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The Role of TLR4 in Chemotherapy-Induced Toxicity, Immunity and Tumour Growth

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Thesis Abstract

Chemotherapy, while highly effective, causes collateral damage to a range of healthy tissues, especially those with highly proliferative cell populations. The gastrointestinal (GI) tract is especially vulnerable to damage, resulting in a constellation of GI symptoms including diarrhoea, bleeding and pain. Chemotherapy-induced GI toxicity (CIGT) impacts patient quality of life and treatment adherence, and is therefore associated with significant clinical, psychosocial and financial burden.

The chemotherapeutic drug, irinotecan, is associated with particularly severe CIGT. It has been proposed that this is due to its direct and indirect activation of the pattern recognition receptor, toll-like receptor 4 (TLR4), which governs local and systemic inflammatory responses. TLR4 is widely expressed on epithelial and immune cells along the GI tract, with TLR4 displaying distinct and unique roles depending on its specific site of expression. Despite TLR4s clear role in mediating inflammatory processes that are consistent with the pathobiology of CIGT, it has been difficult to dissect the true contribution of TLR4 to CIGT due to contradictory evidence. Furthermore, recent evidence suggests TLR4 signalling enhances the efficacy of some chemotherapeutic drugs. As such, developing methods of targeting TLR4 to control CIGT has proven challenging. In considering this interaction, it is important to recognise that TLR4's activity is site-specific, meaning its activity differs based on the cell type upon which it is expressed. I therefore hypothesised that the impact of TLR4 on irinotecan GI toxicity and anti-tumour efficacy was dependent on its cellular expression; a hypothesis that has not been previously considered. Prior to this project, a clear gap in knowledge existed, as it was unknown if CIGT was mediated by immune cell or GI epithelial cell TLR4 signalling, or a contribution of both. Given that 7-Ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of irinotecan, is highly abundant in the lumen of the gut due to its enterohepatic recirculation

mechanism causing repeat exposure to GI mucosa, I hypothesised that intestinal epithelial TLR4 was most critical in the aetiology of CIGT, with immune or other cell type expression of TLR4 more likely to be governing its anti-cancer effects. This thesis therefore aimed to investigate the role of intestinal epithelial TLR4 in GI homeostasis and in a mouse model of CIGT in colorectal cancer. This was achieved in four main sections.

Firstly, I aimed to clarify the roles of site-specific TLR4 in host-immune interactions (i.e. during the healthy state) and inflammatory condition. This was achieved through a critical literature review of site-specific TLR4 and its roles in homeostasis and inflammatory disease (Chapter 2). This overview was the first to highlight the lack of specificity in the literature, with site-specific TLR4 analysis remaining a common oversight in medical research. In order to analyse the role of intestinal epithelial TLR4 on homeostatic control of the GI system, I utilised a conditional knockout (KO) model in which TLR4 deletion was restricted to just the intestinal epithelium (*Tlr4*$^{\Delta IEC}$) and compared markers of intestinal function to wild-type (WT) littermates (Chapter 3). Through characterisation of the intestine in regards to structure, cellular turnover and permeability, I confirmed that this model was indeed suitable for the investigation of TLR4's site-specific impact on irinotecan-induced GI toxicity.

Using this model, I aimed to investigate the role of intestinal epithelial TLR4 in irinotecan-induced GI toxicity (Chapter 4). *Tlr4*$^{\Delta IEC}$ and WT mice were injected with either 270 mg/kg irinotecan or vehicle buffer and monitored for 72 hrs post-treatment for *in vivo* markers of CIGT. While no difference was noted between treatment groups and strains for molecular markers of intestinal damage, a significant reduction in acute diarrhoea development was identified in the *Tlr4*$^{\Delta IEC}$ irinotecan group 24 hrs post-treatment compared to WT.

In light of recent speculation that TLR4 and its associated inflammatory responses mediate the anti-tumour efficacy of chemotherapy, I next aimed to clarify this interaction first through systematic review of clinical literature and a paralleled investigation of The Cancer Genome Atlas (TCGA) data analysis (Chapter 5). This analysis revealed that TLR4 expression aligns with cancer staging, however exhibited heterogeneous regulation of survival outcomes depending on the stage of disease.

Finally, the role of intestinal epithelial TLR4 in the efficacy of irinotecan was analysed in *Tlr4*$^{\Delta IEC}$ mice compared to WT, each of which were inoculated with the MC-38 colorectal tumour (Chapter 6). *Tlr4*$^{\Delta IEC}$ and WT vehicle mice showed steady tumour growth over the 72 hrs time period, suggesting intestinal epithelial TLR4 does not impact on tumour growth (i.e. in a treatment naïve setting). While tumours in both strains responded to irinotecan treatment, the durability of the response (i.e. maintenance of tumour control) in *Tlr4*$^{\Delta IEC}$ mice was compromised, suggesting that intestinal TLR4 is important in extending the duration of tumour suppression induced by irinotecan.

The results of my thesis demonstrate the complex and unique roles of intestinal epithelial TLR4 in CIGT development and in the efficacy of irinotecan. Furthermore, these results provide a strong rationale for the importance of site-specific analysis of TLR4 in the cancer and chemotherapy field.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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First author publications arising from this thesis

1. Publication under maiden name: **Bruning, E. E**., Coller, J. K., Wardill, H. R., & Bowen, J. M. (2020). Site-specific contribution of Toll-like receptor 4 to intestinal homeostasis and inflammatory disease. *Journal of Cellular Physiology, 236*(2), 877-888. https://doi.org/10.1002/jcp.29976
2. **Crame, E. E**., Bowen, J. M., Secombe, K. R., Coller, J. K., Francois, M, Leifert, W, Wardill, H. R., (2021). Epithelial-specific TLR4 knockout challenges current evidence of TLR4 homeostatic control of gut permeability. *Inflammatory Intestinal Diseases, 6*(4), 199-209. https://doi.org/10.1159/000519200
3. **Crame, E.E**., Nourmohammadi, S., Wardill, H. R., Coller, J. K., Bowen, J. M., (2022) Contribution of TLR4 to colorectal tumour microenvironment, etiology and prognosis. *Journal of Cancer Research and Clinical Oncology.* *https://doi.org/10.1007/s00432-022-04199-4*

Other publications completed during candidature

1. Secombe, K. R., **Crame, E. E**., Tam, J., Wardill, H. R., Gibson, R. J., Coller, J. K., & Bowen, J. M. (2022). Intestinal toll-like receptor 4 knockout alters the functional capacity of the gut microbiome following irinotecan treatment. *Cancer Chemotherapy and Pharmacology*, *89*(2), 275–281. https://doi.org/10.1007/s00280-021-04382-3

Conference presentations

Florey Postgraduate Research Conference (2019)

Poster presentation title: The role of intestinal TLR4 on chemotherapy toxicity, immunity and tumour growth.

Annual Meeting Gastroenterological Society of Australia (GESA) (2019)

Poster presentation title: Toll-like receptor 4 humanized C57BL/6 mouse intestinal permeability: a translational model for cancer treatment toxicity.

Multinational Association of Supportive Care in Cancer (MASCC) Annual Meeting (2021)

Online poster presentation title: Intestinal epithelial TLR4 are crucial to chemotherapy toxicity development and tumour kill.

Annual Meeting Clinical Oncology Society of Australia (COSA) (2021)

Online poster presentation title: Intestinal epithelial TLR4 are crucial to chemotherapy toxicity development.

Florey Postgraduate Research Conference (2021)

Poster presentation title: Intestinal epithelial TLR4 regulated irinotecan-induced diarrhoea.

Annual Australian Society for Medical Research (ASMR) SA Scientific Meeting (2022)

Oral presentation title: Intestinal epithelial TLR4 exerts control over chemotherapy-induced gastrointestinal toxicity.

List of abbreviations

For ease of reading, these abbreviations are also introduced within each chapter.

AB-PAS: Alcian Blue, Periodic acid–Schiff Stain

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

BALB/c: Albino, immunodeficient laboratory-bred strain of the house mouse

BSA: Bovine serum albumin

C57Bl/6: C57 black 6 mouse strain

CaCC: Calcium-activated chloride channels

$CaCl_2$: Calcium chloride

cAMP: cyclic adenosine monophosphate

CCL20: C-C motif ligand 20

CCL28: C-C motif ligand 28

CD: Crohn's disease

CD11b/CD11b$^+$: Leukocyte immune marker, Integrin alpha M

CD4: Cluster of differentiation 4

CD8: Cluster of differentiation 8

CD14: Cluster of differentiation 14

CD45: Cluster of differentiation 45

CD68: Cluster of differentiation 68

CD80+: Cluster of differentiation 80

cDNA: Complementary DNA

CI: Confidence interval

CIGT: Chemotherapy-induced gastrointestinal toxicity

CO_2: Carbon dioxide

cm: Centimetre

CNS: Central nervous system

CRC: Colorectal cancer

CT: Cycle threshold values for PCR genetic analyses

CXCL10: C-X-C motif chemokine 10

DAB: 3, 3′-Diaminobenzidine

DAMP: Damage-associated molecular pattern

DAPI: 4′, 6-diamidino-2-phenylindole

DFS: Disease-free survival

dH_20: Deionised water

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

DSS: Dextran sulphate sodium

dsRNA: Double-stranded RNA

EDTA: Ethylenediaminetetraacetic acid

Elane: Elastase, Neutrophil Expressed

ENS: Enteric nervous system

5-FU: 5-fluorouracil

F4/80: Mature mouse macrophage cell surface glycoprotein, by the Adgre1 locus

FBS: Foetal Bovine Serum

FC: Flow cytometry

FITC: Fluorescein isothiocyanate

FOLFIRI: fluorouracil, leucovorin and irinotecan combined treatment

FOLFOX6: folinic acid, fluorouracil, and oxaliplatin combined treatment

g: Gram

GI: Gastrointestinal

H&E: Haematoxylin and eosin

HCl: Hydrochloric acid

HMGB1: High mobility group box chromosomal protein 1

HR: Hazard ratio

hrs: Hour

HSP: Heat shock protein

HSP70: Heat shock protein 70

IBC: Institutional Biosafety Committee

IBD: Inflammatory bowel disease

IBS: Irritable bowel syndrome

IBS-C: Constipation-dominant IBS

IBS-D: Diarrhoea-dominant IBS

IBS-M: Mixed symptoms IBS phenotypes

ICD: Immunogenic cell death

IDO: Indoleamine 2, 3-dioxygenase

IEC: Intestinal epithelial cells

IF: Immunofluorescence

IFL: Fluorouracil

IFN-β: Interferon-beta

IFN-γ: Interferon-gamma

IgA: Immunoglobulin A

IHC: Immunohistochemistry

IκB: I kappa B

IKK: IκB kinase

IL: Interleukin

IL-1β: Interleukin 1 beta

IL-6: Interleukin 6

IL-8: Interleukin 8

IL-18: Interleukin 18

i.p.: Intraperitoneal

IRAK: Interleukin 1 receptor-associated kinase

IRF3: Interferon-regulating transcription factor 3

I_{sc}: Short-circuit current

i.v.: Intravenous

KCl: Potassium chloride

kg: Kilograms

Ki-67: Marker of active cell proliferation, derived from city of Kiel (Ki) and number of original clone (67)

KO: Knockout

L: Litre

LPS: Lipopolysaccharide

loxP: Locus of Crossover in P1

LV5FU2: Folinic acid

min: Minutes

µg: Microgram

µL: Microlitre

µm: Micrometres

µM: Micromolar

m: Metres

mm: Millimetres

MC-38: Mouse Colon-38

Mcp1: Monocyte chemoattractant protein-1

MD-2: Myeloid differentiation factor 2/ Protein lymphocyte antigen 96

MDSC: Myeloid-derived suppressor cells

mg: Milligrams

$MgCl_2$: Magnesium chloride

$MgSO_4$: Magnesium sulphate

mL: Millilitre

MLCK: Myosin light chain kinase

mM: Millimolar

MPO: Myeloperoxidase

mRNA: Messenger RNA

MTX: Methotrexate

MyD88: Myeloid differentiation primary response 88

MyD88$^{-/-}$: Global MyD88 knockout

n: Number

NaCl: Sodium chloride

$NaHCO_3$: Sodium bicarbonate

NaH_2PO_4: Monosodium phosphate

NEC: Necrotising enterocolitis

NF-κB: Nuclear factor-kappa B

ng: Nanograms

NHS: Normal horse serum

NLRP3: NOD-like receptor Family Pyrin Domain Containing 3

nm: Nanometres

Ωcm^2: Ohms/centimetre squared

O_2: Oxygen

OS: Overall survival

P, *p*: P value

PAMP: Pathogen-associated molecular pattern

PAMPs: Pathogen-associated molecular patterns

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PD-1: Programmed death 1 receptor

PD-L1: Ligand to programmed death 1 receptor

PFS: Progression-free survival

PKC: Protein kinase C

pmol: Picomoles

PPAR: Peroxisome proliferator-activated receptor

PRR: Pattern recognition receptor

PRRs: Pattern recognition receptors

pTh17: Pathogenic T helper 17 cells

RR: Risk ratio

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT-PCR: Real-time polymerase chain reaction

s: Seconds

SEM: Standard error of the mean

SERT: Serotonin transporter

SN-38: 7-Ethyl-10-hydroxycamptothecin / Active metabolite of irinotecan

SN-38G: SN-38 glucuronide

ssRNA: Single-stranded RNA

TIR: Toll-interleukin-1 receptor

TIRAP: TIR-domain-containing adaptor protein

TLR: Toll-like receptor

TLRs: Toll-like receptors

TLR2: Toll-like receptor 2

Tlr2$^{-/-}$: Global TLR2 knockout

TLR4: Toll-like receptor 4

Tlr4$^{-/-}$: Global TLR4 knockout

Tlr4$^{\Delta IEC}$: Intestinal epithelial conditional TLR4 knockout

T_m: Melting point for double-stranded nucleic acid

TNF-α: Tumour necrosis factor alpha

TOPI: Topoisomerase I

TRAM: TRIF-related adaptor molecule

TRIF: TIR-domain-containing adaptor protein inducing interferon-β

UC: Ulcerative colitis

VEGF: Vascular endothelial growth factor

Vil1-cre: Villin protein expressing Cre recombinase

WB: Western blot

WT: Wild-type

ZO-1: Zonula occludens-1

ZNF160: Zinc Finger Protein 160

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Chapter 1: General introduction

This thesis investigates the role of intestinal epithelial TLR4 expression on chemotherapy-induced gastrointestinal (GI) toxicity (CIGT), tumour development and treatment efficacy. CIGT is a widespread oncological concern characterised by DNA damage, cell death, oxidative stress and inflammation, which together produce profound destruction of the intestinal mucosa, leading to increased intestinal permeability and impaired functional capacity of the gut (Wardill et al. 2016). Symptoms include severe diarrhoea, pain and bleeding, which predispose to secondary complications including systemic infection, graft versus host disease and renal failure (Peinert et al. 2010). Of the many anti-cancer agents known to induce GI toxicity, the chemotherapeutic drug, irinotecan, is associated with particularly severe GI damage. Evidence suggests that this is due to its direct and indirect activation of the pattern recognition receptor (PRR), toll-like receptor 4 (TLR4) (Wardill et al. 2016). Previous research in mice has examined the effect of a global TLR4 KO (*Tlr4*$^{-/-}$) on CIGT development, indicating that *Tlr4*$^{-/-}$ mice were protected from irinotecan-induced GI toxicity (Wardill et al. 2016). In contrast, newer findings have suggested *Tlr4*$^{-/-}$ exacerbates irinotecan-induced toxicity and worsens intestinal damage, however this was investigated in a different mouse strain making direct comparison difficult (Wong et al. 2021). Adding further complexity, TLR4 activity has been linked to the development of immunogenic cell death (ICD) pathways, known to aid in the anti-tumour response of many chemotherapeutic agents (Fang et al. 2014). These findings need to be considered within context, as TLR4 is widely expressed on a range of cells, including epithelial (Dheer et al. 2016), immune (Li & Cherayil 2003) and neuronal cells (Caputi et al. 2017). Given that current TLR4-based CIGT research is contradictory in nature, it is hypothesised that toxicity development may be reliant on a site-specific expression of TLR4 (meaning the specific cell type in

which TLR4 is expressed), rather than global TLR4 signalling. TLR4 expressed on both epithelial and immune cells has been previously implicated in GI disease development (Bruning et al. 2021), suggesting that these sites of expression may be the cause of CIGT. However, at the time this project was commenced it was unknown whether CIGT is mediated by immune or epithelial TLR4 signalling, or a contribution of both. Therefore, this thesis characterised the intestinal phenotype of an intestinal epithelial conditional TLR4 KO (*Tlr4*$^{\Delta IEC}$) model and examines the impact of *Tlr4*$^{\Delta IEC}$ on irinotecan-induced GI toxicity development. Given recent identification of immune-mediated cell death (immunogenic death) in the context of chemoefficacy (Fang et al. 2014), this work also determined the impact of *Tlr4*$^{\Delta IEC}$ on irinotecan efficacy for colorectal cancer (CRC).

Human intestinal anatomy and physiology

The adult small intestine is an approximately 6 m long hollow tube, sectioned into the duodenum, jejunum and ileum (Volk & Lacy 2017). The primary objective of the small intestine is the transport of nutrients from the intestinal lumen into the interstitial tissue. The inner most lining of the small intestine, the mucosa, is comprised of three distinct layers; the epithelium, lamina propria and muscularis mucosae (Volk & Lacy 2017). The epithelial layer is the first line of contact between the intestinal lumen and body, and is organised in crypt and villi-like structures, allowing for greater nutrient reabsorption, immune system regulation and homeostatic intestinal cell turnover (Volk & Lacy 2017). Columnar epithelial cells are constantly proliferating at the crypt base, and differentiating into one of seven potential cells; enterocytes, Paneth, goblet, enteroendocrine, tuft, cup, and M cells (Volk & Lacy 2017). Collectively, these cells form a semi-permeable barrier that controls the passage of substances from the lumen in addition to other neuroendocrine functions. The intestinal barrier is maintained largely by proteins on the apical-lateral cell surface called tight junctions,

which dictate paracellular permeability from the gut lumen to sub-epithelial tissue (Ma, Anderson & Turner 2012). Tight junction proteins are highly plastic structures, able to undergo changes in their structure and function in response to a variety of physiological and pathological mediators. This makes the intestinal barrier easily augmented and often implicated in the aetiology of various GI diseases (Wardill et al. 2014; Lee 2015).

While communication between the epithelium and immune system is vital to host tolerance and response to pathogens (Shang et al. 2008), the second mucosal layer, the lamina propria, is home to substantial lymphocyte and mast cell populations (Volk & Lacy 2017). This makes the lamina propria one of the largest immunologically active tissues in the human body (Volk & Lacy 2017). The final layer of the mucosa is the muscularis mucosae, comprised of smooth muscle and partly responsible for local intestinal motility (Volk & Lacy 2017). Below the mucosal layer of the small intestine, lies the submucosa; a layer of connective tissue, inclusive of fibroblasts and mast cells, which contains dense networks of arteries, veins and lymphatic vessels (Volk & Lacy 2017). The lymphatic system is composed of small lymphatic capillaries within the villi of the small intestine, which drain directly into mesenteric lymph nodes along the superior mesenteric artery (Volk & Lacy 2017). Finally, the muscularis propria, the outermost layer of the small intestine, comprised of innervated muscle fibres primarily responsible for intestinal motility and peristalsis (Volk & Lacy 2017).

Upon leaving the small intestine, luminal contents enter the proximal end of the colon via the ileocaecal junction (Heitmann et al. 2021). The healthy adult colon is a tubular organ of approximately 1.5 m in length, with a luminal width starting at 80 mm in the caecum, narrowing to only 25 mm in the sigmoid colon (Heitmann et al. 2021). The primary functions of the colon are water and electrolyte transport and elimination of wastes via the rectum (Kiela & Ghishan 2016). The colonic mucosa in structured in

large crypts, containing goblet cells and varying immune cell presence (James et al. 2020). Colonic motility is controlled by the combination of the enteric nervous system (ENS), lumbar nerves, the Vagus nerve and pelvic splanchnic nerves (Heitmann et al. 2021). A distinct feature of the colon is the ability for its cells to undergo epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions, which have been indicated in the development of colon cancer, chronic inflammation-related fibrosis and in mucosal healing (Sipos & Galamb 2012).

Throughout the entire intestinal tract, the mucosa constantly interacts with trillions of commensal microorganisms and diverse pathogens (Perez-Lopez et al. 2016). The collection of these microorganisms is termed the GI microbiota, whereby the interactions between the host epithelium and microbiota helps to, in part, shape homeostatic immunity (Perez-Lopez et al. 2016). One group of proteins involved in the recognition of microbes within the intestines are toll-like receptors, or TLRs (Abreu 2010). TLRs are pattern recognition receptors (PRR) of the innate immunity, which recognise a wide range of pattern-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) depending on TLR type (Kawai & Akira 2011). These immunosurveillance proteins are type I transmembrane proteins comprising of a leucine-rich ectodomain, a transmembrane region and a cytosolic Toll-IL-1 receptor (TIR) domain which activated downstream signalling pathways (Kawai & Akira 2011). Each TLR detects distinct PAMPs or DAMPs depending on type and protein location. Many TLR studies are carried out through the use of animal models, specifically in carefully-designed mouse and rat models. It is important to note, that while TLRs have similar structures between species, only 10 TLRs have been identified in humans, compared to 12 function TLRs identified in mice (Table 1.1) (Kawai & Akira 2011). While each TLR type is unique in their structure and function, Toll-like receptor 4 (TLR4) has gained particular interest due

to its proven roles in both homeostasis and GI disease development, as seen in chapter 2 of this thesis (Bruning et al. 2021).

Table 1.1: TLR type, key ligands and downstream effects.

TLR	Species	Key ligands	Downstream effects
TLR1	Human and mouse	Triacyl lipoproteins	MyD88, TIRAP producing pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-18
TLR2	Human and mouse	Lipoproteins, Peptidoglycan and lipoteichoic acid	Forms heterodimers with TLR1 and TLR6. MyD88, TIRAP producing pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-18
TLR3	Human and mouse	Viral dsRNA. mRNA	TRIF producing pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-18 and Type I or Type III IFN or IFN-inducible genes
TLR4	**Human and mouse**	**LPS, heat shock proteins, SN-38 and HMGB1**	**MyD88 and TRIF pathways, which increases NF-κB and IRF3 producing pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-18 and Type I or Type III IFN or IFN-inducible genes**
TLR5	Human and mouse	Flagellin	MyD88 producing pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-18
TLR6	Human and mouse	Diacyl lipoproteins and lipotechoic acid	MyD88 and TIRAP pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-18
TLR7	Human and mouse	Viral and bacterial ssRNA	MyD88 producing pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-18 and Type I or Type III IFN or IFN-inducible genes

TLR8	Human and mouse	Viral and bacterial ssRNA	MyD88 producing pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-18 and Type I or Type III IFN or IFN-inducible genes
TLR9	Human and mouse	Viral and bacterial DNA	MyD88 producing pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-18 and Type I or Type III IFN or IFN-inducible genes
TLR10	Human	Unknown	MyD88 producing pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-18
TLR11	Mouse	Profilin and flagellin	MyD88, produces pro-inflammatory cytokines and stimulates immune cells to produce IFN-ɣ
TLR12	Mouse	Profilin	Homodimer or forms heterodimer with TLR11. Similar downstream targets as TLR11
TLR13	Mouse	Bacterial ribosomal RNA	Overexpression of TLR13-induced activity of IFN-stimulated response (Type I or Type III IFN or IFN-inducible genes) but not promoter activity of NF-κB

*Information adapted from R&D Systems Inc. (2012), Kawasaki & Kawai (2014), De Nardo (2015), and Wang et al. (2016). Abbreviations are: double stranded DNA (dsDNA), interferon (IFN), interferon beta (IFN-β), interferon gamma (IFN-ɣ), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 18 (IL-18), lipopolysaccharide (LPS), myeloid differentiation primary response 88 (MyD88), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), messenger RNA (mRNA), positive-sense single-stranded RNA (ssRNA), tumour necrosis factor alpha (TNF-α), toll-Interleukin 1 Receptor Domain-Containing Adapter Protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF). Key TLR in inflammatory disease, cancer and treatment efficacy, TLR4, highlighted in bold.

TLR4: a key player in inflammation

TLR4 is a member of the highly conserved TLR family of pattern recognition receptors, and is characterised by its distinct tripartite structure. This immunosurveillance protein includes a leucine rich extracellular sensing domain, transmembrane region and an intercellular signalling domain (Takeda & Akira 2004; De Nardo 2015). Critically, TLR4 requires the accessory proteins myeloid differentiation factor 2 (MD-2, encoded by the human Lymphocyte Antigen 96 gene) and cluster of differentiation 14 (CD14) to efficiently bind to ligands including, LPS from gram-negative bacteria, heat shock proteins and HMGBI (Santaolalla, Sussman & Abreu 2011; Cheng et al. 2015). Combined, MD-2 and CD14 allow TLR4 to be a key protein for sensing subepithelial and luminal signals of infection and initiating an appropriate inflammatory response. Upon ligand recognition and binding, TLR4 activates the myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) pathways, which increases nuclear factor kappa B (NF-κB) and interferon regulatory transcription factor 3 (IRF3) production, respectively (Cheng et al. 2015). TLR4 is expressed on a range of different cells types throughout the body and specifically within the GI tract. This includes immune expression of TLR4 on dendritic cells, myeloid cells and macrophages (Vaure & Liu 2014), nervous system expression on microglia (Vaure & Liu 2014) and epithelial expression of TLR4 in the intestinal tract (McClure & Massari 2014).

Under homeostatic circumstances, TLR4 sensing is crucial for immune development and maintaining homeostasis within the GI tract ensuring rapid initiation of an innate immune response upon recognition of tissue injury or invading pathogens (Ohto et al. 2012). However, while functional TLR4/MD-2 surveillance of the host-microbe environment is essential to homeostasis, current evidence has also shown TLR4

involvement in the development of various GI diseases, especially those characterised by dysregulated inflammatory processes. Previous findings strongly suggest TLR4 activity is a major driving factor in the development of GI conditions, including irritable bowel syndrome (IBS) (Belmonte et al. 2012) and CIGT (Wardill et al. 2016), with recent studies finding that the active metabolite of irinotecan, SN-38, interacts with the TLR4 signalling pathway by directly binding to TLR4 (Wong et al. 2019). Interestingly, new evidence suggests that GI disease development mediated by TLR4 expression may be dependent on the site of TLR4 expression, as explained in chapter 2 of this thesis (Bruning et al. 2021), further strengthening the importance of site-specific approaches for future TLR4-based research, including investigations into the role of TLR4 in colorectal cancer (CRC).

CRC aetiology, treatment and GI consequences of cancer therapy

CRC is a commonly diagnosed cancer with a large economic, clinical and personal burden (Feletto et al. 2020). The traditional pathway for the development of CRC (adenoma-carcinoma pathway) includes a multi-stage process, starting from genetic mutation causing a single abnormal cell in the epithelial lining of the colon, leading to formation of a benign polyp and finally to malignant tumour growth (Figure 1.1) (Hankey & Groden 2013; Australian Institute of Health and Welfare 2021). An alternate pathway of CRC development, serrated CRC, is estimated to account for 15-30% of CRCs, with the cancerous tumour originating from serrated lesions among the glandular crypts of the intestinal epithelial layer (De Palma et al. 2019). Common symptoms of CRC including abdominal pain, change in stool frequency and consistency, weight loss, rectal bleeding, vomiting and fatigue, are shared between both benign pathology and malignancy (Tsai & Gearhart 2011) Unfortunately, the often vague nature of early-stage CRC symptoms may lead to delayed diagnoses, further highlighting the importance of preventative measures and regular stool testing

via the National Bowel Cancer Screening Program in Australia (Tsai & Gearhart 2011; Feletto et al. 2020). Therefore, CRC is often treated with chemotherapy due to the advanced stage at which it is identified. While the direct cause of CRC is unknown, widely accepted risk factors include age, family history of CRC, obesity, physical inactivity, high intake of red meat and processed meats, low intake of fibre-rich foods, tobacco smoking and consumption of alcohol (Australian Institute of Health and Welfare 2021).

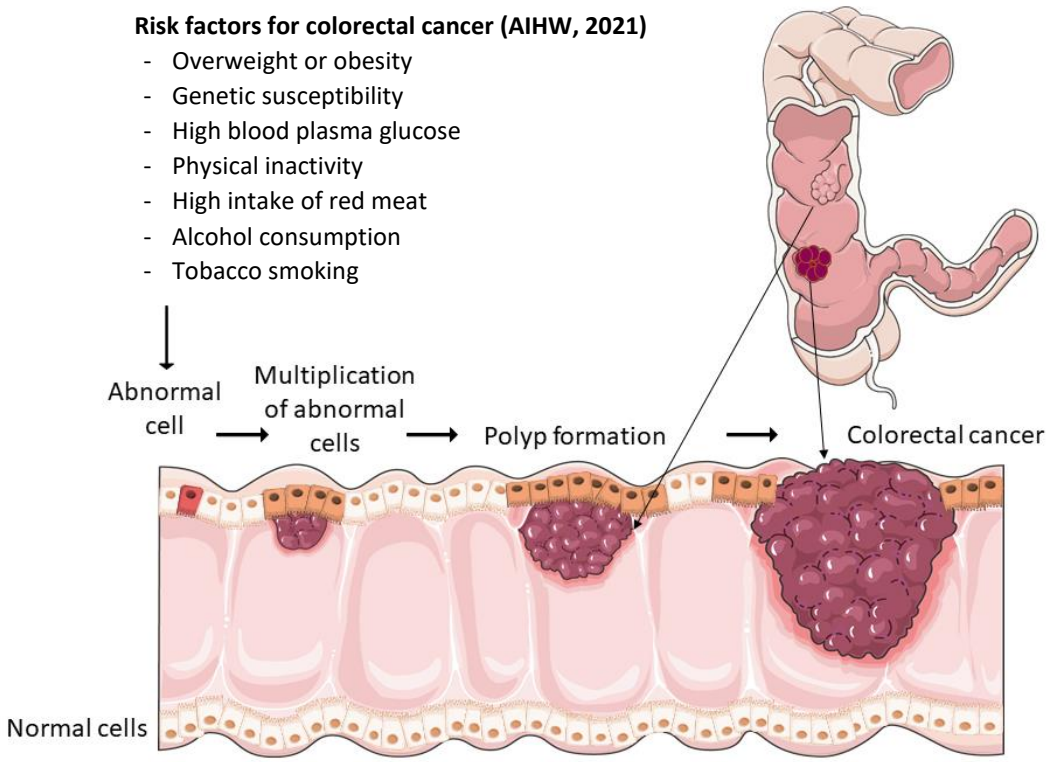

Figure 1.1: Visual representation of colorectal cancer development.

Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

CRC currently ranks as the fourth most commonly diagnosed cancer in Australia, with an age-standardised rate of 99 new CRC cases diagnosed per 100,000 people aged 50-74 (Australian Institute of Health and Welfare 2021). Statistically, this equates to a relative risk of CRC diagnoses being 26 new cases in 1,000 people, for those aged 50-74 (Australian Institute of Health and Welfare 2021). These high rates of CRC diagnoses are shared internationally, with a 2018 study by Ferlay and colleagues finding CRC is the second most prevalent cancer in European countries behind female breast cancer (Ferlay et al. 2018). Furthermore, North American population data mimics CRC rates in Australia, with incidence rates of 37.8 CRC cases per 100,000 people, again placing CRC as the fourth most common cancer diagnoses in the United States of America (National Cancer Institute 2021). While this data highlights the prevalence of CRC in western populations, promising figures have shown that 5 year relative survival in people living with CRC have dramatically improved, from only 55% survival in 1988-1992, to approximately 74% survival in 2013-2017 (Australian Institute of Health and Welfare 2021). The continuous improvements in patient survival are often attributed to the advancements of modern treatment options and the influence of primary prevention interventions, as many risk factors for CRC are modifiable (Feletto et al. 2020).

Treatments for CRC ultimately aim to remove malignant tumour growth from all effected organs, and is often tailored to each individual depending on CRC staging. CRC staging relates to the relative size and spread of CRC at diagnosis, and whether the cancer has metastasised to other sites in the body (Australian Institute of Health and Welfare 2021). CRC staging ranges from Stage I (early stage) where the cancer has invaded several layers of the colon, with no spread outside the colon wall (99% 5-year survival rate) to Stage IV (metastatic) where the cancer has spread to accessory organs, most commonly the liver and lungs, and lymph nodes (13% 5-year

survival rate) (Australian Institute of Health and Welfare 2021). Understandably, treatment protocols vary depending on disease severity at presentation, with more intensive systemic treatments reserved for advanced disease. Treatment options for CRC include surgical resection, radiation, immunotherapy and chemotherapy (Endreseth & Stornes 2021). Chemotherapies that are approved to treat CRC, include 5-fluorouracil (5-FU), leucovorin and the highly potent irinotecan (Fuchs, Mitchell & Hoff 2006; eviQ 2021).

While the introduction of multimodal chemotherapeutic regimens for CRC has undoubtedly led to improved long-term outcomes, these benefits continue to be undermined by the highly toxic and non-selective nature of these drugs. Chemotherapy often causes collateral damage to healthy tissues with highly proliferative cell populations. This includes the GI tract. Almost all chemotherapies are associated with diverse GI toxicities including symptoms such as vomiting, nausea, diarrhoea, constipation, pain and bleeding (Thorpe, Stringer & Gibson 2013). While the mechanisms are based on the specifics of the chemotherapy treatment used, it is well understood that cytotoxic chemotherapy causes profound and irreversible DNA damage in the highly proliferative cells of the GI mucosa (Bowen et al. 2019). This is broadly referred to as chemotherapy-induced GI toxicity (CIGT) which places significant burden on the patient, their care team and the broader healthcare system.

Pathobiological framework and consequences of CIGT

CIGT is a challenging complication of current chemotherapy treatments, affecting up to 80% patients (Blijlevens 2005; Richardson & Dobish 2007). GI toxicity is the most commonly reported side effect of chemotherapy treatments for elderly people living with stage III colon cancer (van Erning et al. 2016). Symptoms of CIGT include severe diarrhoea, pain and bleeding often resulting in increased risk of infection,

leading to delayed, or discontinued, treatment (Fuchs, Mitchell & Hoff 2006; Andreyev et al. 2014). These symptoms are predominantly underpinned by changes to the intestinal barrier, in particular direct injury to enterocytes and weakening of tight junction proteins, leading to unrestricted communication between the luminal contents and underlying immune system, resulting in profound inflammation (Wardill, Bowen & Gibson 2012). Foundational works by Sonis and colleagues (2004) has previously outlined a five-phase model of chemotherapy-induced mucosal injury, being; initiation, upregulation with generation of messenger signals, signalling and amplification, ulceration, and, healing (Sonis et al. 2004). Briefly, the five phase model explains the general sequence of events involved in chemotherapy-induced GI mucosal injury, including; primary initiation of reactive oxygen species (ROS) by chemotherapeutic agents, ROS mediated DNA damage, epithelial cell death and upregulation of NF-κB, TNF-α, IL-1β and IL-6, proinflammatory signalling pathways induced by amplified NF-κB, TNF-α, IL-1β and IL-6 production, tissue ulceration with associated immune infiltrate and bacterial colonisation, and, finally, tissue healing marked by renewal of the epithelial barrier and re-establishment of the host microbiota (Sonis et al. 2004). This early framework has been continually reviewed and updated by the governing body, The Multinational Association of Supportive Care in Cancer (Al-Dasooqi et al. 2013; Bowen et al. 2019), with the most recent review revealing that the current mediators of GI toxicity should include greater emphasis on the influence of the host microbiome and immunity, targeted inflammation pathways and altered functional physiology of the GI tract (Bowen et al. 2019). However, it is generally agreed that chemotherapy treatment has the potential to cause substantial GI injury via increased inflammatory signalling and altered host microbiota composition.

The clinical consequences of CIGT are clear, with symptoms substantially impacting on length of hospitalisation, malnutrition/need for nutritional support, treatment dose reduction (or cessation), patient quality of life and survival (Feliu et al. 2020). These consequences often drive substantial economic burden to the system and the patient (Goldsbury et al. 2021; Hess et al. 2021). Ultimately, the substantial physical, emotional and financial burdens of CIGT are often overwhelming, and can have serious consequences for quality of life. While the impact of CIGT on patient quality of life has been previously reported (Prieto-Callejero et al. 2020), these findings are rarely individualised to CRC-specific chemotherapies. In fact, a recent systematic review has shown that many CRC clinical trials do not include quality of life assessments or show evidence of novel CIGT management strategies (Lombardi et al. 2020). While there are many post-chemotherapy strategies designed to improve patient quality of life, including mindfulness and self-compassion (Garcia et al. 2021), these do not address the underlying biological causes of GI toxicities. Therefore, treatment-specific investigations are required to minimise the pathobiological mechanisms behind toxicity development, which could ultimately improve patient well-being and quality of life.

Irinotecan-induced GI toxicity

Irinotecan is a commonly used chemotherapy drug, often used in combination with other agents (e.g. 5-FU) in the treatment of advanced CRC (eviQ 2021). Irinotecan actively decreases tumour burden via conversion to its active metabolite, SN-38, by carboxylases in the liver. SN-38 is a potent inhibitor of topoisomerase I (Top I), a key enzyme required for DNA ligation and replication (Kawato et al. 1991). Top I inhibition triggers cell death via irreparable DNA damage during S-phase of the cell replication cycle (Chabot 1997). This process makes irinotecan a highly effective chemotherapeutic agent, resulting in a superior response rate (2-fold higher in

regimens containing irinotecan than those without) and increased patient survival, compared to other single chemotherapy regimens (Douillard et al. 2000; Saltz et al. 2000).

While irinotecan is an effective chemotherapeutic agent, its widespread clinical usage is limited by the severe toxicity with which it is associated. The surface of the intestinal tract is highly susceptible to irinotecan damage due to being exposed to high levels of SN-38. This occurs due to enterohepatic recirculation of SN-38, which is required for drug excretion (Chabot 1997). After initial exposure to the GI tract, SN-38 becomes glucuronidated in the liver to form SN-38G, a non-toxic metabolite. SN-38G is recirculated back to the GI tract via bile, for eventual excretion, where it is then cleaved by β-glucuronidase expressed by the intestinal microbiota to reform the toxic metabolite SN-38. This causes a repeat exposure of SN-38 to the intestines, leading to severe damage, inflammation and development of diarrhoea (Chabot 1997). As a result, treatment-induced premature death rates for chemotherapy regimens containing irinotecan, are 3-fold higher than those for chemotherapy protocols not containing irinotecan (Rothenberg et al. 2001).

Emerging role for TLR4 in irinotecan-induced GI toxicity

Although it is well established that irinotecan causes severe gastrointestinal toxicity because of its high GI epithelial exposure (Chabot 1997), additive mechanisms that could be therapeutically exploited remain challenging to dissect. Most recently, attention has focused on the contribution of TLR4 to irinotecan-induced GI toxicity due to several pieces of supportive data: i) irinotecan increases the expression of TLR4 (Formica et al. 2013), ii) irinotecan is associated with innate immune responses consistent with the downstream targets of TLR4 (Logan et al. 2008), and iii) SN-38 inhibits binding of LPS to TLR4 (Wong et al. 2019). Since these findings, several

studies have confirmed a causal role for TLR4 in irinotecan-induced GI toxicity (Secombe et al. 2022; Wong et al. 2021).

Current evidence shows that activation of the innate immune receptor, TLR4, may mediated intestinal barrier dysfunction and irinotecan-induced GI toxicity (Gibson et al. 2016). TLR4-mediated barrier dysfunction is now considered critical in the initiation and potentiation of GI damage, particularly induced by irinotecan, which was evaluated using a BALB/c global TLR4 KO model (*Tlr4*$^{-/-}$) (Wardill et al. 2016). *Tlr4*$^{-/-}$ mice showed reduced GI toxicity with fewer instances of moderate or severe diarrhoea than their WT counterparts, as well as a decrease in weight loss (Wardill et al. 2016). Additionally, serum FITC-dextran was increased in the WT compared to *Tlr4*$^{-/-}$ mice, indicating an increased intestinal permeability (Wardill et al. 2016). Previous research supports these findings, with MyD88 (TLR4 accessory protein) KO mice significantly reducing irinotecan-induced toxicity and tissue injury compared to WT (Wong et al. 2015). Interestingly, this research also found that after irinotecan administration in WT mice, a 200% increase of MyD88 expression was noted, along with typical CIGT symptoms of diarrhoea, increased bacteraemia and reduced survival (Wong et al. 2015). Taken together, these results show that TLR4 is a key mediator of CIGT development.

TLR4 and the complex interplay between chemotherapy efficacy and toxicity

A significant challenge faced in supportive oncology is the overlap that exists between the mechanisms that govern both the efficacy and toxicity of chemotherapies, with many anti-mucotoxic agents failing to translate clinically due to unforeseen impacts on tumour progression/kill (Apetoh et al. 2007; Coller et al. 2017). Similar to chemotherapy's *toxicity*, it was previously thought that its *efficacy* was largely governed by direct DNA damage. However, it is now understood that innate immune activation and inflammation also contribute to tumour kill, and as

such, the interplay between toxicity and efficacy is inherently more complex to navigate (Apetoh et al. 2007; Ghiringhelli et al. 2009).

Of particular interest in understanding the immune system's role in treatment efficacy is the contribution of TLR4. However, current evidence detailing the effects of TLR4 signalling on tumour growth and survival is contradictory in nature. A large body of evidence suggests the TLR4 signalling pathway is associated with enhanced tumour survival (Lee, Wu & Shiau 2010; Lin et al. 2011; Hakim et al. 2014; Zhu et al. 2015), and may even be partially responsible for spontaneous cancer development (Wang et al. 2010; Koliaraki et al. 2019), whereas other work shows anti-tumour functions of TLR4 (Davis et al. 2011; Iida et al. 2013; Fang et al. 2014; Hayes et al. 2018). This complexity, combined with evidence that TLR4 antagonism impairs irinotecan's efficacy (Coller et al. 2017), have challenged attempts to effectively inhibit TLR4 to prevent CIGT. However, what all of these studies fundamentally fail to acknowledge is the site of expression of TLR4, and whether targeting just intestinal TLR4 may be an effective strategy to prevent CIGT without impairing the anti-tumour efficacy of chemotherapy.

Hypotheses and Aims

Given the complex role TLR4 plays in regulating the efficacy and toxicity of chemotherapeutic agents, including irinotecan, the overarching goal of this thesis was to dissect how epithelial-TLR4 differentially affected these distinct, but related, treatment outcomes. I broadly hypothesised that TLR4 expressed on intestinal epithelial cells would selectively control GI toxicity, but not anti-tumour efficacy, resulting in:

- Reduced severity of irinotecan-induced gastrointestinal toxicity in an intestinal epithelial conditional TLR4 KO mouse line (*Tlr4*$^{\Delta IEC}$) compared to WT mice

- Comparable tumour response to irinotecan in *Tlr4*$^{\Delta IEC}$ and WT mice

To investigate these hypotheses, I aimed to:

- Establish and phenotype *Tlr4*$^{\Delta IEC}$ mice to compare against WT mice (Chapter 3)
- Determine the severity of gastrointestinal toxicity induced by irinotecan in *Tlr4*$^{\Delta IEC}$ mice compared to WT mice (Chapter 4)
- Investigate the role of TLR4 in human CRC cohorts through systematic review of literature and genomic data mining (Chapter 5)
- Establish tumour inoculation protocol to produce a CRC tumour-bearing *Tlr4*$^{\Delta IEC}$ model (Chapter 6)
- Compare tumour response to irinotecan in *Tlr4*$^{\Delta IEC}$ mice compared to wild type mice (Chapter 6)

Chapter 2: Site-specific contribution of Toll-like receptor 4 to intestinal homeostasis and inflammatory disease

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This chapter highlights the unique roles of TLR4 activation depending on site-specific expression, and underpins the importance of cell-specific TLR4 investigations in gastrointestinal (GI) research. This chapter is published in the Journal of Cellular Physiology under the following reference; publication under maiden name: **Bruning, E. E.,** Coller, J. K., Wardill, H. R., & Bowen, J. M. (2020). Site-specific contribution of Toll-like receptor 4 to intestinal homeostasis and inflammatory disease. *Journal of Cellular Physiology*, 236(2), 877-888. https://doi.org/10.1002/jcp.29976

Please note that figure numbering and referencing style have been altered to suit thesis formatting style. All spelling has been converted to Australian spelling. All other formatting has remained unchanged from original publication.

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Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 03/08/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Toll-like receptor 4 (TLR4) is a highly conserved protein of innate immunity, responsible for the regulation and maintenance of homeostasis, as well as immune recognition of external and internal ligands. TLR4 is expressed on a variety of cell types throughout the gastrointestinal tract, including on epithelial and immune cell populations. In a healthy state, epithelial cell expression of TLR4 greatly assists in homeostasis by shaping the host microbiome, promoting immunoglobulin A production, and regulating follicle-associated epithelium permeability. In contrast, immune cell expression of TLR4 in healthy states is primarily centred on the maturation of dendritic cells in response to stimuli, as well as adequately priming the adaptive immune system to fight infection and promote immune memory. Hence, in a healthy state, there is a clear distinction in the site-specific roles of TLR4 expression. Similarly, recent research has indicated the importance of site-specific TLR4 expression in inflammation and disease, particularly the impact of epithelial specific TLR4 on disease progression. However, the majority of evidence still remains ambiguous for cell-specific observations, with many studies failing to provide the distinction of epithelial versus immune cell expression of TLR4, preventing specific mechanistic insight and greatly impacting the translation of results. The following review provides a critical overview of the current understanding of site-specific TLR4 activity and its contribution to intestinal/immune homeostasis and inflammatory diseases.

KEYWORDS

immunity, inflammation, intestines, mucous membrane, Toll-like receptor 4

Introduction

The human body processes trillions of microbiological signals daily, from both external non-self pathogens to internal self-derived signs of danger (Pott & Hornef 2012; Comalada & Xaus 2013). Initial avoidance of infection and tissue damage is highly dependent on the evolutionary conserved, innate immune system (Gribar et al. 2008; Pardo-Camacho et al. 2018). Unlike the adaptive immune system, the innate immune system is not specific to particular pathogens. Rather, innate immunity utilises a variety of barriers, protein receptors and phagocytic cells which recognise threats and activate potent inflammatory responses (Pott & Hornef 2012). While each component of innate immunity plays a crucial role in maintaining homeostasis, the relationship between Pattern Recognition Receptors (PRRs) and Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPs) is of great interest. It is this connection that allows for the initial recognition of danger, stimulating signalling cascades which leads to inflammation and elimination of the invading pathogen (Kishore 2009). A widely researched class of PRRs are Toll-like receptors - due to their expansive range of complimentary ligands, complex signalling pathways and significance to disease. While there are 10 human TLR subtypes and 13 mouse TLR subtypes (Nie et al. 2018), TLR4 has received significant attention due to its interaction with bacterial products and relevance to multiple disease states.

While TLR4 expression and signalling is vital for the maintenance of homeostasis and immune tolerance (Chung et al. 2012), this receptor has also been implicated in the development of many inflammatory pathologies, particularly those of gastrointestinal origin. Including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), alcoholic-induced barrier injury and chemotherapy-induced gastrointestinal toxicity (CIGT) (Belmonte et al. 2012; Wardill et al. 2016). In both

IBS/D and CIGT, genetic mutations in the TLR4 pathway have been associated with increased disease risk. Similarly, upregulated expression of TLR4 and its downstream targets have been reported in both clinical studies and preclinical models emphasising the detrimental effects of TLR4 hyper-activation. These findings have prompted enthusiastic investigation of interventions aimed at inhibiting the proinflammatory effects of TLR4 with the goal of controlling chronic inflammatory diseases or preventing intestinal injury. However, reports are highly variable with conflicting evidence regarding the efficacy of TLR4 inhibition, and some reports suggesting detrimental effects on chronic inflammation and tumour growth. These findings clearly highlight an underappreciated level of complexity in TLR4-dependent mechanisms, and a significant gap in our fundamental understanding of how to modify TLR4 signalling in the context of disease prevention.

A critical and often overlooked aspect of TLR4 signalling is its site specificity. TLR4 is expressed primarily on immune cell subsets, however is also expressed on intestinal epithelial cells acting as the first point of immunosurveillance. Despite the intensity of interest in TLR4, few studies acknowledge or address the site-specific implications of TLR4 signalling. This common oversight is likely to impact the true nature and translational impact of results, and is a possible reason for the high degree of variability seen in the efficacy of TLR4-targeted interventions and the growing number of adverse events reported following TLR4 inhibition. As such, the following review will provide a critical overview of the site-specific actions of TLR4, with specific focus on the unique contribution of epithelial- and immune-cell TLR4 signalling in the maintenance of healthy states and contribution to gastrointestinal diseases.

Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) are chronic inflammatory conditions which primarily affect the intestines, resulting in diarrhoea, constipation and pain (Belmonte et al. 2012). Genetic studies into IBD susceptibility

indicate that the TLR4 signalling molecule, Toll/interleukin-1 receptor domain-containing adapter protein (TIRAP), plays a significant role in increased susceptibility to combined IBD risk and Crohn's disease risk (De Jager et al. 2007). Further clinical research using human colon samples showed a significant increase in colonic TLR4 expression in diarrhoea-dominant IBS, suggesting that the pro-inflammatory nature of TLR4 may exaggerate disease severity and progression (Kocak et al. 2016). In the context of alcoholic steatohepatitis and alcohol-induced barrier injury, a 2013 study found that chronic exposure to ethanol significantly increases intestinal permeability of mice via TLR4-dependant downregulation of phosphorylated occludin and increased protein kinase C activity (Li et al. 2013). This is reflected in the setting of CIGT, a severe inflammatory complication of cancer therapy characterised by upregulation of pro-inflammatory cytokines including interleukin 6 (IL-6), interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α); all downstream targets of TLR4 (Bossi et al. 2016; Logan et al. 2008). Further research has shown that TNF-α and IL-6 induce tight junction protein dysfunction and the breakdown of actin filaments (Wardill, Bowen & Gibson 2012), with global TLR4 knockout (KO) shown to mitigate the clinical severity of CIGT in a mouse model via its regulation of claudin-1 internationalisation (Wardill et al. 2016). In fact, TLR4-related genetic mutations have been shown to elevate the risk of CIGT in patients undergoing standard dose chemotherapy, echoing genetic susceptibility results from IBD cohorts (De Jager et al. 2007). Therefore, TLR4-mediated intestinal barrier dysfunction is now considered critical in the initiation and potentiation of gastrointestinal damage (Wardill et al. 2016).

Although TLR4 has been the subject of many studies regarding inflammatory gastrointestinal disease, the distinction between site-specific expression of TLR4 is rarely addressed or acknowledged. This common oversight is likely to impact the true

nature and translational impact of results, as site-specific expression may significantly impact the contextual role of TLR4. The following review will therefore contrast intestinal epithelial and immune cell expressed TLR4, dissecting their unique contribution to the maintenance of healthy states and in gastrointestinal diseases highlighting methods of improving mechanistic insight of TLR4-based signalling and translation of TLR4-targeted therapeutics.

The importance of dissecting site-specific TLR4-dependant mechanisms

The current level of understanding regarding cell-specific involvement of TLR4 in healthy and diseased states is vague, with the majority of research relying on global KO models to demonstrate TLR4-dependant mechanisms or failing to identify target cell populations. The mechanisms of epithelial and immune TLR4 differ and as such, there are likely to be site-specific mechanisms that govern disease initiation and progression. Failure to acknowledge and specifically investigate or target these pathways is a critical oversight in translational research efforts. This is particularly pertinent when considering the dichotomous role of TLR4, which exerts both beneficial and detrimental effects on host physiology depending on its cellular location and degree of activation. When considering this, it is unsurprising to see the degree conflicting data regarding TLR4 manipulation in an attempt to prevent or control disease. For example, TLR4 activation has been widely reported to promote the development of experimental dextran sodium sulfate (DSS)-colitis (Fukata et al. 2007) yet TLR4 deficiency has paradoxically been reported to aggravate symptoms (Shi et al. 2019). Similarly, global TLR4 deletion was robustly demonstrated to mitigate chemotherapy-induced mucosal injury however inhibition with naloxone was unable to control symptoms and reduced the efficacy of chemotherapy. It is therefore critical that we appreciate the contextual roles of TLR4-dependent inflammation to appropriately modulate its activity without inhibiting restorative/healing processes that

are critical in the resolution of chronic inflammatory insult, and without influencing extra-intestinal TLR4-dependent mechanisms. Achieving this requires a greater insight into the site-specific actions of TLR4 in both the maintenance of homeostasis and initiation of disease.

Site-specific TLR4 expression in healthy states

In healthy states, TLR4 contributes to intestinal homeostasis via several distinct and well defined mechanisms: 1) maintenance of the intestinal barrier; 2) recognition and response to invading pathogens; 3) metabolic regulation; and 4) gastric motility (Anitha et al. 2012; Guo et al. 2015). TLR4 is a key sentinel protein for luminal and sub-epithelial signals of infection and sterile injury (Akira, Takeda & Kaisho 2001). This type 1 transmembrane protein has a distinct tripartite structure, including a leucine-rich extracellular domain, transmembrane domain and an intercellular signalling domain (De Nardo 2015). In a healthy condition, TLR4 expression is relatively low, with research showing that in intestinal epithelial cells, the transcription factor, ZNF160, repressed TLR4 expression in order to maintain homeostasis of the intestines and allow for commensal microbiome development (Takahashi et al. 2009). Situated in either the outer cell membrane, Golgi apparatus or within the endosome, TLR4 recognises a variety of ligands including, but not limited to; lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria, heat shock proteins, hyaluronic acid, and high-mobility group protein I (HMGBI) (Cheng et al. 2015; Santaolalla, Sussman & Abreu 2011b). Myeloid differentiation factor 2 (MD-2) is a glycoprotein on the extracellular domain of TLR4 which assists in the binding of LPS and other ligands (Lee, Avalos & Ploegh 2012; Meng, Lien & Golenbock 2010). An example of TLR4 ligand binding and activation is LPS recognition, which is driven by the binding of lipid A, of LPS, to the TLR4/MD-2 complex (Steeghs et al. 2008). Upon binding, TLR4 activates the Myeloid differentiation primary response 88

(MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) pathways, in order to increase expression of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon regulatory transcription factor 3 (IRF3) activation, respectively (Cheng et al. 2015). This cascade results in the production of pro-inflammatory cytokines including IL-6, IL-8 and interferon-β (IFN-β) (Figure. 2.1).

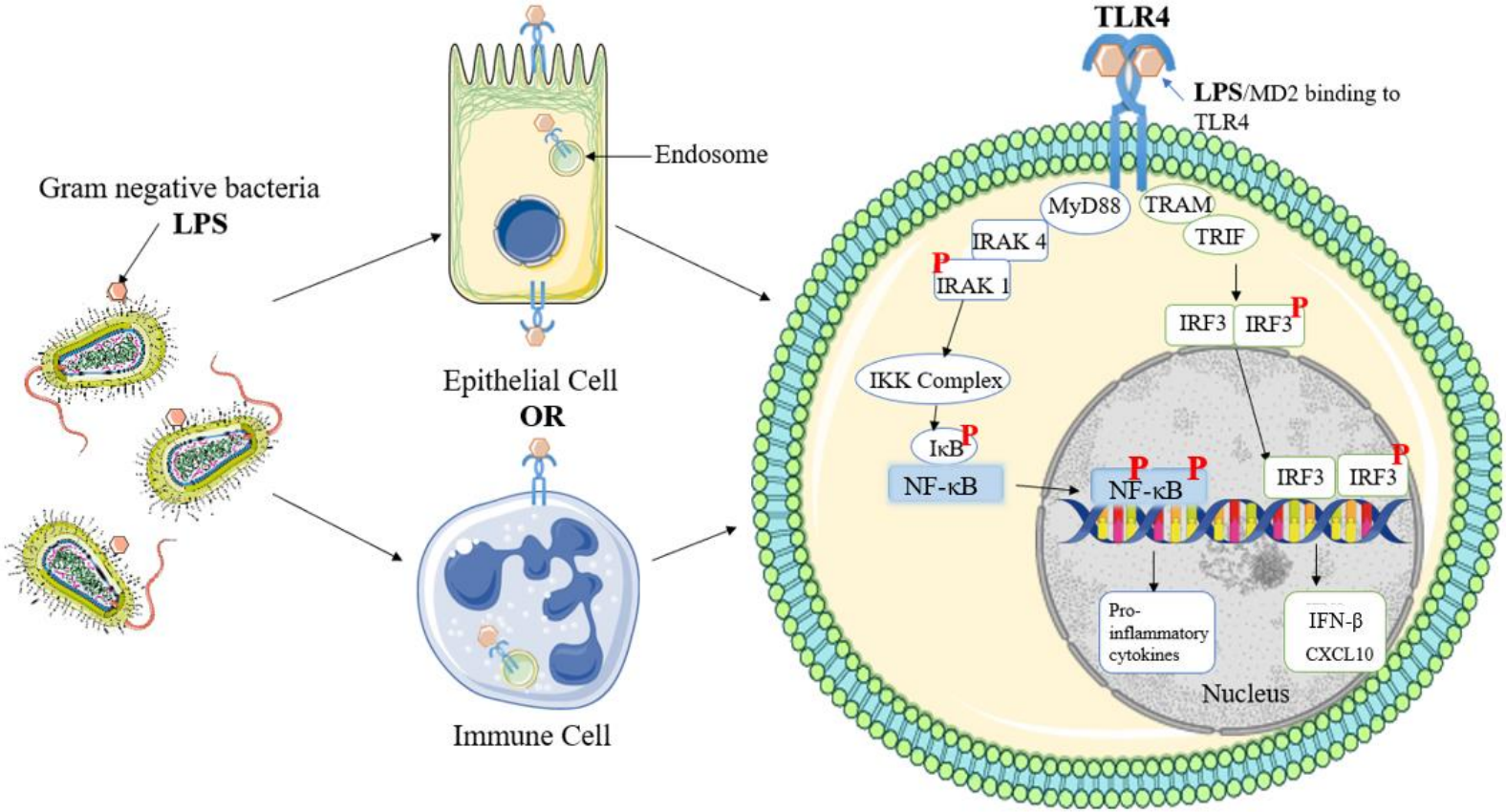
Gram negative bacteria
LPS
Endosome
Epithelial Cell
OR
Immune Cell
TLR4
LPS/MD2 binding to TLR4
MyD88
TRAM
TRIF
IRAK 4
IRAK 1
P
IKK Complex
IκB
NF-κB
IRF3
IRF3
Pro-inflammatory cytokines
IFN-β
CXCL10
Nucleus

Figure 2.1: Intracellular signalling pathways of TLR4 activation by LPS in both epithelial and/or immune cells, resulting in inflammatory responses. LPS expressed on Gram-negative bacterial cells binds to TLR4 on immune and/or epithelial cells causing the activation of an inflammatory cytokine cascade mediated by MyD88 or TRAM/TRIF activation, which leads to the upregulation of NF-κB and IRF3 respectively. This cascade results in the production of pro-inflammatory cytokines including interleukin 6 (IL-6), interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α). C-X-C motif chemokine 10 (CXCL10), interferon beta (IFN-β), interferon regulatory transcription factor 3 (IRF3), kappa B kinase (IκB), inhibitor of kappa B kinase (IKK), Interleukin-1 receptor-associated kinase 1, 4 (IRAK 1, IRAK 4), myeloid differentiation primary response 88 (MyD88), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), tumour necrosis factor alpha (TNF-α), translocating chain-associating membrane (TRAM), TIR-domain-containing adapter-inducing interferon-β (TRIF). Red P indicates phosphorylation.

