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Title: Epigenetic Modifications of the Nuclear Factor Kappa B Signalling Pathway and its Impact on Inflammatory Bowel Disease

Short title: Epigenetic modifications of NF-κB in inflamed bowel

Authors: Stamatia Papoutsopoulou[1, *] and Barry J. Campbell[1, *]

Author ORCID Numbers: 0000-0001-6665-8508 and 0000-0002-7407-012X

Address: [1] Henry Wellcome Laboratories of Molecular & Cellular Gastroenterology, Faculty of Health & Life Sciences, University of Liverpool, Liverpool, L69 3GE, UK

***Corresponding authors:**

Address correspondence to Dr Stamatia Papoutsopoulou or Professor Barry J. Campbell at The Henry Wellcome Laboratories of Molecular & Cellular Gastroenterology, Faculty of Health & Life Sciences, University of Liverpool, Liverpool, L69 3GE, UK. **Tel:** +44-151-794-6829. **E-mail addresses:** **s.papoutsopoulou@liverpool.ac.uk** and **b.j.campbell@liverpool.ac.uk**

ABSTRACT:

Background: Inflammatory bowel disease (IBD) is a multifactorial condition influenced by the immune system, the intestinal microbiota, environmental factors, genetic and epigenetic factors. Genetic- and environment-induced dysregulation of the Nuclear Factor-kappa B (NF-κB) transcription factor pathway has been linked to IBD pathogenesis. **Objective:** To assess the current evidence in relation to the contribution of the classical and alternative NF-κB pathways in IBD and to discuss the epigenetic mechanisms that impact on NF-κB function. **Methods:** A Medline search for 'NF-kappaB/NF-κB', in combination with terms including 'inflammatory bowel disease/IBD', 'intestinal inflammation', 'Crohn's disease', 'ulcerative colitis', 'colitis'; 'epigenetics', 'DNA methylation', 'histones', 'microRNAs/miRNAs' and 'short non-coding/long non-coding RNAs' was performed. **Results:** Both NF-κB pathways contribute to the chronic inflammation underlying IBD by regulating the inflammatory immune responses and homeostasis of the intestinal epithelium (classical pathway) or regulating bowel inflammation and epithelial microfold (M) cell function (alternative pathway). DNA methylation is a common epigenetic modification in intestinal inflammation, including *NFKB1* and *RELA* loci. Conversely, little is understood regarding epigenetic effects on genes encoding other NF-κB subunits, particularly those of the alternative pathway, and in the context of IBD. However, NF-κB interaction with chromatin modifiers is also seen to be an essential mechanism of regulation of downstream target genes relevant to NF-κB-mediated inflammatory responses. **Conclusion:** Further research is clearly warranted in this area, as understanding the cell-specific regulation of the NF-κB pathways will bring researchers into a position to achieve more efficient stratification of IBD patients, and more targeted and effective choice of treatment.

Key words: Epigenetics, NF-κB, inflammation, intestine, Crohn's disease, ulcerative colitis, colon cancer.

1. INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gut encompassing two main phenotypes; Crohn's disease and ulcerative colitis [1, 2]. The hallmark of IBD is chronic, uncontrolled inflammation of the intestinal mucosa driven by the imbalance between pro-inflammatory and anti-inflammatory cytokines [3]. Crohn's disease is characterised by deep tissue inflammation, typically with granulomata and may affect any part of the gastrointestinal tract from the mouth to the anus, although it most commonly affects the terminal ileum and/or colon. Ulcerative colitis is confined to the colon, particularly the sigmoid colon and rectum, and causes more superficial, non-granulomatous ulceration. In patients with IBD the mucosal immune system is also chronically activated, driving and perpetuating inflammation in the intestine [1-4]. One of the main problems in IBD is that the response to treatment can vary immensely and there is no efficient way as yet to predict this. Only 60% of patients with acute severe colitis will respond to therapy with steroids [4, 5] and a similar percentage may respond to rescue therapy, mainly anti-tumour necrosis factor (TNF) antibody treatment; the remainder will undergo colectomy [6]. Therefore, there is a real need to reliably predict response to therapy and develop personalised treatment approaches [7-9].

2. The NF-κB family of transcription factors

At the cellular level, one of the main signaling pathways in the immune system, activated in IBD is the Nuclear Factor kappa B (NF-κB) transcription factor pathway [10-13]. NF-κB pathway activation mediates gene transcription, synthesis and release of many cytokines involved in initiating and perpetuating inflammation [4]. The NF-κB family consists of ubiquitously expressed transcription factors that drive the transcription of a plethora of genes, regulating the development of the immune system, the innate and adaptive immune responses,

inflammation, and cancer [14-18]. Therefore, understanding of the factors that regulate NF-κB can potentially lead towards discovering targeted therapeutic interventions for IBD [19].

2.1. NF-κB dimers

There are five monomer family members; p50 (processed from p105), p52 (processed from p100), p65 (or RelA), c-Rel and RelB, that act as homo- or hetero-dimers [20-22]; see **Figure 1**. The dimers are held in the cytoplasm in an inactive state by the Inhibitors of NF-κB (IκBs) [23]. The IκBs also form a family, with five typical members; p105, p100, IκB alpha (IκBα), IκB beta (IκBβ), IκB epsilon (IκBε), and three atypical members B-cell lymphoma 3-encoded protein (BCL-3), IκB zeta (IκBζ) and IκBNS (also known as IκB delta, IκB-δ) [24]. Upon cell activation, IκBs become phosphorylated, ubiquitinated and then degraded, releasing active dimers that can translocate to the nucleus and drive transcription [25, 26].

