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Irinotecan-induced gastrointestinal dysfunction and pain are mediated by common TLR4-dependent mechanisms

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Abstract

Strong epidemiological data indicates chemotherapy-induced gut toxicity and pain occur in parallel, indicating common underlying mechanisms. We have recently outlined evidence suggesting that TLR4 signaling may contribute to both side effects. We therefore aimed to determine if genetic deletion of TLR4 improves chemotherapy-induced gut toxicity and pain. Forty-two female wild-type (WT) and 42 *Tlr4* null (-/-) BALB/c mice weighing between 18-25 g (10-13 weeks) 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. Bacterial sequencing was conducted on caecal samples of control animals to determine gut microbiome profile. Gut toxicity was assessed using validated clinical and histopathological markers, permeability assays and inflammatory markers. Chemotherapy-induced pain was assessed using the validated rodent facial grimace criteria, as well as immunological markers of glial activation in the lumbar spinal cord. TLR4 deletion attenuated irinotecan-induced gut toxicity, with improvements in weight loss ($p=0.0003$) and diarrhoea ($p<0.0001$). Crypt apoptosis was significantly decreased in BALB/c-*Tlr4*$^{-/-billy}$ mice ($p<0.0001$) correlating with lower mucosal injury scores ($p<0.005$). Intestinal permeability to FITC-dextran (4kDa) and LPS translocation were greater in WT mice compared to BALB/c-*Tlr4*$^{-/-billy}$ ($p=0.01$ and $p<0.0001$, respectively). GFAP staining in the lumbar spinal cord, indicative of astrocytic activation, was increased at 6 and 72 h in WT mice compared to BALB/c-*Tlr4*$^{-/-billy}$ mice ($p=0.008$, $p=0.01$). These data indicate that TLR4 is uniquely positioned to mediate irinotecan-induced gut toxicity and pain, highlighting the possibility of a targetable gut/CNS axis for improved toxicity outcomes.

Key words: chemotherapy-induced gut toxicity, mucositis, pain, toll-like receptor 4, gut/CNS-axis, gut microbiome

1.0 Introduction

Irinotecan-induced gut toxicity remains a priority concern within the field of supportive care in cancer. Typically used to treat a variety of solid tumours, irinotecan can cause severe diarrhoea, rectal bleeding and infection in patients, often resulting in dose reductions and treatment delays (1). Irinotecan-induced diarrhoea is clinically very significant as fluid/electrolyte imbalances can lead to renal insufficiency, malnutrition, and extreme dehydration. More importantly, these side effects have severe psychological impacts for patients and significantly effects the ability to deliver optimal treatment (2). Despite both its prevalence and clinical significance, the precise mechanisms that underpin gut toxicity remain unclear and therapeutic options for patients are limited (1).

The broadly accepted pathophysiology of chemotherapy-induced gut toxicity (CIGT) comprises five continuous and overlapping phases described by Sonis (3). Although this model can be applied to most chemotherapeutic agents, each treatment modality has unique pathological features due to differences in the metabolism and pharmacokinetics of each anticancer drug. In the case of irinotecan, its unique enterohepatic recirculation is thought to be responsible for the high levels of intestinal toxicity. Irinotecan serves as the water-soluble precursor of the lipophilic metabolite SN-38, which is formed by carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and the dipiperdino side chain (4). SN-38 is glucuronidated to the non-toxic SN-38 glucuronide (SN-38G) in the liver via the uridine-diphospho-glucuronosyl transferase (UGT1A) enzyme family, which then releases SN-38G into the intestine for elimination (4). However, in the intestinal lumen, bacterial β-glucuronidases regenerate SN-38 from SN-38G (5). This unique metabolic pathway not only results in high levels of intestinal toxicity, but also highlights the key relationship between toxicity and the gut microbiome (5).

The gut microbiome has received significant attention for its role in the development of gut toxicity following chemotherapy, with documented changes in the balance of commensal and pathogenic bacteria following numerous chemotherapeutic agents (5-7). In light of these findings, the interaction between the gut microbiome and innate mucosal immune system has also gained interest, with particular emphasis on the impact of toll-like receptor (TLR) signaling (8-10). TLRs are a family of transmembrane protein receptors recognising a diverse range of signals on exogenous and endogenous substances considered to be ‘dangerous’, and hence warranting activation of the innate immune system for host survival (11-13). TLR4 has been most extensively characterised as it recognises, and responds

to, lipopolysaccharide (LPS) from gram-negative bacteria. We have shown that TLR4 is overexpressed in the gut during peak injury (14) and is undetectable at later time points associated with healing (15). Further, TLR4 is thought to induce an exacerbated innate immune response resulting in a heightened toxicity profile. This mechanism is particularly relevant in the setting of irinotecan-induced gut toxicity, as our preliminary *in silico* docking data indicate that SN-38 has the potential to act as a ligand for the TLR4/MD-2 complex.

TLR4 has also been hypothesised to mediate chemotherapy-induced pain through central glial activation (9), with strong clinical evidence showing chemotherapy-induced gut toxicity is often paralleled by the symptom of pain (16, 17). This is suggestive of common underlying mechanisms. The ubiquitous involvement of the innate immune system in both chemotherapy-induced pain and gut toxicity therefore makes TLR4 a potentially overlooked candidate in the pathophysiology of these toxicities. We therefore hypothesise that TLR4-mediated signaling plays a central role in the development of irinotecan-induced gut toxicity and pain.

