THE UNIVERSITY of ADELAIDE

SCHOOL OF MEDICINE

Epithelial TLR4 deletion: a balancing act between irinotecan-induced diarrhoea and body weight change

A thesis submitted in partial fulfilment of the

HONOURS DEGREE of BACHELOR OF

HEALTH AND MEDICAL SCIENCES

In

The Discipline of Physiology

Adelaide Medical School

The University of Adelaide

by

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November 2020

Abstract

Background/Aims: Irinotecan, a highly effective chemotherapeutic used to treat colorectal cancer, is associated with chemotherapy-induced gastrointestinal toxicities (CIGT) which limit its widespread use. CIGT pathogenesis involves intestinal toll-like receptors (TLRs), particularly TLR4, whose expression is increased during CIGT. While global TLR4 deletion attenuates symptoms of CIGT, it has yet to be investigated if intestinal deletion of epithelial TLR4 impacts CIGT and how this pathway interacts with the tight junction protein, claudin-2.

Methods: Twenty-four wild-type (WT) and intestinal epithelium-specific TLR4 knock out (*Tlr4*$^{\Delta IEC}$) colorectal-tumour bearing C57BL/6 mice received an intraperitoneal dose of irinotecan (270 mg/kg) or vehicle control and were killed at day 3 post treatment. CIGT was defined by clinically-relevant measures of body weight and diarrhoea severity. Irinotecan efficacy was defined as tumour burden relative to body weight. Claudin-2 expression was quantified using real-time PCR (fold-change), and immunofluorescence (percentage area staining) in the ileum and colon.

Results: *Tlr4*$^{\Delta IEC}$ treated mice had less severe diarrhoea (day 1 WT versus *Tlr4*$^{\Delta IEC}$ p-value <0.0001), however, significantly more weight loss in comparison to WTs (day 3 WT versus *Tlr4*$^{\Delta IEC}$ p-value = 0.0045). Irinotecan efficacy was similar in *Tlr4*$^{\Delta IEC}$ and WT treated mice. Claudin-2 fold-change was significantly greater in *Tlr4*$^{\Delta IEC}$ control versus treated mice in the colon (p-value = 0.0163).

Conclusion: This study demonstrated that different TLR4 mechanisms underlie diarrhoea and weight loss change in CIGT, however, that epithelial TLR4 could be targeted to decrease diarrhoea severity. This has significant clinical application as severity of diarrhoea is strongly correlated to treatment reduction and breaks.

Introduction

Colorectal cancer is the fourth most commonly diagnosed and second leading cause of cancer-related deaths,[1] making it one of Australia's most prevalent and lethal cancers. Irinotecan, a highly effective chemotherapeutic used to treat advanced-stage colorectal cancer, is known to have detrimental toxic side effects, with diarrhoea considered the most common and impactful symptom.[2] This toxicity is clinically referred to as chemotherapy-induced gastrointestinal toxicity (CIGT) and is underpinned by severe intestinal injury and breakdown of the mucosal barrier. Patients who experience CIGT have twice the infection risk, a 3-fold higher chance of hospitalisation and a 4-fold higher chance of death, ultimately necessitating treatment reduction and breaks.[2] Up to 25% of irinotecan-treated patients experience grade 3 or 4 diarrhoea that requires urgent hospitalisation.[3] In addition, to the huge clinical impact, severe CIGT contributes a large economic burden, with it costing AUD~$1,350 per hospitalised diarrhoea episode.[4]

Irinotecan, a topoisomerase I inhibitor,[5, 6] prevents the covalent bonding of the DNA strands,[5] resulting in an irreparable DNA double-strand break which triggers cell death.[5-7] Irinotecan-induced injury and death of highly vulnerable intestinal crypt cells activates an inflammatory cascade, inducing CIGT.[8] Furthermore, amplification of irinotecan-induced CIGT occurs due to its unique enterohepatic recirculation.[9] Initially carboxylesterase converts irinotecan to the active metabolite SN-38 in the liver and intestines.[6] SN-38 is then processed in the liver by hepatic uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) to form the non-toxic metabolite SN-38-glucuronide (SN-38G).[6] SN-38G is transported in bile into the intestine.[9] However, intestinal bacteria, such as *Escherichia coli*, produce β-glucuronidases which converts SN-38G back to SN-38 through deconjugation,[6] resulting in a secondary intestinal exposure, more severe mucosal injury and CIGT.[8, 9] This mechanism has been demonstrated as mice, treated with irinotecan and a β-glucuronidase inhibitor, had decreased irinotecan-induced diarrhoea.[10] The clear link between the intestinal microbiota and their products modulating irinotecan toxicity is further shown as irinotecan-induced diarrhoea is associated with alterations in intestinal microbiota composition.[8, 9] Both the microbial

and cellular factors released during irinotecan-induced damage influence mucosal immune responses,[8] including toll-like receptor (TLR) activation.

TLRs are a family of pattern recognition receptors that recognise various commensal bacterial ligands to maintain gut homeostasis, restraining inflammation and accelerating healing.[8] Thus, TLRs form an important part of the gastrointestinal innate immune response,[2] being expressed on both immune and intestinal epithelial cells, and are implicated in states of intestinal inflammation, including inflammatory bowel disease[11] and CIGT.[9]

There is a strong biological rationale implicating TLR4 in CIGT pathobiology, largely based on its interaction with microbial ligands and regulation of innate immune responses.[12] It is well documented that irinotecan-induced acute cytotoxic injury in CIGT is perpetuated by indirect cellular injury,[5, 7] initiating innate immune activation and pro-inflammatory cytokine production.[7, 12] Here, the cytokine profile is consistent with that of TLR4 activation,[12] including increased interleukin-6 (IL-6), interleukin-1-beta (IL-1β) and tumour necrosis factor-α (TNF-α).[12-14] Furthermore, during CIGT, the conditions favour gram negative bacteria as there is increased lipopolysaccharide (LPS) production, TLR4s main activating ligand in CIGT.[12]

Based on a strong foundation of anecdotal evidence implicating TLR4 in CIGT, it is not surprising that it is documented to be upregulated in the gut following chemotherapy treatment.[8, 9, 15-17] More recently, it has been shown that TLR4 global knockout (*Tlr4*$^{-/-}$) mice are protected from both CIGT[9] and ulcerative colitis-induced colorectal cancer.[18] While insightful, this data is inherently limited in translational capacity as emerging evidence suggests that tumour-bearing mice with diminished TLR4 signalling have decreased chemotherapeutic efficacy, and consequently, increased tumour burden.[19, 20] This suggests tumour response to chemotherapy requires TLR4 signalling.[19] However, little research has sought to understand whether TLR4 regulation of chemoefficacy and toxicity is via its expression on immune or epithelial cells. In villin-TLR4 mice, where *epithelial* TLR4 is over-expressed, impaired epithelial barrier, microbiota alterations and increased predisposition to colitis have been reported.[21] Contrastingly, when *epithelial* TLR4 is diminished, the epithelium is hypo-

responsive to LPS,[22] suggesting a homeostatic role for epithelial TLR4.[23] Together these studies demonstrate *epithelial* TLR4 plays a role in intestinal homeostasis and immune response,[23] implying its contribution to inflammation and therefore the possibility to restrict interventional approaches to epithelial TLR4 to control CIGT.

