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TLR4-dependent claudin-1 internalization and secretagogue-mediated chloride secretion regulate irinotecan-induced diarrhea

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ABBREVIATIONS

TLR4	: Toll-like receptor 4
LPS	: Lipopolysaccharide
IL	: Interleukin
TNF	: Tumor necrosis factor
IFN	: Interferon
ZO	: Zonular occludens
FITC	: Fluorescein isothiocyanate
RNA	: Ribonucleic acid
RT-PCR	: Real time polymerase chain reaction
IF	: Immunofluorescence
WB	: Western blot

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Abstract

We have previously shown increased intestinal permeability, to 4 kDa FITC-dextran, in BALB/c mice treated with irinotecan. Importantly, genetic deletion of Toll-like receptor 4 (TLR4; *Tlr4*$^{-/-}$) protected against loss of barrier function indicating, TLR4 is critical in tight junction regulation. The current study aimed to (1) determine the molecular characteristics of intestinal tight junctions in wild-type and *Tlr4*$^{-/-}$ BALB/c mice, and (2) characterize the secretory profile of the distal colon. Forty-two female wild-type and 42 *Tlr4*$^{-/-}$ BALB/c mice weighing between 18-25 g received a single 270 mg/kg (i.p.) dose of irinotecan hydrochloride or vehicle control and were killed at 6, 24, 48, 72 and 96 h. The secretory profile of the distal colon, following carbachol and forksolin, was assessed using Ussing chambers at all time points. Tight junction integrity was assessed at 24 h, when peak intestinal permeability and diarrhea were reported, using immunofluorescence, western blotting and RT-PCR. Irinotecan caused internalization of claudin-1 with focal lesions of ZO-1 and occludin proteolysis in the ileum and colon of wild-type mice. *Tlr4*$^{-/-}$ mice maintained phenotypically normal tight junctions. Baseline conductance, a measure of paracellular permeability, was increased in irinotecan-treated wild-type mice at 24 h (53.19±6.46 S/cm^2; p=0.0008). No change was seen in *Tlr4*$^{-/-}$ mice. Increased carbachol-induced chloride secretion was seen in irinotecan-treated wild-type and *Tlr4*$^{-/-}$ mice at 24 h (wild-type 100.35±18.37 μA/cm^2; p=0.022; *Tlr4*$^{-/-}$ 102.72±18.80 μA/cm^2; p=0.023). Results suggest TLR4-dependent claudin-1 internalization and secondary anion secretion contribute to irinotecan-induced diarrhea.

1.0 Introduction

Irinotecan is a commonly prescribed chemotherapeutic agent used to treat a variety of solid tumours (1, 2). Despite long-standing clinical efficacy, irinotecan is associated with a host of debilitating off target toxicities which severely impact its widespread implementation (3). Of these side effects, gut toxicity presents as a major clinical obstacle in oncology practice with limited therapeutic avenues. Characterized by severe diarrhea, rectal bleeding, pain and infection, irinotecan-induced gut toxicity is considered the most significant dose-limiting side effect of irinotecan, placing a substantial clinical and economic burden on the provision of optimal cancer care (4, 5). The current understanding of the molecular mechanisms that drive gut toxicity has not yet lead to any advances in its treatment and thus, a better understanding of the underlying biology is required. Recent research has outlined emerging evidence implicating intestinal barrier injury and tight junction disruption in the development of gut toxicity (6, 7), however few studies have investigated their regulation and involvement in diarrhea.

Tight junctions are highly dynamic signaling complexes critical to gastrointestinal homeostasis and the maintenance of barrier function (8). In the setting of chemotherapy-induced gut toxicity, intestinal barrier dysfunction is most commonly associated with augmentation of key tight junction proteins claudin-1, zonular occludens(ZO)-1 and occludin; all of which are integral to maintenance of the tight junction unit (9, 10). A large body of evidence demonstrates disruption of these proteins, and other claudin subtypes, in a number of gastrointestinal pathologies, particularly those characterized by diarrhea (8, 11), with altered barrier function and tight junction integrity seen following a number of chemotherapeutic agents (8). Dual sugar permeability assays are typically used to non-invasively assess barrier function in patients receiving chemotherapy. Elevations in permeability indices have been identified in a number of chemotherapy patient cohorts and have been shown to correlate with the severity of gastrointestinal symptoms (12-16). Despite these findings, the molecular characteristics of tight junctions have been largely ignored clinically, assessed only in *in vivo* studies. These studies consistently show decreased expression, redistribution and phosphorylation of claudin-1, occludin and ZO-1 (7, 17-19), however few report these changes in combination with robust permeability data.

It is well documented that changes in tight junction integrity parallel the onset and severity of gastrointestinal symptoms (20). *In vivo* studies have shown increased intestinal permeability, caused by irinotecan treatment, permits endotoxin and bacterial translocation, thus increasing the risk of secondary infection (7). In patients with ulcerative colitis, loss of intestinal barrier function and tight

junction integrity has been shown to contribute to diarrhea development via passive back-flow of anions and water into the lumen (21). Intestinal barrier dysfunction has also been identified as a key risk factor for secondary complications in patients receiving multi-drug chemotherapy (22), increasing the risk of bacteremia, mucosal-related invasive fungal disease, typhlitis and sepsis (23).

