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Constitutive androstane receptor regulates the intestinal mucosal response to injury

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Running Title – CAR and intestinal mucosal repair

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Abstract

Background and Purpose: The pathogenesis of the inflammatory bowel diseases(IBD), composed of Crohn's disease(CD) and ulcerative colitis(UC), involves aberrant interactions between a genetically susceptible individual, their microbiota and environmental factors. Alterations in xenobiotic receptor expression and function are associated with increased risk for IBD. In the current study, we assessed the role of the constitutive androstane receptor(CAR), a xenobiotic receptor closely related to the pregnane X receptor, in the regulation of intestinal mucosal homeostasis.

Experimental Approach: CAR expression was assessed in intestinal mucosal biopsies obtained from CD and UC patients, and in C57/Bl6 mice exposed to dextran sulphate sodium (DSS; 3.5% w/v in drinking water) to evoke intestinal inflammation and tissue damage. CAR-deficient mice were exposed to DSS and mucosal healing assessed. The modulation of wound healing by the CAR was assessed *in vitro*. Lastly, the therapeutic potential of CAR activation was evaluated, using TCPOBOP, a selective rodent CAR agonist.

Key Results: CAR expression was significantly reduced in CD and UC samples compared healthy controls. This was recapitulated in our DSS studies, where CAR expression was reduced in colitic mice. CAR-deficient mice exhibited reduced healing following DSS exposure. *In vitro*, CAR activation accelerated intestinal epithelial wound healing by enhancing cell migration. Lastly, treating mice with TCPOBOP, following the induction of colitis, enhanced mucosal healing.

Conclusion and Implications: The results of our study support the notion that xenobiotic sensing is altered during intestinal inflammation, and suggest that CAR activation may prove effective in enhancing mucosal healing in patients with IBD.

Abbreviations – **IBD** inflammatory bowel diseases; **AhR** aryl hydrocarbon receptor; **PXR** pregnane X receptor; **CAR** constitutive androstane receptor; **CD** Crohn's disease; **UC** ulcerative colitis; **MPO** myeloperoxidase**; DSS** dextran sulphate sodium **CITCO** 6-(4-Chlorophenyl) imidazo[2,1b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; **EE2** 17α-ethynylestradiol; **TCPOBOP** 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene

Introduction

While the exacts cause(s) of the inflammatory bowel diseases (IBD) have yet to be completely elucidated, it is currently thought that chronic intestinal inflammation arises in a genetically susceptible individual in the context of aberrant interactions with the intestinal microbiota and environmental factors (Xavier & Podolsky, 2007). Although the link to the former is supported by a number of population-based and mechanistic studies (Miyoshi & Chang, 2016), considerably less is understood about the exact role that environmental factors play in the development of IBD (Abegunde, Muhammad, Bhatti & Ali, 2016).

The intestinal mucosa, particularly the epithelium, is the main point of contact for the resident microbiota and ingested substances of environmental origin, providing a physical and "metabolic" barrier that prevents the microbiota and noxious compounds from entering the internal tissues (Turner, 2009). Much is known about the host receptors that mediate key interactions with the constituents of the intestinal microbiota to confer appropriate mucosal defense and maintain intestinal mucosal homeostasis (Davies & Abreu, 2015). However, less is known about how the intestinal mucosa senses and responds to substances of environmental origin, including pharmaceutical drugs, natural compounds in foods, pesticides and other environmental/industrial pollutants. Interestingly, conserved mechanisms exist for multicellular organisms to sense and respond to such xenobiotic compounds and are thought to add an additional level of defense in the liver and gastrointestinal tract of multicellular organisms (Dussault & Forman, 2002).

Within the liver, the aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) play key roles in the response to chemicals of environmental origin. Recent reports suggest that metabolic products released from the intestinal microbiota may also evoke the activation of these receptors (Hubbard et al., 2015; Venkatesh et al., 2014), suggesting their function may also constitute an additional dimension of host-microbe interaction at sights of microbial colonization. Accumulating evidence suggests that the AhR and PXR play important roles within the gastrointestinal tract; enhancing intestinal epithelial barrier function (Garg et al., 2016; Venkatesh et al., 2014); modulating mucosal inflammatory signaling (Ji et al., 2015; Venkatesh et al., 2014); and regulating the function of resident immune cell populations (Chng et al., 2016; Qiu et al., 2013). In experimental models of colitis or inflammation-induced mucosal damage, genetic ablation of the AhR or PXR enhance the severity of tissue damage (Arsenescu et al., 2011; Furumatsu et al., 2011), whereas their selective

activation affords protection (Monteleone et al., 2011; Takamura et al., 2010). In IBD patients, the function/expression of the PXR (Dring et al., 2006; Glas et al., 2011) and AhR (Monteleone et al., 2011; Singh, Singh, Singh, Price, Nagarkatti & Nagarkatti, 2011) are altered, suggesting that defects in xenobiotic sensing may contribute to disease pathogenesis. While the CAR's expression has been confirmed in the intestinal epithelium (Arnold, Eichelbaum & Burk, 2004; Martin, Riley, Back & Owen, 2008), its role in the intestinal mucosa has yet to be assessed.

In the current study, we sought to test the hypothesis that the CAR would function as a protective entity in the intestinal mucosa, much like the AhR and PXR. We found that CAR gene expression was reduced in intestinal mucosal biopsies obtained from patients with UC and CD with active mild/moderate inflammation, an observation that was paralleled in tissues isolated from colitic mice. Selective activation of the CAR enhanced wound healing in intestinal epithelial cell monolayers, an effect driven by enhanced cell migration. Lastly, deletion of CAR delayed mucosal healing after the induction of experimental colitis; whereas its pharmacological activation accelerated recovery. Taken together, our data support novel role for the CAR as a xenobiotic sensor that contributes to reparative processes in the intestinal mucosa.