Intestinal epithelial TLR4 expression in the maintenance of healthy states

While TLR4 is expressed on a range of cells types throughout the body, including dendritic cells, myeloid cells, macrophages (Vaure & Liu 2014) and microglia (Vaure & Liu 2014), its expression in the gastrointestinal tract is particularly significant due to the high microbial load and interaction with exogenous stimuli (McClure & Massari 2014). TLR4 is widely expressed along the small and large intestines under normal physiological conditions (Cario & Podolsky 2000), with higher rates of epithelial expression found in the distal ileum and colon (Cario & Podolsky 2000; Gourbeyre et al. 2015). Epithelial TLR4 is expressed on both the apical and basolateral cell membrane, as well as, within endosomes and the Golgi apparatus (Abreu 2010; Hornef et al. 2002). This variety of epithelial expression has been shown to change the site-specific role of TLR4, with basolateral TLR4 expression on epithelial cells recognising LPS and stimulating an inflammatory response, whereas, apical expression promotes tolerance to LPS, without causing inflammation (Vamadevan et al. 2010).

Lotz and colleagues were the first to explain the role of TLR4 in intestinal epithelial cells immediately after birth. Using both TLR4 KO mice and cell culture stimulation assays, this research found that intestinal epithelial TLR4 is the first to respond to exogenous endotoxins, like LPS, rather than immune expressed TLR4 on macrophages (Lotz et al. 2006). This initial site-specific recognition helps to establish the microbial colonisation of the intestine and is fundamental in the development of a healthy microbiome and host immune tolerance (Lotz et al. 2006). Previous evidence shows that the absence of intestinal epithelial TLR4 resulted in a hypo-responsiveness to LPS (Abreu et al. 2001; Naik et al. 2001), therefore demonstrating a clear link between intestinal homeostasis and epithelial TLR4 expression.

Furthermore, a 2008 study using a Caco-2 cell culture and monocyte derived dendritic cells found that intestinal epithelial cells ready the immune system response to commensal bacteria via partially TLR4 driven mechanisms, leading to a tolerogenic phenotype to commensals (Zeuthen, Fink & Frokiaer 2008). This study supports the importance of epithelial TLR4 and its role in establishing immune tolerance to intestinal bacteria and development of a healthy microbiome.

In addition to immunosurveillance, epithelial TLR4 signalling is involved in a variety of essential functions for the establishment and maintenance of a healthy epithelial function, including; inflammation control, regulation of host microbiome, B cell recruitment, IgA production, follicle-associated epithelial permeability and antimicrobial peptide expression (Abreu 2010; Chabot et al. 2006). Of particular relevance to intestinal homeostasis are interactions between TLR4 and the mucosal barrier, a highly regulated interface between the luminal environment and mucosal compartment comprised of intestinal epithelial cells and basement membrane.

Mucosal barrier integrity is maintained by the apical junction complex (tight junction, desmosome, adherens junction), with tight junctions maintaining the semi-selective state of the paracellular mucosal route. Careful control of the molecular structure of the tight junction allows for absorption of solutes across their respective electro-osmotic gradients and also serves as an immunosurveillance checkpoint (Anderson, Van Itallie & Fanning 2004; Blijlevens 2005; Hooper, Littman & Macpherson 2012; Wells et al. 2017). The tight junction complex was first identified at the apico-lateral surface of adjacent enterocytes in 1963 by Farquhar and Palade (Farquhar & Palade 1963). This foundational study utilised haemoglobin as a mass tracer in rat and Guinea pig tissue to assess permeability (Farquhar & Palade 1963). Combined with electron microscopy techniques, this protocol showed that the tight junction is responsible for the diffusion of the tracer along the intercellular space, providing the

first evidence highlighting the importance of tight junctions in regulating intestinal permeability (Farquhar & Palade 1963). Since this pivotal study, the specific proteins that comprise this complex have been identified, including occludin, zonular occludens 1 (ZO-1) and claudins (Figure. 2.2). The tight junction is indicated in the healthy functioning of the intestinal barrier, where damage to tight junction proteins often results in increased permeability and progression of illness (Salim & Soderholm 2011; Wardill, Bowen & Gibson 2012; Lee 2015). Previous evidence concluded that TLR4 expression did not influence tight junction protein development (Wardill et al. 2016) although tight junctions were primarily assessed after a mucosal challenge of irinotecan treatment. Of note, a recent study found that the colon epithelial cells treated with 5-HT or commensal *E.coli* showed increased expression of TLR4 which correlates with the downregulation of E-cadherin and claudin-2 (Banskota et al. 2017). Similarly, increased TLR4 signalling (transgenic villin TLR4 hyperactivity) was reported to increase intestinal permeability as detected by FITC-dextran and tight junction protein expression (Dheer et al. 2016). These findings implicate a functional role for epithelial TLR4 in tight junction protein control. These results were supported by Bein and colleagues whose study into necrotising enterocolitis (NEC) in neonates found that the decrease of TLR4 expression seen in NEC is associated with a decrease in tight junction proteins, occludin, cingulin, claudin-4 and ZO-1 (Bein et al. 2018). While this may be a by-product of NEC pathophysiology, this could also suggest a homeostatic role of TLR4 in tight junction maintenance and TLR4 impact on intestinal permeability. Furthermore, recent research has shown that increased TLR4 expression negatively influences the function of the adherens junction. The adherens junction is comprised of cadherin transmembrane receptors and their associate binding proteins, forming a junction that allows for cell-to-cell adhesion and maintenance of tissue integrity (Pinheiro & Bellaiche 2018). Research conducted by Ralls and colleagues using human inflamed small bowel tissue, found that in

underfed sections of bowel, TLR4 is significantly increased and E-cadherin expression is decreased resulting in loss of epithelial barrier function (Ralls et al. 2015). This evidence shows the dual impact of TLR4 expression on the intestinal junctional complexes, often leading to exacerbated intestinal disruption.

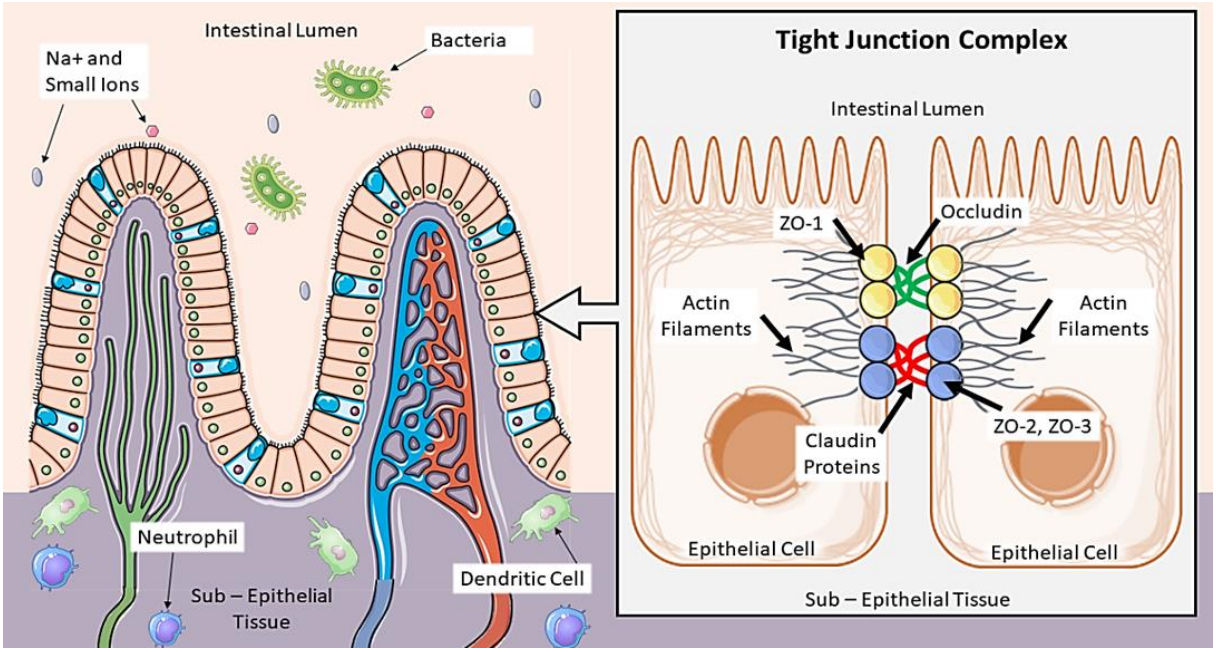

Figure 2.2: Representative diagram of the intestinal epithelial barrier and tight junction protein complex. The junctional complex, including tight junction proteins (pictured), adherens and desmosomes, is located at the apical lateral junction of epithelial cells and acts to bind cells together to form a selective barrier. In health, physiological solutes and ions are able to cross this barrier; however, pathogenic bacteria are unable to cross, therefore maintaining the sterile sub-epithelial environment.

In addition to its pro-barrier properties, epithelial TLR4 also influences the structural development of the intestine and the intestinal immune environment (Lu, Sodhi & Hackam 2014; Meng et al. 2015). A transgenic mouse model, constitutively expressing TLR4 in the intestinal epithelium, found links between epithelial TLR4 signalling and B cell recruitment and IgA production (Shang et al. 2008). In the transgenic model B cells and B cell tropic chemokines were increased with CCL20 protein being 5-fold higher in transgenic mice than wild-type (WT) counterparts, and CCL28 being 1.6-fold higher than WT (Shang et al. 2008). Furthermore, the continuous overexpression of epithelial TLR4 resulted in increased IgA^+ cells in the small intestine lamina propria and produced higher faecal IgA levels as compared to WT (Shang et al. 2008). Finally, this study also found that TLR4 signalling in intestinal epithelial cells induces immunoglobulin class switching to IgA in the small intestinal lamina propria (Shang et al. 2008). Taken together, this evidence supports the important role intestinal epithelial cells have in response to external signalling and presents a mechanism by which epithelial TLR4 prevent attachment of pathogens via the regulation of luminal IgA production (Shang et al. 2008).

The importance of epithelial TLR4 to intestinal function was convincingly demonstrated using intestinal epithelial conditional KO mice (*Tlr4*$^{\Delta IEC}$) in investigations into the regulatory contribution of epithelial TLR4 to altered metabolic symptoms and disease (Lu et al. 2018). *Tlr4*$^{\Delta IEC}$ mice were shown to develop metabolic syndrome faster than their WT counterparts, showing significant weight gain independent of food intake or hormone levels and the presence of macrophage accumulation within adipose tissue via histologic analyses (Lu et al. 2018). Furthermore, intestinal epithelial TLR4 was shown to significantly impact the composition of the intestinal microbiota in mice, where *Tlr4*$^{\Delta IEC}$ mice showed both a lower diversity of bacteria and altered bacterial clustering (Lu et al. 2018).

Additionally, *Tlr4*$^{\Delta IEC}$ mice showed an altered expression of metabolic and inflammatory genes in comparison to WT. Metabolic pathway genes associated with peroxisome proliferator-activated receptor (PPAR) signalling were significantly downregulated in the ileum of *Tlr4*$^{\Delta IEC}$ mice (Lu et al. 2018). Finally, the conditional KO of intestinal epithelial TLR4 resulted in a pro-inflammatory phenotype, including upregulation of macrophage markers (*CD68, F4/80 and Mcp1*) and neutrophil markers (*Mpo and Elane*). Together, these results show that intestinal epithelial TLR4 expression in healthy states assists with the regulation of metabolic control, microbiota composition and function, metabolic gene expression and prevention of unnecessary inflammation.

In contrast, the impact of TLR4 to microbial invasion was elegantly demonstrated using a C57BL/6 villin-TLR4 transgene model which constitutively expressed intestinal epithelial TLR4. Interestingly, where intestinal epithelial TLR4 was consistently overexpressed, an increase in microbial invasion, alterations to luminal and mucosal microbiota and disruptions to epithelial barrier function were found (Dheer et al. 2016). This evidence emphasises the dichotomous role of TLR4, with its physiological benefits restricted within a threshold of expression/activity (Abreu 2010). Importantly, it is consistently shown that site-specific overexpression is detrimental to intestinal homeostasis resulting in breakdown of the mucosal barrier and exaggerated immune activation, which is likely to be mediated by the basolaterally-expressed epithelial TLR4. Hyperactivation of TLR4 is therefore a key driver of many diseased states affecting gastrointestinal function, many of which will be discussed further in later sections of this review.

Immune cell TLR4 expression in healthy states

The majority of our fundamental knowledge regarding TLR4 signalling has been gained from studying its activity in immune cell subsets, likely reflecting higher expression of TLR4 on immune cells compared to enterocytes. Gourbeyre and colleagues state a 2.5-fold increase of TLR4 expression within the mesenteric lymph nodes of pigs compared to intestinal tissue, and a 1.5-fold increase of TLR4 expression in the intestinal lumen compared to the jejunal villi (Gourbeyre et al. 2015). This increase of TLR4 abundance is due to the high immune cell presence within these organ systems with TLR4 expressed on dendritic cells, macrophages and myeloid cells (Vaure & Liu 2014). While immune-derived TLR4 signalling is crucial to the recognition and response to dangerous PAMPs and DAMPs (Cario & Podolsky 2000; Fukata et al. 2005; Apetoh et al. 2007; Leaphart et al. 2007; Hajjar et al. 2012), immune cell TLR4 expression also helps to regulate the immune and intestinal environment in healthy states. Arguably, one of the most important immune cells to express TLR4 are dendritic cells.

Dendritic cells are a highly specialised form of antigen-presenting cells, which provide an important link between innate immunity and activation of the adaptive immune system (Pufnock et al. 2011). Upon capture and recognition of an antigen, mature dendritic cells migrate from the site of recognition to the lymphoid organs and initiate the development of effector and memory T cells from primary T cells (Michelsen et al. 2001; Pufnock et al. 2011). Maturation of dendritic cells is the process whereby the role of dendritic cells changes from immature phagocytic actions to cytokine production and efficient T cell antigen-presenting. This process occurs before reaching the lymphoid organs and is highly dependent on the recognition and digestion of pathogens (Michelsen et al. 2001). A foundational study using mice with a mutated TLR4 protein, examined the role of immune expressed

TLR4 on dendritic cell maturation (Michelsen et al. 2001). Results showed that both TLR2- and TLR4-related mechanisms were crucial for the normal maturation of dendritic cells in response to bacterial ligands (Michelsen et al. 2001). A further immunological study by Pufnock and colleagues found that co-activation of both TLR4 and TLR7/8 provides an increased maturation of dendritic cells and increased generation of CD8+ memory T cells (Pufnock et al. 2011). Together, this evidence supports the importance of immune cell expression of TLR4 for dendritic cell maturation and successful priming of the T cell response to invading pathogens.

Current research has also indicated a strong relationship between TLR4 expressed on dendritic cells and immunological tolerance via the release of indoleamine 2, 3-dioxygenase (IDO) (Salazar et al. 2017). Apart from the role of IDO as an enzyme which catalyses the amino acid tryptophan, IDO also plays an important role in immune regulation (Chen 2011). IDO produced by dendritic cells promotes immunosuppression and helps establish tolerance to commensal microbiota (Harden & Egilmez 2012). Critically, LPS-primed human dendritic cells produced a higher IDO than unprimed cells, and that the primed cells resulted in a tolerogenic phenotype (Salazar et al. 2017). Furthermore, unprimed cells challenged with LPS also resulted in increased IDO and a tolerogenic phenotype (Salazar et al. 2017). Interestingly, when monocytes were challenged with LPS, there was no increase in IDO production, suggesting that this is a unique trait of dendritic cells (Salazar et al. 2017). This evidence characterises the role of TLR4 expression on dendritic cells and its importance in homeostasis and supports previous research in which the TLR4 ligand, LPS, induced expression of IDO in dendritic cells which contributed to an immunogenic tolerance (Von Bubnoff et al. 2011). While the links between TLR4-mediated dendritic cell IDO expression and immune tolerance are clearly stated, a 2008 study found a further mechanism whereby dendritic-expressed TLR4 assists in

homeostatic tolerance (Albrecht et al. 2008). This work using *in vitro* LPS pre-cultured human dendritic cells found that further stimulation with high dose LPS produced an immune tolerance phenotype in these cells, marked by the ablation of the IRAK-1 adaptor protein and decreased NF-κB activation (Albrecht et al. 2008).

While a well-defined link between dendritic-expression of TLR4, immune tolerance and adaptive immune activation is clear, the role of TLR4 expressed on monocytes remains uncertain. Current evidence shows that monocytic TLR4 expression may not contribute to enhanced immune tolerance (Salazar et al. 2017), however current evidence is sparse and further research is therefore required to dissect monocytic-specific TLR4 signalling and its contribution to healthy and disease states. With this said, the monocyte-derived macrophage expression of TLR4 has been well-characterised to engage the NF-κB pathway, leading to active cytokine release and promotion of inflammation in response to pathogenic bacteria (Li & Cherayil 2003). The innate immune response to pathogens is highly dependent on ligand binding to TLR4 on macrophages, which enables a maximised release of TNF-α, resulting in beneficial inflammation and elimination of harmful bacteria (Li & Cherayil 2003). Furthermore, previous research indicates the role of TLR4 in increasing oxidative stress in activated macrophages, in order to aid in removing pathogens from the body (Matsebatlela et al. 2015). In contrast, this relationship is also implicated in the development and progression of renal hypertension, with research showing that TLR4 deficiency reduces oxidative stress and therefore improves the hypertensive condition (Pushpakumar et al. 2017). Therefore, TLR4 on macrophages seem to play both homeostatic roles, as well as, contributing roles in disease

Disease– specific impact of TLR4 expression

Role of TLR4 and LPS response

As outlined, TLR4 signalling is best known for its well-established role in LPS recognition from Gram-negative bacteria and initiation of inflammation (Abreu et al. 2001; Hajjar et al. 2012). Historically, this process was believed to be heavily dependent on immune expression of TLR4. However, research by Abreu and colleagues found that TLR4 expression is significantly increased in response to LPS with T cell-derived cytokines, IFN-ɣ and TNF-α, increasing TLR4 expression in intestinal epithelial cells, therefore increasing the inflammatory response to LPS administration (Abreu et al. 2002). Furthermore, a study conducted by Hornef and colleagues produced strong evidence highlighting the importance of intestinal epithelial TLR4 in LPS recognition (Hornef et al. 2002). Using isolated murine small intestinal crypt epithelial cells, this study showed pro-inflammatory mediator secretion and CD14 upregulation in response to LPS administration (Hornef et al. 2002). Furthermore, epithelial cell TLR4 expression was found within the Golgi apparatus (Hornef et al. 2002), identical to the site of internalised LPS, showing that intestinal epithelial cells may be responsible for the initial recognition and signalling in response to LPS. It is this which then attracts the accumulation and stimulation of the more advanced immune response (Hornef et al. 2002). The damaging inflammatory response to LPS from Gram-negative bacteria in the intestinal tract is partially characterised by increased intestinal permeability. Research by Guo and colleagues found a significant decrease in epithelial resistance in response to LPS administration in Caco-2 cell monolayers from 100% relative epithelial resistance in untreated control versus 70% relative epithelial resistance in LPS treated monolayers (Guo et al. 2013). Furthermore, in an *in vivo* C57BL/6 mouse model, LPS administration was shown to increase permeability flux of the macromolecular

Dextran 10K across the intestinal epithelium of WT mice with 3-fold higher permeability in LPS treated mice, however, in a global TLR4 KO mouse, permeability and flux did not increase from baseline with LPS treatment (Guo et al. 2013). This study concluded that LPS administration results in an increased intestinal permeability mediated in part by increased TLR4 and CD14 expression and localisation along intestinal enterocytes (Guo et al. 2013).

While current evidence strongly suggests the role of epithelial TLR4 in recognition and response to LPS, many foundational studies have investigated the relationship between LPS and TLR4, finding that immune TLR4 is essential for the development of inflammation and the elimination of bacteria (Poltorak et al. 1998). A study conducted by Hoshino and colleagues investigated the impact of TLR4 elimination on LPS responsiveness (Hoshino et al. 1999). This study utilised an immune-specific TLR4 KO mouse model, which was deemed successful due to the harvested macrophages and B cells showing no response to LPS administration (Hoshino et al. 1999). In the TLR4 KO model, LPS response was greatly decreased compared to WT counterparts (Hoshino et al. 1999). This study is further supported by Qureshi and colleagues, who found that endotoxin hypo-responsive mice showed unique mutations within the *Tlr4* gene coding (Qureshi et al. 1999). More recent evidence showed that the mechanism of the LPS-derived increased gastrointestinal permeability is caused primarily by the TLR4/MyD88 signalling pathway (Guo et al. 2015). Two strains of KO mice were utilised including a TLR4 KO and MyD88 KO. Results found that permeability was increased as a result of injection of LPS to mice and that this was strongly dependant on TLR4 expression (Guo et al. 2015). These findings were supported by Nighot and his group further clarifying the role of the TLR4/MyD88 pathway in increased permeability (Nighot et al. 2017). This study introduced LPS to Caco-2 monolayers and C57BL/6 mice and found an increase in

permeability, both *in vitro* and *in vivo*, via upregulation of myosin light chain kinase (MLCK). While these more recent studies do provide evidence of TLR4 involvement in LPS recognition, they did not distinguish between epithelial versus immune cell expression, negatively impacting the translatability of results.

Role of TLR4 in altered intestinal permeability, IBD and IBS

While the TLR4/LPS mediated inflammatory pathway has been well-characterised, recent literature has indicated the involvement of epithelial TLR4 in the development of many intestinal diseases and illnesses. The pathogenesis of many inflammatory bowel diseases and intestinal toxicities are characterised by increased permeability and disruption to the tight junction complex. A study by Li and colleagues investigated the role of intestinal epithelial TLR4 on the phosphorylation of the tight junction protein, occludin, via protein kinase C (PKC) and its contribution to increased permeability in alcoholic steatohepatitis (Li et al. 2013). Using both a Caco-2 monolayer cell cultures and C57BL/6 WT mice, this study found that the neutralisation of TLR4 using mAB pre-treatment reduced ethanol-induced paracellular permeability. Furthermore, in the presence of ethanol, TLR4 expression was increased and phosphorylated occludin was decreased (Li et al. 2013). This decrease in functional occludin mediated by TLR4 may explain why with high concentrations of ethanol, a significant increase of intestinal permeability is seen. This study further supports the involvement of epithelial TLR4 in the development of intestinal barrier dysfunction in several disease states.

Altered intestinal permeability is also a hallmark of IBD, which is includes Crohn's disease (CD) and ulcerative colitis (UC), each a chronic inflammatory diseases which significantly impact wellbeing and an individual's ability to thrive due to symptoms of pain, diarrhoea and constipation. A large genetic study collated 1539 DNA samples from human IBD patients and included pooled analysis of 4805 cases of IBD (De

Jager et al. 2007). Focussing on 23 genes related with TLR4 expression and associated signalling pathways, this study genetically identified TLR4 as a disease risk factor for both CD and overall IBD (De Jager et al. 2007). Due to the large sample sizes and well-tested methodologies used, this study provides strong genetic evidence that TLR4 plays a role in the development of gastrointestinal pathologies. However, due to the genetic nature of this study, it is unable to provide any distinguishing information regarding cell-specific TLR4 involvement as is common in the current literature. An early study by Cario and Podolsky investigated the differential expression of different Toll-like receptors in the intestinal epithelia in IBD (Cario & Podolsky 2000). This patient-based study utilised human tissues of colon and terminal ileum, from those with active IBD. Western blot and immunohistochemistry analyses revealed that epithelial TLR4 was greatly upregulated in both UC and CD and may contribute to disease progression (Cario & Podolsky 2000). Interestingly, a significant difference in positioning of epithelial cell TLR4 expression was noted between UC and CD. Higher expression of TLR4 on the basolateral cell surface was noted in UC patients whereas, higher expression of TLR4 on the apical cell surface was observed in CD patients (Cario & Podolsky 2000). As expected, an increase in TLR4 expression was also found on immune cells of the lamina propria due to the inflammatory nature of these diseases. Knowing that intestinal epithelial TLR4 are overexpressed in IBD patients, a study conducted by Dheer and colleagues investigated the impact of constitutive TLR4 expression on epithelial function and the microbiota composition (Dheer et al. 2016). Increased TLR4 signalling resulted in invasion of intestinal microbiota and significant alterations to bacterial composition within the intestines (Dheer et al. 2016). This led to an exacerbation of DSS-mediated colitis in animal models (Dheer et al. 2016).

Another common gastrointestinal condition is irritable bowel disease (IBS), which effects one in five Australians (Belmonte et al. 2012). IBS is often characterised by bloating, abdominal pain, diarrhoea and/or constipation, which significantly impacts quality of life (Belmonte et al. 2012). A study conducted by Belmonte and colleagues, investigated the impact and prevalence of TLR4 expression on IBS development (Belmonte et al. 2012). In this clinical study, a total of 48 IBS patients (including similar rates of diarrhoea-dominant (IBS-D), constipation-dominant (IBS-C) and mixed symptoms phenotypes (IBS-M)) and 31 control patients were enrolled, and colonic biopsy samples were collected via colonoscopy (Belmonte et al. 2012). A 2-fold increase of TLR4 expression was found in IBS-M patients when compared to healthy controls. Additionally, TLR4 expression was highest in IBS patients with a disease duration of over five years (Belmonte et al. 2012). Furthermore, increased TLR4 expression in IBS patients was predominantly found in intestinal epithelial cells, with a higher TLR4 presence in the crypts of intestinal tissue compared to surface epithelial layers (Belmonte et al. 2012). These results are supported by a more recent study, which also analysed human colonic tissue from both IBS patients and healthy control patients (Kocak et al. 2016). This study only evaluated two IBS subgroups, being IBS-D and IBS-C, omitting the IBS-M group. However, results showed significantly increased expression of TLR4 in colonic mucosa for both subgroups of IBS patients when compared to control (Kocak et al. 2016). These findings support the detrimental role of TLR4 hyper-expression in the context of IBD and IBS, and help to shape our understanding of disease pathophysiology and progression. However, due to the non-specific reporting of TLR4 expression states, it is difficult to distinguish between epithelial and immune TLR4 contextual roles, and thus translation of these findings is challenging.

Role of TLR4 in necrotising enterocolitis

NEC is a leading cause of death in premature infants, where intestinal injury permits bacterial translocation into the usually sterile sub-epithelial space (Anand et al. 2007; Leaphart et al. 2007). This disease mechanism results in necrosis of the small intestine leading to sepsis, multi-organ failure and death (Hackam et al. 2005). A study by Leaphart and colleagues investigated the role of intestinal mucosal TLR4 on NEC development. TLR4 expression within the mucosa is significantly increased in NEC and in TLR4 mutant mice, the severity of NEC was significantly reduced compared to WT counterparts (Leaphart et al. 2007). However, interestingly, this study also identified a potential role of TLR4 in mucosal repair via increased proliferation and association with intestinal focal adhesion kinases to induce healing (Leaphart et al. 2007). Together, these findings show that TLR4 expression in the mucosa is important in the context of NEC. A major limitation of this study was the uncertainty of which specific cell types were examined, as mucosal tissue includes both epithelial and immune cell lines. A more recent study conducted by Sodhi and colleagues further investigated the role of TLR4 in NEC, and found that it is epithelial expression of TLR4 which contributes to NEC development and goblet cell regulation (Sodhi et al. 2012). This ambiguity is mimicked across many fields of TLR4 and intestinal disorders, including both intestinal disease and chemotherapy-induced gastrointestinal toxicity (CIGT).

TLR4 regulates CIGT risk and severity

CIGT is a serious complication of current anti-cancer treatments, affecting up to 80% of people treated with chemotherapy (Blijlevens 2005; Richardson & Dobish 2007). Symptoms of CIGT include severe diarrhoea, pain, bleeding and ulceration often resulting in increased risk of infection, leading to delayed, or discontinued, treatment (Andreyev et al. 2014; Fuchs, Mitchell & Hoff 2006). These symptoms are

predominantly underpinned by changes to the intestinal barrier, which is comprised of polarised epithelial cells acting as a highly selective barrier between the intestinal lumen and the sub-epithelial tissue (Pott & Hornef 2012; Wardill, Bowen & Gibson 2012). Irinotecan is a common chemotherapy associated with CIGT development, due to its unique enterohepatic recirculation which causes a double exposure to its toxic metabolite, SN-38, in the intestines (Chabot 1997; Kawato et al. 1991). A 2016 study examined the link between irinotecan-induced CIGT and TLR4 involvement, demonstrating reduced clinical indicators of toxicity in the TLR4 KO mice versus WT. Specifically, TLR4 KO mice showed decreased diarrhoea and lost significantly less body weight compared to their WT counterparts (Wardill et al. 2016). Furthermore, authors reported less severe intestinal barrier dysfunction, with TLR4 KO mice demonstrating lower serum FITC-dextran (Wardill et al. 2016). Together with intestinal injury, CIGT is also characterised by an increase of pain and discomfort hypothesised to occur via neuroinflammation and neurotoxicity, controlled via the gut-brain axis (Li et al. 2015). Results from an *in vivo* mouse study found that administration of the chemotherapy, paclitaxel, activated TLR4 which sensitised transient receptor potential vanilloid subtype 1 to dorsal root ganglia neurons, creating adverse physiological effects (Li et al. 2015). This evidence emphasises the importance of TLR4 mediated mechanisms in governing intestinal barrier function and its secondary consequences. However, given the ubiquitous expression of TLR4 on epithelial cells, enteric glia, CNS supportive cells and immune cells, it remains challenging to decipher the exact mechanisms at play, thus hindering the design of suitable interventions. Despite these challenges, inhibition of TLR4 (via naloxone has been investigated in the setting of CIGT (Coller et al. 2017). Naloxone was administered orally, with the goal of inhibiting epithelial TLR4 and thus prevent both direct SN-38-dependant TLR4 activation as well as host-microbiome interactions that govern mucosal injury (Coller et al. 2017). While naloxone was unable to attenuate

CIGT, it was also shown to impact tumour growth, with adjuvant TLR4 inhibition decreasing the efficacy of irinotecan against adenocarcinoma cells. This parallels findings of Apetoh (2007) and Fukata (2007), with increased basal tumour growth and decreased chemoefficacy both shown in TLR4 KO mice (Apetoh et al. 2007; Fukata et al. 2007). Together, these findings highlight the potential hazards of broad-spectrum antagonism of TLR4 in conjunction with chemotherapies and emphasises the importance of dissecting immune and epithelial mechanisms prior to transitioning to interventional studies.

Concluding remarks and future directions

TLR4 is widely expressed on epithelial and immune cells along the gastrointestinal tract, and is responsible for a range of homeostatic control mechanisms including immune system development. Recent evidence indicates the role of TLR4 signalling in the development, and progression, of many gastrointestinal diseases and toxicities. In normal physiology, both epithelial and immune expression of TLR4 is required to maintain host microbiome (Abreu 2010), B cell recruitment, immune tolerance (Salazar et al. 2017) and maturation of dendritic cells (Michelsen et al. 2001). While it is crucial to understand the role of TLR4 in a healthy condition, the impact of site-specific TLR4 expression in gastrointestinal diseases is of great importance. New disease mechanisms involving the activation of TLR4, and its associated signalling pathways, are continually emerging in this field. While TLR4-based research continues to grow, there is a significant lack of specificity in the literature concerning cell-specific TLR4 signalling and its contribution to both health and disease. This common oversight almost certainly introduces translational errors and clouds interpretation of preclinical evidence. The use of novel intestinal-specific KO models will provide much needed resolution into the site-specific roles of TLR4. These new models aim to better inform the development of new interventions

targeting TLR4, providing greater specificity in augmenting immune or epithelial TLR4 without compromising core homeostatic mechanisms.

Chapter 3: Epithelial-specific TLR4 knockout challenges current evidence of TLR4 homeostatic control of gut permeability

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This chapter details the breeding strategy and baseline intestinal characteristics of the intestinal epithelial conditional TLR4 KO (*Tlr4*$^{\Delta IEC}$) mice compared to wild-type (WT) in health. Results supported the use of the *Tlr4*$^{\Delta IEC}$ model in my chemotherapy-induced gastrointestinal toxicity (CIGT) and colorectal cancer (CRC) modelling experiments (chapters 4 and 6, respectively). This chapter is published in Inflammatory Intestinal Diseases under the following reference; Crame, E. E., Bowen, J. M., Secombe, K. R., Coller, J. K., Francois, M, Leifert, W, Wardill, H. R., (2021). Epithelial-specific TLR4 knockout challenges current evidence of TLR4 homeostatic control of gut permeability. *Inflammatory Intestinal Diseases*, 6(4), 199-209. https://doi.org/10.1159/000519200

Please note that figure numbering and referencing style have been altered to suit thesis formatting style. All spelling has been converted to Australian spelling. All other formatting has remained unchanged from original publication.

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Name of Principal Author (Candidate)	Mrs. Elise Ellen Crame		
Contribution to the Paper	Developed research question and proposal. Conducted *in-vivo* animal work and molecular benchtop experimental work. Together with Dr. Kate Secombe, created the conditional TLR4 knockout mouse model. Primary author of manuscript, responsible for writing manuscript, data analysis and creation of all figures.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	03/08/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	21/09/2022

Abstract

Introduction

Toll-like receptor 4 (TLR4) is a highly conserved immunosurveillance protein of innate immunity, displaying well-established roles in homeostasis and intestinal inflammation. Current evidence shows complex relationships between TLR4 activation, maintenance of health and disease progression, however, commonly overlooks the importance of site-specific TLR4 expression. This omission has the potential to influence translation of results, as previous evidence shows the differing and distinct roles that TLR4 exhibits is dependent on its spatiotemporal expression.

Methods

An intestinal *epithelial* TLR4 conditional knockout mouse line (*Tlr4*$^{\Delta IEC}$, n = 6 - 8) was utilised to dissect the contribution of *epithelial* TLR4 expression to intestinal homeostasis with comparisons to wild-type (WT, n = 5 - 7) counterparts. Function of the intestinal barrier in the ileum and colon were assessed with tissue resistance in Ussing chambers. Molecular and structural comparisons in the ileum and colon were assessed via histological staining, expression of tight junction proteins (occludin and zonular occludens 1) and presence of CD11b positive immune cells.

Results

There was no impact of the intestinal epithelial TLR4 knockout, with no differences in: 1. tissue resistance - ileum (mean ± SEM): WT 22 ± 7.2 vs *Tlr4*$^{\Delta IEC}$ 20 ± 5.6 (Ωcm^2) *p* = 0.831, colon WT 30.8 ± 3.6 vs *Tlr4*$^{\Delta IEC}$ 45.1 ± 9.5 *p* = 0.191; 2. histological staining (overall tissue structure); and 3. tight junction protein expression (% area stain, mean ± SEM) - ZO-1: ileum - WT 1.49 ± 0.155 vs *Tlr4*$^{\Delta IEC}$ 1.17 ± 0.07, *p* = 0.09; colon - WT 1.36 ± 0.26 vs *Tlr4*$^{\Delta IEC}$ 1.12 ± 0.18 *p* = 0.47; occludin: ileum - WT 1.07 ± 0.12 vs *Tlr4*$^{\Delta IEC}$ 0.95 ± 0.13, *p* = 0.53; colon - WT 1.26 ± 0.26 vs *Tlr4*$^{\Delta IEC}$ 1.02 ± 0.16 *p* = 0.45.

CD11b positive immune cells (% area stain, mean ± SEM) in ileum were mildly decreased in WT mice: WT 0.14 ± 0.02 vs *Tlr4*$^{\Delta IEC}$ 0.09 ± 0.01 p = 0.04. However, in the colon there was no difference in CD11b positive immune cells between strains: WT 0.53 ± 0.08 vs *Tlr4*$^{\Delta IEC}$ 0.49 ± 0.08 p = 0.73.

Conclusions

These data have two important implications. Firstly, these data refute the assumption that epithelial TLR4 exerts physiological control of intestinal physiology and immunity in health. Secondly, and most importantly, these data support the use of the *Tlr4*$^{\Delta IEC}$ line in future models interrogating health and disease, confirming no confounding effects of genetic manipulation.

Introduction

Polarized epithelial cells covering the intestinal tract form a highly selective barrier between the bacteria-filled gut lumen and the comparatively sterile sub-epithelial tissue (Pott & Hornef 2012). This barrier maintains homeostasis within the gastrointestinal tract, allowing for nutrient absorption and regulation of water exchange (Wells et al. 2017). Crucially, the intestinal epithelial lining is also a first-line of defence from pathogens, whereby innate immune pattern recognition receptors (PRR) recognize harmful bacteria and promote protective inflammatory cascades (Pott & Hornef 2012). Toll-like receptor 4 (TLR4) is a type of PRR expressed on a variety of cell types including immune (Vaure & Liu 2014) and epithelial cells (McClure & Massari 2014). TLR4, and its accessory proteins MD-2 and CD14, are widely researched due to the dual roles in homeostatic control and suspected involvement in multiple conditions, including inflammatory bowel diseases and chemotherapy-induced gastrointestinal toxicity (CIGT) (Belmonte et al. 2012; Wardill et al. 2016).

Based on its consistent implication with diseased states that are characterized by intestinal dysfunction, TLR4 has been regularly reported to be a key regulator of mucosal barrier function and thus intestinal permeability under physiological conditions (Abreu 2010; Chabot et al. 2006). Intestinal permeability via the paracellular route is dictated via tight junction proteins located on the apical-lateral cell surface (Anderson, Van Itallie & Fanning 2004). The multiple intercellular and bridging proteins of the tight junction, including occludin, zonular occludens 1 (ZO-1) and claudins, allow for the movement of solutes across their electro-osmotic gradient to maintain intestinal homeostasis (Ma, Anderson & Turner 2012). A considerable body of evidence anecdotally supports TLR4-mediated barrier control, with TLR4

expression strongly correlating with functional assessments of intestinal permeability and molecular characteristics of tight junction proteins (Wardill et al. 2016). For example, a 2018 study by Bein and colleagues found that in a necrotizing enterocolitis model, a decrease of TLR4 expression was significantly associated with a decrease in occludin, ZO-1 and claudin-4 and resulted in increased permeability (Bein et al. 2018). While this suggests a connection between functional TLR4 and the preservation of the tight junction complex, these findings are only secondary to original aims and do not fully explain the role of TLR4 in homeostasis. Previous research using global TLR4 knockout (KO) mice also shows that a lack of TLR4 expression does not impact tight junction protein development and barrier function, however, this study only analysed tight junction expression post-chemotherapy challenge (Wardill et al. 2016). While these studies implicate TLR4 in the pathobiological control of the mucosal barrier, the majority of these data have been generated in models of disease and as such, conclusions regarding its physiological control cannot be made.

Another major oversight in the literature regarding TLR4's regulatory control of the intestinal barrier is the lack of site-specific interrogation. TLR4 is not only expressed on epithelial cells of the intestinal mucosa, but also immune cells of the submucosa. In fact, immune expression of TLR4 is considerably higher than epithelial expression (Vaure & Liu 2014) and as such, its impact on mucosal homeostasis and disease is arguably higher. A failure to address site-specific TLR4 mechanisms hampers our ability to dissect causative mechanisms, and thus impairs translation of fundamental findings. This has the potential to misguide new interventions targeting TLR4 that may not be delivered in a manner that optimally targets TLR4. This paradox is particularly important in cancer research, where TLR4-dependent mechanisms are central to both the efficacy and toxicity of therapy; yet a lack of site-specific

interrogation has resulted in highly variable and contradictory findings in studies attempting to augment its activity.

There is a clear need to study TLR4-dependent control of the mucosal barrier in a manner that dissects epithelial versus immune mechanisms. As such, we have utilised a conditional intestinal epithelial-specific TLR4 KO mouse line (*Tlr4*$^{\Delta IEC}$) (Sodhi et al. 2012), with epithelial deletion of TLR4 and unimpaired immune cell expression TLR4. In characterizing this mouse line, we are given the unique opportunity to rigorously define the regulatory role of epithelial TLR4 on the intestinal barrier under physiological conditions. As such, we aimed to characterize the potential intestinal differences of this *Tlr4*$^{\Delta IEC}$ line compared to wild-type (WT) mice, using structural, molecular and electrophysiological assessments.

Materials and Methods

Animal husbandry

Male and female WT C57BL/6 (n = 5 - 7) and intestinal epithelial conditional TLR4 KO C57BL/6 (*Tlr4*$^{\Delta IEC}$, n = 6 - 8) mice aged 8 – 12 weeks, were housed in ventilated cages in groups of 3 - 6 animals per cage with a 12- hour light/dark cycle and access to irradiated standard mouse chow and sterile water. Mice were euthanized via CO_2 exposure and cervical dislocation prior to dissection, in accordance with ethical approval of the University of Adelaide Animal Ethics Committee (M-2019-020) and the University of Adelaide Institutional Biosafety Committee (IBC approval number 14254). The study complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2014).

Breeding strategy and Genetic Confirmation

The intestinal epithelial conditional TLR4 KO C57BL/6 (*Tlr4*$^{\Delta IEC}$) mouse model was created by following a transgenic *Vil1-cre/Tlr4loxP* breeding strategy (The Jackson Laboratory, Maine, USA). By crossing a homozygous *Tlr4loxP*/*Vil1-cre* WT with a homozygous *Tlr4loxP*/hemizygous *Vil1-cre*, this breeding strategy resulted in 1 in 2 offspring being the desired conditional KO. Conditional KO of TLR4 was confirmed via polymerase chain reaction (PCR) analysis, as per protocols provided by The Jackson Laboratory (Maine, USA) (Madison et al. 2002), for *TLR4flox* (Laboratory) and *Vilcre* genes (Laboratory), where DNA was extracted from mouse ear notches using the Nucleospin Tissue DNA extraction kit, and used at a working concentration of 20 ng/µL (Machery-Nagel, Duren, Germany). Primer sequences used for confirmation of genotype were as follows: Vil-cre forward GCTTTCAAGTTTCATCCATGTTG, Vil-cre WT reverse TTCATGATAGACAGATGAACACAGT, Vil-cre mutant reverse GTCTTTGGGTAAAGCCAAGC, TLR4 floxed forward TGACCACCCATATTGCCTATAC, and TLR4 floxed reverse TGATGGTGTGAGCAGGAGAG. Cycling conditions for Vil-cre were as follows: denaturing at 95 °C for 3 minutes, then 95 °C for 5 seconds, then 60 °C for 30 seconds. The final two steps were repeated for 40 cycles. The mutant band, representing presence of hemizygous Vil-cre was at 85 base pairs, compared to WT Vil-cre at 119 base pairs. Cycling conditions for TLR4 flox were 94 °C for 2 minutes, then the following steps being repeated for 10 cycles: 94 °C for 20 seconds, 65 °C for 15 seconds (decreasing by 0.5 °C each cycle), 68 °C for 10 seconds. Following from this, samples were cycled 28 times at 94 °C for 16 seconds, 60 °C for 15 seconds then 72 °C for 10 seconds. The final step was 2 minutes at 72 °C. Homozygous TLR4 floxed samples produced a band of 285 base pairs, heterozygous samples

were at 234 base pairs and 285 base pairs, and WT TLR4 produces a band at 234 base pairs. For visualisations, samples were run in 4 % agarose and visualised using Midori Green Advance DNA stain (Nippon Genetics, Japan). Conditional knockout of intestinal epithelial TLR4 (*Tlr4*$^{\Delta IEC}$) resulted in PCR showing mutant Vil-cre (85 base pairs) and homozygous TLR4 floxed (285 base pairs).

Further confirmation of successful knockout was conducted by real-time PCR where small intestinal tissue for both WT and *Tlr4*$^{\Delta IEC}$ was harvested and scraped to separate the submucosal layer for epithelial specific TLR4 analyses. For both strains, the whole small intestine was dissected and opened longitudinally. Using a rounded scalpel, a light feather-like scraping was undertaken to separate out an epithelial-dominant sample. Ten nanograms of RNA extracted from these scrapings for both WT and *Tlr4*$^{\Delta IEC}$ mice was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, New South Wales, Australia; #1708890) as per manufacturer's instructions. RT-PCR was performed using the Rotor-Gene 3000 (Corbett Research Sydney, Australia). Amplification mixes contained 1 µL of cDNA sample (100 ng/µL), 5 µL of SYBR green fluorescence dye (QuantiTect Qiagen, Hilden, Germany), 3 µL of RNase-free water (Macherey Nagal, Duren, Germany), and 0.5 µL of each forward and reverse primers (50 pmol/µL), to make a total reaction volume of 10 µL. Primer details were as presented in table 3.1.

Table 3.1: Real-time PCR primer sequences for TLR4 and β-actin.

	Forward Primer	Reverse Primer
TLR4 (Merck)	5'-CTCTGCCTTCACTACAGAGAC-3' T_m 58.3 °C	5'-TGGATGATGTTGGCAGCAATG-3' T_m 69.1 °C
β-actin (Integrated DNA Technologies)	5'-CTCTTCCAGCCTTCCTTCCT-3' T_m 56.4 °C	5'-AGCACTGTGTTGGCGTACAG-3' T_m 57.9 °C

Thermal cycling conditions were: 95 °C for 10 minutes; 40 cycles of 95 °C for 10 seconds, 59 °C for 30 seconds and 72 °C for 45 seconds and a final melt step of 60 - 95°C changing 1 °C per step, holding for 5 seconds each. Samples were run in triplicate, including negative controls (no cDNA template). Experimental threshold (CT) values were calculated by the Rotor Gene 6 programme. CT values were used to quantify relative mRNA expression of TLR4 and β-actin using the ΔC_T method, where relative expression = $2^{-(CT\ TLR4 - CT\ \beta\text{-actin})}$ (Wardill, Bowen, et al. 2016).

Ex vivo electrophysical assessments

Ussing chambers (EM-CSYS-8 with DM-MC8 voltage clamp/electrode input, Physiological Instruments, San Diego, USA) were used to assess intestinal electrophysiology in WT and *Tlr4*$^{\Delta IEC}$ mice as previously described (Wardill et al. 2016). Briefly, segments of ileum and colon were dissected from mice and flushed with ice-cold 1 x phosphate-buffered saline (PBS). One cm segments were opened longitudinally along the mesenteric attachment line, mounted into 0.1 cm^2 aperture sliders (Physiologic Instruments; P2303A, San Diego, USA) and inserted into chambers filled with a glucose-fortified Ringers solution consisting of (in mM): NaCl 115.4; KCl 5; $MgCl_2$ 1.2; NaH_2PO_4 0.6; $NaHCO_3$ 25; $CaCl_2$ 1.2; and glucose 10, bubbled with carbogen gas (95% O_2, 5% CO_2) and warmed to 37 °C (Wardill, Bowen, et al. 2016). Ileal segments had the mucosal side bathed in mannitol-fortified Ringers (10mM) to maintain osmotic balance. Once mounted, tissue was voltage-clamped to zero potential difference, establishing baseline readings. Tissue was allowed to equilibrate for 20 minutes before short circuit current (Isc, marker of net ion transport/secretion) and transepithelial electrical resistance (marker of barrier integrity) were measured using Acquire and Analyse Revision II (Physiologic Instruments; San Diego, USA, v2.3).

Histopathological analyses:

Hematoxylin and Eosin (H&E) Staining

Mouse ileum and colon samples were fixed in 10% formalin and embedded into paraffin wax blocks. Formalin-fixed paraffin embedded blocks were sectioned (4 µm) and mounted on SuperFrost White slides (Menzel-Gläser, Braunschweig, Germany). Slides were then fixed on a 37 °C heat block, for a minimum of 1 hour. Standard H&E staining procedures were followed (Wardill et al. 2016). In brief, slides were dewaxed in 3 x washes in 100 % histolene for 5 minutes each and then rehydrated with graded ethanol as previously described (Wardill et al. 2016). Slides were then stained in Lille-Mayers hematoxylin for 5 minutes and rinsed until clear in running tap water. Slides were then quickly dipped twice in 1 % acid alcohol (5 mL HCl + 500 mL 70 % ethanol) and washed in running tap water until clear. Tissue was then placed in Scott's Tap Water (in mM) ($MgSO_4$ 166.2; $NaHCO_3$ 23.7 in 1 L dH_20) for 2 minutes and washed. Counterstaining with alcoholic eosin (Sigma-Aldrich, St Louis USA) occurred for 2 minutes and slides were washed with running tap water until clear. Slides were then treated with 90 % ethanol (30 seconds) and 100 % ethanol (30 seconds) and finally cleared with 3x washes in 100 % histolene for 5 minutes each. Slides were coverslipped with D.P.X neutral mounting medium (Sigma-Aldrich, St Louis USA).

Slides were imaged using an Olympus BX51 light microscope and Olympus DP20 microscope camera (Olympus, Tokyo, Japan). A modified version of well-established intestinal injury scoring criteria (Howarth G S et al. 1996) was used to quantify possible differences between groups, with 1 representing the presence and 0 representing the absence of the pathophysiological marker. The criteria included: 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; oedema; villous fusion; and villous atrophy. The latter two criteria were not assessed in colon samples, therefore the total possible score for ileum samples was 8 and colon samples was 6. Furthermore, ileum villus height and crypt depth, and colonic mucosal thickness was assessed by a blinded researcher (EEB) using the Olympus cellSens Standard imaging program (Olympus, Tokyo, Japan).

Alcian Blue and Periodic acid - Schiff (AB-PAS stain)

AB-PAS was used to quantify goblet cells in the mucosal of the ileum and colon from formalin-fixed paraffin-embedded tissue blocks. Tissue was cut into 4 µm sections and placed on SuperFrost White slides (Menzel-Gläser, Braunschweig, Germany). Slides were dewaxed in 3x washes in 100% histolene for 5 minutes each and then rehydrated with graded ethanol. Slides were stained in alcian blue for 5 minutes (194 mL dH_2O + 6 mL 100% acetic acid + 2 g Alcian Blue) before being incubated in 0.5% periodic acid for 5 minutes. Slides were again washed with dH_20 and then incubated in Schiff's reagent for 15 minutes before being counterstained with haematoxylin for 30 seconds. Slides were cleared in 100 % histolene (3x 5 min), and coverslipped with D.P.X neutral mounting medium. Slides were imaged using the Nanozoomer Digital slide scanner, with goblet cell counts conducted by a blinded researcher (KRS).

Immunofluorescence of tight junction proteins and immune cells

To investigate molecular determinants of barrier function, immunofluorescence for ZO-1 and occludin was performed. Briefly, the intestine was removed and immediately flushed with ice-cold 1 x PBS. Segments of ileum and colon were fixed in 10% neutral buffered formalin, processed and embedded into paraffin wax. Tissues were then cut into 4 µm sections and placed onto FLEX IHC microscope

slides (Flex Plus Detection System, Dako; #K8020) and heated on a heat pad. Slides were deparaffinised via 3 x washes with 100% histolene and rehydrated with graded ethanol (100 % ethanol for 30 seconds, 90 % ethanol for 30 seconds and 70 % ethanol for 30 seconds) (Wardill, Bowen, et al. 2016). Antigen retrieval was via the PT Link bath (pre-treatment module; Dako; #PT101) using an EDTA/Tris buffer consisting of (in mM): Tris 9.9; and EDTA 1.3; and 0.5 mL Tween 20 in 1.5 L dH_20, pH = 9 at 97 °C for 20 minutes.

Tissue samples were stained using the DakoCytomation Autostainer (AutostainerPlusTM, Dako, Næstved, Denmark, serial number: AS1271F1104). The primary antibodies used were as follows: ZO-1 (Invitrogen 61-7300, California, USA, 0.25 mg/mL, 1:100 dilution); occludin (Invitrogen 33-1500, 0.5 mg/mL, 1:200 dilution); and CD11b (Abcam ab133357, Cambridge, UK, 1:1000 dilution). Primary antibody was diluted in 5% normal horse serum (NHS) (Sigma-Aldrich, St Louis USA), 1 x PBS for tight junction analyses and 1% bovine serum albumin (BSA) for immune cell analyses (Sigma-Aldrich, St Louis USA). The secondary antibodies were AlexaFluor 488 anti-mouse (occludin) AlexaFluor 488 anti-rabbit (CD11b) and AlexaFluor 568 anti-rabbit (ZO-1) (Thermo Fisher, Massachusetts USA). The secondary antibody was diluted in 1 x PBS, 1% BSA and 2% fetal bovine serum. DAPI (1 µg/mL) (Sigma-Aldrich, St Louis USA) counter staining was utilised to visualize the nucleus of cells in sample, with 1 x PBS as the diluent. A protein block of 10% NHS for tight junctions and 4% BSA for immune cells was used to reduce non-specific antibody binding during the procedure.

Post-staining, a drop of Fluoroshield (Sigma-Aldrich, St Louis USA) was applied to each slide and coverslipped. Slides were then stored in the dark at 4°C to await imaging. Slides were imaged using the Nikon A1 Confocal Microscope using a 40x

objective. Fluorescent staining was quantified via % area stain on the Fiji Image J program as previously described (Abramoff, Magalhaes & Ram 2004).

Statistics

All data were compared using Prism version 8.0 (GraphPad Software, San Diego, USA). Data were first assessed was for normality using the Shapiro-Wilk test. Parametric data was analysed using a one-way ANOVA or t-test and presented as mean ± standard error of mean (SEM). Non-parametric data was analysed using a Kruskal-Wallis test and presented as median and range. *p* values lower than 0.05 were deemed significant.

Results

Epithelial TLR4 does not control intestinal barrier function, tight junction integrity or immune cell infiltration in healthy mice

Successful conditional knockout of TLR4 on intestinal epithelial cells was confirmed by both genotyping and RT-PCR analysis, with 8-fold higher TLR4 expression in WT intestinal epithelial-dominant scrapings compared to $Tlr4^{\Delta IEC}$ scrapings (mean ± SEM): WT 0.29 ± 0.02 vs. $Tlr4^{\Delta IEC}$ 0.04 ± 0.02, $p = 0.012$.

No difference between WT and $Tlr4^{\Delta IEC}$ was observed in ileum or colon in baseline short-circuit current: ileum (mean ± SEM): WT 113.3 ± 69.8 vs $Tlr4^{\Delta IEC}$ 75.3 ± 34.9 mA p = 0.607, colon: WT 28.4 ± 21.1 vs $Tlr4^{\Delta IEC}$ 36.7 ± 15.5 mA $p = 0.752$ (Figure. 3.1A and B). Similarly, there was no difference in baseline transepithelial tissue resistance: ileum (mean ± SEM): WT 22 ± 7.2 vs $Tlr4^{\Delta IEC}$ 20 ± 5.6 Ωcm^2, $p = 0.831$, colon WT 30.8 ± 3.6 vs $Tlr4^{\Delta IEC}$ 45.1 ± 9.5 Ωcm^2, $p = 0.191$ (Figure. 3.1C and 3.1D).

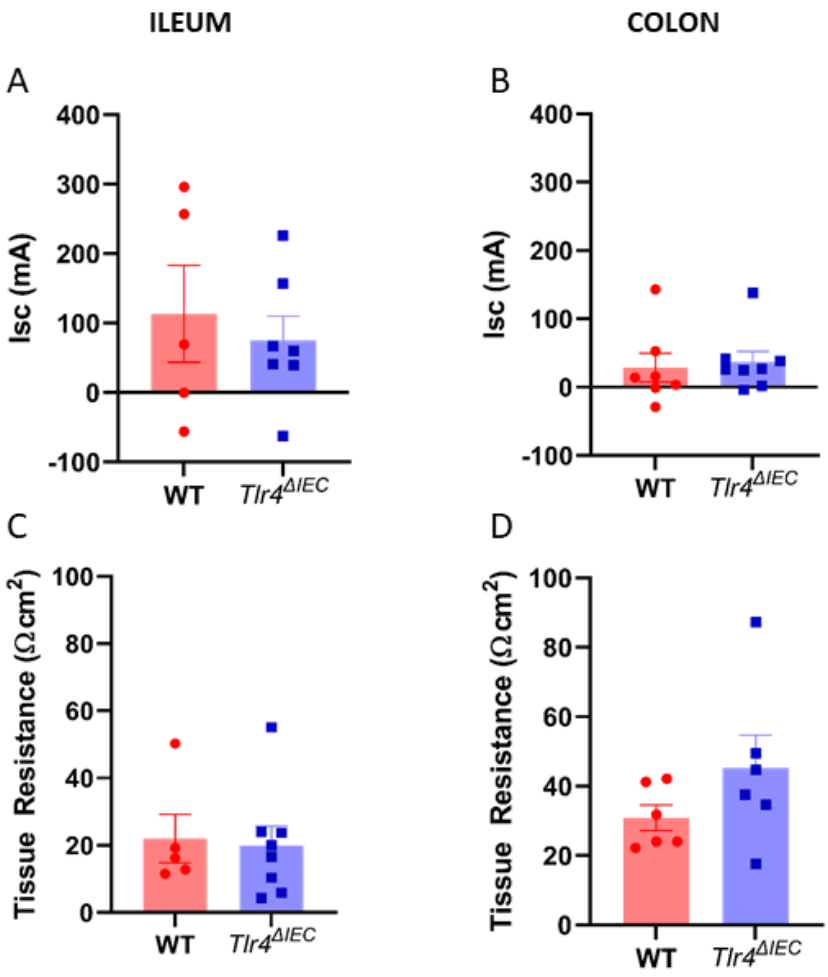

Figure 3.1: Intestinal electrophysiology is not dependent on TLR4 expression. Baseline short-circuit current (Isc, mA) for ileum (A) and colon (B), and transepithelial tissue resistance (Ωcm^2) for ileum (C) and colon (D) samples in WT and *Tlr4*$^{\Delta IEC}$ mice. No difference between groups (WT n = 5-7 and *Tlr4*$^{\Delta IEC}$ n = 6-8, $p > 0.05$). Data presented as mean ± SEM.

