Cancer Chemotherapy and Pharmacology (2022) 89:275–281
https://doi.org/10.1007/s00280-021-04382-3

SHORT COMMUNICATION

Intestinal toll-like receptor 4 knockout alters the functional capacity of the gut microbiome following irinotecan treatment

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Received: 30 August 2021 / Accepted: 22 November 2021 / Published online: 2 December 2021

Abstract

Purpose Irinotecan can cause high levels of diarrhea caused by toxic injury to the gastrointestinal microenvironment. Toll-like receptor 4 (TLR4) and the gut microbiome have previously been implicated in gastrointestinal toxicity and diarrhea; however, the link between these two factors has not been definitively determined. We used a tumor-bearing, intestinal epithelial cell (IEC) TLR4 knockout model ($Tlr4^{\Delta IEC}$) to assess microbiome changes following irinotecan treatment. We then determined if a fecal microbiota transplant (FMT) between $Tlr4^{\Delta IEC}$ and wild-type (WT) mice altered irinotecan-induced gastrointestinal toxicity.

Methods MC-38 colorectal cancer cells were injected into WT and $Tlr4^{\Delta IEC}$ mice. Fecal samples were collected prior to tumor inoculation, prior to irinotecan treatment and at cull. 16S rRNA gene sequencing was used to assess changes in the microbiome. Next, FMT was used to transfer the microbiome phenotype between $Tlr4^{\Delta IEC}$ and WT mice prior to irinotecan treatment. Gastrointestinal toxicity symptoms were assessed.

Results In study 1, there were no compositional differences in the microbiome between $Tlr4^{\Delta IEC}$ and WT mice at baseline. However, predicted functional capacity of the microbiome was different between WT and $Tlr4^{\Delta IEC}$ at baseline and post-irinotecan. In study 2, $Tlr4^{\Delta IEC}$ mice were protected from grade 3 diarrhea. Additionally, WT mice who did not receive FMT had more colonic damage in the colon compared to controls ($P = 0.013$). This was not seen in $Tlr4^{\Delta IEC}$ mice or WT mice who received FMT ($P > 0.05$).

Conclusion $Tlr4^{\Delta IEC}$ and WT had no baseline compositional microbiome differences, but functional differences at baseline and following irinotecan. FMT altered some aspects of irinotecan-induced gastrointestinal toxicity.

Keywords Toll-like receptor 4 · Fecal microbiota transplant · Chemotherapy · Mucositis

Introduction

Irinotecan is a chemotherapeutic agent used to treat a wide range of cancers. Unfortunately, it causes high levels of gastrointestinal toxicity that is characterized by diarrhea and abdominal pain, leading to dose delays and reductions [1]. Previous research has shown that the innate immune receptor toll-like receptor 4 (TLR4), widely expressed throughout the gastrointestinal tract, is implicated in the development of this toxicity, although findings have differed as to whether TLR4 function has a positive or negative effect on toxicity [2, 3]. Additionally, the gut microbiome is emerging as a key factor in how this toxicity develops and persists, with inter-individual differences a possible determining factor in the severity of gastrointestinal toxicity that each person develops [4]. TLR4 can sense a range of pathogen and damage associated

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molecular patterns (e.g., bacterial lipopolysaccharide), and, therefore, has an important regulatory role over the microbiome. However, it remains unclear how the interactions between TLR4 and the microbiome could be exploited to develop effective preventative or alternative treatments for irinotecan-induced gastrointestinal toxicity. We have previously developed a conditional TLR4 knockout model [5], where TLR4 is specifically knocked out in the intestinal epithelial cells (IEC, $Tlr4^{\Delta IEC}$) to study these interactions.

Fecal microbiota transplant (FMT) is an evolving method used clinically to treat refractory *Clostridioides difficile* infections, and pre-clinically to assess microbiome-disease links [6]. It has also previously been used pre-clinically in cancer therapy-induced toxicity settings [7, 8] to further understand how the gut microbiome influences the development of toxicity. Therefore, our aims were to use our existing $Tlr4^{\Delta IEC}$ model to determine gut microbial changes in $Tlr4^{\Delta IEC}$ and wild-type (WT) mice following irinotecan administration in a colorectal tumor model, and to determine whether FMT between the two mouse genotypes altered irinotecan-induced gastrointestinal toxicity.