Methods

Reagents

To assess the role of the CAR in epithelial wound healing and migration assays, we used the selective human CAR agonist 6-(4-Chlorophenyl)imidazo[2,1b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO; Sigma Aldrich). CITCO was dissolved in sterile DMSO and diluted in media to reach the experimental concentrations of 25-50 nM. Matching volumes of sterile DMSO were added to culture media for experimental vehicles controls. To assess the role of the CAR in mediating the basal and CITCO-induced wound closure, we used the selective CAR inverse agonist 17α-ethynylestradiol (EE2; 10 μM; Sigma Aldrich). To characterize the signaling events associated with CAR-driven wound healing and cell migration, we used SB202190 (10 μM; Sigma Aldrich), a selective p38 MAP kinase inhibitor. SB202190 was added 30 minutes prior to activating the CAR. To determine the role of the CAR in tissue repair following inflammation-associated tissue damage, mice were treated with the selective rodent CAR agonist 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene (TCPOBOP; 3 mg/kg/oral gavage; Sigma Aldrich) dissolved in sterile corn oil. Matched volumes of sterile corn oil were administered as an experimental control.

Human Biopsies

All patients were consented through the Intestinal Inflammation Tissue Bank according to a protocol approved through the University of Calgary's Conjoint Health Research Ethics Board (Study ID: REB14-2429). Intestinal mucosal biopsies were taken from the colon of patients undergoing clinically indicated colonoscopy procedures at a single tertiary care centre. Biopsies from patients with Crohn's disease (CD) and ulcerative colitis (UC) were taken from sites of active inflammation within the colon (without ulceration) and were all obtained through the Foothills Medical Centre endoscopy outpatient unit. Control colonic samples were taken from patients without inflammation and with no history of IBD who were undergoing routine screening at the Foothills Forzani & MacPhail Colon Cancer Screening Centre.

RNA isolation, cDNA generation and real-time PCR

Human colonic mucosal biopsies and whole mouse colonic tissues were placed immediately into RNAlater (Thermo Fisher Scientific), stored overnight at 4^{o}C and kept at –80 °C for long-term storage. For RNA extraction, biopsies and whole colonic sections were transferred

from RNA later into TRIzol reagent (Thermo Fisher Scientific) and homogenized by stainless steel-bead-based lysis using a Bullet Blender BBX24 (Next Advance – Laboratory Instruments). RNA was isolated following the standard TRIzol procedure. RNA pellets were resuspended in water and further purified through RNeasy spin columns (Qiagen) according to the RNeasy Mini kit instructions, including an on-column DNase digestion step. RNA isolated from mouse colonic tissues was incubated with lithium chloride to remove dextran sulphate sodium, as described previously (Viennois, Chen, Laroui, Baker & Merlin, 2013). Quantity and purity of RNA was determined using a Nanodrop spectrophotometer. For all RNA, 260/280 ratios were all ≥1.8 and 260/230 ratios were all ≥ 1.7. cDNA was synthesized using the RT2 First Strand Kit (SaBiosciences/Qiagen) according to the manufacturer's instructions using 1000 ng of input RNA per sample. Human NR1I3 (primer - Cat. No. PPH01278E; Refseq Accession #: NM_005122; SaBiosciences/Qiagen), mouse Nr1i3 (primer Cat. No. PPM24908B; Refseq Accession #: NM_009803; SaBiosciences/Qiagen), human ABCB1/MDR1 (primer Cat. No. PPH01527B; Refseq Accession #: NM_000927) and mouse Abcb1a/Mdr1 (primer Cat. No. PPM03898C; Refseq Accession #: NM_011076; SaBiosciences/Qiagen) were amplified with using the Quantitect SYBR Green PCR kit on an ABI StepOnePlus real-time PCR cycler (Applied Biosystems) using β-actin (human; primer - Cat. No. PPH00073G; Refseq Accession #: NM_001101; mouse; primer - Cat. No. PPM02945B; Refseq Accession #: NM_007393; SaBiosciences/Qiagen) as the reference gene for each species.

Animal studies

All experiments were approved by the Health Sciences Animal Care Committee, University of Calgary and conform to the guidelines set by the Canadian Council on Animal Care (protocol # AC12-0146). Mice were used throughout the current study because they represent an experimental system that encompasses the complex interplay between the intestinal epithelium, immune system and microbiota in the maintenance of intestinal mucosal homeostasis. Furthermore, the mouse model allows for the incorporation of transgenic mice to better characterize the mechanisms the contribute to mucosal healing and assessment of pre-clinical targets for the treatment of intestinal inflammation. To control for intestinal microbiota influences, mice from the same vendor/source were used for all comparisons. Male wild-type (C57Bl/6; Taconic) and Nr1i3-/- (Taconic; designated CAR-/- throughout the manuscript) mice were used between 8-10 weeks of age (20-25 grams) for the experiments presented in Figure 2.

For our CAR agonist intervention studies (presented in Figures 7-8), male wild-type (C57Bl/6; in-house colony) were used between 8-10 weeks of age (20-25 grams) for the experiments. Mice were housed in Tecniplast ventilated cage systems under specific-pathogen free conditions (standard 12 hr light/dark cycle), with wood shaving-based bedding, free access to chow and autoclaved water, and housed with no more than 5 animals per cage. All mice were randomly allocated to cages designated for specific treatment groups by vivarium staff, upon transfer from the breeding barrier unit into the animal housing room. All mice purchased from vendors or transferred from our breeding barrier unit were acclimatized in the animal housing room for 7 days prior to commencing experiments.

Colitis was induced by the addition of dextran sulphate sodium (DSS; 2.5% w/v, molecular weight, 40,000; ICN Biomedical) to the drinking water, as described previously (Hirota et al., 2011). Animals were assessed and body weights recorded daily. For the first study, CAR-/- and WT mice were exposed to DSS for 5 days, and then allowed to recover from colitis for 7 days (consuming normal drinking water) (Terc, Hansen, Alston & Hirota, 2014). For the second study, WT mice were exposed to DSS for 7 days while being treated concurrently with TCPOBOP (3 mg/kg; daily; oral gavage) or vehicle (matched volume of sterile corn oil). In the final study, WT mice were exposed to DSS for 5 days and then allowed to recover from colitis for 7 days while being treated with TCPOBOP (3 mg/kg; daily; oral gavage) or vehicle (matched volume of sterile corn oil) (Terc, Hansen, Alston & Hirota, 2014). At the end of each experiment, mice were anaesthetized with isoflurance (5%) and euthanized by cervical dislocation. Following this, colonic tissues were removed and snap-frozen to be assessed for tissue myeloperoxidase (MPO) activity (an inflammatory index of granulocyte infiltration), as we have described previously (Hirota et al., 2011; Terc, Hansen, Alston & Hirota, 2014); or formalin-fixed, stained with hematoxylin and eosin, and scored histologically in a blinded fashion for inflammation and architectural changes (Hirota et al., 2012; Hirota et al., 2011). An inflammation score was tabulated using the following criteria: 0 - no inflammation; 1 - mild infiltration of inflammatory cells in the lamina propria, 2 - moderate infiltration of inflammatory cells in the lamina propria; 3 - increased inflammatory cells noted above the muscularis mucosa only; 4, increased inflammatory cells involving the submucosa and above; 5, increased inflammatory cells involving the muscularis and/or serosa. A histological score was tabulated using the following criteria: 0 - normal; 1 - loss of the basal 1/3 of the crypt; 2 - loss of the basal 2/3 of the crypt; 3 - loss of the

entire crypt but intact epithelium; 4 - loss of the entire crypt with mild epithelial damage; 5 - complete loss of crypt architecture and severe epithelial damage.

