Noncanonical NF-κB and Gastrointestinal Disease

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ABSTRACT

Noncanonical NF-κB is an alternative NF-κB pathway that is critically involved in the development and maturation of the adaptive immune system. As such, it has typically been studied in B and T cell biology without application to complex organ systems such as the gut. The following work explores the contribution of noncanonical NF-κB to inflammatory and neoplastic disease in the gastrointestinal (GI) system, as well as the effects of its loss on GI health. Chapter 1 opens with an overview of gastrointestinal homeostasis and inflammation, with emphasis on the particular diseases studied in this body of work. Chapter 2 focuses on a review of noncanonical NF-κB function and components, as well as its applications in inflammatory bowel disease (IBD), a quintessential example of disruption of intestinal homeostasis. In Chapter 3 we determine the role of noncanonical NF-κB in allergic disease of the upper gastrointestinal tract, namely a novel model of the disease eosinophilic esophagitis. Our studies revealed that loss of NF-κB-inducing kinase (NIK), the bottleneck molecule in noncanonical NF-κB signaling, results in targeted esophageal inflammation, remodeling, and gene expression changes that are comparable to the human disease. In Chapter 4, we examine the role of noncanonical NF-κB in inflammatory bowel disease using biopsy samples from human IBD patients, and compare the expression of various components of the pathway to inflammation status and treatment response. Noncanonical NF-κB is upregulated in IBD patients, and also appears to be specifically upregulated in patients that have lost response to anti-TNF inhibitors, which are potent drugs that are widely used to treat IBD. In Chapter 5 we focus on NIK and its effects on stem cell function, growth, and inflammation-induced cancer in the gut. Loss of NIK in mice results in alterations in colonic stem cell function and changes in colonic microbiome, which predisposes them to the development of inflammation-induced carcinogenesis. Indeed, in human patients with colorectal cancer, noncanonical NF-κB is also suppressed. Overall, we have discovered multiple novel roles of noncanonical NF-κB signaling at multiple levels in the gut and in the context of a variety of diseases, and have greatly expanded the current body of knowledge as to the functions and effects of this pathway.
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GENERAL AUDIENCE ABSTRACT

The gastrointestinal system has a complex set of checks and balances to maintain overall health. If factors involved in the promotion or suppression of inflammation, the regulation of growth, or the prevention of tumor formation become dysregulated, there can be catastrophic consequences for the human body. The aim of this work is to investigate a pathway called noncanonical NF-κB in the development of various diseases in the GI tract. Noncanonical NF-κB is not a well-understood pathway and to date has mostly been studied in the context of white blood cell development. However, we discovered that noncanonical NF-κB has several very important functions in the GI tract that have implications in conditions such as inflammatory bowel disease and colorectal cancer. First we explored the role of noncanonical NF-κB in the upper GI tract, namely the esophagus, and found that this signaling pathway is critically involved in the movement of white blood cells called eosinophils to the esophagus, resulting in throat inflammation in both mouse models and human patients. Secondly, we determined that this same pathway also has effects in the lower GI tract. Human patients with inflammatory bowel disease, especially those who develop resistance to popular medications, see an upregulation of this pathway in their colon tissue. Loss of this pathway in the colons of mice also causes changes in growth of the colonic epithelium, and predisposes them to the formation of colon cancer. Interestingly, in human colon cancer patients, we also see similar changes in expression of genes associated with this pathway. Overall, we have found many new and exciting roles for this underappreciated pathway in the gut.
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Attributions

Chapter 2: Chapter 2 is a manuscript that was co-first authored with Dylan K. McDaniel and Veronica Marie Ringel-Scaia. My primary contributions were the sections on negative regulation of noncanonical NF-κB, NIK biology and regulation, noncanonical chemokines and experimental relevance, and mouse models of colonic inflammation involving noncanonical signaling, all of which are the most relevant to the focus of this dissertation. I also generated Figure 3 and provided input and general editing of remaining sections. The remaining sections and figures were written by Dylan McDaniel and Veronica Ringel-Scaia. Irving C Allen provided both funding and editing of the manuscript.

Chapter 3: I am the primary author and wrote all sections, as well as generated all experimental data represented in Figures 1, 2, 3, 4, 5, and 7. For Figure 6, I performed the IHC quantification and real-time PCR in Figure 6C and 6D. The immunohistochemistry in Figure 6A and 6B was performed by the paper’s second author, Daniel Rothschild. Dylan McDaniel and Bettina Heid contributed to the maintenance of the Nik⁻/⁻ mouse line. Irving C Allen provided funding, experimental design advice, and editing for these projects and manuscript.

Chapter 4: I am the primary author and writer, categorized patients, processed tissues for downstream assays, and performed all superarray analyses, with contributions by Irving C. Allen, who also performed the IPA analysis. Vu Nguyen and Dario Sorrentino were responsible for the collection of patient data and biopsies. Rebecca Brock and Siena Sorrentino assisted with extraction of RNA from patient tissues. Marissa Lang and Kristin Knight were responsible for patient database management and transport of samples. Douglas Grider was the attending pathologist on patient cases. Irving C Allen provided funding, experimental design advice, and editing for these projects and manuscript.

Chapter 5: I am the primary author and wrote all sections, as well as generating all experimental data represented in Figures 1, 3, 6, 7, and 8. For Figure 2, I extracted all fecal DNA and microbiome analysis was performed by Yufeng Qin and Paul Wade. In Figure 4, I generated the cytology represented in panel 4C, while Daniel Rothschild generated organoid data and panels 4A and 4B. For Figure 5, Daniel Rothschild assisted with cell preparation and performed real-time PCR for data in panel 5C and 5E, while I performed and quantified all immunocytochemistry and performed the Western blot represented in 5A, B, D, and F. Megan Sammons assisted in colonic crypt harvest. Eda Holl provided RNA from human colorectal cancer biopsy patients. Dario Sorrentino provided control colonic tissue. Irving C Allen provided funding, experimental design advice, and editing for these projects and manuscript.
Chapter 1

Introduction

The gastrointestinal (GI) system is truly a microcosm inside the human body, with an immense number of complex mechanisms that maintain epithelial barrier integrity, mucosal immunity, cell division and growth, and the various populations of microorganisms that inhabit it. This complexity makes the study of disturbances in inflammation and growth in the GI tract particularly challenging due to so many intersecting signaling pathways. Of course, depending on both the instigating factors and the surrounding environment, the appearance and pathogenesis of gastrointestinal disease can take many forms. The molecular pathway involved in GI homeostasis that is discussed in this dissertation is an understudied and, until recently, underappreciated cascade known as noncanonical NF-κB. In general, NF-κB has long been studied as an immensely powerful pathway in a large variety of cellular processes including inflammation, cell growth and division, and neoplastic change[1]. The vast majority of focus over the last several decades has been on the more well-known side of the pathway, called canonical NF-κB [1, 2]. Canonical NF-κB is defined by the liberation of protein subunits RelA (also called p65) and p50 in the cell cytoplasm from their respective inhibitory proteins, called IκBα proteins[3, 4]. These subunits then travel into the cell nucleus to initiate transcription of a large number of genes associated with inflammation and growth, such as interleukin-6 (IL-6) and tumor necrosis factor (TNF). Since canonical NF-κB controls such a large number of inflammatory mediators, it has been the focus of intense study in gastrointestinal inflammation and cancer. Chapter 2 of this manuscript provides a comprehensive overview of the second side of this signaling pathway, noncanonical NF-κB signaling, along with the many potential biological connections of inflammation in the gut.
Noncanonical NF-κB is a different arm of NF-κB signaling that is unique from its canonical counterpart, and has received much less attention in research and development. While canonical NF-κB is quickly activated by a large number of stimulatory factors and promotes the transcription of a large variety of genes, noncanonical NF-κB is slowly, persistently activated and is defined by a much more targeted array of initiation factors as well as chemokine products [5-7]. The lynchpin of noncanonical NF-κB signaling is a molecule called NIK, or NF-κB inducing kinase. NIK is a tightly regulated protein kinase that functions to phosphorylate IKKα, which then catalyzes the processing of the protein p100 to its functional product p52[6]. This is unique from canonical signaling, where only simple liberation of p65 is needed to function. p52 then moves to the nucleus to initiate transcription of four major chemokines (CXCL12, CXCL13, CCL19, and CCL21) involved in development of lymphoid structures such as lymph nodes and spleen due to their critical nature in B and T cell development[6]. Due to this essential role in lymphoid development, it was unsurprising that mice which harbor various loss-of-function mutations in NIK develop immune abnormalities such as poorly organized splenic architecture, undersized or absent lymph nodes and Peyer’s patches, and poor adaptive immune responses [8-11]. A particularly interesting phenotype in Nik⁻/⁻ mice is the development of a hypereosinophilic disease that appears to target the skin with accumulations of eosinophils and major filtering organs such as the spleen[12]. It appears that this accumulation is due to a prominent Th2 bias of Nik⁻/⁻ T cells[12]. Prior to our work here, characterization of the gut of these noncanonical NF-κB deficient animals as well as the systematic assessment of the effect of noncanonical signaling in all levels of the GI tract had not been attempted. Given these important immunological effects, we hypothesize in this body of work that dysregulation of noncanonical
NF-κB can disrupt mucosal homeostasis and result in alterations in susceptibility to gastrointestinal disease.

Our investigations into noncanonical signaling in the gut begin in Chapter 3, where we investigate the effects of this hypereosinophilia caused by lack of NIK in mice on the gastrointestinal tract. Interestingly, we found that eosinophilic inflammation in the GI tract of \( \text{Nik}^{-/-} \) mice is limited to the esophagus, with very few eosinophils located in any other section of the tract. Other morphologic changes such as esophageal fibrosis and hyperplasia followed, in addition to a pattern of inflammation that was remarkably similar to the human disease eosinophilic esophagitis (EoE), a localized mucosal hypersensitivity in the esophagus[13-15]. Typically patients exhibit a dietary sensitivity to one or more foodstuffs such as wheat, soy, or dairy, and the esophagus undergoes chronic inflammatory and fibrotic changes that can result in difficult swallowing, pain, and stricture formation. This disease is most often diagnosed in childhood and requires lifelong management[16]. The molecular mechanisms involves a T helper cell-2 (Th2) bias in the tissue, sometimes augmented by a gain of function mutation in thymic stromal lymphopoietin (TSLP), that results in eosinophils targeting the esophagus[13, 17]. In addition to the many morphologic similarities, gene expression analyses of esophageal tissue from \( \text{Nik}^{-/+} \) mice mimicked the expression patterns of human patients with EoE, including upregulation of TSLP and many Th2 mediators. Using metadata analysis, we found that EoE patients also showed disruption of normal noncanonical signaling that indicated a potential failure of proper NIK signaling. Overall, this work was the first to characterize the effects of NIK and noncanonical signaling in the upper GI tract and discovered a novel spontaneous animal model for this disease.
One of the most common and well-characterized GI diseases is inflammatory bowel disease (IBD), a chronic and progressive condition that leads to inflammation of the gut, dysbiosis, and even predisposition towards cancer[18, 19]. Inflammatory bowel disease is comprised of two main subtypes deemed Crohn’s disease (CD)[20] and ulcerative colitis (UC)[21]. Crohn’s disease is typically present along the entire length of the GI tract and is marked by large influxes of inflammatory cells, mucosal thickening, and scarring. Ulcerative colitis, as the name implies, is characterized by massive ulceration of the colonic epithelium, along with inflammatory infiltrate, and is typically confined to the colon. Therapy for inflammatory bowel disease often utilizes biologic medications such as monoclonal antibodies directed against the proinflammatory cytokine tumor TNF, which shut down TNF-mediated inflammatory cascades[22, 23]. These drugs have drastically improved modern inflammatory bowel disease therapy and have helped innumerable patients manage their disease. For example, during the ACCENT clinical trial for infliximab (also known as Remicade®, one of the most well-known of these anti-TNF therapies) 69% and 46% of patients treated with 10 and 5 mg/kg respectively achieved remission, compared to the placebo group of 23%[24]. However, a significant percentage of patients become refractory to treatment with these medications, and both diagnosis of and management of nonresponse is a continual challenge for clinicians[25-27].

Chapter 4 of this body of work discusses the role we have discovered for noncanonical NF-κB in inflammatory bowel disease, and potential development of unique biomarkers for treatment nonresponse. We obtained intestinal biopsies from inflammatory bowel disease patients through a collaboration with the Carilion Clinic in Roanoke VA, and stratified patients based on disease subtype, treatment, and treatment response. We analyzed gene expression patterns in all of these patients subsets using a custom-designed PCR superarray containing over
80 genes involved in noncanonical NF-kB signaling and related pathways. We found that noncanonical signaling pathways were upregulated in biopsies from IBD patients as compared to controls, and that treatment also affected expression values. Specifically, it appears that noncanonical signaling was especially elevated in patients who had lost response to anti-TNF inhibitors, which are common and important medications in IBD management. The noncanonical chemokine CXCL13 in particular appears to be a potential biomarker for nonresponse in these patients. Analysis of these data using bioinformatics software indicated several other important pathways that noncanonical signaling might be affecting in the gut, such as lymphocyte trafficking and Nod-like receptor activity.

Interestingly, individuals with inflammatory bowel disease are more prone to the development of a serious neoplastic condition: colorectal adenocarcinoma (CRC)[19, 28, 29]. CRC is a slow-growing but very serious neoplastic condition that can spread not only to regional lymph nodes but to other solid organs if left unchecked. Neoplastic change in the colon is not only encouraged by the chronic inflammation associated with IBD, but also by alterations in cell growth and metabolism[30, 31]. One of the most important cell types in cancer as a disease, including CRC, is the stem cell[32, 33]. While typically we think of cancer being caused by an overexpression of “stem-like” factors in resident tissue which then give rise to unchecked and damaging growth patterns, this is not the only way cancer can form. In circumstances where normal cell growth and turnover is suppressed, mature cells may also revert to acquire stem-like properties and also give rise to neoplasia despite their good intentions of replenishing the healing and regenerative niche[34, 35]. In Chapter 5, we explore effects of stem cell suppression and determine its relevance in the development of cancer in the lower GI tract in both mouse models and human patients.
Given the connection between chronic inflammation and the development of cancer in the lower gastrointestinal tract, we also investigated the role of noncanonical signaling in colorectal cancer as described in Chapter 5. Interestingly, in patients with colorectal cancer, we found that noncanonical signaling is suppressed. In mouse models, loss of NIK resulted in disruptions in the stem cell compartment of the colon and altered growth patterns as well as changes in colitic microbiome species. We developed a conditional knockout \( \text{Nik}^{-/-} \) mouse that lacked NIK signaling only in the colonic epithelium in order to further dissect NIK’s role in colonic growth homeostasis. We found that this cell-specific loss of NIK is correlated in mouse models with increased development of inflammation-induced colorectal cancer. These data suggest an extremely novel role for NIK and noncanonical signaling, and may further enhance our understanding of the stem cell niche in colon cancer.

Collectively, all of these projects produce a much deeper understanding of noncanonical signaling in the context of the GI tract. Indeed, our research goes beyond lymphoid development and intersects with multiple disease processes including mucosal inflammation, allergy, and cancer development. At the center of these studies is the critical noncanonical NF-\( \kappa \)B kinase NIK, which is proving to be a protein with far more functionality than originally thought. We hope to increase awareness of the importance and versatility of NIK and the noncanonical pathway it controls, as well as present new biomarkers, signaling cascades, therapeutic targets, and mechanisms for a variety of gastrointestinal diseases.
References:


Chapter 2

Literature Review: Emerging Roles for Noncanonical NF-κB Signaling in the Modulation of Inflammatory Bowel Disease Pathobiology

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Abstract:

Crohn’s disease and ulcerative colitis are common and debilitating manifestations of inflammatory bowel disease (IBD). IBD is characterized by a radical imbalance in the activation of pro-inflammatory and anti-inflammatory signaling pathways in the gut. These pathways are controlled by NF-κB, which is a master regulator of gene transcription. In IBD patients, NF-κB signaling is often dysregulated resulting in overzealous inflammation. NF-κB activation occurs through two distinct pathways, defined as either canonical or noncanonical. Canonical NF-κB pathway activation is well studied in IBD and is associated with the rapid, acute production of diverse pro-inflammatory mediators, such as COX-2, IL-1β, and IL-6. In contrast to the
canonical pathway, the noncanonical or “alternative” NF-κB signaling cascade is tightly regulated and is responsible for the production of highly specific chemokines that tend to be associated with less acute, chronic inflammation. There is a relative paucity of literature regarding all aspects of noncanonical NF-κB signaling. However, it is clear that this alternative signaling pathway plays a considerable role in maintaining immune system homeostasis and likely contributes significantly to the chronic inflammation underlying IBD. Noncanonical NF-κB signaling may represent a promising new direction in the search for therapeutic targets and biomarkers associated with IBD. However, significant mechanistic insight is still required to translate the current basic science findings into effective therapeutic strategies.
Introduction:

Ulcerative Colitis (UC) and Crohn’s Disease (CD) are jointly defined as Inflammatory Bowel Disease (IBD) and are both chronic and debilitating disorders. The specific cause of IBD remains unknown. However, it is clear that both UC and CD are associated with complex genetic, immunological, and environmental interactions. Overzealous inflammation is the most prominent feature associated with IBD pathobiology. As such, immunosuppressive and anti-inflammatory drugs remain the first line of treatment for patients. Recently, the use of biologics targeting inflammatory signaling pathways have proven to be a highly successful therapeutic strategy. Indeed, a significant amount of basic and translational IBD research over the last decade has been focused on identifying and characterizing specific immune signaling pathways that may serve as therapeutic targets.

The transcription factor nuclear factor kappa B (NF-κB) is a central regulator of inflammation and modulates a diverse spectrum of biological processes. The NF-κB signaling cascade has been well studied in IBD pathogenesis and is often dysregulated in patients, resulting in aberrant cytokine and chemokine production in the gut. NF-κB activation occurs through two distinct pathways, defined as the canonical pathway and the noncanonical or “alternative” pathway. The vast majority of studies have thus far focused on the canonical NF-κB signaling cascade. Signal processing in the canonical pathway is rapid and constitutive. In the canonical pathway, (RelA/p65)/p50 heterodimers are maintained in the cytoplasm in an inactive state by a family of inhibitors of NF-κB (IκBα). Activation is mediated through a large IκB kinase complex consisting of the regulatory subunit IκB kinase γ (IKKγ; NF-κB essential modulator; NEMO), and catalytic subunits IκB kinase α (IKKα) and IκB kinase β (IKKβ). Upon upstream kinase activity, the IKK complex phosphorylates IκBα leading to its degradation and
the subsequent release of the RelA/p50 heterodimer. Newly liberated RelA/p50 heterodimers rapidly translocate to the nucleus to activate the transcription of a diverse range of inflammatory mediators, such as COX2, TNF, IL-1β, and IL-6 (Figure 1).

In contrast to the canonical pathway, there is a relative paucity of data pertaining to noncanonical NF-κB signaling during IBD. Similar to the canonical cascade, the noncanonical NF-κB pathway involves an NF-κB heterodimer which comprises p52 and RelB, instead of p50 and RelA (1). However, before p52 is activated via the degradation of its C-terminal ankyrin repeat-containing domain, it is maintained in its precursor form, known as p100 where the DNA binding domain is not easily accessible (1). The p100 molecule acts as an IκB-like molecule, similar to p105 in the canonical pathway, and holds RelB in the cytoplasm (2). Upon receptor stimulation, p100 is targeted for proteasome degradation and subsequently processed to p52 (1, 3). This processing of p100 and degradation of the ankyrin repeat-containing domain unmasks the nuclear localization sequence (NLS), which facilitates the translocation of p52 to the nucleus (1).

Processing of p100 is tightly regulated by NF-κB-inducing kinase (NIK) and negatively regulated by a processing-inhibitory domain (PID) within p100. Under normal unstimulated conditions, NIK is constitutively degraded via the binding of a complex comprised of TNF-receptor associated factor 3 (TRAF3), TRAF2 and cellular inhibitor of apoptosis 1 and 2 (cIAP1/2), which together prevent basal activation (1, 2, 4). Upon recognition of a TNF family signaling molecule, such as CD40L, lymphotoxin β, or BAFFR, TRAF3 is degraded and NIK is stabilized. NIK is then available to phosphorylate IKKα (3, 5). The activation of IKKα leads to the phosphorylation of p100, causing its ubiquitination and targeting by proteasomes that cleave it into the active form of p52. The N-terminal portion of the p100 molecule is considered to be
the active form, which translocates to the nucleus along with RelB. Meanwhile, the inhibitory C-terminal portion of p100 is degraded (2). Once in the nucleus, NF-κB initiates the transcription of a diverse, but limited repertoire of target genes, such as the chemokines CXCL12 and CXCL13 (3, 6) (Figure 2).

One of the major biological functions of the noncanonical NF-κB pathway is the development and organization of secondary lymphoid structures, such as Peyer’s patches in the gut (3). Mice lacking the lymphotoxin β receptor (LTβR) and mice with a mutation in NIK (Aly), which completely blocks noncanonical NF-κB signaling, lack lymph nodes and Peyer’s patches (7, 8). Peyer’s patches are critical for the maintenance of immune system homeostasis in the gastrointestinal tract. For example, dendritic cells isolated from Peyer’s patches are significantly more efficient in T cell activation compared with dendritic cells from other tissues. Indeed, murine dendritic cells isolated from Peyer’s patches have been shown to prime OVA TCR transgenic T cells to secrete fivefold higher levels of the anti-inflammatory cytokines IL-4 and IL-10 compared to dendritic cells from the spleen (9, 10). In addition to dendritic cells, gut macrophages from Peyer’s patches are also important for the regulation of the immune response to commensal components of the microflora. This was best illustrated using macrophages isolated from the jejunum and Peyer’s patches of normal patients undergoing tissue resection. These macrophages were challenged with LPS, which revealed that gut macrophages had much lower levels of the pro-inflammatory cytokines IL-1, IL-6, and TNF compared to monocytes (10, 11).

Beyond the generation of these secondary lymphoid structures in the gut, the noncanonical NF-κB signaling pathway is also associated with a myriad of inflammatory disorders. For example, it has been shown that loss of noncanonical NF-κB signaling through the
disruption of NIK can lead to the development of a systemic inflammatory condition in mice known as hypereosinophilic syndrome-like disease (12). Hypereosinophilic syndrome (HES) is a family of inflammatory diseases characterized by an increase in eosinophils without a known cause. Hacker et al. (2012) showed that Nik−/− mice develop a progressive HES-like disorder characterized by eosinophilia, tissue destruction and premature death. Interestingly, they found that this disease progresses independent of IKKα phosphorylation because mice containing a point mutation in IKKα (IKKαAA/AA) did not show the classical signs of HES characteristic of the NIK deficient mice (12).

The noncanonical NF-κB signaling cascade is relatively understudied in the context of IBD. However, as new data emerges related to this alternative signaling cascade, the importance of this pathway in maintaining immune system homeostasis in the gut is becoming more evident. In addition to controlling the development of secondary lymphoid structures in mucosal tissues, recent studies have also found that noncanonical NF-κB signaling regulates T-cell differentiation and function (13, 14), IgA class switching (15, 16), cell migration (17), chemokine production (18), and interferon signaling (19) through mechanisms that are distinct from canonical NF-κB signaling. In essence, this signaling cascade is likely to influence IBD pathobiology through multiple mechanisms. This review focuses on our current knowledge of emerging concepts associated with the activation, regulation, and clinical relevance of noncanonical NF-κB signaling in maintaining immune system homeostasis in the gut. In addition to synthesizing recent findings related to the noncanonical NF-κB pathway and IBD, we also discuss potential therapeutic strategies and targets associated with this understudied signaling cascade.

**Starting the engine: Noncanonical NF-κB stimulatory ligands and receptors**
The first step of the noncanonical NF-κB pathway involves the recognition of a TNF family signaling molecule, such as TNF, CD40L, lymphotoxin β, or BAFFR. TNF is a potent pro-inflammatory cytokine produced by many leukocytes, including macrophages, lymphoid cells and mast cells. TNF is a type II transmembrane protein that signals through the TNF receptor (TNFR) family membrane receptors. Two receptors are known to bind TNF, TNFR1 and TNFR2. TNFR1 is constitutively expressed; whereas TNFR2 is highly regulated. TNF signaling is associated with diverse biological effects, including inflammation, cell differentiation and cell death. TNF signaling is typically associated with canonical NF-κB activation. However, TNF also activates the noncanonical pathway via TNFR1 stimulation, which leads to TRAF2 degradation, NIK stabilization, IKKα phosphorylation and p100 processing (20). There is clearly functional interplay between the canonical and noncanonical NF-κB signaling pathways following TNFR1 stimulation. This appears to be, at least in part, mediated by the receptor-interacting protein 1 (RIP1), which promotes TNF-mediated activation of the canonical pathway while functioning as a negative regulator of the noncanonical pathway (20). Mouse embryonic fibroblasts from Rip1−/− mice are highly sensitive to TNF induced cell death at early timepoints due to deficient canonical NF-κB signaling; whereas late activation of the noncanonical NF-κB cascade in these Rip1−/− cells appears to protect against cell death (20). Thus, it appears that RIP1 may function as a molecular switch between TNF induced canonical and noncanonical NF-κB signaling. It is clear that TNF and its receptors play a role in IBD pathogenesis. In both human patients and in IBD animal studies, increased colonic expression of TNF and both TNF receptors are routinely observed and heavily involved in IBD pathogenesis. In fact, compared to healthy controls, TNF levels are much higher in the stool and serum of patients with both UC and CD (21-23).
Traditionally, corticosteroids and immunomodulators were the preferred treatment strategies for patients with IBD (24). However, recently the use of anti-TNF monoclonal antibodies (mAbs) have shown significant promise (22, 24, 25). Infliximab was one of the first anti-TNF treatment to be used for IBD and was found to be very effective at inducing mucosal healing in patients with UC (22, 24-26). One of these early clinical trials revealed that 69% of patients who received 5 mg of infliximab and 61% who received 10mg had a clinical response after 8 weeks of treatment. In contrast, only 37% of those who received placebo had a clinical response (26). Mucosal healing was significantly improved in patients who were given infliximab at 8, 30 and 54 weeks (26). In addition to Infliximab, adalimumab and certolizumab are also highly effective anti-TNF mAbs currently in use to treat IBD. The effects of these anti-TNF therapeutics on noncanonical NF-κB signaling has not been directly explored. However, the reduction in TNF signaling mediated by these therapeutics would almost certainly impact both canonical and noncanonical NF-κB signaling.

