Review: Importance of the alternative NF-κB activation pathway in inflammation-associated gastrointestinal carcinogenesis.

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Running Head: Alternative pathway NF-κB in GI carcinogenesis

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Abstract

Chronic inflammation is a common factor in the development of many gastrointestinal malignancies. Examples include inflammatory bowel disease predisposing to colorectal cancer, Barrett's esophagus as a precursor of esophageal adenocarcinoma and *Helicobacter pylori*-induced gastric cancer.

The classical activation pathway of NF-κB signaling has been identified as regulating several sporadic and inflammation-associated gastrointestinal tract malignancies. Emerging evidence suggests that the alternative NF-κB signaling pathway also exerts a distinct influence on these processes. This review brings together current knowledge of the role of the alternative NF-κB signaling pathway in the gastrointestinal tract, with a particular emphasis on inflammation-associated cancer development.

Keywords: NFκB, gastric cancer, inflammation, Helicobacter pylori, colitis

Introduction

Members of the Nuclear Factor κ-light-chain-enhancer of activated B cells (NF-κB) family were initially described as transcription factors in B lymphocytes in 1986(68). Since then, they have been shown to be widely expressed and are conserved across both vertebrates and invertebrates(6, 27).

The conventional model of NF-κB signaling proposes two main arms of the pathway. These share similar features, but are triggered independently and activate different target genes(76). The classical (canonical) NF-κB activation pathway is triggered by Th1 cytokines and is typified by the action of RelA(p65)–NF-κB1(p50) heterodimers, whilst the alternative (non-canonical) activation pathway signals through the adaptor protein NF-κB-inducing kinase (NIK). Activation of this mechanism leads to nuclear translocation of transcriptionally active v-rel avian reticuloendotheliosis viral oncogene homolog B (RelB)–NF-κB2(p52) heterodimers.

Signaling through either pathway can influence multiple different cellular functions and can exert effects that may appear contradictory. For example, both pro- and anti-apoptotic effects, as well as proliferation(18) and senescence(70) signals have been attributed to the classical activation pathway of NF-κB signaling. Because of the wide variation in outcomes following pathway activation, it is difficult to extrapolate the effects of NF-κB signaling from one context to another. Classical pathway NF-κB signaling has been identified as a key regulator of inflammation associated carcinogenesis in several tissues since the early 2000s when Greten *et al* demonstrated increased sensitivity to colitis associated carcinogenesis in mice lacking IKK-ß in intestinal epithelial cells(31), and, almost simultaneously Pikarsky *et al* identified a similar increase in tumour burden in Mdr2 mice lacking IKK-ß in

hepatocytes(60). More recent evidence has established that alternative activation pathway NF-κB signaling is also important during the development of several gastrointestinal (GI) pathologies in mouse and man. This article seeks to review this evidence and to establish questions for future research.

Literature search strategy

A systematic search of the English language literature listed in the PubMed database up to 5th November 2015 was performed using the following search terms:

(NF-κB2 OR NFκB2 OR NF-kappaB2 OR NFkappaB2 OR NF-kB2 OR NFkB2 OR p52 OR p100 OR "alternative NF-κB" OR "alternative NFκB" OR "alternative NF-kappaB" OR "alternative NFkappaB" OR "alternative NF-kB" OR "alternative NFkB" OR "non-canonical" OR relB OR NIK OR IKKa OR IKKalpha OR MAP3K14 OR CHUK) AND (stomach OR gastric OR intestine OR intestinal OR gastrointestinal OR colon OR colonic OR colorectal OR colitis OR Crohn's OR bowel OR oesophagus OR oesophageal OR esophagus OR esophageal OR Barrett's).

This search generated 449 results, of which 159 articles were retained following review of titles and abstracts. The full texts of these articles were acquired and used as the basis of this article; retrieved article reference lists were searched manually for other relevant literature. The scope of the article has been limited to the importance of the alternative NF-κB signaling pathway in the gastrointestinal tract, particularly during the development of cancers and pre-malignant pathologies. Articles discussing the liver and pancreas only have been excluded.

The alternative NF-κB activation pathway

The NF-κB/Rel family includes five members: RelA(p65), c-Rel, RelB, NF-κB1(p50/p105), and NF-κB2(p52/p100). Each possesses a structurally conserved 300 amino-acid sequence; the *REL* homology domain (RHD). In the unstimulated state, these proteins pool as homo- or heterodimers (of which there are at least 15 known combinations), predominantly within the cytoplasm(71). Some of these dimers function exclusively in the classical NF-κB signaling pathway (figure 1a) and are beyond the scope of this article. Alternative NF-κB pathway activation leads to translocation of NF-κB2(p52)/RelB heterodimers into the nucleus, following which NF-κB2(p52) and RelB directly influence gene transcription.

NF-κB mediated transcription is regulated by the inhibitors of NF-κB (IκB) (IκBα, IκBβ, IκBε, and Bcl-3) which are peptides that bind NF-κB protein dimers through residues containing 6 to 7 ankyrin repeats. Interaction of IκB proteins with NF-κB protein dimers prevents the nuclear translocation of the NF-κB protein dimers. NF-κB2 is synthesized as a 100kDa protein, p100, which has a RHD within its N-terminus and a seven ankyrin repeat domain at its C-terminus which confers intrinsic IκB activity (figure 1b)(55).

