Title: Ron receptor signaling is protective against DSS-induced colitis in mice.

Running Title: Ron receptor signaling and colitis

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ABSTRACT

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the intestine that result in painful and debilitating complications. Currently no cure exists for IBD and treatments are primarily aimed at reducing inflammation to alleviate symptoms. Genome-wide linkage studies have identified the Ron receptor tyrosine kinase and its ligand, hepatocyte growth factor-like protein (HGFL), as genes highly associated with IBD. However, only scant information exists on the role of Ron or HGFL in IBD. Based on the linkage of Ron to IBD, we directly examined the biological role of Ron in colitis. Wild type mice and mice lacking the tyrosine kinase signaling domain of Ron (TK-/- mice) were utilized in a well-characterized model of chronic colitis induced by cyclic exposure to dextran sulfate sodium. In this model, TK-/- mice were more susceptible to injury as judged by increased mortality compared to control mice and developed more severe colitis. Loss of Ron led to significantly reduced body weights and more aggressive clinical and histopathologies. Ron loss also resulted in a dramatic reduction in colonic epithelial cell proliferation and increased proinflammatory cytokine production, which was associated with alterations in important signaling pathways known to regulate IBD. Examination of human gene expression data further supports the contention that loss of Ron signaling is associated with IBD. In total, our studies point to important functional roles for Ron in IBD by regulating healing of the colonic epithelium and by controlling cytokine secretion.

KEYWORDS

Ron receptor, HGFL, Colitis, Inflammatory Bowel Disease, Met receptor
INTRODUCTION

Inflammatory bowel disease (IBD) is a multifactor, chronic inflammatory disorder of the colon and small intestine. Crohn’s disease (CD) and ulcerative colitis (UC) are the two main types of IBD and affect over 1.4 million people in the United States (12, 17). Complications from IBD include fistulas, malnutrition, bowel obstruction and an increased risk of colorectal cancer (30). The development and course of IBD are affected by genetic susceptibility of the host, the intestinal microbial flora, environmental factors and the status of the host immune system (17). The pathogenesis of IBD is complex and involves intestinal epithelial cell death, immune cell activation and increased proinflammatory cytokine production. Perturbation of a tightly controlled cytokine network, with abnormal crosstalk between several mucosal cell types within the colon is an important step in progression of chronic IBD. While genome-wide association studies have identified several candidate genes in IBD (17, 27), current treatments for IBD are aimed at the management of symptoms and decreasing inflammation through immunomodulators (12). Key gaps exist in our knowledge of genes that control IBD and in the development of novel targeted therapies to lessen disease pathogenesis.

Genome-wide linkage studies identified a region on chromosome 3 (3p21-22) as an IBD locus (5, 13, 27). This region contains genes including the Ron receptor tyrosine kinase (MST1R) and its ligand, hepatocyte growth factor-like protein/macrophage-stimulating protein (HGFL, MST1). Further, two single nucleotide polymorphisms (SNPs) in the Ron receptor (R523Q and G133R) and a SNP in HGFL (R689C) have been identified in IBD patients with strong linkage disequilibrium (5, 13, 27). The Ron receptor is a membrane spanning protein that belongs to the c-Met family of receptor tyrosine kinases. Ron is expressed on many cell-types (47). Ron mRNA and protein expression has been reported in the intestine and colon as well as in colon epithelial cells in both mice and humans (18, 32, 33). Ron is also expressed in tissue resident macrophages including peritoneal macrophages and Kupffer cells but not in circulating monocytes (16, 43). In epithelial cells, Ron functions to promote proliferation, survival,
migration and differentiation (47). Macrophage Ron expression has been shown to be critical in suppressing M1 activation limiting inflammation and immunity to pathogens while stimulating M2 activation which promotes the resolution of inflammation and wound healing (14, 40). HGFL, the sole ligand for Ron, is predominantly produced by the liver and is secreted into the plasma as a pro-protein which is cleaved by a variety of serum and membrane-bound proteases to form a biologically active protein (47). Binding of HGFL to Ron leads to the activation of multiple downstream signaling cascades, in a cell-type dependent manner, which induces pleotropic effects. The HGFL and Ron SNPs identified in IBD patients are predicted to interfere with the ability to activate Ron signaling and a recent report, while finding no activation differences, showed that the R689C SNP in HGFL reduced serum levels of HGFL in IBD patients (5, 13, 18). These data suggest that loss or inhibition of Ron signaling may play an important role in IBD.