It is clear that TNF is a major contributing factor to IBD pathogenesis. However, this inflammatory mediator is only one piece of the puzzle that links the noncanonical NF-κB pathway with IBD. For example, in addition to TNF, CD40 is also a potent initiator of noncanonical NF-κB signaling and can dramatically impact IBD pathogenesis through multiple mechanisms. CD40 is a type I transmembrane protein and a member of the TNF-receptor superfamily that is primarily found on B cells and antigen-presenting immune cells, such as dendritic cells and macrophages. CD40 is activated through interactions with CD40 ligand (CD40L), which is a type II transmembrane protein expressed primarily by activated CD4+ T cells and platelets. The interaction between CD40 on B cells and CD40L on T cells is critical for the proliferation and differentiation of B cells and plays an important role in the regulation of
inflammation. Meanwhile, ligation of CD40 on macrophages and dendritic cells increases the production of cytokines and acts as a survival signal (27). In the context of IBD, intestinal lamina propria T cell (LP-T cell) expression of CD40L plays an important role in pathogenesis (27). LP-T cells from inflamed mucosa do indeed express functional CD40L, which has been shown to induce production of IL-12 and TNF in monocytes, leading to increased inflammation in the gut (27). This LP-T cell mediated increase in monocyte cytokine expression can be attenuated using mAbs that block the CD40-CD40L interaction (27). For example, blockade of CD40 and CD40L in LP-T cell and monocyte co-cultures with the mAbs M90 and 5D12, respectively, resulted in a decrease in expression of IL-12 and TNF (27). The treatment with M90 led to a 39-100% inhibition of IL-12 and 34-100% inhibition of TNF. While the inclusion of 5D12 was associated with a 26-100% inhibition of IL-12 and a 33-100% inhibition of TNF production (27). This indicates a potential therapeutic strategy for IBD involving attenuation of the CD40-CD40L interactions. Indeed, it is clear that CD40 signaling underlies many diverse biological processes that are associated with IBD. Due to the potency of CD40 activation on noncanonical NF-κB signaling, it is highly likely that dysregulation of the noncanonical pathway underlies many of the pathobiological effects that have been attributed to CD40L-CD40 during disease.

The contribution of this CD40L/CD40/non-canonical NF-κB axis in maintaining gastrointestinal health, immune system homeostasis in the gut, and as a mechanism associated with IBD has not been adequately explored. This is particularly true for cell populations beyond the immune system. While the best characterized functions for CD40/CD40L are associated with their roles in leukocyte function, expression of these proteins are not limited to immune cells. For example, there are many non-immune cell types, such as epithelial cells, that also express CD40 (28). One indication of this is seen in the increased expression of CD40 and CD40L in
endoscopically obtained biopsies from patients with IBD (27, 29). In these studies, intestinal epithelial cells (IECs) of the colon lacked fluorescent staining of CD40 in healthy control patients. However, CD40 expression was significantly upregulated in IECs from patients with active IBD (29). In addition to IEC expression of CD40, platelets express CD40L and play a role in the inflammatory response and tissue injury in the gut (30). This is indicated by the fact that unstimulated platelets isolated from patients with active IBD have significantly increased levels of CD40L compared to platelets from normal healthy controls (30). In fact, unstimulated platelets from healthy subjects showed low expression of CD40L (30). Following thrombin stimulation, CD40L expression increased in both groups; however, a significant increase was observed between UC and CD groups compared to platelets isolated from healthy controls (30).

It has been suggested that this interaction between platelets and IBD is most likely via the activation of the mucosal microvasculature. Indeed, changes in the vasculature often accompany inflammation in response to various factors including vascular endothelial growth factor (VEGF). Angiogenesis and increased permeability are among the most common changes that occur during an inflammatory response. Typically, IBD pathogenesis is accompanied by these vasculature changes, mostly in the form of increased expression of cellular adhesion molecules (CAMs) and chemokine secretion via endothelial cells (31). This inflammatory-driven angiogenesis has been shown to occur in both CD and UC (29). As evidence of this, mucosal extracts and plasma of patients with active IBD have been reported to have higher levels of VEGF-A, as indicated by enzyme-linked immunosorbent assay (ELISA) (31). It is postulated that the CD40-CD40L interaction is crucial in mediating vascular changes in the inflamed mucosa. These changes are also accompanied by an increase in cellular adhesion molecules to allow leukocyte-endothelial cell interactions. Human intestinal microvascular endothelial cells
(HIMEC) co-cultured with CD40L-positive platelets from normal donors increases the expression of both intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (30). These adhesion molecules are expressed on endothelial and immune cells and are responsible for binding integrins, which allow the translocation of immune cells from the circulation to sites of inflammation. This increase in CAM expression is even stronger in co-cultures that contain platelets from IBD patients, indicating an important role for platelet-activated endothelium in IBD. Interestingly, blockade of CD40L with a neutralizing antibody resulted in a decrease in the upregulation of these adhesion molecules (30).

In addition to TNF and CD40, Lymphotoxin β-receptor (LTβR) is another TNF superfamily receptor involved in the activation of noncanonical NF-κB signaling (3). As mentioned earlier, one of the functions of LTβR is the development of Peyer’s patches in the gut. This was shown in prior studies utilizing mice deficient in LTβR (7). These mice also show a failure of normal B and T cell segregation, indicating a role of LTβR in the development of peripheral lymphoid tissue (7, 32). The role of LTβR in IBD has been previously characterized in the *Citrobacter rodentium* infection model of IBD mucosal inflammation. *C. rodentium* infection results in epithelial hyperplasia and mild to moderate inflammation of the colonic mucosa that can be used as a murine model for some aspects of IBD (33). *C. rodentium* infection causes RORγt+ cells to synthesize lymphotoxin (LT) which binds to LTβR on epithelial cells, resulting in the release of chemokines, such as CXCL-1 and CXCL-2, that recruit pro-inflammatory neutrophils and macrophages (34, 35). When mice conditionally deficient in LTβR in intestinal epithelial cells are infected orally with *C. rodentium* they display a deficiency in clearing the infection and increased pathology (34). This indicates a role for LTβR in the defense against
mucosal pathogens in the gut. These data could be extended to IBD, suggesting that LTβR plays a protective role in the human condition.

CD40, LTβR, and TNFR are the most commonly studied initiators of noncanonical NF-κB signaling and most likely to directly influence IBD pathogenesis. However, another TNF superfamily receptor-ligand interaction is worth noting and has also been shown to be correlated with IBD, albeit through a more indirect mechanism. Receptor Activator of Nuclear factor κB (RANK) and its ligand RANKL also stimulate noncanonical NF-κB signaling (36). The stimulation of the noncanonical pathway via this route is most notable for promoting osteoclastogenesis and is associated with bone loss in humans particularly with relation to chronic gut inflammation (37, 38). Consistent with these prior findings, aly/aly mice that lack NIK and noncanonical signaling, show a baseline osteopetrosis, suggesting a defect in osteoclasts and bone remodeling (39). This defect was traced to the bone marrow in the mice, with RANK-L mediated generation of osteoclasts being impaired. Transfection of p100 or p52 coupled with RANKL stimulation abolished this impairment. Overexpression of RelB also abolished these changes in another aly/aly model (40). The RANKL-controlled osteoprotegerin (OPG) system has been shown to be dysregulated in UC and CD patients, and is correlated with increased bone loss in patient sub-groups (41). Indeed, bone loss is not uncommon in patients with chronic IBD, along with a variety of concurrent risk factors being identified including corticosteroid use, smoking, and vitamin D deficiency. Interestingly, treatment with infliximab is associated with improvements in bone metabolism (42). The exact mechanism linking RANKL/RANK/OPG, mucosal immunology, and bone remodeling has been investigated in several mouse models. Using IL-2 deficient mice, which develop a spontaneous autoimmune chronic colitis, bone loss was shown to be mediated via upregulation of RANKL, which resulted
in promotion of intestinal dendritic cell survival and immune system activation (43). Recently, RANKL-RANK has also been shown to be critical in the development of regulatory T cells in models of chronic colitis (44). Immunodeficient mice were administered adoptively transferred CD4+ effector T cells (CD4(+)) in order to induce T cell driven colitis, as well as, doses of CD4+CD25+CD45RBlo T regulatory cells, which function to suppress the effector-mediated colitis. The administration of anti-RANK mAbs negated the ability of T regulatory cells to suppress the effector cell driven colitis. Further underlining RANKL/RANKs effects in dendritic cells of the gut as suggested in IL-2 deficient mice (44). As with the other activating signals, there is a high likelihood that dysregulated noncanonical NF-κB signaling underlies many aspects of osteoclastogenesis associated with RANKL/RANK and may also be associated with increased bone loss in IBD patient sub-groups.

Finally, B cell Activating Factor (BAFF) and its partner BAFFR, are critical for noncanonical NF-κB signaling and are associated with B cell development and maturation (45). Although classically considered a stimulatory ligand for the canonical NF-κB pathway when it interacts with its normal partner BAFFR, BAFF can have additional regulatory effects on noncanonical signaling when it interacts with its other partner, the transmembrane activator and CAML interactor (TACI) (46). TACI signaling upon BAFF binding results in suppression of noncanonical signaling and upregulation of cIAP1, apparently by the formation of a multicomponent interaction with TANK, cIAP1, and TRAF2, which inhibits NIK (46). Studies focusing on BAFF and BAFFR specifically in the context of IBD are scarce, despite the involvement of B cells in IBD. However, suppression of BAFFR and TACI at the mRNA level, with concurrent hypo-methylation of their respective genes TNFRSF13B and TNFRSF13C, has been seen in the duodenal mucosa of dogs with IBD using methylation specific PCR (47). While
BAFFR expression has been reported to be suppressed in canine IBD patients, BAFF itself has been observed to be upregulated at the mRNA level and is correlated with decreased in mucosal IgA and TGF-β (48). It would be highly interesting if the findings from these veterinary patients can be extended to human IBD.

Overall, the major ligands and receptors of noncanonical NF-κB signaling have a variety of effects ranging from adaptive immune cell development to skeletal remodeling; however, they all share a common link back to mucosal immunology, with several having been already implicated in IBD pathogenesis. The interaction between ligand and receptor is only the beginning of the signaling cascade. Noncanonical signaling also has an eclectic repertoire of positive and negative regulators that serve to fine-tune the pathway as needed, and are essential given the tightly-controlled nature of this alternative pathway. Thus, the failure of one or more molecules downstream may lead to significant loss of immune system homeostasis in the gut.

**Working like a well-oiled machine: Positive regulation of noncanonical NF-κB signaling**

Noncanonical signaling is maintained by a variety of positive regulators that can affect various points of the pathway. These positive regulators typically target receptor-ligand interactions at the cell membrane, remove negative regulatory complexes, stabilize pathway components, and directly activate key signaling molecules. For example, a member of the tumor necrosis factor family, tumor necrosis factor-like weak inducer of apoptosis (TWEAK), activates NF-κB via interactions with the inducible cell-surface receptor Fn14 (Figure 3). TWEAK/Fn14 signaling can upregulate both canonical and noncanonical NF-κB signaling. However, under physiological conditions TWEAK/Fn14 signaling appears to primarily target the noncanonical pathway (49, 50). Specifically, TWEAK triggers p100 processing via Fn14 and has been associated with the production of noncanonical cytokines, such as CCL21 (50, 51). TWEAK signaling acts as a
balance to cIAP1/2, inducing the degradation of the TRAF2, TRAF3, and cIAP1/2 complex to re-sensitize the cells to TNF (52) (Figure 3). Indeed, a TWEAK signaling complex consisting of FN14, TRAF2, and cIAP1 has been suggested (52).

TWEAK/Fn14 has been implicated in IBD (53, 54). In human UC patients, TWEAK levels are upregulated at the mRNA level in the intestinal mucosa along with Fn14 and IL-13 (55). Mechanistically, TWEAK appears to modulate damage to the intestinal epithelium associated with the production of IL-13 (55). In the gut, increased IL-13 has been shown to be associated with the loss of β-catenin in mouse primary intestinal explants and shown to disturb tight junction formation between epithelial cells (55). However, these responses were not seen in FN14 KO mice, suggesting that a loss of TWEAK/Fn14 signaling may actually be protective during IBD (55). Further support of this hypothesis was shown when TWEAK signaling was ablated, either through gene KO or mAbs. In both cases, TWEAK attenuation resulted in decreased progression of experimental colitis in a TNBS mouse model (56).

Similar to TWEAK, A20 represents another well studied regulatory protein in canonical and noncanonical NF-κB signaling. A20 is a ubiquitin-editing enzyme and can function to actually suppress signaling in the canonical NF-κB pathway, often along with a second subset of molecules termed A20 binding inhibitors of NF-κB or ABINS (57, 58). Although this mechanism isn’t fully understood, it is thought that A20 exerts its inhibitory effects via the disruption of TNFRI (59). However, in the non-canonical NF-κB signaling cascade, A20 is associated with the activation of signaling. Under normal unstimulated conditions, the cIAP1/cIAP2/TRAF2/TRAF3 complex functions to continuously poly-ubiquitinate NIK in order to induce its degradation and repress the noncanonical pathway (Figure 2). However, A20 activation is induced by TNF family members and results in the suppression of the complex (60).
Upon activation, A20 binding results in the interruption and disassociation of the cIAP1/cIAP2/TRAF2/TRAF3 complex and subsequent stabilization of NIK, which facilitates the activation of noncanonical signaling (60). Mice deficient in A20 develop severe hyper-inflammation and are extremely sensitive to both LPS and TNF (61). Mice that lack A20 in their enterocytes display increased susceptibility to experimental colitis and their enterocytes are particularly sensitive to TNF-driven apoptotic signals (62). In humans, the A20 locus contains several mutations that are associated with a diverse range of autoimmune and inflammatory conditions, including IBD (63, 64).

As with TWEAK and A20, B-cell CLL/lymphoma 10 (BCL10) is another molecule that significantly regulates noncanonical NF-κB signaling. BCL10 promotes NIK phosphorylation, rather than controlling the level of NIK, resulting in downstream p100/p52 processing (65). Mutations in BCL10, particularly those affecting the critical phosphorylation site at Ser(138), can directly affect downstream noncanonical NF-κB signaling (66, 67) (Figure 3). Studies in human intestinal epithelial cells have repeatedly shown BCL10’s necessity for inflammatory cytokine production and both canonical and noncanonical NF-κB activation. These effects are also seen in in vivo models using Bcl10−/− mice. When exposed to carrageenan, an inflammatory food additive that stimulates both the canonical and noncanonical NF-κB pathways, Bcl10−/− mice display decreased noncanonical activity, including decreased nuclear RelB and NIK levels (68). Additionally, the paracaspase MALT1, which acts as a partner to BCL10, also contributes to this noncanonical regulation. Lack of MALT1 in B cells results in decreased p100 phosphorylation and processing that is normally induced by BAFF, resulting in decreased survival of marginal zone B cells (69). Interestingly, in this same study, MALT activity was shown to be largely dispensable for canonical NF-κB activation.
Several noncanonical NF-κB regulatory proteins have roles in maintaining gastrointestinal homeostasis and cell growth, but have not been directly evaluated in the context of IBD. One of these is X-linked ectodermal dysplasia receptor (XEDAR, also known as EDAR2 or TNFRSF27), a lesser-known TNFR family member that has recently been shown to be an activator of the noncanonical NF-κB pathway (70). XEDAR requires the kinase activity of NIK and IKKα to bind TRAF2 and TRAF6 (70). This results in the processing of p100 and is negatively regulated by molecules such as cIAP1, A20, and TRAF3 (Figure 3). Although XEDAR has not been investigated in IBD, it has been shown to be greatly upregulated in the gastrointestinal mucosa of nonhuman primates and mice after radiation exposure (71, 72). This may have functional implications in IBD, where mucosal epithelial cell turnover and barrier integrity are critical events in the inflammatory process. In addition it serves as a link between constant renewal, proliferation and dysplastic potential. Another regulator of non-canonical NF-κB signaling, but not directly evaluated in the context of IBD, is Zinc finger protein 91 (ZFP91) (73). ZFP91 is an E3 ubiquitin ligase that has the ability to bind directly to NIK, promoting its stabilization and downstream noncanonical signaling (Figure 3). ZFP91 is also essential in CD40-mediated NIK activation in vitro and plays a critical role in LIGHT (homologous to lymphotoxins, inducible expression, competes with herpesvirus glycoprotein D for herpesvirus entry mediator, a receptor expressed on T lymphocytes/LTβR interactions, leading to activation of noncanonical NF-κB signaling (74). In fact, knockdown of ZFP91 resulted in cessation of nuclear P52/RelB DNA binding, but has no effect on canonical p65 signaling. It remains unclear whether the mechanism of action is a direct promotion of NIK’s kinase activity, or rather a result of NIK stabilization. ZFP91 has not been investigated in mucosal immunology; however, due to
its role in T-cell activation, it is highly likely that this pathway contributes to gastrointestinal immune responses and inflammation.

**Maintenance and inspection: Noncanonical NF-κB processing and effector molecules**

The processing of p100 into p52 and subsequent translocalization of the p52/RelB heterodimer into the nucleus to activate gene transcription is the final step in the noncanonical NF-κB signaling cascade (75). This process is highly regulated compared to p105/p65 signaling, with only minimal constitutive action (76). In common mouse models of experimental colitis, wild type mice given DSS show a significant up-regulation of p100/p52 processing that is indicative of increased activation of the noncanonical NF-κB signaling cascade (77). Likewise, the generation of p100 is controlled by the gene Nfkb2, which has also been implicated in the pathobiology of IBD. The majority of data supporting a role for Nfkb2 in IBD was generated using mouse models of experimental colitis and colitis associated tumorigenesis. For example, Nfkb2−/− mice develop less severe colitis and dampened cytokine responses following DSS exposure, which results in fewer colonic tumors and attenuated tumorigenesis in the AOM/DSS model of colitis-associated cancer (78). Not surprisingly, aberrant p100/p52 expression has been seen in a variety of neoplasms and has been shown to interact with the critical proliferation protein cyclin D1, cooperating with p53 to express targeted proliferation and cell cycle genes (79). Together, these results are strong indicators that noncanonical signaling can have significant effects on both IBD and inflammation-driven tumorigenesis in the colon.

In addition to p100/p52, RelB is also an essential molecule in this final step of noncanonical NF-κB signaling. RelB dimerizes with p52 and functions to translocate p52 into the nucleus. All Rel proteins share a highly conserved 300 amino acid Rel homology domain (RHD), which includes domains involved in dimerization, nuclear localization, and DNA-
binding. Significant inflammatory cell infiltration has been reported in several organs, including the GI tract, in Relb−/− mice (80). RelB deficiency is associated with significant defects in acquired and innate immunity, increased T-cell infiltration in the organs and severe skin inflammation (80-82). Together, these data illustrate the importance of RelB in maintaining immune system homeostasis and indicates that a lack of RelB function cannot be compensated for by any of the other Rel family members. Because of the specificity for RelB to noncanonical NF-κB signaling, it is commonly used as a cellular marker of activation of the alternative pathway.

RelB/p52 nuclear translocation results in the upregulation of a diverse group of pro-inflammatory cytokines and chemokines that are commonly associated with IBD pathobiology. The best characterized chemokines associated with IBD and attributed to noncanonical NF-κB activation are Chemokine (C-X-C Motif) Ligand 12 (CXCL12) and 13 (CXCL13) (6, 83). Indeed, both human and mouse studies have provided evidence supporting a strong role for the pleiotropic chemokine CXCL12 (Stromal-cell Derived Factor-alpha; SDF1-α) and its receptor CXCR4 in the pathogenesis of IBD. The genes encoding both CXCL12 and CXCR4 are ubiquitously expressed in a diverse range of cell types, including intestinal epithelial cells (84, 85). In the context of IBD, CXCL12 and CXCR4 are expressed in intestinal epithelial cells during homeostasis and differentially up-regulated in IBD patients, suggesting that CXCL12 is both constitutive and inflammatory during IBD (85). In addition to the intestinal epithelial cells, increased numbers of circulating immature plasma cells from IBD patients have also been found to have significantly higher expression of CXCR4 compared to healthy controls (86). Beyond gene expression studies, findings from genetic association studies have also identified polymorphisms in CXCL12 associated with disease progression in a Polish IBD patient.
population (87). This study reported a significant association between 3 mutations associated with the CXCL12/CXCR4 axis between UC patients and CD patients, compared to healthy subjects (87). Here, the authors concluded that having combinations of these 3 polymorphisms in the CXCL12/CXCR4 axis may significantly predispose individuals to the development of IBD.

Two notable studies have investigated the CXCL12 and CXCR4 interaction within the context of experimental murine colitis. The first employed a blockade of this pathway using a CXCR4 antagonist, which resulted in amelioration of disease in wild-type mice in the DSS experimental colitis model and in the IL-10 knockout mouse model (88). In both models, colitic mice not given the CXCR4 antagonist exhibited increased disease progression (88). However, the administration of the CXCR4 antagonist reduced TNF and IFN-γ production, independently of any effects on IL-10 generation and T cell differentiation (88). The second study also utilized pharmacological blockade of the CXCL12/CXCR4 axis. Here, the authors used a non-peptide antagonist AMD3100 (89). Administration of AMD3100 reduced colitis, prevented defects in intestinal permeability, and lowered cytokine production, including IL6 and TNF (89). Together, these mouse studies imply that increased CXCL12/CXCR4 signaling contributes to worsening of IBD pathogenesis. Conversely, a rat study that used lentiviral transduction to overexpress CXCR4 in mesenchymal stem cells, which were subsequently engrafted, showed a protective function for CXCR4 in a 2,4,6-trinitrobenzene sulfonic acid (TNBS) model (90). Engraftment of CXCR4 overexpressing bone marrow-derived mesenchymal stem cells (BMSCs) resulted in significant amelioration of both clinical and microanatomical severity of TNBS induced colitis (90). The mechanism associated with these findings indicated that CXCR4 overexpression resulted in more efficient migration of BMSCs to the inflamed colon and a suppression of pro-inflammatory cytokines in the injured colon associated with reduced STAT3 phosphorylation.
The seemingly contradictory findings could simply reflect the systematic attenuation of the CXCL12/CXCR4 axis in the mouse models versus the immune cell specific overexpression of the axis in the rat model. Regardless, together with the human data, these findings indicate a complex and highly significant role for CXCL12/CXCR4 in IBD.

Similar to CXCL12, CXCL13 (BCA-1; BLC) and its receptor CXCR5 have also been shown up-regulated in IBD patients. A study evaluating CXCL13 (BCA-1) expression in frozen biopsy sections of normal and UC patients, and found that expression of CXCL13 was not only increased in inflamed tissue, but also highly expressed in both the peripheral elements of normal GALT patches and the abnormal lymphoid aggregates of UC patients (91). Consistent with these findings, mouse studies have shown that CXCL13 and CXCR5 are significantly involved in normal secondary lymphoid organ development (92). In mouse models, CXCL13 is significantly upregulated in various colitis models (93, 94). For example, increased transient pup consumption of n-6 fatty acid has recently been shown to reduce the severity of DSS induced experimental colitis in mice, likely through significantly altering the microbiome composition (94). In the transiently n-6-fed mice with attenuated disease progression, decreased numbers of CXCR5+ CD4+ T cells were detected in the mesenteric lymph nodes (94). Subsequent follow-up studies using this model revealed that antibody treatment with anti-CXCL-13 decreased the severity of DSS colitis and revealed critical roles for the CXCL13/CXCR5 axis in the progression of IBD (94). Indeed, elevated levels of CXCL13 were found in the serum of untreated pediatric IBD patients, linking the mouse findings to human subjects (94). Together, these data suggest that CXCL13/CXCR5 play an important role in the gastrointestinal tract, particularly in local mucosal lymphoid tissue, and in the pathogenesis of IBD.
In addition to CXCL12 and CXCL13, several C-C motif ligand chemokines are also regulated through the noncanonical NF-κB signaling pathway. For example, Chemokine (C-C motif) ligand 19 (CCL19; MIP-3-β; ELC) transcription is mediated through the noncanonical pathway and signals through the CCR7 receptor (95). The binding of CCL19 to CCR7 results in T cell and DC homing to T cell zones of lymphoid tissue. Little is known about CCL19’s importance in IBD, as the majority of research into this chemokine has focused on its function in the lymphoid system and various neoplastic conditions, including colorectal cancer. CCL19 expression is decreased in colorectal cancer, which is highly interesting, given the propensity of IBD patients to develop dysplasia (96). However, in CD patients, dendritic cells show increased CCL19 and CCR7 expression (97). Specifically, inflamed colonic tissue showed increased numbers of mature myeloid dendritic cells that expressed high levels of both CCR7 and CCL19 (97). Similar to CCL19, Chemokine (C-C motif) ligand 21 (CCL21) is another product of the noncanonical pathway. CCL21 also binds to CCR7 and functions as a potent lymphocyte chemoattractant. Interestingly, CCL21 has been shown to be upregulated in the serum of IBD patients, as well as, in reticular cells and lymphatic vessels in CD patients in concert with the CCR7/CCL19-expressing dendritic cells mentioned previously (97, 98). However, while the dysregulation of CCL19 and CCL21 appear to be associated with IBD, mechanistic insight associated with their exact roles in modulating disease pathogenesis is currently lacking.

