Published in final edited form as:
Gastroenterology. 2008 June ; 134(7): 2036–2048.e3. doi:10.1053/j.gastro.2008.03.009.

Hypoxia-Inducible Factor Augments Experimental Colitis Through a MIF-Dependent Inflammatory Signaling Cascade

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Abstract

Background & Aims—Colon epithelial cells are critical for barrier function and contain a highly developed immune response. A previous study has shown hypoxia-inducible factor (HIF) as a critical regulator of barrier protection during colon epithelial injury. However, the role of HIF signaling in colon mucosal immunity is not known.

Methods—With the use of cre/loxP technology, intestinal specific disruption of Vhl, Hif-1α, and Arnt were generated. Colon inflammation was induced using a dextran sulfate sodium (DSS)-induced colitis model and the mice analyzed by histology, western blot analysis, and quantitative polymerase chain reactions.

Results—In mice, colonic epithelium disruption of Vhl resulted in constitutively expression of HIF which initiated an increase in inflammatory infiltrates and edema in the colon. These effects were ameliorated in mice by disruption of both Vhl and Arnt/Hif1β (which inactivates HIF). In a DSS-induced colitis model, increased HIF expression correlated with more severe clinical symptoms and an increase in histological damage, while disruption of both Vhl and Arnt in the colon epithelium inhibited these effects. Furthermore, colons with constitutive activation of HIF displayed increased expression of pro-inflammatory mediators which were synergistically potentiated following DSS administration and reduced by inhibition of the pro-inflammatory and direct HIF-target gene macrophage migration inhibitory factor (MIF).

Conclusion—The present study demonstrates that a chronic increase in HIF signaling in the colon epithelial cells initiates a hyper-inflammatory reaction that may have important implications in developing therapeutic strategies for inflammatory bowel disease.

Introduction

Hypoxia, a deficiency in oxygen availability, was shown to regulate a large subset of genes critical in both oxygen delivery and adaptation to oxygen deprivation [1, 2]. Regulation of hypoxia-mediated genes are dependent on the heterodimeric nuclear transcription factor, hypoxia inducible factor (HIF) consisting of an oxygen sensitive alpha subunit, where three

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The authors have no conflict of interest to disclose.

isoforms have been identified HIF-1α [3, 4], HIF-2α [5] and HIF-3α [6], and a ubiquitously expressed beta subunit, also referred to as aryl hydrocarbon nuclear translocator (ARNT) [2]. In the presence of adequate oxygen levels (normoxia), HIF alpha subunits are rapidly degraded via post-translational hydroxylation and ubiquitination. Oxygen-dependent prolyl-hydroxylation is necessary for binding to the von Hippel-Lindau tumor suppressor protein (VHL) and consequently to the E3 ubiquitin ligase complex [7, 8]. Thus the absence of a functional VHL results in constitutively active HIF *in vivo* [9]. HIF signaling was shown to activate transcription of genes critical in cell survival, angiogenesis, glycolysis and iron homeostasis [10–13]. The central role of HIF signaling in normal development and physiology is underscored by the embryonic lethality observed in mice lacking HIF-1α, HIF-2α, ARNT and VHL due to various vascular abnormalities [14–17].

Recently, using a two-step 2,4,6-trinitrobenzene sulphonic acid (TNBS) or oxazolone-induced inflammatory bowel disease (IBD) model, it was shown that HIF-1α and VHL are critical factors in maintaining intestinal epithelial integrity during increased local inflammation [18]. The two-step model initiates a delayed hypersensitive reaction. First, an epicutaneous treatment with TNBS primes T-cells. A subsequent inter-rectal instillation of TNBS results in a haptenization of the epithelial mucosa leading to a massive Th1 driven immune response to self cells [19, 20]. Mice containing an epithelial specific disruption of HIF-1α demonstrated an increase in the intestinal permeability and clinically more severe colitis as compared to their wild-type counterparts, whereas conditional targeting of Vhl in epithelial cells was protective. The mechanism by which HIF-1α maintains colonic mucosal integrity was shown to be through the induction of a number of barrier-protective genes [18]. However, IBD is thought to be a combination of a disturbance in function of the intestinal epithelial barrier and a dysregulation of the mucosal immune system [21, 22]. Intestinal epithelial cells that are critical in mucosal immunity by expressing several immunomodulatory genes, act in concert with other immune mediators to elicit a pro-inflammatory signal [23]. Using the TNBS or oxazolone-induced colitis model, it is difficult to assess the immunomodulatory role of HIF and VHL in mucosal immunity due to a direct robust immune response caused by primed T-cells. Therefore, the present study used a DSS-induced acute colitis model where the immune response is secondary to disruption of the epithelial barrier [20]. In addition, to gain a better insight into HIF signaling in mucosal immunity, the present study used intestinal epithelial cell conditional knockouts of HIF-1α, ARNT and VHL by use of the cre/loxP technology where the Cre transgene is under the control of the murine villin promoter. The villin promoter was shown to target expression of transgenes to the small and large intestine in both differentiated and undifferentiated cells of the crypt [24].

The present study demonstrates that a chronic increase in HIF signaling in colon epithelial cells triggers inflammatory response as assessed by an increase in pro-inflammatory mediators and colon histology that were dramatically potentiated by administration of DSS in the drinking water. Disruption of both VHL and ARNT in intestinal epithelial cells prevented development of intestinal inflammation indicating a HIF-dependent mechanism. Moreover, the inhibition of MIF activity, a direct HIF target [25], ameliorated the increase in pro-inflammatory mediators demonstrating MIF as a critical factor in the HIF-induced pro-inflammatory cascade.

Methods

Animals

Vhl-floxed ($Vhl^{F/F}$) [9], Hif-1α-floxed ($Hif\text{-}1\alpha^{F/F}$) [26] and Arnt-floxed ($Arnt^{F/F}$) [27] mice containing *loxP* sites flanking exons 1, 13–15, and 6 respectively, were crossed with mice harboring the Cre recombinase under control of the villin promoter (villin-cre mice) [24]. The intestine specific knockout mice for Vhl, Hif-1α, and Arnt were designated $Vhl^{\Delta IE}$ $Hif\text{-}1\alpha^{\Delta IE}$ $Arnt^{\Delta IE}$ mice, respectively. Mice were housed in temperature and light controlled rooms, were

given water and pelleted NIH-31 chow ad libitum. All animal studies were carried out in accordance with Institute of Laboratory Animal Resources (ILAR) guidelines and approved by the National Cancer Institute Animal Care and Use Committee.

Recombination efficiency and genotype determination

To assess recombination efficiency in the $Vhl^{F/F}$ and $Vhl^{\Delta IE}$ mice, intestine epithelium and all other tissues were harvested and kept in liquid nitrogen. DNA was isolated using the DNeasy kit (Qiagen, Valencia, CA) and the following primers were used to assess recombination Vhl-FWD1 PRIMER 5′-CTGGTA CCCACGAAACTGTC-3′ Vhl-FWD2 PRIMER 5′ CTAGGCACCGAGCTTAGAGGTTTGCG-3′ Vhl-RVS PRIMER 5′-CTGACTTCCACTGATGCTTGTCACAG-3′. The same primers were used for routine genotyping of the mice. Routine genotyping for the Arnt and Hif-1α allele were previously described [28].

Induction and assessment of colitis

Mice (6- to 8-weeks-old) were administered 2.5% or 5% (wt/vol) DSS (MW, 35,000–44,000) (MP Biomedicals, Aurora, OH) in the drinking water for five days. In experiments using ISO-1 (Calbiochem, San Diego, CA), the inhibitor was resuspended in 5% DMSO and delivered to the mouse daily via intraperitoneal injection at 20mg/kg and 5% DMSO was used as vehicle. Daily changes in body weight, diarrhea, bleeding, and histological damage were assessed and reported as previously described [29].

