

Supplementary Materials

Materials & Methods

Cell culture.

Caco-2 human intestinal epithelial cells (passage 10-30), T84 human intestinal epithelial cells and HMEC-1 human microvascular endothelial cells were cultured as previously described.[20, 21, 22] Caco-2 and T84 cells were treated on Transwell® membranes (3.0µm pore;Costar) in triplicate with a combination of recombinant human TNF-α (10ng/ml; R&D Systems), recombinant human IL-1β (10ng/ml; R&D Systems) and recombinant human IFN-γ (10ng/ml; R&D Systems), or vehicle for 2 and 6 hours and harvested for mRNA analysis. HMEC-1 cells were treated identically on 6 well tissue culture plates (Falcon) and harvested for mRNA analysis (described below).

DSS colitis.

Netrin-1 heterozygous mice (CD1 mice), A2B adenosine receptor deficient (*Adora2b^{-/-}*) mice and corresponding littermate controls matched in gender, age and weight were used in DSS (dextran sulphate sodium) studies. For netrin-1 treatment studies, C57BL/6 at 10-12 weeks of age, matched in gender and weight were implanted with a subcutaneous osmotic pump (Alzet) for constant infusion of recombinant mouse netrin-1 (R&D Systems) at 1µg/mouse/day one day prior to DSS exposure. For antibody blocking studies, anti-UNC5B antibody (R&D systems) or control IgG (Goat IgG; R&D Systems) was administered at 800µg/kg/mouse intraperitoneally (I.P) two days prior to DSS (1 day prior to commencement of netrin-1 administration) and every second day thereafter until day 6 of DSS exposure. Treatment studies in *Adora2b^{-/-}* mice were performed with vehicle or netrin-1 treatment as previously described. Dextran sulfate sodium (DSS 36,000-50,000 MW; MP biomedical) induced colitis was performed using 4.5% DSS over a time period of 6-7 days, with measurement of disease activity parameters as described.[23]

Baseline weight and disease activity were determined relative to values collected on day 0. No weight loss was scored as 0, 1-3% as 1, 3-6% as 2, 6-9% as 3, and greater than 9% as 4. Stool consistency was scored as follows, 0 points for well formed stool, 2 points for loose stool with associated 3-6% weight loss, 3 points for loose stool with associated 6-9% weight loss and 4 points for diarrhea. Presence of blood was scored as 0 for none present, 2 points for in/on stool with associated 3-6% weight loss, 3 points for in/on stool with associated 6-9% weight loss and 4 points for active bleeding. All animals were handled according to procedures approved by the institutional committee for animal use at the University of Colorado.

Colon histology.

Post mortem colons were harvested by blunt dissection and measured. A 1 cm segment of transverse colon was fixed in 10% buffered-formalin (Sigma Aldrich) prior to staining of paraffin sections with haemotoxylin and eosin. Histological scoring was performed by a blinded pathologist. The scoring system consists of three categories, scored 0-3 for a total index of 9 adapted from a previously described system.[24] Inflammation index gives 0 points for no lamina propria inflammation, 1 represents increased lamina propria (LP) inflammatory cells, 2 represents confluence of inflammatory cells extending into the submucosa, 3 represents transmural inflammation. Injury index awards 0 points for no injury, 1 point for discrete lymphoepithelial lesions, 2 points for surface erosion/focal ulceration, 3 points for extensive mucosal damage and extension into deeper structures. Modified injury index gives 0 points for no crypt damage, 1 point for partial (up to 50%) crypt drop-out, 2 points for partial to complete crypt drop-out and 3 points for complete crypt drop-out.

Immunohistochemistry.