Despite strong clinical evidence indicating that barrier dysfunction permits secondary toxicity and infectious consequences, the mechanisms by which barrier dysfunction and tight junction disruption contribute to irinotecan-induced diarrhea remain unclear. We have recently demonstrated increased intestinal permeability to 4 kDa FITC-dextran in BALB/c mice treated with irinotecan, indicating poor tight junction integrity. Importantly, our findings demonstrated that genetic deletion of Toll-like receptor 4 (TLR4; *Tlr4*$^{-/-}$) protected against the development of barrier dysfunction (24) and reduced the duration and severity of diarrhea. We hypothesise that the ability of TLR4 to recruit powerful downstream inflammatory signals, recognized for their ability to disrupt tight junction proteins (25), makes it a potential driver of barrier dysfunction following chemotherapy. Research shows that TLR4, and its ligand lipopolysaccharide (LPS), are both upregulated following irinotecan (26). This parallels other models of barrier dysfunction and supports the idea that TLR4 activation promotes barrier disruption (27, 28).

The current study therefore aims to investigate the morphology and molecular integrity of intestinal tight junctions in a model of irinotecan-induced diarrhea, and gauge the importance of TLR4 in tight junction regulation. In addition, this study aims to assess the secretory profile of the intestine using Ussing chambers to determine the impact of altered anion secretion, secondary to barrier disruption, on diarrhea development.

2.0 Materials and methods

2.1 Animal Model and Ethics

The study was approved by the Animal Ethics Committee of the University of Adelaide and complied with National Health and Research Council (Australia) Code of Practice for Animal Care in Research and Training (2014). Mice were group housed in ventilated cages with three to five animals per cage. They were housed in approved conditions on a 12 h light/dark cycle. Food and water were provided *ad libitum*.

2.1.1 Experimental Design

All mice were on a BALB/c background. Forty-two female BALB/c-wild-type (WT) and BALB/c-*Tlr4*$^{-/-billy}$ mice (n_{total}=84) weighing between 18-25 g (10-13 weeks) were used. WT BALB/c mice were obtained from the University of Adelaide Laboratory Animal Service (SA, Australia), and BALB/c-*Tlr4*$^{-/-billy}$ mice, back-crossed onto BALB/c for more than 10 generations, were kindly provided by Professor Paul Foster from the University of Newcastle (NSW, Australia) and were originally sourced from Osaka, Japan (29). All BALB/c-*Tlr4*$^{-/-billy}$ mice were homozygous null mutants and hence expressed no detectable TLR4 mRNA or protein (24). Mice were treated with a single 270 mg/kg intraperitoneal (i.p.) dose of irinotecan hydrochloride (kindly provided by Pharmacia/Pfizer, Michigan, USA) prepared in a sorbitol/lactic acid buffer (45 mg/ml sorbitol / 0.9 mg/ml lactic acid; pH 3.4; Sigma-Aldrich, NSW, Australia; D-sorbitol #S1876, lactic acid #252476), which was shown in our previous work to cause reproducible diarrhea with no mortality (24). Control mice received the sorbitol/lactic acid buffer only. All mice received 0.03 mg/kg of atropine subcutaneously immediately prior to treatment to reduce the cholinergic response to irinotecan. Mice were randomly assigned to treatment groups and killed at 6, 24, 48, 72 and 96 h. Mice were anaesthetized using 200 mg/kg (intraperitoneal) ilium sodium pentobarbital (60 mg/ml) and blood was collected from the facial vein. They were killed via transcardial perfusion with cold, sterile 1 X PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4).

2.1.2 Clinical assessment of gut toxicity

Mice were assessed four times daily for response to irinotecan treatment as per Wardill et al., (2016). For clinical data please refer to Wardill et al., 2016 (24).

2.1.3 Tissue preparation

The entire gastrointestinal tract from the pyloric sphincter to the rectum was dissected prior to perfusion with 4% PFA and flushed with chilled 1 X PBS (pH 7.4) to remove intestinal contents. Both the small and large intestines were weighed immediately after resection. Samples (1 cm in length) of jejunum, ileum and colon were collected and (1) drop-fixed using 10% neutral buffered saline for processing and embedding into paraffin wax, or (2) stored in RNAlater® (Sigma Aldrich, NSW, Australia; #R0901) at -20ºC for molecular analyses. Mucosal scrapings were also collected from the jejunum, ileum and colon, snap frozen and stored at -80ºC.

2.2 Tight junction analysis

Tight junction analysis was performed on the jejunum, ileum and colon taken from WT and BALB/c-*Tlr4*$^{-/-billy}$ mice 24 h after irinotecan treatment as peak diarrhea severity and serum FITC-dextran were seen at this time point. Tight junction analysis was also performed on six vehicle control mice from each genotype to ensure TLR4 deletion did not affect tight junction morphology.