Intracellular MIF tautomerase activity assay

Colon extracts were prepared by homogenizing colon epithelial cells from $Vhl^{F/F}$ and $Vhl^{\Delta IE}$ mice treated with vehicle or ISO-1 in non-denaturing tris buffer. L-Dopachrome methyl ester was generated by mixing equal volumes of L-dopa methyl ester (4mM) and sodium periodate (8mM) (Sigma). Colon extracts (0.7mL) were mixed with freshly prepared L-dopachrome methyl ester (0.3mL) and the decay in absorbance was measured at 475nm.

RNA analysis

RNA was extracted from colon epithelium and qPCR was performed as previously described [29]. All primers sequences are available upon request.

Western blot analysis

Colon epithelium or HCT116 cells were lysed using NE-PER nuclear extraction kit for nuclear extract(Pierce, Rockford, IL) or RIPA buffer for whole cell extract. The membranes were incubated with an antibody against Hif-1α, Hif-2α (Novus Biologicals, Littleton, CO), and Arnt and MIF (Santa Cruz Biotechnology Inc, Santa Cruz, CA) the signals obtained were normalized to HNF4α (Santa Cruz) or GAPDH (Chemicon International, Temecula, CA).

Data analysis

Results are expressed as mean ± S.D. P values were calculated using multifactorial Anova test and Independent T Test. $p < 0.01$ was considered significant.

Results

Intestine specific disruption of Vhl, Arnt and Hif-1α genes via Cre-loxP–mediated recombination in mice

To estimate the extent of cell-specific disruption of the *Vhl* locus, PCR analysis was used. The *Vhl* null allele amplifies as a 260 bp product, and was detected in genomic DNA isolated from

intestinal epithelium cells of *Vhl*$^{\Delta IE}$ mice and was not detected in intestinal epithelium DNA isolated from *Vhl*$^{F/F}$ mice (Fig 1A). In contrast, the intact floxed allele was the only band evident in the intestinal epithelium from *Vhl*$^{F/F}$ mice and from all non-gut derived tissues in *Vhl*$^{\Delta IE}$ mice (Fig 1A). The expression of the Vhl mRNA was markedly decreased in the colon and throughout the small intestine of *Vhl*$^{\Delta IE}$ mice (Fig 1B), whereas, Vhl mRNA levels were unchanged in non-gut-derived tissues (data not shown). Next, western blot analysis was performed using nuclear protein extracts isolated from colonic epithelium from *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice. Interestingly, while no specific signal was observed for HIF-1α from colonic epithelium extracts, a robust HIF-1α expression was detected in the HCT116 colon cancer-derived cell line following a 24 hour incubation in 1% O_2. However, induction of HIF-2α was observed in *Vhl*$^{\Delta IE}$ compared to their wild-type littermate *Vhl*$^{F/F}$ mice, hepatic nuclear factor 4 alpha (HNF4α) used as a loading control for the nuclear fraction (Fig 1C). qPCR analysis demonstrated an increase in mRNAs encoding well-characterized HIF target genes (Fig 1D). As previously shown [28], Hif-1α and Arnt expression was significantly impaired in the colon epithelium from *Hif-1α*$^{\Delta IE}$ and *Arnt*$^{\Delta IE}$ mice when compared to their wild-type counterparts *Hif-1α*$^{F/F}$ and *Arnt*$^{F/F}$, and no decrease was observed in non-gut-derived tissues (Fig 1E and F). Together these data demonstrate that inactivation of the *Vhl* gene leads to an increase in HIF signaling. Interestingly, no HIF-1α protein expression was observed suggesting the increase in HIF gene expression may be due solely to HIF-2α.

Colon inflammation as assessed by histology and pro-inflammatory gene expression was increased in Vhl$^{\Delta IE}$ mice

Macroscopic examination of the colons in *Hif-1α*$^{F/F}$, *Hif-1α*$^{\Delta IE}$, *Arnt*$^{F/F}$ and *Arnt*$^{\Delta IE}$ mice demonstrated no apparent abnormalities (Fig 2A). However, histological examination of colons from *Vhl*$^{\Delta IE}$ mice, demonstrated edema in the submucosa layer and marked increase in inflammatory infiltrates (Fig 2A). In addition, 6-month-old *Vhl*$^{\Delta IE}$ mice demonstrated inflammatory polyps in the colon. Of the 72 *Vhl*$^{\Delta IE}$ mice assessed at 6-months of age, 27% contained polyps consisted of regenerative epithelium and were accompanied by ulceration and covered by exudates including cell debris, and fibrin. Infiltration of inflammatory cells and micro hemorrhage were also noted. The incidence increased to over 50% in 1-year-old mice, whereas no colon abnormalities were observed in *Vhl*$^{F/F}$ wild-type littermate mice (Fig 2B). The inflammatory polyps were similar to those described in patients with Crohn's Disease and other IBDs such as ischemic colitis and ulcerative colitis. Consistent with the increase in the histological signs of inflammation, all pro-inflammatory mediators assessed by qPCR were increased in the *Vhl*$^{\Delta IE}$ compared to Vhl$^{F/F}$ (Fig 2C). Interestingly, no changes were observed in *Hif-1α*$^{\Delta IE}$ or *Arnt*$^{\Delta IE}$ mice compared to their wild-type littermates (Fig 2C). These results clearly indicate that disruption of *Vhl* in the colon epithelium results in a marked increase in inflammation.

Exacerbation of colitis in *Vhl*$^{\Delta IE}$ mice treated with low-dose (2.5%) DSS

Vhl$^{\Delta IE}$ mice showed an increased susceptibility to DSS-induced colitis and the study was stopped following five days of 2.5% DSS administration due to marked increase in the pathological phenotype of *Vhl*$^{\Delta IE}$ mice. *Vhl*$^{\Delta IE}$ mice had significantly more severe disease as assessed by rectal bleeding, diarrhea, body weight, and colon length as compared to *Vhl*$^{F/F}$ mice (Fig 3A and B). Furthermore, histological analysis revealed increased inflammation in the mucosa, thickening and edema in the submucosa, and muscularis propria with complete loss of the crypts and surface epithelia, and increased recruitment of inflammatory infiltrates in *Vhl*$^{\Delta IE}$ mice when compared to the *Vhl*$^{F/F}$ mice (Fig 3C). Consistent with the increase in pathological phenotype and histological score following 5 days of DSS administration in *Vhl*$^{\Delta IE}$ mice, several pro-inflammatory mediators demonstrated robust potentiation in expression following DSS administration (Fig 3D). These data suggest that Vhl is critical in maintaining the mucosal immune response homeostasis following an inflammatory insult.

Colon specific double disruption of Vhl and Arnt genes via Cre-loxP–mediated recombination in mice

