Identification of a novel therapeutic agent for Inflammatory Bowel Disease guided by systems medicine

One sentence summary: Novel bioinformatic analyses and laboratory validation demonstrate the anti-inflammatory effects of clarithromycin and suggest that it may be repositioned for the management of inflammatory bowel diseases.

Authors:

Katie Lloyd[1]*, Stamatia Papoutsopoulou[1,2]*, Emily Smith[2], Philip Stegmaier[3], Francois Bergey[4], Lorna Morris[4], Madeleine Kittner[4], Hazel England[2], Dave Spiller[2], Mike HR White[2], Carrie A Duckworth[1], Barry J Campbell[1], Vladimir Poroikov[5], Vitor AP Martins dos Santos[4], Alexander Kel[3], Werner Muller[2], D Mark Pritchard[1], Chris Probert[1], Michael D Burkitt[1,2], the SysmedIBD consortium[6].

* These authors contributed equally

Affiliations:

1: Department of Cellular and Molecular Physiology, University of Liverpool, Liverpool, UK

2: Faculty of Biology Medicine and Health, University of Manchester, Manchester, UK

3: geneXplain GmbH, Wolfenbuettel, Germany

4: LifeGlimmer GmbH, Berlin, Germany

5: Institute of Biomedical Chemistry, Moscow, Russia

6: www.sysmedIBD.eu

Contact Details:

Dr. Michael Burkitt
Division of Diabetes, Endocrinology
& Gastroenterology
School of Medical Sciences
Faculty of Biology, Medicine & Health
Michael Smith Building,
Dover Street,
Manchester,
M13 9PT

Email: michael.burkitt@manchester.ac.uk

Abstract:

Inflammatory bowel diseases cause significant morbidity and mortality. Aberrant NF-κB signalling is strongly associated with these conditions, and several established drugs influence the NF-κB signalling network to exert their effect.

To identify drugs which may be repositioned for inflammatory bowel disease, the SysmedIBD consortium combined text-mining and network development tools to enhance published NF-κB signalling network diagrams.

Up-stream network analysis identified novel therapeutic targets, which were analysed using *in-silico* drug discovery software to identify candidate drugs with the potential for repositioning for use in inflammatory bowel disease. The highest-ranked candidate drug was clarithromycin.

Clarithromycin's effects were validated in several experimental contexts: it influenced NF-κB mediated transcription in murine peritoneal macrophages and intestinal enteroids, it suppressed NF-κB protein shuttling in murine reporter enteroids, it suppressed NF-κB (p65) DNA binding in the small intestine of mice exposed to LPS, and it reduced the severity of dextran sulphate sodium-induced colitis in C57BL/6 mice. Clarithromycin also suppressed NF-κB (p65) nuclear translocation in human intestinal enteroids.

These findings demonstrate that *in-silico* drug repositioning algorithms can viably be allied to laboratory validation assays in the context of inflammatory bowel disease; and that further clinical assessment of clarithromycin in the management of inflammatory bowel disease is required.

Keywords:

Inflammatory Bowel Diseases

NF-kappa B

Drug Repositioning

Interdisciplinary research

Organoids

Macrolide

Ulcerative colitis

Crohn's disease

Introduction:

Inflammatory bowel diseases (IBD) affect 0.5-1.0% of people in the Western world and cause substantial morbidity with associated costs to society (1, 2). Whilst the underlying causes of IBD are complex and multifactorial, a common feature involves aberrant inflammatory responses, leading to mucosal damage and the disease phenotype.

The heterogeneity of disease presentations has resulted in the development of diverse therapeutic approaches. For example, patients with mild ulcerative colitis may be treated with 5-aminosalicylic acid preparations, which predominantly act topically on the colonic mucosa to suppress inflammatory responses (3), whilst other patients require systemic therapy with highly specific biologic agents that target inflammatory mediators including TNF (4, 5). Whilst these drugs have very different mechanisms of action, a common factor is that they influence host inflammatory responses and either directly or indirectly alter NF-κB signalling.

NF-κB signalling describes a tightly controlled, dynamically regulated signal transduction network with several well-described transcription regulatory feedback loops (6): this orchestrates innate immune responses by regulating transcription through dimers of the five NF-κB proteins (RelA(p65), RelB, NF-κB1(p50), NF-κB2(p52) and c-Rel). Signalling through this network is characterised by oscillation of NF-κB proteins between the cytoplasm and nucleus; most clearly demonstrated for the RelA(p65) subunit (7). In addition to changes in the inflammatory milieu, this network also affects several other processes which are dysregulated during chronic gastrointestinal inflammation including cell turnover, DNA damage responses and cell senescence (8). Several studies have associated aberrant NF-κB signalling with IBD (reviewed in refs (9-11)).

Because of the complexity of NF-κB signalling, targeting specific components of the network has not been a successful drug development strategy to date. One of the principal reasons

for this failure is the diverse nature of NF-κB signalling. The impact of gross inhibition of key members of the NF-κB signalling cascade has been complicated by undesirable off-target effects. This has led to calls for highly specific inhibitors which target precise components of the NF-κB signalling network in specifically selected cell types (12). This approach may be useful in certain circumstances, for instance, multiple myeloma, where targeting a specific cancer cell lineage is desirable, but for complex benign inflammatory diseases, including IBD, this strategy is less likely to be successful

The reasons for this include difficulties in identifying a target cell population in complex multi-system inflammatory diseases, meaning that highly selective NF-κB inhibition in a specified tissue is unlikely to yield successful therapeutic strategies.

We, therefore, propose an alternative strategy, to enable 'nudging' an individual's NF-κB signalling network towards an idealised healthy phenotype. This approach may offer a more pragmatic way of targeting the aberrant NF-κB signalling that has been described and extensively investigated in many inflammatory conditions, without the limitations of selective tissue targeting.

In order to investigate this in the context of inflammatory bowel disease, our consortium used a combination of novel bioinformatic analyses and laboratory studies to identify agents that are likely to impact NF-κB signalling and inflammatory bowel disease. We then validated the efficacy of the most highly ranked agent, both in terms of inhibiting NF-κB activity, and modulating an *in-vivo* model of colitis.

Results:

Annotation of the existing NF-κB network diagram

Text mining was used to develop an enhanced NF-κB signalling network incorporating the most recent literature, whilst existing ChIP-seq datasets were analysed to predict previously unrecognised transcription regulatory feedback loops. The text mining analysis was

combined with querying the TRANSPATH™ database – the largest collection of manually curated mammalian signal transduction and metabolic pathways.

Text mining approach

The MedlineRanker tool (13) was used to fetch the 1000 most relevant in the PubMed database using the MeSH terms "Inflammatory bowel diseases" and "NF-κB". Protein-protein interactions were identified in these abstracts using the PESCADOR (14) tool. PESCADOR detects genes, proteins and their interactions, and rates them based on co-occurrences in an abstract. In our analysis we used concepts to generate species-specific text-mining for *Homo sapiens* and *Mus musculus*. As a result, 827 interactions common for both *Homo sapie*ns and *Mus musculus*, 2 for *Mus musculus* alone, and 26 uniquely for *Homo sapiens* were extracted (Supplementary Table S1). After querying the TRANSPATH® database for known direct interactions, we identified 127 novel co-occurrences of genes and/or proteins in the abstracts analysed

Novel transcription regulatory feedback loop discovery

Our approach to predict previously unrecognised transcription regulatory feedback loops found 38 transcription factors that potentially contribute to transcription regulation. Intersection of consensus transcription factors with consensus target genes revealed that 26 of these transcription factors (~ 68%) were among the target genes (Table 1). These 26 transcription factors were therefore targeted by NF-κB whilst simultaneously being predicted cooperating with NF-κB to regulate transcription, suggesting that they may contribute to regulatory cascades within the NF-κB signalling network. We tested the overlap between 284 NF-κB pathway genes and consensus target genes and found that 90 (> 30%) of the pathway genes were represented among the target genes (Supplementary Table S2).

Functional analysis of inferred target genes and prioritized transcription factor motifs suggests that there are two broad groups of target genes supported by topics of associated

GO categories as well as transcription factor motifs enriched in corresponding target gene promoters (Figure 1). The larger group of functional classes mainly reflects immune regulatory processes, whereas the other group appears to be dominated by processes of protein production such as translation, protein targeting and localization or complex assembly. These functional groups strikingly coincide with stronger enrichment of NF-κB-type motifs in the former and with ETS-type motifs in the latter group.

In-silico prediction of small molecules likely to influence NF-κB signalling and IBD activity

To identify drugs with potential for repurposing as NF-κB targeted anti-IBD agents, we performed a multi-step, *in-silico* analysis of existing transcriptomic datasets.

To create an extensive list of IBD associated genes, we retrieved information from the HumanPSD database including known IBD biomarkers and drug targets. We also collected two lists of IBD-related genes from two independent genome wide association studies, one focused on revealing genes of IBD prognosis (15), and another on IBD susceptibility (16). As a result, we established a summary table of 159 genes with, an indication of the source of evidence about their relevance to IBD (Supplementary Table S3).

Next, we proposed that key-node proteins of the IBD-related signal transduction and gene regulatory network that show a topology leading to the potential control of the activity of NF-κB components would be potential drug targets. To obtain these key-node proteins, we performed a master-regulator search of the signal transduction network contained in the TRANSPATH database considering the IBD related protein-protein interactions obtained by text-mining.

We searched upstream of the various molecular components of NF-κB complex including: NF-κB1-isoform1, NF-κB1-isoform2, NF-κB2-isoform4, NF-κB2-p100, NF-κB2-p49, RelA-p35, RelA-p65-delta, RelA-p65-delta2, RelA-p65-isoform1, RelA-p65-isoform4, RelB, c-Rel.