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 sourced from Professor Paul Foster from the University of Newcastle (NSW, Australia) and were originally sourced from Osaka, Japan (18). All BALB/c-*Tlr4*$^{-/-billy}$ mice were homozygous null mutants and hence expressed no detectable TLR4 mRNA or protein (personal communication MRH). Mice were treated with a single 270 mg/kg intraperitoneal (i.p.) dose of irinotecan hydrochloride (kindly provided by Pharmacia/Pfizer, Mich, USA) prepared in a sortibol/lactic acid buffer (45mg/ml sorbitol/0.9mg/ml lactic acid; pH 3.4; Sigma-Aldrich, NSW, Australia; D-sorbitol #S1876, lactic acid #252476), which was shown in pilot work to cause reproducible diarrhoea with no mortality. Control mice received the sorbitol/lactic acid buffer only. All mice received 0.03 mg/kg of atropine subcutaneously (s.c.) immediately prior to treatment to reduce the cholinergic response to irinotecan. Mice were randomly assigned to treatment groups and killed at 6 h, 24 h, 48 h, 72 h and 96 h. Mice were anaesthetised using 200 mg/kg i.p. ilium sodium pentobarbital (60 mg/ml) and blood was collected from the facial vein. They were then 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

All mice were monitored four times daily for the presence of diarrhoea and other clinical parameters. Diarrhoea was quantified (by two independent assessors) using a validated grading system where 0 = no diarrhoea, 1 = mild perianal staining, 2 = moderate staining covering hind legs, and 3 = severe

staining covering hind legs and abdomen with continual anal leakage (19). Mice were weighed daily to track weight loss/gain. Mice were killed if they displayed ≥15% weight loss or significant distress and clinical deterioration, in compliance with animal ethical requirements.

2.1.3 Facial grimace criteria

Chemotherapy-induced pain was measured by two independent assessors, in a blinded manner, 4 times daily in all mice using the validated rodent facial grimace criteria (20), as previously published by our group (17). Briefly, the scoring method consists of five distinct criteria: orbital tightening, cheek bulge, nose bulge, ear position and whisker position. Each criterion was scored as 0 = absent, 1 = moderate and 2 = severe. Please refer to Table S1 for detailed breakdown of scoring criteria.

2.1.4 Tissue preparation

Gastrointestinal Tract Following anesthesia with sodium pentobarbital, mice with perfused with chilled, sterile 1 X PBS (pH 7.4). The entire gastrointestinal tract from pyloric sphincter to 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 -20^{o}C for molecular analyses.

Central Nervous System Mice were perfused with 4% PFA and the vertebral column dissected. Vertebral bodies were removed to expose the entire spinal cord. The entire spinal cord from cervical to lumbar regions was removed and the lumbar region prepared for further analysis (L3/L4). The mice were then decapitated and the brain extracted. All tissue stored in 4% PFA overnight for processing and embedding in paraffin wax.

2.2 Bacterial diversity profiling

It is well established that the gut microbiome is involved in the metabolism of irinotecan (21). To confirm both WT and BALB/c-*Tlr4*$^{-/-billy}$ contain similar bacterial profiles, the caecal contents of 12 control animals (WT n=6, BALB/c-*Tlr4*$^{-/-billy}$ n=6) were aseptically collected and sent for genetic sequencing at the Australian Genomics Research Facility (Brisbane, Australia).

The sequencing details are as follows: Target: 341F-806R, Forward primer (341F): 5'-CCTAYGGGRBGCASCAG-3'; Reverse primer (806R): 5'-GGACTACNNGGGTATCTAAT-3'; Read Length: 300bp

2.2.1 Bioinformatics Method

Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (22) (version 0.9.5). Primers were trimmed using Seqtk (version 1.0). Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (23) USEARCH (24, 25) (version 8.0.1623) and UPARSE software.

Using USEARCH tools, sequences were quality filtered, full length duplicate sequences were removed and sorted by abundance. Singetons or unique reads in the data were discarded. Sequences were clustered followed by chimera filtering using the "rdp_gold" database as a reference. To obtain number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Using QUIIME, taxonomy was assigned using Greengenes database (version 13_8, Aug 2013).

PEAR assembly read statistics were as follows: WT BALB/c control 59892 / 67175 (89.16%); BALB/c-*Tlr4*$^{-/-billy}$ control 57982 / 65950 (87.92%).

2.3 Histopathological analysis

Haematoxylin and eosin (H&E) staining was performed on 5 μm sections of jejunum, ileum and colon cut on a rotary microtome and mounted onto glass Superfrost® microscope slides (Menzel-Gläser, Braunschweig, Germany). Slides were scanned using the NanoZoomer™ (Hamamatsu Photonics, Japan) and assessed with NanoZoomer™ Digital Pathology software view.2 (Histalim, Montpellier, France). The occurrence of eight histological criteria in the jejunum and ileum were examined to generate a total tissue injury score (26). These criteria were villous fusion, villous atrophy, disruption of brush border and surface enterocytes, crypt loss/architectural disruption, disruption of crypt cells, infiltration of polymorphonuclear cells and lymphocytes, dilation of lymphatics and capillaries, and oedema. In the colon, the latter six criteria were examined. Each parameter was scored as present = 1 or absent = 0 in a blinded fashion by two independent assessors (HRW/KRS).