Disruption of the intestinal barrier is a prominent feature of CIGT, as the severity and duration of intestinal barrier injury is associated with increased symptoms and infection risk.[17, 24] TLR4 has been indicated to be imperative in intestinal barrier injury in CIGT, as *Tlr4*$^{-/-}$ mice were protected from barrier injury, and hence, had reduced duration and severity of diarrhoea in comparison to wild-type (WT) mice.[9] Furthermore, TLR4-produced cytokines have been implicated in inducing tight junction dysfunction,[25] as *Tlr4*$^{-/-}$ mice had decreased pro-inflammatory cytokines and retained tight junction expression.[9] Apical tight junction proteins, particularly claudins, play a key role in intestinal barrier function.[25, 26] The tight junction proteins most commonly associated with CIGT are claudin-1, zonula occludens (ZO-1) and occludin.[17, 27, 28] After irinotecan treatment, their expression is known to remain unchanged, however their relocation contributes to poor barrier function and leakiness.[17] Other tight junction proteins implicated in intestinal inflammation are yet to be investigated.

Claudin-2 is a pore-forming protein responsible for increasing intestinal permeability to promote transport across the mucosa.[26] In addition, claudin-2 directly decreases the barrier function of the claudin-1 and -4 strands.[26] Previously, in an inflammatory state, of dextran sodium sulfate-induced colitis, claudin-2 overexpression protected barrier function and decreased inflammation.[29] Opposingly, claudin-2 has also been shown to upregulate and contribute to symptomology in intestinal inflammatory states, including ulcerative colitis[30] and FOLFOX-induced CIGT (a combination of 5-fluorouracil, leucovorin, and oxaliplatin).[15] This literature demonstrates that little research has sought to understand claudin-2 expression in intestinal inflammatory states, particularly irinotecan-induced CIGT.

The literature clearly covers TLRs role in gut homeostasis, particularly by identifying the implication of TLR4 in CIGT. Despite a clear biological rationale and translational potential, few studies have

explored whether CIGT manifestations are a result of epithelial or immune TLR4 signalling. Furthermore, it is unknown whether mice lacking TLR4 in intestinal epithelial cells (*Tlr4*$^{\Delta IEC}$) display differences in irinotecan efficacy and response. Lastly, whilst the clear link between TLR4 and tight junction disruption is defined, TLR4's interaction with claudin-2 remains entirely unexplored, thus producing a knowledge gap.

Therefore, from the literature it was hypothesised that:

- Deletion of intestinal epithelial TLR4 will result in **decreased** severity of irinotecan-induced CIGT.
- There will be **no effect** of epithelial TLR4 intestinal deletion on tumour growth.
- A lack of epithelial TLR4 signalling will **prevent** changes in claudin-2 expression following irinotecan treatment.

As guided by the hypotheses, this study aimed:

- To measure the effect epithelial TLR4 intestinal deletion has on severity of irinotecan-induced CIGT manifestations, including, diarrhoea, weight loss and organ atrophy.
- To determine the effect epithelial TLR4 intestinal deletion has on tumour growth.
- To characterise the change in expression of claudin-2 before and after irinotecan treatment in WT and *Tlr4*$^{\Delta IEC}$ mice.

Materials & Methods

Ethics

This study was approved by the University of Adelaide Animal Ethics Committee (M-2020-028) and complied with the National Health and Research Council Australian Code for the care and use of animals for scientific purposes (8th edition: 2013).[31]

Experimental Design

Animal Model

The study was conducted in male and female C57BL/6 mice. Hemizygous vil1-cre and homozygous floxed TLR4 mice were crossed to obtain *Tlr4*$^{\Delta IEC}$ mice. *Tlr4*$^{\Delta IEC}$ and WT littermates were used in all experiments. All mice were subcutaneously implanted with the colorectal tumour cell line MC-38, in the right flank, according to methods previously established by Milczarek et al (2013), who demonstrated reproducible responses of the cell line to irinotecan treatment in C57BL/6 mice.[32] Briefly, 24 mice, aged between 6-10 weeks, were injected with 200 µL of MC-38 cell suspension at a concentration of 1×10^7 cells/mL.[33] Tumours were grown until they reached 0.2 cm^3 in volume before being treated,[33] taking ~7 to 10 days. Daily measurements were taken with digital callipers. Tumour volume was calculated with the formula:[32]

$$\frac{((\text{tumour width}^2) \times \text{tumour length}))}{2}$$

Tumour burden was assessed by calculating tumour volume as a percentage of body weight.

Treatment

WT and *Tlr4*$^{\Delta IEC}$ mice were randomly divided into two treatment groups: Control (sorbitol lactic acid buffer; WT n = 6, *Tlr4*$^{\Delta IEC}$ n = 6) or irinotecan hydrochloride (kindly provided by Pfizer; WT n = 6, *Tlr4*$^{\Delta IEC}$ n = 6).

Irinotecan was dissolved in sorbitol lactic acid buffer (pH 3.4) and administered in a single 270 mg/kg intraperitoneal dose.[9] Mice were humanely killed at 72 hours after treatment, via CO^2 inhalation and cervical dislocation (*figure 1*).