Our search of the network upstream of the NF-κB components extended to a maximal radius of 10 steps, used a false discovery rate (FDR) cut-off 0.05 and Z-score (reflecting how specific each master regulator is) cut-off of 1.0. The list of interacting proteins obtained by our text-mining efforts was used as "Context proteins" in our algorithm for master-regulator acquisition (17). This revealed 325 controlling nodes that may exert signalling activity to the NF-κB components (Supplementary Table S4). We intersected this list of genes with the list of IBD-related genes to obtain 62 genes that represented potential therapeutic targets (defined as IBD key-nodes, Supplementary Table S5).

In order to predict drugs which may interact with our putative therapeutic targets, we used the PASS software package: this software predicts the ability of a chemical structure to interact and influence the activity of defined molecular targets and biological activities: 46 PASS activities were predicted to influence the activity of our IBD key-nodes (Supplementary Table S6).

To identify compounds with potential for clinical re-positioning, we analysed the Top 200 drugs library to predict the probability that they may interfere with IBD key-nodes. For each compound tested, we calculated the cumulative score across all 46 PASS activities related to IBD targets and arbitrarily defined a combined score of 3.0 as a cut off for selecting agents with potential for repurposing. We also required that the Pa (probability to be active) of the PASS activity "Transcription factor NF κB inhibitor" was higher than 0.0.

29 compounds achieved these criteria (Table 2); importantly, this table included several corticosteroids (budesonide (ranked 2), dexamethasone (4), triamcinolone acetonide (5), methylprednisolone (6), prednisone (7)), which are already in clinical use for IBD, supporting the validity of our discovery strategy.

The highest-ranked drug was a macrolide antibiotic: clarithromycin. This agent is of particular interest because macrolides have an established role in treating aseptic

inflammatory conditions including chronic rhinosinusitis (18) and panbronchiolitis (19), and because clarithromycin has previously been trialled in IBD with divergent outcomes (20, 21). We, therefore, considered it to be an excellent candidate for strategic repurposing and have used clarithromycin as a paradigm molecule for the development of a mechanism-led drug validation pathway.

Clarithromycin suppresses stimulus-induced luciferase activity in primary cell cultures.

To validate the efficacy of clarithromycin as an inhibitor of NF-κB mediated transcription, we used primary cultures from the hTNF.LucBAC mouse, a transgenic line that expresses firefly luciferase under the control of the entire human *TNF* promoter (22).

Luciferase activity was reliably triggered by ligand binding to the TNF receptor and pattern recognition receptors including TLR4 (lipopolysaccharide), TLR5 (flagellin) and NOD2 (muramyl dipeptide, MDP) (Figures 2A-D) in peritoneal macrophages harvested from this mouse. When cells were pre-treated for 30 minutes with 10µM or 100µM clarithromycin a dose-dependent reduction in luciferase activity was observed, independent of the stimulus applied.

The effect of clarithromycin on NF-κB activation in murine peritoneal macrophages was confirmed by isolating peritoneal macrophages from 4 WT mice, and treating them with 10µM clarithromycin or vehicle before stimulating with 1µg/ml LPS. Cells were fixed, stained for phosphorylated-p65 and quantified by flow-cytometry. Median fluorescence intensity (MFI) increased on stimulation with LPS (p=0.03, ANOVA); co-administration of LPS and clarithromycin suppressed this response (p=0.008, Figure 2E).

To determine whether clarithromycin could also alter NF-κB responses in gastrointestinal epithelial cell cultures, small intestinal organoids (enteroids) were established from hTNF.LucBAC mice. In response to 100ng/mL TNF administration, luciferase activity was reliably detectable in these cultures (Figure 3A and B). Moreover, luciferase activity was

significantly suppressed, by pre-treatment for 30 minutes with either 1µM clarithromycin (p=0.014) or 10µM clarithromycin (p=0.001) (Figure 3B and C).

Clarithromycin suppresses TNF-induced NF-κB(p65) shuttling in enteroids

To assess whether clarithromycin influenced NF-κB protein shuttling dynamics, we established small intestinal organoid cultures from reporter mice expressing p65-DsRedxp/IκBalpha-eGFP. This mouse strain has bacterial artificial chromosomes that express human p65-DsRedxp and human IκBalpha-eGFP fusion proteins. Primary cultures of gastrointestinal mucosal origin from this mouse strain express these fluorophore fusion proteins and using live-cell time-lapse confocal microscopy the shuttling of p65 to and from the nucleus can be observed.

Administration of 100ng/mL TNF-induced synchronised p65 translocation to the nucleus of cells within enteroids with a periodicity of approximately 50 minutes. This was observed as a highly damped shuttling response, with a single wave of synchronised nuclear translocation, followed by a second wave of partially-synchronised translocation. Beyond the second oscillatory wave, further nuclear localisation occurred in an apparently stochastic fashion (Figure 4A and B). When cultures were pre-treated with 10µM clarithromycin for 30 minutes, p65-DsRedxp nuclear translocation was markedly suppressed.

To quantify these events more precisely we compared mean area under the curve (AUC) during the first oscillatory wave (Figure 4D) and demonstrated decreased nuclear intensity of p65-DsRed fluorescence in clarithromycin pre-treated enteroids (p=0.005). We also calculated the time to peak nuclear fluorescence after 100ng/mL TNF administration for each cell quantified (figure 4E). In DMSO vehicle-treated cells, peak fluorescence occurred at a median time of 47.2 (IQR 41.0-47.2) minutes. This was not significantly different for clarithromycin treated enteroids, but there was significantly more cell to cell variability

observed in clarithromycin treated than vehicle-treated cells (p<0.0001), demonstrating the loss of a synchronised pulse of p65 nuclear translocation (Figures 4C and 4F).

Clarithromycin suppresses LPS-induced NF-κB (p65) DNA binding in-vivo

To determine whether the effects of clarithromycin on stimulus-induced NF-κB activity observed *in-vitro* also occurred *in-vivo*, we administered 0.125mg/kg LPS i.p. to groups of six C57BL/6 male mice either with or without clarithromycin co-administration. This stimulus induces small intestinal epithelial cell shedding, regulated by both NF-κB1 and NF-κB2 signalling pathways (23).

Ninety minutes after administration of LPS lysates of proximal small intestinal mucosa were generated and p65 DNA binding activity was measured by DNA-binding ELISA. LPS administration induced a 2-fold increase in p65 DNA binding, compared to saline vehicle control (p<0.001, 1-way ANOVA and Dunnett's post-hoc test); pre-treatment with clarithromycin suppressed this effect by approximately 51% (p=0.007, Figure 5A).

Clarithromycin suppresses DSS-induced colitis

To determine whether clarithromycin had an effect on murine colitis *in-vivo* we co-administered either clarithromycin or vehicle to mice receiving DSS to induce colitis. Animals received clarithromycin or saline vehicle daily for 4 days by oro-gastric gavage, after a 4 day washout period 2.5% w/v DSS in drinking water *ad-libitum* was commenced for 5 days, followed by allowed to recovery for a further 3 days. In this context pre-treatments was performed to assess whether there was any prolonged effect of pre-treating mice with a macrolide antibiotic (clarithromycin) on the severity of DSS colitis.

Mice pre-treated with clarithromycin had similar weight loss, composite inflammation scores and crypt survival parameters, to mice that received saline vehicle pre-treatment.

Mice co-administered DSS and clarithromycin lost significantly less weight than other groups (p=0.039, 1-way ANOVA and Dunnett's post-hoc test, Figures 5B and 5C) and had lower

compound histology scores (p=0.004, Figures 5D and 5F) and a higher number of surviving colonic crypts than mice treated with vehicle (p=0.017, Figure 5E). This suggests that clarithromycin at least partially ameliorates this model of colitis.

Clarithromycin suppresses TNF-induced NF-κB (p65) nuclear localisation in human enteroids

To determine whether the effects that were identified in murine primary culture and *in-vivo* experiments were also relevant to humans, passaged human ileal organoids from individuals who had no evidence of IBD were pre-treated with 10µM clarithromycin or DMSO vehicle for 30 min prior to stimulation with 100ng/ml TNF. Paraformaldehyde fixed cultures were immunostained for p65, and the percentage of cells expressing nuclear-localised p65 was quantified. In untreated human enteroids, 0.6% (SEM 0.37) of cells demonstrated p65 nuclear localisation, in keeping with low basal levels of NF-κB activation. Administration of TNF induced a 57-fold increase in cells expressing nuclear p65 (33%, +/- 3.2 SEM, p<0.0001, 1-way ANOVA and Dunnett's post-hoc test, N=6 per group). Pre-treatment with clarithromycin suppressed TNF-induced nuclear localisation of p65 to a level comparable with DMSO vehicle-treated enteroids. (1%, +/- 0.36 SEM, p<0.0001, Figure 6).

To validate the findings in the human organoid model, which relies on static measurement of NF-κB activation, we also examined the effects of clarithromycin in a stably transfected human cell line (HeLa) which expresses luciferase regulated by a promoter containing NF-κB DNA binding motifs. When cells were stimulated with 100ng/ml TNF, modest increases in luciferase activity were observed at 60 minutes, with more pronounced luciferase activity being observed at 180 minutes. Luciferase activity 3 hours after TNF stimulation was significantly suppressed in a dose-dependent manner, by pre-treatment with 1µM (p=0.01) or 10µM (p=0.0001) clarithromycin (Supplementary Figure S2).

Discussion:

This article demonstrate that the macrolide antibiotic clarithromycin is an effective modifier

of NF-κB signalling in the gastrointestinal tract. The identification of clarithromycin using an *in-silico* screen of drugs with potential for repositioning demonstrates how novel bio-informatics can used to progress drug-repurposing, a strategy that has the potential to reduce the cost of future drug development.