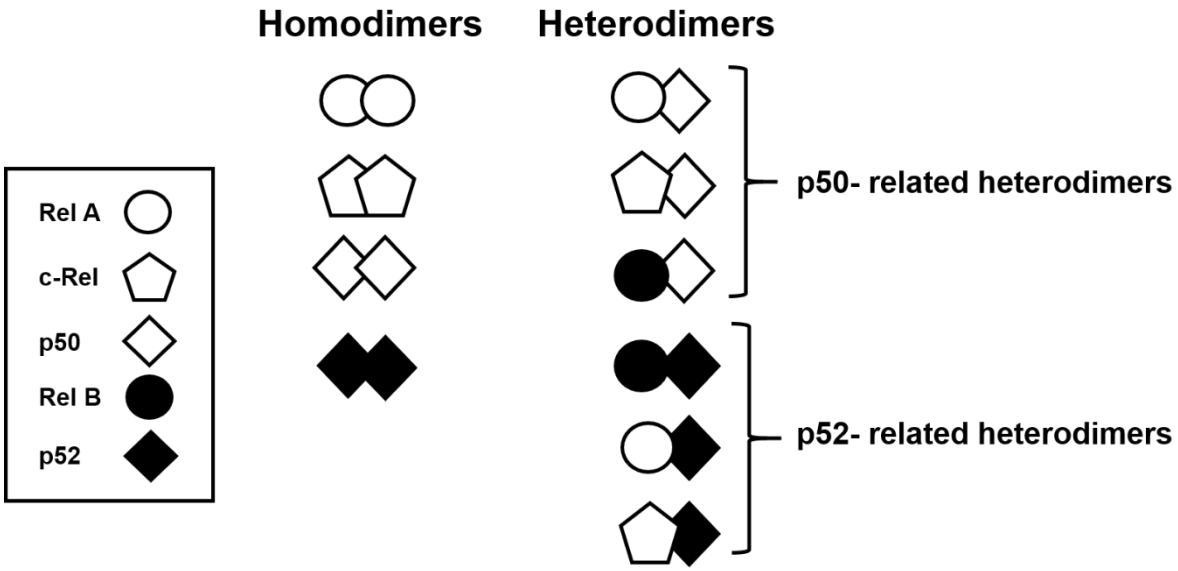

Figure 1: NF-κB dimers. Representations of Nuclear Factor kappa B (NF-κB) transcription factor homodimer and heterodimer combinations found in mammalian cells. The predominant dimer involved in classical signalling is p50:RelA(p65) heterodimer. Heterodimer p52:RelB is the predominant NF-κB dimer of alternative NF-κB pathway signalling.

2.2. NF-κB signalling pathways

There are two NF-κB pathways, the classical (canonical) and alternative (non-canonical) pathways, that regulate different dimers and are being regulated by different upstream kinases, the IκB kinases (IKKs) [27-29]; see **Figure 2.**

2.2.1 Classical NF-κB signaling

The classical NF-κB pathway is activated downstream of a variety of receptors, including Toll-like receptors (TLRs), T-cell receptor (TCR), B-cell receptor (BCR) or TNF-receptors (TNFR1/2). In brief, activated TLRs recruit adapter proteins, such as myeloid differentiation primary response 88 adaptor protein (MyD88). The exception is TLR3, which instead recruits and activates members of the Interleukin 1 (IL-1) receptor-associated kinase (IRAK) family [30]. The IRAK-MyD88 association triggers hyperphosphorylation of IRAK leading to its interaction with the downstream adaptor, TNF receptor-associated factor 6 (TRAF-6). The interaction of IRAK with TRAF-6 leads to activation of transforming growth factor beta (TGFβ)-activated kinase 1 (TAK1) and subsequently activation of the IKK complex [31]. The TNF receptor superfamily represents another collection of receptors that activate classical NF-κB signalling, and represents an important receptor-ligand pathway within the intestinal epithelium. Engagement of TNFR1 by TNF leads to the recruitment of TNF-receptor-associated death domain protein (TRADD), which then associates with TRAF-2 and receptor-interacting protein kinase (RIP). TRAF-2 subsequently recruits the IKK complex to the TNFR1 complex, where RIP activates the catalytic IKK subunits via mitogen-activated protein kinase kinase kinase 3 (MEKK3). TCR and BCR signalling involve recruitment of signalosome complex (CARMA1–BCL10–MALT1–TRAF6) that leads to TAK1 activation [32]. The IKK complex consists of two kinases IKK1 and IKK2 (also known as IKKα and IKKβ), and a regulatory subunit named NF-κB essential modulator (NEMO, or IKKγ) [33]. Consequently,

IKK2 phosphorylates IκBα and targets it for ubiquitination and proteasomal degradation, allowing the classical NF-κB pathway dimers to translocate to the nucleus and activate transcription. Further information regarding the detailed complexities of the adaptor/modulator interactions of the classical NF-κB pathway can be found in a recent article published by Yu and colleagues [34].

2.2.2. Alternative NF-κB signalling

The alternative NF-κB pathway, unlike the classical pathway, is normally held inactive by the continuous proteasomal degradation of NF-κB-inducing kinase (NIK) mediated by a ubiquitin-ligase complex. Upon ligand-receptor binding (e.g., CD40L to CD40, B cell activating factor (BAFF) to BAFFR, or lymphotoxin to LTβR), TRAF3 is polyubiquitinated and targeted for proteasomal degradation, leading to NIK accumulation in the cytoplasm. NIK phosphorylation results in activation of its kinase activity, allowing phosphorylation and homodimerization of IKK1. IKK1 homodimers further phosphorylate p100, resulting in ubiquitination and degradation, followed by release of active RelB-p52 dimers that are able to enter the nucleus and regulate transcription. For further detailed information regarding the adaptor/modulator complexities of the alternative NF-κB pathway, see [34].