Alternative pathway NF-κB activation is characterized by processing of p100 into p52, both of which dimerize with RelB (Figure 1b). p100 is processed by at least two kinases, NIK and IκB kinase α (IKKα). These kinases can be activated by ligand interaction with several cell-surface receptors, including CD40 ligand binding to CD40, B-cell activating factor (BAFF) binding to its receptors, Lymphotoxin β interacting with LTβR, lipopolysaccharide (LPS) binding to Toll-like receptor 4 (TLR4) and Receptor activator of nuclear factor kappa-B ligand (RANKL) binding to RANK(21). The pathway can also be activated by oncogenic viruses including Epstein-Barr virus(48).

Upon ligand binding, tumor necrosis factor receptor-associated factors (TRAFs; such as TRAF2 and TRAF3) are recruited either directly or via other adaptor proteins to the intracellular domain of the activated receptor. Unlike TRAF2 and TRAF5 that also regulate classical NF-κB signaling, TRAF3 only regulates the alternative NF-κB signaling pathway and directly binds to NIK, regulating NIK turnover(31). NIK is continually degraded by the proteasome as a result of polyubiquitination by the cellular inhibitors of apoptosis (c-IAP 1,2)/E2 ligase complex that is also recruited to TRAF3 (figure 1b). As a result of TRAF3 complex recruitment to the activated receptor, TRAF3 is targeted for degradation by the proteasome which allows NIK to accumulate in the cytosol (figure 1c)(60). Cytosol accumulated NIK becomes phosphorylated at threonine 559 by an incompletely described mechanism that may involve both autophosphorylation(31), and phosphorylation via zinc-finger-protein-91(60). Subsequently, Thr559-phospho-NIK phosphorylates p100 at serine (Ser) residues 866 and 870 acting as a docking molecule for recruitment of IKKα to p100. Recruitment of IKKα leads to phosphorylation of the N-terminus of NF-κB2(p100) at Ser 99, 108, 115 and 123 and at C-terminal Ser 872. Beta-transducin repeat-containing protein (beta-TrCP), a component of the SCF ubiquitin ligase complex is then recruited to phosphorylated NF-κB2(p100) leading to polyubiquitination of NF-κB2(p100) at lysine (Lys) 855 which targets NF-κB2 for partial proteasomal degradation, leading to generation of NF-κB2(p52)(82).

Activation of the alternative NF-κB signaling pathway is generally slower (~6-8 hours) than the classical activation pathway, leading to a delayed but sustained response which integrates with the acute-phase (~1.5 hours) response driven by classical NF-κB pathway activation(21, 64). Emerging evidence supports the concept that classical and alternative NF-

κB activation pathways interact to induce coordinated and sustained immunological responses. Banoth and colleagues recently used systems level analysis to identify stimulus-specific crosstalk between the TLR4-activated canonical NF-κB activation pathway and LTβR-induced alternative NF-κB activation pathway signaling. They identified a positive-feedback loop that sustained the NF-κB response. Using mouse embryonic fibroblasts, they demonstrated that LTβR prolonged the TLR4-induced RelA response, by targeting newly-synthesized p100 for processing to p52, allowing formation of p65(RelA)-p52(NF-κB2) dimers. This led to increased expression of pro-inflammatory cytokines and chemokines(10). Understanding the integration of different mechanisms of NF-κB activation is a key challenge for this field.

Role of the alternative NF-κB activation pathway in gut development and repair

Whilst the classical NF-κB activation pathway components RelA and c-Rel can be found in many tissues and cell types, in health the expression of alternative pathway NF-κB sub-units has more frequently been demonstrated in cells of the immune system where there is strong evidence that they play pivotal roles in the development of an effective immune system. For examble *RelB*$^{-/-}$ mice are deficient in dendritic cells and develop a spontaneous multi-organ autoimmune phenotype(16, 60, 80), whilst *Nfkb2*$^{-/-}$ mice have structural defects in the spleen and lymph nodes with irregular B-cell areas and an absence of perifollicular and mantle zones. This leads to a reduced number of B-cells, but with normal B-cell maturation. In keeping with this, *Nfkb2*$^{-/-}$ mice also exhibit impaired antigen specific antibody production(17, 80).

Observations from unstimulated transgenic mice have also offered insight into an epithelial role for the alternative activation pathway of NF-κB signaling. Mice that lack *Ikka*, which

encodes the pathway regulating kinase IKKα, survive until birth, but die shortly after delivery. These animals are born with marked cutaneous and skeletal deformities including markedly thickened skin(36, 44, 74). A similar phenotype is observed in mice with keratinocyte specific deletion of *Ikka*(26) suggesting that this phenotype is due to the disruption of alternative pathway NF-κB activation in the epithelial compartment.

More recent data have also demonstrated constitutive expression of RelB within small intestinal Peyer's patches(45) and of RelB and NF-κB2 (p100) in murine gastric epithelium(15).