The SysmedIBD consortium integrated diverse skill-sets to enable the development of this approach which has the potential to be applied in different ways in the future. The same bioinformatic analysis could be applied to panels of novel small molecules to identify potential novel therapeutics for IBD. Similarly, the system has potential for adaptation for use in other clinical contexts where NF-κB signalling is of paramount importance, or in different biological contexts focussing on other signalling networks.

The list of drugs predicted to influence IBD outcomes by *in-silico* analysis identified several relevant agents. Most prominent are the corticosteroids, which are used to treat the acute relapse of inflammatory bowel disease (24, 25), and the macrolide antibiotics, which we have investigated further in this manuscript. Intriguingly the analysis also identified sex hormones such as medroxyprogesterone and estradiol which have been shown to modulate coli (26)tis, and colitis associated adnoma development *in vivo* (27); and non-steroidal anti-inflammatory drugs, which, in clinical practice, are identified as agents that exacerbate IBD (28). However, NSAIDs non-selectively inhibit cyclooxygenase (COX) activity resulting in inhibition of constitutively expressed COX-1 in the gastrointestinal epithelium, causing epithelial damage and ulceration; and inhibition of COX-2, which is upregulated at sites of inflammation. Our drug discovery approach deliberately inserted a bias for agents that influence NF-κB signalling, it is likely that the NSAIDs have been selected at least in part because of this bias.

These observations demonstrate the importance of interdisciplinary working. The *in-silico* drug discovery model is a powerful tool to identify drugs that may be repurposed, but it is only by comparing those drugs to the clinical literature that decisions about which agents to pursue for further analysis can be made.

The aim of the laboratory evaluation of clarithromycin was to demonstrate proof of principal that a drug identified by *in-silico* testing can be shown to have the mechanism of action that was predicted and show efficacy *in-vivo*.

The initial drug testing assays could be developed into a higher throughput compound screening system. For example, the hTNF.LucBAC macrophage assay can be performed in a 96-well plate format, and peritoneal macrophages are abundant, simple to extract and highly sensitive to stimulus. Reporter enteroids generated from hTNF.LucBAC mice allowed us to validate the findings seen in peritoneal macrophages in a relevant, untransformed epithelial model using analogous technology, but are unlikely to be amenable to high-throughput assay development due to the challenges (and cost) of maintaining a 3D culture in a small well in a culture plate.

The visualisation of p65.DsRed translocation between the nucleus and cytoplasm in enteric organoids is technically challenging, and not immediately scalable; but it generated fascinating data demonstrating fundamentally different NF-κB signalling dynamics compared to cancer cell lines (7, 29), with TNF induced p65 oscillations being heavily damped in organoids. The mechanisms that underly these differences will be the subject of further investigation.

The evidence that intestinal NF-κB signalling is altered by clarithromycin *in-vivo* is in keeping with earlier work using gastric cancer cell lines (30), but it is the first demonstration that macrolides alter this signalling pathway in untransformed gastrointestinal epithelium.

Similarly, we provide the first evidence that macrolides influence NF-κB activation in gastrointestinal mucosae *in-vivo*.

Previous studies have demonstrated that macrolides may influence models of colitis in the mouse (31) but have not specifically studied clarithromycin. Specifically, CSY0073, a non-antibiotic macrolide, ameliorated acute DSS colitis (31).

Our final validation was to characterise whether clarithromycin influences human epithelial NF-κB signalling. We initially investigated this using a HeLa reporter cell-line model, which is fast, inexpensive, and commercially available. This assay could be adapted as a screening test for novel compounds to assess their effect on epithelial NF-κB activity, but it is inferior d to the hTNF.LucBAC mouse primary culture models as it relies on a cancer cell line, and NF-κB signalling is dysregulated in numerous cancer cell lines. By generating human enteroids, we were able to assess the effect of clarithromycin on a primary, untransformed 3D human epithelial cell culture. Unfortunately, the assay used for NF-κB activation in this model was less specific, but this was necessary in order to use genetically unaltered human organoid cultures.

Our drug discovery algorithms were established to identify drugs that are likely to influence NF-κB signalling and IBD, and we have reported the results of our analysis of the Top 200 drugs, in order to prioritise already clinically licensed agents that have potential for repurposing. It was serendipitous that our highest-ranked agent for repurposing is a drug that has already been trialled in IBD. Importantly the outcomes of previous trials of clarithromycin in IBD have been heterogeneous (20, 21, 32-34), suggesting that there may be context dependent factors that determine whether clarithromycin is effective in a group of patients.

There have been four published papers, and a conference abstract (33) that have examined the effect of clarithromycin in IBD. All of these studies focussed on Crohn's disease and were

predicated on an antibiotic effect of clarithromycin, either targeting intra-macrophage killing of *E. coli (20, 21)* or attempting to eradicate *Mycobacterium avium paratuberculosis (32-34)*.

There are several potential reasons why these studies have had discordant outcomes. In the case of the Selby (32) and Goodgame studies (34), the aim was clearly to assess the long-term effect of bacterial eradication. Patient inclusion was determined based on clinical definitions of active IBD and response assessed by clinical outcome measures. These measures lack objectivity and would not be acceptable endpoints or selection criteria for a current study. Our work demonstrates that in addition to its anti-biotic effect clarithromycin also has anti-inflammatory properties. In order to test the effects of anti-inflammatory treatment with clarithromycin, further carefully designed clinical studies will be needed.

The observation that previous trials of clarithromycin in IBD yielded discordant results raises questions about trial design and patient selection. In the current era of precision medicine it is increasingly important that patients are very carefully selected for treatments, and that agents that have previously had both positive and negative results in unselected clinical trials are revisited to assess their efficacy in defined patient sub-populations. In order to do this, the mechanisms responsible for drug efficacy need to be fully understood. This study has helped us to understand how clarithromycin affects NF-κB signalling dynamics and based on this we hypothesise that it may also be possible to select a group of clarithromycin responsive patients based on their NF-κB signalling status.

In conclusion, our findings strongly suggest that clarithromycin may be a viable, novel, anti-inflammatory therapeutic agent for IBD. In order to progress to a personalised medicine trial of clarithromycin in IBD a partner diagnostic test which can demonstrate altered NF-κB dynamics in patients' peripheral blood monocyte derived macrophages is under

development (35, 36), once established this will be used to inform a personalised drug repurposing trial for clarithromycin in IBD.

Methods:

Text mining for pathway annotation

Relevant abstracts were retrieved using appropriate MeSH terms and evaluated using MedlineRanker, a tool which attempts to find most relevant publications for a topic based on abundantly appearing keywords. The resulting abstracts were analysed using a text-mining algorithm based on PESCADOR software (14) which was adapted for the purpose. The algorithm recognizes genes or proteins using the NLProt program, which is based on Support Vector Machines and a protein-name dictionary, subsequently the terms recognized by NLProt are normalized using species-specific gene/protein dictionaries. Biointeraction terms (e.g. activate, inhibit, induce … etc) were identified according to an interaction-term dictionary. All identified terms and biointeractions were then analysed using a co-occurrence analysis trained to find genes, proteins and user-defined concepts that co-occurred within sentences. Shell scripts were implemented to search for the most frequently found protein-protein interactions and highly populated concepts.

Inference of NF-kappaB-regulated transcriptional feedback loops

Transcription regulatory feedback loops driven by NF-kappaB/RelA and cooperating transcription factors were inferred based on existing knowledge about signalling pathways involving NF-kappaB/RelA transcription factor binding motifs and ChIP-seq experiment data targeting NF-kappaB/RelA-bound sites in the human genome. The analyses were mainly conducted using the geneXplain platform (37).

Our analysis aimed to identify feedback loops that encompass components of NF-kappaB/RelA pathways as well as cooperating transcriptional regulators that are themselves regulated by NF-kappaB/RelA (Figure 7).

First, we applied a novel method for motif enrichment analysis using logistic regression (MEALR). MEALR was developed to analyse binding site combinations in sets of DNA sequences that are likely to be bound by a common set of transcriptional regulators, as obtained through ChIP-seq experiments. The algorithm chooses a small set of DNA-sequence motifs from a possibly large library of such models. Given N sequences assigned to classes $y_i \in \{0,1\}$ and a library of M positional weight matrices (PWMs), MEALR estimates a sparse logistic regression (LR) model

$$P(y|x) = \frac{1}{1 + e^{-(\beta_0 + \sum \beta_k x_k)}}$$

using the vector of sequence scores calculated for the ith sequence

$$x_{ik} = \log\left(\frac{1}{L_i}\sum_{w} \exp\left(S_w\right)\right); i = 1..N, k = 1..M$$

where S_w is the log-odds score of the PWM assigned to the wth sequence window. The model coefficients can be used to prioritize motifs of transcriptional regulators with respect to their importance for experimentally observed binding events. Combinations of motifs prioritized by MEALR tend to coincide with transcription factors that are known to cooperate.

We applied MEALR to reveal discriminative DNA-sequence motifs in genomic regions bound by NF-kappaB/RelA after TNF-alpha stimulus. Sequence scores were calculated for a subset from the TRANSFAC(R) database, release 2014.4, consisting of 1429 motifs for transcription

factors from vertebrate organisms. Genomic NF-kappaB/RelA binding sites were identified by analysis of ChIP-seq experiments carried out by the ENCODE project [Encode] and deposited in GEO [Gm10847, Gm12878, Gm12891, Gm12892, Gm15510, Gm18505, Gm18526, Gm18951, Gm19099, and Gm19193 (GEO series GSE31477)]. MEALR models were estimated to distinguish ChIP-seq peak sequences from genomic background sequences that were randomly sampled from gene promoter regions not overlapping with peaks of the respective experiment. The analysis was conducted five times for each ChIP-seq data set with different background sequence sets. We retained motifs that were incorporated in the model in all five runs. As MEALR may select similar binding motifs with different identifiers in separate runs, we considered DNA-sequence motif similarity quantified by our method m2match (38).