2.4 Immunohistochemistry

2.4.1 Immunohistochemical assessment of cellular markers of apoptosis and proliferation

Immunohistochemistry (IHC) was carried out on 5 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). Immunohistochemical analysis was performed for caspase 3 (Abcam, Vic, Australia; #ab4051), a marker of apoptosis, and Ki67 (Abcam, Vic, Australia; #ab16667), a marker of proliferation. Changes in both parameters are validated markers for altered tissue kinetics and an excellent way to assess the subclinical severity of gut toxicity (27). Immunohistochemical analysis was performed using Dako reagents on an automated machine (AutostainerPlus™, Dako, Denmark; #AS480) following standard protocols supplied by the manufacturer. Briefly, sections were deparrafinised in histolene and rehydrated through graded ethanols before undergoing heat mediated antigen retrieval using an EDTA/Tris buffer (0.37 g/L EDTA, 1.21 g/L Tris; pH 9.0). Retrieval buffer was preheated to 65^{o}C using the Dako PT LINK™ (pre-treatment module; Dako, Denmark; #PT101). Slides were immersed in the buffer and the temperature raised to 97^{o}C for 20 min. After returning to 65^{o}C, slides were removed and placed in the Dako AutostainerPlus™ (Dako, Denmark; #AS480) and stained following manufacturer's guidelines. Negative controls had the primary antibody omitted. Slides were scanned using the NanoZoomer™ (Hamamatsu Photonics, Japan) and assessed with NanoZoomer™ Digital Pathology software view.2 (Histalim, Montpellier, France). Apoptosis was quantified by counting the number of positively stained cells for 15 crypts. Data were presented as average positively stained cells per crypt. Ki67 data was represented as the percentage of positively staining cells relative to total cells in the intestinal crypts. Only well oriented, non-oblique crypts were included for analysis. All staining was analysed by two independent assessors (HRW/KRS).

2.4.2 Immunohistochemical assessment of microglia and astrocyte reactivity expression markers

Immunostaining was conducted on 5 μm sections of lumbar spinal cord (L3/4), cut on a rotary microtome and mounted onto Superfrost® microscope slides (Menzel-Gläser, Braunschweig, Germany). Immunohistochemical analysis was performed for astrocytic Glial Fibrillary Acidic Protein (GFAP), Clone 6F2, (DakoCytomation, Dako, Denmark; #M0761) and microglial Iba-1 (Wako, Virginia, USA; #019-19741). Briefly, sections were dewaxed on a hot air blower and in xylene, then dehydrated in 100% ethanol before being quenched for endogenous peroxidase activity with 0.5% hydrogen peroxide in methanol for 30 min. Slides were then washed in 0.1 M PBS (pH 7.4, 2 x 3 min) before being subjected to heat-mediated antigen retrieval using 0.1 M citrate buffer (pH 6.0). Non-specific binding was blocked by 3% normal horse serum (NHS; Sigma-Aldrich, NSW, Australia) for

30 min at room temperature. Primary antibodies were applied, using 3% NHS as the diluent, overnight at room temperature in a humid chamber (GFAP 2 μg/ml; Iba-1 0.1 μg/ml). Following incubation with the primary antibody, a secondary goat biotinylated anti-mouse/rabbit IgG (6 μg/ml) was applied to sections for 30 min at room temperature (Vector Laboratories, California, USA; anti-mouse #BA-9200; anti-rabbit #BA-1000). After a further PBS wash (2 x 3 min), slides were incubated with pierce streptavidin peroxidase conjugate at 2 μg/ml (ThermoFisher Scientific, Vic, Australia; #21130) for 30 min at room temperature followed by another rinse with 0.1 M PBS. The immunocomplex was then visualised with precipitation of DAB (Sigma-Aldrich, NSW Australia; #D-5637) in the presence of hydrogen peroxide (3%). Slides were washed to remove excess DAB and lightly counterstained with haematoxylin, dehydrated and mounted with DePeX from histolene. Slides were scanned using the NanoZoomer™ (Hamamatsu Photonics, Japan) and assessed with NanoZoomer™ Digital Pathology software (Histalim, Montpellier, France). Staining was assessed in the dorsal column of the lumbar spinal cord using ImageJ 1.49 software and the validated colour deconvolution method (28).

2.4.3 Immunohistochemical assessment of blood brain barrier permeability

Immunohistochemical analysis was also performed on 5 μm sections of brain, cut (mid-sagitally) on a rotary microtome and mounted onto Superfrost® microscope slides (Menzel-Gläser, Braunschweig, Germany). Immunostaining was performed using a rabbit polyclonal anti-human albumin antibody (Dako, Denmark; #A0001) as per the method described in 2.3.2. No antigen retrieval was required. Staining was assessed using a semi-quantitative grading system where 0 = no staining, 1 = mild staining with leakage localised to one region, 2 = moderate staining with two unrelated sites of leakage and 3 = intestine staining with ≥3 unrelated sites or global leakage. Staining was assessed in a blinded fashion by two independent assessors (HRW, JM).

2.5 Assessment of *in vivo* intestinal permeability

2.5.1 FITC-dextran assay

Three hours prior to kill time points, mice received a 500 mg/kg dose (75 mg/ml) of 4 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich, NSW, Australia; #46944) via oral gavage. Blood was collected from the facial vein into Multivette® 600 Serum-Gel with Clotting Activator capillary tubes (Sarstedt, Numbretcht, Germany; #15.1670.100) and stored on ice for 30 min. Samples were centrifuged at 11,000 x g for 5 min at room temperature and the serum isolated. Serum samples were

diluted 1:3 with 1 X PBS and quantified using the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA) and Gen5 version 2.00.18 software relative to a standard curve (range 0.0001-10 μg/ml).

