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IFN-γ-mediated induction of an apical IL-10 receptor on polarized intestinal epithelia

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Abstract

Cytokines secreted at sites of inflammation impact the onset, progression and resolution of inflammation. Here we investigated potential pro-resolving mechanisms of IFN-γ in models of inflammatory bowel disease (IBD). Guided by initial microarray analysis, *in vitro* studies revealed that IFN-γ selectively induced the expression of IL-10R1 on intestinal epithelia. Further analysis revealed that IL-10R1 was expressed predominantly on the apical membrane of polarized epithelial cells. Receptor activation functionally induced canonical IL-10 target gene expression in epithelia, concomitant with enhanced barrier restitution. Furthermore, knockdown of IL-10R1 in intestinal epithelial cells results in impaired barrier function *in vitro*. Colonic tissue isolated from murine colitis revealed that levels of IL-10R1 and SOCS3 were increased in the epithelium and coincided with increased tissue IFN-γ and IL-10 cytokines. In parallel, studies showed that treatment of mice with rIFN-γ was sufficient to drive expression of IL-10R1 in the colonic epithelium. Studies of DSS colitis in intestinal epithelial-specific IL-10R1-null mice revealed a remarkable increase in disease susceptibility associated with increased intestinal permeability. Together, these results provide novel insight into the crucial and underappreciated role of epithelial IL-10 signaling in the maintenance and restitution of epithelial barrier and of the temporal regulation of these pathways by IFN-γ.

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Introduction

The inflammatory bowel diseases (IBD), including both Crohn's disease and ulcerative colitis, are debilitating disorders of unknown etiology (1). Recent evidence suggests IBD results from an inappropriately directed inflammatory response to the intestinal microbiota in a genetically susceptible host. Epithelial cells are crucial in the maintenance of colonic tissue homeostasis, as IBD is characterized by a breakdown of the intestinal epithelial barrier leading to increased exposure of the mucosal immune system to antigenic luminal material. Since the epithelium functions as the critical interface between the intestinal lumen and the sub-epithelial mucosa, they are thereby anatomically positioned as a central coordinator of mucosal inflammatory response.

Studies to date indicate that cytokines and chemokines that are produced locally at sites of inflammation play an important role in onset and progression of IBD. Interferon (IFN)-γ is a signature Th1 cytokine and has been shown to have a pro-inflammatory role in a number of autoimmune and inflammatory diseases including IBD (1). There is mounting evidence, however, that IFN-γ also displays inflammation mitigating properties and has been shown to be protective in a number of disease models (2). More recently it was shown that lack of IFN-γ exacerbates disease in a genetic mouse model of IBD, suggesting a protective role for IFN-γ in modeled intestinal inflammation (3). Indeed, IFN-γ induces IDO1 expression, which has been shown to play a protective role in IBD models (4). Additionally, our lab has demonstrated that IFN-γ upregulates cellular methylation pathways that play a protective role in intestinal epithelium in a murine colitis model (5). Therefore, recent evidence suggests that IFN-γ plays a complicated part in the etiology of IBD, displaying both protective and pathogenic properties.

Another cytokine that has been demonstrated to have a crucial role in IBD is interleukin-10 (IL-10). IL-10 is an anti-inflammatory cytokine that acts to limit inflammation by inhibiting the secretion of pro-inflammatory cytokines such tumor necrosis factor (TNF)-α and IFN-γ (6). IL-10 is produced by a number of cell types, including intestinal epithelial cells, and is known to exert effects on lymphocytes, monocytes and polymorphonuclear cells (PMNs) (6). The IL-10 receptor (IL-10R) is comprised of two alpha subunits (IL-10R1, IL-10RA), the IL-10-specific ligand-binding portion, and two beta subunits (IL-10R2, IL-10RB), which are shared with other IL-10 receptor family members (7). Expression of IL-10R has been identified on multiple cell types, including intestinal epithelial cells (8, 9). Knockout mice deficient in IL-10 or IL-10R develop spontaneous severe colitis (10-13). Similarly, mutations in both IL-10 and the IL-10R have been implicated as causative factors in human IBD (14-16). Despite these findings, systemic treatment with recombinant IL-10 has been found to provide no benefit to IBD patients in randomized human trials (17-19).

Based upon our previous findings (5), we hypothesized that IFN-γ may activate additional pathways promoting inflammation resolution and tissue restitution in the intestinal epithelium. To define these principles, we utilized microarray data dovetailed with qPCR analysis of an *in vitro* model of mucosal inflammation. We found that IFN-γ induces expression of the ligand-binding subunit of the IL-10R, IL-10R1. We further characterized the localization of IL-10R1 expression and demonstrate that the receptor is apically

displayed on intestinal epithelial cells. We confirmed these findings utilizing immunohistochemistry in both mouse and human tissue. Additionally, we demonstrate that loss of epithelial IL-10R1 significantly worsens disease in a mouse colitis model and impairs epithelial barrier function both *in vitro* and *in vivo*. We propose that IFN-γ temporally coordinates the progression of inflammation and primes the tissue for pro-resolving IL-10 signaling.

Materials and Methods

Cell culture

Human intestinal epithelial cells (T84) epithelial cells were grown and maintained in T175 cell culture flasks (Costar Corp., Cambridge MA) in DMEM/F12 (Ham) media (Gibco, Grand Island, NY) as described previously (20). Where indicated, cells were cultured on polyester transwell inserts (Costar Corp., Cambridge MA). Transepithelial resistances (TEER) were monitored using an EVOM voltohmmeter (World Precision Instruments). All cytokines were purchased from R&D Systems (Minneapolis, MN) and used at indicated concentrations.