ZO-1 and occludin staining was evident at the apicolateral border of epithelial cells of villous and crypt structures of the ileum and colon (Figure. 3.2 and 3.3). Quantification of tight junction protein staining (% area stain) showed no differences for ZO-1: ileum (mean ± SEM) WT 1.49 ± 0.155 vs *Tlr4*$^{\Delta IEC}$ 1.17 ± 0.07 $p = 0.09$; colon WT 1.36 ± 0.26 vs *Tlr4*$^{\Delta IEC}$ 1.12 ± 0.18 $p = 0.47$. Similarly, there was no difference in occludin expression: ileum (mean ± SEM) WT 1.07 ± 0.12 vs *Tlr4*$^{\Delta IEC}$ 0.95 ± 0.13 $p = 0.53$; colon WT 1.26 ± 0.26 vs *Tlr4*$^{\Delta IEC}$ 1.02 ± 0.16 $p = 0.45$. Positive CD11b staining (Figure. 3.4) was evident in both ileum mucosa and colon mucosa and sub mucosa. CD11b positive immune cells in ileum were mildly decreased in WT mice: (% area stain, mean ± SEM) WT 0.14 ± 0.02 vs *Tlr4*$^{\Delta IEC}$ 0.09 ± 0.01 $p = 0.04$. However, there were no differences in CD11b positive immune cells in the colon between strains: (% area stain, mean ± SEM) WT 0.53 ± 0.08 vs *Tlr4*$^{\Delta IEC}$ 0.49 ± 0.08, $p = 0.73$.

Epithelial TLR4 deletion does not affect intestinal morphometry

No histological differences were observed between WT and *Tlr4*$^{\Delta IEC}$ mice in ileum or colon (Figure. 3.5A-D), with no change in villus height ($p = 0.49$), ileum crypt depth ($p = 0.66$) or colonic crypt depth ($p = 0.52$) (Figure. 3.5 E-G, $p > 0.05$). Furthermore, to ensure a comprehensive and translational assessment of intestinal structure, a tissue injury score was assessed. No evidence of microscopic injury was detected in either WT or *Tlr4*$^{\Delta IEC}$ ileum or colon tissue (ileum $p = 0.617$, colon $p = 0.529$). Finally, no difference in goblet cell abundance in ileum villi, ileum crypt and colon crypt between WT and *Tlr4*$^{\Delta IEC}$ was observed (Figure. 3.6A-G, $p > 0.05$ for all groups).

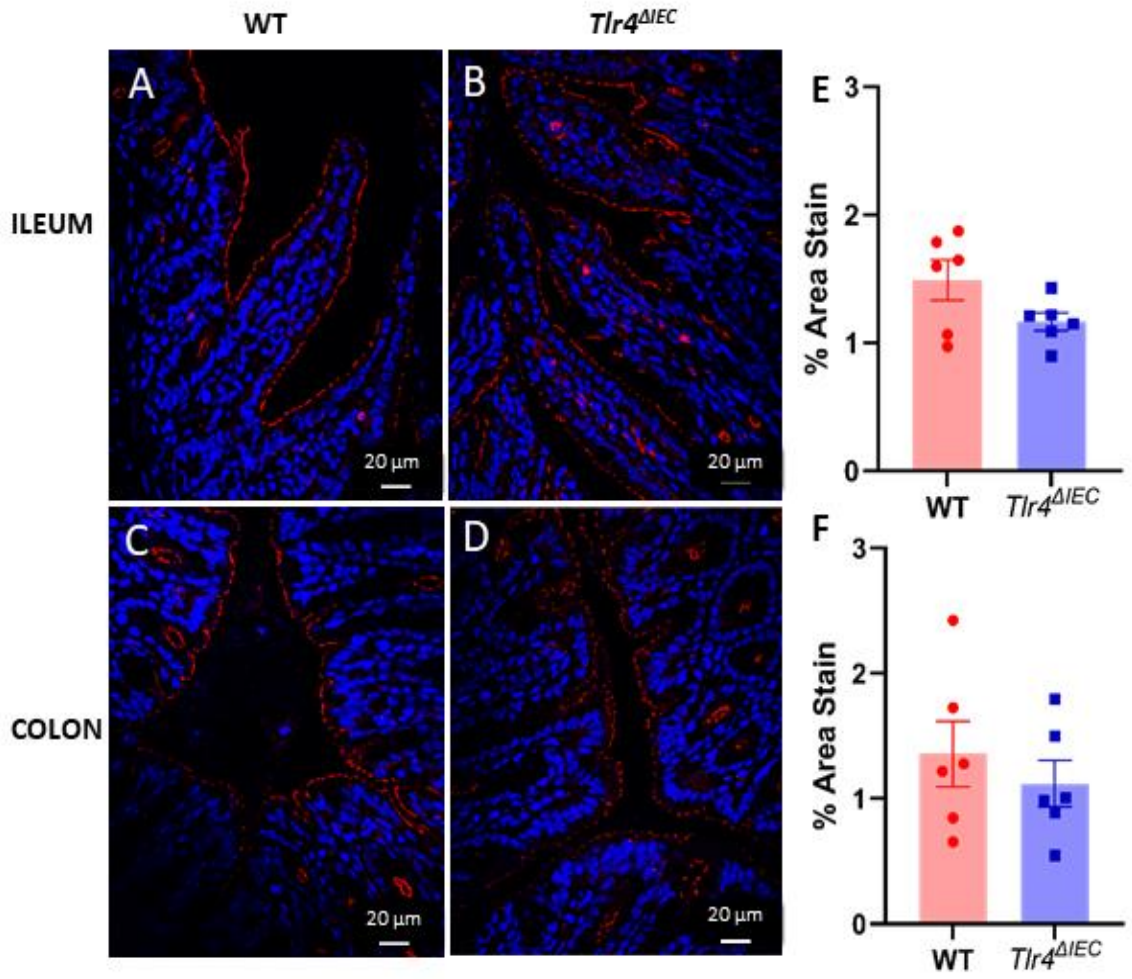

Figure. 3.2: Epithelial TLR4 deletion does not influence ZO-1 expression. ZO-1 expression (red) in WT and *Tlr4*$^{\Delta IEC}$ mice, ileum and colon with DAPI (blue) counterstain of nuclei; (A) WT ileum, (B) *Tlr4*$^{\Delta IEC}$ ileum, (C) WT colon, (D) *Tlr4*$^{\Delta IEC}$ colon, 40x magnification. No difference in ZO-1 expression for either (E) ileum or (F) colon (% area stain, n = 6, $p > 0.05$). Data presented as mean ± SEM.

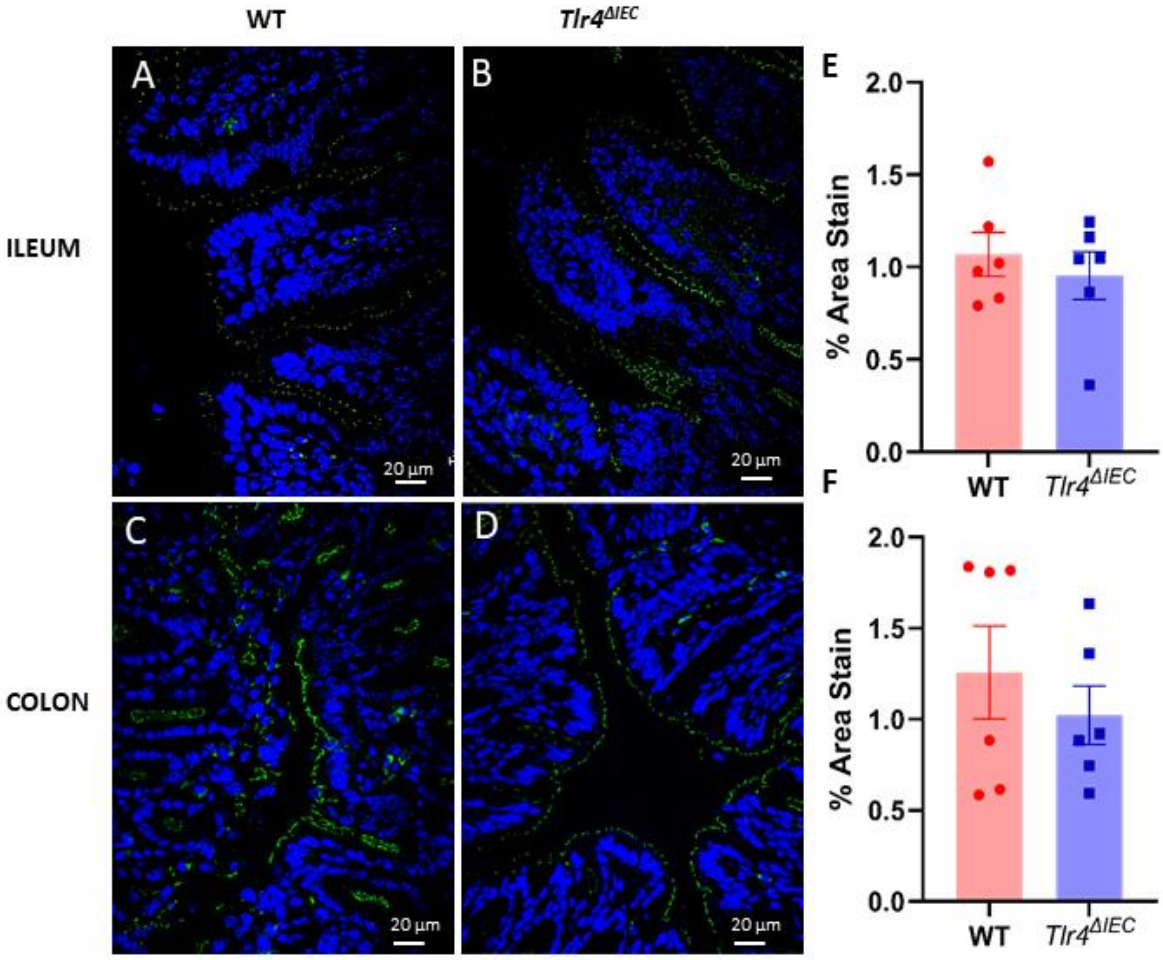

Figure. 3.3: Occludin expression is independent of epithelial TLR4 expression.

Occludin expression (green) in WT and *Tlr4*$^{\Delta IEC}$ mice, ileum and colon with DAPI (blue) counterstain of nuclei; (A) WT ileum, (B) *Tlr4*$^{\Delta IEC}$ ileum, (C) WT colon, (D) *Tlr4*$^{\Delta IEC}$ colon, 40x magnification. No difference in occludin expression for either (E) ileum or (F) colon (% area stain, n = 6, $p > 0.05$). Data presented as mean ± SEM.

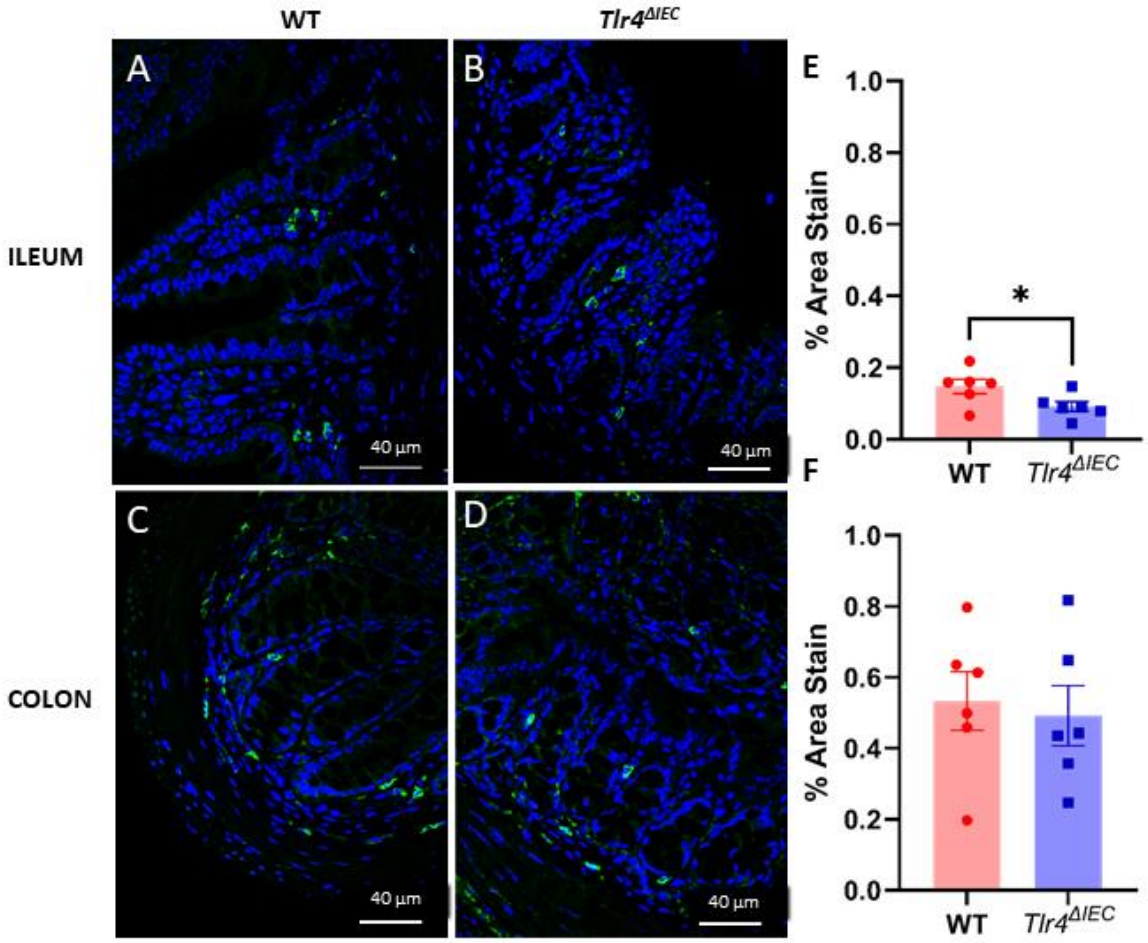

Figure. 3.4: Immune cell infiltration does not depend on epithelial TLR4 expression. CD11b expression (green) in WT and *Tlr4*$^{\Delta IEC}$ mice, ileum and colon with DAPI (blue) counterstain of nuclei; (A) WT ileum, (B) *Tlr4*$^{\Delta IEC}$ ileum, (C) WT colon, (D) *Tlr4*$^{\Delta IEC}$ colon, 40x magnification. Difference in CD11b immune cells in (E) ileum (* $p = 0.04$), no difference in (F) colon (% area stain, n = 6, $p > 0.05$). Data presented as mean ± SEM.

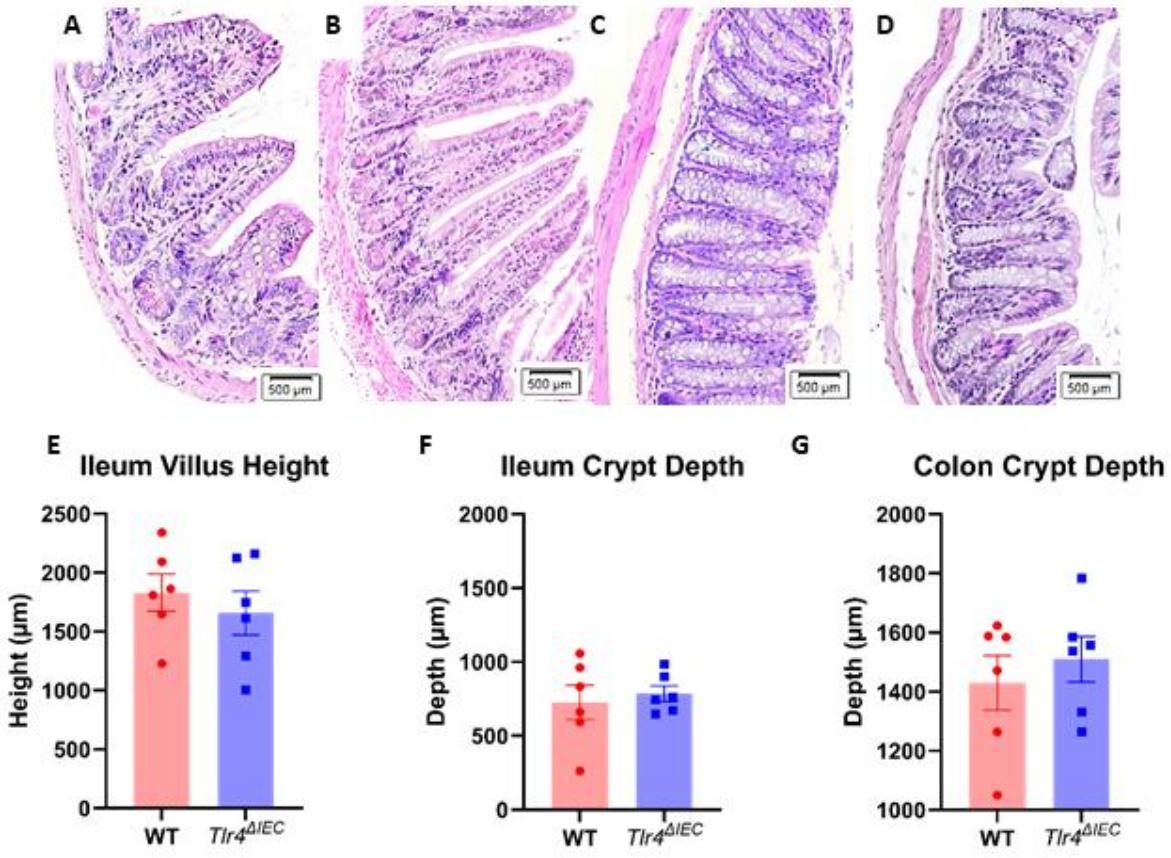

Figure. 3.5: Epithelial TLR4 deletion does not affect intestinal architecture. H&E stain of ileum and colon from WT and *Tlr4$^{\Delta IEC}$* mice. (A) WT ileum, (B) *Tlr4$^{\Delta IEC}$* ileum, (C) WT colon, (D) Tlr4$^{\Delta IEC}$ colon, 20x magnification. No difference in ileum villus height (E, µm), ileum crypt depth (F, µm) or colon crypt depth (G, µm) ($n = 6$, $p > 0.05$). Data presented as mean ± SEM.

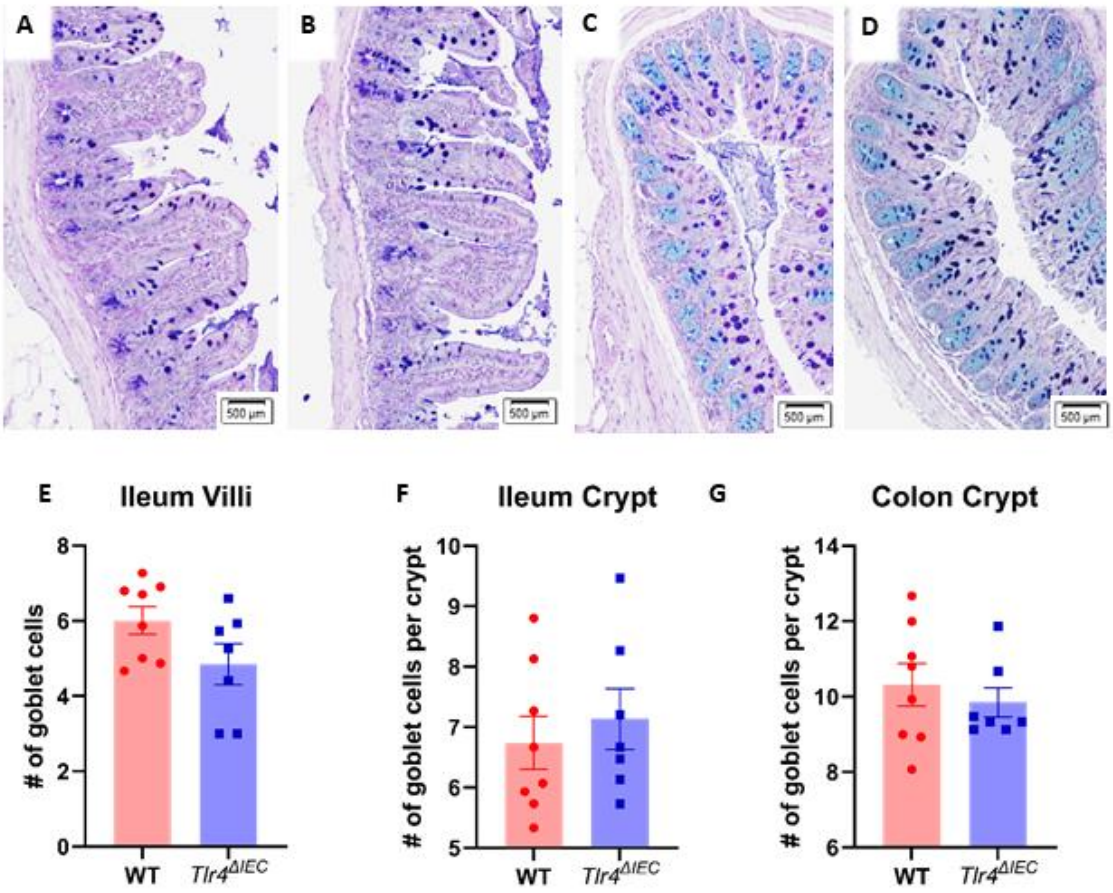

Figure. 3.6: Goblet cell abundance is not affected by TLR4 expression in the intestinal epithelium. AB-PAS stain of ileum and colon from WT and *Tlr4*$^{\Delta IEC}$ mice to visualize goblet cells (dark purple/blue stain). (A) WT ileum, (B) *Tlr4*$^{\Delta IEC}$ ileum, (C) WT colon, (D) *Tlr4*$^{\Delta IEC}$ colon, 20x magnification. No differences in goblet cells in ileum villi (E), ileum crypt (F) or colon crypts (G) (# of goblet cells per structure, WT n = 8, *Tlr4*$^{\Delta IEC}$ n = 7, $p > 0.05$). Data presented as mean ± SEM

Discussion/Conclusion

TLR4 has received a significant amount of attention for its homeostatic control and therapeutic applications based on its assumed regulation of mucosal barrier function. This is the first study to present the baseline intestinal characteristics of epithelial-specific TLR4 KO mice (*Tlr4*$^{\Delta IEC}$) and has found that contrary to expectations, the absence of epithelial TLR4 did not alter intestinal homeostasis. These data highlight the importance of site-specific TLR4 investigation and underscore the limitations of extrapolating evidence from disease models to healthy states.

The concept that TLR4 is involved in homeostatic control is clearly outlined in studies investigating the nervous control of intestinal tissue (Forcen et al. 2015; Caputi et al. 2017) and immune tolerance (Lu, Sodhi & Hackam 2014). However, TLR4 involvement in homeostatic control of barrier function has been inferred from disease modelling studies. Shi, Hu and colleagues (2019) found that intestinal injury in response to dextran sulfate sodium induced colitis, was significantly aggravated in a global TLR4 KO mouse model (Shi et al. 2019). This suggests TLR4 expression is a protective component of the intestines and supports the healthy functioning of the intestinal barrier (Shi et al. 2019), however, this is contradictory to earlier findings which show that TLR4 over-expression leads to impaired intestinal epithelial cell differentiation and barrier dysfunction (Dheer et al. 2016). Since not all studies distinguish between site-specific expression of TLR4, contradicting evidence is expected. Our study is one of the first to entirely focus on how intestinal epithelial TLR4 expression influences the healthy state of the intestines.

As outlined, our data suggest that epithelial TLR4 is not essential to the regulation of the intestinal environment, namely the role of tight junction protein expression, goblet cell populations and functional tissue permeability in healthy development. While minor non-significant differences have been noted between groups, this is most likely

due to stochastic variation. Previous research conducted by our group has demonstrated intestinal permeability and morphology changes in a positive control of epithelial tissue disruption. In mice treated with the chemotherapy, irinotecan, baseline intestinal conductance (a measure of intestinal permeability, the opposite of tissue resistance) was significantly increased (53.19 ± 6.46 S/cm2, +105.62% relative to WT controls; p = 0.0008) (Wardill et al. 2016). Furthermore, irinotecan-treated small intestinal and colonic tissue showed severe damage, including villous blunting and crypt degeneration (Wardill et al. 2014). There is a substantial difference between these positive control outcomes and the minor changes in the present study, therefore suggesting that current minor variability in the *Tlr4*$^{\Delta IEC}$ versus WT data is inconsequential. Our current findings indicate that there may be compensatory mechanisms controlling the gastrointestinal microenvironment in the absence of epithelial TLR4. A possible mechanism could be that immune TLR4 is responsible for modulating barrier function and intestinal homeostasis. This aligns with the higher TLR4 expression on immune cell populations and acknowledges the profound immune infiltrate of the gut (Gourbeyre et al. 2015). TLR4 is expressed on a range of immune cells including, macrophages, myeloid cells and dendritic cells (Vaure & Liu 2014), and has proven roles in dendritic cell maturation (Pufnock et al. 2011) and immune tolerance (Salazar et al. 2017). Considering that immune TLR4 has been shown to control immune system functioning and development of the healthy microbiome, it could be also deduced that immune TLR4 aids in controlling intestinal permeability and barrier function in mice. This notion is supported by our data, which showed no difference in intestinal characteristics between the *Tlr4*$^{\Delta IEC}$ and WT mice. To confirm this role of exclusive immune TLR4 signalling, future work could be conducted in conditional mice where there is a deletion of immune TLR4 expression.

An alternative mechanism possibly responsible for this compensation could include the recognition of pathogens and tolerance of the commensal microbiota via different TLRs, notably TLR2 (Abreu 2010). Upon ligand binding, TLR2 activates the MyD88-dependent inflammatory pathway (Mukherjee, Karmakar & Babu 2016). This pathway is also activated in response to TLR4 activation, therefore a distinct overlap in TLR4 and TLR2 signalling exists (Mukherjee, Karmakar & Babu 2016). The similarity between TLR2 and TLR4 is best shown in healthy states, with an early study finding that activation of TLR2 and TLR4 primes dendritic cell tolerance to commensal organisms (Albrecht et al. 2008). Furthermore, the combination of TLR2 and TLR4 signalling is indicated in the healthy control of spontaneous and serotonin-induced contractile responses of mouse ileum (Forcen et al. 2015). It is the commonality between TLR2 and TLR4 signalling pathways, which may explain why intestinal homeostasis was maintained in our *Tlr4*$^{\Delta IEC}$ model. Previous research has investigated disruption of TLR pathways, including TLR2 knockout, not MD-2, and have shown in intestinal models of chemotherapy-induced intestinal mucositis that deletion of TLR2 alone increased intestinal inflammation and damage, suggesting TLR2 is a potential therapeutic target (Frank et al. 2015). While this evidence indicates importance of TLR2, rather than TLR4, in intestinal regulation, unfortunately epithelial deletion of TLR2 has not been previously studied in either healthy or diseased states. Therefore, future research could be centred on a TLR2 epithelial-specific knockout mouse model, to further investigate this complex relationship between different TLR expression and intestinal function.

Overall, our findings support the use of this *Tlr4*$^{\Delta IEC}$ mouse line in the investigation of gastrointestinal disease where TLR4 may be of interest. These mice showed no difference in baseline intestinal characteristics compared to WT, therefore displaying no inherent variability of intestinal function caused by genetic modification of

intestinal epithelial TLR4. This is a promising sign for the ongoing viability of this model, as the retention of normal intestinal function suggests that the *Tlr4*$^{\Delta IEC}$ model is reliable. This could allow for future disease models in the *Tlr4*$^{\Delta IEC}$ mice to dissect the contribution of epithelial TLR4 to disease development. Translationally, the use of these *Tlr4*$^{\Delta IEC}$ mice in models of gastrointestinal disease will provide much greater insight into the site-specific contribution of TLR4. This would allow for the guiding of future therapeutics, including nanoparticle delivery systems allowing epithelial TLR4 to be augmented in a manner that prevents any systemic effect (de Groot et al. 2018). This could possibly include TLR4 agonist or antagonist delivery to the site-specific area, meaning that only the intestinal epithelial population of TLR4 would be altered, leaving immune and nervous TLR4 functioning uninterrupted. This is especially important where site-specific TLR4 expression shows distinct and potentially contradicting mechanisms, for example following cancer treatments (Wardill et al. 2016; Coller et al. 2017). However, while these results shed further insight into the mechanistic roles of epithelial TLR4, they should be approached with caution as this study utilised a small sample size where further functional data, such as metabolic and absorptive capacity, was not assessed. A further limitation of the current study is the exclusion of other related knockout models, such as immune-specific TLR4 or TLR2 knockout. As data presented in the *Tlr4*$^{\Delta IEC}$ mice revealed no differences in intestinal functioning, it can be deduced that epithelial TLR4 is likely to have a minor role in intestinal homeostasis. Consequently, future research including these alternative knockout models would greatly enhance this field of knowledge.

In conclusion, TLR4 is an important immunosurveillance protein to many areas of current medical research, including inflammatory gastrointestinal diseases and CIGT (Belmonte et al. 2012; Wardill et al. 2016). While there is a large body of research surrounding the dual roles of TLR4 in both healthy states and disease, currently there

is very little distinction of cell-specificity in research outcomes. This oversight has the potential to influence the translation of results to clinical practice. To facilitate the emergence of research that considers cell-specific TLR4 expression, a well-validated intestinal epithelial TLR4 conditional mouse model (*Tlr4*$^{\Delta IEC}$) must exist. The current study verified that *Tlr4*$^{\Delta IEC}$ mice are not fundamentally altered prior to future disease modeling studies. These results both support the use of this model in future studies and has presented novel insights into the role of intestinal epithelial TLR4 in homeostatic control.

Chapter 4: Intestinal Epithelial TLR4 Controls Severity of Chemotherapy-Induced Diarrhoea

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This chapter is my second original research chapter and details the characterisation of chemotherapy-induced gastrointestinal toxicity (CIGT) in intestinal epithelial TLR4 knockout (KO) mice (*Tlr4*$^{\Delta IEC}$) versus wild-type (WT) littermates. This chapter is written in publication-style format, but has not yet been submitted.

Statement of Authorship

Title of Paper	Intestinal Epithelial TLR4 Controls Severity of Chemotherapy-Induced Diarrhoea
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Details the characterisation of chemotherapy-induced gastrointestinal toxicity (CIGT) in intestinal epithelial TLR4 knockout (KO) mice (*Tlr4*$^{\Delta IEC}$) versus wild-type (WT) littermates. This chapter is written in publication-style format, but has not yet been submitted.

Principal Author

Name of Principal Author (Candidate)	Mrs. Elise Ellen Crame		
Contribution to the Paper	Developed research question and proposal. Conducted *in-vivo* animal work and molecular benchtop experimental work. Primary author of manuscript style chapter, responsible for writing manuscript, data analysis and creation of all figures.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/10/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor Joanne M. Bowen		
Contribution to the Paper	Provided crucial feedback and significantly helped forming research approach. 5%		
Signature		Date	12/10/2022

Name of Co-Author	Dr. Janet K. Coller		
Contribution to the Paper	Provided crucial feedback and helped to shape research. 5%		
Signature		Date	12/10/2022

Name of Co-Author	Dr. Hannah R. Wardill		
Contribution to the Paper	Provided crucial feedback and helped in the formation of the research question and experimental approach. 5%		
Signature		Date	12/10/2022

Abstract

Irinotecan, a commonly used chemotherapy agent, is known to cause severe gastrointestinal (GI) toxicity that is dependent on activation of the immune receptor, toll-like receptor 4 (TLR4). While previous research shows that global TLR4 knockout (KO) (*Tlr4*$^{-/-}$) mice are protected from irinotecan-induced GI toxicity, it is unknown if chemotherapy-induced gastrointestinal toxicity (CIGT) development is dependent on TLR4 signalling expressed on epithelial or immune cells. This study therefore aimed to determine the effect of epithelial-specific TLR4 on irinotecan-induced GI toxicity using a novel transgenic mouse model. Male and female conditional (intestinal epithelial) TLR4 KO (*Tlr4*$^{\Delta IEC}$) mice and wild-type (WT) littermates, bearing subcutaneous colorectal tumours received a single intraperitoneal dose of irinotecan (270 mg/kg) or vehicle (n = 6-8/group). GI toxicity was assessed over 72 hrs via evaluation of body weight and diarrhoea severity. At termination (72 hrs), functional analysis of colon permeability using Ussing chambers, histological analysis of the ileum and colon, and immunofluorescence for tight junction proteins (occludin and ZO-1) and CD11b^{+} immune cells was performed. Irinotecan caused moderate GI toxicity characterised by weight loss and diarrhoea in both *Tlr4*$^{\Delta IEC}$ and WT mice. *Tlr4*$^{\Delta IEC}$ mice were protected from high-grade diarrhoea compared to WT 24 hrs after irinotecan ($P < 0.0001$) but were not protected from weight loss at any time point. Irinotecan treatment resulted in significant disruption to ileum morphology in both WT and *Tlr4*$^{\Delta IEC}$ mice compared to vehicle (*Tlr4*$^{\Delta IEC}$ $P = 0.0020$, WT $P = 0.0033$). No difference in colon tissue resistance, tight junction protein expression or CD11b^{+} immune cell expression was observed between all groups. Our findings confirm a role for intestinal epithelial TLR4 in dictating specific consequences of irinotecan treatment, however, the lack of total protection against symptoms may suggest that

multiple TLR4 expression sites are required to control irinotecan-induced GI toxicities.

Introduction

Gastrointestinal (GI) toxicity is a common and challenging consequence of chemotherapy reflecting the non-selective and highly cytotoxic nature of these drugs (Blijlevens 2005; Richardson & Dobish 2007). Characterised by breakdown of the mucosal lining, chemotherapy-induced gastrointestinal toxicity (CIGT) is initiated by direct cytotoxic injury and perpetuated by inflammation, resulting in ulcerative and atrophic changes that impact GI function (Pott & Hornef 2012; Wardill, Bowen & Gibson 2012). The consequence of this impaired function is a constellation of GI symptoms including, diarrhoea, pain, and bleeding, which predispose to infection, dehydration, and malnutrition and weight loss (Gibson et al. 2007). With limited interventions available for CIGT, it is often managed through dose-reductions, delays or complete discontinuation of treatment which inevitably impact treatment efficacy and survival (Andreyev et al. 2014).

The chemotherapeutic agent, irinotecan, is known to cause GI toxicity through a range of increasingly sophisticated mechanisms, in particular its enterohepatic recirculation which sees the active metabolite of irinotecan (7-ethyl-10-hydroxycamptothecin, otherwise known as SN-38) directly excreted into the GI lumen (Chabot 1997). SN-38 is a potent topoisomerase I inhibitor which decreases tumour burden via irreparable DNA damage during the S-phase of the cell replication cycle (Kawato et al. 1991; Chabot 1997). SN-38 is approximately 1000x more potent than irinotecan and highly mucotoxic when exposed to the GI mucosa, where it causes profound tissue destruction characterised by intestinal barrier dysfunction (i.e. hyper-permeability), disruption of the GI microbiota and activation of the host immune system (Wardill et al. 2014). The highly toxic nature of irinotecan is a major challenge in supportive oncology, with treatment schedules that include irinotecan associated

with a 3-fold higher rate of premature death compared to protocols not containing irinotecan (Rothenberg et al. 2001).

Although still under investigation, one distinctive feature of irinotecan treatment is disrupted microbiota associated with diarrhoea (Secombe et al. 2022). The notion that irinotecan treatment disrupts the microbiota composition is well characterised across both pre-clinical and clinical studies, with results showing increased abundance of pathogenic microbes belonging to the Proteobacteria phyla (Wang et al. 2019; Shuwen et al. 2020). Expansion of these pathogenic microbes results in a higher production of danger signals, including lipopolysaccharide (LPS) (Santaolalla, Sussman & Abreu 2011). It is now well understood that irinotecan's toxicity is dependent on interaction between these microbial products and the host immune system, with this interaction thought to be responsible for the intense inflammatory cytokine storm that dictates the duration and severity of mucosal injury and GI symptomology (Wardill et al. 2016). Mechanistically, the microbiota communicates with the host immune system to elicit these inflammatory responses through pattern recognition receptors, especially toll-like receptors (TLRs). Of particular interest is TLR4 as it recognises and responds to LPS (Santaolalla, Sussman & Abreu 2011; Cheng et al. 2015; De Nardo 2015). Of interest, it is also known that in addition to LPS, SN-38 also acts as a ligand for TLR4 and therefore has the capacity to directly and indirectly (through microbial disruption) activate its downstream inflammatory responses (Wong et al. 2019). As such, TLR4 activation is now considered critical in the initiation and potentiation of GI damage caused by irinotecan.

In support of this hypothesis, global *Tlr4* knockout (KO) (*Tlr4*$^{-/-}$) mice are protected from GI toxicity induced by irinotecan (Wardill et al. 2016). However, in interpreting previous findings, it is critical to appreciate that TLR4 is expressed on a range of cells, including the intestinal epithelium (Dheer et al. 2016) and a range of immune

cells (Li & Cherayil 2003). As such, given the global TLR4 KO in the *Tlr4*$^{-/-}$ model, it remains unclear if the mechanism of protection is mediated at the mucosal interface (i.e. by epithelial cell-expressed TLR4) or by TLR4 expressed on immune cells. This complexity is further amplified in light of new data that show *Tlr4*$^{-/-}$ mice were in fact more susceptible to irinotecan-induced GI toxicity (Wong et al. 2021). Although there were inherent differences in the experimental design that may explain the conflicting data (e.g. strain and irinotecan treatment), the use of global KO models does not provide the granularity needed to appropriately dissect and target TLR4's involvement in GI toxicity caused by irinotecan. Here, we use a transgenic, intestinal epithelial conditional TLR4 KO model (*Tlr4*$^{\Delta IEC}$, where only GI epithelial TLR4 has been deleted) to better understand how TLR4 contributes to irinotecan-induced GI toxicity. I hypothesised *Tlr4*$^{\Delta IEC}$ would display decreased GI toxicity caused by irinotecan compared to wild-type (WT).

Methods

Animal husbandry

An intestinal epithelial conditional TLR4 KO C57BL/6 (*Tlr4*$^{\Delta IEC}$) line was established by following a transgenic Vil1-cre/Tlr4loxP breeding strategy (The Jackson Laboratory, Bar Harbor, ME, USA) (please refer to chapter 3 of this thesis) (Crame et al. 2021; Secombe et al. 2022). Successful deletion of TLR4 in intestinal epithelial cells was determined by real-time polymerase chain reaction (please refer to chapter 3 of this thesis) (Crame et al. 2021). Male and female WT C57BL/6 (n = 6-7) and *Tlr4*$^{\Delta IEC}$ (n = 6-8) mice aged 8–12 weeks were housed in ventilated cages in groups of 3–6 animals per cage with a 12 hrs light/dark cycle and access to irradiated standard mouse chow and sterile water *ad libitum*. Mice were euthanised via CO_2 exposure and cervical dislocation prior to dissection, in accordance with ethical approval of the University of Adelaide Animal Ethics Committee (M-2020-028) and

the University of Adelaide Institutional Biosafety Committee (IBC approval number 14254). The study complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2013).

Mouse model of irinotecan-induced GI toxicity

To investigate the effect of epithelial TLR4 on CIGT, a tumour-bearing model was established using the colorectal cancer cell line, MC-38. The MC-38 mouse colon cancer cell line was kindly provided by A/Professor Michele Teng of the Cancer Immunoregulation and Immunotherapy Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, Australia. MC-38 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS) (Sigma-Aldrich, St Louis USA), 2 mM glutamine (Sigma-Aldrich), 0.1 mM nonessential amino acids (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 50 µg/mL gentamycin sulphate, penicillin/streptomycin (Sigma-Aldrich) (2016 MC-38 Kerafast Protocol, Boston USA). Cells were passaged at 80% confluency.

For initiation of tumour formation in mice, a cell load of 2 x 10^6 MC-38 cells in 1 x PBS (passage number 7-12) was subcutaneously injected into the right flank (Figure 4.1A). Tumours were allowed to grow and were measured daily using electronic calipers. Once tumour volume reached 0.2 cm^3 (approximately 6-8 days post-injection), mice were randomly assigned to receive irinotecan or vehicle (n = 6-8 mice per group). Irinotecan (Pfizer, New York USA, 20 mg/mL) was administered as a single, 270 mg/kg intraperitoneal (i.p.) dose prepared in a sorbitol/lactic acid buffer, previously shown to cause moderate CIGT in mice (Wardill et al. 2016). Vehicle mice received a volume equivalent dose of the sorbitol/lactic acid buffer as previously described (Wardill et al. 2016) (Figure 4.1A). Mice were monitored twice daily (Wardill et al. 2016) for clinical markers of toxicity including diarrhoea score, body weight,

animal appearance, dehydration and rectal bleeding and tumour volume was measured once daily. Diarrhoea scoring was quantified using a well-established scoring 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 (Gibson et al. 2007). Mice were euthanised via CO_2 exposure and cervical dislocation prior to dissection. All major organs including the entirety of the GI tract and tumour tissue were dissected from all mice, with ileum and colon tissues assessed for toxicity in this study. All other tissues were stored for future use. Tissues were either placed in 10% neutral buffered formalin for processing and embedding in paraffin, or were snap frozen in liquid nitrogen. Mid-colon sections were collected and immediately placed into warmed glucose-fortified Ringer's solution and promptly mounted into Ussing chambers as described in chapter 3 (Crame et al. 2021). The primary outcome measures were *in vivo* diarrhoea scores and body weight.

Electrophysical analysis of mouse mid-colon

The EM-CSYS-8 Ussing chamber system with DM-MC8 voltage clamp/electrode input (Physiological Instruments, San Diego USA) was used to determine the electrophysiological properties of the colon as previously described (Wardill et al. 2016). Briefly, colonic tissue was excised and cut longitudinally along the mesenteric attachment line. Tissue was then mounted into 0.1 cm^2 aperture slider (Physiologic Instruments; P2303A) and mounted into chambers. Tissue was kept under physiological conditions, immersed in warm (37 °C), glucose-fortified Ringers solution consisting of (in mM): NaCl 115.4; KCl 5; $MgCl_2$ 1.2; NaH_2PO_4 0.6; $NaHCO_3$ 25; $CaCl_2$ 1.2; and glucose 10, bubbled with carbogen gas (95% O_2, 5% CO_2) (Wardill et al. 2016).

After equilibrating for 20 min, baseline resistance, conductance and short circuit current were measured via the Acquire and Analyse Revision II program (v2.3; Physiologic Instruments) every 20 s for 5 min. The average value was then calculated. After baseline readings, amiloride (20 µM) was administered to inhibit the epithelial sodium channel, before forskolin (apical and basolateral chambers, 10 µM) and carbachol (basolateral chamber, 100 µM) were added to elicit cyclic adenosine monophosphate (cAMP)-dependent and calcium-activated chloride channels (CaCC) responses, respectively. The maximum peak in short circuit current (I_{sc}) was determined and response defined as the change in I_{sc} relative to pre-drug baseline (ΔI_{sc}) (Wardill et al. 2016).

Haematoxylin and Eosin (H&E) Staining

Dissected distal ileum and colon samples were fixed in 10% formalin and embedded in paraffin blocks. Tissue sections (4 µm) were cut using a rotary microtome and mounted onto glass slides (Menzel-Gläser, Braunschweig Germany). Standard haematoxylin and eosin (H&E) staining was performed as previously described (Wardill et al. 2016). In brief, slides were dewaxed in histolene and rehydrated through graded ethanols. Slides were stained in Lille-Mayers haematoxylin and rinsed until clear in running tap water before being differentiated in 1% acid alcohol (5 mL HCl + 500 mL 70% ethanol). Tissue was then placed in Scott's Tap Water (in mM) ($MgSO_4$ 166.2; $NaHCO_3$ 23.7 in 1 L dH_20) prior to being counterstained with alcoholic eosin (Sigma-Aldrich, St Louis USA). Slides were dehydrated through graded ethanols and cleared with histolene before being coverslipped with D.P.X neutral mounting medium (Sigma-Aldrich, St Louis USA).

Slides were imaged using a NanoZoomer 2 Digital Slide Scanner (Hamamatsu Photonics, Japan). A modified version of well-established intestinal injury scoring criteria (Howarth et al. 1996) was used to quantify differences between groups, with 1

representing the presence and 0 representing the absence of the pathophysiological marker. The criteria included: 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, oedema, villous fusion, and villous atrophy. The latter 2 criteria were not assessed in colon samples; therefore, the total possible score for ileum samples was 8 and for colon samples was 6 (Crame et al. 2021). Furthermore, ileum villus height and crypt depth and colonic mucosal thickness were assessed by a blinded researcher (E.E.C.) using the NDP.view2 image viewer program (Hamamatsu Photonics, Japan).

Immunofluorescence (IF) of Intestinal Tissue

Immunofluorescent staining was performed to evaluate the expression of tight junction proteins, occludin and zonular occludens 1 (ZO-1), and leukocyte immune marker, integrin alpha M ($CD11b^+$) immune cells using the DakoCytomation Autostainer (AutostainerPlusTM, Dako, Denmark, serial number: AS1271F1104) as previously reported (chapter 3 of this thesis) (Crame et al. 2021). Briefly, paraffin-embedded tissue was cut on a rotary microtome into 4 µm sections and mounted onto glass slides (Flex Plus Detection System, Dako; #K8020). Slides were deparaffinised in histolene and rehydrated through graded ethanols. Antigen retrieval was performed using the PT Link bath (pre-treatment module; Dako; #PT101) using an ethylenediaminetetraacetic acid (EDTA)/Tris buffer consisting of (in mM): Tris 9.9; EDTA 1.3; and 0.5 mL Tween 20 in 1.5 L dH_20, pH = 9 at 97°C for 20 mins.

The primary antibodies used are presented in Table 4.1. DAPI (1 µg/mL) (Sigma-Aldrich, St Louis USA) counterstaining was used to visualise the nucleus of associated cells in sample, with 1 x PBS as diluent. Post-staining, slides were coverslipped with Fluoroshield (Sigma-Aldrich, St Louis USA), sealed and stored in

the dark at 4°C prior to imaging. Slides were imaged using the Nikon A1 Confocal Microscope (Nikon, Tokyo Japan) using a 40 x objective. IF staining was quantified via % area stained on the Fiji Image J program as previously described, where the average value of three areas of interest was used for final analysis (Abramoff, Magalhaes & Ram 2004). Manual cells counts of $CD11b^{+}$ positive cells were taken for three random areas of interest at 40 x objective, where the average score per tissue was used for final analysis.

Table 4.1: IF protein blocking agents and antibody information

Protein	Blocking Solution	Primary antibody	Secondary antibody
Occludin	10% NHS	Invitrogen #33-1500, 0.5 mg/mL, 1:200 dilution in 5% NHS	AlexaFluor 568 anti-mouse in 1 x PBS, 1% BSA and 2% FBS
ZO-1	10% NHS	Invitrogen #61-7300, 0.25 mg/mL, 1:100 dilution in 5% NHS	AlexaFluor 568 anti-rabbit in 1 x PBS, 1% BSA and 2% FBS
$CD11b^{+}$	4% BSA	Abcam #ab133357, 1:1000 dilution in 1% BSA	AlexaFluor 488 anti-rabbit in 1 x PBS, 1% BSA and 2% FBS

*Abbreviations: BSA bovine serum albumin, FBS foetal bovine serum, NHS normal horse serum, PBS phosphate buffered saline.

Statistical Analysis

All data were analysed using Prism version 9.0.0 (GraphPad Software, San Diego USA). All parametric data were deemed to be normally distributed from the D'Agostino-Pearson test and therefore analysed using a one-way analysis of variance (ANOVA) or two-way ANOVA and presented as mean ± standard error of mean (SEM). Diarrhoea data was analysed using a Chi-square test. P-values < 0.05 were deemed significant.

Results

Intestinal epithelial TLR4 is required for severe diarrhoea following irinotecan

A moderate toxicity profile was achieved in all animals characterised by acute diarrhoea and weight loss in response to irinotecan. While no significant differences in weight loss were observed between the irinotecan-treated strains (i.e. group-effect), longitudinal changes in weight were observed within strains (i.e. time-effect). No dehydration, rectal bleeding, pica or changes to animal appearance was observed in any of the mice, regardless of strain or treatment group.

All mice treated with irinotecan lost weight (Figure 4.1B). WT mice treated with irinotecan lost a significant amount of weight at 24 hrs post-treatment compared to WT vehicle treated mice (P = 0.015). There were no differences in WT mice +/- irinotecan at other time points. In contrast, *Tlr4*$^{\Delta IEC}$ mice had significant weight loss at all time points post-treatment compared to vehicle treated *Tlr4*$^{\Delta IEC}$ mice (24 hrs P = 0.0004, 48 hrs P =0.0006 and 72 hrs P = 0.037). Diarrhoea occurred in both strains treated with irinotecan, peaking at 24 hrs. The severity of diarrhoea was significantly lower in *Tlr4*$^{\Delta IEC}$ compared to WT mice at 24 hrs (P < 0.0001) (Figure 4.1C). No diarrhoea was observed in either WT or *Tlr4*$^{\Delta IEC}$ vehicle groups (data not shown).

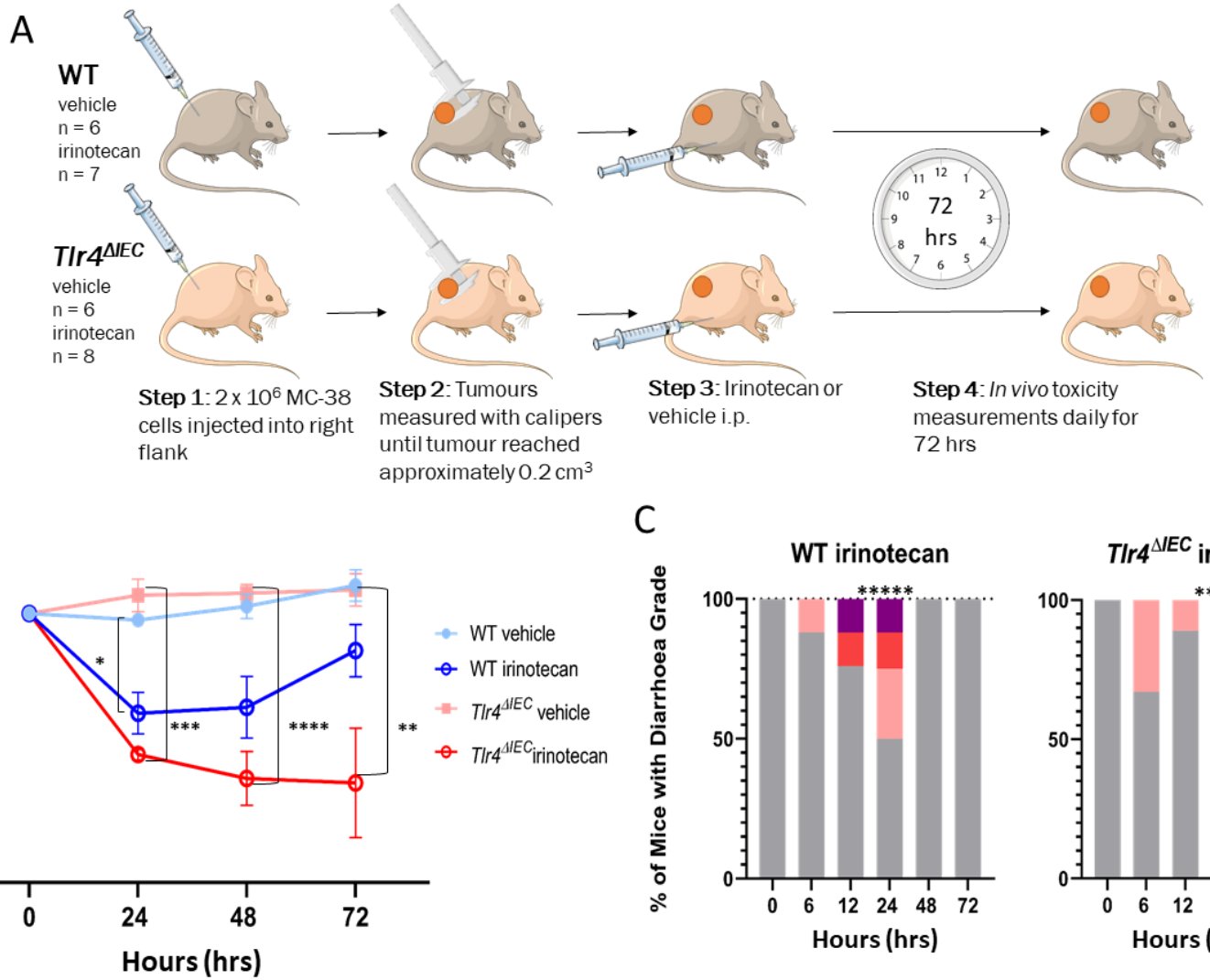
A
WT
vehicle
n = 6
irinotecan
n = 7
Tlr4ΔIEC
vehicle
n = 6
irinotecan
n = 8
72
hrs
Step 1: 2 x 10⁶ MC-38 cells injected into right flank
Step 2: Tumours measured with calipers until tumour reached approximately 0.2 cm³
Step 3: Irinotecan or vehicle i.p.
Step 4: In vivo toxicity measurements daily for 72 hrs
B
% Weight Change
5
0
-5
-10
-15
0
24
48
72
Hours (hrs)
*
***
****
**
WT vehicle
WT irinotecan
Tlr4ΔIEC vehicle
Tlr4ΔIECirinotecan
C
WT irinotecan
Tlr4ΔIEC irinotecan
% of Mice with Diarrhoea Grade
100
50
0
*****
0
6
12
24
48
72
Hours (hrs)
Grade 3
Grade 2
Grade 1
Grade 0

Figure 4.1: Experimental study design and *in vivo* outcomes. (A) Representative experimental study design for MC-38 tumour formation, intervention and monitoring of WT and *Tlr4*$^{\Delta IEC}$ mice, (B) % body weight change from baseline and (C) diarrhoea scores for WT and *Tlr4*$^{\Delta IEC}$ mice over 72 hrs period post-irinotecan treatment. Weight change was significantly different between WT irinotecan vs WT vehicle at 24 hrs post-treatment (*P = 0.015) and *Tlr4*$^{\Delta IEC}$ irinotecan vs *Tlr4*$^{\Delta IEC}$ vehicle at all time points post-treatment (24 hrs ***P = 0.0004, 48 hrs ****P =0.0006 and 72 hrs **P = 0.037). Diarrhoea severity scoring represented as grade 0 (no diarrhoea, grey), grade 1 (mild, light pink), grade 2 (moderate, dark pink) and grade 3 (severe, purple). Significant difference between WT irinotecan and *Tlr4*$^{\Delta IEC}$ irinotecan mice 24 hrs post-treatment (***** P < 0.0001). Weight data presented as mean ± SEM, diarrhoea data presented as percentage of affected mice per group, n = 6-8 mice per group.

Deletion of intestinal epithelial TLR4 does not impact colonic tissue permeability or secretagogue-mediated chloride secretion

Ussing chambers were used to investigate the electrophysiological properties of the colon, where resistance was used as a marker of intestinal permeability/barrier function and I_{sc} a marker of secretory profiles. There was no significant change in tissue resistance (Ωcm^2, Figure 4.2A) or I_{sc} (Figure 4.2B) across treatment groups. Similarly, strain or irinotecan did not change I_{sc} responses to chloride channel stimulants, forskolin and carbachol (Figure 4.2C and D, respectively).