Animal models suggest that the alternative NF-κB activation pathway components NIK, NF-κB2 and RelB are required for the normal development of small intestinal Peyer's patches(24, 83). Peyer's patches consist predominantly of immune cells which exist in close association with the specialized 'dome'-like epithelium described as follicle-associated epithelium (FAE). The presence of RelB-expressing cells also appears essential for the differentiation of enterocytes into microfold cells (M-cells) which represent approximately 5% of epithelial cells within FAE. Functionally, M-cells are specialized to perform luminal antigen sampling and immune priming(49). M-cells have been implicated in the initiation of granulomatous lesions in Crohn's disease(11) and are thought to influence responses to gastrointestinal infections(23, 73), and colorectal carcinogenesis(3, 51). Tahoun and colleagues found that stimulation of RelB-expressing FAE enterocytes with *Salmonella enterica* Typhimurium triggered autocrine activation by RANK ligand-RANK interaction induced the transcription factor Snail family zinc finger-2 (Slug) and resulted in transdifferentiation into M-cells(73). The expression of glycoprotein-2 (GP2), a key receptor

expressed on mature M-cells also appears to be dependent on the nuclear translocation of RelB(41).

Whilst there is substantial evidence that RelB is involved in the development of Peyer's patches and M-cells within FAE, the mechanisms involved remain unclear. Wang and colleagues demonstrated that Peyer's patch development was dependent on stimulation of TNFR and LTβR(78), however the precise ligands for these receptors and the downstream effects of signaling via this pathway during the development of Peyer's patches have not yet been defined.

There is also evidence that alternative pathway NF-κB signaling may influence gastric epithelial homeostasis. Mice lacking the COOH-terminal ankyrin repeat domain of p100 synthesize a truncated form of NF-κB2, which lacks the IκB activity of p100, but is able to dimerize with other NF-κB protein family members and can translocate to the nucleus to influence transcription. This models unimpeded alternative pathway NF-κB activation and leads to persistent translocation of NF-κB2-containing dimers to the nucleus. These animals exhibit massive gastric antral hyperplasia, have increased lymphocyte proliferation and die during early postnatal life due to complications following gastric outlet obstruction(38).

Alternative pathway NF-κB signaling in cancers of the gastrointestinal tract

Inflammatory mediators influence many cellular functions that may promote development of a malignant phenotype(32). Since NF-κB signaling regulates innate immune responses, influencing the expression of a set of target genes in epithelial and immune compartments including cytokines (e.g. TNF, interleukin (IL)-6 and IL-1β)(35, 46, 70), their receptors (TNF superfamily receptors) and other inflammatory mediators, including cell adhesion molecules

such as vascular cell adhesion protein 1 (VCAM-1)(37, 65) as well as genes involved in regulating the cell cycle such as cyclins and cyclin-dependent kinases(33, 39, 43), it is unsurprising that these processes also influence tumorigenesis.

The majority of studies investigating NF-κB and cancers of the gastrointestinal tract have focused upon the classical activation pathway family members NF-κB1, RelA (p65) and c-Rel. However there is emerging evidence that alternative NF-κB activation pathway family members also play important roles during carcinogenesis throughout the GI tract.

Esophageal cancer

Two histological subtypes of esophageal cancer exist, both of which develop on a background of chronic inflammation. Esophageal squamous carcinoma is associated with inflammation induced by extrinsic stimuli including cigarette smoking and alcohol consumption. Esophageal adenocarcinoma is also associated with these extrinsic risk factors, but is also associated with chronic gastroesophageal reflux, and occurs on a background of columnar metaplasia (Barrett's esophagus). The epidemiology and pathophysiology of these conditions are distinct, with the incidence of both conditions increasing in western populations.

In normal squamous esophageal tissue, the expression of NF-κB proteins is relatively low. However tissue samples from patients with esophageal squamous cell carcinoma showed increased expression of NF-κB1(p50/105), NF-κB2(p52/100) and RelA up to 18-fold(40). In addition the anti-tumor activity of the hypo-methylating chemotherapeutic drug decitabine has been associated with increased expression of NF-κB2(47).

Few investigators have addressed the role of alternative pathway NF-κB signaling in gastro-esophageal reflux disease, Barrett's esophagus or esophageal adenocarcinoma in humans, however a recent study examined the role of reflux and smoking on the expression of alternative pathway NF-κB signaling components in the esophageal epithelium of mice. Using immunohistochemistry it was demonstrated that the abundance of NIK was increased in the presence of either cigarette smoke alone or reflux alone(2). One of the challenges of investigating the function of NF-κB pathways is the need to differentiate the abundance of proteins that signal within the pathway and their functional effects. These data provide inconclusive evidence that alternative pathway NF-κB signaling is involved in esophageal adenocarcinoma development, but support the need for further investigation of alternative pathway NF-κB signaling in this context.

Gastric Cancer

The most important etiological factor during gastric carcinogenesis is infection with *Helicobacter pylori*(1, 25, 30, 56, 58). *H. pylori* infection results in stereotypical pre-malignant gastric pathology progressing from atrophic gastritis through metaplasia into dysplasia and cancer. In humans this process develops over several decades; mouse infection with the closely related bacterium *Helicobacter felis* recapitulates this sequence of pathology over an accelerated timescale. We recently showed that, despite heavy colonization with *H. felis*, *Nfkb2*$^{-/-}$ mice developed minimal inflammatory cell infiltration, demonstrated a blunted cytokine response and did not develop the pre-malignant lesions associated with chronic *H. felis* infection(15) (Figure 2a-d).

