-inflammatory protein heme oxygenase-1 (HO-1).

## 3.3 MATERIALS AND METHODS

**Mice.** Male C57BL/6 (10-12 weeks old) and female BALB/c (12-13 weeks old) mice were obtained from The Jackson Laboratory. An *IL-10*$^{-/-}$ colony (breeder pairs from The Jackson Laboratory) was maintained in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and was approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh School of Medicine.

**Ethyl pyruvate treatment in *IL-10*$^{-/-}$ mice.** 10-week old male and female *IL-10*$^{-/-}$ mice were grouped randomly and treated with either lactated Ringer's solution alone (vehicle) or 40 mg/kg ethyl pyruvate (Acros Organics) in sterile lactated Ringer's solution. Treatment was administered in a total volume of 500 μl intraperitoneally every other day for two weeks. Weight changes were monitored every other day and compared to pretreatment values. At the end of the

study period, animals were euthanized using excess $CO_2$ inhalation and intestinal tissue was harvested (as described below).

**Ethyl pyruvate treatment in TNBS-induced colitis.** To induce colitis, 2.5 mg 2,4,6-trinitrobenzene sulfonic acid, (TNBS, Sigma) in 50% ethanol (to break the intestinal epithelial barrier) was administered for studies of acute TNBS colitis. Fasted BALB/c mice were lightly anesthetized with isoflurane and then administered the haptenating agent TNBS intrarectally via a PE20 polyethylene tubing catheter (BD) equipped with a 1 ml syringe. The lubricated catheter tip was carefully inserted into the colon and advanced such that the tip was located 4 cm proximal to the anus, at which time the TNBS was administered in a total volume of 100 µl. To ensure distribution of the haptenating agent within the entire colon, mice were held in a vertical position for 30 seconds after the intrarectal injection. Mice were then grouped randomly and treated with either lactated Ringer's solution alone (vehicle) or 40 mg/kg ethyl pyruvate dissolved in sterile lactated Ringer's solution. Vehicle or ethyl pyruvate was delivered at 4 hours, 2 days, and 4 days after the administration of TNBS. Weight changes were monitored daily and compared to pretreatment values. At the end of five days, animals were euthanized using excess $CO_2$ inhalation and intestinal tissue was harvested (as described below).

**Intestinal tissue explant cultures.** Colons were isolated from individual mice, cut open longitudinally, and feces were collected for ELISA (described below). Intestinal tissue was washed with PBS to remove residual fecal content. Intestinal sections were cut in half longitudinally, and one half was shaken at 250 rpm at room temperature for 30 min in RPMI1640 supplemented with 1% antibiotic/antimycotic. Tissue fragments (0.05 g dry weight)

were cut into small pieces and incubated in 1 ml RPMI1640 supplemented with 1% antibiotic/antimycotic and 10% FBS. Supernatants were collected after 24 hours, assayed for spontaneous cytokine production via ELISA, and normalized to dry gut weight.

**Grading of histologic changes.** Removed colonic tissue was fixed in 10% buffered formalin and embedded in paraffin. 5 μm thick sections were stained with hematoxylin and eosin. Colitis scores were determined for each high-powered field (100× magnification) viewed by a pathologist (Dr. Antonia R. Sepulveda, University of Pittsburgh) blinded to the treatment groups. For *IL-10*$^{-/-}$ mice, colitis scores were graded from 0 to 4 using a modified scoring criteria reported by Berg *et al.* (33). For the TNBS colitis model, the degree of inflammation was graded from 0 to 3 (0, no signs of inflammation; 1, mucosal neutrophils or increased mucosal lymphocytes present; 2, mucosal and submucosal or transmural neutrophils or increased lymphocytes present; 3, mucosal regenerative features with crypt distortion and increased crypt proliferation or ulcers or erosions). Scores were presented as the average sum of ten fields using a composite scoring system.

**Fecal samples for HMGB1 and RAGE ELISA.** Fecal samples were removed from longitudinally cut large intestines of sacrificed mice. Samples were extracted in PBS by vigorous shaking followed by rotation at 4°C for 48 hours, cleared at high speed, and analyzed for HMGB1 and RAGE concentration by specific sandwich ELISAs. For the HMGB1 ELISA, the capture antibody was a mouse anti-HMGB1 monoclonal (R&D Systems) and the detection antibody was a polyclonal affinity purified anti-HMGB1 generated in rabbits (from Dr. Michael T. Lotze, University of Pittsburgh, epitope used for immunization: aa 166-181

KPDAAKKGVVKAEKSC) and a $F(ab')_2$ donkey anti-rabbit HRP (Jackson Immunoresearch). A standard curve was generated using a recombinant full-length HMGB1. For the RAGE ELISA (each component from R&D Systems), the capture antibody was a rat anti-mouse RAGE monoclonal and the detection antibody was a biotinylated goat anti-mouse RAGE polyclonal along with a streptavidin-HRP reagent. A standard curve was generated using a recombinant murine RAGE/Fc chimera. Following the addition of a chromogenic substrate, reactions were stopped with sulfuric acid and absorbance was read at $\lambda$=450nm. Values were normalized to total protein concentration determined by the Bradford assay (Coomassie Protein Assay Kit, Pierce).

**Immunohistochemistry.** Colonic tissue sections were fixed in 2% paraformaldehyde in PBS and then incubated in 30% sucrose in PBS at 4°C overnight. Samples were snap-frozen in isopentane and cut into 6 μm-thick frozen sections and placed on microscope slides. Sections were washed in 0.5% bovine serum albumin (BSA) in PBS (wash buffer) three times to remove sucrose and then blocked with 2% BSA in PBS for 45 minutes. Sections were stained with rabbit anti-HMGB1 (BD Pharmingen), rabbit anti-RAGE (gift from Dr. Tim Oury, University of Pittsburgh) (195), or rabbit anti-HO-1 (StressGen) polyclonal antibodies at a 1:500 dilution in wash buffer for 1 hour at room temperature. Samples were washed three times with wash buffer, and then incubated for 1 hour at room temperature with secondary antibody (1:500 dilution of goat anti-rabbit Alexa 488; Molecular Probes), rhodamine phalloidin (1:500 dilution; Molecular Probes) to visualize F-actin, and Draq5 (Biostatus Limited, Leicestershire, United Kingdom) to visualize nuclei. Rabbit nonimmune serum or secondary antibody alone was used as controls and nonspecific immunostaining was not observed (data not shown). Slides were washed three

times with wash buffer, three times with PBS, and then coverslipped with Gelvatol (23 g polyvinyl alcohol 2000, 50 ml glycerol, 0.1% sodium azide, 100 ml PBS). Slides were viewed on an Olympus Flouview 1000 confocal microscope (Olympus America, Melville, NY).

**Murine macrophages.** Bone marrow (BM) from femurs of C57BL/6 mice were flushed with washing medium (RPMI1640 with 1% Pen/Strep), passed through a 70 μM nylon cell strainer into a 50 ml conical tube, and spun down at 1500 rpm for 5 minutes. RBCs were lysed using sterile-filtered 0.8% ammonium chloride, washed twice with washing medium, and resuspended in complete medium (washing medium with 10% FBS). BM cells were seeded in complete medium in a 150 mm dish and differentiated using recombinant murine GM-CSF (20 ng/ml) (R&D Systems). At day three, another 25 ml fresh culture medium containing GM-CSF was added to the culture plates. At day seven, the cells, representing the BM-derived macrophage population, were harvested. Cells were seeded at $4\times10^6$ cells/well for RNA, $2\times10^5$ cells/well for ELISA, or $25\times10^6$ cells/plate for nuclear extracts, pretreated with ethyl pyruvate dissolved in media, and subsequently stimulated with 100 ng/ml extracted LPS (147) for the times indicated in the figure legends.

**RNA isolation and real time RT-PCR.** Total RNA was isolated using the TRIzol reagent (Invitrogen). cDNA was derived from 1.5 μg total RNA by RT using Superscript II (Invitrogen). Real-time RT-PCR was performed using specific primers for IL-12 p40, HO-1, and GAPDH, as described previously (196). Quantitation of mRNA was expressed as relative fold increase in transcript level with respect to unstimulated cells.

**Cytokine ELISAs and NO Assay.** Murine IL-12 p40, TNF, and IL-10 immunoassay kits were used according to the manufacturer's instructions (BD Pharmingen). The stable NO metabolite, nitrite, was measured using the Griess reagent as previously described (150). Values were measured using a plate reader and SOFTMax Pro v4.8 software (Molecular Devices).

**Nuclear extracts and DNA binding assay (EMSA).** Nuclear extracts were isolated from stimulated BM macrophages using the NE/PER Reagents by following the manufacturer's protocol (Pierce). Protein concentration was determined using the Bradford assay. The electrophoretic mobility shift assay (EMSA) was performed on nuclear extracts using a NF-κB DNA binding element probe, as previously described (197). Briefly, 200 ng of double-stranded oligonucleotide NF-κB DNA probes (murine Igκ NF-κB consensus 5′ - CAGAGGGGACTTTCCGAGA - 3′ or the NF-κB element of the murine IL-12 p40 promoter -141 to -107) were labeled with [α-$^{32}$P]dGTP and [α-$^{32}$P]dCTP by Klenow enzyme and purified with Quick Spin columns (Sephadex G-50, Roche). Probes were incubated with 5 μg of nuclear extracts on ice for 30 min prior to electrophoresis. For supershift assays, 5 μg nuclear extract was incubated with 2 μg of anti-p65, anti-c-Rel, or anti-p50 polyclonal antibodies (Santa Cruz Biotechnology, Inc.) for 45 min at RT prior to addition of the $^{32}$P labeled probe. Gels were electrophoresed for 150 min at 150 V.

**Western blotting.** Western blot analyses were performed on nuclear extracts as described previously (149). Anti-p65, anti-c-Rel, anti-p50, and anti-Nrf2 were obtained from Santa Cruz Biotechnology, Inc., anti-HO-1 was from StressGen, and anti-actin was from Sigma.

**Luciferase assay.** HEK293 cells stably transfected with a multimerized NF-κB DNA binding element-luciferase reporter were seeded at $2\times10^5$ cells/well. Cells were pretreated for one hour with ethyl pyruvate dissolved in media and activated for two hours with 10 ng/ml recombinant human TNF (R&D Systems). RAW264.7 cells were seeded at $2.5\times10^5$ cells/well and transfected with a HSP promoter-β-galactosidase reporter (148) and either pHO15luc, a luciferase reporter construct under the control of a 15 kb mouse HO-1 promoter fragment, or pHO15lucΔE1, a luciferase reporter lacking the E1 enhancer (kindly provided by Dr. Jawed Alam, LSU Medical Center, New Orleans, LA). Cells were pretreated for one hour with ethyl pyruvate dissolved in media, and stimulated for 36 hours with 1000 ng/ml LPS. The cells were lysed in reporter lysis buffer and luciferase activity was measured with a luciferase assay system (Promega) using a Turner Designs Luminometer TD20/20. In RAW264.7 cells, transfection efficiency was assessed by β-galactosidase activity in the same cell lysates. The ratio of the two parameters (luciferase/β-galactosidase) was used for data presentation as previously reported (148).

**Statistical Analysis.** Statistical significance between groups was assessed by two-tailed Student's *t* test. A *p*-value equal or less than 0.05 was considered to be statistically significant.

## 3.4 RESULTS

**Systemic ethyl pyruvate treatment ameliorates colitis in *IL-10*$^{-/-}$ mice.** Ethyl pyruvate is an experimental therapeutic that can rescue animals from various pathologic conditions mediated by inflammatory cytokines. We first sought to determine whether systemic administration of ethyl

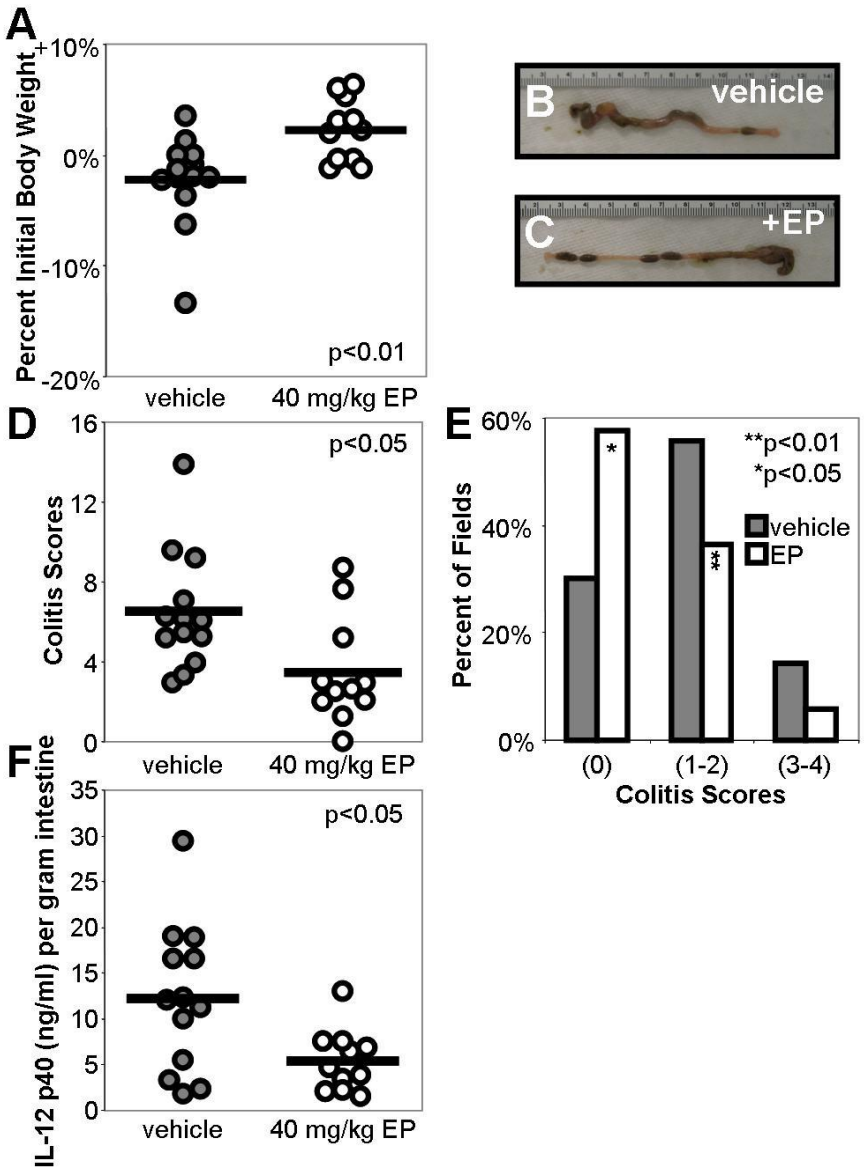


**Figure 3.1: Systemic ethyl pyruvate administration ameliorates colitis in *IL-10*$^{-/-}$ mice.**

10-week old *IL-10*$^{-/-}$ mice were treated intraperitoneally for two weeks every other day with (white, n=11) or without (gray, n=13) 40 mg/kg ethyl pyruvate (EP) in lactated Ringer's solution. (A) Ethyl pyruvate-treated mice demonstrated an overall increase in body weight versus the vehicle-treated group. (B-C) Representative photographs of a colon from an ethyl pyruvate-treated mouse demonstrated increased length, decreased tissue thickening, and formed stool pellets compared to a vehicle-treated mouse. (D-E) Colitis scores were significantly lower in the ethyl pyruvate-treated mice. Histologic improvement in colitis is presented as (D) the average colitis score sum of five fields and (E) percent of histologic fields that demonstrate scores of 0 (no inflammation), 1-2 (mild inflammation), or 3-4 (severe inflammation). (F) Intestinal explants from vehicle- or ethyl pyruvate-treated mice were cultured for 24 hours for measurement of spontaneous IL-12 p40 secretion by ELISA. Values were normalized to weight of intestinal explant.

pyruvate could ameliorate chronic colitis in *IL-10*$^{-/-}$ mice. 10-week old *IL-10*$^{-/-}$ mice were grouped randomly and treated with either lactated Ringer's solution alone (vehicle, n=13) or 40 mg/kg ethyl pyruvate (n=11) dissolved in lactated Ringer's solution. Treatment was administered in a total volume of 500 µl intraperitoneally every other day for two weeks. Ethyl pyruvate-treated mice demonstrated an overall increase in body weight compared to initial body weight versus the vehicle treatment group (2.2% weight increase vs. 2.2% weight decrease, $p<0.01$; Figure 3.1A). On gross inspection of the intestines, colons from ethyl pyruvate-treated mice demonstrated increased lengths, decreased tissue thickening, and formed stool pellets compared to vehicle-treated mice (Figure 3.1B-C).

Histologic severity of the colitis was determined by a pathologist blinded to treatment intervention. Assessment of histologic scores in *IL-10*$^{-/-}$ mice is compromised by incomplete penetrance and a segmental, patchy pattern of colitis. Therefore, we depict colitis scores in two different ways. First, results are presented as the averaged sum total of five fields over the length of the colon (33). Ethyl pyruvate treatment demonstrated about a 50% improvement in histological scores when compared to the vehicle control group (Figure 3.1D). Second, the scores are presented as the percentage of fields that demonstrate no histological inflammation (colitis score of 0), mild to moderate inflammatory changes (colitis score of 1 and 2), and severe inflammation (colitis score of 3 and 4). This method provides a better representation of the spectrum of disease encountered over the entire length of the colon. Compared to the vehicle group, ethyl pyruvate-treated mice had significantly more fields demonstrating no evidence of histologic inflammation, and consequently, fewer fields with inflammatory changes (Figure 3.1E).

We next asked if ethyl pyruvate treatment *in vivo* alters mucosal inflammatory cytokine production. Spontaneous IL-12 p40 and TNF levels were measured in supernatants from colonic mucosal tissue. Intestinal explants from ethyl pyruvate-treated mice secreted significantly less IL-12 p40 compared to vehicle controls (Figure 3.1F), whereas TNF was not significantly decreased (data not shown).

**Fecal HMGB1 levels are increased in *IL-10*$^{-/-}$ mice and decrease with ethyl pyruvate treatment.** In a model of sepsis, ethyl pyruvate treatment improved survival, and reduced circulating levels of HMGB1 (184). HMGB1 was undetectable in the sera of wild-type or *IL-10*$^{-/-}$ mice by specific ELISA (data not shown). Recent data demonstrate HMGB1 is secreted in an apical direction in stimulated intestinal epithelial cells (IECs) (125). We, therefore, investigated the effect of ethyl pyruvate on HMGB1 levels within the intestinal lumen. Feces was collected at the time of sacrifice and assayed for levels of HMGB1 (normalized to total protein). *IL-10*$^{-/-}$ mice with active colitis (n=11, colitis score 5.2) demonstrated approximately a five-fold higher level of fecal HMGB1 (51.9±6.2 ng/mg total protein) compared to wild-type controls (n=5, colitis score 0.0, 7.2±6.0 ng/mg total protein) (Figure 3.2A). Ethyl pyruvate treatment strongly reduced the levels of HMGB1 (Figure 3.2B). This finding suggests that ethyl pyruvate mechanistically may decrease fecal HMGB1 levels, and that fecal HMGB1 may be a marker of disease activity in IBD.

**Ethyl pyruvate modifies intestinal RAGE and HMGB1 expression.** To corroborate the above result and to further investigate mechanisms through which ethyl pyruvate inhibits intestinal

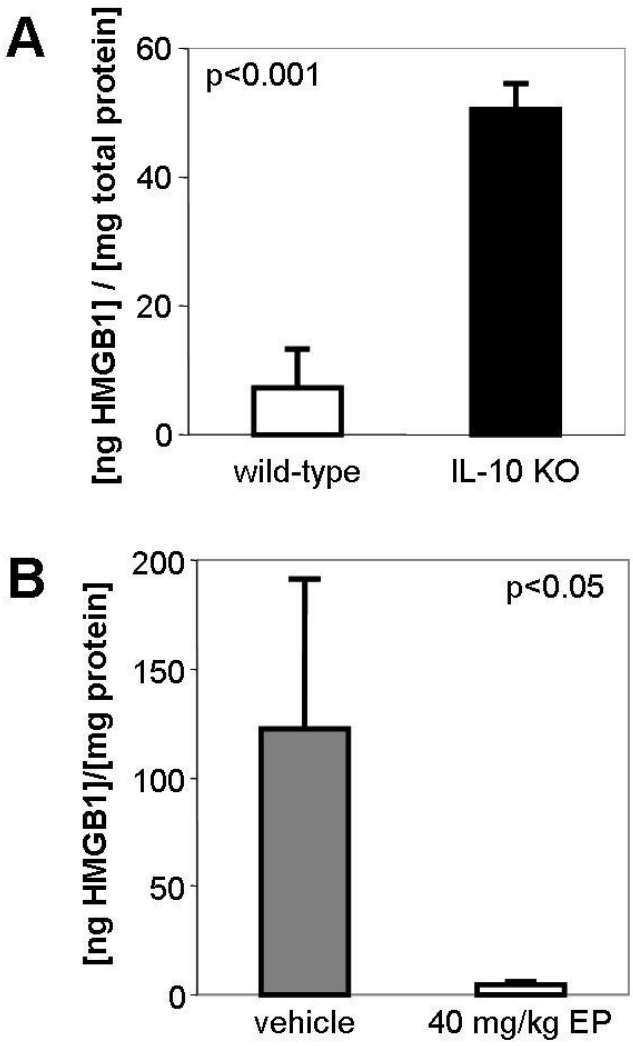


**Figure 3.2: Fecal HMGB1 levels are increased in *IL-10*$^{-/-}$ mice and decrease with ethyl pyruvate treatment.**

(A) Fecal HMGB1 is increased in *IL-10*$^{-/-}$ mice. Colonic stool samples of *IL-10*$^{-/-}$ mice (n=11, colitis score 5.2, black bars) and wild-type strain matched controls (n=5, colitis score 0.0, white bars) were collected in PBS, filtered, and analyzed for HMGB1 concentration by specific ELISA (mean ± SD). Values were normalized to total fecal protein. (B) Ethyl pyruvate (EP) treatment decreases fecal HMGB1 levels in *IL-10*$^{-/-}$ mice. HMGB1 levels were analyzed from vehicle- (n=4) or ethyl pyruvate- (n=4) treated mice as described in Figure 3.1. Values were normalized to total protein.

inflammation, the distribution of HMGB1 and one of its receptors, the receptor for advanced glycation end products (RAGE) was determined by immunohistochemistry in wild-type, *IL-10*$^{-/-}$ mice, and *IL-10*$^{-/-}$ mice treated with ethyl pyruvate. In *IL-10*$^{-/-}$ mice, HMGB1 immunostaining was markedly upregulated in the colonic epithelium, with nuclear and cytoplasmic staining evident compared to wild-type mice where nuclear staining predominated (Figure 3.3A-B). In ethyl pyruvate-treated *IL-10*$^{-/-}$ mice, compared to vehicle-treated controls, IEC immunostaining for HMGB1 is predominantly nuclear (Figure 3.3C-D). Therefore, ethyl pyruvate may prevent cytoplasmic redistribution and secretion of HMGB1.

We next analyzed intestinal RAGE expression by immunohistochemistry. In wild-type mice, RAGE staining appears to be diffuse in the epithelium, with prominent staining on the luminally-oriented microvillus border that co-localized with actin (Figure 3.4A). In *IL-10*$^{-/-}$ mice, there is notable downregulation of RAGE expression in the epithelium (Figure 3.4B-C). However, there are individual epithelial cells that strongly stain for RAGE, and RAGE immunoreactivity is detected within the lamina propria. In *IL-10*$^{-/-}$ mice treated with ethyl pyruvate, RAGE staining appears similar to wild-type intestine (Figure 3.4D). As RAGE expression has not been previously described in the intestine, protein expression was confirmed in fecal samples by a specific ELISA. Similar to our immunohistochemical analysis, there appears to be an inverse correlation with the development of IBD as RAGE is detectable in fecal samples from wild-type mice, but is undetectable in *IL-10*$^{-/-}$ mice with active colitis (Figure 3.5). Possibly due to low levels of detection (5-10 pg/ml RAGE) and the sensitivity of the ELISA, fecal RAGE levels did not significantly increase in *IL-10*$^{-/-}$ mice treated with ethyl pyruvate.

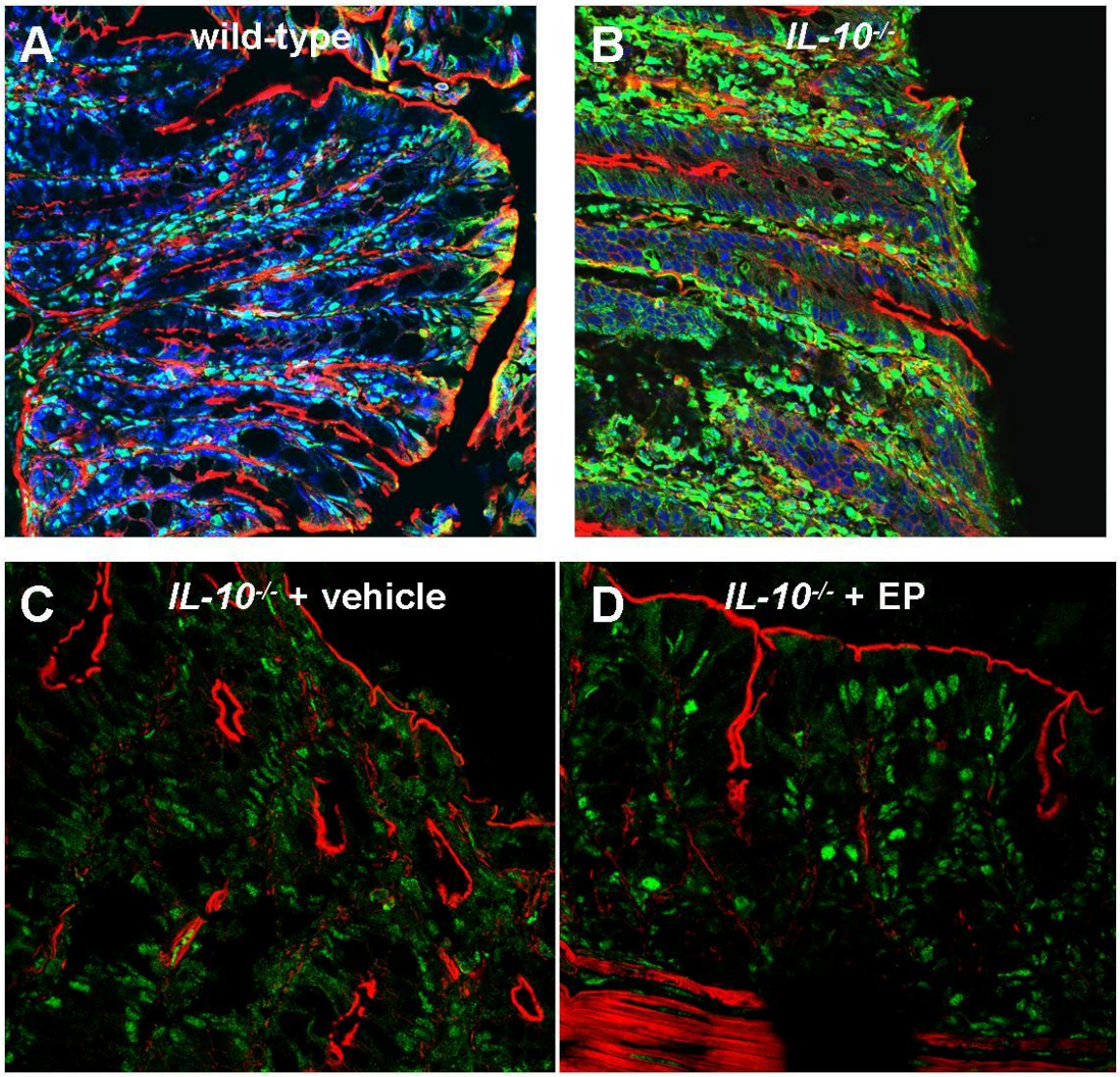


**Figure 3.3: Increased HMGB1 nuclear staining pattern in *IL-10*$^{-/-}$ mice treated with ethyl pyruvate.**

Immunofluorescence demonstrates an increase in nuclear and cytoplasmic distribution of HMGB1 in the colonic epithelium of *IL-10*$^{-/-}$ mice. In ethyl pyruvate (EP)-treated *IL-10*$^{-/-}$ mice, compared to vehicle-treated controls, IEC immunostaining for HMGB1 is predominantly nuclear. Paraformaldehyde-fixed colons of (A) wild-type C57BL/6, (B) *IL-10*$^{-/-}$ mice, (C) *IL-10*$^{-/-}$ mice treated with vehicle, or (D) *IL-10*$^{-/-}$ mice treated with ethyl pyruvate were stained for actin (red), HMGB1 (green), and/or Draq5-nuclear stain (blue) and viewed by confocal microscopy. Staining controls did not show nonspecific binding. Each image is a representative result from at least five different mice.

