POTENTIAL MECHANISMS UNDERLYING OXALIPLATIN-INDUCED ENTERIC NEUROPATHY

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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ABSTRACT

Cisplatin, carboplatin and oxaliplatin are platinum-based agents that are amongst the most widely used drugs for the treatment of cancer in the clinical setting. Despite their therapeutic efficacy, these platinum-based drugs are associated with a myriad of dose-limiting side-effects. These include acute and chronic peripheral neuropathies (paraesthesias, dysaesthesias), and gastrointestinal complications (nausea, vomiting, constipation and diarrhoea). These side-effects decrease quality of life and cause life-threatening cardiac and renal sequeale consequent to malnutrition, dehydration and fluid and electrolyte imbalances, which in severe cases, can lead to death.

Extensive research into the mechanisms underlying chronic peripheral neuropathies associated with platinum-based agents has focused on drug accumulation within the dorsal root ganglia (DRG). Only recently, a few studies have demonstrated damage to the enteric nervous system (ENS) following platinum-based chemotherapy. The ENS is an intrinsic and complex orchestration of nerves embedded throughout the entirety of the gastrointestinal tract innervating the musculature and mucosa. The mechanisms underlying ENS toxicity remain unknown. Furthermore, the gastrointestinal tract receives extrinsic innervations and it is also unknown if these nerves are vulnerable to platinum-based drugs.

Platinum-based drugs mediate their cytotoxic effects through the formation of interstrand and intrastrand DNA adducts, particularly binding

to the N7 position of guanine nucleotides. Essentially, these platinum lesions inhibit DNA replication through the distortion of the helical structure. DNA damage typically results in the induction of canonical apoptotic cascades. Until recent years apoptosis was deemed an immunologically ‘silent’ or ‘tolerogenic’ event. However, unlike cisplatin and carboplatin, there is substantial evidence shown in models of cancer that oxaliplatin prompts a fatal immune response against cells committed to apoptosis. This phenomenon is termed ‘immunogenic cell death’. Oxaliplatin-induced cytotoxicity results in the hallmark presentation of damage-associated molecular patterns (DAMPs) which can be recognised by antigen-presenting cells, and thus, stimulating phagocytosis of apoptotic cells and/or debris. The gastrointestinal tract harbours ~70% of the body’s immune system, and so it is unknown whether oxaliplatin treatment can induce changes in immunological responses which may directly or inadvertently induce ENS damage. Given the bi-directional communication between the immune and nervous systems, exploring the consequences of oxaliplatin-induced cytotoxicity and potential immunogenicity may provide insight into the multifaceted mechanisms underlying neuronal damage and death which impact gastrointestinal function.

CANDIDATE DECLARATION

I, Vanesa Stojanovska, declare that the PhD thesis entitled 'Potential Mechanisms Underlying Oxaliplatin-Induced Enteric Neuropathy' is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature: 

Date: 11-09-17

DECLARATIONS OF CONTRIBUTED WORK

Chapter 1: Sarah Miller and Rachel McQuade assisted with immunohistochemistry of the nerve fibres calcitonin gene-related peptide (CGRP), tyrosine hydroxylase (TH), and vesicular acetylcholine transporter (VAChT).

Chapter 2: Dr Matthew Stewart provided training and assistance with atomic absorption spectrophotometry (AAS); Rachel McQuade assisted with some animal injections; Cara Timpani provided training for subcellular fractionation technique; Dr Berin Boughton and Dr Dominic Hare provided expertise in metal detection and conducted laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) imaging of platinum ions within the longitudinal muscle myenteric plexus preparation; and Dr Craig Goodman provided advice and training for Western Blotting.

Chapter 3: Dr Matthew Stewart provided assistance with AAS; Rachel McQuade assisted with some animal injections; Cara Timpani provided training for subcellular fractionation technique; Dr Ahmed Rahman provided training for intracardiac perfusions; Dr Berin Boughton and Dr Dominic Hare provided expertise in metal detection and conducted LA-ICP-MS imaging of platinum ions within the forebrain and brainstem; and Dr Craig Goodman provided advice and training for Western Blotting.

Chapter 4: Rachel McQuade collected fecal pellets from animals and isolated DNA for microbiota analysis; microbiota results were obtained commercially from the Australian Genome Research Facility, Dr Shaku Gondalia and Prof Enzo Palombo assisted with 16s rRNA analysis of fecal microbiota; Dr Samy Sakkal provided flow cytometry training. Dr Sarah Fraiser assisted with polymerase chain reaction (PCR) experiments.

Chapter 5: Dr Monica Prakash assisted with some flow cytometry. Dr Sarah Fraiser assisted with PCR experiments.

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PUBLICATIONS

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1. **Stojanovska V**, 2014. Neurotoxicity to the Myenteric Plexus Associated with the Anti-Cancer Drug 5-Fluorouracil., *Australian Society for Medical Research*, Melbourne, Australia. ***Special Oral Session for Highest Ranked Abstracts**

2. McQuade RM, **Stojanovska V**, Bornstein JC, Nurgali K, 2014. Damage to Submucosal Neurons as a Result of Treatment with Anti-Cancer Treatment. *Victoria University Post-Graduate Research Symposium*, Melbourne, Australia.
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3. **Stojanovska V**, 2015. Effects of Colorectal Cancer and Anti-Cancer Chemotherapy on the Enteric Nervous System. *Combined Enteric Neuroscience Seminar*, University of Melbourne, Melbourne, Australia.
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1. **Stojanovska V**, Sakkal S, Nurgali K, 2013. Oxaliplatin-Induced Enteric Neuronal Damage and Changes in Neuro-Immune Interactions. *Australasian Neuroscience Society*, Melbourne, Australia.
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SUCCESSFUL PROPOSALS AND AWARDS

1. **Australian Synchrotron – X-Ray Fluorescence Microscopy, 2017**

   Awarded beamtime: 72 hours. Project title: “X-ray microscopy detection of platinum and investigation of trace metal distribution in enteric neurons in a small animal model of oxaliplatin therapy”. Proposal ID: 12452; reference no: AS173/XFM/12452.
2. **PhD scholarship and academic position**, 2013-2016. College of Health and Biomedicine, Victoria University.
3. **People’s Choice Award** for oral presentation, 2015. Platinum-based chemotherapy: drug accumulation in neurons controlling gastrointestinal functions. *Victoria University Post-Graduate Conference,* Melbourne, Australia.
4. **Best Oral Presentation Award**, 2016. Platinum drug accumulation in the longitudinal muscle-myenteric plexus and the presentation of damage-associated molecular patterns. *Victoria University Post-Graduate Conference,* Melbourne, Australia.

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1. Chemotherapy destroys more than just the cancer, 2014. The Australian Society for Medical Research, Melbourne, Australia. http://www.asmr.org.au/MRW%20Media/2014Vic%20Meeting.pdf
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ABBREVIATION	DEFINITION
AAS	Atomic absorption spectrophotometry
Aicda	Activation-induced cytidine deaminase
ADCC	Antibody-dependant cell-mediated cytotoxicity
APCs	Antigen presenting cells
ATOX1	Antioxidant protein 1
ATP	Adenosine triphosphate
ATP7A	ATPase 7A
ATP7B	ATPase 7B
Bcl2l1	Bcl2 like 1 gene
BER	Base excision repair
Ccl	Chemokine ligand
Ccr	Chemokine receptor
CCS	Copper chaperone for superoxide dismutase
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyltransferase
CNS	Central nervous system
COX17	Cytochrome *c* oxidase 17
CRC	Colorectal cancer
CTLA-4	Cytotoxic T lymphocyte-asscoiated protein 4
CTR1	Copper transporter receptor 1
CTR2	Copper transporter receptor 2
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DMNX	Dorsal motor nucleus of the vagus nerve
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglia
DTNB	5,5'-dithio-bis-[2-nitrobenzoic acid]

ECL	Enhanced chemiluminescence
ENS	Enteric nervous system
FACS	Fluorescence-activated cell sorting
FOLFOX	Folinic acid, fluorouracil, oxaliplatin
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GSH	Glutathione
H2-D1	Histocompatibility 2, D region locus 1
HMGB1	High mobility group box 1 protein
HSPs	Heat-shock proteins
ICCs	Interstitial cells of Cajal
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Induicible nitric oxide synthase
IPANS	Intrinsic primary afferent neurons
IR	Immunoreactive
LA-ICP-MS	Laser ablation inductively coupled plasma mass spectrometry
LMMP	Longitudinal muscle-myenteric plexus
MHC	Major histocompatibility
MLNs	Mesentric lymph nodes
MMR	Mismatch repair
MPO	Myeloperoxidase
MT	Netallothionein
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NER	Nucleotide excision repair
NK	Neurokinin
NK cells	Natural killer cells
NKT cells	Natural killer T cells
nNOS	Neuronal nitric oxide synthase

NO	Nitric oxide
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
OTUs	Operational taxonomic units
OxPhos	Oxidative phosphorylation
PACAP	Pituitary adenylate cyclase-activating polypeptide
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline + Triton X-100
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
PPs	Peyer's patches
PVDF	Polyvinylidene difluoride
SCO1	Synthesis of cytochrome *c* oxidase 1
SCO2	Synthesis of cytochrome *c* oxidase 2
SDS-PAGE	Sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SOD1	Superoxide dismutase 1
TBST	Tris-buffered saline containing + Tween 20
TGFβ	Transforming growth factor β
TH	Tyrosine hydroxylase
Th cells	Helper T cells
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor α
Tregs	Regulatory T cells
VAChT	Vesicular acetylcholine transporter
VIP	Vasoactive intestinal peptide
γδ T cells	Gamma delta T cells