Materials and methods

These studies were approved by the Animal Ethics Committee of the University of Adelaide (M-2017-114, M-2020-028, M-2020-026), and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2013). All mice were maintained under standard conditions in ventilated cages with ad libitum access to food and water, with a 12-h light/dark cycle.

Generation of conditional TLR4 knockout mouse model

C57BL/6 J mice with a knockout of the *Tlr4* gene in IECs specifically were generated ($Tlr4^{\Delta IEC}$) through breeding *Tlr4* floxed mice (Jackson Laboratories, B6(Cg)-Tlr4tm1.1Karp/J, #024872) with transgenic mice expressing cre recombinase in IECs under the control of the villin 1 promoter (Jackson Laboratories, B6.Cg-Tg(Vil1-cre)997Gum/J, #004586).

Mouse genotypes were determined using genomic DNA isolated from ear notches taken upon weaning. Isolated DNA was used in specific polymerase chain reactions to confirm epithelial TLR4 knockout (methods in supplementary information). Mice not displaying the $Tlr4^{\Delta IEC}$ genotype were used as WT controls.

IEC knockout of TLR4 has previously been confirmed at the tissue level using quantitative RT-PCR on intestinal mucosal scrapings [5].

Study 1—colorectal tumor model

MC-38 colorectal cancer cells [9] (kind gift from Michelle Teng, QIMR Berghofer) were cultured, and 2×10^6 cells were subcutaneously injected into the right flank of WT and $Tlr4^{\Delta IEC}$ mice, that had been co-housed since weaning (total $n = 31$, aged 12 ± 4 weeks). When the tumor reached approximately 0.2 cm^3 (approximately 7 ± 1 days), mice were intraperitoneally injected with a 270 mg/kg dose of irinotecan hydrochloride (Pfizer, 20 mg/mL) or vehicle (sorbitol–lactic acid buffer, 45 mg/mL sorbitol, 0.9 mg/mL lactic acid, pH = 3.4) [2]. Mice were weighed once daily (in the morning), with a $3 \times$ daily comprehensive clinical symptom recording. Diarrhea was graded according to an established grading system [2]. Mice were culled if displaying $\geq 15\%$ weight loss from baseline or significant distress and clinical deterioration. At the experiment end, mice were culled 72 h after irinotecan via CO_2 inhalation and cervical dislocation.

16S ribosomal RNA (rRNA) sequencing

Fecal samples were collected directly from each mouse at the time of tumor inoculation ($n = 8$) and irinotecan/vehicle injection ($n = 8$). Fecal contents from the distal colon were collected at cull ($n = 15$, Fig. 1A). DNA was subsequently extracted using the Qiagen DNeasy PowerLyzer PowerSoil Kit with the semi-automated QIAcube Connect and eluted in nuclease free water. Samples were sent to the Australian Genome Research Facility for 16S rRNA gene region analysis (methods in supplementary information). The program BURRITO was used to visualize the links between taxonomic composition and predicted function in the dataset using KEGG pathways [10].

Study 2—FMT model

WT and $Tlr4^{\Delta IEC}$ mice (total $n = 29$) were separated into genotype- and sex-specific cages upon weaning. Housing, food and bedding were kept consistent throughout the study.

Our FMT protocol is described following the GRAFT framework for the reproducible reporting of animal FMT studies [11]. Fecal samples were collected for FMT preparation the day prior to the first administration. Samples were collected directly from the mice (aged 8 weeks) into sterile containers, 2 h after the light cycle began. Samples from cage mates (1–3 mice) were pooled together. Containers were kept closed except when adding fecal samples to reduce oxidative stress. Sterile, room temperature $1 \times$ PBS, pH = 7.4, was added to the fecal samples within 20 min of collection at a concentration of 100 mg of feces per 1 mL of PBS. These tubes were vortexed using the Bio-Rad BR-2000