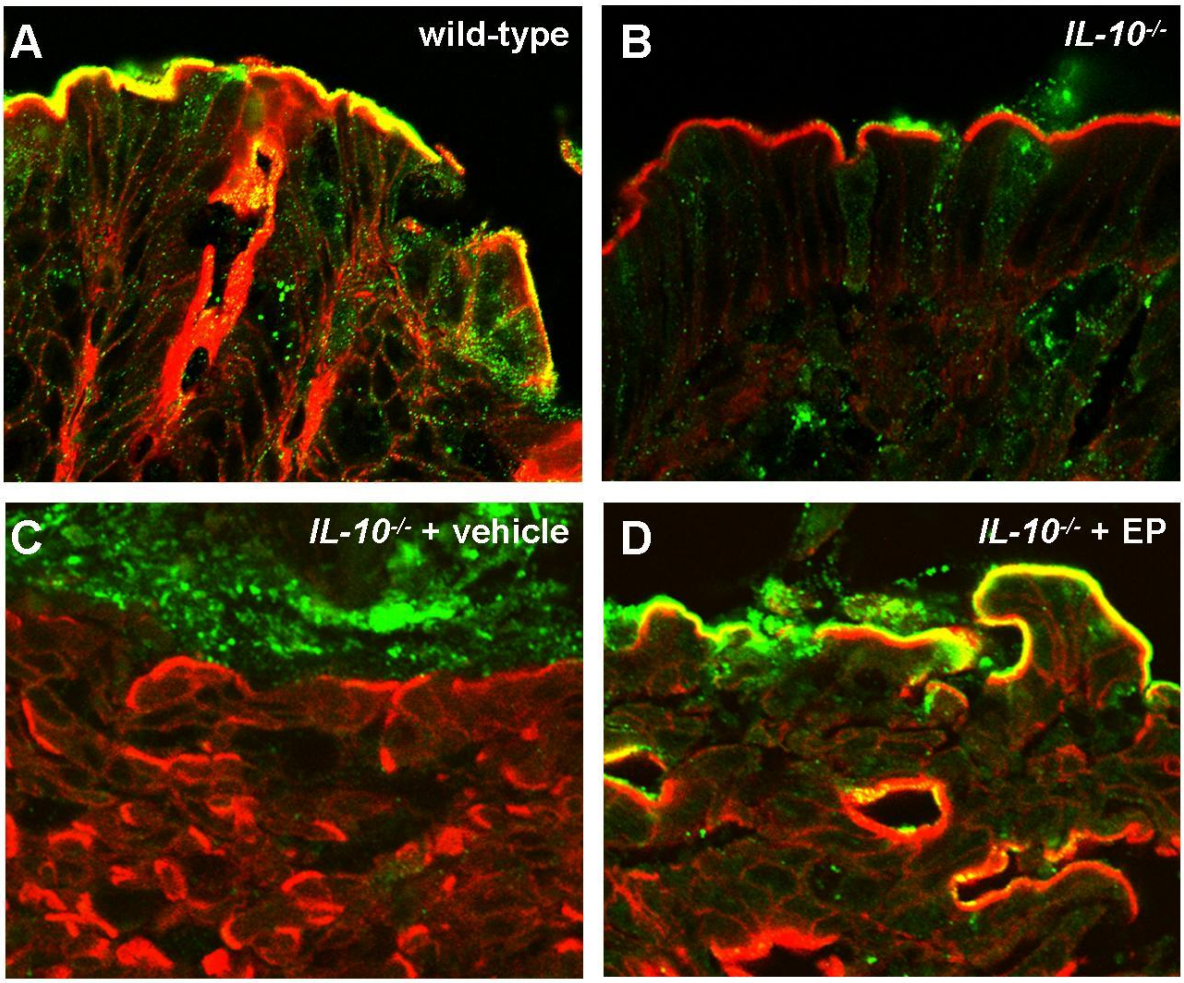


**Figure 3.4: Ethyl pyruvate modifies intestinal RAGE expression.**

Immunofluorescence demonstrates a downregulation of RAGE expression in the colonic epithelium of *IL-10*$^{-/-}$ mice compared to wild-type controls. In ethyl pyruvate (EP)-treated *IL-10*$^{-/-}$ mice, RAGE staining appears similar to wild-type tissue. Paraformaldehyde-fixed colons of (A) wild-type C57BL/6, (B) *IL-10*$^{-/-}$ mice, (C) *IL-10*$^{-/-}$ mice treated with vehicle, or (D) *IL-10*$^{-/-}$ mice treated with ethyl pyruvate were stained for actin (red) and RAGE (green) and viewed by confocal microscopy. Colocalization between actin and RAGE is represented by a yellow color observed at the luminally-oriented microvillus border. Staining controls did not show nonspecific binding. Each image is a representative result from at least five different mice.

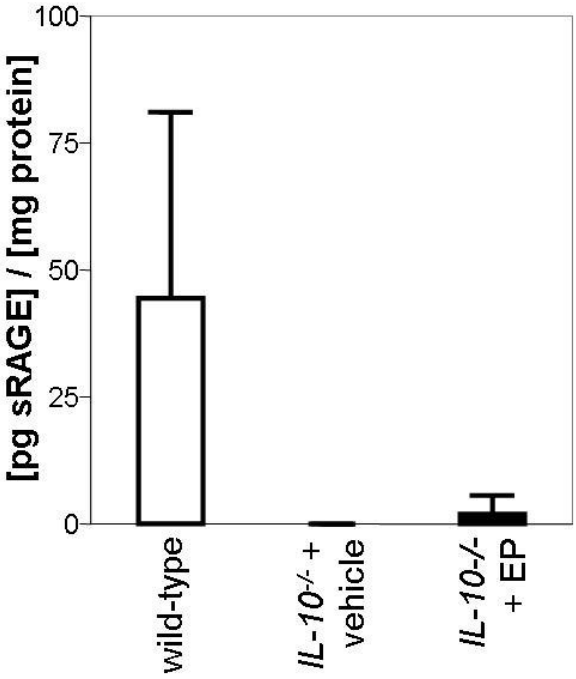


**Figure 3.5: RAGE is detectable in fecal samples from wild-type mice, but is undetectable in *IL-10*$^{-/-}$ mice with active colitis.**

Fecal extracts isolated from wild-type (n=5), *IL-10*$^{-/-}$ mice treated with vehicle (n=4), or *IL-10*$^{-/-}$ mice treated with ethyl pyruvate (EP, n=4) was analyzed for RAGE via a specific ELISA as described in the materials and methods section. Levels were normalized to total fecal protein. Each result represents the mean ± standard deviation.

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To summarize, in *IL-10*$^{-/-}$ mice treated with ethyl pyruvate, histological improvement correlated with decreased mucosal IL-12 p40 expression. Furthermore, abundant quantities of HMGB1 are detected in feces, and upregulated HMGB1 expression is demonstrated in the intestinal mucosa of *IL-10*$^{-/-}$ mice. With ethyl pyruvate treatment, downregulation of HMGB1 expression in the intestine with decreased cytoplasmic to nuclear ratios is demonstrated. Upregulation of apical epithelial RAGE expression correlates with histological improvement.

**Ethyl pyruvate induces HO-1 expression in the intestinal epithelium.** Heme oxygenase (HO) catalyzes the first and rate-limiting step in the degradation of heme to yield equimolar quantities of biliverdin, carbon monoxide, and iron (198). Three isoforms of HO exist; HO-1 is highly inducible while HO-2 and HO-3 are constitutively expressed (199). HO-1, besides its role in heme degradation, also plays a vital function in maintaining cellular homeostasis and has emerged as a key anti-inflammatory pathway (200-202). We have recently demonstrated that specific induction of HO-1 ameliorates colitis in *IL-10*$^{-/-}$ mice (196). Therefore HO-1 expression was assessed in colonic sections of *IL-10*$^{-/-}$ mice. An increase in HO-1 expression throughout the epithelial crypt-villus axis was observed in ethyl pyruvate-treated mice compared to vehicle-treated controls (Figure 3.6A-B).

**Local delivery of ethyl pyruvate ameliorates TNBS-induced colitis.** In *IL-10*$^{-/-}$ mice, systemically administered ethyl pyruvate by the intraperitoneal route improved chronic mucosal inflammation. To determine whether ethyl pyruvate may function mechanistically through direct mucosal effects, we next asked if local delivery of ethyl pyruvate influences the course of acute colitis following intrarectal administration of the hapten 2,4,6-trinitrobenzene sulfonic acid (TNBS) to BALB/c mice. Mice were grouped randomly and treated with either lactated Ringer's solution alone (vehicle, n=9) or 40 mg/kg ethyl pyruvate (n=13) in sterile lactated Ringer's solution by intrarectal administration. Vehicle or ethyl pyruvate was delivered at 4 hours, 2 days, and 4 days after the administration of TNBS. Weight changes were monitored daily and compared to pretreatment values. Mice treated intrarectally with ethyl pyruvate demonstrated an increase in body weight compared to vehicle-treated mice (8.5% weight increase vs. 6.1% weight

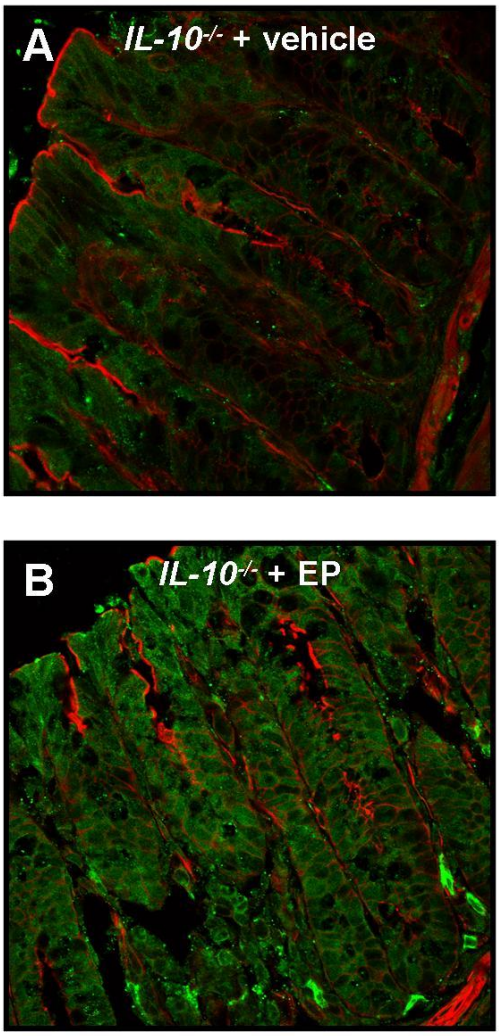


**Figure 3.6: Ethyl pyruvate induces intestinal HO-1 in *IL-10*$^{-/-}$ mice.**

Immunofluorescence with a specific HO-1 antibody demonstrates an increase in intestinal HO-1 staining in the colonic epithelium of ethyl pyruvate (EP)-treated *IL-10*$^{-/-}$ mice. Paraformaldehyde-fixed colons of (A) vehicle- or (B) 40 mg/kg ethyl pyruvate-treated *IL-10*$^{-/-}$ mice were stained for actin (red) and HO-1 (green) and viewed by confocal microscopy. Staining controls did not demonstrate nonspecific binding. Each image is a representative result from at least five different mice.

loss, $p<0.05$; Figure 3.7A). Gross inspection of the intestines showed increased colonic length, less tissue thickening, and increased stool pellets in ethyl pyruvate-treated mice (Figure 3.7B-C). Severity of colitis was graded using a modified scoring system by a pathologist blinded to treatment intervention and presented as the average sum of ten fields. Ethyl pyruvate treatment compared to vehicle demonstrated a significant improvement in histological scores (Figure 3.7D). Lastly, spontaneous inflammatory cytokine production in supernatants from colonic mucosal explants was assayed. Local ethyl pyruvate administration significantly reduced TNF production compared to vehicle controls (Figure 3.7E).

**Ethyl pyruvate inhibits IL-12 p40 and NO production in LPS-stimulated murine macrophages.** To further determine molecular mechanisms through which ethyl pyruvate inhibits innate immune responses, cytokine expression in ethyl pyruvate-treated murine macrophages was next determined. Bone marrow (BM)-derived macrophages preincubated with ethyl pyruvate for 1 hour prior to stimulation with LPS demonstrated a significant inhibition of IL-12 p40 mRNA (Figure 3.8A) and protein production (Figure 3.8B), without affecting cell viability (>95% by Trypan blue exclusion, data not shown). Also, NO levels (nitrite by the Griess reagent) were inhibited by ethyl pyruvate (Figure 3.8C). Ethyl pyruvate did not affect the production of TNF or IL-10 from LPS-stimulated macrophages (Figure 3.8D-E). To begin to determine how ethyl pyruvate regulates cytokine expression, activation of important downstream signal transduction pathways involved in proinflammatory cytokine gene expression, the MAP kinases, were assessed. Ethyl pyruvate did not inhibit LPS-stimulated activation of MAP kinases p38, JNK, or ERK (data not shown). Furthermore, the transcription factor, interferon regulatory

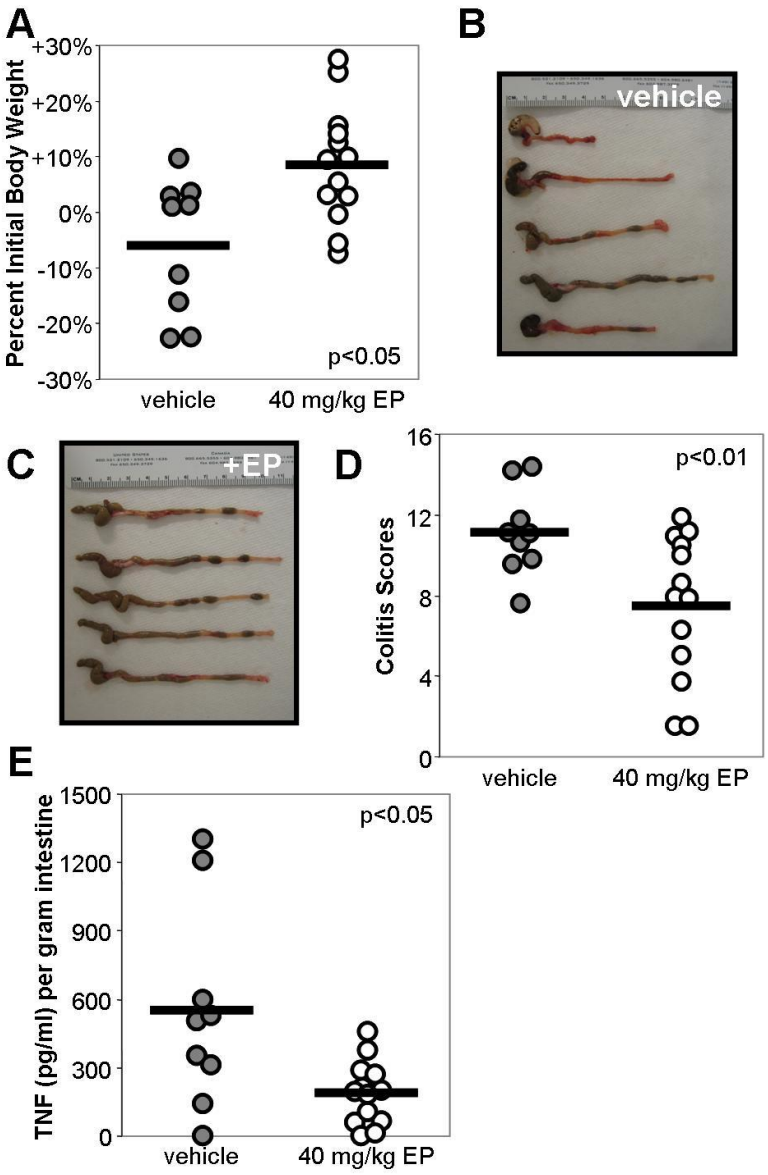


**Figure 3.7: Local ethyl pyruvate administration ameliorates TNBS-induced colitis.**

Colitis was induced in fasting female BALB/c mice by intrarectal delivery of TNBS. Administration of lactated Ringer's solution with (white circles, n=13) or without (gray circles, n=9) 40 mg/kg ethyl pyruvate (EP) was given locally (intrarectally) at 4 hours, 2 days, and 4 days after TNBS and sacrificed on day 5. (A) Ethyl pyruvate-treated mice demonstrated an overall increase in body weight compared to initial body weight versus the vehicle-treated group. (B-C) Representative photographs of colons from ethyl pyruvate-treated mice demonstrated increased lengths, decreased tissue thickening, and formed stool pellets compared to vehicle-treated mice. (D) Colitis scores were significantly lower in the ethyl pyruvate-treated mice. Histologic improvement in colitis is presented as the average sum of ten fields using a composite scoring system. (E) Intestinal explants from vehicle- or ethyl pyruvate-treated mice were cultured for 24 hours for measurement of spontaneous TNF secretion by ELISA. Values were normalized to weight of intestinal explant.

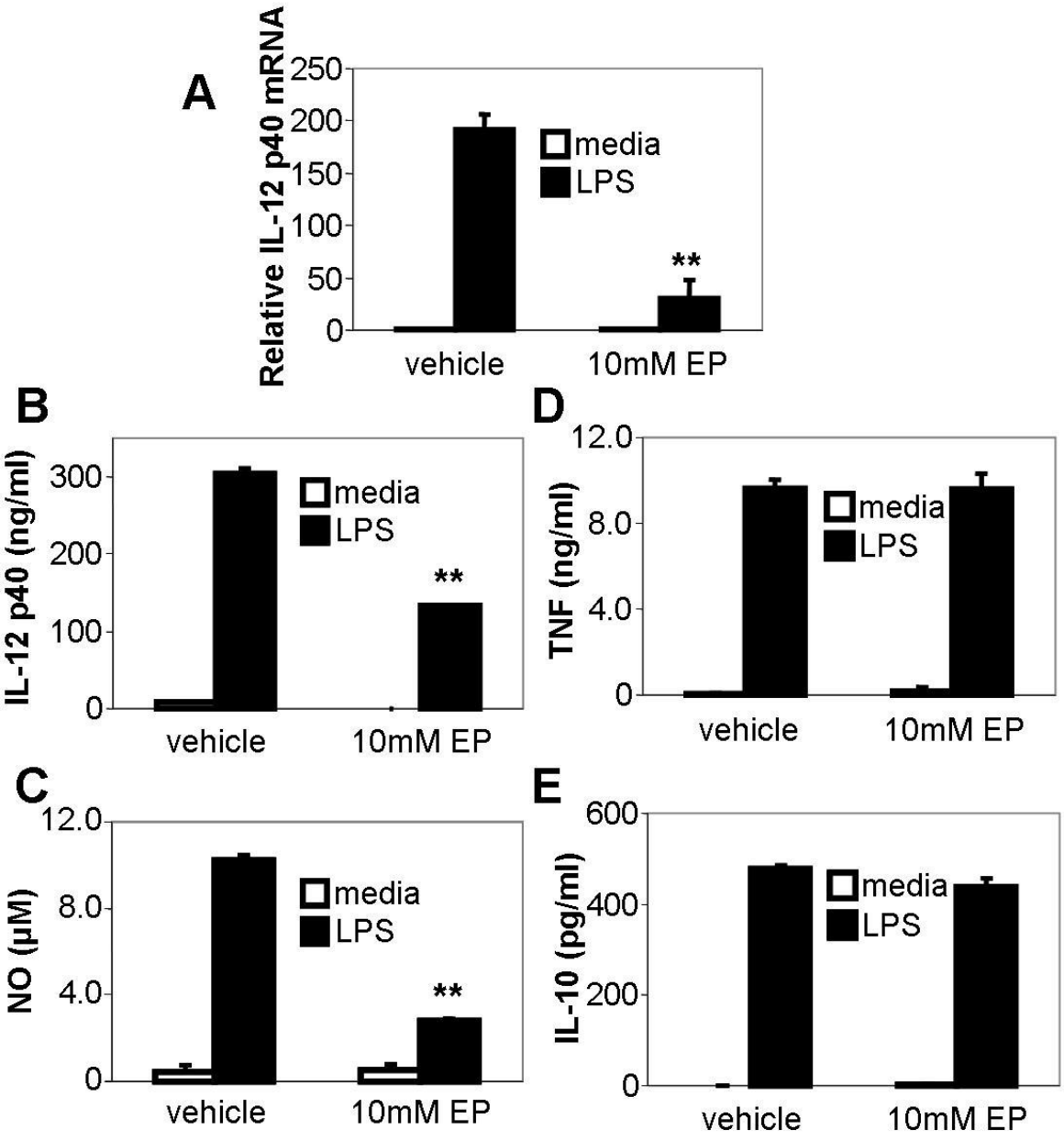


**Figure 3.8: Ethyl pyruvate inhibits LPS-induced IL-12 p40 and NO production in murine macrophages.**

BM-derived murine macrophages were incubated with 10 mM ethyl pyruvate (EP) for 1 hour prior to activation with 100 ng/ml LPS. (A) Cells were harvested at 4 hours for mRNA and real time RT-PCR assay for IL-12 p40 was performed using the housekeeping gene GAPDH as an internal control. Real time RT-PCR was performed in duplicate and repeated three times (mean ± standard deviation). Fold induction is compared to values in vehicle/media samples (=1). (B-E) Supernatants were harvested at 24 hours and assayed for (B) IL-12 p40, (C) NO, (D) TNF, and (E) IL-10. Experiments were performed in duplicate and repeated three times. A representative result is shown (mean ± standard deviation). **, $p<0.01$ compared to LPS-stimulated samples without ethyl pyruvate.

factor 8 (IRF-8), which is involved in IL-12 p40 and iNOS transcription (148, 203), is not inhibited by ethyl pyruvate (data not shown).

**Ethyl pyruvate inhibits NF-κB DNA binding, but does not inhibit nuclear translocation of NF-κB.** Activation of the NF-κB family of transcription factors has been shown to be important for inflammatory gene expression in many chronic inflammatory diseases such as asthma, rheumatoid arthritis, and IBD (103). In unstimulated cells, NF-κB proteins are localized in the cytoplasm through their association with members of a family of inhibitory proteins known as IκB proteins. Proinflammatory cytokines such as TNF and IL-1, and bacterial products such as LPS induce phosphorylation of IκB proteins. Phosphorylated IκB proteins are then ubiquitinated and degraded by the proteosome, releasing bound NF-κB which translocates to the nucleus, binds to NF-κB DNA binding sites on enhancer elements of target genes, and induces transcription (101-103). Ethyl pyruvate has been previously described to inhibit NF-κB (187), and accordingly, NF-κB activation was determined in a cell line model. Ethyl pyruvate effectively abrogates TNF-induced NF-κB activity in a dose dependent manner in the HEK293 cell line stably transfected with a multimerized NF-κB DNA binding element that drives expression for a luciferase reporter gene (Figure 3.9).

To determine how ethyl pyruvate inhibits NF-κB activity, nuclear fractions from LPS-activated and unstimulated BM-derived macrophages were obtained. By Western immunoblot, stimulation of cells with LPS resulted in an increased nuclear expression of NF-κB family members p65 and c-Rel compared to extracts from unstimulated cells. Ethyl pyruvate pretreatment of cells did not inhibit nuclear NF-κB expression (Figure 3.10).

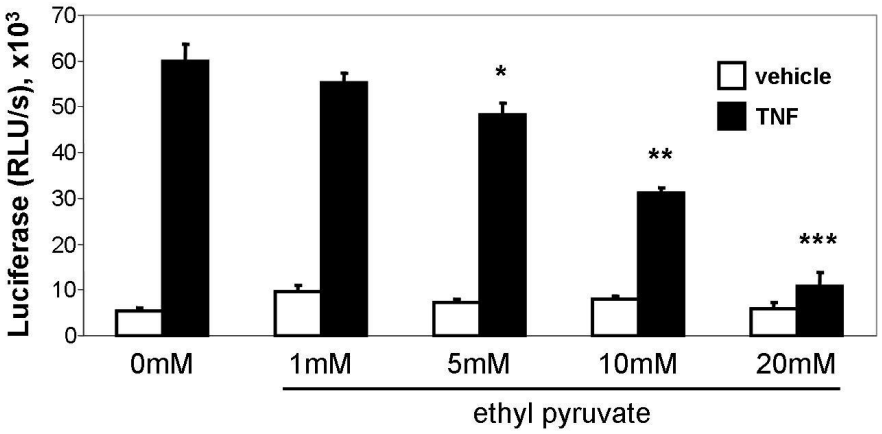


**Figure 3.9: TNF-induced NF-κB activation is inhibited by ethyl pyruvate.**

HEK293 cells stably transfected with a multimerized NF-κB DNA binding element-luciferase reporter were preincubated for 1 hour with an increasing dose of ethyl pyruvate. Cells were subsequently stimulated for 2 hours with 10 ng/ml rhTNF. Cells were harvested and lysates were analyzed for luciferase activity. Results are expressed as relative light units per second (RLU/s). Experiments were performed in triplicate and repeated three times. A representative result is shown (mean ± standard deviation). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared to TNF-stimulated samples without ethyl pyruvate.