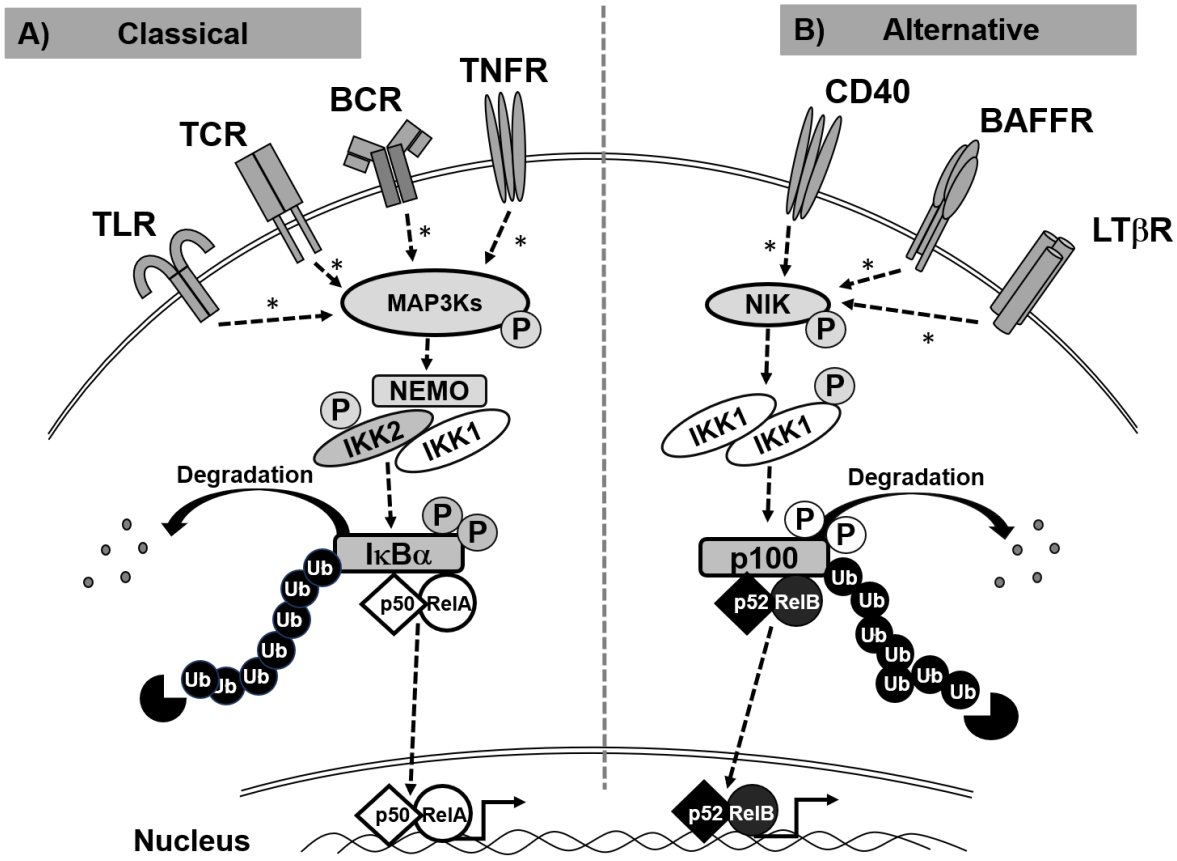

Figure 2. Classical (canonical) and alternative (non-canonical) NF-κB signalling pathways. (A) The classical NF-κB pathway is activated downstream of a variety of receptors, such as Toll-like receptors (TLRs), T-cell receptor (TCR), B-cell receptor (BCR) or TNF-receptor (TNFR). Ligand-receptor binding triggers downstream cascades that lead to autophosphorylation/activation of a family of receptor pathway-specific mitogen-activated protein kinase kinase kinases (MAP3Ks), and subsequently activation of the IKK complex (consisting of IKK1, IKK2 and NEMO). Activation of IKK2 then leads to phosphorylation of IκBα and targets it for ubiquitination and proteasomal degradation, allowing classical pathway NF-κB dimers (such as p50:RelA) to translocate to the nucleus and activate transcription. **(B)** The alternative pathway is normally held inactive but upon ligand-receptor binding (e.g., CD40L to CD40, B cell activating factor (BAFF) to BAFFR, or lymphotoxin to LTβR), NF-κB-inducing kinase (NIK) is stabilised leading to its accumulation in the cytoplasm. NIK phosphorylation results in activation of its kinase activity, allowing subsequent phosphorylation of IKK1. IKK1 homodimers further phosphorylate p100, resulting in ubiquitination and degradation followed by release of active p52:RelB dimers that are able to enter the nucleus and regulate transcription. * For information on upstream receptor-specific adaptor/modulators please refer to the article text.

3. NF-κB signalling in inflammatory bowel disease

Hyperactivation of NF-κB has been associated with increased levels of pro-inflammatory cytokines such as TNF, Interleukin-1 beta (IL-1β) and Interleukin-6 (IL-6) which promote intestinal inflammation and gastrointestinal carcinogenesis [13, 35, 36]. In pre-clinical models, NF-κB has been suggested to be a central player linking IBD to colorectal cancer (CRC) [37] by inducing cellular transformation, mediating cellular proliferation and preventing the elimination of pre-neoplastic cells [38, 39]. Apart from the immune system, the pathogenesis of IBD is also influenced by environmental factors within the exposome and genetic predispositions [40, 41]. The majority of research has been focused on the classical NF-κB pathway, which is known to regulate inflammatory responses. Nevertheless, there is emerging interest in the contribution of the alternative pathway in the development of IBD and IBD-associated cancer. The two pathways are discussed separately below, and for an overview of the role of NF-κB pathway signalling in intestinal inflammation, see **Figure 3**.

3.1. Classical NF-κB pathway activation

The classical NF-κB pathway is activated by the upstream IKKs, mainly IKK2 (or IKKβ) and shows a rapid and transient activation, typically generating an active heterodimer (p50:RelA) that regulates transcription of many genes encoding pro-inflammatory factors.