Cell Culture

To model the intestinal epithelium *in vitro*, we used Caco-2 cells (adult human colonic epithelial cell line derived from a colorectal adenocarcinoma: American-Type Culture Cell (ATCC)). Caco-2 cells were propagated in Dulbecco's modified Eagle medium with 20% fetal bovine serum and penicillin-streptomycin (100ug/mL, 1nmol/L; Invitrogen, Carlsbad, CA). Cells were split 1:10 upon reaching 75% confluence. All experiments were performed on cell passages 15-30.

Western Blots

To confirm the expression of the CAR in our Caco-2 cell line and murine intestinal tissues, we performed western blots using a polyclonal anti-CAR antibody (ab62590; Abcam). For Caco-2 samples, total cytosolic protein was isolated from cells at 1 to 14-days post confluence. Human liver tissue lysate (ab29889; Abcam) was used as a positive control for Caco-2 blots. Mouse small and large intestinal crypts, and whole colonic tissue lysates were isolated, as we have described previously (Hirota et al., 2011), and total cytosolic protein isolated. In both cases, cells were lysed in buffer (150mM NaCl, 20mM Tris, pH 7.5, 1mM EDTA, 1mM EGTA, 1%Triton X-100, and protease inhibitor cocktail; phosphatase inhibitor cocktail - Complete Minitab; Complete PhoStoP, Roche), total protein quantified using the Precision Red Advanced Protein Assay (Cytoskeleton) and sample protein concentration equalized. Samples were separated via SDS-PAGE, transferred to a PVDF membrane, blocked with 5% w/v bovine serum albumin (BSA), and probed with a 1.25 µg/mL of primary antibody (ab62590) (Abcam), followed by incubation with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), and then treated with West Femto detection reagents (Thermo Fisher Scientific). Blots were imaged using a Bio-Rad ChemiDoc XRS (Bio-Rad). In whole colonic lysates, the band intensity was quantified using ImageJ (NIH) and data expressed as a percentage of β-actin loading control.

To assess p38 MAP kinase activation, cells were seeded at a density of 5 x 10^7 cells/mL onto 12-well plates and allowed to grow to 3-days post confluence. Cells were treated with the selective human CAR agonist, CITCO, or vehicle control. Following treatment, total cytosolic

protein was isolated, as described above. Samples were separated via SDS-PAGE and transferred to nitrocellulose membranes (0.2 μm pores; BioRad), blocked with 5% milk in PBS-T, and blotted with antibodies. Active and total p38 MAP kinase expression was assessed using anti-phospho-p38 MAP kinase (Thr180/Tyr182; D3F9; Cell Signaling) and anti-total p38 MAP kinase (D13E1; Cell Signaling) antibodies, respectively. Active and total ERK MAP kinase expression was also examined using anti-phospho-p44/42 MAP kinase (ERK 1/2) (Thr202/Tyr204) (9106, Cell Signaling) and anti-total p44/42 MAP kinase (ERK 1/2) (L34F12) (4696; Cell Signaling) antibodies. Blots were imaged using a Bio-Rad ChemiDoc XRS (Bio-Rad) and band intensity quantified using ImageJ (NIH). Data are expressed as percentage of phospho-p38/phospho ERK1/2 compared to total p38/ total ERK1/2 in each sample.

Wound Healing

To examine the effect of CAR activation on wound healing, Caco-2 (ATCC) cells were seeded at a density of 3 x 10^5 cells/mL in ibidi cell culture inserts (IBIDI) using standard 12-well plates (Corning). At 3-days post-confluence, the culture inserts were removed to expose a 500 μm gap, to model a wound between cell fronts, as we have done previously (Terc, Hansen, Alston & Hirota, 2014). Cells were then incubated with 5% FBS-OptiMEM (Invitrogen) containing either CITCO or matching volumes of sterile DMSO vehicle control. Wound closure was assessed continuously using the Evolve Widefield microscope and Nikon NIS Elements software (Nikon), capturing magnified images of cell fronts at 4 time points per hour for 24 hr following addition of reagents. Data were analyzed as a percentage of wound closure compared to initial wound using FIJI/ImageJ software (NIH).

To assess the role of the p38 MAP kinase pathway in the CAR accelerated wound healing, Caco-2 cells (ATCC) were propagated and plated, as described above. Prior to CAR activation, cells were incubated with media containing either the p38 MAP kinase inhibitor SB202190 (10 μM; Sigma Aldrich) or sterile DMSO vehicle control. Wound healing was then assessed and quantified, as described above.

Migration Assays

To determine if CAR activation enhanced cell migration, two methods were employed. First, individual cells from the live cell wound healing videos were tracked over 24 hours to examine and quantify cell movement. MTrackJ software was utilized and eight cells were tracked

per treatment group and the corresponding DMSO vehicle control. Cell movement was quantified and expressed as arbitrary units of length.

Next, cell migration was assessed using the xCELLigence RTCA DP Instrument (ACEA Biosciences Inc.) (Shelfoon, Shariff, Traves, Kooi, Leigh & Proud, 2016). Prior to cell plating, a background impedance measurement was performed and each well set to a value of zero. Caco-2 cells were then plated on CIM-Plates 16 (modified Boyden chamber plates with electrodes imbedded within the basolateral aspect of the porous membrane; median pore size of 8 μm; ACEA Biosciences Inc.) at a concentration of 1 x 10^4 cells per well, and treated with the selective CAR agonist CITCO, or sterile DMSO vehicle control (in both the upper and lower chambers to avoid the generation of a concentration gradient). Cell migration across the porous membrane over time was then assessed by continuous measurement of electrical impedance on the basolateral aspect of the membrane and the data normalized after the first 20 minutes of data collection to account for minor electrical differences between wells. Impedance was assessed over the course of 24 hours and data expressed as Normalize Cell Index.