To assess the influence of the HIF-dependent pathways on the hyper-inflammatory response in the colons of $Vhl^{\Delta IE}$ mice, mice with a disruption of both Vhl and Arnt were made. To use background matched littermate control mice, $Vhl^{F/F}Arnt^{F/+}$ mice hemizygous for villin-cre were mated to each other and four genetic littermate strains were used for comparison; 1) $Vhl^{F/F}Arnt^{+/+}$ 2) $Vhl^{\Delta IE}Arnt^{+/+}$ 3) $Vhl^{F/F}Arnt^{F/F}$ 4) $Vhl^{\Delta IE}Arnt^{\Delta IE}$. The expression level of Arnt and Vhl mRNAs were assessed in the four-littermate genetic strains. As expected, no difference in Arnt mRNA expression levels were seen in the $Vhl^{F/F}Arnt^{+/+}$, $Vhl^{\Delta IE}Arnt^{+/+}$ and $Vhl^{F/F}Arnt^{F/F}$ mice, however in the $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice, Arnt expression was abolished. No change in Vhl expression was observed in the $Vhl^{F/F}Arnt^{+/+}$ and $Vhl^{F/F}Arnt^{F/F}$, but the mRNA expression was completely reduced in $Vhl^{\Delta IE}Arnt^{+/+}$ and $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice (Fig 4A). Next, western blot analysis was performed on nuclear extracts for HIF-1α, HIF-2α, ARNT, and HNF4α as a loading control. No HIF-1α expression was observed with $Vhl^{F/F}Arnt^{+/+}$ (lanes 1 and 2) and $Vhl^{F/F}Arnt^{F/F}$ mice (lanes 5 and 6) and consistent with what was observed n the $Vhl^{\Delta IE}$ mice (Fig 1C), no induction of HIF-1α was observed in $Vhl^{\Delta IE}Arnt^{+/+}$ mice (lanes 3 and 4). Interestingly, HIF-1α expression was increased in the $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice (lanes 7 and 8) (Fig 4B). As expected, HIF-2α expression was induced in both the $Vhl^{\Delta IE}Arnt^{+/+}$ and $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice, whereas ARNT protein expression was abolished only in the $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice (Fig 4B). Interestingly, Hif-1α mRNA levels were reduced in the colons of $Vhl^{\Delta IE}Arnt^{+/+}$ mice, whereas no change in expression was observed in $Vhl^{F/F}Arnt^{+/+}$, $Vhl^{F/F}Arnt^{F/F}$ and $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice (Fig 4C). The expression of Hif-1α mRNA in $Vhl^{\Delta IE}Arnt^{+/+}$ mice was similar to that observed in $Hif\text{-}1\alpha^{\Delta IE}$ mice (Fig 1E), suggesting a complete disruption of expression. In addition, no change in Hif-2α expression was seen in any genotype (Fig 4C). The data suggest a HIF-dependent negative feedback, which will be further discussed below. As observed with the $Vhl^{\Delta IE}$ mice (Fig 1D), $Vhl^{\Delta IE}Arnt^{+/+}$ mice demonstrated an increase in several HIF target genes, which were inhibited in the $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice (Fig 4D), indicating that HIF alpha subunit function is dependent on binding to its obligate heterodimer partner ARNT.

Colon inflammation induced in $Vhl^{\Delta IE}$ mice is dependent on HIF signaling

Examination of the colon in $Vhl^{\Delta IE}Arnt^{+/+}$ mice demonstrated an increase in the histological signs of inflammation (Fig 5A). In addition, of the 54 $Vhl^{\Delta IE}Arnt^{+/+}$ mice assessed at 6-months of age, 25% displayed inflammatory polyps in the colons similar to what was observed in $Vhl^{\Delta IE}$ (data not shown). These effects were ameliorated in the $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice (Fig 5A and data not shown). The induction of pro-inflammatory mediators observed in $Vhl^{\Delta IE}Arnt^{+/+}$ mice were inhibited in the $Vhl^{\Delta IE}Arnt^{\Delta IE}$ confirming that HIF signaling is required for the increase in inflammation following *Vhl* disruption (Fig 5B). To clarify whether the increase in susceptibility to DSS-induced colitis in $Vhl^{\Delta IE}$ mice was dependent on intact HIF signaling, $Vhl^{F/F}Arnt^{+/+}$, $Vhl^{\Delta IE}Arnt^{+/+}$, $Vhl^{F/F}Arnt^{F/F}$, and $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice were subjected to 2.5% DSS treatment. $Vhl^{\Delta IE}Arnt^{+/+}$ mice developed severe bloody diarrhea and body weight loss, while the body weight loss, diarrhea, and rectal bleeding were only marginally changed in $Vhl^{F/F}Arnt^{+/+}$, $Vhl^{F/F}Arnt^{F/F}$ and $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice (Fig 6A). The colon length of $Vhl^{\Delta IE}Arnt^{+/+}$ mice treated with 2.5% DSS was dramatically decreased compared to 2.5% DSS-treated colons from $Vhl^{F/F}Arnt^{+/+}$, $Vhl^{F/F}Arnt^{F/F}$ and $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice (Fig 6B). The histological injury score obtained from $Vhl^{\Delta IE}Arnt^{+/+}$ mice was significantly higher than that from $Vhl^{F/F}Arnt^{+/+}$, $Vhl^{F/F}Arnt^{F/F}$ and $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice (Fig 6C). In addition, using a 5% DSS dose demonstrated that $Vhl^{\Delta IE}Arnt^{+/+}$ mice had a decreased survival when compared to $Vhl^{F/F}/Arnt^{+/+}$, $Vhl^{F/F}Arnt^{F/F}$ and $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice demonstrating 100% survival following seven days of 5% DSS treatment (Fig 6D). These findings indicate that the disruption of *Vhl* in the colon epithelium exacerbates colitis, due to an increase in HIF signaling.

MIF inhibition decreased the expression of pro-inflammatory mediators in *Vhl*$^{\Delta IE}$ mice

MIF was shown to be a direct target of HIF signaling [25]. To assess whether MIF is critical in HIF-induced colonic inflammation, the protein expression and enzyme activity were measured. Western analysis demonstrated an increase in MIF protein expression from colonic extracts (Fig 7A). In addition, MIF activity was measured by utilizing the unique catalytic domain capable of tautermirizing L-dopachrome to indole derivates [30]. Colon extracts from *Vhl*$^{\Delta IE}$ mice demonstrated an 8-fold increase in MIF tautomerase, and the specific MIF tautomerase inhibitor ISO-1 completely inhibited MIF activity thus demonstrating the specificity of the assay (Fig 7B). *Vhl*$^{\Delta IE}$ mice treated with ISO-1 exhibited a significant decrease in pro-inflammatory mediator expression (iNOS, IFNγ, TNFα, IL-6, IL-10 COX-2, ICAM-1, TLR2 and IL-1β) when compared to vehicle treated *Vhl*$^{\Delta IE}$ mice. As expected, ISO-1 had no effect on MIF expression as it functions as an enzymatic inhibitor. Moreover, MIF antagonism was shown to be specific for HIF-induced pro-inflammatory mediators; no decrease in mRNA expression was observed for any pro-inflammatory mediators in *Vhl*$^{F/F}$ mice treated with ISO-1 compared to vehicle treated *Vhl*$^{F/F}$ mice (Fig 7C). The enzymatic activity of COX-2, a direct pro-inflammatory target of HIF [31], was also shown to be increased in *Vhl*$^{\Delta IE}$ mice (Supplemental Fig 1A and B). However when *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice were treated with nimelsulide, a COX-2 specific inhibitor, no decrease in any pro-inflammatory expression was observed when compared to vehicle treated *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice (Supplemental Fig 1A and B). Interestingly IL-1β levels were significantly increased in nimesulide treated *Vhl*$^{\Delta IE}$ mice, possibly due to intestinal side effects known to be associated with COX-2 inhibitors [32]. In addition, *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice treated with ISO-1 were protected from DSS-induced colitis. Due to robust response observed in both *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice on 5% DSS, the study was stopped following five days DSS administration. *Vhl*$^{\Delta IE}$ mice had a significantly more severe response as assessed by rectal bleeding, diarrhea, body weight, and colon length as compared to *Vhl*$^{F/F}$ mice. However ISO-1 administration protected both *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice (Fig 7D and E). Mortality was also slightly higher (2/10) in *Vhl*$^{\Delta IE}$ mice, when compared to *Vhl*$^{\Delta IE}$ mice administered ISO-1 (0/10) following DSS treatment (data not shown). The pathological findings were consistent with histological analysis revealing an improvement in mucosal inflammation, thickening and edema in the submucosa, and muscularis propria loss of the crypts and surface epithelia, following ISO-1 treatment (data not shown). These data demonstrates a critical role for MIF in the HIF-induced pro-inflammatory cascade and colitis.