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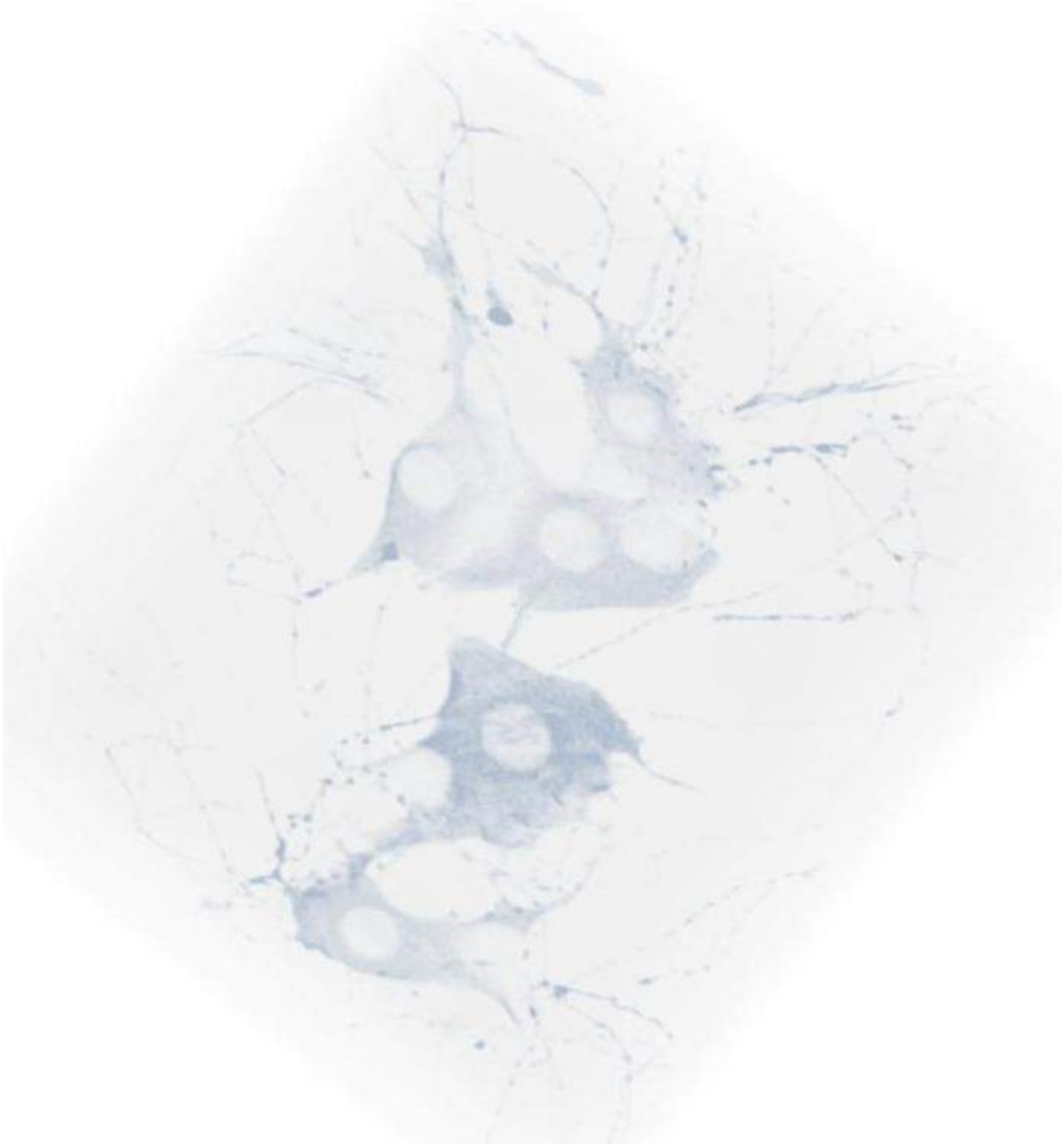
LITERATURE REVIEW



THE MATERIAL PRESENTED IN THIS CHAPTER HAS BEEN PUBLISHED AND REPRODUCED HERE WITH MINOR ALTERATIONS, WITH THE PERMISSION OF THE PUBLISHERS

1. **Stojanovska V**, Sakkal S, Nurgali K, 2014. Platinum-Based Chemotherapy: Gastrointestinal Immunomodulation and Enteric Nervous System Toxicity. *Am J Physiol Gastrointest Liver Physiol,* 308: G223–G232.
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1.1 Introduction

Colorectal cancer is one of the leading causes of cancer-related death worldwide (Jemal et al., 2011, Siegel et al., 2013, Ferlay et al., 2015). It is the fourth highest cause of morbidity and mortality in men, and the third highest in women (Parkin et al., 2005, Center et al., 2009). Epidemiological factors associated with colorectal cancer include age, gender and race/ethnicity (Amersi et al., 2005, Haggar and Boushey, 2009). Geographic variations have also demonstrated a trend for higher incidence rates of colorectal cancer in developed countries that have adopted a Westernised culture (Boyle and Langman, 2000, Haggar and Boushey, 2009). Risk factors for colorectal cancer include but are not limited to, nutritional practices, obesity, physical inactivity, smoking, heavy alcohol consumption, inflammatory bowel disease, inherited genetics, and familial history of adenomatous polyps (Center et al., 2009, Haggar and Boushey, 2009). Colorectal cancer is typically asymptomatic in the early stages of development, whereas weight loss, rectal bleeding, altered bowel habits and abdominal pain can present at the later stages of disease progression (Cappell, 2005, Adelstein et al., 2011). Treatment strategies for colorectal cancer include surgical resection for patients diagnosed at stages I-II and chemotherapy for patients diagnosed at stages III-IV when metastasis to secondary locations has occurred (Chibaudel et al., 2012, Johnston et al., 2012). Oxaliplatin is a third-generation platinum-based drug with proven efficacy against colorectal cancer when used as a single agent, or in combination with the anti-

metabolite, 5-fluorouracil, and topoisomerase inhibitor, irinotecan (Sharif et al., 2008, Jeon et al., 2011, Wang and Li, 2012, Marschner et al., 2015).

1.2 Structure, metabolism, and mechanism of action of platinum-based chemotherapeutic agents

The platinum drugs cisplatin, carboplatin and oxaliplatin, contain a double charged platinum ion that is surrounded by four ligands (Dasari and Bernard Tchounwou, 2014)**.** These surrounding ligands consist of stable amine or bidentate complexes that form strong interactions with the platinum ion (Marques, 2013). The chloride ligands and carboxylate complexes of these platinum drugs are considered as “leaving groups” since they dissociate during the aquation process (Marques, 2013). This aquation process (aquation and chlorination in the case of oxaliplatin only) results in the formation of several reactive platinum metabolites **(Figure 1.1 A-C'''')**. The cleavage of the leaving groups allows the exposure of the platinum ion to forge a bond with purine bases of nuclear and mitochondrial DNA, forming intra- and inter-strand crosslinks or DNA adducts (Marques, 2013). Although these three platinum-based agents form similar DNA adducts, they are quite different in terms of systemic toxicity and tolerance.