**Putting on the brakes: Negative regulation of noncanonical NF-κB signaling.**

Control of overzealous inflammation is critical to maintain immune system homeostasis in the gastrointestinal system. Attenuation of hyper-inflammation can be achieved through a variety of mechanisms, including the attenuation of NF-κB. Over the last decade, a variety of proteins have been characterized that function to negatively regulate both canonical and
noncanonical NF-κB signaling. For example, a sub-group of the nucleotide-binding domain and leucine-rich-repeat containing (NBD-LRR; NLR) family of pattern recognition receptors have been shown to function as negative regulators of inflammation through attenuation of NF-κB signaling. NLRP12 is a member of this regulatory NLR sub-group that has been shown to reduce both canonical and noncanonical pathways. In the context of noncanonical NF-κB signaling, NLRP12 has been shown to bind to NIK and TRAF3 (6, 83). This binding is thought to result in the stabilization of TRAF3 and ultimately NIK degradation (6, 83). This mechanism is critical during IBD, where Nlrp12−/− mice have significantly increased noncanonical NF-κB signaling in models of experimental colitis and during colitis associated tumorigenesis (83). In acute and chronic models of experimental colitis utilizing DSS, Nlrp12−/− mice demonstrated increased morbidity and mortality, with significantly increased inflammation and damage to the epithelial barrier compared to wild type animals (83). While some changes were observed in the canonical NF-κB signaling cascade, a significant increase in noncanonical NF-κB signaling was observed in the Nlrp12−/− mice and strongly associated with disease pathogenesis (83, 99). Both NIK levels and p100 processing to p52 were found to be significantly increased in colonic explants from Nlrp12−/− mice (83). Furthermore, primary murine myeloid dendritic cells taken from Nlrp12−/− mice showed sustained and increased levels of NIK following activation with TNF compared to wild type cells. Downstream of NIK and p100 to p52 processing, a significant increase in noncanonical NF-κB associated chemokines were detected in the Nlrp12−/− mice, including CXCL12 and CXCL13 (83). Together, these data strongly support a role for NLRP12 in the attenuation of overzealous inflammation associated with IBD and further identify the critical nature of noncanonical NF-κB signaling in disease pathobiology.
The interaction between NLRP12, NIK, and TRAF3 support the hypothesis that NLRP12 negatively regulates NF-κB signaling through the formation of a multiprotein complex. Indeed, other NLRs in this functional subgroup, such as NLRC3, have also been suggested to form a multiprotein complex termed the “TRAFasome” (100). Multiprotein complexes are common players in major immunologic pathways, including the noncanonical NF-κB cascade. For example, a central inhibitory complex has been described that includes cIAP1/2, which act as negative regulatory molecules that suppress TNF-mediated NF-κB activation (101, 102). Mechanistically, a ubiquitin ligase complex consisting of TRAF2, TRAF3, and cIAP1/2 catalyzes the ubiquitination and degradation of NIK (103, 104). This is a potent negative regulatory mechanism, as stabilization of cIAP1 through this multi-protein complex limits downstream noncanonical activity, even during active TNF stimulation (105). cIAP1 and cIAP2 have become increasingly associated with IBD (106). For example, upregulation of cIAP2 has been seen in the colonic epithelium of ulcerative colitis patients (107, 108). Specifically, cIAP2 mRNA was detected at a significantly elevated rate in primary human cells isolated from colonocytes grown from biopsies of UC patients compared to control patients and UC patients in remission (108). In another study, biopsies from patients with active UC and control patients were evaluated and cIAP2 was upregulated in the regenerating colonocytes of the UC group (107, 108). Interestingly, the activity of cIAP1 and cIAP2 can be modulated by another subgroup of regulatory NLRs that function as positive regulators of inflammation. This NLR subgroup includes NOD1 and NOD2. Both of these NLRs are pro-inflammatory and function through the formation of a large macromolecular complex termed the “NODosome” (109). This complex modulates a variety of inflammatory signaling pathways, including activation of the NF-κB cascade. In human IBD patients, mutations in NOD2 have been highly associated with
disease pathogenesis in specific sub-populations (110). These mutations in NOD2 are some of the best characterized in the context of disease and are routinely evaluated in IBD patients. In vitro work showed that macrophages from cIAP1 and cIAP2 deficient mice displayed defective NOD signaling, as did colonocytes deprived of cIAP1 or cIAP2 by iRNA (111). In mouse studies done by the same group, administration of the NOD2 agonist Muramyldipeptide (MDP) protected wild type mice, but not cIAP2-deficient mice, in models of experimental colitis (111), suggesting the protective role of NOD2 in IBD may be cIAP2 dependent.

TRAF3 is associated with the TRAF2-TRAF3-cIAP1-cIAP2 complex and has been shown to directly interact with NIK (103). In human IBD patients, TRAF3 was detected at significantly higher levels in plasma, peripheral blood mononuclear cells (PBMCs), and biopsies of inflamed and normal colon tissue from patients with IBD compared to healthy controls (112). In mouse models, depletion of TRAF3 results in constitutively activated noncanonical NF-κB signaling. For example, TRAF3 depleted B cells demonstrate constitutive p100 processing (113). Traf3−/− mice do not survive into adulthood. However, fetal liver transplants have been successfully utilized to study TRAF3 function and the postnatal lethality of Traf3−/− mice could be rescued by the additional loss of p100. Traf3−/− p100−/− mice were reported to grow at normal rates and survive into adulthood (113). In addition to these conventional TRAF3 deficient mice, cell type specific Traf3−/− animals have also been generated. For example, myeloid cell-specific Traf3−/− mice have been characterized as developing spontaneous and chronic inflammation and increased tumorigenesis in multiple organs, including the colon (114). Similar to TRAF3, TRAF2 deletion has also been demonstrated to result in hyperactive noncanonical NF-κB signaling. Western blot analysis detected elevated p100 processing and RelB/p52 DNA binding in B cells isolated from mice lacking TRAF2 (115). Traf2−/− mice develop spontaneous, severe
colitis as early as 10 days after birth, and succumb within 3 weeks of age (116). Interestingly, the administration of antibiotics to the drinking water of nursing mothers was able to partially rescue the Traf2−/− pups from the colitis symptoms (116). Together these data emphasize the importance of the interactions between the TRAF2/TRAF3/cIAP1/cIAP2 complexes with elements of the noncanonical NF-κB signaling cascade in IBD.

Positive and negative modulation of ubiquitination is an important post-translational regulatory mechanism for the control of noncanonical NF-κB signaling. While cIAP1/2, TRAF2, and TRAF3 all play important roles in ubiquination, deubiquitination is also highly relevant. For example, CYLD is a deubiquitinase that acts as a negative regulator of the NF-κB pathway (Figure 3). It plays a role in the regulation of canonical signaling via its interactions with NEMO (117, 118). Likewise, CYLD is also a significant negative regulator of the noncanonical signaling cascade via modulation of TRAF2 (119). A negative regulatory role of CYLD for NF-κB signaling was suggested after it was proven that expression of CYLD in HEK cells inhibited the activity of co-expressed NF-κB activators (119). Ubiquitination of TRAF2 by CYLD was found to be nearly abolished in the presence of a dominant negative ubiquitin-specific protease mutant deficient in CYLD (119). Indeed there appears to be a reciprocal autoregulation between NF-κB and CYLD, with induction of the latter also requiring the former (120). Upon stimulation with NF-κB activators TNF and nontypeable *Haemophilus influenzae* (NTHi), CYLD expression increased in the presence of functional IKKβ and NEMO (120). Because of the implications of TRAF2 as a negative regulator of CYLD, a TRAF2 dominant negative mutant was utilized and shown that when stimulated with TNF, the induction of CYLD was negated (120). CYLD’s role in mucosal immunity is also gaining traction. Its expression is reduced in human colorectal carcinomas (121). Likewise, genome wide association studies (GWAS) have detected mutations
associated with CYLD as a risk factor for IBD (122). In animal studies, $Cyld^{-/-}$ mice showed prominent increases in the size and number of mucosal lymphoid tissue proliferation and hypertrophy, and eventually these animals develop spontaneous colitis with inflammatory cell infiltrate, crypt damage, and increases in pro-inflammatory cytokines (123). The spontaneous colitis observed in these $Cyld^{-/-}$ mice appeared consistent with features associated with human disease, including histological tissue damage, increased expression of pro-inflammatory cytokines IL-4 and IL-12, and weight loss and clinical symptoms. CYLD’s regulatory effects seem to be primarily via T cells, given that $Cyld^{-/-}$ T cells are hyper-responsive to TCR stimuli and $Rag1^{-/-}$ mice given $Cyld^{-/-}$ T cells develop a significant immune-mediated colitis compared to those given wild type T cells (123). Of note, the $Rag1^{-/-}$ mice adoptively transferred $Cyld^{-/-}$ T cells demonstrated increased lymphocyte infiltration into the periportal regions of the liver, infiltration of inflammatory cells in the colon, thickening of mucosa, and goblet cell depletion compared to mice transferred wild type T cells (123). Consistent with the expression data from human colorectal carcinoma patients, $Cyld^{-/-}$ mice also show increased sensitivity in AOM/DSS models of inflammation driven colon tumorigenesis (124). This was observed as an increase in leukocyte infiltration, histologic damage, mucosal ulcers, and dysplasia to colonic epithelium in the $Cyld^{-/-}$ mice compared to the wild type animals after one 21-day cycle exposure to AOM/DSS. Following a second 21-day cycle with AOM/DSS, the $Cyld^{-/-}$ mice presented with an increase in colonic tumors compared to wild type mice (124). Increased NF-κB signaling was associated with the mechanism underlying the increase in colitis and cancer in these CYLD deficient animals (124).

Heat shock 70 kDa (Hsc70) is another regulator of noncanonical NF-κB signaling that appears to function through the modulation of ubiquitination. Hsc70 is a positive regulator of cell
cycle transition and carcinogenesis, regulating nuclear accumulation of cyclin D1 along with Connexin43 (Cx43) (125). It is a stress-induced molecule that is both constitutively expressed (Hsc70c) and inducible (Hsc70i), and interacts with an E3 ubiquitin ligase partner, called C-terminus of Hsc70 Interacting Protein (CHIP) (126). CHIP has been shown to a downregulate NIK and the noncanonical NF-κB pathway (127). Hsc70 has been linked to IBD both at the cellular and clinical level, particularly with ulcerative colitis (128). Both CD and UC patients show strong immunostaining of this protein in intestinal biopsies, especially in epithelial cells (128) and expression is decreased during treatment and remission (129, 130). This epithelial cell expression is mimicked in vitro, with induction of Hsc70 resulting in protection of intestinal epithelial cells from oxidative damage (131). In the mucosa of CD patients, but not UC, there is additional increased expression of Hsc70 in submucosal and mucosal mononuclear cells, potentially indicating a unique leukocyte-focused mechanism of Hsc70 in CD versus UC (128). Hsc70 expression is also associated with increasing colonic dysplasia (129), a significant lesion in IBD that can lead to colorectal cancer. Compared to tissue biopsies taken at initial diagnosis, six months after treatment there was a significant increase in Hsc70 expression in the colonic mucosa of patients who were unresponsive to treatment in both inflamed and dysplastic tissue (129). Overall, these findings suggest that during active damage and disease, Hsc70 may be activated in an attempt to curtail noncanonical signaling. In addition to Hsp70 inhibiting NIK signaling, other Heat shock proteins can actually stabilize NIK and are suggested to have a role in IBD. For example, Hsp90 binds to and stabilizes NIK in order to protect it from autophagy (132). Geldanamycin, an anti-tumor agent that inhibits Hsc90, was found to decrease p100 processing in a dose-dependent manner and was associated with decreased levels of NIK (132).
Hsp90 is elevated in UC patients suggesting that it may prolong noncanonical NF-κB signaling and augment IBD pathogenesis (129).

Although there is no current literature directly detailing an association between TRAF and NIK-associated protein (TNAP) and F-Box and WD Repeat Domain Containing 7, E3 Ubiquitin Protein Ligase (FBXW7) in IBD, these two additional molecules also negatively regulate noncanonical NF-κB activity. TNAP has been shown to be down-regulated during both canonical and noncanonical signaling (133). In regards to noncanonical signaling, TNAP interacts with NIK, TRAF2, and TRAF3 to inhibit NIK’s kinase activity, resulting in suppression of downstream p100 processing through both knockdown and overexpression studies (133) (Figure 3). It does not, however, appear to affect NIK stability, only its activity. Although TNAP has not been characterized in immune-mediated disease, it is interesting to speculate that its ability to inhibit NIK plays a major role in the attenuation of disease pathology. Likewise, little is known regarding the role of FBXW7 in IBD. FBXW7 binds to NF-κB 2 and has previously been characterized as a tumor suppressor (134). FBXW7 interacts with and binds to substrates via interaction between the motif on the substrate and its WD40 repeats (134). FBXW7 ubiquitinates p100 and promotes its degradation in a GSK3β-dependent manner (135). The inactivation of FBXW7 at the cellular level was shown to result in elevated p100 levels, and may therefore be interpreted as an increase in noncanonical NF-κB activity (135). Despite the fact that there is currently no evidence in the literature evaluating its involvement in IBD, some data have concluded that FBXW7 is downregulated in gastric cancer (136) and is inversely correlated with the tumor promoter ENO1 in colorectal cancer (137). In addition to TNAP and FBXW7, an assortment of other miscellaneous molecules have been shown to bind to NIK including epidermal growth factor (EGF) and its receptor (EGFR) (138), caspase death domains (139) and
the protein of the proto-oncogene Cot/Tpl-2 (140), although only the effects on the canonical NF-κB pathway has been fully characterized for these additional proteins.

Conclusions

Given the powerful effects of TNF family members on inflammation, it is unsurprising that the majority of approved therapies for IBD target one or more of these molecules. Infliximab, adalimumab, and certolizumab are all anti-TNF mAbs currently approved for Crohn’s disease and are often used when more moderate medications, such as immunosuppressants, fail to result in a better quality of life for the patient. Under optimal conditions, these drugs have great efficacy in changing the clinical course of the disease. However, the effects are variable and often temporary. Indeed, the high risk of patients becoming nonresponsive to these treatments is of great concern amongst clinicians. Although the majority of patients initially respond well, only one-third of patients on biologics are still in remission after 1 year without steroids (141). Even in those patients who do not completely lose responsiveness, increasing dosages in order to maintain clinical remission is often required. Losing this efficacy is exceptionally problematic, as IBD is a chronic and progressive disease that can result in significant surgical intervention and complications over the course of the patient’s lifetime.

In addition, as TNF ligand-receptor interactions are important in noncanonical NF-κB signaling, the effects of these treatments on noncanonical signaling is a question worthy of investigation. For example, it is not currently clear if these current therapeutics target or suppress the noncanonical NF-κB cascade to the same extent as the canonical pathway. Given that this signaling cascade is tightly regulated, as opposed to the rapid and constitutive expression of the canonical signaling pathway, it is not unreasonable to assume that the noncanonical NF-κB
cascade significantly contributes to IBD at multiple stages of disease pathogenesis. It is our opinion that the contribution of this pathway to IBD pathobiology has been significantly overlooked, due to the high level of interest in canonical signaling. Because of the paucity of data regarding noncanonical signaling in IBD, it is our belief that a deeper investigation of this pathway and its components is warranted. Further research will certainly provide additional insight into disease pathogenesis. It is interesting to speculate that further mechanistic studies associated with the noncanonical NF-κB pathway will identify novel therapeutic targets, something that is greatly in demand for patients and clinicians alike.
References:


64. Wellcome Trust Case Control C Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature. 2007;447:661–678.


75. Scheinman RI, Beg AA, Baldwin AS., Jr. kappa B p100 (Lyt-10) is a component of H2TF1 and can function as an I kappa B-like molecule. Mol Cell Biol. 1993;13:6089–6101.


**Figure 1:** The Canonical NF-κB Signaling Pathway. This schematic demonstrates some of the major steps associated with the canonical NF-κB signaling pathway under both unstimulated and stimulated conditions. The canonical pathway is triggered by a variety of stimuli that activate diverse receptors, such as pattern recognition receptors, TNF receptors, and proinflammatory cytokine receptors. In this representative image, the TNF receptor is shown. When unstimulated, the IKK complex composed of NEMO (IKKγ), IKKβ, and IKKα, along with the heterodimer
composed of NF-κB proteins RelA and p50 are inactive and located in the cytoplasm. The binding of a ligand to the cell surface receptor, such as TNF binding to TNF receptor, leads to the recruitment of adaptor proteins, such as TRAF2 or TRAF5 and TAK1. This upstream activity leads to the phosphorylation and activation of the regulatory subunit of the IKK complex, NEMO, which in turn leads to the phosphorylation of the catalytic subunit of the IKK complex, IKKβ. IKKβ then mediates the phosphorylation and induction of proteosomal degradation of IκBα, which then allows for nuclear localization of the heterodimer RelA/p50. Nuclear localization leads to the transcription of proinflammatory cytokines such as TNF, IL-1β, and IL-6.
Figure 2: The Noncanonical NF-κB Pathway. NF-κB inducing kinase (NIK) is constantly being translated. However, under normal unstimulated conditions, NIK is ubiquitinated and degraded via the TRAF3/TRAF2/cIAP1/cIAP2 complex. Upon stimulation by TNF family ligands, this complex is degraded via K48 ubiquitination, which allows NIK to interact with and phosphorylate IKKα. IKKα then phosphorylates p100, leading to its cleavage to p52. The processing of p100 allows the RelB/p52 dimer to enter the nucleus and initiate transcription of noncanonical NF-κB associated genes, such as CXCL12 and CXCL13.
Figure 3: Major Positive and Negative Regulators of Noncanonical NF-κB Signaling

Much of the regulation of the noncanonical NF-κB pathway revolves around stabilization or degradation of NIK. cIAP1/cIAP2 are the most well-known suppressors of NIK activity, forming a complex with TRAF2 and TRAF3, resulting in ubiquitination and proteosomal degradation. NLRP12 performs a similar function by binding to NIK and enhancing the formation of this complex. HSP70 also downregulates NIK via its partner CHIP, and TNAP interacts with NIK.
TRAF2, and TRAF3 and inhibits NIK’s kinase activity. CYLD negatively regulates noncanonical NF-κB signaling further upstream, by ubiquinating TRAF2 and therefore inhibiting CD40 and XEDAR signaling. In terms of activators, XEDAR activates noncanonical NF-κB signaling and stimulates NIK by binding to TRAF6 and TRAF2. Also at the receptor level, TWEAK/Fn14 binding disassociates the ubiquitination complex itself and frees NIK. A20 binds to cIAP1/2 and also prevents proper NIK degradation. BCL10 controls the phosphorylation of NIK and promotes its activity, while ZPF91 binds directly to NIK to augment function.
Chapter 3

Noncanonical NF-κB Signaling and the Essential Kinase NIK Modulate Critical Features Associated with Eosinophilic Esophagitis Pathogenesis

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Abstract

Eosinophilic esophagitis (EoE) is an allergic disease of the esophagus driven by T cell and eosinophil responses to dietary allergens, resulting in chronic mucosal inflammation. Few spontaneous animal models of esophageal eosinophilia exist, with most studies relying on artificial sensitization procedures. NF-κB-inducing kinase (NIK; MAP3K14) is a key signaling molecule of the noncanonical NF-κB pathway, an alternative signaling cascade producing chemokines involved in lymphoid stroma development and leukocyte trafficking. Nik⁻/⁻ mice have been shown to develop a hypereosinophilic syndrome in peripheral blood and major
filtering organs; however, the gastrointestinal mucosa of these mice has not been well characterized. We show that Nik−/− mice develop significant, localized eosinophilic esophagitis that mimics human EoE, including features such as severe eosinophil accumulation, degranulation, mucosal thickening, fibrosis and basal cell hyperplasia. The remainder of the GI tract, including the caudal stomach, small intestine and colon, in mice with active EoE are unaffected, also similar to human patients. Gene expression patterns in esophageal tissue of Nik−/− mice mimics human EoE, with thymic stromal lymphopoetin (TSLP) in particular also elevated at the protein level. In gene expression data sets from human biopsy specimens, we further show that many genes associated with noncanonical NF-κB signaling are significantly dysregulated in EoE patients, most notably a paradoxical upregulation of NIK itself with concurrent upregulation of powerful protein-level destabilizers of NIK. These findings suggest that Nik−/− mice could be useful as a spontaneous model of specific features of EoE and highlight a novel role for noncanonical NF-κB signaling in human patients.
Introduction

Over the last 20 years, eosinophilic esophagitis (EoE) has emerged as a significant cause of upper gastrointestinal (GI) morbidity with significant quality of life implications for patients [1-5] and an estimated $1.4 billion annual healthcare cost [6]. This disorder can affect both children and adults with symptoms that vary by age, but typically include difficulty eating, failure to thrive, dysphagia, and food impaction. EoE is considered to be an allergic disease of the esophageal mucosa and is most often associated with immune responses to food such as wheat, eggs, and dairy [3, 7-9]. Consistent with these findings, T helper 2 (Th2) cells and their related cytokines are prominently involved in EoE pathogenesis [10] and, interestingly, other atopic diseases such as allergic dermatitis or rhinitis can be comorbidities of EoE [11]. Currently the most successful treatment is through induction and maintenance of a strict elimination diet, although drugs such as steroids and biologics are sometimes employed as well as physical methods such as esophageal dilation for fibrotic stenosis [12-15].

EoE must be differentiated clinically and histologically from gastroesophageal reflux disease and proton-pump responsive esophageal eosinophilia which can be difficult given the overlap of clinical presentation, endoscopic assessment, and sometimes even response to certain treatments [16-18]. EoE has several characteristics that distinguish it as a separate disease entity, such as the presence of >15 eosinophils per high power field (HPF) and typical lack of response to proton pump inhibitors [19], although there may well be synergy between the various diseases. EoE also differentiates itself from other more general eosinophilic GI disorders such as eosinophilic gastroenteritis by having a restricted distribution to the esophageal mucosa. The vast majority of current animal models of EoE rely on sensitization procedures to allergens or exposure with pathogens such as Aspergillus fumigatus, similar to protocols originally designed
for pulmonary models of asthma [20, 21]. Indeed, the existing animal models used to study EoE can be problematic because this disease can present heterogeneously with a spectrum of inflammatory characteristics and additional extra-esophageal manifestations [22]. Few murine models of spontaneous esophageal eosinophilia currently exist, and those that have been described are limited to molecules that are already well-studied in Th2 biology and allergy such as IL-5, eotaxins, and IL-13 [23, 24].

Canonical NF-kappa-B (NF-κB) signaling is a prominent and widely studied inflammatory signaling cascade in the field of immunology. The central process of the canonical cascade is the liberation of p65 and p50 from their inhibitor, IκB, when it is post-translationally modified by the IKK group of kinases. This allows p65/p50 translocation to the nucleus and the subsequent transcription of a multitude of gene targets. In general, the canonical pathway underlies the rapid and acute cellular response to a wide variety of pathogenic and pro-inflammatory stimuli. However, while the canonical NF-κB signaling cascade is well characterized and regulates a large repertoire of downstream mediators, there is a relative paucity of data associated with the lesser studied noncanonical NF-κB pathway. Canonical and noncanonical NF-κB signaling are quite different in several crucial ways in terms of their individual cascades and downstream effects. For example, ligand-receptor interactions in noncanonical signaling are much more restricted compared to the canonical pathway and are primarily composed of a specific subset of TNF-related receptors [25]. Noncanonical signaling also depends selectively on the activity of its central molecule NF-κB inducing kinase (NIK) and the IKKα subunit. In contrast to the model of simple cytoplasmic liberation of p65, as seen in canonical NF-κB signaling, the noncanonical pathway relies heavily on protein processing for preparation of its unique unit p100, encoded by the NFKB2 gene. The IKKα subunit, activated
by NIK, drives the proteolytic removal and degradation of a segment of p100, converting it to p52. Only then can the nuclear translocation of p52 and initiation of gene transcription proceed. Protein-level dynamics are not only important in positive regulation of noncanonical NF-κB; NIK is a target of a wide array of suppressor proteins that work to destabilize and ubiquitinate it rather than affect it simply at the gene expression level. The downstream chemokine targets for gene transcription in noncanonical signaling are less defined, but appear to be both more limited in number and more specific in function. To date, four chemokines, CXCL12, CXCL13, CCL19, CCL21, are associated with noncanonical NF-κB activation [26, 27]. While these chemokines can function as proinflammatory mediators and chemotactic factors for immune cell trafficking, they are typically associated with lymphoid development and maintenance. Given this targeted effect and the prominent role of mucosal-associated lymphoid tissue in the gut, it is tempting to speculate that noncanonical signaling may play an important role in maintaining immune system homeostasis at GI mucosal surfaces [25]. Indeed, prior research from our team revealed that loss of negative regulators of noncanonical NF-κB signaling are associated with increased progression of experimental colitis and colitis associated tumorigenesis in mice [26].

Mice having either a functional mutation (aly/aly) or completely lacking this essential noncanonical kinase MAP3K14 (NIK; Nik−/−) have been previously described and display a variety of phenotypes, such as improper formation of secondary lymphoid organs, altered immune responses and, in Nik−/− mice specifically, a hypereosinophilic-like syndrome [28-31]. Common gross and clinical observations in these mice include dermatitis, ocular keratitis, urticaria, weight loss, and microscopic deposits of eosinophils into major organs such as liver, lung, and spleen. The eosinophilia in Nik−/− mice seems to be dependent on T cells, as lack of
lymphocytes results in prevention of the syndrome [29]. Indeed, T cells from $Nik^{-/-}$ mice appear to be naturally more prone to a Th2 phenotype, which may be an additional driving force behind the hyperesinophilia [29]. While these animals have been previously characterized, the presence and patterns of eosinophilic influx in the GI system and the role of NIK and noncanonical NF-κB signaling in human eosinophilic GI disease has not been explored.

Here we characterized the gastrointestinal tract of $Nik^{-/-}$ mice and determined that there is a significant and localized targeting of the esophageal mucosa by eosinophils with sparing of the rest of the GI tract. Morphologic changes such as microabscessation, basal cell hyperplasia, and fibrosis all mimicked the progression of EoE in human patients. Esophageal inflammation and remodeling in these mice was accompanied by upregulation of key cytokines involved in the microenvironmental signature of EoE, along with increased transcription and protein expression of thymic stromal lymphopoietin (TSLP), which is a key player in human EoE. Metadata analysis of publically available gene expression datasets from patient and control biopsies revealed significant increases in genes associated with both the activation and repression of noncanonical NF-κB signaling in EoE afflicted subjects. The significance of our findings go beyond identification of a spontaneous pre-clinical mouse model of EoE to additionally implicate a novel pathway in the development of the disease itself.