Epithelial macromolecule permeability assay

Epithelial paracellular permeability was assessed exactly as described previously (21) using T84 cells grown on polycarbonate permeable inserts (0.4 μm pore, 6.5 mm diameter; Costar, Cambridge, MA). Permeability of FITC-labeled dextran (3 kDa, Molecular Probes, Eugene, OR) was assessed on washed monolayers (HBSS) by sampling serosal fluid. Fluorescence intensity of each sample was measured (excitation, 485 nm; emission, 530 nm) (Cytofluor 2300, Millipore, Bedford, MA), and FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran. Paracellular flux rates were calculated by linear regression.

Western Blot Analysis

Cells or colonic tissues were harvested and processed as described previously (5). Proteins were separated by SDS-PAGE electrophoresis and transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA) for immunoblotting. Antibodies utilized for this study were: anti-IL-10R1 (1:1000, Novus), anti-pSTAT3 (1:2000, Cell Signaling Technologies), anti-integrin b1 (1:1000, Abcam), anti-ALPI (1:1000, Novus) and anti-β–actin (1:10000, Abcam). Cell surface biotinylation to examine proteins associated with the apical and/or basolateral membrane were performed as previously described (22).

RNA isolation and transcriptional analysis

RNA was isolated and processed for real-time PCR as described previously. Analysis of IL-10R1 in human patient samples was performed using the OriGene Crohn's/Colitis cDNA tissue array I (OriGene Technologies, Rockville, MD). All qPCR reactions were performed using Power SYBR Green Master Mix and analyzed using 7900HT Fast Real-Time PCR System (Applied Biosystems). The following primers were used to quantify expression in intestinal epithelial cells: IL10-R1: forward 5'-CCCTGTCCTATGACCTTACCG-3' and reverse 5'-CACACTGCCAACTGTCAGAGT-3'; IL-10R2: forward 5’-

TCTCCTTTCCATTGTCGGATGA-3’ and reverse 5’-TCACCAGGATTCTGCTTGCC-3’; SOCS3: forward 5'-GGCCACTCTTCAGCATCTC-3' and reverse 5'-ATCGTACTGGTCCAGGAACTC-3'; IL-10: forward 5’-AATAAGGTTTCTCAAGGG-3’ and reverse 5’-AGAACCAAGACCCAGACA-3’; TBF- 1; forward 5'-GCGTGCTAATGGTGGAAAC-3' and reverse 5'-CGGTGACATCAAAAGATAACCAC-3' ; β-actin: forward 5’-GCACTCTTCCAGCCTTCCTTCC-3’ and reverse 5’-CAGGTCTTTGCGGATGTCCACG-3’. For mouse experiments primers were: IL-10R1: forward 5’-CCCATTCCTCGTCACGATCTC-3’ and reverse 5’-TCAGACTGGTTTGGGATAGGTTT-3’; SOCS3: forward 5’-ATGGTCACCCACAGCAAGTTT-3’ and reverse 5’-TCCAGTAGAATCCGCTCTCCT-3’ ; β-actin: forward 5’-TACGGATGTCAACGTCACAC-3’ and reverse 5’-AAGAGCTATGAGCTGCCTGA-3’.

Mice

Wild-type (WT) C57/B6 and *Villin*^{cre} mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Il10r1*^{fl} mice have been previously described (10, 12). Colitis was induced on day 0 by the addition of 3% DSS (MW = 36,000-50,000, MP Biomedicals, Illkirch, France) solution in drinking water (23). Control animals received water alone. For IFN-γ treatment experiments, mouse recombinant IFN-γ (R&D Systems) was injected intra-peritoneally (IP) at 5 g/mouse/day. Mice were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols, and experiments were approved by the Institutional Animal Care and Use Committee of the University of Colorado.

Histology

Samples were fixed in 10% formalin before staining with H&E. All histological quantitation was performed blinded by the same individual using a scoring system previously described (24). The three independent parameters measured were severityof inflammation (0– 3: none, slight, moderate, severe), extent of injury (0–3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0–4: none, basal 1/3 damaged, basal 2/3 damaged, only surface epithelium intact, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (x1: 0–25%, x2: 26–50%, x3: 51–75%, x4: 76–100%) and all numbers were summed. Maximum possible score was 40. All histological examinations were performed by a trained pathologist.

Intestinal permeability in vivo

Intestinal permeability was examined using a FITC-labeled-dextran method, as described previously (25, 26). Briefly, mice were gavaged with 0.6 mg/g body weight of FITC-dextran (mol wt 4,000 kDa, at a concentration of 80 mg/ml; Sigma-Aldrich) and left undisturbed for 4 hours (n = 4–6 per condition). At the time of animal harvest, cardiac puncture was performed and serum analysis of FITC concentration performed.

Quantification of cytokines in murine colonic tissue and serum

For cytokine analysis, colonic tissue was extracted in Tris lysis buffer by sonication and protein homogenates were stored at -80°C until use. Serum was prepared from whole blood using microvette tubes (Sarstedt) and stored at -80°C until use. Tissue and serum concentrations of cytokines were measured using a pro-inflammatory cytokine screen (Meso Scale Discovery) as described previously (27). Briefly, these arrays incorporate electrochemiluminescence on patterned arrays. For these experiments we utilized the Mouse ProInflammatory 7-Plex Ultra-Sensitive Kit. Assays were performed per manufacturer's instructions. Cytokine concentrations were normalized to total protein concentration.

Confocal Microscopy

T84 human epithelial cells were plated, fixed and processed for confocal microscopy as described elsewhere(5). Cells were localized with anti-IL-10R1 followed by AlexaFluor 488 secondary Ab and counter-stained with AlexaFluor 546 phalloidin (Invitrogen). Fluorescence images were obtained using Zeiss Axiovert 200M laser-scanning confocal/ multiphoton-excitation fluorescence microscope with a Meta spectral detection system (Zeiss NLO 510 with META, Zeiss, Thornwood, NY).