Discussion

Individuals in a chronic hypoxic state, such as patients with chronic obstructive pulmonary disease demonstrate systemic inflammation [33]. Interestingly, when a focused case report review was performed on respiratory disorders leading to whole-body hypoxia, cardio-pulmonary disorders were more frequent among patients with IBD than previously considered [34]. In addition, hyperbaric oxygen treatment has been demonstrated to be effective in animal models of acute colitis and patients with severe IBD [35], however the molecular mechanism leading to either observation is currently unclear. The present study provides evidence demonstrating a molecular link between dysregulation of oxygen signaling and an increase in inflammation. Utilizing conditional *Vhl*$^{\Delta IE}$, *Hif-1α*$^{\Delta IE}$, *Arnt*$^{\Delta IE}$ and *Vhl*$^{\Delta IE}$*Arnt*$^{\Delta IE}$ mutant mice, a chronic increase in colon epithelial HIF-2α signaling resulted in a hyper-inflammatory response with an increase in colon inflammation and pro-inflammatory mediators. *Hif-1α*$^{\Delta IE}$ and *Arnt*$^{\Delta IE}$ mice demonstrated no significant difference in response to the susceptibility of DSS-induced colitis compared to their wild-type littermates possibly due to a limited role of hypoxia during the initial stage of DSS-induced colitis (Supplemental Fig 2). An increase in HIF signaling was shown to be a late event following low dose DSS administration (Supplemental Fig 2), therefore suggesting a mechanism whereby increased physiological HIF signaling predisposes mice to an inflammatory insult such as DSS. This hypothesis is further

supported by the finding that all basal level pro-inflammatory mediators measured are increased in *Vhl*$^{\Delta IE}$ mice, whereas no change in basal level expression was observed in *Hif-1α*$^{\Delta IE}$ and *Arnt*$^{\Delta IE}$ mice.

Previous work has revealed that HIF-1α signaling is critical in the intestinal epithelial barrier and several HIF-1α target genes are also barrier protective [18]. Paradoxically, the loss of VHL in the epithelial cells demonstrated a protective role in TNBS-induced colitis. This observation is different from that of the present study, and several possibilities may account for the differences in phenotype. A protective role for HIF in colitis was shown by use of a Cre-recombinase under transcriptional control of the fatty acid–binding protein (Fabp) promoter demonstrating less than 60% cre-mediated recombination of the Vhl allele, whereas in the present study using the villin promoter to drive the Cre gene, almost complete recombination was observed and the residual floxed allele in total gut tissue was thought to be due to inflammatory cells within the colon epithelium. Interestingly, gene dosage effects of Hif-1α have indeed been noted; heterozygous null mice demonstrated an impaired response to chronic hypoxia [36]. Therefore, the increase in recombination efficiency of the villin-cre promoter may have uncovered the hyper-inflammatory response that was not seen utilizing the FABP promoter-driven Cre recombinase that yields incomplete gene disruption in the gut.

Interestingly, loss of VHL in myeloid cells and overexpression of a degradation resistant HIF-1α in the skin epithelium results in a hyper-inflammatory response in 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced acute skin inflammation model [37 38]. The results of these studies are consistent with the present work and may suggest that the intrinsic differences between the inflammatory models may account for some of the differences in phenotype following initiation of colitis. In the TNBS-induced colitis model, disruption of Vhl was shown to be protective via HIF-1α-mediated induction of mucosal barrier genes. However, in the DSS model, the primary insult overrides the role of barrier protection, and thereby revealing the pro-inflammatory role of HIF signaling in colon epithelia.

Lastly, the *Vhl*$^{\Delta IE}$ mouse model used in the present study was HIF-2α mediated; no protein expression of HIF-1α was detected in the colon epithelium. Therefore, the pro-inflammatory phenotype observed was mainly due to HIF-2α signaling, whereas Karhausen et al.[18] demonstrated that the protective role of HIF signaling, was due to HIF-1α expression in the colon. Recently, two reports demonstrate a protective function in mouse colitis models using pharmacological inhibitors of prolyl hydroxylases, which activate HIF signaling by inhibiting its degradation [39, 40]. The HIF prolyl hydroxylase inhibitor FG-4497 was shown to decrease intestinal permeability thereby protecting the intestine. Interestingly, the pharmacological inhibitor displayed no effects on barrier function in the intestine specific *Hif-1α*-null mice, confirming that the protective role HIF signaling in colon homeostasis is primarily dependent on HIF-1α [39]. In addition, HIF-1α was shown to directly regulate several barrier protective genes, such as intestinal trefoil factor, CD73 and multidrug resistance gene 1 [41–43], however in present study, HIF-2α does not increase their expression in the colon epithelium (data not shown). Due to high sequence similarity between HIF-1α and HIF-2α, these transcription factors share many similar functions, however it is becoming apparent that HIF-1α and HIF-2α can regulate unique sets of target genes with distinct functions. The roles of HIF-1α and HIF-2α have diverged in respect to cellular growth. In cell lines, HIF-2α can promote cycle progression, whereas HIF-1α inhibits cell proliferation [44]. Therefore the differences observed in present study and in Karhausen et al.[18] may reflect the divergent roles of HIF-1α and HIF-2α in colon homeostasis.

Currently, the mechanism by which Hif-1α is downregulated is unclear. The present study suggests that Hif-1α gene expression is under a negative feedback regulation following prolonged HIF signaling mediated by HIF-2α or its respective target genes. Several *in vitro*

studies have described this phenomenon [45–47]. In lung epithelial-derived cell lines, prolonged hypoxia decreased HIF-1α protein expression via a decrease in the Hif-1α mRNA, whereas no change in Hif-2α expression was observed [46]. Inhibiting HIF signaling using a dominant negative HIF-2α, prevented the down regulation of Hif-1α expression following a prolonged hypoxia treatment [46]. Currently this is major focus and the molecular mechanism will be assessed in future studies.

MIF was identified as a primary mediator downstream of HIF-signaling responsible for the increase in pro-inflammatory gene expression following induction of HIF-2α. MIF is a well-characterized pro-inflammatory mediator secreted by several different cell types including colon epithelium and has been demonstrated to be critical in the pathogenesis of colitis. MIF serum concentrations were shown to be elevated in Crohn's patients [48], and a decrease in MIF activity by anti-MIF antibody administration or MIF-null mice were highly protective in DSS and TNBS-induced colitis models [48, 49 50]. Furthermore, mice transgenically overexpressing MIF demonstrated an increase in susceptibility to DSS-induced colitis [51]. Consistent with the above reports inhibiting MIF activity decreased pro-inflammatory cytokines and protected $Vhl^{F/F}$ and $Vhl^{\Delta IE}$ mice in DSS-induced colitis model. The importance of the catalytic domain in modulating MIF activity is well documented [52]; the present study provides further evidence demonstrating the utility in small-molecule inhibitors of MIF as a therapeutic modality and clearly demonstrates that the increase in MIF expression and activity are critical in the HIF-induced pro-inflammatory cascade in the colon.

In conclusion, the present study demonstrates an increase in colonic inflammation in murine models with constitutive epithelial HIF signaling, which is mediated by HIF-2α activation of the pro-inflammatory gene MIF. Taken together with the previous study [18], the present working model suggests that an increase in HIF signaling may be protective early in the pathogenesis of IBD via HIF-1α-mediated maintenance of the epithelial barrier. However, as the epithelial barrier breaks down, HIF-2α may potentiate the chronic inflammatory reactions, worsening disease progression. Currently several HIF modulators are either in pre-clinical or clinical trials for a variety ischemic diseases and cancers [53]. Therefore the present study provides important implications for using HIF modulators as therapeutic modalities and suggests that caution should be exercised in the long-term use of these compounds in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Grant Support: This study was funded by the National Cancer Institute Intramural Research Program and Y.M.S was supported by a postdoctoral fellowship from the American Cancer Society PF-06-014-01-CNE.