2.2.1 Immunofluorescent analysis of tight junction protein distribution (claudin-1, ZO-1, occludin)

Immunofluorescence (IF) was carried out on 4 μm sections of jejunum, ileum and colon, cut on a rotary microtome and mounted onto FLEX IHC microscope slides (Flex Plus Detection System, Dako, Denmark; #K8020). IF analysis was performed for key tight junction proteins: claudin-1, ZO-1 and occludin. IF was performed using Dako reagents on an automated machine (AutostainerPlus™, Dako, Denmark; #AS480) following standard protocols supplied by the manufacturer. Briefly, sections were deparaffinized in histolene and rehydrated through graded ethanols before undergoing heat-mediated antigen retrieval using an ethylenediaminetetraacetic acid-sodium bicarbonate (EDTA-NaOH) buffer (0.37 g/L EDTA, pH 8.0). Retrieval buffer was preheated to 65ºC using Dako PT LINK™ (pre-treatment module; Dako, Denmark; #PT101). Slides were immersed in the buffer and the temperature raised to 97ºC for 20 min. After returning to 65ºC, slides were placed in the Dako AutostainerPlus™ and tissue was blocked using 10% normal horse serum (NHS) in 1 X phosphate buffered saline (PBS). The primary antibodies were applied for 1 h using 5% NHS as a diluent. A fluorescently labeled secondary antibody (Donkey anti-rabbit or mouse IgG (H+L) Secondary Antibody, AlexaFluor® 568 or 488 conjugate, Invitrogen, Vic, Australia; #A10042) was applied at 0.8 μg/ml for a further 1 h, using 1 X PBS + 1% bovine serum albumin (BSA; Sigma-Alrich, NSW, Australia; #A2058) and 2% foetal

bovine serum (FBS; Sigma-Aldrich, NSW, Australia; #F2442) as a diluent. Slides were washed using 1 X PBS, counterstained using 1 μg/ml 4',6-diamidino-2-phenylidole (DAPI; Life Sciences, Vic, Australia; #D1306) and coverslipped using an aqueous mounting medium (Fluorshield™, Sigma Aldrich, NSW, Australia; #F6182). Negative controls had the primary antibody omitted. Slides were visualized using the SP5 Spectral Scanning Confocal Microscope (Leica, Wetzlar, Germany). Immunofluorescence was assessed qualitatively for staining intensity and distribution in a blinded fashion.

IF antibody details are as follows: claudin-1 (Abcam ab15098; 2 μg/ml; 1:100; AlexaFluor® anti-rabbit 568 nm); ZO-1 (Invitrogen 61-7300; 2.5 μg/ml; 1:100; AlexaFluor® anti-rabbit 568 nm); occludin (Invitrogen 33-1500; 5 μg/ml; 1:100; AlexaFluor® anti-mouse 488 nm).

2.2.2 Western blotting for tight junction proteins (claudin-1, ZO-1, occludin)

2.2.2.1 Protein extraction and quantification

Total protein was isolated from jejunal, ileal and colonic tissue samples. Tissue samples (30 mg) were immersed in 300 μl of Radio-Immunoprecipitation Assay buffer containing 150 mM sodium chloride, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.1% protease inhibitor cocktail and 50 mM Tris, pH 8.0 (Sigma-Aldrich, NSW, Australia; #R0278). Samples were homogenized using the QIAGEN® Tissue LyserLT™ (Qiagen, NSW, Australia) at 50 Hz for 5 min. Homogenates were centrifuged at 11,000 x *g* for 15 min at 4°C, and supernatant was collected, aliquoted and stored at -80°C. Total protein was quantified using the Pierce™ BCA protein quantification kit (ThermoFisher Scientific, Vic, Australia; #23225) relative to a 8-point standard curve (BSA: 25 μg/ml – 2 mg/ml).

2.2.2.2 Western blot

Total protein lysates were quantified and 30 μg supplemented with 4 μl Bolt® reducing agent (ThermoFisher Scientific, Vic, Australia; #B0008) and 10 μl Bolt® lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific, Vic, Australia; #B0007). The total volume was then adjusted to 40 μl with milliQ water. Samples were denatured for 10 min at 70°C before being loaded into precast Bolt® 4-12% Bis Tris Plus, SDS-PAGE Gels (12-well) (ThermoFisher Scientific, Vic, Australia; #NW04122BOX). Samples were separated using the Bolt® mini gel tank (ThermoFisher

Scientific, Vic, Australia; #A25977) at 150 V for 45 min. Proteins were transferred to a polyvinylidene difluoride membrane using the iBlot® transfer stacks (ThermoFisher Scientific, Vic, Australia; #IB24002) and compatible iBlot® 2 gel transfer device (ThermoFisher Scientific, Vic, Australia; #IB21001). The membrane was washed with 1 X tris-buffered saline and Tween20 (TBST) and stained with Ponceau S red staining solution (Sigma-Aldrich, NSW, Australia; #09276) to confirm equal loading. The membrane was blocked and probed with primary and secondary antibodies using the iBind™ western device (ThermoFisher Scientific, Vic, Australia; #SLF1000) as per manufacturers guidelines. Western blots were assessed using ImageStudio® Lite, version 4.0. Signal intensity was determined relative to local background. Data were presented relative to loading control (GAPDH) and vehicle treated controls.

Antibody details are as follows: claudin-1 (Invitrogen 51-9000; 5 μg/ml; 1:50; IRDye® anti-rabbit 800 CW); ZO-1 (Invitrogen 61-7300; 6.25 μg/ml; 1:40; IRDye® anti-rabbit 800 CW); occludin (Invitrogen 71-1500; 1.25 μg/ml; 1:400; IRDye® anti-rabbit 800 CW); GAPDH (Abcam ab15822; 0.5 μg/ml; 1:2000; IRDye® anti-chicken 680 CW).