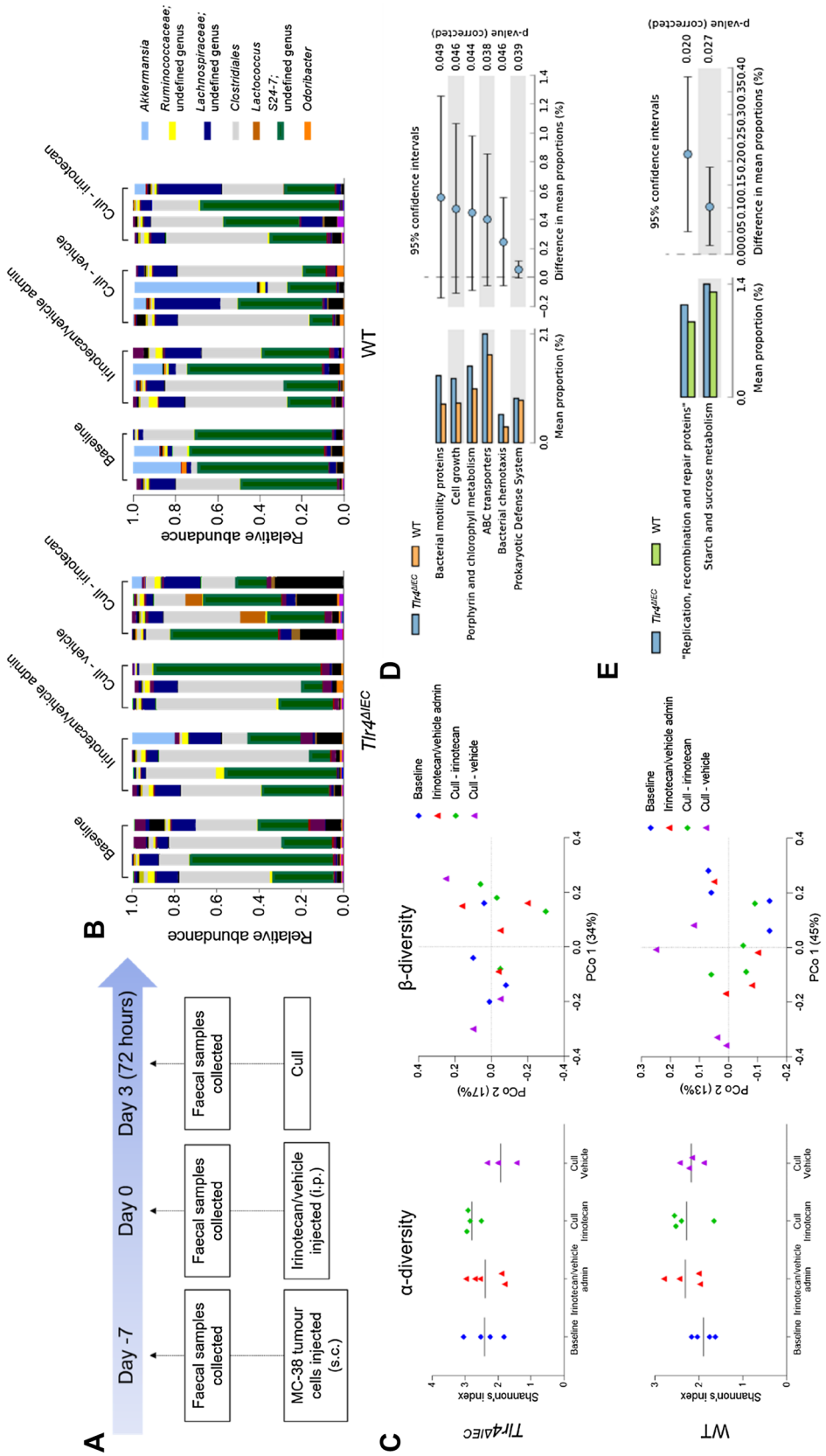

Fig. 1 (**A**) Colorectal tumor model timeline. Approximately 7 days after MC-38 tumor cells were injected, irinotecan or vehicle was given. Mice were culled 72 h after irinotecan/vehicle. Fecal samples were collected at each time point. (**B**) Relative abundance at the genus level for $Tlr4^{\Delta IEC}$ and WT mice. Baseline, at irinotecan/vehicle (following MC-38 tumor cell injection) and at cull time points presented. Highly abundant bacteria represented in figure legend. *S24-7* and *Clostridiales* most abundant across all samples. (**C**) Alpha and beta diversity measures for WT and $Tlr4^{\Delta IEC}$ at each study time point. No differences between groups of WT mice in Shannon's index. Lines represent mean. PCoA plot of WT mice, with no differences in clustering (PERMANOVA, $P > 0.05$). No differences between groups of $Tlr4^{\Delta IEC}$ mice in Shannon's index. Lines represent mean. PCoA plot of $Tlr4^{\Delta IEC}$ mice, with no differences in clustering (PERMANOVA, $P > 0.05$). (**D**) Significant predicted functional differences in the microbiome of $Tlr4^{\Delta IEC}$ and WT mice at ***baseline*** (FDR-corrected $P < 0.05$). (**E**) Significant predicted functional differences in the microbiome of $Tlr4^{\Delta IEC}$ and WT mice ***after*** irinotecan treatment (FDR-corrected $P < 0.05$)

Vortexer at top speed (3000 rpm) for 60 s, and left to settle by gravity for 5 min at room temperature. The supernatant was collected, and glycerol was added to the supernatant at 10% v/v. This mixture was aliquoted into 150 µL aliquots and frozen at − 80 °C. The product was thawed at ambient temperature just prior to use. This procedure was not conducted under anaerobic conditions.