In intestinal epithelial cells, NF-κB has a key protective role to support barrier homeostasis [42, 43]. Studies utilising genetically modified animals have highlighted important functions of NF-κB in regulating cell survival, barrier integrity, and the immunological and anti-microbial responses of intestinal epithelial cells [44, 45]. NF-κB inhibition can be harmful and trigger the development of inflammation and disease [46] and as discussed by deFonseca and Kaunitz, IKK/NF-κB signalling is a major mechanism of gastrointestinal defense [47]. Also, *in vivo* intestinal epithelium-specific deletion of both IKK1 and IKK2 has been shown to result

in spontaneous development of intestinal inflammation in mice [48]. In IBD, classical NF-κB signalling has been shown to influence the severity of intestinal inflammation [11]. Elevated RelA (p65) protein levels or high DNA-binding activity of NF-κB has been detected in intestinal biopsies of Crohn's disease patients [49-51]. More recently, Yao *et al.* showed that in IBD the chronic NF-κB activation sensitises intestinal epithelial cells to TNF-induced apoptosis in a ripoptosome-dependent manner [52]. Mice lacking the classical NF-κB signalling pathway in intestinal epithelial cells demonstrate increased susceptibility to colitis, indicating a protective role for NF-κB in intestinal epithelium during oxidative stress/hypoxic inflammation [53, 54]. Similarly, *Nfkb1*$^{-/-}$ mice, are also show higher susceptibility to *Helicobacter felis*-driven carcinogenesis [55] and to colitis-associated adenoma development [56]. In humans, population-based studies have demonstrated that single nucleotide polymorphisms (SNPs) in *NFKB1*, the gene that encodes the p105/p50 subunit, and NF-κB target genes indicative of high inflammatory response, were associated with increased risk of Crohn's disease [57, 58].

In activated innate immune cells, such as macrophages and dendritic cells, classical NF-κB pathway signalling drives transcription of genes encoding pro-inflammatory cytokines, such as TNF, IL-6 and IL-1β [31, 59-61]. It is well-established that intestinal macrophages show inflammatory anergy and this phenotype seems to be due to multiple factors [62, 63]. As an example, human intestinal macrophages do not express the pathogen-associated molecular pattern CD14 (cluster of differentiation 14) and they have very low levels of MyD88 and TNF receptor-associated factor 6 (TRAF6); both key adaptor molecules that relay signal crucial for activation of NF-κB downstream of Toll-like receptors (TLRs) [62]. In addition, mast and stromal cells present in the intestinal mucosa typically produce higher levels of transforming growth factor beta (TGF-β), known to block NF-κB pathway activation downstream of TLR2, TLR4 and TLR5 via ubiquitination and degradation of MyD88 [63]. We have shown activation

dynamics of the classical NF-κB pathway to be significantly elevated in peripheral blood mononuclear cell-derived macrophages from Crohn's disease patients when compared to macrophages derived from both healthy donors and patients with ulcerative colitis [64].

Regarding adaptive immune cells, T lymphocyte differentiation into effector cells (e.g. Th1 and Th17) and their maintenance is also classical NF-κB signalling pathway dependent [65-69]. *Nfkb1* knock-in mice that express p50 but not its precursor, p105, display constitutive NF-κB activation and spontaneously develop colitis characterized by hyperproduction of Th17 cells [70]. T cell profiling studies on biopsies taken at time of diagnosis have revealed that inflammatory bowel disease patients have higher numbers of $CD4^+$ T cells, T regulatory cells (Tregs) and central memory T cells (TCMs), and lower observed proportions of $CD8^+$ T cells [71]. Different adaptive immune response are seen in Crohn's disease and ulcerative colitis though. Crohn's disease patients show a Th1/Th17 response with increased levels of pro-inflammatory cytokines, such as TNF, interferon-gamma (IFN-γ) and interleukin 17A (IL-17A) [1].

NF-κB activation is also important in the endothelium since it regulates the expression of adhesion molecules that play a pivotal role in leukocyte–endothelium interactions [72]. The gut endothelium plays a key contributory role in progression of IBD because it also releases pro-inflammatory cytokines, and upregulates adhesion molecules, e.g. E-selectin and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), that facilitate adhesion and migration of pro-inflammatory and cytotoxic leukocytes expressing CX3CL1/fractalkine, a chemokine that attracts dendritic cells and macrophages [73]. The majority of these molecules are regulated by the canonical NF-κB pathway [74].

In our own studies, hyperactivation of the classical NF-κB pathway in Crohn's disease patients was not seen to correlate with disease severity, activity, nor disease status or type of medication,

but rather was seen to correlate with the smoking status, although this was not the case in healthy donors [64]. Smoking is well-known to cause epigenetic modifications on DNA or histones that predispose to adverse health outcomes, such as respiratory and cardiovascular diseases, and cancer [75]. There is a lot of literature showing that smoking has a protective role in ulcerative colitis but has detrimental effects in Crohn's disease and yet there is no clear explanation for this discrepancy [76]. Another level of complexity here, is that cigarette smoke contains more than 5000 ingredients, some of which are known to influence barrier integrity of the gut epithelium, immune responses and intestinal microbiota composition, and cause epigenetic modifications influencing gene expression relevant to development and progression of IBD [41, 77, 78]. It has been also shown that cigarette smoking promotes inflammation by mainly activating the NF-κB family of transcription factors [79]. Conversely, Wang and colleagues have shown that 'side-stream' smoking (i.e. smoke emitted from the burning end of a cigarette, rather than the direct inhaled 'mainstream' smoke) reduces intestinal inflammation and activation of the classical pathway [80].