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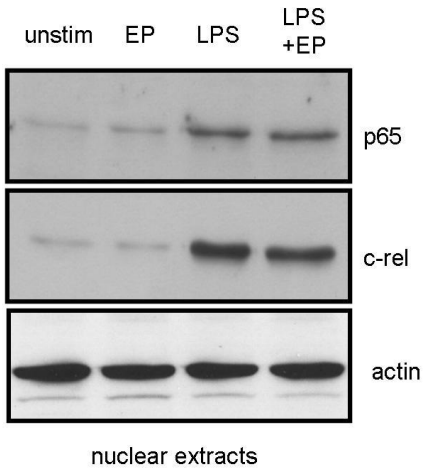


**Figure 3.10: Nuclear translocation of NF-κB is not inhibited by ethyl pyruvate.**

BM-derived murine macrophages were incubated with 10 mM ethyl pyruvate (EP) for 1 hour before stimulation with 100 ng/ml LPS for 4 hours. Nuclear extracts were isolated as described in the materials and methods section. NF-κB protein expression of p65 and c-Rel in nuclear extracts were determined by Western blot. Actin was used as loading control. This result is representative of three independent experiments.

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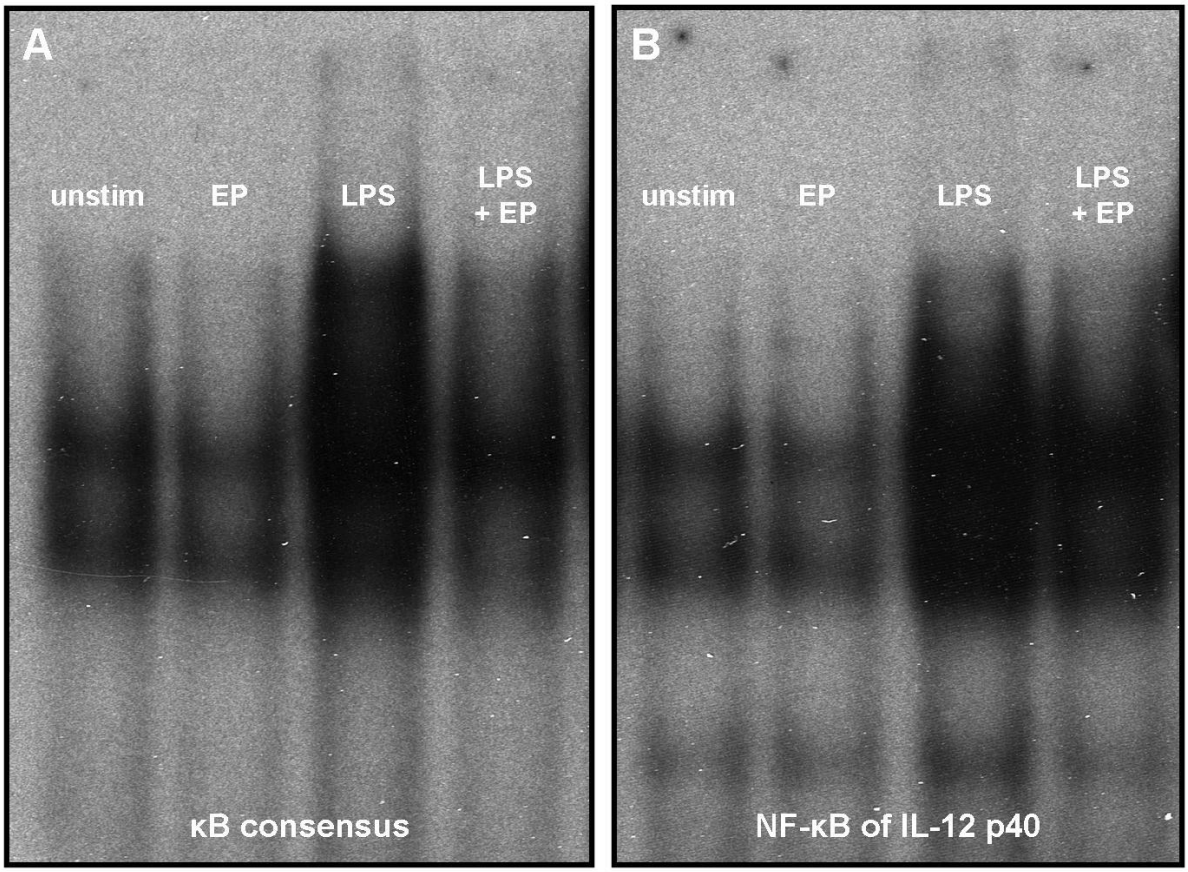


**Figure 3.11: Ethyl pyruvate decreases NF-κB DNA binding.**

BM-derived murine macrophages were incubated with 10 mM ethyl pyruvate (EP) for 1 hour before stimulation with 100 ng/ml LPS for 4 hours. Nuclear extracts were isolated as described in the materials and methods section. Nuclear extracts were incubated with either (A) a murine Igκ NF-κB consensus DNA binding element probe or (B) the NF-κB DNA binding element of the IL-12 p40 promoter radiolabeled probe. This result is representative of three independent experiments.

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To investigate NF-κB protein-DNA interactions in murine macrophages, electrophoretic mobility shift assays (EMSAs) were performed with a DNA probe containing the murine immunoglobulin kappa NF-κB consensus sequence (Figure 3.11A) or the NF-κB element of the murine IL-12 p40 promoter (Figure 3.11B). Specific protein-DNA complexes were observed in unactivated-cell extracts and were enhanced in extracts from LPS-activated cells. Extracts from

cells treated with ethyl pyruvate showed significantly less DNA binding. Supershift experiments with antibodies against the p50 or c-Rel proteins demonstrated the presence of these NF-κB family members in the DNA binding complex suggesting that ethyl pyruvate treatment may target DNA binding of p50 containing homodimers and/or heterodimers in murine macrophages (data not shown).

As ethyl pyruvate attenuates NF-κB DNA binding without inhibiting nuclear translocation of NF-κB family members in activated cells, ethyl pyruvate may induce a nuclear protein that restricts NF-κB DNA binding. Recently, peroxisome proliferator-activated receptor γ (PPAR-γ), a regulator of the inflammatory response, has been shown to complex with NF-κB RelA and shuttle it out of the nucleus (204, 205). However, ethyl pyruvate did not affect nuclear PPAR-γ expression in unstimulated or LPS-activated macrophages (data not shown).

**Ethyl pyruvate induces HO-1 mRNA, protein, and transcriptional activity.** Ethyl pyruvate induces the anti-inflammatory gene HO-1 *in vivo*. Accordingly, mechanisms of induction of HO-1 by ethyl pyruvate were determined in murine macrophages. Ethyl pyruvate alone and ethyl pyruvate plus LPS induced HO-1 mRNA and protein in BM-derived macrophages (Figure 3.12A-B). Next, transcriptional activation of the HO-1 gene by ethyl pyruvate was studied in the murine macrophage RAW264.7 cell line transfected with pHO15luc, a luciferase reporter under the control of a 15 kb murine HO-1 promoter fragment (Figure 3.12C) (206). Ethyl pyruvate alone and ethyl pyruvate plus LPS augmented HO-1 promoter activity by about three-fold. A previously identified enhancer region, E1, contains an antioxidant response element (ARE) important for HO-1 induction (207, 208). To implicate a role of the E1 enhancer, a mutant

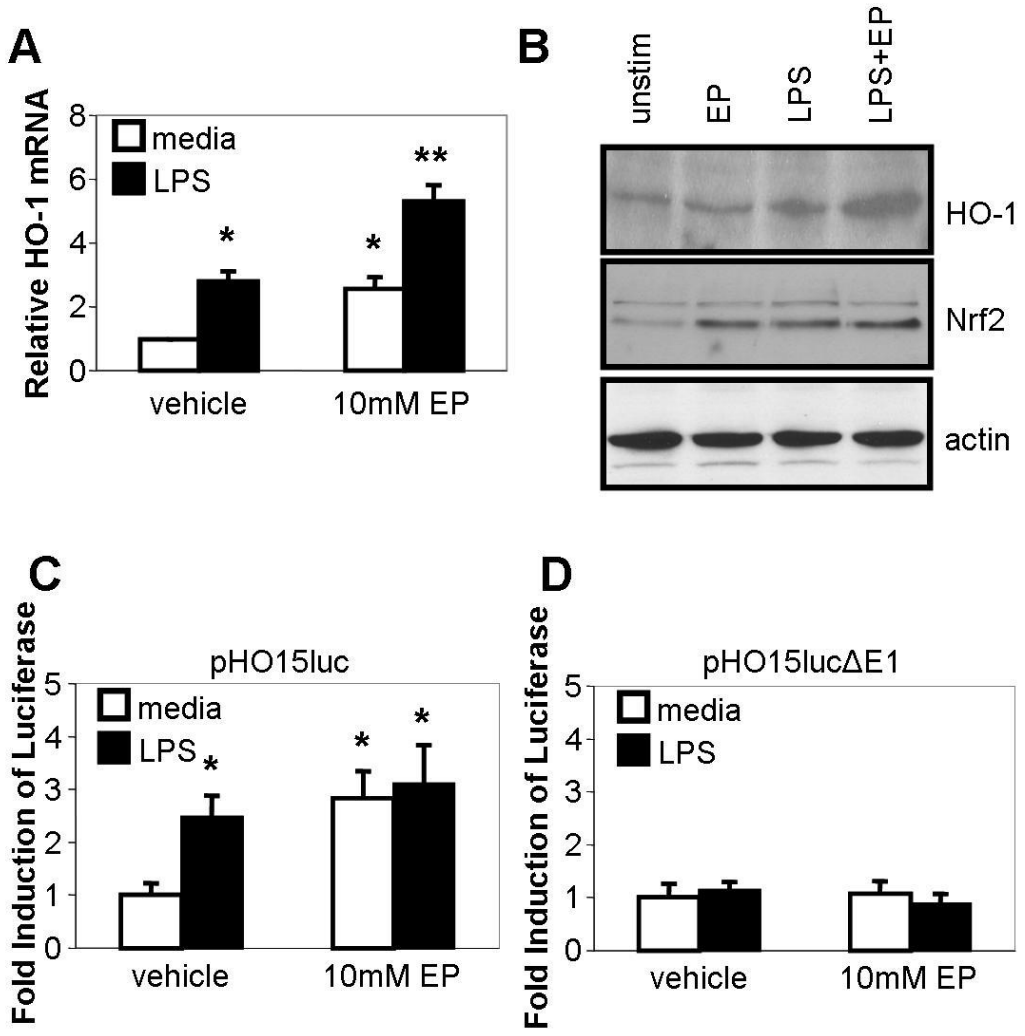


**Figure 3.12: Ethyl pyruvate induces HO-1 mRNA, protein, and transcriptional activity.**

BM-derived murine macrophages were incubated with 10 mM ethyl pyruvate (EP) for 1 hour prior to activation with 100 ng/ml LPS. (A) Cells were harvested at 4 hours for mRNA and real time RT-PCR assay for HO-1 was performed using the housekeeping gene GAPDH as an internal control. Real time RT-PCR was performed in duplicate and repeated three times (mean ± standard deviation). Fold induction is compared to values in vehicle/media samples (=1). (B) Cells were harvested at 4 hours for nuclear extracts. Western blots were performed for HO-1 and Nrf2 protein. RAW264.7 cells were transiently transfected with the (C) wild-type pHO15luc or (D) pHO15lucΔE1 mutant reporter. Results are expressed as relative light units normalized to β-galactosidase activity from a co-transfected HSP promoter-β-galactosidase plasmid to correct for transfection efficiency. Fold induction of the reporter is compared to values in unstimulated cells (=1) for each group. Each result represents the mean ± standard deviation of an experiment performed in triplicate. *, $p<0.05$; **, $p<0.01$ compared to unstimulated samples.

promoter construct lacking the E1 enhancer site (pHOlucΔE1) was transfected into RAW264.7 cells. The mutant promoter was only minimally responsive to ethyl pyruvate compared to the wild-type construct (Figure 3.12D). Recent evidence suggests that antioxidants, such as ethyl pyruvate, activate the ARE via a Keap1/Nrf2-dependent pathway (209). By Western immunoblot, ethyl pyruvate increased levels of the Nrf2 transcription factor in the nucleus, correlating with increased expression of HO-1 (Figure 3.12B).

## 3.5 DISCUSSION

In the present study, we have demonstrated that ethyl pyruvate ameliorates colitis in the acute model of TNBS-induced colitis, and in the chronic *IL-10*$^{-/-}$ IBD model. Moreover, we show that local administration (intrarectal) of ethyl pyruvate is effective in the TNBS model suggesting that ethyl pyruvate can exert its immunomodulatory effects directly in the gastrointestinal mucosa. *In vivo* and in cells, ethyl pyruvate appears to have pleiotropic anti-inflammatory effects which include downregulation of proinflammatory cytokine expression and NF-κB activation, inhibition of HMGB1 expression and/or secretion, and upregulation of anti-inflammatory pathways mediated by HO-1.

We demonstrate anti-inflammatory effects of ethyl pyruvate in two different mouse models of IBD using two different routes of administration. Intrarectal administration of the haptenating agent TNBS results in an induced model of colitis that has immunologic similarities to human CD: In genetically predisposed mouse strains, TNBS has been described to result in Th1-mediated inflammation (71). However, for short term studies as performed in this analysis, it is more accurate to describe this model as an acute intestinal injury. Nevertheless, this model

provides several advantages for testing local administration of ethyl pyruvate by the intrarectal route, compared to a chronic model like *IL-10*$^{-/-}$ mice. First, there is 100% penetrance of colitis in the BALB/c mouse strain. Furthermore, the distribution of colonic injury will parallel the distribution of locally applied therapy as TNBS and vehicle/ethyl pyruvate are both given intrarectally. This model is well validated for such therapeutic trials. Finally, given that ethyl pyruvate has been shown to augment altered intestinal barrier function, local delivery of ethyl pyruvate in TNBS colitis is an ideal model to study this notion *in vivo* as altered barrier function has been well described (35, 135-137). Ultimately, the TNBS and *IL-10*$^{-/-}$ models will both be useful to address specific mechanistic questions about ethyl pyruvate, including durability of clinical responses, alterations in immune cell phenotype and function, and alterations in barrier function following systemic and local administration.

As a lipophilic agent, ethyl pyruvate can be contemplated as a therapeutic modality that may be mucosally active, i.e. can be delivered directly to the gastrointestinal tract by the oral or intrarectal route. It is likely to be safe as ethyl pyruvate is a common additive in beverages and confectionary products and its safety profile has been studied in animals (210, 211). Moreover, ethyl pyruvate has entered phase II clinical trials in high-risk patients undergoing cardiac surgery and cardiopulmonary bypass, having successfully demonstrated safety during phase I (Critical Therapeutics, Inc., Lexington, MA) (212). Interestingly, there is a report that enteric bacteria of the *Enterobacter* species can metabolize endogenous ethyl pyruvate (213), leading to the hypothesis, akin to other bacterial metabolites with putative anti-inflammatory properties like butyric acid, that ethyl pyruvate may have a physiologic role in maintaining mucosal homeostasis. Accordingly, ethyl pyruvate was tested in preclinical IBD models as both a systemic and a local agent. An advantage of a locally administered therapy is selective inhibition

of intestinal as opposed to systemic immune responses. However, it is also possible that local effects of therapeutic interventions may exert global mucosal immunologic effects. This may be achieved through the specific alteration and trafficking of specific mucosal cell populations. Furthermore, it has been shown that animals pretreated or transiently-treated with ethyl pyruvate demonstrated durable protection against inflammation induced intestinal epithelial barrier dysfunction (~6 hours) (185). It is plausible that ethyl pyruvate may exert durable immunologic effects in IBD following local or systemic administration through alterations in immune cell populations or phenotypes. These studies are currently being investigated.

Specific inflammatory mediators are secreted by host cells during inflammation and cellular stress and necrosis, termed damage associated molecular pattern (DAMP) danger signals (92, 119, 120). DAMPs subsequently trigger an inflammatory response in neighboring cells, signifying the presence of danger and/or tissue damage. HMGB1, originally described as a non-histone, chromatin-associated nuclear protein, has emerged as a critical DAMP, or "endogenous danger signal" produced by host cells (122, 123). HMGB1 induces inflammatory mediators (127) and has been implicated in cytokine-induced pathologies, such as endotoxemia and polymicrobial bacterial sepsis, ischemia and reperfusion, acute lung injury, and arthritis (184, 214-217). HMGB1 is actively secreted by immunostimulated macrophages, natural killer cells, and enterocytes and released by necrotic, but not apoptotic, cells (124-126, 218, 219). In cell line models, HMGB1 also alters intestinal epithelial cell permeability (131). Moreover, in septic mice, ethyl pyruvate treatment demonstrated a significant decrease in HMGB1 levels that correlated with improved mortality (184). In *IL-10*$^{-/-}$ mice, we show increased fecal levels of HMGB1 (Figure 3.2A), suggesting apical secretion of HMGB1 from IECs and/or IEC necrosis as a consequence of chronic inflammation. Treatment with ethyl pyruvate decreased fecal levels

of HMGB1 (Figure 3.2B), which correlated with disease activity. Similar to HMGB1, the S100 family of proteins has been characterized as putative DAMPs and may have diagnostic and pathogenic implication in human IBD. An increase in serum levels of S100A12 (en-RAGE) was described in patients with active CD and UC. This was correlated with expression of S100A12 in inflamed intestinal tissue from IBD patients, but not healthy controls (220). Furthermore, S100A8/A9 (calprotectin), a calcium binding protein contained in the cytoplasm of neutrophils, macrophages, and epithelial cells, is a marker of inflammation and is increased in the serum and stool of several chronic inflammatory disorders, including IBD (221). Calprotectin is also detectable in feces, and this has been used as a non-invasive marker of disease activity and has a positive predictive value for inflammatory gastrointestinal conditions (222). Likewise, HMGB1 may have pathogenic significance and diagnostically may serve as a marker of disease activity. Immunohistochemistry and fecal ELISA for HMGB1 in ethyl pyruvate-treated and vehicle-treated mice suggest that ethyl pyruvate may mechanistically function in part to prevent HMGB1 release/secretion as has been previously demonstrated (184).

The first described receptor for HMGB1 was RAGE (128). Recent studies have demonstrated that HMGB1 also utilizes pattern recognition receptors of the innate immune system, TLR2 and TLR4 (129, 130). As recognition of enteric microbes by the innate immune system is an underlying defect in human IBD, our present findings further suggest that HMGB1 may also have a direct involvement in mucosal inflammation. Given the prominent apical location of RAGE immunostaining, it is interesting to speculate that IEC RAGE may be produced in a secreted form, soluble RAGE (sRAGE). The detection of RAGE in fecal samples by ELISA could represent a secreted sRAGE isoform, and/or it may represent membrane RAGE expressed on sloughed epithelial cells. As HMGB1 is secreted apically into the gastrointestinal

lumen by IECs and its receptor RAGE has an apical location, it is possible that HMGB1-RAGE interactions on the apical surface of epithelia modulate immune responses in the gut. RAGE is promiscuous for other S100 family members and DAMPs, and therefore is an attractive target for therapeutic intervention in IBD. A small study has demonstrated amelioration of colitis by sRAGE administration in *IL-10*$^{-/-}$ mice (223). Furthermore, a recent study demonstrated that a specific antibody that recognizes carboxylated glycans on RAGE attenuates murine colitis in an adoptive T cell transfer model (224). This study demonstrated expression of carboxylated glycans on human colonic endothelium and lamina propria macrophages. Although IEC immunostaining was not described, carboxylated glycans are only expressed on a subpopulation of RAGE molecules and modify additional proteins. These results, taken as a whole, support an important immunoregulatory role for the HMGB1/S100/RAGE axis in IBD.

NF-κB proteins have been implicated as key functional activators for IL-12 p40 and NO (225, 226). We observed substantial inhibition of the NF-κB DNA binding by ethyl pyruvate. Therefore, it is not surprising that ethyl pyruvate is able to inhibit target genes, such as IL-12 p40 and NO (Figure 3.8). It is of interest that ethyl pyruvate inhibits NF-κB DNA binding activity but not nuclear translocation. Although mechanisms for these phenomena are not clear, we show that it is not a result of induction of the nuclear inhibitor of NF-κB, PPAR-γ (204, 205). We speculate that NF-κB regulation by ethyl pyruvate may be related to its antioxidant properties. This is supported by evidence that antioxidants impair NF-κB p50 DNA binding as p50 DNA binding can be rescued by the addition of the reducing agent, DTT (227). This suggests that redox-sensitive amino acids are important for NF-κB DNA binding. Cysteine 62 in the DNA-binding domain of NF-κB p50 has been shown to be sensitive to antioxidants and ROS, presumably resulting in a protein conformational change and the decreased ability to bind DNA

(228, 229). There is one report suggesting that ethyl pyruvate specifically targets NF-κB p65 (187).

The importance of the anti-inflammatory HO-1 pathway in mucosal inflammation has recently been demonstrated (196). Using pharmacologic inducers of HO-1, hemin and cobalt protoporphryin, amelioration of colitis was demonstrated in *IL-10*$^{-/-}$ mice. Hemin has been shown to induce HO-1 through the transcription factor Nrf2 (230, 231). This transcription factor is also important in antioxidant driven HO-1 expression mediated through the E1 enhancer site that contains the ARE and the Nrf2 binding site (207-209, 232). Therefore, we speculate that ethyl pyruvate induces HO-1 promoter activity, mRNA and protein in cells (Figure 3.12) and *in vivo* (Figure 3.6) through mechanisms involving its antioxidant properties (180). This is likely accomplished through increased translocation of the redox-sensitive transcription factor Nrf2.

In conclusion, ethyl pyruvate, a simple aliphatic ester derivative of pyruvate, demonstrated notable anti-inflammatory effects *in vivo* in acute and chronic murine colitis. Local intrarectal administration as well as intraperitoneal administration was associated with downregulation of mucosal inflammation. Ethyl pyruvate appears to have pleiotropic anti-inflammatory effects, including modulating HMGB1 secretion/release and/or receptor activation, attenuating NF-κB DNA binding, and inducing HO-1. As ethyl pyruvate is currently under investigation in a phase II trial for cardiac disease, it will be of clinical interest to further investigate a therapeutic role for ethyl pyruvate in the human IBDs.

# 4.0 AMELIORATION OF CHRONIC MURINE COLITIS BY PEPTIDE MEDIATED TRANSDUCTION OF THE IKB KINASE (IKK) INHIBITOR NEMO BINDING DOMAIN (NBD) PEPTIDE

## 4.1 ABSTRACT

The NF-κB family of transcription factors is a central regulator of chronic inflammation. The phosphorylation of IκB proteins by the IκB kinase (IKK) complex (IKKα, IKKβ, and NF-κB essential modulator, or NEMO) is a key step in NF-κB activation. Peptides corresponding to IKK's NEMO binding domain (NBD) blocks NF-κB activation without inhibiting basal NF-κB activity. In this report, we determined the effects of the IKK inhibitor peptide (NBD) in a model of murine colitis, the IL-10-deficient (*IL-10*$^{-/-}$) mouse. Utilizing a novel cationic peptide transduction domain (PTD) consisting of eight lysine residues (8K), we were able to transduce the NBD peptide into cells and tissues. In a NF-κB reporter system, 8K-NBD dose-dependently inhibits TNF-induced NF-κB activation. Furthermore, 8K-NBD inhibited nuclear translocation of NF-κB family members. *In vivo*, *IL-10*$^{-/-}$ mice treated systemically with 8K-NBD demonstrate amelioration of colitis and a reduction in spontaneous intestinal IL-12 p40 and TNF production. These results demonstrate that inhibitors of IKK, in particular a PTD-NBD peptide, may be therapeutic in the treatment of inflammatory bowel disease.

## 4.2 INTRODUCTION

The NF-κB family of transcription factors is considered to be a "master switch" for inflammatory gene expression (101, 102). NF-κB represents a group of structurally related proteins that includes five members in mammals: p65, c-Rel, Rel-B, p50, and p52. In unstimulated cells, NF-κB proteins are localized in the cytoplasm through their association with members of a family of inhibitory proteins known as IκB proteins. IκB proteins bind to NF-κB and mask their nuclear localization signals. Proinflammatory cytokines such as TNF and IL-1, and bacterial products such as lipopolysaccharide (LPS)[4] induce phosphorylation of IκB proteins at specific N-terminal serine residues. Phosphorylated IκB proteins are then ubiquitinated and degraded by the proteosome, releasing bound NF-κB which translocates to the nucleus, binds to NF-κB DNA binding sites on enhancer elements of target genes, and induces transcription. NF-κB plays an essential role in the inflammatory response through the regulation of genes encoding proinflammatory cytokines (IL-1β, TNF, IL-12), chemokines (IL-8, MIP-1α, MCP-1), and adhesion molecules (ICAM-1, VCAM, E-selectin). Cytokines that are stimulated by NF-κB such as IL-1β and TNF, can also directly activate the NF-κB pathway, thus establishing an auto-regulatory loop that may be essential in the perpetuation of chronic inflammation (103).

Activation of NF-κB has been shown to be important in many chronic inflammatory diseases such as asthma, rheumatoid arthritis, and inflammatory bowel disease (IBD) (103). The critical role of NF-κB in chronic intestinal inflammation is best illustrated by numerous reports

[4]Abbreviations used in this chapter: 6CF, 6-carboxyfluorescein; BM, bone marrow; CARD, caspase recruitment domain; CD, Crohn's disease; CIA, collagen induced arthritis; EAE, experimental allergic encephalomyelitis; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IKK, IκB kinase; *IL-10*$^{-/-}$, IL-10-deficient; LPS, lipopolysaccharide; MDP, muramyl dipeptide; mNBD, mutant NBD; NBD, NEMO binding domain; NEMO, NF-κB essential modulator; NOD2, Nucleotide oligomerization domain 2; PTD, protein transduction domain; TLR, toll-like receptor; TNBS, 2,4,6-trinitrobenzene sulfonic acid; UC, ulcerative colitis.

of non-specific as well as selective blockade of NF-κB activation demonstrating therapeutic efficacy in animal models of IBD (134, 135, 137). Furthermore, activated NF-κB is found in the human IBDs, Crohn's disease (CD) and ulcerative colitis (UC). A significant increase in p65 protein in lamina propria macrophages and epithelial cells from CD patients was correlated with increased production of the inflammatory cytokines IL-1β, IL-6, and TNF (138-140). Many of the standard agents used to treat human IBD, including sulfasalazine, 5-aminosalicylates, and corticosteroids, have been postulated to exert some of their anti-inflammatory effects through NF-κB inhibition (103, 233-236). Thus, downregulation of NF-κB activity emerges as a potential key event in the control of chronic intestinal inflammation in humans.

The phosphorylation of IκB proteins is a key step in the regulation of NF-κB. Phosphorylation is mediated by a specific IκB kinase (IKK) complex. IKK is made up of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit named "NF-κB essential modulator" (NEMO; also named IKKγ) (132). An N-terminal region of NEMO associates with a hexapeptide sequence within the C-terminus of both IKKα and IKKβ, named the NEMO binding domain (NBD). Activation of the IKK complex in response to proinflammatory mediators depends critically on the presence of the NEMO subunit of the IKK complex, as NEMO-deficient cells lack detectable NF-κB binding activity in response to TNF, IL-1β, and LPS (133). Because of the importance of the IKK complex in inflammation, the identification of selective IKK inhibitors as potential therapeutic agents is of considerable interest. A short peptide spanning the NBD disrupted the association of NEMO with IKKs *in vitro* and blocked TNF induced NF-κB activation *in vivo*. Notably, the NBD peptide did not affect basal activity of the IKK, important for cell viability, but only suppressed the induction of activity in response to proinflammatory cytokines (237). Recent studies have shown that continuous administration of

the NBD peptide effectively ameliorates inflammatory responses in animal models of inflammation (238).