Proliferation Assay

To examine cell proliferation in response to treatment with the selective CAR agonist CITCO, confluent Caco-2 cells were split and plated in a 6-well plates (Corning) and allowed to grow to 3 days post-confluence. At this point, the cells were trypsinized, and 6 x 10^4 cells plated per well in standard media (Dulbecco's modified Eagle medium supplemented with 20% fetal bovine serum and penicillin-streptomycin; 100μg/mL, 1nmol/L; Invitrogen) in a 96-well plate (Corning). After 24 hours, wells were loaded with 5% FBS-Opti-MEM or FBS-free-Opti-MEM (Invitrogen) containing increasing concentrations of CITCO, an FBS-free negative control, or a 5% positive FBS control. After 24 hours of treatment, the growth medium was removed, the cells fixed with PBS-buffered formalin (3.7% v/v) and stained with crystal violet dye (0.1%w/v solution). Wells were then rinsed with tap water for 15 min and the dye solubilized with 33% acetic acid. The absorbance of each well was then measured at 570 nm via utilizing a spectrophotometer.

Statistical analysis

All data were assessed for Gaussian distribution using the D'Agostino & Pearson omnibus normality test. Multiple comparisons of parametric data were assessed using either a 1-way or

2-way ANOVA. Bonferroni post-tests were performed when the respective F achieved $P<0.05$ and no significant variance inhomogeneity detected. Multiple comparisons of data that were not deemed normally distributed were assessed using the Kruskal-Wallis test followed by the Dunns post-test. GraphPad Prism 5.0 was used for all analyses and to plot data points graphically. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

Results

NR1I3 expression is reduced during intestinal inflammation

The CAR, encoded by *NR1I3* is highly expressed in the liver (Moore et al., 2006) and exhibits modest expression in the gastrointestinal tract (Arnold, Eichelbaum & Burk, 2004). Considerably less is known about how its expression may change during active intestinal inflammation. To determine what effect active mucosal inflammation has on the expression of the CAR, intestinal mucosal biopsies (obtained from the colon) were collected from patients with active UC and CD patients (non-ulcerated; endoscopically confirmed inflammation), and compared to samples collected from non-IBD healthy controls. Real-time PCR assessment of *NR1I3*, revealed significantly reduced expression in the samples obtained from UC and CD patients with active inflammation (Fig. 1A), compared to non-IBD healthy control samples. Furthermore, the expression of *ABCB1*, which encodes the MDR1 protein and is a known transcriptional target of the CAR, was also significantly reduced in UC and CD samples (Fig. 1B).

To parallel this experimentally, we assessed the expression of the *Nr1i3* in samples isolated from mice exposed to DSS. Using western blot, we first confirmed the expression of the CAR protein in colonic and ileal crypts freshly isolated from naïve non-inflamed C57/Bl6 mice (Fig. 1E). Following 7-days of DSS exposure, we found that *Nr1i3* and *Abcb1a* expression were significantly reduced in intestinal tissues isolated from colitic mice (Fig. 1C-D). This correlated with a reduction in CAR protein in whole colonic lysates isolated from DSS-treated mice (Fig. 1F-G). Taken together, these data suggest that CAR expression, and its downstream signaling, is reduced during intestinal inflammation.

CAR-/- mice display delayed mucosal healing following DSS-induced intestinal tissue damage and inflammation

To assess the role of the CAR in the intestinal mucosa, we exposed WT and CAR-/- mice to a course of DSS to trigger intestinal mucosal damage, inflammation and ensuing reparative processes, as we have done previously (Terc, Hansen, Alston & Hirota, 2014). Exposure to DSS triggered significant weight loss and increased colonic tissue MPO levels (an index of neutrophilic infiltration) in both WT and CAR-/- mice, compared to naïve WT and CAR-/- mice (Fig. 2A-B). There was no significant difference in either of these measures in DSS-treated WT and CAR-/- mice, suggesting that the loss of CAR signaling does not increase the sensitivity to DSS exposure (Fig. 2A-B). However, our histological analysis of colonic tissues isolated following 7 days of

recovery revealed that DSS-treated CAR-/- mice exhibited delayed mucosal healing (Fig. 2C-D). Our scoring of tissue inflammation and mucosal architecture quantified these effects, revealing that the colonic tissues isolated from DSS-treated CAR-/- mice exhibited significantly more residual inflammation and mucosal damage compared to their DSS-treated WT counterparts (Fig. 2E-F).

The CAR is expressed in intestinal epithelial cells (IECs) and its activation enhances wound closure

We reported previously that activation of the PXR, a close relative to the CAR, enhanced intestinal epithelial wound healing (Terc, Hansen, Alston & Hirota, 2014). The CAR and PXR share similar pharmacology and some overlapping signaling properties (Banerjee, Robbins & Chen, 2015). Given the reduced healing observed in DSS-treated CAR-/- mice, we hypothesized that the CAR, like the PXR, may regulate intestinal epithelial wound healing and mucosal restitution following damage. While the expression and signaling function of the CAR in IECs has been reported previously (Martin, Riley, Back & Owen, 2008), some have reported that its expression is variable and depends on cell differentiation and the degree of monolayer confluence (Arnold, Eichelbaum & Burk, 2004). Thus, we first sought to assess the expression of the CAR in Caco-2 IECs over the course of post-confluence differentiation. We found that Caco-2 IECs express the CAR as early as 1 day post-confluence, and that its expression does not change over the course of 14 days of differentiation (Fig. 3A).