Hypoxic challenge has also been shown to modulate NF-κB activation in IBD [81] and known to affect gene expression through epigenetic modification of histones and chromatin remodelling [82]. Hypoxia-induced NF-κB activation in turn induces transcription of hypoxia-inducible factor 1 alpha (HIF-1α), a key downstream regulator of many genes involved in angiogenesis and vacularisation, enhanced oxygen delivery and adaptation to a hypoxic state, and in regulation of cellular apoptosis and energy metabolism [83, 84].

3.2. Alternative NF-κB pathway activation

The alternative NF-κB pathway mediates activation of the p52/RelB heterodimer in response to signals mediated by a subset of TNF receptor superfamily members and regulate immune

cell differentiation and maturation and secondary lymphoid organogenesis [28, 85, 86]. Whilst much less is known about the role and importance of the alternative NF-κB pathway in IBD patients, there is some indication of its contribution to the chronic inflammation underlying IBD and inflammation-associated gastrointestinal cancer [87-89]. Recent studies using *Nfkb2*$^{-/-}$ mice that do not synthesize p100/p52, show they are resistant to dextran sodium sulphate (DSS)-induced colitis and to colitis-associated colon adenoma development [56]. Moreover, several other key studies have shown that alternative NF-κB pathway signalling plays a role in regulating colonic inflammation [89] and in promotion of defence against enteric infection [85, 90-92]. This has suggested that p100/p52 inhibition could be a potential therapeutic approach to improve inflammation-associated intestinal disease [93]. Recently, Ramakrishnan and colleagues, using NIK knockout (*Nik*$^{-/-}$) mice, identified a role for the alternative NF-κB pathway in intestinal homeostasis, with this upstream protein kinase, that induces proteolytic processing of NF-κB2/p100, being required for maintenance of key bacteria/antigen sampling microfold cells (M cells) that constitute about 5-10% of the dome or follicle-associated epithelium overlying Peyer's patches in the distal ileum and smaller lymphoid follicles of the colon [94]. Constitutive intestinal epithelium NIK signalling is active in mouse models of colitis and patients with ulcerative colitis [94] .

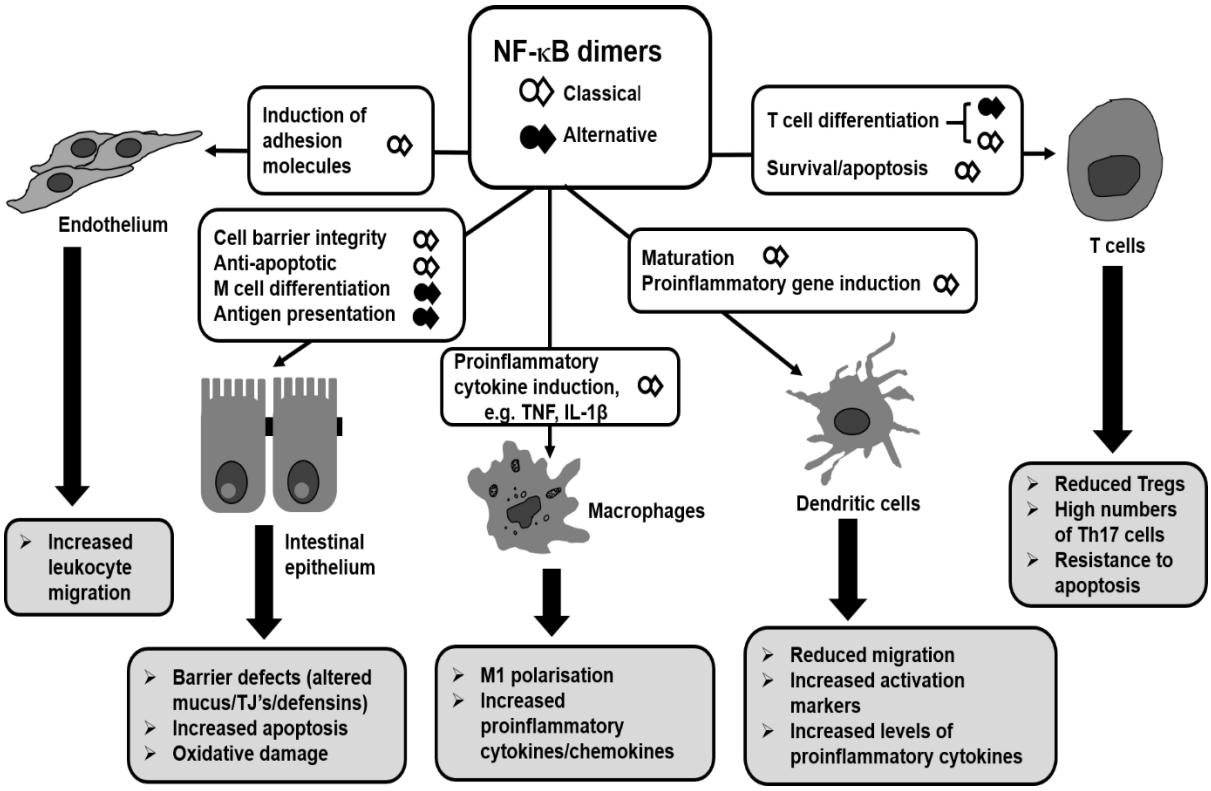

Figure 3. NF-κB and its role in intestinal inflammation development and progression. The pathway (classical or alternative) and functional role of NF-κB signalling relevant to the intestinal epithelium, endothelium, mucosal macrophages, dendritic cells and T lymphocytes (thin arrows and white boxes) are illustrated here, as are the cell-specific changes that have been observed in inflammatory bowel disease due to NF-κB signal pathway dysregulation (thick arrows and grey boxes). Note, only the predominant NF-κB dimer involved in classical NF-κB signalling, heterodimer p50:RelA (white symbols), and that of the alternative NF-κB pathway, heterodimer p52:RelB (black symbols), are shown for simplicity.