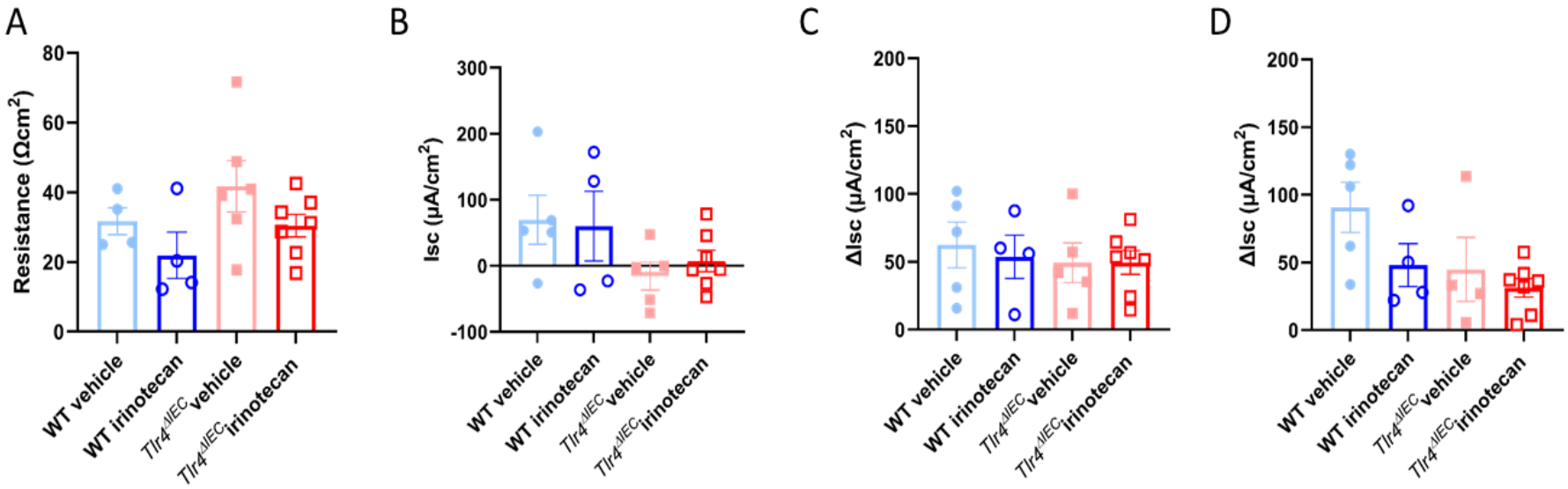

Figure 4.2: Ussing chamber analysis of mid-colon tissue. (A) Baseline tissue resistance, a marker of paracellular intestinal permeability, post-equilibration (Ωcm^2), (B) Baseline current post-equilibration (I_{sc} ($\mu A/cm^2$)). (C) Forskolin response as change in short circuit current post-treatment (ΔI_{sc} ($\mu A/cm^2$)), (D) Carbachol response as change in short circuit current post-treatment (ΔI_{sc} ($\mu A/cm^2$)). No differences observed between groups in all data sets ($P > 0.05$). Data presented as mean ± SEM, n = 4-8 mice per group.

Deletion of intestinal epithelial TLR4 does not protect against disruption of ileum morphology post-irinotecan

Irinotecan caused an increase in the tissue injury score in the ileum for both WT and *Tlr4*$^{\Delta IEC}$ groups (*Tlr4*$^{\Delta IEC}$ P = 0.0020, WT P = 0.0033) (Figure 4.3A, B). There was no significant difference between any treatment groups or strains in distal colon tissue injury scores (P > 0.05). No significant difference in ileum villus height (Figure 4.3D), ileum crypt depth (Figure 4.3E) or distal colon crypt depth (Figure 4.3F) were observed between treatment groups and strains.

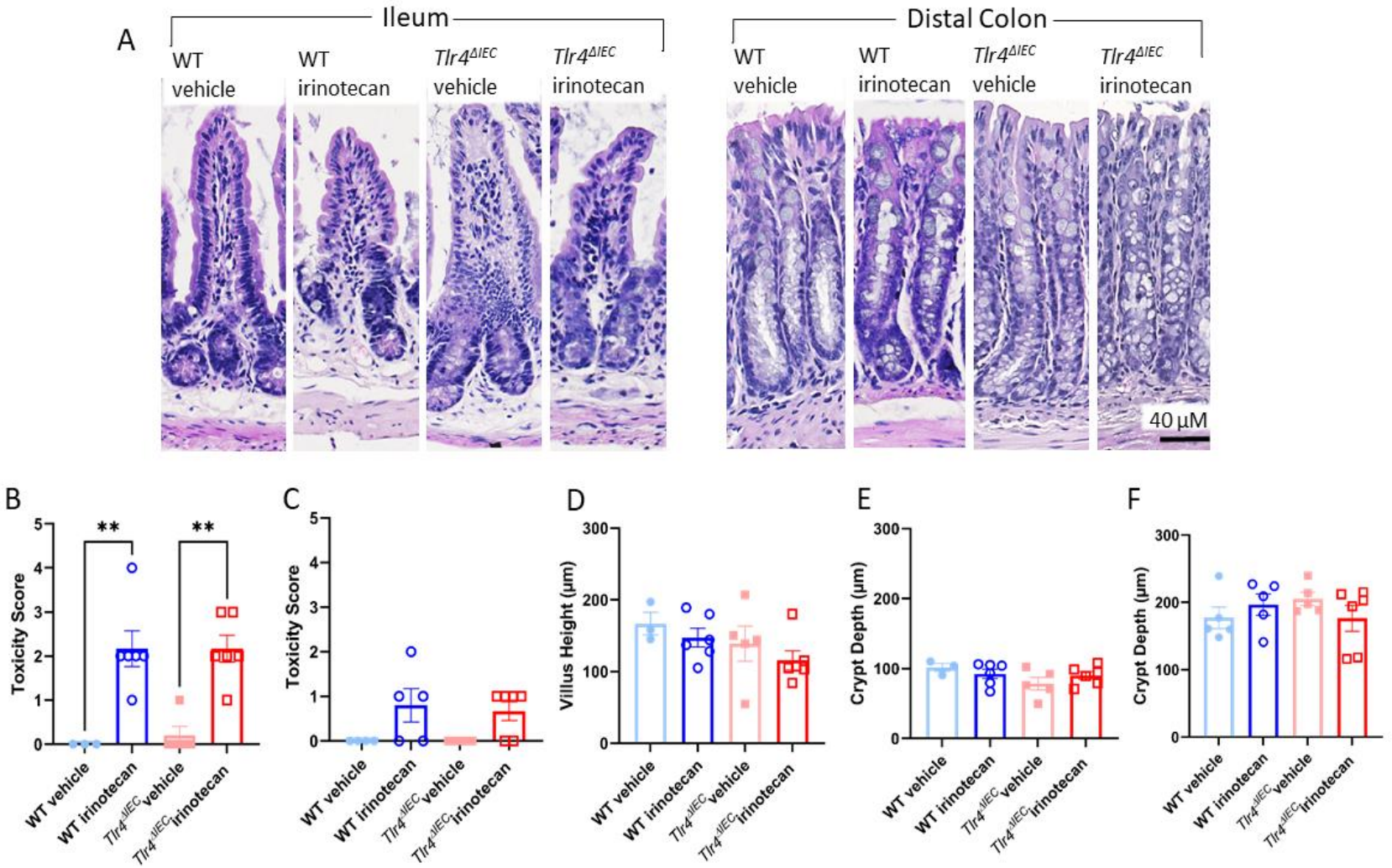
A
Ileum
Distal Colon
WT vehicle
WT irinotecan
Tlr4ΔIEC vehicle
Tlr4ΔIEC irinotecan
40 µM
B
Toxicity Score
**
**
C
Toxicity Score
D
Villus Height (µm)
E
Crypt Depth (µm)
F
Crypt Depth (µm)
WT vehicle
WT irinotecan
Tlr4ΔIEC vehicle
Tlr4ΔIEC irinotecan

Figure 4.3: Histological analysis of WT and *Tlr4*$^{\Delta IEC}$ mice ileum and distal colon (n = 6 per group). (A) H&E staining of ileum and distal colon tissue. Images presented at 20 x magnification. Intestinal tissue destruction assessed via well-established toxicity scoring. Total toxicity scoring for all groups presented for (B) ileum (WT vehicle vs WT irinotecan and *Tlr4*$^{\Delta IEC}$ vehicle vs *Tlr4*$^{\Delta IEC}$ irinotecan ** $P < 0.003$) and (C) distal colon. No difference observed in (D) ileum villus height, (E) ileum crypt depth and (F) distal colon crypt depth. All data presented as mean ± SEM, n = 5-6 mice per group.

Intestinal epithelial TLR4 expression did not alter tight junction protein expression or CD11b^{+} cell abundance post-irinotecan treatment

Expression of tight junction proteins occludin and ZO-1 was assessed in ileum and distal colon tissue for all groups (Figures 4.4 and 4.5, respectively). No difference in overall protein abundance (via % area stain) of either ZO-1 or occludin was observed between treatment groups and strains for ileum and colon ($P > 0.05$). Similarly, there was no difference in CD11b^{+} immune cell count in the ileum and colon between treatment groups and strains (Figure 4.6).

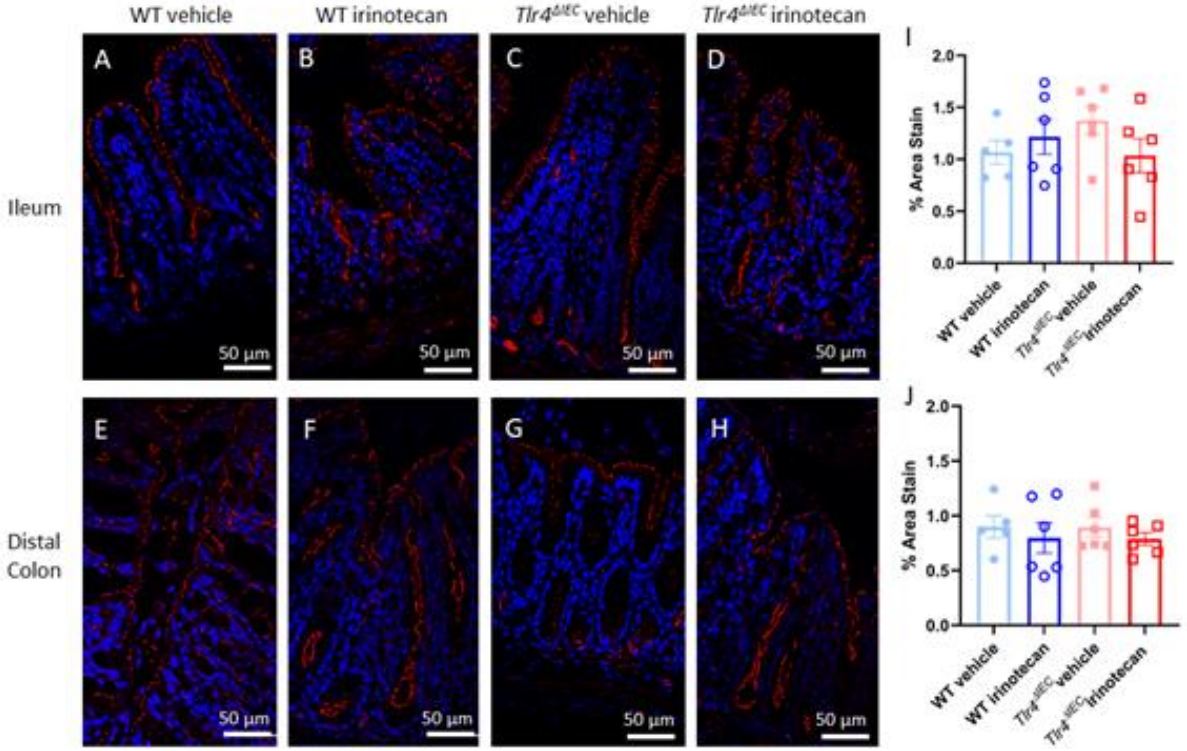

Figure 4.4: IF of ZO-1 protein expression in ileum and distal colon tissues (n = 6 per group). Positive ZO-1 expression indicated by red staining with counterstaining of cell nuclei with DAPI (blue). Ileum samples as follows; (A) WT vehicle, (B) WT irinotecan, (C) *Tlr4*$^{\Delta IEC}$ vehicle, (D) *Tlr4*$^{\Delta IEC}$ irinotecan. Distal colon samples as follows; (E) WT vehicle, (F) WT irinotecan, (G) *Tlr4*$^{\Delta IEC}$ vehicle, (H) *Tlr4*$^{\Delta IEC}$ irinotecan. Images presented at 40 x magnification. No difference observed in ZO-1 protein quantification in either (I) ileum or (J) distal colon, data presented as mean ± SEM, n = 5-6 mice per group.

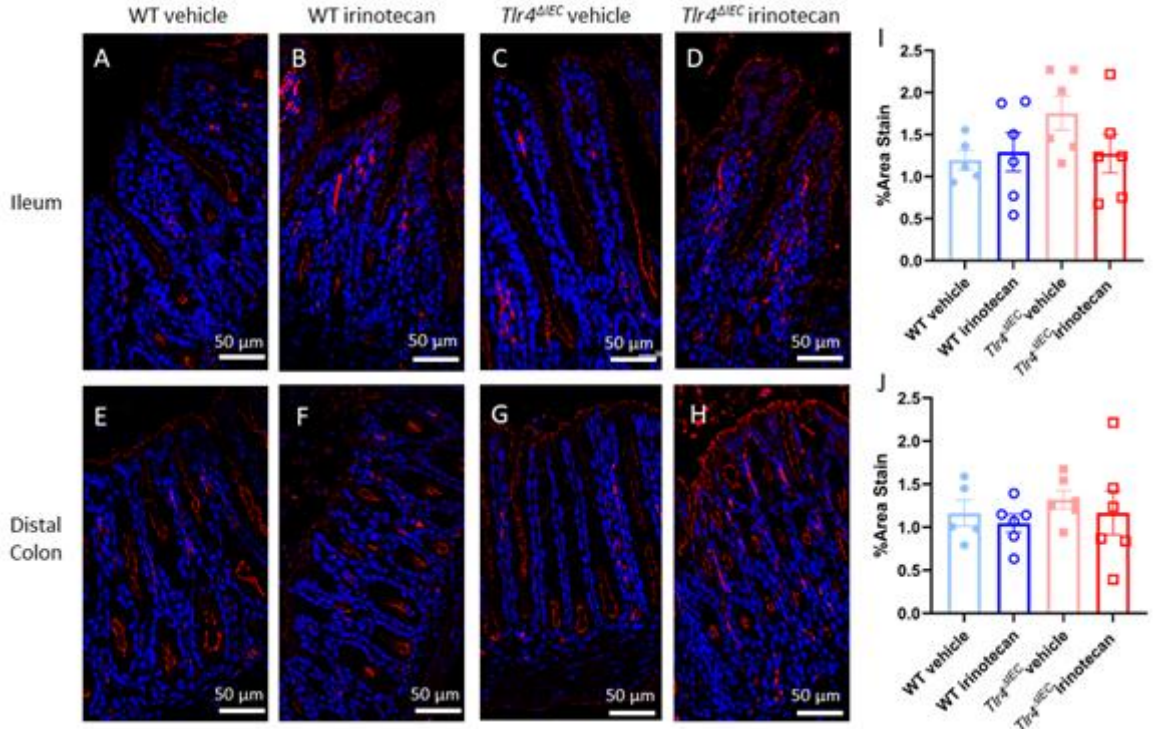

Figure 4.5: IF of occludin protein expression in ileum and distal colon tissues (n = 6 per group). Positive occludin expression indicated by red staining with counterstaining of cell nuclei with DAPI (blue). Ileum samples as follows; (A) WT vehicle, (B) WT irinotecan, (C) *Tlr4*$^{\Delta IEC}$ vehicle, (D) *Tlr4*$^{\Delta IEC}$ irinotecan. Distal colon samples as follows; (E) WT vehicle, (F) WT irinotecan, (G) *Tlr4*$^{\Delta IEC}$ vehicle, (H) *Tlr4*$^{\Delta IEC}$ irinotecan. Images presented at 40 x magnification. No difference observed in occludin protein quantification in either (I) ileum or (J) distal colon, data presented as mean ± SEM, n = 5-6 mice per group.

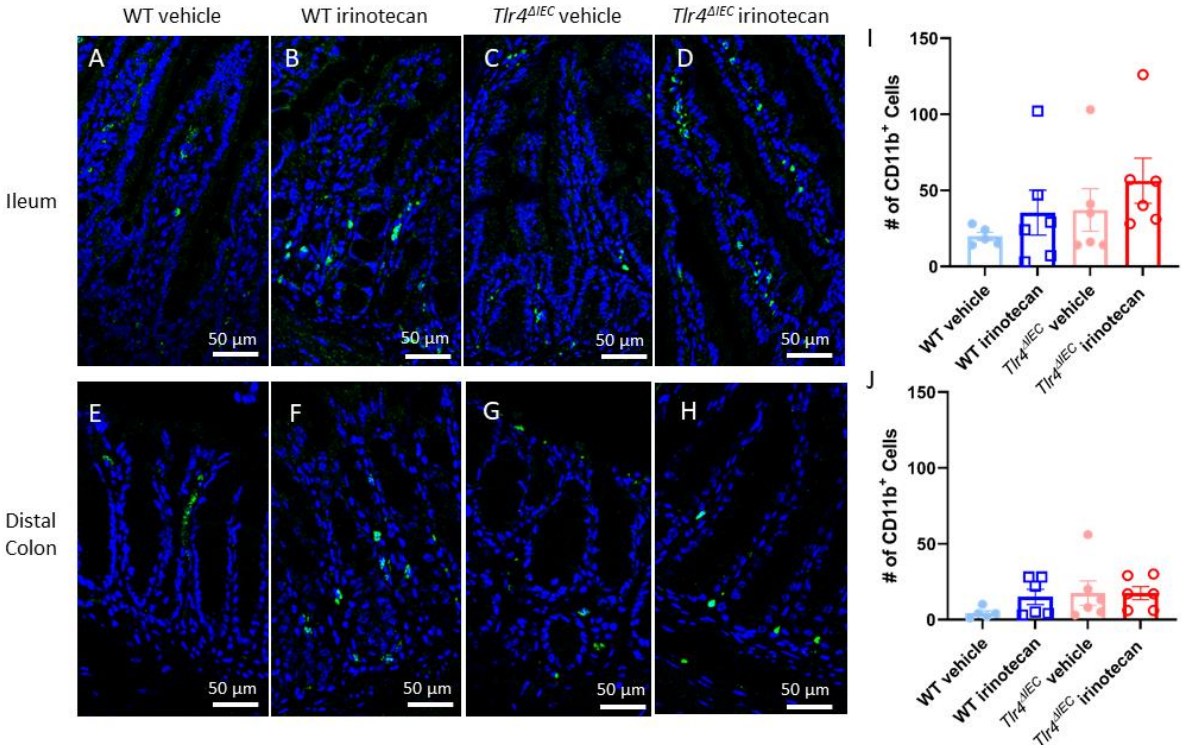

Figure 4.6: IF of CD11b+ protein expression in ileum and distal colon tissues (n = 6 per group). Positive CD11b expression indicated by green staining with counterstaining of cell nuclei with DAPI (blue). Ileum samples as follows; (A) WT vehicle, (B) WT irinotecan, (C) *Tlr4*$^{\Delta IEC}$ vehicle, (D) *Tlr4*$^{\Delta IEC}$ irinotecan. Distal colon samples as follows; (E) WT vehicle, (F) WT irinotecan, (G) *Tlr4*$^{\Delta IEC}$ vehicle, (H) *Tlr4*$^{\Delta IEC}$ irinotecan. Images presented at 40 x magnification. No difference observed in CD11b^{+} immune cell count from one field of view in either (I) ileum or (J) distal colon, data presented as mean ± SEM, n = 5-6 mice per group.

Discussion

Irinotecan is an effective chemotherapeutic drug that is limited by its highly mucotoxic nature and resulting GI symptoms (Gibson et al. 2007). There is mounting evidence that microbial-mucosal cross talk is critical in dictating these symptoms, which appears to be mediated by the pattern recognition receptor TLR4 (Wardill et al. 2016). Using a transgenic mouse model with selective KO of TLR4 in the intestinal epithelium ($Tlr4^{\Delta IEC}$), it was shown that intestinal epithelial TLR4 may control acute, severe diarrhoea development caused by irinotecan.

While this data certainly supported a beneficial effect of TLR4 deletion on diarrhoea development, consistent with previous reports (Wardill et al. 2016), it was clear that this was not consistent across other parameters used to assess GI toxicity. In particular, we identified clear disparity in TLR4's effect on irinotecan-induced diarrhoea and weight loss, suggesting core differences in their underlying mechanisms despite these symptoms long being considered manifestations of the same underlying pathobiology. When considering these findings, we must appreciate the variety of mechanisms that can cause diarrhoea. By and large, it is hypothesised that diarrhoea development is driven by anatomical changes in the intestinal mucosa that impair fluid absorption. However, we identified no changes in the histopathological features of the ileum or colon between experimental strains treated with irinotecan.

Similarly, functional analyses of colonic electrophysiology showed no difference in baseline tissue resistance or short-circuit current (I_{sc}) between groups. This differs significantly from previous global TLR4 KO animals which found changes in baseline I_{sc} in irinotecan-treated WT mice, and a significant decrease in baseline I_{sc} in global TLR4 KO mice compared to WT 24 hrs post-irinotecan (Wardill et al. 2016). Furthermore, ΔI_{sc} increased in response to carbachol and forskolin in global TLR4

KO and WT mice following irinotecan (Wardill et al. 2016), which was not observed in the current study. This discrepancy could be due to differing time points, as Wardill et al. reports changes to baseline I_{sc} and ΔI_{sc} at 24 hrs and 48 hrs, however the current study analysed tissue 72 hrs post-irinotecan treatment when diarrhoea was not present in these mice (Wardill et al. 2016). However, when looking specifically at baseline measurements of colonic permeability, it has previously been shown that baseline I_{sc} and ΔI_{sc} data does not associate with diarrhoea severity in global TLR4 KO or WT mice (Wardill et al. 2016). This may suggest that the diarrhoea observed may be caused by more subtle changes to colonic mucosa, including dysbiosis of host microbiome.

These findings must be considered in context of mouse models used, as Wardill and colleagues used BALB/c mice (Wardill et al. 2016), which have been shown to exhibit slightly different immune and toxicity profiles to C57Bl/6 mice. However, it is important to note that while the current study found diarrhoea prevention in *Tlr4*$^{\Delta IEC}$ mice at 24 hrs compared to WT, *ex vivo* analysis of intestinal tissue occurred at 72 hrs post-treatment. This may not accurately reflect the intestinal pathology at the 24 hrs time-point, suggesting the need for time-matched *in vivo* biomarkers, including quantification of serum citrulline (a marker of functional enterocytes) (Shen et al. 2016) or faecal calprotectin (a marker of intestinal inflammation) (Stringer et al. 2013). Furthermore, cholinergic diarrhoea within the first 24 hrs post-irinotecan treatment is common (Blandizzi et al. 2001), which may account for the diarrhoea severity at this time-point for the WT irinotecan treated mice. However, if cholinergic diarrhoea was the cause of the diarrhoea observed at 24 hrs, then it could be deduced that *Tlr4*$^{\Delta IEC}$ mice were potentially associated with reduced cholinergic response. Evidence supports this theory, as irinotecan-treated WT mice have been found to have increased proportion of cholinergic neurons (McQuade et al. 2017),

however the absence of TLR4 reduces the number of enteric neurons and lowers the cholinergic excitatory response delaying gastrointestinal transit (Caputi et al. 2017). Further investigations distinguishing cholinergic diarrhoea response versus CIGT would aid in clarification.

Host-microbe interaction at the mucosal interface appears to control CIGT-mediated diarrhoea development in a unique way, independent of mucosal injury. Antibiotic-induced diarrhoea poses a well-defined example of microbial-mediated diarrhoea development, where diarrhoea occurs in the absence of gross mucosal injury (Mekonnen et al. 2020). In line with this concept, recent evidence has shown that disruption of the microbiota prior to methotrexate chemotherapy increases diarrhoea severity, but does not alter the degree of mucosal injury (Wardill et al. 2021). This suggests that the microbiota may dictate diarrhoea independent of mucosal integrity, but dependent on mucosal TLR4. Irinotecan-specific GI changes have the potential to impact the host microbiome composition and increase diarrhoea severity (Stringer et al. 2008; Pedroso et al. 2015; Alexander et al. 2017). Upon microbiota dysbiosis, increased levels of Gram-negative bacteria have been associated with increased diarrhoea severity (Stringer et al. 2008). Given intestinal epithelial expression of TLR4 is known to regulate host microbiota populations and aid in host microbiological tolerance (Abreu et al. 2001; Lotz et al. 2006), the observed attenuation of diarrhoea in the *Tlr4*$^{\Delta IEC}$ mice may have been due to changes in the host-microbiota relationship and should be examined in future research. This is supported by Abreu (2001) which found site-specific absence of intestinal epithelial TLR4 in cell lines resulted in hypo-responsiveness to the Gram-negative protein, LPS (Abreu et al. 2001). More recently, Secombe and colleagues (2022) found significant differences in five functional groups of the intestinal microbes between *Tlr4*$^{\Delta IEC}$ and WT mice at baseline, and significant differences in two functional groups of the microbiome in

Tlr4$^{\Delta IEC}$ and WT mice 72 hrs post-irinotecan treatment (Secombe et al. 2022). This key finding suggests that absence of intestinal epithelial TLR4 expression may stabilise the host microbiota, and is therefore less likely to drive a diarrhoea-producing enterotype.

Our data suggests that intestinal epithelial TLR4 expression drives diarrhoea development post-irinotecan, independent of mucosal injury. Therefore, it is likely that the underlying mechanism behind acute diarrhoea severity may lie in the role of TLR4 on intestinal motility. Intestinal motility is controlled by the enteric nervous system (ENS), where ENS dysfunction has been shown to contribute to the development of CIGT symptoms (McQuade et al. 2017). Although not examined with irinotecan, a 2016 study investigated the effects of various chemotherapy treatments (inclusive of 5-fluorouracil (5-FU) and oxaliplatin) on myenteric nerves in the colon, demonstrating that myenteric S neurons were hyperexcitable and the threshold to activate an action potential was lower in the chemotherapy group than in the non-treated control group (Carbone et al. 2016). Importantly, evidence suggests potential interactions between host microbes and ENS neuronal survival, involving a relationship between intestinal TLR4 activation, increased enteric neuronal survival and increased GI motility (Anitha et al. 2012). Early work suggests that the proximity of enteric neurons and glial cells to the microbial rich intestinal lumen, may explain the increased expression of ENS TLR4 (Barajon et al. 2009), where TLR4 activation has been shown to regulate intestinal motility via inhibition of pacemaker currents through interstitial cells of Cajal (Zuo et al. 2013). Interestingly, the role of TLR4 KO in reducing GI motility is supported by recent research showing TLR4 global KO decreased colonic contractile activity (Grasa et al. 2019). Given decreased colonic contractions imply a reduced propensity for diarrhoea development, our data agrees with these findings, as the *Tlr4*$^{\Delta IEC}$ mice had reduced severity and instances of

diarrhoea post-irinotecan. Combined, this evidence suggests that TLR4 KO, both global and intestinal epithelial specific, results in decreased colonic motility, potentially reducing diarrhoea development. Future investigations using the *Tlr4*$^{\Delta IEC}$ mouse model would strengthen the understanding behind TLR4 involvement in motility and irinotecan-induced diarrhoea.

Contrary to expectations, body weight was decreased and remained low in *Tlr4*$^{\Delta IEC}$ irinotecan mice compared to *Tlr4*$^{\Delta IEC}$ vehicle mice. This was not seen in WT mice, with WT irinotecan mice only displaying significant weight loss compared to WT vehicle at 24 hrs post-treatment before partially recovering at 72 hrs. This may be regulated by differences in food intake or appetite. Currently, limited data exists regarding the association between TLR4 signalling and appetite/anorexia, however a 2004 study reported a resistance to anorexia (induced by LPS) in TLR4 KO mice (von Meyenburg et al. 2004).

Similarly, given the important role inflammatory cytokines play in cachexia (a muscle wasting syndrome commonly seen after chemotherapy (Suzuki et al. 2013)), it is also important to consider its role in this model. Heightened inflammation is known to drive cachexia (Suzuki et al. 2013), and as such, one would naturally assume that TLR4 deletion would mitigate cachexia. Accordingly, ADIPOQ, IL-6, NFKB1 and TLR4, have been identified as possible markers for cachexia risk (Tan et al. 2011). A 2017 study by Zhang et al. argues that TLR4 signalling mediates cachexia in Lewis lung carcinoma, with global TLR4 KO mice exhibiting reduced muscle wastage (Zhang et al. 2017). To date, there has been a lack of site-specific analysis in this area, and outlines great potential for extended investigations using the *Tlr4*$^{\Delta IEC}$ model. Another suggested cause for lack of weight gain could be due to a decrease in appetite. Recent research has shown that intact TLR4 signalling is required for food reward via stimulating dopamine neurons in the ventral tegmental area of the

brain (Li et al. 2021). While the average meal size and frequency has been shown to increase in TLR4 KO mice, interestingly, hedonic feeding (or feeding for pleasure, not to sustain energy requirements) was significantly decreased (Li et al. 2021). While this reduction in feeding motivation *may* explain the inability for the *Tlr4*$^{\Delta IEC}$ mice to regain weight post-irinotecan, like their WT littermates, the role of GI epithelial TLR4 has not been investigated in feeding studies. Therefore, further iterations of this study should include food intake as an outcome measure to investigate this relationship between TLR4 and appetite in greater detail.

While this research highlights a potential role of intestinal epithelial TLR4 signalling on GI toxicity post-irinotecan, it is not without its limitations. As previously discussed, this work was unable to fully investigate the food and water intake, and excretion patterns of mice while undergoing treatment. This should be a focus of future investigations, in order to better understand the site-specific role of TLR4 in appetite and weight management post-irinotecan. Ideally, these measures may arise from the use of metabolic caging of mice and analysis of appetite control hormones ghrelin and leptin (Malik & Yennurajalingam 2019). Furthermore, microbiota analysis was out of scope for this study and it is recommended that a detailed functional group analysis, based on the microbiota work conducted by Secombe and colleagues (Secombe et al. 2022), be considered for future investigations. Finally, due to the nature of our research questions, we were unable to collect intestinal tissue for analysis at acute injury time-points (24 hrs and 48 hrs post-irinotecan). This may be addressed in future work using varied end-point times or, as previously mentioned, inclusion of time-matched *in vivo* biomarkers, including quantification of serum citrulline (a marker of functional enterocytes) (Shen et al. 2016) or faecal calprotectin (a marker of intestinal inflammation) (Stringer et al. 2013).

Taken together, the current study data highlight the importance of intestinal epithelial TLR4 in the complex control of irinotecan-induced diarrhoea. Overall, our findings shed new light onto the intricate and complex roles of site-specific TLR4 expression in the CIGT context, and emphasise the importance of understanding the unique contribution of cell-specific TLR4 on the symptoms of CIGT. This work challenges the previously held conception that chemotherapy-associated weight loss and diarrhoea are governed by the same feature of mucosal atrophy in rodent models of CIGT. This provides unique opportunities for future investigations and challenges our existing understanding the role of intestinal epithelial TLR4 in CIGT development.

Chapter 5: Contribution of TLR4 to Colorectal Tumour Microenvironment, Aetiology and Prognosis

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This chapter discusses the impact of TLR4 expression in human colorectal cancer (CRC) survival and clinical outcome. This chapter is published in the Journal of Cancer Research and Clinical Oncology under the following reference; Crame, E.E., Nourmohammadi, S., Wardill, H. R., Coller, J. K., Bowen, J. M., (2022) Contribution of TLR4 to colorectal tumour microenvironment, etiology and prognosis. *Journal of Cancer Research and Clinical Oncology*. https://doi.org/10.1007/s00432-022-04199-4

Please note that figure numbering and referencing style have been altered to suit thesis formatting style. All spelling has been converted to Australian spelling. All other formatting has remained unchanged from original publication.

Statement of Authorship

Title of Paper	Contribution of TLR4 to Colorectal Tumor Microenvironment, Etiology and Prognosis
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Crame, E.E., Nourmohammadi, S., Wardill, H. R., Coller, J. K., Bowen, J. M., (2022) Contribution of TLR4 to colorectal tumor microenvironment, etiology and prognosis. Journal of Cancer Research and Clinical Oncology. https://doi.org/10.1007/s00432-022-04199-4

Principal Author

Name of Principal Author (Candidate)	Mrs. Elise Ellen Crame		
Contribution to the Paper	Conducted journal database searches and systematic literature review of previous colorectal cancer/TLR4 research in human cohorts.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	03/08/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Mr. Saeed Nourmohammadi		
Contribution to the Paper	Conducted TCGA database search and data analysis. 10%		
Signature		Date	16/05/2022

Name of Co-Author	Dr. Hannah R. Wardill		
Contribution to the Paper	Provided crucial feedback and helped to shape research. 5%		
Signature		Date	21/09/2022

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Abstract

Purpose

Toll-like receptor 4 (TLR4) is increasingly recognised for its ability to govern the aetiology and prognostic outcomes of colorectal cancer (CRC) due to its profound immunomodulatory capacity. Despite widespread interest in TLR4 and CRC, no clear analysis of current literature and data exists. Therefore, translational advances have failed to move beyond conceptual ideas and suggestions.

Methods

We aimed to determine the relationship between TLR4 and CRC through a systematic review and analysis of published literature and datasets. Data was extracted from nine studies that reported survival, CRC staging and tumour progression data in relation to TLR4 expression. Primary and metastatic tumour samples with associated clinical data were identified through the Cancer Genome Atlas (TCGA) database.

Results

Systematic review identified heterogeneous relationships between TLR4 and CRC traits, with no clear theme evident across studies. A total of 448 datasets were identified through the TCGA database. Analysis of TCGA datasets revealed TLR4 mRNA expression is decreased in advanced CRC stages ($P < 0.05$ for normal vs Stage II, Stage III and Stage IV). Stage-dependent impact of TLR4 expression on survival outcomes were also found, with high TLR4 expression associated with poorer prognosis (stage I vs III (HR = 4.2, $P = 0.008$) and stage I vs IV (HR = 11.3, $P < 0.001$)).

Conclusion

While TLR4 mRNA expression aligned with CRC staging, it appeared to heterogeneously regulate survival outcomes depending on the stage of disease. This underscores the complex relationship between TLR4 and CRC, with unique impacts dependent on disease stage.

Keywords: Toll-Like Receptor 4, Colorectal Neoplasms, Systematic Review, Humans

Introduction

Colorectal cancer (CRC) remains one of the most prevalent cancer diagnoses worldwide, with incidence rates in the United States of America of 37.8 per 100,000 (National Cancer Institute 2021). This places CRC as the fourth most common cancer in western populations (Australian Institute of Health and Welfare 2020; National Cancer Institute 2021) which when coupled with its high mortality rates, cements this disease as a major healthcare burden. While significant advances have been made in identifying high level risk factors for CRC, heterogeneity in tumour progression and treatment response continues to challenge the understanding of its etiology (Buikhuisen, Torang & Medema 2020). Few factors remain significant when traditional, largely unmodifiable risk factors (e.g. age, sex) are adjusted for, pointing to complex mechanisms governing tumour microenvironment which dictate growth trajectory and vulnerability to anti-cancer therapy (Buikhuisen, Torang & Medema 2020).

The tumour microenvironment is a complex system of molecular and cellular components, produced by both host and tumour (Wang, Lei & Han 2018). The microenvironment's contribution to prognosis and clinical outcome has proven controversial, although evidence supports both beneficial and inhibitory roles. For example, the microenvironment facilitates immune invasion and destruction of tumour tissue (Fang et al. 2014). In contrast, it also contributes to tumour development, cancer cell survival and treatment resistance (Zhao et al. 2019). Irrespective of this complexity, it is clear that infiltration of peripheral immune cells into the tumour microenvironment is related to CRC progression and prognosis. A 2019 study using the cancer genome atlas (TCGA) and gene expression omnibus (GEO) databases reported that M_0 macrophages, M_1 macrophages and $CD4^+$

memory T cells were more abundant in CRC tissue compared to healthy tissues ($P < 0.02$) (Ge et al. 2019). Furthermore, higher infiltration of M_1 macrophage populations in CRC tissue correlated with lower participant survival ($P = 0.04$) (Ge et al. 2019). This underscores the involvement of the host immune system in CRC.

In light of the strong immune-mediated mechanisms that appear to be linked with CRC etiology and treatment response, there has been substantial interest in the potential role of the innate immune surveillance protein, toll-like receptor 4 (TLR4). TLR4 is a pattern recognition receptor, which upon activation, initiates a strong inflammatory response (Takeda & Akira 2004). TLR4 requires the accessory proteins myeloid differentiation factor 2 (MD-2) and cluster of differentiation 14 (CD14) to efficiently bind to ligands including, LPS, heat shock proteins (Hsp70 and Hsp90) and high-mobility group protein I (HMGBI) (Cheng et al. 2015). TLR4 signalling is vital to intestinal homeostatic maintenance, as previously reviewed (Bruning et al. 2021). TLR4 is notably upregulated in the intestine under inflammatory states including in people with ulcerative colitis, and this is further linked to ulcerative colitis-associated CRC risk and development (Fukata et al. 2007). Furthermore, genetic variants of *TLR4* (rs10116253, rs192791 1, rs7873784) have been linked to CRC (Huang et al. 2018).

TLR4 is expressed on a range of different cell types within the tumour microenvironment, including dendritic, stromal, macrophage and epithelial cells (Li et al. 2017). The importance of site-specificity of TLR4 expression in healthy and diseased states, including CRC, is well documented (Bruning et al. 2021). Pre-clinical CRC models indicate that TLR4 has both pro- and anti- tumour roles, with expression sites being a possible differentiating factor between whether TLR4 aids in cancer destruction or survival (Li et al. 2017). To add further complexity, TLR4 has also been identified to modulate toxicity following cancer therapy, including diarrhea and pain

(Wardill et al. 2016). As such, it is currently unclear whether TLR4 is beneficial, or, potentially harmful in the CRC microenvironment, and whether it is a rationale target for intervention. We therefore aimed to systematically review current published evidence and datasets to crystalize the relationship between TLR4 and CRC staging, treatment toxicity and survival.

Methods

Search strategy, study selection and data retrieval

PubMed, Cochrane Library and Embase were searched between January and February 2022 for peer-reviewed journal publications using keywords listed in Supporting Information Table 1 and were screened for inclusion based on specific criteria; original research, clinical trials and studies conducted between 2010-2021; archival human tissue; CRC; participant survival; tumour recurrence; prognosis; toxicity; and TLR4 expression. Exclusion criteria included: animal models; cell lines; and cancer types other than CRC. Eligible publications were reviewed with the following data being extracted manually by two independent authors (EEC, JKC) using a computer-based template: sample size; CRC stage; chemotherapy treatments; participant demographics; type of TLR4 analysis; TLR4 specific outcomes (including expression rates and site-specificity); survival data (overall survival (OS), progression-free survival (PFS) or disease-free survival (DFS)); and tumour progression data. Summary outcomes are presented in Table 5.1.

Table 5.1: Summary of studies investigating impacts of TLR4 expression on human CRC clinical outcome

Author (Date)	Archival/ Clinical Study	Sample Size (n)	CRC Stage	Anti-Cancer Treatments	TLR4 Analysis	Site-Specific TLR4 (Y/N)	Survival Outcomes (Y/N)	Type of Survival	Cancer Recurrence (Y/N)	Key Findings
Cammarota et. al. (2010)	Archival	132	Mixed stage, stages I - IV	NR	IHC	Y	Y	DFS	Y	- ↑ TLR4 cells = ↑ grade of dysplasia. - ↓ % of TLR4+ cells in the tumour stromal compartment = ↑ DFS and later relapse compared to ↑ % of TLR4+ cells in the stromal compartment (RR 2.36; log rank chi-square 4.25, $p < 0.05$).
Tesniere et. al. (2010)	Clinical trial	668	Non-resectable metastases of colorectal adenocarcinoma and Stage II non-metast	LV5FU2 followed by FOLFOX 6 to FOLFOX 6 and by FOLFIRI, or, surgical removal	PCR *TLR4*	N	Y	PFS, or, progression 5 years after diagnosis	Y	- WT *TLR4* allele = ↑ PFS (HR: 0.73; CI=0.53–1.00; P<0.05) and OS (HR=0.72; CI =0.52–1.01); P=0.05), compared to loss-of-function *TLR4* allele following treatment.

			atic CRC	of the tumour						
Wang et. al. (2010)	Clinical trial	138	Mixed stage, stages I - IV	Surgery, chemotherapy and/or radiation treatment details NR.	IHC	N	Y	DFS and OS	N	- ↑ TLR4 = ↓ 5-year DFS (HR (95% CI) 1.62 (0.87 – 2.99), P = 0.1213) and ↓ 5-year OS (HR (95% CI) 2.17 (1.15 – 4.07), P = 0.015). - ↑ TLR4+MyD88 = ↓ 5-year DFS (HR (95% CI) 2.25 (1.27 – 3.99) P = 0.0053) and ↓ 5-year OS (HR (95% CI) 2.97 (1.64 – 5.38) P = 0.0003). - ↑ TLR4 expression was significantly associated with liver metastasis (P=0.0001) - ↑ co-expression of TLR4/MyD88 was significantly associated with vascular invasion (P=0.0186), liver metastasis (P=0.0002), and TNM stage (P=0.0036).
Eiro et. al. (2013)	Clinical trial	104	Resectable, mixed stage, tumour stages I - IV	Surgery, varying chemotherapy and radiation treatments througho	IHC	Y	Y	OS	Y	- ↑ TLR4 expression by tumour cells = ↓ rate of tumour recurrence (P=0.01) - ↑ TLR4 expression by fibroblasts = ↑ tumour recurrence (P=0.019)

				ut sample populatio n						- TLR4 expression by fibroblasts = ↓ OS (P=0.022). - TLR4 expression by fibroblasts was an independent factor associated with relapse-free survival (P=0.0001), and OS (P=0.013).
Formica et. al. (2013)	Clinical trial	31	Mixed stage, stages I to III	FOLFIRI with bevacizu mab	FC of TLR4 on neutro phils	Y	Y	PFS and OS	N	- No association between baseline or one-month post-treatment neutrophilic TLR4 expression and PFS or OS (P = 0.30 and P = 0.34 respectively).
Sussman et. al. (2014)	Archival	279	Mixed stage, stages I - IV	NR	IHC	Y	Y	OS	N	- No difference in TLR4 stromal staining and OS (P = 0.16), no difference in epithelial TLR4 staining and OS (P = 0.11). - ↑ TLR4 tumour stroma intensity score in stages 3 and 4 compared to stage 1 (Stage 1 = 2.80, Stage 2 = 3.24,Stage 3 = 4.36, Stage 4 = 3.75; p = NS, 0.0004, and 0.04, respectively). - ↑ TLR4 tumour epithelium intensity score for stages 2 and 3

										compared to stage 1 (Stage 1 = 0.17, Stage 2 = 0.64, Stage 3 = 0.64, Stage 4 = 0.92; p =0.01, 0.002, and NS, respectively).
Gray et. al. (2019)	Clinical trial	4877	Mixed stages inclusive of stage II, stage III and stable or responding metastatic CRC	SCOT trial (ISRCTN59757862): oxaliplatin-based adjuvant chemotherapy COIN trial (ISRCTN27286448): cetuximab added to oxaliplatin-based chemotherapy	PCR *TLR4*	N	Y	DFS and OS	N	- SCOT trial: no statistically significant association of any *TLR4* SNP and OS or DFS. - COIN trial: no statistically significant association of either *TLR4* SNP with OS or DFS.
Zhang et. al. (2019)	Clinical trial	94	Advanced stage, stages II and III	Standard 5-Fu-based adjuvant chemotherapy after	WB, IHC	N	Y	DFS	Y	- ↑ Fn (P = 0.028) and ↑ BIRC3 expression (P = 0.046) correlated with ↓ DFS. - TLR4 expression was independent of DFS; TLR4 was not a factor in

				radical surgery						univariate or multivariate cox regression analyses for DFS. - ↑ TLR4 (p = 0.036) and ↑ BIRC3 (p = 0.008) resulted in ↑ recurrence.
Wong et. al. (2021)	Clinical trial	46	Mixed stage, stages III–IV	Irinotecan monotherapy or in combination with 5-Fu and IFL regimen	PCR *TLR4*	N	N	NR	N	- Participants with *TLR4* SNPs rs4986790, rs4986791 ↑ severe diarrhoea (50%) than wild-type homozygous (15%). - Participants with TLR4 SNPs presented any grade of diarrhoea, contrasting with one half of the AA and CC WT groups (20 patients each, AG + GG, P = 0.012 vs. AA; and CT + TT, P = 0.012 vs. CC) that showed no signs of gastrointestinal toxicity - No impact of TLR4 polymorphisms on occurrence / severity of nausea

* Calcium leucovorin, citrovorum factor, folinic acid (LV5FU2), folinic acid, fluorouracil, and oxaliplatin (FOLFOX6), fluorouracil, leucovorin and irinotecan (FOLFIRI), Fluorouracil (5-FU), irinotecan, folinic acid, and fluorouracil (IFL). Immunohistochemistry (IHC), polymerase-chain reaction (PCR), flow cytometry (FC), western blot (WB). Overall survival (OS), disease-free survival (DFS), progression-free survival (PFS). No record (NR).

TCGA clinical CRC cases database extraction and statistical analysis

RNA sequencing data and associated clinical metadata with a total of 512 samples in read counts (HTSeq-Counts) of CRC were obtained from the TCGA data portal (https://portal.gdc.cancer.gov/, accessed in December 2020). Data related to TLR4 mRNA expression, CRC staging and OS were extracted. TLR4 mRNA expression was dichotomized into high and low expression using the tertile cut point. The OS curve was constructed using Kaplan–Meier and log-rank test analysis, comparing high and low TLR4 expression groups for all cases and within each CRC stage. Statistical analyses were performed using GraphPad Prism 8.3.1 (GraphPad Software Inc., CA, USA) and R. studio 1.2.5033 (Inc., Boston, MA).

Multivariate analysis was also performed to determine whether mRNA expression was associated with OS in each tumour stage where variables included tumour stage (I: IV), sex and age. To avoid using potentially biased cut-points splitting low and high TLR mRNA expressing participant groups, a two sample t-test using continuous TLR4 mRNA expression values (with no cut-point required) compared mRNA expression between alive and deceased participants. Finally, TLR4 mRNA expression between normal tumour adjacent tissue and tumour samples from different stages were analysed with a one-way ANOVA (normal vs stage I, stage II, stage III and stage IV).

Results

180 publications were initially identified, with 9 meeting inclusion criteria for final analysis (Figure 5.1). 6 publications were clinical trials with a combined participant total of 1081. The remaining 3 publications used archival tissue from previous clinical research. Only 2 publications analysed advanced stage CRC (non-resectable tumour stage II – IV), whereas 7 publications included mixed analysis of varying CRC stage. Participant survival data was extracted from 8 publications, inclusive of DFS, PFS

and OS dependant on individual study outcomes. Only 1 publication included data regarding toxicity in relation to TLR4 expression. Finally, CRC recurrence was analysed in 3 publications. TLR4 expression in the publications was assessed using immunohistochemistry (5/9, all of which used different primary antibodies), polymerase chain reaction (PCR) (3/9) and flow cytometry (1/9). Only 4 publications included site-specific analysis of TLR4 expression in CRC (Table 5.1) (Cammarota et al. 2010; Eiro 2013; Formica et al. 2013; Sussman et al. 2014). Of the 9 publications, 4 analysed formalin fixed and paraffin embedded tissue blocks, 4 analysed peripheral blood samples and 1 (Sussman et al. 2014) analysed tumour tissue microarray slides provided by the NCI Cancer Diagnosis Program (CDP).

Impact of TLR4 genotype and expression on CRC survival

Of the 8 publications to report on CRC survival, one reported that wild-type (WT) *TLR4* genotype was beneficial to CRC participant survival rates (Tesniere et al. 2010). Metastatic CRC participants with the WT *TLR4* allele had higher PFS (hazard ratio (HR): 0.73; 95% confidence interval (CI) = 0.53 – 1.00; $P < 0.05$) and OS (HR = 0.72; 95% CI = 0.52 – 1.01; $P = 0.05$) compared with participants bearing the *TLR4* loss-of-function (Asp299Gly) variant post-oxaliplatin chemotherapy treatment (Tesniere et al. 2010). No differences in DFS among participants bearing the WT versus the variant *TLR4* alleles were observed.

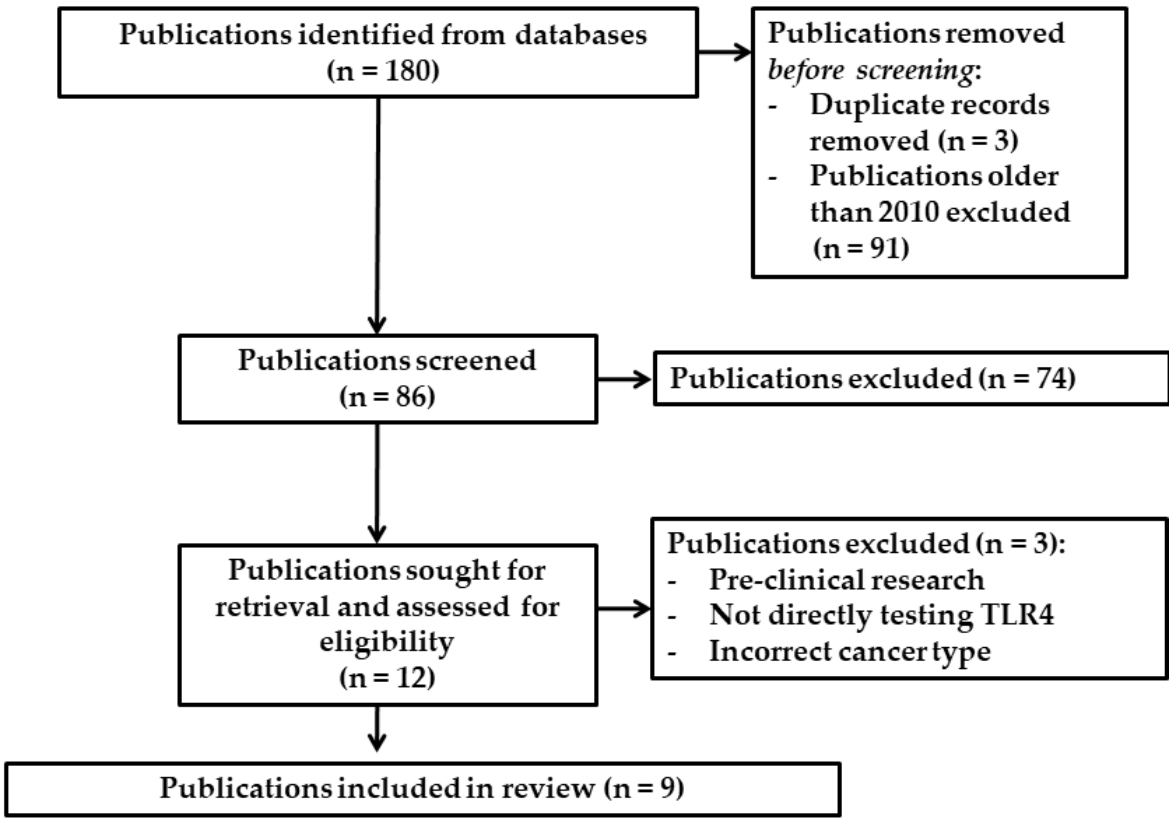

Figure 5.1: Flow diagram of literature search results for systematic review.

In contrast, 2 publications suggested that increased TLR4 expression is detrimental to participant survival (Cammarota et al. 2010; Wang et al. 2010). Cammarota et al. found that in mixed stage CRC tissue, participants with lower TLR4 expression in the tumour stroma compartment had improved DFS compared to participants with higher TLR4 expression (risk ratio (RR) 2.36; log-rank Chi-square 4.25, $P < 0.05$) (Cammarota et al. 2010). Furthermore, participants with pT_3 adenocarcinoma with high TLR4 expression (over 50% positive cells) relapsed sooner (14 months) compared to participants with low TLR4 expression (40 months, RR 3.15; log-rank Chi-square 4.03, $P < 0.05$) (Cammarota et al. 2010). This is supported by Wang and colleagues, who confirmed that CRC tissue displayed expression of TLR4 in 78 of 108 samples (72%), of which 22 displayed high TLR4 expression (Wang et al. 2010). In addition, increased TLR4 expression was associated with liver metastasis ($P = 0.0015$) and advanced tumour stage (stage IV) ($P = 0.0197$). Upon univariate analysis there was no difference in 5-year DFS rate for low versus high TLR4 expression, but OS was reduced with high TLR4 expression (HR (95% CI) 2.17 (1.15 – 4.07), $P = 0.015$) (Wang et al. 2010). However, this was not retained in multivariate analysis. In contrast, when samples exhibited high expression of both TLR4 and the adapter protein MyD88, DFS and OS were poorer (HR (95% CI) 2.11 (1.05 – 4.23) $P = 0.0352$) (Wang et al. 2010).

The conflicting nature of outcomes may be reflective of the lack of site-specific TLR4 investigations throughout human CRC research. Eiro and colleagues reported TLR4 expression by fibroblasts, not tumour cells themselves, was associated with a shortened OS of CRC participants ($P = 0.022$). Furthermore, TLR4 expression in fibroblasts was a significant and independent factor associated with DFS ($P = 0.0001$), and OS ($P = 0.013$) (Eiro 2013).

Four publications reported that TLR4 expression does not impact upon CRC survival. Formica and colleagues found that in 31 metastatic CRC participants, neutrophil TLR4 expression at baseline, or 1-month post-chemotherapy, had no association with PFS ($P > 0.05$) (Formica et al. 2013). This is supported by Sussman and colleagues who, in n = 279, found no associated between TLR4 expression in stromal tissue and OS after correcting for both CRC stage and grade. Furthermore, epithelial TLR4 expression was also not associated with OS (Sussman et al. 2014).

More recently, Zhang and colleagues found that in an advanced CRC cohort (n = 94) post-standard Fluorouracil-based adjuvant chemotherapy and radical surgery, the measured level of TLR4 expression was independent of DFS; hence no impact of TLR4 on overall DFS (Zhang et al. 2019). In addition, TLR4 was not a significant factor in survival outcomes following univariate or multivariate analyses (Zhang et al. 2019). However, high amounts of Fusobacterium (*Fn*), an anaerobic bacterium known to activate the TLR4 pathway in CRC cells, correlated with poor DFS ($P = 0.028$) (Zhang et al. 2019). Finally, Gray and colleagues analysed previously collected tissues from two large-scale clinical trials, the SCOT (ISRCTN59757862) trial and COIN (ISRCTN27286448) trial (Gray et al. 2019). Data generated from SCOT showed no association of any *TLR4* single nucleotide polymorphism (SNP) with survival (Gray et al. 2019). There was also no association of the *TLR4* SNP, rs867228, with DFS in cases with functional polymorphisms (Gray et al. 2019). Data from COIN showed no association of any tested *TLR4* SNP with OS by either log-rank test or univariate or multivariable Cox regression (Gray et al. 2019).

CRC Recurrence

Three publications reported on TLR4s contribution to CRC recurrence, with 2 publications identifying a detrimental role of TLR4 in CRC recurrence (Wang et al. 2010; Zhang, S et al. 2019). Wang and colleagues (2010) report upon 5 year follow-

up of 108 mixed stage CRC participants, 53 participants had tumour recurrence (DFS rate: 49%), with participants exhibiting high expression of TLR4 and its accessory protein MyD88 displaying increased recurrence rates compared to those with low expression (TLR4+MyD88 (low vs high) 5-year DFS HR (95% CI) = 2.25 (1.27 – 3.99) P = 0.0053) (Wang et al. 2010). Furthermore, participants with CRC and liver metastasis showed higher TLR4 and MyD88 expression versus CRC without liver metastasis (Wang et al. 2010). Among the 14 liver metastases obtained by hepatectomy, 12 were TLR4 positive and 6 showed a high expression (Wang et al. 2010). These findings are supported by Zhang and colleagues who showed high expression of TLR4 (P = 0.036) were more likely detected in participants with CRC recurrence, compared with participants without recurrence (Zhang et al. 2019).

In contrast, Eiro and colleagues observed that recurrence was dependent on the site of TLR4 expression, not its overall quantitative expression such that TLR4 expression by tumour cells was associated with a lower rate of recurrence in tumours from left colon/rectum compared to those from right colon/rectum (P = 0.028) (Eiro 2013). Further, TLR4 expression by fibroblasts was associated with a high rate of recurrence (P = 0.0001) in left colon/rectum tumours (Eiro 2013).

Toxicity post-chemotherapy in participants with CRC

Only 1 publication investigated the role of TLR4 in relation to post-chemotherapy toxicity outcomes, including diarrhea and nausea. Wong and colleagues investigated a cohort of 46 advanced stage CRC (stage III – IV), treated with first cycle of irinotecan-based chemotherapy (irinotecan monotherapy or in combination with fluorouracil and leucovorin - IFL regimen) (Wong et al. 2021). Participants the variant *TLR4* SNPs rs4986790 and rs4986791 had more severe diarrhoea (50%) compared to those without the variants (15%) (Wong et al. 2021). When looking at diarrhea of all severities, all participants (100%) with the variant *TLR4* SNPs developed diarrhea,

compared to only 50% of those without the variants (20 participants each, rs4986790, P = 0.012 vs. rs4986791, P = 0.012).(Wong et al. 2021) There was no association with nausea (Wong et al. 2021).

TCGA Database Results

TLR4 expression differs due to cancer stage

Summary of participant clinical data is presented in supporting information Table 5.2. Although TLR4 expression was not statistically different between normal and stage I, significantly higher TLR4 expression was observed in normal tissues vs Stage II, Stage III and Stage IV (Figure 5.2A).

TLR4 expression is associated with survival in respect to tumour stage

Number of participants per tumour stage is presented in Figure 5.2C. OS of participants with CRC with respect to TLR4 expression (low vs high) was conducted. TLR4 expression was not a significant prognostic factor (HR = 1.1, P = 0.64) when all stages were combined (Figure 5.2B) or compared between stages (Figure 5.3). In contrast, multivariate analysis revealed high TLR4 expression prior to treatment conferred worse prognosis, with the strength of the effect increasing with tumour stage (stage I vs II (HR = 2.2, P = 0.138), stage I vs III (HR = 4.2, P = 0.008) and stage I vs IV (HR = 11.3, P < 0.001); Figure 5.4). Sex and age had no impact on OS (Figure 5.4). In stage I disease, those that were alive had lower TLR4 expression at diagnosis (P = 0.034). For all other stages TLR4 expression at diagnosis was higher in those still alive (P = 0.035) (Figure 5.5).