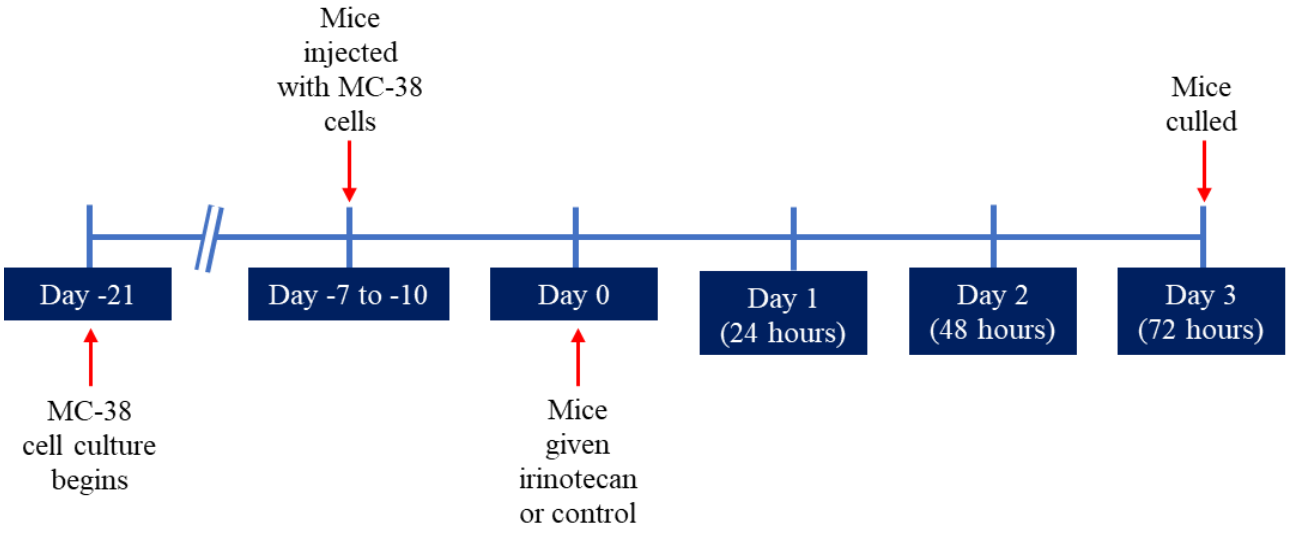


Figure 1. Experimental Design

Clinical Outcomes

Animals were weighed once daily. Diarrhoea was graded as none (G0), mild diarrhoea with staining of the anus (G1), moderate diarrhoea with staining spreading over the top of the legs (G2), and, severe diarrhoea with staining over the legs and abdomen, often with continual anal leakage (G3).[9, 16] Once tumours reached a measurable size, they were measured daily throughout the experiment. At time of cull the spleen, small intestine and colon were removed and weighed.

Tissue Preparation

Cold phosphate-buffered saline (PBS) was used to flush the gastrointestinal tract after removal, before weighing. Samples of the ileum and proximal colon were cut into ~2 cm length pieces and fixed in 10% neutral buffered formalin for 24 hours. Samples were transferred to 70% ethanol, before being processed and embedded in paraffin wax. Remaining sections of the ileum and colon were snap-frozen and stored at -80°C.

Immunofluorescence Staining

All histopathological analysis was performed on samples of ileum and proximal colon. All samples were cut to 4 μm using a microtome and mounted onto FLEX IHC microscope slides (Flex Plus Detection System, DAKO, China; #K8020) for immunofluorescence analysis, as described previously.[17] In brief, immunofluorescence analysis was performed for claudin-2, using DAKO reagents on an automated machine, AutostainerPlus (DAKO, China; #AS480), following manufacturers protocols. Sections were dewaxed in histolene, before slowly being rehydrated through graded ethanol (100%, 90% and 70%) and introduced into water. Antigen retrieval used EDTA-NaOH buffer (0.37 g/L ethylenediaminetetraacetic acid (EDTA), pH 9), in a preheated 65°C DAKO PT LINK water bath (DAKO; #PT10126). Samples were immersed and raised in temperature to 97°C for 20 minutes. After returning to 65°C, slides were placed into the DAKO AutostainerPlus and blocked using 10% normal horse serum (NHS). The claudin-2 rabbit polyclonal primary antibody was diluted with 5% NHS at concentration of 5 μg/ml. The fluorescently labelled secondary antibody, donkey anti-rabbit (AlexaFluor 568) at a concentration of 0.8 μg/mL, was diluted with 1% PBS, 1% bovine serum albumin (Sigma-Aldrich, #1002440032) and 2% foetal bovine serum. Similarly, slides were counterstained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Life Sciences; #D1306), which was diluted with 1 x PBS. Slides were cover slipped with Fluoroshield mounting medium (Sigma-Aldrich, #1003019312), and sealed with nail polish. The primary antibody was omitted for all negative controls. Slides were imaged on the Axio Scan.Z1 (ZEISS). Immunofluorescence was assessed by quantification of percentage area staining, using ImageJ (Fiji) (technical note 07, area and intensity of stain).[34]

Real-Time PCR (qPCR)

RNA Extraction

Thirty mg of tissue (either ileum or colon) was placed into 350 μL of lysis buffer and 3.5 μL of beta-mercaptoethanol and homogenised, using the Qiagen Tissue Lyser LT (Germany). The protocol was followed in accordance with the manufacturer's instructions (Macherey-Nagel, 2013) to achieve

RNA that was eluted in 60 µL of RNase-free water. RNA yield and purity were measured using the BioTek® multi-mode microplate reader, with the Take3 micro-volume plate (BioTek® Instruments Inc. USA). A 260/280 ratio of ~≥2.0 was accepted as RNA with a high purity yield (ThermoFisher USA, 2012). All RNA was stored at -20°C. Randomly RNA samples were chosen and underwent RNA integrity number (RIN) analysis to ensure quality (Adelaide Microarray Centre).

cDNA Conversion

1 µg of RNA was converted to cDNA using the iScript cDNA Synthesis Kit, as per manufacturer's instructions (Bio-Rad, #1708891). cDNA yield and purity were measured using the BioTek®Take3 micro-volume plate. A 260/280 ratio of ~1.8 was accepted as cDNA with a high purity yield (ThermoFisher USA, 2012). cDNA was diluted to 200 ng/µL concentration and stored at -20°C.

qPCR

Primers were used from the literature or designed, by accessing the National Centre of Biotechnology Information (NCBI) and retrieving the sequence of the gene of interest. The sequence was placed into Primer 3 (version 4.0) to produce the forward and reverse primer sequences. The quality of all primers used was checked using Net Primer (Premier Biosoft). *Table 1* outlines the mRNA sequences for primers, all of which were synthesised by Sigma-Aldrich.

Table 1: qPCR Primers

Primer Name	Sequence	Primer Length (bp)	Reference
GAPDH forward	5'-CCTCGTCCCGTAGACAAAATG-3'	21	(Wardill et al 2016)
GAPDH reverse	5'-TCTCCACTTTGCCACTGCAA-3'	20	
Claudin-2 forward	5'-GGAGATCTGTCCCCAAACCA-3'	20	As designed for this study
Claudin-2 reverse	5'-AAGCTTCAGGGCCCATTACT-3'	20	

qPCR was performed on the Rotor Gene Q (Qiagen, Australia). Each well contained 10 μL which included 0.5 μL of the appropriate forward and reverse primers (50 pmol), 1 μL (200 ng) of cDNA, 3 μL of nuclease-free water, and, 5 μL of fluorescent SYBR green dye (QuantiTect SYBR® Green, Qiagen). The claudin-2 forward and reverse primer were optimised to the thermal cycling conditions. All samples were run with the following thermal cycling conditions, hold at 95°C for 10 minutes and amplification through 40 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 45 seconds. GAPDH, cited from Wardill et al (2016), was chosen as the appropriate housekeeping gene for normalising expression.[17] Transcript expression was calculated by the delta delta CT method and presented as a fold-change.[35]

Statistical Analyses

Prism 8.0 (GraphPad Software) was used for all statistical analysis. Data was tested for normality using the Kolmogorov-Smirnov test. Normally distributed data was analysed with a one-way or two-way ANOVA with Tukey's post-hoc test, while non-parametric data was analysed with a Kruskal-Wallis with Dunn's post-hoc test. This excluded diarrhoea severity, which was analysed with a chi-square test. In all cases a p-value of ≤ 0.05 was considered statistically significant.

Results

Irinotecan-treated mice lost weight, reaching nadir at day 2

WT and *Tlr4*$^{\Delta IEC}$ mice lost significant weight following treatment compared to control counterparts (*figure 2*). On day 3, treated *Tlr4*$^{\Delta IEC}$ were significantly lighter than treated WT mice (*figure 2*).