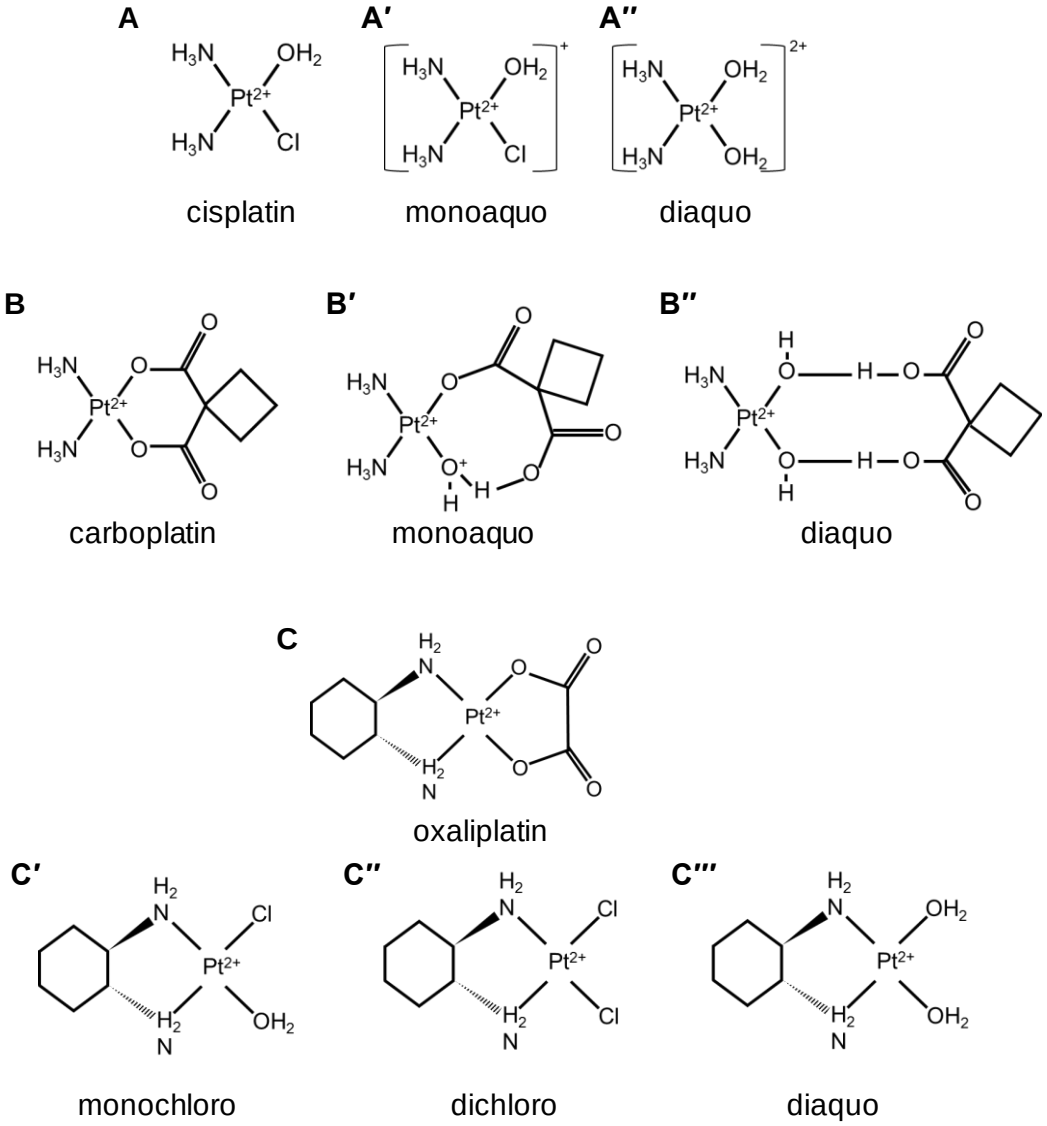


Figure 1.1. Chemical structures of the platinum-based chemotherapeutic agents cisplatin, carboplatin and oxaliplatin and their metabolites.

Cisplatin is transformed into a reactive complex upon cell entry, whereby water molecules displace the chloride atoms, resulting in a hydrolysed compound (Dasari and Bernard Tchounwou, 2014). This compound can then react with adenine and guanine nucleotides forming intra- and inter-strand crosslinks (Dasari and Bernard Tchounwou, 2014). The rapid aquation of cisplatin may explain the myriad of side-effects associated with its use.

The hydrolysis of carboplatin into its reactive form is thought to occur via a biphasic process. The first hydration step leads to the ring opening, followed by a second hydration step where the malonate group is displaced (Hay and Miller, 1998, Pavelka et al., 2007). The rate-limiting step of carboplatin hydrolysis is the first hydration stage during ring opening; this process is timely and thus, results in a slow activation course of the drug. This might explain the milder systemic toxicity caused by carboplatin when compared to cisplatin and oxaliplatin (Pavelka et al., 2007).

Oxaliplatin is comprised of a central platinum atom enclosed by a 1,2-diaminocyclohexane group and a bidentate oxalate ligand. Hydrolysis of this drug occurs non-enzymatically following the cleavage of the leaving group (the bidentate oxalate ligand) and subsequent aquation or chlorination. The key metabolites formed during oxaliplatin hydrolysis include monochloro-, dichloro- and diaquo-diaminocyclohexane complexes, with dichloro-diaminocyclohexane being the most reactive (Alcindor and Beauger, 2011).

1.3 Neurotoxic and gastrointestinal side-effects associated with platinum-based chemotherapeutic agents

Despite their efficacy, cancer treatment with platinum-based agents is often compromised due to the serious side-effects associated with these cytotoxic drugs leading to dose limitation, total treatment cessation, and, in severe cases, death (Denlinger and Barsevick, 2009, Di Fiore and Van Cutsem, 2009). The side-effects induced by platinum-based anti-cancer agents include, but are not limited to, central and peripheral neuropathies as well as gastrointestinal toxicities (Gregg et al., 1992, Ling et al., 2007, Liu et al., 2009, Weickhardt et al., 2011). Vomiting and diarrhoea in particular, are serious gastrointestinal complications which cause dehydration, malnutrition and weight loss, in addition to fluid and electrolyte depletion which can progress to life-threatening cardiac and renal sequelae (Stein et al., 2010, McQuade et al., 2014). The gastrointestinal side-effects can have a delayed onset, and may be long lasting, even for up to 10 years following chemotherapeutic treatment (Denlinger and Barsevick, 2009).

Cisplatin induces central and peripheral neurotoxicities, ototoxicity, severe gastrointestinal side-effects such as nausea, vomiting, constipation and diarrhoea, nephrotoxicity and mild hematologic toxicity (McWhinney et al., 2009, Boussios et al., 2012). Cisplatin-associated central neurotoxicities include leukoenecephalopathy characterised by bilateral reversible abnormalities in the frontal, parietal and occipital white matter, cerebral herniation, coma, seizures, tremor, loss of coordination,

Parkinsonian symptoms, ataxia, anxiety, insomnia and vision loss of varying indices **(Figure 1.2)** (Hartmann and Lipp, 2003, Sioka and Kyritsis, 2009, Amptoulach and Tsavaris, 2011). Cisplatin-induced ototoxicity is caused by DNA platinum adduct formation within the organ of Corti, spiral ganglion cells and the cochlear lateral wall (Thomas et al., 2006, Rybak et al., 2009). DNA platinum adducts have been visualised using immunohistochemical methods via the conjugation of fluorophores to cisplatin or antibodies which recognise platinum adducts (Rybak et al., 2007). This suggests that cisplatin is small enough and able to pass through the blood-brain barrier, and explains why central nervous system (CNS) toxicities are a common side-effect of cisplatin treatment.

Peripheral neuropathies associated with cisplatin occur in 50% of patients, presenting as tingling or numb paraesthesias of the distal extremities, burning pain and loss of sensations, as well as Lhermitte's phenomenon (electrical sensations descending the back and lower extremities) (Amptoulach and Tsavaris, 2011, Grisold et al., 2012). It has been observed that 3-4 cycles of cisplatin therapy induce paraesthesia in 11% of patients, and this proportion increases to 65% after 3 months, indicating delayed neurotoxicity (Schlippe et al., 2001). In some cases, peripheral neuropathy gradually reduces over time, but for many patients it often persists chronically, manifesting as permanent neurological damage (Amptoulach and Tsavaris, 2011).