Immunohistochemistry

Mouse tissue was fixed in 4% PFA, followed by passing the tissue through sucrose gradients. Tissues were then embedded in OCT and frozen tissue sections were prepared and analysed as previously described (28). Blank sections for human tissue were cut from formalin fixed, paraffin embedded samples. Sections from archived human tissue from patients definitively diagnosed with IBD or from screening colonoscopy were obtained under research protocols approved by the Colorado Multi-Institutional Review Board. Sections were stained with anti-IL-10R1 Ab and anti-ZO-1 Ab (Invitrogen) followed by incubation with AlexaFluor 555 and 488 secondary Ab, respectively. Sections were counterstained with DAPI (Invitrogen).

Statistics

Unpaired T-test and/or analysis of variance (ANOVA) test were used to determine differences between groups, as indicated, where $p<0.05$ was considered significant.

Results

IFN-γ selectively induces IL-10R1 on intestinal epithelial cells

Recent studies from our lab have demonstrated that IFN-γ induces endogenously protective cellular methylation pathways in epithelial cells *in vitro* and *in vivo* (5). Further analysis of these studies implicated protective pathways beyond that of methylation, and therefore, we pursued the identity of additional anti-inflammatory targets. Guided by microarray analysis of cultured epithelial cells exposed to IFN-γ (5); accession no. GSE33880, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33880), we identified the somewhat surprising induction of the ligand-binding alpha-subunit of the IL-10R (IL-10R1) in epithelia. These microarray results were confirmed by qPCR and western blot analysis

demonstrating that, while untreated T84 cells express very low basal levels of IL-10R1, IFN-γ-exposed T84 cells demonstrate a time-dependent induction in IL-10R1 expression (Fig. 1A and 1B). IFN-γ treatment demonstrated no alteration in the expression of the beta subunit of IL-10R, IL-10R2 (Fig 1C), in support previously published of data showing constitutive expression of this subunit (6). Importantly, IL-10R1 induction was specific for IFN-γ. Indeed, exposure of T84 cells to a panel of cytokines including IL-1, IL-6, IL-8, TNF-α and IFN-β revealed no significant change in IL-10R1 expression following 24 hr exposure (Fig. 1D), suggesting at least some degree of specificity for IFN-γ.

We next sought to localize IL-10R expression in intestinal epithelial cells. To this end, T84 cells were grown to confluent, high resistance monolayers, treated with IFN-γ for 12 hours and IL-10R localization was determined using confocal microscopy. As shown in Fig. 1E, IL-10R was found to be localized almost exclusively to the apical membrane of T84 cells. We were unable to detect IL-10R1 staining associated with the basolateral membrane in these studies (Fig. 1E). These results were confirmed using cell surface biontinylation to examine proteins associated with the apical and/or basolateral membrane (22). T84 cells were grown to confluent, high resistance monolayers followed by treatment with IFN-γ for 12 hours and then assayed for IL-10R1 localization. These studies defined localization of IL-10R largely to the apical membrane (Fig. 1F). Such findings provide compelling evidence for the expression of IL-10 receptor expression in response to modeled inflammatory conditions.

IL-10R signaling activates protective mechanisms in intestinal epithelial cells

Having demonstrated IL-10R1 induction on the apical surface of epithelial cells, we next examined its functionality. IL-10 signaling has been shown to act predominantly through the activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) (6). STAT3 is phosphorylated and translocates to the nucleus to activate transcription of IL-10-responsive genes. However, pro-inflammatory cytokines such as IL-6 have also been shown to activate STAT3 (29, 30). Additionally, IFN-γ can activate STAT3 under certain circumstances (31). Therefore, we examined STAT3 activation in T84 cells in response to potential STAT3 activators. As shown in Figure 2A, while IL-10 alone did induce some pSTAT3, pre-treatment of T84 cells with IFN-γ for 24 hr followed by IL-10 exposure resulted in a nearly 6-fold increase in STAT3 phosphorylation relative to untreated controls. Treatment with IFN-γ alone or IL-6 did not significantly induce pSTAT3 in this model, indicating IL-10 to be a more potent STAT3 activator in this setting. We next examined the consequences of IL-10R1 up-regulation on an IL-10-responsive gene, suppressor of cytokine signaling 3 (SOCS3), which has been found to be protective in intestinal inflammation (32). SOCS3 expression was increased by less than 3-fold with IFN-γ, IL-6 or IL-10 exposure, in strong agreement with studies showing up-regulation of SOCS3 by these cytokines (33)(Fig 2B). However, pre-treatment of cells with IFN-γ followed by IL-10 exposure resulted in an 8±0.9-fold increase in transcript ($p<0.01$, Fig. 2B), strongly implicating a functional IL-10R complex in response to IFN-γ. We next examined the impact of increased IL-10 signaling on the expression of IL-10 and transforming growth factor β (TGF-β). These cytokines have been shown to promote epithelial barrier (34, 35). Following pre-treatment with IFN-γ, IL-10 induced a time-

dependent increase of both IL-10 ($p<0.05$) and TGF-β ($p<0.05$), with minimal changes evident without IFN-γ pre-treatment. Following these experiments, we determined the impact of IL-10 on IFN-γ induced barrier disruption in T84 cell monolayers. IFN-γ exposure has been shown disrupt epithelial barrier function (36), and for these purposes, T84 cells were grown to high resistance (TEER (transepithelial electrical resistance) >2000 $\Omega \cdot cm^2$). Cells were then treated with vehicle, IL-10, IFN-γ or to IFN-γ (36hr) followed by IL-10. As shown in Fig. 2C, IL-10 alone did not significantly influence barrier function compared to control ($p>0.05$). Cells treated with IFN-γ showed a progressive loss of barrier over 48 hr. IFN-γ treated cells replenished with media alone did not recover over the course of 72hr (TEER = 0.3175 ± 0.096 $\Omega \cdot cm^2$, Fig 2C). However, IFN-γ-treated cells exposed to media containing IL-10 exhibited significant restitution at 72hr (TEER = 1.33 ± 0.718 $\Omega \cdot cm^2$, Fig 2C) demonstrating that IL-10 signaling promotes restitution of epithelial barrier following IFN-γ-induced barrier disruption. Such findings identify a functional IL-10R complex on intestinal epithelial cells.