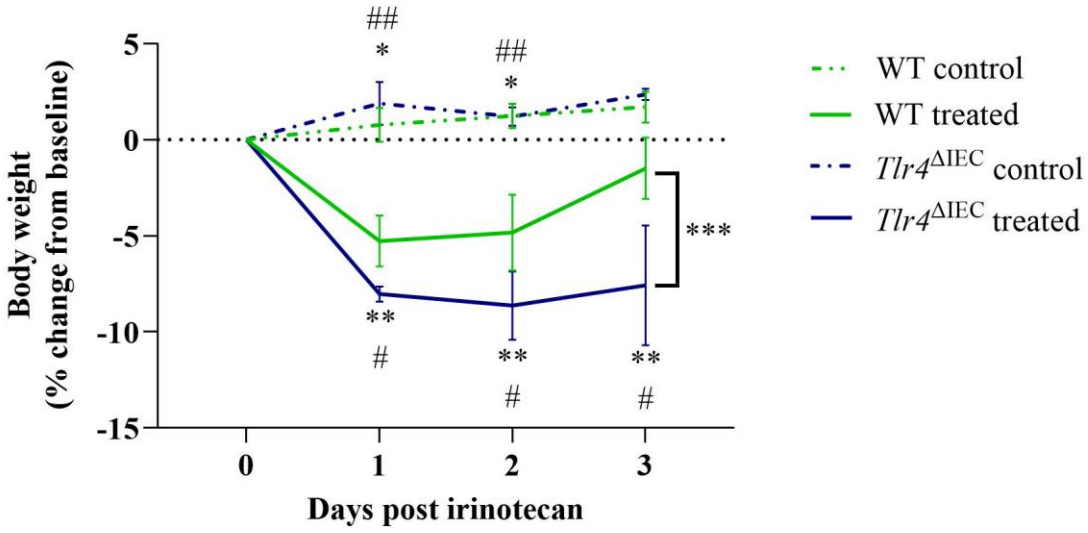


Figure 2. Change in body weight (%) from baseline (day 0). Treated mice weighed significantly less then control (*WT control versus treated p-value = 0.0050, 0.0047 (day 1 and 2, respectively), **$Tlr4^{\Delta IEC}$ control versus treated p-value <0.0001 (day 1, 2, and, 3), #WT control versus $Tlr4^{\Delta IEC}$ treated p-value <0.0001 (day 1, 2, and, 3), ##$Tlr4^{\Delta IEC}$ control versus WT treated p-value = 0.0006, 0.0050 (days 1 and 2, respectively), and, ***WT treated versus $Tlr4^{\Delta IEC}$ treated day 3 p-value = 0.0045). Data was presented as mean ± SEM.

Tlr4$^{\Delta IEC}$ mice are protected from irinotecan-induced diarrhoea

40% of WT and 17% of *Tlr4*$^{\Delta IEC}$ mice treated with irinotecan experienced diarrhoea on day 1 (*figure 3B*). The percentage of WT treated mice with a higher grade of diarrhoea was significant in comparison to other groups on day 1 (*figure 3B*). Diarrhoea improved on day 2 and was completely resolved by day 3 (*figure 3C & D*).

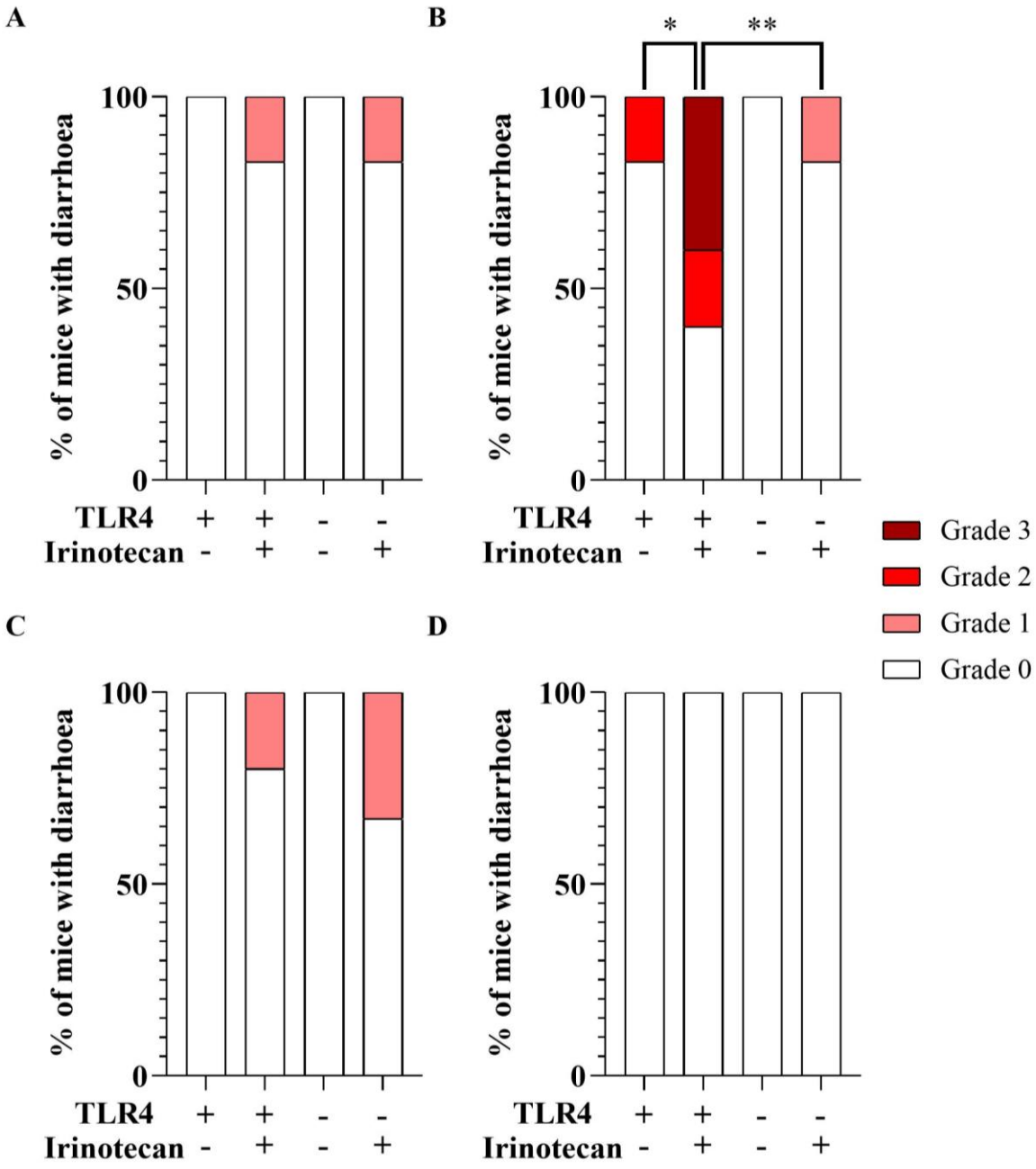


Figure 3. Percentage (%) of mice with diarrhoea grade at (A) 6 hours, (B) day 1, (C) day 2, and (D) day 3. On day 1 WT treated (+/+) had significantly more diarrhoea than WT control (+/-) mice (*p-value <0.0001) and *Tlr4*$^{\Delta IEC}$ treated (-/+) mice (**p-value <0.0001).