To test our hypothesis, we first performed wound healing assays in Caco-2 monolayers, treated with the selective human CAR agonist CITCO (Maglich et al., 2003). We found that selective activation of the CAR enhanced wound closure (Fig. 3B-C). Furthermore, CITCO-treatment enhanced the expression of the CAR target gene *ABCB1* in Caco-2 cells (Fig. 3D). To confirm that the CITCO-induced effect was driven by CAR activation, we employed the CAR inverse agonist EE2 (Jyrkkarinne et al., 2005). Treating our monolayers with EE2, had a modest, but statistically significant, effect on basal wound closure (Fig. 3E). On the other hand, EE2 significantly reduced CITCO-induced wound closure approximately 30% (Fig. 3F). Interestingly, while the acceleration of CITCO-induced wound closure appears rapidly (Fig. 3C), the effects of EE2 manifest later in the response (~16 hr post-CAR activation; Fig. 3F).

CAR activation enhances IEC migration, an effect that is associated with p38 MAP kinase activation

The intestinal epithelial wound healing response involves both cell migration and proliferation (Iizuka & Konno, 2011). We previously reported that selective activation of the PXR enhances Caco-2 IEC migration, a response that contributed to the enhancement of wound closure (Terc, Hansen, Alston & Hirota, 2014). Thus, we first assessed whether CAR activation could enhance IEC migration. When analyzing our live-cell imaging experiments, we found that stimulation of the CAR with 50 nM CITCO significantly enhanced the distance travelled by individually tracked cells (Fig. 4A-B), suggesting that CAR activation may be enhancing wound closure by inducing cell migration. To quantify cell-migration differently, we performed modified Boyden chamber experiments using the xCELLigence RTCA DP Instrument, which quantifies the movement of cells by detecting a change in impedance on electrodes on the basolateral aspect of the porous filter (Shelfoon, Shariff, Traves, Kooi, Leigh & Proud, 2016). In support of our cell-tracking data, treating Caco-2 IECs with 50 nM CITCO significantly increased the migratory response (Fig. 4C).

Since the activation of xenobiotic receptors in hepatocytes has been associated with enhanced proliferation and liver hypertrophy (Ross et al., 2010), we next sought assess whether CAR activation could be driving Caco-2 proliferation. In contrast to our migration studies, no concentration of CITCO enhanced cell proliferation, either in the presence or absence of FBS (Fig. 5A-B).

p38 MAP kinase signaling has been implicated in mediating agonist-induced IEC migration (Frey, Golovin & Polk, 2004). We reported previously that PXR-induced Caco-2 migration was associated with increased activation of p38 MAP kinase (Terc, Hansen, Alston & Hirota, 2014). Thus, we sought to assess whether activation of the CAR could evoke a similar response. Indeed, treating Caco-2 IECs with 50 nM CITCO significantly increased the detection of phosphorylated activated p38 MAP kinase (Fig. 4D-E). In contrast, selective activation of the CAR had no effect on the phosphorylation of ERK1/2 MAP kinase (Fig. 5C-D).

Inhibition of p38 MAP kinase attenuates CAR-associated enhancement of wound healing

Since CAR activation enhanced cell migration and p38 MAP kinase activation, we sought to assess the role of this signaling cascade in the enhancement of intestinal epithelial wound healing. We, and others, have reported previously that selective inhibition of p38 MAP kinase signaling attenuates PXR-dependent cell migration (Kodama & Negishi, 2011; Terc, Hansen,

Alston & Hirota, 2014). We found that pretreating cells with SB202190, a selective p38 MAP kinase inhibitor, attenuated CITCO-induced enhancement of wound closure (Fig. 6A-B), an effect that was associated with reduced cell movement, as evidenced by a reduction in the distance travelled by individually tracked cells (Fig. 6C-D).

Selective activation of the CAR enhances mucosal repair following DSS-induced intestinal tissue damage and inflammation

To assess the therapeutic potential of CAR activation on intestinal mucosal healing, we employed the selective mouse CAR agonist TCPOBOP (Tzameli, Pissios, Schuetz & Moore, 2000). We first sought to assess whether treating mice with TCPOBOP (3 mg/kg; daily; oral gavage) would alter their sensitivity to DSS-induced colitis. We found that concurrent treatment with TCPOBOP had no effect on the acute response to DSS exposure. TCPOBOP-treated mice exhibited a weight loss trajectory and tissue MPO levels that were no different than those observed in vehicle-treated mice (Fig. 7A-B). We next sought to assess whether CAR activation could enhance mucosal healing and inflammatory resolution following DSS exposure. In this experiment, WT mice were exposed to DSS for 5 days and then allowed to recover for 7 days. During the recovery phase, mice were treated with either TCPOBOP (3 mg/kg; daily; oral gavage) or vehicle (matched volume of sterile corn oil). While both groups followed a similar weight loss trajectory (Fig. 8A), TCPOBOP-treated mice exhibited significant reductions in colonic MPO levels (Fig. 8B), enhanced mucosal healing and accelerated inflammatory resolution (Fig. 8C-D). Our histological scoring revealed that TCPOBOP treatment reduced the colonic inflammatory and architecture scores to levels exhibited by non-DSS-treated mice (Fig. 8E-F; naïve and TCPOBOP alone).

Discussion

Previous studies have implicated xenobiotic receptors as key regulators of intestinal mucosal homeostasis, based on their ability to modulate the function of the intestinal epithelium and resident mucosal immune cell populations (Chng et al., 2016; Garg et al., 2016; Ji et al., 2015; Qiu et al., 2013; Venkatesh et al., 2014). We have previously reported that the PXR, a receptor closely related to the CAR, enhances intestinal epithelial wound healing and protects the mucosal barrier during inflammatory stress (Garg et al., 2016; Terc, Hansen, Alston & Hirota, 2014). Furthermore, others have characterized the anti-colitic effects of PXR signaling (Cheng et al., 2010; Shah, Ma, Morimura, Kim & Gonzalez, 2007). In the current study, we have identified a novel role for the CAR in regulation of intestinal mucosal healing. We found that CAR transcript expression was significantly reduced in mild to moderately inflamed colonic mucosa from patients with either UC or CD. Using the DSS model of experimental colitis, we were able to recapitulate these findings *in vivo*. Subsequent to this, we were able to use *in vitro* and *in vivo* approaches to provide functional evidence that the CAR can regulate intestinal epithelial wound healing and mucosal restitution following inflammation-associated tissue damage.

