NOVEL CHANGES IN NF-κB ACTIVITY DURING PROGRESSION AND REGRESSION PHASES OF HYPERPLASIA: ROLE OF MEK, ERK AND p38

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Running Title: Sustained activation of NF-κB in vivo

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Utilizing the Citrobacter rodentium (CR)-induced transmissible murine colonic hyperplasia (TMCH) model, we measured hyperplasia and NF-κB activation during progression (days 6 and 12 post-infection) and regression (days 20-34 post-infection) phases of TMCH. NF-κB activity increased at progression in conjunction with bacterial attachment and translocation to the colonic crypts and decreased 40% by day 20. NF-κB activity at days 27 and 34 however, remained 2-3 fold higher than uninfected control. Expression of the downstream target gene CXCL-1/KC in the crypts correlated with NF-κB activation kinetics. Phosphorylation of cellular IKKα/β (Ser176/180) was elevated during progression and regression of TMCH. Phosphorylation (Ser32/36) and degradation of IκBα however, contributed to NF-κB activation only from days 6 to 20 but not at later time points. Expression of the downstream target gene CXCL-1/KC in the crypts correlated with NF-κB activation kinetics. Phosphorylation of cellular IKKα/β (Ser176/180) was elevated during progression and regression of TMCH. Phosphorylation (Ser32/36) and degradation of IκBα however, contributed to NF-κB activation only from days 6 to 20 but not at later time points. Phosphorylation of MEK1/2 (Ser217/221), ERK1/2 (Thr202/Tyr204) and p38 (Thr180/Tyr182) paralleled IKKα/β kinetics at days-6 and 12 without declining with regressing hyperplasia. siRNAs to MEK, ERK and p38 significantly blocked NF-κB activity in vitro while MEK1/2-inhibitor (PD 98059) also blocked increases in NF-κB activity by blocking increases in p65 abundance and nuclear translocation thereby down-regulating CXCL-1/KC expression in the crypts. Thus, NF-κB activation persists despite lack of bacterial attachment to colonic mucosa beyond peak hyperplasia. MEK/ERK/p38 pathway therefore seems to modulate sustained activation of NF-κB in colonic crypts in response to CR infection.

The intestinal epithelium is a self-renewing tissue that represents a unique model for studying interconnected cellular processes such as proliferation, differentiation, cell migration and carcinogenesis. Increased rates of proliferation have been described both as a precursor to cancer and as an integral part of the malignant transformation of the epithelium (1, 2) and are associated with a multitude of changes in cell signaling molecules and oncogenes (3-5). Proliferative zone of the normal colorectal mucosa is confined to the lower two-thirds of the colonic crypts whereas in conditions of high risk of cancer, proliferating cells are observed throughout the whole length of the gland.

Pathogenic strains of Escherichia coli, including enteropathogenic E. coli (EPEC) and enterohemorrhagic E. coli (EHEC) and a closely related mouse pathogen Citrobacter rodentium (CR) are classified as attaching and effacing (A/E) pathogens based on their ability to adhere to intestinal epithelium, destroy
microvilli, and induce actin filled membranous protrusions called “pedestals” at the site of attachment. In the mouse colon, pedestal formation is associated with the formation of A/E lesions, breach of the epithelial barrier by the bacteria, and development of disease (6,7).

Transmissible murine colonic hyperplasia (TMCH), caused by *Citrobacter rodentium* (CR) infection is characterized by significant hyperplasia accompanied by expansion of the proliferative compartment throughout the longitudinal crypt axis (8,9). The epithelial cell hyperproliferation that results from CR infection promotes the development of colonic adenomas after administration of the carcinogen 1,2-dimethylhydrazine (DMH) (10). CR infection, in the absence of carcinogen administration however, does not result in adenoma formation and the mucosa reverts back to normal in 4-6 weeks.

CR colonizes preferentially the murine colon with over $10^9$ bacteria present during the peak of infection. However, by day 21 post-oral challenge, CR is cleared from the gastrointestinal tracts of normal adult mice (11). Studies have shown that both innate and adaptive immune responses are required for immunity (11-15), with CD4 T-cell-dependent antibody responses believed to be central to clearance (12). Infection of mice with CR elicits a mucosal Th-1 immune response (16), very similar to mouse models of inflammatory bowel disease.

Immune and inflammatory responses in the gut and other immunocompetent tissues often involve the transcription factor NF-κB (17). Multiple stimuli, including cytokines, mitogens, environmental particles, toxic metals, and viral or bacterial products, activate NF-κB, mostly through IκB kinase (IKK)-dependent phosphorylation and subsequent degradation of its inhibitor(s), the IκB family of proteins (17). Activated NF-κB translocates to the nucleus, binds to its sequence recognition motif on promoters of target genes, and activates their transcription. The transcriptional activity of NF-κB is also controlled by various post-translational modifications, including phosphorylation (18,19) and acetylation of the p65 subunit (20,21).

Utilizing the TMCH model, we showed previously that it was associated with a robust activation of β-catenin and NF-κB in the colonic crypts. NF-κB activation in TMCH followed the canonical pathway including IKKα/β phosphorylation and IκBα degradation (22), but was also characterized by atypical mechanism that enhances NF-κB activity including phosphorylation and acetylation of the p65 subunit of NF-κB.

The epithelial hyperplasia induced by CR infection in TMCH is resolved within 4-6 weeks after infection, but the transient hyperplastic axis induced by CR infection promotes the development of carcinogen-induced colorectal tumors. Since inflammation in the gut and activation of NF-κB are often associated with an increased susceptibility to colon cancer, we hypothesized that some signals that contribute to NF-κB activation in TMCH remain elevated/altered in the mucosa even after bacterial clearance. Therefore, in the present study we investigated the mechanistic basis of NF-κB activity during progression and regression of TMCH. We report that NF-κB activation was highest at peak hyperplasia which coincided with maximal colonization of the colon by CR. However its activation, though reduced at later time points remained elevated over that of normal mucosa after bacterial clearance and even when the hyperplasia was completely resolved. Activation of NF-κB at time points that coincided with bacterial colonization (TMCH progression) was regulated primarily through the canonical mechanism (IκBα phosphorylation and degradation). In contrast, sustained activity after bacterial clearance (TMCH regression) was regulated by persistent phosphorylation events of the MEK/MAPK,ERK/RSK-1 pathway. We conclude that CR infection caused irreversible changes in colonic epithelium that could contribute to increased susceptibility to carcinogenesis.

**EXPERIMENTAL PROCEDURES**
**TMCH and Diets:** TMCH was induced in Helicobacter-free Swiss-Webster mice (15-20 g; Harlan) by oral inoculation with a 16-h culture of *C. rodentium* (CR), as previously described (9, 22-29). Age- and sex-matched control mice received sterile culture medium only. For dietary intervention, mice were randomized to receive either a control AIN-93 diet (30) or 6% pectin (Cat#TD97202) and 1% calcium (Ca) (Cat#TD97200) diet synthesized by Harlan Teklad (Madison, WI, USA). All dietary interventions began 3-days post-CR infection as described previously (23). Animals were euthanized at 0, 6, 12, 20, 27, and 34-days-post-infection and distal colons removed. Animals on various dietary regimens were killed at 12 days post-infection and their colons harvested. To isolate crypts, distal colons were attached to a paddle, and immersed in Ca²⁺-free standard Krebs-buffered saline (in mmol/l: 107 NaCl, 4.5 KCl, 0.2 NaH2PO4, 1.8 Na2HPO4, 10 glucose, and 10 EDTA) at 37°C for 10-20 min, gassed with 5% CO₂/95% O₂. Individual crypt units were then separated from the submucosa/musculature by intermittent (30-s) vibration into ice-cold potassium gluconate-HEPES saline (in mmol/l: 100 potassium gluconate, 20 NaCl, 1.25 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, and 5 sodium pyruvate) and 0.1% BSA. The isolated crypts were processed for biochemical and molecular assays as described previously (9, 22-29).