Epithelial TLR4 deletion has no effect on organ or tumour weight

There was no difference in small intestine or colon weight between control and irinotecan treated groups in both WT and *Tlr4*$^{\Delta IEC}$ animals (*figure 4*). Both treated groups had a significant decrease in spleen weight in comparison to control groups (*figure 4C*).

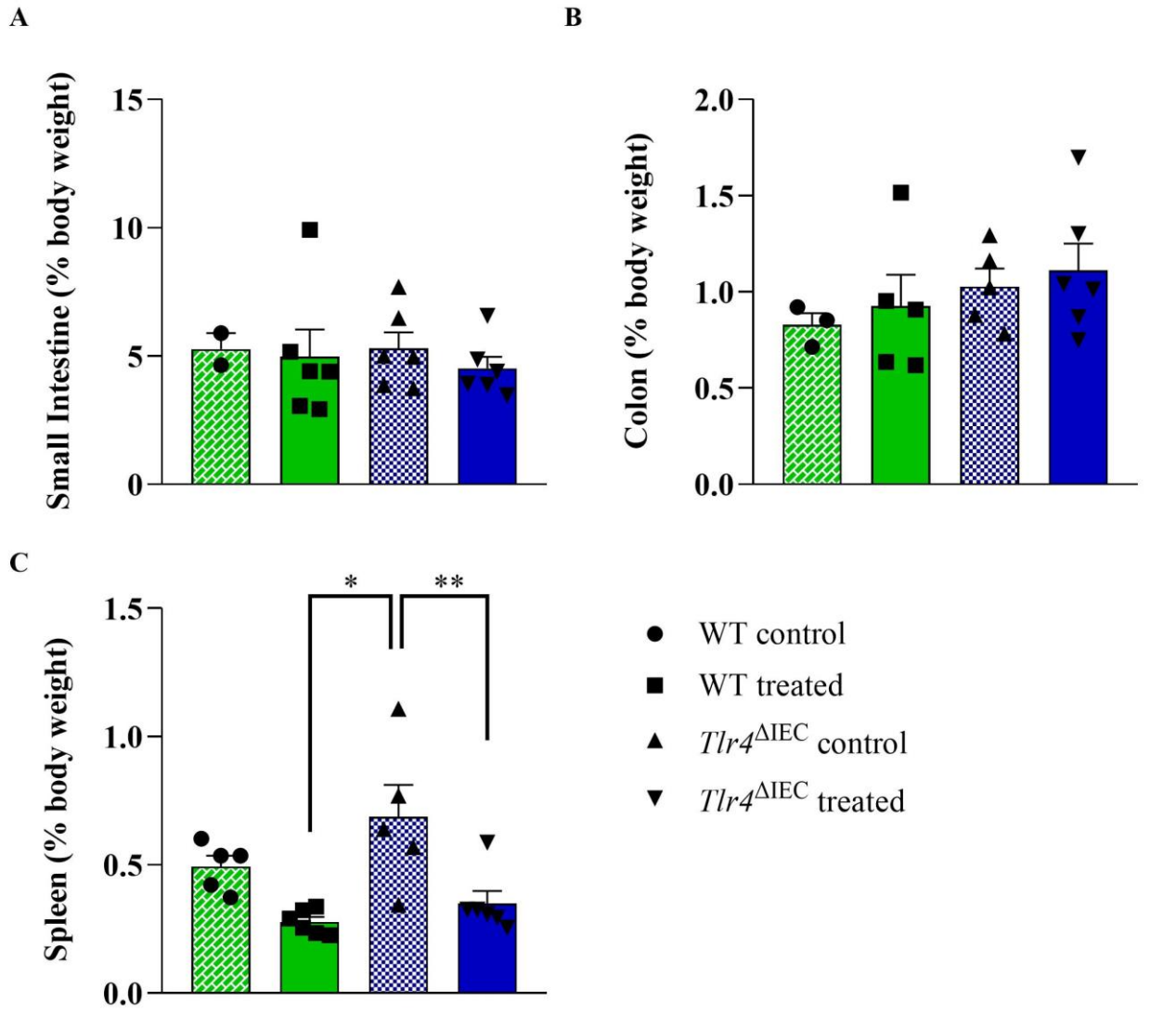


Figure 4. The (A) small intestine, (B) colon, (C) spleen weight relative to body weight. The splenic weight of treated groups was significantly less than that of the *Tlr4*$^{\Delta IEC}$ controls (p-value, *WT treated = 0.0020, **$Tlr4^{\Delta IEC}$ treated = 0.0105). Data was presented as mean ± SEM.

Irinotecan efficacy does not require epithelial TLR4

On day 3, tumour burden was significantly less in treated mice compared to their control counterparts, for both WT and *Tlr4*$^{\Delta IEC}$ (*figure 5*).