To begin to define the role of IL-10 signaling in IEC barrier formation we knocked down IL-10R1 expression in human IEC cell lines using a lentiviral transduction system. Figure 2D displays qPCR analysis demonstrating that the IL-10R1 knockdown cells express approximately 75% less IL-10R1 than either untransfected cells or non-targeting control (shNTC) cells. As shown in Fig. 2E knockdown of IL-10R1 resulted in significantly diminished capacity to form barrier in IEC compared to shNTC cells as measured by TEER over a 7-day period. To further examine barrier in these cells we utilized a FITC-dextran paracellular flux assay. For these experiments shNTC and IL-10R1 knockdown cells were left untreated or treated with IFN-γ for 48 hr prior to FITC-dextran analysis. As shown in Fig. 2F, IL-10R1 knockdown IEC exhibit altered paracellular flux. Importantly, the knockdown cells display significantly increased paracellular flux in the untreated state, corroborating the TEER experiments. Taken together, these results demonstrate an important role of epithelial IL-10 signaling in the maintenance and restitution of IEC barrier *in vitro*.

IL-10R1 is induced by IFN-γ in mouse models of colitis

After finding that IL-10R1 is induced in response to IFN-γ in epithelial cell models, we extended these findings to a mucosal inflammatory model, namely the dextran sodium sulfate (DSS) model of murine colitis. We began by investigating the temporal expression of IFN-γ and IL-10 in this model. For these purposes, mice were administered DSS (3%) for 5 days. Following this DSS was removed and mice were monitored for an additional 14 days. A cohort (n=3) of control and DSS-treated animals were sacrificed on days 2, 3, 6, 9 and 19 of the experiment. As shown in Fig. 3A, IFN-γ was detectable in colonic tissue beginning at day 3, peaking at day 6 and declining thereafter. IL-10 levels in the tissue were delayed compared to IFN-γ with levels being detectable at day 6, peaking at day 9 and then declining (Fig 3A). In parallel, we examined IL-10R1 expression in the tissue at day 6, as this represented the time point of most significant overlap between these cytokines. In both whole tissue and in isolated colonic epithelial cells, expression of IL-10R1 was significantly up-regulated on day 6, as was the IL-10 gene target, SOCS3 (Fig 3B). Examining IL-10R1 protein levels over the time course demonstrated increased protein levels at day 6 with

expression declining at day 9 (Fig 3C). Immunohistochemical localization of IL-10R1 expression in colonic tissue harvested at day 6 revealed a clear up-regulation of IL-10R1 in the diseased tissue relative to control (Fig 3D). Importantly, the vast majority of the IL-10R1 localized to the apical membrane in the epithelium (Fig 3D). Finally, to define the relative contribution of IFN-γ to induction of IL-10R1 *in vivo*, C57/B6 mice were administered rIFN-γ (5 μg/mouse) by IP injection daily and harvested on days 2 or 3. As shown in Fig. 3E, IL-10R1 gene expression was increased by day 2 ($p<0.05$) and further induced on day 3 ($p<0.01$). Examination of protein levels in isolated epithelial cells corroborated these results, showing an increase in IL-10R1 protein at day 2 and more evidently at day 3 of IFN-γ exposure. These data suggest that IFN-γ may act to "prime" the mucosal tissue for IL-10-mediated restitution of the epithelium in a mouse model of colitis.

Colitis is exacerbated in epithelial-specific Il-10r1-null mice

As an extension of our *in vitro* studies, we next initiated experiments in an epithelial-specific IL-10R1-knockout (KO) mouse model. To this end, we induced intestinal epithelial cell-specific ablation of IL-10R1 by breeding mice harboring a conditional allele encoding IL-10R1 (*Il10r1*^{fl}) (10, 12) with *Villin*^{cre} mice. The loss of IEC IL-10R1 expression was confirmed by genotyping analysis and qPCR of isolated epithelial cells (Supplemental Fig. 1). The *Vil*^{cre}/*Il-10r1*$^{fl/fl}$ (IEC Il10r1$^{-/-}$) mice appeared healthy and did not overtly demonstrate spontaneous onset of intestinal inflammation. However, administration of DSS to IEC Il10r1$^{-/-}$ animals resulted in significantly exacerbated colitic disease. Mice were administered DSS (3%) for 5 days, followed by DSS removal and a 3 day recovery. As shown in Fig. 4, these mice display significantly increased weight loss compared to DSS control animals colon shortening (Fig. 4A), significantly increased colon shortening (Fig. 4C), and more severe histological score (Fig. 4D). Importantly, the IEC Il10r1$^{-/-}$mice also demonstrate significantly higher baseline epithelial permeability (Fig. 4E) both in diseased and non-diseased animals compared to control mice. Finally, histological analysis revealed that IEC Il-10r1$^{-/-}$ water fed mice (Fig. 4B) appear very similar to control samples (Fig. 4B). Wild-type DSS mice demonstrated inflammatory infiltration, epithelial damage and partial loss of normal architecture consistent with this model (Fig 4B). However, the IEC Il-10r1$^{-/-}$ mice display an almost complete loss of epithelium and tissue architecture (Fig. 4B), implicating a significant role for epithelial IL-10R1 in mucosal integrity and likely barrier function.