Formalin fixed paraffin embedded tissues were sectioned, deparaffinised and re-hydrated through descending series of ethanol/water baths. Tissues were antigen retrieved in citrate buffer (Vector Labs, CA), blocked with 5% bovine serum albumin/PBS (Sigma). Tissues were

incubated with polyclonal chicken anti-mouse netrin-1 antibody (Abcam) diluted 1:100. Following overnight incubation at 4°C, slides were rinsed in PBS and incubated with goat anti-chicken biotinylated secondary antibody (Vector Labs, CA). Slides were rinsed in PBS and incubated with Vectastain ABC Reagent (Vector Labs, CA) for 30 min. Immunopositive cells were visualised by addition of a 3,3-diaminobenzidine substrate (DAB; Vector Labs, CA), were counterstained with Methyl Green (Vector Labs, CA) and mounted in Cytoseal media (Thermo Fisher, PA). Negative controls were performed by omitting the primary antibody. In these slides no signal was observed. Images were acquired at 10X using a Nikon Eclipse Ti-S microscope.

Immunofluorescence.

Netrin-1 heterozygous mice or wildtype controls were anaesthetised using sodium pentobarbital (Nembutal, 100mg/kg) and perfused transcardially with cold 4% paraformaldehyde (PFA) in 0.1M sodium phosphate buffer (PB). Colon tissue was removed, cleaned of fecal matter, post fixed for three hours at room temperature and then cryoprotected overnight at 4°C in 20% sucrose in 0.1M PB. Sections of 18µm were sliced on a Lieca cryostat to super frost slides (Fisher Scientific) and stored overnight at -20°C. Slides were brought to room temperature, dried on a slide warmer, and washed three times in phosphate buffered saline (PBS). Sections were blocked in antibody media (0.1M PBS, 0.3M NaCl, 0.3% triton, 1% BSA) with 1% normal horse and 1% normal goat serums. Rabbit anti-PGP9.5 (Protein gene product; 1:500, ABD Serotec) or rabbit anti-CGRP (calcitonin gene related peptide; 1:5000, Peninsula Labs), were incubated in blocking solution overnight at 4°C. Three washes in PBS were followed by incubation in the secondary antibody goat anti-rabbit Alexa Flour 568 (1:400, Invitrogen) in blocking solution for two hours at room temperature. Slides were washed three more times in 0.1M PBS and then cover slipped using DAPI mounting media (Vector Labs). Images were acquired with a QImaging Retiga-400RV camera connected to an Olympus BX41 microscope. Confocal Images were acquired by an Olympus Fluoview Laser Scanning Confocal Microscope.

Acquired images were modified in Photoshop. Slides were analysed by two blinded experts in peripheral neuronal anatomy.

Western blotting.

Whole colonic tissue or mucosal scrapings were homogenised in tissue protein extraction reagent (Pierce). SDS-PAGE of 20-40µg of total protein followed by specific immunoblotting was performed as previously described.[2]

Real-time RT-PCR.

Total RNA was extracted from whole colonic tissue or cell pellets using the RNeasy Mini kit (Qiagen) according to the manufacturer. cDNA synthesis was performed using High capacity cDNA reverse transcription kit as described by the manufacturer (Applied Biosystems). Transcript quantification was performed with PowerSybr (Applied Biosystems) using specific primers for mouse netrin-1, TNF- α and IL-1 β (QuantiTect Primer assays, Qiagen) on the AB7900 real-time PCR system (Applied Biosystems). Quantitect primers (Qiagen) were used for netrin-1 transcript analysis in Caco-2 cells. Specific β -actin primers were used as internal control as described.[2]

Colonic lamina propria leukocyte harvest and flow cytometry.

Lamina propria leukocytes were isolated as previously described.[26] Intraepithelial lymphocytes and epithelial cells were removed from whole colonic tissue using 1mM EDTA, tissues were digested in collagenase VIII (Sigma Aldrich), filtered and viability assessed prior to cell counting and staining. Cells were incubated with fluorescent rat anti-mouse antibodies against GR-1(RB6-8C5; BD Pharmigen), anti-SiglecF (E50-2440; BD Pharmigen), anti-Ly-6G (1A8; BD Pharmigen), anti-Ly-6C (HK1.4; eBiosciences), CD45 (30-F11; BioLegend) and LIVE/DEAD® fixable aqua dead cell stain or their respective isotype controls. Cells were washed and fixed with 2% paraformaldehyde and analysed using the FACS® Canto system

(Beckton-Dickinson Immunocytometry Systems). Post-analyses were performed using FLOWJo software (Tree Star Inc). Percentage of live CD45 positive cells of each population were calculated and multiplied by the total cell number retrieved from organ harvest to calculate actual cell number of distinct sub-populations. For specific identification of monocytes/macrophages Ly-6G positive cells were excluded prior to analysis of Ly-6C and CD11b expression.