A total of 62 TRANSFAC(R) motifs were selected by MEALR in at least 9 of the 10 ChIP-seq data sets. These motifs mapped to 38 transcription factor genes including NF-κB-type factors as well as other transcription factors which our analysis suggests cooperate with NF-κB (Supplementary Table S7).

To identify potential consensus target genes of NF-kappaB/RelA we grouped overlapping peak regions and mapped groups with peaks in at least 6 out of 10 ChIP-seq experiments to nearby genes. This part of the analysis focused on peaks with a maximal length of 3000 bases (>99.1% of all peak regions in the ten ChIP-seq experiments). 4835 consensus ChIP-seq regions were identified in the vicinity of 6329 genes. These genes were defined as consensus target genes.

Among the target genes we identified 24 transcription factors inferred by MEALR as well as 90 of 284 genes encoding components of signalling pathways and cascades involving NF-kappaB/RelA collected from the TRANSPATH(R) database, release 2013.3. These comprise the feedback loops with NF-kappaB/RelA consensus targets described in Figure 7.

Functional analysis of NF-kappaB/RelA target genes

To gain more insight into the functional classes and biological significance of potential target genes we analysed their associations with Gene Ontology (GO) biological processes, molecular functions and cellular components and assessed their significance using the Fisher test. We selected GO categories with at most 1500-member genes, 50 to 500 target gene hits and an adjusted P-value ≤ .001, in order to discard the broadest categories as well as those representing only a few target genes.

The enrichment of binding sites in promoters of target genes associated with selected GO functional classes was analysed using the algorithms developed by Koschmann *et al.* (37). A subset of 190 positional weight matrices for the 24 transcription factors identified through our feedback loop analysis was extracted from TRANSFAC(R), release 2018.3. Enrichment was determined for five different background gene sets sampled from non-target genes of Ensembl 91. Motifs were considered relevant for target genes associated with a certain GO term if enrichment was detected with every background set.

Pathway analysis for master regulator search

Molecules that regulate the expression of differentially expressed genes through concerted control of the activity of transcription factors which act on the gene loci were defined as master regulators., and were identified by applying a key-node analysis algorithm (39), using the TRANSPATH® database of gene regulatory and signal transduction pathways (40). All signal transduction reactions from TRANSPATH® were considered.

Key-nodes were prioritized according to a score based on the weighted ratio between the number of molecules from the input set that can be reached from the key-node in a limited number of steps (radius parameter) and the total number of reachable nodes in the graph. The higher the score the greater is the chance that the key-node plays a master regulatory role in the process.

In-silico drug discovery

In order to discover potential new drugs for IBD treatment, we used a computational tool (PASS) for large-scale prediction of activity spectra (41, 42); http://genexplain.com/pass/. This tool analyses relationships between the chemical structure of compounds and their biological activities. 2D structural formulae of compounds are used as input data, in order to obtain estimates of biological activity profiles, even for virtual molecules, prior to chemical synthesis or biological testing. Molecular structures are represented in PASS by a set of unique multilevel neighbourhoods of atoms (MNA) descriptors (43). The PASS algorithm of biological activity spectrum prediction is based on Bayesian estimates of probabilities of molecules belonging to the classes of active and inactive compounds, respectively (44). The predicted activity spectrum is presented in PASS by the list of activities with probabilities "to be active" Pa and "to be inactive" Pi calculated for each activity.

In our work, for each compound of the tested library, we computed a cumulative score which sums up the values of Pa across the 46 PASS activities that were selected in the previous step of the analysis.

$$Score = \sum_{i=1}^{46} p_a(i)$$

here $p_a(i)$ is the probability for the given compound to be active for the activity *i* computed by PASS (for the 46 selected PASS activities).

For each compound tested we required the cumulative score to be higher than 3.0. We also required that the Pa (probability to be active) of the PASS activity “Transcription factor NF-κB inhibitor” was higher than 0.0.

Patient recruitment and ethics

Samples used for the generation of human enteroids were donated by patients without evidence of IBD attending for colonoscopy at the Royal Liverpool and Broadgreen University

Hospitals NHS Trust. Samples were collected following written informed consent and favourable ethical opinion from North West - Liverpool East Research Ethics Committee (15/NW/0045).

Animal maintenance and welfare

All animal breeding, maintenance and procedures were performed under a UK Home Office licence, with local Animal Welfare and Ethics Review Board approval. Transgenic animals were maintained at the University of Manchester. Wild-type C57BL/6 mice were purchased from Charles River (Margate, UK) and maintained either at the University of Manchester or University of Liverpool. All animals were maintained in SPF facilities with access to standard chow and drinking water *ad-libitum* unless otherwise specified. Standard 12-hour light/dark cycles were used; standard temperature and humidity levels were maintained.

Transgenic mouse strains

Human TNFα luciferase mice (hTNF.LucBAC)

This mouse has been engineered to have a bacterial artificial chromosome (BAC) expressing luciferase under the regulation of the entire human TNF promoter. Primary cultures established from this mouse enable direct measurement of TNF promoter activity in a non-transformed *ex-vivo* system (22).

Human p65-DsRedxp/IκBalpha-eGFP mouse (p65-DsRedxp/IκBalpha-eGFP).

This mouse expresses fusion proteins of NF-κB(p65) and the *Discosoma* red fluorescent protein – Express (DsRedxp) under the regulation of the native human p65 promoter, and IκBalpha and enhanced green fluorescent protein (eGFP), regulated by the human IκBalpha promoter (45). Primary cultures established from this mouse enable direct visualisation of these fusion proteins.

Peritoneal macrophages isolation, in vitro culture and luciferase assay

Resident peritoneal macrophages were obtained from mice by standard methods. After plating, cultures were left to rest overnight. Cells were stimulated with either 10ng/mL LPS (*Salmonella enterica* serovar Minnesota R595; Calbiochem), TNF (R&D Systems) at 40 ng/mL, muramyl dipeptide (MDP, Invivogen) at 10µg/mL or Flagellin (Novus Bio) at 500ng/ml. Luciferase activity was measured over time in a CO_2 luminometer (Lumistar Omega, BMG Labtech).

Flow Cytometry

Peritoneal macrophages cultures were stimulated with 1µg/ml LPS for 20min. The reaction was terminated by fixation, before staining for phosphor-p65 using the PE Mouse anti-NF-κB p65 (pS529) antibody (BD Bioscience, clone K10-895.12.50). Stained cells were analysed by flow cytometry using an LSRII Cytometer (BD). Data analyses were performed with FlowJo887 software (Tree Star).

Primary Epithelial cell culture

Enteroids were generated from human and murine tissue using modifications of established protocols. Briefly, tissue samples were disaggregated by calcium chelation in EDTA followed by mechanical disaggregation in a sucrose/sorbitol solution. Crypt pellets were resuspended in Matrigel (Corning, UK) in order to maintain 3-dimensional structures and were maintained in media containing a combination of recombinant growth factors and growth factor containing conditioned media. Details of media and disaggregation solutions are included as supplementary methods. All enteroids were passaged at least once prior to use in experiment.

Enteroid immunohistochemistry

Enteroids were pre-treated with 10µM clarithromycin or 1% v/v DMSO (vehicle control) for 30mins. Following pre-treatment, 100ng/mL recombinant human TNF (Peprotech) was applied for a further 30mins. Following fixation, enteroids were transferred into Richard-Allan™ Histogel™ (Thermofisher), and processed for histology. Immunohistochemical analysis was performed using a rabbit monoclonal p65 primary antibody (Cell Signaling Technologies, #8242).

Murine intestinal organoid confocal microscopy

Proximal enteroids derived from p65-DsRedxp/IκBalpha-eGFP dual-reporter mice were passaged into glass bottom dishes (Thermo Scientific™ Nunc™ Glass Bottom Dishes) and left to grow in phenol red-free complete media for 3 days. Time series and Z-stack images were taken for 6-8 enteroids per dish using a Zeiss Laser Scanning Microscope (LSM880) with a C-Apochromat 40x/1.2 W Korr FCS M27 objective. Enteroids were imaged for 30mins prior to treatment with 10µM clarithromycin or 1% v/v DMSO vehicle. Thirty minutes later cultures were stimulated with 100ng/ml TNF and imaged for a further 3hrs. Images and videos were processed using Zen 2011 software and CellTracker (Warwick Systems Biology Centre).

LPS-induced NF-κB activation in mice

Groups of adult (8-10-week-old) male C57BL/6 mice were administered 50mg/kg clarithromycin i.p, or vehicle daily for 3 days. Following a 3-day washout period, animals received either 50mg/kg clarithromycin i.p., or 0.9% w/v saline vehicle, on the next day either 50mg/kg clarithromycin i.p. or vehicle was followed by either 0.125mg/kg ultrapure LPS from E. coli K12 (Invivogen) i.p. or 0.9% w/v saline vehicle. Animals were culled 90 minutes after LPS / vehicle administration; small intestinal mucosal scrapes were prepared in RIPA buffer with protease inhibitors (Sigma, Gillingham, UK). NF-κB p65 DNA binding was quantified using a TransAM DNA-binding ELISA (ActiveMotif, La Hulpe, Belgium).

Dextran sulphate sodium-induced colitis

Groups of adult (8-10-week-old) male C57BL/6 mice were administered either 10 mg/kg clarithromycin, or vehicle by orogastric gavage daily for 4 days. Following a washout period, animals received 2.5% DSS in drinking water, for 5 days. Animals recovered while receiving normal drinking water for a further 3 days. From the start of DSS treatment to termination of the experiment either 10 mg/kg clarithromycin or normal saline vehicle was administered by daily orogastric gavage. Tissue samples were harvested and prepared for histological analysis, including quantitative histology, as previously described (46).