No pre-conditioning methods (e.g., antibiotics or fasting) were used in this study. Mice were individually housed from the first FMT treatment onwards. Under isoflurane anesthesia, FMT was rectally administered (100 µL) via a flexible 20-gauge gavage needle inserted 35 mm into the colon. FMT was administered once daily for 3 consecutive days, 4 h prior to the lights off cycle beginning. Vehicle groups received an equivalent amount of PBS + 10% v/v glycerol. Mice were individually transferred into clean cages following each FMT.

Six days following the final FMT, mice were intraperitoneally injected with irinotecan hydrochloride or vehicle as described above. Mice were weighed once daily and monitored as above. In addition, chemotherapy-induced pain was measured 3 × daily in a blinded manner, using a validated rodent facial grimace scoring system [2, 12]. In brief, each criterion (orbital tightening, cheek bulge, nose bulge, ear, and whisker position) was scored as: 0 = absent; 1 = present; or 2 = severe. The maximum total score was 10.

Mice were culled 72 h after irinotecan via CO_2 inhalation and cervical dislocation. At necropsy, the gastrointestinal tract was removed from the pyloric sphincter to the rectum. The small and large intestines were flushed with chilled, sterile 1 × PBS. Samples from each region of the gastrointestinal tract were collected and drop-fixed in 10% formalin before embedding in paraffin.

Tissue analysis

Hematoxylin and eosin (H&E) staining and analysis were completed as previously described to assess irinotecan-induced gastrointestinal damage in the distal colon [2]. There were six criteria: 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 edema; with a 0–2 scale, where 0 = absent, 1 = present and 2 = severe. The maximum total score was 12.

Statistical analysis

Data were compared using GraphPad Prism version 9.0. The assumptions of equality of variance for each group and normally distributed data were tested using Bartlett's test and D'Agostino & Pearson omnibus normality test, respectively. If data were normally distributed, *t* tests or 2-way ANOVA tests were used, and data were represented as mean ± SEM. If these assumptions were violated, non-parametric equivalent tests were performed, including Kruskal–Wallis tests for independent data and data were represented as median. Diarrhea proportions were analyzed by a Chi squared test. Permutational multivariate analysis of variance (PERMANOVA) was used to compare principal coordinate analysis (PCoA) clustering with generalized UniFrac distances. The program STAMP was used to assess the predicted metagenome using Welch's *t* test with the Benjamini–Hochberg correction for the false-discovery rate (FDR) [13]. *P* values less than 0.05 were considered statistically significant.

Results

Study 1: Colorectal tumor model: no compositional, but functional differences in the microbiome of Tlr4$^{\Delta IEC}$ and WT mice

Fecal samples were collected from *Tlr4*$^{\Delta IEC}$ and WT mice at the time of MC-38 tumor inoculation (baseline), irinotecan or vehicle injection and at cull (Fig. 1a). Relative abundance at the genus level was assessed following 16S rRNA gene sequencing (Fig. 1b). There were no significant differences in abundance of any genera between *Tlr4*$^{\Delta IEC}$ and WT mice at any time point, as well as no significant differences longitudinally in *Tlr4*$^{\Delta IEC}$ mice or WT mice. In addition, there were no significant differences in alpha or beta diversity between the groups (Fig. 1c). As there were no compositional differences in the microbiome at genus level (longitudinally or between genotypes), we then assessed potential predicted functional differences between the microbiome of *Tlr4*$^{\Delta IEC}$ and WT mice at specific time points. When assessing differences between *Tlr4*$^{\Delta IEC}$ and WT mice at baseline, there were five functional groups that were significantly different between the genotypes (bacterial motility proteins, cell growth, porphyrin and chlorophyll metabolism, ABC transporters, bacterial chemotaxis, prokaryotic defense system; Fig. 1d). Finally, there were two significantly different functional groups between *Tlr4*$^{\Delta IEC}$ and WT mice after irinotecan treatment (Fig. 1e). These differences were in starch and sucrose metabolism, and replication, recombination and repair proteins. These were not seen at baseline or between *Tlr4*$^{\Delta IEC}$ and WT mice treated with vehicle (data not shown).