2.5.2 Serum Limulus Amebocyte Lysate (LAL) endotoxin assay

The Limulus Amebocyte Lysate (LAL) endotoxin assay was run on serum samples isolated from blood collected from the facial vein into Multivette® 600 Serum-Gel with Clotting Activator capillary tubes (Sarstedt, Numbretcht, Germany; #15.1670.100). The LAL QCL-1000™ endotoxin detection kit (Lonza, Basel, Switzerland; #50-647U, 50-648U) was then used to quantify serum endotoxin, as per manufacturer's guidelines. Endotoxin concentration was determined relative to a linear standard curve (range 0.1-1 EU/ml).

2.6 Tissue cytokine protein quantification

2.6.1 Protein extraction

Irinotecan causes global gastrointestinal damage, however damage is typically most severe in the ileum and colon. Proinflammatory cytokine expression was therefore assessed using 30 mg of ileal and colonic tissue samples. Intestinal tissue samples were homogenised at room temperature using the QIAGEN TissueLyser LT (QIAGEN, NSW, Australia) for 5 min at 50 Hz in 500 μl of Radio-Immunoprecipitation Assay (RIPA) buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) (Sigma Aldrich, NSW, Australia; #R0278) supplemented with Roche protease inhibitor cocktail (Sigma Aldrich, NSW, Australia; #04693116001). Homogenates were centrifuged at 11,000 x g for 15 min at 4ºC and the supernatant isolated, aliquoted and stored at -80ºC. Total protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, VIC, Australia; #23225). A working concentration of 1 mg/ml was used for cytokine analysis.

2.6.2 Luminex multiplex cytokine assay

Cytokine concentration was measured in individual ileal and colonic homogenates using Luminex xMAP technology (Milliplex Mouse Cytokine Kit, Merck Millipore, Darmstadt, Germany; #MPMCYTOMAG70K08) as per manufacturer's instructions. The cytokines analysed were: IL-1β, IL-6, TNFα, IL-10, IFN-γ, IL-2, IL-17α and MCP-1. Each 96-well plate included a 6-point standard curve and two quality controls provided by Merck Millipore.

2.7 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, a 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. Diarrhoea data was assessed using a Chi-square test (29). A p-value of <0.05 was considered significant.

3.0 Results

3.1 Bacterial diversity profiling

Bacterial profiling showed comparable microbiome composition in both WT and BALB/c-*Tlr4*$^{-/-billy}$ mice (Table 1). The majority of the microbiome comprised of Fermicutes (WT 76.66 ± 5.98%; BALB/c-*Tlr4*$^{-/-billy}$ 71.33 ± 2.66%) and Bacteroidetes (WT 22.46 ± 6.01%; BALB/c-*Tlr4*$^{-/-billy}$ 24.34 ± 3.00%) phyla. A two-tailed t-test with Welch's correction showed increased expression of the Proteobacteria and TM7 phyla in BALB/c-*Tlr4*$^{-/-billy}$ mice (Proteobacteria: WT 0.44 ± 0.12%; BALB/c-*Tlr4*$^{-/-billy}$ 1.93 ± 0.61%, p=0.046; TM7: WT 0.10± 0.06%; BALB/c-*Tlr4*$^{-/-billy}$ 0.55 ± 0.16%, p=0.028).

3.2 BALB/c-*Tlr4*$^{-/-billy}$ mice have attenuated clinical manifestations of irinotecan-induced gut toxicity

Irinotecan caused diarrhoea in all mice from as early as 6 h (Figure 1A and B). Diarrhoea severity was significantly improved in BALB/c-*Tlr4*$^{-/-billy}$ mice compared to WT (*p<0.0001). No diarrhoea was seen in any vehicle control animals (Figure 1C and D). Weight loss following irinotecan treatment was most severe at 72 h in WT (-9.96 ± 0.98% from baseline) and *Tlr4*$^{-/-}$ mice (-5.68 ± 0.64% from baseline), the weight loss in BALB/c-*Tlr4*$^{-/-billy}$ mice was significantly less than that seen in WT (*p<0.0001) (Figure 1E).

3.3 BALB/c-*Tlr4*$^{-/-billy}$ mice have improved histological architecture in the small intestine

BALB/c-*Tlr4*$^{-/-billy}$ mice treated were protected against irinotecan-induced mucosal tissue injury most effectively in the jejunum (Figure 2A), with improvements seen in BALB/c-*Tlr4*$^{-/-billy}$ mice compared to WT at 48 (*p=0.003) and 72 h (*p=0.023). Despite improvements in diarrhoea, architectural tissue injury remained evident at 96 h in the jejunum (Figure 2A WT #p<0.0001; BALB/c-*Tlr4*$^{-/-billy}$ ^p=0.003) and ileum (Figure 2B WT #p<0.0001; BALB/c-*Tlr4*$^{-/-billy}$ ^p<0.0001). This late histopathology was not evident in the colon (Figure 2C p>0.05) suggesting colonic histopathology may be more indicative of diarrhoea severity. Representative images (Figure 2J) show villus blunting/fusion (arrow), crypt disruption (arrow head) and inflammatory infiltrate (subset panel).

Peak apoptosis was seen at 6 h in both WT and BALB/c-*Tlr4*$^{-/-billy}$ mice (Figure 2D, E and F) with paralleled decreased in proliferation seen in the jejunum and ileum (Figure 2G and H). BALB/c-*Tlr4*$^{-/-billy}$ mice had reduced apoptotic counts at 6 h in the jejunum (Figure 2D ***p<0.0001) and ileum

(Figure 2E *p=0.002). Representative immunostaining (Figure 2K) shows crypt caspase 3 positive cells in the jejunal crypts. No change was seen in proliferation between WT and BALB/c-*Tlr4*$^{-/-billy}$ mice in any region at any time point (p>0.05) (Figure 2G, H and I).