**Cell Culture, transfection and bacterial infection:** Young adult mouse colon (YAMC) cells are derived from colonic crypts from the immortomouse, such that they are conditionally immortalized with an SV40 large T-antigen with a temperature-sensitive IFNγ (interferon-γ) inducible promoter (31). The YAMC cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM glutamine, 50µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 units/ml IFNγ in a humidified incubator with 5%CO₂ at 33°C. For experiments, cells were incubated at 33°C in IFNγ-containing medium for 24 h and then transferred to 37°C in IFNγ-free RPMI 1640 medium for 24 h. To delineate the role(s) of MAPK signaling in NF-κB activation in response to CR infection, YAMC cells (5x10⁵) were seeded in 35 mm dishes and incubated at 37°C for 24 hr followed by growth in serum- and antibiotic-free medium for 24 hr. The cells were then transfected with either 100nmol/L of scrambled siRNA or siRNAs specific for MEK1/2 (sc-35904 and sc-75906), ERK1/2 (sc-39308 and sc-35336) and p38α/β (sc-29434 and sc-39117) using 2-8µl of transfection reagent (sc-29528) from Santa Cruz Biotechnology Inc., Santa Cruz, CA. CR strain DBS 100 (ATCC cat.# 51459™) was grown under aerobic conditions on Luria-Bertani (LB) agar plates for 24 h at 37°C and cultured in LB broth O/N at 37°C. RPMI, containing 0.45% glucose was inoculated with a 1:20 dilution of a standard LB overnight culture and incubated for 2h at 37°C. Monolayers of 5x10⁵ YAMC cells at ~50% confluence or cells transfected with various siRNAs after 36 hr were infected with CR at a multiplicity of infection (MOI) of 90 or the medium alone (as a control) for 3h at 37°C. Medium was tested negative for colony formation after 24 h (data not shown). At 24, 48 and 72 hr, cellular/nuclear proteins were extracted and processed for either Western blotting or DNA binding assay as described elsewhere.

**TLR4/NF-κB/SEAPorter Activity Assay:** To directly assess the role of toll-like receptor-4 (TLR4) in NF-κB activation in response to CR infection, we utilized a stably co-transfected HEK 293 cell line (IML-104, Imgenex Corp., San Diego, CA) which expresses full-length TLR4 and the secreted alkaline phosphatase (SEAP) reporter gene under the transcriptional control of an NF-κB response element (hereby designated as HEK 293-reporter cell line). The functionality of this cell line has been validated by measuring SEAP levels after LPS (lipopolysaccharide; a component of the outer membrane of Gram negative bacteria) stimulation. The HEK-293 cells were maintained in DMEM supplemented with
4.5g/L glucose, 10% FBS, 4mM L-glutamine, 1mM sodium pyruvate, 100 units/ml penicillin, 100μg/ml streptomycin, 10μg/ml blasticidin and 500μg/ml G418 (geneticin). Cells were infected with CR at a MOI of 90 for 3 hr, washed and incubated in fresh medium. The SEPorter activity was measured with the Imgenex SEPorter assay kit (cat. #10055K) as described by the manufacturer. An increase in SEAP levels indicates activation of NF-κB.

**RNA Isolation and polymerase chase reaction:** For measuring expression levels of CXCL-1/KC in the colonic crypts, cDNA was synthesized from total RNA by using superscript II and random primers. Specific gene products were identified by performing semiquantitative PCR. Primer sequences are provided in Supplementary Table 1. The PCR products were separated by polyacrylamide gel electrophoresis and visualized by ethidium bromide staining of the gels under UV light. Gel data were recorded with the BioRad FluorS Imaging System, and relative densities of the bands were determined with Quantity One software (BioRad, Hercules, Ca). Gene expression was normalized against Actin.

**Electrophoretic Mobility Shift and DNA Binding Assays:** Crypt nuclear extracts were prepared from normal or *Citrobacter*-infected mouse distal colon essentially as described (22-29). 10 μg of nuclear extract in 10μl buffer was mixed with 2μg of poly (dl-dc) and 1μg BSA to a final volume of 19 μl. After 15- min incubation on ice, 1μl of [γ-32P] ATP end-labeled double-stranded NF-κB consensus oligonucleotide (TGAGGGGACTTTCCCAGGC) was added to each reaction and incubated at room temperature for an additional 15 min. The reaction products were separated on a 4% native-polyacrylamide-0.5%x Tris-borate-EDTA gel and analyzed by autoradiography. Supershift antibodies (1 μl) were included in the binding reaction as indicated (all supershift antibodies were obtained from Santa Cruz Biotechnology) (26). For DNA binding assay, relative levels of activated p65NF-κB in nuclear extracts of normal or *Citrobacter*-infected mouse distal colonic crypts were measured using the TransAm p65 NF-κB Chemi Transcription Factor Assay Kit from Active Motif (Carlsbad, CA) (27).

**Immunoprecipitation (IP) and Western Blotting:** For IP studies, crypt cytosolic extracts were normalized for protein concentration and pre-cleared for 1 h at 4°C with 30μl of protein A-coated Sepharose beads. IP was carried out at 4°C by incubating the fractions O/N with antibody recognizing p90RSK-1. Immune-complexes were captured by incubation with 50μl of protein A/G-Sepharose beads for 2 h at 4°C. Control experiments were performed by carrying out immunoprecipitations in the presence of the immunizing peptides, or with control IgG antisera. The immunoprecipitated proteins were recovered by boiling the sepharose beads in 2X SDS sample buffer.

Total crypt cellular extracts, subcellular fractions (30-100μg protein/lane) or immunoprecipitated proteins were subjected to SDS-PAGE and electrotransferred to nitrocellulose membrane. The efficiency of electrotransfer was checked by back staining gels with Coomassie Blue and/or by reversible staining of the electrotransferred protein directly on the nitrocellulose membrane with Ponceau S solution. No variability in transfer was noted. De-stained membranes were blocked with 5% non-fat dried milk in TBS [20 mM Tris-HCl and 137 mM NaCl (pH 7.5)] for 1 h at room temperature (21 °C) and then overnight at 4 °C. Immunoantigenicity was detected by incubating the membranes for 1-2 h with the appropriate primary antibodies [0.5-1.0 μg/ml in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS/Tween); Sigma]. After washing, membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies, and developed using the ECL detection system (Amersham Corp., Arlington Heights, IL, USA) according to the manufacturer’s instructions.

**Immunofluorescence (IMF) and Immunohistochemistry (IHC):** IMF or IHC studies for anti-LPS as a surrogate to detect *Citrobacter rodentium*’s presence in the colonic mucosa was performed on 5-μm-thick paraffin sections from distal colons of normal...
and TMCH mice (days 6-34) utilizing the HRP labeled polymer conjugated to secondary antibody using Envision+ System-HRP (DAB; DakoCytomation, Carpinteria, CA) with microwave accentuation as described previously (9,22,27,28). Slides were washed and incubated with 4-6-diamidino-2-phenylindole for 5 min at room temperature to stain the nuclei. The visualization was carried out either via fluorescent or light microscopies, respectively. Controls included either omission of primary antibody or detection of endogenous IgG staining pattern with goat anti-mouse or anti-rabbit IgG (Calbiochem, San Diego, CA, USA).