Statistical analysis of laboratory work

All statistical analyses were performed using GraphPad Prism v.7.0 software. Specific statistical tests are annotated in the text and figure legends. All reported *p*-values are two-tailed. Normality testing was performed with D'Agostino & Pearson test.

Author Contributions:

Katie Lloyd:

KL performed experiments, acquired original data, analysed data and drafted part of the manuscript. SP performed experiments, acquired original data, analysed data and drafted part of the manuscript. ES performed experiments and acquired original data. PS developed analytical tools, analysed data and drafted part of the manuscript. FB analysed data and drafted part of the manuscript. LM analysed data and drafted part of the manuscript. MK developed analytical pathways and analysed data. HE performed experiments, acquired original data and analysed data. DS supported imaging experiments and reviewed the manuscript. MW devised the project and contributed to funding applications. CD supported organoid work and reviewed the manuscript. BC contributed to funding applications and reviewed the manuscript. VP developed analytical tools and analysed data. VMdS

contributed to funding applications and supported the development of analytical pathways. AK contributed to funding applications, supported the development of novel analytical tools, performed data analyses and contributed to the drafting of the manuscript. WM contributed to funding applications and study design, reviewed the manuscript and supported laboratory work. DMP reviewed the manuscript and supported laboratory work. CP contributed to funding applications, designed the study, reviewed the manuscript and supported laboratory work. MB designed the research study, conducted experiments, analysed data, drafted the manuscript and co-ordinated the project.

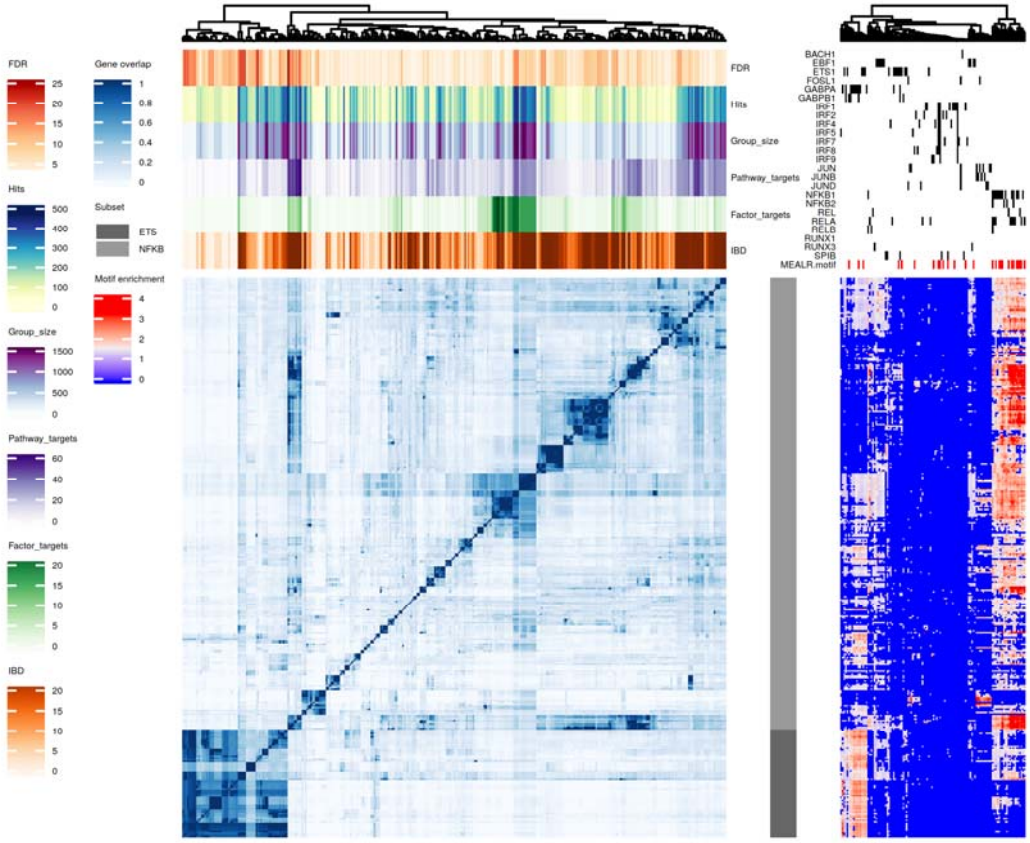
Acknowledgments: This work was supported by the European Union Seventh Framework Programme [FP7/2012–2017] under the SysmedIBD grant, agreement no. 305564

References

1. Feagan BG, Kozma CM, Slaton TL, Olson WH, and Wan GJ. Healthcare costs for Crohn's disease patients treated with infliximab: a propensity weighted comparison of the effects of treatment adherence. *J Med Econ.* 2014;17(12):872-80.
2. van Deen WK, van Oijen MG, Myers KD, Centeno A, Howard W, Choi JM, et al. A nationwide 2010-2012 analysis of U.S. health care utilization in inflammatory bowel diseases. *Inflamm Bowel Dis.* 2014;20(10):1747-53.
3. Marteau P, Probert CS, Lindgren S, Gassul M, Tan TG, Dignass A, et al. Combined oral and enema treatment with Pentasa (mesalazine) is superior to oral therapy alone in patients with extensive mild / moderate active ulcerative colitis: a randomised, double blind, placebo controlled study. *Gut.* 2005;54(7):960-5.
4. Baert FJ, D'Haens GR, Peeters M, Hiele MI, Schaible TF, Shealy D, et al. Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. *Gastroenterology.* 1999;116(1):22-8.
5. Targan SR, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, et al. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med.* 1997;337(15):1029-35.
6. Lev Bar-Or R, Maya R, Segel LA, Alon U, Levine AJ, and Oren M. Generation of oscillations by the p53-Mdm2 feedback loop: a theoretical and experimental study. *Proc Natl Acad Sci U S A.* 2000;97(21):11250-5.
7. Nelson DE, Ihekwaba AE, Elliott M, Johnson JR, Gibney CA, Foreman BE, et al. Oscillations in NF-kappaB signaling control the dynamics of gene expression. *Science.* 2004;306(5696):704-8.
8. Perkins ND. The diverse and complex roles of NF-kappaB subunits in cancer. *Nat Rev Cancer.* 2012;12(2):121-32.
9. Merga YJ, O'Hara A, Burkitt MD, Duckworth CA, Probert CS, Campbell BJ, et al. Importance of the alternative NF-kappaB activation pathway in inflammation-associated gastrointestinal carcinogenesis. *Am J Physiol Gastrointest Liver Physiol.* 2016;310(11):G1081-90.
10. Atreya I, Atreya R, and Neurath MF. NF-kappaB in inflammatory bowel disease. *J Intern Med.* 2008;263(6):591-6.
11. McDaniel DK, Eden K, Ringel VM, and Allen IC. Emerging Roles for Noncanonical NF-kappaB Signaling in the Modulation of Inflammatory Bowel Disease Pathobiology. *Inflamm Bowel Dis.* 2016;22(9):2265-79.
12. Baud V, and Karin M. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. *Nat Rev Drug Discov.* 2009;8(1):33-40.
13. Gijon-Correas JA, Andrade-Navarro MA, and Fontaine JF. Alkemio: association of chemicals with biomedical topics by text and data mining. *Nucleic Acids Res.* 2014;42(Web Server issue):W422-9.
14. Barbosa-Silva A, Fontaine JF, Donnard ER, Stussi F, Ortega JM, and Andrade-Navarro MA. PESCADOR, a web-based tool to assist text-mining of biointeractions extracted from PubMed queries. *BMC Bioinformatics.* 2011;12:435.
15. Lee JC, Biasci D, Roberts R, Gearry RB, Mansfield JC, Ahmad T, et al. Genome-wide association study identifies distinct genetic contributions to prognosis and susceptibility in Crohn's disease. *Nat Genet.* 2017;49(2):262-8.
16. Hasler R, Sheibani-Tezerji R, Sinha A, Barann M, Rehman A, Esser D, et al. Uncoupling of mucosal gene regulation, mRNA splicing and adherent microbiota signatures in inflammatory bowel disease. *Gut.* 2017;66(12):2087-97.