In this report, the in vivo significance of the HGFL-Ron signaling axes was investigated utilizing a well-established murine model of chronic colitis induced by administration of dextran sodium sulfate (22, 51). Following the induction of colitis, mice lacking the Ron signaling exhibited more severe colonic injury characterized by heightened pathologies, diminished colonic epithelial cell proliferation, and increased production of proinflammatory mediators. These studies provide strong evidence of an important role for this signaling cascade in colitis whereby Ron signaling in the colon is required for the healing of the colonic epithelium and for limiting inflammation, which may have wide therapeutic implications for the treatment of IBD.
MATERIALS AND METHODS

Mice and induction of chronic colitis. Ron tyrosine kinase (TK) deficient mice (TK-/- mice) were generated as previously described and backcrossed into a C57BL/6 background (48). Age-matched 8-10 week old male mice were used for all experiments. Chronic colitis was induced by administering 2.5% dextran sodium sulfate (DSS), (molecular weight 36-50 kDa; MP Biomedicals, Solon, OH) in the drinking water for 7 days, followed by 14 days of regular water. This DSS cycle was repeated three times (22, 51). Mice were weighed every other day and a clinical score was calculated based on well-established criteria including stool score, stool blood score and mouse appearance. Stool score was scored as 0, normal; 1, moist; 2, soft; 3, diarrhea; stool blood was scored as 0, no blood; 1, blood in stool or around anus; 2, severe bleeding; and mouse appearance was scored as 0, normal; 1, ruffled fur or altered gait; 2 moribund (22). Mice were euthanized 56-62 days after starting treatment. Two hours prior to sacrifice, mice were injected with BrdU. All mice were maintained under specific pathogen-free conditions and were treated and euthanized in accordance with protocols approved by the Institutional Animal Care and Use Committee of University of Cincinnati.

Tissue histology and immunohistochemical analyses. Colons were isolated, fixed and embedded in paraffin for histological assessment. The severity of colitis was assessed on stained colonic sections in a blinded fashion using established criteria based on crypt damage and ulceration. Crypt damage was scored as follows: 0, intact crypts; 1, loss of the basal one-third; 2, loss of the basal two-thirds; 3, entire crypt loss; 4, change of epithelial surface with erosion; 5, confluent erosion. Ulceration was scored as follows: 0, absence of ulcer; 1, one or two foci of ulcerations; 2, two to four foci of ulcerations; 3, confluent or extensive ulceration. Values were added to give a maximal histological score of 8 (19). Immunohistochemical analyses were performed on colon sections with antigen retrieval performed using citrate buffer or Proteinase K. The sections were incubated overnight with primary antibodies p-STAT1 and p-IKK-α/β (Cell Signaling Technology, Danvers, MA), Ron and HGFL (Santa Cruz...
Biotechnology, Dallas, TX), plectin, laminin and F4/80 (AbCam, Cambridge, MA). Biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) were used to detect the primary antibodies and visualized using an HRP conjugated ABC system (Vector Laboratories). Diaminobenzidine or Nova red (Vector Laboratories) were used as the chromogen. For the quantification of F4/80, p-STAT1 and p-IKKα/β positive cells in colonic sections, positive staining epithelial or stromal cells were counted from 5-7 non-overlapping (400x magnification) fields from 3-4 mice per genotype.

Mitotic and cell death indices were assessed by BrdU incorporation and TUNEL staining according to the manufacture’s instructions (Invitrogen, Carlsbad, CA). A minimum of three animals were assessed per genotype per treatment, with five to eight fields per section and a minimum of 300 epithelial cells assessed per slide. The percentage of crypt epithelial cells that labeled positive was calculated.