RESULTS

Colonic pithelial Cell proliferation accompanies NF-κB activation during progression and/or regression phases of hyperplasia:

To determine the effect of Citrobacter rodentium (CR) infection on colonic crypt hyperplasia, we stained the distal colonic sections from Swiss-Webster outbred mice for Ki-67 as a marker of proliferation by immunohistochemistry. In normally proliferating crypts, only cells at the base exhibited nuclear staining (Fig 1). At Day 6 and particularly at Day 12 TMCH, intense nuclear immunoreactivity extended throughout the longitudinal crypt axis (Fig 1). Intense Ki-67 nuclear immunoreactivity persisted till Day 27 before returning to base line by Day 34 (Fig 1). We define in this study, day 6 and day 12 post infection as “TMCH progression” and days 20 through 34 post-infection as “TMCH regression”. When NF-κB activity in the distal colonic crypt nuclear extracts were measured via DNA binding assay, the activity increased reproducibly during TMCH progression, declined 40% at day 20 and then plateaued off at days 27 and 34. However, the residual activity, even at day 34 however, was 2-fold higher than uninfected control (Fig 2A). It needs to be emphasized that even though we have previously reported the mechanism of NF-κB activation during progression of TMCH (22), we considered it extremely critical to include these time points in order to understand how progression and regression phases of hyperplasia associated with TMCH affect NF-κB activity in colonic crypts in vivo. To further investigate the effect of NF-κB activation on downstream signaling, we focused on chemokine (C-X-C motif) ligand 1 (CXCL-1/KC), one of the targets of NF-κB signaling and a cognate ligand for CXC chemokine receptor, CXCR2. Time course studies paralleled NF-κB activation kinetics to certain extent wherein expression of CXCL-1 increased significantly at days 6 and 12 post-infection, declined at day 20 but was still detectable at days 27 and 34 albeit at lower levels (Fig 2B). As proof-of-principle, we used YAMC (Young Adult Mouse Colon) cells to study sustained activation of NF-κB in response to CR infection. YAMC cells were infected with CR at 90:1 MOI for three hr at 37°C, washed to remove bacteria and incubated in fresh medium with antibiotics for 24, 48 and 120 hrs. Expression analysis revealed significant increase in CXCL-1 levels at 24 and 48 hr compared to uninfected control with sustained overexpression even at 120 hr (Fig 2C). Data from three separate experiments are presented as bar graph in Fig 2Cii as a ratio of GAPDH in the samples. Since CXCL-1 is a downstream target of NF-κB signaling, these findings clearly indicate that sustained NF-κB activation may indeed be regulating expression of CXCL-1 over extended period of time both in vivo and in vitro.

To rule out the possibility that the NF-κB activity during regression of TMCH may be a result of prolonged bacterial attachment to the colonic mucosa, we performed LPS immunofluorescence (IMF) in deparaffinized sections from distal colon at different time points as a surrogate to detect Citrobacter attachment to the colonic mucosa. These IMF studies showed robust bacterial binding to the surface epithelial cells at days 6 and 12 wherein bacterial binding could be seen along the entire length of the colonic crypts (Fig 3A). However, at days 20 through 34, almost complete loss of bacterial binding to the surface mucosa was observed (Fig 3A) consistent with earlier report of bacterial
clearance at day 21 post-infection (11). Bar graph in Fig 3B represents percent increase in fluorescence intensity for LPS staining at indicated time points. Thus, although some residual uptake of bacteria by cells of the immune system at later time points cannot be ruled out, we conclude that despite lack of detectable bacterial attachment to colonic mucosa beyond day 12, NF-κB remains active albeit at reduced level during TMCH regression.

We have shown recently that NF-κB activation by CR infection during progression of TMCH occurs via the canonical pathway including phosphorylation of cellular IKKα/β and phosphorylation and degradation of IκBα (22). In the present study, we assessed/compared these molecular events during progression and regression of TMCH. As shown in Figure 4A, phosphorylation of IκBα at Ser-32/36 increased at days 6, 12 and 20 compared with uninfected control but decreased to baseline levels at later time points. Since phosphorylation of IκBα results in its degradation, we next measured total IκBα at these time points. Indeed, substantial decrease in the abundance of total IκBα was recorded at days 6 and 12 as reported earlier (22). At days 20, 27 and 34 however, total IκBα was restored to baseline suggesting that NF-κB activity seen at these time points (Fig 2A) may be independent of IκBα degradation. Bar graph in 4Aii represents the ratio of phosphorylated versus total IκBα from three independent experiments. Since these changes in phosphorylation and degradation of IκBα depend on activation of IKKα/β, we also analyzed the phosphorylation status of IKKα/β during TMCH, and found that relative levels of phosphorylated cellular IKKα (Ser176/180) and IKKβ (Ser187/181) increased substantially at day 6 and remained elevated throughout progression and regression of TMCH (Fig 4B). While the level of unphosphorylated IKKα did not change during TMCH, IKKβ levels progressively increased during TMCH regression but the significance of this remains unknown. Bar graph in 4Bii represents the ratio of phosphorylated and total IKKα/β levels normalized to actin (n = 3). These studies suggest that activated IKKs in the hyperproliferating crypts regulate IκBα function during progression but not during regression of TMCH.

**Signaling via components of the TLR4 pathway**: Based on recent report that TLR4 signaling contributes to inflammation induced by CR and to identify signaling cascades responsible for NF-κB activation during TMCH, we next examined sequential changes in the components of the TLR pathway in the colonic crypts in vivo. Signaling cascades initiated by engagement of TLRs with their ligands require many adaptor and accessory proteins including MyD88, TRAF-6 and TAK-1 which are directly or indirectly involved in activating downstream kinases such as MEK1/2, ERK and p38 (32). When cellular extracts were probed with antibodies for MyD88, TRAF6 and TAK-1, all the three proteins were detected in the colonic crypts (Suppl. Fig 1A-C). However, we did not detect any change in their cellular abundance during the course of TMCH as was revealed by densitometry following normalization with actin (n = 3). Cellular and nuclear levels of phosphorylated and hence activated MEK1/2 (Thr202/Tyr204; MEK1/2) and p44/42-ERK (Thr180/Tyr182; p44/42-ERK) on the other hand, increased substantially at day 6 and remained elevated throughout the course of progression and regression of TMCH (Fig 4Ci, Di) as was revealed by densitometry following normalization with actin or laminB respectively (Figs 4Cii, Dii; n = 3). This sustained elevation in the levels of cellular and nuclear pMEK1/2 or pp44/42-ERK correlated with the sustained phosphorylation status of IKKα/β (see Fig 4B) as well as with sustained activation of NF-κB at these time points (see Fig 2A). Similarly, cellular levels of phosphorylated p38 (Thr180/Tyr182; pp38) increased significantly at day 6 and remained elevated throughout TMCH (Fig 4Ei). Nuclear pp38 levels however, increased at days 6 through 20 but returned to baseline at days 27 and 34 (Fig 4Ei) as was revealed by
densitometry following normalization with total p38 (Fig 4Eii; n = 3). Thus, phosphorylated, hence activated pMEK1/2, pp44/42-ERK and pp38 may be regulating NF-κB activity both during progression and regression of TMCH.

We next utilized a HEK 293-reporter cell line in order to definitively establish the role of TLR4 in NF-κB activation in response to CR infection in vitro. HEK 293 cells in general, do not express TLR4 (33) and therefore utilization of a TLR4-reporter cell line allows direct assessment of TLR4 function during LPS-induced activation of NF-κB signaling pathway. The HEK 293-reporter cells were infected with CR at 90:1 MOI for 3 hr at 37°C, washed to remove bacteria and incubated in fresh medium with antibiotics for 24, 48 and 72 hr. Measurement of secreted alkaline phosphatase (SEAP) in the spent medium via the SEAPorter assay kit revealed sequential increases in SEAP levels at 24, 48 and 72 hr indicating NF-κB activation via TLR4 in response to CR infection (Fig 5A). At the same time, SEAP levels in the cell extract sequentially declined thereby correlating with its secretion in the medium (Fig 5B).

