Supplemental material to:
Bacteria penetrate inner colon mucus layer in both murine colitis models and in patients with ulcerative colitis

MATERIALS AND METHODS

Animals

Experiments were performed using wild type C57/Bl6, IL10$^{-/-}$, Slc9a3$^{-/-}$, C1galt$^{-/-}$, Muc2$^{-/-}$and Tlr5$^{-/-}$ mice all on a C57/Bl6 background (male 8-12 weeks) according to local ethics committee guidelines, All experiments included controls from the same animal facility as the knock-out strains. DSS experiments were performed as described (1;2). The animals were housed in standardized conditions of temperature (21-22°C) and illumination (12h light and 12 dark) with food and water provided ad libitum.

Human subjects and biopsies

Subjects were recruited among patients referred for colonoscopy at Sahlgrens' University Hospital, Gothenburg, Sweden. Biopsies from the sigmoid colon were assessed from 28 patients with UC. Disease activity was determined by the endoscopic Mayo Score (3) and histological evaluation was performed by clinical pathologist. Clinical information regarding the UC patients is presented in Table 1. Biopsies were also obtained from 12 patients referred for colonoscopy for reasons such as anemia, bleeding and altered bowel habits. These subjects all had macroscopically normal mucosa at colonoscopy, and were used as a control group. Clinical information on these patients is presented in Table S1. Written and informed consent was obtained from all study subjects and approval for this study was granted by the Human Research Ethical Committee, University of Gothenburg. Biopsies were collected one at the time using single-use large capacity forceps (Olympus) and instantly put into ice-cold oxygenated Krebs buffer for studies of mucus penetrability or fixed in Carnoy's fixative (4) for immunohistological analysis.

FISH and immunostaining

Animals were euthanized by cervical dislocation and the colon dissected. A piece of the distal colon containing a fecal pellet was cut and fixed in Carnoy's fixative (60% dry methanol, 30% chloroform, 10% glacial acetic acid). Paraffin embedded sections were dewaxed and stained with H&E, Alcian blue/PAS or hybridized with 10 ng/µl of a general bacterial 16S rRNA probe (EUB 338) and immunostained for Muc2 using the MUC2C3 antisera or DNA was stained by DAPI (4).

Images were obtained with an Axio Examiner Z1 LSM 700 confocal microscope using a plan apochromat 40x/1.3 oil DIC objective and the ZEN 2010 software (Zeiss, Germany). In the immunohistological analysis of mouse colon the pictures were taken with a resolution of 2048x2048 pixels, 129 µm pinhole, averaging 4 pictures in two tracks (excitation: 555 nm and 488 nm) . Immunohistochemistry of human biopsies were examined using the same instrument with 1024x1024 resolution, 92 µm pinhole, averaging 8 pictures in two tracks (excitation: 405 nm and 488 nm). Brightness and contrast adjustments were made for the whole image and all samples were equally processed.

Mucus penetration score and histology score. Mucus penetration by bacteria was blinded evaluated on DNA stained sections by two independent observers. The IL10$^{-/-}$ and WT groups (n=5) were used for statistical analysis. Mucus penetration by bacteria was in addition scored in the other genotype groups (n=3). The scores (0-3) were based on observations from 3 whole intestinal cross section per sample. No bacteria in contact with the epithelium and no bacteria in the inner mucus layer was assigned a value of 0. Bacteria found in the inner mucus layer with up to 20% of the surface epithelial cells in contact with bacteria was assigned a value of 1. Bacteria in the inner mucus layer with about 50% of the surface epithelial cells in contact with bacteria was assigned a value of 2, and bacteria in the inner mucus layer with more than 80% of the cells in contact with bacteria or bacteria located in the crypts was assigned a value of 3. Data is presented as mean±SEM.