3.4 TLR4-dependent signaling contributes to intestinal barrier disruption

Serum FITC-dextran was elevated in WT mice at 24 (#p<0.0001), 48 (#p=0.0043) and 72 h (#p=0.01) compared to vehicle controls (Figure 3A), indicating compromised intestinal barrier function. No statistically significant change was seen in BALB/c-*Tlr4*$^{-/-billy}$ mice at any time point (p>0.05). At 24 h post-irinotecan, WT mice had significantly greater serum FITC-dextran concentrations compared to BALB/c-*Tlr4*$^{-/-billy}$ mice (3209.59 ± 1020.88 ng/ml vs. 1373.97 ± 303.56 ng/ml; *p=0.01).

Serum endotoxin (LAL), a measure of LPS translocation, was elevated at all time points in WT mice treated with irinotecan (#p<0.005), with most significant peaks at 24 and 72 h (both #p<0.0001) (Figure 3B). Serum endotoxin was highest at 24 (^p=0.001), 48 (^p=0.003) and 96 h (^p=0.02) in BALB/c-*Tlr4*$^{-/-billy}$ mice treated with irinotecan compared to control. There was a significant difference in serum endotoxin between WT and BALB/c-*Tlr4*$^{-/-billy}$ mice at 72 h post irinotecan treatment (33.35 ± 2.19 EU/ml vs. 13.96 ± 5.87 EU/ml; ***p<0.0001).

3.5 BALB/c-*Tlr4*$^{-/-billy}$ mice exhibit a muted inflammatory response

BALB/c-*Tlr4*$^{-/-billy}$ mice treated with irinotecan showed no statistically significant increase in IL-1β, IL-6 or TNFα expression in the ileum or colon when compared to vehicle controls (Figure 4). There were significant increases in the expression of IL-1β in the ileum of WT mice treated with irinotecan (#p=0.04 24 h; #p=0.004 48 h). No statistically significant increase was seen in TLR4 deficient mice (Figure 4, p>0.05). BALB/c-*Tlr4*$^{-/-billy}$ mice lacked the IL-6 response seen in WT animals at 6 h (#p=0.003, *p=00002 ileum; #p=0.0004, *p=0.0005 colon) (Figure 4). WT mice also showed increased ileal expression of TNFα (#p=0.01) 24 h after treatment with irinotecan and this was significantly elevated relative to treated BALB/c-*Tlr4*$^{-/-billy}$ mice (*p=0.02). No change was seen in IL-10.

3.6 Irinotecan-induced pain is associated with TLR4-dependent astrocytic GFAP expression

Facial grimace criteria peaked at 6 h in both treated animals groups, reducing steadily for the remainder of the experimental time-course (Figure 5A). From 6 to 72 h, BALB/c-*Tlr4*$^{-/-billy}$ mice had reduced facial grimace criteria compared to WT mice (***p<0.0001). Elevated GFAP staining, indicative of astrocyte activation, was seen at 6 h in WT animals compared to controls (#p=0.004) (Figure 5B).

GFAP staining was significantly greater in WT compared to BALB/c-*Tlr4*$^{-/-billy}$ mice at 6 (*p=0.008) and 72 h (*p=0.01). No change was seen in Iba-1 staining in any animals (Figure 5C; p>0.05). Representative images (Figure 5D) support activation of astrocytes with obvious changes in phenotype 6 h after irinotecan in WT mice.

3.7 Irinotecan increases blood brain barrier permeability to albumin

Elevated albumin staining was seen in WT (#p=0.0001) and BALB/c-*Tlr4*$^{-/-billy}$ mice (^p=0.03) at 24 h, and in WT mice at 48 and 72 h (#p=0.006 and #p=0.03, respectively) (Figure 6A), although there was no difference between WT and BALB/c-*Tlr4*$^{-/-billy}$ mice (p>0.05). Both parenchymal (Figure 6C) and perivascular (Figure 6D) albumin was evident in WT and BALB/c-*Tlr4*$^{-/-billy}$ mice treated with irinotecan, with minimal leakage in control animals (Figure 6B).

4.0 Discussion

TLR4 has been hypothesised to play a key role in the development of both chemotherapy-induced gut toxicity and pain (9, 17). Results from the current study support this newly proposed hypothesis, highlighting significant improvements in symptomatic parameters of gut toxicity and histopathological markers in BALB/c-*Tlr4*$^{-/-billy}$ mice treated with irinotecan. This study is also the first to show paralleled improvements in *in vivo* pain markers and central glial reactivity following irinotecan.

The gut microbiota is critical in regulating the severity of gut toxicity, with increased levels of LPS-producing, gram-negative bacteria correlating with diarrhoea severity (5, 30). Comparable levels of major phylogenies (fermicutes, bacteroidetes) were seen in WT and BALB/c-*Tlr4*$^{-/-billy}$ mice at baseline. However, small variations were seen in two relatively low abundance microbes. These differences seen in the composition of the gut microbiome in WT and BALB/c-*Tlr4*$^{-/-billy}$ mice are not surprising given the wealth of emerging evidence indicating that both genetic and environment factors, such as breeding rooms//facilities, weigh significantly on the composition of the gut microbiome (31).