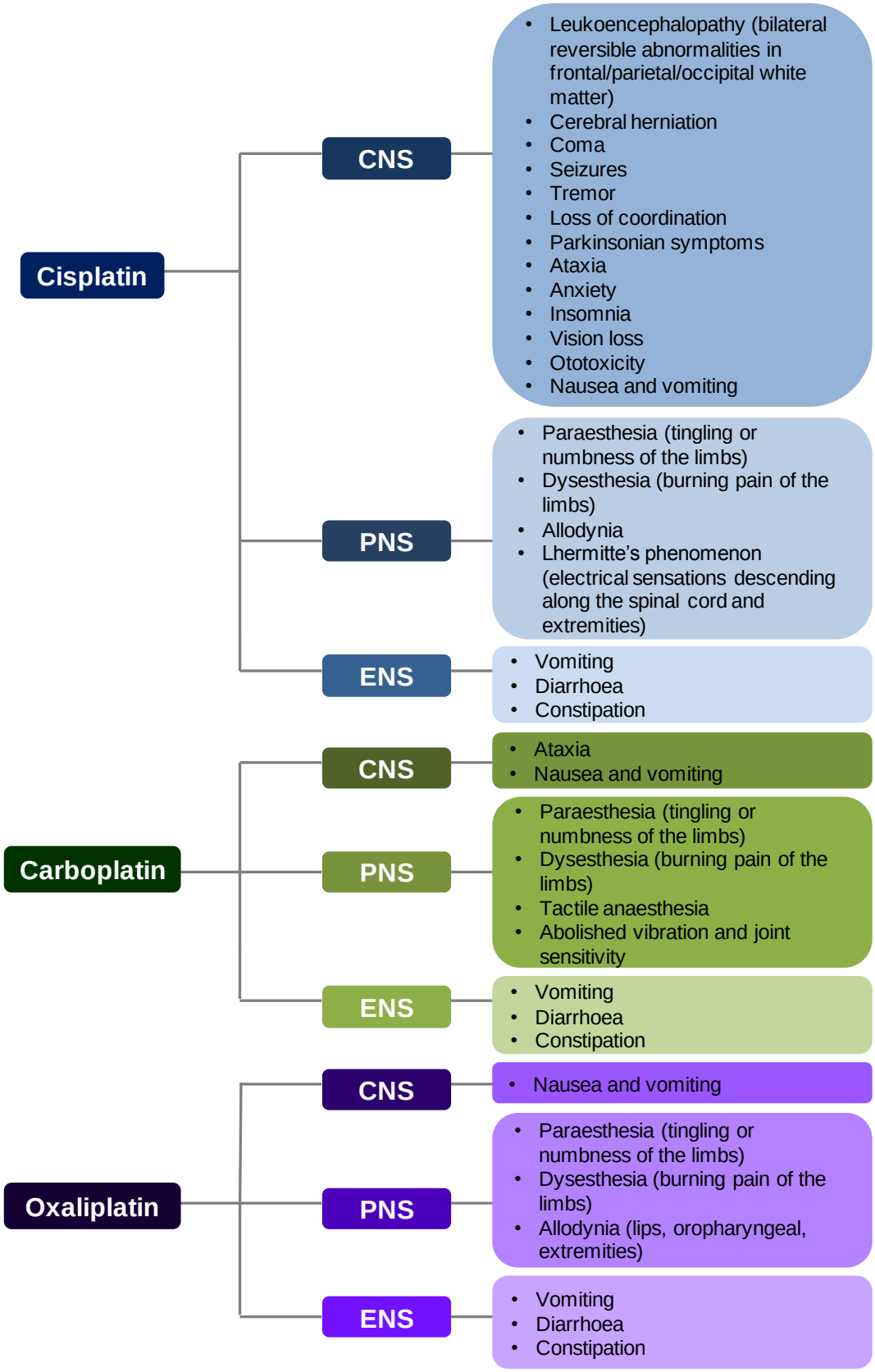


Figure 1.2. Summary of the neurotoxic and gastrointestinal side-effects associated with cisplatin, carboplatin and oxaliplatin.

The chronic form of cisplatin-induced neurotoxicity has been attributed to the long-term retention of reactive platinum species, and many studies to date have focused on correlating tissue platinum accumulation to CNS toxicity, ototoxicity, peripheral neuropathy and nephrotoxicity (Gregg et al., 1992, Krarup-Hansen et al., 1999, Gietema et al., 2000, Liang et al., 2005, Brouwers et al., 2006, Ta et al., 2006, Brouwers et al., 2008, Bouslimani et al., 2010, Ding et al., 2011, Sprauten et al., 2012). Long-term peripheral neuropathy has been shown to range from 5 up to 20 years post-cisplatin treatment (Sprauten et al., 2012). Circulating platinum complexes have also been detected systemically well after treatment cessation, sometimes for decades subsequent to platinum-based therapy administration (Tothill et al., 1992, Gietema et al., 2000, Gelevert et al., 2001, Sprauten et al., 2012, Hjelle et al., 2015). Furthermore, it has been established that platinum species recovered from the plasma of cancer patients are still reactive against nuclear DNA, as determined by the formation of platinum adducts (Brouwers et al., 2008). Thus, platinum retention is considered the primary mechanism for permanent neuropathy.

Cisplatin also causes severe vomiting in 60-90% of patients (Boussios et al., 2012). Platinum-based chemotherapeutic agents are known to stimulate the release of serotonin by gastrointestinal enterochromaffin cells (Schroder et al., 1995). Serotonin stimulates vagal afferents projecting to the gastrointestinal tract, sending impulses to the emetic centre in the medulla oblongata which may impact the onset and severity of vomiting at various time points (Janelsins et al., 2013).

Furthermore, increased serotonin levels have been observed in the area postrema, the jejunum of the intestines, as well as blood plasma following cisplatin administration (Cubeddu, 1996, Sioka and Kyritsis, 2009, Boussios et al., 2012). Alongside serotonin, the neurotransmitter substance P, an excitatory neurotransmitter involved in nociceptive and emetic signalling, has been implicated in the pathophysiology of nausea and vomiting (Rojas and Slusher, 2012). Antagonists of serotonin and substance P receptors have been used with varying degrees of success. These antagonists cause additional side-effects such as dizziness, headaches, anxiety, fever, ataxia, twitching, arrhythmia, heart failure, renal disorders, and can worsen constipation and diarrhoea (Feyer and Jordan, 2011, Rojas and Slusher, 2012).

Intestinal mucosal damage is implicated in the acute stages of gastrointestinal dysfunction following platinum-based chemotherapy, however, given that this tissue regenerates quite rapidly but the side-effects can be delayed or remain persistent, it is plausible to suggest that circulating platinum may affect other cell types that regulate gastrointestinal functions. This could include the serotoninergic neurons within the enteric nervous system innervating the gastrointestinal tract. Although the ENS chiefly regulates gastrointestinal functions it has been largely overlooked in regards to chemotherapy-induced toxicity. One study conducted by Vera et al. (2011) revealed that cisplatin induced a proportional increase in inhibitory neuronal nitric oxide synthase (nNOS)-expressing neurons within the myenteric plexus, alongside a significant

decrease in the proportion of calcitonin gene-related peptide-immunoreactive (IR) neurons and varicosities which are involved in gastrointestinal reflexes and motility (Vera et al., 2011). Altered neuronal subpopulations within the myenteric plexus following cisplatin have been linked to intestinal dysmotility leading to chronic constipation.

Carboplatin typically induces dose-limiting thrombocytopenia and bone-marrow suppression, whereas neurotoxicity is less prevalent, occurring in only 4-6% of patients compared to 15-60% of patients undergoing cisplatin or oxaliplatin therapy (McWhinney et al., 2009, Amptoulach and Tsavaris, 2011). Patients who are more susceptible to carboplatin-induced neuropathy are typically those who are undergoing high-dose therapy with this agent, or who received cisplatin chemotherapy in the past (Heinzlef et al., 1998). Symptoms of neuropathy associated with carboplatin include burning and aching sensations in the feet, paraesthesias such as numbness and tingling, clumsiness, ataxia, distal motor deficits, amyotrophy in the upper and lower extremities, tactile anaesthesia as well as abolished vibration and joint sensitivity (Heinzlef et al., 1998). Furthermore, carboplatin treatment causes vomiting in 65% of patients, diarrhoea in 17%, and constipation in 6% (Boussios et al., 2012).

The main dose-limiting side-effect associated with oxaliplatin therapy is peripheral neuropathy which manifests as acute or chronic forms, depending on the cumulative dose received (Hartmann and Lipp, 2003, McWhinney et al., 2009, Amptoulach and Tsavaris, 2011, Beijers et al., 2014). The acute form of oxaliplatin-induced neuropathy affects

approximately 85-95% of patients and is characterised by paraesthesias, dysesthesias, and allodynia of the lips, oropharyngolaryngeal area and the extremities (Hartmann and Lipp, 2003, Alcindor and Beauger, 2011, Beijers et al., 2014). These symptoms are often triggered upon exposure to cold, and typically subside within a few days (Alcindor and Beauger, 2011, Beijers et al., 2014). The acute form of peripheral neuropathy is thought to be caused by the chelation of calcium by the oxaliplatin metabolite oxalate, contributing to the failed inhibition of voltage-gated sodium currents (Adelsberger et al., 2000). An increase in amplitudes and duration of action potentials, as well as prolonged refractory periods have been electrophysiologically observed in rat nerve preparations following oxaliplatin treatment (Adelsberger et al., 2000). The chronic form of oxaliplatin-induced neuropathy is thought to result from platinum accumulation and the formation of nuclear DNA adducts within the DRG neurons (Ta et al., 2006).

Moreover, the prevalence of oxaliplatin-induced gastrointestinal side-effects is quite high. Approximately 74% of patients feel nauseous, 47% experience vomiting, and 56% and 32% experience diarrhoea and constipation, respectively (Boussios et al., 2012). Histological damage of the gastrointestinal tract following oxaliplatin therapy has not been studied extensively, although, mucosal insult following chemotherapeutic agents is well-established. It has been demonstrated that cumulative oxaliplatin treatment causes damage to the ENS. Oxaliplatin induces neuronal loss within the murine myenteric plexus, and a proportional increase in the

number of nNOS neurons, causing gastrointestinal dysmotility (constipation) (Wafai et al., 2013). Furthermore, treatment of patients with combination chemotherapy including oxaliplatin and 5-fluorouracil causes the translocation of Hu protein **(Figure 1.3)** (Stojanovska et al., 2015). Hu proteins are important for the regulation of mRNA in the nucleus and cytoplasm. The loss of cytoplasmic Hu protein contributes to mRNA degradation, which is indicative of neuronal stress and damage (Hinman and Lou, 2008). Electrophysiological studies on human myenteric neurons in resected colon specimens from patients following combined oxaliplatin and 5-fluorouracil treatment demonstrated hyperexcitability of neurons, and failure to generate action potentials from those with Hu translocation (Carbone et al., 2016). Thus, platinum-based drugs have the capacity to induce ENS damage, however, the mechanisms underlying toxicity remain unknown. Thus, investigating the potential for platinum accumulation within the ENS as a mechanism of neurotoxicity is plausible. This hypothesis is supported by the fact that long-term retention of platinum in the plasma and tissues has been observed 8–75 months after treatment with cisplatin and oxaliplatin (Brouwers et al., 2008). Whether damage/death induced by oxaliplatin is specific to certain enteric neuronal subtypes should be identified as this may be correlated with gut dysfunctions.