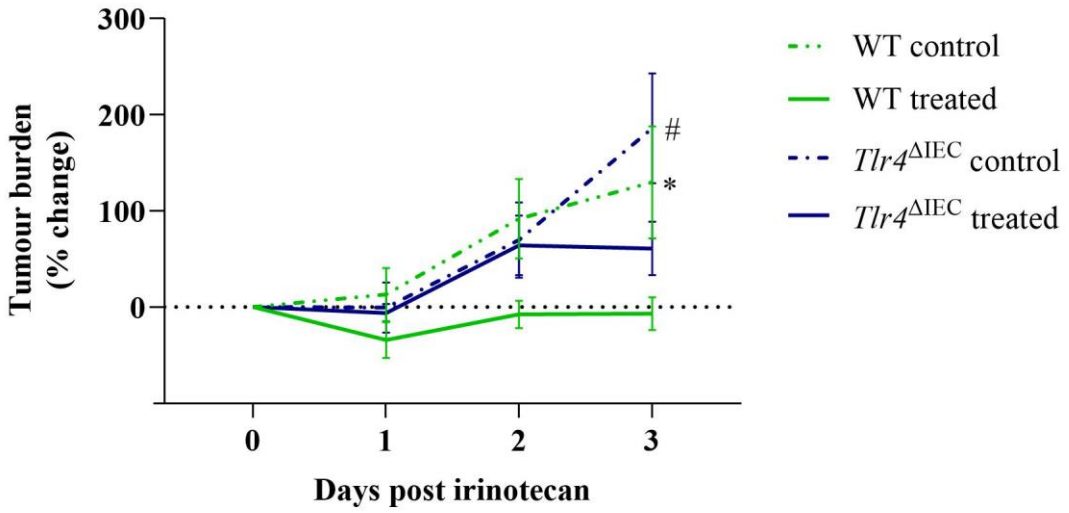
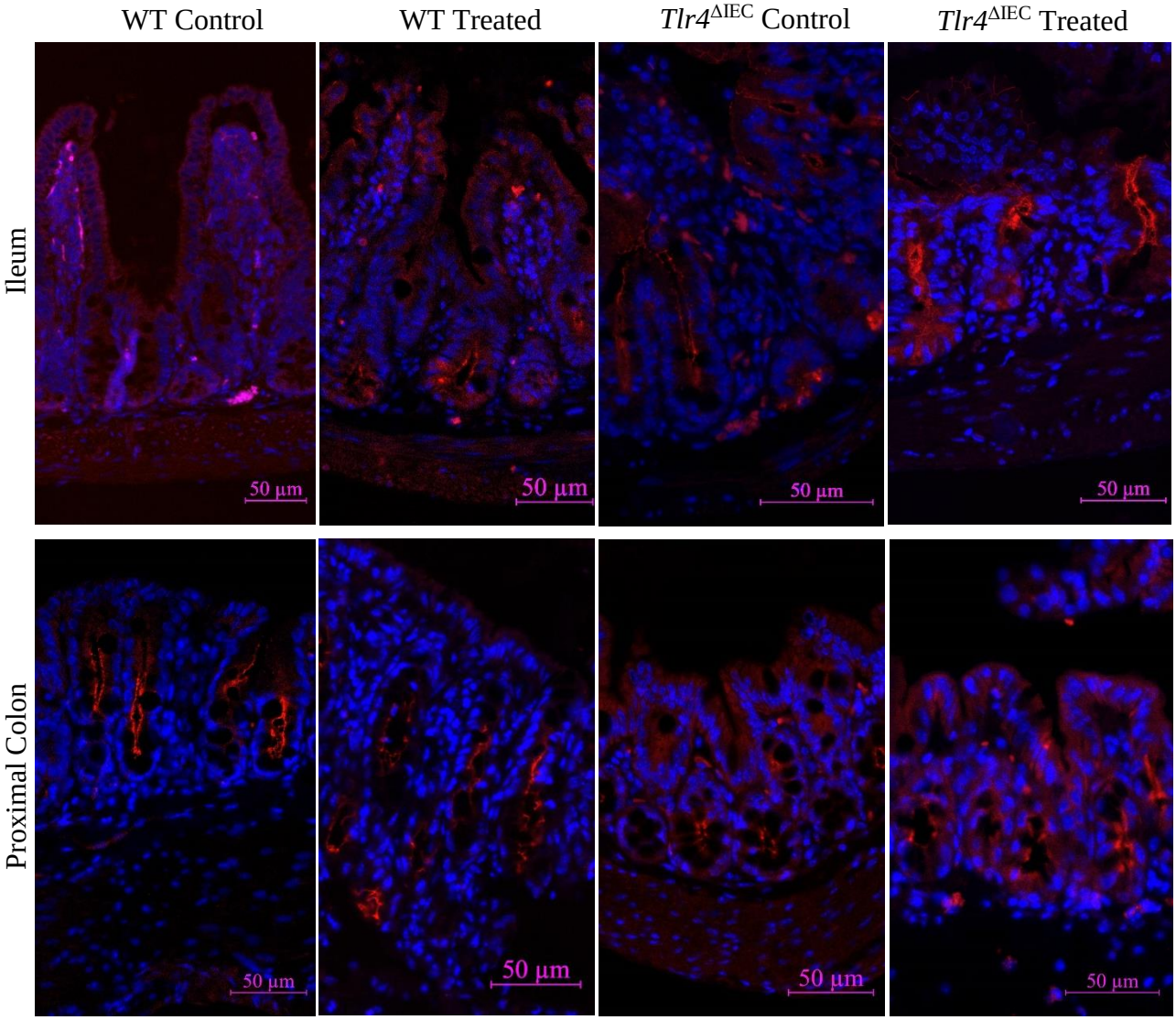


Figure 5. Tumour burden expressed as % change from baseline (day 0). Irinotecan caused decreased tumour growth in WT treated (*p-value = 0.0086), and *Tlr4*$^{\Delta IEC}$ treated (#p-value = 0.0192) mice in comparison to controls. Data presented as mean ± SEM.

Irinotecan impacts claudin-2 expression differently in the small intestine and colon

Representative immunofluorescence images are shown in *figure 6A.* There were no differences in ileum claudin-2 staining or transcript expression between the groups (*figure 6B, C & D*). There were increased claudin-2 colon transcript levels in *Tlr4*$^{\Delta IEC}$ control, in comparison to *Tlr4*$^{\Delta IEC}$ treated mice (*figure 6E*).