**Primary colonic cell isolation and immunocytochemistry.** Colon epithelial cells and lamina propria macrophages were isolated based on standard protocols (49). Briefly, colons were cut into small pieces and placed in calcium and magnesium free HBSS containing 5 mM EDTA and 1 mM DTT, shaken vigorously at 37°C for 30 min, followed by filtration through a 70 μm cell strainer. The filtrate was centrifuged and the supernatant was removed. The pellet was washed and the resulting epithelial cells were used for immunofluorescence and RNA isolations. For isolating lamina propria macrophages, colon fragments remaining on the 70 μm strainer were collected and digested with collagenase (Roche Diagnostics, Indianapolis, IN) and DNAse I (Sigma, St. Louis, MO) for 30 min. The solution was filtered, centrifuged, and the pellet resuspended in a 40% fraction of Percoll solution (GE Healthcare Biosciences, Piscataway, NJ), which was overlaid on an 80% Percoll solution. The Percoll solution was centrifuged and the cells at the interphase were removed. Colon macrophages were enriched from the immune cell fraction by magnetic beads coated with mouse CD11b according to manufacturer's instructions (Miltenyi Biotech, Auburn, CA). The resulting population was used for
immunofluorescence and RNA isolations. Immunocytochemistry on isolated primary cells was performed as described (43) with antibodies against E-cadherin (BD Biosciences, San Jose, CA), F4/80 and Ron. The isolated epithelial cells and macrophages were 90%-95% pure based on E-cadherin and F4/80 immunostaining respectively.

Isolation of bone marrow derived macrophages (BMDMs). Bone marrow cells were isolated, cultured and differentiated into macrophages using standard protocols (44). Briefly, femurs and tibia were obtained from 10-12 week old C57BL/6 wild type (TK+/+) and TK-/- mice. Bones epiphyses were removed and the bones were flushed with DMEM to extrude bone marrow. The marrow was passed through a 40μm filter, and the cells were plated in cell culture dishes. Cells were differentiated into BMDMs using CMG-14-12 cell conditioned medium for 3 days. To elucidate the changes in chemokine/cytokine secretion, equal numbers of BMDMs from TK+/+ and TK-/- mice were plated and treated with 500 μg/mL lipopolysaccharide (E. coli serotype 0111:B4; Sigma, St. Louis, MO) for 1 hour for RNA isolation or for the indicated time points for the collection of cell supernatants for cytokine measurements. IL-6 and IL-10 levels were measured by ELISA analyses according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

RNA isolation and quantitative real time (qRT-)PCR: Total RNA was isolated from colon tissues and cells using TriZol reagent (Invitrogen) and converted to cDNA using a high capacity cDNA kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed using FastStart SYBR Green. The following murine genes and their corresponding primers were chosen: IL-6 (5'-TAGTCCCTTCCTACCCCAATTTCC-3'; 5'-TTGGTCCTTAGCCACTCCTTC-3'), Ron (5'-TCCCATTGCAGGTCTGTGTAGA-3'; 5'-CGGAAGCTGTATCGTTGATGTC-3'), TNF-α (5'-CATCTTCTCAAAAATTCTGAGTAGACA-3'; 5'-TGGGAGTAGACAAAGGTACACCC-3'), IL-17A (5'-TTAAACTCCCTTGGCGCAAAA-3'; 5'-CTTCCCCTCCGCATTGACAC-3'), iNOS (5'-
TGCCCCTTAATGTTGGTA-3';  5'-ACTGGAGGGACCAGCCAAAT-3'), IL-10 (5'-
GGTGCCAAGCCTATCGGA-3';  5'-ACCTGCTCCACTGCTTCTG-3'), HGFL (5'-
TGTTACAGTTCTCAAGGCTCTTT-3';  5'-GCATGGCTGGCCTGCTA-3') and 18S (5'-
AGTCCCTGCCCCCTTGACTACA-3';  5'-GATCGAGGGCCTCACTAAAC-3'). Expression levels
were normalized to 18S as internal control and relative gene expression reported.

Western blotting: Protein was isolated from tissues as described previously (43), separated by SDS-
PAGE and transferred to Immobilon-P membranes (BD Biosciences). Membranes were probed with α-6
integrin, β-4 integrin (Santa Cruz Biotechnology, Dallas, TX), laminin (AbCam, Cambridge, MA), p-
STAT3, total STAT3 (Cell Signaling Technologies) and c4-actin (Seven Hills Bioreagents, Cincinnati,
OH). Densitometry was used to quantitate protein band intensities with Image J software (NIH,
Bethesda, MD).

Myeloperoxidase (MPO) activity. Frozen colons were homogenized in phosphate buffered saline and
centrifuged at 20,000g. MPO activity was performed as previously described (21).