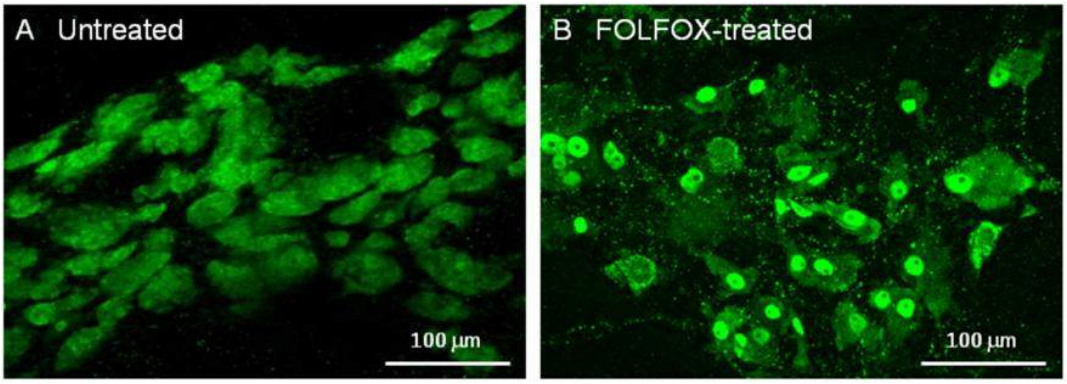


Figure 1.3. Effect of treatment with folinic acid, 5-fluorouracil, and oxaliplatin (FOLFOX) on human myenteric neurons. Confocal images of the colon wholemount preparations labelled with an antibody to the pan-neuronal marker, human neuronal protein (Hu). **A.** Hu-IR myenteric neurons in the colon tissue resected from colorectal cancer patient untreated with chemotherapy. **B.** Translocation of Hu protein to the nuclei of myenteric neurons in the colon tissue from a colorectal cancer patient treated with FOLFOX prior to surgery.

1.4 Pathophysiology and treatment of chemotherapy-induced nausea, vomiting, diarrhoea, constipation, and peripheral neuropathy

1.4.1 Nausea and Vomiting

Chemotherapy-induced nausea and vomiting are serious and debilitating side-effects experienced by patients undergoing anti-cancer therapy (Hawkins and Grunberg, 2009, Janelsins et al., 2013). These side-effects present hurdles in potentially curative cancer treatments, and thus, pharmacologically alleviating the adverse events to continue anti-cancer drug administration remains of high importance (Hawkins and Grunberg, 2009, Janelsins et al., 2013). Nausea and vomiting are controlled by the emetic centre in the lateral medullary reticular formation in the pons, as well as the receptors lining the floor of the fourth ventricle of the brain, the area postrema which is referred to as a chemoreceptor trigger zone (Di Fiore and Van Cutsem, 2009, Rojas and Slusher, 2012). The medulla oblongata is a circumventricular organ, allowing noxious stimuli within the blood such as metabolites from anti-cancer drugs to provoke nausea and vomiting (Di Fiore and Van Cutsem, 2009). Additionally, vagal afferent fibres propagate sensory information regarding the physiological milieu of the gastrointestinal tract directly to the brainstem in order to regulate gastric and intestinal functions (Travagli et al., 2006). Nausea and vomiting are believed to be part of a protective mechanism through which the body rids itself of toxins. Nausea essentially acts as a warning sign

that results in food/substance aversion, allowing for the confinement of toxins in the upper gastrointestinal tract for eventual expulsion through the act of vomiting (Andrews and Horn, 2006, Horn, 2014, Navari, 2016, Singh et al., 2016). Ingested toxins may produce delays in gastric emptying, leading to the onset of nausea which is thought to be sensed by vagal afferents projecting to the brainstem (Andrews and Horn, 2006, Babic and Browning, 2014, Horn, 2014). Investigating the mechanisms underlying nausea is extremely difficult, and the majority of human studies examining the severity of chemotherapy-induced nausea relies solely on self-reporting by patients, particularly through the means of a Likert scale outlining varying indices of severity (0= no nausea to 10= severe nausea). Nauseous episodes are thought to occur in a 'wave-like' fashion through increases in sympathetic and decreases in parasympathetic activity (LaCount et al., 2011, Farmer et al., 2015, Navari, 2016). Functional magnetic resonance imaging of healthy volunteers with visually-induced nausea has highlighted additional key brain areas that increase in activity during this event. These areas include the anterior insula, anterior/mid-cingulate, inferior frontal and middle occipital gyri and medial prefrontal cortex (Napadow et al., 2013, Farmer et al., 2015). Additionally, a decrease in posterior cingulate activity is also reported (Navari, 2016). This further highlights the 'wave-like' presentation of nausea as the anterior areas are known to control sympathetic outflow, and the posterior areas control parasympathetic output. Moreover, vomiting is a complex and coordinated process potentiated by the brainstem and carried out

through the autonomic and somatic divisions of the PNS **(Figure 1.4)** (Janelsins et al., 2013, Rapoport, 2017). Afferent inputs from the stomach converge within the nucleus tractus solitarius (NTS), and neurons from this region can act on those within the dorsal motor nucleus of the vagus nerve (DMNX), which in turn, regulate gastric and intestinal motility and function (Travagli et al., 2006).

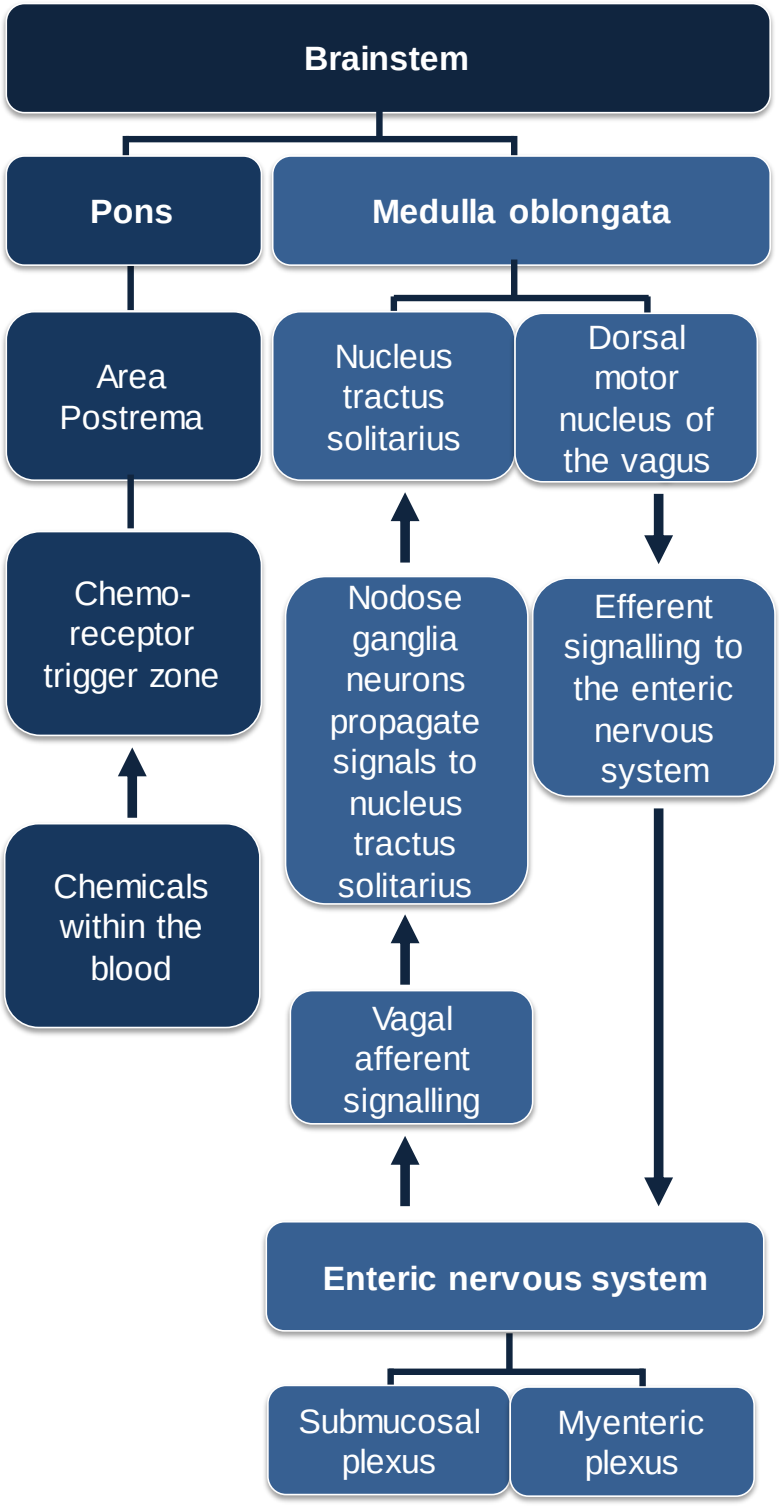


Figure 1.4. Neuroanatomical areas of the brainstem and ENS which regulate gastric and intestinal function.

Prior to vomiting, the gastric fundus is relaxed and contractile activity of the antrum declines. Increased sympathetic activity initiated during nausea and vomiting ultimately reduces gastric motility and secretion, as well as blood flow to the stomach and intestines. This serves as a protective measure as it limits the propulsion and absorption of contents further down the gastrointestinal tract. Mechanoreceptive abdominal vagal afferent terminals detect changes in distension and contractility of smooth muscle (Grundy, 1993, Grundy, 2004). Changes in gastric antrum distension, wall tension and motor quiescence stimulate mechanosensitive vagal afferents to induce nausea and vomiting. These afferents also lie anatomically close to enteroendocrine cells which produce mediators that can influence the function of these neurons. Chemotherapy-induced mucosal insult within the gastrointestinal tract can activate vagal afferents via the release of serotonin from enterochrommaffin cells (Schworer et al., 1991, Fukui et al., 1992).