To assess the role of MEK, ERK and p38 in regulating NF-κB activity in response to CR infection, YAMC cells were transfected with either control or specific siRNAs for these kinases and infected with CR as described in Experimental Procedures. As can be seen from Fig 5Ci, CR infection caused significant increase in cellular levels of MEK1/2, ERK1/2 and p38α/β thereby correlating with in vivo data (see Fig 4C). Cells transfected with siRNAs to MEK-1, ERK1 and p38β exhibited almost complete loss of these proteins (Fig 5Ci) while cellular levels of MEK2, ERK2 and p38α also decreased in response to intervention with specific siRNAs. Bar graph in 5Cii shows relative changes in the levels of these kinases when normalized with actin (n = 3).

To determine if these interventions affected the NF-κB activity in response to CR infection, nuclear extracts prepared from control or specific siRNA-treated YAMC cells were subjected to DNA binding assay as described elsewhere (22,27). As expected, CR infection led to significant increase in NF-κB activity compared to uninfected control (Fig 5D). Interestingly, while siRNAs to all three MAPKs inhibited the NF-κB activity (Fig 5D), isoform-specific siRNAs to MEK1, ERK2 and p38 were relatively more effective in reversing the activation of NF-κB in response to CR infection (Fig 5D).

To further assess the role of MEK/ERK pathway in NF-κB activation, both uninfected (data not shown) and CR-infected mice were treated with specific MEK inhibitor, PD98059 for 10 days starting 2 days post-CR infection as described in Experimental Procedures. PD98059 significantly blocked increases in relative levels of MEK1/2 at peak hyperplasia as was revealed by densitometry following normalization with actin (Fig 6Ai, ii) and, inhibited NF-κB activity compared to untreated mice (Fig 6Aiii) thereby confirming MEK’s role in NF-κB activation during TMCH. To examine downstream pathways affected due to MEK inhibition, we next examined the phosphorylation status of both ERK1/2 and IKKα/β in colonic crypts. MEK1/2 inhibition in vivo led to significant blockade of phosphorylated ERK1/2 alongwith phosphorylated IKKα/β (Figs 6B, C). Bar graphs in Bii and Cii represent the ratio of phosphorylated versus total ERK1,2/IKKα,β from three independent experiments. Given that IKKα/β catalyzes phosphorylation and subsequent degradation of IκBα, inhibition of IKKα/β may have directly affected the NF-κB activity in response to MEK1/2 inhibition at peak hyperplasia.

p65 phosphorylation, acetylation and interaction with ribosomal S6 kinase-1 (RSK-1) accompany NF-κB activation in vivo: Besides the phosphorylation and subsequent degradation of IκBα, protein kinases are also required for optimal NF-κB activation by targeting functional domains of NF-κB proteins themselves. Phosphorylation of p65
subunit for example, enhances its ability to recruit histone acetyltransferases such as cAMP response element-binding (CREB)-binding protein (CBP) and p300 (20,21). We therefore investigated phosphorylation status of p65-NF-κB in order to determine if post-translational modification of this subunit contributed towards sustained activation of NF-κB during regression of hyperplasia. Utilizing an antibody that detects phosphorylation of p65 at Ser-536 (p65536), we evaluated this phosphorylation event in cellular and nuclear extracts during TMCH. Levels of both cellular and nuclear p65536 along with total p65 increased reproducibly at days 6 and 12 of TMCH compared to uninfected control, remained elevated till day 27 and then declined by day 34 (Fig 7Ai). Bar graph in 7Aii represents the ratio of phosphorylated/total p65 versus actin/laminB from three independent experiments. To assess the acetylation status of p65 at these time points, we utilized an antibody that detects endogenous levels of p65 only when it is acetylated at lysine-310. Indeed, the kinetics of this acetylation event of p65 (Fig 7B) paralleled that of p65536 (Fig 7A) suggesting that phosphorylation of p65 at Ser-536 may have facilitated its acetylation at Lysine-310. These studies clearly show that NF-κB activation during the progression and regression phases of hyperplasia is associated with significant phosphorylation, nuclear translocation and acetylation of p65 subunit ensuring NF-κB’s functional activation.

We previously reported that NF-κB-p65 subunit co-immunoprecipitated with RSK-1 and that p65/RSK-1 interaction kinetics overlapped with increases in p65 phosphorylation at Ser-536 (22). In the current study, we utilized an antibody that detects endogenous levels of RSK-1 only when phosphorylated at Thr359/Ser363 in order to directly measure activation status of RSK-1 during TMCH. Relative levels of RSK-1 phosphorylated at Thr-359 and Ser-363 (pp90RSK) increased in both cellular and nuclear extracts during the time course of TMCH (Fig 7Ci) and overlapped to certain extent with the changes in p65536 kinetics (Fig 7A). Bar graph in 7Cii represents the ratio of phosphorylated/total RSK-1 versus actin/laminB from three independent experiments. RSK-1 also co-immunoprecipitated with p65 subunit and the kinetics paralleled increases in p65536 during the time course of TMCH (Fig 7Di, ii). Thus, RSK-1/p65 interaction may be integral to sustained NF-κB activation during TMCH.

**Dietary intervention to block NF-κB in vivo:** We have shown previously that pectin [a soluble fiber and a short-chain fatty acid (SCFA) delivery system to the colon], abrogates TMCH by blocking increases in cell census (23). SCFA butyrate has been shown to modulate NF-κB activity in human colonic epithelial cell line (34). Since pectin provides SCFAs to the colon, we aimed at investigating whether pectin would duplicate the effects of butyrate in vivo.

Both 6% pectin and 1% calcium diets abrogated CR-induced hyperplasia (Suppl. Fig 2). To examine the effects of these dietary ingredients on NF-κB, NF-κB activity in nuclear extracts were measured by EMSA using nuclear extracts prepared from uninfected normal (N) or TMCH crypts (D12) isolated from mice receiving either regular chow or high pectin and high calcium diets, respectively. Pectin in the absence of TMCH, had no effect on NF-κB (Fig 8A). High pectin diet in the presence of TMCH however, inhibited increases in NF-κB activity but high calcium diet had no effect (Fig 8A). Super shift studies were performed by p50 and p65 antibodies, respectively. While antibody to p50 supershifted the NF-κB signal, no supershift was observed with antibody to p65 (Fig 8A) suggesting that pectin may specifically be targeting p65 subunit of NF-κB. When the abundance of NF-κB subunits was measured in isolated crypts under different dietary conditions, we found that high pectin diet blocked increases in p65 cellular abundance (Fig 8Bi), while p50 subunit abundance was not affected. High calcium diet (1%) on the other hand, did not alter the abundance of any of these proteins (Fig 8Bi). Data from three separate mouse experiments.
samples are presented as bar graph in Fig 8Bii as ratio of p50/p65 versus actin.

Since NF-κB activation requires nuclear translocation of p65, we next examined the effect of either high pectin or high calcium diet on nuclear translocation of p65 during TMCH. Significant increases in nuclear p65 were observed in infected mice on control or high calcium diet (Fig 8Ci) as reported previously (22). In contrast, high pectin diet substantially decreased the abundance of nuclear p65 (Fig 8Ci); bar graph in 8Cii shows relative changes in levels of p65 vs. laminB from three mouse samples. This led to significant inhibition of NF-κB activity (Fig 8A) and subsequent downregulation of downstream target gene CXCL-1 (Fig 8Di). Bar graph in Fig 8Dii shows relative changes in CXCL-1 in relation to GAPDH (n = 3). Finally, to ascertain that pectin did not interfere with CR’s ability to bind to colonic mucosa, the tissue sections prepared from the distal colons of untreated or pectin-treated mice were stained with antibody to LPS. As shown in Fig 8E, sections prepared from infected mice fed a control diet and high pectin diet exhibited significant bacterial binding to the colonic mucosa at 12 days post-infection when compared to uninfected colon thereby confirming that this dietary change did not alter the CR attachment to colonic epithelium. Thus, pectin inhibits NF-κB activity by modulating p65 cellular abundance and nuclear translocation eventually affecting expression of downstream target genes.