We also examined tissue and serum cytokine levels in these mice. As shown in Fig. 5, cytokine levels in the tissue were dramatically altered in the IEC Il10r1$^{-/-}$ mice compared to control mice. These include KC (Fig. 5A), TNF-α (Fig. 5B), IL-6 (Fig. 5C), IL-1β (Fig. 5D), IL-12p70 (Fig. 5E) and IL-10 (Fig. 5F). Importantly, IL-6, IL-1β and IL-10 levels were significantly higher in the IEC Il10r1$^{-/-}$ animals, even in the absence of inflammation. Similar cytokine trends were observed in the systemic protein analysis (Supplemental Figure 2). Taken together, these results corroborate the *in vitro* findings that IEC IL-10 signaling is crucial to barrier homeostasis and restitution following acute inflammation.

Increased apical IL-10R1 expression in the mucosa of IBD patient samples

Finally, we explored the expression of IL-10R1 in human biopsy samples from patients with IBD. Initially, IL-10R1 expression was examined by immnofluorescence in biopsy samples from UC patients compared to healthy controls. As shown in Figure 6A, healthy control tissue showed minimal specific staining for IL-10R1. UC tissue samples showed prominent localization of IL-10R1 within the epithelium (Fig. 6A, arrows). Co-staining with the tight junction marker ZO-1 revealed that IL-10R1 localizes to the apical surface of the intact epithelium (Fig 6A, arrows). To extend thee findings, we screened IL-10R1 gene expression in a cohort of IBD patients compared to non-diseased tissue. As shown in Figure 6B, IL-10R1 transcript was significantly up-regulated in biopsy samples from IBD patients ($p<0.05$). For the samples analyzed here, the vast majority of signal was from the mucosa (range 40-100% mucosa with an average of 73% for all samples examined). Thus, human biopsies recapitulate our findings in both cultured cells and in murine colitis.

Discussion

This study aimed to elucidate mechanisms by which the pro-inflammatory cytokine IFN-γ promotes inflammation resolution and tissue restitution in models of mucosal inflammation. This concept is not without precedence. Indeed, IFN-γ has previously been shown to have protective properties in models of autoimmune disease such as experimental autoimmune encephalomyelitis and collagen-induced arthritis (37, 38). While IFN-γ has been demonstrated to play a causative role in the induction of inflammation in the DSS murine colitis model (39), recent evidence also suggests that IFN-γ-regulated pathways do play protective roles in models of IBD (3-5). The studies presented here, we identify the existence of the ligand-binding subunit of the IL-10 receptor (IL-10R1) as inducible within the epithelium in response to IFN-γ. This induction is specific to IFN-γ, as a number of other cytokines involved in the inflammatory response do not significantly alter IL-10R1 expression. Importantly, studies presented here suggest that the IL-10 receptor is associated with the apical membrane of intestinal epithelial cells. We further demonstrate that IL-10 signaling through the up-regulated receptor produces an amplified response, inducing expression of SOCS3 as well as the epithelial protective cytokines IL-10 and TGF- . These cytokines have been shown to have barrier protective properties both *in vitro* and *in vivo* (34, 35, 40, 41) and studies from our group and others indicate that the epithelium represents an important source of these cytokines (34, 42). We hypothesize that low level stimulation of IL-10R through multiple sources of IL-10 (including IEC) at baseline sets a homeostatic tone for both barrier development and maintenance. This is supported by our findings that loss of epithelial IL-10R1 results in baseline barrier dysfunction both *in vitro* and *in vivo*. Additionally, we demonstrate that treatment of T84 monolayers ameliorates the loss of transepithelial resistance induced by IFN-γ.

As a proof of principle, we extended these findings to models of mucosal inflammation. Given our understanding of inflammation-associated changes within the epithelium, we selected DSS colitis as an appropriate animal model to study colitis. DSS functions primarily as an epithelial irritant to drive permeability-induced colonic inflammation (43). Time course analysis of the cytokines IFN-γ and IL-10 in the DSS colitis model

demonstrated that IFN-γ levels peak in the tissue at day 6, which corresponds to a significant increase in tissue IL-10. Analysis of IL-10R1 expression in these tissues demonstrates that at day 6, IL-10R1 is significantly up-regulated in the epithelium and correlated with expression of SOCS3. This supports previous experiments which suggested up-regulation of SOCS3 as important in tissue repair (32). Examination of IL-10R1 localization in tissue taken from animals harvested on day 6 of the time course again demonstrated that a large portion of the IL-10 receptor staining appears to be associated with the apical surface of the mucosa. Lastly, we show that treatment of mice with IFN-γ alone is sufficient to induce the expression of IL-10R1 in the colonic epithelium.

IL-10 receptors have been shown to be expressed on mouse IEC (9) and more recently on IEC from non-human primates (44). Interestingly, in this latter study of rhesus macaques, consistent with our findings in human and mice, the authors revealed that the IEC IL-10R is positioned at the apical junctional complex (44). These studies did not, however, examine in detail the function of epithelial IL-10R in disease models. Thus, we turned to conditional deletion of the Il10r1 using the villin-Cre promoter to target deletion in IEC. This approach resulted in deletion of Il10r1 in greater than 90% colonic epithelial cells. Use of these mice in a mucosal inflammation model (DSS colitis) was immensely revealing from several perspectives. First, IEC-specific $Il10r1^{-/-}$ mice showed significant increases in baseline permeability *in vivo*. Such findings suggest that epithelial IL-10 signaling sets a basal tone for homeostasis in the intestine. Such findings are reminiscent of other apical junction complex proteins (e.g. JAM-A(45)) where genetic deletion results in increased basal permeability and significant increases in disease susceptibility. Second, these mice were highly susceptible to DSS-induced colitis. Standard doses of DSS resulted in remarkably enhanced disease onset and little to no resolution of disease. These findings suggest that without a counter-regulatory expression of a functional epithelial IL-10R complex, inflammatory resolution is not initiated. Third, the disease-associated loss of barrier function is associated with intense systemic inflammatory responses that coincided with increases in intestinal permeability. Marked increases in circulating TNF-α, IL-1β and IL-6 were indicative of an overall systemic response to localized intestinal inflammation. Taken together, the IEC IL-10R appears to function centrally in the control of homeostatic mucosal barrier function.