The functional importance of the CAR has been widely characterized in the liver, where it acts as a xenobiotic receptor that can sense and respond to chemicals of environmental origin (Banerjee, Robbins & Chen, 2015; Burk, Arnold, Geick, Tegude & Eichelbaum, 2005). In this capacity, the CAR's activation enhances hepatic detoxification and efflux processes by increasing the expression of phase I/II enzymes and chemical transporters. More recently, studies have begun to implicate the CAR as a regulator of additional physiological processes, such as energy metabolism. In these reports, pharmacological activation of the CAR improved the metabolic parameters in an experimental model of type II diabetes by improving glucose tolerance, insulin sensitivity, inducing β-oxidation and reducing lipid deposition (Dong et al., 2009; Gao, He, Zhai, Wada & Xie, 2009). Interestingly, little is known about whether the CAR can regulate inflammatory processes as they relate to metabolic syndromes.

In the current study, our data suggest that targeting the CAR may prove valuable in the clinical management of the inflammation-associated mucosal damage observed in patients with IBD. Interestingly, altered expression of genes related to xenobiotic detoxification processes has been observed in IBD patients. Mencarelli *et al.* (2010) reported that selective activation of the PXR could normalize the expression of a number of genes related to xenobiotic metabolism (Mencarelli et al., 2010), many of which can also be transcriptionally regulated by the CAR. For

instance, the *MDR1* gene (*ABCB1*), which encodes P-glycoprotein and efflux pump that protects against exogenous toxins, is associated with increased IBD susceptibility (Zhao, Wang, Yao, Sun & Li, 2015). *MDR1* is a common downstream target of the CAR (Burk, Arnold, Geick, Tegude & Eichelbaum, 2005), PXR (Geick, Eichelbaum & Burk, 2001) and AhR (Mathieu, Lapierre, Brault & Raymond, 2001) and its genetic deletion in mice results in spontaneous intestinal inflammation (Banner, Cattaneo, Le Net, Popovic, Collins & Gale, 2004). Kabakchiev *et al.* (2014) used gene-network analysis and discovered that the expression of genes involved in xenobiotic metabolism, including the *MDR1* gene, were reduced in IBD patients that exhibited a CD-like disease recurrence following ileal pouch-anal anastomosis (Kabakchiev, Tyler, Stempak, Milgrom & Silverberg, 2014). More recently, Weiser *et al.* (2016) reported that adult and pediatric CD phenotypes exhibit differential expression of genes related to distinct Reactome-defined pathways, including a gene-cluster associated with xenobiotic metabolism (Weiser et al., 2016).

While a role for the CAR in intestinal tissues is not well characterized, some have reported that local and systemic inflammatory processes may regulate the CAR's expression in the gastrointestinal tract. Inflammatory mediators that activate downstream NFκB signaling have been reported to repress the expression of several nuclear receptor family members, including the PXR and CAR. In human and mouse hepatocytes, CAR transcript expression is reduced by exposure to inflammatory mediators, such as IL-1β, IL-6 and lipopolysaccharide (LPS) (Assenat et al., 2004; Beigneux, Moser, Shigenaga, Grunfeld & Feingold, 2002). Using the TNBS model of experimental colitis, Zhou *et al*. (2013) reported that intestinal inflammation was associated with reduced expression of the CAR transcript the small intestine, and to a lesser extent the colon (Zhou et al., 2013). Furthermore, Liu *et al.* (2011) reported that intestinal *Helicobacter bilis* infection and the subsequent increased susceptibility to DSS-induced colitis was associated with reduced CAR transcript expression in the colon (Liu et al., 2011). In our study, we found that CAR transcript expression was significantly reduced in inflamed tissue obtained from patients with UC and CD compared to samples isolated from non-inflamed healthy controls. This was paralleled by a decrease in CAR transcript expression in colonic tissue isolated from DSS-exposed mice. Interestingly, IL-1β and IL-6 are significantly increased in patients with IBD (Grottrup-Wolfers, Moeller, Karbach, Muller-Lissner & Endres, 1996), and within the tissues of DSS-exposed mice (Murano et al., 2000). These data suggest that inflammation may exert negative effects on xenobiotic defense mechanisms within the intestinal mucosa, effects that have been well described in the liver during hepatic inflammation (Zhou et al., 2006).

The functional significance of reduced xenobiotic sensing in the intestinal mucosal has yet to be completely clarified. In our study, we found that reduced CAR expression was associated with decreased expression of MDR1, suggesting that xenobiotic defenses may be compromised in the inflamed intestinal mucosa of IBD patients and colitic mice. The PXR and AhR, which are both expressed in the intestinal tissues and resident immune cells, have each been implicated in the regulation of mucosal homeostasis and their altered expression/function linked to UC and CD susceptibility, suggesting that dysfunctional xenobiotic response/protection mechanisms may contribute to IBD pathogenesis. To understand the functional impact of altered CAR expression during intestinal mucosal inflammation, we sought to first characterize its physiological role using the DSS model of colitis. We, and others, have used this model previously to assess aspects of mucosal restitution and intestinal epithelial wound healing (Ross et al., 2010; Terc, Hansen, Alston & Hirota, 2014). In our study, we found that CAR-/- mice exhibited significantly more mucosal damage and inflammation following 7 days of recovery from DSS-induced colitis, suggesting the CAR may regulate mucosal restitution and repair following intestinal injury. While these mice were not littermates, both WT and CAR-/- mice were purchased from Taconic and housed under the same conditions in our vivarium to best control for microbiota effects.

To determine how the CAR might be regulating the repair response following colitis, we assessed whether its activation could modulate intestinal epithelial wound healing. Treating wounded epithelial monolayers with the selective human CAR agonist CITCO significantly enhanced intestinal epithelial wound closure, an effect that was associated with increased cell migration, but no change in cell proliferation. While this is the first report outlining the role of the CAR in IEC migration, others have reported that CAR-dependent events contribute to hepatocyte migration. Kamino *et al.* (2011; 2011) reported that CAR-induced expression of FAM84A (Kamino et al., 2011) and TUBA8 (Kamino et al., 2011) increased the migration of HepG2, but not Huh7 cells. Furthermore, CAR activation increases the expression of GADD45beta (Columbano et al., 2005), an early response gene whose product can directly activate p38 MAP kinase and is associated with xenobiotic receptor-mediated cell migration (Kodama & Negishi, 2011). In agreement with the latter, selective activation of the CAR was associated with p38 MAP kinase phosphorylation. Furthermore, we found that selective inhibition of p38 MAP kinase signaling attenuated CITCO-induced wound healing and IEC migration.