Histological scoring of inflammation was performed on H&E and Alcian blue/PAS sections in a blinded fashion by two independent observers evaluating a whole tissue section per sample. The IL10$^{-/-}$ and WT groups (n=5) were used for statistical analysis. Inflammatory histology scores were also evaluated in the other genotype groups (n=3). The sum of scores for inflammatory cell infiltration (score, 0–4), goblet cell depletion or decreased mucus accumulation (score, 0–4), mucosa thickening (score, 0-4), destruction of architecture (score, 0 or 3-4) and loss of crypts (score, 0 or 3-4) was calculated (maximum 20). Inflammatory cell infiltration (0-4): The presence of no or occasional inflammatory cells in the lamina propria was assigned a value of 0, increased number of inflammatory cells in the lamina propria was assigned a value of 1, high numbers of number of inflammatory cells in the lamina propria was assigned a value of 2, confluent inflammatory cells in the lamina propria was assigned a value of 3 and confluent inflammatory cells in the lamina propria, extending into the submucosa was assigned a value of 4. Goblet cell depletion or decreased mucus accumulation (0-4): Normal goblet cell distribution with densely filled goblet cells was assigned a value of 0. 5-20% decrease in number of densely filled goblet cells was assigned a value of 1. 20-40% decrease was assigned a value of 2, 40-50% decrease was assigned a value of 3, and more than 50% reduction was assigned a value of 4. Mucosa thickening and crypt elongation (0-4): Normal thickness was assigned a value of 0, 20-40% increased thickness was assigned a value of 1, 40-80% increased thickness was assigned a value of 2, 80-100% increased thickness was assigned a value of 3, and more than 100% increased thickness was assigned a value of 4. Destruction of architecture (0, 3-4): Normal epithelial structure was assigned a value of 0, changed architecture of the epithelium with altered crypt structure and irregular luminal surface was assigned a value of 3 and gross structural changes including ulcers was assigned a value of 4. Crypt loss (0, 3-4): Normal crypt frequency was assigned a value of 0, 5-20% decreased number of crypts was assigned a value of 3 and more than 20% loss of crypts was assigned a value of 4. Data is presented as mean±SEM.

Goblet cell number and theca area measurement.

Number of goblet cells were counted for a defined distance (100 µm of mouse samples and 150 µm for human samples) from the surface epithelium of longitudinally cut crypts. A total of 12 crypts (4 crypts per section) were analyzed for each mouse and 3 crypts (from 3 different sections) were analyzed for every human subject. Data is presented as area±SEM

with 34 human samples (10 controls, 14 UC patients in remission, 10 UC patients with active disease) and 10 mouse samples (5 WT, 5 $IL10^{-/-}$).

Mucus filled theca area of goblet cells were measured on pictures of Anti-MUC2C3 stained sections using the Volocity software (version 6.1, Perkin Elmer). The pictures were acquired with the same settings, and the intensity threshold was set to 15% and a size limit exclusion of 30 μm^2 was used. Region of interests were chosen to only include goblet cells of the upper third of the crypt and 25 goblet cells (in 4 sections of 1 biopsy) were measured per human sample and 60 goblet cells (20 goblet cells per section in 3 sections) per mouse sample Data is presented as area±SEM (μm^2) with 34 human samples (10 controls, 14 UC patients in remission, 10 UC patients with active disease) and 10 mouse samples (5 WT, 5 $IL10^{-/-}$).

Preparation of mouse tissue for in vivo and ex vivo experiments

For the *in vivo* experiments mice were anesthetized by spontaneous inhalation of 3.5% isoflurane (Isoba vet, Schering Plough) and moved to a breathing mask (AgnThos) for continuous isoflurane administration. Body temperature was kept at 37°C by a heating pad and controlled by a rectal thermometer. The abdomen was opened through a midline incision, and the distal colon positioned on a holder (in house) at the level of the abdomen. The intestinal segment was cut open (1 cm). Fecal pellets were removed and the mucosal surface was rinsed with warm saline. A cup exposing 0.07 cm^2 of the tissue was gently positioned on the mucosa and filled with warm saline. The isoflurane dosage was lowered to 2% prior to mucus thickness measurement.