The final set of experiments involved examining the expression of IL-10R1 in human IBD patient samples. Initial analysis concerned exploring IL-10R1 expression using immunofluorescent microscopy utilizing non-diseased and UC biopsy samples. The results of these studies demonstrated increased expression of IL-10R1 in the UC biopsy specimen. Additionally, these studies once again suggest that the IL-10 receptor is associated with the apical aspect of epithelium. IL-10R1 expression was also analyzed by qPCR in a larger sample set of IBD biopsy specimens. The data show a significant increase in IL-10R1 expression in disease versus control tissue.

Taken together, these studies provide insight on a new and compelling protective role for IFN-γ in the intestinal epithelium. The data presented suggest that IFN-γ induces inflammatory adaptive mechanisms which promote epithelial restitution and repair. While not known at the present time, it is likely that IL-10 provides other functional roles than

barrier restitution within the epithelium. Up-regulation of the IL-10R1 in epithelial cells results in amplification of IL-10 signaling in the mucosa, which has been shown to play a vital role in restoration of epithelial barrier (34, 42). The significance of the localization of the IL-10 receptor to the apical membrane remains unclear at this time. It is enticing to speculate about this finding, particularly given the lack of efficacy of systemic IL-10 in clinical trials and in light of data showing that luminal administration of IL-10 ameliorates disease in mouse models of colitis and in human disease (46, 47). However, additional studies will be necessary to allow definitive conclusions. Moving forward, the findings presented herein provide tractable evidence to re-examine IL-10, albeit by a different delivery route, as a potential localized therapy for the treatment of mucosal diseases such as IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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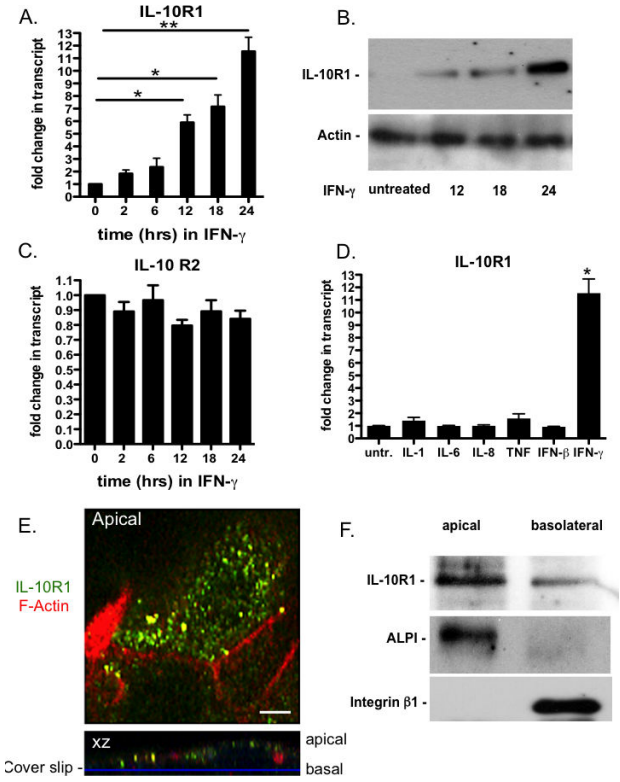

Figure 1. Induction of IL-10R1 during modeled inflammation

All experiments were performed using T84 human intestinal epithelial cells. Cytokine treatments were performed at 10 ng/ml for the indicated times. Panel A displays qPCR data for IL-10R1 in T84 cells treated with IFN-γ. Panel B displays western blot analysis of IL-10R1 expression in response to IFN-γ. Panel C displays qPCR analysis for IL-10R2 and panel D shows qPCR analysis for IL-10R1 treated with indicated cytokines for 24 hr (n=3, *p<0.05, student t test; western blot n=2). Panel E, confocal microscopy showing IL-10R1 localization in T84 cells grown to confluency on cover slips and treated with 10 ng/ml IFN-γ for 12 hr prior to staining. Apical, basolateral and cover slip are indicated. Bar indicates 10 μm (results representative of n=3). Panel F, cell surface biotinylation assay showing IL-10R1 localization. Cells were grown to confluency on transwell inserts and treated with IFN-γ for 12 hr. ALPI represents the control for apical localization, integrin β1 for basolateral (n=3).