**Materials and Methods:**

**Mouse Models**

$Nik^{-/-}$ mice ($Map3k14tm1Rds$) originally created by Yin et al.[30] were generously provided by Amgen. Heterozygous mice were bred and littermates genotyped upon weaning (Supplementary Figure 1). Knockout animals and wild-type littermates were then separated by
sex and genotype and all mice were housed under SPF conditions. Mice were fed standard Tekland rodent diet (16% protein with ingredients in order of inclusion: wheat, corn, wheat middlings, soybean meal, corn gluten meal, soy oil) and given water ad libitum. Enrichment was provided in the form of red plastic huts, paper twists, and Nestlets. All studies were conducted in accordance with the Institutional Animal Care and Use Committee guidelines of Virginia Maryland College of Veterinary Medicine and National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were sacrificed between 3 - 20 weeks of age depending on appearance of dermatitis, the most consistent and recognizable sign of disease onset. Age-matched wild-type animals were sacrificed at identical timepoints.

**Tissue Collection and Histological Analysis**

Mice were euthanized by carbon dioxide narcosis followed by cervical dislocation. The entire esophagus from the level of the gastroesophageal junction to the pharynx was dissected out, flushed, opened, and “Swiss rolled” for histopathology evaluation. Intestine and colon were similarly prepared. Samples were fixed in 10% buffered formalin, paraffin embedded, sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Esophagi were scored using designations of severity of eosinophilic inflammation (0 = absent, 1 = scattered eosinophils within the epithelium with or without occasional submucosal extension, 2 = moderate and consistent presence of eosinophils in both the epithelium and submucosa with disruption of normal architecture, 3 = severe, dense eosinophilic infiltrate with significant obscuring of the basal layer and submucosal expansion, and 4 = very severe, dense infiltrate with transmural extension), keratin loss (0 = absent, 1 = <25% of section, 2 = 26-50% of section, 3 = >50% of section), and microabscessation (0 = none, 1 = 1-2 per esophageal roll cross-section, 2 = 3+ per esophageal cross-section with degranulation). Eosinophil count was performed based on
eosinophil morphology (correct size, bilobed nucleus, prominent and distinct magenta granules) as well as positive MBP staining in the esophagus, small intestine (ileum) and colon (entire length). For esophagus, 5 randomly selected fields were counted and averaged; for the lower GI tract, the total number in 10 40x fields was reported. Mucosa thickness in the stomach and esophagus was quantified using five randomly selected areas of mucosa surrounding the junction of each individual specimen. Basal cell hyperplasia was evaluated as a percentage of total epithelial thickness measured at 5 independent points within each individual sample. To evaluate collagen deposition, additional slides were stained with Masson’s trichrome. Thickness of the submucosal collagen deposition was measured using the tunica muscularis and the basal cell layer of the epithelium as borders and was quantified averaging five measurements at randomly determined points within each individual section. Toluidine blue staining was performed using a 1% solution of toluidine blue O (Sigma) dissolved in 70% EtoH and adjusted to pH 2.3. All histopathology assessments were conducted by a board-certified veterinary pathologist (K.E.) who was blinded to the identity of the samples.

**Immunohistochemistry**

Tissue sections (5 μM) were deparaffinized, hydrated, and subjected to heat-mediated enzymatic retrieval using pH 6.0 citrate buffer for anti-TSLP protein staining. For anti-major basic protein staining, enzymatic antigen retrieval was changed to a 0.1% trypsin for 20 minutes at 37°C. Endogenous peroxidase activity blocking, general serum blocking, and washings were performed using reagents from the Pierce Peroxidase IHC Detection Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Sections were incubated with rabbit anti-mouse major basic protein antibody (My BioSource, MBS2004321) or rabbit anti mouse/human TSLP (Cell Signaling Technology, PA5-20320) at 4°C overnight. Sections were then incubated
with SignalStain® Boost IHC Detection Reagent (HRP, Rabbit; Cell Signaling Technology #8114) for 30 minutes at room temperature and staining was detected using the Pierce Peroxidase Substrate solution and 3,3’-diaminobenzidine (DAB) following the manufacturer’s protocol. The sections were counterstained with Mayer’s Hematoxylin (Sigma-Aldrich), dehydrated, and mounted. Degree of immunoreactivity was measured as a percentage contribution of positive pixels per image using identical magnifications and orientations of esophageal tissue using the IHC Profiler plugin for ImageJ, and 5 independent measurements were taken and averaged for each individual sample.

**Gene Expression**

Esophageal tissue was finely minced, total RNA extracted using Trizol (Thermo Fisher), and quantity/quality assessed via Nanodrop. Complimentary DNA was made using an ABI High Capacity cDNA kit, in accordance with the manufacturer's protocols and 1 μg amplified using the Taqman-based rtPCR platform (Thermo Fisher) on an ABI 7500 Fast Block Thermocycler. Gene expression was determined using the ΔΔCt method\[64\]. All data were normalized to Gapdh and the fold change in gene expression was determined for Tslp, Il4, Il13, Il5, IFNγ, TNF, TGFβ, and Il1b. All samples were run in triplicate.

**Human Gene Expression Analysis**

Gene expression from human patient and control biopsies was evaluated using publically accessible datasets (NIH GEO) as previously described [65, 66]. Datasets were analyzed using Ingenuity Pathways Analysis (IPA) software to identify genes and pathways that were significantly dysregulated in EoE patients and specimens. Specific genes of interest identified in the IPA analysis associated with noncanonical NF-κB signaling were further evaluated. Gene
expression data was normalized to the average expression of 6 housekeeping genes (18s, ACTB, RPLP0, HPRT, B2M, and GAPDH) for each specimen and the fold change in gene expression between EoE patient and control specimens were determined. Gene expression analysis was conducted using the array data series GSE58640 and GSE8853.

**Statistical Analysis**

Data was analyzed using GraphPad Prism, version 7 (GraphPad Software, Inc., San Diego, CA). Student’s two-tailed t test was used for comparison of two experimental groups. Multiple comparisons were done using one-way and two-way ANOVA where appropriate followed by Mann-Whitney or Tukey post-test for multiple pairwise examinations. Correlation was also computed using GraphPad Prism. Changes were identified as statistically significant if p was less than 0.05. Mean values were reported together with the standard error of the mean (SEM).

**Results:**

*Nik−/−* mice develop a spontaneous hypereosinophilic-like syndrome (HES) characterized by ulcerative dermatitis and weight loss.

Previous studies have characterized the development of HES in *Nik−/−* mice [29]. However, the underlying mechanism associated with this phenotype is unclear and a variety of local environmental factors could potentially impact disease progression. Thus, we first sought to confirm and characterize the progression of HES in the *Nik−/−* mice under our institutional conditions. Consistent with the previous reports, we observed significant tissue eosinophilia, tissue injury, and increased morbidity and mortality. HES in these animals resulted in
progressive loss of body condition (Figure 1A) and a scaling dermatitis that most commonly affected the tail (Figure 1B), extremities (Figure 1C) and eventually the head, ear, and shoulder region. Indeed, our animals typically require premature euthanasia between weeks 9-12, specifically due to the progressive nature of the dermatitis and associated morbidity (Figure 1D). Onset of the dermatitis is variable with some animals displaying lesions by as little as 3 weeks of age, whereas others remain outwardly unaffected until several months of age. Both male and female animals appeared to be equally affected.

The GI tract of Nik−/− mice displays a localized pattern of eosinophilic inflammation that targets the esophageal mucosa.

Despite the previous reports of generalized eosinophil presence in multiple filtering organs, inflammatory pathology in the GI system of the Nik−/− mouse appears to be restricted to the esophagus and the immediately adjacent gastroesophageal junction, with the mucosa of the esophagus being particularly affected. In contrast with the wild-type littermates, the esophagus of Nik−/− mice is affected by a florid influx of eosinophils that invade the mucosa and submucosa and distort esophageal architecture (Figure 2A and 2B) along with a secondary, smaller population of mature lymphocytes. This change is often accompanied by mucosal thickening (Supplementary Figure 2) and significant basal cell hyperplasia (Figure 2B, arrows) with basal layers in some cases composing >30% of the total epithelial thickness (Figure 2C). Eosinophils are quite prominent in the border of the squamous epithelial layer and the submucosa, and occasionally form small intraepithelial microabscesses and degranulate (Figure 2D, arrow). These eosinophils were formally quantified and were present in densities far greater than 15 per HPF, a diagnostic criterion for the human disease (Figure 2E). The identity of these cells as eosinophils was confirmed under high magnification where the characteristic distinct
magenta granules and bilobed nucleus can be appreciated (Figure 2F) as well as immunohistochemical staining with anti-mouse major basic protein (Figure 2G-H). Inflammation, keratin loss/erosion, and microabscessation were also histologically graded, with Nik−/− mice showing significant increases in all of these characteristics (Figure 2I).

**Esophageal fibrosis and gastroesophageal junction hyperplasia are key sequelae to inflammation in Nik−/− mice.**

Given that fibrosis and stricture of the esophagus is a serious complication of eosinophilic esophagitis in human patients [32-34], we investigated the degree of collagen deposition in the submucosa of esophageal tissue from wild-type and Nik−/− mice. Grossly, Nik−/− esophagi were thicker than wild-type littermates (Figure 3A). Using trichome staining, massive expansion of the submucosa in the Nik−/− animals was apparent, with extensive collagen deposition as compared to wild-type mice (Figure 3B-D). The degree of submucosal fibrosis was quantified using ImageJ software to measure the average submucosal diameter, with Nik−/− mice having approximately twice as much collagen deposition as wild type mice (Figure 3E). The mucosa of the gastroesophageal junction (GEJ) of Nik−/− mice with eosinophilic esophagitis is also circumferentially hyperplastic and contains scattered inflammation suggestive of local irritation (Figure 4A-D). Mucosal thickness was again quantified from the level of the apical mucosa to the basement membrane (Figure 4E), with Nik−/− animals showing significant thickening. While the majority of GEJ-focused inflammation was located only in that area, there was occasional mild extension into the cranial forestomach. This restricted pattern of eosinophil distribution in the GI tract is strikingly similar to human EoE patients. It is also interesting to note the concurrent dermatitis and esophagitis in the Nik−/− mouse, as atopic dermatitis (AD) can be a co-morbidity of human EoE patients; however, whether the dermatitis of the Nik−/− mouse is
primarily an allergic phenomenon as it is in AD is unclear. Mast cells, another important inflammatory cell in EoE, were identified by toluidine blue staining (Figure 4F) and counter in the esophagus and GEJ. Counts were not significantly different in the esophagus of Nik−/− mice compared to wild-type (Supplementary Figure 4); however, the GEJ of Nik−/− mice showed a significant mastocytosis compared to wild-type (Figure 4G). The caudal glandular stomach is unaffected by inflammation, as well as the small and large intestines in age-matched controls (Supplementary Figure 3) where all mucosal architecture was within normal limits. The only instance in which mild eosinophilic inflammation is seen in the lower GI tract is in older animals (>6 mo) and remains both mild and more consistent with vascular spillover from generalized hypereosinophilia rather than any sort of specific mucosal targeting, such as what is seen in the esophageal lesions.

**Gene expression profiles in the esophagus of Nik−/− mice are similar to those observed in human EoE patients with special emphasis on thymic stromal lymphopoietin (TSLP)**

EoE is classically thought of as a Th2-mediated disorder characterized by increased cytokines, such as IL-4 and IL-13. Additionally, thymic stromal lymphopoietin (TSLP), a potent attractor and promoter of Th2 activity, has also been implicated in the pathogenesis of EoE with some patients showing gain of function mutations [35]. We evaluated esophageal mRNA expression of a wide panel of cytokines in our Nik−/− mice to further dissect the inflammatory microenvironment of their eosinophilic esophagitis. Esophageal tissue in Nik−/− mice displayed significant upregulation of prominent Th2 cytokines IL-4 and IL-13 (Figure 5A and 5B); however, there was no significant difference in IL-5 expression (Figure 5C). IL-1β, IFNγ and TNF were also upregulated (Figure 5D-F) potentially indicating an additional Th1 contribution to disease processes as well as support for generalized inflammation and fibrosis. There was no
significant difference in expression of TGFβ1, despite the impressive fibrosis seen in the
\(Nik^{-/-}\) mice. Thymic stromal lymphopoietin (TSLP) was evaluated at both the gene level and the
protein level due to its importance in Th2-mediated immune responses and eosinophilic
esophagitis in particular. Significant elevation of TSLP mRNA transcripts was detected in the
esophagi of \(Nik^{-/-}\) mice (Figure 6C) and subsequently immunohistochemistry was performed in
order to both confirm this finding at the protein level and evaluate the distribution and
localization of TSLP within esophageal tissue. \(Nik^{-/-}\) mice exhibited significant TSLP
immunoreactivity within the esophageal epithelium (Figure 6A-B), that was quantitatively
greater than wild-type (Figure 6D). This overall increase in TSLP immunoreactivity appears to
be due to the hyperplastic nature of the \(Nik^{-/-}\) esophageal mucosa, with increased numbers of
epithelial cells producing TSLP and therefore accounting for increased overall positivity of the
tissue as a function of area.

The noncanonical NF-\(\kappa\)B signaling pathway is significantly dysregulated in human EoE
patients.

There is currently a paucity of data pertaining to the role of the noncanonical NF-\(\kappa\)B
signaling cascade in modulating inflammation in the GI tract. The data from our \(Nik^{-/-}\) mice
suggest that inhibition of the noncanonical NF-\(\kappa\)B signaling pathway results in increased EoE
pathogenesis. However, the role of this pathway in human patients is un-explored and the
activation state of noncanonical NF-\(\kappa\)B signaling in EoE has yet to be evaluated. To address
these shortcomings and evaluate the relevance of animal model findings in human patients, we
conducted a retrospective analysis of gene expression data archived as a NIH GEO dataset from
human EoE patients and control patients [36]. This study evaluated gene expression on >31,666
transcripts from 6 healthy controls and 10 EoE patients. Data was normalized to the average
expression of 5 housekeeping genes and the fold change in gene expression in the EoE patients were compared against the healthy control biopsies. Our analysis revealed a significant up-regulation in specific genes associated with noncanonical NF-κB signaling (diagrammed in Figure 7A) in biopsies from EoE patients compared to the healthy controls. While several genes were significantly up-regulated, defined as >2 fold change in expression, in the EoE subjects, we found several genes greater than 100 fold increased such as NIK itself (629.16 fold), and NFkB2/p100 (412.31 fold) (Supplementary Table 1 and Figure 7B), and noncanonical receptors CD40 (180.17 fold) and LTβR (3010.012 fold) (Supplementary Table 1). Other key components of noncanonical signaling such as RELB and CHUK (IKKα) were also significantly increased (Supplementary Table 1 and Figure 7B).

Together, these data would suggest a significant increase in the transcription of downstream chemokines CXCL12, CXCL13, CCL19, and CCL21. Likewise, these observations run counter to our findings in the Nik−/− mice, where reduced noncanonical NF-κB signaling is correlated with increased disease progression. However, we did not observe a significant difference in genes directly downstream of noncanonical NF-κB pathway activation in the human EoE patients (Figure 7C). These findings can be reconciled by the corresponding findings that powerful negative regulators of the noncanonical pathway (diagrammed in Figure 7D) were also significantly up-regulated in the patient specimens (Figure 7E). Specifically, our data revealed significantly increased transcription of the genes encoding cIAP1/2, CYLD, NLRP12, HSP1, and CHIP in human EoE patients (Figure 7E). These regulators are known to potently inhibit multiple levels of the noncanonical NF-κB signaling cascade, including functioning either directly or indirectly as post-translational degraders and destabilizers of NIK, reviewed in [25]. Thus, it is possible that despite up-regulation at the gene level, blockade and
subsequent degradation of NIK at the protein level is able to outpace its transcription. This scenario would agree with the loss of functional NIK in our knockout mice and the similar EoE phenotype between the animal model and human patients. Also of note, we also found a significant upregulation in MAP3K14 (NIK) antisense RNA in the biopsy samples from the EoE patients, which may also be interfering with translation and further repressing this pathway (Figure 7F). Lastly, we analyzed NIK expression in the gastric antrum of eosinophilic gastritis patients and found that there was no significant different between patient populations (Figure 7G), highlighting the potential specificity of the esophageal mucosa in this disease process. Together, these data reveal the presence of a complex, dynamic regulatory environment associated with noncanonical NF-κB signaling during EoE and identify a large number of previously undefined mediators that appear to modulate disease progression in human patients.

Discussion

The noncanonical NF-κB pathway remains an understudied contributor to GI disease in general and particularly in its modulation of eosinophilic inflammation. Here, we not only present the Nik−/− mouse as a potential spontaneous model of eosinophil influx during EoE, but also identify dysregulation of the noncanonical NF-κB signaling cascade as a potentially significant contributor in disease pathogenesis. The similarity in microscopic features and severity in this spontaneous model is on par with the disease classically induced by Aspergillus and other allergen/agent induced models of EoE [21, 37, 38]. However, while the Nik−/− mice are excellent models of specific aspects of disease progression, there are certainly limitations as there are with all rodent models. For example, the Nik−/+ mice have increased circulating eosinophils [29] whereas human EoE patients typically do not present with high levels of systemic eosinophilia. Indeed, the ultimate progression of the Nik−/− mice to systemic HES
reflects broader mechanistic defects beyond the esophagus and is a limitation of this proposed model. However, the \( \text{Nik}^{-/-} \) mouse remains one of the few spontaneous models of esophageal eosophagitis, and demonstrates significant microscopic and gene expression patterns similar to the human disease.

The eosinophilic targeting of the esophagus and the concurrent sparing of the caudal GI system of the \( \text{Nik}^{-/-} \) mouse is intriguing given the categorization of HES as a systemic disease. It is possible that, given the fact that severe dermatitis is most often the clinical sign that leads to euthanasia, that lack of noncanonical NF-\( \kappa \)B signaling is essential for homeostasis in areas containing stratified squamous epithelium, such as the esophagus and skin in the mouse. This would be consistent with prior studies that revealed local hypersensitivity in transgenic mice that overexpress IL-5 in squamous epithelial cell compartments [39]. Since esophageal epithelial cells can act as nonprofessional APCs in EoE [40], lack of NIK may well be key in the epithelial-driven promotion of a Th2 microenvironment and the concurrent accumulation of eosinophils. Alternatively, lack of noncanonical signaling in either eosinophils or T lymphocytes may result in excessive targeting of squamous structures, with inflammation of other organ systems being a product of vascular spillover from peripheral hypereosinophilia. Given that the HES is abrogated when \( \text{Nik}^{-/-} \) mice are backcrossed onto immunodeficient mice lacking T and B cells, it appears that the implications of NIK loss in the adaptive immune system is the primary issue [29]. Since noncanonical NF-\( \kappa \)B signaling is intricately involved in lymphoid development and structure, it is tempting to speculate that dysregulated T cell biology is a major player in NIK-mediated EoE development. This concept is supported by recent studies focused on CD40L, a ligand in the noncanonical NF-\( \kappa \)B signaling cascade and is expressed on T cells. These recent studies revealed a strong correlation between CD40L and EoE pathogenesis [41]. Indeed, CD40L
expression, along with the expression of an array of additional, well-known proinflammatory cytokines, such as IL-4, IL-5, IL-13, and IL-17, were found to be highly efficient biomarkers of EoE pathogenesis [41].

A baseline structural or junctional defect in \( \text{Nik}^{-/-} \) squamous epithelial cells which attracts eosinophilic inflammation as a damage control mechanism, may also be a contributor to this process and remains to be investigated. Barrier defects in the esophagus have also been seen in human EoE [42, 43]. However, the lack of significant inflammation (compared to the esophagus and skin) in the squamous epithelium of the \( \text{Nik}^{-/-} \) mouse forestomach distal to the GEJ junction implies that there may be other factors at play besides simple epithelial type. Finally, EoE develops as a response to a dietary allergen or allergens, which on the surface is not a feature of our model. However, the potential sensitivity of \( \text{Nik}^{-/-} \) mice to allergens commonly found in standard rodent chow such as wheat and corn (allergens that are also human EoE triggers) has not been investigated. Given the Th2-prone, hyperinflammatory profile of the \( \text{Nik}^{-/-} \) mouse, predisposition to allergy may well be a feature. Future studies using diet alteration and oral allergen exposure in these mice would be interesting to pursue.

In terms of gene expression, the upregulation of inflammatory cytokines such as IL-4 and IL-13 that we observed in the \( \text{Nik}^{-/-} \) mice correlated well overall with expression patterns in human EoE, with the exception of a lack of significant differences IL-5 and TGFβ. Our characterization of TSLP in these mice was particularly rewarding given the importance of TSLP in both EoE and eosinophilic diseases as a whole. TSLP is primarily produced by non-hematopoietic cells such as fibroblasts and epithelial cells, although there is also evidence of mast cell, dendritic cell, and airway smooth muscle cell expression [44]. Upon introduction of an allergen, TSLP stimulates epithelial and stromal cells to produce T-cell-attracting substances and
promote neighboring dendritic cell maturation and activity. In EoE patients, TSLP expression is generally localized to the suprabasal layer of the esophageal epithelium and can be overexpressed or mutated in EoE patients [35, 45-48]. Indeed, cultures of differentiated esophageal epithelial cells alone can produce TSLP in reaction to food allergens, such as ovalbumin in vitro [45]. In terms of other tissues composed of stratified squamous epithelium, such as skin, expression of TSLP has been shown to contribute to disease in allergic dermatitis in both human patients and mouse models [49, 50]. In our mice, TSLP was strongly expressed in all layers of the epithelium in both wild-type and Nik null animals. Given the epithelial hyperplasia seen in the knockout animals, it appears that increased numbers of TSLP-expressing epithelial cells is the reason for the upregulation seen at the gene level rather than an increase in intensity per epithelial cell as seen by the similar staining density. Additionally, there appear to be small numbers of stromal cells, most likely fibroblasts or dendritic cells, within the submucosa of inflamed Nik−/− animals that may also be contributing to the increased expression.

While we have shown a strong Th2 phenotype in our mice that corresponds directly with human EoE, we also saw changes in other cytokines that are worthy of note, such as Th1 mediators IL-1β, IFNγ, and TNF, all of which can also contribute to inflammation and EoE pathogenesis. TNF is upregulated in EoE patients and tissue [10, 51], and is thought to play a role in fibrotic change along with IL-1β [52, 53], although these markers are not specific for EoE [41]. Similarly, IFNγ has been reported to be upregulated in EoE patients [54] and was increased in more modern transcriptomic studies as well [36]. Interestingly, CD8+ T cells from EoE patients also produce increased levels of this cytokine along with TNF [55]. Despite the significant fibrosis associated with this disease, there was not a significant upregulation in TGFβ, a very powerful and well-studied cytokine in esophageal remodeling and fibrosis in EoE [33, 34,
56], in our mice. However, even in classical EoE patients, TGFβ is not always upregulated [57, 58]. It is possible that fibrosis in the inflamed esophagus of \( \text{Nik}^{-/-} \) mice may occur through a TGFβ-independent mechanism, such as fibroblast growth factor 9 (FGF9) which is elevated in EoE patients [57, 59]. It is also possible that the fibrosis in our animals could be a primary defect associated with NIK loss rather than a cytokine-driven reaction to eosinophilic inflammation.

The upregulation of several key components of noncanonical NF-κB signaling in human EoE patients including NFκB2 (which encodes the p100 subunit), RELB (a p52 chaperone), NIK itself, and the essential kinase IKKα (encoded by CHUK) in human EoE biopsy samples initially appeared contrary to our findings in our \( \text{Nik}^{-/-} \) mice. This was further confounded by the lack of upregulation of noncanonical effector chemokines, which one would think to be quite upregulated given the increased expression of their promoters. One of the distinguishing features of noncanonical NF-κB signaling is its extensive reliance on post-translational events, including protein processing, stabilization, and ubiquitination that are not always correlated or reflected at mRNA levels. For example, NIK is regulated at the protein level by several other molecules, such as cIAP1/2 and NLRP12, and this stabilization (rather than expression) is key for downstream p100-p52 processing and subsequent nuclear translocation [26, 60, 61]. Interestingly, in the EoE patient data, we found a significant increase in gene expression for a diverse range of these negative regulators of noncanonical NF-κB signaling, especially those that target NIK. The most notable are cIAP1 and cIAP2, which bind to NIK to form a ubiquitin ligase complex along with TRAF proteins that eventually undergo proteosomal degradation [60, 62]. This degradation is potent enough that even in the face of active TNF stimulation, noncanonical NF-κB activity is suppressed [63]. Similar to increased cIAP1/cIAP2, we also found significant
upregulation of CYLD, HSPA1B, and FN14, which also exert significant negative pressure on noncanonical NF-κB signaling either directly or indirectly via targeting NIK. Given this large array of post-translational destabilizers that are upregulated, it is reasonable to suggest that the gene overexpression we see in NIK is a compensatory reaction to overactive and continual degradation of the NIK protein. This is in line with the lack of response we see in chemokine production associated with the noncanonical NF-κB cascade and would be consistent with the findings from our Nik−/− mice. Of course, analysis of NIK stability and additional studies of these negative regulators at the protein level in human specimens would be needed to confirm this potential mechanism, requiring the analysis of lysates from human patient biopsy samples. Another possibility for the discrepancy may be the method of tissue acquisition. Biopsy samples from human patients tend to be composed of epithelial layers only, while our sections involved the entire esophagus including submucosa and tunica muscularis and could therefore include changes exerted by stromal cells as opposed to a purely epithelial signature.