Various antiemetic drugs are available in clinical practice: corticosteroids and drugs acting on various neurotransmitter receptors including dopaminergic, histaminic, muscarinic and serotoninergic (Navari, 2009, Rojas and Slusher, 2012). A new class of antiemetics, a selective neurokinin 1 (NK_1) receptor antagonist, aprepitant, inhibits cytochrome P450 isoenzyme 3A4 and can lead to significant drug interactions, resulting in need for dose modification of concomitant therapy (Navari, 2004, Rojas and Slusher, 2012). Thus, more effective treatment strategies are needed to treat chemotherapy-induced nausea and vomiting.

1.4.2 Diarrhoea

Normal gastrointestinal motility requires coordinated neurogenic input and myogenic output, mucosal transport, as well as defecation reflexes (Mancini and Bruera, 1998, McQuade et al., 2016d). The incidence of chronic post-treatment diarrhoea amongst cancer survivors varies from 14% to 49% and episodes of diarrhoea can persist for more than 10 years (Denlinger and Barsevick, 2009). Diarrhoea is thought to result from chemotherapy-induced mucosal damage, increased intestinal permeability and a reduction in absorptive function (Andreyev et al., 2012, McQuade et al., 2014). Chemotherapy-induced crypt ablation, epithelial atrophy and villus blunting within the ileum and colon have been reported previously (Logan et al., 2008, McQuade et al., 2016a). Such mucosal damage is thought to induce gastrointestinal inflammation, and ultimately result in chemotherapy-induced mucositis (McQuade et al., 2016d). Furthermore, intestinal mucosal insult is also thought to contribute to fluid and electrolyte imbalance through crypt apoptosis resulting in malabsorption and mucous hypersecretion by goblet cells (Richardson and Dobish, 2007, Stringer et al., 2009a, Stein et al., 2010). Moreover, intestinal mucositis is typically associated with an increase in cyclooxygenase-2 and prostaglandin E2 levels following chemotherapy treatment (McQuade et al., 2016d). Prostaglandin E2 release stimulates colonic motility and enhances chloride secretion, further contributing to diarrhoea (Riviere et al., 1991). The ENS also has the capacity to regulate the functions of gastrointestinal epithelial cells and intestinal motility (Bornstein et al.,

2004, Snoek et al., 2010, Furness, 2012). Fluid secretion and the absorption of electrotyes are typically controlled by secretomotor neurons within the submucosal plexus, but their function may also be modulated by myenteric neurons (Costa et al., 2000, Furness, 2012). Thus, damage to enteric neurons within the submucosal or myenteric plexi may exacerbate or underlie chemotherapy-induced gastrointestinal dysfunction.

Despite the number of clinical trials evaluating therapeutic or prophylactic measures in chemotherapy-induced diarrhoea, the most common current treatment is the μ-opioid receptor agonist, loperamide, which can cause abdominal pain, bloating, nausea, vomiting, constipation, paralytic ileus, dizziness, rashes and anaphylaxis (Perez-Calderon and Gonzalo-Garijo, 2004, Sharma et al., 2005).

1.4.3 Constipation

Chemotherapy-induced constipation is recognised as infrequent bowel movements and increased stool consistency (McQuade et al., 2016d). Constipation is a prevalent side-effect associated with anti-cancer treatment, affecting 50-87% of patients (Abernethy et al., 2009). Severe constipation can result in abdominal pain and distension, haemorrhoids, rectal fissures, life-threatening faecal impaction and bowel obstruction, intestinal ischemic necrosis, perforation and bleeding (MacDonald et al., 1991, Leung et al., 2011). Furthermore, constipation can also result in poor absorption of oral drugs which may be used to alleviate other chemotherapy-induced side-effects.

The gastrointestinal epithelium and mucosa has a high cellular turnover rate and the capacity to regenerate quite rapidly, so the mechanisms underlying delayed or chronic constipation and diarrhoea caused by chemotherapeutic agents appear to be more complex and perhaps involve damage of post-mitotic cells such as enteric neurons. Neuronal damage can impair neurally-mediated gastrointestinal functions which may underlie long-term dysmotility. A few studies have demonstrated ENS damage characterised by neuronal loss and proportional changes of neuronal subpopulations which can be associated with long-term dysmotility following cisplatin and oxaliplatin, as well as 5-fluorouracil treatments (Vera et al., 2011, Wafai et al., 2013, McQuade et al., 2016a, McQuade et al., 2016b).

1.4.4 Acute and chronic peripheral sensory neuropathy

Acute peripheral sensory neuropathy associated with oxaliplatin in particular is thought to be caused by alterations in voltage-gated sodium channel kinetics through the chelation of calcium ions by oxalate (Adelsberger et al., 2000, Saif et al., 2001), whereas the chronic form of neuropathy is thought to be caused by platinum drug accumulation (Gregg et al., 1992, Ta et al., 2006, Sprauten et al., 2012). There are differing forms of precautionary measures and treatment options for neurotoxicity which include education, dose modifications and neuropharmacological methods.

Education, as a form of precaution and treatment, has become paramount for patients and their care givers. Knowledge about the side-effects associated with platinum-based drugs is important in order to avoid aggravating stimuli. These include avoiding exposure to cold objects, liquids and temperatures (Saif and Reardon, 2005). Furthermore, routine questioning of patients regarding the onset of symptoms, duration, body location, sensations experienced, relationship to cold temperatures (if any), and persistence should be monitored (Saif and Reardon, 2005). These details fall in line with modifications of drug dose and timecourse of delivery. A dose reduction is the first treatment measure for patients presenting with transient neurological symptoms, whereas those with persistant side-effects will not receive their next scheduled cycle. This 'stop and go' model has shown beneficial effects in reducing the severity of neurotoxic complications (André et al., 2004). However, retrospective studies have shown that increasing chemotherapeutic dose significantly improves patient response rates to cancer treatment without intensifying neurotoxicity (Maindrault-Gæbel et al., 2000). Further research is required to delineate the benefits of dose modifications on neuropathological outcomes and anti-cancer efficacy.

Neuropharmacological treatment methods have also been trialled to alleviate sensory peripheral neuropathy. Given that research has shown neuronal hyperexcitability following oxaliplatin treatment, and the possibility of calcium chelation by oxalate, calcium and magnesium infusions have been trialled. Calcium in particular has the capacity to

promote closure of voltage-gated sodium channels (Armstrong and Cota, 1999). Thus, divalent cations such as calcium and magnesium may reduce neuronal hyperexcitability by altering voltage-gated sodium channel kinetics. Two studies have demonstrated the benefits of calcium and magnesium infusions in reducing the severity of oxaliplatin-induced peripheral neuropathy (Gamelin et al., 2004, Saif, 2004). Conversely, a phase III randomised, placebo-controlled, double-blind study revealed that calcium and magnesium infusions do not particularly protect against neurotoxicity (Loprinzi et al., 2014).

Platinum-based drugs have shown the capacity to induce oxidative stress in neuronal and non-neuronal cell types (Carozzi et al., 2010, Florea and Büsselberg, 2011, Marullo et al., 2013, McQuade et al., 2016b). Glutathione (GSH) supplementation has also shown protective effects against cisplatin and oxaliplatin-induced neuropathy (Cascinu et al., 2002, Milla et al., 2009). However, the efficacy of GSH supplementation is also called into question as there is evidence to suggest this antioxidant does not alleviate neurotoxicity (Dong et al., 2010). The treatment options for alleviating peripheral neuropathy following platinum-based drug treatments remain limited.

1.5 Autonomic control of the gastrointestinal tract

The gastrointestinal tract is innervated by both extrinsic and intrinsic neural pathways (Lundgren, 2000, Furness, 2012, Browning and Travagli, 2014). Extrinsic innervations of the gastrointestinal tract are supplied

through the vagus nerve, DRG, spinal and paravertebral ganglia, and lumbosacral nerves (Browning and Travagli, 2014, Furness et al., 2014, Travagli and Anselmi, 2016). The eosophagus and stomach are primarily controlled by sympathetic and parasympathetic nerves stemming from the caudal brainstem, spinal cord and paravertebral ganglia (Furness et al., 2014). The intestines however, are chiefly controlled by the ENS, an intrinsic and complex orchestration of sensory neurons, interneurons and motor neurons, which allows for ample autonomous control of ileal and colonic functions (Travagli et al., 2006, Furness et al., 2014). The gastrointestinal tract also receives extrinsic innervation (Furness et al., 2014).