A



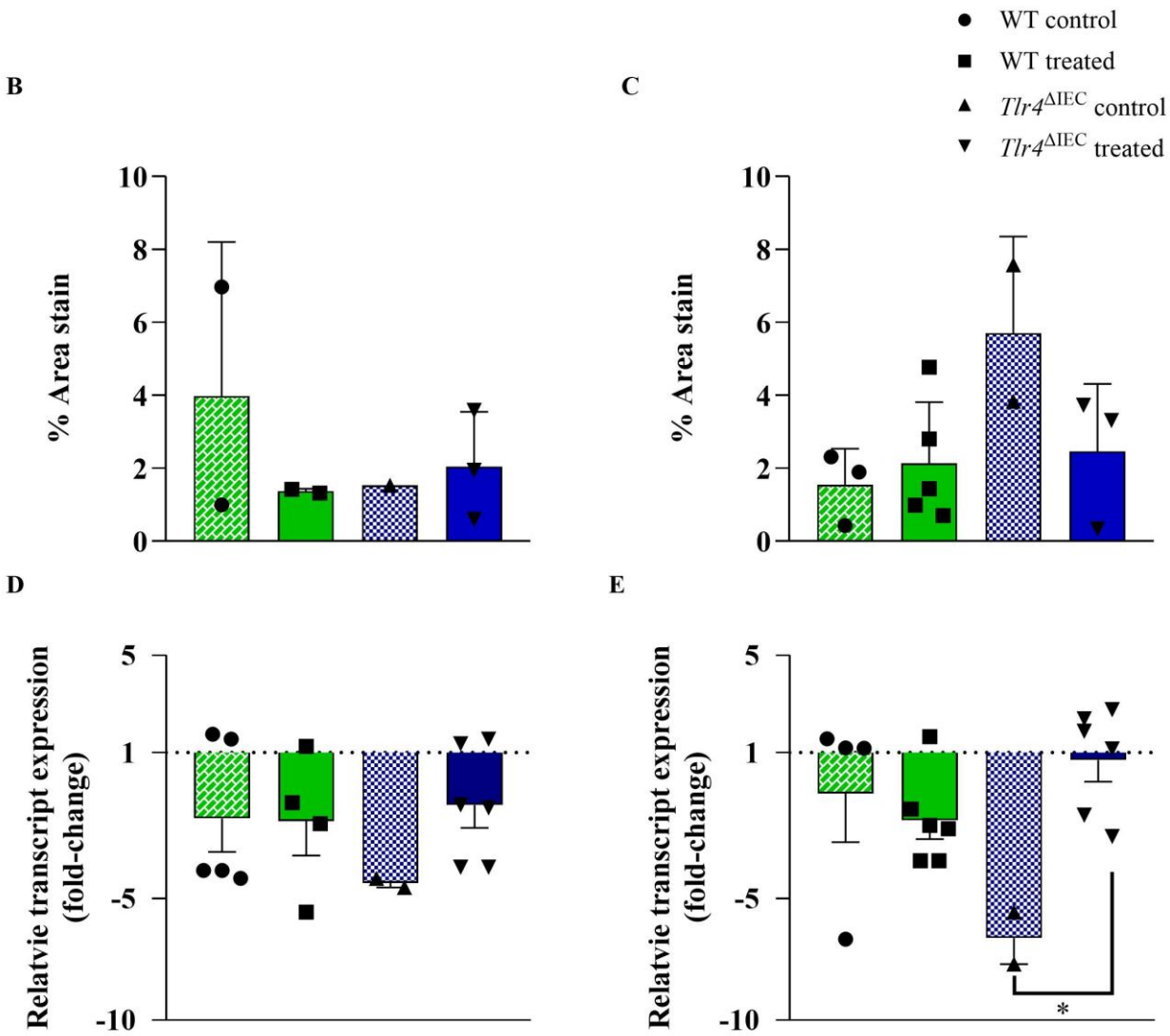


Figure 6. Claudin-2 expression in the ileum and colon. (A) Sections of ileum and proximal colon were stained for claudin-2 and visualised using an AlexaFluor 568. Blue counterstain (DAPI) shows nuclei. Original magnification 40x. Percentage (%) area of claudin-2 staining in (B) ileum and (C) proximal colon, and claudin-2 expression as a fold-change in (D) ileum and (E) colon. In the colon there was increased claudin-2 transcript expression in *Tlr4*$^{\Delta IEC}$ control relative to treated group (*p-value = 0.0163). Data presented as mean ± SEM.

Discussion

Gastrointestinal complications of cancer therapy remain poorly prioritised and without effective intervention. Strong anecdotal evidence suggests supportive care interventions targeting TLR4 may be of clinical benefit, however, its dual regulation of mucosal toxicity and chemoefficacy challenge translation of TLR4-based therapeutics. This study proposed to understand the relationship between intestinal TLR4 signalling, intestinal inflammation, and response to irinotecan treatment, in a mouse model of colorectal cancer.

This study found disparate effects in irinotecan-induced manifestations. While treated *Tlr4*$^{\Delta IEC}$ had less diarrhoea compared to WT mice, they showed increased weight loss and delayed body weight recovery. This finding is of interest since previous work in *Tlr4*$^{-/-}$ mice has shown reduced weight loss following irinotecan compared to WT counterparts.[9] Alternatively, other models of inhibited TLR4 signalling in CIGT and acute colitis showed no weight difference in comparison to WT.[20, 36] Contrastingly metabolic syndrome studies, utilising *Tlr4*$^{\Delta IEC}$ mice with no intestinal inflammation, showed a significant weight gain response in the *Tlr4*$^{\Delta IEC}$ mice in comparison to WT.[37] This suggests different weight governing mechanisms in *Tlr4*$^{\Delta IEC}$ and WTs.[37] It is important to note that whilst these studies cannot be discounted, all compared studies used different strains of mice and, since there are immune response differences between mice strains,[38] the comparison of the current study to others needs to be interpreted with caution. Nevertheless, these studies allude to a critical role of TLR4 in weight regulation, however the underlying mechanisms and how the weight change may present itself are unknown. My results demonstrate, in irinotecan-induced CIGT, that epithelial TLR4 potentially regulates weight-loss recovery after treatment.

The day 1 diarrhoea response is supported by previous literature, as *Tlr4*$^{-/-}$ mice have displayed this response to irinotecan.[9] However, this is contrasting to TLR4 antagonism studies, which reported no differences between the TLR4 antagonist group and control rats in irinotecan-induced diarrhoea.[20] To support the current studies response, it has been suggested that *Tlr4*$^{-/-}$ mice have decreased intestinal motility and delayed emptying, in comparison to WT,[39] which could contribute to attenuated

diarrhoea. Therefore, as supported by *Tlr4*$^{-/-}$ models, this study shows that *Tlr4*$^{\Delta IEC}$ mice are protected from irinotecan-induced diarrhoea, which could be a result of decreased intestinal motility and emptying.

Furthering this, the small intestine and colon weight response in both WT and *Tlr4*$^{\Delta IEC}$ treatment groups does not support the current literature, as previously conducted unpublished data from our lab showed small intestine and colon wet weight decreased after one dose of irinotecan, at 24 hours (Wardill 2016). This is thought to be a result of irinotecan targeting highly rapid proliferating cells. In light of this conflicting evidence, it could be suggested the response was seen as the explored timepoint was after some indicators of toxicity had lessened, such as no groups had diarrhoea on day 3. This limitation could be improved with the employment of a more sensitive marker of microscopic architectural changes to measure mucosal thickness, such as crypt depth and villus height ratios. In contrast, the spleen weight response was as expected, as previously irinotecan treated groups have had a reduced spleen weight.[40] Mechanisms underlying irinotecan's splenic effect is currently unknown, however other chemotherapeutics, oxaliplatin, also showed decreased spleen weight.[41] This was suggested to be the result of the chemotherapeutics immunogenic effects, particularly spleen B-cell depletion.[41]

The current study found that *epithelial* TLR4 deletion did not significantly impact irinotecan efficacy. This is contrasting to previous work, where global *Tlr4*$^{-/-}$ have shown decreased doxorubicin efficacy against mouse colon carcinomas.[19] Furthermore, our lab previously showed that TLR4 antagonism significantly reduced irinotecan efficacy against mammary adenocarcinomas.[20] Taken together, this data suggest that *immune* TLR4 is more likely to mediate irinotecan efficacy, at least for certain tumour types. This is highly plausible as the immune system is critical in anti-cancer responses, with TLR4 facilitating immunogenic cell death that amplifies chemotherapy efficacy.[19] Taken with previous findings, this enables the potential for targeting of *epithelial* TLR4 to reduced diarrhoea severity, without inhibiting chemoefficacy.