4. EPIGENETIC MODIFICATION OF NF-κB AND IκB FAMILY GENES

There are numerous genetic studies that link mutations in the NF-κB signalling pathway and human diseases [95], but very little is known about the epigenetic modifications of the NF-κB family members, with DNA methylation being the most studied modification to date. Epigenetic modifications are changes that affect the regulation of chromatic structure and gene expression or cellular characteristics and processes. There are three major categories of epigenetic modification; 1) DNA methylation, 2) histone modification – such as acetylation, deacetylation, phosphorylation and methylation, and 3) non-coding RNA, all of which have been associated with the development of human diseases [96-98]; see **Figure 4**.

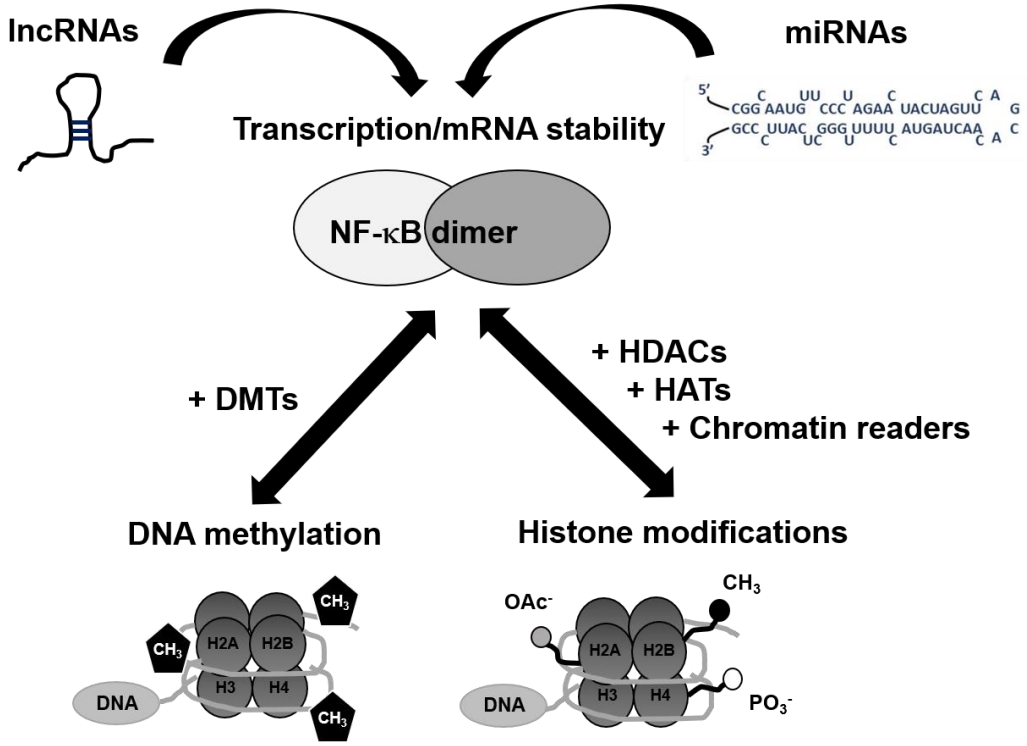

Figure 4: Epigenetic mechanisms that regulate the NF-κB transcription factor pathway. Non-coding RNAs, such as micro RNAs (miRNAs) and long non-coding RNAs (lncRNAs) regulate the transcription levels and the mRNA stability of the NF-κB family members. On the other hand, NF-κB dimers interact with enzymes that induce epigenetic modifications. Interaction with DNA methyltransferases (DMTs) induce DNA methylation. Instead, interaction with histone deacetylases (HDACs), histone acetylases (HATs) and chromatin readers, e.g. bromodomain and extraterminal proteins (BETs), induce histone modifications. Abbreviations: CH_3, methyl group; PO_3^-, phosphate group; OAc^-, acetate functional group.

4.1. Classical pathway genes

Studies on primary immune cells derived from healthy individuals, have shown that the methylation status of the promoter of *NFKB1* varies among monocytes, neutrophils and lymphocytes and this could potentially impact on the inflammatory response during sepsis [99]. Several other studies have also observed strong correlation of altered methylation status of genes regulating inflammation and with high levels of pro-inflammatory cytokines. A controlled, pilot study showed that in older women, high dose of milk product intake post-exercise induced hypermethylation of many genes driving inflammation, including *NFKB1* and *NFKB2* [100]. Hypomethylation of the *NFKB1* gene promoter was also seen in peripheral blood mononuclear cells of type 2 diabetes mellitus patients and correlated with increased levels of IL-1β [101]. Moreover, changes in the methylation levels of the *NFKB1* gene were correlated to decreased blood pressure after bariatric surgery [102]. Jeong and colleagues have shown that there was no significant difference in methylation status of *NFKB1* and also for *RELA,* the gene that encodes the p65 subunit, when comparing tumours versus normal breast tissue [103]. It was notable in this study, that methylation of the *RELA* was upregulated in those tumour tissues demonstrating high levels of pro-inflammatory TNF [103]. There is therefore the potential for this to be important also in IBD, especially given the high intestinal mucosal pro-inflammatory cytokines levels seen, such as TNF, but no similar human studies that have been conducted to date. There is however some evidence from *in vivo* experiments where increased tri-methylation of lysine 27 on histone 3 (H3K27me3) in the promoter of *Nfkb1* has been observed in the colonic tissue of mice fed with indigestible resistant starches known to attenuate intestinal inflammation [104]. This was due to an increase in butyrogenic intestinal bacteria and was supported by *in vitro* studies showing that exogenous addition of sodium butyrate produced similar tri-methylation of histone 3 in cultured human colonocytes [104].

Using an intestinal epithelium-specific knockout mouse model of the transcriptional repressor of inflammatory genes, retinoic acid-related orphan receptor alpha (RORα$^{\Delta IEC}$), induction of colitis using dextran sodium sulphate (DSS) demonstrated that *Nfkb1* was among the most upregulated genes the intestinal epithelium in the absence of RORα [105].