These differences suggested this effect was specific to irinotecan treatment, and hence may help to explain the role of TLR4 and the microbiome in reducing irinotecan-induced toxicity. Therefore, we next undertook an FMT study to assess whether altering the microbiome would reduce irinotecan-induced gastrointestinal toxicity.

Study 2: FMT model: Tlr4$^{\Delta IEC}$ FMT led to less weight loss and grade 2 and 3 diarrhea in WT mice

Tlr4$^{\Delta IEC}$ and WT mice were given three FMT treatments prior to irinotecan treatment (Fig. 2a). WT mice received FMT produced from *Tlr4*$^{\Delta IEC}$ fecal samples and vice-versa (Fig. 2b). FMT was tolerated well, with no adverse events, e.g. intestinal perforation or bleeding. There was no weight change from baseline, diarrhea, or facial pain scoring in the period between the final FMT and irinotecan treatment (data not shown).

Irinotecan caused weight loss in all mice (Fig. 2c, d). At 72 h post-irinotecan, WT mice who received irinotecan only had lost more weight than WT mice who received *Tlr4*$^{\Delta IEC}$ FMT and irinotecan ($P = 0.045$). There were significant differences in the diarrhea profiles of WT mice who received irinotecan (with or without FMT) versus vehicle ($P = 0.03$), and *Tlr4*$^{\Delta IEC}$ mice who received irinotecan (with or without FMT) versus vehicle ($P = 0.01$). *Tlr4*$^{\Delta IEC}$ mice did not develop grade 3 diarrhea following irinotecan treatment; however, WT mice did (Fig. 2e). Additionally, WT mice who received a *Tlr4*$^{\Delta IEC}$ FMT developed less grade 2 and 3 diarrhea compared to WT mice who did not receive an FMT. There were no differences in facial pain scores in any group following treatment (data not shown). Finally, there was an increase in histopathological damage in the distal colon (H&E scores) due to irinotecan in the WT group ($P = 0.013$, Fig. 2f, g). This difference was not seen in the *Tlr4*$^{\Delta IEC}$ mice or in any mice treated with FMT and was characterized by crypt cell loss and architectural derangement (Fig. 2f, g). The maximum total histopathology score (12) was not reached in any sample, with no samples displaying edema, and no instances of severe dilation of lymphatics.

Discussion

This study aimed to use a conditional intestinal epithelial TLR4 knockout (*Tlr4*$^{\Delta IEC}$) model to determine the links between the gut microbiome and TLR4 in the setting of irinotecan-induced gastrointestinal toxicity. There were no compositional differences in the gut microbiome following irinotecan administration in *Tlr4*$^{\Delta IEC}$ and WT mice. This was surprising, as changes in microbiome composition and diversity following chemotherapy treatment has been previously reported [14]. However, the predicted functional capacity of the microbiome was altered between the genotypes at baseline and following irinotecan treatment.

Intestinal TLR4 has an important interaction with the gut microbiome [15], and TLR4 knockout can mediate less irinotecan-induced gastrointestinal toxicity [2]. Here, the microbiome of *Tlr4*$^{\Delta IEC}$ mice had higher functional capacity for replication, recombination and repair proteins than WT mice following irinotecan treatment. This increased capacity would certainly allow a better response to oxidative stress stemming from irinotecan treatment [16], suggesting that intestinal TLR4 knockout may allow a more favorable microbiome to limit irinotecan-induced damage.

Recent research by Wong et al. [3] showed that in a patient population, TLR4 polymorphisms increased patients' susceptibility to chemotherapy-associated diarrhea. This study investigated the impact of multiple irinotecan doses, and hence differed from the present study that utilized one irinotecan dose. This suggests a potential fundamental difference in the function of TLR4 following an acute insult compared to an ongoing treatment. In a recent publication by our laboratory [5], we showed that the conditional knockout mice had no differences in intestinal structure or function to WT mice, confirming that these types of changes were not the reason the microbiome was functionally altered. Future research could do further immune profiling on the gastrointestinal tract of *Tlr4*$^{\Delta IEC}$ and WT mice to confirm this.