**DISCUSSION**

TMCH, atleast in the context of outbred genetic background, is a self-limited disease and contrasts with the ongoing proliferation that occurs in colonic neoplasia. However, development of colonic hyperplasia during TMCH is associated with an increased susceptibility to carcinogenesis in response to either a chemical mutagen (10) or in the absence of a functional Apc gene product (35). As a non-invasive organism, CR lends itself to the study of how the host recognizes and eliminates pathogens in the intestinal lumen and distinguishes such pathogens from the normal flora. Signaling via TLR-4 has been implicated in the protective host responses that lead to clearance of CR infection (33,36). Thus, innate immune signaling in limiting mucosal damage is necessary to protect the host while an adaptive immune response develops resulting in bacterial clearance.

NF-κB is an important component of the innate and adaptive immunity. Utilizing the CR-induced TMCH model, we showed previously that CR-infection is associated with dramatic increase in NF-κB activation at peak hyperplasia and this activation involved both phosphorylation and degradation of IκBα and phosphorylation and nuclear translocation of p65 subunit (22). In the current study, we report that activation of NF-κB at time points that coincided with bacterial colonization (TMCH progression) was regulated primarily through the canonical pathway (IκBα phosphorylation and degradation). In contrast, we also observed sustained NF-κB activity after bacterial clearance (TMCH regression) which was regulated by persistent phosphorylation events of the MEK/ERK/p38/RSK-1 pathway. These changes correlated with hyperplasia of the colonic crypts wherein, sustained Ki-67 immunoreactivity was recorded between day 6-27 followed by decline by day 34. Thus, continuous bacterial attachment or colonization per se is not required to keep NF-κB active for an extended period which corroborates well with the non-invasive nature of CR (37). Instead, the CR-induced cytokinetic changes due to intracellular signaling as discussed below may be sufficient to regulate NF-κB activity for an extended period of time.

In response to diverse stimuli, IκB kinase (IKK), a complex composed of the regulatory IKKγ (NEMO) subunit and two enzymatically active subunits IKKα and IKKβ, undergoes phosphorylation at consensus phosphorylation sites (Ser176 and Ser 180 in IKKα, Ser 177 and Ser181 in IKKβ) leading to its activation. Canonical IKK activation involves IKKβ and results in phosphorylation of IκBα, dissociation of IκBα from NF-κB, ubiquitination and proteasomal degradation.
We observed significant increases in the relative levels of phosphorylated IKKs not only at days 6 and 12 of TMCH leading to increases in IκBα phosphorylated at Ser 32/36 and proteasomal degradation as reported earlier (22), but the increased levels were sustained at days 20, 27 and 34 of TMCH. However, NF-κB activation at later time points was probably less dependent on IκBα degradation despite continued presence of phosphorylated, hence activated IKKs at these time points.

Positional cloning work and subsequent biochemical analyses have revealed that TLR4 transduces the lipopolysaccharide (LPS) signal, alerting the host to infection by Gram-negative bacteria. The interaction of TLR4 with its ligand LPS (lipopolysaccharide) results in the recruitment of the adaptor molecule MyD88 and phosphorylation of the IRAK (38). Phosphorylated IRAK1 undergoes ubiquitination and proteasomal degradation which is followed by TRAF-6 activation (38). TRAF-6, in association with the proteins TAB-1 [transforming growth factor-β-activated kinase-1 (TAK-1)-binding protein-1] and TAB-2, leads to the activation of the MAPK pathway following ubiquitination of both TRAF-6 and TAK-1 (39). TAK-1 then activates both MEK1,2/ERK and p38/JNK pathways which catalyzes activation of both IKK complex and NF-κB. In the current study, utilizing a TLR4-reporter cell line, we established the direct involvement of TLR4 in NF-κB activation in response to CR infection (see Fig 5A, B) and also detected all three major proteins involved in signaling via TLR-4: MyD88, TRAF-6 and TAK-1 in the colonic crypts. Despite CR infection being unable to affect cellular changes in these proteins, their expression in colonic crypts suggests an important role for these proteins in transducing the TLR-4 signaling in response to CR infection resulting in the activation of the MEK/ERK/MAPK pathway.

Three major MAP kinase cascades have been described in mammalian cells, the ERK, p38, and the JNK pathways. They are linked to activation by LPS and subsequent cytokine gene expression. In the current study, we observed significant increases in cellular and nuclear levels of phosphorylated and hence activated MEK1/2, ERK and p38 at peak hyperplasia. Interestingly, the levels of these kinases did not decline with regressing hyperplasia suggesting that they may be regulating sustained levels of IKKα/β and NF-κB activity during TMCH. To that end, two different approaches were undertaken to establish direct involvement of these MAPKs in NF-κB activation in response to CR infection: i) an in vitro approach wherein, YAMC cells were transfected with siRNAs specific to MEK1/2, ERK1/2 and p38α/β and NF-κB activity was examined. siRNAs specific to all three MAPKs significantly inhibited NF-κB activity although isoform-specific inhibition was also recorded for MEK1, ERK2 and p38β MAPKs; ii) an in vivo approach wherein, a proof-of-principle experiment was performed in vivo with an inhibitor of MEK1/2 and ERK pathway in order to further validate in vitro finding. MEK1/2 inhibitor PD98059 blocked increases in phosphorylated MEK1/2 and ERK leading to significant inhibition of NF-κB activity in the day 12 crypts. Since siRNA to MEK1 was more effective in reversing the effects of CR-induced NF-κB activation in vitro (see Fig 5C), it is tempting to speculate that MEK1 and not MEK2 may be predominantly involved in regulating NF-κB activity in colonic crypts. We showed previously that inhibition of NF-κB via proteasomal inhibitor Velcade did not abrogate hyperplasia (22). Moreover, preliminary studies in TLR-4−/− mice show significant hyperplasia of the colonic crypts in response to CR infection despite attenuated NF-κB activity. Hyperplasia in these mice is driven by β-catenin-mediated increases in downstream targets, cyclin D1 and c-myc (manuscript under preparation). These studies clearly suggest that β-catenin is not downstream to TLR-4/MEK/ERK pathway and that β-catenin rather than NF-κB, regulates crypt hyperplasia in response to CR infection (9,22). Nevertheless, MEK1,2/ERK pathway may be critical in regulating NF-κB activity at least at peak hyperplasia. Similarly, suppressor of cytokine signaling-1 (SOCS-1),
a negative regulator of NF-κB activity in the TLR-4/ERK/p38 pathway, failed to completely abolish NF-κB activity in regressing hyperplasia (data not shown). These studies suggest that ERK/p38 pathway may have overwhelmed the inhibitory cues in order to maintain NF-κB in an activated state in regressing crypts. While more studies are needed to clearly understand how the sustained levels of MEK/ERK/p38 are regulated in colonic crypts in response to CR infection in vivo, the current study looking at sustained activation of NF-κB due to MEK1/ERK1,2/p38β may eventually prime the colonic mucosa towards neoplasia in response to a second hit. Given that NF-κB is an obvious target for newer treatments to block the inflammatory response in instances where this process becomes chronic or dysregulated, targeting these kinases to regulate NF-κB could be useful in blocking prolonged activation of this pathway. It is important to keep in mind however that it may not be feasible to block NF-κB pathway for prolonged periods since NF-κB plays an important role in the maintenance of protective immunity. Based on our studies, short-term treatment with pathway specific inhibitors may reduce such potential side-effects and may enhance the efficacy of cancer chemotherapy and reducing abnormal cytokine production eventually blocking growth and progression of colon carcinogenesis.