Other data have demonstrated *in-vitro* relevance for this pathway in response to *H. pylori* in both gastric epithelial and immune cell culture systems(15, 56, 58). Hirata and colleagues

showed that IKKα accumulates in AGS cells following *H. pylori* infection, but this did not lead to NF-κB2(p100) processing to NF-κB2(p52). The consequences of this are not clear, however it has been postulated that accumulation of cytosolic NF-κB2(p100) may inhibit nuclear NF-κB2(p52) interaction with chromatin, leading to an altered transcriptional fingerprint(34).

Separately, *H. pylori* activation of NF-κB was shown to occur via a pathway requiring IKKα, IKKβ, and NIK in MKN45 and KATO III human gastric cancer cell lines. In this context, activation of NIK also required signaling via the adapter proteins TRAF2 and TRAF6(50). Further evidence for the involvement of NIK was provided by Neumann and colleagues[41] who demonstrated that activation of NF-κB by *H. pylori* required the formation of a complex between p21-activated kinase 1 (PAK1) and NIK(54).

In addition to the evidence that *H. pylori* can activate NF-κB via the alternative activation pathway in epithelial tissue, *in-vitro* processing of NF-κB2(p100) to NF-κB2(p52) has also been demonstrated in B lymphocytes in response to *H. pylori*(57). This may have implications for the development of gastric MALT lymphomas.

Further evidence that classical and alternative pathway NF-κB activation is relevant to *Helicobacter*-induced pathology comes from the evidence that clarithromycin, one of the antibiotics used most frequently to eradicate *H. pylori*, modulates *H. pylori*-induced activation of NF-κB via both activation pathways in gastric cancer cells(59).

Colorectal cancer

Sporadic colorectal cancers most commonly develop from adenomatous polyps(9). These lesions develop as a consequence of the acquisition of stereotypical mutations over time.

They occur in an otherwise non-inflamed colonic mucosa, but an inflammatory response to adenoma development is frequently identified. Despite this, few data regarding the importance of the alternative activation pathway of NF-κB signaling during sporadic colorectal carcinogenesis exist.

Amongst the best characterized pathways involved in colorectal carcinogenesis is the WNT signaling pathway. Activation of this pathway leads to stabilization of β-catenin, which is observed in many colonic adenomas. There is increasing evidence for crosstalk between the NF-κB and WNT signaling pathways, with evidence that activation of NF-κB pathways may accelerate the loss of APC during colorectal adenoma development(69). However there is little direct evidence to suggest that alternative pathway NF-κB signaling is involved.

There is circumstantial evidence that alternative pathway NF-κB signaling may exert an effect on sporadic colonic carcinogenesis. MicroRNA-518a-3p was observed to be down-regulated in colon cancers in proportion to their size and TNM stage. This microRNA targets NIK mRNA for degradation, therefore its down-regulation leads to increased NIK abundance and subsequent activation of NIK dependent NF-κB pathways(61). In support of this are data that have demonstrated an increase in IKKα abundance in tissue samples from patients with sporadic colon cancer (19).

In contrast to sporadic colon cancer, more has been published regarding the impact of alternative pathway NF-κB signaling during the development of colitis-associated colorectal cancer. Individuals with chronic inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, have an increased risk of colorectal cancer(75). In these patients, colorectal tumors develop on the background of colonic inflammation and rather than developing as discrete polyps, usually arise within fields of flat dysplastic mucosa(9).

The most established murine model of colitis-associated cancer uses a single dose of the DNA-damaging agent azoxymethane (AOM) followed by several doses of dextran sulfate sodium (DSS) to induce chronic colitis (AOM/DSS)(20). We have reported that *Nfkb2*$^{-/-}$ mice are resistant to AOM/DSS-induced colitis-associated adenoma development(14). Transgenic mice developed fewer dysplastic colonic lesions, were less susceptible to DSS-induced colitis (Figure 2i-p), and had a more robust apoptotic response following DNA damage compared to wild-type controls.

In contrast to *Nfkb2*$^{-/-}$ mice, *Nlrp12*$^{-/-}$ mice exhibited increased susceptibility to AOM/DSS induced colitis-associated colorectal cancer; polyps isolated from these mice had increased activation of the alternative NF-κB activation pathway. *Nlrp12*$^{-/-}$ primary dendritic cells showed elevated NIK expression, p100 processing to p52 and reduced abundance of TRAF3. This provides evidence for NLRP12 acting as a checkpoint on signaling via the alternative NF-κB activation pathway during inflammation and tumorigenesis(4).

Alternative pathway NF-κB signaling in conditions of the GI tract that predispose to cancer

Gastrointestinal tract infections

Chronic bacterial and helminth infections are established risk factors for the development of malignancy. The best-characterized organism is *H. pylori*, which plays a major role in gastric carcinogenesis as discussed above. Several lines of evidence have identified that alternative pathway NF-κB signaling is important in the host response to gastrointestinal pathogens.

Infection with the foodborne pathogen *Salmonella* Typhimurium has been shown to promote the differentiation of M-cells in the small intestine through induction of RANK, and its ligand RANKL, leading to increased differentiation of M-cells and increased uptake of the

pathogen. In addition to the evidence described above that *S*. Typhimurium may induce M-cell transdifferentiation via an alternative activation NF-κB pathway dependent mechanism Tahoun and colleagues(73) have also demonstrated that translocation of *S*. Typhimurium was significantly reduced in the presence of the NF-κB inhibitor SN50, suggesting that NF-κB signaling plays a role in enhancing *S*. Typhimurium translocation across the epithelium. Whilst this organism is not directly associated with gastrointestinal carcinogenesis, these mechanisms offer a paradigm by which other carcinogenic organisms may influence epithelial events via NF-κB signaling.