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Abbreviations

ARNT, aryl hydrocarbon nuclear translocator
COX-2, cyclooxygenase-2
DSS, dextran sulfate sodium
HIF, hypoxia inducible factor
HNF4α, hepatic nuclear factor 4 alpha
IBD, inflammatory bowel disease
iNOS, inducible nitric oxide synthase
MIF, macrophage migration inhibitory Factor
PDK1, pyruvate dehydrogenase kinase 1
PGK1, phosphoglycerate kinase 1
PGE_2, prostaglandin E_2
qPCR, quantitative RT-PCR
TNBS, 2,4,6-trinitrobenzene sulphonic acid
VEGF, vascular endothelial growth factor

Vhl, von Hippel-Lindau tumor suppressor protein

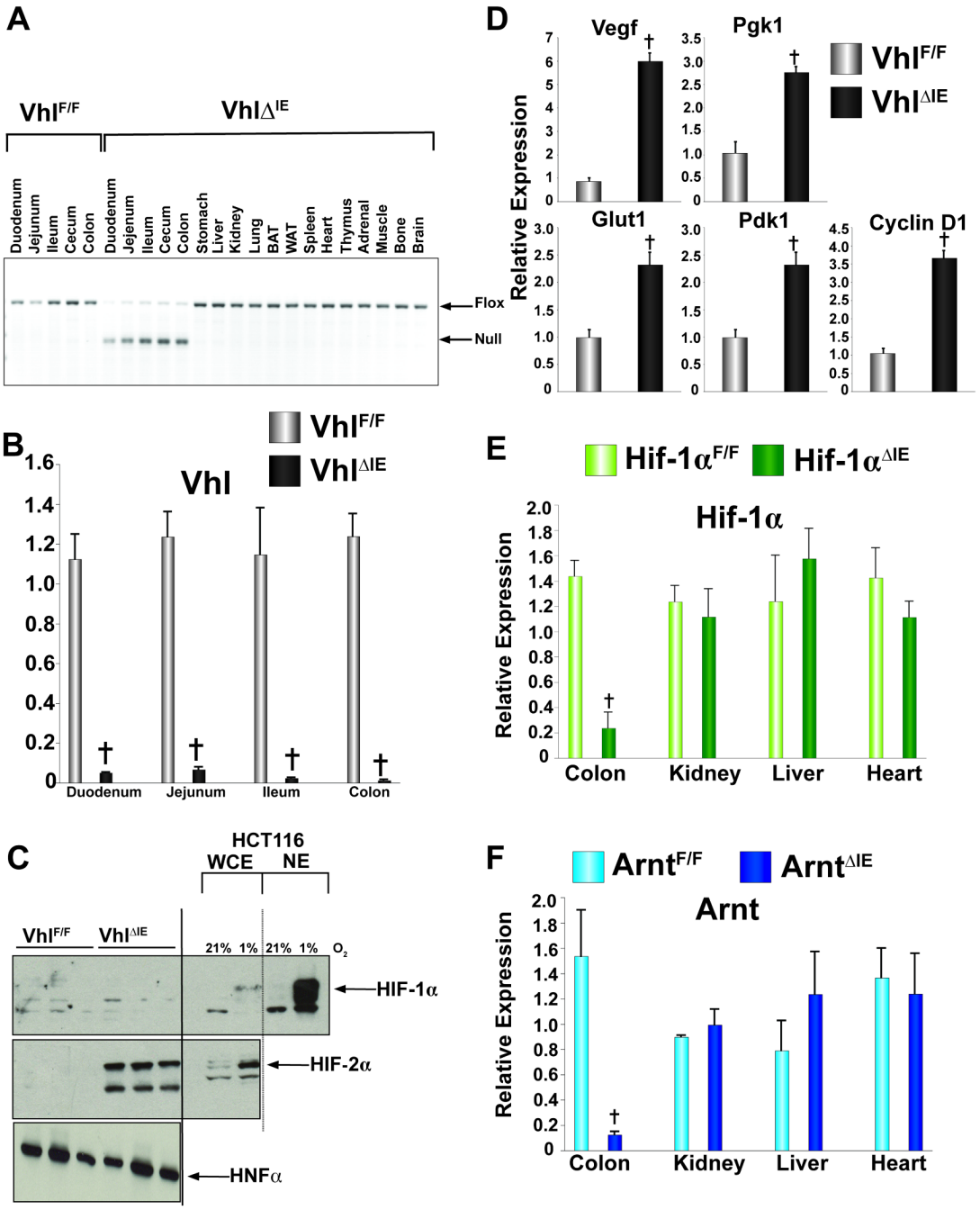

Figure 1. Colon Specific disruption of Vhl

(A) PCR diagnostic for Cre-mediated recombination of the Vhl allele in genomic DNA isolated from *Vhl*$^{\Delta IE}$ or *Vhl*$^{F/F}$ mice. (B) qPCR analysis measuring Vhl mRNA expression in intestinal epithelium from *Vhl*$^{\Delta IE}$ or *Vhl*$^{F/F}$ mice. (C) Western blot analysis measuring Hif-1α or Hif-2α expression in colon epithelial cells isolated from *Vhl*$^{\Delta IE}$ or *Vhl*$^{F/F}$ mice. Expression was normalized to HNF4α protein expression, and HCT116 cells treated with 1% O_2 for 24 hours served as positive control. (D) qPCR analysis of HIF target genes. (E) qPCR analysis measuring Hif-1α mRNA expression in total RNA from colon epithelium from *Hif1α*$^{\Delta IE}$ or *Hif1α*$^{F/F}$ mice. (F) qPCR analysis measuring Arnt expression in total RNA from colon epithelium from

Arnt$^{\Delta IE}$ or *Arnt*$^{F/F}$ mice. For qPCR analysis the expression was normalized to β-actin and each bar represents the mean value ± S.D. (†)= $P<.01$ compared to wild-type littermates.

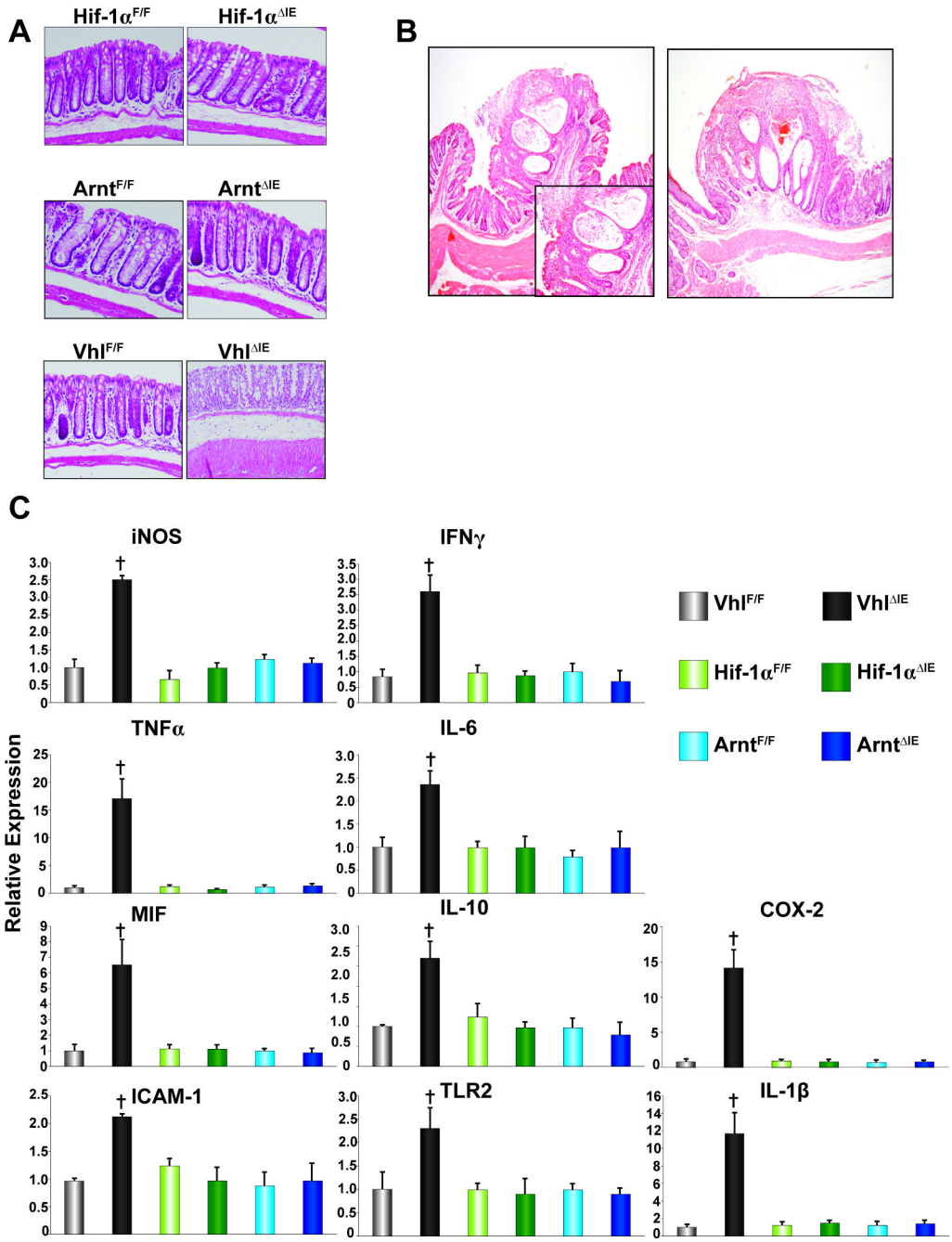

Figure 2. Pro-inflammatory gene expression in the Hif1α, Arnt or Vhl disrupted colon epithelium