For the *ex vivo* experiments mice were anaesthetized with isoflurane and euthanized by cervical dislocation. The distal colon (approximately 2 cm) was dissected, flushed with ice-cold oxygenized Krebs' buffer and opened along the mesenteric border. The longitudinal muscle layer was removed by blunt dissection and the tissue was mounted in horizontal perfusion chambers for measurements of mucus thickness or mucus penetrability.

Perfusion chamber characteristics and buffer compositions

Horizontal perfusion chambers with an open apical chamber and a closed basolateral chamber were used in both the mucus thickness measurements and mucus penetrability measurements. The chamber characteristics were identical except for two features. The apical chamber volume was 150 µl for the mucus thickness measurements and 1.5 ml for the mucus penetrability measurements. The diameter of the circular opening was 2.5 mm in the thickness measurements and 1.5 mm in the penetrability measurements. The chambers were mounted in a heating block and kept at 37°C during the whole experiment. The apical Krebs' buffer was kept unstirred during the experiment to limit disturbance to the mucus layer while the serosal chamber (volume 165 µl) was perfused at a rate of 5ml/h. The buffer compositions were the same in both chambers; Krebs' buffer (mM): NaCl 116, $CaCl_2$ 1.3, KCl 3.6, KH_2PO_4 1.4, $NaHCO_3$ 23 and $MgSO_4$ 1.2 (Merck, Germany). The apical Kreb-Mannitol buffer also contained Na-Pyruvate (5.7 mM) (Sigma-Aldrich, Germany), Na-L-Glutamate (5.1 mM) (Merck, Germany) and D-Mannitol (10 mM) (Sigma-Aldrich, Germany) whereas the serosal Kreb-Glucose buffer contained D-Glucose (10mM) (Sigma-Aldrich, Germany) instead of D-

Mannitol. All solutions were oxygenized (95% O_2, 5% CO_2) for 20 min prior to the experiment and pH was set to 7.4 using 37% HCl.

Mucus thickness

The thickness of the colonic mucus was measured as described previously (2). Briefly, the upper surface of the colonic mucus was visualized by addition of charcoal particles to the apical surface of the tissue. The thickness of the mucus layer was then determined by measuring the distance between the epithelial surface and the mucus surface by a micropipette (tip diameter ~10 µm) connected to a digimatic indicator (Mitutoyo, Japan) and viewed through a stereomicroscope (Leica MZ12). For the *in vivo* experiments, the initial mucus thickness was measured followed by aspiration of mucus and measurement of the remaining layer thickness. For the *ex vivo* experiments, the initial mucus thickness was measured followed by repeated measurements in 15 min intervals for 60 min.

Mucus penetrability

Mucus penetrability was measured as described previously (2). Briefly, human colonic biopsies or mouse colonic explants were mounted in the perfusion chamber as for mucus thickness measurements, and incubated for 20 min followed by addition of a suspension of 2 µm green beads and 0.5 µm red beads to the apical surface (Fluospheres, Invitrogen). The beads were left to sediment through the mucus for 40 min after which the position of the beads in relation to the epithelium was analyzed by taking confocal images in XY stacks (320x320 µm).

An LSM 700 Axio Examiner Z.1 confocal imaging system with a Plan- Apochromat 20x/1.0DIC water objective (Zeiss, Germany) was used in the penetrability studies. XY sections were obtained with an optical thickness of 2.8 µm in 10 µm intervals. The pictures were obtained at a 512x512 pixels resolution using bidirectional scan, 12 bit depth, 45 µm pinhole, 6.3 µs pixel dwell time and an average of 2 pictures. In track 1 the excitation was at 405 nm and emission at SP 490 nm, DBS 493 nm and master gain 730-820. In track 2 the excitation was at 488 nm and emission at SP 555 nm, DBS 604 nm and master gain 329. In track 3 the excitation was at 555 nm the emission at SP 640 nm, DBS 626 nm and master gain was set to 350. Data was analyzed using the Volocity 5.5.1 software (Perkin-Elmer). The density parameter was set to 70% in track 1 and (track 1) and 50% in track 2 and 3 and the brightness was set to 2x and 3x respectively. Data was obtained as total intensity measurements in ROI of 20 µm sections of the Z-stack consisting of the raw data from the ZEN 2010 acquisition software. The level of the tissue surface was set manually from the XY sections and values above the tissue surface in track 1 were considered background.