2.2.3 RT-PCR for tight junction proteins (occludin, ZO-1, claudin-1)

2.2.3.1 RNA extraction and quantification

Total RNA was isolated from jejunal, ileal and colonic mucosal scrapings samples using the Macherey-Nagel NuceloSpin® RNA/protein purification kit as per manufacturer's instructions (Macherey-Nagel, Düren, Germany; #740933.250). Once eluted, RNA was stored at -80ºC. Total RNA yield and purity were assessed using the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA), TAKE3 plate and Gen5 (version 2.00.18) software.

2.2.3.2 Reverse transcription and RT-PCR

500 ng of total RNA was reverse transcribed using the iScript™ cDNA synthesis kit (BioRad, NSW, Australia; #1708890) as per manufacturer's instructions. RT-PCR was performed using the Rotor-Gene 3000 (Corbett Research, NSW, Australia). Amplification mixes contained 1-2 μl of cDNA sample (100 ng/μl), 5 μl of SYBR green fluorescence dye, 2-3 μl of RNase-free water and 0.5 μl of each forward and reverse primers, prediluted to 50 pmol/μl, to make a total volume of 10 μl.

Primer details are as follows: ***Mouse claudin-1*** (NM_016674.4) QIAGEN Mm_Cldn1_1_SG QuantiTect Primer Assay (QT00159278); T_m 55ºC; ***Mouse ZO-1*** (NM_001163574.1) F: 5'-ACTCCCACTTCCCCAAAAAC-3'; R: 5'-CCACAGCTGAAGGACTCACA-3'; 166 bp; T_m 52/54ºC; (30); ***Mouse occludin*** (NM_008756.2) F: 5'-ACTGGGTCAGGGAATATCCA-3'; R: 5'-TCAGCAGCAGCCATGTACTC-3'; 192 bp; T_m 52/54ºC; (30); ***Mouse GAPDH*** (NM_008084.2) F: 5'-CCTCGTCCCGTAGACAAAATG-3'; R: 5'-TCTCCACTTTGCCACTGCAA-3'; T_m 52/54ºC; (31); ***Mouse 18S rRNA*** (NR_003278.3) F: 5'-TCGGAACTGAGGCCATGATT-3'; R: 5'-TTTCGCTCTGGTCCGTCTTG-3'; 100 bp; T_m 52/54ºC; (31).

Thermal cycling conditions for ZO-1 and occludin included a denaturing step at 95ºC for 15 min, followed by 45 cycles of denaturation at 95ºC for 10 s, annealing at 52ºC for 15 s and extension at 72ºC for 20 s. Claudin-1 cycling conditions were as follows: denaturing step at at 95ºC for 15 min, followed by 45 cycles of denaturation at 94ºC for 15s, annealing at 55ºC for 30 s and extension at 72ºC for 30 s. All samples were run in triplicate with the inclusion of blank, negative controls (no cDNA template). Primer efficiency was evaluated using standard curves and experimental threshold (C_T) values were calculated by the Rotor Gene 6 programme. C_T values were used to quantify relative mRNA expression of each tight junction protein using the ΔC_T method, where relative expression = $2^{-[C_T(target)-C_T(housekeeper)]}$. 18S and GAPDH were assessed for their suitability as housekeeping/reference genes (32).

2.3 Electrophysiological studies using Ussing chambers

Immediately following dissection, samples of distal colon were mounted into Ussing chambers (Physiologic Instruments, CA, USA; #EM-CSYS-8) for electrophysiological analyses. Briefly, the colon was cut longitudinally along the mesenteric attachment and placed onto a 0.1 cm^2 apeture slider (Physiologic Instruments, CA, USA; P2303A). The tissue was mounted and continually bathed in an oxygenated, glucose-fortified Ringer's solution at 37ºC with the following composition (composition in mM/L: NaC1 115.4; KCl 5; $MgCl_2$ 1.2; NaH_2PO_4 0.6; $NaHCO_3$ 25; $CaCl_2$ 1.2 and glucose 10). Tissues were voltage-clamped to zero potential difference by the application of short-circuit current (Isc) and baseline was established. Tissues were allowed to equilibrate for 20 min and baseline Isc ($\mu A/cm^2$) and conductance (S/cm^2) were recorded. Tissues were pretreated with amiloride (20 μM) to inhibit the apical epithelial sodium channel before being treated with forskolin (adenosine 3'5'-cyclic monophosphate agonist; 10 μM) and carbachol (Ca^{2+} agonist; 100 μM), applied to the apical chamber.

The Isc response was then measured, determined as the change in Isc following agonist administration ($\Delta\mu A/cm^2$), representing stimulated chloride secretion.

2.4 Statistical analysis

Data were compared using Prism version 7.0 (GraphPad® Software, San Diego, USA). A D'Agostino-Pearson omnibus test was used to assess normality. When normality was confirmed, two-way analysis of variance (ANOVA) with appropriate post-hoc testing were performed to identify statistical significance between groups. In other cases, a Kruskal-Wallis test with Dunn's multiple comparisons test and Bonferroni correction was performed. A p-value of <0.05 was considered significant.