Although we hypothesized that p38 MAP kinase acts downstream from the CAR, others have reported a complex interaction between the two proteins that directly modulates CAR-

dependent gene transcription (Hori, Moore & Negishi, 2016; Saito, Moore & Negishi, 2013). Recently, Hori *et al.* (2016) reported that p38 MAP kinase forms a complex with the CAR regulating its ability to bind DNA. Through its binding to the CAR, nuclear p38 MAP kinase enhances CAR/RXR heterodimer binding to phenobarbital-responsive enhancer modules within CAR-induced genes, subsequently phosphorylating the CAR, leading to its eventual inactivation (Hori, Moore & Negishi, 2016). Interestingly, p38 MAP kinase inhibition with SB202190, the inhibitor used in our study, has been reported to negatively regulate the expression of *MDR1*, a CAR target gene, in other systems (Guo et al., 2008). While our data support a role for p38 MAP kinase in the enhanced wound healing observed in our system, additional work will be required to determine whether it functions upstream or downstream from CAR activation in IECs.

Previous reports have outlined a reciprocal interaction between xenobiotic receptor signaling and tissue inflammation. Interestingly, activation of the PXR (Zhou et al., 2006) and AhR (Lee et al., 2015) can modulate inflammatory signaling by interacting with NFκB signaling cascade. Little is known about how the CAR interacts with inflammatory signaling pathways. In our study, we found that CAR-/- mice exhibited a similar weight loss profile during the 5 days of DSS exposure. Furthermore, selective activation of the mouse CAR with TCPOBOP during the initiation of acute colitis had little effect on colonic inflammation or disease severity. Taken together these data suggest that, unlike the PXR and AhR, the CAR does not exhibit direct anti-inflammatory effects that dampen the response to DSS exposure. However, treating mice with TCPOBOP during recovery from colitis enhanced mucosal healing and reduced tissue inflammation, the latter reflected by reduced tissue MPO levels and reduced histological inflammation scores. In this context, activation of the CAR may be reducing tissue inflammation by accelerating mucosal repair and restitution. On the other hand, CAR expression was recently reported in tumor-associated macrophages in the airways, suggesting its activation may regulate inflammation (Fukumasu et al., 2015). While these findings have yet to be confirmed by others, it is apparent that additional studies are required to determine whether the CAR plays a direct role in modulating mucosal immune cell function.

To our knowledge this is the first report implicating the CAR in the regulation of intestinal epithelial wound healing and mucosal restitution following inflammation-associated injury. We, and others, have reported that the PXR, a closely related receptor, plays an important role in gastrointestinal tract, regulating barrier function, inflammatory signaling and mucosal repair following injury (Cheng et al., 2010; Garg et al., 2016; Shah, Ma, Morimura, Kim & Gonzalez, 2007;

Terc, Hansen, Alston & Hirota, 2014; Venkatesh et al., 2014). These studies and others, in conjunction with the data presented herein, suggest that xenobiotic receptors play a key role in the maintenance of intestinal mucosal homeostasis, and that their dysfunction may contribute to the pathogenesis of IBD. Furthermore, given the interest in targeting the CAR for the treatment of metabolic disorders, including obesity and diabetes, our data support developing gut-specific agents to target this receptor for the treatment of IBD.

Author contributions

GMH, KLF, AZ and LA performed experiments, analyzed data and drafted portions of the manuscript.

CA was involved in obtaining and processing tissue samples from DSS-exposed colitic mice.

CLH, SG and KPR were involved in obtaining tissue samples from human subjects, isolating RNA, generating cDNA and running qPCR experiments.

SM, TKH and SAH designed experiments, supervised the study team, analyzed data and wrote/edited the manuscript.

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Figure Legends

Figure 1: The expression of the constitutive androstane receptor (CAR) is reduced during intestinal inflammation. A) The expression of *NR1I3*, the gene that encodes the CAR, is significantly reduced in intestinal mucosal biopsies obtained from UC and CD patients (N = 8 health control; N = 8 UC; N = 10 CD) with active mild to moderate inflammation. B) The expression of ABCB1, a CAR target gene which encodes the MDR1 protein, is significantly reduced in samples from UC and CD patients. C-D) The expression of *Nr1i3* and *Abcb1a* is significantly reduced in colonic tissues isolated from DSS-treated mice (male mice; 10 weeks of age; 2.5% w/v in drinking water for 7 days) compared to non-DSS treated control mice. (N = 5 control; N = 5 DSS-treated mice). E) The CAR is expressed in intestinal crypts isolated from the colon and ileum of male C57/Bl6 mice (10 weeks of age). F) The expression of the constitutive androstane receptor (CAR) is reduced in the colonic tissue of DSS-treated mice as assessed by western blot. G) The pooled densitometry of CAR expression in control vs. DSS-treated mice expressed as a percentage of β-actin expression (N = 5 control; N = 5 DSS-treated mice). * denotes $p<0.05$; human data are expressed as mean +/- SD; mouse data are expressed as mean +/- SEM.

Figure 2: CAR-/- mice exhibit delayed recovery from DSS-induced colitis. A) The body weight of WT C57/Bl6 and CAR-/- mice treated with DSS (male; 10 weeks of age; 2.5% w/v; in the drinking water) for 5 days and then allowed to recovery for 7 days with normal drinking water. * denotes $p<0.05$ for WT + DSS and CAR-/- + DSS compared to Naïve WT and Naïve CAR-/- groups. B) Colonic tissue MPO levels assessed at experimental day 12, after 7 days of recovery from DSS-induced colitis. * denotes $p<0.05$ WT + DSS and CAR-/- compared to Naïve WT and Naïve CAR-/-. C, D) Representative images of colonic tissues (distal colon nearest the centre of the roll) isolated from mice at experimental day 12. Blind histological scoring of the histological samples assessing the magnitude of colonic tissue inflammation (E) and architectural changes (F). # denotes $p<0.05$ compared to Naïve WT and Naïve CAR-/-; * denotes $p<0.05$ compared to DSS WT; (N = 8 Naïve WT; N = 8 Naïve CAR-/-; N = 8 DSS-treated WT mice; N = 8 DSS-treated CAR-/- mice).