In conclusion, we have identified a novel signaling pathway in EoE that is dysregulated in human patients and associated with spontaneous development of eosinophilic esophageal inflammation in mice. We anticipate that future studies will better define the role of NIK and noncanonical NF-κB in EoE. Here, we have uncovered an extensive repertoire of genes that have not previously been associated with EoE that may serve as future therapeutic targets or biomarkers of disease progression. Likewise, the Nik−/− mice will be highly beneficial to study eosinophil, lymphocyte, and epithelial cell/stromal interactions associated with disease pathobiology. Noncanonical NF-κB signaling remains an under-characterized pathway in mucosal biology, particularly in GI disorders. However, it is becoming more apparent that this
pathway underlies a variety of mechanisms associated with aberrant inflammation in the gut and deserves greater scrutiny.
References


p. 2799-807.

29. Hacker, H., et al., NIK prevents the development of hypereosinophilic syndrome-like

30. Yin, L., et al., Defective lymphotoxin-beta receptor-induced NF-kappaB transcriptional

31. Shinkura, R., et al., Defects of somatic hypermutation and class switching in

32. Dellon, E.S., et al., A phenotypic analysis shows that eosinophilic esophagitis is a


34. Nhu, Q.M. and S.S. Aceves, Tissue Remodeling in Chronic Eosinophilic Esophageal
Inflammation: Parallels in Asthma and Therapeutic Perspectives. Front Med (Lausanne), 2017.
4: p. 128.

35. Sherrill, J.D., et al., Variants of thymic stromal lymphopoietin and its receptor associate

36. Sherrill, J.D., et al., Analysis and expansion of the eosinophilic esophagitis transcriptome


Figure 1: Lack of NIK in mice results in a hyperinflammatory phenotype. (A) *Nik−/−* mice develop progressive inflammation that leads to loss of subcutaneous fat stores and overall body condition (A; wild-type sibling, left, and *Nik−/−* right). They also develop a scaling, ulcerative dermatitis that most commonly affects the tail (B; WT left, *Nik−/−* right) and (C; WT left, *Nik−/−* right) the extremities. (D) A large proportion of *Nik−/−* mice require euthanasia by 12 weeks of age due to these changes. n = 25.
Figure 2: The Nik−/− mouse esophagus is specifically targeted by eosinophils and mimics human EoE histologically. (A-B; 20X, bar = 100µm) Compared to (A) wild-type littermates, (B) Nik−/− mice display marked eosinophilic inflammation in the esophagus in both the submucosa and epithelium accompanied by scattered lymphocytes. This change is often accompanied by basal cell hyperplasia (B, arrows) which was quantified as a percentage of overall epithelial thickness(C). (D; 20X, bar = 100µm) Eosinophils are quite prominent in the epithelial layer and occasionally form small microabscesses (arrow). (E) Eosinophils were enumerated at 5 independent points in each section and averaged, and were far above the 15-20 per HPF deemed necessary for diagnosis of EoE. (F; 100x, bar = 10µm) The identity of the cells
as eosinophils was based on nuclear morphology (biloced to donut-shaped), correct size (9-12µm), distinct, well-defined magenta granules, and positive reactivity to major basic protein (G; WT and H; Nik/-, 10X, bar = 200µm). Other features of esophagitis that were measured were severity and distribution of eosinophilic inflammation, esophageal erosion and keratin loss, and microabscess frequency, with Nik/- mice showing significantly increase histopathologic scores in all categories (I). Photomicrographs and graphs are representative of n = 5 WT and n= 9 Nik/- mice. H&E stain. Statistics were performed using the Mann-Whitney U test and significance set at p ≤ 0.05.
Figure 3: Fibrosis is a significant sequelae to eosinophilic esophagitis in Nik−/− mice. (A) Grossly, the esophagus of the Nik−/− mouse is circumferentially thicker than that of WT (arrows). (B-D; 20X, bar = 100µm) Esophageal sections were stained with trichrome, revealing a consistent and significant expansion of the submucosa (arrows) by fibrous connective tissue in (C-D) the Nik−/− mice as compared to (B) wild-type. (E; 20X, bar = 100µm) Thickness of the submucosal collagen deposition was measured using the tunica muscularis and the basal cell layer of the epithelium as borders (arrows). Trichrome stain. n = 5 WT, 7 Nik−/−. Statistics were performed using the Mann-Whitney U test and significance set at p ≤ 0.05
Figure 4: Microscopic changes of upper GI inflammation in Nik-/- mice is limited to the esophagus and gastroesophageal junction. (A-B; 10X, bar = 200µm) Compared to (A) wild-type littermates, (B) Nik-/- mice show gastric hyperplasia and localized foci of eosinophilic inflammation (C; 100X, bar = 10 µm). These changes are limited to the (D; 4X, bar =500µm) gastroesophageal junction and the immediately adjacent mucosa, and the thickening was statistically significant when quantified (E). Upon toluidine blue staining for mast cells (F; 100x, bar = 10µm) there were significantly increased mast cells present in the GEJ area. (G) For H&E staining and quantifications, n = 5 WT, 7 Nik-/- . For toluidine blue staining, n = 5 WT, 5 Nik-/- . Statistics were performed using the Mann-Whitney U test and significance set at p ≤ 0.05.
Figure 5: Gene expression in the Nik-/- esophagus reveals a gene expression profile that mimics human EoE. (A-D) Whole esophageal tissue was used for mRNA extraction and gene amplification. Nik-/- mice have significantly elevated expression of important Th2/EoE-relevant cytokines (A) IL-4 and (B) IL-13; however, there was no change in (C) IL-5. (D-F) IFNγ, IL-1β, and TNF expression levels were also significantly increased. Despite the fibrosis seen in the model, the expression of (G) TGFβ was not significantly different. For all genes, n = 8 WT, 14 Nik-/- . Statistics were performed using the Mann-Whitney U test and significance set at \( p \leq 0.05 \).
Figure 6: Thymic stromal lymphopoietin plays a significant role in NIK-mediated EoE. (A-B; 20x, bar = 100µm) Immunohistochemical staining for TSLP reveals significant expression in both WT and NIK-null epithelium. Upon quantification using image software (C) Nik−/− esophagi has greater overall expression, potentially due at least in part to epithelial hyperplasia. (D) TSLP was also upregulated at the gene expression level in whole esophageal tissue. Histology and image quantification; n = 5 WT, 6 Nik−/−. Gene expression; n = 8 WT, 14 Nik−/−. Statistics were performed using the Mann-Whitney U test and significance set at p ≤ 0.05.
Figure 7: Noncanonical gene expression patterns in human EoE patients implies blockage of NIK function. (A) Upon ligand-receptor interaction, NIK activates IKKα resulting in the proteolytic processing of p100 into p52 with subsequent nuclear translocation and promotion of effector chemokine gene expression. (B) Esophageal biopsies from human EoE patients show significantly upregulated expression of main noncanonical components NFKB2 (p100), NIK, RELB, and CHUK (IKKα); (C) however, there is no significant effector chemokine response, indicating a possible signaling disconnect. (D) Negative regulation of NIK takes place at the
protein level via ubiquitination and degradation and (E) all but one of molecules are simultaneously upregulated in human patients. (F) In addition, there is also significant upregulation of the MAP3K14 (NIK) antisense RNA, which may be interfering with proper transcription. (G) There is no significant change in MAP3K14 expression in the gastric antrum of eosinophilic gastritis patients. EG dataset = GSE54043 (n = 5 control, 5 EG). EoE dataset = GSE58640 (n = 6 control, 10 EoE). Statistics were performed using the Mann-Whitney U test and significance set at p ≤ 0.05.
Supplementary Information:

Table 1: Significant fold changes in molecules directly and peripherally related to noncanonical signaling in EoE patients

<table>
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<tr>
<td>RELB</td>
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Supplementary Figure 1: *Nik*−/− mice were bred heterozygously for the generation of wild-type and knockout littermates and routinely genotyped. An example 1.5% agarose gel showing several groups of mice (Lanes 1-16) with appropriate no template controls (Lane 17) and control/known wild-type DNA (Lane 18). “Wt” signifies *Nik*+/*+*, “het” signifies *Nik*−/+*, and “ko” signifies *Nik*−/−. Wild-type band = 270bp, knockout = 500bp.
Supplementary Figure 2: Mucosal proliferation is a feature of EoE in $Nik^{+/}$ mice

$Nik^{+/}$ mice exhibit and overall thickening of the esophageal mucosa. $N = 5$ WT, $9$ $Nik^{+/}$.

Statistics were performed using the Mann-Whitney U test and significance set at $p = 0.05$. 
Supplementary Figure 3: Eosinophilic inflammation in the GI tract is localized to the esophagus in $\text{Nik}^{-/-}$ mice. (A-D) Compared to (A) wild-type colon (20X bar = 100μm) and (C) small intestine (20X, bar = 100mm), the lower gastrointestinal tract of $\text{Nik}^{-/-}$ mice including the (B) colon (20X) and (D) small intestine (20X) was within normal limits. (E) Eosinophil counts (total number per 10 40X fields, n = 8 per group) in the lower GI were not significantly different between WT and $\text{Nik}^{-/-}$ and there was no mucosal architecture disruption. N = 8 WT, 8 $\text{Nik}^{-/-}$. H&E stain. Statistics were performed using the Mann-Whitney U test and significance set at p = 0.05.
Supplementary Figure 4: Mast cell densities in the inflamed esophagi of \( \text{Nik}^{-/-} \) mice are not significantly different that wild-type mice. Esophageal mast cell counts based on toluidine blue staining were not significantly different between WT and \( \text{Nik}^{-/-} \) mice. \( N = 5 \) WT, 7 \( \text{Nik}^{-/-} \). Mast cell counts are expressed as total number of mast cells in 5 40x fields for each individual sample. Statistics were performed using the Mann-Whitney U test and significance set at \( p = 0.05 \).
Chapter 4

Noncanonical NF-κB Signaling Is Upregulated in Inflammatory Bowel Disease Patients and is a Factor Associated with the Loss of Anti-TNF Therapy Response

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Abstract

Administration of anti-tumor necrosis factor (TNF) antibodies such as infliximab and adalimumab is a widely used and effective therapeutic strategy for inflammatory bowel disease (IBD). Unfortunately, a significant number of patients fail to respond or lose response to these agents. Previous studies have defined multiple complex roles for canonical NF-κB signaling in the pathogenesis of IBD. However, preliminary evidence presented here suggests that the lesser defined noncanonical NF-κB signaling pathway also contributes to disease pathogenesis and anti-
TNF antibody responsiveness. A total of 27 subjects with IBD (19 CD and 8 UC) and 15 non-UC/CD control subjects were collected at the Carilion Clinic in Roanoke, VA. Clinical criteria, patient history, and endoscopic disease activity were used to categorize patients and define therapeutic response. Biopsy specimens were collected and expression was determined for 89 target genes associated with NF-κB signaling and IBD. Noncanonical NF-κB signaling is significantly upregulated in IBD patients and is associated with increased gastrointestinal inflammation, lymphocyte migration, and Nod-like receptor signaling. Noncanonical NF-κB signaling is further upregulated in patients that are not responsive to anti-TNF therapeutics and suppressed in responsive patients. MAP3K14, NFKB2, CCL19, CXCL12, and CXCL13 are significantly dysregulated, as are genes that encode pathway regulators, such as NLRP12 and BIRC2/3. Our study identifies a previously uncharacterized role for the understudied noncanonical NF-κB signaling pathway in the pathogenesis of IBD and infliximab responsiveness. The genes and pathways identified may ultimately prove useful in future IBD therapeutic or biomarker applications.
Introduction

Inflammatory bowel disease (IBD) is a chronic and progressive disease characterized by ongoing inflammation in the gastrointestinal tract that is driven by elements of both the innate and adaptive immune systems in genetically susceptible individuals. Idiopathic IBD can be subdivided into two distinct forms, Crohn’s disease (CD) and ulcerative colitis (UC). Together these diseases afflict approximately 1.4 million Americans and over 4 million people worldwide, which makes IBD a significant global health care and economic burden.[1] The nuclear factor kappa B (NF-κB) family of transcription factors are master regulators of inflammation and drive a diverse spectrum of biological processes.[2] This signaling cascade is well studied in IBD pathogenesis and is commonly found dysregulated in IBD patients, leading to dysfunctional cytokine and chemokine production in the gastrointestinal tract. NF-κB signaling occurs through two distinct pathways defined as the canonical pathway and the noncanonical (or alternative) pathway.

In the context of IBD, the overwhelming majority of studies have focused on the canonical NF-κB signaling cascade. In the canonical pathway, signaling is rapid, constitutive, and relies on RelA/p65-p50 heterodimers. Activation results in the transcription of a wide range of well characterized inflammatory mediators such as IL-1β, TNF, and IL-6.[3] Unlike the canonical pathway, there is minimal data pertaining to the noncanonical NF-κB signaling cascade in IBD. Noncanonical NF-κB signaling is highly regulated at multiple levels, resulting in a slower, more controlled, and chronic signaling response as compared to canonical.[2, 3] The noncanonical pathway also relies on a different NF-κB heterodimer, comprised of p52 and RelB.[3] Lastly, noncanonical NF-κB initiates the transcription of a limited repertoire of target genes, including the chemokines CXCL12, CXCL13, CCL19, and CCL21[4, 5] compared to the wide range of
mediators produced by canonical NF-κB. While many of the biological functions associated with noncanonical NF-κB signaling are undefined, particularly in the context of mucosal immunology, its essential role in the development and organization of secondary lymphoid structures such as Peyer’s patches in the gut has been characterized.[6]

Prior research from our team using mouse models has revealed roles for dysregulated noncanonical NF-κB signaling in multiple gastrointestinal disorders. For example, in mice lacking the essential kinase NIK, the noncanonical NF-κB signaling cascade is functionally ablated.[7, 8] These animals spontaneously develop a hypereosinophilic-like syndrome that targets the upper gastrointestinal tract and effectively models many features of human eosinophilic esophagitis, or EoE.[7] Conversely, mice lacking negative regulators of noncanonical NF-κB signaling, such as the Nod-like receptor NLRP12, demonstrate increased pathway activation and are more susceptible to models of IBD and inflammation driven colon tumorigenesis.[3, 4] Based on the findings from these mouse models, it is clear that the noncanonical NF-κB signaling cascade significantly contributes to immune system homeostasis in the gut. However, additional mechanistic insight, clinical relevance in human patients, and the full realization of the utility of components of this pathway in improved therapeutics are still unknown.

Current therapies for IBD are continuously improving, as evidenced by the emergence of new classes of biologics including the anti-tumor necrosis factor (anti-TNF) targeted therapeutics. However, while anti-TNF therapies are effective in treating various forms of IBD, the approach remains suboptimal in many patient populations due to unpredictable remission rates.[9-11] Lack of remission can result from either primary non-response (10% - 40% in clinical trials and real-life cohorts) or from the loss of response associated with immunogenicity of the antibodies themselves and subsequent anti-TNF clearance (13% - 24% of patients within 1 year).[11] The
mechanisms underlying this resistance appear to be complex and are relatively undefined. Multiple studies have attempted to identify novel genetic and microbiome signatures for improved IBD diagnostics, prognostics, and biomarker identification that could be used to characterize patient responsiveness to anti-TNF therapeutics.[12-17] Taken together, there is a critical need for predicting therapeutic response and effectiveness in IBD patients to assist clinicians in treatment decision-making, minimize healthcare costs, and improve quality of life in IBD patients.

Several studies have explored the role of individual components associated with the noncanonical NF-κB signaling cascade in CD or ulcerative colitis (UC).[3] However, these individual components have always been explored in isolation with minimal exploration of the noncanonical pathway as a whole.[3] Based on our previous mouse studies[4], we hypothesized that noncanonical NF-κB signaling is significantly upregulated during IBD and is a factor associated with the lack of anti-TNF responsiveness in patients. Here, we used a combined analysis of both metadata and gene expression profiles acquired directly from our own IBD patient biopsies to evaluate our hypothesis. Collectively, our data provides evidence linking dysregulation of noncanonical NF-κB signaling with both IBD and anti-TNF responsiveness, which together further our knowledge regarding the underlying mechanisms involved in IBD.

Materials and Methods:

**Protection of Human Subjects:** All studies were conducted following the regulations, policies, and guidelines set forth by the National Institutes of Health for research involving human subjects. All studies were conducted under the approval of the Carilion Clinic Institutional Review Board (IRB) and Virginia Tech Carilion School of Medicine.
**Patient Selection:** A total of 27 subjects with IBD (19 CD and 8 UC) and 15 non-UC/CD control subjects deemed appropriate for colon biopsy (for inclusion and exclusion criteria, see Supplementary Table S1) were identified from patients currently being treated at Carilion Clinic by the clinical investigators. The 15 non-UC/CD controls were patients undergoing colonoscopy for conditions other than UC/CD (such as abdominal pain or bleeding) with the colonoscopy being negative for any pathology. Patients were asked if they are willing to receive information regarding participation in this study. Those wishing to participate were counseled and consented. Samples from each patient were obtained both from lesion and non-lesion areas as defined by the presence of endoscopically visible inflammation. Deidentified patient biopsies were stored in RNAlater at -80 °C. Patients were sorted into groups based on diagnosis (Crohn’s disease or ulcerative colitis), medication use, and response history (Supplemental Table S2). Control tissues were obtained from patients who were undergoing colonoscopy for reasons other than IBD, and samples were taken from endoscopically normal areas.

**Tissue Processing and Data Analysis:** Biopsy samples were removed from RNAlater, finely minced, and homogenized in RLT buffer (Qiagen) with 2-mercaptoethanol. Tissue was processed using an AllPrep Kit (Qiagen) following manufacturers protocols. RNA was analyzed for quality and concentration using a NanoDrop™. Pools were made using equal amounts of RNA from each patient sample for each experimental group. The total amount of RNA used for each cDNA reaction was 600ng and cDNA was generated using a First Strand Kit (Qiagen) following manufacturer’s protocols. Following cDNA preparation, gene expression was evaluated using a custom Qiagen RT² superarray following the manufacture’s protocols. The gene list for the custom superarray platform is provided in Supplemental Table S3. For each array, gene expression was determined utilizing the ΔΔCt method, using a panel of 5
housekeeping genes and internal controls provided on each array. Gene expression was determined using a 7500 Fast Block Real-Time PCR System (ABI). Data was analyzed using SA Biosciences Data Analysis Center and Ingenuity Pathway Analysis (IPA). Prior to assessments of pooled samples, quantitative rtPCR was also evaluated on select genes of interest for a selection of individual specimens. Complimentary DNA was generated from each specimen using an ABI High Capacity cDNA kit, in accordance with the manufacturer's protocols and 1 μg amplified using the Taqman-based rtPCR platform (ThermoFisher) on a 7500 Fast Block Real-Time PCR System (ABI). Gene expression was determined using the ΔΔCt method. All data were normalized to 18s and the fold change in gene expression was determined.

**Metadata Analysis:** Multiple publically available datasets were mined for this study using the following array data series (available through the National Center for Biotechnology Information: https://www.ncbi.nlm.nih.gov/): GSE6731; GSE10714; GSE16879; GSE107865; GSE10616; GSE72780; GSE75214.[18-24] Initial analysis on each dataset was conducted using either Nextbio™ or Genevestigator™. Data from the following 3 studies were further analyzed using IPA: GSE16879 (Biopsy, 61 IBD patients, 12 control patients, classified for response to infliximab); GSE107865 (PBMC, 22 CD patients, classified for response to infliximab); or GSE75214 (Biopsy, 75 CD patients, 11 control patients, classified by IBD type and activity).[20, 21, 24] All data was converted into fold change and log2 values using ACTB, B2M, and GAPDH to normalize between samples as previously described.[7, 25] Pathway analysis was conducted using IPA with settings limited to 2000 – 4000 genes for optimal performance and network generation, per software instructions.

**Statistical Analysis:** The number of patients were determined based on a power analysis conducted using findings from a retrospective metadata analysis of publically accessible gene
expression data from dataset GSE16879.[20] Gene expression was determined for a single marker of noncanonical NF-κB signaling (CXCL13) and the average and standard deviation of expression for this gene was used to determine the number of patients to target in our clinical study presented here. Based on our power analysis, 7 patients/group would provide a power of 92.4%, where a power over 80% is considered to be statistically acceptable. For gene expression analysis, data was analyzed using GraphPad Prism, version 6 (GraphPad Software, Inc., San Diego, CA). Student’s two-tailed t test was used for comparison of two experimental groups. Multiple comparisons were conducted using one-way and two-way ANOVA where appropriate followed by Tukey post-test for multiple pairwise examinations. Spearman correlation was computed using GraphPad Prism. Changes were identified as statistically significant if P was less than 0.05. Mean values were reported together with the standard error of mean (SEM).

Results:

Noncanonical NF-κB Signaling is Significantly Upregulated in CD Patients and is a Factor Associated with Loss of Response to the Anti-TNF therapeutic Infliximab

Dysregulated canonical NF-κB signaling has been well studied in IBD pathogenesis.[3] Indeed, the perpetuated activation of NF-κB signaling in human patients has been an attractive therapeutic target for various IBD intervention strategies and high NF-κB signaling activity has been associated with more severe histologic scores in CD patients.[26, 27] To initially address our hypothesis that noncanonical NF-κB is also significantly upregulated in CD patients, we evaluated data from 8 independent studies deposited in the GEO Database (Figure 1). These studies were selected based on the accessibility, type, and completeness of data deposited and the
availability of patient clinical data to support the expression analysis.[20, 21, 23, 24, 28-31] These studies evaluated gene expression in either biopsy specimens or peripheral blood mononuclear cells.[20, 21, 23, 24, 28-31] For each study, the complete datasets were collated, gene expression was normalized to internal controls, housekeeping genes, and control specimens as appropriate for each dataset/study and fold change in gene expression was determined for all genes as detailed in the Methods section. The data from each study were analyzed individually using Ingenuity Pathway Analysis (IPA) as previously described by the research team. [7, 25, 32]

Consistent with prior findings, our initial retrospective metadata analysis of publically available datasets found that NF-κB signaling was upregulated in all of the CD patient datasets evaluated, compared to either control subjects or biopsies from areas of the GI tract that were unaffected at the time of assessment (Figure 1). For example, we evaluated data from GSE75214, which consisted of microarray data obtained from mucosal biopsies from 8 CD patients and 11 controls.[24] The data from this study was originally utilized to show genetic and transcriptomic dysregulations of epithelial barrier genes, including mucus genes (MUC1 and MUC4), in IBD pathogenesis.[24] Our retrospective analysis of these data further expanded on the previous findings by revealing a link to NF-κB signaling (Figure 1A). Here we find that NF-κB signaling is the third most dysregulated pathway in the CD patient population with multiple genes directly or indirectly associated with the signaling cascade significantly upregulated (red) or downregulated (green) compared to the controls (Figure 1A). Significance in IPA was defined as genes with a +/- 5 fold change in gene expression, with limits set at +/- 135 fold in order to restrict the analysis to <6000 genes and optimize the computational assessments. Incidentally, consistent with the original study, we did find that MUC1 and MUC4 were significantly
dysregulated as previously described, but fell outside of the limits of our IPA analysis (data not shown). As expected, the majority of genes and pathways that were dysregulated were associated with canonical NF-κB signaling. However, a higher resolution analysis of the NF-κB signaling cascade revealed a significant upregulation of both the canonical pathway and noncanonical pathway (Figure 1A). Here, we were able to identify specific genes in each arm of the signaling cascade that were significantly upregulated or downregulated. Consistent with our hypothesis, multiple genes in the noncanonical cascade, including CD40L/CD40, LTA, BAFF, NIK, IKKα, NF-κB2, and RELB were significantly upregulated between 5- and 28-fold in CD patients (Figure 1A).

In addition to grouping patients by IBD sub-type, several datasets used in the present analysis additionally categorized patients by their responsiveness to the anti-TNF agent infliximab (Remicade®). Thus, we next sought to define the role of noncanonical NF-κB signaling in CD patients in the context of therapeutic response, specifically towards infliximab. Here, we focused on dataset GSE16879 (Biopsy, 61 IBD patients, 12 control patients, classified for response to infliximab).[20] Similar to the original analyses of these datasets, we also found significantly differences in gene expression between infliximab responders and non-responders (Figure 1B-E). Using the commercially available database analysis programs NextBio™ and Genevestigator™, we found that the chemokine CXCL13, which is a chemokine regulated by noncanonical NF-κB signaling[4, 5] was significantly upregulated in both CD (55 fold increase) and UC (33 fold increase) patients that were categorized as infliximab non-responders compared to the increases in this gene observed in the corresponding IBD patients that were responsive (Figure 1B). To build upon these initial findings, we analyzed dataset GSE107865 (PBMC, 22 CD patients, classified for response to infliximab) via IPA using the same cutoffs described
above. Here, we identified 2143 genes significantly dysregulated and unique to patients responding to therapy, 433 genes significantly dysregulated and unique to patients that were classified as infliximab non-responders, and 135 genes significantly dysregulated and shared between the 2 groups of patients (Figure 1C). Consistent with our original hypothesis, we identified 28 genes associated with the noncanonical NF-κB signaling cascade that were significantly dysregulated in CD patients that were classified as non-responsive to infliximab (Figure 1D). These genes encode mediators at all levels of the signaling pathway, including several positive and negative regulators of NIK (MAP3K14) (Figure 1E). MAP3K14 was significantly upregulated in patients that were not responsive to infliximab compared to responsive patients (Figure 1E). Conversely, several genes that encode proteins that negatively regulate noncanonical NF-κB signaling through impacting NIK function were significantly upregulated, while several gene associated with pathway activation were significantly downregulated in non-responsive patients (Figure 1E). These genes included NLRP12 (+61.734 fold); A20 (TNFAIP3; -44.073 fold); cIAP1 (BIRC2; -9.984 fold); cIAP2 (BIRC3; +9.067 fold), and STUB1 (CHIP; +18.181 fold) (Figure 1D-E). Together, these data illustrate the complexity of the level of regulation exerted on this pathway and the convergence of these regulatory factors on NIK in CD patients during infliximab treatment. Ultimately the 4 downstream chemokines best associated with noncanonical NF-κB signaling are all significantly upregulated in CD infliximab non-responder patients with CCL19 (+16.785 fold), CCL21 (+4.435 fold), CXCL12 (+6.221 fold), and CXCL13 (+4.184 fold) each showing significant changes. Together, these data show increased noncanonical NF-κB signaling across multiple previous studies and IBD patient populations, and this pathway is even further upregulated in CD patients that are unresponsive to infliximab.
Noncanonical Signaling is Upregulated at Multiple Levels in Our Crohn’s Disease Patient Population.