3.0 Results

3.1 Cytoplasmic redistribution of tight junction proteins contributes to irinotecan-induced barrier disruption

Genetic deletion of TLR4 does not affect morphology of tight junction proteins

Qualitative analysis of IF for tight junction proteins showed membranous staining for all tight junction proteins in vehicle treated WT and BALB/c-*Tlr4*$^{-/-billy}$ mice. No apparent differences were noted in any tight junction protein between genotypes receiving vehicle (Figure 1-3; vehicle control panels).

Claudin-1 undergoes cytoplasmic translocation in response to irinotecan

Claudin-1 staining presented with sharp apical intensities (Figure 1B/H; arrow heads; red staining) and membranous staining down the apico-lateral border of the enterocyte. Marked claudin-1 internalization was evident at 24 h after irinotecan treatment in WT mice (Figure 1C/D, red staining; arrows). This was particularly evident in the ileum (Figure 1C/D), with complete loss of membranous staining specificity in some areas. Claudin-1 internalization was seen as uniform cytoplasmic staining. Evidence of claudin-1 internalization was also seen in the colon of WT animals following irinotecan treatment, characterized by cytoplasmic staining and loss of membrane specificity (Figure 1K/L). The degree of claudin-1 redistribution was comparatively less in BALB/c-*Tlr4*$^{-/-billy}$ mice treated with irinotecan in both gut regions. Membranous staining remained intact, although loss of apical staining intensity was evident (Figure 1G/H, O/P).

Irinotecan causes focal areas of ZO-1 and occludin proteolysis

IF staining for ZO-1 (Figure 2; red staining) showed focal areas of proteolysis in the ileum (Figure 2D; arrow heads) and colon (Figure 2L; arrow heads) of WT mice 24 h post-irinotecan. These focal areas of protein disruption were particularly evident in areas of epithelial injury (identified in our previous study(24)), often occurring alongside phenotypically normal tight junction staining (Figure 2D/L; arrows). Similar changes in occludin expression were also seen in the ileum and colon of WT mice, with focal areas of proteolysis corresponding with frank epithelial damage (Figure 3D/L, arrow head; green staining). Staining appeared uniform for BALB/c-*Tlr4*$^{-/-billy}$ mice treated with irinotecan (Figure 2G/H, O/P; Figure 3G/H, O/P).

Western blotting shows no change in total protein expression of claudin-1, ZO-1 and occludin

Despite significant redistribution of claudin-1 and focal areas of ZO-1/occludin disruption following irinotecan treatment, western blot analysis revealed no quantitative changes in tight junction protein expression in any mouse group (Figure 4).

RT-PCR shows no change in the mRNA expression of tight junction proteins

Average RNA yield using the Macherey-Nagel NuceloSpin® Protein/RNA isolation kit was 464.44±276.47 ng/µl with an average $A^{260/280}$ of 2.07±0.06. cDNA conversion resulted in an average yield of 2077.69±347.44 ng/µl with an average $A^{260/280}$ of 1.76±0.11. Linear regression slope analysis revealed suitable primer efficiencies for ZO-1 (relative to 18S: 0.083; relative to GAPDH -0.080) and occludin (relative to 18S: -0.084; relative to GAPDH -0.080). Linear regression slope analysis for claudin-1 showed disparate primer efficiencies relative to each housekeeper. Housekeeping stability was analysed using the ΔC_T method, where relative expression = $2^{-[C_T(HK\ in\ controls)-C_T(HK\ in\ treated)]}$. 18S was eliminated as a suitable housekeeper based on differential expression in vehicle- and irinotecan-treated mice (data not shown).

Data showed no change in the relative mRNA expression of occludin or claudin-1 in WT or BALB/c-*Tlr4*$^{-/-billy}$ mice following irinotecan treatment (Figure 5). ΔC_T analysis revealed a significant decrease in the mRNA expression of ZO-1 (relative to GAPDH) in the colon 24 h after irinotecan treatment in WT mice (Figure 5H).

3.2 Irinotecan increases chloride secretion in the distal colon via TLR4-independent mechanisms

Ussing chamber studies indicated changes in baseline short-circuit current (Isc) and conductance in irinotecan-treated WT mice (Figure 6A-D). There was a significant difference in baseline Isc between WT and BALB/c-*Tlr4*$^{-/-\ billy}$ mice 24 h after irinotecan treatment (Figure 6A; WT 103.65±36.32 µA/cm^2; BALB/c-*Tlr4*$^{-/-billy}$ 28.6727±10.03; *p=0.0168). Increased baseline conductance, a measure of paracellular permeability, was seen in WT mice at 24 h post-irinotecan treatment (Figure 6B/D; 53.19±6.46 S/cm^2, +105.62% relative to WT vehicle controls; #p=0.0008). There was no change seen in BALB/c-*Tlr4*$^{-/-billy}$ mice at any time after irinotecan compared to vehicle controls.