(A) Representative H & E stained colon sections from wild-type littermates and *Hif1α*$^{\Delta IE}$, *Arnt*$^{\Delta IE}$, and *Vhl*$^{\Delta IE}$ mice. (B) Representative H & E stained pro-inflammatory polyp from two individual *Vhl*$^{\Delta IE}$ mice (inset indicates higher magnification). (C) qPCR analysis of pro-inflammatory mediators in the colon epithelium from wild type littermates and *Hif1α*$^{\Delta IE}$, *Arnt*$^{\Delta IE}$, and *Vhl*$^{\Delta IE}$ mice.± S.D. (†)= *P*<.01 compared to vehicle treated wild-type mice.

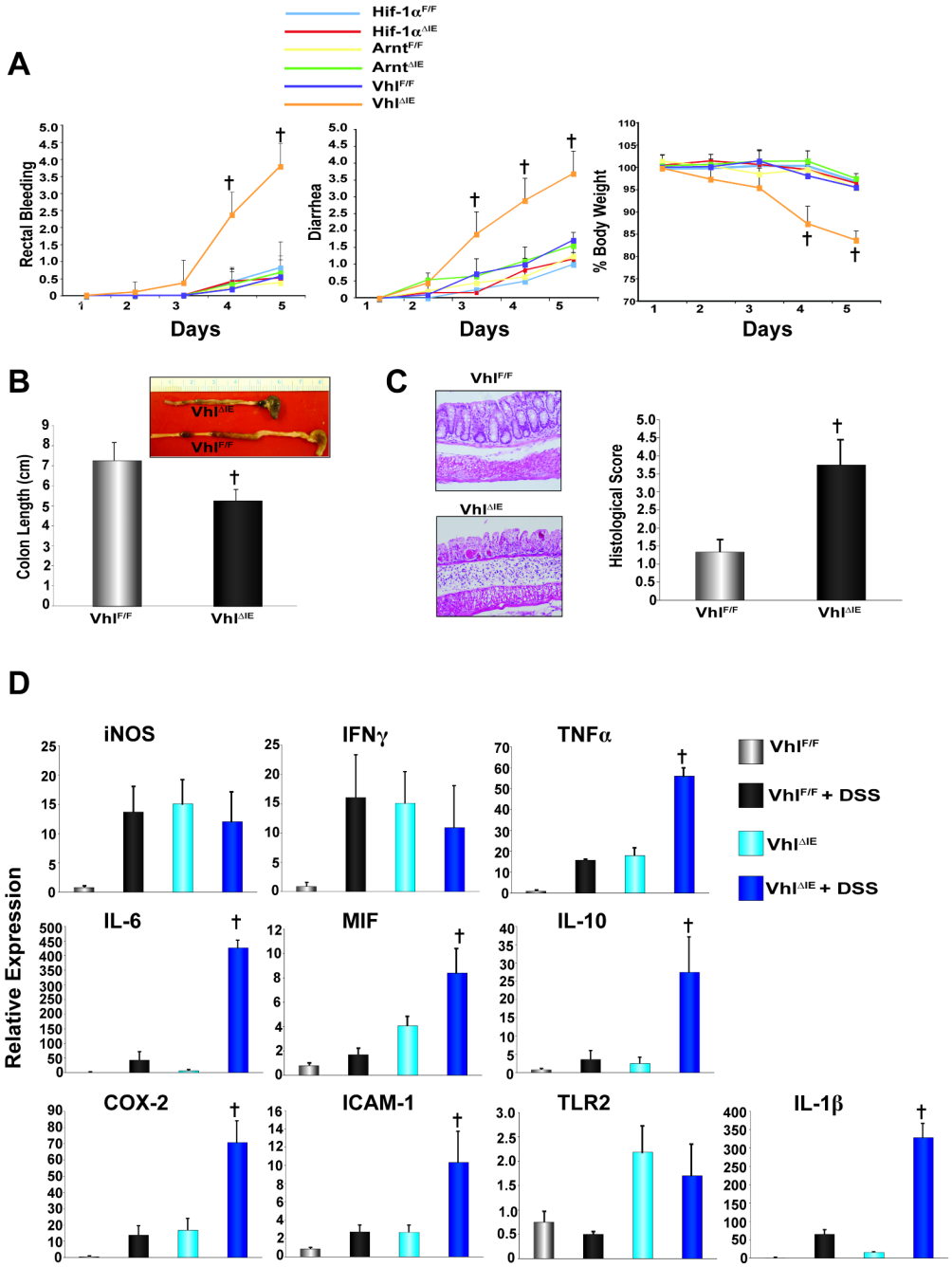

Figure 3. Clinical assessment of DSS-induced colitis in *Hif1α*$^{\Delta IE}$, *Arnt*$^{\Delta IE}$, and *Vhl*$^{\Delta IE}$ mice

(A) Rectal bleeding, diarrhea, and body weight changes following DSS-induction of colitis, (B) colon length, (C) representative H & E stained colon sections and histology score. (D) qPCR analysis of pro-inflammatory mediators in the colon epithelium from *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice treated with control H_2O or 2.5% DSS H_2O. Data represent the mean value ± S.D, (†)= $p < 0.01$ compared to *Vhl*$^{F/F}$ DSS treated mice.