Banoth and colleagues demonstrated that C57BL/6 mice infected with *Citrobacter rodentium* developed colitis with epithelial accumulation of p100. *Nfkb2*$^{-/-}$ mice infected with *C. rodentium* were more susceptible to developing colitis than wild-type mice. Using reciprocal bone marrow transfer experiments, *Nfkb2*$^{-/-}$ mice given wild-type mouse bone marrow lost weight and had 100% mortality before day 10, whereas wild-type mice given either wild-type or *Nfkb2*$^{-/-}$ bone marrow did not lose weight or show increased mortality. These findings indicate that NF-κB2 expression within the non-myeloid compartment is involved in limiting the severity of *C. rodentium* associated colitis(10).

Alternative pathway NF-κB signaling is also implicated in the orchestration of immune responses to gastrointestinal helminth infestation. C57BL/6 mice generate a marked Th2 immune response following colonization with the whipworm *Trichuris muris.* This induces rapid expulsion of the helminth and clearance of infestation within 35 days of colonization. In contrast, *Nfkb2*$^{-/-}$ mice develop a chronic, persistent infestation with an impaired Th2 cytokine response(8). Recently, an immunosuppressed man with histoplasmosis and extra-intestinal *Hymenolepis nana* infection was found to possess abnormal proliferative cells

which displayed malignant characteristics. Upon investigation, the cells were found to be consistent with tapeworm stem cells; this was the first report of parasite-derived cancer cells in a human, further demonstrating the link between infection and cancer, and highlighting its relevance when considering GI exposure to helminths(52).

Inflammatory bowel disease

Chronic idiopathic inflammatory bowel diseases that affect the colon increase an individual's risk of developing colorectal cancer in proportion to the duration of disease and the extent of inflammation(22, 28, 62, 63). Evidence suggests that treatments that decrease an individual's inflammatory burden, including azathioprine(29) and 5-amino salicylic acid preparations(77) may decrease the risk of developing colitis-associated colon cancer.

The alternative NF-κB activation pathway has not to date been targeted specifically for the development of novel therapeutics for IBD. This is likely due to the fact that most studies in IBD have found activation of the classical activation pathway, but not the alternative pathway(7, 66, 67). For example, Ardite and colleagues used electromobility shift assays to detect NF-κB components in colonic biopsy samples from patients with active IBD who had been treated with steroids. The classical NF-κB activation pathway subunit p50 was found in inflamed tissue from IBD patients, but p65, p52, c-Rel and RelB were not detected(7). Conversely Andresen *et al* examined NF-κB activity by EMSA and supershift assays and demonstrated p52 DNA binding activity in colonic mucosal samples from patients with both ulcerative colitis and collagenous colitis(5). These articles demonstrate the heterogeneity of NF-κB signaling in tissues. Part of this variability is likely to come from the heterogeneity of tissue samples, which contain cells of many different types that may have different NF-κB signaling states. Another element of this heterogeneity may arise from the dynamic

shuttling of NF-κB sub-units between the nucleus and cytoplasm, which has been shown to be a feature of NF-κB activation *in-vitro*(53). As individual cells of the same lineage in a tissue will be in different phases of the shuttling process, this will add to the heterogeneity of the readout from EMSA assays performed using whole tissue samples.

In contrast to the findings in humans, mouse models of colitis induced by carrageenan and DSS have demonstrated that classical and alternative NF-κB signaling pathways are activated(12, 13, 81, 84) during colonic inflammation. One of the earliest events in IBD is thought to be impairment of enteric mucosal barrier function. We have modeled this by administration of LPS from *Escherichia coli* to C57BL/6 mice and mice lacking NF-κB2. We demonstrated that *Nfkb2*$^{-/-}$ mice were more resistant to LPS-induced small intestinal epithelial cell shedding compared to wild-type mice(81) (Figure 2e-h). Separately and as described above, we demonstrated that the severity of DSS-induced acute colitis was reduced in *Nfkb2*$^{-/-}$ mice relative to wild-type mice(14)(figure 2i-p).

Our studies, and those of Banoth suggest that there may be a function for these pathways in the regulation of enteral inflammation, suggesting that targeting alternative pathway NF-κB signaling in IBD may be a fruitful avenue for further research.

One element of IBD therapy in which tangential evidence for alternative pathway NF-κB signaling has been accrued is the role of functional foods. This is of particular interest in the context of Crohn's disease, where dietary modification with liquid feeding has been shown to offer therapeutic benefits, particularly in pediatric patients.

A study examining the role of parenteral nutrition in IBD demonstrated that a lack of enteral stimulation in mice led to globally reduced expression of NF-κB proteins in Peyer's

patches(42). Given the data that implicate RelB in Peyer's patch development, and the role that Peyer's patches play in immune priming, these findings suggest that diet-induced NF-κB signaling may influence GI immune tolerance.

In contrast the use of dietary flaxseed to attempt to ameliorate experimental colitis was found to exacerbate DSS-induced colitis in mice, which was associated with an increase in expression of RelB in colonic tissue(84). Together these studies highlight the functional effect that foods may have on NF-κB alternative pathway signaling in the gastrointestinal tract.