**Phosphorylation and Acetylation of p65 subunit may regulate sustained activation of NF-κB in vivo:** Phosphorylation of the p65 subunit plays a key role in determining both the strength and duration of the NF-κB-mediated transcriptional response (40,41). Sites of phosphorylation reported to date are serines 276 and 311 in the Rel-homology domain, and serines 468, 529 and 536 in the transactivation domain. Moreover, acetylation of phosphorylated p65 subunit at Lys 310 is essential for NF-κB’s transcriptional activity (40,42,43). We previously showed significant increases in phosphorylation of p65 subunit at Ser 536 (ppp65\textsuperscript{536}), which translocated to the nucleus and interacted with transcriptional coactivator CREB binding protein (CBP). In the current study, the relative levels of both cellular and nuclear p65\textsuperscript{536} alongwith total p65 increased reproducibly at days 6 and 12 of TMCH and remained elevated till day 27 before declining by day 34 (see Fig 7). p65 subunit also underwent significant acetylation at lysine 310 (Ac-p65\textsuperscript{Lys310}) at peak hyperplasia with sustained levels even at day 34 of TMCH (see Fig 7B) suggesting that phosphorylation of p65 subunit at Ser-536 in the colonic crypts may have facilitated recruitment of transcriptional coactivator CBP with intrinsic acetyl-transferase activity to catalyze acetylation of p65 at lysine 310. Since phosphorylation at Ser-536 also reduces the ability of p65 to bind IkBα in cell lines (44), both ppp65\textsuperscript{536} and Ac-p65\textsuperscript{Lys310} rather than degradation of IkBα may be regulating sustained and functional activation of NF-κB in the colonic crypts beyond peak hyperplasia.

The p90 ribosomal S6 kinases (RSKs) are a family of serine/threonine kinases that lie at the terminus of the ERK pathway (45-48). In mammals, four isoforms are known, RSK1 to RSK4. Each one has two catalytically functional kinase domains, the N-terminal kinase domain (NTKD) and C-terminal kinase domain (CTKD) as well as a linker region between the two. The NTKD is responsible for phosphorylation of exogenous substrates, and the CTKD and linker region regulate RSK activation (45,46,48). In quiescent cells, ERK binds to the docking site in the C-terminus of RSK (49-51). Upon mitogen stimulation, ERK is activated by its upstream MAPK/ERK kinase, MEK1/2 as discussed in the previous sections. The active ERK phosphorylates Thr-359/Ser-363 of RSK-1 in the linker region and Thr-573 in the CTKD activation loop. Through a series of trans- and autophosphorylations, RSK is finally activated. We also observed significant and sustained increases in relative levels of both cellular and nuclear RSK-1 phosphorylated at Thr-359 and Ser-363 as well as co-immunoprecipitation of RSK-1 with p65 subunit which paralleled p65\textsuperscript{536} kinetics during the time course of TMCH. Thus, RSK-1 and
not necessarily IKKα/β seems to be involved in phosphorylating p65 at Ser-536 both at peak hyperplasia and more importantly when hyperplasia is regressing thereby regulating sustained activation of NF-κB in colonic crypts. It remains to be determined whether MEK1,2/ERK inhibition in vivo affected RSK-1 levels or activation in colonic crypts and how sustained activation of RSK-1 is regulated during TMCH. Recent studies have shown that Kaposi sarcoma-associated herpes virus (KSHV) infection activates multiple MAPK pathways and promotes interaction with RSKs (52-54). More importantly, the ERK/RSK activation was sustained both during KSHV primary infection and during reactivation from latency (52,53,55,56). Mechanistically, Kuang E. et al. (56) showed that ORF-45, an immediate early and also virion tegument protein of KSHV, interacted with RSK-1 and strongly stimulated its kinase activity. ORF45 also increased the association of RSK-1 with ERK and protected them from dephosphorylation, causing their sustained activation (56). Whether similar mechanism exists for sustained phosphorylation and/or activation of pp44/42-ERK, pp90RSK or pp38 during TMCH remains to be determined and will be investigated in future studies.

**Dietary intervention to block NF-κB in vivo:** Given the diverse processes involved in activating the NF-κB pathway, it is not surprising that a plethora of chemical inhibitors or biologics have been utilized and implicated in blocking activation of this pathway (57). Butyrate and other short-chain fatty acids (SCFAs) are generated by the bacterial metabolism of dietary fiber and are putative modulators of the beneficial effects of fiber on the colon. Intracellular mechanisms that mediate the beneficial aspects of butyrate however, are not well elucidated. We showed previously that 6% pectin diet, as a butyrate delivery system to the colon, abrogated increases in β-catenin abundance and reduced its downstream effectors, cyclin D1 and c-myc thereby abrogating hyperplasia of colonic crypts in response to CR infection (38). Several natural compounds including polyphenols have been reported to suppress NF-κB activity and are suggested to be useful for the inhibition of cancer cell growth (57). For any intervention to be successful however, it is critical to pin-point exact cell-type responding to a particular intervention. In the current study, we discovered that 6% pectin diet significantly inhibited NF-κB activity in the epithelial cells of the colonic crypts of day 12 animals by blocking increases in cellular levels of p65 subunit and preventing its nuclear import without altering CR’s ability to bind to colonic mucosa. To our knowledge, this is the first demonstration of a particular cell type responding to a dietary intervention. It has been shown by Yin et al. (58) that butyrate inhibits NF-κB by suppressing degradation of IκBα and cellular proteasome activity in vitro. Since bacterial fermentation of pectin generates butyrate and other SCFAs and our own observation that pectin treatment of infected animals increased generation of butyrate in the colon by 2-fold (data not shown), it is likely that pectin-induced inhibition of NF-κB activity in vivo is mediated by butyrate. The butyrate suppression of IκBα degradation and proteasome activity may derive from its ability to inhibit histone deacetylases as is reported previously (58). Thus, modulation of both β-catenin (23) and NF-κB pathways by luminal factors such as pectin and SCFAs during TMCH may be a physiologically relevant mechanism to regulate colonic crypt hyperplasia. Since a decrease in intestinal proliferation is associated with a reduced colon cancer risk, treatments or diets that increase colonic levels of SCFAs such as pectin, may be beneficial for preventing the risk for colon carcinogenesis.

**TMCH in context:** Transmissible murine colonic hyperplasia (TMCH) induced by *Citrobacter rodentium* (CR) causes significant hyperproliferation and hyperplasia of the distal colonic crypts and increases the risk of subsequent neoplasia. In adult animals, infection is typically self-limited, and life-long immunity is provided after recovery. In a recent study (59), it has been shown that NF-κB-p50/− mice failed to promote bacterial clearance despite higher levels of anti-
Citrobacter IgG and IgM, due to lack of NF-κB activation in these mice in response to CR infection suggesting that non-NF-κB-dependent defenses are insufficient to control CR infection. Thus, sustained activation of NF-κB during TMCH, driven by a combination of both IkBx-phosphorylation and degradation (at peak hyperplasia; Fig 9) and MEK1,2/ERK/p38-dependent regulation of p65/RSK-1 interaction (both during progression and regression; Fig 9), besides working in tandem with β-catenin to regulate proliferatory activity of the colonic crypts, may be better suited to regulate bacterial clearance thereby keeping TMCH a self-limiting infection. From a disease standpoint however, NF-κB has been reported to become constitutively active in tumor cells and therefore blocking NF-κB can cause tumor cells to stop proliferating, to die, or to become more sensitive to the action of anti-tumor agents. In that context, TMCH model with its entire repertoire of activated Wnt/β-catenin (9,26) and MEK/ERK/p38/NF-κB pathways (Fig 9), basically mimics human conditions wherein, CR-induced cytokinetic alterations in colonic epithelia may promote mucosal priming for neoplasia. Thus, methods of blocking NF-κB signaling in vivo either via chemical inhibitors (such as shown in Fig 6) or through dietary intervention (see Fig 8), have therapeutic implications in cancer and inflammatory diseases.

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FOOTNOTES

This work was supported by National Institutes of Health Grants R01 CA131413 (to SU) and R01 CA 97959 and CA114264 (to PS) from the NCI.