17. Kel AE, Stegmaier P, Valeev T, Koschmann J, Poroikov V, Kel-Margoulis OV, et al. Multi-omics "upstream analysis" of regulatory genomic regions helps identifying targets against methotrexate resistance of colon cancer. *EuPA Open Proteom.* 2016;13:1-13.
18. Oakley GM, Harvey RJ, and Lund VJ. The Role of Macrolides in Chronic Rhinosinusitis (CRSsNP and CRSwNP). *Curr Allergy Asthma Rep.* 2017;17(5):30.
19. Lin X, Lu J, Yang M, Dong BR, and Wu HM. Macrolides for diffuse panbronchiolitis. *Cochrane Database Syst Rev.* 2015;1:CD007716.
20. Leiper K, Martin K, Ellis A, Watson AJ, Morris AI, and Rhodes JM. Clinical trial: randomized study of clarithromycin versus placebo in active Crohn's disease. *Aliment Pharmacol Ther.* 2008;27(12):1233-9.
21. Leiper K, Morris AI, and Rhodes JM. Open label trial of oral clarithromycin in active Crohn's disease. *Aliment Pharmacol Ther.* 2000;14(6):801-6.
22. Faisal Minshawi MW, Werner Muller, Neil Humphreys, Dean Jackson, Barry J. Campbell, Antony Adamson, and Stamatia Papoutsopoulou. Human TNF-Luc reporter mouse: A new model to quantify inflammatory responses. *Scientific Reports.* 2018;Accepted Manuscript.
23. Williams JM, Duckworth CA, Watson AJ, Frey MR, Miguel JC, Burkitt MD, et al. A mouse model of pathological small intestinal epithelial cell apoptosis and shedding induced by systemic administration of lipopolysaccharide. *Dis Model Mech.* 2013.
24. Turner D, Walsh CM, Steinhart AH, and Griffiths AM. Response to corticosteroids in severe ulcerative colitis: a systematic review of the literature and a meta-regression. *Clin Gastroenterol Hepatol.* 2007;5(1):103-10.
25. Truelove SC, and Witts LJ. Cortisone in ulcerative colitis; final report on a therapeutic trial. *Br Med J.* 1955;2(4947):1041-8.
26. Armstrong CM, Allred KF, Weeks BR, Chapkin RS, and Allred CD. Estradiol Has Differential Effects on Acute Colonic Inflammation in the Presence and Absence of Estrogen Receptor beta Expression. *Dig Dis Sci.* 2017;62(8):1977-84.
27. Son HJ, Sohn SH, Kim N, Lee HN, Lee SM, Nam RH, et al. Effect of Estradiol in an Azoxymethane/Dextran Sulfate Sodium-Treated Mouse Model of Colorectal Cancer: Implication for Sex Difference in Colorectal Cancer Development. *Cancer Res Treat.* 2018.
28. Long MD, Kappelman MD, Martin CF, Chen W, Anton K, and Sandler RS. Role of Nonsteroidal Anti-Inflammatory Drugs in Exacerbations of Inflammatory Bowel Disease. *J Clin Gastroenterol.* 2016;50(2):152-6.
29. Harper CV, Woodcock DJ, Lam C, Garcia-Albornoz M, Adamson A, Ashall L, et al. Temperature regulates NF-kappaB dynamics and function through timing of A20 transcription. *Proc Natl Acad Sci U S A.* 2018;115(22):E5243-E9.
30. Peng YC, Ho SP, Shyu CL, Chang CS, and Huang LR. Clarithromycin modulates Helicobacter pylori-induced activation of nuclear factor-kappaB through classical and alternative pathways in gastric epithelial cells. *Clin Exp Med.* 2014;14(1):53-9.
31. Mencarelli A, Distrutti E, Renga B, Cipriani S, Palladino G, Booth C, et al. Development of non-antibiotic macrolide that corrects inflammation-driven immune dysfunction in models of inflammatory bowel diseases and arthritis. *Eur J Pharmacol.* 2011;665(1-3):29-39.
32. Selby W, Pavli P, Crotty B, Florin T, Radford-Smith G, Gibson P, et al. Two-year combination antibiotic therapy with clarithromycin, rifabutin, and clofazimine for Crohn's disease. *Gastroenterology.* 2007;132(7):2313-9.
33. Graham DY, Al-Assi M, and Robinson M. Prolonged remission in Crohn's disease following therapy for Mycobacterium paratuberculosis infection. *Gastroenterology.* 1995;108(4):A826.

34. Goodgame RW, Kimball K, Akram S, Ike E, Ou CN, Sutton F, et al. Randomized controlled trial of clarithromycin and ethambutol in the treatment of Crohn's disease. *Aliment Pharmacol Ther.* 2001;15(12):1861-6.
35. Papoutsopoulou S, England H, Burkitt M, Merga Y, Schmidt L, Hough R, et al. Screening of IBD patients based on NF-kappa B activation in PBMC-derived macrophages. 2016.
36. Sellge G, Papoutsopoulou S, Verdier J, Trautwein C, Bergey F, Burkitt M, et al. Inflammatory dyskinesis: defects of NF-kappa B dynamic behaviour as a new potential biomarker for personalized medicine in inflammatory bowel disease. *Journal of Crohn's and Colitis.* 2017;11(S1):S55-6.
37. Koschmann J, Bhar A, Stegmaier P, Kel AE, and Wingender E. "Upstream Analysis": An Integrated Promoter-Pathway Analysis Approach to Causal Interpretation of Microarray Data. *Microarrays (Basel).* 2015;4(2):270-86.
38. Stegmaier P, Kel A, Wingender E, and Borlak J. A discriminative approach for unsupervised clustering of DNA sequence motifs. *PLoS computational biology.* 2013;9(3):e1002958.
39. Kel A, Voss N, Jauregui R, Kel-Margoulis O, and Wingender E. Beyond microarrays: find key transcription factors controlling signal transduction pathways. *BMC Bioinformatics.* 2006;7 Suppl 2:S13.
40. Krull M, Pistor S, Voss N, Kel A, Reuter I, Kronenberg D, et al. TRANSPATH: an information resource for storing and visualizing signaling pathways and their pathological aberrations. *Nucleic Acids Res.* 2006;34(Database issue):D546-51.
41. Filimonov DA, Poroikov VV, Karaicheva EI, Kazarian RK, Budunova AP, Mikhailovskii EM, et al. [The computerized prediction of the spectrum of biological activity of chemical compounds by their structural formula: the PASS system. Prediction of Activity Spectra for Substance]. *Eksp Klin Farmakol.* 1995;58(2):56-62.
42. Poroikov V, Akimov D, Shabelnikova E, and Filimonov D. Top 200 medicines: can new actions be discovered through computer-aided prediction? *SAR QSAR Environ Res.* 2001;12(4):327-44.
43. Filimonov D, Poroikov V, Borodina Y, and Gloriozova T. Chemical Similarity Assessment through Multilevel Neighborhoods of Atoms:⍰ Definition and Comparison with the Other Descriptors. *Journal of Chemical Information and Computer Sciences.* 1999;39(4):666-70.
44. Filimonov DA, Lagunin AA, Gloriozova TA, Rudik AV, Druzhilovskii DS, Pogodin PV, et al. Prediction of the Biological Activity Spectra of Organic Compounds Using the Pass Online Web Resource. *Chemistry of Heterocyclic Compounds.* 2014;50(3):444-57.
45. Dudek M, Yang N, Ruckshanthi JP, Williams J, Borysiewicz E, Wang P, et al. The intervertebral disc contains intrinsic circadian clocks that are regulated by age and cytokines and linked to degeneration. *Ann Rheum Dis.* 2017;76(3):576-84.
46. Williams JM, Duckworth CA, Vowell K, Burkitt MD, and Pritchard DM. Intestinal Preparation Techniques for Histological Analysis in the Mouse. *Curr Protoc Mouse Biol.* 2016;6(2):148-68.



Figures

Figure 1: Graphical representation of the functional analysis of NF-κB target genes (see also Material and methods). The heatmap on the left (shades of blue) represents the gene ontological (GO) categories that are enriched in NF-κB ChIP-seq target genes clustered according to the number shared target genes. The annotations indicate (from top to bottom) the FDR (-log10) significance of enrichment with target genes, number of target gene hits, size of the GO category, number of pathway target genes, number of cooperating TF target genes and number of known IBD biomarkers in the HumanPSD database. The heatmap to the right (blue/red) demonstrates how enriched motifs of the cooperating factors that we inferred are in the promoters of target genes associated with the GO categories. In the top right (black and red) the cooperating transcription factors, and their associated motifs are represented. The grey/dark grey row annotation highlights the two

main functional and regulatory groups revealed by our analysis. A high definition version of this figure is included as supplementary Figure S1.

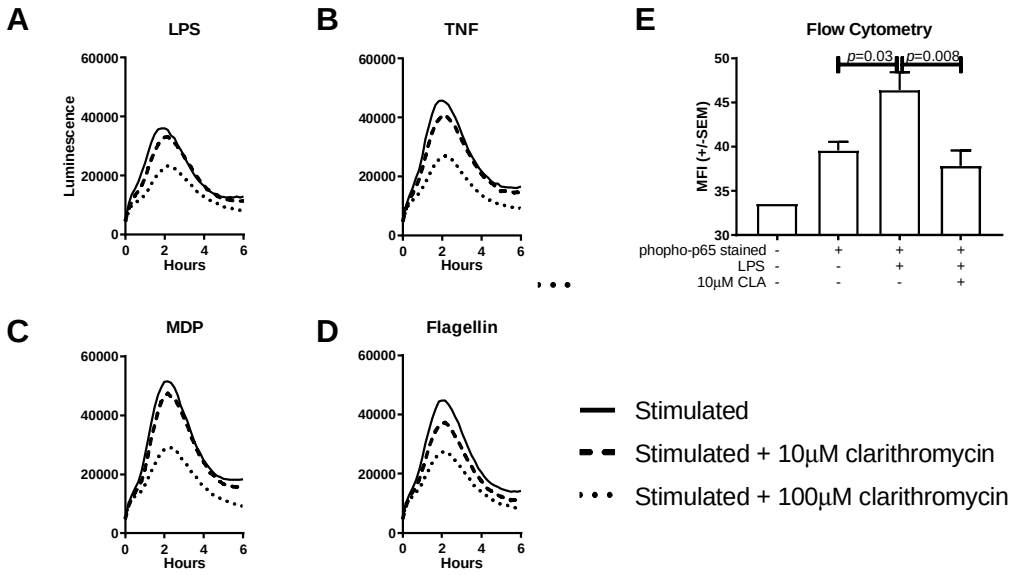


Figure 2. A-D: Luciferase activation curves for peritoneal macrophages extracted from HTNF.LucBAC mice stimulated with 10ng/mL LPS (**A**), 40 ng/mL TNF (**B**), 10µg/mL MDP (**C**) or 500ng/ml flagellin (**D**). Solid lines indicate cells response of pre-treated with drug vehicle (DMSO), dashed and dotted lines responses generated from cells pre-treated with 10µM or 100 µM clarithromycin, respectively. **E:** Median fluorescence intensity for anti-phopsho-p65 antibody stained peritoneal macrophages from C57BL/6 mice either unstimulated or stimulated with LPS and pre-treated with 10µM clarithromycin or DMSO vehicle. N=4 mice. Statistically significant differences tested by 1-way ANOVA and Dunnett's post hoc test.