Celiac Disease

Gluten sensitive enteropathy (celiac disease) confers an increased risk of small bowel adenocarcinoma and small bowel lymphoma, however due to the rarity of these cases it is difficult to accurately quantify the degree of increased risk(17). Recent data have identified increased expression of NFKB2 in patients with celiac disease, irrespective of their adherence to a gluten free diet(80). Currently however it is not clear whether this association has any impact on the prevalence or biology of small bowel malignancies.

Potential for alternative pathway NF-κB signaling as a therapeutic drug target

One of the aspirations of research into this field is to identify potential therapeutic drug targets within the alternative NF-κB activation pathway. NF-κB signaling has been identified as a potentially useful therapeutic target in cancer due in part to the data arising from animal models of carcinogenesis reviewed here, and in part from the *in-vitro* evidence that signaling via NF-κB can influence each of the hallmarks of cancer. Much of the interest to

date has been dominated by targeting the classical NF-κB activation pathway, however direct targeting of the NF-κB proteins has so far been of little value.

There are existing drugs which influence alternative pathway NF-κB signaling, largely by "off target" effects. There are several established examples in which the mechanism of action of the drug has not been fully characterized, and in which multiple off-target responses may be expected. Such drugs include sulfasalazine, which has been used for the treatment of ulcerative colitis since the 1940s (72), and which has been shown to inhibit both IKKα and IKKβ (79). This phenomenon is not however limited to established drugs with imprecisely defined molecular effects; drugs developed in the precision medicine era also have diverse effects on NF-κB signaling. The most well studied example is the 26S proteasome inhibitor Bortezomib. This drug non-selectively inhibits the kinase targeted breakdown of signaling molecules by the proteasome, and has marked effects on both classical and alternative NF-κB activation pathways. Bortezomib is effective in multiple myeloma, and a specific subset of patients with a defined NF-κB dependent pattern of transcription has been identified in whom Bortezomib is particularly effective. However as this agent impacts both classical and alternative NF-κB pathways, it is not possible to interpret how effective drugging the alternative pathway is in this context.

The development of novel drugs that specifically target alternative pathway NF-κB signaling will be relatively challenging due to the vast number of downstream effects. It is therefore likely that to develop rational therapeutic strategies that target this signaling network, careful modelling of upstream regulatory components which combine to trigger specific NF-κB responses will be required. For this reason, future investigations of potential therapies in this area will need to move beyond simple strategies targeting an individual component of

the pathway towards an understanding of how the NF-κB signaling network is altered in different disease states. Understanding this, and the regulatory pathways that lead to these differences, will allow the identification of drugs that interfere with NF-κB signaling to promote a more physiological state.

Discussion

Several recent studies have highlighted the importance of alternative pathway NF-κB signaling in addition to the more widely studied classical NF-κB signaling pathway during the development and progression of both inflammation-associated cancers of the GI tract and sporadic cancers which have an inflammatory element. Some elements of NF-κB signaling pathways and their implications for disease have been well characterized, but investigation in other areas is lacking (table 1). In particular, gaps exist in our understanding of these pathways in the formation of esophageal cancers and the role, if any, played by the gastrointestinal microbiome in regulating them.

Most of the studies reported to date have relied on relatively imprecise tools to characterize the function of NF-κB signaling pathways, for instance the observation that NF-κB activation events may be localized to specific anatomical structures within in the GI tract (e.g. Peyer's patches) suggests that previous studies which have assessed NF-κB activation events in whole tissue preparations should be interpreted with caution. Many investigations have also relied on either transgenic mice in which the stoichiometry of different pathway members is fundamentally altered, or have used semi-quantitative analyses of protein abundance to try to explain the complexities of these pathways.

Banoth and colleagues have demonstrated a different approach to research in this field by employing systems medicine techniques to characterize interactions between multiple components of this complex transcriptional regulating machinery. In the future, increasingly complex mathematical modeling will be required to better understand how inflammation is regulated by NF-κB signaling in real-time, and to apply these models to complex systems, including the pathogenesis of cancer. To achieve this, close collaboration between researchers with diverse backgrounds including clinician scientists, molecular biologists and systems biologists will be necessary.