Protein transduction domains (PTDs) have been shown to deliver a wide variety of therapeutic agents into cells including peptides, proteins, nucleic acids, antibodies and small drugs. The first protein reported with transductional properties was the HIV transactivator protein, TAT in which the 11 amino acid PTD was identified by virtue of its cationic content (239). Recently, PTDs have been characterized that mediate efficient and rapid receptor-independent internalization of peptide-protein conjugates (240). These cationic transduction peptides transduce a wide variety of cells similar to the HIV TAT, mediating highly efficient transduction *in vitro* and *in vivo* (241, 242). Screening of a panel of cationic peptides has demonstrated that peptides of 8 or 10 lysines are highly efficient transduction domains, working as or more effectively than other cationic PTDs for delivery of peptides and proteins to numerous cell types, including mucosal cells and antigen presenting cells (241).

Although immunological research in IBD has focused on the central role of adaptive immunity, in particular the T cell, a convergence of recent findings suggests that innate immunity to enteric microbes may ultimately direct the adaptive immune response. Key participants in innate immunity are macrophages and dendritic cells. These cells recognize microbial products through pattern recognition receptors and elaborate proinflammatory cytokines that recruit other inflammatory cells and activate T cell responses (243). Of the proinflammatory genes that are induced in macrophages and dendritic cells by the microbial environment, NF-κB dependent genes such as IL-12 p40 play a central role in mucosal inflammation and bridging innate and adaptive immune responses. Accordingly, we postulated that a novel cell permeable peptide IKK inhibitor, NBD, (8K-NBD) may efficiently target

macrophages and dendritic cells, and therefore may represent a novel therapeutic approach in chronic murine colitis in IL-10-deficient (*IL-10*$^{-/-}$) mice. In this report, we show 8K-NBD dose-dependently inhibits TNF-induced NF-κB activation and translocation in cells and ameliorates chronic colitis *in vivo*.

## 4.3 MATERIALS AND METHODS

**Peptides.** Peptides 8K-NBD (acetyl-KKKKKKKKGGTALDWSWLQTE-amide), inactive mutant, 8K-mNBD (acetyl-KKKKKKKKGGTALDASALQTE-amide), random-NBD (ARPLEHGSDKAT-GGTALDWSWLQTE), 8K-biotin (KKKKKKKK-biotin), and random peptide-biotin (ARPLEHGSDKAT-biotin) were synthesized by the peptide synthesis facility at the University of Pittsburgh. The random-NBD and 8K-NBD peptides were made fluorescent using an N-terminal conjugated 6-carboxyfluorescein (6CF, Molecular Probes). Peptides were purified and characterized by reversed-phase high performance liquid chromatography and mass spectrometry.

**Murine macrophages.** The murine macrophage cell line, RAW264.7, was maintained in DMEM/10% FBS/1% Pen/Strep. Primary bone marrow (BM) murine macrophages were isolated from femurs of C57BL/6 mice. BM were flushed with washing medium (RPMI1640 with 1% Pen/Strep), passed through a 70 μM nylon cell strainer into a 50 ml conical tube, and spun down at 1500 rpm for 5 minutes. RBCs were lysed using sterile-filtered 0.8% ammonium chloride, washed twice with washing medium, and resuspended in complete medium (washing medium with 10% FBS). BM cells were seeded in complete medium in a 150 mm dish and

differentiated using recombinant murine GM-CSF (20 ng/ml) (R&D Systems). At day three, another 25 ml fresh culture medium containing GM-CSF was added to the culture plates. At day seven, the cells, representing the BM-derived macrophage population, were harvested for experiments.

**NF-κB Luciferase assay.** The HEK293 cells stably transfected with a multimerized NF-κB DNA binding element-luciferase reporter (DMEM/10% FBS/1% Pen/Strep) were pretreated for one hour with 8K-NBD dissolved in OptiMEM media (Invitrogen) and activated for two hours with 10 ng/ml TNF (R&D Systems). The cells were lysed in reporter lysis buffer and luciferase activity was measured with a luciferase assay system (Promega) using a Turner Designs Luminometer TD20/20.

**Nuclear extracts and Western blotting.** HEK293 cells were pretreated for one hour with media, 8K-NBD, or 8K-mNBD dissolved in OptiMEM media (Invitrogen) and activated for 15 minutes with 10 ng/ml TNF (R&D Systems). Nuclear extracts from treated HEK293 cells were isolated following manufacturer's protocol (NE/PER Reagents, Pierce). Protein concentration was determined using the Bradford assay (Pierce). Western blot analyses were performed on nuclear extracts as described previously (149). Anti-p65 and anti-c-Rel antibodies were obtained from Santa Cruz Biotechnology, Inc., and anti-PARP and anti-phospho-p65 (p-p65) antibodies were obtained from Cell Signaling.

**Mice.** Male C57BL/6 (10-12 weeks old) and female BALB/c (12-13 weeks old) mice were obtained from The Jackson Laboratory. An *IL-10*$^{-/-}$ colony (breeder pairs from The Jackson

Laboratory) was maintained in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and was approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh School of Medicine.

***In vivo* PTD transduction.** C57BL/6 or BALB/c mice were grouped randomly and treated either intraperitoneally or intrarectally with biotinylated peptides (random or 8K) linked to streptavidin-Cy3. After 30 minutes of treatment, organs were harvested, fixed in 2% paraformaldehyde, and then incubated in 30% sucrose in PBS at 4°C overnight. Samples were snap-frozen in isopentane and cut into 6 μm-thick frozen sections and placed on microscope slides.

**Immunohistochemistry.** After transduced macrophages or cut tissue sections were placed on coverslips, slides were blocked in BSA and stained for nuclei with either Draq5 (Biostatus Limited, Leicestershire, United Kingdom) or Propidium Iodide (PI, Molecular Probes), for 30 min. Phalloidin was used to visualize F-actin (Molecular Probes). After extensive washing, slides were mounted and viewed on an Olympus Flouview 1000 confocal microscope (Olympus America, Melville, NY).

***In vivo* NBD peptide treatment.** 10-week old *IL-10*$^{-/-}$ mice were grouped randomly and treated with either mutant (10 mg/kg) or wild-type (2 or 10 mg/kg) NBD peptide linked to 8K. Treatment was administered in PBS in a total volume of 500 μl intraperitoneally for 10 out of 14 days. At the end of the study period, animals were euthanized using excess $CO_2$ inhalation and intestinal tissue was harvested.

**Intestinal tissue explant cultures.** Colons were isolated from individual mice, cut open longitudinally, and cleaned of fecal matter. The intestinal tissue was washed with PBS to remove residual fecal content. Intestinal sections were cut in half longitudinally, and one half was shaken at 250 rpm at room temperature for 30 min in RPMI 1640 supplemented with 1% antibiotic/antimycotic. Tissue fragments (0.05 g dry weight) were incubated in 1 ml RPMI supplemented with 1% antibiotic/antimycotic and 10% FBS. Supernatants were collected after 24 hours, assayed for spontaneous cytokine production via ELISAs, and normalized to dry gut weight.

**Histology.** Colons were isolated from individual mice, cut open longitudinally, and cleaned of fecal matter. Intestinal sections were cut in half longitudinally, and one half was fixed in 10% buffered formalin and embedded in paraffin. 5 μm thick sections were stained with hematoxylin and eosin. Colitis scores (0-4) were determined by a staff pathologist using the criteria reported by Berg *et al.* (33). At least 20 separate microscopic fields (10×) were evaluated for each mouse by a pathologist (Dr. Antonia R. Sepulveda, University of Pittsburgh) blinded to the treatment groups.

**Cytokine ELISAs.** Murine IL-12 p40 and TNF immunoassay kits were used according to the manufacturer's instructions (BD Pharmingen). Values were measured using a plate reader and the SOFTMax Pro v4.8 software (Molecular Devices).

**Statistical Analysis.** Statistical significance between groups was assessed by two-tailed Student's *t* test. A *p*-value equal or less than 0.05 was considered to be statistically significant.

## 4.4 RESULTS

**Transduction of 8K-PTD peptide into macrophages.** As previously reported (241), a panel of cationic protein transduction domains were screened for the ability to efficiently transduce a variety of cell types. Eight to 10 amino acid polylysine tracts efficiently transduce a wide array of cell lines and primary cells, including islet β-cells, synovial cells, polarized airway epithelial cells, tumor cells, and dendritic cells (241). Dendritic cells and macrophages are particularly important cell types in mucosal innate immune responses and the IBDs. Accordingly, we postulated that the 8K PTD linked to the NBD peptide may efficiently target macrophages and dendritic cells, and therefore may represent a novel therapeutic approach in chronic murine colitis.

To assess transduction of the 8K PTD specifically into macrophages, murine BM-derived macrophages were incubated with biotinylated 8K PTD linked to streptavidin-Cy3 and the murine macrophage cell line RAW264.7 was incubated with 8K PTD conjugated to the fluorescent label 6-carboxyfluorescein (6CF). Murine macrophages were efficiently transduced with the 8K PTD (>90% of cells demonstrate fluorescence) compared to a negative control peptide containing a random peptide sequence in place of the PTD (Figure 4.1). This result demonstrates that macrophages are efficiently transduced with the 8K-PTD.

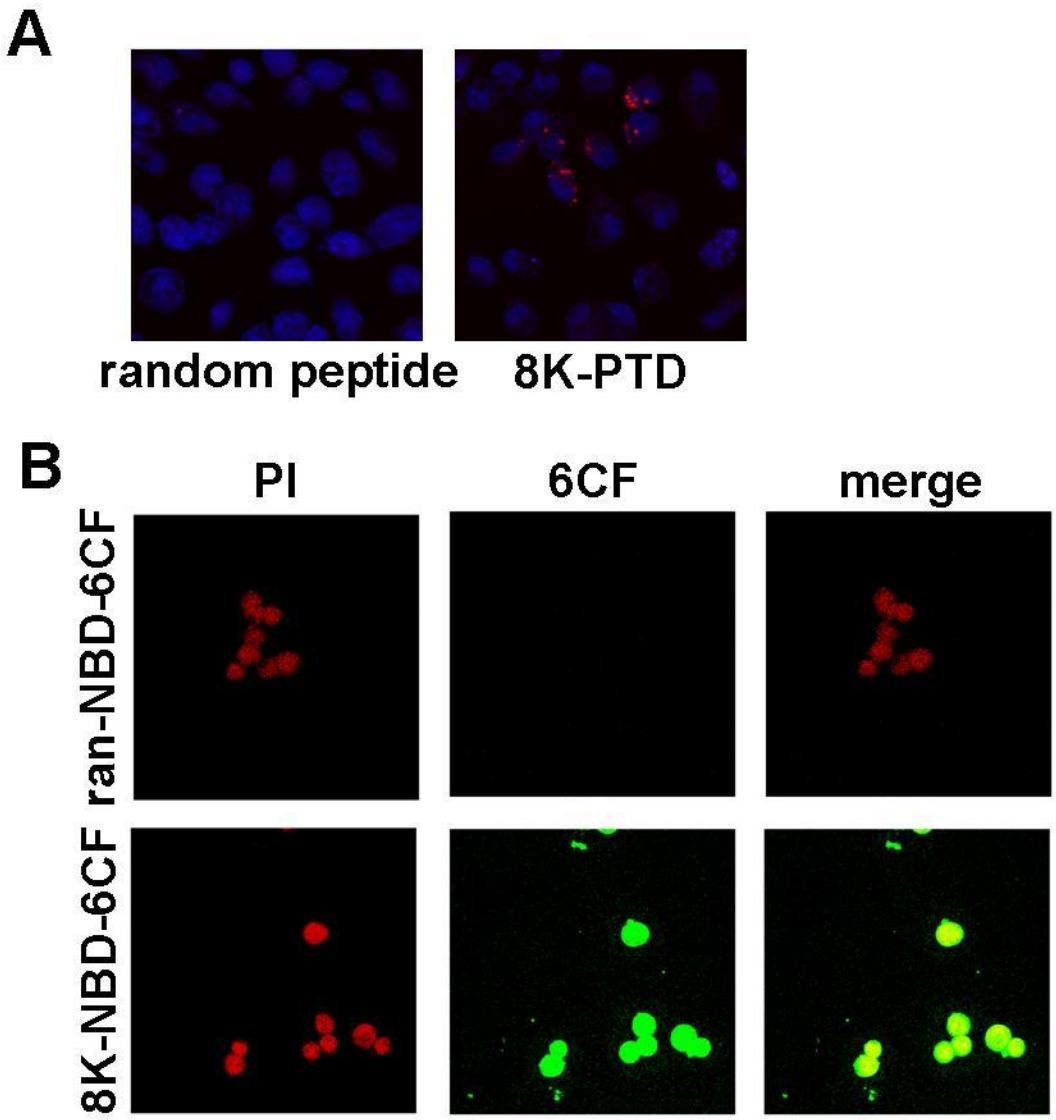


**Figure 4.1: 8K efficiently transduces murine macrophages.**

(A) Biotinylated random (ran) peptide linked to streptavidin-Cy3 or 8K PTD linked to streptavidin-Cy3 were added to BM-derived macrophages. (B) The fluorescently labeled random peptide-NBD-6CF or 8K-NBD-6CF were added to RAW264.7 macrophages. Following incubation for 1 hour, cells were fixed, stained for nuclei (Draq5 in A, PI in B), and placed on a microscope slide. Localization of peptide was visualized by a confocal microscope system. Results were repeated three times and representative images are shown.

**8K-NBD inhibits TNF-stimulated NF-κB activation and nuclear translocation in cells.** Next, 8K-NBD peptide was preincubated with HEK293 cells expressing a stably transfected multimerized NF-κB DNA binding element-luciferase reporter gene. Cells were subsequently stimulated with TNF. Pretreatment with 8K-NBD demonstrated a dose-dependent inhibition in TNF-stimulated NF-κB activity (Figure 4.2A). Moreover, 8K-NBD pretreatment without TNF stimulation did not alter basal levels of NF-κB activity, indicating that the peptide specifically targets activated NF-κB. Nuclear translocation of NF-κB family members in activated cells was next assessed. By Western immunoblot analysis on nuclear extracts, decreased nuclear quantities of the NF-κB family members p65 (and its phosphorylated form, p-p65), c-Rel, and p50 (Figure 4.2B) are demonstrated in 8K-NBD pretreated TNF-activated HEK293 cells.

**Transduction of 8K-PTD *in vivo*.** 8K PTD efficiently transduces cells and inhibits NF-κB activity. We next investigated whether 8K PTD transduces cells *in vivo* in mice. Mice were administered biotinylated peptides (random sequence or 8K PTD) linked to streptavidin-Cy3 either intraperitoneally or intrarectally. *In vivo* uptake of peptide is observed as early as 30 minutes after administration. Intraperitoneal administration of 8K PTD revealed uptake in the spleen (Figure 4.3A-B) and mesenteric lymph nodes (Figure 4.3C-D). Intrarectal administration of 8K PTD revealed uptake in colonic tissue (Figure 4.3E-F). These results demonstrate important intestinal immune compartments could be targeted by 8K peptide-mediated transduction *in vivo*.

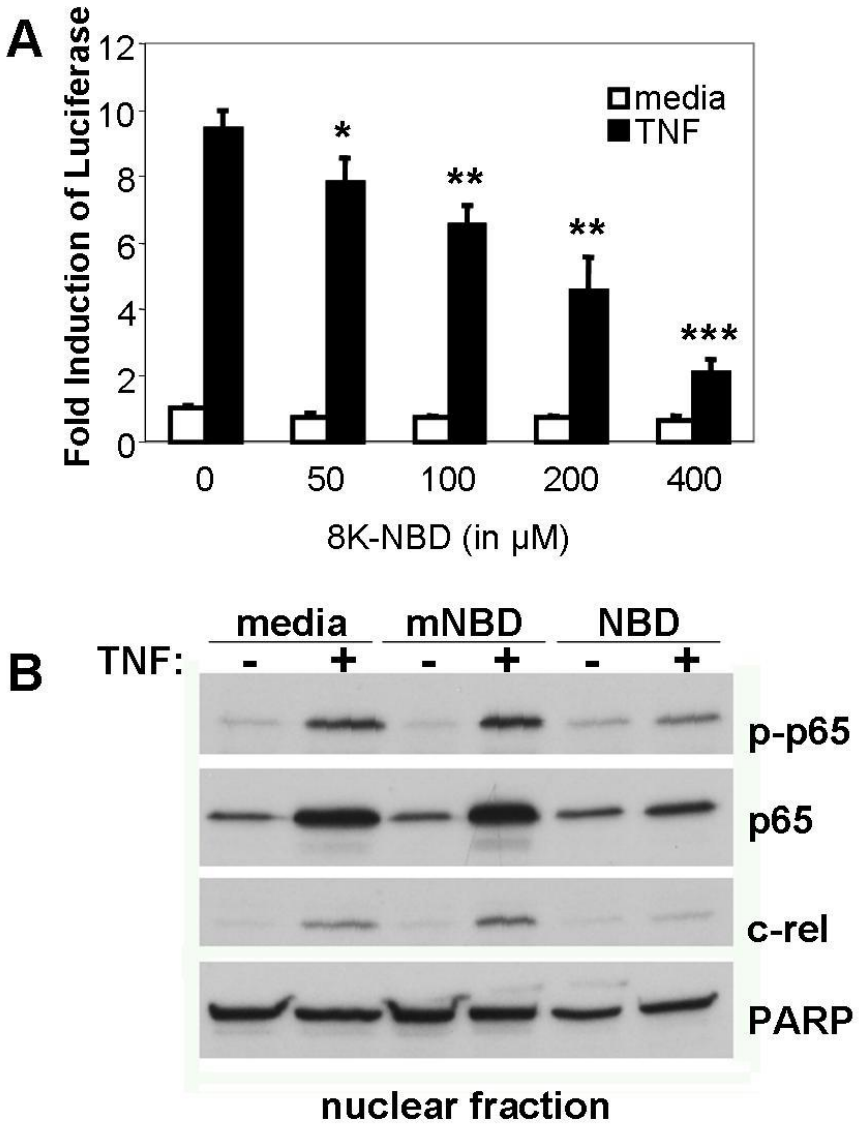


**Figure 4.2: TNF-induced NF-κB activation is inhibited by 8K-NBD transduction in cells.**

(A) HEK293 cells stably transfected with a multimerized NF-κB DNA binding element-luciferase reporter were preincubated for 1 hour with an increasing dose of 8K-NBD peptide in media. Cells were subsequently stimulated for 2 hours with 10 ng/ml rhTNF. Cells were harvested and lysates were analyzed for luciferase activity. Results are expressed as fold induction of luciferase compared to unstimulated + 0 μM peptide lysates (=1). Experiments were performed in triplicate and repeated three times. A representative result is shown (mean ± standard deviation). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared to unstimulated sample. (B) Nuclear extracts of HEK293 cells stimulated with or without 10 ng/ml rhTNF in the presence of media, 8K-mNBD, or 8K-NBD were isolated and run out for Western blotting. Blots were probed with p-p65, p65, and c-Rel. Blots were probed with PARP to assess equal loading. Experiments were repeated three times and a representative blot is shown.

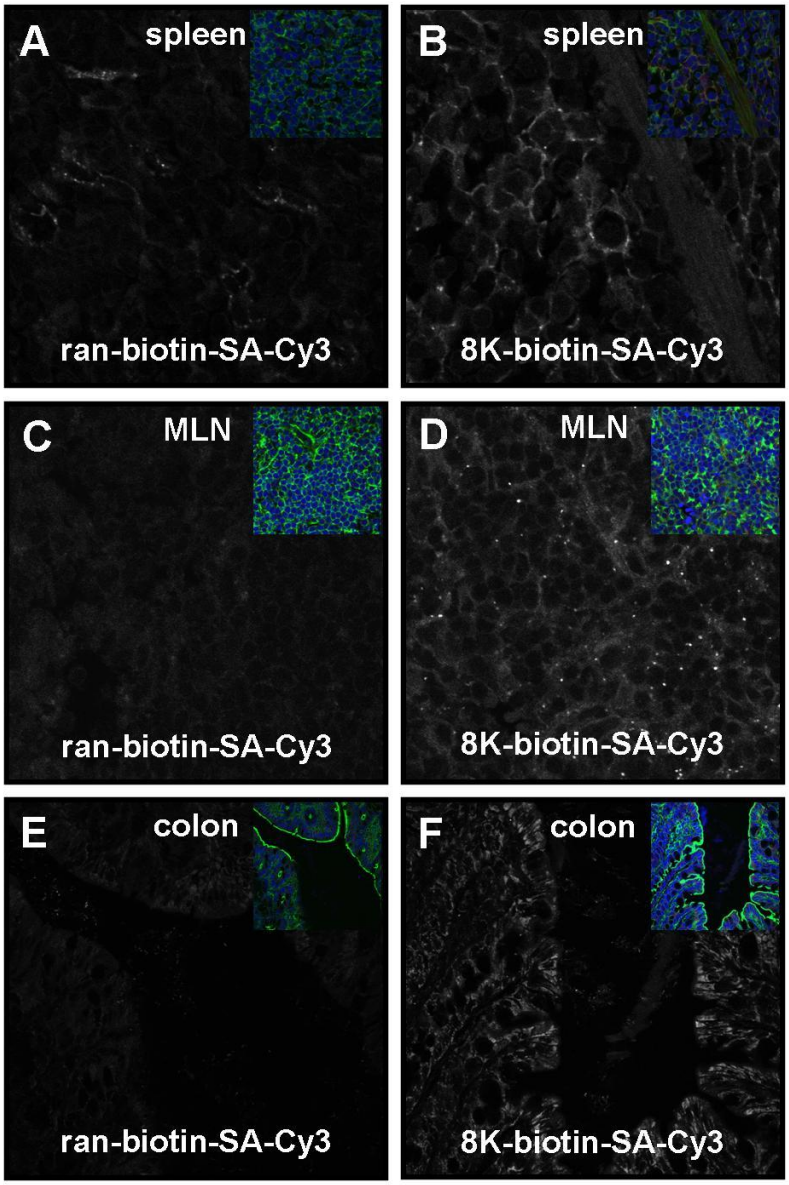


**Figure 4.3: *In vivo* transduction of the 8K PTD.**

Biotinylated (A, C, E) random (ran) peptide linked to streptavidin (SA)-Cy3 or (B, D, F) the 8K PTD linked to streptavidin (SA)-Cy3 were injected (A-D) intraperitoneally or (E-F) intrarectally for 30 minutes. Paraformaldehyde-fixed (A and B) spleens, (C and D) mesenteric lymph nodes (MLN), and (E and F) colons were stained for actin (green) and nuclei (blue, Draq5) and viewed by confocal microscopy. Inset represents the three-color image for each sample. To specifically visualize the location of the peptide (Cy3, red), the signal from the red channel was given a false white color, while the other channels were turned off. This is represented in the larger black and white image. Background threshold levels were adjusted to random peptide samples for each organ.

**8K-NBD treatment ameliorates colitis in *IL-10*$^{-/-}$ mice.** As a next step, we tested the hypothesis that the 8K-NBD may ameliorate active chronic colitis *in vivo* in *IL-10*$^{-/-}$ mice. *IL-10*$^{-/-}$ mice were treated from 10 to 12 weeks of age with either 8K-NBD at 2 or 10 mg/kg or 8K-mutant NBD (mNBD) at 10 mg/kg by intraperitoneal injection for 10 of 14 days. Gross inspection of the intestines revealed increased colonic lengths, decreased colonic wall thickening, and formed fecal pellets in the 8K-NBD treatment groups (Figure 4.4B-C) compared to the 8K-mNBD controls (Figure 4.4A). Histologic severity of colitis was graded over the entire length of the colon for each mouse by a single pathologist blinded to treatment groups. Due to the incomplete penetrance and the segmental, patchy pattern of colitis in *IL-10*$^{-/-}$ mice, colitis scores are depicted in two different ways. First, composite scores are represented using a modified standard scoring system described by Berg *et al.* (33) (Figure 4.5A). Mice treated with 8K-NBD at 2 mg/kg and 10 mg/kg demonstrated a 50% improvement in histological scores compared to the control treated group (mNBD). Second, the scores are presented as the percentage of fields that demonstrate no histological inflammation (colitis score of 0), mild to moderate inflammatory changes (colitis score of 1 and 2), and severe inflammation (colitis score of 3 and 4). This method provides a better representation of the spectrum of disease encountered over the entire length of the colon. This depiction of histological differences between treatment subgroups best exemplifies the magnitude of changes during therapeutic intervention trials as well as the patchy nature of colitis, similar to that often seen in human CD. Compared to the 8K-mNBD group (Figure 4.5B, black bars), 2 mg/kg and 10 mg/kg 8K-NBD treated mice (Figure 4.5B, gray and white bars, respectively) displayed more fields demonstrating no evidence of histologic inflammation and concomitantly fewer fields with inflammatory changes.

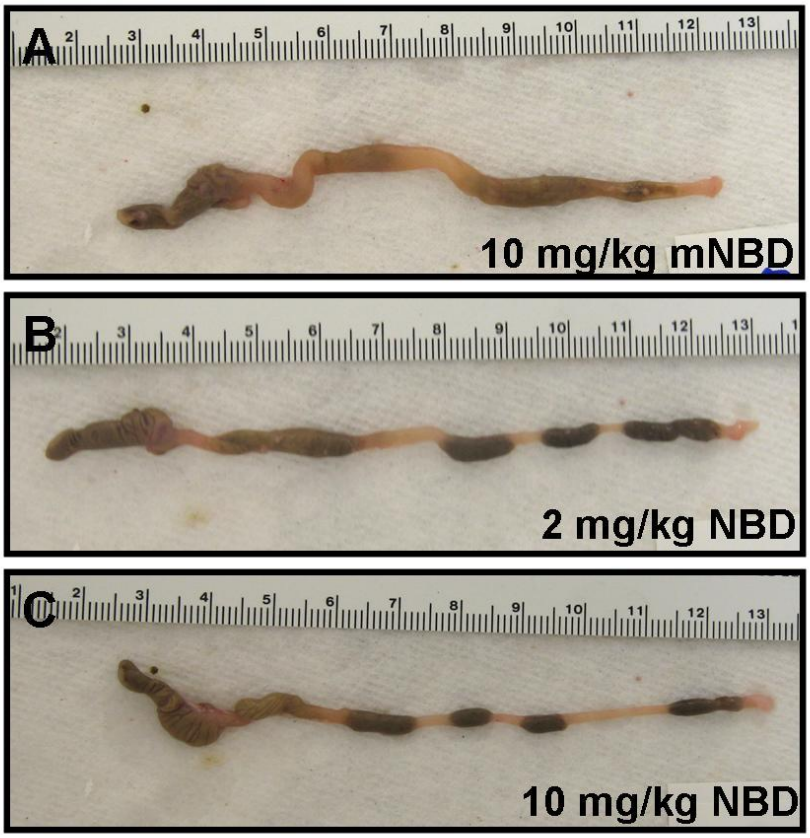


**Figure 4.4: Improvement in gross colonic appearance in 8K-NBD-treated mice.**

Representative photographs of colons from the control group (A) 10 mg/kg 8K-mNBD and treatment groups (B) 2 mg/kg 8K-NBD and (C) 10 mg/kg 8K-NBD. Colons from mice treated with 8K-NBD peptide demonstrated increased length, decreased tissue thickening, and formed stool pellets compared to the control group.