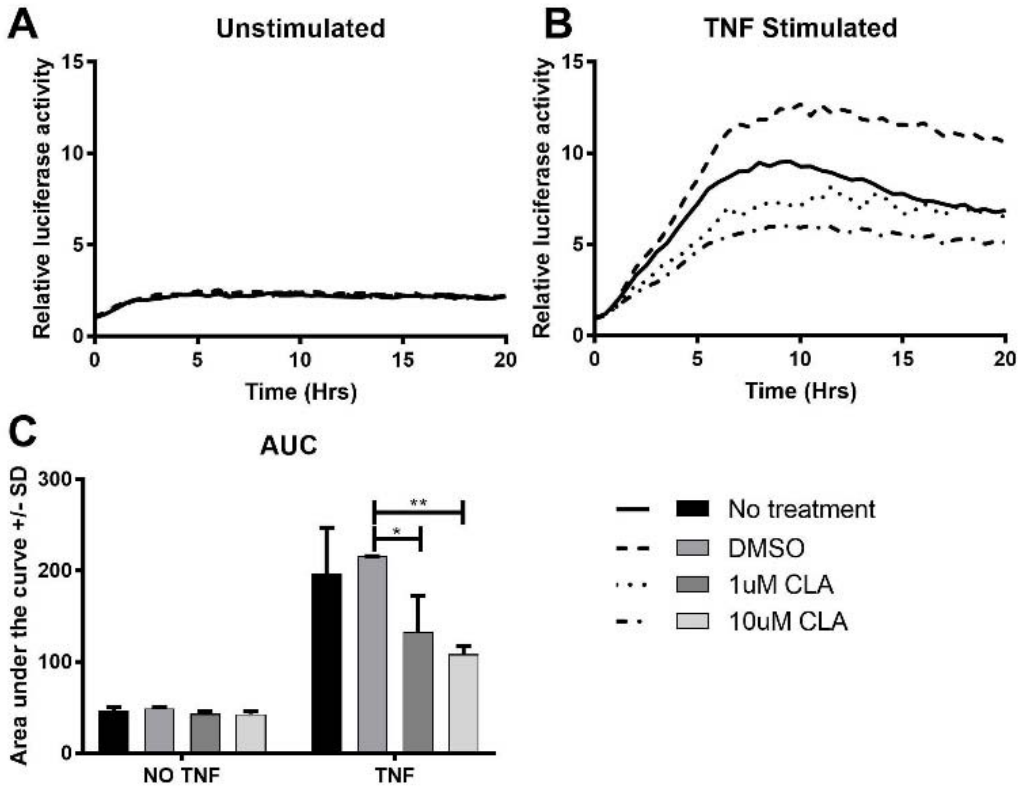


Figure 3: Representative luciferase activation curves for enteroids derived from HTNF.LucBAC mice either unstimulated (**A**) or stimulated with 100ng/mL TNF (**B**), and without pre-treatment (solid line) or 30 min pre-treatment with, DMSO vehicle (dashed line), 1µM clarithromycin (dotted line) or 10µM clarithromycin (dotted and dashed line). **C:** Area under the curve (AUC) calculations for the same experiment. N=3. Statistically significant differences tested by 2-way ANOVA and Dunnett's post-hoc test.

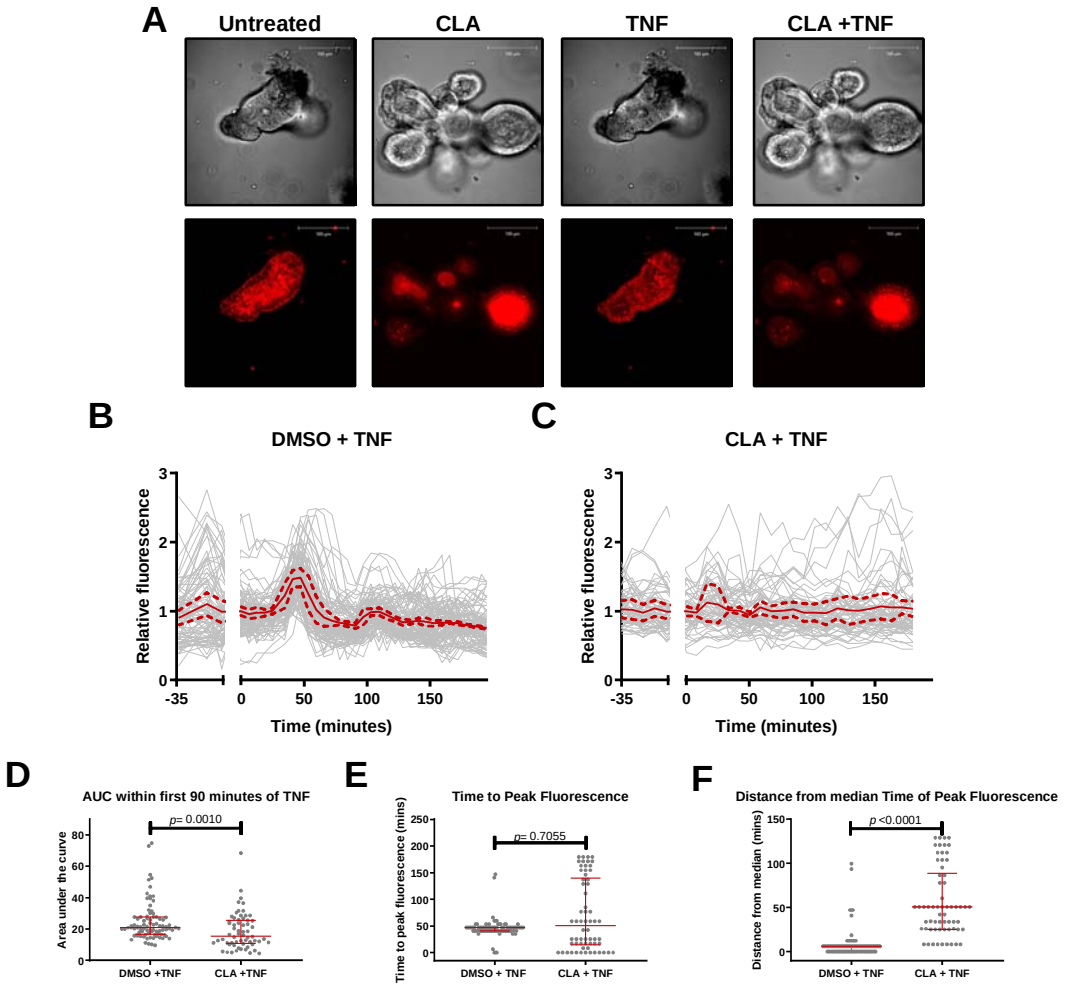


Figure 4 A: Representative images of bright field (upper panels) and red channel (lower panels) images of enteroids derived from p65-DsRedxp/ IκBalpha-eGFP mice, either untreated, treated with clarithromycin alone, treated with 100ng/mL TNF alone, or pre-treated with 10µM clarithromycin and subsequently stimulated with TNF. **B and C:** Relative nuclear red fluorescence curves for individual cells (grey lines), mean (solid red line) and 1 SD above and below the mean (dashed red lines) over time. Cells pre-treated with DMSO vehicle **(B)** or clarithromycin **(C)** at time -35 min and stimulated with TNF at time 0 min. **D:** Area under the curve calculations for individual cells between time 0 and time 90 min for

panels B and C. Statistically significant differences tested by Mann-Whitney U test. **E:** Time to peak fluorescence, post-TNF stimulation for individual cells, statistically significant differences tested by Mann-Whitney U test. **F:** Distance of individual cells peak fluorescence from the median value, statistically significant differences tested by Mann-Whitney U test. N=88 vehicle pre-treated, 62 clarithromycin pre-treated cells for panels D-F, lines represent median and IQR for these panels.

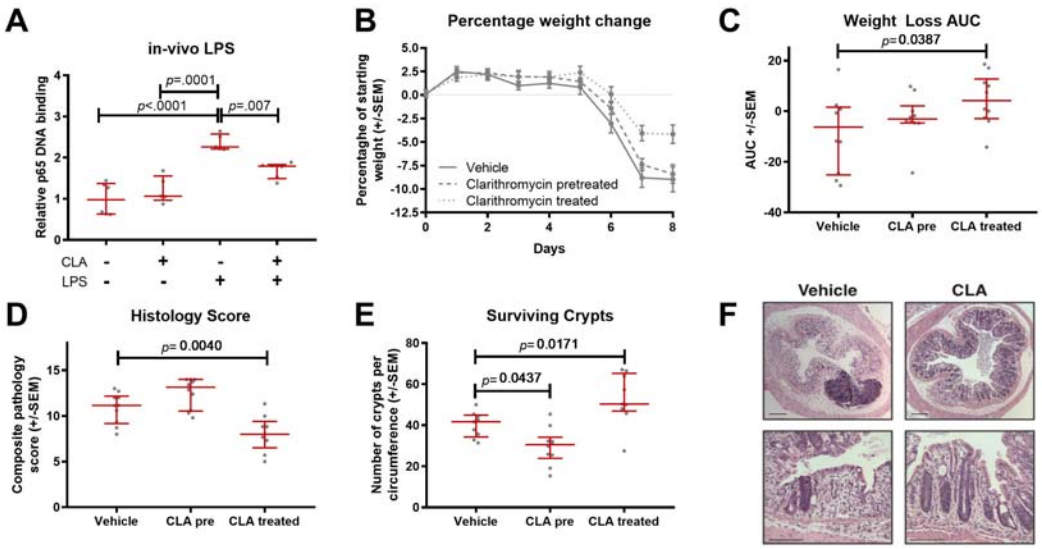


Figure 5A: Relative p65 DNA binding activity in whole cell lysates from proximal small intestine of C57BL/6 mice pre-treated for 3 days with 50mg/kg clarithromycin or saline vehicle, and subsequently injected i.p. with 0.125mg/kg LPS or vehicle. N=5-6. **B-F:** Effect of clarithromycin on outcomes of DSS colitis in C57BL/6 mice. **B:** Weight loss plotted over time. **C:** Area under the curve analysis of weight loss. **D:** Histology severity scores. **E:** Number of surviving crypts per colonic circumference. **F:** Representative photomicrographs of the colonic mucosa of DSS treated mice co-treated with saline vehicle or 10mg/kg clarithromycin by oro-gastric gavage. N=9-10. Statistically significant differences tested by 1-way ANOVA and Dunnett's post hoc test in all panels.