WT and BALB/c-*Tlr4*$^{-/-\ billy}$ mice exhibited increased ΔIsc after carbachol administration 24 h (Figure 7A; WT 100.35±18.37 µA/cm^2; #p=0.022; BALB/c-*Tlr4*$^{-/-\ billy}$ 102.72±18.80 µA/cm^2; ^p=0.023) and WT at 48 h (99.75±25.22 µA/cm^2; #p=0.0244) after irinotecan. Irinotecan-treated WT mice also showed increased ΔIsc in response to forskolin at 72 h (82.18±16.54 µA/cm^2; #p=0.025). No change

was seen in irinotecan-treated BALB/c-*Tlr4*$^{-/-\ billy}$ mice in response to forskolin compared to vehicle controls (65.60±18.28 μA/cm^2; p=0.999). Unstimulated (baseline) current and secretory responses to both carbachol/forskolin did not correlate with diarrhea severity (Figure 7C/D; baseline r^2=0.08, carbachol r^2=0.04, forskolin r^2=0.04). # denotes a change relative to untreated vehicle controls in WT mice; ^ denotes a change relative to untreated vehicle controls in BALB/c-*Tlr4*$^{-/-billy}$ mice; * denotes a significant difference between WT and BALB/c-*Tlr4*$^{-/-billy}$ mice, where $p<0.05$.

4.0 Discussion

Irinotecan is a commonly prescribed chemotherapeutic agent, however its therapeutic efficacy is often limited by its severe gastrointestinal side effects, notably late-onset diarrhea (4). Diarrhea significantly impacts on patients' clinical outcomes (5), however the underlying mechanisms remain unclear and therapeutic interventions limited (33). It is becoming increasingly clear that irinotecan causes significant intestinal barrier disruption, characterized by increased paracellular permeability and tight junction breakdown (7, 24). Our previous research has shown that BALB/c-*Tlr4*$^{-/-\ billy}$ mice are protected from developing irinotecan-induced intestinal barrier disruption, supporting the idea that TLR4-dependent mechanisms are critical in tight junction disruption (24). Results from the current study support this hypothesis, showing improved tight junction integrity in irinotecan-treated BALB/c-*Tlr4*$^{-/-\ billy}$ mice compared to their WT counterparts. This is the first study to compare functional *in vivo* permeability, *ex vivo* electrophysiological measures of barrier function and morphological assessment of tight junction proteins in the setting of irinotecan-induced gut toxicity.

Tight junctions are critical in maintaining gastrointestinal health and homeostasis. Despite this, they are highly plastic structures vulnerable to post-transcriptional and -translational modification by a variety of pathological cues (34, 35). Tight junction disruption has been identified following treatment with a number of chemotherapeutic agents, both preclinically (6, 7) and clinically (12, 15, 36), however the mechanisms that underpin their breakdown remain unclear. To date, many studies have shown architectural abnormalities (15), functional alterations (12, 37) and downregulation of key tight junction proteins such as claudin-1, ZO-1 and occludin (7, 18). The present study did not identify any alterations in the protein expression of these tight junction units in any region of the gut. Instead, significant derangement of these proteins was identified, characterized by severe cytoplasmic redistribution and disassembly of the tight junction unit. Internalization of tight junction proteins is well recognized to contribute to poor barrier function and loss of tight junction apposition (35). In the current study, cytoplasmic redistribution of claudin-1 was seen at 24 h; where peak barrier dysfunction and diarrhea were detected.

In addition to demonstrating tight junction disruption in response to irinotecan, this study has also provided evidence implicating TLR4-dependent mechanisms in tight junction regulation. This is a growing area of research, with the interaction between TLR4 and its ligand, LPS, as well as its powerful downstream effects on inflammation providing a strong rationale for its involvement.

Evidence suggests that LPS/TLR4-dependent tight junction disruption occurs via direct epithelial processes (27). This has been shown using *in vitro* models of LPS-induced barrier dysfunction, with LPS administration resulting in TLR4-dependent activation of focal adhesion kinase (FAK) and tight junction disruption in Caco-2 monolayers. Importantly, small interfering RNA silencing of TLR4 prevented LPS-induced disruption. This was also confirmed with *in vivo*, intestinal epithelial-specific knockdown of TLR4.

Despite a growing body of evidence showing direct TLR4-mediated regulation of tight junctions, it is likely that this mechanism occurs in concert with cytokine-mediated tight junction disruption. Evidence exists supporting a role for proinflammatory cytokine-mediated tight junction disruption (35, 38) explaining the observation of intestinal barrier disruption in diseases characterized by inflammation (21, 39). Conversely, evidence also suggests that loss of anti-inflammatory cytokines can be detrimental in regulation of barrier function (40, 41). This molecular crosstalk between inflammatory cytokines and tight junctions is compelling with regards to irinotecan-induced gut toxicity as peak interleukin(IL)-1β, interferon (IFN)γ and tumour necrosis factor (TNF) levels coincide with intestinal barrier dysfunction and cytoplasmic redistribution of claudin-1 in WT mice treated with irinotecan (24). Although associative links have been identified between a proinflammatory state and tight junction disruption, much of the research has been conducted in *in vitro* models as *in vivo* models can be complicated by cytokine-dependent immune cell recruitment and activation within the mucosa (11).

A particularly strong case for IL-1β-mediated tight junction disruption exists given TLR4 acts as its main upstream regulator (42). Addition of IL-1β to growth media has been shown to directly increase epithelial permeability and decreased expression of key tight junction proteins in Caco-2 cells (representative of the small intestinal epithelium) (43). Similarly, TNF and IFNγ have been shown to synergistically disrupt tight junctions through protein rearrangement and subsequent phosphorylation of myosin II regulatory light chain (MLC) (44, 45). Given that BALB/c-*Tlr4*$^{-/-billy}$ mice showed no significant change in IL-1β, TNF and IFNγ (24), it is possible that proinflammatory cytokine production drives barrier dysfunction in a TLR4-dependent manner. Further, the barrier protective effects of anti-inflammatory cytokines, such as IL-10, also warrant further investigation as research suggests these cytokines are downregulated following chemotherapy (46).