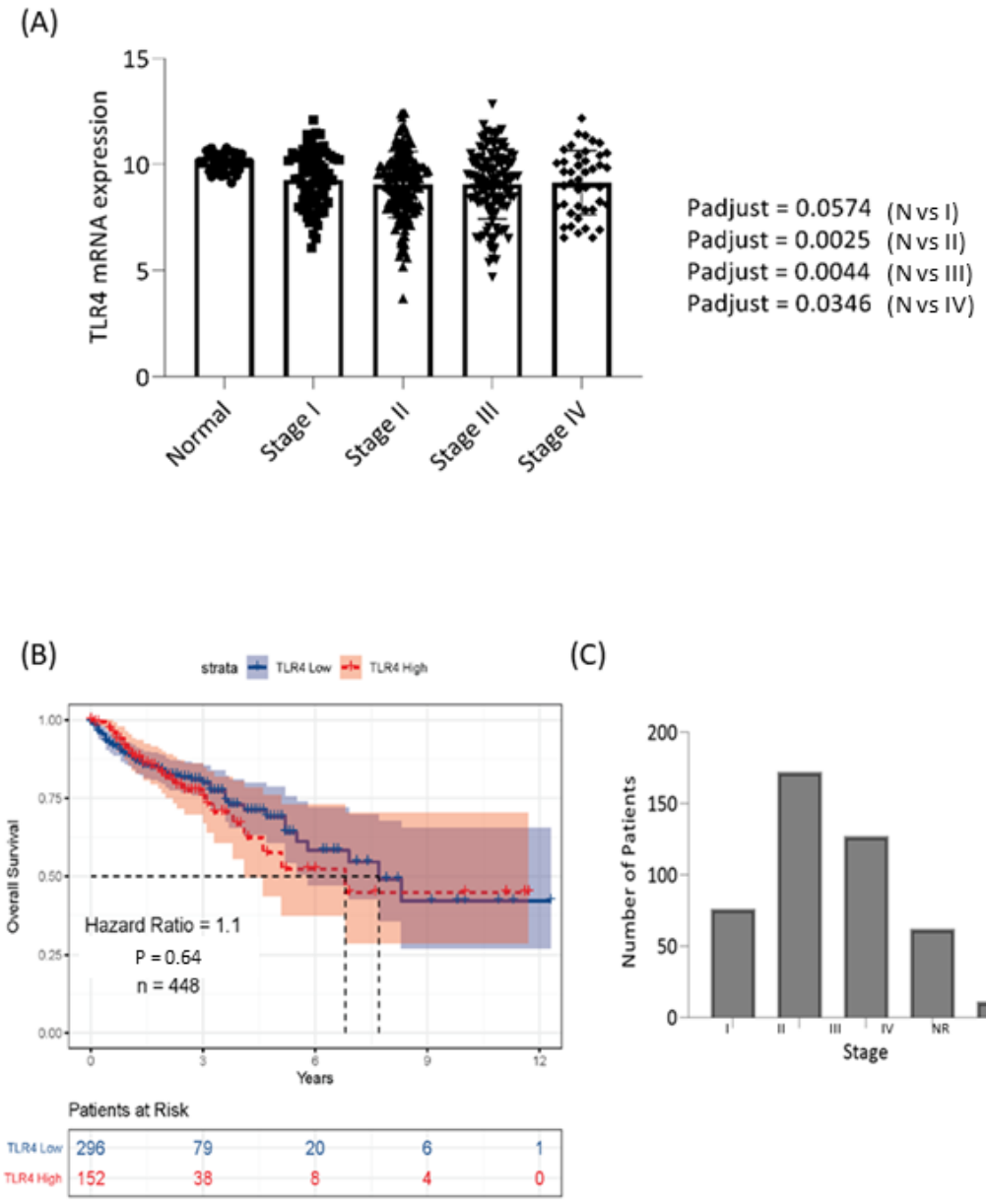

Figure 5.2: (A) Comparison of TLR4 expression between stage specific tumour and adjacent normal tissues from TCGA cohort. One-way ANOVA was performed by comparing solid tissue normal vs stage I, stage II, stage III, and stage IV participants. Statistical significance was represented as $P < 0.05$. (B, C) Assessment of TLR4 mRNA expression using the tertile cut-point. (B) Kaplan-Meier curves of overall survival (OS) in TCGA cohort. (C) Bar plot depicting the stage distribution of the cohort.

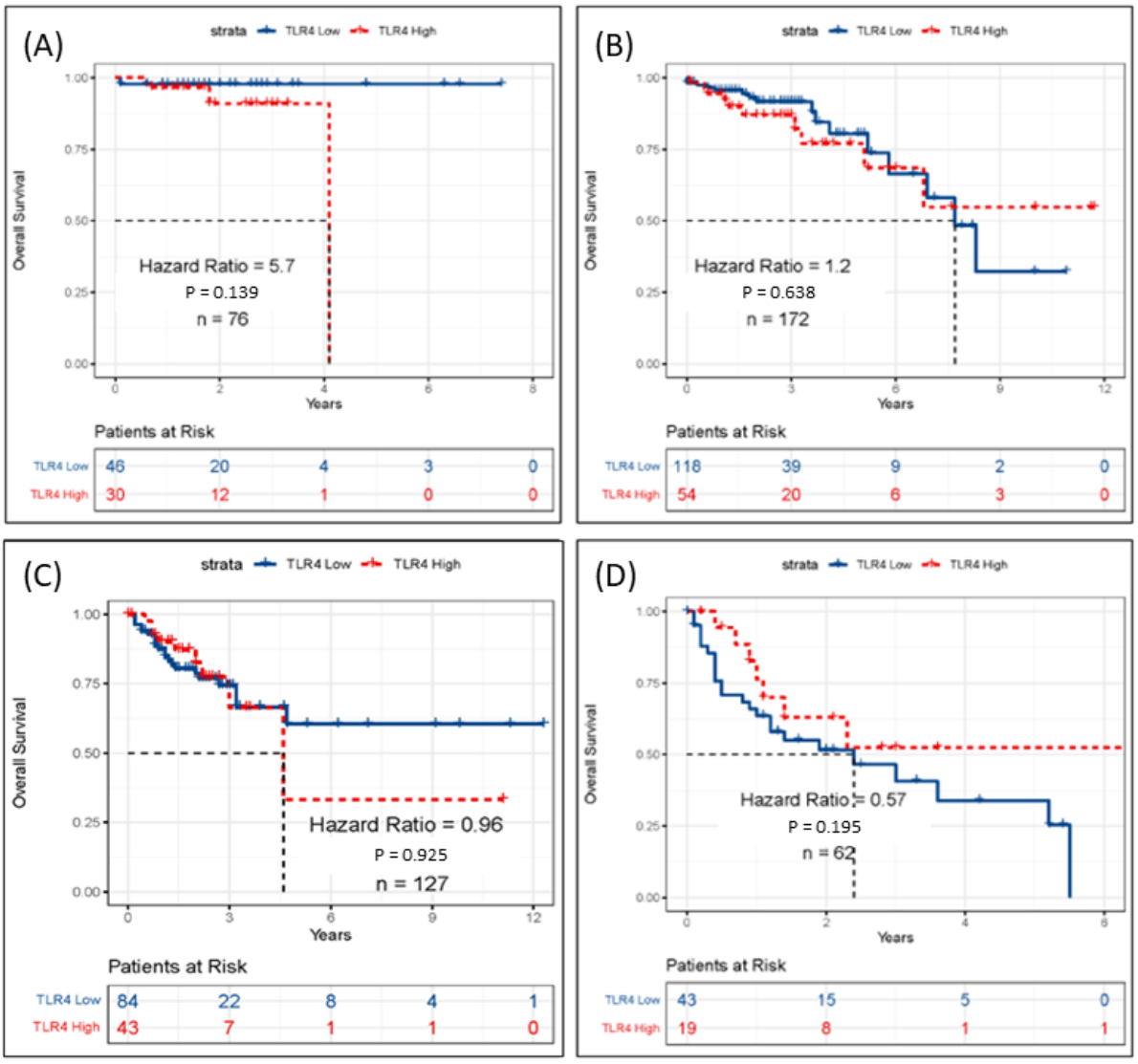

Figure 5.3: Assessment of TLR4 mRNA expression in stage specific CRC participants from TCGA cohort. (A) Kaplan-Meier curves depicting the OS in stage I participants (B) stage II participants, (C) stage III participants and (D) stage IV participants using the tertile cut point. No significant difference between groups.

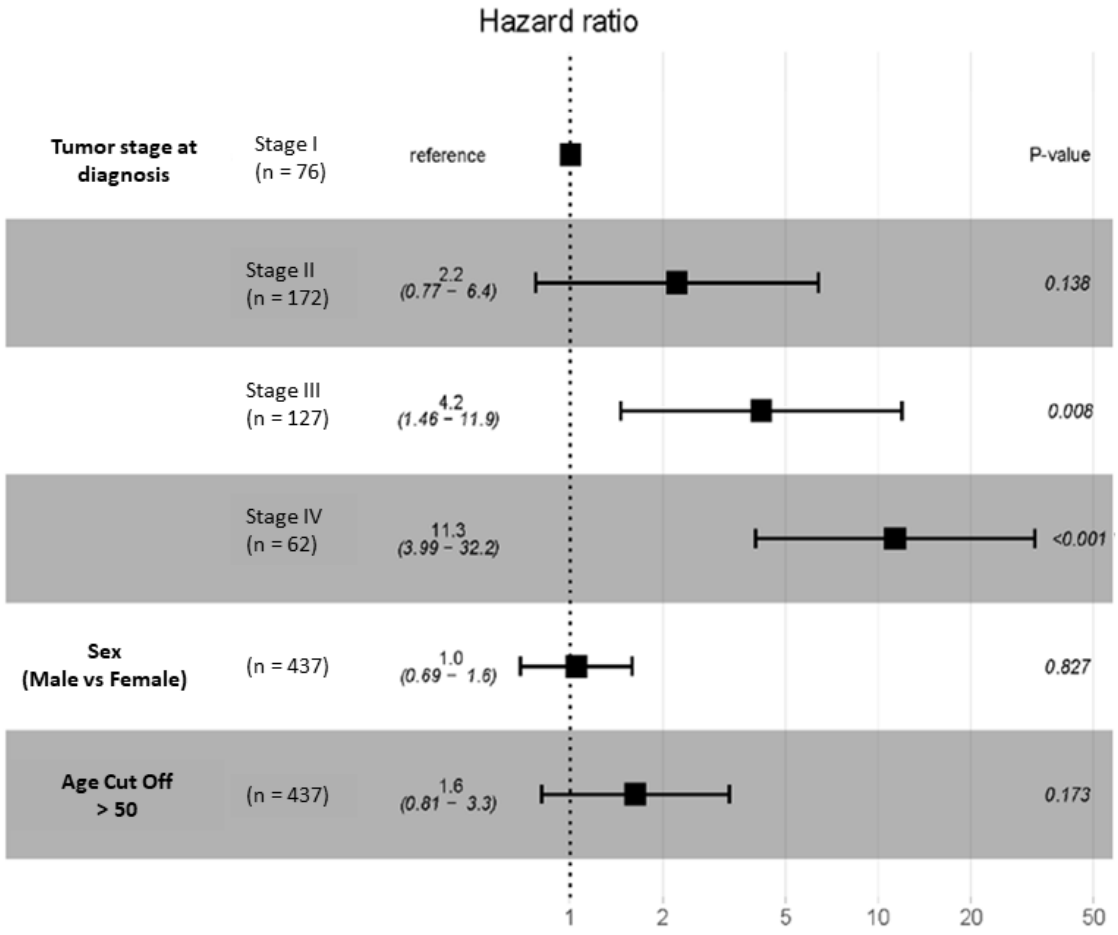

Figure 5.4: Forest plot of OS in stage specific participants. The tertile cut-point, the p-values and HRs with 95% CI derived for measurement of the cohorts from assessing the cut-point were shown. Statistical significance was represented as $P < 0.05$.

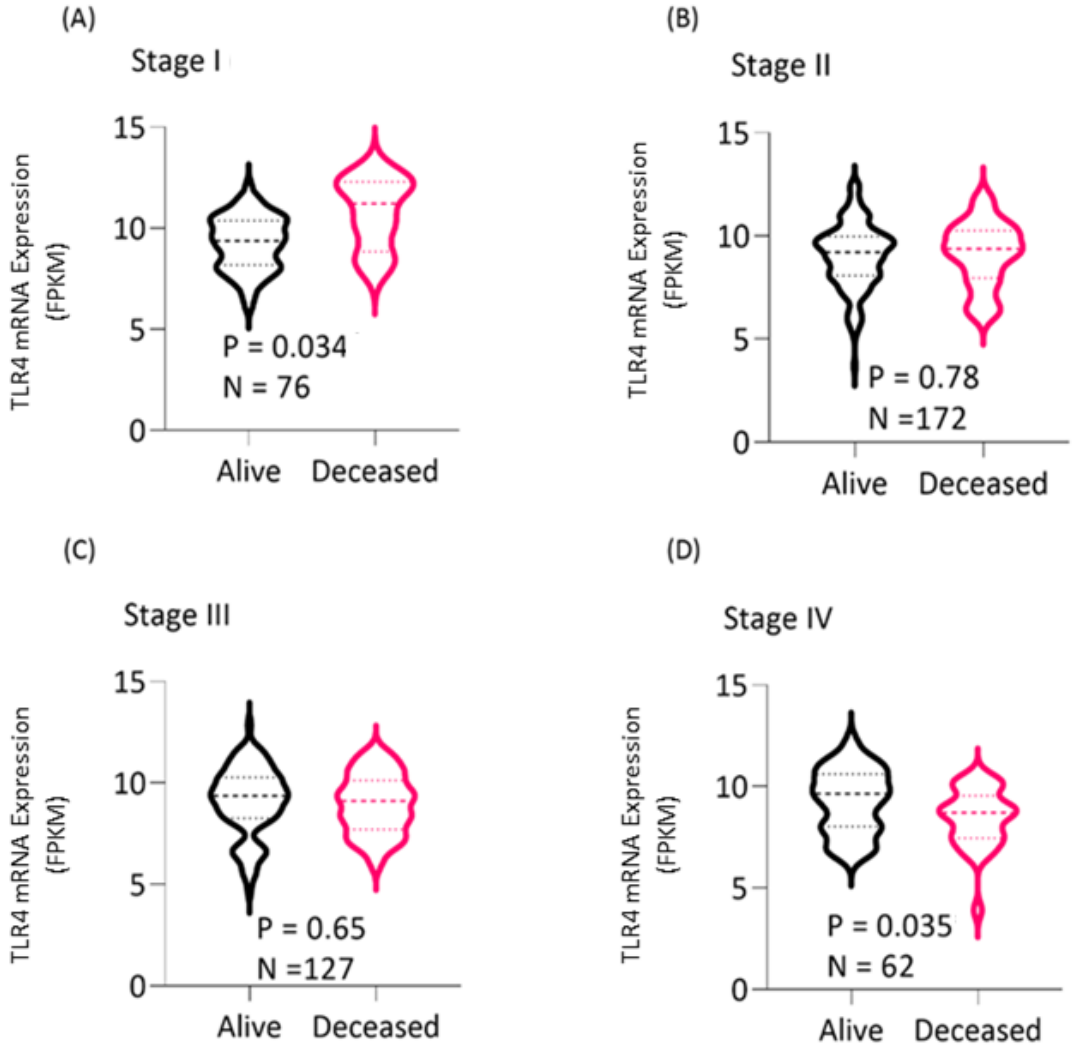

Figure 5.5: Comparison of TLR4 expression in Fragments per Kilobase of transcript, per Million mapped reads (FPKM) with respect to OS. Analysis of TLR4 expression using two sample t-test based on participants' survival in (A) stage I, (B) stage II, (C) stage III, and (D) stage IV participants. Statistical significance was represented as $P < 0.05$.

Discussion

TLR4 is an attractive target for controlling cancer development and optimizing treatment response due to its potent regulation of systemic immune responses. Our analysis exposes the significant heterogeneity in CRC outcomes linked with TLR4 expression. We have shown that TLR4 expression decreases with increasing CRC tumour stage at prognosis, and appears to have stage-dependent associations with participant outcomes. We highlight two novel findings related to high TLR4 expression in early- and late-stage CRC being; (1) in stage I CRC results in worse participant outcomes, and (2) in stage IV CRC results in improved participant outcomes. With TLR4 expression decreasing in higher grade CRC, this potential reduction of innate immune signalling may prove to be the causative mechanism behind unfavourable treatment responses and reduced survival.

TLR4 expression relative to tumour stage is well documented in the literature (Li et al. 2019; Omrane et al. 2014). These patterns of TLR4 expression reflect its core physiological mechanism of inducing inflammation, a process known to be carcinogenic. Our data showed a significant decrease in TLR4 expression in later stage CRC (stages II – IV) compared to normal tissue. This decrease in TLR4 expression was not found in stage I tumours, suggesting that the slightly higher TLR4 expression in early CRC may align with the well-defined concept that inflammatory processes are involved in the early development of CRC (Karin & Greten 2005). However, our analysis did show that non-tumour comparative tissue had the highest TLR4 expression. As this tissue was primarily collected from adjacent tissue in the same participants, systemic inflammatory responses may have impacted on interpretation. The finding that TLR4 expression decreases with tumour growth is also consistent with the current understanding of tumour development, with tumours often adapting to evade immune detection and control. Activation of the receptor,

programmed death 1 (PD-1), has been found to inhibit immune control of tumour growth, with the PD-1 ligand, PD-L1, being significantly upregulated in solid tumours like CRC (Hino et al. 2010). Therefore, this upregulation of PD-L1 is suggested to play a crucial role in the tumours ability to evade host immune system (Dong et al. 2002). This is of particular interest in the context of TLR4 research, as PD-L1 has also been shown to block the cytolytic activity of PD-1+ tumour infiltrating $CD4^+$ and $CD8^+$ T cells, which are reliant on dendritic cell-TLR4 interaction (Brahmer et al. 2012; Fife et al. 2009). In addition, Xiao et. al. (2016) reported that inhibition of TLR4 signalling via a blocking antibody significantly reduced the number of PD-1+ B cells in human hepatoma tissues, where PD-1+ B cell populations promoted cancer growth (Xiao et al. 2016). Furthermore, Huang (2018) found that improvement in clinical outcome is resultant of cytosolic HMGB1 triggering dendritic cell maturation through TLR4 activation, whereby consequently recruiting PD-1+ tumour-infiltrating lymphocytes to the tumour site (Huang et al. 2018). These findings highlight the importance of TLR4 to this particular tumour kill pathway and outlines the importance for TLR4 expression for improved clinical outcomes of people living with CRC.

While our findings suggest a likely relationship between TLR4 expression and tumour stage, the relationship between TLR4 and long-term outcome was less clear cut in both our systematic review and genetic analyses. When looking at all tumour stages, there was no significant impact on OS in low vs high TLR4 expressing tumours. This contradicts existing data, as a metanalysis of 212 people living with CRC found that high TLR4 expression associated with a significantly reduced OS and poorer prognosis (HR (95% CI) 2.30 (1.41,3.75), P = 0.001) (Hao et al. 2018). However, this analysis did not classify the cohort based on CRC stage which may have masked some findings and increased bias towards advanced stage disease. While our initial analyses showed no effect of TLR4 expression on OS, analysis of this relationship within specific tumour stages revealed that TLR4 may in fact have an impact but, in a

stage-specific manner. Specifically, we showed that TLR4 expression in Stage IV disease was higher in tumours from people still alive compared to those that were not. While we weren't able to show this in our longitudinal OS analyses, this may reflect the lack of power when breaking down our cohort of 488 into specific stages. This heterogeneity in how TLR4 may act to regulate overall survival for Stage I vs Stage IV disease is likely to reflect the differences in how these disease stages are treated. Stage I disease is almost always treated with surgery, but no cytotoxic therapy, whereas stage IV disease will certainly contain cytotoxic therapy. TLR4 is considered to exert its impact on treatment outcomes via its ability to modulate immunogenic cell death (Fang et al. 2014; Kroemer et al. 2013). Immunogenic cell death acts in concert with direct cytotoxicity, and collectively results in more thorough tumour clearance, and thus long-term survival. As such, higher TLR4 expression would theoretically confer a larger immune response and thus better response in late-stage CRC. This is supported by the Isambert et al. study (2013) which found that increased activation of TLR4 via a lipid A analogue (OM-174) enhanced inflammatory anti-tumour response in metastatic CRC and improved clinical outcomes (Isambert et al. 2013). Furthermore, data from Huang and colleagues (2018) showed improved DFS in people living with late-stage rectal cancer with increased activation of TLR4 via HGMB1 binding (Huang et al. 2018).

Despite new interpretation of stage-specific roles of TLR4, we must acknowledge some limitations of our approach. Firstly, the studies included within the literature review were varied, often with low sample sizes and differing approaches to measuring TLR4 expression. Furthermore, our genetic analysis relied on previously collected data and exhibited low power when analysing within the specific CRC stages. It is also important to acknowledge that we relied solely on TLR4 tumour-expression data; whereas evidence from pre-clinical work suggests expression of TLR4 in host tissues (typically non-cancerous) may be critical in setting immune tone

of host and thus response (Li et al. 2017). Nonetheless, our findings indicate a general trend towards higher TLR4 expression being associated with favourable OS outcomes in stage IV CRC suggesting its ability to induce immunogenic cell death is critical in CRC prognosis.

Supporting information Table 5.1: literature database search strategies and publication results.

Database	Date Accessed	Search Strategy	Identified Literature
PubMed	January 2022 – February 2022	“Toll-Like Receptor 4”[mh] OR TLR4[tiab] OR Toll 4 Receptor[tiab] OR Toll Like Receptor 4[tiab] AND “Antineoplastic Protocols”[mh] OR chemotherap*[tiab] NOT ("Animals"[Mesh] NOT ("Animals"[Mesh] AND "Humans"[Mesh])) and (((((“Toll-Like Receptor 4”[mh] OR TLR4[tiab] OR Toll 4 Receptor[tiab] OR Toll Like Receptor 4[tiab]) AND (“Antineoplastic Protocols”[mh] OR chemotherap*[tiab])) AND (colon OR colo* OR bowel)) AND (cancer OR cancer* OR tumour* OR tumour)) NOT (("Animals"[Mesh] NOT ("Animals"[Mesh] AND "Humans"[Mesh])))) AND ((((“Toll-Like Receptor 4"[mh] OR TLR4[tiab] OR Toll 4 Receptor[tiab] OR Toll Like Receptor 4[tiab]) AND ("Antineoplastic Protocols"[mh] OR chemotherap*[tiab])) AND (colon OR colo* OR bowel)) AND (cancer OR cancer* OR tumour* OR tumour)) NOT (("Animals"[Mesh] NOT ("Animals"[Mesh] AND "Humans"[Mesh]))).	36 individual publications - 34 when within 2010 – 2021 timeframe - only 2 conformed with eligibility criteria
Cochrane Library	January 2022 – February 2022	“Trials AND TLR4 AND cancer and colorectal”	5 individual publications - No publications conformed with eligibility criteria

Embase	January 2022 – February 2022	“Toll Like Receptor 4”/de OR TLR4:ti,ab OR “Toll 4 Receptor*”:ti,ab OR “Toll Like Receptor 4”:ti,ab AND “Antineoplastic Protocols”:ti,ab OR chemotherap*:ti,ab OR chemotherapy/exp NOT ([animals]/lim NOT [humans]/lim) and ('toll like receptor 4'/de OR tlr4:ti,ab OR 'toll 4 receptor*':ti,ab OR 'toll like receptor 4':ti,ab) AND ('antineoplastic protocols':ti,ab OR chemotherap*:ti,ab OR 'chemotherapy'/exp) AND (colon OR colorectal OR bowel OR intestine) NOT ([animals]/lim NOT [humans]/lim) AND (cancer OR tumour OR tumour OR 'malignant neoplasm'). and ((Toll Like Receptor 4 or TLR4 or Toll 4 Receptor* or Toll Like Receptor 4) and (Antineoplastic Protocols or chemotherap* or chemotherapy) and (toll like receptor 4 or tlr4 or toll 4 receptor* or toll like receptor 4) and (antineoplastic protocols or chemotherap* or 'chemotherapy) and (colon or colorectal or bowel or intestine) and (cancer or tumour or tumour or malignant neoplasm)).af	139 individual publications - 52 when within 2010 – 2021 timeframe - 3 duplicates removed - 4 articles removed due to ineligibility with inclusion and exclusion criteria - Only 7 conformed with eligibility criteria

Supporting Information Table 5.2: Summary of clinical participant data (n = 448) from TCGA analyses. Data are n (%) unless otherwise stated. Data extracted on 6/6/2020.

Characteristic	**n (%)**
Age	
Median (Range)	68.00 (31-90)
Sex	
Male	239 (53)
Female	209 (47)
CRC stage at prognosis	
I	76 (17)
II	172 (38)
III	127 (28)
IV	62 (14)
not reported	11 (2)
TLR4 (CPM)	
Median (Range)	9.23 (3.7-2.8)
Site of resection or biopsy	
Ascending colon	87 (19)
Cecum	90 (20)
Colon, NOS	98 (22)
Descending colon	16 (4)
Hepatic flexure of colon	15 (3)
Recto sigmoid junction	8 (2)
Sigmoid colon	110 (25)
Splenic flexure of colon	5 (1)
Transverse colon	19 (4)
Vital Status	
Alive	352 (79)
Deceased	96 (21)
History of neo-adjuvant treatment	
Yes	0 (0)
No	448 (100)

Chapter 6: Intestinal TLR4 is required for tumour suppression achieved by irinotecan treatment

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The investigation on the effect of TLR4 on survival in a clinical database (chapter 5 of this thesis) identified heterogenous data and confounding variables. Furthermore, clinical data lacked information regarding the impact of the host's TLR4 expression (i.e. only presenting tumour TLR4 expression without considering host immune cell or epithelial cell TLR4 expression details). Therefore, this chapter is my third original research chapter and details the response of MC-38 tumour post-irinotecan treatment in intestinal epithelial TLR4 knockout (KO) mice (*Tlr4*$^{\Delta IEC}$) versus wild-type (WT) littermates. This work allowed for the relationship between site-specific TLR4 expression, tumour growth and chemoefficacy to be assessed in a controlled environment. This chapter is written in publication-style format, but has not yet been submitted.

Statement of Authorship

Statement of Authorship

Title of Paper	Intestinal TLR4 is required for tumour suppression achieved by irinotecan treatment
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Details the chemoefficacy response of MC-38 tumour post-irinotecan treatment in intestinal epithelial TLR4 knockout (KO) mice (*Tlr4*$^{\Delta IEC}$) versus wild-type (WT) littermates. This chapter is written in publication-style format, but has not yet been submitted

Principal Author

Name of Principal Author (Candidate)	Mrs. Elise Ellen Crame		
Contribution to the Paper	Developed research question and proposal. Conducted *in-vivo* animal work and molecular benchtop experimental work. Primary author of manuscript style chapter, responsible for writing manuscript, data analysis and creation of all figures.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/10/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor Joanne M. Bowen		
Contribution to the Paper	Provided crucial feedback and significantly helped forming research approach. 5%		
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Abstract

The efficacy of chemotherapeutic agents, including irinotecan, is increasingly linked to immune activity of the host and the tumour. This represents as a possible obstacle for emerging supportive care strategies that aim to modulate immune responses that exacerbate chemotherapy-induced side effects, especially those targeting TLR4. While previous evidence has shown the promising potential of TLR4 as a target to reduce gastrointestinal (GI) toxicity caused by irinotecan, this is undermined by impaired treatment efficacy when TLR4 is indiscriminately inhibited or globally knocked out (*Tlr4*$^{-/-}$). This overlap in the mechanistic control of both the toxicity and efficacy of irinotecan underscores the importance of dissecting TLR4's contribution to each treatment outcome. In particular, the differential contribution of TLR4 expressed in various cell populations should be investigated to understand if its site-specific expression can be exploited to improve GI toxicity without impacting treatment efficacy. As such, this study examined tumour growth and response to irinotecan in wild-type (WT) and intestinal epithelial conditional TLR4 knockout (KO) (*Tlr4*$^{\Delta IEC}$) mice. Male and female WT and *Tlr4*$^{\Delta IEC}$ mice, bearing MC-38 subcutaneous colorectal tumours received a single intraperitoneal dose of irinotecan (270 mg/kg i.p.) or vehicle (n = 6 per group). The primary outcome for chemoefficacy was change in tumour burden (cm^3/g body weight) evaluated over 72 hrs, with secondary outcomes including quantification of the proliferation marker Ki-67, apoptosis marker caspase-3, and immune markers CD45, $CD11b^+$ and TLR4 in tumour tissue via immunohistochemical staining. Irinotecan caused significant decrease in tumour burden in WT mice compared to vehicle mice (P = 0.0022 (48 hrs), P = 0.0055 (72 hrs)). In contrast, no difference in tumour burden was noted in *Tlr4*$^{\Delta IEC}$ treated with irinotecan versus vehicle controls. No difference was observed in Ki-67, caspase-3, CD45 or TLR4 staining, however a significant reduction in $CD11b^+$ immune cell

infiltration in tumour tissue was identified in *Tlr4*$^{\Delta IEC}$ treated with irinotecan compared to vehicle controls and WT mice treated with irinotecan (P = 0.0478 and 0.0197, respectively). In conclusion, TLR4 expressed on intestinal epithelial cells may modulate the efficacy of irinotecan.

Introduction

The overlap in mechanisms that govern both the efficacy and toxicity of chemotherapies has been, and continues to be, a significant challenge in the development of interventions that aim to prevent or manage the side effects of chemotherapy. Interventions designed to protect intestinal mucosa during chemotherapy treatment commonly fail to translate clinically due to unforeseen impacts on tumour kill (Apetoh et al. 2007; Coller et al. 2017). While historically this has largely been attributed to direct cytotoxic damage, which drives tumour kill and collateral tissue injury, it is becoming increasingly clear that these events share other mechanisms. In particular, the contribution of innate immune responses and inflammation to chemoefficacy and toxicity has become better characterised, and as such, the interplay between toxicity and efficacy is inherently more complex to navigate (Apetoh et al. 2007; Ghiringhelli et al. 2009).

Immunogenic cell death (ICD), a process in which immune-mediated mechanisms result in cell death (Apetoh et al. 2007; Ghiringhelli et al. 2009), is increasingly recognised to enhance the anti-tumour efficacy of a range of chemotherapeutic agents (Rapoport & Anderson 2019). Previous works in colorectal cancer (CRC) models have shown a clear relationship between ICD and chemoefficacy. Early pre-clinical evidence found oxaliplatin treatment triggers ICD through immune exposure to calreticulin and high mobility group box 1 (HMGB1), resulting in activation of the NOD-like receptor Family Pyrin Domain Containing 3 (NLRP3) inflammasome in dendritic cells (Ghiringhelli et al. 2009). This resulted in an increased anti-tumour response by the host adaptive immunity (Ghiringhelli et al. 2009). Recent evidence has solidified this notion, showing oxaliplatin activates ICD pathways in both *in vitro* colorectal cancer cell lines and *in vivo* CRC tumour-bearing mice (Limagne et al. 2019). Interestingly, the addition of the immune regulating protein associated with

ICD, anti-programmed cell death protein 1 (PD-1), to trifluridine/tipiracil plus oxaliplatin significantly improved mouse survival, compared to each treatment individually (Limagne et al. 2019). In addition, associations have also been identified between single nucleotide polymorphisms in hallmark genes of ICD (*ANXA1* rs1050305 and *LRP1* rs1799986) and oxaliplatin efficacy (Arai et al. 2020). While no associations between ICD genetic markers and irinotecan efficacy were found in this study (Arai et al. 2020), a separate body of evidence suggests that irinotecan does trigger ICD in CRC. In a multi-treatment study, various chemotherapies including 5-FU, oxaliplatin and irinotecan combined with X-ray, were highly effective in increasing expression of ICD markers receptor interacting protein, interferon regulatory factor 5 and tumour protein p53 (Frey et al. 2012). Recent evidence has shown irinotecan induces common ICD pathways, with irinotecan-treated CT26 cells displaying increased release of calreticulin, HMGB1 and adenosine triphosphate (ATP) compared to untreated cells (He et al. 2021). Collectively these data strongly suggest that irinotecan's efficacy relies at least in part on the host's immune response. Importantly, a clear relationship between the intestinal microenvironment, GI microbiota and effective anti-cancer ICD has emerged. Previous research using germ-free mouse models have found that having a disrupted GI microbiota reduced the infiltration of myeloid-derived immune cells into the tumour, which resulted in decreased chemoefficacy (Iida et al. 2013).

Whilst the mechanisms that govern ICD continue to be unravelled, the contribution of toll-like receptor 4 (TLR4) is increasingly clear. TLR4 is an pattern recognition receptor of the innate immunity, known to recognise lipopolysaccharide (LPS) from gram-negative bacteria among a host of varying ligands (Kawasaki & Kawai 2014). Upon ligand binding, TLR4 activation initiates a strong inflammatory response, commonly suggested to regulate host immunity (Comalada & Xaus 2013) and

potentially mediate the development of CRC tumours (Lee, Wu & Shiau 2010). Due to this potent immune regulation, TLR4 has been implicated in the efficacy of chemotherapy, especially in the treatment of CRC. However, while a range of studies have looked at this, the current evidence base is contradictory. Previous work highlights the role of TLR4 in anti-cancer immunity driven by dendritic cell activation. A crucial *in vivo* study used a cell-based vector combined with LPS to investigate the TLR4-based anti-tumour response in mice (Davis et al. 2011). Intra-tumoral addition of LPS to the cell-based vector increased anti-tumour immunity compared to the vector alone. Furthermore, mice treated with the LPS-enriched vector showed increased $CD4^+$ and $CD8^+$ T cell infiltration and lymph tissue showed increased numbers of activated $CD80^+$ dendritic cells (Davis et al. 2011). A later study by Fang and colleagues found that TLR4 expression in dendritic cells is crucial for the activation of the anti-tumour immune response, caused by CRC cell-derived danger associated molecular patterns including HMGB1 and heat shock protein 70 (Fang et al. 2014). Collectively these findings suggest TLR4 activation is strongly linked to induction of ICD pathways, which may highlight a potential link between innate immune response and efficacy of chemotherapy.

While these findings outline the anti-tumour potential of TLR4, the majority of current evidence examining the effects of TLR4 signalling on tumour survival is contradictory in nature. We have previously highlighted the heterogeneous impact of TLR4 signalling on patient survival and tumour recurrence (chapter 5 of this thesis) (Crame et al. 2022). While the anti-tumour potential of TLR4 signalling is clear, the complexity of TLR4 signalling in CRC is mirrored in pre-clinical research, with TLR4 also being associated with pro-tumour outcomes. For example, the contribution of TLR4 and its downstream signalling molecule MyD88 to spontaneous tumorigenesis in the GI tract was recently investigated using global TLR4 knockout (KO) (*Tlr4*$^{-/-}$) and

global MyD88 KO (*MyD88*$^{-/-}$) C57BL/6 mouse models (Koliaraki et al. 2019). Of interest, the TLR4/MyD88 signalling pathway was shown to activate intestinal mesenchymal cells and cancer-associated fibroblasts, promoting carcinogenesis and spontaneous tumour growth in the intestine (Koliaraki et al. 2019). This is supported by further research showing that HMGB1 activation of immune expressed TLR4 initiates a cascade of angiogenic mediators stimulating vascularisation of the tumour and enhancing CRC survival (Zhu et al. 2015).

These data highlight the significant heterogeneity in TLR4s regulation of tumour growth and treatment efficacy. However, a glaring omission from all these studies is the lack of appreciation of TLR4s activity in relation to its cellular expression. It was hypothesised that site-specific TLR4 activity is an overlooked aspect in the contribution of TLR4 to tumour growth and treatment efficacy, and dissecting this control may shed light on the complexity of TLR4-tumour interactions. As such, this study aimed to investigate the impact of intestinal epithelial TLR4 on irinotecan efficacy using the MC-38 tumour-bearing, intestinal epithelial conditional TLR4 KO mouse model (*Tlr4*$^{\Delta IEC}$).

Methods

Ethics and husbandry

This study was conducted in accordance with ethical approval of the University of Adelaide Animal Ethics Committee (M-2020-028) and the University of Adelaide Institutional Biosafety Committee (IBC approval number 14254). The study complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2013). Male and female WT C57BL/6 (n = 6-7 per treatment group) and *Tlr4*$^{\Delta IEC}$ (n = 6-8 per treatment group) mice aged 8–12 weeks were housed in ventilated cages in groups of 3–6 animals per cage with a 12 hrs light/dark cycle and access to irradiated standard mouse chow and sterile water

ad libitum. Mice were euthanised via CO_2 exposure and cervical dislocation prior to dissection.

Cell culture and tumour model development

We used the mouse MC-38 cell line as a representative colorectal tumour with immune infiltration (Grasselly et al. 2018; Xu et al. 2019). Based on the growth patterns of subcutaneous MC-38 tumours by Grasselly and colleagues (2018), mice were inoculated with 2 x 10^6 MC-38 cells administered subcutaneously. For detailed description of MC-38 cell growth conditions and tumour model development please refer to chapter 4 of this thesis.

In vivo study design

MC-38 tumour-bearing male and female (8-12 weeks old) *Tlr4*$^{\Delta IEC}$ and wild-type (WT) mice of a C57Bl/6 background were used for this study. For detailed information regarding *Tlr4*$^{\Delta IEC}$ breeding and animal husbandry please refer to chapter 3. A total of 27 mice were assessed, and randomly assigned to the following groups, WT vehicle (n = 6), WT irinotecan (n = 7), *Tlr4*$^{\Delta IEC}$ vehicle (n = 6) and *Tlr4*$^{\Delta IEC}$ irinotecan (n = 8), with equal numbers of male and female mice per group. Irinotecan treated groups received a single 270 mg/kg intraperitoneal dose of irinotecan hydrochloride prepared in a sorbitol/lactic acid buffer as previously described (chapter 4). Vehicle treated mice received a volume equivalent dose of the sorbitol/lactic acid buffer only. Tumours were assessed daily using digital callipers and their volume (cm^3) determined using the following equation: (width squared x length)/2. *Tumour burden* was defined as tumour volume (cm^3) adjusted for body weight (g) and represented as a percentage (%, cm^3/g). Mice were euthanised by CO_2 exposure and cervical dislocation 72 hrs after irinotecan/vehicle treatment. Tumours were excised, weighed and prepared for processing in which half the tumour was fixed in 10% neutral-

buffered saline and embedded into paraffin wax for histological analysis and the other half snap frozen in liquid nitrogen and stored at -80°C.

Haematoxylin and eosin (H&E) staining

Formalin fixed, paraffin embedded (FFPE) tumours were cut on a rotary microtome (4 µm), mounted onto glass slides (Menzel-Gläser, Braunschweig Germany) and stained with haematoxylin and eosin (H&E) as previously described in chapter 3 of this thesis (Crame et al. 2021). Slides were imaged using a NanoZoomer 2 Digital Slide Scanner (Hamamatsu Photonics, Shizuoka Japan). Tissue analyses were conducted using the NDP.view2 image viewer program (Hamamatsu Photonics, Shizuoka Japan). Analyses included assessment of mitotic index (cell count of mitotic figures in up to 10, 2 mm^2 fields of view, average per tissue used for statistical analysis), apoptotic index (cell count of apoptotic bodies in 10, 2 mm^2 fields of view, average per tissue used for statistical analysis) and assessment of necrotic tissue (as % necrotic tissue of whole tumour).

Immunohistochemisty and Immunofluorescence

Immunohistochemistry (IHC) was performed on 4 µm sections of tumour tissue mounted onto FLEX microscope slides (Flex Plus Detection System, Dako, Denmark; #K8020). IHC analysis was performed for Ki-67, a marker of cell proliferation, and proenzyme and cleaved caspase-3, a marker of apoptosis. IHC analysis was performed using Dako reagents on an automated machine (AutostainerPlus, Dako, Denmark) following standard protocols supplied by the manufacturer.

Immunofluorescent (IF) staining of 4 µm sections of tumour tissue mounted onto FLEX microscope slides (Dako) for immune markers $CD11b^+$, CD45 and TLR4 was also conducted. Please see Table 6.1 for protein block and antibody details. For both IHC and IF stains, sections were deparaffinised in histolene and rehydrated through graded ethanols before undergoing heat-mediated antigen retrieval using either an

EDTA/Tris buffer (Ki-67, caspase-3 and $CD11b^+$, 0.37 g/L EDTA, 1.21 g/L Tris; pH 9.0) or citrate buffer (CD45 and TLR4, citric acid 1M, trisodium citrate 1M; pH 6.0). Retrieval buffer was preheated to 65°C using the Dako PT LINK (pre-treatment module). Slides were immersed in the buffer and the temperature raised to 97°C for 20 min. After returning to 65°C, slides were removed and placed in the Dako AutostainerPlus and stained following manufacturer's guidelines.

Table 6.1: Immunostaining blocking solutions and antibodies

	Blocking Solutions	Primary Antibody	Secondary Antibody
IHC – **Ki-67**	FLEX peroxidase block plus a serum-free protein block (Dako, Denmark; #X0909).	Abcam #ab16667, 1:100 dilution in EnVision™ FLEX Antibody Diluent (Dako, Denmark; #K8006)	EnVision™ FLEX+ Anti-Rabbit (Dako, Denmark; #K8019)
IHC – **Caspase-3**	FLEX peroxidase block plus a serum-free protein block (Dako, Denmark; #X0909).	Abcam #ab4051, 1:100 dilution in EnVision™ FLEX Antibody Diluent (Dako, Denmark; #K8006)	EnVision™ FLEX+ Anti- Rabbit (Dako, Denmark; #K8019)
IF – **CD11b^{+}**	4% BSA	Abcam #ab133357, 1:1000 dilution in 1% BSA	AlexaFluor 488 anti-rabbit in 1 x PBS, 1% BSA and 2% FBS
IF – **CD45**	5% goat serum, 2.5% BSA in 1 x PBS	Invitrogen #14-0451-82, 1:200 dilution in 2.5% goat serum and 1.25% BSA	AlexaFluor 657 anti-rat in 1 x PBS, 1% BSA and 2% FBS
IF – **TLR4**	5% goat serum, 2.5% BSA in 1 x PBS	Abcam #ab13556, 1:100 dilution in 2.5% goat serum and 1.25% BSA	AlexaFluor 488 anti-rabbit in 1 x PBS, 1% BSA and 2% FBS

*Abbreviations: BSA bovine serum albumin, FBS foetal bovine serum, PBS phosphate buffered saline.

For IHC, endogenous peroxidase was blocked using the FLEX peroxidase block followed by a serum-free protein block (Dako, Denmark; #X0909). Primary antibodies were suspended in the EnVision™ FLEX Antibody Diluent (Dako, Denmark; #K8006) and applied for 60 min. Negative controls had the primary antibody omitted. The EnVision™ FLEX+ Rabbit (Dako, Denmark; #K8019) was then applied for 60 min before DAB was used to visualise the target protein. Slides were then counterstained in Lillie-Mayers Haematoxylin, dehydrated in graded ethanols and coverslipped with D.P.X neutral mounting medium (Sigma-Aldrich, St Louis USA). Slides were scanned using a NanoZoomer (Hamamatsu, Japan) and analysed using the NDP.view2 image viewer program (Hamamatsu Photonics, Japan).

IF stains were counterstained with DAPI (1 µg/mL) (Sigma-Aldrich, St Louis USA) coverslipped with Fluoroshield (Sigma-Aldrich, St Louis USA), sealed and stored in the dark at 4°C prior to imaging. Slides were imaged using the Nikon A1 Confocal Microscope using a 10 x objective. Fluorescent staining was quantified by % area stained (CD45 and TLR4) on the Fiji Image J program as previously described, in chapter 3 of this thesis. The average value of three areas of interest were used for final analysis for all IF imaged, except for $CD11b^{+}$ where the average cell count of labelled cells per three random areas of interest was taken.

Data Analysis

All data were analysed using Prism version 9.0.0 (GraphPad Software, San Diego USA). All data were deemed to be normally distributed from the D'Agostino-Pearson test and therefore analysed using a one-way or two-way analysis of variance (ANOVA) and presented as mean ± standard error of mean (SEM). Correlation between vehicle treated mice tumour TLR4 expression and percentage change in tumour burden (percent change relative to baseline) at 72 hrs was determined using simple linear regression of transformed data (X = 1/X). Correlation between

irinotecan treated mice tumour TLR4 expression and percentage change in tumour burden at 72 hrs was determined using simple linear regression of transformed (X = 1/X), normalised data. Finally, correlation between tumour TLR4 expression and Ki-67 expression for both vehicle and irinotecan groups were performed using simple linear regression of transformed data (X = 1/X). P-values < 0.05 were deemed significant.

Results

Irinotecan induces significant reduction in tumour burden in WT but not *Tlr4*$^{\Delta IEC}$ treated mice

Successful creation of a stable MC-38 tumour model was established in both *Tlr4*$^{\Delta IEC}$ and WT mice, with steady tumour growth observed in vehicle treated mice without impacting on animal welfare (Figure 6.1A). We first compared tumour growth dynamics over time in all groups. While all tumours in vehicle treated mice increased in volume over time (Figure 6.1A), this was only significant in WT mice when anlaysed using a liner mixed model with post-hoc correction for multiple comparisons (Table 6.2, row 1). Next, we compared change in tumour burden at specific time points across our treatment groups (Figure 6.1B). WT mice treated with irinotecan showed a significant decrease in tumour burden (relative to baseline) at all time points compared to vehicle treated WT mice (Figure 6.1B, P = 0.007, P= 0.0007, P = 0.002, 24, 48, 72 hrs respectively Table 6.3). In contrast, *Tlr4*$^{\Delta IEC}$ mice treated with irinotecan showed no significant change in tumour burden (relative to baseline) at any time point compared to *Tlr4*$^{\Delta IEC}$ vehicle mice (Table 6.3). *Tlr4*$^{\Delta IEC}$ irinotecan-treated mice showed a significant reduction in tumour burden at 24 and 48 hrs (P = 0.001 and P = 0.02, respectively), when compared to vehicle-treated WT mice. These findings reflect the more variable nature of the tumour growth in the *Tlr4*$^{\Delta IEC}$ mice. Of note, this significance was lost at 72 hrs (Table 6.3). Most importantly, no

difference in tumour burden was found after irinotecan in WT and *Tlr4*$^{\Delta IEC}$ mice at any time point (Table 6.3, row 5).

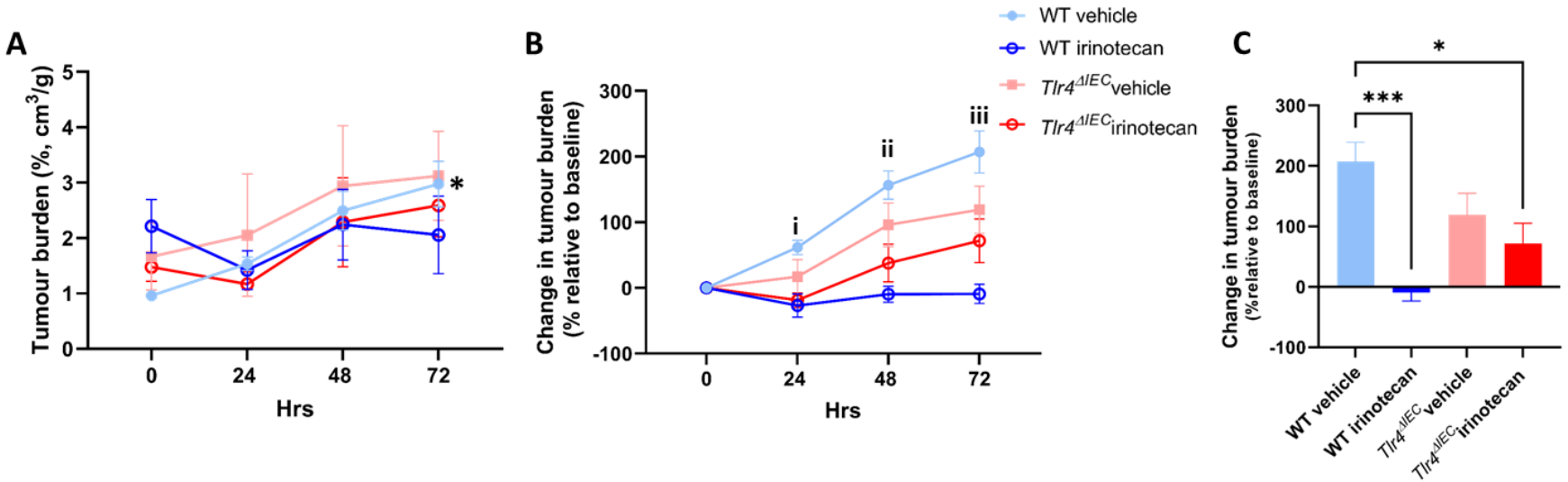


Figure 6.1: In vivo tumour growth and response to irinotecan. (A) Tumour burden (%cm^3/g) from treatment (0 hrs) to 72 hrs post-treatment *= WT vehicle (0 hrs to 72 hrs) P = 0.022. (B) Percentage change in tumour burden post-treatment in all groups, i = 24 hrs (WT vehicle vs WT irinotecan P = 0.007, WT vehicle vs *Tlr4*$^{\Delta IEC}$ irinotecan P = 0.001), ii = 48 hrs (WT vehicle vs WT irinotecan P = 0.0007, WT vehicle vs *Tlr4*$^{\Delta IEC}$ irinotecan P = 0.02) and iii = 72 hrs (WT vehicle vs WT irinotecan P = 0.002). No significant differences between other strains or treatment groups. (C) Percentage change in tumour burden at 72 hrs post-treatment, ***P = 0.0003, *P = 0.019. All data presented as mean ± SEM, n = 6 – 8 per treatment group.

Table 6.2: Change in tumour burden per strain, over time

	0 vs 24 hrs	**0 vs 48 hrs**	**0 vs 72 hrs**
WT vehicle	0.96 ± 0.77 vs 1.53 ± 0.12, **P = 0.013***	0.96 ± 0.77 vs 2.4 ± 0.34, **P = 0.025***	0.96 ± 0.77 vs 2.9 ± 0.41, **P = 0.022***
WT irinotecan	2.2 ± 0.48 vs 1.41 ± 0.35, P = 0.33	2.2 ± 0.48 vs 2.2 ± 0.64, P = >0.99	2.2 ± 0.48 vs 2.05 ± 0.69, P = 0.99
***Tlr4*$^{\Delta IEC}$ vehicle**	1.6 ± 0.6 vs 2.05 ± 1.1, P = 0.99	1.6 ± 0.6 vs 2.9 ± 1.1, P = 0.38	1.6 ± 0.6 vs 3.1 ± 0.81, P = 0.21
***Tlr4*$^{\Delta IEC}$ irinotecan**	1.5 ± 0.26 vs 1.1 ± 0.21, P = 0.49	1.5 ± 0.26 vs 2.3 ± 0.80, P = 0.81	1.5 ± 0.26 vs 2.6 ± 0.57, P = 0.26

Data presented as mean percentage change in tumour volume (cm^3) ± SEM and P value. ***** = indicating significance.

Table 6.3: Comparative tumour burden as percentage relative to baseline between groups over time

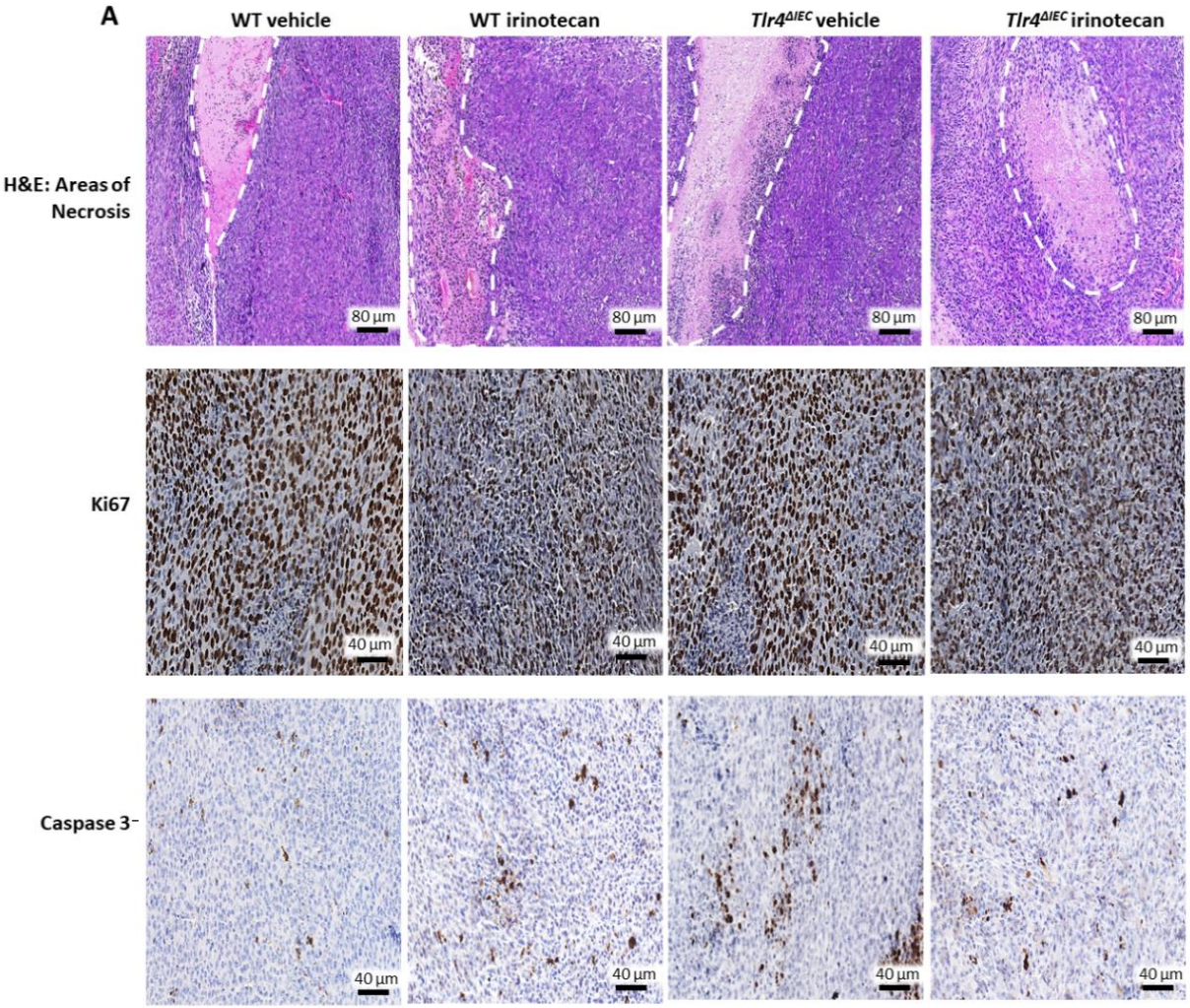
	24 hrs	48 hrs	72 hrs
WT vehicle vs WT irinotecan	61.7 ± 10.8 vs -26.8 ± 17.6, **P = 0.007***	156.6 ± 21.5 vs -9.6 ± 12.2, **P = 0.0007***	207.2 ± 32 vs -9.02 ± 14.6, **P = 0.002***
***Tlr4*$^{\Delta IEC}$ vehicle vs *Tlr4*$^{\Delta IEC}$ irinotecan**	17.2 ± 25.9 vs -18.5 ± 10.9, P = 0.61	96.3 ± 33 vs 37.8 ± 28.8, P = 0.56	119.1 ± 35.8 vs 71.8 ± 33.3, P = 0.77
WT vehicle vs *Tlr4*$^{\Delta IEC}$ irinotecan	61.7 ± 10.8 vs -18.5 ± 10.9, **P = 0.001***	156.6 ± 21.5 vs 37.8 ± 28.8 **P = 0.028***	207.2 ± 32 vs 71.8 ± 33.3, P = 0.31
WT vehicle vs *Tlr4*$^{\Delta IEC}$ vehicle	61.7 ± 10.8 vs 17.2 ± 25.8, P = 0.44	156.6 ± 21.5 vs 96.3 ± 33, P = 0.46	207.2 ± 32 vs 119.1 ± 35.86, P = 0.31
WT irinotecan vs *Tlr4*$^{\Delta IEC}$ irinotecan	-26.8 ± 17.6 vs -18.5 ± 10.9, P = 0.97	-9.6 ± 12.2 vs 37.8 ± 28.8 P = 0.466	-9.02 ± 14.6 vs 71.8 ± 33.3, P = 0.18

Data presented as mean percentage change in tumour burden relative to baseline ± SEM and P value. ***** = indicating significance.

Change in irinotecan efficacy in *Tlr4*$^{\Delta IEC}$ mice is not mediated by gross tumour cell proliferation or cell death

Histopathological assessment of the tumour microenvironment identified significant nuclear polymorphism, with numerous mitotic figures (Figure 6.2A). Nuclei ranged from round to ovoid in shape, with a moderate amount of basophilic cytoplasm and indistinct cell borders. Apoptotic bodies (the end stage of apoptosis) were found as single bodies and characterised by rounded, condensed cytoplasm with nuclear remnants, surrounded by a clear, non-staining space. Areas of necrosis were characterised by cellular shrinkage and cytoplasmic eosinophilia with pyknotic nuclei (small, round, hyperchromatic nuclei), which progressed to nuclear fragmentation (karyorrhexis) and eventual disappearance, the cytoplasm becoming a coagulated mass with little evidence of cellular detail and, sometimes, small nuclear fragments (Figure 6.2A, H&E areas of necrosis). A significant difference was noted in the number of mitotic figures in vehicle and irinotecan treated *Tlr4*$^{\Delta IEC}$ mice (Figure 6.2B, *Tlr4*$^{\Delta IEC}$ vehicle versus irinotecan (mean ± SEM): 14 ± 3.14 versus 4.3 ± 1.3, P = 0.0106). No significant difference was found between treatment groups or strains for apoptotic bodies count (Figure 6.2C) or percentage area of tumour tissue undergoing necrosis (Figure 6.2D).