At baseline, TLR4 knockout mice exhibited higher levels of the TM7 bacterial phyla. Little is known about this bacterial phylogeny, however it has been suggested to contribute to inflammatory pathologies within the gastrointestinal tract (32). More importantly, BALB/c-*Tlr4*$^{-/-billy}$ mice had elevated levels of β-glucuronidase-producing proteobacteria, likely increasing the rate of SN-38 reactivation, and thus worsened gut toxicity. Despite this predisposition, BALB/c-*Tlr4*$^{-/-billy}$ mice showed improvements in both the duration and severity of symptoms compared to WT mice. This finding compliments recent research showing that germ-free mice experienced less severe irinotecan-induced gut toxicity compared to conventional mice (33). Most importantly, the germ-free mice also had higher levels of unbound SN-38 and higher β-glucuronidases activity. Comparatively, depletion of the gut microbiome with oral antibiotics has shown to be effective in reducing irinotecan-induced diarrhoea (34). It is now essential to determine if these improvements are the results of reduced microbial metabolism and SN-38 reactivation, or the result of reduced TLR4-mediated signaling. Determination of which factor contributes more significantly to clinical outcomes would therefore better direct therapeutic research efforts.

Extensive literature exists showing the protective effect of TLR4 deletion in an inflammatory setting, however this appears to be limited to only acute insults, with TLR4 deficiency exacerbating chronic

inflammatory diseases (35). For example, significant improvements in acute inflammation have been shown in the absence of TLR4 and MyD88, a downstream signaling molecule of TLR, following acute infection with *Citrobacter rodentium* (35). Similar results have also been demonstrated in methotrexate-induced gut toxicity, with MD-2 (TLR4 accessory protein) deletion improving clinical and histological parameters of toxicity (36). Importantly, this study showed that TLR4 and TLR2 appear to have opposing roles, with both genetic deletion and pharmacological inhibition of TLR2 worsening methotrexate-induced damage (36). It appears that TLR2 has paradoxical roles in chemotherapy-induced gut toxicity, with improvements seen in *Tlr2*$^{-/-}$ mice treated with irinotecan. This study also showed that *Myd88*$^{-/-}$ mice were also protected from developing severe irinotecan-induced gut toxicity, reiterating the importance of TLR4-dependent signaling (36). Despite support for TLR4 deletion providing protection against chemotherapy-induced gut toxicity, this does not appear to be the case for acute and chronic colitis, with *Tlr4*$^{-/-}$ mice more susceptible to ulceration and bleeding (35). Although the unique mechanisms to each effect are not understood, these data do imply ambivalent roles for TLR4 in different inflammatory-based pathologies in the gastrointestinal tract.

It is well established that irinotecan-induced gut toxicity occurs through apoptosis of crypt epithelial cells through the gastrointestinal tract and consequently apoptosis is an established marker of toxicity severity (27). Our results showed significantly decreased levels of apoptosis in the jejunum and ileum of irinotecan-treated BALB/c-*Tlr4*$^{-/-billy}$ mice. This supports recent research suggesting that TLR4 signaling contributes to intestinal stem cell apoptosis through endoplasmic reticular stress-related mechanisms (37). We also saw levels of proliferation inversely parallel these changes in cellular dynamics. This is of great clinical significance as apoptosis is one of the initial steps in the cascade of biological events that results in the development of gut toxicity. If TLR4 deletion is able to profoundly impact such an early mediator of toxicity, it provides an excellent opportunity to intervene prior to architectural tissue damage, inflammation and bacterial translocation.

In this study, increased FITC-dextran and endotoxin permeability were seen in irinotecan-treated WT mice, indicative of altered intestinal barrier function. Importantly, *Tlr4*$^{-/-}$ maintained intestinal barrier function with no significant changes in FITC-dextran permeability and decreased LPS translocation. Surprisingly, BALB/c-*Tlr4*$^{-/-billy}$ mice only showed mild improvements in serum endotoxin compared to WT mice and this did not appear to reflect the differences in intestinal damage. The failure to show

differences at most time points in this study could be explained by evidence suggesting that TLR4 on hepatocytes is required for complete endotoxin clearance (38).

Reducing bacterial translocation has profound implications for systemic inflammatory responses and the exacerbation of direct mucosal cytotoxicity. Highlighting this pathobiological mechanism, BALB/c-*Tlr4*$^{-/-billy}$ mice, displayed less severe intestinal inflammation compared to WT mice. The most significant difference was seen for IL-6, in which BALB/c-*Tlr4*$^{-/-billy}$ mice showed no increase after treatment, supporting the idea that TLR4-dependent NFκB activation is required for the release of IL-6 from macrophages (39). Stunted IL-6 production has also been seen in MyD88 deficient mice (40) and *Tlr4*$^{-/-}$ macrophages (41).