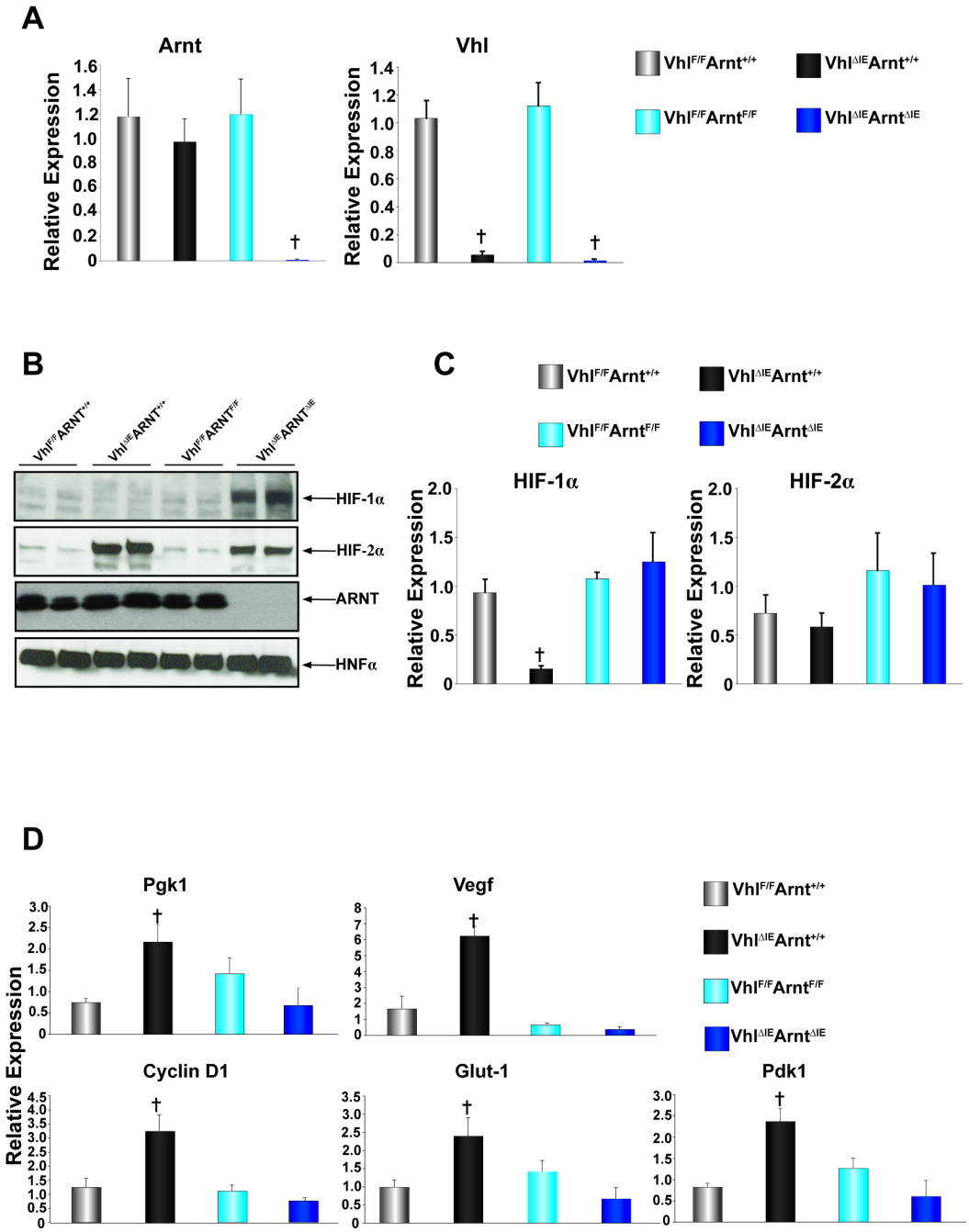

Figure 4. Colon-specific double-disruption of Vhl and Arnt

(A) qPCR analysis measuring Arnt and Vhl expression in total RNA from colon epithelium. (B) Western blot analysis measuring Hif-1α, Hif-2α and Arnt expression in colon epithelial cells. Expression was normalized to HNF4α protein expression. (C) qPCR analysis of Hif-1α and Hif-2α in colon epithelium. (D) qPCR analysis of HIF target genes in colon epithelium. For qPCR analysis the expression was normalized to β-actin and each bar represents the mean value ± S.D.(†)= *P*<.01 compared to wild-type littermates.

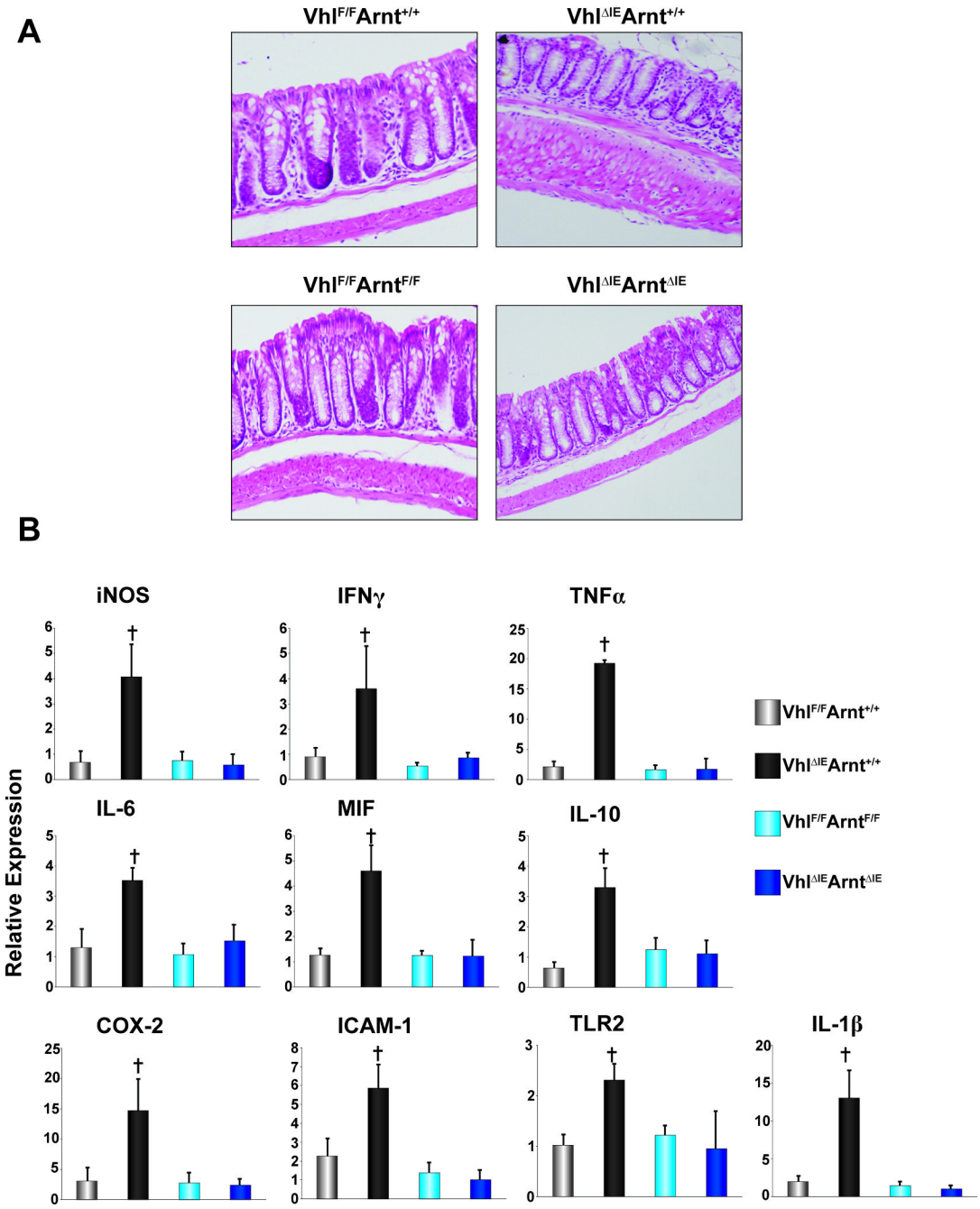

Figure 5. Pro-inflammatory gene expression in the double Vhl and Arnt disrupted colon epithelium

(A) Representative H & E stained colon sections. (B) qPCR analysis of pro-inflammatory mediators in the colon epithelium. Expression was normalized to β-actin and each bar represents the mean value ± S.D. (†)= *P*<.01 compared to wild-type littermates.