To complement the histological analysis of cell division and death, immunohistochemistry for the cell proliferation marker, Ki-67, and the apoptosis marker, caspase-3, was conducted. No difference was found between groups for either % area stained of Ki-67, or, caspase-3 positive cells (Figure 6.2E, F respectively).


A
WT vehicle
WT irinotecan
Tlr4ΔIEC vehicle
Tlr4ΔIEC irinotecan
H&E: Areas of Necrosis
Ki67
Caspase 3
80 μm
40 μm

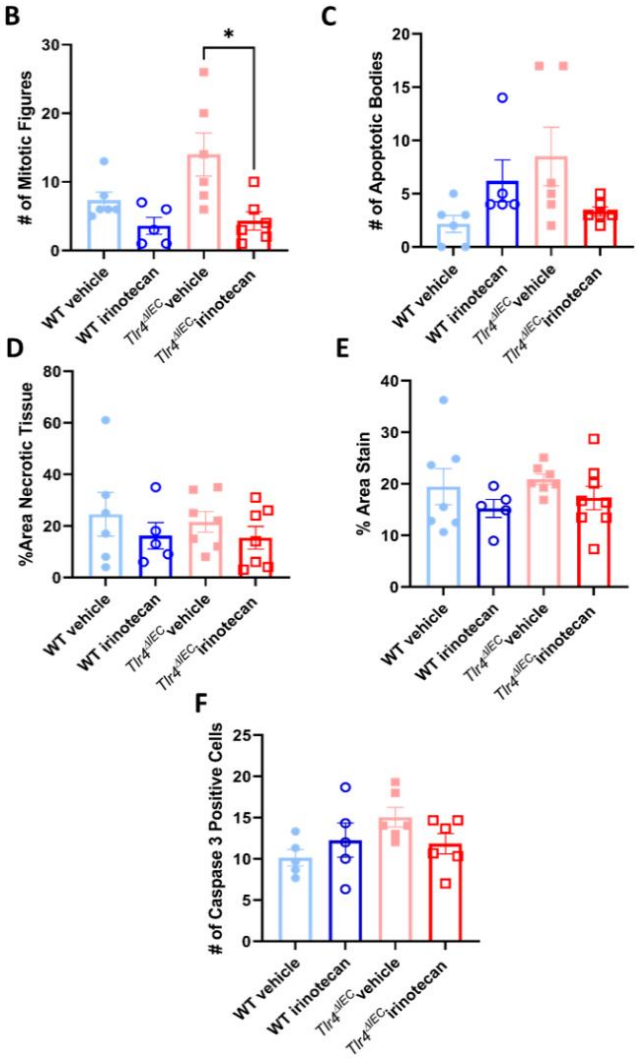

B
# of Mitotic Figures
C
# of Apoptotic Bodies
D
%Area Necrotic Tissue
E
% Area Stain
F
# of Caspase 3 Positive Cells
WT vehicle
WT irinotecan
Tlr4ΔIEC vehicle
Tlr4ΔIEC irinotecan
*

Figure 6.2: Histological analysis of tumour tissue cell survival (Ki-67 (A, E) and mitotic figures (B)) versus cell death (caspase-3 (A, F), apoptotic bodies (C) and necrotic tissue (A, D)). (A) H&E staining of tumour tissue (images taken at 10 x magnification, dashed white line areas indicating areas of necrosis) and visual representation of IHC staining for Ki-67 and caspase-3 (images taken at 20 x magnification), (B) cell count of mitotic figures, average of 10 x 2mm^2 fields of view (*P = 0.0106), (C) cell count of apoptotic bodies average of 10 x 2mm^2 fields of view, (D) % area of necrotic tissue, (E) Ki-67 quantification via % area stained and (F) cell count of caspase-3 positive cells. All data presented as mean ± SEM, n = 5-6 assessable per treatment group

Loss of intestinal TLR4 may reduce efficacy of irinotecan via reduced infiltration of CD11b^{+} immune cells in tumour tissue

Co-localisation of CD45 and TLR4 was noted via IF staining (Figure 6.3A, B), however separate staining of CD45 and TLR4 was also observed in tumour tissues as shown in representative low magnification images (Figure 6.4). Quantitative expression of CD45 and TLR4 did not differ between treatment groups or strains (Figure 6.3C and 6.3D, respectively). A significant decrease in CD11b^{+} immune cell infiltration in tumour tissue was observed in irinotecan treated *Tlr4*$^{\Delta IEC}$ mice compared to vehicle *Tlr4*$^{\Delta IEC}$ mice (P = 0.0291) and irinotecan treated WT mice (P = 0.0256) (Figure 6.5). A significant positive correlation was observed between TLR4 tumour expression and percent change in tumour burden compared to baseline in WT and TLR4 vehicle mice (Fig 6.6A, P = 0.037). No significant correlation was found between TLR4 expression and Ki-67 expression in vehicle treated mice (Figure 6.6C). No significant correlation was found between TLR4 tumour expression and percent change in tumour burden at 72 hrs, or for TLR4 expression and Ki-67 expression, for irinotecan treated mice (Figure 6.6B, D respectively).

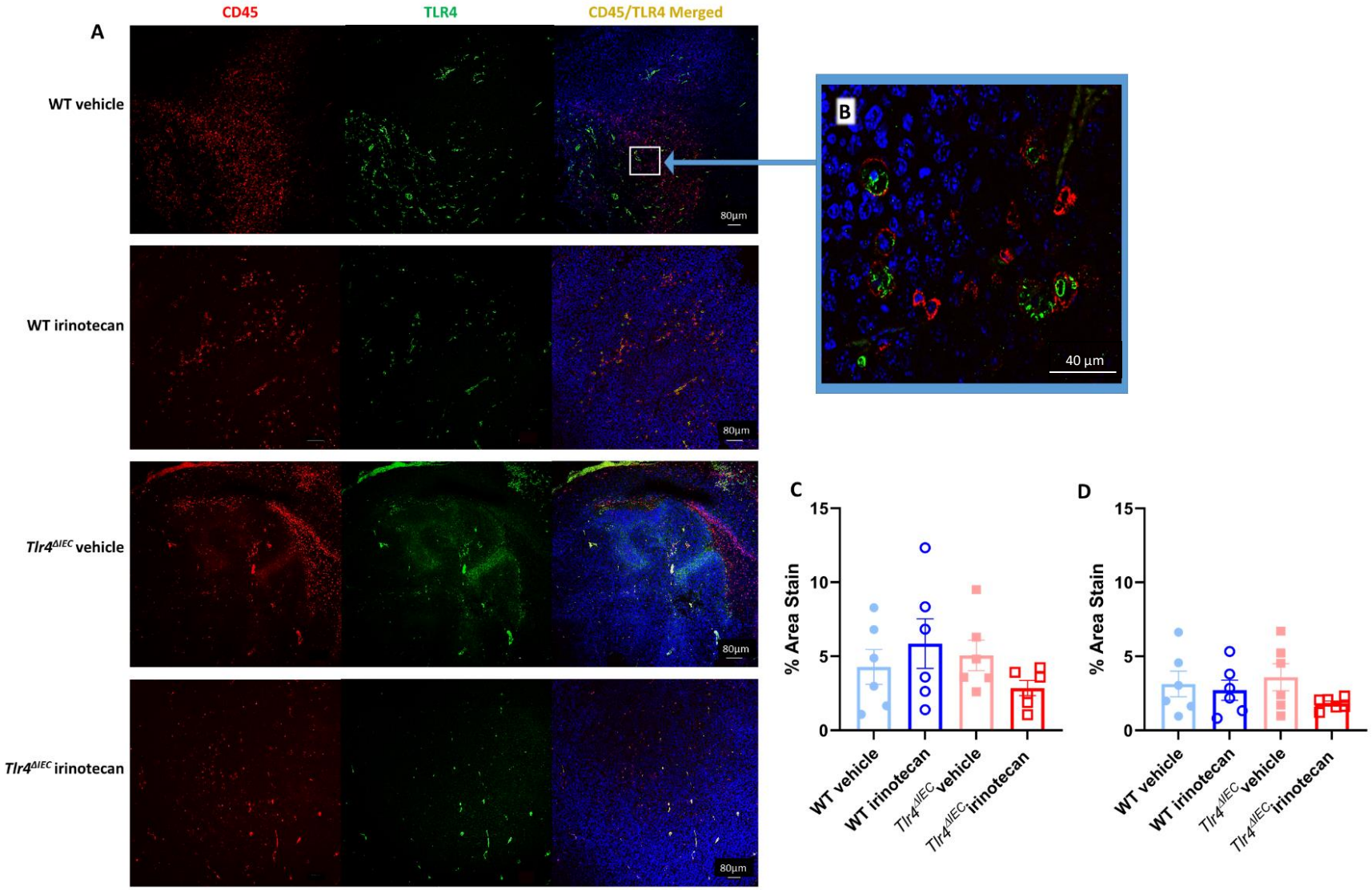

A
CD45
TLR4
CD45/TLR4 Merged
WT vehicle
WT irinotecan
Tlr4ΔIEC vehicle
Tlr4ΔIEC irinotecan
80µm
B
40 µm
C
% Area Stain
15
10
5
0
WT vehicle
WT irinotecan
Tlr4ΔIEC vehicle
Tlr4ΔIEC irinotecan
D
% Area Stain
15
10
5
0
WT vehicle
WT irinotecan
Tlr4ΔIEC vehicle
Tlr4ΔIEC irinotecan

Figure 6.3: IF staining of tumour tissue for immune markers, CD45 (red) and TLR4 (green) with DAPI counterstain of nuclei (blue) (A), 10 x magnification. White boxes indicate co-labelled cells (B) High magnification representative image of co-labelled cells, 40 x magnification. Quantification of CD45 (C) and TLR4 (D) via % area stained, average score of 3 fields of view at 10 x magnification use for analysis. All data presented as mean ± SEM, n = 6 assessable per treatment group.

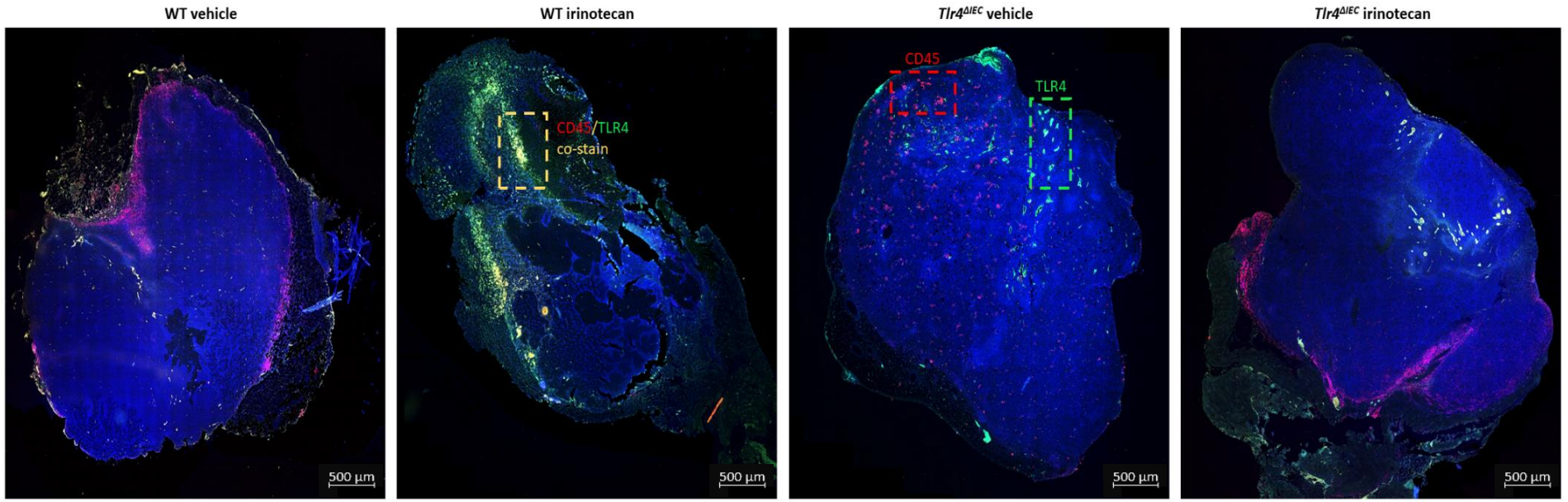


Figure 6.4: Representative low magnification IF images of whole tumour for CD45 (red) and TLR4 (green) co-staining for WT vehicle, WT irinotecan, *Tlr4*$^{\Delta IEC}$ vehicle and *Tlr4*$^{\Delta IEC}$ irinotecan, to highlight variability of tumour shape and immune infiltration between tumour samples. Tissues counterstained with DAPI (blue) for visualisation of cell nuclei. Areas of independent CD45 (red box) and TLR4 (green box) and co-localised CD45/TLR4 (orange box) staining indicated.

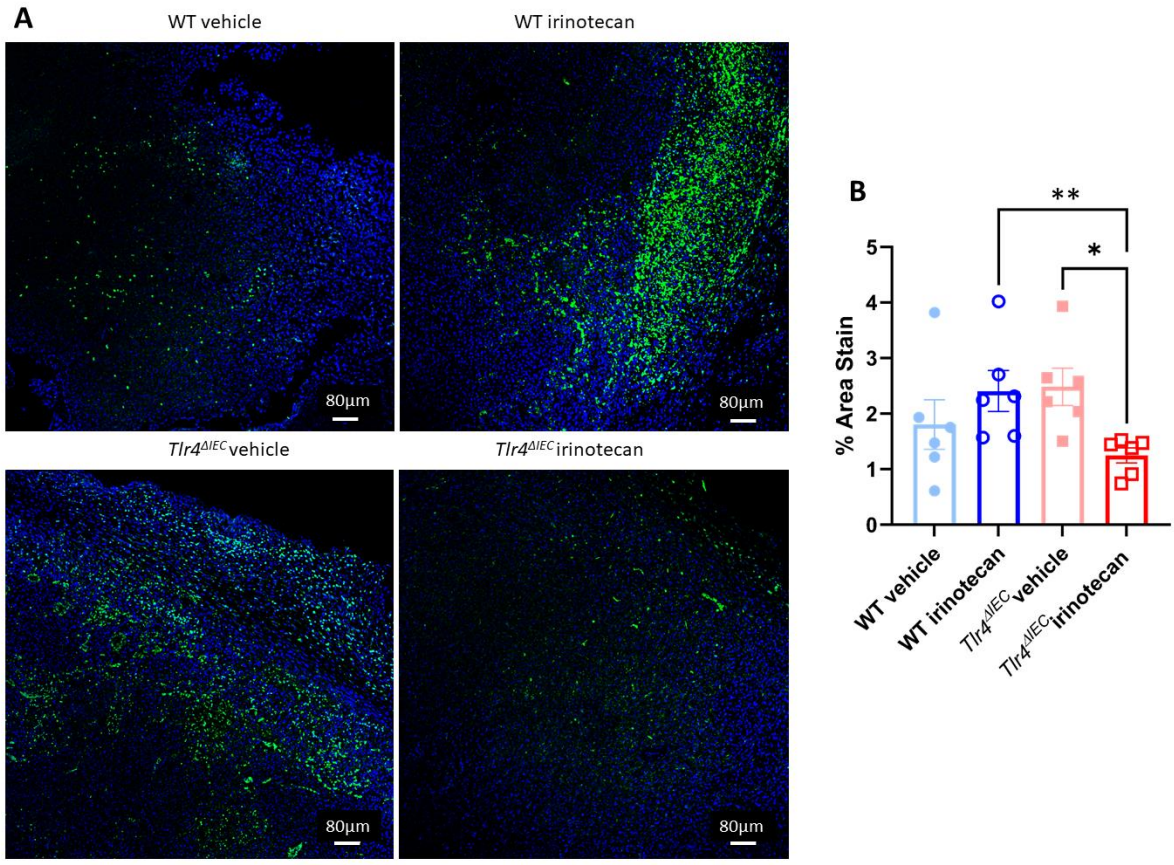


Figure 6.5: Immunofluorescent staining of tumour tissue for immune marker, CD11b+ (green) with DAPI counterstain of nuclei (blue) (A). (B) Quantification of $CD11b^{+}$ positive cells via % area stained, average score of 3 fields of view at 10 x magnification use for analysis (*P = 0.0291, **P = 0.0256). All data presented as mean ± SEM, n = 6 assessable per treatment group.

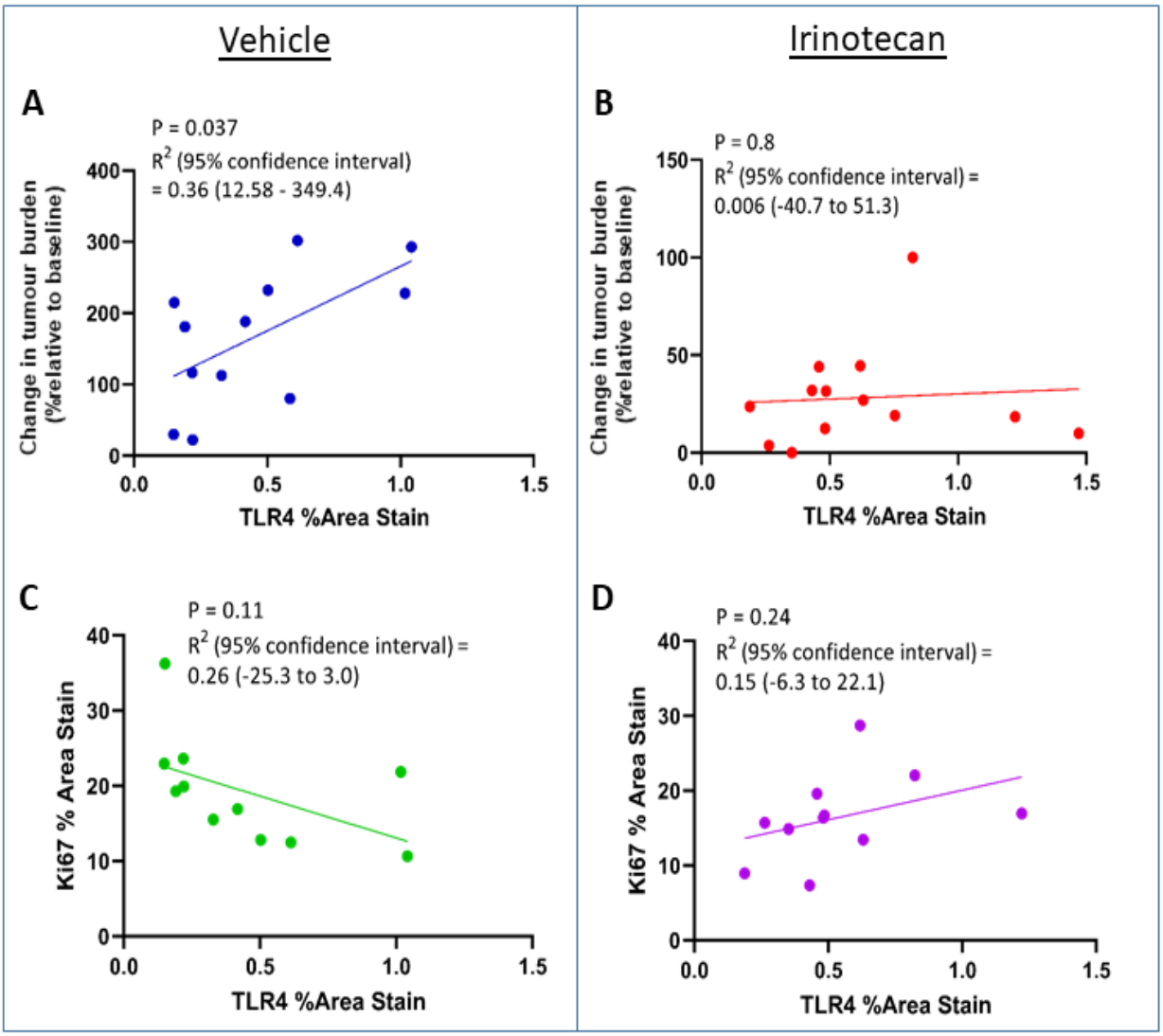


Figure 6.6: Correlation analysis between TLR4 and a marker of cell proliferation (Ki-67) and clinical tumour outcome (percentage change in tumour burden, % relative to baseline at 72 hrs post-intervention). Correlation between tumour burden and TLR4 for vehicle (A, P = 0.037, R^2 (95% CI) 0.36 (12.58 – 349.4)) and irinotecan-treated mice (B, P = 0.8, R^2 (95% CI) 0.006 (-40.7 – 51.3)). Correlation between TLR4 and Ki-67 for vehicle (C, P = 0.11, R^2 (95% CI) 0.26 (-25.3 – 3.0)) and irinotecan-treated mice (D, P = 0.24, R^2 (95% CI) 0.15 (-6.3 – 22.1).

Discussion

Ensuring that chemotherapy efficacy is maintained while investigating supportive care interventions must be at the forefront of current research, as failure to acknowledge this has the potential to substantially impact the translation of findings into safe clinical practice. In the case of TLR4-directed interventions, this is especially critical as mounting evidence suggests its activation enhances chemoefficacy to enhance tumour response. Here we show, for the first time, that intestinal TLR4 appears to potentially modulate tumour growth dynamics and reduce the durability of irinotecan's anti-tumour efficacy in mice.

This research was the first to characterise MC-38 tumour growth patterns pre- and post-irinotecan treatment in *Tlr4*$^{\Delta IEC}$ and WT mice. This work also shows that MC-38 CRC tumours contain tumour-infiltrating leukocytes, including those specific to the innate immune system. This *in vivo* data suggests deletion of intestinal epithelial TLR4 may reduce chemoefficacy of single dose irinotecan for MC-38 CRC in mice. Using this model, I have made substantial inroads into understanding the complex interplay between the host, the tumour and chemotherapy. A key finding from this study was reduced efficacy of irinotecan in *Tlr4*$^{\Delta IEC}$ mice. It was observed that both *Tlr4*$^{\Delta IEC}$ and WT mice had comparable initial responses to irinotecan, no significant difference in tumour burden at 24 hrs after irinotecan treatment between the strains. However, while this response was maintained in WT mice, the tumours in *Tlr4*$^{\Delta IEC}$ mice regained their growth from 48-72 hrs. This suggests that the ***acute cytotoxic*** effect of irinotecan (i.e. within 24 hours) is ***independent*** of intestinal TLR4. Instead, the ***maintenance*** of that response is ***dependent*** on intestinal TLR4. This suggests that intestinal TLR4 sets the immune tone that ultimately dictates the duration of tumour suppression and the durability of irinotecan's efficacy.

When considering the mechanisms by which intestinal TLR4 may modulate irinotecan's anti-tumour efficacy, two potential mechanisms can be considered. Firstly, it is known that irinotecan's active metabolite, SN-38, directly binds to TLR4 (Wong et al. 2019). It is known that SN-38 is excreted into the lumen of the GI tract in high concentrations, as such, this is a plausible mechanism by which intestinal TLR4 is augmenting irinotecan's efficacy through its downstream inflammatory signals (Parvez et al. 2021). Secondly, other intestinal luminal products that are either: i) physiologically produced to determine baseline immune tone (James et al. 2020), or ii) pathologically increased in response to irinotecan to dictate an immune response (Formica et al. 2013; Peng, H et al. 2021), may be also be activating TLR4. In any case, it is the downstream impacts of intestinal TLR4 activation that appear to dictate the maintenance of irinotecan's initial effect on the tumour. In light of the finding that *Tlr4*$^{\Delta IEC}$ mice treated with irinotecan has lower expression of CD11b^{+} compared to WT irinotecan treated mice and *Tlr4*$^{\Delta IEC}$ vehicle mice, I suggest the latter is most likely. It appears that intestinal TLR4 has the capacity to modulate immune cell infiltration into the tumour and hence it's endogenous immune response. Without this response, there is potentially a less potent inflammatory response, allowing the tumour cells to re-populate at a higher rate. This clearly supports a role for intestinal TLR4 influencing ICD, however, more in-depth investigation of ICD markers is required.

When investigating how intestinal TLR4 influences tumour responses, the role of the intestinal barrier must be considered. We now know that intestinal barrier dysfunction and consequent influx of gastrointestinal microbiota to the luminal space is linked to tumour development (Gori et al. 2019; Lu et al. 2019). This phenomenon may also be heightened by intestinal bacteria also binding to TLR4 receptors on epithelial, immune and nerve cells (Wardill et al. 2015). In addition, this mechanism would

further explain the importance of a homeostatic level of intestinal barrier permeability, allowing for bacterial translocation for immune activation and ICD (Hayes et al. 2018). Interestingly, anecdotal evidence supports this notion, with mucosal barrier injury associated with increased chemotherapy treatment efficacy, presumably by enhancing bacterial translocation and systemic immune activation (Iida et al. 2013).

While our data strengthens a potential relationship between intestinal epithelial TLR4 and chemoefficacy in CRC, this study does present with some limitations. We first recognise the small sample size within each treatment group. However, upon further power analyses it has been confirmed that all studies were adequately powered to detect differences between groups. Furthermore, we recognise the need for more in-depth immune investigations using a comprehensive immune panel to better characterise the immune profiles in these tumours, through the use of flow cytometry, as $CD11b^{+}$ and CD45 markers are inclusive of various immune subsets. In addition, ICD pathways would need to be directly measured in this model, in order to further understand the causative mechanisms behind the observed change in tumour burden between irinotecan treated groups. This would be done via analysis of ICD markers including; NLRP3, caspase-1, calreticulin, HMGB1, ATP, ANXA1, and type I IFN.

Furthermore, while significant to foundational research, analysing a single-dose model of irinotecan on CRC response does not reflect the clinical scenario, which often includes multiple rounds of combined chemotherapy regimens (Fuchs, Mitchell & Hoff 2006). Testing tumour response to multiple doses of chemotherapy combined with a longer time frame would more accurately represent the clinical setting (eviQ 2021), and may allow for more in-depth chemoefficacy and survival analyses. Furthermore, the metabolism of irinotecan and its metabolite SN-38, was not assessed in the *Tlr4*$^{\Delta IEC}$ model in this thesis. Future investigations of blood serum and

tumour concentrations of irinotecan and SN-38, are required to ensure that deletion of TLR4 from the intestinal epithelial layer does not impact on the rates of drug metabolism. This would ensure that the observed differences in chemoefficacy of irinotecan were wholly due to the site-specific expression of TLR4 and not a drug exposure effect. In addition, recent clinical literature has highlighted the importance of tumour stage and TLR4 expression, with high TLR4 expression being detrimental to CRC patient survival in Stage I disease, but beneficial to survival in Stage IV disease, please see chapter 5 of this thesis for details (Crame et al. 2022). Given irinotecan is most commonly prescribed for late-stage and metastatic CRC (eviQ 2021), repeating this study in a model of metastatic cancer may better represent the clinical scenario and result in better translational findings.

We note that further in-depth investigations into cell-specific immune invasion (for example, macrophage versus dendritic cell populations) is required to gain more specific understanding of the exact immune cell types involved in the anti-tumour response. The knowledge gained in this study supports enhancing ICD pathways in order to improve irinotecan efficacy, as greater quantification of $CD11b^+$ immune cells were found in tumours which responded well to irinotecan (WT irinotecan). However, in contrast, increased macrophage (a subset of immune cells included in bulk $CD11b^+$ analysis) invasion in the tumour microenvironment of ovarian cancers has been linked with poor patient prognosis (Hong et al. 2018). While contradictory to our findings, it is important to note that this previous research was conducted in ovarian cancer, not CRC. It is therefore possible that increased macrophage invasion in the tumour may result in different outcomes depending on cancer type. Furthermore, myeloid-derived suppressor cells (MDSC, immature myeloid cells which are induced by pro-inflammatory cytokines including TNFα and IL-1β) have been shown to promote immunosuppressive functions which assist in the development of

tumours, by inducing apoptosis of T cells and transforming host immunity from tumour-rejecting type 1 to tumour-promoting type 2 (Gabrilovich & Nagaraj 2009). Based on these findings, further research including MDSC, tumour-associated macrophage and T cell-specific analysis in *Tlr4*$^{\Delta IEC}$ mice would aid in clarifying outcomes and distinguishing between cells types included in bulk CD11b^{+} analysis. Finally, additional analysis of non-apoptotic cell death pathways including pyroptosis, necroptosis and ferroptosis in these tumour samples may help to distinguish specific differences in cell death between samples (Westman et al. 2020), not yet captured by our broader investigations.

In conclusion, this study was the first to compare the tumour growth patterns of MC-38 CRC subcutaneous tumours in WT and *Tlr4*$^{\Delta IEC}$ mice and suggests that epithelial TLR4 signalling within the intestine plays an important role in the maintenance of irinotecan's anti-tumour efficacy via CD11b^{+} infiltration.

Chapter 7: General Discussion

Introduction

Despite significant research effort, chemotherapy-induced gastrointestinal toxicity (CIGT) remains a debilitating and costly problem in supportive oncology (Andreyev et al. 2014). Activation of the immune protein toll-like receptor 4 (TLR4) has been implicated in the development of CIGT, however previous findings of TLR4-based pre-clinical toxicity research have proven inconclusive or contradictory in nature (Wardill et al. 2016; Wong et al. 2021). This trend is also seen in clinical trials, where the role of TLR4 in colorectal cancer (CRC) and patient survival is also inconclusive, please see chapter 5 of this thesis for details (Crame et al. 2022). Therefore, translation of results into actionable clinical practice has been stunted in this field. At the beginning of this project, I hypothesised that the observed contradictions were due to an oversight in the importance of site-specific TLR4 expression.

We know that the functional outcomes of TLR4 signalling are dependent on site-specificity for both health and disease, thesis chapter 2 (Bruning et al. 2021). This has been shown in previous research, for example while both epithelial and immune TLR4 signalling contributes to the tolerance of commensal gastrointestinal (GI) microbiota (Zeuthen, Fink & Frokiaer 2008; Salazar et al. 2017), only intestinal epithelial TLR4 expression has been shown to impact healthy metabolic control of the intestines (Lu et al. 2018). Furthermore, a significant upregulation in intestinal epithelial TLR4 expression has been found in patient's living with inflammatory bowel disease (IBD), where the increased TLR4 presence was shown to exacerbate inflammation and mucosal damage (Dheer et al. 2016). Mice that overexpress intestinal TLR4 have increased risk of colitis, while mice that lack TLR4 are protected from inflammatory-associated CRC development (Fukata et al. 2007). The

importance of site-specific TLR4 expression, and particularly the unique roles of intestinal epithelial TLR4, has been supported by this work in CIGT and CRC tumour.

The work carried out in this thesis aimed to:

1) De-couple the role of immune-based and GI epithelial-based TLR4-mediated inflammation in the development of CIGT and

2) Elucidate the contribution of intestinal epithelial TLR4 signalling in CRC growth and response to irinotecan.

Through the use of CRC tumour-bearing, intestinal epithelial specific knockout (KO) mice (*Tlr4*$^{\Delta IEC}$) and online patient databases it was shown that;

- TLR4 expressed on the intestinal epithelium dictates diarrhoea development caused by irinotecan, independent of mucosal injury
- There is a clear disparity in TLR4's effects on irinotecan-induced toxicity, with results showing that weight loss can occur without mice experiencing severe diarrhoea
- High TLR4 expression in humans with stage I CRC results in worse clinical outcomes, whereas high TLR4 expression in humans with stage IV CRC results in improved clinical outcomes
- TLR4 expressed on the intestinal epithelium potentially modulates MC-38 CRC tumour growth dynamics and reduces the durability of irinotecan efficacy in mice through altered host immune response

Taken together, we have found that the balance of CIGT and tumour control between intestinal epithelial TLR4 and TLR4 expressed on other cell types is more complex that originally assumed. While unsurprising given the established complexity of TLR4 signalling pathways, previous research had suggested a clear distinction in the downstream outcomes dependent on the site of TLR4 expression. For example, the

established contradiction between whether TLR4 expression improved (Shi et al. 2019) or worsened (Fukata et al. 2007) dextran sodium sulfate (DSS)-mediated colitis, was one of the original areas to highlight the impact of TLR4 site-specificity to explain findings. These findings combined with the unsuccessful outcomes of non-specific antagonism of TLR4 in the CIGT-context (Coller et al. 2017), presented a strong basis for why site-specific TLR4 analysis would shed new light on GI toxicity and disease.

The outcomes of this project highlight the true complexity of site-specific TLR4 signalling and are the first to explain the role of intestinal epithelial TLR4 in homeostasis, CIGT and irinotecan efficacy. In this chapter, I will discuss our findings in light of previous research and suggest future recommendations which have the potential to crystallise our understanding of TLR4 in CIGT and CRC tumour growth.

Intestinal epithelial TLR4 plays little role in GI homeostasis but is necessary for full GI toxicity response

The studies presented in chapters 3 and 4 investigated the roles of intestinal epithelial TLR4 in GI homeostasis and CIGT, respectively. Upon starting this project, I broadly hypothesised that intestinal epithelial TLR4 does not impact on baseline intestinal homeostasis and would selectively control GI toxicity. Together, our findings are generally supportive of this initial hypothesis. Based on chapter 3 results, we showed that conditional KO of intestinal epithelial TLR4 does not impact on homeostatic control of the GI mucosal barrier or associated immune cell infiltration in healthy mice (Crame et al. 2021). These initial findings challenge previously held assumptions that TLR4 expression is required for homeostatic control of the intestines (Pott & Hornef 2012). It is suggested that these previous assumptions were based on inferences from disease modelling (Shi et al. 2019), rather than investigations purely focussing on baseline characteristics and careful phenotyping of

animal models. Importantly, this initial work highlights no inherent bias in the *Tlr4*$^{\Delta IEC}$ model, therefore suggesting that the results of the CIGT study (chapter 4) and tumour response study (chapter 6) are valid and less likely to be influenced by underlying differences in the GI microenvironment between the two strains.

Irinotecan-induced GI toxicity is a widely recognised complication, characterised by moderate to severe diarrhoea, weight loss and pain (Fuchs, Mitchell & Hoff 2006). Through the use of the *Tlr4*$^{\Delta IEC}$ mouse model, we have identified clearly divergent roles of intestinal epithelial TLR4 in acute diarrhoea development and weight maintenance post-irinotecan. Acute diarrhoea was attenuated in *Tlr4*$^{\Delta IEC}$ mice compared to WT post-irinotecan treatment. This finding alone suggests that intestinal epithelial TLR4 may selectively control diarrhoeal symptoms of the toxicity response, potentially via reducing the number of enteric neurons and lowering the cholinergic excitatory response (Caputi et al. 2017). However, *Tlr4*$^{\Delta IEC}$ animals were not protected from weight loss post-irinotecan, suggesting that diarrhoea and weight are controlled by divergent mechanisms. It was previously suggested that diarrhoea and weight loss were largely driven by the same structural changes in the intestinal mucosa, impairing fluid and nutrient absorption (Richardson & Dobish 2007). Results do not support this, with no histopathological differences observed in either the ileum or colon of irinotecan-treated mice. This differs from previous global TLR4 KO (*Tlr4*$^{-/-}$) models, where *Tlr4*$^{-/-}$ irinotecan-treated animals showed reduced diarrhoea, improved mucosal integrity of the small intestine and improved weight maintenance (Wardill et al. 2016). This suggests that TLR4 expressed on the intestinal epithelium independently controls diarrhoea, most likely through altered host-microbe interactions or impaired motility. It is worth nothing that *ex vivo* electrophysiological assessment of the intestinal tissue using Ussing chambers showed no evidence of altered chloride secretory response in *Tlr4*$^{\Delta IEC}$ animals, confirming no secretory

mechanisms were responsible for the evident diarrhoea. However, the Ussing chamber set up was not optimised for sodium absorption since tissues were treated with amiloride prior to analysis, which is known to inhibit these pathways. While this project still concludes that the observed changes in diarrhoea was unlikely to be caused by mucosal injury (based on combined structural, molecular and functional analysis), it is recommended that un-interrupted secretory analysis of colonic tissue in the irinotecan *Tlr4*$^{\Delta IEC}$ model is explored in future research to confirm these conclusions.

When considering causes of diarrhoea that occur in the absence of gross histopathological changes in the GI tract, antibiotic-induced diarrhoea may offer some insight (Mekonnen et al. 2020). Diarrhoea is commonly seen after antibiotic administration, and is suggested to be caused by a dysbiosis in the gut microbiota (McFarland et al. 2016; Rajkumar et al. 2020). Dysbiosis of the gut microbiota is also shown to contribute to chemotherapy-induced diarrhoea, where microbial disruption before methotrexate (MTX) increased diarrhoea severity without altering the intestinal mucosa (Wardill et al. 2021). Interestingly, recent evidence suggests that site-specific expression of TLR4 on intestinal epithelial cells may be partly responsible for host microbiota disruption post-irinotecan treatment (Secombe et al. 2022). While novel, these findings propose a potential role of intestinal epithelial TLR4 destabilising the host microbiota and contributing to the development of chemotherapy-induced diarrhoea.

Given the microbiota's potent control over the enteric nervous system (ENS), this is also a possible mechanism by which intestinal TLR4 may regulate diarrhoea (Gu et al. 2022). ENS dysfunction and impaired motility has previously been linked to the development of CIGT, where chemotherapy treatments resulted in hyperexcitable myenteric S neurons and increased intestinal motility (Carbone et al. 2016). The

relationship between altered host microbiota and altered intestinal motility is most evident in the field of irritable bowel syndrome (IBS). IBS is a common disorder, with symptoms including constipation, diarrhoea and abdominal pain (Yin et al. 2021). A multifactorial disease, IBS is known to be regulated by visceral hypersensitivity, increased GI motility and an altered host microbiota (Ahluwalia et al. 2021). Importantly, symptoms of IBS are commonly present in the absence of gross pathological changes in the mucosa, suggesting that diarrhoea development may be caused by alternate mechanisms (Ahluwalia et al. 2021). A retrospective human trial comparing the intestinal microbiota and metabolite profiles of 40 people living with IBS and 18 healthy controls, found a significant difference in intestinal microbial populations between healthy individuals versus those with IBS, and, between people with constipation-dominant IBS versus those with diarrhoea-dominant IBS (Ahluwalia et al. 2021). Multiple intervention studies have proven that the ability to re-adjust the host-microbiota towards the healthy microbial profile results in alleviation of diarrhoea-dominant IBS symptoms through control of intestinal motility and modulation of intestinal tight junctions (Peng et al. 2022; Yin et al. 2021). A recent study conducted by Gu and colleagues (2022) found that the supernatant of the fungal probiotic *Saccharomyces boulardii* (Sb) relieved IBS diarrhoea development via serotonin transporter (SERT) upregulation and epidermal growth factor receptor activation caused by significant changes to host microbiota populations (Gu et al. 2022). Furthermore Sb supernatant decreased diarrhoea severity via modulation of intestinal motility via the upregulated SERT pathway (Gu et al. 2022). Taken together, these findings solidify the notion that chemotherapy-induced alterations to the host-microbiome may mediate the development of diarrhoea post-treatment via disrupted intestinal motility.

Given our *Tlr4*$^{\Delta IEC}$ mice showed improved diarrhoea severity compared to WT post-irinotecan with no structural or molecular changes to the mucosal barrier at the time investigated, we suggest that intestinal epithelial TLR4 has the potential to modulate host-microbiota and intestinal motility, in turn controlling chemotherapy-induced diarrhoea. While these factors were not independently analysed in this project, our group has previously shown significant differences in five functional groups of the intestinal microbes between *Tlr4*$^{\Delta IEC}$ and WT mice at baseline, and significant differences in two functional groups of the microbiome in *Tlr4*$^{\Delta IEC}$ and WT mice 72 hrs post-irinotecan treatment (Secombe et al. 2022). This finding combined with the established links between microbial disruption, TLR4 and motility in IBS research, strongly supports our statement and data. While these findings are promising, it is important to recognise the lack of overlap in regards to timing of chemotherapy-induced diarrhoea and time that tissue was collected. Colonic tissue was collected from mice at 72 hrs post-treatment, which does not necessarily align with peak diarrhoea development (24-48 hrs post-irinotecan). Therefore, while the modulation of host-microbiota and intestinal motility is a highly likely cause of observed diarrhoea changes, no definitive conclusions can be made without confirming a lack of tissue injury at the time of peak diarrhoea. Finally, it is important to note that *Tlr4*$^{\Delta IEC}$ irinotecan treated mice still showed mild diarrhoea for the entire 72 hrs period, whereas, WT irinotecan mice had fully recovered by 72 hrs. This finding may suggest a possible role of intestinal epithelial TLR4 in recovery and repair mechanisms of the intestine. Further research examining the relationship between intestinal epithelial TLR4 and intestinal repair post-chemotherapy is required to clarify.

Intestinal epithelial TLR4 modulates CRC growth and reduces durability of treatment response

Through the use of tumour-bearing *Tlr4*$^{\Delta IEC}$ mice, we have shown a potential mechanism whereby intestinal epithelial TLR4 is required for CRC growth and efficacy of irinotecan treatment response. It was originally hypothesised that there would be comparable tumour response to irinotecan in *Tlr4*$^{\Delta IEC}$ and WT mice. This was not supported by findings, as *Tlr4*$^{\Delta IEC}$ mice were relatively more resistant to irinotecan, as they had no significant drop in tumour burden compared to *Tlr4*$^{\Delta IEC}$ mice treated with vehicle. Furthermore, *Tlr4*$^{\Delta IEC}$ mice treated with vehicle showed a relatively reduced CRC tumour growth pattern and greater variability in tumour burden between mice when compared to WT mice. This work is the first to find that the acute cytotoxic effect of irinotecan (i.e. within 24 hours of administration) ***is not*** dependent on intestinal epithelial TLR4, and rather that longer-term maintenance of irinotecan efficacy ***is*** dependent on intestinal epithelial TLR4. Furthermore, our data suggest that intestinal epithelial TLR4 impacts on the prevalence of tumour infiltrating immune cells, which may dictate the overall success of irinotecan treatment for CRC in mice via impacting on immunogenic cell death (ICD) pathways. TLR4 is essential for chemotherapy treatment efficacy via its impact on ICD. ICD is governed by T-cell priming and release of IFN-ɣ, which has been linked to TLR4-dependent mechanisms (Spiller et al. 2008). Mechanistically, this phenomenon is driven by intestinal bacteria which initiate the innate immune response by binding to TLR4 receptors on epithelial, immune and nerve cells (Wardill et al. 2015). It is this interaction within immune cells which has shown strong connections to ICD and tumour kill (Apetoh et al. 2007). Given our data showed altered immune cell presence in tumour tissue of *Tlr4*$^{\Delta IEC}$ mice, this may suggest that intestinal epithelial TLR4 are required for successful ICD and peak irinotecan efficacy.

These findings are consistent with outcomes of the TCGA database analyses and systematic literature review (chapter 5), which also found that immune tone and quantity of tumour TLR4 expression per CRC stage were important for treatment success and improved clinical outcomes for people with CRC (Crame et al. 2022). It is important to note, that a key difference between findings are that the *in vivo* mouse data focussed primarily on the absence of intestinal epithelial TLR4, whereas the TCGA database and systematic literature review examined *tumour* expressed TLR4 on CRC response. Given the lack of clinical literature which considers the host (normal) tissue expression of TLR4, as well as site-specific expression, the role of intestinal epithelial TLR4 on long-term survival outcomes in CRC patients remains unclear (chapter 5). This again highlights a call to action for clinical cancer research to consider the impact of the host on tumour dynamics, and delineate the site of TLR4 expression being investigated to allow for more pointed discussion of results. However, taken together, the outcomes of this PhD project supports a potential role of TLR4 expression influencing the ICD pathways in CRC response to chemotherapy. Moreover, we suggest that the underlying connections between site-specific TLR4 and ICD are highly complex and most likely dependent on CRC stage.

Implications of findings and opportunities for future research

The work presented within this PhD project substantially improves our understanding of intestinal epithelial TLR4 expression on CIGT development and CRC treatment. These novel findings are the first to uncover the true complexity of these pathways within this context, and highlight the need for further action in site-specific TLR4 research. This research is the first to suggest an uncoupling of the underlying mechanisms which mediate GI toxicities, and suggests that intestinal epithelial TLR4 may hold greater control over acute diarrhoea development post-irinotecan and may

dictate irinotecan efficacy via influencing immune tone within the tumour microenvironment.

Due to the wide-reaching applications of TLR4-based research, these findings have the potential to influence many different medical fields, including projects focussing on infectious disease, IBS, IBD, cancer and predictive modelling analysis of cancer and GI toxicities. Importantly, this study highlights potential safety concerns for TLR4-based therapeutics in supportive oncology and CIGT research, as CIGT and chemoefficacy seem to be mediated by similar mechanisms. While the absolute requirement for site-specific consideration of TLR4 expression in CIGT and CRC research has been strengthened by our findings, one implication of these findings suggests that intestinal epithelial TLR4 may prove an attractive target for diarrhoea control for both GI toxicity and disease. It is important that caution is taken when attempting to create novel interventions to combat the GI side-effects of chemotherapy, as modulating intestinal epithelial TLR4 appears to influence the efficacy of chemotherapy. This begs the question, how can we mediate the TLR4-dependent side effects of chemotherapy *without* impairing treatment efficacy? I suggest that this is the key question vital for future research and must be considered before the translation of TLR4-based interventions into practice. One possible solution could be the timing of TLR4-based, anti-toxicity interventions in relation to chemotherapy administration. This is already seen in clinical practice, between the administration of MTX and folinic acid. MTX is a versatile chemotherapy administered in high doses for a range of cancer diagnoses including lymphoblastic leukaemia, brain tumours and lymphoma (Hansson et al. 2021). High dose MTX is highly toxic and associated with side effects of nephrotoxicity, GI toxicity and immune suppression (Stoller et al. 1977). The administration of rescue folinic acid (5-formyl-tetrahydrofolate or leucovorin) post-MTX has been shown to alleviate toxicity without

impacting on treatment efficacy (Howard et al. 2016). Interestingly, folinic acid is also given to amplify the effects of 5-FU in the treatment of GI cancers without causing toxicity (Hartmann et al. 2003). It is possible that this type of framework may be possible for TLR4-based interventions, as immediate TLR4 stimulation with monophosphoryl lipid A combined with the proteasome inhibitor, bortezemib, has been shown to induce ICD and increase acute tumour cell death (Tang et al. 2018). This mimics our findings, where intact TLR4 expression seems to be required for effective tumour kill in the acute phase of treatment (chapter 6). Therefore, a delayed TLR4-based, anti-toxicity intervention may allow time for efficient tumour kill and also reduce the severity of CIGT symptoms. However, adding further complexity to this approach, clinical data suggests that the role of TLR4 is also dependent on tumour stage (chapter 5) (Crame et al. 2022). This may suggest that the timing of TLR4-based interventions would need two distinct considerations, 1) timing of intervention in relation to chemotherapy administration, and 2) timing of the intervention in relation to disease stage. These findings would hold great clinical relevance and may advance this field into translational results.

While clinically relevant, investigating the timing of interventions would not aid in distinguishing the true roles between cell-specific expressions of TLR4. As previously stated, TLR4 signalling holds distinct and unique control over health and disease depending on site of expression. While this PhD project has uncovered the potential of intestinal epithelial TLR4 in the CIGT and CRC context, a reasonable future direction would be to target immune expressed TLR4 to determine whether this site of TLR4 expression holds similar overlapping effects on CIGT and chemoefficacy. It is known that dendritic cell TLR4 expression has control over anti-tumour efficacy of chemotherapies (Apetoh et al. 2007), however little is understood about the role of dendritic TLR4 in CIGT. Conversely, it is known that macrophage expressed TLR4 is

involved in development of some chemotherapy-induced toxicities including systemic inflammation and peripheral neuropathy (Wang et al. 2016; Zhang et al. 2016), however currently the role of macrophage expressed TLR4 in treatment efficacy is not well understood (Son et al. 2019). Considering these findings, suggested future investigations would include analysis of different transgenic animal lines, potentially cell-specific KO of TLR4 in innate immune cells (e.g. dendritic cells, macrophages, natural killer cells, neutrophils or cytotoxic T cell populations) to further differentiate the unique pathways of site-specific TLR4 signalling in CIGT and CRC chemoefficacy.

Finally, further future gazing opportunities could examine whether the intestinal epithelial site-specific KO of TLR4 would protect mice from dextran sulphate sodium (DSS) and azoxymethane (AOM)-induced CRC (Ibrahim et al. 2019). While this PhD project investigated the change in injected CRC tumour in *Tlr4*$^{\Delta IEC}$ mice compared to WT, we were unable to provide information regarding the possibility of a reduction in inflammation-induced CRC. The DSS model of colitis-induced CRC is widely used in pre-clinical research and serves as a prime example of inflammation-induced tumour development (Ibrahim et al. 2019). TLR4 has been implicated in the promotion of colitis-induced CRC development previously (Fukata et al. 2007). Undertaking pointed investigations into the impact of intestinal epithelial specific TLR4 and colitis-induced CRC would not only provide information regarding risk of CRC development in colitis, but could also inform future IBD/IBS research targets.

Conclusion

It is clear that TLR4 expression and its associated downstream signalling pathways are associated with development of substantial GI toxicity post-chemotherapy treatments in both humans and mice. Prior to this project, it was unknown whether site-specific expression of TLR4 influenced CIGT severity or efficacy of the

chemotherapy drug, irinotecan. The studies presented in this thesis have uncovered potential roles of intestinal epithelial TLR4 in controlling GI toxicity symptoms, and suggests that intestinal TLR4 expression may mediate host anti-tumour immune response impacting irinotecan efficacy. While promising, future research using immune-specific transgenic KO models would provide greater insight into the unique and complex roles of TLR4 signalling in the CIGT and CRC contexts.

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Appendix 1: Publications arising from this thesis

Chapters 2, 3 and 5 have been published in peer-reviewed journals. These chapters are presented in this thesis in the original format, except for spelling and table/figure number changes to ensure consistency and referencing style. Here, the chapters are included in the original published formats.

Chapter 2 Publication

Site-specific contribution of Toll-like receptor 4 to intestinal homeostasis and inflammatory disease

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REVIEW ARTICLE

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Site-specific contribution of Toll-like receptor 4 to intestinal homeostasis and inflammatory disease

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Abstract

Toll-like receptor 4 (TLR4) is a highly conserved protein of innate immunity, responsible for the regulation and maintenance of homeostasis, as well as immune recognition of external and internal ligands. TLR4 is expressed on a variety of cell types throughout the gastrointestinal tract, including on epithelial and immune cell populations. In a healthy state, epithelial cell expression of TLR4 greatly assists in homeostasis by shaping the host microbiome, promoting immunoglobulin A production, and regulating follicle-associated epithelium permeability. In contrast, immune cell expression of TLR4 in healthy states is primarily centred on the maturation of dendritic cells in response to stimuli, as well as adequately priming the adaptive immune system to fight infection and promote immune memory. Hence, in a healthy state, there is a clear distinction in the site-specific roles of TLR4 expression. Similarly, recent research has indicated the importance of site-specific TLR4 expression in inflammation and disease, particularly the impact of epithelial-specific TLR4 on disease progression. However, the majority of evidence still remains ambiguous for cell-specific observations, with many studies failing to provide the distinction of epithelial versus immune cell expression of TLR4, preventing specific mechanistic insight and greatly impacting the translation of results. The following review provides a critical overview of the current understanding of site-specific TLR4 activity and its contribution to intestinal/immune homeostasis and inflammatory diseases.

KEYWORDS

immunity, inflammation, intestines, mucous membrane, Toll-like receptor 4

1 | INTRODUCTION

The human body processes trillions of microbiological signals daily, from both external non-self-pathogens to internal self-derived signs of danger (Comalada & Xaus, 2013; Pott & Hornef, 2012). Initial avoidance of infection and tissue damage is highly dependent on the evolutionary conserved innate immune system (Gribar, Richardson, Sodhi, & Hackam, 2008; Pardo-Camacho, Gonzalez-Castro, Rodino-Janeiro, Pigrau, & Vicario, 2018). Unlike the adaptive immune system, the innate immune system is not specific to particular pathogens. Rather, innate immunity utilises a variety of barriers, protein receptors, and phagocytic cells which recognise threats and activate potent inflammatory responses (Pott & Hornef, 2012). Although each component of innate immunity plays a crucial role in maintaining homeostasis, the relationship between pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) is of great interest. It is this connection that allows for the initial recognition of danger, stimulating signalling cascades which leads to inflammation and elimination of the invading pathogen (Kishore, 2009). A widely researched class of PRRs are Toll-like receptors (TLRs)—due to their expansive range of

complimentary ligands, complex signalling pathways, and significance to disease. Although there are 10 human TLR subtypes and 13 mouse TLR subtypes (Nie, Cai, Shao, & Chen, 2018), TLR4 has received significant attention due to its interaction with bacterial products and relevance to multiple disease states.

Although TLR4 expression and signalling are vital for the maintenance of homeostasis and immune tolerance (Chung et al., 2012), this receptor has also been implicated in the development of many inflammatory pathologies, particularly those of gastrointestinal origin, including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), alcoholic-induced barrier injury, and chemotherapy-induced gastrointestinal toxicity (CIGT; Belmonte et al., 2012; Wardill, Gibson et al., 2016). In both diarrhoea-dominant (IBS-D) and CIGT, genetic mutations in the TLR4 pathway have been associated with increased disease risk. Similarly, upregulated expression of TLR4 and its downstream targets have been reported in both clinical studies and preclinical models emphasising the detrimental effects of TLR4 hyperactivation. These findings have prompted an enthusiastic investigation of interventions aimed at inhibiting the proinflammatory effects of TLR4 with the goal of controlling chronic inflammatory diseases or preventing intestinal injury. However, reports are highly variable with conflicting evidence regarding the efficacy of TLR4 inhibition, with some reports suggesting detrimental effects on chronic inflammation and tumour growth. These findings clearly highlight an underappreciated level of complexity in TLR4-dependent mechanisms and a significant gap in our fundamental understanding of how to modify TLR4 signalling in the context of disease prevention.

A critical and often overlooked aspect of TLR4 signalling is its site specificity. TLR4 is expressed primarily on immune cell subsets; however, it is also expressed on intestinal epithelial cells acting as the first point of immunosurveillance. Despite the intensity of interest in TLR4, few studies acknowledge or address the site-specific implications of TLR4 signalling. This common oversight is likely to impact the true nature and translational impact of results and is a possible reason for the high degree of variability seen in the efficacy of TLR4-targeted interventions and the growing number of adverse events reported following TLR4 inhibition. As such, the following review will provide a critical overview of the site-specific actions of TLR4, with a specific focus on the unique contribution of epithelial- and immune-cell TLR4 signalling in the maintenance of healthy states and contribution to gastrointestinal diseases. IBD and IBS are chronic inflammatory conditions that primarily affect the intestines, resulting in diarrhoea, constipation, and pain (Belmonte et al., 2012). Genetic studies on IBD susceptibility indicate that the TLR4 signalling molecule, Toll/interleukin-1 (IL-1) receptor domain-containing adapter protein, plays a significant role in increased susceptibility to combined IBD risk and Crohn's disease (CD) risk (De Jager et al., 2007). Further clinical research using human colon samples showed a significant increase in colonic TLR4 expression in IBS-D, suggesting that the proinflammatory nature of TLR4 may exaggerate disease severity and progression (Kocak, Akbal, Koklu, Ergul, & Can, 2016). In the context of alcoholic steatohepatitis and alcohol-induced barrier injury, X. Li et al. (2013) study found that chronic exposure to ethanol significantly increases intestinal permeability of mice via TLR4-dependant downregulation of phosphorylated occludin and increased protein kinase C (PKC) activity. This is reflected in the setting of CIGT, a severe inflammatory complication of cancer therapy characterised by upregulation of proinflammatory cytokines including IL-6, IL-1β, and tumour necrosis factor-α (TNF-α), and all downstream targets of TLR4 (Bossi et al., 2016; Logan et al., 2008). Further research has shown that TNF-α and IL-6 induce tight junction protein dysfunction and the breakdown of actin filaments (Wardill, Bowen, & Gibson, 2012), with global TLR4 knockout (KO) shown to mitigate the clinical severity of CIGT in a mouse model via its regulation of claudin-1 internationalisation (Wardill, Bowen et al., 2016). In fact, TLR4-related genetic mutations have been shown to elevate the risk of CIGT in patients undergoing standard-dose chemotherapy, echoing genetic susceptibility results from IBD cohorts (De Jager et al., 2007). Therefore, TLR4-mediated intestinal barrier dysfunction is now considered critical in the initiation and potentiation of gastrointestinal damage (Wardill, Gibson et al., 2016).

Although TLR4 has been the subject of many studies regarding the inflammatory gastrointestinal disease, the distinction between site-specific expression of TLR4 is rarely addressed or acknowledged. This common oversight is likely to impact the true nature and translational impact of results, as site-specific expression may significantly impact the contextual role of TLR4. The following review will, therefore, contrast intestinal epithelial and immune cell-expressed TLR4, dissecting their unique contribution to the maintenance of healthy states and in gastrointestinal diseases highlighting methods of improving mechanistic insight of TLR4-based signalling and translation of TLR4-targeted therapeutics.