1.5.1 Extrinsic innervation of the gastrointestinal tract

The brainstem which consists of the midbrain, pons and medulla oblongata plays a role in regulating autonomic gastrointestinal functions (Travagli et al., 2006). The vagus nerve (cranial nerve X) stems from the medulla oblongata and extensively innervates the head, neck, thorax and abdomen (Gibbins, 2013). Two distinct ganglia emerge from the vagus nerve; the superior jugular ganglion and the inferior nodose ganglion (Zhuo et al., 1997, Travagli et al., 2006). Neuronal cell bodies of vagal afferent fibres which innervate the upper gastrointestinal tract are found within the nodose ganglia (Travagli and Anselmi, 2016). Vagal afferent fibres propagate sensory information regarding the physiological milieu and activity of the stomach and gastrointestinal tract through glutaminergic and gamma-aminobutyric acid (GABA)ergic synapses with second-order

neurons at the level of the NTS within the medulla oblongata (Browning and Travagli, 2011, Hermes et al., 2014). The second-order neurons receive sensory information from the vagal afferents, as well as higher brain centres. From thereon, NTS neurons project to the neighbouring DMNX which contains parasympathetic preganglionic neurons supplying vagal motor output to the gastrointestinal tract and stomach (Travagli et al., 2006).

Furthermore, pelvic splanchnic nerves originating from the sacrum also provide parasympathetic outflow to the colon and rectum (Nadelhaft and Booth, 1984, Bessant and Robertson-Rintoul, 1986, Sengupta and Gebhart, 1994). Although the ileum and colon contain complete neural circuitry which allows for sufficient intrinsic and autonomous control of gastrointestinal functions, the oesophagus and stomach lacks the ability for such autonomy. Therefore, the oesophagus and stomach receive the greatest parasympathetic innervation from the vagus nerve, whilst innervation diminishes caudally towards the ileum and colon (Furness et al., 2014). Parasympathetic postganglionic neurons synapse with myenteric neurons of the ENS to control gastric tone, motility and secretion through an excitatory cholinergic pathway (Berthoud et al., 1991, Zheng and Berthoud, 2000, Berthoud et al., 2001, Travagli et al., 2006). Inhibition of gastric function is mediated through a non-adrenergic, non-cholinergic pathway, primarily through the use of nitric oxide (NO) and vasoactive intestinal peptide (VIP) (Lefebvre et al., 1995, Takahashi and Owyang, 1995, Curro et al., 2008).

Additionally, the gastrointestinal tract is innervated by sympathetic thoracolumbar spinal afferents from the splanchnic nerves, as well as thoracolumbar and lumbosacral spinal afferents from the pelvic and rectal nerves innervating the distal colon (Brierley et al., 2004, Furness et al., 2014, Spencer et al., 2014).

The thoracolumbar afferents run parallel to sympathetic efferents innervating the gastrointestinal tract, and the vagal and sacral afferents run parallel to the parasympathetic efferents (Brookes et al., 2013). Sympathetic afferent fibres also stem from the prevertebral ganglia, namely, the celiac, superior mesenteric and inferior mesenteric (Lomax et al., 2010). The cell bodies of spinal afferents are found within the DRG. Primary afferent sensory fibres from DRG neurons project alongside the mesenteric arteries, within the mucosa and around submucosal and myenteric neurons. DRG innervation is typically involved in the regulation of blood flow, modulating enteric neuronal function, as well as gastrointestinal secretion and motility (Ratcliffe, 2011, Furness et al., 2014).

Sympathetic postganglionic fibres originate from the paravertebral sympathetic ganglia which are noradrenergic and release norepinephrine (Browning and Travagli, 2014). These noradrenergic fibres synapse with neurons in the submucosal and myenteric plexi of the ENS, and they also innervate smooth muscle, blood vessels, and endocrine cells of the gastrointestinal tract (Furness and Costa, 1974, Phillips and Powley, 2007).

1.5.2 Intrinsic innervation of the gastrointestinal tract

The ENS is a complex orchestration of neurons innervating the gastrointestinal tract and controlling its motor, absorbptive and secretory functions (Costa et al., 2000, Furness, 2012). Several different classes of neurons reside in the ENS and they differ in terms of cell morphology, electrophysiological properties, neurotransmitter synthesis and release, and the types of synaptic inputs received (Furness, 2012). Neurons and glia are arranged into ganglia forming two major plexi: myenteric and submucosal. The myenteric plexus located between the circular and longitudinal muscle regulates the movement of the contents along the gut (motility). The submucosal plexus located between the circular muscle layer and submucosa regulates secretion, fluid and electrolyte balance as well as vascular tone (Costa et al., 2000, Furness, 2012). Networks of interganglionic fibres connect ganglia within myenteric and submucosal plexi. Additionally, nerve fibres stemming from the myenteric and submucosal plexi innervate non-neuronal cell types throughout the gastrointestinal tract such as smooth muscles, mucosa, epithelium and blood vessels (Costa and Furness, 2010). There are distinct neuronal phenotypes within the myenteric and submucosal plexi which can be identified using various immunohistochemical markers, with respect to their functionality, morphology and electrophysiological properties

Generally, the immunohistochemical labelling of neurotransmitters has revealed a number of distinct neuronal phenotypes within the ENS (Furness et al., 1994, Furness, 2000). The ENS contains

neurotransmitters found within the CNS and others which include, but are not limited to: acetylcholine, glutamate, GABA, monoamines (serotonin, norepinephrine, dopamine), NO, VIP, neuropeptide Y (NPY), calretinin, somatostatin, calcitonin gene-related peptide (CGRP) and substance P (Furness, 2000, Bornstein et al., 2004, Costa and Furness, 2010). Neurons can co-express multiple transmitters and have varying functions (McConalogue and Furness, 1994, Beck et al., 2009). Moreover, there are also a few distinct glial cell phenotypes within the ENS. Enteric glial cells play a role in neuronal support, both structurally and metabolically. They are implicated in neurotransmitter modulation as well gastrointestinal epithelial barrier and mucosal functions (Yu and Li, 2014, Coelho-Aguiar Jde et al., 2015, Sharkey, 2015). Enteric glia can also be characterised based on their expression of sox10, glial fibrillary acidic protein (GFAP), and s100β (Rühl, 2005, Soret et al., 2013). Sox10 is a transcription factor which plays a role in glial cell differentiation and maturation (Kuhlbrodt et al., 1998, Britsch et al., 2001). GFAP is an intermediate filament involved in maintaining the cytoskeleton of glial cells, as well as their mechanical strength which is important for supporting neighbouring neurons (Yang and Wang, 2015). s100β is a calcium-binding protein that may interact with a range of proteins, enzymes and transcription factors. s100β is involved in calcium homeostasis, and has demonstrated the capacity to modulate cellular energy metabolism, cell differentiation, proliferation, survival, protein phosphorylation, and may also function as a damage-associated molecular pattern (DAMP) (Sorci et al., 2013).

Within the last decade several studies have been done to characterise the ENS in mice as they are widely used in biomedical research (Sang and Young, 1996, Qu et al., 2008, Foong et al., 2014). Furthermore, genetically manipulated strains can provide significant insight into mechanisms of many disorders. Although there is very limited knowledge regarding the various neuronal phenotypes within the mouse and human ENS, it is thought that neuronal functions are conserved between different species (Grider, 2003, Hao and Young, 2009).

Within the myenteric plexus of the mouse gastrointestinal tract, only a few classes of neurons and their abundance have been identified. Approximately 26% of neurons innervating the circular muscle, submucosa and mucosa are IR for CGRP, whereas 29% of neurons innervating the musculature express NOS with or without VIP colocalisation (Qu et al., 2008). Furthermore, 10% of excitatory neurons supplying the longitudinal muscle IR for ChAT, and 52% express calretinin (with 20% co-expressing ChAT or tachykinins) (Qu et al., 2008). Similar to the guinea-pig, there are regional differences of neuronal phenotypes throughout the mouse intestine. The proportions of nNOS-IR neurons in the mouse proximal and distal colon are 51% and 44% respectively (Wattchow et al., 2008). In the proximal and distal colon, 41% and 48% of neurons are ChAT-IR respectively (Wattchow et al., 2008). Approximately 90% of submucosal neurons within the mouse ileum express calretinin, 51% express VIP (with most coexpressing NPY), 41% express ChAT (in

which half are IR for CGRP), and 40% of neurons which express TH (Mongardi et al., 2009).

The CNS and ENS work harmoniously to regulate normal gastrointestinal motility, secretion, absorption and vasomotor tone. Therefore, any potential damage to this neural circuitry by platinum-based chemotherapeutic agents may induce unfavourable gastrointestinal complications which compromise optimal anti-cancer treatment efficacy through the onset of nausea, vomiting, diarrhoea and constipation. It remains unclear how platinum-based drugs exert their cytotoxic effects on the nervous system, and how these agents gain entry to the intracellular environment of nerves. However, there is emerging work that platinum-based drugs may utilise metal transporters and chaperones, such as those belonging to the copper family, as a means of influx and intracellular distribution (Holzer et al., 2006, Howell et al., 2010, Arnesano et al., 2011, Zhao et al., 2014). The gastrointestinal tract is directly involved in the homeostatic regulation of copper (Lönnerdal, 2008, Zimnicka et al., 2011). High expression of copper transporters and chaperones may therefore render the gastrointestinal tract particularly vulnerable to platinum-based drugs.