The metadata analysis identified multiple genes of interest in the noncanonical NF-κB signaling cascade that are dysregulated in CD patients and are factors associated with infliximab responsiveness. To build upon these initial retrospective findings, we collected biopsies from 27 IBD patients and 15 control subjects and began our focus with Crohn’s disease patients. We began with a brief initial assessment via individual real-time PCR of 4 IBD (CD or UC) patients and 4 control patients and performed individual real-time PCR on five genes associated with noncanonical NF-κB signaling: TNF, NIK, CXCL13, CXCL12, and a CXCR4 (the receptor for CXCL12) in both lesion and non-lesion tissue. We found that all of these genes has a strong trend towards upregulation in lesion tissue compared to non-lesion, indicating that noncanonical NF-κB is likely upregulated in our patient set (Figure 2A). We then used our full cohorts for superarray analysis, categorizing patients based on IBD diagnosis, clinical disease parameters, and anti-TNF antibody (infliximab or adalimumab) responsiveness (Supplemental Table S2). We chose to begin with assessing CD patients, as much of our initial metadata was from this IBD subtype, and focused initially on untreated CD patients in order to avoid any skewing of the data by treatment. RNA was extracted from each biopsy and gene expression (ΔΔCt) was evaluated using a custom designed superarray that evaluated ~80 genes, chosen based on the findings from the metadata analysis (Supplemental Table S3). We identified 33 genes associated with noncanonical NF-κB signaling that were significantly upregulated in the untreated CD population and 3 genes significantly downregulated (greater than 2 or less than -2 fold change) (Figure 2B). Consistent with the metadata analysis, these dysregulated genes encode for proteins
impacting every aspect of the signaling cascade, as represented in Figure 2C. In additional to gene relationships and pathways, IPA also has the ability to identify additional gene regulatory networks that are affected based on gene data entered. As expected, the top gene expression profiles significantly increased in our CD patients were gastrointestinal inflammation and IBD, which are consistent with the CD diagnosis (data not shown). However we also identified a significant upregulation in gene expression networks associated with naïve lymphocyte migration (Figure 2D), indicating that this pathway is a key effect of elevated noncanonical signaling in CD patients.

**Signaling Networks Associated with Pattern Recognition Receptor Signaling are Also Significantly Upregulated in CD Patients**

In addition to these altered signaling networks, we also observed a significant increase in pattern recognition receptor signaling in the untreated CD patients that were directly impacted by the changes in noncanonical NF-κB signaling (Figure 3). Specifically, signaling networks associated with the Nod-Like Receptor (NLR) family of pattern recognition receptors was significantly upregulated, ranking in as the fifth most impacted signaling network (Figure 3A). The NLR family can be functionally divided into 3 distinct sub-groups, including inflammasome forming NLRs, positive regulatory NLRs, and negative regulatory NLRs.[33] Members from all three sub-groups have been implicated in IBD pathogenesis.[34] In our CD patients, the dysregulation of TNF activation resulted in a significant increase in gene transcription associated with the NLRP1, NLRP3, NLRC5, and NLRP6 inflammasomes (Figure 3A). Previous studies have found that inflammasomes are associated with IBD pathogenesis through the regulation of IL-1β and IL-18 generation, cell death, and maintenance of the microbiome composition.[34]
Likewise, NOD1 (NLRC1) and NOD2 (NLRC2) were also found upregulated in our CD patients (Figure 3A). Multiple studies have previously associated these NLRs with IBD pathogenesis, with NOD2 being one of the most cited genes associated with CD.[34] Both NOD1 and NOD2 function as regulatory NLRs and augment NF-κB signaling following TNF activation.[34] Conversely, NLRP12 and NLRC3 were also found significantly upregulated in our patients. Both of these NLRs function as negative regulators of either canonical NF-κB (NLRC3) or noncanonical NF-κB (NLRP12) signaling, with dysfunctional NLRP12 also found to be associated with experimental colitis.[34] In sum, the identification of multiple NLRs upregulated during CD and associated, either directly or indirectly, with increased noncanonical NF-κB signaling suggests a strong connection between pathways.

In addition to our initial assessments of NLR signaling in CD, we also found that changes in the NLR pathway was strongly associated with anti-TNF antibody responsiveness (Figure 3). The expression of each NLR was individually evaluated based on patient treatment and responsiveness (Figure 3B). Here, we observed a significant decrease in the inflammasome associated NLRs, NLRP1, NLRP3, and NLRP6 in patients that were responsive to anti-TNF antibodies compared to other CD patients (Figure 3B). Conversely, in patients that were classified as anti-TNF antibody nonresponders, we observed a significant increase in NLRP6 and NLRP3 compared to other CD patients (Figure 3B), with the exception of a significant increase in NLRP3 signaling in patients treated with other non-TNF targeted treatments (Figure 3B). Conversely, we observed a significant decrease in NLRP12 in anti-TNF antibody nonresponder patients (Figure 3B). This loss of NLRP12, which is a potent negative regulator of noncanonical NF-κB signaling, was strongly associated with the loss of anti-TNF antibody responsiveness in our CD patient populations. Interestingly, we also observed a significant decrease in NLRX1
expression in all of the CD patients compared to control subjects (Figure 3B). NLRX1 functions as a negative regulator of canonical NF-κB signaling and mice lacking NLRX1 demonstrate increased experimental colitis severity associated with dysfunctional effector and metabolic functions in CD4+ T cell populations.[35]

The IPA network predictions further confirmed the association between dysregulated NLR signaling and anti-TNF antibody responsiveness. As shown in Figure 3C, in CD patients with no treatment we observed a significant increase in specific members of all three NLR sub-groups. Likewise, we observed a similar increase in CD patients being treated with non-TNF targeted therapeutics (e.g. corticosteroids) (Figure 3C). However, in this group of patients, we observed a significant increase in NLRP3 inflammasome signaling, over the levels observed in the untreated patient population (Figure 3C). The most significant differences in NLR signaling were associated with patients in the anti-TNF antibody responder and nonresponder populations (Figure 3C). Here, we observed significant increases in NLRP6 inflammasome signaling pathways in patients that were unresponsive to anti-TNF antibodies, whereas NLRP3 inflammasome signaling was up regulated but not significantly different between groups of responsive and unresponsive patients (Figure 3C). Conversely, we also observed a significant decrease in NLRP12 signaling in CD patients that were unresponsive to anti-TNF antibody treatment (Figure 3C). Interestingly, in addition to NLRP6 and NLRP12, we also observed a significant difference in NLRP4 signaling in nonresponders (Figure 3C). NLRP4 is an under-characterized NLR that has been implicated in inflammasome formation, but better defined for its role as a regulator of type I interferon signaling.[36] Together, these data identify multiple members of the NLR family that are dysregulated during CD and in the context of anti-TNF antibody responsiveness. Many of the NLRs identified function as potent regulators of canonical
and noncanonical NF-κB signaling, suggesting some potential mechanisms underlying signaling dysfunction in these pathways.

Noncanonical NF-κB Signaling is Significantly Upregulated in IBD Patients that are Unresponsive to Anti-TNF Therapy

Our data identified 38 genes both directly and indirectly related to noncanonical NF-κB signaling cascade that are significantly upregulated in CD and the pathway was identified as a significant gene regulatory network associated with IBD pathogenesis (Figures 1 - 2). Likewise, our metadata analysis further indicates that upregulation of noncanonical NF-κB signaling over levels observed in other CD patients is associated with anti-TNF antibody responsiveness, as is the dysregulation of genes encoding both positive and negative regulators of this signaling cascade (Figure 1 and 4). Here, we evaluated these observations in our own patient populations, and combined both CD and UC patients under the heading of inflammatory bowel disease (IBD) patients, given the fact that anti-TNF therapy is a component of treatment for both diseases. All fold changes can be found in Supplementary Table S4. Consistent with the metadata findings, we observed a significant upregulation of genes in the noncanonical NF-κB signaling cascade in IBD patients that were unresponsive to anti-TNF antibody therapy (Figure 4A). We also identified 17 genes significantly upregulated and 7 genes significantly downregulated in IBD patients that were responsive. Downregulated genes included noncanonical chemokine CXCL13 (-3.25 fold change) and noncanonical receptor for CXCL12, CXCR4 (-2.84 fold change) (Figure 4A). Conversely in nonresponder patients, we identified 39 genes associated with noncanonical NF-κB signaling that were significantly upregulated and only 2 genes significantly downregulated (TLR3, -2.43 fold change and BCL2L1, -2.16 fold change) (Figure 4A). Notably,
in contrast to responder patients, CXCL13 was significantly upregulated in nonresponders (+18.77 fold). When evaluated together, pathway analysis revealed significant downregulation of noncanonical NF-κB signaling in the anti-TNF antibody responsive IBD patients compared to IBD patients that were not being treated with any medications (Figure 4B). Indeed, this responder population also showed significant decreases in noncanonical NF-κB signaling compared to the IBD patients treated with non-TNF targeted treatments and the anti-TNF antibody nonresponders (Figure 4B). The decreased expression occurred in genes across all levels of the pathway, and specifically included reduced expression of MAP3K14, NFKB2, TRAF3, STAT3, CXCL12, CXCL13, CXCR4, and CCR7 that were not observed in the other IBD patient groups (Figure 4B). While noncanonical NF-κB signaling was significantly downregulated in responders, the converse was true for nonresponders compared to the IBD patients that were in the no treatment group (Figure 4B). Here, the pathway analysis revealed a significant upregulation of noncanonical NF-κB signaling in these nonresponder IBD patients that was strongly associated with downregulation of NLRP12 and upregulation of MAP3K14, CXCL12 and CXCL13 (Figure 4B). Together, these data confirm many aspects of the metadata analysis and reveal that noncanonical NF-κB signaling is both significantly decreased in patients responsive to anti-TNF antibody therapy and conversely increased in unresponsive IBD patients.

**CXCL12 and CXCL13 Expression is Strongly Correlated with Anti-TNF antibody Responsiveness**

Currently, only four major chemokines have been associated with the noncanonical NF-κB signaling cascade, CCL19, CCL21, CXCL12, and CXCL13.[3] Throughout our studies, we found these chemokines significantly upregulated and correlated with disease (Figures 1-4).
Additionally, our metadata analysis revealed that CXCL13 upregulation was strongly associated with infliximab responsiveness in both CD and UC patients (Figure 1B). Consistent with the metadata analysis, the pathway analysis of the gene expression patterns from our IBD patients further confirmed that all four of these chemokines were significantly upregulated in IBD patients that were in either untreated or treated with non-TNF targeted therapeutics (Figure 4B). Likewise, IBD patients that were responsive to anti-TNF therapy demonstrated significant decreases in CXCL12, CXCL13, and CCL21 compared to those in the no treatment group (Figure 5A). Again consistent with the metadata analysis, CXCL12 and CXCL13 were significantly upregulated in patients that were non-responsive to anti-TNF antibodies (Figure 4B and 5A). However, CCL19 and CCL21 were downregulated in our nonresponder patient populations (Figure 4B and 5A). Based on the expression profile associated with these chemokines, migration of naïve lymphocytes is the signaling network most likely impacted by the combination of expression patterns associated with anti-TNF antibody therapy responsiveness (Figure 5B). Responders are predicted to have a decrease in lymphocyte migration, whereas nonresponsive patients are predicted to have an increase, which is highly consistent with the pathogenesis of IBD in these patient populations. Both CXCL12 and CXCL13 are relatively understudied in IBD pathogenesis, with only a small number of studies evaluating these chemokines. However, our findings suggest that they are significant factors in disease progression and therapeutic responsiveness.

Discussion:

Noncanonical NF-κB signaling has been recognized as a key regulator and promoter of both this innate and adaptive immune systems, particularly in the development of secondary
lymphoid organs. However, its role in inflammation of mucosal tissues and clinical presentation of gastrointestinal disease has not been explored. Here, we show that noncanonical NF-κB signaling and the chemokines associated with pathway activation are associated not only with inflammatory bowel disease itself, but also patient response to treatment. Molecules associated with all levels of the noncanonical NF-κB signaling cascade are upregulated in lesion tissue from IBD patients, and remain upregulated in those patients unresponsive to anti-TNF antibody treatment. The key markers identified were CXCL12 and CXCL13, which were both identified in retrospective metadata analyses as well as within our own patient population. Given the complexities with managing loss of response to anti-TNF inhibitors, novel targets or markers for therapeutic effectiveness are desperately needed. Here, we present noncanonical NF-κB and the products of pathway activation as unique and novel markers associated with responsiveness to anti-TNF therapy in IBD patients, as well as identify this signaling pathway as a new contributor to the pathogenesis of IBD. Given this pathway’s involvement in both immune cells and epithelial cells, it follows that upregulation may have a variety of effects not only on the severity or behavior of lesion formation, but also the patient response to treatment. Indeed, multiple levels of noncanonical NF-κB signaling have been associated with inflammatory bowel disease, from ligand-receptor interaction to chemokine products[3]; in this work, we tie together that pathway as a whole and emphasize potential clinical relevance to treatment response

CXCL12 and CXCL13, two of the most well-known noncanonical effector molecules, are expressed in many cell types including intestinal epithelial cells.[37, 38] CXCL13 has been studied predominantly in the area of B cell development, dendritic cell activation, germinal center management, and T cell trafficking.[39-43] Its association with B cells is especially strong, as its interaction with the receptor CXCR5 has been shown to be essential for B cell
migration in lymphoid follicles and is highly expressed in Peyer’s patches, lymph nodes, and the spleen.[39] Given these essential physiologic effects, CXCL13 has become a protein of interest in diseases such as lupus, where B cells play an important role.[44-46] In fact, CXCL13 has been proposed as a biomarker for lupus nephritis, one of the most severe manifestations of the disease.[44] Increased CXCL13 expression has also been seen in other autoimmune diseases such as rheumatoid arthritis[47, 48], Sjögren's disease[49], myasthenia gravis[50], and autoimmune thyroiditis[51]. In rheumatoid arthritis, both CXCL13 and CCL19 are being pursued as biomarkers for drug response, B cell kinetics, and remission likelihood.[47, 52]. In fact, an anti-CXCL13 antibody was effective in reducing inflammation in mouse models of rheumatoid arthritis, multiple sclerosis, and experimental autoimmune encephalitis, further underscoring its potential utility as a treatment target in inflammatory bowel disease.[53] In ulcerative colitis, CXCL13 expression has been found to be upregulated in inflamed tissue and both normal and aberrant lymphoid tissue[54] as well as serum of untreated pediatric IBD patients.[55] However, its exact role and importance in IBD and treatment methodologies has not been pursued until now.

CXCL12, which is also known as stromal cell-derived factor-1 (SDF-1) or pre-B-cell-growth-stimulating factor (PBSF) was first recognized as a chemokine important to the growth and stimulation of B cell precursors[56], similar to CXCL13. CXCL12 and its receptor CXCR4 are essential in B cell development, hematopoiesis, and HSC migration[57], with deficient mouse models showing significant defects in these processes.[58, 59] More recently, this CXCL12-CXCR4 axis has been implicated in mesenchymal and epithelial cell behavior. In particular, its relationship with the phenomenon known as epithelial to mesenchymal transition and its relation to various cancers, including epithelial cancers.[60-62] EMT has been long
studied in cancer pathobiology, and is characterized by a change in a normal static, polarized epithelial cell to assume a more migratory and mesenchymal physiology. Characteristics of this switch include increased production of extracellular matrix, increased migratory ability, and, in the case of cancer cells, invasion and metastasis[63]. EMT does not only play a role in cancer metastasis, but is also important in wound healing, inflammatory responses, and fibrosis of inflamed tissue.[63]. Indeed, there is upregulation of EMT related molecules in fibrotic tissue and fistula formation from Crohn’s disease patients[64, 65], which increased CXCL12 expression may well be connected to given the risk of fibrosis in nonresponder patients.

CXCL12 has, interestingly, been found to have 3 significant polymorphisms associated with IBD in a cohort of Polish IBD patients[66]. During active disease, the mucosa of IBD patients has been shown to have increased CXCL12, and circulating plasma cells increased CXCR4[37] [38]; however, as with CXCL13, it has not been well-characterized in IBD and not in the context of the major pathway it is a product of.

We also found that Nod-like receptors were significantly involved in the pathogenesis of IBD, and found that upregulation of these diverse pattern recognition receptors may play a important part in disease in our patient population. Nod-like receptors function as sentinels for sensing pathogen-associated molecular patterns (PAMPS) and damage-associated molecular patterns (DAMPS) in the gut. The most well-know IBD-related Nod-like receptor that has been studied in IBD that was upregulated along with noncanonical signaling in our model and patient population are is NOD2. NOD2 mutations are most typically associated with an increased risk of Crohn’s disease[67-69] and patients with these mutations are at increased risk for downstream sequelae such as increased risk for requiring surgical intervention and development of stricture.[70] The connection between NOD1 and IBD is slightly more nebulous. A
insertion/deletion variant of NOD1 has been shown to increase IBD risk[71, 72], however results are inconsistent.[73] Several inflammasome-forming NLRs were also noted as upregulated in our patient population, including NLRP3, NLRP6, and NLRC4. Variants in NLRP3 have been linked to susceptibility to Crohn’s disease[74], however these associations are not always strong.[75] NLRP6, on the other hand, has also been shown to control susceptibility to colitis as well as microbiome.[76] Indeed, mice lacking NLRP6 display increased susceptibility to DSS-induced colitis as well as develop a spontaneous colitis [76, 77]. NLRP4 is a little-known NLR that has been associated with interferon signaling and negative regulation of autophagy[36, 78], although its relation to inflammatory bowel disease may be of future interest. We also found changes in the expression of the important noncanonical regulatory NLR NLRP12, which is downregulated in responder patients and upregulated in nonresponders along with its downstream noncanonical molecules. Lack of NLRP12 and increased noncanonical signaling has been shown to exacerbate colitis and colitis-associated cancer in mice when it is deleted.[4] This phenotype appears to be due to the resulting increase in noncanonical signaling due to lack of negative regulation, particularly through overexpression of NIK and noncanonical chemokines.

In humans, mutations in NLRP12 have been associated with autoimmune disease[79-81], however its effects in IBD as a regulator of noncanonical signaling have not been explored. Given its importance in regulating noncanonical signaling, it may well have a significant influence on our patient population via its ability to rein in noncanonical chemokine expression, such as that of CXCL13 and CXCL12.

As TNF is a major activator of noncanonical NF-κB, anti-TNF antibody failure due to overwhelming TNF production or interference from entities such as anti-drug antibodies may be responsible for noncanonical stimulation due to excess TNF availability. Another factor at play
in these patients may be hyper-responsiveness of the lymphoid populations in the colon as a whole. Given noncanonical NF-κBs close relationship with the maintenance of Peyer’s patches and lymphoid organization, its increased expression may be a reflection of mucosal hyper-reactivity in these patients which would predispose them to continued inflammation, immune cell recruitment, and tissue damage. In particular, elevated levels of CXCL13 and CXCL12 can result in a vicious cycle of increasing positive feedback loops of T and B cell activation, which may not only result in generalized inflammation caused by resident cells but also increased trafficking of these cells to the colon from peripheral lymph nodes. This change is supported by our findings of a predicted increase in lymphocyte trafficking via IPA. This increase in the adaptive immune response may also be a factor in the development of anti-drug antibodies, which is a significant negative prognostic indicator of treatment responsiveness in patients. Of course, these findings are somewhat of a chicken-and-egg scenario. It is plausible that increased in noncanonical signaling is due to extrinsic reasons such as drug failure and nonspecific inflammatory response, or also that it is an innate overactivity of noncanonical signaling that causes patients to become nonresponsive. However, it remains a fact that noncanonical signaling is a robust marker of nonresponse.

As with any study, ours has several limitations. Our data relies on gene expression profiling which, while thorough, does not address protein levels and post-translational modifications of the proteins produced by the genes of interest. Given the importance of post-translational processing in noncanonical signaling, including the ubiquitin-mediated degradation of NIK and the conversion of p100 to p52, this may be an area of further study. Another limitation is the relatively small pools of patients used to generate our own data. However, through the use of metadata from other peer-reviewed studies, we supported our findings in light
of other larger studies. Biopsies from human IBD patients also contain a mix of cell types, including the epithelial cells of the colon itself, mesenchymal cells, and inflammatory cells. Based on the complexity of tissue biopsy structure, it cannot be fully determined if the effects in noncanonical signaling are due to changes in expression in epithelial cells, infiltrating immune cells, or support cells. Given that the vast majority of biopsy tissue is composed of epithelial cells, we propose that this may well be a major component of the expression profile, which opens many new doors into noncanonical function outside of immune cells. Further studies using organoids grown from human biopsy samples may allow us to fully determine noncanonical signaling’s role in an epithelial-cell-only model from these patients in the future. From a clinical perspective, molecules such as CXCL13 have already become of interest in other autoimmune disease such as lupus. Both it and the noncanonical pathway that controls its expression may have utility not only as a biomarker, but also as a target for immunotherapy for other diseases of immune dysregulation such as IBD.

**Conclusions:**

Noncanonical NF-κB signaling has been recognized as a key regulator and promoter of the adaptive immune system, particularly in the development of secondary lymphoid organs. However, its role in inflammation of mucosal tissues and clinical presentation of gastrointestinal disease remains understudied. Here we show that noncanonical NF-κB and the chemokines it produces are associated not only with inflammatory bowel disease itself, but also patient response to treatment. Noncanonical molecules are upregulated in lesion tissue from CD patients, and remain upregulated in CD and UC patients unresponsive to anti-TNF antibody inhibitors. This effect is particularly strong in Crohn’s disease patients, where the key marker CXCL13 was
identified both in retrospective metadata analysis as well as within our own patient population. Given the complexities with managing loss of response to anti TNF inhibitors, novel targets or markers for therapeutic effectiveness are always under investigation. Here we present noncanonical NF-κB and CXCL12/CXCL13 as unique and novel new markers for loss of response to biologic treatment in IBD, as well as a new contributor to the pathogenesis of the disease as a whole.
References:


Figure 1. Metadata analysis revealed a significant upregulation of genes associated with noncanonical NF-κB signaling in CD and treatment response. Using data from GSE75214 (8 CD patients and 11 controls) we determined that there were significant dysregulation (upregulated in red, or downregulated in green) in signaling in both the canonical and noncanonical NF-κB pathways (A). Using NextBio™ and Genevestigator™ to analyze GSE16879 (Biopsy, 61 IBD patients, 12 control patients, classified for response to infliximab) we found that CXCL13 was significant upregulated in both patient subsets that were nonresponders to infliximab (B) Using GSE107865 (PBMC, 22 CD patients, classified for response to infliximab) we identified 2143 genes significantly dysregulated (either upregulated or downregulated) in responder patients, 433 genes dysregulated in nonresponder patients, and 135 genes significantly dysregulated in both categories of patients (C). Of these dysregulated genes in CD patients, 28 were associated with noncanonical NF-κB signaling cascade and were unique to nonresponder patients (D). NIK, the central molecule of noncanonical signaling (also called MAP3K14, +25.933 fold change) as well as NLRP12 (+61.734 fold); A20 (TNFAIP3; -44.073 fold); cIAP1 (BIRC2; -9.984 fold); cIAP2 (BIRC3; +9.067 fold), and STUB1 (CHIP; +18.181 fold) were also affected in this dataset (D-E). Data are presented as the fold-change values of affected tissue from patients compared to normal controls. *, +, #, $ = p<0.01.
Figure 2. Noncanonical NF-κB Signaling, under the control of NIK, is upregulated in intestinal biopsies from Crohn’s disease patients. Using biopsies from our own patient population, we conducted an initial scan of noncanonical signaling patterns using 4 IBD patients.
(lesion and non-lesion tissue from the same patient) and 4 control patients (A). There were strong trends towards upregulation in TNF, NIK, noncanonical chemokines CXCL13 and CXCL12, and the chemokine receptor CXCR4 in IBD lesion tissue. We then analyzed noncanonical NF-κB expression from the tissue of untreated Crohn’s disease patients (n = 2, one pool) compared to controls (n = 15, 3 pools). We found over 30 genes related to noncanonical NF-κB to be upregulated, with yellow indicating upregulation and blue indicating downregulation (B; genes outside of dashed lines are considered significantly upregulated). We analyzed all levels of noncanonical signaling in these untreated CD patients, from ligand-receptor interaction to chemokine production, and found multiple areas of upregulation including the bottleneck molecule NIK (MAP3K14) which regulates noncanonical signaling (C). Additionally IPA predicted a significant (orange = upregulation) increase in lymphocyte migration in our untreated CD patient tissues (D).
Figure 3

A.

B.

C.

No Treatment

Inflammation (NLRP3, ASC, Casp1)
Inflammation (NLRP3, ASC, Casp1, Casp9)
Inflammation (NLRP3, ASC, Casp1, Casp9, Casp9)

Non-TNF Targeted Treatment

Inflammation (NLRP3, ASC, Casp1)
Inflammation (NLRP3, ASC, Casp1, Casp9)
Inflammation (NLRP3, ASC, Casp1, Casp9, Casp9)

Anti-TNF Responders

Anti-TNF Nonresponders
Figure 3. Nod-like receptors and their related pathways are upregulated in untreated Crohn’s disease patients and reflect treatment response. Here we analyzed the gene expression networks involved in NLR signaling, and found that a large number of inflammasome-forming, non-inflammasome forming, and negative regulatory NLRs are dysregulated (A). When separated out into treatment groups, anti-TNF nonresponder CD patients (n = 8, one pool) showed consistent upregulation in NLR signaling including NLRP1, NLRP3, and NLRP6 compared to control (n = 15, three pools) (B). These elevations were also present in untreated CD patients (n = 2, one pool) and non-anti-TNF treated CD patients (n = 5, one pool); however, in responder CD patients (n= 4, one pool), this elevation was ablated (B, C). NLRP12, a negative regulator of noncanonical signaling, was downregulated in nonresponder patients, consistent with our previous data of increased noncanonical signaling (B, C).
Figure 4. Nonresponder IBD patients show increased noncanonical signaling. Lesion tissue from nonresponder IBD (CD and UC combined) patients (n = 10, two pools) displayed over 30 upregulated genes related to noncanonical signaling, compared to only 17 in responder IBD patients (n = 5, two pools) (A). We then focused specifically on the core of noncanonical signaling, using the bottleneck molecule NIK as our entry point. In nonresponder IBD patients, expression levels of NIK, CXCL12, and CXCL13 were similar to both untreated patients (n = 2, one pool) and patients treated with non-anti-TNF medications (n=10, two pools). Responder
patients (n=5, two pools), however, showed a distinct attenuation of expression of all four noncanonical chemokines along with NIK itself (B).
Figure 5

A. 