The clinical consequences of intestinal barrier dysfunction are becoming increasingly recognised in the

setting of chemotherapy-induced gut toxicity. We have shown significant barrier dysfunction at 24 h which precedes serum detection of LPS (24), supporting Blijlevens et al., (2000) who describes that loss of barrier function is critical in preventing secondary, systemic toxicity. LPS translocation has also been suggested to underpin cancer-related sickness behaviour (47) and secondary toxicities such as chemotherapy-induced pain (48). In addition to permitting LPS translocation, loss of barrier integrity has also been shown to result in bacterial translocation and colonization in mesenteric lymph nodes and the spleen following treatment with irinotecan, thus increasing the risk for infection, graft versus host disease (49) and sepsis (22). It is also likely that loss of paracellular integrity allows greater exposure, and subsequent activation, of the innate mucosal immune system, driving a heightened inflammatory response following cytotoxic insult. Barrier dysfunction is therefore likely to exacerbate direct cytotoxic injury in the gut, thus worsening clinical outcomes for patients. By understanding the mechanisms that lead to barrier dysfunction, therapeutic interventions may be targeted to prevent local toxicity transitioning to systemic toxicity, reducing the associated risk such as infection, sepsis and pain.

Our previous research shows that intestinal permeability best reflects the clinical progression of diarrhea following irinotecan treatment (24). This parallels clinical findings in patients with inflammatory bowel disease, with barrier function and tight junction integrity correlating with the onset, severity and duration of diarrhea (20). A wealth of studies show associative links between barrier dysfunction and diarrhea, however the mechanisms by which barrier dysfunction contributes to diarrhea remain unclear. It has been suggested that passive ‘leak flux’ mechanisms may be involved (21), where loss of barrier integrity allows passive leakage of solute and water into the lumen of the gut. The current study did not assess passive ion and/or water movement following irinotecan treatment, however electrophysiological analyses of the distal colon revealed hyper-responsiveness following treatment with chloride secretagogues in both animal strains following irinotecan. This suggests an active secretory component, independent of TLR4, may be involved in the development of irinotecan-induced gut toxicity. However, dissecting its role in diarrhea development is difficult as neither secretagogue-induced chloride secretion nor unstimulated baseline current correlated with diarrhea. Additionally, conclusions can not be drawn regarding paracellular sodium movement, regulated by the pore-forming claudin-2 protein (50). Future studies should therefore focus on the synergism between barrier dysfunction, active chloride secretion and paracellular sodium/water fluxes

in chemotherapy-induced diarrhea.

5.0 Conclusion

Tight junction disruption is a hallmark trait of many pathological states. A wealth of research now implicates poor tight junction integrity following treatment with various chemotherapeutic agents. We have shown that irinotecan treatment causes tight junction disruption, characterized by claudin-1 internalization, and barrier dysfunction via TLR4-dependent mechanisms. We have outlined mechanisms by which TLR4 may regulate tight junction disruption; 1) through direct epithelial events mediated through luminal LPS, and 2) through its downstream effects on inflammation. In reality, it is likely that these mechanisms occur in concert with one another given the highly multifactorial biology of irinotecan-induced gut toxicity. Nonetheless, TLR4 appears to be critical in the development of barrier dysfunction, with overarching effects on gut toxicity. In addition, this study shows active chloride secretion following irinotecan treatment, irrespective of TLR4, and provides a novel avenue for the treatment of irinotecan-induced diarrhea.

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8.0 Figure legends

Figure 1 **Representative images of claudin-1 immunofluorescence in the ileum and colon of vehicle- and irinotecan-treated WT and BALB/c-*Tlr4*$^{-/-billy}$ mice at 24 h.** Claudin-1 staining was evident at the apico-lateral boundaries of epithelial cells, with distinct apical intensities (Panel A/B, E/F, I/J and M/N arrow heads). This was seen in vehicle-treated WT and BALB/c-*Tlr4*$^{-/-billy}$ mice. No distinct changes in staining intensity were identified, although tight junction abnormalities typified by claudin-1 internalization were seen in the ileum (Panel C/D; red staining) and colon (Panel K/L) of irinotecan treated WT. BALB/c-*Tlr4*$^{-/-billy}$ mice show only mild changes in staining following irinotecan, maintaining membrane specificity (Panel G/H and O/P). Sections of ileum and colon were stained with a primary antibody for claudin-1 and visualized using an AlexaFluor anti-rabbit (680 nm, red). Blue counterstaining (DAPI, 405 nm) shows nuclei. GC = goblet cell. Original magnification 40 X.