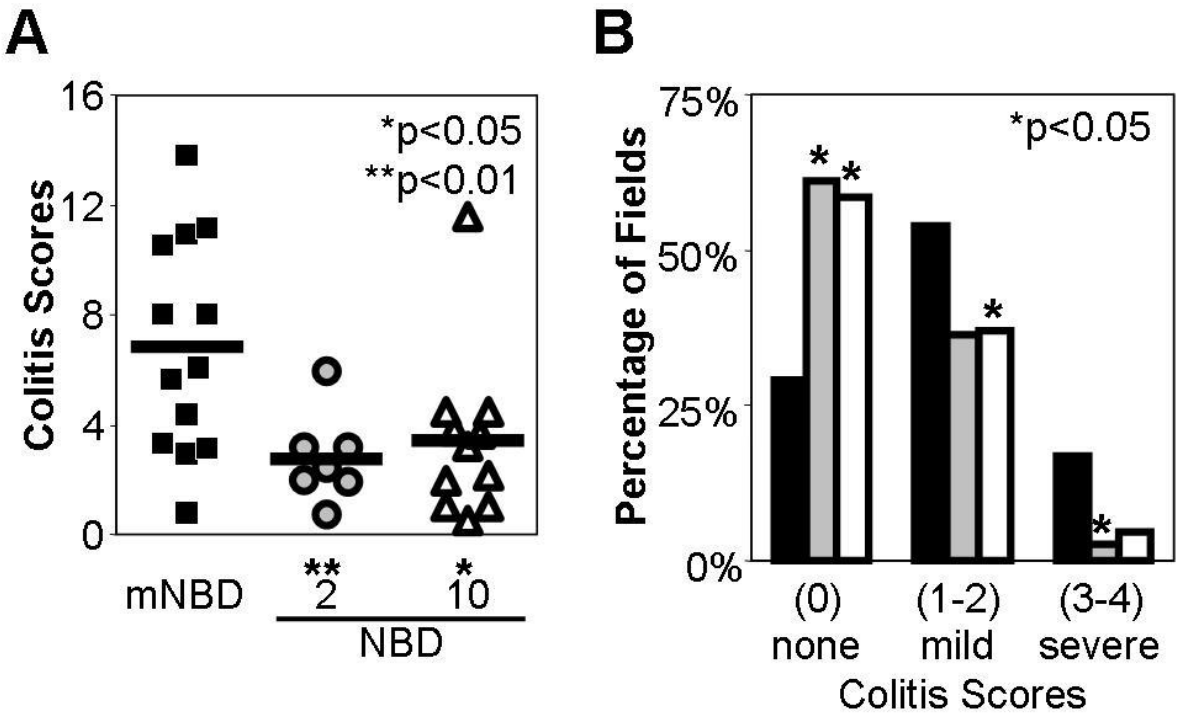


**Figure 4.5: 8K-NBD treatment ameliorates histologic colitis.**

Colons were isolated from individual mice (colors: black, 10 mg/kg mNBD; gray, 2 mg/kg NBD; white, 10mg/kg NBD), cleaned, fixed in formalin, and embedded in paraffin. Sections were stained with hematoxylin and eosin and colitis scores were determined using a modified scoring system (0-4) as described in the materials and methods section. At least 20 separate microscopic fields (10×) were evaluated for each mouse by a pathologist blinded to the treatment groups. Colitis scores were significantly lower in the 8K-NBD-treated mice. Histologic improvement in colitis is presented as (A) a composite score (the average colitis score sum of five fields) and (B) percent of histologic fields that demonstrate scores of 0 (no inflammation), 1-2 (mild inflammation), or 3-4 (severe inflammation).

Lastly, we investigated the effect of 8K-NBD treatment on mucosal inflammatory cytokine production in *IL-10*$^{-/-}$ mice. The spontaneous release of the NF-κB regulated proinflammatory cytokines IL-12 p40 (Figure 4.6A) and TNF (Figure 4.6B) were determined in cell free supernatants from colonic mucosal tissue explants. Explants of 8K-NBD-treated mice secreted significantly less IL-12 p40 and TNF compared to 8K-mNBD-treated control mice. This observation correlates with histological findings, suggesting that specific targeting of the IKK complex with cell permeable NBD peptides may be effective in treating chronic IBD.

## 4.5 DISCUSSION

In the present study, we investigated the systemic delivery of NBD, an IKK inhibitor peptide, employing a novel PTD, 8K. This peptide efficiently transduces cells including macrophages in culture, and transduces intestinal lymphoid tissue following intraperitoneal and intrarectal administration *in vivo*. In cells, 8K-NBD inhibits TNF-induced NF-κB transcriptional activity and nuclear translocation. Moreover, we demonstrate that 8K-NBD ameliorates chronic colitis in *IL-10*$^{-/-}$ mice, and histological improvement correlated with the reduction in levels of the mucosal inflammatory cytokines IL-12 p40 and TNF.

The critical role of NF-κB in chronic intestinal inflammation is perhaps best illustrated by non-specific as well as selective blockade of NF-κB activation in animal models of IBD (134, 135, 137). The first studies demonstrating activation of NF-κB in chronic intestinal inflammation were performed in the 2,4,6-trinitrobenzene sulfonic acid (TNBS) mouse model of induced colitis. Increased NF-κB DNA binding activity nuclear extracts from lamina propria

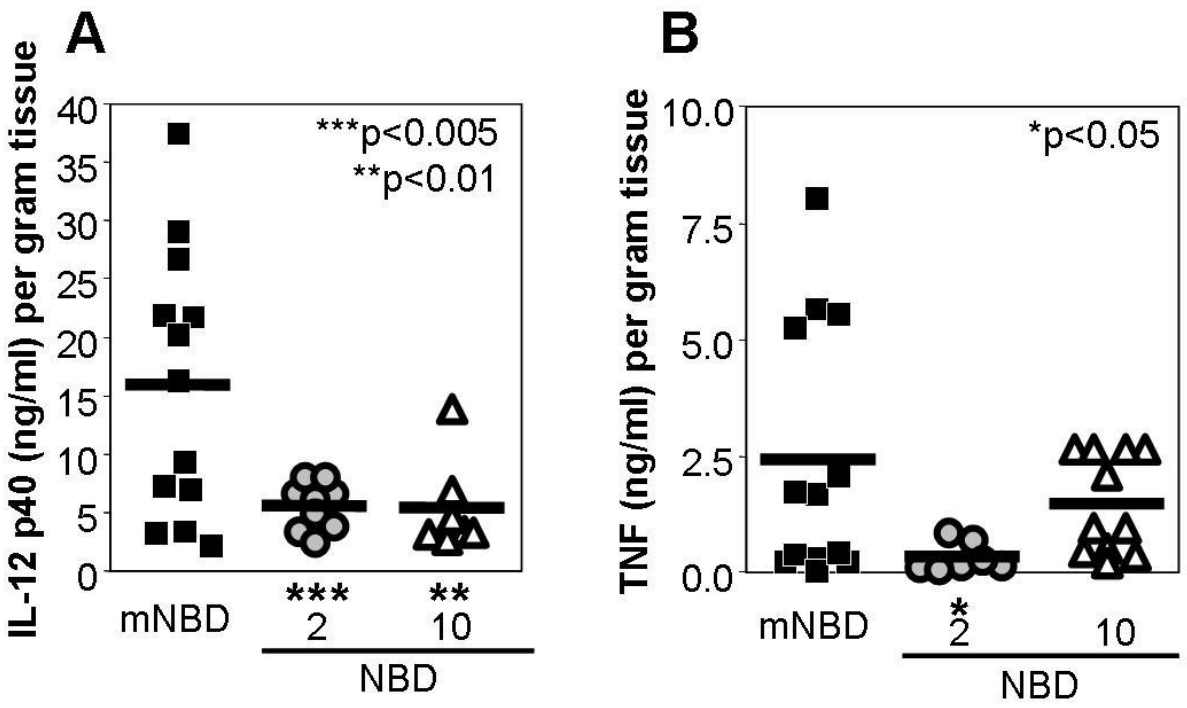


**Figure 4.6: 8K-NBD inhibits NF-κB-dependent cytokine production in intestinal explants.**

Colons were isolated from individual mice (colors: black, 10 mg/kg mNBD; gray, 2 mg/kg NBD; white, 10mg/kg NBD), cleaned, and processed for intestinal tissue explant cultures, as described in the materials and methods section. Tissue fragments (0.05 g dry weight) were incubated in 1 ml RPMI supplemented with 1% antibiotic/antimycotic and 10% FBS. Supernatants were collected after 24 hours and spontaneous secretion of (A) IL-12 p40 and (B) TNF were measured by ELISA. Values were normalized to dry weight of the intestinal explant.

macrophages of mice with TNBS colitis was described. A p65 antisense phosphorothioate oligonucleotide specifically and dose-dependently downregulated of p65 mRNA and protein, which was accompanied by reduced secretion of IL-1, IL-6, and TNF by LPS-stimulated lamina propria macrophages from mice with TNBS colitis. Strikingly, *in vivo* administration of p65 antisense abrogated established TNBS induced colitis following a single intravenous injection. Moreover, local administration of p65 antisense oligonucleotides into the colon of mice with TNBS colitis successfully treated established colitis (134, 135). Recently, local administration of antisense p65 oligonucleotides blocked intestinal inflammation as well as fibrosis in the TNBS model (137). In spontaneously occurring colitis in *IL-10*$^{-/-}$ mice, increased NF-κB DNA binding activity and increased p65 protein expression were found in lamina propria macrophages. Again, the essential role of p65 in maintaining chronic intestinal inflammation was demonstrated by successful treatment of established colitis in these mice with p65 antisense oligonucleotides (135).

Based on animal model results, it is not surprising that activated NF-κB is found in human IBD. A significant increase in p65 protein in lamina propria macrophages and epithelial cells from CD patients was correlated with increased production of the inflammatory cytokines IL-1β, IL-6, and TNF (138). *In vitro* treatment of lamina propria macrophages from CD patients with p65 antisense oligonucleotides was more effective in down-regulating cytokine production than treatment with 5-aminosalicylates or glucocorticoids, suggesting a key role for NF-κB p65 in inflammatory cytokine expression in CD (138). Subsequently, higher levels of NF-κB p65 were demonstrated in nuclear extracts of lamina propria biopsy specimens from CD patients compared to normal controls or patients with UC. In this study, increased nuclear p65 from CD patients was attributed to activation and nuclear translocation of NF-κB as total levels of NF-κB

p65 from whole cell extracts did not differ between controls and IBD patients. Importantly, increased DNA binding activity of NF-κB was demonstrated in nuclear extracts from biopsy specimens as well as isolated lamina propria mononuclear cells from CD as well as UC patients which confirmed the activation of NF-κB in IBD (139). Activated NF-κB has been reported in macrophages and epithelial cells from inflamed mucosa of patients with IBD *in situ* using a specific p65 antibody that exclusively detects the activated form of NF-κB (140). No significant differences were found between sections of inflamed mucosa from patients with CD, UC, or diverticulitis (140). This study indicated that activation of NF-κB is not necessarily specific for the pathophysiology of IBD: NF-κB activation could represent an important step in mucosal inflammation regardless of the etiology.

Because of the importance of the IKK complex in inflammation, the identification of selective IKK inhibitors as potential therapeutic agents is of considerable interest. A concern about the use inhibitors that completely suppress IKK activity (such as inhibitors that block the catalytic activity of IKK) is that they may also inhibit the ability of basally active NF-κB to act as a physiologic survival factor, thereby raising the possibility of toxicity. Activation of the IKK complex in response to proinflammatory mediators depends critically on the presence of the NEMO subunit of the IKK complex. For example, NEMO-deficient cells lack detectable NF-κB binding activity in response to TNF, IL-1β, and LPS (133). Furthermore, recent studies have shown that continuous administration of the NBD peptide effectively ameliorates inflammatory responses in animal models of inflammation without overt side effects or liver or kidney toxicity (238, 244).

Additionally, specific inhibition of IKK activity by NBD peptides has other theoretic advantages. In mouse models of chronic inflammation, including collagen induced arthritis

(CIA) (244, 245) and experimental allergic encephalomyelitis (EAE) (246), *in vivo* treatment with NBD peptides blocked disease activity, proinflammatory cytokine expression, and homing of cells to inflammatory sites due to inhibition of expression of cellular adhesion molecules. In EAE, clinical recovery was correlated with a durable alteration of the T cell phenotype, as NBD treated mice demonstrated Th2 cytokine production rather than disease-associated Th1 cytokine production (246). Furthermore, mice treated systemically with an NBD peptide for five days after induction of CIA maintained clinical and histological improvement for nearly three weeks following termination of peptide administration (244). Therefore, the therapeutic effect of the NBD peptide in disease models may far outlast the pharmacokinetic properties of this short-lived protein. Thus, selective IKK inhibition by NBD peptides may be an effective therapeutic intervention in chronic inflammatory diseases such as IBD, may lead to durable alterations in immune responses that correlate with durable clinical efficacy, and may minimize potential toxicity concerns associated as basal NF-κB activity remains intact as does the alternative pathway of NF-κB activation necessary for B cell development and lymphoid organogenesis (247-249). Future studies in IBD models will be necessary to characterize changes in immune cell populations and durability of clinical responses with the 8K-NBD.

When contemplating strategies to inhibit NF-κB in IBD, it will be critical to dissect protective from detrimental properties of NF-κB activation in mucosal immunity and mucosal inflammation. Although increased activation of NF-κB is implicated in the pathogenesis of numerous chronic disorders, studies have elucidated NF-κB activation pathways that may actually be protective and serve to maintain homeostasis in the intestine (110, 250). For example, the toll-like receptor (TLR) family recognizes extracellular microbial constituents resulting in the downstream activation of NF-κB. TLR-deficient mice or signaling intermediate

knock-outs, such as MyD88, demonstrate a decrease in survival compared to wild-type mice when colitis is induced with dextran sodium sulfate (110, 250). In these mice, intestinal epithelial proliferation was shown to be markedly decreased in TLR- or MyD88-deficient animals. This study suggested that TLRs, potentially expressed on intestinal epithelial cells (IECs), may recognize signals afforded by the luminal bacteria and provide a protective response manifested by normal epithelial barrier function and mediated through NF-κB activation. Furthermore, the description of mutations in the *Nucleotide oligomerization domain 2/Caspase recruitment domain 15* (*NOD2/CARD15*) gene conferring susceptibility to human CD provides further evidence for a potential protective role for NF-κB activation in IBD (19, 20). NOD2/CARD15 is expressed in the cytoplasm of cell types including macrophages, dendritic cells, and intestinal epithelial Paneth cells. The C-terminal leucine rich repeat is required for signaling in response to the bacterial cell wall product muramyl dipeptide (MDP). Following exposure to MDP, oligomerization of NOD2/CARD15 leads to the recruitment of the CARD containing protein RICK/RIP2 (for RIP-like interacting CLARP kinase/receptor interacting protein-2). RICK then interacts with NEMO to activate the IKK complex (2). Functional characterization of CD-associated NOD2 mutations in cell lines and human mononuclear cells from CD patients show that these mutations were associated with loss of function, i.e. cells containing mutant NOD2 demonstrated diminished MDP-induced NF-κB activation (111, 115). Although the notion that decreased NF-κB activation through NOD2/CARD15 signaling leads to IBD is still controversial, recent work suggested a possible mechanism. NOD2 expression and NF-κB activation in intestinal epithelial Paneth cells likely mediate innate immune responses against enteric microbes. The impaired capacity of CD associated NOD2 mutations in the epithelium to sense luminal bacteria and to secrete antimicrobial peptides such as α-defensins

into the gut lumen may result in increased susceptibility to certain gut microbes (251). In summary, several lines of evidence suggest that NF-κB inhibition, particularly in the intestinal epithelium, may lead to abrogation of mucosal protective effects.

Conversely, the preponderance of evidence already discussed suggests that inhibiting NF-κB in lamina propria macrophages and dendritic cells may be of therapeutic benefit in IBD. A recent study demonstrated that the development of colitis in *IL-10*$^{-/-}$ mice is completely dependent on TLR signaling pathways (252). In *IL-10*$^{-/-}$ × *MyD88*$^{-/-}$ mice, colitis is abrogated and intestinal IL-12 p40 levels are markedly decreased. Furthermore, bone marrow chimera experiments reveal that bone marrow derived cells are responsible for recognition of commensal microbial signals and mucosal innate immune activation (252).

Taken as a whole, the spectrum of NF-κB biology in the gut is complex. Our results and many others suggest that inhibition of activated NF-κB in mucosal macrophages and dendritic cells may ameliorate innate immune responses that underlie chronic IBD; however, NF-κB may play a protective role in the epithelium. Thus, in contemplating new therapeutic strategies such as 8K-NBD, many interrelated factors may be important for ultimate success, including inhibition of activated versus basal NF-κB, targeting specific cell types (macrophages versus gut epithelium), and route of delivery (systemic versus local).

This study supports the proof of concept that selective inhibition of activated NF-κB by 8K-NBD is an effective strategy for suppressing intestinal inflammatory responses. Compared to other NF-κB inhibitors tested in chronic inflammatory diseases, 8K-NBD has the theoretic advantages of inhibiting activation of NF-κB, a hallmark of chronic inflammation while not inhibiting basal NF-κB activity which may be involved in fundamental cellular processes, thus correlating with toxicity. Future studies in IBD models will investigate immunologic

pharmacodynamic properties of this peptide, durability of clinical responses, as well as compare systemic versus local administration. Our results suggest that 8K-NBD peptide may represent a novel therapeutic approach in IBD.

# 5.0 DISCUSSION

## 5.1 GENERAL DISCUSSION

Inflammatory bowel disease (IBD)[5] is a complex process that involves the interplay of genetic, immune, and environmental contributions (10). The prevailing hypothesis driving current research is that the onset of IBD is thought to be dependent on an aberrant immune response in a genetically susceptible individual against the normal intestinal flora. As a result, new targets associated with this disease involved in innate immune responses against microbes, such as TNF, have been identified. However, even the most aggressive and rational therapeutic interventions are effective in a minority of patients and are associated with short and long term toxicities. These observed failures substantiate the heterogeneity of this disease only amongst human patients. Significant unmet medical needs in patients with IBD have opened the door for new areas of research in patients with this debilitating disease. As precise immune mechanisms are described, they have provided new directed therapeutic approaches and expanded the understanding of IBD pathogenesis.

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[5] Abbreviations used in this chapter: CCK, cholecystokinin; DAMP, damaged associated molecular pattern; DSS, dextran sodium sulfate; EEC, enteroendocrine cell; HMGB1, high mobility group box 1; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IKK, IκB kinsase; *IL-10*$^{-/-}$, IL-10-deficient; LPS, lipopolysaccharide; MRP, multidrug resistance protein; NBD, NEMO binding domain; NEMO, NF-κB essential modulator; NOD2, Nucleotide oligomerization domain 2; PAMP, pathogen associated molecular pattern; PTD, protein transduction domain; RAGE, receptor of advanced glycation end products, (RAGE); sRAGE, soluble RAGE; TLR, toll-like receptor; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

The underlying hypothesis of our research is damaged associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) drive the inflammatory process contributing to IBD. To address this hypothesis, attention was focused on DAMP and PAMP signaling pathways in mucosal and innate immune activation. This dissertation provides further evidence of the importance of DAMPs and PAMPs in the mucosal inflammatory response in macrophages and *in vivo* in mouse models of IBD. We first have identified an intestinal epithelial cell (IEC) population, namely the enteroendocrine lineage, which strongly express toll-like receptors (TLRs) that may recognize danger signals. We suggest functional consequences of TLR activation in enteroendocrine cells (EECs), including production of inflammatory mediators, growth factors, and the neuropeptide cholecystokinin (CCK). Next, we demonstrate a role for high mobility group box 1 (HMGB1), a prototype endogenous danger signal, in macrophage activation and IBD. We show treatment with a small organic molecule, ethyl pyruvate, decreases levels of HMGB1 while improving murine colitis. These results suggest that DAMPs may also contribute to IBD by providing a second source of danger signals. Lastly, we show that targeting common DAMP and PAMP signaling effector molecules, the NF-κB family of transcription factors, results in amelioration of chronic murine colitis. In the following sections we will expand on these findings and speculate about future directions and implications of these studies.

## 5.2 IMMUNOLOGICAL FUNCTION OF ENTEROENDOCRINE CELLS

Current studies in IBD are reliant on the use of IEC lines to recapitulate *in vivo* expression and function, and attempt to reconcile *in vivo* findings. This is necessitated by the fact that primary

IEC cultures have not been established despite decades of effort, and even short term culture is fraught with difficulty, as detached intestinal epithelium are in an immediate state of programmed cell death (253). These obstacles prevent effective short term investigation in *ex vivo* cultures for cellular and/or immune responses in IECs. Most of the immortalized IEC lines are derived from tumors and have been selected over time to grow on plastic. Thus, when one interprets data derived from IEC lines, one must ask the question: how similar are these cell lines to actual intestinal cells? Until an accepted methodology is developed and substantiated to allow isolated cells to remain alive for extended periods of time, experiments will out of necessity rely on cell lines.

The expression of TLRs have been described on IECs, albeit at low levels (104, 105, 145, 146). Having low expression of TLRs may be important due to the inability of IECs to discriminate pathogenic bacteria from commensal bacteria based on PAMPs alone. The location of TLR expression on cells may also play an important role in microbial recognition. TLR5, which recognizes flagellin, appears basolateral, while other TLRs are apical (109). The importance of this separation is that luminal bacteria are located apically; basolateral localization of bacteria would signify a breach of the epithelial barrier, thus appropriately mandating innate immune recognition. Likewise, other TLRs such as TLR9 have an intracellular location in IECs, perhaps in the endoplasmic reticulum where it may encounter CpG DNA from invading intracellular pathogens (254, 255). In the setting of chronic inflammation, as in IBD, TLR expression is upregulated, perhaps for the purpose of responding to various danger signals from host cells and the pathogenic and, commensal bacteria (105, 106, 256).

Studies in the literature investigating intestinal TLR expression have presented discrepant findings. Thus, our results demonstrating EECs express TLRs offers another as of yet

undescribed view of innate immune responses in the intestine. Numerous studies have phenotypically characterized EECs based on their anatomic location and the neuropeptides they express. However, there have not been studies describing immunological functions for EECs. Here, we have demonstrated that TLR expression on IECs in human and mouse is confined to an EEC population. We accordingly looked at an EEC line to recapitulate the observed *in vivo* TLR staining pattern and to postulate a function for TLR activation on this cell type.

TLR activation does not necessarily lead to inflammation. It is becoming increasingly evident that intestinal homeostatic functions may be regulated through TLR activation. Molecular analysis of commensal-host interactions showed induction of a complex pattern of gene expression (257). The results of this study revealed that commensal bacteria modulates expression of genes involved in several important intestinal functions, including nutrient absorption, xenobiotic metabolism, angiogenesis, postnatal intestinal maturation, and mucosal barrier strengthening. This last observation was confirmed using IEC lines stimulated with bacterial PAMPs. Stimulation resulted in apical recruitment and fortification of the tight junctional protein ZO-1, while increasing transepithelial electrical resistance (258). This result may explain previous observations that certain probiotic strains increased barrier function *in vitro* (66) and ameliorated murine colitis (65, 67). However, there are some TLR interactions that may have a negative result on the intestinal function. In mice with necrotizing enterocolitis, TLR4 engagement inhibited migration of IECs across a wound during healing and repair (259). Therefore, TLR signaling may aid in certain aspects of barrier functions and can be deleterious in others.

Furthermore, TLR function has been related to protection from intestinal epithelial injury and maintenance of homeostasis. Oral administration of dextran sodium sulfate (DSS) to induce

acute colitis, results in a transient epithelial injury. However, DSS administered to TLR2-, TLR4-, or MyD88-deficient mice, demonstrated a decrease in survival compared to wild-type mice (110). Moreover, administration of broad spectrum antibiotics to deplete the resident microflora resulted in wild-type mice having an increased disease index when challenged with DSS, similar to that of knock-out mice. At the cellular level, intestinal epithelial proliferation was shown to be markedly decreased in knock-out animals. This suggested that TLRs recognize signals afforded by the luminal bacteria, providing a protective response manifested in normal epithelial barrier function and healing of the gut.

If TLR signaling is beneficial to the gut, specifically what role might the EEC play? EEC products, such as CCK, are important in aiding in digestion (260). CCK in particular is the principal stimulus to contract the gallbladder to release bile for emulsification of fats and activate the pancreas to produce digestive enzymes to breakdown macromolecules. CCK has also been shown to increase contractions of the intestines and allows for quicker intestinal emptying to expedite the transit of foods in the gut. Furthermore, injection of CCK into the brain induces satiety in laboratory animals, suggesting a function in food intake.

In view of these physiologic roles, it might make immunologic sense that this hormone may participate in an indirect innate immune response. In the setting of an intestinal infection, the goal would be to remove the infectious agent as quickly as possible. Increased production of CCK might accomplish this by accelerating the transit time of intestinal contents to expunge pathogens. Additionally, increased CCK may communicate to the brain a satiety signal, which may prevent further entry of pathogens that are contributing to the infection in the gastrointestinal tract.

To explicate an immunological role for CCK (and other EEC products), future experiments will investigate expression and function of TLRs in mice. For instance, mice depleted of their enteric flora (raised germ-free and/or antibiotic-treated) could be compared to specific pathogen-free mice and resting CCK levels determined. Based on our results, we hypothesize that resting levels of CCK should be decreased in germ-free mice since tonic stimulation of enteric flora is absent. If bacteria were reintroduced or TLR ligands were injected into the intestinal lumen, we would expect a transient rise in blood CCK levels. This has been suggested through one reported case of acute upper gastrointestinal infection, where plasma CCK concentrations were elevated in the setting of *Giardia* infection (174). This would support a role for CCK in the innate immune response in an attempt to expel foreign pathogens from the gut.

A genetic knockout strategy would be an attractive approach to study TLR function in EECs. Global gene deletion of TLRs or the signaling intermediate MyD88 has already been described in the context of an inducible model of acute colitis (110). Their results demonstrate TLRs may recognize enteric commensals and orchestrate a protective response, which may be important in intestinal homeostasis. However, in this study they did not link the expression of TLRs to a specific cell type responsible for the observed biological effects. Therefore, it would be interesting to examine the role of TLRs specifically in EECs. However, specific ablation of EEC populations *in vivo* has been challenging. For example, knocking out EECs through global deletion of the transcription factor *Math1* (required for intestinal stem cell differentiation into EECs) results in death shortly after birth (261). To overcome deleterious developmental consequences, the use of cell-specific deletions, which can be implemented in adult life, will be of investigational benefit. Taking advantage of the specific transgenes expressed within EECs

(167-169), one could design transient knock-out approaches by using the Cre/loxP recombination system to target specific EECs. For example, a mouse line could be engineered in which specific TLR genes were flanked by loxP sites. Cell-specific gene knockout can then be achieved by breeding these animals with a line of mice carrying a tetracycline-sensitive enteroendocrine CCK promoter-enhancer, specifically driven by the *BETA2* transcription factor (262) driving the Cre gene. The usage of the tetracycline system ensures temporal activation of the system to allow for proper development of tissue and cells before TLR expression is targeted. Taken together, this approach is specific for knocking out TLRs in CCK-expressing EECs and, ideally, a similar strategy could be used to target other neuropeptide expressing EECs (serotonin, somatostatin, etc.). By using an inducible model of colitis, either 2,4,6-trinitrobenzene sulfonic acid (TNBS) or DSS, or crossing these mice onto genetically susceptible strains, such as IL-10-deficient (*IL-10*$^{-/-}$), one can effectively determine the role of TLRs on EECs in the setting of IBD.