Figure 3: The constitutive androstane receptor (CAR) is expressed in the Caco-2 cell line and its activation enhances intestinal epithelial wound closure. A) Representative western blot depicting the expression of the CAR in Caco-2 cells at various time-points post-confluence compared to the positive control (human liver tissue lysate - ab29889; Abcam). B)

Representative images depicting the extent of wound closure at 12 and 24 hours post-treatment with various concentration of CITCO, a selective CAR agonist, or the DMSO vehicle (Control). C) The pooled data from the experiments depicted in panel B expressed as a percentage of the original wound. * denotes $p<0.05$ 50 nM CITCO compared to all other groups; ^ denotes $p<0.05$ 25 nM nM compared to Control; N = 8 separate experiments. D) 50 nM CITCO evokes the expression of *ABCB1* in Caco-2 cells. * denotes $p<0.05$ compared to all groups; N = 5. E) The CAR selective inverse agonist 17α-ethynyl-3,17β-estradiol (EE2; 10 μM) reduced the basal/non-stimulated wound closure between 19-24 hr. F) EE2 pretreatment significantly attenuates CITCO-induced responses. In panel E, * denotes $p<0.05$ compared to control; In panel F, * denotes $p<0.05$ compared to 50 nM CITCO group; N = 6 separate experiments.

Figure 4: Activation of the CAR enhances cell movement and p38 MAP kinase activation in Caco-2 intestinal epithelial cells. A) Representative images of cell movement tracks in Caco-2 monolayers over the course of 24 hours of treatment with various concentration of CITCO, a selective CAR agonist, or the DMSO vehicle (Control). B) The average length of cell movement tracks quantified from the images depicted in panel A. Units of Length were assessed in 6-8 cells per separate experiment and the average plotted in this graph. * denotes $p<0.05$ compared to Control and 25 nM CITCO; N = 8 separate experiments. C) Caco-2 cells exhibit enhanced migration across a porous membrane (8 μm pore diameter) when treated with CITCO (50 nM), as assessed with xCELLigence Real Time Impedance Analysis. Vehicle control or CITCO were place in both the apical and basolateral chambers to avoid the generation of a concentration gradient. * denotes $p<0.05$ compared to Control; N = 6 separate experiments. D) Representative western blot depicting the induction of p38 MAP kinase phosphorylation following stimulation of the CAR by CITCO (50 nM). This blot is representative of 4 separate experiments. E) The pooled densitometric analysis of CITCO-induced p38 MAP kinase phosphorylation in Caco-2 cells. * denotes $p<0.05$ compared to Control (DMSO vehicle); N = 6 separate experiments.

Figure 5: Activation of the CAR in Caco-2 cells does not induce proliferation, nor does it activate the ERK1/2 MAP kinase signaling cascade. Proliferation of Caco-2 cells in response to varying concentrations of CITCO, a selective CAR agonist, as assessed by crystal violet staining. In A), the Control group was treated with serum-free OptiMEM (A) while all other groups were assayed in 5% FBS-containing OptiMEM with the depicted CITCO concentration for 24 hours. * denotes

p<0.05 compared to Control (serum-free media); N = 7 separate experiments. In B) the Control group was treated with serum-free OptiMEM while the experimental groups were assayed in serum-free OptiMEM with the depicted CITCO concentration for 24 hours. * denotes p<0.05 compared to Control (serum-free media); N = 5 separate experiments. B) Representative western blot depicting the levels of ERK1/2 MAP kinase phosphorylation following stimulation of the CAR by CITCO (50 nM). This blot is representative of 6 separate experiments. C) The pooled densitometric analysis of CITCO-induced ERK1/2 MAP kinase phosphorylation in Caco-2 cells. N = 6 separate experiments.

Figure 6: Inhibition of p38 MAP kinase signaling attenuates CITCO-induced enhancement of wound closure and cell movement in Caco-2 intestinal epithelial monolayers. A) Representative images depicting the extent of wound closure at 12 and 24 hours post-treatment with CITCO (50 nM) in the presence and absence of SB202190 (10 μM), a selective inhibitor of p38 MAP kinase. B) The pooled data from the experiments depicted in panel A expressed as a percentage of the original wound. * denotes p<0.005 CITCO + SB202190 compared to CITCO alone; N = 7 separate experiments. C) Representative images of cell movement tracks in Caco-2 monolayers over the course of 24 hours of treatment with CITCO (50 nM) in the presence and absence of SB202190 or the DMSO vehicle (Control). D) The average length of cell movement tracks quantified from the images depicted in panel A. Units of Length were assessed in 6-8 cells per separate experiment and the average plotted in this graph. * denotes p<0.05 compared to all groups; # denotes p<0.05 compared to CITCO (50 nM); N = 7 separate experiments.

Figure 7: Activation of the CAR does not modify the acute response to DSS-induced colitis. Mice were treated with the selective CAR agonist TCPOBOP (male; 10 weeks of age; 3 mg/kg/oral gavage in corn oil) or vehicle and exposed to DSS (2.5% w/v in drinking water) for 7 days. A) The body weight of C57/Bl6 mice treated with DSS for 7 days; N = 7 per group. B) Colonic tissue MPO levels assessed at after 7 days of DSS exposure; N = 7 per group.

Figure 8: Activation of the CAR enhanced recovery from DSS-induced colitis. A) The body weight of C57/Bl6 mice treated with DSS (male; 10 weeks of age; 2.5% w/v; in the drinking water) for 5 days and then allowed to recovery for 7 days with normal drinking water while treated with a selective mouse CAR agonist (TCPOBOP; 3 mg/kg; oral gavage; daily) or vehicle. # denotes

$p<0.05$ for DSS + Vehicle and DSS + TCPOBOP compared to Naïve and TCPOBOP groups. B) Colonic tissue MPO levels assessed at experimental day 12, after 7 days of TCPOBOP treatment during recovery from DSS-induced colitis. # denotes $p<0.05$ compared to Naïve and TCPOBOP; * denotes $p<0.05$ compared to DSS + Vehicle; N = 5 per group. C, D) Representative images of colonic tissues (distal colon nearest the centre of the roll) isolated from mice at experimental day 12. Blind histological scoring of the histological samples assessing the magnitude of colonic tissue inflammation (E) and architectural changes (F). # denotes $p<0.05$ compared to Naïve and TCPOBOP; * denotes $p<0.05$ compared to DSS + Vehicle; N = 5 per group.

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