B. 

Migration of naive lymphocytes

Anti-TNF Responders

Anti-TNF Nonresponders
Figure 5. Major noncanonical chemokines show distinct patterns based on treatment response in IBD patients. CXCL12, CXCL13, CCL19, and CCL21 all show significant upregulation in both untreated IBD patients (n = 2, one pool) and patients treated with medications other than anti-TNF biologics (n = 10, two pools) (A). When examining the expression patterns between responder (n = 5, two pools) and nonresponder (n = 10, two pools) patients, we discovered that CXCL12 and CXCL13 display significant and opposite expression profiles based on response, with upregulation occurring in nonresponder patients and downregulation occurring in responder patients (A). CCL21 remained downregulated in anti-TNF therapy regardless of responsiveness. As many of these noncanonical chemokines are involved in lymphocyte trafficking, we then evaluated the predicted effect on the migration of naïve lymphocytes to tissue based on response. We found that in responder patients, lymphocyte trafficking was reduced (B). In nonresponder patients, we found that this increase in noncanonical signaling molecules resulted in a large predicted increase in lymphocyte migration (B). Blue and orange dashed lines with arrows indicate indirect inhibition and activation, respectively. Yellow and gray dashed lines with arrows depict inconsistent effects and no prediction, respectively.
Supplemental Table S1.

**Inclusion Criteria:**
(1) Subject is >18 years of age (IBD and non-IBD patients);
(2) Subject diagnosed with Crohn’s disease or Ulcerative colitis as established by standard criteria (IBD patients only);
(3) Subject not on NSAID’s or other GI toxic medications (IBD and non-IBD patients);
(4) Subject has been scheduled for a colonoscopy and is deemed fit for the biopsy procedure (IBD and non-IBD patients);
(5) Subject has no other known upper and/or lower GI tract diseases (IBD and non-IBD patients), including celiac disease, food allergies, microscopic colitis, infections;
(6) Subject has no contraindications to endoscopy (IBD and non-IBD patients)

**Exclusion Criteria:**
(1) Subject is <18 years of age;
(2) Subject is pregnant, nursing or planning a pregnancy during the study period;
(3) Subject has any medical problems deemed by the investigator to interfere with the study
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Chapter 5

Noncanonical NF-κB Controls Stem Cell Signatures in the Colonic Mucosa and Affects Susceptibility to Inflammation-Induced Carcinogenesis

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Abstract:

Nuclear factor kappa-b (NF-κB) signaling has long been established as a major player in not only inflammation but also cell growth, division, and neoplasia. While the canonical arm of NF-κB is the most well-studied component of this pathway with effects in a variety of cell types, there is a second alternative pathway that until recently has remained somewhat of a mystery in terms of its part to play in mucosal immunology. Noncanonical NF-κB signaling is a unique offshoot of the typical canonical NF-κB pathway with a distinct cascade compared to its canonical counterpart. Noncanonical signaling has been classically thought of as a pathway with
importance restricted to the lymphoid system. Indeed, many of the chemokines it produces have been primarily investigated in the context of the development of the adaptive immune system and secondary lymphoid structures such as lymph nodes and Peyer’s patches. Recently, interest in noncanonical NF-κB’s association with mucosal and epithelial immunology has been increasing, with emphasis on its bottleneck molecule NF-κB inducing kinase (NIK). Our data shows that lack of noncanonical signaling through knockout of NIK in mice results in an aberrant large intestine phenotype characterized by reduced stem cell marker expression, altered regeneration and differentiation capacity under stress, changes in microbiome, and predisposition to inflammation-associated tumorigenesis. Dysregulation of noncanonical signaling is also seen in human colorectal cancer patients, which have marked suppression of this pathway in colonic biopsy samples. Here we present a novel and as of yet undescribed role for the alternative NF-κB pathway and NIK in the stem cell niche of the colon that could have many downstream implications in gastrointestinal disease.
Introduction:

The NF-κB pathway, which is responsible for the production of a host of effector molecules involved in inflammation, proliferation and survival, is a well-studied pathway in a large variety of human diseases[1-5]. The most commonly studied arm of NF-κB signaling is the canonical arm, which has been implicated in many inflammation-induced cancers including colorectal cancer[6, 7]. Canonical NF-κB signaling is initiated by a wide variety of stimuli including cytokines, TLR ligands, and other antigens, and is most often quickly initiated in response to the cellular environment. After liberation of the protein dimer RelA/p65-p50 from its inhibitory IκBα proteins through the kinase activity of IκB kinase (IKK), there is movement of RelA-p50 into the nucleus of the cell to control gene transcription. The repertoire of downstream effector molecules that are a result of canonical NF-κB stimulated transcription as similarly varied, with wide ranging effects in not only inflammation and repair but tumorigenesis. The noncanonical arm of the NF-κB pathway is a unique alternative signaling pathway that is stimulated by a specific array of ligands and produces unique chemokines[8]. It is generally activated in a much more controlled, specific manner than canonical signaling. The products of noncanonical signaling are distinct from the canonical pathway in that they are not general inflammatory mediators but rather very specific chemokines that modulate immune cell development and trafficking[8]. At the center of noncanonical signaling is NF-κB inducing kinase (NIK) encoded by the MAP3K14 gene. This critical kinase is required for activation of the Iκb kinase IKKα, which then phosphorylates the protein p100 which is encoded by the gene NFKB2. This results is targeted proteosomal processing of p100 to its active form p52, which then associates with the protein RelB and enters the nucleus to promote the transcription of downstream genes CXCL12, CXCL13, CCL19, and CCL21 [8].
Inflammation is intricately intertwined with carcinogenesis, with inter- and intra-cellular mediators affecting a wide variety of downstream neoplasia-associated processes such as differentiation, proliferation, apoptosis, and migration[3, 4, 9-13]. Therefore it is unsurprising that a large number of cancers are also associated with chronic relapsing inflammatory diseases in addition to genetic and environmental predispositions. A classic example of this inflammation-tumorigenesis axis is colorectal cancer (CRC), which occurs in patients suffering from IBD at a much higher rate than the normal population and accounts for 15% of all IBD-related deaths [14-17]. According to the Centers for Disease Control and Prevention, colorectal cancer is the second leading cause of cancer-related deaths in the United States and also the third most common cancer overall in both men and women. Inflammation, lack of repair, and acquisition of mutation are only part of the story in colon cancer development. Epithelial regeneration in the gut depends on a miniscule population of cells within the crypts of the small and large intestine deemed the ISCs (intestinal stem cells)[18-21]. This small population is responsible for the regeneration, differentiation, and maintenance of thousands of square feet of surface area in the human small and large intestines. When stem cells are unable to perform their functions, downstream events such as epithelial barrier dysregulation, progressive bacterial translocation, accumulation of DNA damage, and progressive systemic inflammation can occur and be catastrophic for host health. ISCs are most commonly identified by expression of the marker Lgr5[22]. Lgr5 is a G-protein coupled receptor that is expressed in a diverse range of tissue and is highly conserved in mammalian species. It acts as a putative receptor for R-spondin proteins, which have been shown to interact with its extracellular domain[23]. After this binding, the Lgr5 and R-spondin complex binds to and internalizes two transmembrane E3 ligases called RNF43 and ZNRF3. These ligases, when active, ubiquitinate a receptor deemed Frizzled which
is involved in Wnt signaling, a potent growth factor pathway in cellular biology\[24, 25\]. With this internalization, Wnt signaling, cell growth, and division are free to proceed, underscoring Lgr5’s role as a stemness factor\[26\]. In the gut, Lgr5 stem cells are the source of all other differentiated cell types including enterocytes, Paneth cells, goblet cells, and M cells\[22\]. When the intestine is damaged, the Lgr5+ stem cells are critical for replenishing lost cells. Of course, their ability to proliferate, when dysregulated, can result in deleterious downstream issues for the host in the wrong circumstances.

As previously discussed, canonical NF-κB signaling has been well-studied in the context of CRC, especially its effects on inflammation and cellular proliferation. However, noncanonical signaling in stem cell biology and colorectal cancer has been less well defined. Some studies have shown changes in a variety of chemokine products of noncanonical signaling relative to cancer progression\[27-30\], but their connection with the entire noncanonical pathway which produces them has not been fully evaluated or organized into a meaningful stepwise progression. Determining the upstream reason for the changes in these chemokines will lead to additional potential therapeutic targets, as well as a deeper understanding of how noncanonical NF-κB signaling functions in homeostasis of the colonic crypt and colorectal cancer. Here we evaluate the effects of noncanonical signaling in both mouse models of inflammation-induced colon cancer and human CRC patients themselves, with the goal of determining this alternative pathway’s contribution to the development of colorectal neoplasia.

Materials and Methods:
Acquisition of Metadata: Using the Oncomine® Platform (www.oncomine.org) we identified microarray studies of human colorectal cancer patients and evaluated noncanonical gene expression in biopsy tissue. Relative expression was reported as log-transformed median-centered data used in the following datasets (CRC/control): Gaedcke (65/65)[31], Skrzypczak (81/24)[32], Hong (70/12)[33], TCGA colon cancer (215/22)[18], Kaiser (100/5)[34], Graudens (18/12)[35], Ki (50/28)[36], Gaspar (56/22)[37], Notterman (18/18)[38] and Sabates-Bellver (32/32)[39]. Figures were created using GraphPad Prism software v.7 and statistical significance was determined using the unpaired Mann-Whitney U test. Significance was set at p ≤ 0.05.

Clinical Sample Acquisition: CRC biopsy samples were collected from 6 patients at Duke University who were diagnosed with colon or rectum adenocarcinoma that had undergone surgical resection. Control samples were collected at the Roanoke Carilion Hospital’s Gastroenterology Department from endoscopically and histologically unaffected tissue in patients presenting for conditions other than inflammatory bowel diseases. Sections had been previously confirmed by a pathologist to contain either neoplasia (CRC) or lack of histologic change (control). For tissue processing, RNA was extracted from 50 micron sections of formalin-fixed paraffin-embedded tissue for the CRC patients, and from RNAlater®-preserved tissue in the case of controls. All extractions were performed using the Qiagen AllPrep kit according to the manufacturer’s protocols.

RNA Extraction and PCR: RNA was quantified via Nanodrop and converted to cDNA using a ThermoFisher Hi Capacity cDNA kit. Individual real-time PCR was performed using Taqman primers for human CXCL13, CXCL12, CCL19, CCL21, and NIK, with 18s as an internal control and run on an ABI 7500 Fast Block Thermocycler. Fold change was calculated using the ΔΔCt
method[40] and all changes were log-transformed. Statistics were performed using Graphpad Prism v. 7 and the unpaired Mann-Whitney U test. Significance was set at p > 0.05.

Superarray Data: A custom RT-PCR superarray (Supplementary Table 1) was designed and purchased from SA Biosciences. RNA from patient samples was converted to cDNA using the Qiagen First Strand kit, and RT-PCR was performed on an ABI 7500 Fast Block Thermocycler using Qiagen SyberGreen Mastermix. Fold change using the ΔΔCt value was computed using SA Biosciences online RT-PCR analysis and the arithmetic mean of five internal control genes.

Mouse Breeding and Maintenance: All studies were conducted in accordance with the IACUC guidelines of Virginia Maryland College of Veterinary Medicine and NIH Guide for the Care and Use of Laboratory Animals. All experiments were conducted with Nik−/− and Nik+/+ mice housed under SPF conditions that were age and sex-matched on a C57Bl/6J background.

Generation of conditional knockout mice and colitis-associated cancer model: Mice containing a floxed sequence of the kinase domain of the Map3k14 gene were bred with B6.Cg-Tg(Vil1-cre)1000Gum/J (Villin-Cre) mice (Jackson Labs, Bar Harbor Maine). All mice used in experiments were littermates and only male mice were used for studies involving DSS administration. For CRC studies, mice were injected at Day 0 with 10mg/kg azoxymethane intraperitoneally. Beginning on Day 1, the mice were administered 2.5% dextran sulfate sodium (Affymetrix) in drinking water for 5 days. Clinical scores for mice during periods of DSS administration was based on weight loss, fecal consistency, presence and amount of blood in stool, and overall behavior as previously described[41]. Mice were then rested on regular water for two weeks. This was repeated for a total of 3 DSS cycles, after which the mice were sacrificed at day 70. For acute studies, mice were administered 2.5% DSS in drinking water for four days, and were then allowed to recover for 4 days.
**Histology:** Mice were euthanized by carbon dioxide narcosis followed by cervical dislocation. The entire colon from the level of the rectum to the cecum was dissected out, flushed, opened, and swiss-rolled as previously described[42]. Samples were fixed in 10% buffered formalin, paraffin embedded, processed routinely, sectioned at 5μm and stained with H&E. Tissues were evaluated by a board-certified veterinary pathologist. Grading was performed using a standard scheme including both inflammatory and hyperplasia parameters[43].

**Colon crypt harvest and organoid generation:** Colonic crypts were harvested from mice as previously described[44]. Briefly, the entire colon from rectum to cecum was excised, flushed of its contents, and dissected into small pieces. Tissue was incubated with sterile 2mM EDTA solution in 1X PBS (without Ca or Mg), placed on a rocking platform on ice for 45 minutes, and then washed with sterile 1X PBS. Tissue was then incubated with TrypLE Express for 45 minutes at 37°C with agitation every 5-10 minutes in 15mL tubes. Tissue was then rinsed with sterile 1x PBS and resuspended in a 5% w/v bovine serum albumin/PBS solution. To dislodge whole crypts, tissue suspensions were forcefully shaken at least ten times, and larger tissue pieces allowed to settle. After each round of shaking and rest, the supernatant was removed as a crypt fraction and additional 5% BSA solution was added to the tissue fragments. This was repeated 6 times for 6 individual crypt fractionations per mouse. Organoids were created from isolated colonic crypts following established protocols[44]. Diameter was measured using from randomly chosen organoids from multiple wells by a blinded investigator. For growth tracking pictures, the same organoids were measured over time by marking the outside of the plate in order to consistently identify the same organoid.

**IHC and quantification:** Smears for immunocytochemistry were made by placing a drop of concentrated crypt suspension on a frosted microscope slide and allowed to air-dry. Smears were
then fixed in 4% paraformaldehyde for 10 minutes and then rinsed with 1x PBS. Enzymatic antigen retrieval was performed using a 0.5% trypsin solution in distilled water. Immunocytochemistry was performed according to the manufacturer’s protocol using the Pierce IHC kit and an anti-mouse Ki-76 antibody (CST Product #12202) at a 1:1000 dilution. Image quantification was performed using ImageJ.

**Stool collection and microbial taxa analysis:** Colonic contents were collected in a sterile fashion from the entire colon length of mice into a sterile microcentrifuge tube and immediately frozen at -80°C. Bacterial DNA was extracted using the QIAamp DNA Stool Mini Kit. 20ng DNA from each sample was used to generate the libraries for 16s rDNA sequencing on an Illumina Miseq. Each samples had more than 200,000 reads and 99% percent of reads passed Q20. After QC and trimming the adaptors, mothur (http://www.mothur.org/) was used to analyze the data. Paired-end reads were joined and mapped to the Greengenes 13.8 release database. OTUs were picked against Greengenes database, using a 97% similarity threshold. To avoid the different sequencing depth, all samples were normalized to the same number of reads in the following analysis. Lefse (https://huttenhower.sph.harvard.edu/galaxy/) was used to compare the different bacteria abundance in knockout and wild-type mice. α diversity was calculated with 95% CI. Heat map plots with bacterial OTU were created with more than 1000 reads.

**Statistics:** Data was analyzed using GraphPad Prism, version 7 (GraphPad Software, Inc., San Diego, CA). Student’s two-tailed t test was used for comparison of two experimental groups. Multiple comparisons were done using one-way and two-way ANOVA where appropriate. Changes were identified as statistically significant if p was less than 0.05. Mean values were reported together with the standard error of the mean (SEM) or standard deviation (SD), as appropriate.
Results:

Colons from $Nik^{-/-}$ mice display minimal changes from wild-type mice in the resting state

To begin our investigation, we first evaluated the colonic morphology of $Nik^{-/-}$ and wild-type littermates at baseline. Histologically there were no significant differences between wild-type mice and $Nik^{-/-}$ mice throughout all levels of the colon (Figure 1A and 1B). In both cases there appeared to be normal distributions of enterocytes with no evident inflammatory reaction. At the gene expression level, there was no change in the major proinflammatory cytokine TNF (Figure 1C). Although there was a slight trend towards a lower amount of IL-10 expression, the change was not significant (Figure 1D). However, there was a mild but significant attenuation in IL-6 expression (Figure 1E). Although IL-6 is typically thought of as a proinflammatory cytokine, it also is a marker of epithelial cell health and is involved in intestinal epithelial regeneration and repair[45]. In order to evaluate any potential epithelial barrier compromise, we measured serum endotoxin. Serum endotoxin is an indirect method of determining peripheral bacteremia, which can occur when there is gut barrier dysfunction. Both wild-type and $Nik^{-/-}$ mice displayed similar and extremely low endotoxin burden (Figure 1F) suggesting that the barrier itself was intact.

The microbiome of $Nik^{-/-}$ mice is distinct from that of wild-type littersmates and contains an overabundance of disease-associated bacteria

We then moved on to investigate the microbial differences in the colons of these mice. Fecal pellets collected from $Nik^{-/-}$ and wild-type littermates displays distinct patterns of bacterial taxa (Figure 2A), although general abundance and diversity indices were unchanged between the
two genotypes (data not shown). Using linear discriminant analysis (LDA), we found significant differences (i.e. greater than 2 LDA score) in several important bacteria species (Figure 2B, C). Clostridial species appeared to be a major family of interest. While unclassified members of the class Clostridium were identified as being overexpressed in wild-type mice, there were two specific genera of Clostridium as well as one unclassified Clostridiales family member that were overabundant in the Nik<sup>−/−</sup> mice. Clostridial bacteria are immensely important in colonic homeostasis, and can have both protective and proinflammatory effects depending on species[46-48]. Another family of note overexpressed in the Nik<sup>−/−</sup> colon is Helicobacteraceae. Helicobacter has been implicated in many gastrointestinal diseases, including cancer formation[49, 50]. A third bacteria type linked to colonic inflammation, the order Campylobacterales, was also upregulated in the Nik<sup>−/−</sup> mice (Figure 2B, C). Other elevated microbiota in the Nik<sup>−/−</sup> mice included the phylum Proteobacteria, which has been associated with Crohn’s disease[51] and compromised mucous barriers in mouse colons[51], as well as Moryella, Candidatus arthromitus, Rikinella, and Epsilonproteobacteria (Figure 2B, C).

**Nik<sup>−/−</sup> mice are significantly more sensitive to a model of ulcerative colitis**

We hypothesized that any large-scale changes in the Nik<sup>−/−</sup> colon might not be apparent until the mucosa itself was challenged. Therefore, we placed male wild-type and Nik<sup>−/−</sup> littermate mice through a dextran sodium sulfate (DSS) model of ulcerative colitis to evaluate both the degree of colonic damage and any potential regeneration. DSS acts as a detergent to the colonic epithelium, breaking down cell membranes and causing ulceration, and is a widely used chemical model of inflammatory bowel disease. Mice were administered DSS for four days, and then allowed to recover for four additional days in order for the regeneration process to begin.
Nik\(^{-}\) mice consistently had increased clinical scores (a composite score of behavior changes, fecal consistency, blood in stool, and weight loss) throughout the experiment (Figure 3A). Upon necropsy, the colons of Nik\(^{-}\) mice were shrunken, short, and contained poorly formed fecal material (Figure 3B, C). Histologically, wild-type mice showed significant ulceration and inflammation, but also the beginnings of crypt reformation (Figure 3D). The colons of Nik\(^{-}\) mice, however, remained very ulcerated (Figure 3E) and these changes were quantified histologically (Figure 3F). Additionally, the systemic effects of this ulceration and inflammation were more significant in the Nik\(^{-}\) mice as evidenced by increased serum levels of IL-6 (Figure 3G).

**Colonic organoids from Nik\(^{-}\) mice fail to grow and develop at a normal rate in vitro, indicating an epithelial cell-intrinsic defect**

Given the results from the DSS model, we turned out attention towards the possible role of NIK in the colonic epithelium which may have resulted in the in vivo phenotype we observed. Colonic organoids are a way to create an epithelial cell-only population that can be studied outside of the effects of whole-body knockout animals. Organoids arise from stem cells of the gut and will differentiate into all the different cell types of the colonic mucosa including enterocytes, goblet cells, and Paneth cells. Here we harvested colonic crypt cells from wild-type and Nik\(^{-}\) mice, created single-cell suspensions, grew them in culture, and measured their growth over time. As a positive control, we also cultured organoids from the colons of Apc\(^{min}\) mice, which harbor a mutation that causes constitutive Wnt signaling and the development of spontaneous intestinal neoplasia. As expected, these organoid grew very large, while the wild-type organoids grew at a more even pace (Figure 4A). However, organoids from Nik\(^{-}\) mice
grew very little. After 6 days in culture, \(\text{Nik}^{-/-}\) organoids remained significantly smaller than their wild-type counterparts (Figure 4B). Cytologically, although \(\text{Nik}^{-/-}\) organoids were smaller, they appeared quite similar in terms of overall cellularity and density to the wild-type (Figure 4C).

**Whole crypts isolated from \(\text{Nik}^{-/-}\) mice display altered stem cell and differentiated cell profiles at the gene and protein level**

In order to further investigate why we saw this difference in organoid growth, we harvested whole colonic crypts from wild-type and \(\text{Nik}^{-/-}\) mice and stained with the proliferation marker Ki-67 to evaluate proliferative capacity of the crypt where the stem cells typically reside. Our hypothesis was that the lack of growth seen in our organoids may be due to a defect in stem cells in the \(\text{Nik}^{-/-}\) mice, as organoids arise from the stem cells of the colon. Wild-type crypts showed intense staining near the bottom of the crypts, consistent with the replicative niche. However, \(\text{Nik}^{-/-}\) crypts displayed reduced staining that was not consistently localized to the crypt base (Figure 5A). Using image quantification software (ImageJ), the overall intensity of Ki-67 was attenuated in \(\text{Nik}^{-/-}\) crypts (Figure 5B). Due to this reduced proliferation, we therefore performed gene expression analysis for \(\text{Lgr5}\), a marker of colonic stem cells. Expression of this critical gene was significantly downregulated in \(\text{Nik}^{-/-}\) crypts (Figure 5C). Additionally, \(\text{Nik}^{-/-}\) crypts were elongated compared to wild-type (Figure 5D). Based on the aforementioned decreased proliferation as well as an increase in the expression of mature enterocyte marker cytokeratin 20 (\(\text{Krt20}\)) (Figure 5E), this elongation appears to be composed of mature, non-dividing colonic epithelial cells. It also appears that there is a lack of normal apoptotic schedule of these mature cells, as there was a decreased expression of poly-ADP ribose (PARP1) and its cleaved product (cPARP1) within the colonic crypts of \(\text{Nik}^{-/-}\) mice as well (Figure 5F).
Conditional knockout Nik<sup>−/−</sup> mice, which lack noncanonical signaling in colonic epithelial cells, are more susceptible to inflammation-associated colorectal tumorigenesis

NF-κB signaling is a prominent pathway in a variety of cell types, and its effects in different compartments may have different effects on cancer development. For example, in a preliminary study, we discovered that when canonical NF-κB is removed in myeloid cells via the deletion of p65/RelA (Supplementary Figure 1A), there is a significant reduction in inflammation induced tumorigenesis in mice. Mice lacking p65 in their myeloid cell lines (RelA<sup>fl/fl</sup> LysCre<sup>+</sup>) performed better clinically than their RelA<sup>fl/fl</sup> counterparts (Supplementary Figure 1B) in an azoxymethane/dextran sodium sulfate model, and showed a decrease in both colonic polyp formation (Supplementary Figure 1C, 1E (RelA<sup>fl/fl</sup>LysCre<sup>+</sup>, left and RelA<sup>fl/fl</sup>LysCre<sup>+</sup>, right) and 1F) and a maintenance of normal colonic length, indicating decreased fibrosis and inflammation (Supplementary Figure 1D). Given our previous data in this work regarding noncanonical NF-kB and epithelial cell biology, we chose to further investigate this pathways role in colonocytes via the same model

Nik<sup>−/−</sup> x Villin-cre mice and Nik<sup>−/−</sup> littermates were subjected to a 70 day inflammation-induced colorectal cancer model. Briefly, animals were administered a single dose of azoxymethane, which is metabolized by colonic bacteria into a carcinogenic substance that affects the colonic epithelium, followed by multiple rounds of dextran sodium sulfate (DSS). Throughout the experiment, both genotypes displayed similar clinical signs (Figure 6A) and weigh loss patterns (Figure 6B). The length of the colon at sacrifice, a marker of inflammation and fibrosis causing tissue shrinkage and stenosis, was similar as well (Figure 6C). However, intestinal epithelial cell-specific Nik<sup>−/−</sup> mice displayed a marked increase in colonic polyp
formation (**Figure 6D and 6G; representative arrowheads**) and a trend towards increased polyp size (**Figure 6E**). Histology grading of the colon showed no significant differences in inflammation, epithelial ulceration, or edema between the two groups (composite score deemed the “IBD” or inflammatory bowel disease index); however, the conditional knockout mice scored significantly higher in hyperplasia and dysplasia (composite score deemed the “CRC” or colorectal cancer index) as shown in **Figure 6F**. Often, these polyps extended up to the transverse colon, whereas in typical models they are confined to the descending (lower) colon (**Figure 6G; asterisks**). Histologically, these polyps were composed of disorganized, arborizing dysplastic tubules separated by thick fibrovascular stroma, admixed with mononuclear cells (**Figure 6H**).