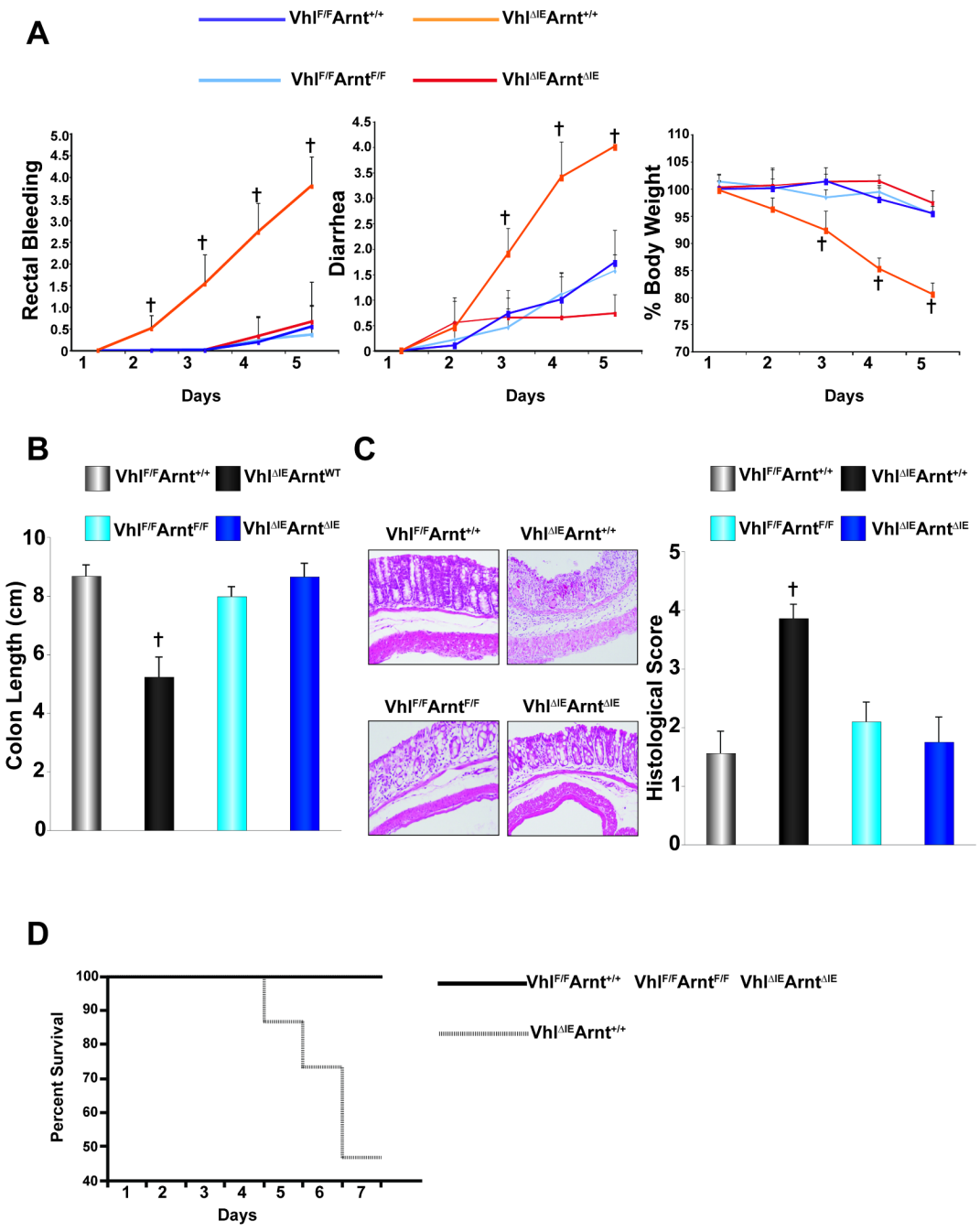

Figure 6. Clinical assessment of DSS-induced colitis in the double Vhl and Arnt disrupted colon epithelium

(A) Rectal bleeding, diarrhea, and body weight changes following DSS-induction of colitis, (B) colon length, (C) representative H & E stained colon sections and histology score. (D) Survival of $Vhl^{F/F}Arnt^{+/+}$, $Vhl^{\Delta IE}Arnt^{+/+}$, $Vhl^{F/F}Arnt^{F/F}$, and $Vhl^{\Delta IE}Arnt^{\Delta IE}$ mice. Data represent the mean value ± S.D, (†)= $p < 0.01$ compared to $Vhl^{F/F}$ DSS treated mice.

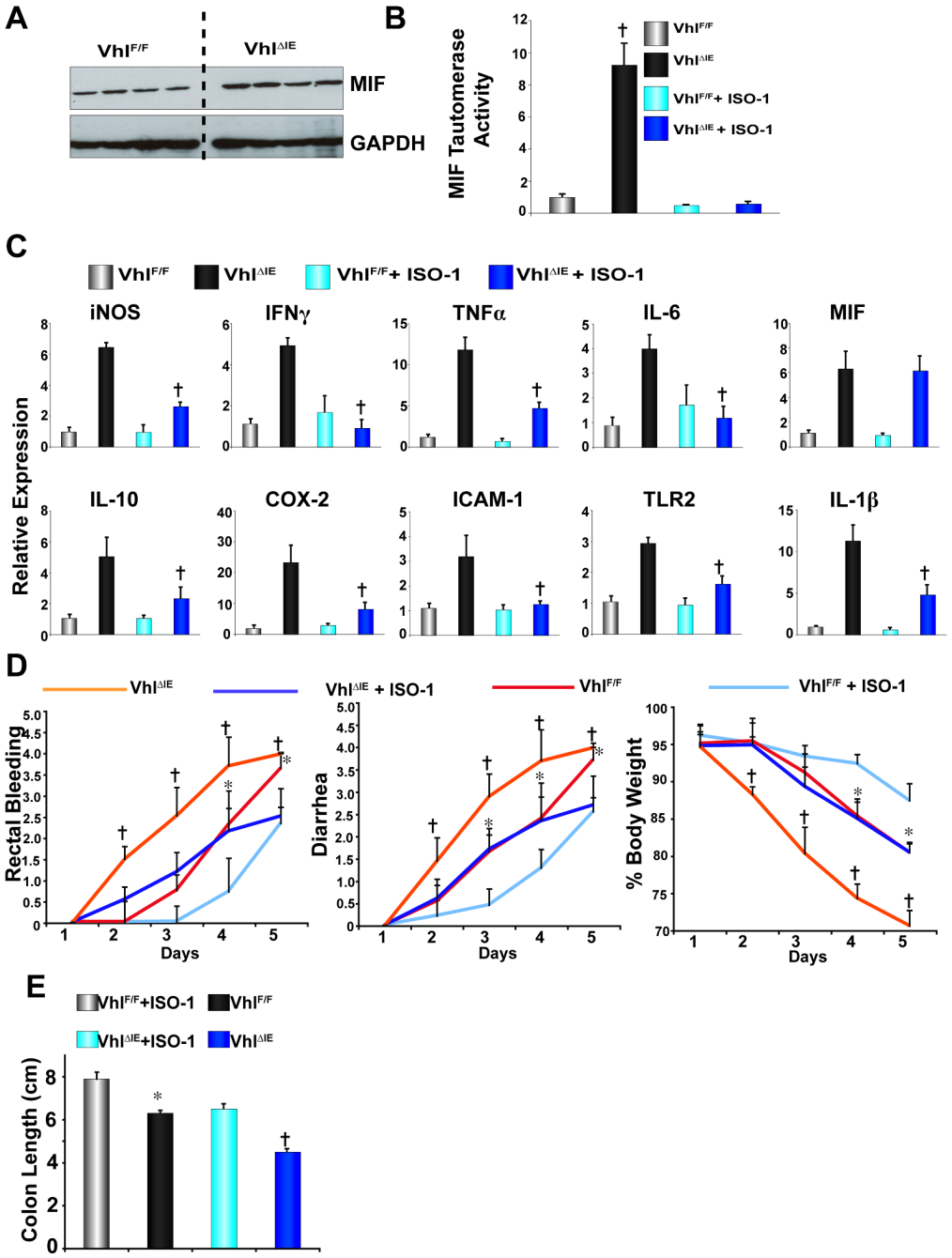

Figure 7. Pro-inflammatory gene expression in *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice following nimesulide or ISO-1 administration

(A) Western blot analysis for MIF expression in colon extracts. (B) MIF tautomerase activity measured in colon extracts of *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice treated with vehicle or 20mg/kg of ISO-1. Data represent the mean value ± S.D, (†)= $p < 0.01$ compared to wild-type littermates. (C) qPCR analysis of pro-inflammatory mediators in the colon epithelium from *Vhl*$^{F/F}$ and *Vhl*$^{\Delta IE}$ mice treated with vehicle or 20mg/kg of ISO-1. Expression was normalized to β-actin and each bar represents the mean value ± S.D. (†)= $P<.01$ compared to vehicle treated *Vhl*$^{\Delta IE}$ mice. (D) Rectal bleeding, diarrhea, body weight changes, and (E) colon length

following DSS-induction of colitis. Data represent the mean value ± S.D, (*)= $p < 0.01$ compared to $Vhl^{F/F}$ MIF treated mice and (†)= $p < 0.01$ compared to $Vhl^{\Delta IE}$ MIF treated mice