Our study raises the possibility that EEC-bacteria interactions through TLRs may in fact maintain intestinal immune homeostasis mediated through CCK. Chronic stimulation through this pathway may yield overabundant cytokine production overwhelming the beneficial effects of CCK and TGF-β. Nevertheless, it is safe to say that the understanding of the EEC in the innate immune response is still early and further investigations will be necessary before we understand its role in disease.

## 5.3 TARGETING ENDOGENOUS DANGER SIGNALS

The complexities of the commensal enteric microbial flora notwithstanding, studies have attempted to implicate specific microbes as initiators of disease in IBD. Attention has been

given to *Mycobacteria*, *Listeria*, and measles virus. However, immune responses to these microbes have been variable and not reproducible. Moreover, these and other inciting microbes were no longer present during the chronic stages of disease (10), suggesting the presence of another signal that must perpetuate the inflammatory process of IBD.

Several endogenous molecules (DAMPs), such as HMGB1, heat shock proteins, and uric acid, have the ability to initiate inflammatory responses by interacting with signaling receptors. They represent families of proteins that reside intranuclear and/or possess intracellular functions that can also act extracellularly as an immunostimulant on their release from necrotic cells. These proteins can be released during infection or tissue damage, and can activate receptor-expressing cells ready for host defense and/or tissue repair.

HMGB1 was originally described as a nuclear, non-histone DNA-binding protein that functions as a structural co-factor critical for proper transcriptional regulation in cells. The physiological role associated with HMGB1 is primarily in the nucleus where it binds to the minor groove of linear DNA and is able to bend it into a helical structure (263). After binding to DNA, HMGB1 can also interact with and recruit various transcription factors, including p53, homeobox-containing proteins, recombination activating gene 1/2 proteins, and steroid hormone receptors. The nuclear functions of HMGB1 are critical for survival as HMGB1-deficient mice are born alive, but die within 24 hours due to hypoglycemia (264). Therefore, in a non-immune situation, this DAMP protein has an important role within cells to promote health and homeostasis.

HMGB1 can reach the extracellular environment in one of two ways (119). First, it is released passively as a soluble molecule from dying, necrotic cells, functioning as a diffusible signal of unprogrammed cell death. Second, HMGB1 is actively secreted following

hyperacetylation of lysine residues from inflammatory cells. Both situations result in signaling tissue injury and initiating the inflammatory response and/or repair. In contrast, apoptotic cells retain HMGB1 in their nuclei and do not activate the inflammatory response (265). This suggests that apoptotic cells modify their chromatin and/or HMGB1 so that HMGB1 binds irreversibly to it. Furthermore, it has been described that apoptotic bodies can inhibit proinflammatory cytokine production (266-268). Therefore, the context and nature of HMGB1 may play a role in health and disease. Perhaps this partly explains the observations of necrotic death resulting in a destructive inflammatory process, while cells that have undergone apoptosis results in a role to modulate the immune response.

Current research has begun to look at mechanisms involved in the release of DAMPs, including HMGB1 (269). The phosphorylation status of serine-14 on histone H2B has been implicated in keeping HMGB1 on chromatin during apoptosis. Furthermore, treating cells with a histone deacetylase inhibitor, such as trichostatin A, inhibits HMGB1 binding to apoptotic chromatin, resulting in apoptotic cells becoming inflammatory. This observation suggests that although hyperacetylation of HMGB1 may be important in its secretion during necrosis, simple hypoacetylation of histone proteins is not sufficient to keep HMGB1 onto chromatin. Thus, characterizing HMGB1 isoforms and their relative contribution to infectious and inflammatory disorders has become an important goal. It is reported that HMGB1 purified from calf thymus exists in several distinct isoforms, including at least 10 acetylated species which may contain different degrees of ADP ribosylation, glycosylation, phosphorylation, and methylation (270). It is becoming apparent that each of these may contribute differently in the final biological and inflammatory activity of HMGB1. Future molecular characterization of isolated HMGB1 will

reveal the state of modifications important for differentiating nonstimulatory versus pathologic and/or inflammatory processes.

HMGB1 secretion appears to follow an alternative secretory pathway where it is loaded into secretory lysosomes for eventual exocytosis (271). The secretion was inhibited by pharmacologic blockade of the adenosine 5'-triphosphate (ATP)-binding cassette transporter-1, a member of the multidrug resistance protein (MRP) family (272). In support of this, preliminary experiments involving peritoneal macrophages from *Mrp1*$^{-/-}$ mice have suggested suboptimal HMGB1 secretion (269). Further analysis from this preliminary work has suggested that glutathionylation, a modification required for the transport of several drugs by MRP1, is important and necessary for HMGB1 secretion. Understanding the complete modifications of HMGB1 and the process of its secretion during inflammation will certainly identify new molecular targets for therapeutic intervention.

As HMGB1 has received attention as an important DAMP protein, there is an increased interest in understanding its signaling pathways. Receptor of advanced glycation end products (RAGE) has been identified to recognize extracellular HMGB1 (273). Ligand binding to the receptor has been shown to activate numerous intracellular signaling molecules. Studies have shown recruitment of p21$^{ras}$, p44/p42 MAP kinase, p38 and SAPK/JNK, cdc42/rac, and the JAK/STAT pathways. In certain settings, RAGE activation appears to result in chronic activation of NF-κB and increase reactive oxygen species through NADPH oxidase (273). In animal models, deletion of RAGE protects about 80% of mice from sepsis caused by cecal ligation and puncture compared to 20% of wild-type controls (274). Protection was reversed when cells were reconstituted with RAGE. The RAGE signaling pathway is also quite diverse, especially in different cell types. For example, it has been shown that HMGB1-RAGE

interaction can cause axonal sprouting associated with neuronal development (275, 276), whereas HMGB1-RAGE interaction on macrophages results in immune activation (130). The HMGB1 signaling network represents a complex system where consequences of environment, ligand, and cell type need to be integrated to properly assess the context in which to respond and contribute to health and disease.

As mentioned, DAMPs also can be recognized by the TLR family and can activate the inflammatory transcription factor NF-κB. HMGB1 is no different, and has been shown to signal through TLR2 and TLR4 (130). Recent work has found that recognition of HMGB1 by TLRs is more complex than first appreciated. Data suggest that there is differential usage of TLR2 and TLR4 in HMGB1 signaling in primary cells and established cells lines (129). Using human whole blood and macrophages, neutralizing antibodies against TLR4 but not TLR2 dose-dependently inhibited HMGB1 stimulation. This was also confirmed using TLR4- and TLR2-deficient murine macrophages, where only TLR4-deficient cells did not respond to HMGB1. However, the human embryonic kidney 293 cell line overexpressing TLR2 demonstrated HMGB1 responsiveness as opposed to TLR4 transfected cells. Findings such as these clearly invite a more thorough analysis of the intestinal epithelia to determine the recognition of HMGB1 by specific TLR members. Although the families of receptors engaged by PAMPs and DAMPs overlap, further experiments will clarify biologically important differences involved in the recruitment of specific signaling molecules. Microarray and proteomic analyses represent potential experimental approaches directed at revealing differences between PAMP- and DAMP-stimulated cells.

Although HMGB1 has been depicted as an inflammatory molecule, there is emerging work describing a beneficial role of HMGB1 as a mediator of tissue regeneration (269). As

described above, HMGB1 is an inducer of neuronal growth (275, 276). In nerve crush injuries, the RAGE-HMGB1 axis is important in nerve regeneration (277). It has also been reported that HMGB1 can induce myocardial regeneration after infarction (278). Injection of exogenous HMGB1 into mouse hearts after ischemic damage resulted in the formation of new myocytes. These observations suggest that the context in which HMGB1 presents itself to cells, may drive the final gene program. This point seems to be particularly relevant to the intestine. Akin to the previous discussion of TLR biology in the gut, it is intriguing to speculate that HMGB1 may have beneficial or deleterious effects in mucosal immunity depending on context.

One of the emerging aspects of HMGB1 biology is that it may act as a cofactor for several TLR ligands to activate specific TLR functions. It is being postulated that HMGB1 may act as a carrier molecule to deliver various PAMPs to or into cells for intracellular pathways, such as through the Nucleotide oligomerization domain 2 (NOD2) protein. This idea has emerged due in part to the following observations (269). *E. coli*-derived HMGB1 (rHMGB1) has been shown to induce inflammatory cytokine production. On the other hand, groups are beginning to show that thymus-derived HMGB1 does not induce cytokines. The ability of rHMGB1 to induce cytokines was implicated to its bacterial nucleic acid content. If this foreign entity was removed by benzonase, a RNA/DNA endonuclease, cytokine production was diminished. To demonstrate biological activity with thymus-derived HMGB1, incubation with TLR2, TLR4, TLR7, and TLR9 ligands resulted in a synergistic activation compared to TLR ligands alone. It was also shown that rHMGB1 treated with Triton X-114 (Tx-HMGB1), a detergent to remove hydrophobic/lipophilic contaminants, no longer was able to stimulate cytokines. However, cells treated with very low concentrations of lipopolysaccharide (LPS) along with Tx-HMGB1 strongly induced cytokine secretion. This was shown to be more than

that induced by low levels of LPS or Tx-HMGB1 alone. These observations suggest that HMGB1 lacks a direct proinflammatory effect, but can prime cells to augment the response by delivering distinct PAMPs. In the context of health and disease, HMGB1 might have different biological effects depending on whether it is part of a complex with bacterial products. Perhaps if HMGB1 is released in the presence of TLR ligands, such as in the intestinal lumen or setting of microbial sepsis, it may promote inflammation. On the other hand, if HMGB1 is released in an environment lacking TLR ligands, such as in the myocardium or neuron, it may lead to tissue repair and healing.

Because genetic knock-out animals of HMGB1 are not viable, using directed biological therapy is of clinical interest to investigate the role of HMGB1 in the context of IBD. Several of the following experimental approaches targeting HMGB1 have been successfully carried out in other disease models, but have not been extensively described in murine models of colitis. The first is the use of monoclonal and/or polyclonal antibodies against HMGB1. Second, two important functional domains in the HMGB1 molecule have been identified: the proinflammatory B-box and the anti-inflammatory A-box. In biochemical studies, the A-box domain has been shown to bind to and inhibit the activity of the B-box domain, preventing inflammatory responses. Theoretically, administering A-box could bind to the B-box domain of HMGB1 to nullify its inflammatory effects. Lastly, HMGB1 delivers an inflammatory signal through its membrane bound receptor, RAGE, and can be blocked by competing with a soluble receptor. The soluble form of RAGE, sRAGE, lacks the intracellular signaling domain, preventing a signal from being transduced. All three approaches, though different, are similar in that it provides the immune system a break in the perpetuating nature of the inflammation caused

by HMGB1. Moreover, these therapies will further the investigations involved with HMGB1 and IBD.

Therapeutic use of HMGB1 antibody, A-box, sRAGE, or ethyl pyruvate administration will require further assessment of mucosal cell functions. First, cell phenotypes and cell trafficking populations will need to be analyzed. Will treatment alter the immune cell populations in the gut? Do we see a shift in T cell phenotype so that inflammatory cells are decreased through increased apoptosis or does a different cell population traffic to the mesenteric lymph nodes, such as T regulatory cells? Furthermore, the durability of immune response changes will need to be analyzed to assess if the treatment provides short term or long term changes locally and/or systemically. FACS analysis will be a strong tool to assist in answering these questions and will provide insight into immunologic differences between various treatment regimens.

## 5.4 THE STRESSED GUT AND NF-KB

The stressed gut is able to recognize inflammatory signals generated by DAMPs and PAMPs through TLRs and RAGE. Cells integrate these signals into an inflammatory response through the activation and translocation of NF-κB. Many of the inflammatory cytokines elevated in IBD are regulated through NF-κB activation. Therefore, this family of transcription factors would seem to be an ideal therapeutic target.

Strategies to inhibit NF-κB message, protein, and DNA binding activity have proven to be effective in colitis. It is also important to keep in mind that another important function of NF-κB is that it has an anti-apoptotic role. Therefore, once again, we are elucidating and

manipulating a pathway that may have beneficial and deleterious effects depending on context. As an obvious concern, therapeutic strategies targeting NF-κB should be cautiously monitored in IBD models: Inhibition of inflammatory cytokines and/or apoptosis of inflammatory cell populations as a result of NF-κB inhibition are desired outcomes, however, accelerated apoptosis of IECs may compromise barrier function and exacerbate mucosal inflammation. Further studies to compare systemic versus local delivery of NEMO binding domain (NBD) peptides may help clarify these opposing possibilities.

Furthermore, creation of an inhibitor which can differentiate between NF-κB members important for cell survival and those important for the immune response would be the ideal therapeutic approach with the most benefit and lowest risk. The NBD peptide is an attractive candidate to accomplish this discrimination by selectively inhibiting the activated form of the IκB kinase (IKK) complex and not the basal pathway. As we show in chapter 4, by administering NBD into cells and tissues using the 8K protein transduction domain (PTD), we were able to inhibit TNF-stimulated NF-κB activation *in vitro* and improve colitis *in vivo*.

Though this preliminary study identified an optimal dosing window via the intraperitoneal route and suggested the effectiveness for 8K-NBD, many questions remain to be answered. First, what are the effects of delivering this peptide locally? As IBD is a local inflammatory response of the intestines, the ideal administration route would be locally administered by mouth. This would be important to minimize potential toxicity as well as to increase patient compliance and quality of life. Second, what is the durability of clinical and histological response after the therapy is discontinued? Do we see benefits of the NBD peptide extending beyond the treatment period? That is, do pharmacodynamic effects outlast its pharmacokinetic properties, similar to what has been observed in the collagen induced arthritis

model (244)? Lastly, do we see specific alterations and trafficking of mucosal cell populations? Durable changes in T cell phenotype and function were observed in murine experimental allergic encephalomyelitis as NBD treatment induced Th2 cytokine production (246). These important mechanistic questions need to be addressed; however the data provided here establishes the use of the 8K-NBD as a potential therapeutic compound in IBD.

## 5.5 CONCLUSIONS

The human IBDs are disorders of innate immunity with an exaggerated inflammatory response and loss of tolerance to the normal microbial constituents of the gut lumen. Much of the work in IBD pathogenesis has focused on the external environmental factors, such as PAMPs from enteric bacteria, in the initiation and perpetuation of chronic mucosal inflammation. Our studies depicted in this dissertation aimed to broaden the scope of initiating factors as we hypothesize that PAMPs and DAMPs, alone or in combination, orchestrate inflammation through activation of innate immune responses in IBD.

The findings from this dissertation are summarized in Figure 5.1. Briefly, our studies reveal basal TLR expression in normal healthy human and murine intestinal tissue (Figure 5.1A). We show that the enteroendocrine lineage is the prominent cell population which strongly expresses TLRs (chapter 2) in the intestine. In the normal state of "controlled inflammation," there does not appear to be highly detectable levels of TLRs on neighboring IECs [chapter 2 and Ref. (106)]. Therefore, in health, the EECs may be the main cell type in the gut to express and respond to the PAMPs present in the enteric microflora. Conversely, intestinal macrophages

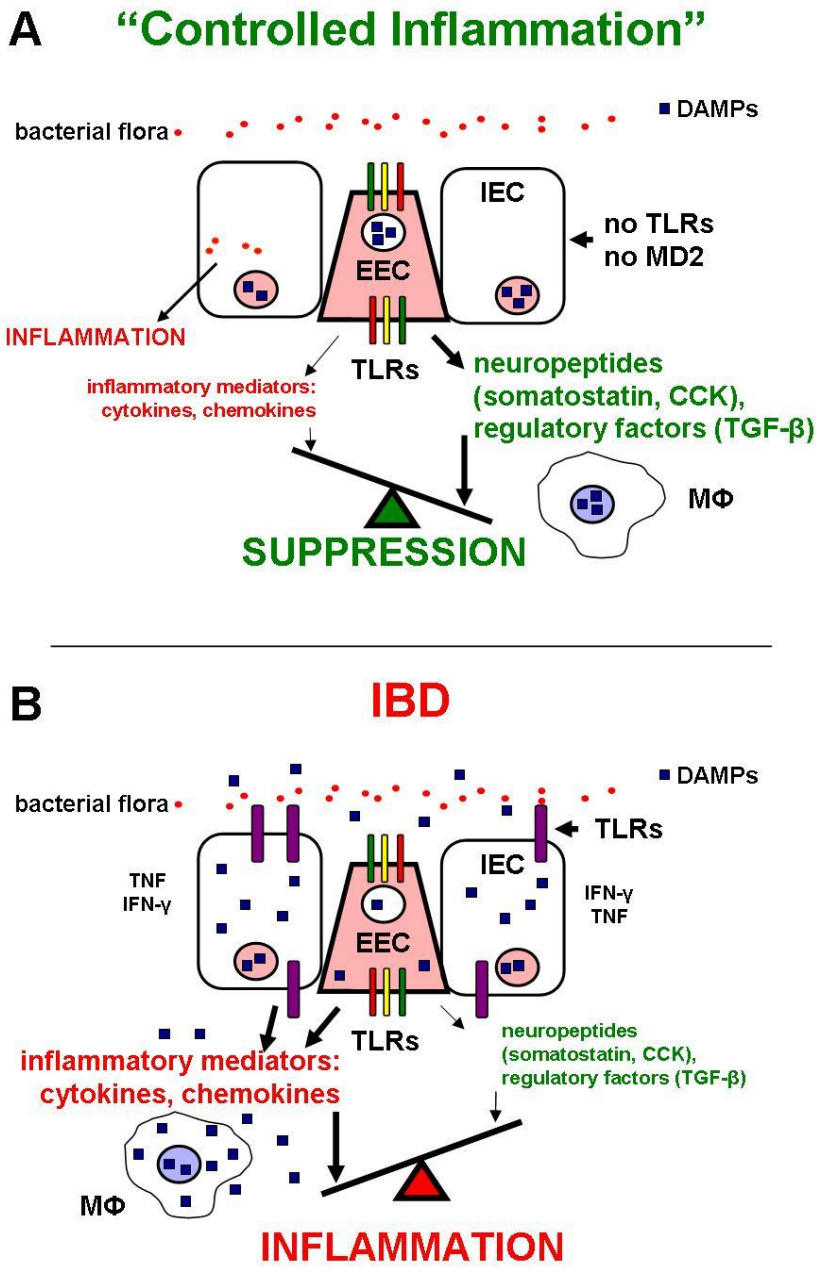


**Figure 5.1: Schematic depicting role of the intestinal epithelia in health and disease.**

(A) During physiologic events of "controlled inflammation" the enteroendocrine (EEC) lineage is the prominent cell population which strongly expresses TLRs. EECs can secrete inflammatory mediators, as well as produce regulatory factors, which contributes towards an immunosuppressive state. (B) During IBD, cytokines such as IFN-γ and TNF upregulate expression of TLRs on neighboring IECs. Additionally, there is an increase in the amount of DAMPs released from cells dying of necrosis and cells activated by inflammatory mediators and/or PAMPs (macrophages, MΦ). The increase in DAMPs, perhaps directly or by binding to the PAMPs in the intestines, activates inflammatory pathways through TLRs on EECs, IECs, and resident macrophages in the gut mucosa. This further activates the innate immune response and tips the balance towards a state of inflammation.

(MΦ) and IECs also must respond to PAMPs and recognize invasive pathogens, such as *Salmonella*, *Shigella*, *Yersinia*, and *Listeria*. Lamina propria macrophages are located in the gut mucosa and provide a link to the adaptive immune response. These macrophages, however, have been shown to have low TLR expression levels rendering them unresponsive to many danger signals (279). In the setting of an invasive bacterial infection, IECs express and secrete a number of proinflammatory cytokines and chemokines (86, 87). Intestinal macrophages and IEC may recognize pathogens by expression of TLRs and other pattern recognition receptors (prominently NOD family members) intracellularly or in the case of IECs basolaterally. Nonetheless, we show that the EECs can secrete inflammatory mediators in response to bacterial ligands. However, this is counterbalanced by the production of regulatory factors, namely the anti-inflammatory growth factor TGF-β, and neuropeptides, CCK (162, 163, 279). EECs may therefore establish an immunosuppressive state in the intestine.

During chronic IBD (Figure 5.1B), there is an increase of inflammatory mediators in the intestinal milieu, including IFN-γ and TNF. These proinflammatory factors have been shown to upregulate innate immune pathways, specifically TLR and NOD2 expression in IECs (105, 106, 256, 280). This effectively increases the total pool of TLRs on the IECs making them more available to recognize danger signals and activate the innate immune response, through the major inflammatory transcription factor NF-κB. Concomitantly, there is an increase in the amount of DAMPs released from cells activated by inflammatory mediators and/or PAMPs and cells dying of necrosis. We show that in our murine model of IBD there are significantly elevated levels of HMGB1 in the stool (chapter 3). The increase in DAMP signaling, perhaps directly or by binding to PAMPs in the intestines, through TLRs on EECs, IECs, and the resident macrophages in the gut mucosa may amplify the inflammatory process. Damaged cells release more DAMPs

and inflammatory mediators. This second wave of danger signals perpetuates inflammation through the activation of innate immune responses, leading to an overt immunologic response and to the pathogenesis of IBD. Taken together, increased TLR expression and release of DAMPs activates the innate immune response and NF-κB (chapter 4), and tips the balance towards a state of inflammation.

Our work provides the first steps towards implicating PAMPs and DAMPs in IBD. We demonstrate that TLRs are expressed on EECs and can activate immune responses. We also show that levels of HMGB1, a putative DAMP, are elevated in a murine model of colitis. By targeting HMGB1 or the NF-κB pathway of the stressed gut, we demonstrate significant improvements in a murine model of colitis. Future studies will build upon these results, dissect molecular pathways, and provide a stronger insight into the pathogenesis of IBD.

# BIBLIOGRAPHY


1. Fiocchi, C. 1998. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology 115:182*.
2. Bouma, G., and W. Strober. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol 3:521*.
3. Podolsky, D. K. 2002. Inflammatory bowel disease. *N Engl J Med 347:417*.
4. Matsumoto, T., S. Nakamura, Y. Jin-No, Y. Sawa, J. Hara, N. Oshitani, T. Arakawa, H. Otani, and H. Nagura. 2001. Role of granuloma in the immunopathogenesis of Crohn's disease. *Digestion 63 Suppl 1:43*.
5. Sandborn, W. J., and S. R. Targan. 2002. Biologic therapy of inflammatory bowel disease. *Gastroenterology 122:1592*.
6. Rutgeerts, P. J. 2001. Review article: the limitations of corticosteroid therapy in Crohn's disease. *Aliment Pharmacol Ther 15:1515*.
7. Larson, D. W., and J. H. Pemberton. 2004. Current concepts and controversies in surgery for IBD. *Gastroenterology 126:1611*.
8. Hanauer, S. B., B. G. Feagan, G. R. Lichtenstein, L. F. Mayer, S. Schreiber, J. F. Colombel, D. Rachmilewitz, D. C. Wolf, A. Olson, W. Bao, and P. Rutgeerts. 2002. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet 359:1541*.
9. Rutgeerts, P., W. J. Sandborn, B. G. Feagan, W. Reinisch, A. Olson, J. Johanns, S. Travers, D. Rachmilewitz, S. B. Hanauer, G. R. Lichtenstein, W. J. de Villiers, D. Present, B. E. Sands, and J. F. Colombel. 2005. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med 353:2462*.
10. Sartor, R. B. 1995. Current concepts of the etiology and pathogenesis of ulcerative colitis and Crohn's disease. *Gastroenterol Clin North Am 24:475*.
11. O'Hara A, M., and F. Shanahan. 2006. The gut flora as a forgotten organ. *EMBO Rep 7:688*.
12. Bonen, D. K., and J. H. Cho. 2003. The genetics of inflammatory bowel disease. *Gastroenterology 124:521*.
13. Kurata, J. H., S. Kantor-Fish, H. Frankl, P. Godby, and C. M. Vadheim. 1992. Crohn's disease among ethnic groups in a large health maintenance organization. *Gastroenterology 102:1940*.
14. Yang, H., C. McElree, M. P. Roth, F. Shanahan, S. R. Targan, and J. I. Rotter. 1993. Familial empirical risks for inflammatory bowel disease: differences between Jews and non-Jews. *Gut 34:517*.

15. Roth, M. P., G. M. Petersen, C. McElree, C. M. Vadheim, J. F. Panish, and J. I. Rotter. 1989. Familial empiric risk estimates of inflammatory bowel disease in Ashkenazi Jews. *Gastroenterology 96:1016*.
16. Tysk, C., E. Lindberg, G. Jarnerot, and B. Floderus-Myrhed. 1988. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut 29:990*.
17. Thompson, N. P., R. Driscoll, R. E. Pounder, and A. J. Wakefield. 1996. Genetics versus environment in inflammatory bowel disease: results of a British twin study. *Bmj 312:95*.
18. Orholm, M., V. Binder, T. I. Sorensen, L. P. Rasmussen, and K. O. Kyvik. 2000. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. *Scand J Gastroenterol 35:1075*.
19. Hugot, J. P., M. Chamaillard, H. Zouali, S. Lesage, J. P. Cezard, J. Belaiche, S. Almer, C. Tysk, C. A. O'Morain, M. Gassull, V. Binder, Y. Finkel, A. Cortot, R. Modigliani, P. Laurent-Puig, C. Gower-Rousseau, J. Macry, J. F. Colombel, M. Sahbatou, and G. Thomas. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature 411:599*.
20. Ogura, Y., D. K. Bonen, N. Inohara, D. L. Nicolae, F. F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R. H. Duerr, J. P. Achkar, S. R. Brant, T. M. Bayless, B. S. Kirschner, S. B. Hanauer, G. Nunez, and J. H. Cho. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature 411:603*.
21. Schreiber, S., R. P. MacDermott, A. Raedler, R. Pinnau, M. J. Bertovich, and G. S. Nash. 1991. Increased activation of isolated intestinal lamina propria mononuclear cells in inflammatory bowel disease. *Gastroenterology 101:1020*.
22. Monteleone, G., L. Biancone, R. Marasco, G. Morrone, O. Marasco, F. Luzza, and F. Pallone. 1997. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology 112:1169*.
23. Fuss, I. J., M. Neurath, M. Boirivant, J. S. Klein, C. de la Motte, S. A. Strong, C. Fiocchi, and W. Strober. 1996. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol 157:1261*.
24. Mullin, G. E., A. J. Lazenby, M. L. Harris, T. M. Bayless, and S. P. James. 1992. Increased interleukin-2 messenger RNA in the intestinal mucosal lesions of Crohn's disease but not ulcerative colitis. *Gastroenterology 102:1620*.
25. Fuss, I. J., F. Heller, M. Boirivant, F. Leon, M. Yoshida, S. Fichtner-Feigl, Z. Yang, M. Exley, A. Kitani, R. S. Blumberg, P. Mannon, and W. Strober. 2004. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest 113:1490*.
26. Trinchieri, G., and P. Scott. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. *Res Immunol 146:423*.
27. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature 383:787*.
28. Magram, J., S. E. Connaughton, R. R. Warrier, D. M. Carvajal, C. Y. Wu, J. Ferrante, C. Stewart, U. Sarmiento, D. A. Faherty, and M. K. Gately. 1996. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity 4:471*.