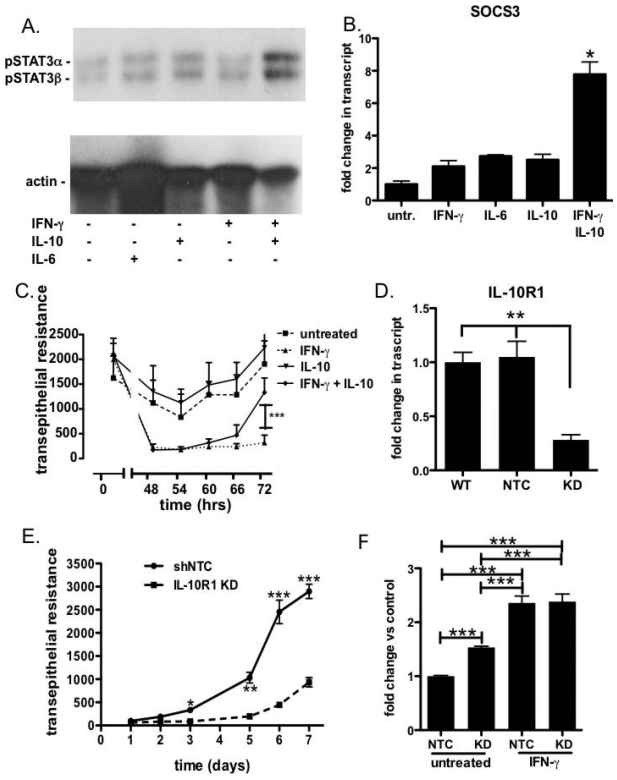

Figure 2. IL-10 signaling in intestinal epithelial cells

Panel A, STAT3 phosphorylation in T84 human epithelial cells cultured in the presence of indicated cytokines (10 ng/ml). All cytokine treatments were for 30 min except IFN-γ + IL-10 where cells were cultured overnight in IFN-γ to allow for receptor expression followed by 30 min exposure to IL-10 (n=3). Panel B shows qPCR data for SOCS3. Cytokine treatments were for 6 hr, except again for the IFN-γ/IL-10 dual treatment where cells were exposed to IL-10 for 6 hr following incubation with IFN-γ overnight (n=3). Panel C, TEER measurement in T84 monolayers. Cells were treated with cytokine for 36 hr, followed by media replenishment. IFN-γ treated cells were then either untreated or exposed to IL-10 for the remainder of the experiment. TEERs monitored at the indicated times; all cytokine treatments at 10 ng/ml (n=3). Panel D, qPCR analysis of IL-10R1 expression in untreated, shNTC and IL-10R1 knockdown T84 monolayers confirming the reduced expression of IL-10R1 in these cells (n=3). Panel E, TEER measurement of control (shNTC) and IL-10R1 knockdown T84 cells. TEER measurements were monitored daily beginning 1 day post plating (n=6). Panel F, FITC-dextran (3 KD) paracellular flux assay of control vs. IL-10R1 knockdown cells. Cells were treated with vehicle or IFN-γ (10 ng/ml) for48 hrs. Measurements were collected at 30 min increments after application of FITC-dextran to the apical aspect of IEC monolayers (units are ng/ml/min/$cm^{2,}$ n=6) (*p<0.05, **p<0.01, ***p<0.005, student t test).

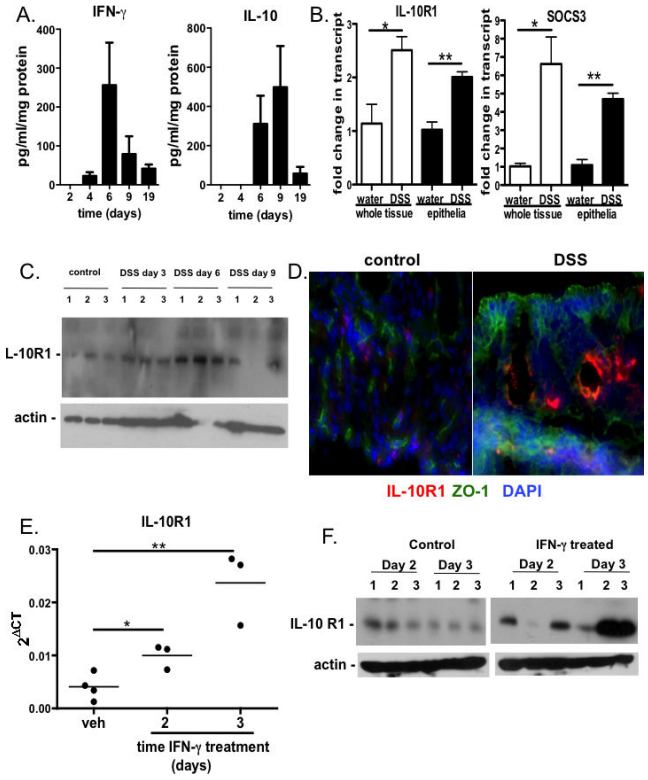

Figure 3. IL-10R1 expression in mouse intestinal epithelium

Panel A, cytokine measurement in colonic tissue. Mice were exposed to 3% DSS in drinking water for 5 days, followed by removal of DSS. Mice (n=3) were sacrificed at the indicated time points and colonic protein isolates were prepared and subjected to MesoScale analysis. Panel B, qPCR analysis of IL-10R1 and SOCS3 in whole colonic tissue and isolated epithelial cells from animals harvested on day 6. Panel C, western blot analysis of IL-10R1 expression in colonic tissue harvested at the indicated time points. Panel D, immunofluorescence microscopy of colonic tissue harvested on day 6. Frozen sections were cut and stained with IL-10R1 (red), ZO-1 (green) and DAPI (blue). Panel E qPCR analysis of IL-10R1 in isolated colonic epithelial cells harvested from mice treated with IFN-γ (5 μg/mouse/day IP) for the indicated time points (*p<0.05, **p<0.01, student t test). Panel F, western blot analysis of IL-10R1 in isolated epithelial cells from control and IFN-γ-treated mice. Results representative of 2 separate experiments.