To further understand how these TLR4-mediated functional microbial alterations may cause irinotecan-induced gastrointestinal toxicity, we investigated 'swapping' the microbiome of WT and *Tlr4*$^{\Delta IEC}$ mice via FMT. FMT was tolerated well and did not, by itself, cause any diarrhea, weight loss, facial pain or histological damage. Overall, WT mice who received a *Tlr4*$^{\Delta IEC}$ FMT had less severe symptoms than WT mice who did not receive an FMT. At 72 h following irinotecan treatment, WT mice who received a *Tlr4*$^{\Delta IEC}$ FMT lost less weight than WT mice who did not receive FMT. Following irinotecan, there were differences in diarrhea profile between mice of both strains who received irinotecan versus vehicle. In addition, there were less WT mice who received FMT and irinotecan that developed grade 2 and grade 3 diarrhea than WT mice who received only irinotecan. Histopathology scoring indicated an increase in colonic damage in WT mice post-irinotecan versus vehicle. This increase was not seen in *Tlr4*$^{\Delta IEC}$ mice or mice who additionally received FMT.

The fact that, irrespective of its origin, FMT had a beneficial effect on irinotecan-induced gastrointestinal toxicity was unexpected, but inherently interesting. It may be explained by the FMT procedure itself and its potential to transfer beneficial metabolites [17] that could be protective against irinotecan-induced gastrointestinal toxicity. Alternatively, the introduction of new microbes may cause a protective immune response [18, 19] that primes the gastrointestinal microenvironment prior to irinotecan treatment. To explore these possibilities, future studies could additionally assess the use of autologous FMT to reduce active irinotecan-induced diarrhea, a technique that has successfully improved gastrointestinal function in pre-clinical and clinical settings [20, 21]. An autologous FMT could be prepared from fecal samples taken prior to irinotecan treatment, and