29. Sieling, P. A., and R. L. Modlin. 1994. Cytokine patterns at the site of mycobacterial infection. *Immunobiology 191:378*.
30. Zhang, M., M. K. Gately, E. Wang, J. Gong, S. F. Wolf, S. Lu, R. L. Modlin, and P. F. Barnes. 1994. Interleukin 12 at the site of disease in tuberculosis. *J Clin Invest 93:1733*.
31. Chehimi, J., S. E. Starr, I. Frank, A. D'Andrea, X. Ma, R. R. MacGregor, J. Sennelier, and G. Trinchieri. 1994. Impaired interleukin 12 production in human immunodeficiency virus-infected patients. *J Exp Med 179:1361*.
32. Seder, R. A., B. L. Kelsall, and D. Jankovic. 1996. Differential roles for IL-12 in the maintenance of immune responses in infectious versus autoimmune disease. *J Immunol 157:2745*.
33. Berg, D. J., N. Davidson, R. Kuhn, W. Muller, S. Menon, G. Holland, L. Thompson-Snipes, M. W. Leach, and D. Rennick. 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest 98:1010*.
34. Stallmach, A., T. Marth, B. Weiss, B. M. Wittig, A. Hombach, C. Schmidt, M. Neurath, M. Zeitz, S. Zeuzem, and H. Abken. 2004. An interleukin 12 p40-IgG2b fusion protein abrogates T cell mediated inflammation: anti-inflammatory activity in Crohn's disease and experimental colitis in vivo. *Gut 53:339*.
35. Duchmann, R., E. Schmitt, P. Knolle, K. H. Meyer zum Buschenfelde, and M. Neurath. 1996. Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. *Eur J Immunol 26:934*.
36. Ehrhardt, R. O., B. R. Ludviksson, B. Gray, M. Neurath, and W. Strober. 1997. Induction and prevention of colonic inflammation in IL-2-deficient mice. *J Immunol 158:566*.
37. Parronchi, P., P. Romagnani, F. Annunziato, S. Sampognaro, A. Becchio, L. Giannarini, E. Maggi, C. Pupilli, F. Tonelli, and S. Romagnani. 1997. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *Am J Pathol 150:823*.
38. Plevy, S. E., C. J. Landers, J. Prehn, N. M. Carramanzana, R. L. Deem, D. Shealy, and S. R. Targan. 1997. A role for TNF-alpha and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. *J Immunol 159:6276*.
39. Breese, E. J., C. A. Michie, S. W. Nicholls, S. H. Murch, C. B. Williams, P. Domizio, J. A. Walker-Smith, and T. T. MacDonald. 1994. Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. *Gastroenterology 106:1455*.
40. Reinecker, H. C., M. Steffen, T. Witthoeft, I. Pflueger, S. Schreiber, R. P. MacDermott, and A. Raedler. 1993. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clin Exp Immunol 94:174*.
41. Murch, S. H., C. P. Braegger, J. A. Walker-Smith, and T. T. MacDonald. 1993. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. *Gut 34:1705*.
42. Kusugami, K., K. R. Youngman, G. A. West, and C. Fiocchi. 1989. Intestinal immune reactivity to interleukin 2 differs among Crohn's disease, ulcerative colitis, and controls. *Gastroenterology 97:1*.

43. Cominelli, F. 1994. Specific mucosal imbalance of IL-1 and IL-1 receptor antagonist (IL-Ira) in IBD: a potential mechanism of chronic inflammation. *Gastroenterology 106*.
44. Koizumi, M., N. King, R. Lobb, C. Benjamin, and D. K. Podolsky. 1992. Expression of vascular adhesion molecules in inflammatory bowel disease. *Gastroenterology 103:840*.
45. Smith, K. M., A. D. Eaton, L. M. Finlayson, and P. Garside. 2000. Oral tolerance. *Am J Respir Crit Care Med 162:S175*.
46. Duchmann, R., M. F. Neurath, and K. H. Meyer zum Buschenfelde. 1997. Responses to self and non-self intestinal microflora in health and inflammatory bowel disease. *Res Immunol 148:589*.
47. Cong, Y., C. T. Weaver, A. Lazenby, and C. O. Elson. 2002. Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. *J Immunol 169:6112*.
48. Weaver, C. T., L. E. Harrington, P. R. Mangan, M. Gavrieli, and K. M. Murphy. 2006. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity 24:677*.
49. Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M. A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D. J. Cua, R. A. Kastelein, and D. Rennick. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest 116:1310*.
50. Papadakis, K. A., and S. R. Targan. 1999. Serologic testing in inflammatory bowel disease: its value in indeterminate colitis. *Curr Gastroenterol Rep 1:482*.
51. Sartor, R. B. 1997. The influence of normal microbial flora on the development of chronic mucosal inflammation. *Res Immunol 148:567*.
52. Sartor, R. B. 1997. Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. *Am J Gastroenterol 92:5S*.
53. Rath, H. C., H. H. Herfarth, J. S. Ikeda, W. B. Grenther, T. E. Hamm, Jr., E. Balish, J. D. Taurog, R. E. Hammer, K. H. Wilson, and R. B. Sartor. 1996. Normal luminal bacteria, especially Bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. *J Clin Invest 98:945*.
54. Rath, H. C., M. Schultz, R. Freitag, L. A. Dieleman, F. Li, H. J. Linde, J. Scholmerich, and R. B. Sartor. 2001. Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. *Infect Immun 69:2277*.
55. Rath, H. C., K. H. Wilson, and R. B. Sartor. 1999. Differential induction of colitis and gastritis in HLA-B27 transgenic rats selectively colonized with Bacteroides vulgatus or Escherichia coli. *Infect Immun 67:2969*.
56. Sellon, R. K., S. Tonkonogy, M. Schultz, L. A. Dieleman, W. Grenther, E. Balish, D. M. Rennick, and R. B. Sartor. 1998. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun 66:5224*.
57. Mow, W. S., E. A. Vasiliauskas, Y. C. Lin, P. R. Fleshner, K. A. Papadakis, K. D. Taylor, C. J. Landers, M. T. Abreu-Martin, J. I. Rotter, H. Yang, and S. R. Targan. 2004. Association of antibody responses to microbial antigens and complications of small bowel Crohn's disease. *Gastroenterology 126:414*.
58. Kim, S. C., S. L. Tonkonogy, C. A. Albright, J. Tsang, E. J. Balish, J. Braun, M. M. Huycke, and R. B. Sartor. 2005. Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. *Gastroenterology 128:891*.

59. Prantera, C., F. Zannoni, M. L. Scribano, E. Berto, A. Andreoli, A. Kohn, and C. Luzi. 1996. An antibiotic regimen for the treatment of active Crohn's disease: a randomized, controlled clinical trial of metronidazole plus ciprofloxacin. *Am J Gastroenterol 91:328*.
60. Gionchetti, P., F. Rizzello, A. Venturi, P. Brigidi, D. Matteuzzi, G. Bazzocchi, G. Poggioli, M. Miglioli, and M. Campieri. 2000. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. *Gastroenterology 119:305*.
61. Gionchetti, P., F. Rizzello, U. Helwig, A. Venturi, K. M. Lammers, P. Brigidi, B. Vitali, G. Poggioli, M. Miglioli, and M. Campieri. 2003. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology 124:1202*.
62. Mimura, T., F. Rizzello, U. Helwig, G. Poggioli, S. Schreiber, I. C. Talbot, R. J. Nicholls, P. Gionchetti, M. Campieri, and M. A. Kamm. 2004. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut 53:108*.
63. Rutgeerts, P., K. Goboes, M. Peeters, M. Hiele, F. Penninckx, R. Aerts, R. Kerremans, and G. Vantrappen. 1991. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. *Lancet 338:771*.
64. Duchmann, R., I. Kaiser, E. Hermann, W. Mayet, K. Ewe, and K. H. Meyer zum Buschenfelde. 1995. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol 102:448*.
65. Madsen, K. L., J. S. Doyle, L. D. Jewell, M. M. Tavernini, and R. N. Fedorak. 1999. Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. *Gastroenterology 116:1107*.
66. Madsen, K., A. Cornish, P. Soper, C. McKaigney, H. Jijon, C. Yachimec, J. Doyle, L. Jewell, and C. De Simone. 2001. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology 121:580*.
67. Schultz, M., C. Veltkamp, L. A. Dieleman, W. B. Grenther, P. B. Wyrick, S. L. Tonkonogy, and R. B. Sartor. 2002. Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflamm Bowel Dis 8:71*.
68. Lammers, K. M., U. Helwig, E. Swennen, F. Rizzello, A. Venturi, E. Caramelli, M. A. Kamm, P. Brigidi, P. Gionchetti, and M. Campieri. 2002. Effect of probiotic strains on interleukin 8 production by HT29/19A cells. *Am J Gastroenterol 97:1182*.
69. Lodes, M. J., Y. Cong, C. O. Elson, R. Mohamath, C. J. Landers, S. R. Targan, M. Fort, and R. M. Hershberg. 2004. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest 113:1296*.
70. Elson, C. O., R. B. Sartor, G. S. Tennyson, and R. H. Riddell. 1995. Experimental models of inflammatory bowel disease. *Gastroenterology 109:1344*.
71. Strober, W., I. J. Fuss, and R. S. Blumberg. 2002. The immunology of mucosal models of inflammation. *Annu Rev Immunol 20:495*.
72. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell 75:263*.
73. Madsen, K. L., J. S. Doyle, M. M. Tavernini, L. D. Jewell, R. P. Rennie, and R. N. Fedorak. 2000. Antibiotic therapy attenuates colitis in interleukin 10 gene-deficient mice. *Gastroenterology 118:1094*.

74. Hoentjen, F., H. J. Harmsen, H. Braat, C. D. Torrice, B. A. Mann, R. B. Sartor, and L. A. Dieleman. 2003. Antibiotics with a selective aerobic or anaerobic spectrum have different therapeutic activities in various regions of the colon in interleukin 10 gene deficient mice. *Gut 52:1721*.
75. Fedorak, R. N., A. Gangl, C. O. Elson, P. Rutgeerts, S. Schreiber, G. Wild, S. B. Hanauer, A. Kilian, M. Cohard, A. LeBeaut, and B. Feagan. 2000. Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. The Interleukin 10 Inflammatory Bowel Disease Cooperative Study Group. *Gastroenterology 119:1473*.
76. Ouellette, A. J. 2004. Defensin-mediated innate immunity in the small intestine. *Best Pract Res Clin Gastroenterol 18:405*.
77. Cunliffe, R. N., and Y. R. Mahida. 2004. Expression and regulation of antimicrobial peptides in the gastrointestinal tract. *J Leukoc Biol 75:49*.
78. Chowers, Y., L. Cahalon, M. Lahav, H. Schor, R. Tal, S. Bar-Meir, and M. Levite. 2000. Somatostatin through its specific receptor inhibits spontaneous and TNF-alpha- and bacteria-induced IL-8 and IL-1 beta secretion from intestinal epithelial cells. *J Immunol 165:2955*.
79. Abad, C., C. Martinez, M. G. Juarranz, A. Arranz, J. Leceta, M. Delgado, and R. P. Gomariz. 2003. Therapeutic effects of vasoactive intestinal peptide in the trinitrobenzene sulfonic acid mice model of Crohn's disease. *Gastroenterology 124:961*.
80. Yang, G. B., and A. A. Lackner. 2004. Proximity between 5-HT secreting enteroendocrine cells and lymphocytes in the gut mucosa of rhesus macaques (Macaca mulatta) is suggestive of a role for enterochromaffin cell 5-HT in mucosal immunity. *J Neuroimmunol 146:46*.
81. Farthing, M. J. 2000. Enterotoxins and the enteric nervous system--a fatal attraction. *Int J Med Microbiol 290:491*.
82. Madara, J. L., S. Nash, R. Moore, and K. Atisook. 1990. Structure and function of the intestinal epithelial barrier in health and disease. *Monogr Pathol:306*.
83. Clayburgh, D. R., L. Shen, and J. R. Turner. 2004. A porous defense: the leaky epithelial barrier in intestinal disease. *Lab Invest 84:282*.
84. Goke, M., and D. K. Podolsky. 1996. Regulation of the mucosal epithelial barrier. *Baillieres Clin Gastroenterol 10:393*.
85. Mashimo, H., D. C. Wu, D. K. Podolsky, and M. C. Fishman. 1996. Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. *Science 274:262*.
86. Kagnoff, M. F., and L. Eckmann. 1997. Epithelial cells as sensors for microbial infection. *J Clin Invest 100:6*.
87. Jung, H. C., L. Eckmann, S. K. Yang, A. Panja, J. Fierer, E. Morzycka-Wroblewska, and M. F. Kagnoff. 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J Clin Invest 95:55*.
88. Bry, L., P. G. Falk, T. Midtvedt, and J. I. Gordon. 1996. A model of host-microbial interactions in an open mammalian ecosystem. *Science 273:1380*.
89. Collier-Hyams, L. S., H. Zeng, J. Sun, A. D. Tomlinson, Z. Q. Bao, H. Chen, J. L. Madara, K. Orth, and A. S. Neish. 2002. Cutting edge: Salmonella AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kappa B pathway. *J Immunol 169:2846*.

90. Neish, A. S., A. T. Gewirtz, H. Zeng, A. N. Young, M. E. Hobert, V. Karmali, A. S. Rao, and J. L. Madara. 2000. Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science 289:1560*.
91. Janeway, C. 1989. Immunogenicity signals 1,2,3 ... and 0. *Immunol Today 10:283*.
92. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol 12:991*.
93. Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nat Rev Immunol 1:135*.
94. Takeuchi, O., S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R. L. Modlin, and S. Akira. 2002. Cutting edge: Role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol 169:10*.
95. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity 11:443*.
96. Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem 274:17406*.
97. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol 162:3749*.
98. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature 410:1099*.
99. Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med 189:1777*.
100. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu Rev Immunol 21:335*.
101. Baldwin, A. S., Jr. 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol 14:649*.
102. Hayden, M. S., and S. Ghosh. 2004. Signaling to NF-kappaB. *Genes Dev 18:2195*.
103. Tak, P. P., and G. S. Firestein. 2001. NF-kappaB: a key role in inflammatory diseases. *J Clin Invest 107:7*.
104. Abreu, M. T., P. Vora, E. Faure, L. S. Thomas, E. T. Arnold, and M. Arditi. 2001. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J Immunol 167:1609*.
105. Suzuki, M., T. Hisamatsu, and D. K. Podolsky. 2003. Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular Toll-like receptor 4-MD-2 complex. *Infect Immun 71:3503*.
106. Cario, E., and D. K. Podolsky. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun 68:7010*.

107. Hausmann, M., S. Kiessling, S. Mestermann, G. Webb, T. Spottl, T. Andus, J. Scholmerich, H. Herfarth, K. Ray, W. Falk, and G. Rogler. 2002. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. *Gastroenterology 122:1987*.
108. Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H. C. Reinecker, and D. K. Podolsky. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol 164:966*.
109. Gewirtz, A. T., T. A. Navas, S. Lyons, P. J. Godowski, and J. L. Madara. 2001. Cutting edge: Bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol 167:1882*.
110. Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell 118:229*.
111. Ogura, Y., N. Inohara, A. Benito, F. F. Chen, S. Yamaoka, and G. Nunez. 2001. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem 276:4812*.
112. Ogura, Y., S. Lala, W. Xin, E. Smith, T. A. Dowds, F. F. Chen, E. Zimmermann, M. Tretiakova, J. H. Cho, J. Hart, J. K. Greenson, S. Keshav, and G. Nunez. 2003. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut 52:1591*.
113. Lala, S., Y. Ogura, C. Osborne, S. Y. Hor, A. Bromfield, S. Davies, O. Ogunbiyi, G. Nunez, and S. Keshav. 2003. Crohn's disease and the NOD2 gene: a role for paneth cells. *Gastroenterology 125:47*.
114. Inohara, N., Y. Ogura, A. Fontalba, O. Gutierrez, F. Pons, J. Crespo, K. Fukase, S. Inamura, S. Kusumoto, M. Hashimoto, S. J. Foster, A. P. Moran, J. L. Fernandez-Luna, and G. Nunez. 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem 278:5509*.
115. Li, J., T. Moran, E. Swanson, C. Julian, J. Harris, D. K. Bonen, M. Hedl, D. L. Nicolae, C. Abraham, and J. H. Cho. 2004. Regulation of IL-8 and IL-1beta expression in Crohn's disease associated NOD2/CARD15 mutations. *Hum Mol Genet 13:1715*.
116. Kobayashi, K. S., M. Chamaillard, Y. Ogura, O. Henegariu, N. Inohara, G. Nunez, and R. A. Flavell. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science 307:731*.
117. Watanabe, T., A. Kitani, P. J. Murray, and W. Strober. 2004. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat Immunol 5:800*.
118. Maeda, S., L. C. Hsu, H. Liu, L. A. Bankston, M. Iimura, M. F. Kagnoff, L. Eckmann, and M. Karin. 2005. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science 307:734*.
119. Lotze, M. T., and K. J. Tracey. 2005. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol 5:331*.
120. Seong, S. Y., and P. Matzinger. 2004. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol 4:469*.
121. Shi, Y., J. E. Evans, and K. L. Rock. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature 425:516*.
122. Wang, H., O. Bloom, M. Zhang, J. M. Vishnubhakat, M. Ombrellino, J. Che, A. Frazier, H. Yang, S. Ivanova, L. Borovikova, K. R. Manogue, E. Faist, E. Abraham, J. Andersson, U. Andersson, P. E. Molina, N. N. Abumrad, A. Sama, and K. J. Tracey. 1999. HMG-1 as a late mediator of endotoxin lethality in mice. *Science 285:248*.

123. Wang, H., H. Yang, C. J. Czura, A. E. Sama, and K. J. Tracey. 2001. HMGB1 as a late mediator of lethal systemic inflammation. *Am J Respir Crit Care Med 164:1768*.
124. Rendon-Mitchell, B., M. Ochani, J. Li, J. Han, H. Wang, H. Yang, S. Susarla, C. Czura, R. A. Mitchell, G. Chen, A. E. Sama, and K. J. Tracey. 2003. IFN-gamma induces high mobility group box 1 protein release partly through a TNF-dependent mechanism. *J Immunol 170:3890*.
125. Liu, S., D. B. Stolz, P. L. Sappington, C. A. Macias, M. E. Killeen, J. J. Tenhunen, R. L. Delude, and M. P. Fink. 2006. HMGB1 is secreted by immunostimulated enterocytes and contributes to cytomix-induced hyperpermeability of Caco-2 monolayers. *Am J Physiol Cell Physiol 290:C990*.
126. Scaffidi, P., T. Misteli, and M. E. Bianchi. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature 418:191*.
127. Andersson, U., H. Wang, K. Palmblad, A. C. Aveberger, O. Bloom, H. Erlandsson-Harris, A. Janson, R. Kokkola, M. Zhang, H. Yang, and K. J. Tracey. 2000. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J Exp Med 192:565*.
128. Schmidt, A. M., S. D. Yan, S. F. Yan, and D. M. Stern. 2001. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest 108:949*.
129. Yu, M., H. Wang, A. Ding, D. T. Golenbock, E. Latz, C. J. Czura, M. J. Fenton, K. J. Tracey, and H. Yang. 2006. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock 26:174*.
130. Park, J. S., D. Svetkauskaite, Q. He, J. Y. Kim, D. Strassheim, A. Ishizaka, and E. Abraham. 2004. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem 279:7370*.
131. Sappington, P. L., R. Yang, H. Yang, K. J. Tracey, R. L. Delude, and M. P. Fink. 2002. HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice. *Gastroenterology 123:790*.
132. May, M. J., R. B. Marienfeld, and S. Ghosh. 2002. Characterization of the Ikappa B-kinase NEMO binding domain. *J Biol Chem 277:45992*.
133. Rudolph, D., W. C. Yeh, A. Wakeham, B. Rudolph, D. Nallainathan, J. Potter, A. J. Elia, and T. W. Mak. 2000. Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. *Genes Dev 14:854*.
134. Neurath, M. F., and S. Pettersson. 1997. Predominant role of NF-kappa B p65 in the pathogenesis of chronic intestinal inflammation. *Immunobiology 198:91*.
135. Neurath, M. F., S. Pettersson, K. H. Meyer zum Buschenfelde, and W. Strober. 1996. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nat Med 2:998*.
136. Fichtner-Feigl, S., I. J. Fuss, J. C. Preiss, W. Strober, and A. Kitani. 2005. Treatment of murine Th1- and Th2-mediated inflammatory bowel disease with NF-kappa B decoy oligonucleotides. *J Clin Invest 115:3057*.
137. Lawrance, I. C., F. Wu, A. Z. Leite, J. Willis, G. A. West, C. Fiocchi, and S. Chakravarti. 2003. A murine model of chronic inflammation-induced intestinal fibrosis down-regulated by antisense NF-kappa B. *Gastroenterology 125:1750*.
138. Neurath, M. F., I. Fuss, G. Schurmann, S. Pettersson, K. Arnold, H. Muller-Lobeck, W. Strober, C. Herfarth, and K. H. Buschenfelde. 1998. Cytokine gene transcription by NF-

kappa B family members in patients with inflammatory bowel disease. *Ann N Y Acad Sci 859:149*.
139. Schreiber, S., S. Nikolaus, and J. Hampe. 1998. Activation of nuclear factor kappa B inflammatory bowel disease. *Gut 42:477*.
140. Rogler, G., K. Brand, D. Vogl, S. Page, R. Hofmeister, T. Andus, R. Knuechel, P. A. Baeuerle, J. Scholmerich, and V. Gross. 1998. Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology 115:357*.
141. Medzhitov, R., and C. A. Janeway, Jr. 1998. An ancient system of host defense. *Curr Opin Immunol 10:12*.
142. Hoffmann, J. A., F. C. Kafatos, C. A. Janeway, and R. A. Ezekowitz. 1999. Phylogenetic perspectives in innate immunity. *Science 284:1313*.
143. Eckmann, L., H. C. Jung, C. Schurer-Maly, A. Panja, E. Morzycka-Wroblewska, and M. F. Kagnoff. 1993. Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin 8. *Gastroenterology 105:1689*.
144. Otte, J. M., E. Cario, and D. K. Podolsky. 2004. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology 126:1054*.
145. Naik, S., E. J. Kelly, L. Meijer, S. Pettersson, and I. R. Sanderson. 2001. Absence of Toll-like receptor 4 explains endotoxin hyporesponsiveness in human intestinal epithelium. *J Pediatr Gastroenterol Nutr 32:449*.
146. Melmed, G., L. S. Thomas, N. Lee, S. Y. Tesfay, K. Lukasek, K. S. Michelsen, Y. Zhou, B. Hu, M. Arditi, and M. T. Abreu. 2003. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J Immunol 170:1406*.
147. Hirschfeld, M., Y. Ma, J. H. Weis, S. N. Vogel, and J. J. Weis. 2000. Cutting edge: Repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol 165:618*.
148. Zhu, C., K. Gagnidze, J. H. Gemberling, and S. E. Plevy. 2001. Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. Demonstration of novel functional elements by a reductionist approach. *J Biol Chem 276:18519*.
149. Xiong, H., C. Zhu, F. Li, R. Hegazi, K. He, M. Babyatsky, A. J. Bauer, and S. E. Plevy. 2004. Inhibition of interleukin-12 p40 transcription and NF-kappaB activation by nitric oxide in murine macrophages and dendritic cells. *J Biol Chem 279:10776*.
150. Weinstein, S. L., A. J. Finn, S. H. Dave, F. Meng, C. A. Lowell, J. S. Sanghera, and A. L. DeFranco. 2000. Phosphatidylinositol 3-kinase and mTOR mediate lipopolysaccharide-stimulated nitric oxide production in macrophages via interferon-beta. *J Leukoc Biol 67:405*.
151. Rindi, G., S. G. Grant, Y. Yiangou, M. A. Ghatei, S. R. Bloom, V. L. Bautch, E. Solcia, and J. M. Polak. 1990. Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. Heterogeneity of hormone expression. *Am J Pathol 136:1349*.
152. Koyama, S. Y., and D. K. Podolsky. 1989. Differential expression of transforming growth factors alpha and beta in rat intestinal epithelial cells. *J Clin Invest 83:1768*.
153. Lugering, N., T. Kucharzik, H. Gockel, C. Sorg, R. Stoll, and W. Domschke. 1998. Human intestinal epithelial cells down-regulate IL-8 expression in human intestinal

microvascular endothelial cells; role of transforming growth factor-beta 1 (TGF-beta1). *Clin Exp Immunol 114:377*.
154. Letterio, J. J., and A. B. Roberts. 1998. Regulation of immune responses by TGF-beta. *Annu Rev Immunol 16:137*.
155. Strober, W., B. Kelsall, and T. Marth. 1998. Oral tolerance. *J Clin Immunol 18:1*.
156. Del Zotto, B., G. Mumolo, A. M. Pronio, C. Montesani, R. Tersigni, and M. Boirivant. 2003. TGF-beta1 production in inflammatory bowel disease: differing production patterns in Crohn's disease and ulcerative colitis. *Clin Exp Immunol 134:120*.
157. Barg, S. 2003. Mechanisms of exocytosis in insulin-secreting B-cells and glucagon-secreting A-cells. *Pharmacol Toxicol 92:3*.
158. Sudhof, T. C. 2004. The synaptic vesicle cycle. *Annu Rev Neurosci 27:509*.
159. Wang, Y., V. Prpic, G. M. Green, J. R. Reeve, Jr., and R. A. Liddle. 2002. Luminal CCK-releasing factor stimulates CCK release from human intestinal endocrine and STC-1 cells. *Am J Physiol Gastrointest Liver Physiol 282:G16*.
160. Hira, T., A. C. Elliott, D. G. Thompson, R. M. Case, and J. T. McLaughlin. 2004. Multiple fatty acid sensing mechanisms operate in enteroendocrine cells: novel evidence for direct mobilization of stored calcium by cytosolic fatty acid. *J Biol Chem 279:26082*.
161. Ganea, D., and M. Delgado. 2002. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) as modulators of both innate and adaptive immunity. *Crit Rev Oral Biol Med 13:229*.
162. Cong, B., S. J. Li, Y. X. Yao, G. J. Zhu, and Y. L. Ling. 2002. Effect of cholecystokinin octapeptide on tumor necrosis factor alpha transcription and nuclear factor-kappaB activity induced by lipopolysaccharide in rat pulmonary interstitial macrophages. *World J Gastroenterol 8:718*.
163. Meng, A. H., Y. L. Ling, X. P. Zhang, and J. L. Zhang. 2002. Anti-inflammatory effect of cholecystokinin and its signal transduction mechanism in endotoxic shock rat. *World J Gastroenterol 8:712*.
164. Smith, P. D., C. Ochsenbauer-Jambor, and L. E. Smythies. 2005. Intestinal macrophages: unique effector cells of the innate immune system. *Immunol Rev 206:149*.
165. Ortega-Cava, C. F., S. Ishihara, M. A. Rumi, K. Kawashima, N. Ishimura, H. Kazumori, J. Udagawa, Y. Kadowaki, and Y. Kinoshita. 2003. Strategic compartmentalization of Toll-like receptor 4 in the mouse gut. *J Immunol 170:3977*.
166. Hornef, M. W., T. Frisan, A. Vandewalle, S. Normark, and A. Richter-Dahlfors. 2002. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J Exp Med 195:559*.
167. Sjolund, K., G. Sanden, R. Hakanson, and F. Sundler. 1983. Endocrine cells in human intestine: an immunocytochemical study. *Gastroenterology 85:1120*.
168. Roth, K. A., and J. I. Gordon. 1990. Spatial differentiation of the intestinal epithelium: analysis of enteroendocrine cells containing immunoreactive serotonin, secretin, and substance P in normal and transgenic mice. *Proc Natl Acad Sci U S A 87:6408*.
169. Rindi, G., A. B. Leiter, A. S. Kopin, C. Bordi, and E. Solcia. 2004. The "normal" endocrine cell of the gut: changing concepts and new evidences. *Ann N Y Acad Sci 1014:1*.
170. Hocker, M., and B. Wiedenmann. 1998. Molecular mechanisms of enteroendocrine differentiation. *Ann N Y Acad Sci 859:160*.