Whilst there are no direct studies examining epigenetic modification of *RELA* in IBD, there is evidence with respect to its impact on innate immune responses, with endogenous retroviral long non-coding RNAs (lncRNAs) shown to significantly alter transcriptional levels of *RELA* [106]. Similarly micro RNAs (miR) elevated in human colorectal tissues, such as miR-221 and miR-222, have been shown to directly bind to the coding region of the *RELA* gene and increase stability of *RELA* mRNA, a mechanism that could perhaps explain sustained activation of the NF-κB pathway observed in colorectal adenocarcinoma [107, 108]. Another miR-181 also regulates the NF-κB signalling pathway via inhibition of the tumour suppressor CYLD lysine 63 deubiquitinase in inflammation-associated colorectal cancer [109, 110]. A further intestinal study also examined for methylation changes in mucosal biopsy tissue obtained from patients with coeliac disease, where *RELA* was the only member of the NF-κB family that was observed to be significantly hypomethylated [111]. Epigenetic modification of *RELA* has also been studied with respect to aging pathologies [112], and in hyperglycaemia, methylation and acetylation modifications of the *RELA* promoter are also seen, leading to upregulation of *RELA* expression in both peripheral blood cells and endothelium [113, 114]. DNA methylation and acetylation of the *RELA* locus and the implications for type-2 diabetes have also been highlighted by Paneni and colleagues [115].

In a recent epigenome-wide DNA methylation study, the atypical **IκB** family member gene B-cell lymphoma 3 (*BCL3*) was seen to be differentially methylated in newly-diagnosed IBD

cases versus controls [116]. BCL3 is a known regulator of NF-κB mediated gene transcription [117] with levels elevated in the lamina propria of colonic tissues of patients with IBD [118]. Bcl-3 interferes with p50 function in a cell specific manner to drive colitis [118] and interaction of Bcl-3 with histone deacetylases leading to transcriptional termination is speculated as being perhaps a key mechanism of action [118, 119].

4.2. Alternative pathway genes

Regarding the alternative pathway, *RELB*, together with other loci, has been identified as a target of histone 3 increased acetylation in diabetic patients with gastrointestinal symptoms [120]. As was observed for *NFKB1*, high dietary intake of milk products post exercise in older women also induced hypermethylation of the *NFKB2* gene encoding p100/p52 [100]. In contrast, there is no literature to date regarding epigenetic modification of the other NF-κB alternative pathway family member gene, *REL* encoding c-Rel.

Table 1: Epigenetic modifications affecting expression of genes that encode the NF-κB and IκB family members.

Gene	Epigenetic modification	Condition
BCL3	DNA hypomethylation	Inflammatory bowel disease [a]
NFKB1/Nfkb1	DNA hypomethylation	Type 2 diabetes mellitus [b]
	DNA hypermethylation	Decreased blood pressure after bariatric surgery [c] Milk intake after exercise [d]
	Deacetylation by RORα/HDAC3 complex	Maintentance of homeostasis in intestinal epithelium [e]
	Trimethylation of histone 3	Resistant starch-derived butyrate effect on human colonic epithelial cells [f]
NFKB2	Increased DNA methylation	Milk intake after exercise [d]
RELA	DNA hypermethylation	Breast tumours [g]
	Induced mRNA expression by ERV-derived lncRNA	Antiviral responses [h]
	Induced mRNA stability by micro-RNA's miR-221 and miR-222	Colorectal adenocarcinoma [i,j]
	DNA hypomethylation	Coeliac disease [k]
	Differential methylation changes	Hyperglyceamia [l,m]
RELB	Histone acetylation	Diabetes and gastrointestinal symptoms [n]

Footnote: References, [a] [116]; [b] [101]; [c] [102]; [d] [100]; [e] [105]; [f] [104]; [g] [103]; [h] [106]; [i,] [107]; [j] [108]; [k] [111]; [l] [113]; [m] [114] and [n] [120]. Abbreviations: ERV, endogenous retrovirus; HDAC3, histone deacetylase 3; lncRNA, long non-coding RNA; miR, micro RNA; RORα, Retinoid-related orphan receptor alpha;

5. NF-κB SUBUNITS AND THEIR ROLE IN EPIGENTIC MODIFICATION OF DOWNSTREAM TARGET GENES

NF-κB transcriptional activity can be enhanced or inhibited as a consequence of epigenetic modifications on the target genes [121-124]; see **Figure 4**. This is due to the ability of the NF-κB proteins, to attract and form complexes with key chromatin modifiers, such as histone deacetylases interacting with the NF-κB p50 subunit [125], methyltransferases interacting with RelB, or chromatin reader proteins such as Bromodomain-containing protein 4 (BRD4) that recognizes and binds acetylated histones, interacting with the p65 (RelA) subunit [126, 127]. Extensive studies on the regulation of inflammatory responses identified an evolutionary conserved nuclear factor, Akirin 2, that links NF-κB to chromatin remodellers within macrophages and B lymphocytes [128]. Moreover, interactions of NF-κB with bromodomain and extraterminal (BET) proteins, key chromatin readers that recognise acetylated histones [129], are essential in the NF-κB-mediated inflammatory response of Th17-cells during transplantation [130]. It is worth noting that NF-κB is critical for the development of Th17 cells [131], a T cell lineage now known to play an important role in IBD [132]. This is further supported by a recent study that showed that a specific BET inhibitor could block Th17 development and ameliorate colitis in a T cell transfer mouse model [133]. In a DSS-induced colitis model, the p65/BRD4 complex is suggested to be active in the intestinal epithelium during the injury phase, while it upregulates transcription of proinflammatory cytokines TNFα and IL-1β. During the recovery phase though, BRD4 is being replaced by a p65/HDAC3/RORa complex which leads to dampening of inflammation [105]. The p65/BRD4 complex was shown to play an important role in regulating super enhancers during acute proinflammatory activation in human endothelial cells [134]. In that study, chromatin immunoprecipitation sequencing analysis revealed a striking colocalization of p65 and BRD4 to enhancer and promoter regions marked by H3K27ac, in TNF-stimulated human umbilical

vein endothelial cells (HUVECs). The NF-κB/BET protein complexes are therefore attractive pharmacological targets for treatment of inflammatory conditions and very recently a highly specific and potent inhibitor was suggested to block the BRD4/NF-κB/NLRP3 pathway in an acute gout arthritis rat model [135].