Cytokine measurements. Sera isolated from mice were analyzed using the Milliplex Map Mouse
Cytokine/Chemokine Panel #MPXMCYTO-70 (Millipore, Billerica, MA) with Luminex Map detection
and BD cytometric bead array (552364; BD Biosciences) as per manufacturer's instructions.

Statistical Analysis. All data graphs are expressed as mean ± standard error (SE). Statistical significance
was defined as P<0.05 for all analyses and was determined by Student’s t-tests and Kaplan-Meier
survival analyses using GraphPad Prism 4.0 software (La Jolla, CA).
RESULTS

Ron and HGFL are expressed in the normal colon and in colitis. Quantitative data comparing the expression of Ron and HGFL in cell types of normal colon are lacking. To examine Ron and HGFL expression, populations of murine colon epithelial cells and resident macrophages were collected from wild type (TK+/+) mice (49). Ron mRNA expression was detected in isolated epithelial cells and resident macrophages (Fig. 1A). qRT-PCR analyses on colons from mice before and after DSS-induced chronic colitis showed an increase in Ron mRNA following DSS treatment (Fig. 1B). Immunohistochemical (IHC) analyses of normal and DSS-treated colons demonstrated Ron expression on epithelial and stromal cells (Fig. 1C). To examine the expression of HGFL, qRT-PCR on isolated colon epithelial cells and macrophages was performed and showed HGFL expression in both cell types (Fig. 1D). HGFL expression was also present and slightly elevated in the colon following DSS-induced colitis (Fig. 1E). IHC on murine colons showed HGFL expression in crypt epithelial and goblet cells as well as in stromal cells (Fig. 1F), which was maintained following DSS treatment (Fig. 1F). In total, these data demonstrate that Ron and HGFL are expressed locally in multiple cell types of the murine colon and are present during DSS-induced colonic injury.

Loss of Ron receptor signaling hastens colitis following DSS treatment in mice. To investigate the functional role of Ron in colitis, TK+/+ mice and mice lacking the tyrosine kinase signaling domain of Ron (TK-/- mice) were subjected to cyclic DSS-induced colitis. During the course of treatment, the TK-/- mice exhibited decreased survival primarily during the first cycle of DSS-treatment compared to TK+/+ mice (Fig. 2A). In addition, while the surviving mice from both genotypes showed similar weight loss through two cycle of DSS treatment, the weight of TK-/- mice was significantly less than that of TK+/+ mice after the third cycle of treatment (Fig. 2B). The failure to gain weight during the recovery period after DSS treatment was concomitant with TK-/- mice displaying elevated clinical scores (Fig. 2C). At necropsy, colon lengths were measured as a readout for the severity of colitis and
while DSS treatment shortened colons in both genotypes, TK-/- colon lengths were significantly shorter than TK+/+ colons (Fig. 2D). While colonic histology of TK+/+ and TK-/- colons prior to colitis did not differ (data not shown), colons of TK-/- exhibited a significant increase in injury compared to TK+/+ colons following DSS treatment characterized by epithelial cell loss, ulceration and immune cell infiltration (Fig. 2E and F). To examine the extent of epithelial damage in the TK-/- colons, colons from TK+/+ and TK-/- mice were analyzed for differences in proliferation and death by IHC for BrdU incorporation and TUNEL staining respectively. Although no differences in TUNEL staining were observed in the colonic epithelial cells from both genotypes (data not shown), TK-/- colons exhibited significantly less epithelial cell proliferation compared to TK+/+ epithelial cells after DSS-treatment (Fig. 2G). Taken together, these data suggest that Ron receptor signaling promotes epithelial cell healing during chronic colitis and that loss of Ron leads to increased lethality and a more severe colitis phenotype compared to mice with intact Ron.