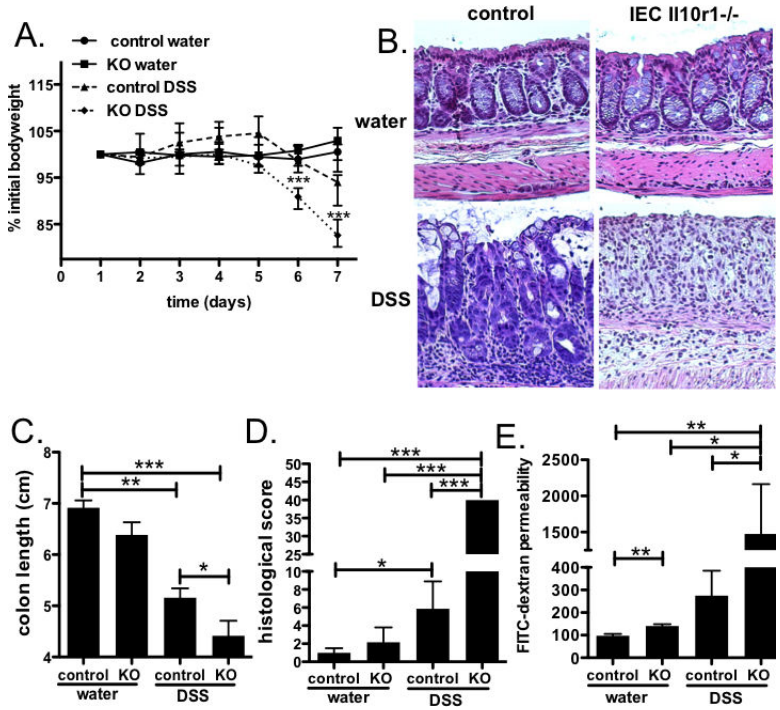

Figure 4. Epithelial IL-10R1 loss exacerbates DSS colitis

12-14 week old control (Cre-Il10r1 fl/fl) or KO (IEC Il10r1-/-) mice were administered water or 3% DSS ad libitum for 5 days. DSS was then removed and mice were allowed to recover for 3 days prior to sacrifice. Panel A displays body weight for control water fed (n=7), KO water fed (n=6), control DSS (n=8) and KO DSS (n=7); Panel B, H&E staining of tissue isolated from control water fed, IEC Il10r1-/- water fed, control DSS and IEC Il10r1-/- DSS; Panel C displays colon length; panel D displays histological score; panel E represents FITC-dextran permeability (data are expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.005; 2-way ANOVA). Results are representative of 3 separate experiments.

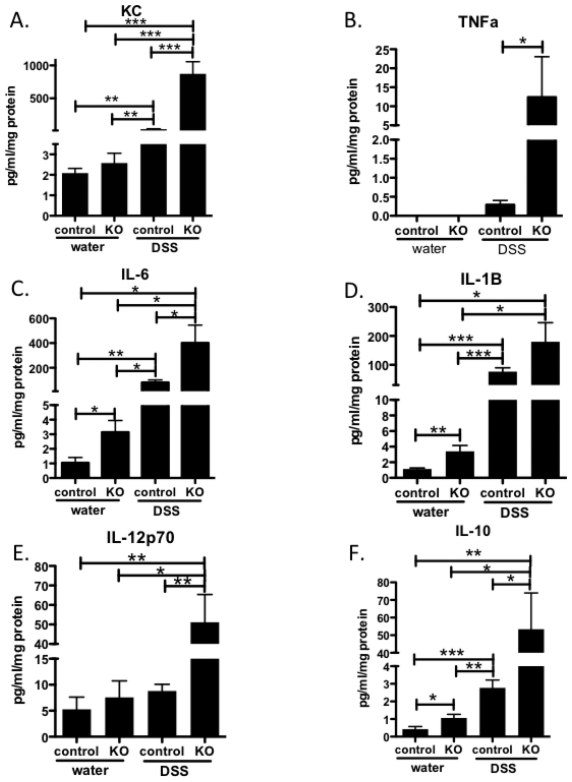

Figure 5. Impact of epithelial IL-10R1 loss on tissue cytokines in vivo

12-14 week old control (Cre- Il10r1 fl/fl) or KO (IEC Il10r1-/-) mice received vehicle or DSS (3%) for 5 days, followed by a 3-day recovery and animal sacrifice. Inflammatory cytokines were measured in colonic tissue lysates. Protein lysates were subjected to MesoScale analysis. All values are mean±SEM with n≥6 animals per group, *p<0.05, **p<0.01, ***p<0.005, 2-way ANOVA; results representative of 3 separate experiments.

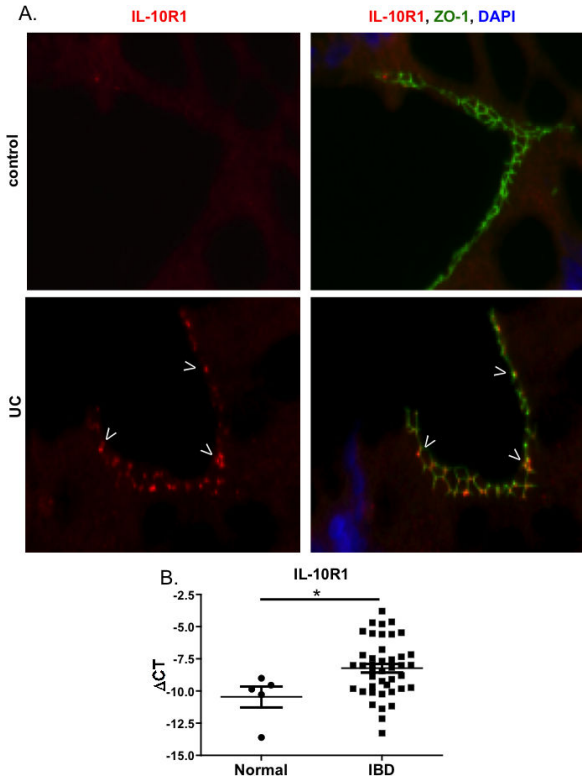

Figure 6. IL-10R1 expression in human biopsy tissue

Panel A, human biopsy specimens from control (screening colonoscopy) and UC patients were subjected to immunofluorescent microscopy. Sections were stained with IL-10R1 (red), ZO-1 (green) and DAPI (blue). Arrows indicate apical IL-10R1 staining; Panel B, qPCR analysis of IL-10R1 in human patient samples. Samples were from OriGene Crohn's/colitis cDNA array I comprised of 6 normal controls, 21 Crohn's, and 21 UC samples (*$p<0.05$, student t test).