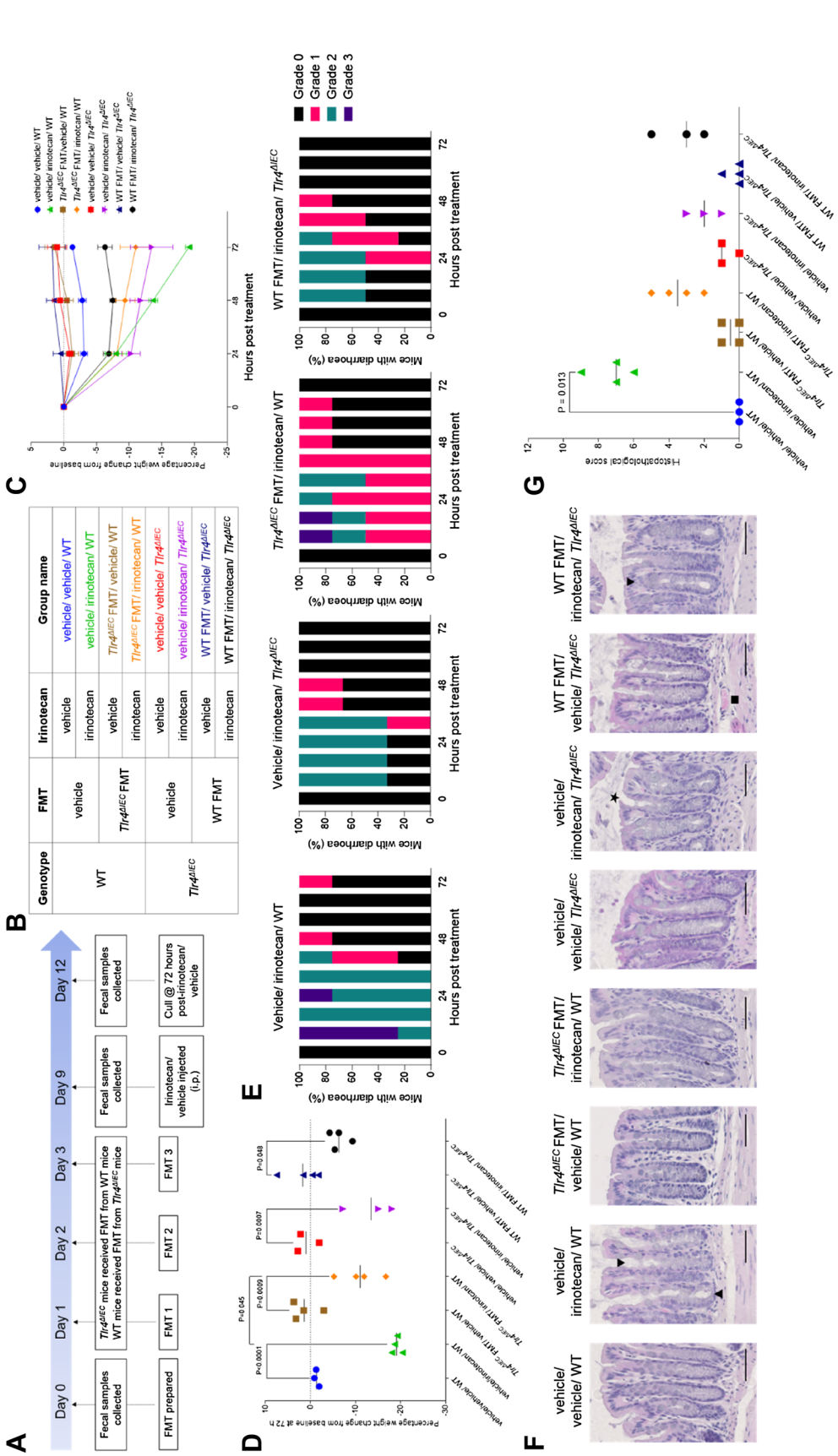

Fig. 2 (**A**) Time course of FMT/irinotecan study. (**B**) Table of treatment groups and nomenclature. (**C**) Weight loss over the 72 h time course. Data displayed as a percentage of weight change from baseline (at irinotecan/vehicle administration), mean ± SEM. (**D**) Percentage weight change at 72 h post-irinotecan treatment. Significant differences between vehicle and irinotecan treated mice of each group, and between the vehicle/irinotecan/WT group and $Tlr4^{\Delta IEC}$ FMT/irinotecan/WT group ($P = 0.045$). Line indicates mean. (**E**) Diarrhea profiles for mice treated with irinotecan, 0–72 h post-irinotecan/vehicle treatment. $Tlr4^{\Delta IEC}$ mice did not develop grade 3 diarrhea. (**F**) Representative images of H&E staining in the distal colon: original magnification is 400×; scale bars represent 50 µm. ▲ represents architectural derangement of crypts, ★ represents shortened crypts, ■ represents dilated capillary. (G) Histological damage scoring in the distal colon from H&E staining, damage scored on a scale of 0–12, lines are medians. There was an increase in damage due to irinotecan in the WT group ($P = 0.013$). This difference was not seen in the $Tlr4^{\Delta IEC}$ mice or in any mice treated with FMT

then administered shortly before peak diarrhea occurs; in this study peak diarrhea occurred in FMT groups between 8 and16 h post-irinotecan. In addition, further functional analysis of the transferred FMT or microbiome contents (e.g., short chain fatty acid analysis) could be useful.

While the results from this study provide justification for further research in this area, it is important to recognize the small sample size. In addition, we chose to not prepare the FMT under anerobic conditions, and did not treat recipient mice with antibiotics prior to receiving the FMT, although there is some conjecture as to whether this is strictly necessary [22]. Future studies could undertake microbiome analysis on fecal samples taken prior to irinotecan treatment to determine how the FMT altered microbial composition or function. Additional studies in the *Tlr4*$^{\Delta IEC}$ model could assess whether FMT could directly alter TLR4 expression in immune cells in the gut lamina propria.

In conclusion, this research suggests that further work on FMT and TLR4 function is warranted to understand their role more fully in irinotecan-induced gastrointestinal toxicity. We have also shown that pre-clinical FMT is a feasible method to investigate the microbiome in chemotherapy-induced gastrointestinal toxicity studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00280-021-04382-3.

Acknowledgements KRS, EEC, and JSYT were in receipt of an Australian Government Research Training Program Scholarship. KRS was in receipt of a Lion's Medical Research Foundation Scholarship. Thank you to Courtney Subramaniam, Ghanyah Al-Qadami, Georgette Radford and Claire Vieyra for assistance with the mouse studies. Thank you to the University of Adelaide Laboratory Animal Services staff for their assistance with the *Tlr4*$^{\Delta IEC}$ model development and breeding.

Funding Not applicable.

Data availability Available upon reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval These studies were approved by the Animal Ethics Committee of the University of Adelaide (M-2017-114, M-2020-028, M-2020-026), and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2013).

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