Cytokine changes in TK-/- colons after chronic colitis. IBD studies in both humans and mice have shown that cytokine production plays a critical role in mediating disease pathogenesis (11, 53). While no differences were observed in resting cytokine levels in sera of TK-/- and TK+/+ mice (data not shown), following cyclic DSS treatment, TK-/- sera exhibited decreased levels of anti-inflammatory cytokines (IL-10 and IL-4) and increased levels of the proinflammatory cytokine, IL-6, compared to control mice (Fig. 3A). qRT-PCR analyses on murine colons after DSS treatment showed that the TK-/- colons had significantly higher IL-6, TNF-α, IL-17A and iNOS mRNA and lower IL-10 mRNA compared to TK+/+ colons (Fig. 3B). To determine whether changes in cytokines were a result of differential macrophage recruitment or activation, macrophage infiltration was assessed by F4/80 IHC. Interestingly, TK-/- colons contained fewer macrophages after chronic colitis compared to TK+/+ colons (Fig. 3C). Colonic neutrophil activity was assessed by myeloperoxidase activity and while increased following colitis, no changes were apparent between genotypes (Fig. 3D). To examine functional differences in macrophages
and since inflammatory cells of the gut are recruited from the bone marrow (54), bone marrow derived macrophages (BMDM) from TK+/+ and TK-/- mice were isolated and examined ex vivo. Western analyses on BMDM from TK+/+ mice showed that these macrophages express Ron (Fig. 3E). BMDM from each genotype were stimulated with bacterial lipopolysaccharide (LPS) and examined by qRT-PCR. TK-/- BMDM expressed significantly more mRNA for the proinflammatory cytokines TNF-α, IL-6 as well as iNOS mRNA compared to TK+/+ BMDM (Fig. 3F). Expression was similar to that observed in colon tissues of DSS treated mice. No differences in the expression of these cytokines were observed in TK-/- and TK+/+ BMDM prior to LPS treatment (data not shown). Consistent with mRNA expression and serum cytokine levels, TK-/- BMDMs produced significantly higher levels of IL-6 (Fig. 3G) and lower levels of IL-10 (Fig. 3H) following LPS stimulation compared to TK+/+ BMDMs. These data show that Ron signaling affects the balance of pro and anti-inflammatory cytokines during colitis.

Ron loss alters the activation of downstream signaling pathways in the epithelium and stroma after chronic colitis. Activation of the NF-κB and STAT signaling pathways are critical components in IBD. Upregulation of NF-κB activity has been shown to promote epithelial barrier dysfunction and inflammation (7). Activation of STAT1 has been shown to worsen colitis in experimental mice (52), while STAT3 activation is important for efficient regeneration of the epithelium and in diminishing inflammation in experimental colitis (26, 50). To determine the effect of Ron loss on the NF-κB and STAT signaling pathways, IHC analysis for the activation of IKKα/β, an upstream marker for NF-κB activation, and STAT1 phosphorylation was performed on colon sections from DSS-treated TK+/+ and TK-/- mice. TK-/- mice displayed increased nuclear STAT1 phosphorylation in colonic epithelial cells compared to TK+/+ mice (Fig. 4A and B). In addition, significantly more STAT1 and IKKα/β phosphorylation was observed in the stroma of the TK-/- colons compared to that of TK+/+ colons (Fig. 4A-D). Western analyses of colons from DSS-treated TK+/+ and TK-/- mice showed a decrease in
STAT3 phosphorylation in the TK-/- compared to TK+/+ colons (Fig. 4E and F). These experiments suggest an important role for Ron in regulating the balance of critical signaling pathways needed to support the resolution of colitis in vivo.

TK-/- mice have reduced expression of cell-matrix interaction molecules after chronic colitis. Cell adhesion molecules and cell-matrix interaction molecules play a vital role in maintaining biological activities of the intestinal epithelium including cell cycle progression as well as barrier function and are required for healing after injury (4). Western blot analyses and IHC were used to examine the expression of various cell-matrix interaction molecules in colons from TK+/+ and TK-/- mice after DSS treatment. Western analyses showed an increase in α-6 integrin, β-4 integrin and laminin expression in TK+/+ colons in the recovery phase following DSS-induced colitis compared to colons from untreated mice. In contrast, this increase was not observed in DSS-treated TK-/- colons (Fig. 5A and B). IHC analyses on colons from TK+/+ and TK-/- mice after chronic colitis showed that the TK-/- colons expressed less laminin and plectin compared to TK+/+ colons (Fig. 5C). These experiments suggest that Ron signaling plays a role in the expression of cell-matrix interacting proteins that are required for healing of colon after injury in colitis.