ABBREVIATIONS

EHEC, Entero-Hemorrhagic E. coli; EPEC, Entero-Pathogenic E. coli; IKK, IκBα Kinase; MAPK, Mitogen Activated Protein Kinase; NEMO, NF-κB Essential Modulator; NF-κB, Nuclear Factor-κB; RSK, Ribosomal S6 Kinase; SCFA, Short Chain Fatty Acid; siRNA, small interfering RNA; TMCH, Transmissible Murine Colonic Hyperplasia; YAMC, Young Adult Mouse Colon.

FIGURE LEGENDS

Figure 1. Crypt hyperplasia as measured by Ki-67 staining. Immunohistochemical labeling of Ki-67 as a marker of proliferation in paraffin-embedded sections prepared from the distal colons of uninfected normal (N) and CR-infected (days 6–34) mice. Bar = 75 μm (n = 5).

Fig. 2. A. NF-κB activity measured via DNA binding assay. Crypt nuclear extracts were prepared from N and days 6-34 post-infected mice and the presence of activated NF-κB p65 in the nuclear extracts was examined by utilizing Trans AM NF-κB p65 Chemi Transcription Factor Assay kit from Active Motif. Significant increases in NF-κB activity was recorded at days 6 and 12 with sustained activation at days 20, 27 and 34 of TMCH (n = 3; p<0.05). B. Expression of downstream target for NF-κB, CXCL-1/KC during TMCH. Total RNA was extracted from colonic crypts isolated from N or days 6-34 post-infected mice. CXCL-1/KC expression was measured via semi-quantitative RT-PCR using actin mRNA as loading control (upper panel). Lower panel represents a bar graph showing relative levels of CXCL-1/KC normalized to actin (P<0.05, n = 3). C. Measurement of CXCL-1/KC expression in YAMC (Young Adult Mouse Colon) cells in vitro. YAMC cells (5x10⁵) were either treated with (+CR) or without (-CR) CR at 100:1 MOI for 3 hr. Cells were washed thoroughly to remove bacteria and incubated in fresh medium containing antibiotics for indicated period of time. Total RNA was examined for the expression of CXCL-1/KC via RT-PCR and GAPDH was used as loading control. A significant increase in the level of CXCL-1 was observed at 24 and 48 h post-infection with sustained expression even at 120 h compared to uninfected control (Ci; n = 3). Cii. Representative bar graph showing the relative expression levels of CXCL-1 normalized to GAPDH. *P<0.05 vs. control (†, n = 3).

Fig. 3. A. Immunofluorescence detection of LPS as a surrogate for Citrobacter presence in the distal colon isolated from uninfected normal (N) and days 6-34 (D₆-D₃₄) post-infected mice. Paraffin-embedded sections were deparaffinized, subjected to antigen retrieval and incubated
Fig. 4. Both canonical and atypical pathways contribute towards NF-κB activation in colonic crypts in vivo. A. Representative bar graph showing the ratio of IκBα phosphorylated at Ser-32/36 vs. total IκBα. *P<0.05 vs. control (†, n = 3); †P<0.05 vs. control (†); ‡P<0.05 vs. day 12 (†●; n = 3). B. Both IKKα and -β undergo increased phosphorylation in vivo. Representative bar graph showing relative levels of phosphorylated and total IKKα/β vs. actin. *P<0.05 vs. control (†, n = 3); ●P<0.05 vs. control (‡, n = 3). C-E. Changes in the relative levels of MEK1/2, p44/42-ERK and p38 during TMCH. Crypt cellular extracts prepared from the distal colons of N and days 6-34 post-infected mice were analyzed for the relative abundance of MEK1/2 (Ci), p44/42-ERK (Di) and p38 (Ei) proteins by Western blot analysis. Relative levels of phospho-MEK1/2 (pMEK1/2), p44/42-ERK (pp44/42) and p38 (pp38) exhibited dramatic increases in both cellular and nuclear extracts at peak hyperplasia and the levels remained elevated during regression phase (days 20-34) of hyperplasia. Bar graphs in Ci [*P<0.05 vs. control (†), †*P<0.05 vs. control (‡)]; ●P<0.05 vs. control (#; n = 3)], Di [*P<0.05 vs. control (†, n = 3); †*P<0.05 vs. control (†, n = 3)] and Ei [*P<0.05 vs. control (†, n = 3); †*P<0.05 vs. control (†, n = 3)] represent relative abundance of phosphorylated cellular/nuclear proteins normalized to actin/laminB.

Fig 5. Signaling via TLR4 and MEK/ERK/p38 regulates NF-κB activity in response to CR infection. A and B: Functional analysis of TLR4. TLR4/NF-κB/SEAPorter HEK293 cells were infected with CR for 3 hr, washed to remove bacteria and secreted alkaline phosphatase (SEAP) levels were measured in the spent medium (A) and cell extracts (B) via SEAP assay kit. Significant and sequential increases in SEAP levels indicating NF-κB activity, were recorded at 24, 48 and 72 hr in the spent medium with concomitant decreases in the cell extracts at these time points (*P<0.05 vs. uninfected control; n = 3). C. Effect of siRNAs on MEK, ERK and p38 levels in vitro. YAMC cells in culture were transiently transfected either with scrambled siRNA (ssiRNA) or siRNAs specific to MEK1/2, ERK1/2 and p38α/β. The transfected cells were treated with or without CR for 3 hr, washed to remove bacteria and processed for Western blotting after 48 hr. Samples in various lanes are: 1. Basal, 2. CR, 3. CR+ssiRNA, 4. CR+siRNA (MEK1, ERK1, p38α), 5. CR+siRNA (MEK2, ERK2, p38β). Please note that siRNAs to MEK1, ERK1/2 and p38β in particular, caused significant reduction in the levels of these proteins (Ci). Cii. Representative bar graph showing relative levels of MEK1/2 (A, B), ERK1/2 (C, D) and p38α/β (E, F) normalized to actin. *P<0.03 vs. control (†, n = 3); †P<0.05 vs. CR/ssiRNA (‡●; n = 3). D. Effect of blocking MEK/ERK/p38 on NF-κB activity. DNA binding assay was performed in YAMC cells transfected either with scrambled siRNA (ssiRNA) or siRNA specific for each kinases and infected with or without CR as described above. Various lane assignments are: 1. Basal, 2. CR infected, 3. CR+ssiRNA, 4-8. CR+siRNAs for MEK1 (4), p38α (5), p38β (6), ERK1 (7) and ERK2 (8) respectively. *P<0.05 vs. control (†, n = 3).
Fig. 6. A. Effect of MEK1/2 inhibition on NF-κB activity in vivo. Swiss-Webster mice were divided into two groups and injected once a day for 10 days with either control or specific MEK1/2 inhibitor, PD98059 (see “Experimental Procedures’’). 2h after the last injection, colonic crypts were isolated and fractionated into cytosolic and nuclear extracts. Representative Western blots for total MEK1/2 in the colonic crypt cellular extracts prepared from uninfected normal (N), day 12 (D12) and day 12+inhibitor (D12+I)-treated mice (Ai). Ai. Representative bar graph showing relative levels of MEK1/2 when normalized to actin. *P<0.05 vs. control (†, n = 3); ‡P<0.05 vs. day 12 (†*, n = 3). Aii. DNA-binding assay. PD98059 significantly inhibited NF-κB activation, measured in a DNA-binding assay with nuclear extracts prepared from day 12+inhibitor (D12+I)-treated mice, compared to levels measured in either control or day 12 (D12) mice alone. Each bar represents mean±S.E.M. values from three measurements from three separate mice. P values (<0.03 for day 12 and <0.007 for day 12+I), respectively, versus corresponding control values. Bi & Ci. Representative Western blots showing relative levels of phosphorylated (pp44/42) and total p44/42-ERK (B) and phospho (pIKKα/β) and total IKKα/β (C) in the colonic crypt cellular extracts prepared from uninfected normal (N), day 12 (D12) and day 12+inhibitor (D12+I)-treated mice. Bar graphs in Bi & Ci represent relative levels of phosphorylated and total p44/42-ERK [Bi; *P<0.05 vs. control (†; n = 3); †P<0.05 vs. day 12 (†*, n = 3)]; ‡P<0.05 vs. control (†; n = 3); *P<0.05 vs. day 12 (†*, n = 3)] and phosphorylated and total IKKα/β [Ci; *P<0.05 vs. control (†, n = 3); †P<0.05 vs. day 12 (†*, n = 3)] normalized to total proteins.