The mucus penetrability was analyzed in two ways. We first determined how large proportion of the total bead intensity that was in close proximity to the epithelial surface. In mouse colon the analysis window was set -20 to +40 µm above the epithelial surface. In human colon the analysis window was set -20 to +120 µm. The total bead intensity in the region was compared to the total bead intensity in the entire Z-stack. In the second analysis we determined how large part of the mucus that was penetrated by the beads. The distance between the outer border of the beads and the epithelial surface was compared to the distance between the inner most beads and the epithelial surface. The lowest point of beads was

defined as the section where the bead intensity was <5% of the maximum bead intensity, representing background fluorescence.

Statistical analysis

Non-parametric tests were chosen as normal distribution could not be verified due to the small sample sizes. Mouse data was analyzed using a two-tailed Mann-Whitney U test. For the human data the Kruskal-Wallis test with Dunns' correction for multiple comparisons was used. A p-value <0.05 was regarded as statistically significant. Mean of each analysis per individual was used as value for the statistical analysis.

REFERENCES

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Table S1: Clinical information about the control patients.

Number	[a]Gender	Age	Clinical indication for colonoscopy
1	M	35	Anemia
2	F	43	Altered bowel habits, constipation
3	F	68	Polyp surveillance
4	M	50	Unspecific bleeding
5	M	65	Altered bowel habits, diarrhea
6	F	63	Unspecific bleeding
7	M	73	Unspecific bleeding
8	M	56	Polyp surveillance
9	F	73	Abdominal pain
10	F	44	Altered bowel habits, constipation
11	F	83	Altered bowel habitsF
12	F	55	Altered bowel habits, diarrhea

[a]M: male, F: Female

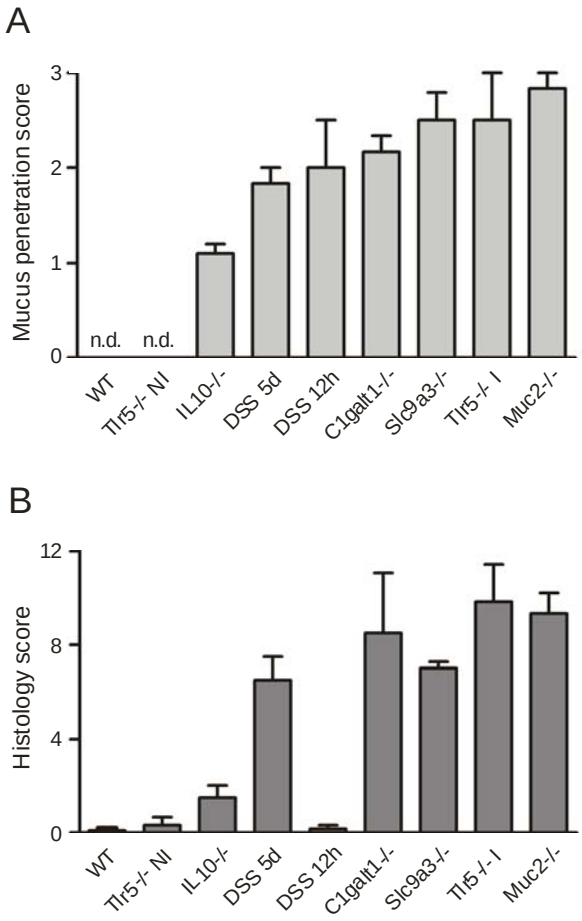

Figure S1. Bacteria penetration and inflammation in the different colitis models. (A) Bacteria penetration of the inner mucus was scored for all the genotypes (n=5 for WT and IL10$^{-/-}$ and n=3 for all other groups). Data is presented as mean±SEM. The WT data is representative for controls from different animal facilities. (B) Inflammation was monitored as histology scores for the different genotypes (n=5 for WT and IL10$^{-/-}$ and n=3 for all other groups). Data is presented as mean±SEM.