In addition, this study revealed different amounts of claudin-2 expression in the ileum and colon. In the ileum, there was no difference in claudin-2 expression at the protein or transcript level. Here, due to lack of TLR4, it is rational to expect that claudin-2 would not increase in *Tlr4*$^{\Delta IEC}$ mice. This is because a link between claudin-2 and TLR4 is implied, as previous studies have found a simultaneous increases in claudin-2 and TLR4 in WT mice following FOLFOX treatment.[15] However, it is interesting the expected claudin-2 increase was not seen in the WT mice. This could be a result of claudin-2 not being implicated in irinotecan-induced CIGT, the small sample size of groups and thus underpowered study, the large variability in the data, or, the analysis technique being unable to recognise more subtle changes. To improve this limitation a more appropriate technique could have included Z-stacks to quantify tight junction placement, as irinotecan treatment can result in relocation away from the membrane and hence render tight junction proteins non-functional for regulating permeability.[17] Contrastingly, in the colon, there were low relative levels of claudin-2 transcript expression in the *Tlr4*$^{\Delta IEC}$ control mice, but levels were more similar to WT control in the *Tlr4*$^{\Delta IEC}$ treated mice. Intriguingly, however, there was no difference observed between the *Tlr4*$^{\Delta IEC}$ and WT groups. This indicates that potential molecular differences between the groups trigger different responses in claudin-2 expression. This is supported in the literature, as when a colonic cell culture was treated with the different inflammatory signalling molecules the expression of claudin-2 differed, as it increased after anti-inflammatory interleukin-13 (IL-13) and decreased after pro-inflammatory interferon gamma (INFγ)/TNFα treatment.[42] It is currently unclear as to why the *Tlr4*$^{\Delta IEC}$ control mice would have such low levels of caludin-2 expression, since from initial analysis they phenotypically have normal barrier function in comparison to WT control, as shown by tissue resistance scores (preliminary unpublished data). Because of the contradictory results in claudin-2 expression further tissue analysis needs to be undertaken before more robust conclusions can be drawn.

Due to the differing severity of CIGT manifestations, particularly weight loss and diarrhoea, in *Tlr4*$^{\Delta IEC}$ mice the hypothesis, *deletion of intestinal epithelial TLR4 will result in* ***decreased*** *severity*

of irinotecan-induced CIGT, was partially supported. Furthermore, due to the observed irinotecan efficacy in *Tlr4*$^{\Delta IEC}$ mice the hypothesis, *there will be* ***no effect*** *of epithelial TLR4 intestinal deletion on tumour growth*, was supported. Lastly, the hypothesis, *a lack of epithelial TLR4 signalling will* ***prevent*** *changes in claudin-2 expression following irinotecan treatment*, was partially supported. This is because, whilst there were no changes in ileum claudin-2 expression, there was in the colon claudin-2 transcript expression between *Tlr4*$^{\Delta IEC}$ groups. Therefore, due to the partial support of hypotheses, further research into this area is warranted to confirm the responses.

In an effort to correlate markers of irinotecan-induced gastrointestinal toxicity and predictors of CIGT, an additional piece of work was conducted to find statistical associations between variables through the use of multivariate logistic regression (*supplementary materials*). Using this approach, we were able to identify that body weight, diarrhoea, intestinal weight and spleen weight, were strongly and significantly associated with histopathologically defined CIGT. These validate the use of these outcomes in the assessment of CIGT, however, further challenge the disparate results obtained in the current study (weight loss vs diarrhoea). This could be due to differences between the study and the model used for the scoring system development, which was different in mouse strain and age. This therefore poses the need for the creation of a universally used robust tool that is modelled off multiple models to accurately and sensitively predict CIGT markers.

Due to result discrepancies, future improvements could be to conduct studies focusing on longitudinal changes across initial injury to full recovery time points, with the addition of histological analysis to quantify architectural differences between groups, using tissue injury scores. This may give further reasoning behind the disparate results in weight change and diarrhoea severity. Additionally, irinotecan dosage could be modified to be more reflective of a clinical setting, with the inclusion of multiple doses. Furthermore, when conducting the animal experiment, future studies should implement a food consumption and activity monitoring system, such as metabolic cages with motion tracking, to potentially aid in the interpretation around weight change.

In addition, to explore the role of TLR4 deletion on response to irinotecan, future studies would need to be powered for the expected tumour burden. Given our variability in growth patterns, as shown by the large error bars, more animals would be needed to adequately power the study. Together, these improvements would further strengthen the conclusion made regarding irinotecan efficacy and be particularly beneficial for model validation, as this was the first time our lab has used *Tlr4*$^{\Delta IEC}$ mice in a tumour bearing model.

Lastly, since TLRs are known to be the interface between the microbiota and downstream processes, it would be beneficial for future research to investigate the implications that *Tlr4*$^{\Delta IEC}$ mice have on microbiota composition, which may further elucidate the manifestations seen in both toxicity and tumour growth.

In conclusion, this study outlined for the first time that different TLR4 mechanisms underlie diarrhoea and weight loss manifestations in CIGT, which have to this point been considered markers of the same underlying disease mechanisms. Importantly, we have confirmed there are differing roles of epithelial and immune TLR4 and that future TLR4 agonist research should account for this when using models of inflammation. In addition, whilst not being directly studied, the findings revealed a novel potential requirement of immune TLR4 on irinotecan efficacy, opening a new research avenue to control TLR4-mediated side effects without impairing tumour kill. Taken together, these findings warrant future research targeting epithelial TLR4 to attenuate irinotecan-induced diarrhoea in tumour-bearing models, with caution exercised when monitoring animal weight. Attenuation of irinotecan-induced diarrhoea would provide immense clinical relief, as this is a key contributor to treatment reductions and breaks in patients with cancer.[2]

Professional & Funding Acknowledgements

The University of Adelaide Development Grant (2017 CIA Bowen) provided funding for this study. I would like to express my sincere gratitude to my primary and secondary supervisors, particularly Associate Professor Joanne Bowen for her continuing support and dedication over the year. Furthermore, to the members of the Cancer Treatment Toxicities Group, particularly the technical/animal model and writing assistance of Elise Bruning and Hannah Wardill. Finally, to Jane Sibbons and Agatha Labrinidis from Adelaide Microscopy, Pacita Wissell from Laboratory Animal Services, and Susan Edwards from Data, Design and Statistics Service at Adelaide Health Technology Assessment for your services and assistance throughout this study.

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Supplementary material

Mucositis Severity Scoring System

Data that has previously been collected from a mouse model of mucositis (n = 23) was statistically evaluated to uncover variables that are significantly associated with mucositis. This was performed with a univariate binary logistic regression where ileum mucositis was the outcome that predictors were tested against. The gold standard used to define ileum mucositis was tissue injury score (TIS), a validated tool across the literature. The following was the given data ranges for ileum mucositis definition:

- Small bowel (jejunum) TIS of 4 or above (out of a possible 8)
- Large bowel (colon) of 3 or above (out of a possible 6)
- Total (i.e. small and large bowel): TIS score of 7 and above (out of a possible 14).

The results are outlined in the table below.

Variables associated with mucositis severity	**Statistical significance**	**p-value**	**Negative or positive correlation with mucositis?**
Weight change from baseline	+ +	0.0090	For every 0.1% increase in weight change the odds of having ileum mucositis decrease by 2%.
Diarrhoea grade	+	0.0102	For every 1 unit increase in diarrhoea grade the odds of having ileum mucositis is multiplied by 15.23.
Small intestine weight	+	0.0281	For every 0.1 unit increase in small intestine weight the odds of having ileum mucositis is multiplied by 1.18.

Large intestine weight	+	0.0108	For every 10 unit increase in large intestine weight the odds of having ileum mucositis is decreased by 13%.
Organ spleen weight	+ +	<0.0001	For every unit increase in organ spleen weight the odds of having ileum mucositis is decreased by 6%.
Welfare score	+ +	<0.0001	For every unit increase the odds of having ileum mucositis is multiplied by 4.47.