2 | THE IMPORTANCE OF DISSECTING SITE-SPECIFIC TLR4-DEPENDANT MECHANISMS

The current level of understanding regarding cell-specific involvement of TLR4 in healthy and diseased states is vague, with the majority of research relying on global KO models to demonstrate TLR4-dependant mechanisms or failing to identify target cell populations. The mechanisms of epithelial and immune TLR4 differ and as such, there are likely to be site-specific mechanisms that govern disease initiation and progression. Failure to acknowledge and specifically investigate or target these pathways is a critical oversight in translational research efforts. This is particularly pertinent when considering the dichotomous role of TLR4, which exerts both beneficial and detrimental effects on host physiology depending on its cellular location and degree of activation. When considering this, it is unsurprising to see the degree of conflicting data regarding TLR4 manipulation in an attempt to prevent or control the disease. For example, TLR4 activation has been widely reported to promote the development of experimental dextran sodium sulfate (DSS)-mediated colitis (Fukata et al., 2007), yet TLR4 deficiency has paradoxically

been reported to aggravate symptoms (Shi et al., 2019). Similarly, global TLR4 deletion was robustly demonstrated to mitigate chemotherapy-induced mucosal injury; however, inhibition with naloxone was unable to control symptoms and reduced the efficacy of chemotherapy. Therefore, it is critical that we appreciate the contextual roles of TLR4-dependent inflammation to appropriately modulate its activity without inhibiting restorative/healing processes that are critical in the resolution of chronic inflammatory insult, and without influencing extraintestinal TLR4-dependent mechanisms. Achieving this requires a greater insight into the site-specific actions of TLR4 in both the maintenance of homeostasis and initiation of disease.

3 | SITE-SPECIFIC TLR4 EXPRESSION IN HEALTHY STATES

In healthy states, TLR4 contributes to intestinal homeostasis via several distinct and well-defined mechanisms: (a) maintenance of the intestinal barrier; (b) recognition and response to invading pathogens; (c) metabolic regulation; and (d) gastric motility (Anitha, Vijay-Kumar, Sitaraman, Gewirtz, & Srinivasan, 2012; Guo et al., 2015). TLR4 is a key sentinel protein for luminal and subepithelial signals of infection and sterile injury (Akira, Takeda, & Kaisho, 2001). This type 1 transmembrane protein has a distinct tripartite structure, including a leucine-rich extracellular domain, transmembrane domain, and an intercellular signalling domain (De Nardo, 2015). In a healthy condition, TLR4 expression is relatively low, with research showing that in intestinal epithelial cells, the transcription factor, ZNF160, repressed TLR4 expression to maintain homeostasis of the intestines and allow for commensal microbiome development (Takahashi, Sugi, Hosono, & Kaminogawa, 2009). Situated in either the outer cell membrane, Golgi apparatus, or within the endosome, TLR4 recognises a variety of ligands including, but not limited to lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria, heat-shock proteins, hyaluronic acid, and high-mobility group protein I (Cheng, Taylor, Ourthiague, & Hoffmann, 2015; Santaolalla, Sussman, & Abreu, 2011). Myeloid differentiation factor 2 (MD2) is a glycoprotein on the extracellular domain of TLR4 which assists in the binding of LPS and other ligands (C. C. Lee, Avalos, & Ploegh, 2012; J. Meng, Lien, & Golenbock, 2010). An example of TLR4 ligand binding and activation is LPS recognition, which is driven by the binding of lipid A, of LPS, to the TLR4/MD2 complex (Steeghs et al., 2008). Upon ligand binding, TLR4 activates the myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β pathways, to increase expression of the nuclear transcription factor κ-light-chain-enhancer of activated B cells (NF-κB) and interferon regulatory transcription factor 3 activations (Cheng et al., 2015). This cascade results in the production of proinflammatory cytokines including IL-6, IL-8, and interferon-β (IFN-β; Figure 1).

3.1 | Intestinal epithelial TLR4 expression in the maintenance of healthy states

While TLR4 is expressed on a range of cell types throughout the body, including dendritic cells, myeloid cells, macrophages (Vaure & Liu, 2014), and microglia (Vaure & Liu, 2014), its expression in the gastrointestinal tract is particularly significant due to the high

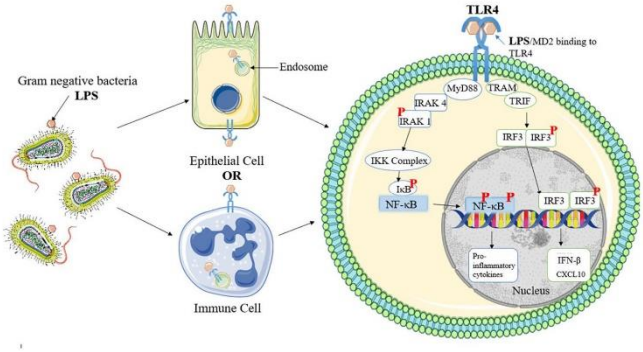

FIGURE 1 Intracellular signalling pathways of TLR4 activation by LPS in both epithelial and/or immune cells, resulting in inflammatory responses. LPS expressed on Gram-negative bacterial cells binds to TLR4 on immune and/or epithelial cells causing the activation of an inflammatory cytokine cascade mediated by MyD88 or TRAM/TRIF activation, which leads to the upregulation of NF-κB and IRF3, respectively. This cascade results in the production of proinflammatory cytokines including interleukin 6 (IL-6), IL-1β, and tumour necrosis factor-α. CXCL10, C-X-C motif chemokine; IFN-β, interferon-β, IκB, κB kinase; IKK, inhibitor of kappa B kinase; IRAK 1, interleukin-1 receptor-associated kinase 1; IRF3, interferon regulatory transcription factor 3; LPS, lipopolysaccharides; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; P, phosphorylation; TLR4, Toll-like receptor 4; TRAM, translocating chain-associating membrane; TRIF, TIR-domain-containing adapter-inducing interferon-β

microbial load and interaction with exogenous stimuli (McClure & Massari, 2014). TLR4 is widely expressed along the small and large intestines under normal physiological conditions (Cairo & Podolsky, 2000), with higher rates of epithelial expression found in the distal ileum and colon (Cairo & Podolsky, 2000; Gourbeyre et al., 2015). Epithelial TLR4 is expressed on both the apical and basolateral cell membranes, as well as within endosomes and the Golgi apparatus (Abreu, 2010; Hornef, Frisan, Vandewalle, Normark & Richter-Dahlfors, 2002). This variety of epithelial expression has been shown to change the site-specific role of TLR4, with basolateral TLR4 expression on epithelial cells recognising LPS and stimulating an inflammatory response, whereas apical expression promotes tolerance to LPS, without causing inflammation (Vamadevan et al., 2010).

Lotz et al. (2006) were the first to explain the role of TLR4 in intestinal epithelial cells immediately after birth. Using both TLR4 KO mice and cell culture stimulation assays, this study found that intestinal epithelial TLR4 is the first to respond to exogenous endotoxins, like LPS, rather than immune-expressed TLR4 on macrophages (Lotz et al., 2006). This initial site-specific recognition helps us to establish the microbial colonisation of the intestine and is fundamental in the development of a healthy microbiome and host-immune tolerance (Lotz et al., 2006). Previous evidence shows that the absence of intestinal epithelial TLR4 resulted in a hyporesponsiveness to LPS (Abreu et al., 2001; Naik, Kelly, Meijer, Petterson & Sanderson, 2001), therefore demonstrating a clear link between intestinal homeostasis and epithelial TLR4 expression. Furthermore, Zeuthen, Fink, and Frokiaer (2008) study using Caco-2 cell culture and monocyte-derived dendritic cells found that intestinal epithelial cells ready the immune system response to commensal bacteria via partially TLR4 driven mechanisms, leading to a tolerogenic phenotype to commensals. This study supports the importance of epithelial TLR4 and its role in establishing immune tolerance to intestinal bacteria and the development of a healthy microbiome.

In addition to immunosurveillance, epithelial TLR4 signalling is involved in a variety of essential functions for the establishment and maintenance of a healthy epithelial function, including inflammation control, regulation of host-microbiome, B cell recruitment, immunoglobulin A (IgA) production, follicle-associated epithelial permeability, and antimicrobial peptide expression (Abreu, 2010; S. Chabot, Wagner, Farrant, & Neutra, 2006). Of particular relevance to intestinal homeostasis are interactions between TLR4 and the mucosal barrier, a highly regulated interface between the luminal environment and mucosal compartment comprised of intestinal epithelial cells and basement membrane.

Mucosal barrier integrity is maintained by the apical junction complex (tight junction, desmosome, and adherens junction), with tight junctions maintaining the semiselective state of the paracellular mucosal route. Careful control of the molecular structure of the tight junction allows for absorption of solutes across their respective electro-osmotic gradients and also serves as an immunosurveillance checkpoint (Anderson, Van Itallie, & Fanning, 2004; Blijevens, 2005; Hooper, Littman & Macpherson, 2012; Wells et al., 2017). The tight junction complex was first identified at the apico-lateral surface of adjacent enterocytes by Farquhar and Palade (1963). This foundational study utilised haemoglobin as a mass tracer in rats and Guinea pig tissue to assess permeability (Farquhar & Palade, 1963). Combined with electron microscopy techniques, this protocol showed that the tight junction is responsible for the diffusion of the tracer along with the intercellular space, providing the first evidence highlighting the importance of tight junctions in regulating intestinal permeability (Farquhar & Palade, 1963). Since this pivotal study, the specific proteins that comprise this complex have been identified, including occludin, zonular occludin 1 (ZO-1), and claudins (Figure 2). The tight junction is indicated in the healthy functioning of the

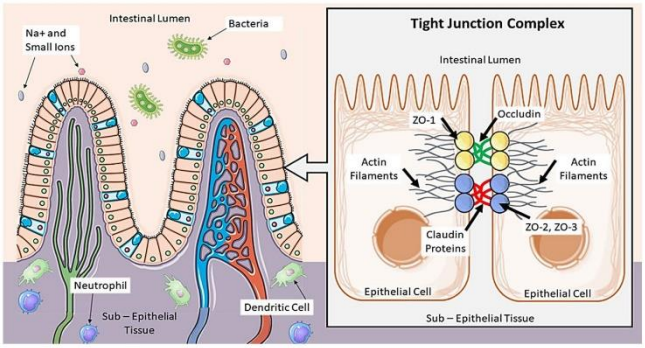

FIGURE 2 Representative diagram of the intestinal epithelial barrier and tight junction protein complex. The junctional complex, including tight junction proteins (pictured), adherens, and desmosomes, is located at the apical lateral junction of epithelial cells and acts to bind cells together to form a selective barrier. In a healthy condition, physiological solutes and ions are able to cross this barrier; however, pathogenic bacteria are unable to cross, therefore maintaining the sterile subepithelial environment. ZO-2, zonular occludin 2

intestinal barrier, where damage to tight junction proteins often results in increased permeability and progression of the illness (S. H. Lee, 2015; Salim & Soderholm, 2011; Wardill et al., 2012). Previous evidence concluded that TLR4 expression did not influence tight junction protein development (Wardill, Bowen et al., 2016), although tight junctions were primarily assessed after a mucosal challenge of irinotecan treatment. Of note, a recent study found that the colon epithelial cells treated with 5-HT or commensal *Escherichia coli* showed increased expression of TLR-4 which correlates with the downregulation of E-cadherin and claudin-2 (Banskota et al., 2017). Similarly, increased TLR4 signalling (transgenic villin-TLR4 hyperactivity) was reported to increase intestinal permeability as detected by fluorescein isothiocyanate (FITC)–dextran and tight junction protein expression (Dheer et al., 2016). These findings implicate a functional role for epithelial TLR4 in tight junction protein control. These results were supported by Bein, Eventov-Friedman, Arbell, and Schwartz (2018) whose study into necrotising enterocolitis (NEC) in neonates found that the decrease of TLR4 expression seen in NEC is associated with a decrease in tight junction proteins, occludin, cingulin, claudin-4, and ZO-1. Although this may be a by-product of NEC pathophysiology, this could also suggest a homeostatic role of TLR4 in tight junction maintenance and TLR4 impact on intestinal permeability. Furthermore, recent research has shown that increased TLR4 expression negatively influences the function of the adherens junction. The adherens junction is comprised of cadherin transmembrane receptors and their associate binding proteins, forming a junction that allows for cell-to-cell adhesion and maintenance of tissue integrity (Pinheiro & Bellaiche, 2018). Research conducted by Ralls, Demehri, Feng, Woods Ignatoski, and Teitelbaum (2015) using human inflamed small bowel tissue found that in underfed sections of bowel, TLR4 is significantly increased and E-cadherin expression is decreased resulting in loss of epithelial barrier function. This evidence shows the dual impact of TLR4 expression on the intestinal junctional complexes, often leading to exacerbated intestinal disruption.

In addition to its probarrier properties, epithelial TLR4 also influences the structural development of the intestine and the intestinal immune environment (Lu, Sodhi, & Hackam, 2014; D. Meng et al., 2015). A transgenic mouse model, constitutively expressing TLR4 in the intestinal epithelium, found links between epithelial TLR4 signalling and B cell recruitment and IgA production (Shang et al., 2008). In the transgenic model B cells and B cell, tropic chemokines were increased with CCL20 protein being fivefold higher in transgenic mice than wild-type (WT) counterparts, and CCL28 being 1.6-fold higher than WT (Shang et al., 2008). Furthermore, the continuous overexpression of epithelial TLR4 resulted in increased IgA^+ cells in the small intestine lamina propria and produced higher faecal IgA levels as compared to WT (Shang et al., 2008). Finally, this study also found that TLR4 signalling in intestinal epithelial cells induces immunoglobulin class switching to IgA in the small intestinal lamina propria (Shang et al., 2008). Taken together, this evidence supports the important role intestinal epithelial cells play in response to external signalling and presents a mechanism by which epithelial TLR4 prevents attachment of pathogens via the regulation of luminal IgA production (Shang et al., 2008).

The importance of epithelial TLR4 in intestinal function was convincingly demonstrated using intestinal epithelial conditional KO mice ($TLR4^{\Delta IEC}$) in investigations into the regulatory contribution of epithelial TLR4 to altered metabolic symptoms and disease (Lu et al., 2018). $TLR4^{\Delta IEC}$ mice were shown to develop metabolic syndrome faster than their WT counterparts, showing significant weight gain independent of food intake or hormone levels and the presence of macrophage accumulation within adipose tissue via histologic analyses (Lu et al., 2018). Furthermore, intestinal epithelial TLR4 was shown to significantly impact the composition of the intestinal microbiota in mice, where $TLR4^{\Delta IEC}$ mice showed both a lower diversity of bacteria and altered bacterial clustering (Lu et al., 2018). In addition, $TLR4^{\Delta IEC}$ mice showed an altered expression of metabolic and inflammatory genes in comparison to WT. Metabolic pathway genes associated with peroxisome proliferator-activated receptor signalling were significantly downregulated in the ileum of $TLR4^{\Delta IEC}$ mice (Lu et al., 2018). Finally, the conditional KO of intestinal epithelial TLR4 resulted in a proinflammatory phenotype, including the upregulation of macrophage markers (*Cd68*, *F4/80*, and *Mcp1*) and neutrophil markers (*Mpo* and *Elane*). Together, these results show that intestinal epithelial TLR4 expression in healthy states assists with the regulation of metabolic control, microbiota composition and function, metabolic gene expression, and prevention of unnecessary inflammation.

In contrast, the impact of TLR4 on the microbial invasion was elegantly demonstrated using a C57BL/6 villin-TLR4 transgene model which constitutively expressed intestinal epithelial TLR4. Interestingly, where intestinal epithelial TLR4 was consistently overexpressed, an increase in microbial invasion, alterations to luminal and mucosal microbiota, and disruptions to epithelial barrier function were found (Dheer et al., 2016). This evidence emphasises the dichotomous role of TLR4, with its physiological benefits restricted within a threshold of expression/activity (Abreu, 2010). Importantly, it is consistently shown that site-specific overexpression is detrimental to intestinal homeostasis resulting in the breakdown of the mucosal barrier and exaggerated immune activation, which is likely to be mediated by the basolaterally expressed epithelial TLR4. Hyperactivation of TLR4 is, therefore, a key driver of many diseased states affecting gastrointestinal function, many of which will be discussed further in later sections of this review.

3.2 | Immune cell TLR4 expression in healthy states

The majority of our fundamental knowledge regarding TLR4 signalling has been gained from studying its activity in immune cell subsets, likely reflecting the higher expression of TLR4 on immune cells compared to enterocytes. Gourbeyre et al. (2015) state a 2.5-fold increase of TLR4 expression within the mesenteric lymph nodes of pigs compared to intestinal tissue and a 1.5-fold increase of TLR4 expression in the intestinal lumen compared to the jejunal villi.

This increase of TLR4 abundance is due to the high immune cell presence within these organ systems with TLR4 expressed on dendritic cells, macrophages, and myeloid cells (Vaure & Liu, 2014). Although immune-derived TLR4 signalling is crucial to the recognition and response to dangerous PAMPs and DAMPs (Apetoh et al., 2007; Cairo & Podolsky, 2000; Fukata et al., 2005; Hajjar et al., 2012; Leaphart et al., 2007), immune cell TLR4 expression also helps us to regulate the immune and intestinal environment in healthy states. Arguably, one of the most important immune cells to express TLR4 is dendritic cells.

Dendritic cells are a highly specialised form of antigen-presenting cells, which provide an important link between innate immunity and activation of the adaptive immune system (Pufnock et al., 2011). Upon capture and recognition of an antigen, mature dendritic cells migrate from the site of recognition to the lymphoid organs and initiate the development of effector and memory T cells from primary T cells (Michelsen et al., 2001; Pufnock et al., 2011). Maturation of dendritic cells is the process whereby the role of dendritic cells changes from immature phagocytic actions to cytokine production and efficient T-cell antigen-presenting. This process occurs before reaching the lymphoid organs and is highly dependent on the recognition and digestion of pathogens (Michelsen et al., 2001). A foundational study using mice with a mutated TLR4 protein examined the role of immune-expressed TLR4 on dendritic cell maturation (Michelsen et al., 2001). Results showed that both TLR2- and TLR4-related mechanisms were crucial for the normal maturation of dendritic cells in response to bacterial ligands (Michelsen et al., 2001). A further immunological study by Pufnock et al. (2011) found that coactivation of both TLR4 and TLR7/8 provides an increased maturation of dendritic cells and an increased generation of CD8+ memory T cells as well. Together, this evidence supports the importance of immune cell expression of TLR4 for dendritic cell maturation and successful priming of the T-cell response to invading pathogens.

Current research has also indicated a strong relationship between TLR4 expressed on dendritic cells and immunological tolerance via the release of indoleamine-2,3-dioxygenase (IDO; Salazar, Awuah, Negm, Shakib, & Ghaemmaghami, 2017). Apart from the role of IDO as an enzyme which catalyses the amino acid tryptophan, IDO also plays an important role in immune regulation (Chen, 2011). IDO produced by dendritic cells promotes immunosuppression and helps us to establish tolerance to commensal microbiota (Harden & Egilmez, 2012). Critically, LPS-primed human dendritic cells produced a higher IDO than unprimed cells, and that the primed cells resulted in a tolerogenic phenotype (Salazar et al., 2017). Furthermore, unprimed cells challenged with LPS also resulted in increased IDO and a tolerogenic phenotype (Salazar et al., 2017). Interestingly, when monocytes were challenged with LPS, there was no increase in IDO production, suggesting that this is a unique trait of dendritic cells (Salazar et al., 2017). This evidence characterises the role of TLR4 expression on dendritic cells and its importance in homeostasis and supports previous research in which the TLR4 ligand, LPS, induced expression of IDO in dendritic cells which contributed to an immunogenic tolerance (Von Bubnoff, Scheler, Wilms, Fimmers, & Bieber, 2011). Although the links between TLR4-mediated dendritic cell IDO expression and immune tolerance are clearly stated, Albrecht, Hofer, Foxwell, Frankenberger, and Ziegler-Heitbrock (2008) study found a further mechanism whereby dendritic-expressed TLR4 assists in homeostatic tolerance. This study using in vitro LPS precultured human dendritic cells found that further stimulation with high-dose LPS produced an immune tolerance phenotype in these cells, marked by the ablation of the IL-1 receptor-associated kinase 1 adaptor protein and decreased NF-κB activation (Albrecht et al., 2008).

While a well-defined link among dendritic expression of TLR4, immune tolerance, and adaptive immune activation is clear, the role of TLR4 expressed on monocytes remains uncertain. Current evidence shows that monocytic TLR4 expression may not contribute to enhanced immune tolerance (Salazar et al., 2017); however, current evidence is sparse and further research is, therefore, required to dissect monocytic-specific TLR4 signalling and its contribution to healthy and diseased states. With this being said, the monocyte-derived macrophage expression of TLR4 has been well-characterised to engage the NF-κB pathway, leading to active cytokine release and promotion of inflammation in response to pathogenic bacteria (Q. Li & Cherayil, 2003). The innate immune response to pathogens is highly dependent on ligand binding to TLR4 on macrophages, which enables a maximised release of TNF-α, resulting in beneficial inflammation and elimination of harmful bacteria (Q. Li & Cherayil, 2003). Furthermore, previous research indicates the role of TLR4 in increasing oxidative stress in activated macrophages to aid in removing pathogens from the body (Matsebatlela, Anderson, Gallicchio, Elford, & Rice, 2015). In contrast, this relationship is also implicated in the development and progression of renal hypertension, with research showing that TLR4 deficiency reduces oxidative stress and, therefore, improves the hypertensive condition (Pushpakumar et al., 2017). Therefore, TLR4 on macrophages seem to play both homeostatic roles, as well as contributing roles in disease.

4 | DISEASE-SPECIFIC IMPACT OF TLR4 EXPRESSION

4.1 | Role of TLR4 and LPS response

As outlined, TLR4 signalling is best known for its well-established role in LPS recognition from Gram-negative bacteria and initiation of inflammation (Abreu et al., 2001; Hajjar et al., 2012). Historically, this process was believed to be heavily dependent on immune expression of TLR4. However, research by Abreu et al. (2002) found that TLR4 expression is significantly increased in response to LPS with T-cell-derived cytokines, IFN-γ, and TNF-α, increasing TLR4 expression in intestinal epithelial cells, therefore increasing the inflammatory response to LPS administration. Furthermore, a study conducted by Hornef et al. (2002) produced strong evidence highlighting the importance of intestinal epithelial TLR4 in LPS recognition. Using

isolated murine small intestinal crypt epithelial cells, this study showed proinflammatory mediator secretion and CD14 upregulation in response to LPS administration (Hornef et al., 2002). Furthermore, epithelial cell TLR4 expression was found within the Golgi apparatus (Hornef et al., 2002), identical to the site of internalised LPS, showing that intestinal epithelial cells may be responsible for the initial recognition and signalling in response to LPS. It is this which then attracts the accumulation and stimulation of the more advanced immune response (Hornef et al., 2002). The damaging inflammatory response to LPS from Gram-negative bacteria in the intestinal tract is partially characterised by increased intestinal permeability. Research by Guo, Al-Sadi, Said, and Ma (2013) found a significant decrease in epithelial resistance in response to LPS administration in Caco-2 cell monolayers from 100% relative epithelial resistance in untreated control versus 70% relative epithelial resistance in LPS-treated monolayers. Furthermore, in an in vivo C57BL/6 mouse model, LPS administration was shown to increase permeability flux of the macromolecular Dextran 10K across the intestinal epithelium of WT mice with threefold higher permeability in LPS-treated mice; however, in a global TLR4 KO mouse, permeability and flux did not increase from baseline with LPS treatment (Guo et al., 2013). This study concluded that LPS administration results in an increased intestinal permeability mediated in part by increased TLR4 and CD14 expression and localisation along intestinal enterocytes (Guo et al., 2013).

Although current evidence strongly suggests the role of epithelial TLR4 in recognition and response to LPS, many foundational studies have investigated the relationship between LPS and TLR4, finding that immune TLR4 is essential for the development of inflammation and the elimination of bacteria (Poltorak et al., 1998). A study conducted by Hoshino et al. (1999) investigated the impact of TLR4 elimination on LPS responsiveness. This study utilised an immune-specific TLR4 KO mouse model, which was deemed successful due to the harvested macrophages and B cells showing no response to LPS administration (Hoshino et al., 1999). In the TLR4 KO model, LPS response was greatly decreased compared to WT counterparts (Hoshino et al., 1999). This study is further supported by Qureshi et al. (1999), who found that endotoxin hyporesponsive mice showed unique mutations within the *Tlr4* gene coding. More recent evidence showed that the mechanism of the LPS-derived increased gastrointestinal permeability is caused primarily by the TLR4/MyD88 signalling pathway (Guo et al., 2015). Two strains of KO mice were utilised including a TLR4 KO and MyD88 KO. Results found that permeability was increased as a result of the injection of LPS to mice and that this was strongly dependant on TLR4 expression (Guo et al., 2015). These findings were supported by Nighot et al. (2017) further clarifying the role of the TLR4/MyD88 pathway in increased permeability. This study introduced LPS to Caco-2 monolayers and C57BL/6 mice and found an increase in permeability, both in vitro and in vivo, via the upregulation of myosin light chain kinase. Although these more recent studies do provide evidence of TLR4 involvement in LPS recognition, they did not distinguish between epithelial versus immune cell expression, negatively impacting the translatability of results.

4.2 | Role of TLR4 in altered intestinal permeability, IBD, and IBS

Although the TLR4/LPS-mediated inflammatory pathway has been well-characterised, recent literature has indicated the involvement of epithelial TLR4 in the development of many intestinal diseases and illnesses. The pathogenesis of many IBDs and intestinal toxicities is characterised by increased permeability and disruption to the tight junction complex. A study by X. Li et al. (2013) investigated the role of intestinal epithelial TLR4 on the phosphorylation of the tight junction protein, occludin, via PKC, and its contribution to increased permeability in alcoholic steatohepatitis. Using both Caco-2 monolayer cell cultures and C57BL/6 WT mice, this study found that the neutralisation of TLR4 using monoclonal antibody pretreatment reduced ethanol-induced paracellular permeability. Furthermore, in the presence of ethanol, TLR4 expression was increased and phosphorylated occludin was decreased (X. Li et al., 2013). This decrease in functional occludin mediated by TLR4 may explain why with high concentrations of ethanol, a significant increase of intestinal permeability is seen. This study further supports the involvement of epithelial TLR4 in the development of intestinal barrier dysfunction in several disease states.

Altered intestinal permeability is also a hallmark of IBD, which includes CD and ulcerative colitis (UC), each being a chronic inflammatory disease which significantly impacts wellbeing and an individual's ability to thrive due to symptoms of pain, diarrhoea, and constipation. A large genetic study collated 1,539 DNA samples from human IBD patients and included a pooled analysis of 4,805 cases of IBD (De Jager et al., 2007). Focussing on 23 genes related to TLR4 expression and associated signalling pathways, this study genetically identified TLR4 as a disease risk factor for both CD and overall IBD (De Jager et al., 2007). Due to the large sample sizes and well-tested methodologies used, this study provides strong genetic evidence that TLR4 plays a role in the development of gastrointestinal pathologies. However, due to the genetic nature of this study, it is unable to provide any distinguishing information regarding cell-specific TLR4 involvement as is common in the current literature. An early study by Cairo and Podolsky (2000) investigated the differential expression of different TLRs in the intestinal epithelia in IBD. This patient-based study utilised human tissues of the colon and terminal ileum, from those with active IBD. Western blot and immunohistochemistry analyses revealed that epithelial TLR4 was greatly upregulated in both UC and CD and may contribute to disease progression (Cairo & Podolsky, 2000). Interestingly, a significant difference in the positioning of epithelial cell TLR4 expression was noted between UC and CD. Higher expression of TLR4 on the basolateral cell surface was noted in patients with UC, whereas higher expression of TLR4 on the apical cell surface was observed in patients with CD (Cairo & Podolsky, 2000). As expected, an increase in TLR4 expression was also found on immune cells of the lamina propria due to the inflammatory nature of these diseases. Knowing that intestinal epithelial TLR4 is overexpressed in patients with IBD, a study conducted by Dheer et al. (2016) investigated the impact of constitutive TLR4

expression on epithelial function and the microbiota composition. Increased TLR4 signalling resulted in the invasion of intestinal microbiota and significant alterations to bacterial composition within the intestines (Dheer et al., 2016). This led to an exacerbation of DSS-mediated colitis in animal models (Dheer et al., 2016).

Another common gastrointestinal condition is irritable bowel disease (IBS), which effects one in five Australians (Belmonte et al., 2012). IBS is often characterised by bloating, abdominal pain, diarrhoea, and/or constipation, which significantly impacts the quality of life (Belmonte et al., 2012). A study conducted by Belmonte et al. (2012) investigated the impact and prevalence of TLR4 expression on IBS development. In this clinical study, a total of 48 patients with IBS (including similar rates of IBS-D, constipation-dominant [IBS-C], and mixed symptoms phenotypes [IBS-M]) and 31 control patients were enrolled, and colonic biopsy samples were collected via colonoscopy (Belmonte et al., 2012). A twofold increase of TLR4 expression was found in patients with IBS-M when compared to healthy controls. In addition, TLR4 expression was highest in IBS patients with a disease duration of over 5 years (Belmonte et al., 2012). Furthermore, increased TLR4 expression in patients with IBS was predominantly found in intestinal epithelial cells, with a higher TLR4 presence in the crypts of intestinal tissue compared to surface epithelial layers (Belmonte et al., 2012). These results are supported by a more recent study, which also analysed human colonic tissue from both IBS patients and healthy control patients (Kocak et al., 2016). This study only evaluated two IBS subgroups, being IBS-D and IBS-C, omitting the IBS-M group. However, results showed significantly increased expression of TLR4 in colonic mucosa for both subgroups of patients with IBS when compared to control (Kocak et al., 2016). These findings support the detrimental role of TLR4 hyperexpression in the context of IBD and IBS and help us to shape our understanding of disease pathophysiology and progression. However, due to the nonspecific reporting of TLR4 expression states, it is difficult to distinguish between epithelial and immune TLR4 contextual roles, and thus a translation of these findings is challenging.

4.3 | Role of TLR4 in NEC

NEC is a leading cause of death in premature infants, where intestinal injury permits bacterial translocation into the usually sterile subepithelial space (Anand, Leaphart, Mollen, & Hackam, 2007; Leaphart et al., 2007). This disease mechanism results in necrosis of the small intestine leading to sepsis, multiorgan failure, and death (Hackam, Upperman, Grishin, & Ford, 2005). A study by Leaphart et al. (2007) investigated the role of intestinal mucosal TLR4 on NEC development. TLR4 expression within the mucosa is significantly increased in NEC and in TLR4 mutant mice, and the severity of NEC is significantly reduced compared to WT counterparts. However, interestingly, this study also identified a potential role of TLR4 in mucosal repair via increased proliferation and association with intestinal focal adhesion kinases to induce healing (Leaphart et al., 2007). Together, these findings show that TLR4 expression in the mucosa is important in the context of NEC. A major limitation of this study was the uncertainty of which specific cell types were examined, as mucosal tissue includes both epithelial and immune cell lines. A more recent study conducted by Sodhi et al. (2012) further investigated the role of TLR4 in NEC and found that it is the epithelial expression of TLR4 which contributes to NEC development and goblet cell regulation. This ambiguity is mimicked across many fields of TLR4 and intestinal disorders, including both intestinal disease and CIGT.

4.4 | TLR4 regulates CIGT risk and severity

CIGT is a serious complication of current anticancer treatments, affecting up to 80% of people treated with chemotherapy (Blijevens, 2005; Richardson & Dobish, 2007). Symptoms of CIGT include severe diarrhoea, pain, bleeding, and ulceration often resulting in an increased risk of infection, leading to delayed, or discontinued treatment (Andreyev et al., 2014; Fuchs, Mitchell, & Hoff, 2006). These symptoms are predominantly underpinned by changes to the intestinal barrier, which is comprised of polarised epithelial cells acting as a highly selective barrier between the intestinal lumen and the subepithelial tissue (Pott & Hornef, 2012; Wardill et al., 2012). Irinotecan is a common chemotherapy associated with CIGT development, due to its unique enterohepatic recirculation which causes a double exposure to its toxic metabolite, SN-38, in the intestines (G. Chabot, 1997; Kawato, Aonuma, Hirota, Kuga, & Sato, 1991). Wardill, Gibson et al. (2016) study examined the link between irinotecan-induced CIGT and TLR4 involvement, demonstrating reduced clinical indicators of toxicity in the TLR4 KO mice versus WT. Specifically, TLR4 KO mice showed decreased diarrhoea and lost significantly less body weight compared to their WT counterparts (Wardill, Gibson et al., 2016). Furthermore, Wardill, Gibson et al. (2016) reported less severe intestinal barrier dysfunction, with TLR4 KO mice demonstrating lower serum FITC–dextran. Together with intestinal injury, CIGT is also characterised by an increase of pain and discomfort hypothesised to occur via neuroinflammation and neurotoxicity, controlled via the gut–brain axis (Y. Li et al., 2015). Results from an in vivo mouse study found that the administration of the chemotherapy, paclitaxel, activated TLR4 which sensitised transient receptor potential vanilloid subtype 1 to dorsal root ganglia neurons, creating adverse physiological effects (Y. Li et al., 2015). This evidence emphasises the importance of TLR4-mediated mechanisms in governing intestinal barrier function and its secondary consequences. However, given the ubiquitous expression of TLR4 on epithelial cells, enteric glia, CNS supportive cells, and immune cells, it remains challenging to decipher the exact mechanisms at play, thus hindering the design of suitable interventions. Despite these challenges, inhibition of TLR4 via naloxone has been investigated in the setting of CIGT (Coller et al., 2017). Naloxone was administered orally, with the goal of inhibiting epithelial TLR4 and thus prevent both direct SN-38-dependant TLR4 activation and host–microbiome interactions that govern mucosal injury (Coller

et al., 2017). Although naloxone was unable to attenuate CIGT, it was also shown to impact tumour growth, with adjuvant TLR4 inhibition decreasing the efficacy of irinotecan against adenocarcinoma cells. This parallels findings of Apetoh et al. (2007) and Fukata et al. (2007), with increased basal tumour growth and decreased chemoefficacy both shown in TLR4 KO mice. Together, these findings highlight the potential hazards of broad-spectrum antagonism of TLR4 in conjunction with chemotherapies and emphasise the importance of dissecting immune and epithelial mechanisms before transitioning to interventional studies.

5 | CONCLUDING REMARKS AND FUTURE DIRECTIONS

TLR4 is widely expressed on epithelial and immune cells along the gastrointestinal tract and is responsible for a range of homeostatic control mechanisms including immune system development. Recent evidence indicates the role of TLR4 signalling in the development, and progression, of many gastrointestinal diseases and toxicities. In normal physiology, both epithelial and immune expression of TLR4 is required to maintain host microbiome (Abreu, 2010), B cell recruitment, immune tolerance (Salazar et al., 2017), and maturation of dendritic cells (Michelsen et al., 2001). Although it is crucial to understand the role of TLR4 in a healthy condition, the impact of site-specific TLR4 expression in gastrointestinal diseases is of great importance. New disease mechanisms involving the activation of TLR4, and its associated signalling pathways, are continually emerging in this field. Although TLR4-based research continues to grow, there is a significant lack of specificity in the literature concerning cell-specific TLR4 signalling and its contribution to both health and disease. This common oversight almost certainly introduces translational errors and clouds interpretation of preclinical evidence. The use of novel intestinal-specific KO models will provide much-needed resolution into the site-specific roles of TLR4. These new models aim to better inform the development of new interventions targeting TLR4, providing greater specificity in augmenting immune or epithelial TLR4 without compromising core homeostatic mechanisms.

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Chapter 3 Publication

Epithelial-specific TLR4 knockout challenges current evidence of TLR4 homeostatic control of gut permeability

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Epithelial-Specific TLR4 Knockout Challenges Current Evidence of TLR4 Homeostatic Control of Gut Permeability

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Keywords
Toll-like receptor 4 · Mucosal barrier function · Gastrointestinal homeostasis · Ussing chamber · Pre-clinical model

Abstract

Introduction: Toll-like receptor 4 (TLR4) is a highly conserved immunosurveillance protein of innate immunity, displaying well-established roles in homeostasis and intestinal inflammation. Current evidence shows complex relationships between TLR4 activation, maintenance of health, and disease progression; however, it commonly overlooks the importance of site-specific TLR4 expression. This omission has the potential to influence translation of results as previous evidence shows the differing and distinct roles that TLR4 exhibits are dependent on its spatiotemporal expression. ***Methods:*** An intestinal epithelial TLR4 conditional knockout (KO) mouse line ($Tlr4^{\Delta IEC}$, $n = 6–8$) was utilized to dissect the contribution of epithelial TLR4 expression to intestinal homeostasis with comparisons to wild-type (WT) ($n = 5–7$) counterparts. Functions of the intestinal barrier in the ileum and colon were assessed with tissue resistance in Ussing chambers. Molecular and structural comparisons in the ileum and colon were assessed via histological staining, expression of tight junction proteins (occludin and zonular occludin 1 [ZO-1]), and presence of CD11b-positive immune cells. ***Results:*** There was no impact of the intestinal epithelial TLR4 KO, with no differences in (1) tissue resistance–ileum (mean ± standard error of mean [SEM]): WT 22 ± 7.2 versus $Tlr4^{\Delta IEC}$ 20 ± 5.6 ($\Omega \times cm^2$) $p = 0.831$, colon WT 30.8 ± 3.6 versus $Tlr4^{\Delta IEC}$ 45.1 ± 9.5 $p = 0.191$; (2) histological staining (overall tissue structure); and (3) tight junction protein expression (% area stain, mean ± SEM)–ZO-1: ileum–WT 1.49 ± 0.155 versus $Tlr4^{\Delta IEC}$ 1.17 ± 0.07, $p = 0.09$; colon–WT 1.36 ± 0.26 versus $Tlr4^{\Delta IEC}$ 1.12 ± 0.18 $p = 0.47$; occludin: ileum–WT 1.07 ± 0.12 versus $Tlr4^{\Delta IEC}$ 0.95 ± 0.13, $p = 0.53$; colon–WT 1.26 ± 0.26 versus $Tlr4^{\Delta IEC}$ 1.02 ± 0.16 $p = 0.45$. CD11b-positive immune cells (% area stain, mean ± SEM) in the ileum were mildly decreased in WT mice: WT 0.14 ± 0.02 versus $Tlr4^{\Delta IEC}$ 0.09 ± 0.01 $p = 0.04$. However, in the colon, there was no difference in CD11b-positive immune cells between strains: WT 0.53 ± 0.08

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versus $Tlr4^{\Delta IEC}$ 0.49 ± 0.08 $p = 0.73$. ***Conclusions:*** These data have 2 important implications. First, these data refute the assumption that epithelial TLR4 exerts physiological control of intestinal physiology and immunity in health. Second, and most importantly, these data support the use of the $Tlr4^{\Delta IEC}$ line in future models interrogating health and disease, confirming no confounding effects of genetic manipulation.

Introduction

Polarized epithelial cells covering the intestinal tract form a highly selective barrier between the bacteria-filled gut lumen and the comparatively sterile subepithelial tissue [1]. This barrier maintains homeostasis within the gastrointestinal tract, allowing for nutrient absorption and regulation of water exchange [2]. Crucially, the intestinal epithelial lining is also a first-line of defense from pathogens, whereby innate immune pattern recognition receptors recognize harmful bacteria and promote protective inflammatory cascades [1]. Toll-like receptor 4 (TLR4) is a type of pattern recognition receptor expressed on a variety of cell types including immune [3] and epithelial cells [4]. TLR4 and its accessory proteins MD2 and CD14 are widely researched due to dual roles in homeostatic control and suspected involvement in multiple conditions, including inflammatory bowel diseases and chemotherapy-induced gastrointestinal toxicity [5, 6].

Based on its consistent implication with diseased states that are characterized by intestinal dysfunction, TLR4 has been regularly reported to be a key regulator of mucosal barrier function and thus intestinal permeability under physiological conditions [7, 8]. Intestinal permeability via the paracellular route is dictated via tight junction proteins located on the apical-lateral cell surface [9]. The multiple intercellular and bridging proteins of the tight junction, including occludin, zonular occludin 1 (ZO-1), and claudins, allow for the movement of solutes across their electro-osmotic gradient to maintain intestinal homeostasis [10]. A considerable body of evidence anecdotally supports TLR4-mediated barrier control, with TLR4 expression strongly correlating with functional assessments of intestinal permeability and molecular characteristics of tight junction proteins [6]. For example, a 2018 study by Bein et al. [11] found that in a necrotizing enterocolitis model, a decrease in TLR4 expression was significantly associated with a decrease in occludin, ZO-1, and claudin-4 and resulted in increased permeability. While this suggests a connection between functional TLR4 and the preservation of the tight junction complex, these findings are only secondary to original aims and do not fully explain the role of TLR4 in homeostasis. Previous research using global TLR4 knockout (KO) mice also shows that a lack of TLR4 expression does not impact tight junction protein development and barrier function; however, this study only analyzed tight junction expression post-chemotherapy challenges [6]. While these studies implicate TLR4 in the pathobiological control of the mucosal barrier, the majority of these data have been generated in models of disease, and as such, conclusions regarding its physiological control cannot be made.

Another major oversight in the literature regarding TLR4's regulatory control of the intestinal barrier is the lack of site-specific interrogation. TLR4 is not only expressed on epithelial cells of the intestinal mucosa but also immune cells of the submucosa. In fact, immune expression of TLR4 is considerably higher than that of epithelial expression [3], and as such, its impact on mucosal homeostasis and disease is arguably higher. A failure to address site-specific TLR4 mechanisms hampers our ability to dissect causative mechanisms and thus impairs translation of fundamental findings. This has the potential to misguide new interventions targeting TLR4 that may not be delivered in a manner that optimally targets TLR4. This paradox is particularly important in cancer research, where TLR4-dependent mechanisms are central to both the efficacy and toxicity of therapy; yet, a lack of site-specific interrogation has resulted in highly variable and contradictory findings in studies attempting to augment its activity.

There is a clear need to study TLR4-dependent control of the mucosal barrier in a manner that dissects epithelial versus immune mechanisms. As such, we have utilized a conditional intestinal epithelial-specific TLR4 KO mouse line ($Tlr4^{\Delta IEC}$) [12], with epithelial deletion of TLR4 and unimpaired immune cell expression TLR4. In characterizing this mouse line, we are given the unique opportunity to rigorously define the regulatory role of epithelial TLR4 on the intestinal barrier under physiological conditions. As such, we aimed to characterize the potential intestinal differences of this $Tlr4^{\Delta IEC}$ line compared to wild-type (WT) mice, using structural, molecular, and electrophysiological assessments.

Materials and Methods

Animal Husbandry

Male and female WT C57BL/6 ($n = 5–7$) and intestinal epithelial conditional TLR4 KO C57BL/6 ($Tlr4^{\Delta IEC}$, $n = 6–8$) mice aged 8–12 weeks were housed in ventilated cages in groups of 3–6 ani-

Table 1. Real-time PCR primer sequences for TLR4 and β-actin

	Forward primer	Reverse primer
TLR4 (Merck)	5′-CTCTGCCTTCACTACAGAGAC-3′ T_m 58.3°C	5′-TGGATGATGTTGGCAGCAATG-3′ T_m 69.1°C
β-Actin (Integrated DNA Technologies)	5′-CTCTTCCAGCCTTCCTTCCT-3′ T_m 56.4°C	5′-AGCACTGTGTTGGCGTACAG-3′ T_m 57.9°C

TLR4, Toll-like receptor 4; PCR, polymerase chain reaction.

mals per cage with a 12-hour light/dark cycle and access to irradiated standard mouse chow and sterile water. Mice were euthanized via CO_2 exposure and cervical dislocation prior to dissection, in accordance with ethical approval of the University of Adelaide Animal Ethics Committee (M-2019-020) and the University of Adelaide Institutional Biosafety Committee (IBC approval number 14254). The study complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2014).

Breeding Strategy and Genetic Confirmation

The intestinal epithelial conditional TLR4 KO C57BL/6 (*Tlr4*$^{\Delta IEC}$) mouse model was created by following a transgenic *Vil1-cre*/*Tlr4loxP* breeding strategy (The Jackson Laboratory, Bar Harbor, ME, USA). By crossing a homozygous *Tlr4loxP*/*Vil1-cre* WT with a homozygous *Tlr4loxP*/hemizygous *Vil1-cre*, this breeding strategy resulted in 1 in 2 offspring being the desired conditional KO. Conditional KO of TLR4 was confirmed via polymerase chain reaction (PCR) analysis as per protocols provided by the Jackson Laboratory [13] for *TLR4flox* [14] and *Vilcre* genes [15], where DNA was extracted from mouse ear notches using the Nucleospin Tissue DNA extraction kit and used at a working concentration of 20 ng/μL (Machery-Nagel, Duren, Germany). Primer sequences used for confirmation of genotype were as follows: Vil-cre forward GCTTTCAAGTTTCATCCATGTTG, Vil-cre WT reverse TTCATGATAGACAGATGAACACAGT, Vil-cre mutant reverse GTCTTTGGGTAAAGCCAAGC, TLR4-floxed forward TGACCACCCATATTGCCTATAC, and TLR4-floxed reverse TGATGGTGTGAGCAGGAGAG. Cycling conditions for Vil-cre were as follows: denaturing at 95°C for 3 min, then 95°C for 5 s, and then 60°C for 30 s. The final 2 steps were repeated for 40 cycles. The mutant band, representing the presence of hemizygous Vil-cre was at 85 base pairs, compared to WT Vil-cre at 119 base pairs. Cycling conditions for TLR4 flox were 94°C for 2 min, then the following steps were repeated for 10 cycles: 94°C for 20 s, 65°C for 15 s (decreasing by 0.5°C each cycle), and 68°C for 10 s. Following from this, samples were cycled 28 times at 94°C for 16 s, 60°C for 15 s, and then 72°C for 10 s. The final step was 2 min at 72°C. Homozygous TLR4-floxed samples produced a band of 285 base pairs, heterozygous samples were at 234 base pairs and 285 base pairs, and WT TLR4 produces a band at 234 base pairs. For visualizations, samples were run in 4% agarose and visualized using Midori Green Advance DNA stain (Nippon Genetics, Tokyo, Japan). Conditional KO of intestinal epithelial TLR4 (*Tlr4*$^{\Delta IEC}$) resulted in PCR showing mutant Vil-cre (85 base pairs) and homozygous TLR4-floxed (285 base pairs).

Further confirmation of successful KO was conducted by real-time PCR, where small intestinal tissue for both WT and *Tlr4*$^{\Delta IEC}$ was harvested and scraped to separate the submucosal layer for epithelial-specific TLR4 analyses. For both strains, the whole small intestine was dissected and opened longitudinally. Using a rounded scalpel, a light feather-like scraping was undertaken to separate out an epithelial-dominant sample. Ten nanograms of RNA extracted from these scrapings for both WT and *Tlr4*$^{\Delta IEC}$ mice was reverse transcribed using the iScript cDNA Synthesis Kit (#1708890; Bio-Rad, Gladesville, NSW, Australia) as per manufacturer's instructions. RT-PCR was performed using the Rotor-Gene 3000 (Corbett Research Sydney, Mortlake, NSW, Australia). Amplification mixes contained 1 μL of cDNA sample (100 ng/μL), 5 μL of SYBR green fluorescence dye (QuantiTect; Qiagen, Hilden, Germany), 3 μL of RNase-free water (Macherey Nagal, Duren, Germany), and 0.5 μL of each forward and reverse primers (50 pmol/μL), to make a total reaction volume of 10 μL. Primer details are as presented in Table 1. Thermal cycling conditions were: 95°C for 10 min, 40 cycles of 95°C for 10 s, 59°C for 30 s, and 72°C for 45 s and a final melt step of 60–95°C changing 1°C per step, holding for 5 s each. Samples were run in triplicate, including negative controls (no cDNA template). Experimental threshold (CT) values were calculated by the Rotor Gene 6 programme. CT values were used to quantify relative mRNA expression of TLR4 and β-actin using the ΔC_T method, where relative expression = $2^{-(CT\,TLR4\,-\,CT\,\beta\text{-actin})}$ [16].

Ex vivo Electrophysical Assessments

Ussing chambers (EM-CSYS-8 with DM-MC8 voltage clamp/electrode input; Physiologic Instruments, San Diego, CA, USA) were used to assess intestinal electrophysiology in WT and *Tlr4*$^{\Delta IEC}$ mice as previously described [16]. Briefly, segments of ileum and colon were dissected from mice and flushed with ice-cold 1× phosphate-buffered saline (PBS). One cm segments were opened longitudinally along the mesenteric attachment line, mounted into 0.1 cm^2 aperture sliders (P2303A; Physiologic Instruments), and inserted into chambers filled with a glucose-fortified Ringers solution consisting of (in millimolar): NaCl 115.4, KCl 5, $MgCl_2$ 1.2, NaH_2PO_4 0.6, $NaHCO_3$ 25, $CaCl_2$ 1.2, and glucose 10, bubbled with carbogen gas (95% O_2, 5% CO_2) and warmed to 37°C [16]. Ileal segments had the mucosal side bathed in mannitol-fortified Ringers (10 mM) to maintain osmotic balance. Once mounted, tissue was voltage-clamped to zero potential difference, establishing baseline readings. Tissue was allowed to equilibrate for 20 min before short circuit current (Isc, marker of net ion transport/secretion), and transepithelial electrical resistance (marker of barrier integrity) was measured using Acquire and Analyse Revision II (v2.3; Physiologic Instruments).

Histopathological Analyses

Hematoxylin and Eosin Staining

Mouse ileum and colon samples were fixed in 10% formalin and embedded into paraffin wax blocks. Formalin-fixed paraffin embedded blocks were sectioned (4 μm) and mounted on SuperFrost White slides (Menzel-Gläser, Braunschweig, Germany). Slides were then fixed on a 37°C heat block, for a minimum of 1 h. Standard hematoxylin and eosin staining procedures were followed [6]. In brief, slides were dewaxed in 3× washes in 100% histolene for 5 min each and then rehydrated with graded ethanol as previously described [6]. Slides were then stained in Lille-Mayers hematoxylin for 5 min and rinsed until clear in running tap water. Slides were then quickly dipped twice in 1% acid alcohol (5 mL HCl + 500 mL 70% ethanol) and washed in running tap water until clear. Tissue was then placed in Scott's Tap Water (in millimolar) ($MgSO_4$ 166.2; $NaHCO_3$ 23.7 in 1 L dH_2O) for 2 min and washed. Counterstaining with alcoholic eosin (Sigma-Aldrich, St Louis, MO, USA) occurred for 2 min, and slides were washed with running tap water until clear. Slides were then treated with 90% ethanol (30 s) and 100% ethanol (30 s) and finally cleared with 3× washes in 100% histolene for 5 min each. Slides were coverslipped with D.P.X neutral mounting medium (Sigma-Aldrich).

Slides were imaged using an Olympus BX51 light microscope and Olympus DP20 microscope camera (Olympus, Tokyo, Japan). A modified version of well-established intestinal injury scoring criteria [17] was used to quantify possible differences between groups, with 1 representing the presence and 0 representing the absence of the pathophysiological marker. The criteria included: 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, edema, villous fusion, and villous atrophy. The latter 2 criteria were not assessed in colon samples; therefore, the total possible score for ileum samples was 8 and for colon samples was 6. Furthermore, ileum villus height and crypt depth and colonic mucosal thickness were assessed by a blinded researcher (E.E.B.) using the Olympus cellSens Standard imaging program (Olympus).

Alcian Blue and Periodic Acid–Schiff

Alcian blue and periodic acid–Schiff was used to quantify goblet cells in the mucosal of the ileum and colon from formalin-fixed paraffin-embedded tissue blocks. Tissue was cut into 4-μm sections and placed on SuperFrost White slides (Menzel-Gläser). Slides were dewaxed in 3× washes in 100% histolene for 5 min each and then rehydrated with graded ethanol. Slides were stained in alcian blue for 5 min (194 mL dH_2O + 6 mL 100% acetic acid + 2 g alcian blue) before being incubated in 0.5% periodic acid for 5 min. Slides were again washed with dH_2O and then incubated in Schiff's reagent for 15 min before being counterstained with hematoxylin for 30 s. Slides were cleared in 100% histolene (3 × 5 min) and coverslipped with D.P.X neutral mounting medium. Slides were imaged using the Nanozoomer Digital slide scanner, with goblet cell counts conducted by a blinded researcher (K.R.S.).

Immunofluorescence of Tight Junction Proteins and Immune Cells

To investigate molecular determinants of barrier function, immunofluorescence for ZO-1 and occludin was performed. Briefly, the intestine was removed and immediately flushed with ice-cold

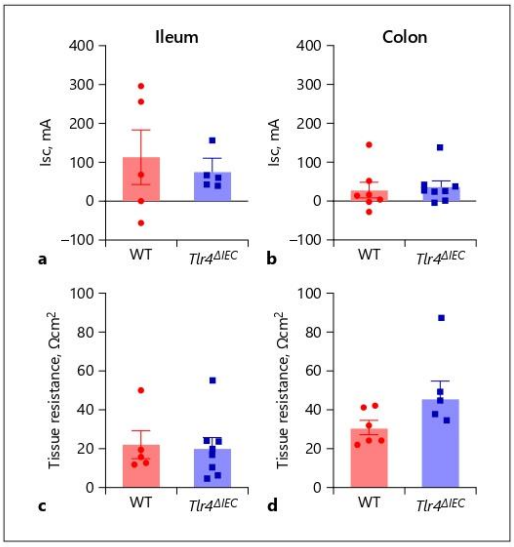

Fig. 1. Intestinal electrophysiology is not dependent on TLR4 expression. Baseline short-circuit current (Isc, mA) for the ileum (**a**) and colon (**b**) and transepithelial tissue resistance ($\Omega \times cm^2$) for the ileum (**c**) and colon (**d**) samples in WT and $Tlr4^{\Delta IEC}$ mice. No difference between groups (WT n = 5–7 and $Tlr4^{\Delta IEC}$ n = 6–8, p > 0.05). Data presented as mean ± SEM. WT, wild-type; SEM, standard error of mean; TLR4, Toll-like receptor 4.

1× PBS. Segments of the ileum and colon were fixed in 10% neutral buffered formalin, processed, and embedded into paraffin wax. Tissues were then cut into 4-μm sections and placed onto FLEX IHC microscope slides (Flex Plus Detection System, #K8020; Dako, Næstved, Denmark) and heated on a heat pad. Slides were deparaffinised via 3× washes with 100% histolene and rehydrated with graded ethanol (100% ethanol for 30 s, 90% ethanol for 30 s and 70% ethanol for 30 s) [16]. Antigen retrieval was via the PT Link bath (pre-treatment module, #PT101; Dako) using an EDTA/Tris buffer consisting of (in millimolar): Tris 9.9 and EDTA 1.3 and 0.5 mL Tween 20 in 1.5 L dH_2O, pH = 9 at 97°C for 20 min.

Tissue samples were stained using the DakoCytomation Autostainer (AutostainerPlusTM; Dako, serial number: AS1271F1104). The primary antibodies used were as follows: ZO-1 (61–7300; Invitrogen, Carlsbad, CA, USA, 0.25 mg/mL, 1:100 dilution), occludin (33–1500; Invitrogen, 0.5 mg/mL, 1:200 dilution), and CD11b (ab133357; Abcam, Cambridge, UK, 1:1,000 dilution). Primary antibody was diluted in 5% normal horse serum (Sigma-Aldrich), 1× PBS for tight junction analyses and 1% bovine serum albumin (BSA) for immune cell analyses (Sigma-Aldrich). The secondary antibodies were AlexaFluor 488 anti-mouse (occludin), AlexaFluor 488 anti-rabbit (CD11b), and AlexaFluor 568 anti-rabbit (ZO-1) (Thermo Fisher, Waltham, MA, USA). The secondary antibody was diluted in 1× PBS, 1% BSA, and 2% fetal bovine serum. DAPI (1 μg/mL) (Sigma-Aldrich) counterstaining was utilized to visual-

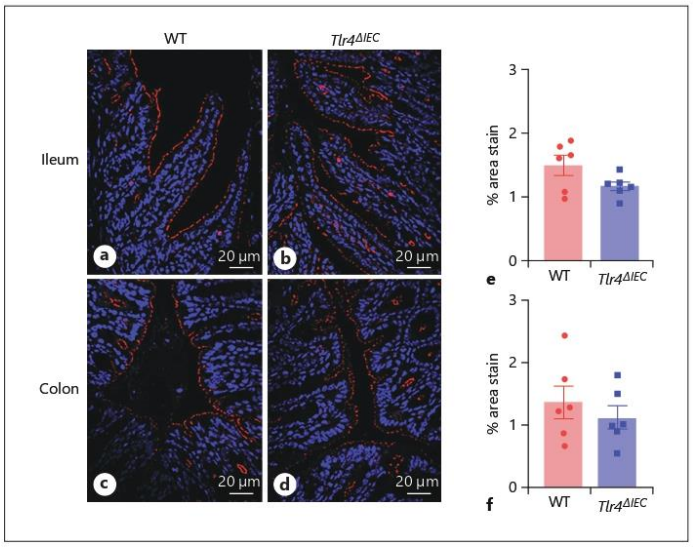

Fig. 2. Epithelial TLR4 deletion does not influence ZO-1 expression. ZO-1 expression (red) in WT and $Tlr4^{\Delta IEC}$ mice ileum and colon with DAPI (blue) counterstain of nuclei; WT ileum (**a**), $Tlr4^{\Delta IEC}$ ileum (**b**), WT colon (**c**), $Tlr4^{\Delta IEC}$ colon (**d**), magnification ×40. No difference in ZO-1 expression for either (**e**) ileum or (**f**) colon (% area stain, $n = 6$, $p > 0.05$). Data presented as mean ± SEM. ZO-1, zonular occludin 1; WT, wild-type; SEM, standard error of mean; TLR4, Toll-like receptor 4.