The current study also identified, for the first time, disruption of the blood brain barrier in animals treated with irinotecan. Blood brain barrier disruption has been hypothesised to contribute to the development of 'chemobrain' and cognitive impairment seen following chemotherapy, allowing cytotoxic agents direct access to the CNS (42). It has also been suggested uncontrolled blood brain barrier transit may potentiate the ability of peripheral inflammation to influence central pain signaling. It is becoming increasingly recognised that TLR4, expressed on centrally located glia, is able to recognise and respond to peripherally derived LPS and inflammatory mediators (9). We have shown translocation of LPS to systemic circulation following chemotherapy treatment, reflecting the swing towards a gram-negative, pathogenic gut microbiome profile following chemotherapy. Despite this, we saw no association serum LPS, glial activation and pain. Instead, astrocytic activation appeared to occur bimodally, with increases in GFAP staining seen at 6 and 72 h. This suggests that cellular events associated with apoptosis (which peaks at 6 h), or inflammation may be more important in TLR4-mediated glial activation. This concept is particularly compelling when looking at recent research by Ji et al., (2013) who reported significant astrocytic hypertrophy and activation in the dorsal horn of vincristine-treated rats with mechanical allodynia (43). Treatment with pentoxifylline, an anti-inflammatory agent, attenuated astrocytic reactivity and mechanical allodynia. Astrocytic reactivity has also been identified in the lumbar spinal cord of rats receiving oxaliplatin treatment, providing evidence linking peripheral inflammation and central gliosis. It is now critical to determine if the irinotecan-induced gut toxicity and pain are independent, yet simultaneously occurring events that are both governed by TLR4, or if there is a true directional mechanism linking one to the other.

Data from the current study have clearly highlighted the involvement of TLR4 in the development of irinotecan-induced gut toxicity and pain, provide a unique opportunity to simultaneously treat irinotecan-induced toxicities. In all cases of TLR4-targeted therapeutic options, the effect on both the efficacy of the anti-cancer therapy and overall tumour kinetics are paramount. This is particularly the case when targeting TLR4, as recent evidence now suggests that it may play critical roles in tumour growth. For example, an Apetoh et al. (2007) showed that TLR4 deficient animals had increased tumour growth under normal, conditions and in response to doxorubicin (44). TLR4 inhibition has also been implicated in tumour regression, with several studies now showing that numerous cancer cell lines (e.g. SW260 [colon], CRC-526 [breast], PC3 [prostate]) overexpress TLR4 and that LPS stimulates their growth (45-47). In addition, although not assessed in the current study, future work will need to clarify the role of TLR4 in normal gastrointestinal motility patterns given recent research implicating TLR4 in altered motility patterns following opioid treatment (48, 49).

5.0 Conclusions

Given the ubiquitous involvement of the innate immune system, particularly TLR4, in gut homeostasis and central pain signaling, it presents as a potentially overlooked candidate in the treatment of chemotherapy-induced gut toxicity and pain. Our research has demonstrated that TLR4 is pivotal in the development of both toxicities. This research not only improves our understanding of the underlying mechanisms involved, but also reveals a promising opportunity to intervene in the complex pathophysiology of these dose-limiting side effects of chemotherapy. Research efforts must now be targeted at tailoring methods of inhibiting TLR4, keeping in mind the potential effects on tumour burden and gastrointestinal function.

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8.0 Tables

Table 1: Mean percentage of each caecal bacterial phyla in vehicle-treated WT and *Tlr4*$^{-/-}$ null mice.

Phylum	**WT**	***Tlr4*$^{-/-}$**	**p-value**
Bacteroidetes	22.45±6.00	24.34±3.01	0.79
Firmicutes	76.65±5.98	71.33±2.66	0.46
Proteobacteria	0.43±0.12	1.93±0.61	***0.05**
TM7	0.10±0.06	0.56±0.16	***0.03**
Actinobacteria	0.25±0.09	0.65±0.19	0.09
Cyanobacteria	0.02±0.01	0.10±0.04	0.08
Acidobacteria	0.002±0.001	0.0004±0.0003	0.26
Verrucomicrobia	0.0005±0.0005	0.13±0.11	0.27
Deferribacteres	0.005±0.003	0.0006±0.0003	0.23
Tenericutes	0.02±0.02	0.92±0.53	0.13
Chloroflexi	0.02±0.005	0.01±0.01	0.58
Nitrospirae	0.003±0.001	0.003±0.002	0.70

9.0 Figure legends

Figure 1 Tlr4$^{-/-}$ **attenuates symptomatic parameters of gut toxicity: diarrhoea and weight loss.** Diarrhoea profiles shown for WT BALB/c mice (A) and BALB/c-*Tlr4$^{-/-billy}$* mice (B) treated with 270 mg/kg of irinotecan, as well as vehicle treated controls (C, D). Diarrhoea data is expressed as the percentage of total animals (per time point) with a particular grade of diarrhoea. Data was analysed using a Chi-Squared test. Diarrhoea was most significant at 24 h post-treatment in both treatment groups. Diarrhoea was significantly improved in BALB/c-*Tlr4$^{-/-billy}$* mice compared to WT (***p<0.0001). Panel E shows weight loss over the 96 h time course. Data displayed as percentage weight change from baseline (0 h). A Kruskal-wallis with post hoc testing was performed to identify statistical significance. BALB/c-*Tlr4$^{-/-billy}$* mice had significantly less body weight loss at 24 h (**p=0.003), 48 h, 72 h and 96 h (***p<0.0001).

Figure 2 **Histopathological parameters in jejunum, ileum and colon of WT and BALB/c-*Tlr4$^{-/-billy}$* mice.** There were significant increases in tissue injury scores in WT and BALB/c-*Tlr4$^{-/-billy}$* mice in the jejunum (A), ileum (B) and colon (C); # denotes a change from baseline in WT mice, where p<0.05; ^ denotes a change from baseline in BALB/c-*Tlr4$^{-/-billy}$* mice, where p<0.05. Data presented as interquartile range±mix/max. TLR4 deletion reduced the severity of jejunal and ileal tissue damage (A *p=0.003 48 h, *p=0.023 72 h); B *p=0.005 96 h). Apoptosis was increased in the jejunum (D), ileum (E) and colon (F) in all treated animals (p<0.05). TLR4 deletion attenuated apoptosis in the jejunum and ileum (D ***p<0.0001 6 h; E *p=0.0023 6 h). Decreased small intestinal proliferation was noted at 6 h in all animals treated with irinotecan (G and H), however no differences were seen between WT and BALB/c-*Tlr4$^{-/-billy}$* mice. No change was seen in the colon (I). Representative histological (J) and caspases-3 immunostaining (K) show clear jejunal tissue injury at 48 h characterised by crypt ablation (arrow head), villous blunting/fusion (arrow), inflammatory infiltrate (subset panel) and crypt apoptosis at 6 h (brown staining [arrow]; Panel K). Scale bars show 30 μm. Original magnification 40 x.