**Noncanonical NF-κB signaling is attenuated in tissue biopsies from human colorectal cancer patients**

In order to maximize the translational relevancy of our studies and determine if suppression of noncanonical signaling is also found in human colorectal cancer patients, we employed a retrospective metadata analysis of gene expression profiles in CRC patients using the Oncomine® platform. We evaluated the expression of noncanonical signaling at multiple levels, including ligand-receptor interaction, main pathway components such as NIK, and effector chemokines. We used the largest dataset available on the platform, The Cancer Genome Atlas (TCGA) dataset which is composed of 215 CRC biopsy samples and 22 control tissues. B-cell activating factor (BAFF) and its receptor BAFFR, which are critical factors in B cell development and activation as well as being potent stimulators of noncanonical NF-κB, were both significantly downregulated in biopsy samples from CRC patients (**Figure 7A, B**).
Noncanonical receptor cluster of differentiation 40 (CD40) and its ligand CD40L, which function to stimulate antigen presenting cells and are associated with proinflammatory pathways in colonic epithelial cells[52], was also downregulated (Figure 7C, D). Finally, lymphotoxin beta, another stimulator of noncanonical NF-κB and contributor to lymphorganogenesis, was suppressed, although its receptor was upregulated (Figure 7E, F). NIK itself, encoded by the gene MAP3K14, was also significantly attenuated (Figure 7G). We then investigated the expression of downstream chemokines of noncanonical signaling not only within the TCGA dataset, but across multiple colorectal cancer datasets available on Oncomine® in which these four genes were expressed and included in the array. We found that not only were CXCL12, CXCL13, CCL19, and CCL21 consistently and significantly downregulated across datasets, they were even downregulated between different subsets of colorectal cancer (Figure 7H).

In order to confirm these findings in our own patient dataset, we acquired RNA extracted from formalin-fixed paraffin-embedded tissue of colonic biopsies of human CRC patients at Duke University, as well as control (non-inflamed, non-neoplastic) biopsies from the colons of patients from Roanoke Carilion Clinic. This RNA was pooled and then analyzed using a custom superarray containing over 80 genes both directly and indirectly involved in noncanonical NF-κB signaling. At a high level, it was evident that there were differences between CRC patients and the controls, as evidenced by a large number of downregulated genes (Figure 8A). NIK and the four noncanonical chemokines were downregulated by significant amounts (NIK, -2.59 fold; CXCL12, -3.67 fold; CXCL13, -7.34 fold; CCL19, -59.28 fold; CCL21, -30.02 fold). In fact, CCL19, CCL21, and CXCL13 were in the top 10% of downregulated genes. Using Ingenuity Pathway Analysis (IPA), we confirmed that noncanonical signaling was suppressed overall in these patients at multiple points (Figure 8B). Specifically, NIK itself, RELB (which functions to
assist p52 translocation into the nucleus), and all four chemokines were the most significant findings. At the chemokine receptor level, there appeared to be some potential compensatory upregulation in CXCR4 and CXCR5, which are the receptors for CXCL12 and CXCL13. Also of special note is the upregulation of NLRP12, which functions as a suppressor of noncanonical signaling. We confirmed these findings with individual real-time PCR on these patients for NIK, as well as the four main chemokines (Figure 8C).

Discussion:

The epithelial lining of the small and large intestine is a stupendously regenerative surface, with complete epithelial renewal occurring every few days under normal circumstances. This speedy renewal is critical given the continual assault on the mucosal lining by microorganisms, metabolites, food antigens, and a wide variety of other stressors. Timely and proper renewal and replacement of intestinal epithelial cells and the barrier they form is paramount not only to overall gastrointestinal homeostasis but to the prevention of chronic, progressive inflammatory disease that can ultimately predispose individuals to mucosal dysplasia and neoplasia. The Nik−/− mouse has been previously characterized as developing a hypereosinophilic syndrome that appears to target epithelial structures such as the skin and esophagus, along with peripheral accumulation in filtering organs[53, 54]. The lower GI tract, however, appears to be mostly unaffected by this syndrome and appears morphologically similar to regular mice at baseline[54]. However, here we show that NIK and noncanonical signaling play a novel role in stem cell maintenance in the gut.

Typically when discussing stem cells and cancer, the natural assumption is that most neoplasms arise from the rapidly dividing stem cells themselves. That is, an aberrant overexpression of these proliferative markers leads to hyperplastic and dysplastic change in the
mucosa. In fact, a notable offshoot of this theory is the incredibly prolific and relevant field of cancer stem cell biology, including those of the colon[55]. Lgr5 itself has been posited to act as a cancer stem cell in this location [56]. This classic progression of cancer formation in the gut is what can be called the “bottom up” development process, where cancer arises from the stem cells of the crypt themselves[57]. In this hyperactive state, often due to a mutation that results in overexpression of growth-related genes, these cells will divide uncontrollably and result in neoplasia formation. For example, Lgr5+ stem cells are key players in the classical model of murine intestinal neoplasia, the Apc\textsuperscript{min} mouse, which develop spontaneous intestinal cancer via overactive Wnt signaling[58]. Other stem cell markers such as Bmi-1[59] and Prominin-1[60] have also been implicated in the bottom-up theory of cancer development.

However, the role of stem cells (and in particular Lgr5) and their niche is not so simple. More and more evidence suggests that cancer can occur not only by overexpression of stem cell markers, but by underexpression of them as well, which leads to a different cascade of dyregulation. For example, \textit{in vitro} studies with colorectal cancer cells show increased tumorigenicity upon Lgr5 ablation, suggesting that Lgr5 might be important in maintaining stemness in the correct niche and preventing other cells from gaining these abilities[61]. This supports the “top down” theory of colon cancer development [62, 63], defined as instances where Lgr5 or other stem cell markers are suppressed and mature cells attempt to de-differentiate to fill in the “gap” left by the lack of normal stem cells. It has been shown that even differentiated cells have plasticity and can be reprogrammed to regain features of stemness and accelerated growth given the right circumstances [64]. Additionally, mature cells that acquire mutations, either from chronic damage such as via inflammation or by lack of proper apoptosis, may result in cancer
spreading in a top-down fashion as they are not replaced by normal stem cell division and renewal.

It may well be that the baseline suppression of normal stem cell activity due to noncanonical NF-κB shutdown may predispose to cancerous development, especially in the face of inflammation, apoptotic failure, and/or colitogenic microbiomes. This would lead to the mature enterocytes, which appear to be “piling up” in the face of decreased apoptosis due to lack of proper renewal schedule in \( \text{Nik}^{+/} \) crypts, to be prone to chronic damage accumulation and potential dedifferentiation. Indeed, this appears to be an epithelial growth-mediated change rather than a simple inflammatory one, as the mice lacking NIK in the colon alone showed similar inflammation and clinical parameters to their wild-type littermates throughout the inflammation-induced colorectal cancer model despite having such a distinct difference in polyp formation at the end of the study. Another complimentary theory is that, since noncanonical chemokines are critical in the recruitment and trafficking of lymphocytes, that underexpression of noncanonical NF-κB may be a mechanism used by tumorigenic cells to escape immune surveillance. It is a well-known fact that cancer cells are exquisitely good at hiding from the immune system, including colon cancer[65]. By suppressing noncanonical NF-κB (or maintaining the suppression), they may be impeding the trafficking of immune cells which would then allow cancerous tissue to grow.

The exact mechanism of how NIK affects Lgr5+ stem cells remains undetermined. However, an excellent candidate for future study is IKKα, the downstream phosphorylation target of NIK. IKKα has been shown to be a promoter of Lgr5 expression in the basal layer of the skin in basal cell carcinoma[66]. In this case, IKKα encourages Lgr5 expression and stemness of the basal cells. It may be that, when NIK is removed as an activating kinase, this has
the opposite reaction on Lgr5 and would produce the phenotype we see. Additionally, in squamous cell carcinoma, which relies more on dedifferentiation and is a “top down” model compared to basal cell carcinoma, there is also a downregulation of IKKα[67]. In fact, in these studies, deletion of IKKα results in spontaneous skin squamous cell carcinomas[67, 68]. It is worthy of note that in Nik−/− mice, one of the major organ targets of their hyperinflammatory syndrome is the skin, producing alopecia, scaling, and ulceration. It would be interesting to investigate if similar lack of repair or tumorigenicity issues arose in Nik−/− skin through an Lgr5 mediated-mechanism, as Lgr5 is also a stem cell marker of the hair follicle[69]. However, NIK is a tightly controlled protein for a reason; lack of regulation of NIK may have consequences as well. For example, mice that lack NLRP12, a negative regulator of NIK, are also predisposed to inflammation-induced tumorigenesis[41].

The intrinsic renewal properties of the stem cells themselves is only part of the equation when it comes to modeling intestinal homeostasis. The gut is home to trillions of microorganisms that, along with the metabolites products they produce, can have major impacts on epithelial repair and stem cell maintenance that can be key for proper defense and healing response. These microorganisms and the molecules they produce are in near-constant contact with the epithelium of the intestine, including the stem cell niche, and provide continual stimuli to the host’s cells. Studied in germ-free animals have confirmed the necessity of the microbiome to proper epithelial renewal, with GF animals showing altered rates of epithelial renewal and proliferation in the intestine[70, 71]. The stem cells themselves express TLR4, and LPS itself can be used to modulate stem cell apoptosis and proliferation[72] Even more specifically, there is a specialized microbiome within the crypt area deemed the crypt-specific core microbiome (CSCM) which has the potential to have major impact on the stem cell niche[73]. Bacterial
species themselves have also been linked to the development of inflammation associated gastrointestinal neoplasia, including colon cancer[74]. Lastly, products of these microorganisms such as butyrate can also directly affect stem cell function. For example, the short chain fatty acid butyrate, which has been well-characterized as a major energy source for enterocytes[75], has been shown to inhibit stem cell proliferation[76].

The investigation of noncanonical signaling in the local microbiome of the large intestine has not been pursued, but there are several hints that there may be an involvement. For example, knockout of NIK has been shown to result in the suppression of the promoter XBP1 and its target genes[77]. XBP1 acts as a promoter for the important butyrate receptor GPR43[78] that is associated with inflammation responses. Loss of noncanonical signaling in a variety of animals models such as aly/aly (“alymphoplasia” mice that harbor a point mutation in NIK)[79, 80] and Relb−/− mice[81], and leads to underdevelopment of Peyer’s patches, critical immunological units of the gut that play a large role in bacterial sensing. Our initial results suggest that there are significant differences between the luminal colonic microbiome of wild-type mice and mice with perturbations in noncanonical signaling. These differences include overgrowth of members of the genera Clostridium and Helicobacter, two bacteria groups that are often implicated in dysregulation of growth and inflammation in the colonic epithelium. Further analysis in terms of exact bacterial species could prove fruitful in determining the effect of noncanonical signaling on the microbial communities in the gut, as well as potential effects on epithelial cell renewal, signaling, and chronic inflammation.

Conclusions:

Here we present a novel role for noncanonical NF-κB signaling in colonic epithelial cells, with implications in both gastrointestinal repair and tumorigenesis. We initially characterized
whole-body knockout mice and focused on the lower GI tract at both resting and inflamed states, finding that there is a susceptibility to ulcerative colitis in the \textit{Nik}^{-/-} mice despite only minimal changes at baseline, as well as a shift in several microbial families important to GI health.

Moving on to a focus on the colonic crypt and its epithelial cells, we isolated crypts and cultured organoids from these mice to investigate the colon mucosa by itself and found that this susceptibility appears to be due to a decrease in stem cell activity that prevented the epithelium from healing; \textit{Nik}^{-/-} crypts displayed a phenotype of decreased stem cell activity and division as well as accumulation of mature enterocytes and defects in apoptosis. In order to define the model further, we used conditional knockout mice lacking NIK in their colonic epithelial cells to define NIK’s role in inflammation-associated tumorigenesis in the gut. Here, we saw that lack of NIK in the colon resulted in significantly increased predisposition to polyp formation, potentially due to disruption of the normal renewal niche. Lastly, we correlated our findings with data from human patients and found that suppression of noncanonical signaling is seen across hundreds of CRC patients from various studies, as well as our own patient dataset. In conclusion, noncanonical signaling appears to have many functions outside of simply immune cells, and may be a useful marker in the study of epithelial carcinogenesis.
References


Figure 1: *Nik*<sup>−/−</sup> mice do not display many significant changes in the resting colon compared to wild-type. Wild-type mice (A) and *Nik*<sup>−/−</sup> mice (B) display almost identical architecture with healthy crypts and lack of inflammation. Scale bars represent 100µm. There are no significant gene expression changes in the proinflammatory cytokine TNF (C) or anti-inflammatory cytokine IL-10 (D). There was a slight decrease in colonic IL-6 (E). Serum endotoxin was not different between the two genotypes (F). n = 10 mice per group. Statistics were performed using the student’s t–test and significant was defined as p ≤ 0.05.
Figure 2:
Figure 2. *Nik*<sup>−/−</sup> mice display an altered microbiome with differential expression of species important to gut health. The OTU heatmap (A) shows a different expression pattern of bacteria taxa in the colonic contents of *Nik*<sup>−/−</sup> mice versus wild-type (KO = *Nik*<sup>−/−</sup>, WT = wild-type). The LDA score is a measure of bacterial species abundance, and a LDA score of more than 2 fold change is considered significant. Again, *Nik*<sup>−/−</sup> and wild-type mice exhibited different patterns, especially in *Clostridia*, *Helicobacter*, and Campylobacter (B). A phylogenic tree is shown in (C) to show the relationships between different orders, families, and genera that were changed. n = 8 mice per group. Statistics were performed using the student’s t test and significant was defined as p ≤ 0.05.
Figure 3: \textit{Nik}^{−/−} mice are exquisitely sensitive to a model of ulcerative colitis. \textit{Nik}^{−/−} mice exposed to DSS had consistently increased clinical scores compared to wild-type (A). Colon length, a typical gross marker of inflammation and damage, was decreased in the \textit{Nik}^{−/−} mice (3B, 3C). Histologically, \textit{Nik}^{−/−} colons (3E) showed increased damage and lack of regeneration compared to wild-type (3D). This damage was translated into a composite score as previously described\cite{43} (F). Serum harvested from the mice showed an increase in systemic IL-6 in the \textit{Nik}^{−/−} mice, consistent with their more inflamed status (G). \textit{n} = 6 (\textit{Nik}^{−/−}) to 7 (WT) mice per group. Statistics were performed using the student’s t test and significant was defined as \( p \leq 0.05 \).
Figure 4: Colonic organoids from *Nik*<sup>−/−</sup> mice remain underdeveloped *in vitro* compared to wild-type. Colonic crypts were isolated and reduced to a single-cell suspension from wild-type, *Nik*<sup>−/−</sup> and *Apc<sup>min</sup>* mice and grown in culture for 6 days. Six days was chosen at the endpoint because typically, organoids are passaged at approximately 7 days. Organoids from *Apc<sup>min</sup>* mice displayed the typically overzealous growth, while wild-type organoids grew at a steady pace and achieved a slightly smaller size (A). *Nik*<sup>−/−</sup> organoids remained small compared to both wild-type and *Apc<sup>min</sup>* (B). Blinded measurement of randomly chosen organoids showed this difference was significant (B). Organoids were also manually disassociated from Matrigel and stained cytologically with Diff-Quik, and showed similar cellular morphology between the two.
genotypes (C; left, wild type and right, \(Nik^+\)) despite the difference in size. Data represents the average of three independent trials with 1 mouse of each genotype. Measurements were taken from 30 randomly chosen organoids spread over a total of 12 wells. Statistics were performed using the student’s t test and significant was defined as \(p \leq 0.05\).
Figure 5: Colonic crypts of $Nik^{-/-}$ mice display decreased levels of proliferation and stemness. Immunocytochemistry of wild-type and $Nik^{-/-}$ crypts reveals decreased Ki-67 expression in the crypt base (A, B). At the mRNA level, $Nik^{-/-}$ crypts also have attenuated expression of Lgr5, a critical stem cell marker in the gut. Additionally, the overall length of $Nik^{-/-}$ crypts was increased compared to wild-type (A, D). Expression levels of cytokeratin 20 (Krt20), a marker of mature colonocytes, was also increased in the $Nik^{-/-}$ crypts (E). Western Blot analysis of the crypt fractions revealed a decrease in poly-ADP ribose (PARP) mediated apoptosis. $n = 8$
mice per group for all gene expression data and measurements. For Ki-67 quantification, each data point represents the average of 10 crypts from 8 mice of each genotype. For Western Blot, image is representative of 4 animals of each genotype. Statistics were performed using the student’s t test and significant was defined as $p \leq 0.05$. 
Figure 6:
**Figure 6: Intestinal epithelial cell specific NIK knockout mice display increased susceptibility to colorectal tumorigenesis.** Clinical scores (composite score of weight loss, fecal consistency, rectal bleeding, and overall behavior; see Methods) and weight loss tracking between $Nik^{fl/fl} \times Villin-cre$ mice and $Nik^{fl/fl}$ littermates were only rarely significantly different (A, B, asterisks) and there were no changes in post-DSS colonic length (C). However, $Nik^{fl/fl} \times Villin-cre$ mice showed significantly increased polyp formation (D) and a slight trend towards increased polyp size (E) compared to $Nik^{fl/fl}$ mice. Grossly, these polyps were large, raised, smooth to slightly cauliflower-like projections from the mucosa (6F, arrowheads) that were typically confined to the distal colon in the $Nik^{fl/fl}$ littermates, but were found up to the level of the transverse colon in the $Nik^{fl/fl} \times Villin-cre$ mice (6G, asterisks). Histologically, these polyps were determined to represent well-differentiated adenocarcinomas. Histology scale bar = 500µm. n = 5-7 mice per group. Statistics were performed using the student’s t test and significant was defined as $p \leq 0.05$. 
Figure 7: Retrospective metadata analysis of noncanonical signaling reveals suppression in human colorectal cancer patients. Using the TCGA dataset, the gene expression levels of multiple levels of noncanonical signaling were analyzed. There was significant downregulation of three important noncanonical molecules, BAFF (A), CD40L (C), and LTB (E). Of the respective receptors, both BAFFR and CD40 were downregulated as well (B, D) although LTBR was slightly upregulated (F). Colorectal cancer patients also showed a significant suppression of the MAP3K14 (NIK) gene (G). In Figure 7H, we show the expression of the four main noncanonical chemokines across multiple datasets. Datasets were only included if they contained all four chemokines. All of the fold change values shown are statistically significant (p < 0.05).
Figure 8:
Figure 8: Analysis of noncanonical signaling in biopsies from CRC patients confirms noncanonical suppression, consistent with previous metadata. Compared to control tissue, tissue from CRC patients showed an overall downregulation of genes related to noncanonical signaling (A; yellow = upregulation, blue = downregulation). The solid line represents no change from control, and each dotted line marks the borders of likely physiologic significance (i.e. change in fold regulation of >2). When analyzed using Ingenuity Pathway Analysis, we see dysregulation of noncanonical signaling at multiple points (B). Suppression of NIK expression as well as chemokine expression was also confirmed via individual real-time PCR (C). n = 6 control, 6 CRC. For superarrays, patients groups were pooled, with each patient contributing equal amounts of mRNA to the reaction. Statistics were performed using the student’s t test and significant was set at p ≤ 0.05.
## Supplementary Table 1

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201
Supplementary Figure 1: Deletion of canonical NF-κB in myeloid cells results in resistance to colitis-associated tumorigenesis. We generated a conditional knockout mouse using a *RelA*^{fl/fl}* x Lysozyme-Cre* breeding scheme. Bone marrow-derived macrophages were isolated and cultured from sibling mice and stimulated with 1μg/mL of LPS and cells were collected for western blotting at various timepoints. RelA/p65 was not detected at any point in cell lysates.
from the Cre+ mice (A) indicating successful knockdown. RelAfl/fl LysCre+ (RelA/p65ΔMYE) mice had decreased clinical scores throughout an AOM/DSS model of inflammation associated tumorigenesis as compared to RelAfl/fl littermates (B). At necropsy, RelA/p65ΔMYE mice showed decreased polyp formation (C, E; RelAfl/fl left and RelA/p65ΔMYE right, asterisks) and maintenance of colonic length (C). A microscopic example of the polyps is shown in (E), displaying branching tubular formations.
Chapter 6

Conclusion

The common theme in all of the discoveries and projects within this body of work is the novel study of noncanonical NF-κB signaling in the gastrointestinal tract. Here we have covered both the upper and lower tracts as well as a variety of different disease processes from inflammation to cancer. GI disease is by nature a complex subject due to the multitude of different cell types, signaling cascades, and environmental factors that all play into disease processes. Therefore, finding new and alternative pathways that may be contributing to said pathogeneses is of the utmost importance. Here we have done in vitro, in vivo, bioinformatic, and clinical projects to evaluate this pathway’s contribution to disease.

This work also highlights the importance of investigating pathways outside of their traditional fields. As discussed in Chapters 1 and 2, noncanonical NF-κB has been thoroughly studied within the context of lymphoid biology. Given that the effector chemokines it produces have such a critical effect in this biological area, it is unsurprising that most of the focus to date has been on those processes. Dysregulation of this pathway, as shown with the Nik\textsuperscript{−/−} mouse, results not only in alterations in physical lymphoid structures such as lymph nodes, but also the immunological bias of these cells. For example, Nik\textsuperscript{−/−} mice are inherently Th2 prone, and develop a hypereosinophilic syndrome that is ultimately progressive and fatal. As discussed in Chapter 3, previous studies of this condition did not focus on the gastrointestinal tract, where one would imagine such changes in immunology would have a marked effect. We found, interestingly, that this Th2-mediated eosinophilic disease specifically targets the esophagus in these Nik\textsuperscript{−/−} mice. Furthermore, gene expression patterns, histologic changes, and downstream sequelae such as fibrosis also mimic human eosinophilic esophagitis (EoE). The localization of
this disease despite global NIK knockdown is interesting indeed, and may have to do with epithelial characteristics of the esophagus such as TSLP overexpression or some other component of squamous cells. This work laid the foundation for future investigations of noncanonical signaling in the upper GI tract, as well as the determination of gene-protein relationships as evidenced by the noncanonical dysregulation we saw in human EoE patients.

Our work on noncanonical signaling and inflammatory bowel disease is also novel and has significant therapeutic implications. As discussed in Chapter 4, inflammatory bowel disease is a condition of management rather than cure. Patients are often on many different medication regimens, and treatment is often difficult due to the large numbers of factors involved in IBD pathogenesis. These factors can include genetic predisposition, environmental factors and behaviors, microbiome, and overall immune dysregulation. First, we found that noncanonical signaling was indeed upregulated in tissues from IBD versus control. Furthermore, we focused our efforts on understanding potential noncanonical NF-κB connections to treatment response. Anti-TNF antibodies have become a critical therapeutic avenue for many IBD patients, with a significant number responding very well to treatment. However, all good things come at a cost; loss of response to these antibodies is a common and frustrating problem for research, clinicians, and of course patients. Finding a consistent marker for loss of response is an area of intense interest, including investigations into drug crest and trough levels as well as anti-drug antibodies. Here we show that noncanonical signaling is another significant marker of loss of response, and possible even a predictive one, particularly the chemokine CXCL13 which was elevated not only in nonresponders of our own patient population but also large retrospective studies.

Lastly, we continued our investigation into the effects of noncanonical signaling on mucosal biology by focusing on its effects in the epithelial cells of the colon. As mentioned
before, most work in NF-κB has been within the realm of lymphoid cells. Given the massive importance of the gastrointestinal epithelium, crypt regeneration, and acquisition of neoplastic phenotypes, we also dissected noncanonical signaling’s contributions to these processes. Here we found that NIK and noncanonical signaling play a significant and previously undescribed role in stem cell biology. Knockout of NIK resulted in depression of stem cell markers, reparative ability, and proper organoid growth in mice. Additionally, there appeared to be an imbalance between the regenerative and mature niches, with associated apoptotic defects. Using an elegant conditional knockout model where NIK loss was confined to the colonic crypts, we discovered that noncanonical signaling acts as a protective force against the development of inflammation-induced colorectal cancer. These findings have direct correlation with human colorectal cancer patients, where we found that noncanonical signaling was also suppressed.

Our disease models delineates many new roles for noncanonical signaling in the gastrointestinal system. We have characterized the effects of noncanonical signaling both in mouse models and human patients, and in three distinct disease processes. These are all new avenues in which noncanonical signaling has not been explored, and all of our results correlate with human disease. These data open up entirely new directions for research into this pathway. Overall, the entirety of this work is both scientifically and clinically impactful, and lays the bedrock for continuing studies that can develop noncanonical signaling as a therapeutic target.
Appendix A

Complete list of published works


*co-first authorship
Appendix B:
Cover Image and Article

Disease Models and Mechanisms, December 2017 Issue, Cover Image. Eosinophilic esophagitis (EoE) is a chronic, localized allergic disease in human patients. In a new spontaneous
model of EoE, mice lacking NF-κB-inducing kinase (NIK) develop severe esophageal
eosinophilia, mucosal hyperplasia and tissue remodeling. In this disease, thymic stromal
lymphopoetin (TSLP) acts as a potent chemoattractant of Th2 lymphocytes and eosinophils.
Nik−/− mice displayed significantly increased expression of TSLP in their hyperplastic
esophageal mucosa, as determined by immunohistochemical staining, as well as several other
mediators associated with the human disease. See article by Eden et al. on page 1517. Cover
image by Daniel E. Rothschild and Kristin Eden is licensed under a Creative Commons
Attribution 4.0 International license.