Ron signaling is diminished in human IBD patients. To examine the expression of Ron and HGFL in settings of IBD, GEO datasets were examined. Analyses of the GEO/GSE9686 database, which includes pediatric patient biopsies with active IBD (6), showed diminished Ron and HGFL in patients with ulcerative colitis (Fig. 6A). Analyses of the GEO/GSE11223 which includes biopsies of adult patients with active as well as quiescent IBD (31), showed diminished expression of HGFL in the colons of ulcerative colitis patients with and without associated inflammation, compared to control colon samples (Fig 6B). These data provide additional support that diminished HGFL-Ron signaling is associated with IBD and provide further evidence on colonic HGFL expression.
DISCUSSION

In the present study, we provide the first direct in vivo evidence for a functional role for the Ron receptor tyrosine kinase in colitis. Similar to previous findings, Ron expression was observed in normal and colitis-affected colons as well as in resident colonic macrophages (15, 18). Further, we have established HGFL expression in the colonic epithelium and macrophages. To our knowledge, this is the first report to document HGFL expression in the colon, although the significance of this localized HGFL production is not known. This novel finding is corroborated by human patient data documenting diminished HGFL and Ron mRNA expression in colon tissue of patients with ulcerative colitis (Fig 6). Combined, these data suggest a possible requirement for local HGFL-mediated Ron activation in the colon for the resolution of intestinal injury. Using mice lacking Ron signaling (48), we demonstrated an exacerbated colitis phenotype after cyclic DSS treatment. We show that Ron loss leads to a decrease in ability of the colonic epithelium to proliferate and heal and is associated with an increased inflammatory environment following injury. These studies suggest that loss or blunting of this signaling cascade, consistent with the proposed role of the SNPs identified in Ron and HGFL through genome-wide association studies, may contribute to the pathogenesis of IBD (5, 13, 27).

Colonic epithelial cell turnover is increased in patients with UC and is required for replenishing the lost epithelium following injury (8). Ron signaling has been shown to promote wound healing by stimulating epithelial cell migration, proliferation and survival at the wound site (25, 36). The decreased epithelial cell proliferation and increased ulceration observed in TK-/- colons following colitis suggest that Ron signaling is required for the proliferation of colon epithelium contributing to the resolution of injury in IBD. Further, as the epithelial barrier remains lost or ulcerated in TK-/- mice, there may be continued interaction between colon immune cells and microbial flora, leading to increased inflammation. Interestingly, studies examining the 689C SNP in HGFL showed that this alteration did not alter binding to Ron but lowered HGFL concentration in the serum of human IBD patients (18). It is interesting to
speculate that the local colonic production of HGFL is also reduced in 689C expressing patients and may decrease the extent of Ron activation in the colon and lessen proliferation and migration needed for healing.

Experimental colitis induced by DSS treatment is characterized by cytokine changes (9). In the TK-/- mice, significant increases were observed in TNF-α, iNOS and IL-6 expression while decreases were found in IL-4 and IL-10 compared to TK+/+ mice (Fig. 3) following DSS treatment. Consistent with this in vivo data, ex vivo LPS treatment of TK-/- bone marrow derived macrophages (BMDMs) led to significant increases in TNF-α, iNOS and IL-6 expression compared to TK+/+ cells. These studies are in accord with numerous publications citing the macrophage-specific regulation of cytokines and iNOS expression by Ron in vivo and in vitro (23, 28, 29, 34, 43). Macrophages play a vital role in inflammation and their activation status determines the repertoire of proinflammatory or anti-inflammatory cytokine production. M1 activated macrophages produce proinflammatory mediators and can worsen the pathology of colitis while M2 or alternately activated macrophages promote angiogenesis, tissue remodeling, and wound healing in chronic inflammatory conditions such as colitis (42). Data in TK-/- BMDM and in TK-/- mice subjected to DSS support prior studies showing Ron signaling as an important determinate in mediating an M2 phenotype with Ron loss associating with the polarization of macrophages to a M1 phenotype (40, 41, 48). Thus, during IBD, Ron may control the polarization status of macrophages during injury with Ron loss expected to contribute to a more severe colitis phenotype. A detailed characterization of the role of Ron in macrophage polarization during colitis is warranted and may suggest opportunities to intervene in IBD pathogenesis through Ron immune cell modulation.