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Figure Captions

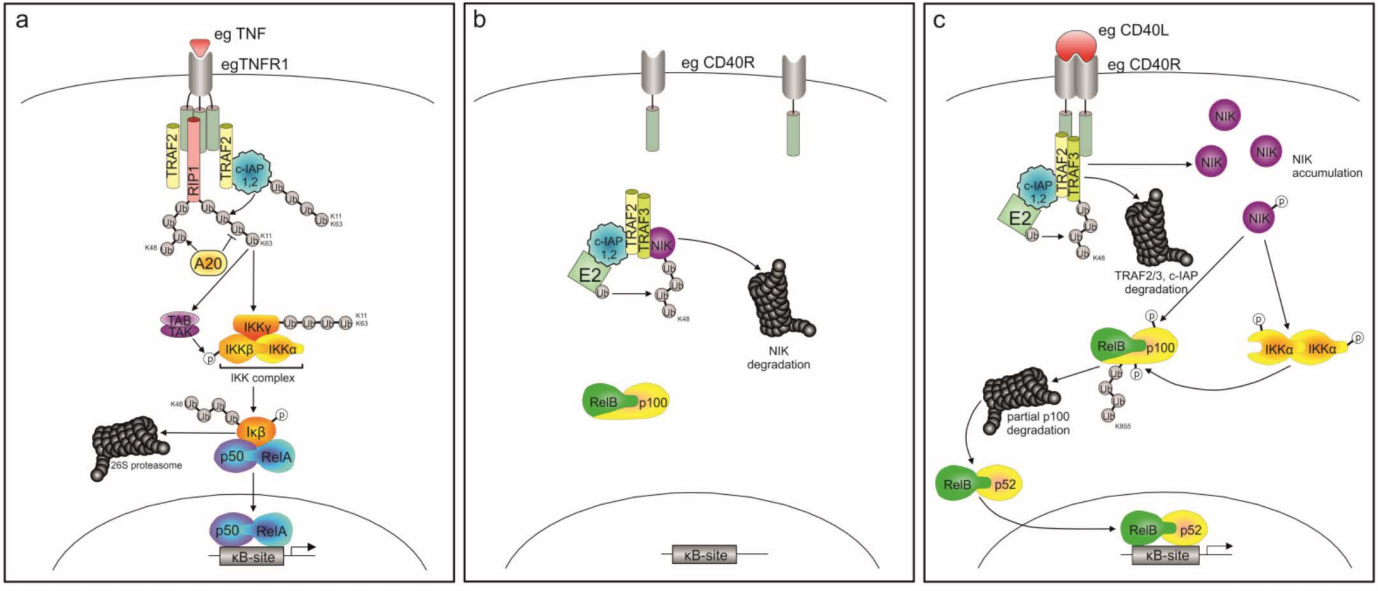
Figure 1 Classical NF-κB signaling pathway activation by receptor binding results in the recruitment of RIP1 (Receptor-interacting protein kinase 1) to the receptor complex and subsequent ubiquitination of IKKγ and phosphorylation of IKKβ resulting in the activation of the IKK complex (consisting of IKKγ, IKKβ and IKKα). The IKK complex targets IκB for proteasomal degradation allowing classical NF-κB pathway dimers to translocate to the nucleus and activate target gene transcription. Regulators of the classical NF-κB signaling pathway also include A20 (also known as TNFAIP3), TAK (transforming growth factor β-activated kinase) and TAB (TAK binding protein) (a). Unlike the classical pathway of NF-κB activation, the alternative pathway is normally held inactive by the continuous proteasomal degradation of NIK mediated by the ubiquitin-ligase complex consisting of E2, c-IAP 1 or 2, TRAF 2 and 3 (b). Upon ligand binding receptor (eg CD40L), TRAF3 is polyubiquitinated by the ubiquitin-ligase complex and targeted for proteasomal degradation, thus releasing NIK to accumulate in the cytoplasm. NIK phosphorylation results in activation of NIK kinase activity and both NF-κB2(p100) and IKKα are then phosphorylated by NIK allowing the homodimerisation of IKKα. IKKα homodimers further phosphorylate p100 at the C- and N- termini resulting in ubiquitination and partial degradation of the p100 NF-κB2 subunit to mature p52 protein. RelB-p100 dimers are transcriptionally inactive, however following p100 proteasomal processing, RelB-p52 dimers are able to enter the nucleus and activate transcription (c).

Figure 2 The gastric corpus (a-d), small intestine (e-h) and colon (i-p) of untreated *Nfκb2*$^{-/-}$ mice (b, f, j, n) are phenotypically normal compared with C57BL/6 wild-type mice (a, e, i, m). The administration of *Helicobacter felis* by oral gavage results in extensive pathology in the gastric corpus of C57BL/6 mice 12 months following indicated by inflammatory infiltrate (arrows), parietal cell atrophy (arrow heads) and cystic glands (open arrow head) (c). The intraperitoneal administration of 0.125mg/kg lipopolysaccharide (LPS) to C57BL/6 mice results in a rapid onset and increased abundance of small intestinal epithelial cell shedding and apoptosis 1.5 hours following and is

indicated by active caspase-3 immunohistochemistry (g). Administration of 2% dextran sulphate sodium in the drinking water of C57BL/6 mice for 5 days followed by 3 days of recovery on normal drinking water results in extensive colonic pathology resembling human colitis (k, o). Arrow heads show inflammatory infiltrate and arrows indicate regenerating colonic crypts (o). However, *Nfκb2*$^{-/-}$ mice administered with the same treatments do not show such severe pathology in gastric corpus (d), small intestine (h) and colon (l, p) and are protected from injury induced by these stimuli. Original magnifications: 16x (a-h), 10x (i-l) and 25x (m-p).