171. Roth, K. A., J. M. Hertz, and J. I. Gordon. 1990. Mapping enteroendocrine cell populations in transgenic mice reveals an unexpected degree of complexity in cellular differentiation within the gastrointestinal tract. *J Cell Biol 110:1791*.
172. Turvill, J. L., F. H. Mourad, and M. J. Farthing. 1998. Crucial role for 5-HT in cholera toxin but not Escherichia coli heat-labile enterotoxin-intestinal secretion in rats. *Gastroenterology 115:883*.
173. Wang, X., V. Soltesz, J. Axelson, and R. Andersson. 1996. Cholecystokinin increases small intestinal motility and reduces enteric bacterial overgrowth and translocation in rats with surgically induced acute liver failure. *Digestion 57:67*.
174. Leslie, F. C., D. G. Thompson, J. T. McLaughlin, A. Varro, G. J. Dockray, and B. K. Mandal. 2003. Plasma cholecystokinin concentrations are elevated in acute upper gastrointestinal infections. *Qjm 96:870*.
175. Rubin, D. C., H. Zhang, P. Qian, R. G. Lorenz, K. Hutton, and M. G. Peters. 2000. Altered enteroendocrine cell expression in T cell receptor alpha chain knock-out mice. *Microsc Res Tech 51:112*.
176. Nemoz-Gaillard, E., M. Cordier-Bussat, C. Filloux, J. C. Cuber, E. Van Obberghen, J. A. Chayvialle, and J. Abello. 1998. Bombesin stimulates cholecystokinin secretion through mitogen-activated protein-kinase-dependent and -independent mechanisms in the enteroendocrine STC-1 cell line. *Biochem J 331 ( Pt 1):129*.
177. McCurdy, J. D., T. J. Olynych, L. H. Maher, and J. S. Marshall. 2003. Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *J Immunol 170:1625*.
178. Beck, P. L., I. M. Rosenberg, R. J. Xavier, T. Koh, J. F. Wong, and D. K. Podolsky. 2003. Transforming growth factor-beta mediates intestinal healing and susceptibility to injury in vitro and in vivo through epithelial cells. *Am J Pathol 162:597*.
179. Fukata, M., K. S. Michelsen, R. Eri, L. S. Thomas, B. Hu, K. Lukasek, C. C. Nast, J. Lechago, R. Xu, Y. Naiki, A. Soliman, M. Arditi, and M. T. Abreu. 2005. Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *Am J Physiol Gastrointest Liver Physiol 288:G1055*.
180. Fink, M. P. 2003. Ethyl pyruvate: a novel anti-inflammatory agent. *Crit Care Med 31:S51*.
181. Montgomery, C. M., and J. L. Webb. 1956. Metabolic studies on heart mitochondria. II. The inhibitory action of parapyruvate on the tricarboxylic acid cycle. *J Biol Chem 221:359*.
182. Sims, C. A., S. Wattanasirichaigoon, M. J. Menconi, A. M. Ajami, and M. P. Fink. 2001. Ringer's ethyl pyruvate solution ameliorates ischemia/reperfusion-induced intestinal mucosal injury in rats. *Crit Care Med 29:1513*.
183. Yang, R., D. J. Gallo, J. J. Baust, T. Uchiyama, S. K. Watkins, R. L. Delude, and M. P. Fink. 2002. Ethyl pyruvate modulates inflammatory gene expression in mice subjected to hemorrhagic shock. *Am J Physiol Gastrointest Liver Physiol 283:G212*.
184. Ulloa, L., M. Ochani, H. Yang, M. Tanovic, D. Halperin, R. Yang, C. J. Czura, M. P. Fink, and K. J. Tracey. 2002. Ethyl pyruvate prevents lethality in mice with established lethal sepsis and systemic inflammation. *Proc Natl Acad Sci U S A 99:12351*.

185. Sappington, P. L., M. E. Fink, R. Yang, R. L. Delude, and M. P. Fink. 2003. Ethyl pyruvate provides durable protection against inflammation-induced intestinal epithelial barrier dysfunction. *Shock 20:521*.
186. Sappington, P. L., X. Han, R. Yang, R. L. Delude, and M. P. Fink. 2003. Ethyl pyruvate ameliorates intestinal epithelial barrier dysfunction in endotoxemic mice and immunostimulated caco-2 enterocytic monolayers. *J Pharmacol Exp Ther 304:464*.
187. Han, Y., J. A. Englert, R. Yang, R. L. Delude, and M. P. Fink. 2005. Ethyl pyruvate inhibits nuclear factor-kappaB-dependent signaling by directly targeting p65. *J Pharmacol Exp Ther 312:1097*.
188. Elson, C. O., R. B. Sartor, S. R. Targan, and W. J. Sandborn. 2003. Challenges in IBD Research: updating the scientific agendas. *Inflamm Bowel Dis 9:137*.
189. Sartor, R. B. 2004. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology 126:1620*.
190. Krieglstein, C. F., W. H. Cerwinka, F. S. Laroux, J. W. Salter, J. M. Russell, G. Schuermann, M. B. Grisham, C. R. Ross, and D. N. Granger. 2001. Regulation of murine intestinal inflammation by reactive metabolites of oxygen and nitrogen: divergent roles of superoxide and nitric oxide. *J Exp Med 194:1207*.
191. Joshi, R., S. Kumar, M. Unnikrishnan, and T. Mukherjee. 2005. Free radical scavenging reactions of sulfasalazine, 5-aminosalicylic acid and sulfapyridine: mechanistic aspects and antioxidant activity. *Free Radic Res 39:1163*.
192. McKenzie, S. J., M. S. Baker, G. D. Buffinton, and W. F. Doe. 1996. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *J Clin Invest 98:136*.
193. Pavlick, K. P., F. S. Laroux, J. Fuseler, R. E. Wolf, L. Gray, J. Hoffman, and M. B. Grisham. 2002. Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. *Free Radic Biol Med 33:311*.
194. Davidson, N. J., S. A. Hudak, R. E. Lesley, S. Menon, M. W. Leach, and D. M. Rennick. 1998. IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J Immunol 161:3143*.
195. Hanford, L. E., J. J. Enghild, Z. Valnickova, S. V. Petersen, L. M. Schaefer, T. M. Schaefer, T. A. Reinhart, and T. D. Oury. 2004. Purification and characterization of mouse soluble receptor for advanced glycation end products (sRAGE). *J Biol Chem 279:50019*.
196. Hegazi, R. A., K. N. Rao, A. Mayle, A. R. Sepulveda, L. E. Otterbein, and S. E. Plevy. 2005. Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. *J Exp Med 202:1703*.
197. Plevy, S. E., J. H. Gemberling, S. Hsu, A. J. Dorner, and S. T. Smale. 1997. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol Cell Biol 17:4572*.
198. Tenhunen, R., H. S. Marver, and R. Schmid. 1970. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. *J Lab Clin Med 75:410*.
199. McCoubrey, W. K., Jr., T. J. Huang, and M. D. Maines. 1997. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem 247:725*.

200. Otterbein, L. E., J. K. Kolls, L. L. Mantell, J. L. Cook, J. Alam, and A. M. Choi. 1999. Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. *J Clin Invest 103:1047*.
201. Poss, K. D., and S. Tonegawa. 1997. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci U S A 94:10925*.
202. Yachie, A., Y. Niida, T. Wada, N. Igarashi, H. Kaneda, T. Toma, K. Ohta, Y. Kasahara, and S. Koizumi. 1999. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest 103:129*.
203. Xiong, H., C. Zhu, H. Li, F. Chen, L. Mayer, K. Ozato, J. C. Unkeless, and S. E. Plevy. 2003. Complex formation of the interferon (IFN) consensus sequence-binding protein with IRF-1 is essential for murine macrophage IFN-gamma-induced iNOS gene expression. *J Biol Chem 278:2271*.
204. Su, C. G., X. Wen, S. T. Bailey, W. Jiang, S. M. Rangwala, S. A. Keilbaugh, A. Flanigan, S. Murthy, M. A. Lazar, and G. D. Wu. 1999. A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response. *J Clin Invest 104:383*.
205. Kelly, D., J. I. Campbell, T. P. King, G. Grant, E. A. Jansson, A. G. Coutts, S. Pettersson, and S. Conway. 2004. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nat Immunol 5:104*.
206. Alam, J., C. Wicks, D. Stewart, P. Gong, C. Touchard, S. Otterbein, A. M. Choi, M. E. Burow, and J. Tou. 2000. Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor. *J Biol Chem 275:27694*.
207. Gong, P., D. Stewart, B. Hu, N. Li, J. Cook, A. Nel, and J. Alam. 2002. Activation of the mouse heme oxygenase-1 gene by 15-deoxy-Delta(12,14)-prostaglandin J(2) is mediated by the stress response elements and transcription factor Nrf2. *Antioxid Redox Signal 4:249*.
208. Prestera, T., P. Talalay, J. Alam, Y. I. Ahn, P. J. Lee, and A. M. Choi. 1995. Parallel induction of heme oxygenase-1 and chemoprotective phase 2 enzymes by electrophiles and antioxidants: regulation by upstream antioxidant-responsive elements (ARE). *Mol Med 1:827*.
209. Kobayashi, A., M. I. Kang, Y. Watai, K. I. Tong, T. Shibata, K. Uchida, and M. Yamamoto. 2006. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol Cell Biol 26:221*.
210. Andersen, P. H., and N. J. Jensen. 1984. Mutagenic investigation of flavourings: dimethyl succinate, ethyl pyruvate and aconitic acid are negative in the Salmonella/mammalian-microsome test. *Food Addit Contam 1:283*.
211. Food and Agriculture Organization of the United Nations and the World Health Organization. 2001. Summary of Evaluations performed by the Joint FAO/WHO expert committee on food additives (http://jecfa.ilsi.org/).
212. US National Library of Medicine. CTI-01 (ethyl pyruvate) safety and complication prevention in cardiac surgery patients on cardiopulmonary bypass (CPB) ( http://www.clinicaltrials.gov/ct/gui/show/NCT00107666).
213. Carballo, J., A. Bernardo, M. J. Prieto, and R. M. Sarmiento. 1993. Kinetics of alpha-dicarbonyls reduction by L-glycol dehydrogenase (NAD+) from Enterobacter aerogenes. *Ital J Biochem 42:79*.

214. Kim, J. Y., J. S. Park, D. Strassheim, I. Douglas, F. Diaz del Valle, K. Asehnoune, S. Mitra, S. H. Kwak, S. Yamada, I. Maruyama, A. Ishizaka, and E. Abraham. 2005. HMGB1 contributes to the development of acute lung injury after hemorrhage. *Am J Physiol Lung Cell Mol Physiol 288:L958*.
215. Tsung, A., R. Sahai, H. Tanaka, A. Nakao, M. P. Fink, M. T. Lotze, H. Yang, J. Li, K. J. Tracey, D. A. Geller, and T. R. Billiar. 2005. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J Exp Med 201:1135*.
216. Yang, H., M. Ochani, J. Li, X. Qiang, M. Tanovic, H. E. Harris, S. M. Susarla, L. Ulloa, H. Wang, R. DiRaimo, C. J. Czura, J. Roth, H. S. Warren, M. P. Fink, M. J. Fenton, U. Andersson, and K. J. Tracey. 2004. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A 101:296*.
217. Andersson, U., and H. Erlandsson-Harris. 2004. HMGB1 is a potent trigger of arthritis. *J Intern Med 255:344*.
218. Bonaldi, T., F. Talamo, P. Scaffidi, D. Ferrera, A. Porto, A. Bachi, A. Rubartelli, A. Agresti, and M. E. Bianchi. 2003. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *Embo J 22:5551*.
219. Semino, C., G. Angelini, A. Poggi, and A. Rubartelli. 2005. NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1. *Blood 106:609*.
220. Foell, D., T. Kucharzik, M. Kraft, T. Vogl, C. Sorg, W. Domschke, and J. Roth. 2003. Neutrophil derived human S100A12 (EN-RAGE) is strongly expressed during chronic active inflammatory bowel disease. *Gut 52:847*.
221. Thjodleifsson, B., G. Sigthorsson, N. Cariglia, I. Reynisdottir, D. F. Gudbjartsson, K. Kristjansson, J. B. Meddings, V. Gudnason, J. H. Wandall, L. P. Andersen, R. Sherwood, M. Kjeld, E. Oddsson, H. Gudjonsson, and I. Bjarnason. 2003. Subclinical intestinal inflammation: an inherited abnormality in Crohn's disease relatives? *Gastroenterology 124:1728*.
222. Tibble, J. A., G. Sigthorsson, R. Foster, I. Forgacs, and I. Bjarnason. 2002. Use of surrogate markers of inflammation and Rome criteria to distinguish organic from nonorganic intestinal disease. *Gastroenterology 123:450*.
223. Hofmann, M. A., S. Drury, C. Fu, W. Qu, A. Taguchi, Y. Lu, C. Avila, N. Kambham, A. Bierhaus, P. Nawroth, M. F. Neurath, T. Slattery, D. Beach, J. McClary, M. Nagashima, J. Morser, D. Stern, and A. M. Schmidt. 1999. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell 97:889*.
224. Srikrishna, G., O. Turovskaya, R. Shaikh, R. Newlin, D. Foell, S. Murch, M. Kronenberg, and H. H. Freeze. 2005. Carboxylated glycans mediate colitis through activation of NF-kappa B. *J Immunol 175:5412*.
225. Murphy, T. L., M. G. Cleveland, P. Kulesza, J. Magram, and K. M. Murphy. 1995. Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. *Mol Cell Biol 15:5258*.
226. Xie, Q. W., Y. Kashiwabara, and C. Nathan. 1994. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem 269:4705*.
227. Tse, H. M., M. J. Milton, and J. D. Piganelli. 2004. Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation-reduction reactions in innate immunity. *Free Radic Biol Med 36:233*.

228. Matthews, J. R., C. H. Botting, M. Panico, H. R. Morris, and R. T. Hay. 1996. Inhibition of NF-kappaB DNA binding by nitric oxide. *Nucleic Acids Res 24:2236*.
229. Matthews, J. R., N. Wakasugi, J. L. Virelizier, J. Yodoi, and R. T. Hay. 1992. Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62. *Nucleic Acids Res 20:3821*.
230. Andreadi, C. K., L. M. Howells, P. A. Atherfold, and M. M. Manson. 2006. Involvement of Nrf2, p38, B-Raf, and nuclear factor-kappaB, but not phosphatidylinositol 3-kinase, in induction of hemeoxygenase-1 by dietary polyphenols. *Mol Pharmacol 69:1033*.
231. Chen, J., and R. F. Regan. 2005. Increasing expression of heme oxygenase-1 by proteasome inhibition protects astrocytes from heme-mediated oxidative injury. *Curr Neurovasc Res 2:189*.
232. Alam, J., E. Killeen, P. Gong, R. Naquin, B. Hu, D. Stewart, J. R. Ingelfinger, and K. A. Nath. 2003. Heme activates the heme oxygenase-1 gene in renal epithelial cells by stabilizing Nrf2. *Am J Physiol Renal Physiol 284:F743*.
233. Yamamoto, Y., and R. B. Gaynor. 2001. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest 107:135*.
234. Scheinman, R. I., A. Gualberto, C. M. Jewell, J. A. Cidlowski, and A. S. Baldwin, Jr. 1995. Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. *Mol Cell Biol 15:943*.
235. Auphan, N., J. A. DiDonato, C. Rosette, A. Helmberg, and M. Karin. 1995. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science 270:286*.
236. Scheinman, R. I., P. C. Cogswell, A. K. Lofquist, and A. S. Baldwin, Jr. 1995. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science 270:283*.
237. May, M. J., F. D'Acquisto, L. A. Madge, J. Glockner, J. S. Pober, and S. Ghosh. 2000. Selective inhibition of NF-kappaB activation by a peptide that blocks the interaction of NEMO with the IkappaB kinase complex. *Science 289:1550*.
238. di Meglio, P., A. Ianaro, and S. Ghosh. 2005. Amelioration of acute inflammation by systemic administration of a cell-permeable peptide inhibitor of NF-kappaB activation. *Arthritis Rheum 52:951*.
239. Wadia, J. S., and S. F. Dowdy. 2002. Protein transduction technology. *Curr Opin Biotechnol 13:52*.
240. Mi, Z., X. Lu, J. C. Mai, B. G. Ng, G. Wang, E. R. Lechman, S. C. Watkins, H. Rabinowich, and P. D. Robbins. 2003. Identification of a synovial fibroblast-specific protein transduction domain for delivery of apoptotic agents to hyperplastic synovium. *Mol Ther 8:295*.
241. Mi, Z., J. Mai, X. Lu, and P. D. Robbins. 2000. Characterization of a class of cationic peptides able to facilitate efficient protein transduction in vitro and in vivo. *Mol Ther 2:339*.
242. Mai, J. C., Z. Mi, S. H. Kim, B. Ng, and P. D. Robbins. 2001. A proapoptotic peptide for the treatment of solid tumors. *Cancer Res 61:7709*.
243. Plevy, S. 2002. The immunology of inflammatory bowel disease. *Gastroenterol Clin North Am 31:77*.
244. Jimi, E., K. Aoki, H. Saito, F. D'Acquisto, M. J. May, I. Nakamura, T. Sudo, T. Kojima, F. Okamoto, H. Fukushima, K. Okabe, K. Ohya, and S. Ghosh. 2004. Selective inhibition

of NF-kappa B blocks osteoclastogenesis and prevents inflammatory bone destruction in vivo. *Nat Med 10:617*.
245. Dai, S., T. Hirayama, S. Abbas, and Y. Abu-Amer. 2004. The IkappaB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks osteoclastogenesis and bone erosion in inflammatory arthritis. *J Biol Chem 279:37219*.
246. Dasgupta, S., M. Jana, Y. Zhou, Y. K. Fung, S. Ghosh, and K. Pahan. 2004. Antineuroinflammatory effect of NF-kappaB essential modifier-binding domain peptides in the adoptive transfer model of experimental allergic encephalomyelitis. *J Immunol 173:1344*.
247. Muller, J. R., and U. Siebenlist. 2003. Lymphotoxin beta receptor induces sequential activation of distinct NF-kappa B factors via separate signaling pathways. *J Biol Chem 278:12006*.
248. Derudder, E., E. Dejardin, L. L. Pritchard, D. R. Green, M. Korner, and V. Baud. 2003. RelB/p50 dimers are differentially regulated by tumor necrosis factor-alpha and lymphotoxin-beta receptor activation: critical roles for p100. *J Biol Chem 278:23278*.
249. Hatada, E. N., R. K. Do, A. Orlofsky, H. C. Liou, M. Prystowsky, I. C. MacLennan, J. Caamano, and S. Chen-Kiang. 2003. NF-kappa B1 p50 is required for BLyS attenuation of apoptosis but dispensable for processing of NF-kappa B2 p100 to p52 in quiescent mature B cells. *J Immunol 171:761*.
250. Araki, A., T. Kanai, T. Ishikura, S. Makita, K. Uraushihara, R. Iiyama, T. Totsuka, K. Takeda, S. Akira, and M. Watanabe. 2005. MyD88-deficient mice develop severe intestinal inflammation in dextran sodium sulfate colitis. *J Gastroenterol 40:16*.
251. Voss, E., J. Wehkamp, K. Wehkamp, E. F. Stange, J. M. Schroder, and J. Harder. 2006. NOD2/CARD15 mediates induction of the antimicrobial peptide human beta-defensin-2. *J Biol Chem 281:2005*.
252. Rakoff-Nahoum, S., L. Hao, and R. Medzhitov. 2006. Role of Toll-like Receptors in Spontaneous Commensal-Dependent Colitis. *Immunity 25:319*.
253. Grossmann, J., S. Mohr, E. G. Lapentina, C. Fiocchi, and A. D. Levine. 1998. Sequential and rapid activation of select caspases during apoptosis of normal intestinal epithelial cells. *Am J Physiol 274:G1117*.
254. Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol 5:190*.
255. Pedersen, G., L. Andresen, M. W. Matthiessen, J. Rask-Madsen, and J. Brynskov. 2005. Expression of Toll-like receptor 9 and response to bacterial CpG oligodeoxynucleotides in human intestinal epithelium. *Clin Exp Immunol 141:298*.
256. Abreu, M. T., E. T. Arnold, L. S. Thomas, R. Gonsky, Y. Zhou, B. Hu, and M. Arditi. 2002. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. *J Biol Chem 277:20431*.
257. Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science 291:881*.
258. Cario, E., G. Gerken, and D. K. Podolsky. 2004. Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. *Gastroenterology 127:224*.

259. Cetin, S., H. R. Ford, L. R. Sysko, C. Agarwal, J. Wang, M. D. Neal, C. Baty, G. Apodaca, and D. J. Hackam. 2004. Endotoxin inhibits intestinal epithelial restitution through activation of Rho-GTPase and increased focal adhesions. *J Biol Chem 279:24592*.
260. Liddle, R. A. 1997. Cholecystokinin cells. *Annu Rev Physiol 59:221*.
261. Yang, Q., N. A. Bermingham, M. J. Finegold, and H. Y. Zoghbi. 2001. Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science 294:2155*.
262. Naya, F. J., H. P. Huang, Y. Qiu, H. Mutoh, F. J. DeMayo, A. B. Leiter, and M. J. Tsai. 1997. Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev 11:2323*.
263. Bianchi, M. E. 2004. Significant (re)location: how to use chromatin and/or abundant proteins as messages of life and death. *Trends Cell Biol 14:287*.
264. Calogero, S., F. Grassi, A. Aguzzi, T. Voigtlander, P. Ferrier, S. Ferrari, and M. E. Bianchi. 1999. The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nat Genet 22:276*.
265. Bianchi, M. E., and A. Manfredi. 2004. Chromatin and cell death. *Biochim Biophys Acta 1677:181*.
266. Lucas, M., L. M. Stuart, J. Savill, and A. Lacy-Hulbert. 2003. Apoptotic cells and innate immune stimuli combine to regulate macrophage cytokine secretion. *J Immunol 171:2610*.
267. Savill, J., I. Dransfield, C. Gregory, and C. Haslett. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat Rev Immunol 2:965*.
268. Stuart, L. M., M. Lucas, C. Simpson, J. Lamb, J. Savill, and A. Lacy-Hulbert. 2002. Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. *J Immunol 168:1627*.
269. Harris, H. E., and A. Raucci. 2006. Alarmin(g) news about danger: workshop on innate danger signals and HMGB1. *EMBO Rep 7:774*.
270. Ulloa, L., and D. Messmer. 2006. High-mobility group box 1 (HMGB1) protein: friend and foe. *Cytokine Growth Factor Rev 17:189*.
271. Gardella, S., C. Andrei, D. Ferrera, L. V. Lotti, M. R. Torrisi, M. E. Bianchi, and A. Rubartelli. 2002. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep 3:995*.
272. Rouhiainen, A., J. Kuja-Panula, E. Wilkman, J. Pakkanen, J. Stenfors, R. K. Tuominen, M. Lepantalo, O. Carpen, J. Parkkinen, and H. Rauvala. 2004. Regulation of monocyte migration by amphoterin (HMGB1). *Blood 104:1174*.
273. Bierhaus, A., P. M. Humpert, M. Morcos, T. Wendt, T. Chavakis, B. Arnold, D. M. Stern, and P. P. Nawroth. 2005. Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med 83:876*.
274. Liliensiek, B., M. A. Weigand, A. Bierhaus, W. Nicklas, M. Kasper, S. Hofer, J. Plachky, H. J. Grone, F. C. Kurschus, A. M. Schmidt, S. D. Yan, E. Martin, E. Schleicher, D. M. Stern, G. G. Hammerling, P. P. Nawroth, and B. Arnold. 2004. Receptor for advanced glycation end products (RAGE) regulates sepsis but not the adaptive immune response. *J Clin Invest 113:1641*.
275. Sajithlal, G., H. Huttunen, H. Rauvala, and G. Munch. 2002. Receptor for advanced glycation end products plays a more important role in cellular survival than in neurite

outgrowth during retinoic acid-induced differentiation of neuroblastoma cells. *J Biol Chem 277:6888*.

276. Huttunen, H. J., J. Kuja-Panula, G. Sorci, A. L. Agneletti, R. Donato, and H. Rauvala. 2000. Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. *J Biol Chem 275:40096*.
277. Rong, L. L., S. F. Yan, T. Wendt, D. Hans, S. Pachydaki, L. G. Bucciarelli, A. Adebayo, W. Qu, Y. Lu, K. Kostov, E. Lalla, S. D. Yan, C. Gooch, M. Szabolcs, W. Trojaborg, A. P. Hays, and A. M. Schmidt. 2004. RAGE modulates peripheral nerve regeneration via recruitment of both inflammatory and axonal outgrowth pathways. *Faseb J 18:1818*.
278. Limana, F., A. Germani, A. Zacheo, J. Kajstura, A. Di Carlo, G. Borsellino, O. Leoni, R. Palumbo, L. Battistini, R. Rastaldo, S. Muller, G. Pompilio, P. Anversa, M. E. Bianchi, and M. C. Capogrossi. 2005. Exogenous high-mobility group box 1 protein induces myocardial regeneration after infarction via enhanced cardiac C-kit+ cell proliferation and differentiation. *Circ Res 97:e73*.
279. Smythies, L. E., M. Sellers, R. H. Clements, M. Mosteller-Barnum, G. Meng, W. H. Benjamin, J. M. Orenstein, and P. D. Smith. 2005. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest 115:66*.
280. Rosenstiel, P., M. Fantini, K. Brautigam, T. Kuhbacher, G. H. Waetzig, D. Seegert, and S. Schreiber. 2003. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology 124:1001*.