NF-κB is expressed on colon epithelial cells and macrophages in normal and IBD colons in humans and mice. The amount of activated NF-κB correlates significantly with the severity of inflammation in
colitis and upregulation of NF-κB activity promotes epithelial barrier dysfunction (1-3, 7, 39). Further, myeloid but not epithelial NF-κB activity has been shown to promote inflammation in a murine model of chronic colitis with deletion of IKK-β, a molecule upstream of NF-κB activation, in myeloid cells diminishing colon inflammation (10). Based on our previous findings that Ron signaling in macrophages suppresses NF-κB activity (43), we postulate that Ron loss in colon macrophages leads to increased NF-κB activity and ensuing proinflammatory cytokine secretion in TK-/- mice. STAT transcription factors play a role in colitis and the expression of STAT1 and STAT3 increases in human and murine colitis (24). The balance of STAT1 and STAT3, especially increased STAT3 over STAT1, controls cell survival, inflammation and drives healing responses in various clinical settings (35, 37). STAT1 activation has been shown to worsen colitis in mice (52), while STAT3 activation aids in the regeneration of epithelium and diminish inflammation in models of experimental colitis (26, 50). In TK-/- colons, decreases in pSTAT3 and increases in STAT1 were observed after DSS treatment compared to TK+/+ colons (Fig. 4). Ron has been shown to activate STAT3 and our data suggests that Ron-mediated STAT3 activation in TK+/+ colons promotes healing following colitis (14). Ron receptor activation has been shown to induce the activation of a number of downstream signaling cascades including MAPK, AKT, PI3K and β-catenin (47). The role of these signaling molecules as mediators of Ron activation in colitis remains to be determined.

Cell-matrix interactions are important to maintain the normal barrier function of the colon epithelium and for renewal of epithelial cells in the normal colon and after injury. Analyses of patient biopsies have shown that expression of laminin in the colon extracellular matrix (ECM) is diminished in active UC and is correlated with clinical severity (38), an observation similar to DSS-induced colitis in TK-/- mice. The decrease in laminin is hypothesized to affect epithelial cell migration, differentiation, and regeneration during colitis leading to more severe disease (38). α-6 and β-4 integrins are expressed on
colon epithelial cells. α6β4 integrin together with plectin are involved in hemidesmosome formation, structures that anchor cells to the ECM by interacting with laminin (4, 45). In a wound-healing model of colon epithelial cells, hemidesmosome formation is the first step, followed by lamellipodia formation, in which the α6β4 integrin complexes relocalize from hemidesmosomes to help cells migrate into denuded regions (20). Ron activation by HGFL has been shown to disrupt plectin-α6β4 integrin interactions in hemidesmosomes and relocalizes α6β4 integrin to lamellipodia promoting keratinocyte migration to promote wound healing (36). The higher amount of plectin and α6β4 integrin in Ron expressing colons may help facilitate healing following DSS-induced injury. Further studies are needed to elucidate the mechanisms associated with diminished expression of cell-matrix interacting proteins in TK-/- mice.

GEO database analyses showed diminished HGFL and Ron expression in human ulcerative colitis patients, which correlates well with our findings suggesting a need for local HGFL-mediated Ron receptor activation in the colon. Based on the data presented here and the established role for Ron in inflammation and wound healing, our data suggest a model wherein Ron expression in the colon epithelium promotes proliferation, and modulates cell-matrix interactions required for epithelial cell migration. Moreover, in macrophages, the Ron receptor suppresses key proinflammatory cytokine secretion by diminishing NF-κB activation during colitis. The net effect of Ron signaling allows for the resolution of colitis and alterations that inhibit or block Ron action, such as SNPs which effect Ron activity, may have deleterious effects. Taken together, these studies have identified a critical role for the Ron receptor tyrosine kinase in chronic colitis, although the cell type specific roles for this receptor remain to be fully characterized. Further investigation of the Ron signaling pathway is warranted and may be an important new target for patients with IBD.

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**FIGURE LEGENDS**

**Fig. 1. Ron and HGFL are expressed in the normal colon and during DSS-induced colitis.** qRT-PCR showing Ron mRNA expression in intestinal epithelium cells and in resident colonic macrophages from the normal colon (A). Kupffer cells, resident liver macrophages, are used as a positive control. qRT-PCR of whole colon tissue demonstrates Ron expression is increased in colons subjected to DSS-induced colitis (B). IHC depicting Ron expression in normal murine colons and in colons subjected to DSS (C). HGFL mRNA is expressed in normal murine colon epithelial cells and macrophages and is increased during experimental colitis by qRT-PCR (D & E). HGFL protein is detected by IHC analysis in normal murine colons and in colons subjected to DSS-induced colitis (F). Histograms represent the mean ± SE of at least 3 isolations/mice per group, *P<0.05.