Table 1

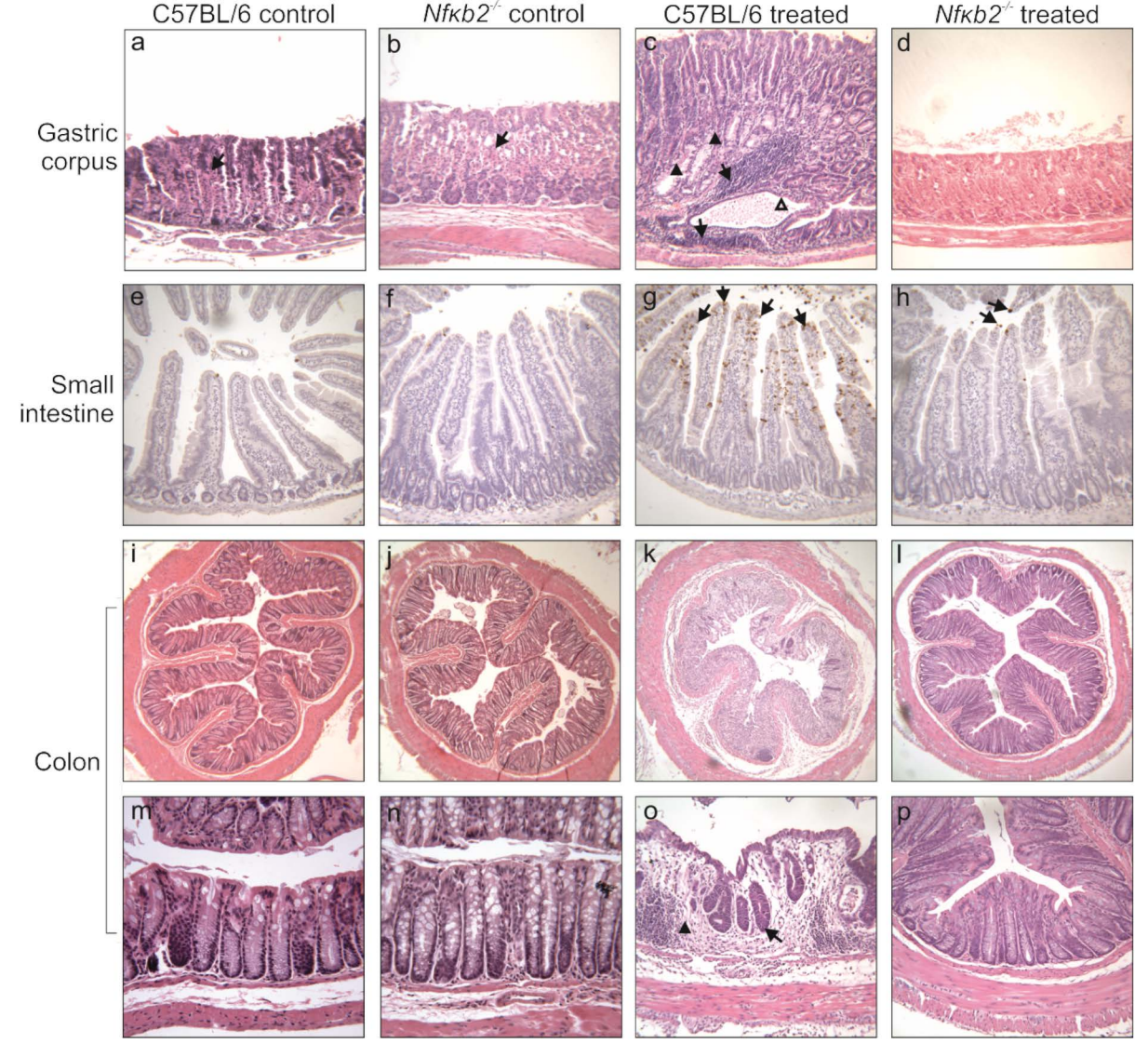
Setting	RELB	NFKB2
Protein expression in the gastrointestinal tract	Murine gastric epithelium [19]. Peyer's patches, including follicle associated epithelium of the small intestine [18].	Murine gastric epithelium [19].
Gastrointestinal phenotype of unstressed transgenic mice	No published data	Mice lacking the COOH-terminal ankyrin repeat domain of p100 exhibit gastric epithelial hyperplasia and die due to gastric outlet obstruction [30].
Esophageal carcinogenesis	No published data	Increased abundance of NFKB2 protein identified in esophageal tissue from patients with squamous cell carcinoma of the esophagus [39].
Gastric carcinogenesis	No published data	Processing of p100 to p52 in B-lymphocytes was induced by *Helicobacter pylori* co-culture *in-vitro* [50]. *Nfkb2*$^{-/-}$ mice were resistant to *H. felis* induced carcinogenesis [19].
Colon carcinogenesis	No published data	*Nfkb2*$^{-/-}$ mice were resistant to azoxymethane and pulsed dextran sulphate sodium induced colitis associated adenoma formation [57].
Gastrointestinal infection and infestation	No published data	*Nfkb2*$^{-/-}$ mice developed more severe colitis in response to *Citrobacter rodentium* [17]. *Nfkb2*$^{-/-}$ mice developed chronic persistent infection with *Trichuris muris*, in contrast to wild-type mice which spontaneously clear the infestation [59].
Inflammatory Bowel Disease	No published data	*Nfkb2*$^{-/-}$ mice were resistant to both DSS induced colitis and lipopolysaccharide induced epithelial cell apoptosis and shedding [71, 57].
Celiac disease	No published data	An increase in the expression of NFKB2 has been identified in tissue from patients with celiac disease, this has yet to be correlated with disease pathogenesis or response to gluten free diet [76].



Classical pathway

Inactive alternative pathway

Active alternative pathway


C57BL/6 control
Nfκb2-/- control
C57BL/6 treated
Nfκb2-/- treated
Gastric corpus
Small intestine
Colon
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