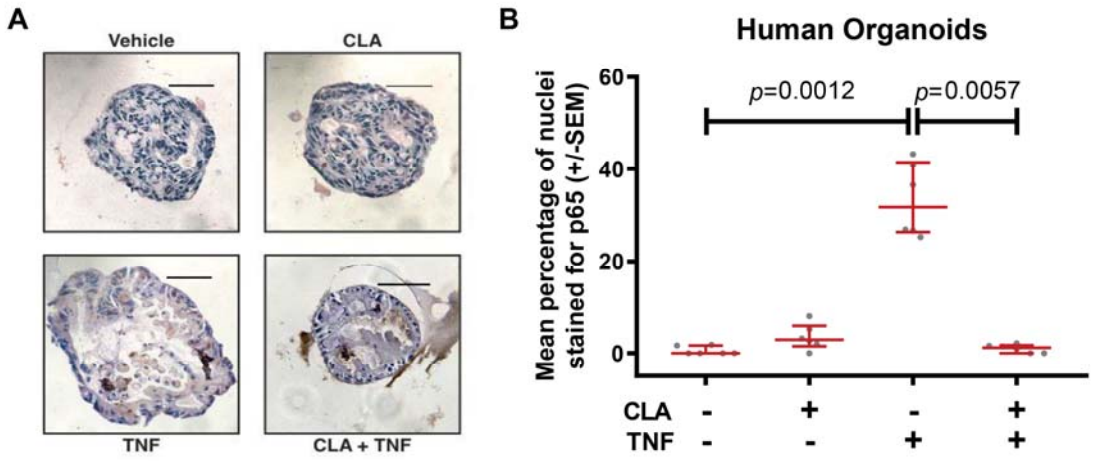


Figure 6A: Representative photomicrographs of human ileal enteroids either unstimulated or stimulated with 100ng/ml TNF, and either co-administered DMSO vehicle or 10µM clarithromycin and immunostained for total p65. **B:** Quantification of nuclear p65 staining of human enteroids. Statistical testing by Kruskal-Wallis 1-way ANOVA and Dunn's post hoc, N=6.

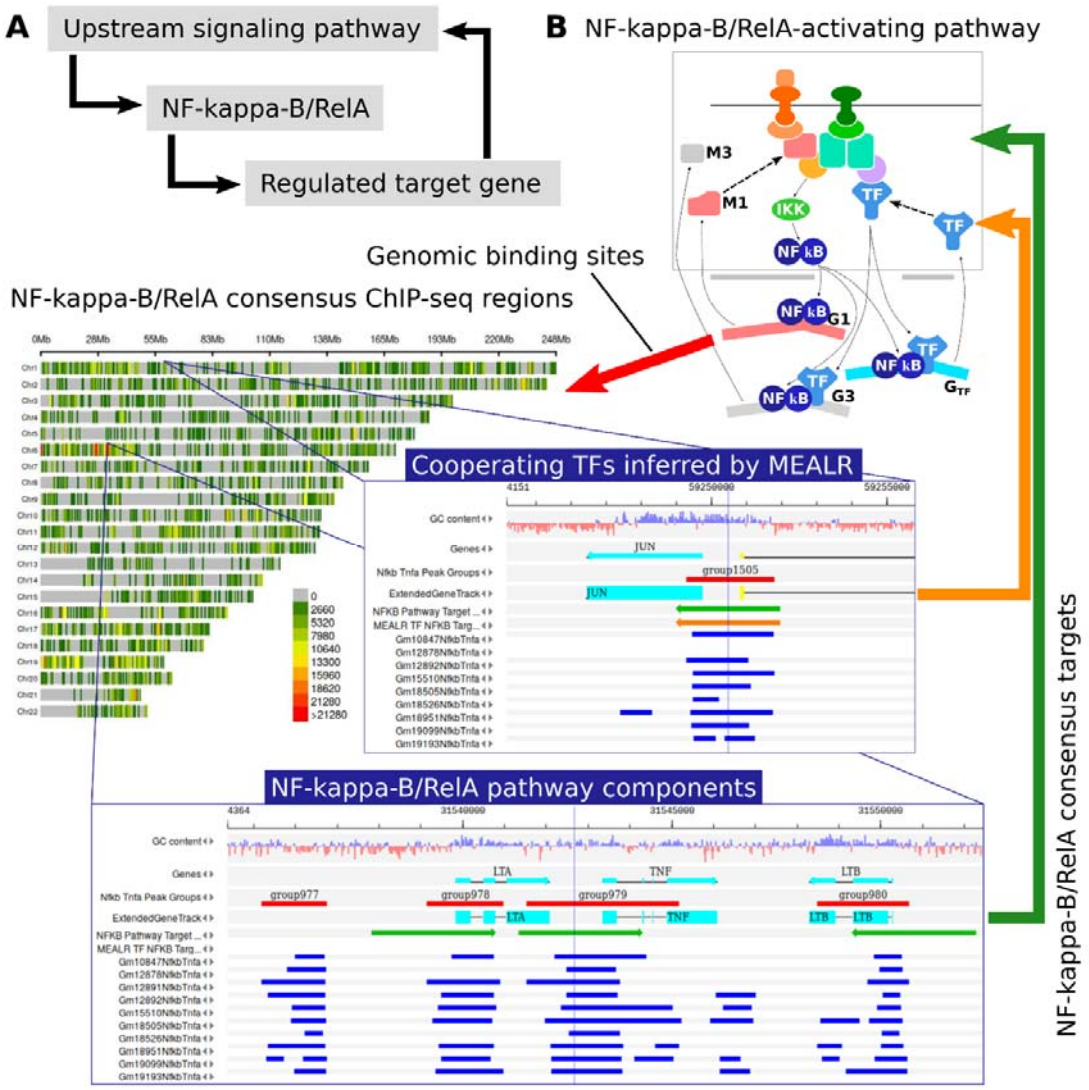


Figure 7A: Schematic of the transcriptional feedback loops sought by our analysis. Upon activation through upstream signalling cascades the transcriptional regulator NF-kappa-B/RelA (and cooperating transcription factors) control expression of target genes which may themselves play a role in signalling cascades regulating the activity of NF-kappa-B/RelA thereby establishing a positive or negative feedback loop depending on whether NF-kappa-B/RelA enhances or interferes with the transcription of respective target genes. **B:** More detailed illustration of identified feedback loops. Using consensus NF-kappa-B/RelA binding sites collected from 10 ChIP-seq experiments we analysed their genomic locations with respect to nearby potential target genes which were known components of relevant pathways and/or transcription factors whose motifs played a role for target sequence

recognition as inferred by MEALR. Details of the compiled and analysed data are exemplified for pathway target genes TNF, LTA and LTB on chromosome 6 and for the cooperating transcription factor JUN on chromosome 1. The consensus peak density presentation was calculated using the CMplot package. The detailed views of genomic regions were created using the genome browser of the geneXplain platform.

Tables:

ID	Gene symbol
ENSG00000156273	*BACH1*
ENSG00000164330	*EBF1*
ENSG00000134954	*ETS1*
ENSG00000175592	*FOSL1*
ENSG00000154727	*GABPA*
ENSG00000104064	*GABPB1*
ENSG00000125347	*IRF1*
ENSG00000168310	*IRF2*
ENSG00000137265	*IRF4*
ENSG00000128604	*IRF5*
ENSG00000185507	*IRF7*
ENSG00000140968	*IRF8*
ENSG00000213928	*IRF9*
ENSG00000177606	*JUN*
ENSG00000171223	*JUNB*
ENSG00000130522	*JUND*
ENSG00000109320	*NFKB1*
ENSG00000077150	*NFKB2*
ENSG00000162924	*REL*
ENSG00000173039	*RELA*
ENSG00000104856	*RELB*
ENSG00000159216	*RUNX1*
ENSG00000020633	*RUNX3*
ENSG00000269404	*SPIB*

Table 1: Transcription factors predicted to contribute to NF-κB regulatory cascades by MEALR analysis

Ranking	Drug
1	Clarithromycin
2	Budesonide
3	Digoxin
4	Dexamethasone
5	Triamcinolone acetonide
6	Methylprednisolone
7	Prednisone
8	Azithromycin
9	Medroxyprogesterone
10	Estradiol
11	Nystatin
12	Progesterone
13	Albuterol
14	Ibuprofen
15	Dicyclomine
16	Naproxen
17	Nabumetone
18	Propranolol
19	Metoprolol
20	Atenolol
21	Acetaminophen
22	Phentermine
23	Divalproex
24	Gabapentin
25	Diclofenac
26	Lisdexamfetamine
27	Pregabalin
28	Methocarbamol
29	Metformin

Table 2: Drugs predicted to influence IBD.