**Fig. 2. Ron loss heightens the severity of colitis following chronic DSS treatment.** Survival curve of TK-/- and TK+/+ mice during chronic colitis is depicted (A). Temporal weight loss in mice following induction of chronic colitis is shown (B). TK-/- mice displayed higher gross clinical pathologic scores in the recovery phase after 3 cycles of DSS treatment compared to TK+/+ mice (C). TK-/- colons exhibited more severe colitis and shortening of the colon (D). Representative colon sections from TK+/+ and TK-/- mice were analyzed by hematoxylin and eosin staining for colitis severity (E) with TK-/- colons exhibiting elevated histopathology scores (F). Arrows denote regions of ulceration. TK-/- colonic crypts had diminished epithelial cell proliferation following chronic colitis assessed by BrdU incorporation (G). n= 5-24 mice per group,* P<0.05, Scale bar=50μm.

**Fig. 3. Ron loss leads to altered cytokine expression and immune cell recruitment following the induction of chronic colitis.** Analyses of sera from TK+/+ and TK-/- mice after DSS-induced chronic colitis over controls (A). qRT-PCR analyses of whole colons from TK+/+ and TK-/- mice after DSS-induced chronic colitis for TNF-α, IL-17A, iNOS, IL-6 and IL-10 mRNA (B). Macrophage counts,
based on F4/80 IHC of colons from control and DSS-treated mice (C). Myeloperoxidase (MPO) assays revealed no significant difference in neutrophil activity between TK+/-+ and TK-/- colons after DSS-induced chronic colitis (D). Western blot analyses for Ron expression in bone marrow derived macrophages (BMDM) from TK+/-+ mice. R7 cells [a murine breast cancer cell line (46)] are used as positive control (E). qRT-PCR analyses of LPS-treated BMDM from TK+/-+ and TK-/- mice for TNF-α, IL-6 and iNOS mRNA (F). Cell culture supernatants of BMDMs from TK++ and TK-/- mice treated with LPS for the indicated times were examined for IL-6 (G) and IL-10 (H) production by ELISA. Data represent the mean ± SE of n=3-6 per group, *P<0.05.

Fig. 4. Signaling differences between TK+/+- and TK-/- colons after the induction of chronic colitis. IHC analyses for phosphorylated (p)-STAT1 and p-IKKα/β (A) in colons of TK+/-+ and TK-/- mice after DSS-induced colitis. Quantification of pSTAT1 positive cells in the colon epithelium (EP) (B) shows more pSTAT1 positive cells in TK-/- mice. Quantification of p-STAT1 (C) and p-IKKα/β (D) cells in the colonic stroma (STR) shows more positive cells in TK-/- colons after DSS treatment. Western analyses of colons from TK+/-+ and TK-/- mice before (Control) and after DSS treatments show more pSTAT3 in TK+/-+ mice after colitis (E). Densitometry analysis for p-STAT3 expression normalized to total STAT3 is shown (F). *P<0.05, scale bar=50μm. Images are representative of n=5-6 mice per group with histograms representing the mean ± SE of n=5-6 per group, *P<0.05.

Fig. 5. Ron mediated changes in cell-matrix molecules following the induction of colitis. Western analyses of colon tissue from TK+/-+ and TK-/- mice before (Ctrl) and after DSS-induced chronic colitis showing changes in the expression of various cell matrix interacting molecules (A). Each lane represents an independent colon sample. Densitometry analyses normalized to β-actin are shown (B). IHC analyses on colons for laminin and plectin (C) showing diminished expression of these molecules in
colons of TK/- mice following the induction of chronic colitis. Scale bar=50μm. Images are representative of n=5-6 mice per group with histograms representing the mean ± SE of n=5-6 per group. *P<0.05.

**Fig. 6. Colonic Ron and HGFL mRNA expression is reduced in patients with ulcerative colitis.**

Analyses of GEO dataset GSE9686 shows significantly lower expression of Ron and HGFL mRNA in colons from pediatric ulcerative colitis (UC) patients with active disease compared to controls (A). Analyses of GEO/GSE11223 dataset documents significantly lower expression of HGFL mRNA in colons from adult UC patients with uninflamed (uninfl) and inflamed (infl) colons compared to controls (B).