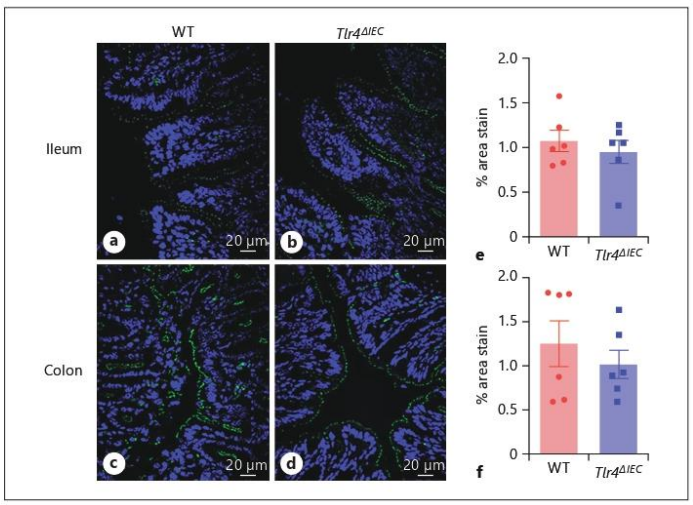

Fig. 3. Occludin expression is independent of epithelial TLR4 expression. Occludin expression (green) in WT and $Tlr4^{\Delta IEC}$ mice ileum and colon with DAPI (blue) counterstain of nuclei; WT ileum (**a**), X ileum (**b**), WT colon (**c**), $Tlr4^{\Delta IEC}$ colon (**d**), magnification ×40. No difference in occludin expression for either (**e**) ileum or (**f**) colon (% area stain, $n = 6$, $p > 0.05$). Data presented as mean ± SEM. WT, wild-type; SEM, standard error of mean; TLR4, Toll-like receptor 4.

ize the nucleus of cells in sample, with 1× PBS as the diluent. A protein block of 10% normal horse serum for tight junctions and 4% BSA for immune cells was used to reduce nonspecific antibody binding during the procedure.

Post-staining, a drop of Fluoroshield (Sigma-Aldrich) was applied to each slide and coverslipped. Slides were then stored in the dark at 4°C to await imaging. Slides were imaged using the Nikon A1 Confocal Microscope using a ×40 objective. Fluorescent staining was quantified via % area stain on the Fiji Image J program as previously described [18].

Statistics

All data were compared using Prism version 8.0 (GraphPad Software, San Diego, CA, USA). Data were first assessed was for

normality using the Shapiro-Wilk test. Parametric data were analyzed using a one-way ANOVA or *t* test and presented as mean ± standard error of mean (SEM). Nonparametric data were analyzed using a Kruskal-Wallis test and presented as median and range. *p* values <0.05 were deemed significant.

Results

Epithelial TLR4 Does Not Control Intestinal Barrier Function, Tight Junction Integrity, or Immune Cell Infiltration in Healthy Mice

Successful conditional KO of TLR4 on intestinal epithelial cells was confirmed by both genotyping and RT-PCR analysis, with 8-fold higher TLR4 expression in WT intestinal epithelial-dominant scrapings than in $Tlr4^{\Delta IEC}$ scrapings (mean ± SEM): WT 0.29 ± 0.02 versus $Tlr4^{\Delta IEC}$ 0.04 ± 0.02, $p = 0.012$.

No difference between WT and $Tlr4^{\Delta IEC}$ was observed in the ileum or colon in baseline short-circuit current: ileum (mean ± SEM): WT 113.3 ± 69.8 versus $Tlr4^{\Delta IEC}$ 75.3 ± 34.9 mA $p = 0.607$, colon: WT 28.4 ± 21.1 versus $Tlr4^{\Delta IEC}$ 36.7 ± 15.5 mA $p = 0.752$ (Fig. 1a, b). Similarly, there was no difference in baseline transepithelial tissue resistance: ileum (mean ± SEM): WT 22 ± 7.2 versus $Tlr4^{\Delta IEC}$ 20 ± 5.6 $\Omega \times cm^2$, $p = 0.831$, colon WT 30.8 ± 3.6 versus $Tlr4^{\Delta IEC}$ 45.1 ± 9.5 $\Omega \times cm^2$, $p = 0.191$ (Fig. 1c, d).

ZO-1 and occludin staining was evident at the apicolateral border of epithelial cells of villous and crypt structures of the ileum and colon (Fig. 2, 3). Quantification of tight junction protein staining (% area stain) showed no differences for ZO-1: ileum (mean ± SEM) WT 1.49 ± 0.155 versus $Tlr4^{\Delta IEC}$ 1.17 ± 0.07 $p = 0.09$; colon WT 1.36 ± 0.26 versus $Tlr4^{\Delta IEC}$ 1.12 ± 0.18 $p = 0.47$. Similarly, there was no difference in occludin expression: ileum (mean ± SEM) WT 1.07 ± 0.12 versus $Tlr4^{\Delta IEC}$ 0.95 ± 0.13 $p = 0.53$; colon WT 1.26 ± 0.26 versus $Tlr4^{\Delta IEC}$ 1.02 ± 0.16 $p = 0.45$. Positive CD11b staining (Fig. 4) was evident in both the ileum mucosa and colon mucosa and submucosa. CD11b-positive immune cells in the ileum were mildly decreased in WT mice: (% area stain, mean ± SEM) WT 0.14 ± 0.02 versus $Tlr4^{\Delta IEC}$ 0.09 ± 0.01 $p = 0.04$. However, there were no differences in CD11b-positive immune cells in the colon between strains: (% area stain, mean ± SEM) WT 0.53 ± 0.08 versus $Tlr4^{\Delta IEC}$ 0.49 ± 0.08, $p = 0.73$.

Epithelial TLR4 Deletion Does Not Affect Intestinal Morphometry

No histological differences were observed between WT and $Tlr4^{\Delta IEC}$ mice in the ileum or colon (Fig. 5a–d), with no change in villus height ($p = 0.49$), ileum crypt depth ($p = 0.66$), or colonic crypt depth ($p = 0.52$) (Fig. 5e–g, $p > 0.05$). Furthermore, to ensure a comprehensive and translational assessment of intestinal structure, a tissue injury score was assessed. No evidence of microscopic injury was detected in either WT or $Tlr4^{\Delta IEC}$ ileum or colon tissue (ileum $p = 0.617$, colon $p = 0.529$). Finally, no difference in goblet cell abundance in ileum villi, ileum crypt, and colon crypt between WT and $Tlr4^{\Delta IEC}$ was observed (Fig. 6a–g, $p > 0.05$ for all groups).

Discussion/Conclusion

TLR4 has received a significant amount of attention for its homeostatic control and therapeutic applications based on its assumed regulation of mucosal barrier function. This is the first study to present the baseline intestinal characteristics of epithelial-specific TLR4 KO mice ($Tlr4^{\Delta IEC}$) and has found that contrary to expectations, the absence of epithelial TLR4 did not alter intestinal homeostasis. These data highlight the importance of site-specific TLR4 investigation and underscore the limitations of extrapolating evidence from disease models to healthy states.

The concept that TLR4 is involved in homeostatic control is clearly outlined in studies investigating the nervous control of intestinal tissue [19, 20] and immune tolerance [21]. However, TLR4 involvement in homeostatic control of barrier function has been inferred from disease modeling studies. Shi et al. [22] found that intestinal injury in response to dextran sulfate sodium induced colitis was significantly aggravated in a global TLR4 KO mouse model. This suggests TLR4 expression is a protective component of the intestines and supports the healthy functioning of the intestinal barrier [22]; however, this is contradictory to earlier findings which show that TLR4 overexpression leads to impaired intestinal epithelial cell differentiation and barrier dysfunction [23]. Since not all studies distinguish between site-specific expression of TLR4, contradicting evidence is expected. Our study is one of the first to entirely focus on how intestinal epithelial TLR4 expression influences the healthy state of the intestines.

As outlined, our data suggest that epithelial TLR4 is not essential to the regulation of the intestinal environment, namely the role of tight junction protein expression, goblet cell populations, and functional tissue permeability in healthy development. While minor nonsignificant differences have been noted between groups, this

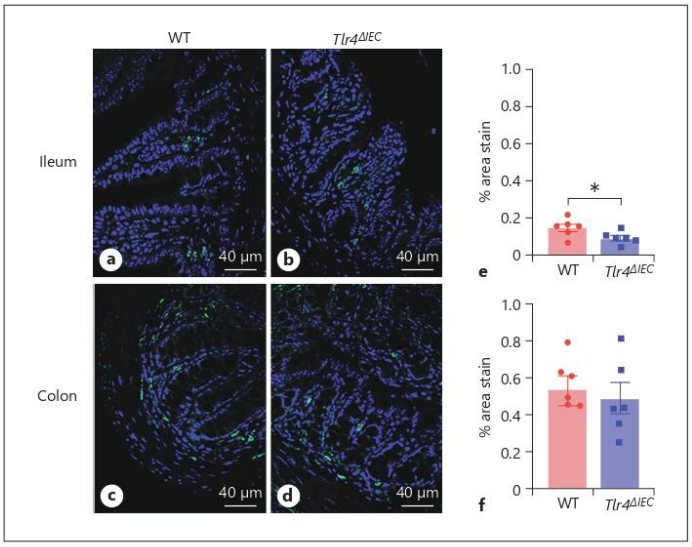

Fig. 4. Immune cell infiltration does not depend on epithelial TLR4 expression. CD11b expression (green) in WT and $Tlr4^{\Delta IEC}$ mice ileum and colon with DAPI (blue) counterstain of nuclei; WT ileum (**a**), $Tlr4^{\Delta IEC}$ ileum (**b**), WT colon (**c**), $Tlr4^{\Delta IEC}$ colon (**d**), magnification ×40. Difference in CD11b immune cells in (**e**) ileum ($^*p = 0.04$), no difference in (**f**) colon (% area stain, $n = 6$, $p > 0.05$). Data presented as mean ± SEM. WT, wild-type; SEM, standard error of mean; TLR4, Toll-like receptor 4.

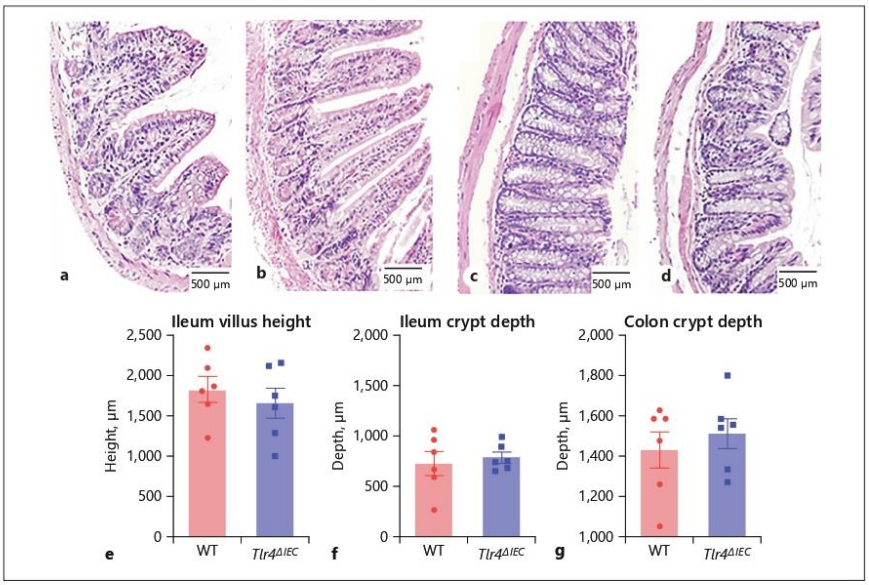

Fig. 5. Epithelial TLR4 deletion does not affect intestinal architecture. H&E stain of the ileum and colon from WT and $Tlr4^{\Delta IEC}$ mice. WT ileum (**a**), $Tlr4^{\Delta IEC}$ ileum (**b**), WT colon (**c**), $Tlr4^{\Delta IEC}$ colon (**d**), magnification ×20. No difference in ileum villus height (**e**, μm), ileum crypt depth (**f**, μm), or colon crypt depth (**g**, μm) ($n = 6$, $p > 0.05$). Data presented as mean ± SEM. H&E, hematoxylin and eosin; WT, wild-type; SEM, standard error of mean; TLR4, Toll-like receptor 4.

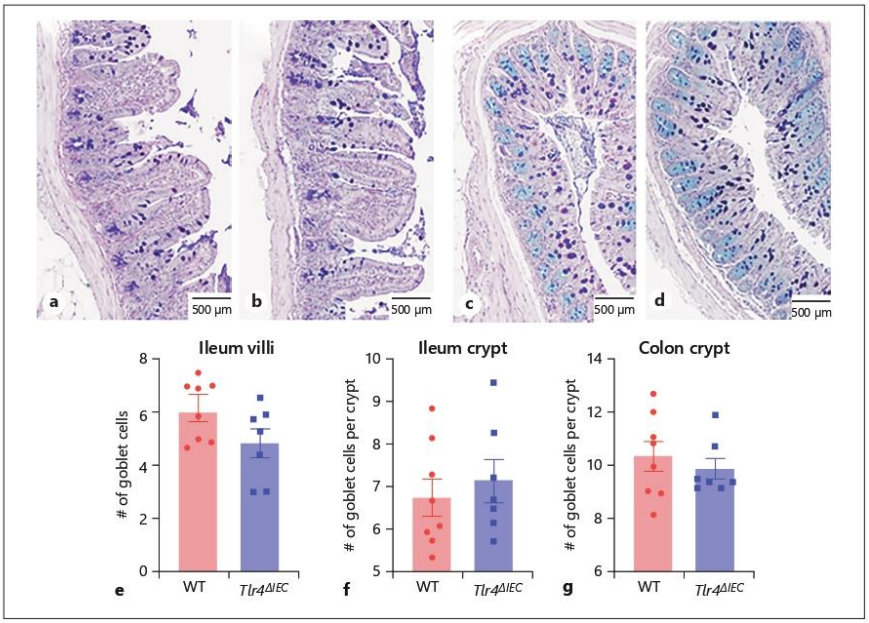

Fig. 6. Goblet cell abundance is not affected by TLR4 expression in the intestinal epithelium. AB-PAS stain of the ileum and colon from WT and $Tlr4^{\Delta IEC}$ mice to visualize goblet cells (dark purple/blue stain). WT ileum (**a**), $Tlr4^{\Delta IEC}$ ileum (**b**), WT colon (**c**), $Tlr4^{\Delta IEC}$ colon (**d**), magnification ×20. No differences in goblet cells in ileum villi (**e**), ileum crypt (**f**) or colon crypts (**g**) (# of goblet cells per structure, WT $n = 8$, $Tlr4^{\Delta IEC} n = 7$, $p > 0.05$). Data presented as mean ± SEM. AB-PAS, alcian blue and periodic acid–Schiff; WT, wild-type; SEM, standard error of mean; TLR4, Toll-like receptor 4.

is most likely due to stochastic variation. Previous research conducted by our group has demonstrated intestinal permeability and morphology changes in a positive control of epithelial tissue disruption. In mice treated with the chemotherapy irinotecan, baseline intestinal conductance (a measure of intestinal permeability, the opposite of tissue resistance) was significantly increased (53.19 ± 6.46 s/cm^2, +105.62% relative to WT controls; $p = 0.0008$) [16]. Furthermore, irinotecan-treated small intestinal and colonic tissue showed severe damage, including villous blunting and crypt degeneration [24]. There is a substantial difference between these positive-control outcomes and the minor changes in the present study, therefore suggesting that current minor variability in the $Tlr4^{\Delta IEC}$ versus WT data is inconsequential. Our current findings indicate that there may be compensatory mechanisms controlling the gastrointestinal microenvironment in the absence of epithelial TLR4. A possible mechanism could be that immune TLR4 is responsible for modulating barrier function and intestinal homeostasis. This aligns with the higher TLR4 expression on immune cell populations and acknowledges the profound immune infiltrate of the gut [25]. TLR4 is expressed on a range of immune cells including, macrophages, myeloid cells, and dendritic cells [3] and has proven roles in dendritic cell maturation [26] and immune tolerance [27]. Considering that immune TLR4 has been shown to control immune system functioning and development of a healthy microbiome, it could be also deduced that immune TLR4 aids in controlling intestinal permeability and barrier function in mice. This notion is supported by our data, which showed no difference in intestinal characteristics between the $Tlr4^{\Delta IEC}$ and WT mice. To confirm this role of exclusive immune TLR4 signaling, future work could be conducted in conditional mice where there is a deletion of immune TLR4 expression.

An alternative mechanism possibly responsible for this compensation could include the recognition of pathogens and tolerance of the commensal microbiota via different TLRs, notably TLR2 [8]. Upon ligand binding, TLR2 activates the MyD88-dependent inflammatory pathway [28]. This pathway is also activated in response to TLR4 activation; therefore, a distinct overlap in TLR4 and TLR2 signaling exists [28]. The similarity between TLR2 and TLR4 is best shown in healthy states, with an early study finding that activation of TLR2 and TLR4 primes dendritic cell tolerance to commensal organisms [29]. Furthermore, the combination of TLR2 and TLR4 signaling is indicated in the healthy control of spontaneous and serotonin-induced contractile responses of mouse ileum [20]. It is the commonality between TLR2 and TLR4 signaling pathways, which may explain why intestinal homeostasis was maintained in our *Tlr4*$^{\Delta IEC}$ model. A previous research study has investigated disruption of TLR pathways, including TLR2 KO, not MD-2, and has shown in intestinal models of chemotherapy-induced intestinal mucositis that deletion of TLR2 alone increased intestinal inflammation and damage, suggesting TLR2 is a potential therapeutic target [30]. While this evidence indicates importance of TLR2, rather than TLR4, in intestinal regulation, unfortunately, epithelial deletion of TLR2 has not been previously studied in either healthy or diseased states. Therefore, future research could be centered on a TLR2 epithelial-specific KO mouse model, to further investigate this complex relationship between different TLR expression and intestinal function.

Overall, our findings support the use of this *Tlr4*$^{\Delta IEC}$ mouse line in the investigation of gastrointestinal disease, where TLR4 may be of interest. These mice showed no difference in baseline intestinal characteristics compared to WT, therefore displaying no inherent variability of intestinal function caused by genetic modification of intestinal epithelial TLR4. This is a promising sign for the ongoing viability of this model as the retention of normal intestinal function suggests that the *Tlr4*$^{\Delta IEC}$ model is reliable. This could allow for future disease models in the *Tlr4*$^{\Delta IEC}$ mice to dissect the contribution of epithelial TLR4 to disease development. Translationally, the use of these *Tlr4*$^{\Delta IEC}$ mice in models of gastrointestinal disease will provide much greater insight into the site-specific contribution of TLR4. This would allow for the guiding of future therapeutics, including nanoparticle delivery systems allowing epithelial TLR4 to be augmented in a manner that prevents any systemic effect [31]. This could possibly include TLR4 agonist or antagonist delivery to the site-specific area, meaning that only the intestinal epithelial population of TLR4 would be altered, leaving immune and nervous TLR4 functioning uninterrupted. This is especially important where site-specific TLR4 expression shows distinct and potentially contradicting mechanisms, for example, following cancer treatments [6, 32]. However, while these results shed further insight into the mechanistic roles of epithelial TLR4, they should be approached with caution as this study utilized a small sample size where further functional data, such as metabolic and absorptive capacity, were not assessed. A further limitation of the current study is the exclusion of other related KO models, such as immune-specific TLR4 or TLR2 KO. As data presented in the *Tlr4*$^{\Delta IEC}$ mice revealed no differences in intestinal functioning, it can be deduced that epithelial TLR4 is likely to have a minor role in intestinal homeostasis. Consequently, future research including these alternative KO models would greatly enhance this field of knowledge.

In conclusion, TLR4 is an important immunosurveillance protein to many areas of current medical research, including inflammatory gastrointestinal diseases and chemotherapy-induced gastrointestinal toxicity [5, 6]. While there is a large body of research surrounding the dual roles of TLR4 in both healthy states and disease, currently, there is very little distinction of cell-specificity in research outcomes. This oversight has the potential to influence the translation of results to clinical practice. To facilitate the emergence of research that considers cell-specific TLR4 expression, a well-validated intestinal epithelial TLR4 conditional mouse model (*Tlr4*$^{\Delta IEC}$) must exist. The current study verified that *Tlr4*$^{\Delta IEC}$ mice are not fundamentally altered prior to future disease modeling studies. These results both support the use of this model in future studies and has presented novel insights into the role of intestinal epithelial TLR4 in homeostatic control.

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Statement of Ethics

The study design was approved by the Animal Ethics Committee of the University of Adelaide and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2014) (animal ethics approval No.: M-2019-020).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All the authors fulfill the ICMJE criteria for authorship. Elise E. Crame is the primary researcher associated with this research and substantially contributed to research design, data acquisition, analysis, and interpretation. She is the primary author of this research, including the drafting and revising process. Joanne M. Bowen, Janet K. Coller, and Hannah R. Wardill provided significant contribution to the study design and conception and had substantial involvement in writing, drafting, and the revision process. Kate R. Secombe provided substantial contribution to data acquisition, analysis, and interpretation. Maxime François and Wayne Leifert provided substantial contribution to data acquisition, analysis, and interpretation.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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Chapter 5 Publication

Contribution of TLR4 to colorectal tumour microenvironment, etiology and prognosis

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RESEARCH

Contribution of TLR4 to colorectal tumor microenvironment, etiology and prognosis

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Abstract

Purpose Toll-like receptor 4 (TLR4) is increasingly recognized for its ability to govern the etiology and prognostic outcomes of colorectal cancer (CRC) due to its profound immunomodulatory capacity. Despite widespread interest in TLR4 and CRC, no clear analysis of current literature and data exists. Therefore, translational advances have failed to move beyond conceptual ideas and suggestions.

Methods We aimed to determine the relationship between TLR4 and CRC through a systematic review and analysis of published literature and datasets. Data were extracted from nine studies that reported survival, CRC staging and tumor progression data in relation to TLR4 expression. Primary and metastatic tumor samples with associated clinical data were identified through the Cancer Genome Atlas (TCGA) database.

Results Systematic review identified heterogeneous relationships between TLR4 and CRC traits, with no clear theme evident across studies. A total of 448 datasets were identified through the TCGA database. Analysis of TCGA datasets revealed TLR4 mRNA expression is decreased in advanced CRC stages ($P < 0.05$ for normal vs Stage II, Stage III and Stage IV). Stage-dependent impact of TLR4 expression on survival outcomes were also found, with high TLR4 expression associated with poorer prognosis (stage I vs III (HR = 4.2, $P = 0.008$) and stage I vs IV (HR = 11.3, $P < 0.001$)).

Conclusion While TLR4 mRNA expression aligned with CRC staging, it appeared to heterogeneously regulate survival outcomes depending on the stage of disease. This underscores the complex relationship between TLR4 and CRC, with unique impacts dependent on disease stage.

Keywords Toll-like receptor 4 · Colorectal neoplasms · Systematic review · Humans

Introduction

Colorectal cancer (CRC) remains one of the most prevalent cancer diagnoses worldwide, with incidence rates in the United States of America of 37.8 per 100,000 (National Cancer and Institute: Surveillance 2021). This places CRC as the fourth most common cancer in western populations (Australian Institute and of Health and Welfare 2020; National Cancer and Institute: Surveillance 2021) which when coupled with its high mortality rates, cements this disease as a major healthcare burden. While significant advances have been made in identifying high level risk factors for CRC, heterogeneity in tumor progression and treatment response continues to challenge the understanding of its etiology (Buikhuisen et al. 2020). Few factors remain significant when traditional, largely unmodifiable risk factors (e.g. age, sex) are adjusted for, pointing to complex mechanisms governing tumor microenvironment which dictate growth trajectory and vulnerability to anti-cancer therapy (Buikhuisen et al. 2020).

The tumor microenvironment is a complex system of molecular and cellular components, produced by both host and tumor (Wang et al. 2018). The microenvironment's contribution to prognosis and clinical outcome has proven controversial, although evidence supports both beneficial and inhibitory roles. For example, the microenvironment

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facilitates immune invasion and destruction of tumor tissue (Fang et al. 2014). In contrast, it also contributes to tumor development, cancer cell survival and treatment resistance (Zhao et al. 2019). Irrespective of this complexity, it is clear that infiltration of peripheral immune cells into the tumor microenvironment is related to CRC progression and prognosis. A 2019 study using the cancer genome atlas (TCGA) and gene expression omnibus (GEO) databases reported that M_0 macrophages, M_1 macrophages and $CD4^+$ memory T cells were more abundant in CRC tissue compared to healthy tissues ($P < 0.02$) (Ge et al. 2019). Furthermore, higher infiltration of M_1 macrophage populations in CRC tissue correlated with lower participant survival ($P = 0.04$) (Ge et al. 2019). This underscores the involvement of the host immune system in CRC.

In light of the strong immune-mediated mechanisms that appear to be linked with CRC etiology and treatment response, there has been substantial interest in the potential role of the innate immune surveillance protein, toll-like receptor 4 (TLR4). TLR4 is a pattern recognition receptor, which upon activation, initiates a strong inflammatory response (Takeda and Akira 2004). TLR4 requires the accessory proteins myeloid differentiation factor 2 (MD-2) and cluster of differentiation 14 (CD14) to efficiently bind to ligands including, LPS, heat shock proteins (Hsp70 and Hsp90) and high-mobility group protein I (HMGBI) (Cheng et al. 2015). TLR4 signaling is vital to intestinal homeostatic maintenance, as previously reviewed (Bruning et al. 2021). TLR4 is notably upregulated in the intestine under inflammatory states including in people with ulcerative colitis, and this is further linked to ulcerative colitis-associated CRC risk and development (Fukata et al. 2007). Furthermore, genetic variants of *TLR4* (rs10116253, rs192791 1, rs7873784) have been linked to CRC (Huang et al. 2018a).

TLR4 is expressed on a range of different cell types within the tumor microenvironment, including dendritic, stromal, macrophage and epithelial cells (Li J et al. 2017). The importance of site-specificity of TLR4 expression in healthy and diseased states, including CRC, is well documented (Bruning et al. 2021). Pre-clinical CRC models indicate that TLR4 has both pro- and anti- tumor roles, with expression sites being a possible differentiating factor between whether TLR4 aids in cancer destruction or survival (Li et al. 2017). To add further complexity, TLR4 has also been identified to modulate toxicity following cancer therapy, including diarrhea and pain (Wardill et al. 2016). As such, it is currently unclear whether TLR4 is beneficial, or, potentially harmful in the CRC microenvironment, and whether it is a rationale target for intervention. We therefore aimed to systematically review current published evidence and datasets to crystalize the relationship between TLR4 and CRC staging, treatment toxicity and survival.

Methods

Search strategy, study selection and data retrieval

PubMed, Cochrane Library and Embase were searched between January and February 2022 for peer-reviewed journal publications using keywords listed in Supporting Information Table 1 and were screened for inclusion based on specific criteria; original research, clinical trials and studies conducted between 2010 and 2021; archival human tissue; CRC; participant survival; tumor recurrence; prognosis; toxicity; and TLR4 expression. Exclusion criteria included: animal models; cell lines; and cancer types other than CRC. Eligible publications were reviewed with the following data being extracted manually by two independent authors (EEC, JKC) using a computer-based template: sample size; CRC stage; chemotherapy treatments; participant demographics; type of TLR4 analysis; TLR4 specific outcomes (including expression rates and site-specificity); survival data (overall survival (OS), progression-free survival (PFS) or disease-free survival (DFS)); and tumor progression data. Summary outcomes are presented in Table 1.

TCGA clinical CRC cases database extraction and statistical analysis

RNA sequencing data and associated clinical metadata with a total of 512 samples in read counts (HTSeq-Counts) of CRC were obtained from the TCGA data portal (https://portal.gdc.cancer.gov/, accessed in December 2020). Data related to TLR4 mRNA expression, CRC staging and OS were extracted. TLR4 mRNA expression was dichotomized into high and low expression using the tertile cut point. The OS curve was constructed using Kaplan–Meier and log-rank test analysis, comparing high and low TLR4 expression groups for all cases and within each CRC stage. Statistical analyses were performed using GraphPad Prism 8.3.1 (GraphPad Software Inc., CA, USA) and R. studio 1.2.5033 (Inc., Boston, MA).

Multivariate analysis was also performed to determine whether mRNA expression was associated with OS in each tumor stage where variables included tumor stage (I: IV), sex and age. To avoid using potentially biased cut-points splitting low and high TLR mRNA expressing participant groups, a two sample t-test using continuous TLR4 mRNA expression values (with no cut-point required) compared mRNA expression between alive and deceased participants. Finally, TLR4 mRNA expression between normal tumor adjacent tissue and tumor samples from different stages were analyzed with a one-way ANOVA (normal vs stage I, stage II, stage III and stage IV).

Table 1 Summary of studies investigating impacts of TLR4 expression on human CRC clinical outcome

Author (Date)	Archival/ clinical study	Sample size (*n*)	CRC stage	Anti-cancer treatments	TLR4 analysis	Site-specific TLR4 (Y/N)	Survival outcomes (Y/N)	Type of survival	Cancer recurrence (Y/N)	Key findings
Cammarota et. al. (2010)	Archival	132	Mixed stage, stages I–IV	NR	IHC	Y	Y	DFS	Y	- TLR4 cells = grade of dysplasia - % of TLR4 + cells in the tumor stromal compartment = DFS and later relapse compared to % of TLR4 + cells in the stromal compartment (RR 2.36; log rank chi-square 4.25, $P < 0.05$)
Tesniere et. al. (2010)	Clinical trial	668	Non-resectable metastases of colorectal adenocarcinoma and Stage II non-metastatic CRC	LV5FU2 followed by FOLFOX6 to FOLFOX6 and by FOLFIRI, or, surgical removal of the tumor	PCR *TLR4*	N	Y	PFS, or, progression 5 years after diagnosis	Y	- WT *TLR4* allele = PFS (HR: 0.73; CI = 0.53–1.00; $P < 0.05$) and OS (HR = 0.72; CI = 0.52–1.01); $P = 0.05$), compared to loss-of-function *TLR4* allele following treatment
Wang et. al. (2010)	Clinical trial	138	Mixed stage, stages I–IV	Surgery, chemotherapy and/or radiation treatment details NR	IHC	N	Y	DFS and OS	N	- TLR4 = 5-year DFS (HR (95% CI) 1.62 (0.87–2.99), $P = 0.1213$) and 5-year OS (HR (95% CI) 2.17 (1.15–4.07), $P = 0.015$) - TLR4 + MyD88 = 5-year DFS (HR (95% CI) 2.25 (1.27–3.99) $P = 0.0053$) and 5-year OS (HR (95% CI) 2.97 (1.64–5.38) $P = 0.0003$) - TLR4 expression was significantly associated with liver metastasis ($P = 0.0001$) - Co-expression of TLR4/MyD88 was significantly associated with vascular invasion ($P = 0.0186$), liver metastasis ($P = 0.0002$), and TNM stage ($P = 0.0036$)

Table 1 (continued)

Author (Date)	Archival/ clinical study	Sample size (*n*)	CRC stage	Anti-cancer treatments	TLR4 analysis	Site-specific TLR4 (Y/N)	Survival outcomes (Y/N)	Type of survival	Cancer recurrence (Y/N)	Key findings
Eiro et. al. (2013)	Clinical trial	104	Resectable, mixed stage, tumor stages I–IV	Surgery, varying chemotherapy and radiation treatments throughout sample population	IHC	Y	Y	OS	Y	- TLR4 expression by tumor cells = rate of tumor recurrence ($P=0.01$) - TLR4 expression by fibroblasts = tumor recurrence ($P=0.019$) - TLR4 expression by fibroblasts = OS ($P=0.022$) - TLR4 expression by fibroblasts was an independent factor associated with relapse-free survival ($P=0.0001$), and OS ($P=0.013$)
Formica et. al. (2013)	Clinical trial	31	Mixed stage, stages I to III	FOLFIRI with bevacizumab	FC of TLR4 on neutrophils	Y	Y	PFS and OS	N	- No association between baseline or one-month post-treatment neutrophilic TLR4 expression and PFS or OS ($P=0.30$ and $P=0.34$ respectively)

Table 1 (continued)

Author (Date)	Archival/ clinical study	Sample size (*n*)	CRC stage	Anti-cancer treatments	TLR4 analysis	Site-specific TLR4 (Y/N)	Survival outcomes (Y/N)	Type of survival	Cancer recurrence (Y/N)	Key findings
Sussman et. al. (2014)	Archival	279	Mixed stage, stages I–IV	NR	IHC	Y	Y	OS	N	- No difference in TLR4 stromal staining and OS ($P=0.16$), no difference in epithelial TLR4 staining and OS ($P=0.11$) - TLR4 tumor stroma intensity score in stages 3 and 4 compared to stage 1 (Stage 1 = 2.80, Stage 2 = 3.24,Stage 3 = 4.36, Stage 4 = 3.75; P = NS, 0.0004, and 0.04, respectively) - TLR4 tumor epithelium intensity score for stages 2 and 3 compared to stage 1 (Stage 1 = 0.17, Stage 2 = 0.64, Stage 3 = 0.64, Stage 4 = 0.92; $P=0.01$, 0.002, and NS, respectively)
Gray et. al. (2019)	Clinical trial	4877	Mixed stages inclusive of stage II, stage III and stable or responding metastatic CRC	SCOT trial (ISRCTN59757862): oxaliplatin-based adjuvant chemotherapy COIN trial (ISRCTN27286448): cetuximab added to oxaliplatin-based chemotherapy	PCR *TLR4*	N	Y	DFS and OS	N	- SCOT trial: no statistically significant association of any *TLR4* SNP and OS or DFS - COIN trial: no statistically significant association of either *TLR4* SNP with OS or DFS

Table 1 (continued)

Author (Date)	Archival/ clinical study	Sample size (*n*)	CRC stage	Anti-cancer treatments	TLR4 analysis	Site-specific TLR4 (Y/N)	Survival outcomes (Y/N)	Type of survival	Cancer recurrence (Y/N)	Key findings
Zhang et. al. (2019)	Clinical trial	94	Advanced stage, stages II and III	Standard 5-Fu-based adjuvant chemotherapy after radical surgery	WB, IHC	N	Y	DFS	Y	- Fn ($P=0.028$) and BIRC3 expression ($P=0.046$) correlated with DFS - TLR4 expression was independent of DFS; TLR4 was not a factor in univariate or multivariate cox regression analyses for DFS - TLR4 ($P=0.036$) and BIRC3 ($P=0.008$) resulted in recurrence
Wong et. al. (2021)	Clinical trial	46	Mixed stage, stages III–IV	Irinotecan monotherapy or in combination with 5-Fu and IFL regimen	PCR *TLR4*	N	N	NR	N	- Participants with *TLR4* SNPs rs4986790, rs4986791 severe diarrhoea (50%) than wild-type homozygous (15%) - Participants with TLR4 SNPs presented any grade of diarrhea, contrasting with one half of the AA and CC WT groups (20 patients each, AG + GG, $P=0.012$ vs. AA; and CT + TT, $P=0.012$ vs. CC) that showed no signs of gastrointestinal toxicity - No impact of TLR4 polymorphisms on occurrence/severity of nausea

LV5FU2 Calcium leucovorin, citrovorum factor, folinic acid; *FOLFOX6* folinic acid, fluorouracil, and oxaliplatin; *FOLFIRI* fluorouracil, leucovorin and irinotecan; *5-Fu* Fluorouracil, *IFL* irinotecan, folinic acid, and fluorouracil. *IHC* Immunohistochemistry, *PCR* polymerase-chain reaction, *FC* flow cytometry, *WB* western blot, *OS* Overall survival, *DFS* disease-free survival, *PFS* progression-free survival, *NR* No record (NR)

Results

180 publications were initially identified, with 9 meeting inclusion criteria for final analysis (Fig. 1). 6 publications were clinical trials with a combined participant total of 1081. The remaining 3 publications used archival tissue from previous clinical research. Only 2 publications analyzed advanced stage CRC (non-resectable tumor stage II–IV), whereas 7 publications included mixed analysis of varying CRC stage. Participant survival data was extracted from 8 publications, inclusive of DFS, PFS and OS dependent on individual study outcomes. Only 1 publication included data regarding toxicity in relation to TLR4 expression. Finally, CRC recurrence was analyzed in 3 publications. TLR4 expression in the publications was assessed using immunohistochemistry (5/9, all of which used different primary antibodies), polymerase chain reaction (PCR) (3/9) and flow cytometry (1/9). Only 4 publications included site-specific analysis of TLR4 expression in CRC (Table 1) (Cammarota et al. 2010; Eiro et al. 2013; Formica et al. 2013; Sussman et al. 2014). Of the 9 publications, 4 analyzed formalin fixed and paraffin embedded tissue blocks, 4 analyzed peripheral blood samples and 1 (Sussman et. al. 2014) analyzed tumor tissue microarray slides provided by the NCI Cancer Diagnosis Program (CDP).

Impact of TLR4 genotype and expression on CRC survival

Of the 8 publications to report on CRC survival, one reported that wild-type (WT) *TLR4* genotype was beneficial to CRC participant survival rates (Tesniere et al. 2010). Metastatic CRC participants with the WT *TLR4* allele had higher PFS (hazard ratio (HR): 0.73; 95% confidence interval (CI) = 0.53–1.00; $P < 0.05$) and OS (HR = 0.72; 95% CI = 0.52–1.01; $P = 0.05$) compared with participants bearing the *TLR4* loss-of-function (Asp299Gly) variant post-oxaliplatin chemotherapy treatment (Tesniere et al. 2010). No differences in DFS among participants bearing the WT versus the variant *TLR4* alleles were observed.

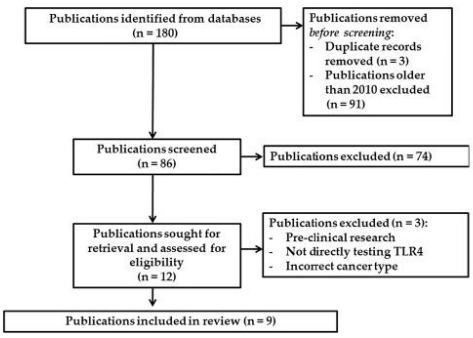

Fig. 1 Flow diagram of literature search results for systematic review

In contrast, 2 publications suggested that increased TLR4 expression is detrimental to participant survival (Cammarota et al. 2010; Wang et al. 2010). Cammarota et al. found that in mixed stage CRC tissue, participants with lower TLR4 expression in the tumor stroma compartment had improved DFS compared to participants with higher TLR4 expression (risk ratio (RR) 2.36; log-rank chi-square 4.25, $P < 0.05$) (Cammarota et al. 2010). Furthermore, participants with pT_3 adenocarcinoma with high TLR4 expression (over 50% positive cells) relapsed sooner (14 months) compared to participants with low TLR4 expression (40 months, RR 3.15; log-rank chi-square 4.03, $P < 0.05$) (Cammarota et al. 2010). This is supported by Wang and colleagues, who confirmed that CRC tissue displayed expression of TLR4 in 78 of 108 samples (72%), of which 22 displayed high TLR4 expression (Wang et al. 2010). In addition, increased TLR4 expression was associated with liver metastasis ($P = 0.0015$) and advanced tumor stage (stage IV) ($P = 0.0197$). Upon univariate analysis there was no difference in 5-year DFS rate for low versus high TLR4 expression, but OS was reduced with high TLR4 expression (HR (95% CI) 2.17 (1.15–4.07), $P = 0.015$) (Wang et al. 2010). However, this was not retained in multivariate analysis. In contrast, when samples exhibited high expression of both TLR4 and the adapter protein MyD88, DFS and OS were poorer (HR (95% CI) 2.11 (1.05–4.23) $P = 0.0352$) (Wang et al. 2010).

The conflicting nature of outcomes may be reflective of the lack of site-specific TLR4 investigations throughout human CRC research. Eiro and colleagues reported TLR4 expression by fibroblasts, not tumor cells themselves, was associated with a shortened OS of CRC participants ($P = 0.022$). Furthermore, TLR4 expression in fibroblasts was a significant and independent factor associated with DFS ($P = 0.0001$), and OS ($P = 0.013$) (Eiro et al. 2013).

Four publications reported that TLR4 expression does not impact upon CRC survival. Formica and colleagues found that in 31 metastatic CRC participants, neutrophil TLR4 expression at baseline, or 1-month post-chemotherapy, had no association with PFS ($P > 0.05$) (Formica et al. 2013). This is supported by Sussman and colleagues who, in $N = 279$, found no associated between TLR4 expression in stromal tissue and OS after correcting for both CRC stage and grade. Furthermore, epithelial TLR4 expression was also not associated with OS (Sussman et al. 2014).

More recently, Zhang and colleagues found that in an advanced CRC cohort ($N = 94$) post-standard Fluorouracil-based adjuvant chemotherapy and radical surgery, the measured level of TLR4 expression was independent of

DFS; hence no impact of TLR4 on overall DFS (Zhang et al. 2019). In addition, TLR4 was not a significant factor in survival outcomes following univariate or multivariate analyses (Zhang et al. 2019). However, high amounts of Fusobacterium (*Fn*), an anaerobic bacterium known to activate the TLR4 pathway in CRC cells, correlated with poor DFS ($P = 0.028$) (Zhang et al. 2019). Finally, Gray and colleagues analyzed previously collected tissues from two large-scale clinical trials, the SCOT (ISRCTN59757862) trial and COIN (ISRCTN27286448) trial (Gray et al. 2019). Data generated from SCOT showed no association of any *TLR4* single nucleotide polymorphism (SNP) with survival (Gray et al. 2019). There was also no association of the *TLR4* SNP, rs867228, with DFS in cases with functional polymorphisms (Gray et al. 2019). Data from COIN showed no association of any tested *TLR4* SNP with OS by either log-rank test or univariate or multivariable Cox regression (Gray et al. 2019).

CRC recurrence

Three publications reported on TLR4s contribution to CRC recurrence, with 2 publications identifying a detrimental role of TLR4 in CRC recurrence (Wang et al. 2010; Zhang et al. 2019). Wang and colleagues (2010) report upon 5 year follow-up of 108 mixed stage CRC participants, 53 participants had tumor recurrence (DFS rate: 49%), with participants exhibiting high expression of TLR4 and its accessory protein MyD88 displaying increased recurrence rates compared to those with low expression (TLR4 + MyD88 (low vs high) 5-year DFS HR (95% CI) = 2.25 (1.27–3.99) $P = 0.0053$) (Wang et al. 2010). Furthermore, participants with CRC and liver metastasis showed higher TLR4 and MyD88 expression versus CRC without liver metastasis (Wang et al. 2010). Among the 14 liver metastases obtained by hepatectomy, 12 were TLR4 positive and 6 showed a high expression (Wang et al. 2010). These findings are supported by Zhang and colleagues who showed high expression of TLR4 ($P = 0.036$) were more likely detected in participants with CRC recurrence, compared with participants without recurrence (Zhang et al. 2019).

In contrast, Eiro and colleagues observed that recurrence was dependent on the site of TLR4 expression, not its overall quantitative expression such that TLR4 expression by tumor cells was associated with a lower rate of recurrence in tumors from left colon/rectum compared to those from right colon/rectum ($P = 0.028$) (Eiro et al. 2013). Further, TLR4 expression by fibroblasts was associated with a high rate of recurrence ($P = 0.0001$) in left colon/rectum tumors (Eiro et al. 2013).

Toxicity post-chemotherapy in participants with CRC

Only 1 publication investigated the role of TLR4 in relation to post-chemotherapy toxicity outcomes, including diarrhea and nausea. Wong and colleagues investigated a cohort of 46 advanced stage CRC (stage III–IV), treated with first cycle of irinotecan-based chemotherapy (irinotecan monotherapy or in combination with fluorouracil and leucovorin—IFL regimen) (Wong et al. 2021). Participants the variant *TLR4* SNPs rs4986790 and rs4986791 had more severe diarrhea (50%) compared to those without the variants (15%) (Wong et al. 2021). When looking at diarrhea of all severities, all participants (100%) with the variant *TLR4* SNPs developed diarrhea, compared to only 50% of those without the variants (20 participants each, rs4986790, $P = 0.012$ vs. rs4986791, $P = 0.012$).(Wong et al. 2021) There was no association with nausea (Wong et al. 2021).

TCGA database results

TLR4 expression differs due to cancer stage

Summary of participant clinical data is presented in supporting information Table 2. Although TLR4 expression was not statistically different between normal and stage I, significantly higher TLR4 expression was observed in normal tissues vs Stage II, Stage III and Stage IV (Fig. 2A).

TLR4 expression is associated with survival in respect to tumor stage

Number of participants per tumor stage is presented in Fig. 2C. OS of participants with CRC with respect to TLR4 expression (low vs high) was conducted. TLR4 expression was not a significant prognostic factor (HR = 1.1, $P = 0.64$) when all stages were combined (Fig. 2B) or compared between stages (Fig. 3). In contrast, multivariate analysis revealed high TLR4 expression prior to treatment conferred worse prognosis, with the strength of the effect increasing with tumor stage (stage I vs II (HR = 2.2, $P = 0.138$), stage I vs III (HR = 4.2, $P = 0.008$) and stage I vs IV (HR = 11.3, $P < 0.001$); Fig. 4). Sex and age had no impact on OS (Fig. 4). In stage I disease, those that were alive had lower TLR4 expression at diagnosis ($P = 0.034$). For all other stages TLR4 expression at diagnosis was higher in those still alive ($P = 0.035$) (Fig. 5).

Discussion

TLR4 is an attractive target for controlling cancer development and optimizing treatment response due to its potent regulation of systemic immune responses. Our analysis

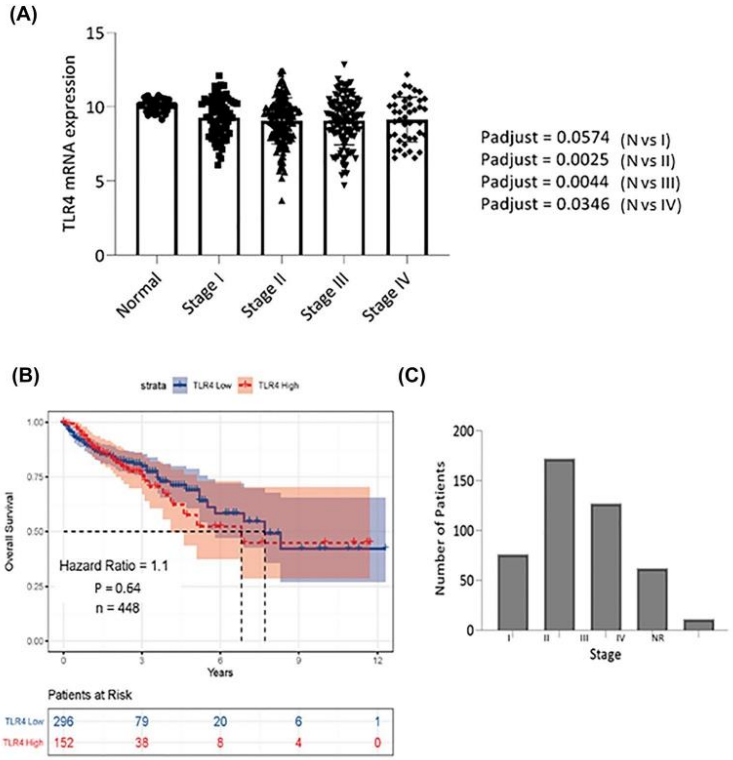

Fig. 2 **A** Comparison of TLR4 expression between stage specific tumor and adjacent normal tissues from TCGA cohort. One-way ANOVA was performed by comparing solid tissue normal vs stage I, stage II, stage III, and stage IV participants. Statistical significance was represented as $P < 0.05$. **B**, **C** Assessment of TLR4 mRNA expression using the tertile cut-point. (B) Kaplan–Meier curves of overall survival (OS) in TCGA cohort. (C) Bar plot depicting the stage distribution of the cohort

exposes the significant heterogeneity in CRC outcomes linked with TLR4 expression. We have shown that TLR4 expression decreases with increasing CRC tumor stage at prognosis, and appears to have stage-dependent associations with participant outcomes. We highlight two novel findings related to high TLR4 expression in early- and late-stage CRC being; (1) in stage I CRC results in worse participant outcomes, and (2) in stage IV CRC results in improved participant outcomes. With TLR4 expression decreasing in higher grade CRC, this potential reduction of innate immune signaling may prove to be the causative mechanism behind unfavorable treatment responses and reduced survival.

TLR4 expression relative to tumor stage is well documented in the literature (Li et al. 2019; Omrane et al. 2014). These patterns of TLR4 expression reflect its core physiological mechanism of inducing inflammation, a process known to be carcinogenic. Our data showed a significant decrease in TLR4 expression in later stage CRC (stages II–IV) compared to normal tissue. This decrease in TLR4 expression was not found in stage I tumors, suggesting that the slightly higher TLR4 expression in early CRC may align with the well-defined concept that inflammatory processes are involved in the early development of CRC (Karin and Greten 2005). However, our analysis did show that non-tumor comparative tissue had the highest TLR4 expression. As this tissue was primarily collected from adjacent tissue in the same participants, systemic inflammatory responses may have impacted on interpretation. The finding that TLR4 expression decreases with tumor growth is also consistent with the current understanding of tumor development, with tumors often adapting to evade immune detection and control. Activation of the receptor, programmed death 1 (PD-1), has been found to inhibit immune control of tumor growth, with the PD-1 ligand, PD-L1, being significantly upregulated in solid tumors like CRC (Hino et al. 2010). Therefore, this upregulation of PD-L1 is suggested to play a crucial role in the tumors ability to evade host immune system (Dong et al. 2002). This is of particular interest in the context of TLR4 research, as PD-L1 has also been shown to block the cytolytic activity of PD-1+tumor infiltrating $CD4^+$ and $CD8^+$ T cells, which are reliant on dendritic cell

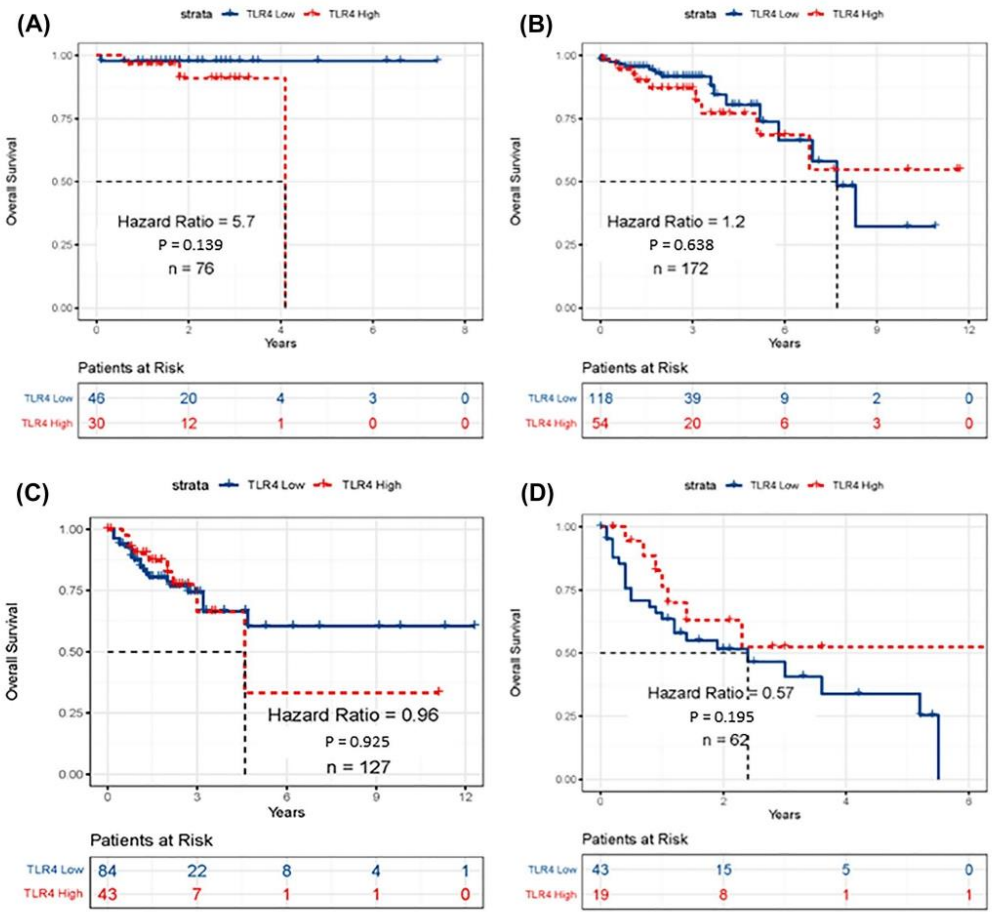

Fig. 3 Assessment of TLR4 mRNA expression in stage specific CRC participants from TCGA cohort. **A** Kaplan–Meier curves depicting the OS in stage I participants **B** stage II participants, **C** stage III participants and **D** stage IV participants using the tertile cut point. No significant difference between groups

-TLR4 interaction (Brahmer et al. 2012; Fife et al. 2009). In addition, Xiao et. al. (2016) reported that inhibition of TLR4 signaling via a blocking antibody significantly reduced the number of PD-1+B cells in human hepatoma tissues, where PD-1+B cell populations promoted cancer growth (Xiao et al. 2016). Furthermore, Huang (2018) found that improvement in clinical outcome is resultant of cytosolic HMGB1 triggering dendritic cell maturation through TLR4 activation, whereby consequently recruiting PD-1+tumor-infiltrating lymphocytes to the tumor site (Huang et al. 2018b). These findings highlight the importance of TLR4 to this particular tumor kill pathway and outlines the importance for TLR4 expression for improved clinical outcomes of people living with CRC.

While our findings suggest a likely relationship between TLR4 expression and tumor stage, the relationship between TLR4 and long-term outcome was less clear cut in both our systematic review and genetic analyses. When looking at all tumor stages, there was no significant impact on OS in low vs high TLR4 expressing tumors. This contradicts existing data, as a metanalysis of 212 people living with CRC found that high TLR4 expression associated with a significantly reduced OS and poorer prognosis (HR (95% CI) 2.30 (1.41, 3.75), $P = 0.001$) (Hao et al. 2018).

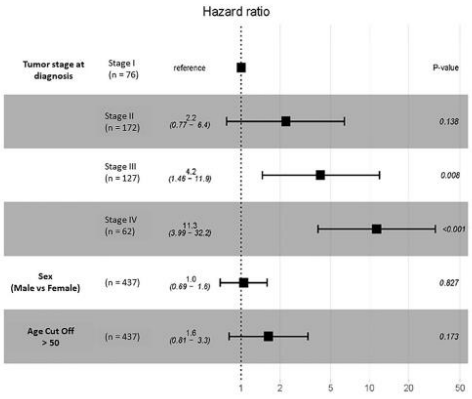

Fig. 4 Forest plot of OS in stage specific participants. The tertile cut-point, the p-values and HRs with 95% CI derived for measurement of the cohorts from assessing the cut-point were shown. Statistical significance was represented as $P < 0.05$

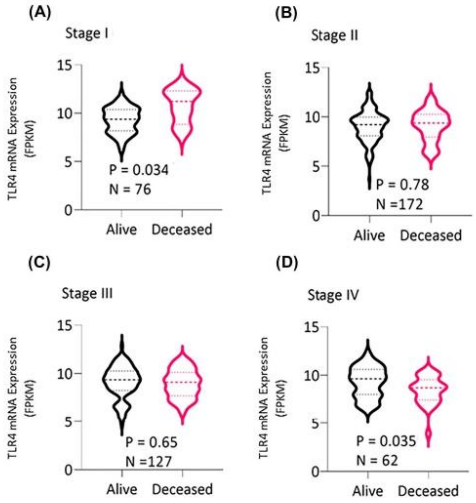

Fig. 5 Comparison of TLR4 expression in Fragments per Kilobase of transcript, per Million mapped reads (FPKM) with respect to OS. Analysis of TLR4 expression using two sample t-test based on participants' survival in **A** stage I, **B** stage II, **C** stage III, and **D** stage IV participants. Statistical significance was represented as $P < 0.05$

However, this analysis did not classify the cohort based on CRC stage which may have masked some findings and increased bias towards advanced stage disease. While our initial analyses showed no effect of TLR4 expression on OS, analysis of this relationship within specific tumor stages revealed that TLR4 may in fact have an impact but, in a stage-specific manner. Specifically, we showed that TLR4 expression in Stage IV disease was higher in tumors from people still alive compared to those that were not. While we weren't able to show this in our longitudinal OS analyses, this may reflect the lack of power when breaking down our cohort of 488 into specific stages.

This heterogeneity in how TLR4 may act to regulate overall survival for Stage I vs Stage IV disease is likely to reflect the differences in how these disease stages are treated. Stage I disease is almost always treated with surgery, but no cytotoxic therapy, whereas stage IV disease will certainly contain cytotoxic therapy. TLR4 is considered to exert its impact on treatment outcomes via its ability to modulate immunogenic cell death (Fang et al. 2014; Kroemer et al. 2013). Immunogenic cell death acts in concert with direct cytotoxicity, and collectively results in more thorough tumor clearance, and thus long-term survival. As such, higher TLR4 expression would theoretically confer a larger immune response and thus better response in late-stage CRC. This is supported by the Isambert et al. study (2013) which found that increased activation of TLR4 via a lipid A analogue (OM-174) enhanced inflammatory anti-tumor response in metastatic CRC and improved clinical outcomes (Isambert et al. 2013). Furthermore, data from Huang and colleagues (2018) showed improved DFS in people living with late-stage rectal cancer with increased activation of TLR4 via HGMB1 binding (Huang et al. 2018b).

Despite new interpretation of stage-specific roles of TLR4, we must acknowledge some limitations of our approach. Firstly, the studies included within the literature review were varied, often with low sample sizes and differing approaches to measuring TLR4 expression. Furthermore, our genetic analysis relied on previously collected data and exhibited low power when analyzing within the specific CRC stages. It is also important to acknowledge that we relied solely on TLR4 tumor-expression data; whereas evidence from pre-clinical work suggests expression of TLR4 in host tissues (typically non-cancerous) may be critical in setting immune tone of host and thus response (Li et al. 2017). Nonetheless, our findings indicate a general trend towards higher TLR4 expression being associated with favorable OS outcomes in stage IV CRC suggesting its ability to induce immunogenic cell death is critical in CRC prognosis.

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Author contributions All authors contributed to the study conception and design. Literature review material preparation, data collection and analysis were performed by EEC and TCGA data collection and analysis were performed by SN. The first draft of the manuscript was

written by EEC and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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