Figure 2 **Representative images of ZO-1 immunofluorescence in the ileum and colon of vehicle- and irinotecan-treated WT and BALB/c-*Tlr4*$^{-/-billy}$ mice at 24 h.** Vehicle-treated mice showed phenotypically normal tight junctions, with apical ZO-1 staining (Panel A/B, E/F, I/J and M/N). No differences were seen between vehicle-treated WT and BALB/c-*Tlr4*$^{-/-billy}$ mice. Following irinotecan treatment, WT mice displayed focal areas of ZO-1 disruption, particularly in areas of epithelial injury (Panel C/D, K/L; arrow heads). These were found alongside areas of phenotypically normal ZO-1 staining (Panel L; arrows). No changes in occludin distribution were noted. BALB/c-*Tlr4*$^{-/-billy}$ mice showed no change in staining intensity following irinotecan (Panel G/H and O/P). Sections of ileum and colon were stained with a primary antibody for ZO-1 and visualized using an AlexaFluor anti-rabbit (680 nm, red). Blue counterstaining (DAPI, 405 nm) shows nuclei. Original magnification 40 X.

Figure 3 **Representative images of occludin immunofluorescence in the ileum and colon of vehicle- and irinotecan-treated WT and BALB/c-*Tlr4*$^{-/-billy}$ mice at 24 h**. Vehicle-treated mice showed phenotypically normal tight junctions, with apical occludin staining (Panel A/B, E/F, I/J and M/N). No differences were seen between vehicle-treated WT and BALB/c-*Tlr4*$^{-/-billy}$ mice. Like ZO-1, irinotecan-treated WT mice displayed focal areas of occludin disruption, particularly in areas of epithelial injury (Panel C/D, K/L; arrow heads). These were found alongside areas of phenotypically normal occludin staining (Panel K/L; arrows). No changes in occludin distribution were noted. BALB/c-*Tlr4*$^{-/-billy}$ mice showed no change in staining intensity (Panel G/H and O/P). Sections of ileum

and colon were stained with a primary antibody for occludin and visualized using an AlexaFluor anti-mouse (488 nm, green). Blue counterstaining (DAPI, 405 nm) shows nuclei. PC = plasma cell; RBC = red blood cells. Original magnification 40 X.

Figure 4 **Relative protein expression of in claudin-1, ZO-1 and occludin the jejunum (A-C), ileum (D-F) and colon (G-I) in vehicle- and irinotecan-treated BALB/c mice at 24 h.** Protein expressed was determined using ImageStudio® Lite software version 4.0 and represented as relative signal intensity (/GAPDH/vehicle controls). No changes were seen in occludin, ZO-1 or claudin-1 in any mouse groups following irinotecan treatment. Representative bands are shown from the ileum for occludin (Panel J), ZO-1 (Panel K) and claudin-1 (Panel L). Data are presented as mean±SEM and were analysed using a one-way ANOVA with Tukey's post-hoc. A p-value of <0.05 was considered statistically significant.

Figure 5 **Relative mRNA expression of claudin-1, ZO-1 and occludin in the jejunum (A-C), ileum (D-F) and colon (G-I) in vehicle- and irinotecan-treated BALB/c mice.** mRNA expression presented relative to GAPDH, an internal housekeeping gene. No changes were seen in occludin or claudin-1 in any animals following irinotecan treatment. There was a decrease in ZO-1 mRNA expression (relative to GAPDH) in the colon of WT mice 24 h after irinotecan treatment (Panel H). Data are presented as median±IQR and were analysed using a one-way ANOVA with Tukey's post-hoc. A p-value of <0.05 was considered statistically significant.

Figure 6 **Baseline short-circuit current (Isc) and conductance of the distal colon at 24 h.** Segments of the distal colon were dissected and opened longitudinally, before being mounted into Ussing chambers. Increased baseline Isc was seen in WT mice compared to BALB/c-*Tlr4*$^{-/-billy}$ mice at 24 h (A; *p=0.0168), indicative of a pro-diarrhea state. Increased conductance was also seen in WT mice 24 h after irinotecan treatment (B; #p=0.0008). # denotes a change relative to untreated vehicle controls in WT mice; # denotes a change relative to untreated vehicle controls in BALB/c mice; * denotes a significant difference between WT and BALB/c-*Tlr4*$^{-/-billy}$ mice, where p<0.05. Data has been presented as absolute values (A/B) and baseline corrected (% relative to vehicle controls; C/D) to account for baseline differences between mouse groups. Data presented as mean±SEM. A two-way ANOVA with Tukey's post-hoc was performed to identify statistical significance where p<0.05.

Figure 7 **Change in short-circuit current (ΔIsc) in response to carbachol (A), a Ca^{2+} agonist, and forskolin (B), an adenosine 3'5'-cyclic monophosphate agonist.** Irinotecan treatment elevated the response to carbachol in irinotecan-treated WT and BALB/c-*Tlr4*$^{-/-billy}$ mice at 24 h (WT #p=0.0222, BALB/c-*Tlr4*$^{-/-billy}$ ^p=0.0229) and only WT at 48 h (#p=0.0244; Panel A) compared to their respective vehicle controls. The change in Isc following administration of forskolin was elevated in WT mice at 72 h post-treatment (#p=0.025; Panel B) compared to vehicle controls. No differences were seen between WT and BALB/c-*Tlr4*$^{-/-billy}$ mice mice in response to either agonist at any time point. Data presented as mean±SEM. A two-way ANOVA with Tukey's post-hoc was performed to identify statistical significance, where $p<0.05$. **Correlation between stimulated current (Panel C) or unstimulated current (Panel D) and diarrhoea severity**. A linear regression model was applied to the data sets and r^2 values calculated.