Figure 3 Ex vivo **markers of barrier permeability, serum 4 kDa FITC-dextran (A) and serum endotoxin (B).** FITC-dextran was administered as a 500 mg/kg dose, via oral gavage, 3 h prior to kill time point. Serum endotoxin was assessed using the Serum Limulus Amebocyte Lysate (LAL) endotoxin assay. Data is expressed as mean±SEM and was analysed using a two-way ANOVA with Tukey's post hoc. Both markers of barrier disruption show elevations following treatment with irinotecan (# denotes a change from baseline in WT mice; ^ denotes a change from baseline in BALB/c-*Tlr4$^{-/-billy}$* mice, where p<0.05). Maximum FITC-dextran permeability coincides with peak diarrhoea in WT animals (24 h). At this time point, TLR4 deletion decreased serum FITC-dextran levels (*p=0.0104) compared to WT. Serum endotoxin, indicative of LPS translocation, was reduced in BALB/c-*Tlr4$^{-/-billy}$* mice at 72 h compared to WT mice treated with irinotecan (***p=0.0001).

Figure 4 **Cytokine expression in the ileum and colon.** Cytokine expression was assessed using Luminex xMAP technology. Data expression as mean±SEM (pg/ml). A two-way ANOVA with Tukey's post hoc was performed to identify statistical significance. IL-1β expression increased in the ileum of WT animals treated with irinotecan compared to control (#p=0.0394 24 h; #p=0.0036 48 h).

Only a significant decrease was seen in BALB/c-*Tlr4*$^{-/-billy}$ mice (# denotes a change from baseline in WT mice; ^ denotes a change from baseline in BALB/c-*Tlr4*$^{-/-billy}$ mice, where $p<0.05$). BALB/c-*Tlr4*$^{-/-billy}$ mice lacked an IL-6 response at 6 h, with significantly lower expression compared to WT mice (**p=0.0002 ileum; **p=0.0005 colon). TNFα expression peaked at 24 h in the ileum of WT mice treated with irinotecan (#p=0.0113). This was significantly elevated relative to BALB/c-*Tlr4*$^{-/-billy}$ mice (*p=0.0166), which showed no elevation in TNFα ($p>0.05$). No change was seen in the anti-inflammatory cytokine, IL-10.

Figure 5 **Facial grimace criteria (A-D), GFAP staining (marker of astrocytic reactivity; E,G) and Iba-1 staining (marker of microglial reactivity; F) following irinotecan treatment.** Facial grimace scores were assessed four times daily using the facial grimace criteria. Most significant facial pain was seen at 6 h following treatment with irinotecan (A,B). Tlr4$^{-/-}$ mice had lower facial pain scores at all time points (***p<0.0001 6-72 h; *p=0.0072 96 h). GFAP and Iba-1 immunostaining was assessed in the dorsal column of the lumbar spinal cord. Increased astrocytic activation (GFAP) was seen in treated WT mice at 6 h compared to controls (E; #p=0.0041). This was not evident in BALB/c-Tlr4$^{-/-billy}$ mice ($p>0.05$). Irinotecan-treated WT mice showed increased GFAP staining compared to BALB/c-Tlr4$^{-/-billy}$ mice at 6 h (*p=0.008) and 72 h (*p=0.012). No change was seen in microglial activity (Iba-1) across the full time course in both WT and Tlr4$^{-/-}$ mice (F; $p>0.05$). Data presented as mean±SEM. Panel G shows representative images of GFAP staining in vehicle control WT mice and 6 h after irinotecan. Scale bars show 50 μm or 10 μm for representative images and subset panels, respectively. Original magnification 40 x. WT mice treated with irinotecan displayed morphological changes in astrocyte phenotype (somatic hypertrophy, thickened and ramified processes) indicative of an activated state.

Figure 6 **Irinotecan causes blood brain barrier dysfunction.** Staining was analysed using a semi-quantitative 0-3 grading system and represented as interquartile range±mix/max. A Kruskal-Wallis with post hoc testing was performed to identify statistical significance in non-parametric data. Scale bars show 20 μm, 100 μm and 50 μm for panels B, C and D, respectively. Original magnification 40 x. Albumin staining, indicative of increased blood brain barrier transit, was elevated compared to untreated controls at 24 h (#p<0.0001), 48 h (#p=0.0063) and 72 h (#p=0.0325) in WT mice; this was only seen at 24 h (^p=0.0325) in BALB/c-*Tlr4*$^{-/-billy}$ mice. No differences were seen between WT and BALB/c-*Tlr4*$^{-/-billy}$ mice ($p>0.05$). Qualitative assessment showed that albumin leakage was not limited to a particular brain region, but affected the vasculature globally with both parenchymal (C) and perivascular (D) albumin staining noted. Minimal leakage was observed in control animals (B).