Fig. 7. Phosphorylation and acetylation of p65 subunit underlies functional activation of NF-κB during TMCH. Relative levels of phosphorylated (p65S356) and total p65 subunit in the cellular and nuclear (Ai) extracts prepared from uninfected normal (N) and days 6-34 post-infected mice were determined by Western blotting. Aii. Representative bar graph showing relative levels of phosphorylated and total p65 subunit in the cellular and nuclear extracts when normalized to actin/laminB. Bars: *P<0.05 vs. control (†, n = 3); ●P<0.05 vs. control (†, n = 3); †P<0.05 vs. control (†*, n = 3). Aiii. DNA-binding assay. Pectin significantly inhibited NF-κB activation, measured in a DNA-binding assay with nuclear extracts prepared from day 12+inhibitor (D12+I)-treated mice, compared to levels measured in either control or day 12 (D12) mice alone. Each bar represents mean±S.E.M. values from three measurements from three separate mice. P values (<0.03 for day 12 and <0.007 for day 12+I), respectively, versus corresponding control values. B. Nuclear accumulation of acetylated p65 subunit overlaps p65S356 kinetics. Relative levels of p65 subunit acetylated at lysine 310 (Ac-p65Lys310) along with total p65 were measured in the nuclear extracts prepared from the distal colons of N and days 6-34 post-infected mice (Bi). Bi. Representative bar graph showing relative levels of acetylated total p65 vs. laminB. *P<0.05 vs. control (†, n = 3); ●P<0.05 vs. control (†, n = 3). C. Phosphorylation status of RSK-1 during TMCH. Ci. Relative levels of RSK-1 phosphorylated at Thr-359/Ser-363 (pp90RSK) and total RSK-1 were measured in the cellular and nuclear extracts prepared from N and days 6-34 post-infected mice by Western blotting. Cii. Representative bar graph showing relative levels of phosphorylated and total p65 subunit in the cellular and nuclear extracts when normalized to actin/laminB. Bars: *P<0.05 vs. control (†, n = 3); ●P<0.05 vs. control (†, n = 3). Di. Co-immunoprecipitation: Crypt cellular extracts prepared from the distal colons of N and days 6-34 post-infected mice were co-immunoprecipitated with anti-p65 and blotted with antibody to RSK-1. Lower panel represents IgG heavy chain. Dii. Representative bar graph showing relative levels of normalized RSK1. *P<0.05 vs. control (†, n = 3).

Fig. 8. Pectin inhibits NF-κB activity in vivo. A. Effect of dietary intervention on NF-κB activity in vivo. NF-κB activity in the nuclear extracts were measured via EMSA in uninfected normal (N) or TMCH crypts (D12) isolated from mice receiving either regular chow or high pectin and high calcium diets, respectively. EMSA showing relative levels of activated NF-κB in colonic crypts from mice treated as indicated: Norm., non-infected (N); P, pectin; CR, C. rodentium-infected (D12). Last 2 lanes: p50 but not the p65 subunit, supershifted with the indicated NF-κB subunit antibodies in the CR+pectin treated crypt nuclear extracts. Pectin in the
absence of TMCH, had no effect on NF-κB. B. Effect of dietary intervention on subunit expression. Crypt cellular extracts prepared from mice treated as indicated in legends to 8A, were subjected to Western blotting with antibodies to p50 and p65 subunits, respectively. Bii. Representative bar graph showing relative levels of p50/p65 when normalized to actin. Bars: *P<0.05 vs. control (†, n = 3); †●P<0.05 vs. control (†, n = 3); ●‡P<0.05 vs. CR (†●, n = 3). C, D. Effect of dietary intervention on nuclear translocation of NF-κB subunits and expression of downstream target CXCL-1. Crypt nuclear extracts prepared as described in legend to 8A were subjected to Western blot analysis with antibody to p65 subunit. 6% pectin but not high calcium diet blocked p65 nuclear translocation [C, *P<0.05 vs. control (†, n = 3); †●P<0.05 vs. CR (†●, n = 3)]. This led to significant inhibition of NF-κB activity (see 8A) and subsequent downregulation of downstream target gene CXCL-1 [D, *P<0.05 vs. control (†, n = 3); †●P<0.05 vs. CR (†●, n = 3)] in the pectin-treated samples (n = 3). E. Effect of pectin treatment on bacterial binding. Paraffin-embedded sections prepared from uninfected (N) and pectin-untreated or pectin-treated 12-days post-infected mouse distal colons were stained with antibody to LPS and counter-stained with hematoxylin to label the nuclei (n = 3; bar = 100μm).

Fig 9. Proposed mechanism of NF-κB activation in response to CR infection in vivo. In response to infection of Swiss Webster mice by Citrobacter rodentium, intracellular signaling via MEK/ERK and p38 pathways may lead to sustained levels of these kinases thereby affecting NF-κB activity in the colonic crypts. The solid arrows represent pathways which are more apparently active in the colonic crypts while broken arrows represent pathways most likely contributing towards NF-κB activation in vivo. A and B represents possible mechanism for activation of NF-κB either during progression or both during progression and regression of hyperplasia. Both MEK1/2 inhibitor PD98059 and 6% pectin diet can significantly inhibit NF-κB activity in the colonic crypts in response to CR infection.
Figure 1

Ki-67

N

D6

D12

D20

D27

D34
Figure 2

A. DNA Binding Assay

Days of Infection

B. Ratio: CXCL-1/Actin

Days of Infection

C.

i) CXCL-1

GAPDH

- CR + CR

- CR + CR

ii) Rel. O.D. (CXCL1 vs. GAPDH)

24h 48h 120h
Figure 3A

LPS staining

N

D6

D12

D20

D27

D34
Figure 3B

Fluorescence intensity (%)

Days of Infection

N 6 12 20 27 34

* * *
Figure 6

**A.**

i) 

![Graph showing MEK1/2 and actin levels](image)

ii) 

![Bar graph showing relative optical density (OD) of MEK1/2 vs. actin](image)

iii) 

![NF-kB activity Abs. (450 nm)](image)

**B.**

i) 

![Graph showing pp44/42 and p44/42 levels](image)

ii) 

![Bar graph showing ratio of phospho p44/42 vs. total p44/42](image)

**C.**

i) 

![Western blot showing pIKKα/β and IKKα/β](image)

ii) 

![Bar graph showing ratio of phospho IKKα/β vs. total IKKα/β](image)
Figure 7

A. i) Cellular
   - p65536
   - p65
   - Actin
   ii) Days of Infection

B. i) Nuclear
   - Ac-p65
   - p65
   - LaminB
   ii) Days of Infection

C. i) Cellular
   - pp90RSK (Thr359/Ser363)
   - RSK1
   - Actin
   ii) Days of Infection

D. i) 50%
   - RSK-1
   - IgG-H
   ii) Days of Infection

Ratio: Phospho/Total p65 vs. Actin/LaminB

Ratio: RSK1 vs. IgG
Figure 8

A. Supershift assay showing NF-κB-DNA complexes.

B. Western blot analysis showing p50 and p65 levels.

C. i) Western blot showing p65 and LaminB.

D. i) Western blot showing CXCL1 and GAPDH.

E. Immunohistochemical staining for D_{12} and D_{12}+P.
Figure 9