In other inflammatory conditions, such as chronic obstructive pulmonary disorder (COPD) of the lung, studies indicate that the oxidative stress resultant of an increased burden of inhaled smoke and pollutants, as well as enhanced levels of reactive oxygen species generated by inflammatory, immune and epithelial cells of the airways, promotes the formation of NF-κB and transcriptional co-activator complexes, which catalyse histone acetylation, leading to increased transcription of pro-inflammatory genes [136-138]. As a consequence, inhibition of histone deacetylases (HDAC) activity, histone deacetylation and downregulation of pro-inflammatory genes has been the target of various anti-inflammatory and antioxidant compounds, including curcumin, resveratrol and glucocorticoids [137]. In a similar approach, sulforaphane has been suggested to prevent aging and neurodegeneration based on its ability to inhibit HDACs and DNA methyltransferases (DMTs) and inhibit the transcriptional activation of NF-κB targets [139]. Sulforaphane has also been shown to supress HDACs in a model of intestinal inflammation and tumorigenesis [140]. MicroRNAs, also regulate the NF-κB pathway, either positively or negatively, and they have been shown to affect the innate immune responses [141], cell adhesion during inflammation [142], or contribute to the development of diseases, such as cancer [143-146]. MicroRNAs and long non-coding RNAs have been also linked to IBD [147, 148]. The caudal homeobox factor 1 (CDX1) is an essential transcription factor for intestinal differentiation and it is highly expressed in intestinal metaplasia, as a result of chronic inflammation of the upper gastrointestinal tract [149, 150]. Rau *et al*. showed that in human gastric cell lines there is methylation-dependent NF-κB

binding on the CDX1 promoter and in human biopsies DNA methylation levels correlate with its expression and inflammation-associated intestinal metaplasia [149].

Table 2: Summary of NF-κB subunit interactions with epigenetic modifiers and their downstream targets.

NF-κB subunit	Epigenetic modifiers	Transcriptional outcome
p50	Histone deacetylases (HDAC1 and Sirt1)	Suppression of Foxp3 renders iTregs permissive to differentiation into Th9 cells.[a,b]
RelA	BET family of bromodomain proteins	Induction of proinflammatory response in respiratory syncytial virus-infected cells [c], murine macrophages [d], or endothelial cells [e]
	Histone acetyltransferases (HATs)/ Histone deacetylases (HDACs)	Redox effect on lung inflammation [f]
	BRD4/CBP and HDAC3/RORα	Inflammation and intestinal epithelial regeneration in a murine DSS-induced colitis model [g]
	Methylation-dependent binding on CDX1 promoter	Gastritis and intestinal metaplasia [f]
	Micro-RNAs (such as miR-29b, miR-146a, miR-181b)	Expression of adhesion molecules in endothelial cells [g]
RelB	Histone methyltransferases (G9a and SETDB1)	Block of *Il17* expression and the suppression of Th17 cell induction [h] [121]
	Acetyltransferase p300/CBP	Mediation of Th9 cell induction [h]

Footnote: References, [a] [121]; [b] [125]; [c] [126]; [d] [130]; [e] [134]; [f] [138]; [g] [105]; [f] [149]; [g] [142]; [h] [121]. Abbreviations: G9a, Euchromatic histone-lysine N-methyltransferase 2; BET, Bromo- and Extra-Terminal domain; BRD4, Bromodomain-containing protein 4; CBP, CREB-binding protein; CDX1, caudal homeobox factor 1; DSS, dextran sodium sulphate; Foxp3, forkhead box P3/scurfin protein; HDAC, histone deacetylase; miR, micro RNA; RORα, Retinoid-related orphan receptor alpha; SETDB1, Histone-lysine N-methyltransferase; Sirt1, sirtuin (silent mating type information regulation 2 homolog); iTregs, induced in cell culture T regulatory cells; Th, T helper cell.

6. CONCLUSIONS

There is a wealth of evidence showing how various epigenetic modifications affect the NF-κB pathways and consequently the expression of downstream target genes. All these studies shed light on how these effects modulate the inflammatory responses, how they can lead to the development of diseases and identify therapeutic targets. Surprisingly, there is very limited literature in the field of gastrointestinal inflammatory diseases, including IBD, although, as discussed, the NF-κB pathways play a major role. It would be of great interest to identify epigenetic modifications in human immune or intestinal epithelial cells that could segregate healthy donors from individuals with IBD. Furthermore, future research should be aimed towards dissecting the complicated links between environmental factors, such as diet, smoking and microbiota composition and the epigenetic profiles of cell types that can be affected. In support of this, recent analysis has shown differential DNA methylation observed in intestinal mucosal cells, whole tissue, whole blood and purified monocytes and T lymphocytes [116, 151-155] obtained from IBD patients. An interesting hypothesis would be that environment-induced epigenetic modifications and NF-κB could be a significant underlying mechanism of IBD, and that once understood, personalised medicine approaches could lead to more effective treatment.

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Not applicable.

CONFLICTS OF INTERESTS:

SP and BJC are employees of the University of Liverpool. BJC is a current advisory board member for Anatara Lifesciences Ltd; Melbourne, Australia.

AUTHOR CONTRIBUTIONS:

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