In vivo permeability assays.

Mice exposed to DSS for three days were administered FITC labeled dextran (4 kDa; Sigma Aldrich) by oral gavage at 0.6mg/g and a concentration of 80mg/ml, as described.[19] 4 hours later blood was drawn by retro-orbital bleed and plasma was separated. Serum-FITC levels were measured at 478nm using a BioTek Synergy 2.

TUNEL assay .

Intestinal epithelial cell apoptosis in formalin fixed paraffin embedded colonic sections following DSS exposure was determined with terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL; DeadEnd Colorimetric TUNEL System; Promega Corporation). Tissues were sectioned, deparaffinised and re-hydrated followed by proteinase K antigen retrieval as per manufacturer’s instructions. Sections were incubated with TUNEL reaction buffer for 1 hour at 37°C in a humidified chamber and were counterstained with hematoxylin. Colonic sections from 6 animals per group were assessed independently. The number of positively stained nuclei per 200 epithelial cells in three randomly selected fields per section were counted (600 cells per mouse). The percentage of positively stained cells were then determined. Counting was performed in an independent blinded fashion. Representative images were acquired at 20X using a Nikon Eclipse Ti-S microscope and quantified using Nikons NIS-Elements BR 3.0 quantitative imaging software.

***In vitro* permeability assays.**

Caco-2 intestinal epithelial cells were plated at 5×10^4 cells per well on 6.5mm polyester permeable Transwell® membranes (3.0µm pore;Costar) and cultured for a minimum of 21 days to allow for formation of a monolayer with appropriate barrier function as measured by transepithelial electrical resistance (TEER;approx 500Ohms.cm²). Quadruplicate wells were incubated basolaterally with vehicle (0.1% BSA in PBS) or a cytokine mix (“cytomix”) of 10ng/ml of each of the following: human recombinant IFN-γ, TNF-α and IL-1β (R&D Systems). Co-treatment was performed with vehicle (0.5% BSA in sterile water) or recombinant human netrin-1 (500ng/ml) in both the apical and basolateral compartment or the basolateral compartment only. TEER measurements were taken at indicated timepoints using an Evom (World Precision Instruments). At 48 hours post-treatment, cells were washed three times with HEPES-buffered DMEM. FITC-dextran (3KDa;Invitrogen) was added at 250µg/ml to the apical compartment and appearance of FITC in the basolateral compartment was measured at 15 minute intervals for 3 hours. The apparent permeability was calculated as described [20]. DSS (36,000-50,000 MW; MP Biomedicals) at increasing concentrations (4.5%) was added to either the apical or both apical and basolateral compartments in the presence of netrin-1 (500ng/ml) or vehicle in both compartments. TEER was measured over a time-course as indicated.

***In vitro* PMN transmigration.**

Caco-2 intestinal epithelial cells were cultured on inverted 6.5mm polyester permeable Transwell® membranes (3.0µm pore;Costar), as previously described.[2] Isolation of human polymorphonuclear leukocytes (PMN;neutrophils) was performed by Histopaque 1077 gradient as described.[6] 1×10^6 PMN were added to the basolateral chamber in the presence of vehicle or recombinant human netrin-1 (50-500ng/ml;Enzo Life Sciences). Formyl-Methinyl-Leucyl-Phenylalanine (fMLP; Sigma Aldrich) was added at a final concentration of 1 µM to the apical

chamber and the assay was incubated at 37c for 1.5 hours. Numbers of transmigrated PMN were determined by myeloperoxidase assay, as described.[2]