1.6 Implications of copper transporters in cellular influx and trafficking of platinum-based chemotherapeutic agents

The copper transport system has been implicated in platinum drug sensitivity and resistance in a number of non-neuronal cells, highlighting it as a likely mode of platinum drug transport into and out of the cellular environment. This system involves influx and efflux transporters, as well as chaperones which deliver copper (or platinum drugs) to various intracellular compartments. Yet, little is known about the copper transport system and its role in platinum drug sensitivity of the nervous system. Platinum drugs can be chaperoned to the nuclei and mitochondria where they can bind to, and damage, DNA; saturation of the copper transport system might disturb intracellular copper homeostasis, and therefore, vital cellular processes.

The cellular uptake of platinum-based drugs was originally thought to result from passive diffusion. Although these agents are hydrophilic, it is unknown whether they are able to enter cells via aquaporins like water does. Most research to date has focused on facilitated cell entry through the use of metal transporters. In particular, there are many studies which have implicated the copper transport system in platinum-based drug influx, distribution, storage and efflux. Copper influx transporters include the copper transporter receptor 1 (CTR1), and the debated copper transporter receptor 2 (CTR2). There are several chaperones involved in the intracellular trafficking of copper which include cytochrome *c* oxidase 17 (COX17), copper chaperone for superoxide dismutase (CCS) and

superoxide dismutase 1 (SOD1). Copper efflux is regulated by the anti-oxidant protein 1 (ATOX1) which resides in the *trans*-Golgi network and transfers metal ions to proteins of the ATPase family (ATP7A and ATP7B). Copper can also be handled by intermediary chaperone proteins such as glutathione (GSH) and metallothionein (MT) **(Figure 1.5)**. Possible pathways for platinum drug influx and intracellular trafficking through the copper transport system will be discussed in detail in sections 1.6.1-1.6.4.

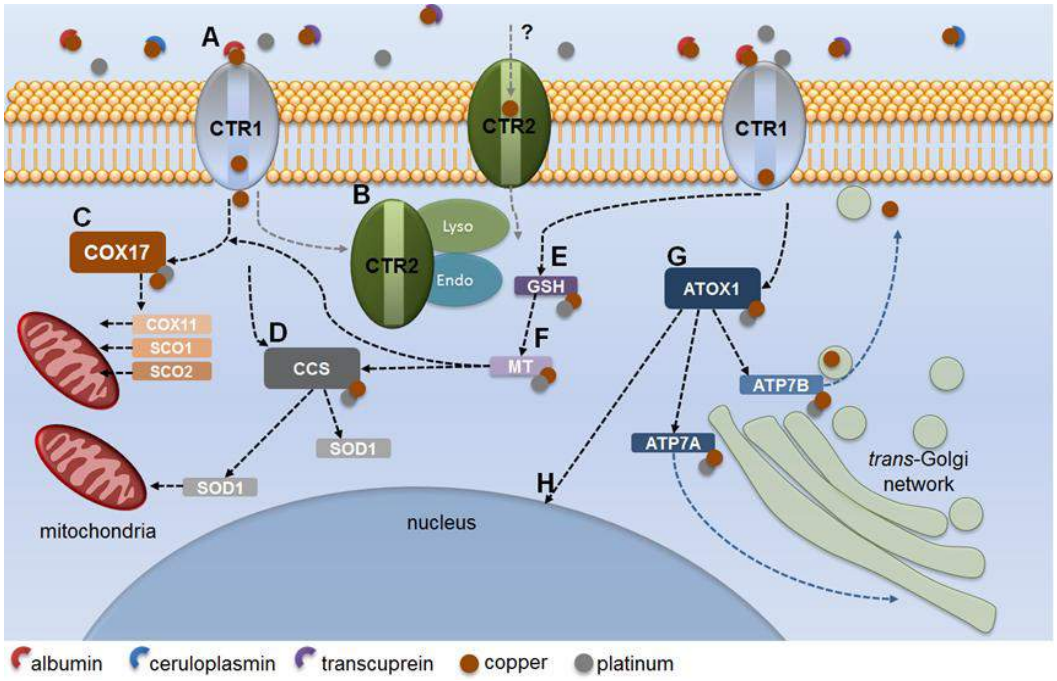


Figure 1.5. Summary of the transport system regulating copper and platinum influx, mobilisation and efflux. As copper is highly reactive, it is bound to albumin, ceruloplasmin and transcurprein in the blood and delivered to the high affinity transporter, CTR1, on the cell membrane **(A)**. Limited studies have demonstrated that CTR2 is localised to the plasma membrane and partakes in copper, however, majority of the research have determined its role in the intracellular mobilisation of copper through lysosomes and endosomes **(B)**. Whether a chaperone delivers copper to CTR2 is unknown. The chaperone COX17 acquires copper and donates it to the acceptor proteins, COX11, Sco1 and Sco2, in the mitochondria **(C)**. CCS aquires copper for delivery to SOD1 within the cytosol and the mitochondria **(D)**. GSH is able to sequester excessive copper, or transfer it to other intermediary handling proteins such as MT **(E)** for redistribution to CCS or COX17 **(F)**. The ATOX1 chaperone sequesters copper to the efflux pathway via ATP7A and ATP7B **(G)**, and can translocate copper to the nucleus **(H)**.

1.6.1 Copper transporter receptor 1 (CTR1)

The main route for cellular copper influx is via the high affinity CTR1, which is encoded by the the *SLC31A1* gene (Prohaska, 2008, Lutsenko, 2010, Wang et al., 2011). CTR1-mediated influx of copper is a non-energy and non-proton gradient-dependent process (Prohaska, 2008). Only the efflux of copper involves the hydrolysis of ATP (Prohaska, 2008). Knockout of CTR1 results in an embryologically lethal phenotype, thus, signifying the importance of copper influx, and the essentiality of this micromineral in many metabolic processes (Wang et al., 2011). CTR1 expression on the plasma membrane is regulated by intracellular copper levels (Wang et al., 2011). Insufficient or excessive intracellular copper levels would provoke the increased or decreased expression of CTR1 on the plasma membrane, respectively (Petris et al., 2003, Gupta and Lutsenko, 2009, Lutsenko, 2010).

There are many studies correlating CTR1 expression with increased platinum-based toxicity in non-neuronal cells (Ishida et al., 2002, Lin et al., 2002, Song et al., 2004, Holzer and Howell, 2006, Jandial et al., 2009, Larson et al., 2010). It has been demonstrated in an ovarian cancer cell line, that the internalisation of CTR1 following cisplatin exposure occurs via macropinocytosis and proteasomal degradation (Holzer and Howell, 2006). The internalisation of CTR1 infers receptor stimulation of drug influx. Inhibition of CTR1 by silver decreases copper uptake at the plasma membrane in HEK293 embryonic kidney cells and mouse embryonic cells (Holzer and Howell, 2006, Holzer et al., 2006). *In*

vitro experiments using murine embryonic fibroblasts have shown that CTR1 receptor mediates platinum-drug influx, however, oxaliplatin at higher concentrations appears to not depend on CTR1 for entry (Holzer et al., 2006). Research linking CTR1 expression in the central and peripheral nervous systems with the extent of platinum-based neurotoxicity is limited **(Table 1.2)**. It has been established that platinum from cisplatin and oxaliplatin accumulates in the DRG (Ta et al., 2006, Ip et al., 2010). This is thought to be the primary mechanism of long-term peripheral neuropathy. CTR1 expression within DRG neurons is characterised by intense immunoreactivity of the plasma membrane and vesicular structures of the cytoplasm (Ip et al., 2010). Larger-sized DRG neurons are highly immunoreactive (IR) for CTR1 and are more vulnerable to oxaliplatin-induced atrophy when compared to small-sized DRG neurons with minimal or no CTR1 expression (Liu et al., 2009, Ip et al., 2010). Whether CTR1 internalisation and degradation occurs in neurons of both the central and peripheral nervous systems in response to platinum-based drugs, as it does in yeast (*Saccharomyces cerevisiae*) and cancer cell lines, requires further elucidation (Ishida et al., 2002, Holzer and Howell, 2006).

1.7 DNA repair and cell death pathways associated with platinum-based chemotherapeutic agents

Mammalian cells have the capacity to recognise and respond to a myriad of noxious factors which include oxidative stress, ultra violet irradiation, chemotherapeutic toxicity and their subsequent DNA distortion and damage. Depending on the type of damaging stimulus, cells are equipped with several systems capable of DNA repair. As discussed in section 1.2, platinum-based drugs induce intrastrand and interstrand DNA adducts or cross-links, particularly on guanine clusters, or guanine-adenine base pairs which heavily distort the helical DNA structure (Alcindor and Beauger, 2011). There are several systems which may modulate DNA repair following platinum-based drugs, or, if cellular damage is too severe, can potentiate apoptotic cell death.

The nucleotide excision repair (NER), base excision repair (BER), and the mismatch repair (MMR) pathways in particular can be recruited upon platinum-based chemotherapeutic challenge (Martin et al., 2008, Alcindor and Beauger, 2011, Kothandapani et al., 2011, Kothandapani et al., 2013). The initial steps in the NER system involves flagging bulky platinum adducts and binding to damaged nucleotides, followed by the unwinding of the affected portion of the DNA nucleotides which are ultimately excised by *Xeroderma pigmentosum* complementation group A, and excision repair cross-complementation group 1 proteins (Reed, 1998, Martin et al., 2008, Earley and Turchi, 2011). Subsequent to nucleotide excision, the damaged DNA fragment is resynthesised and ligated to

continue the helical DNA structure. Platinum-based agents can also induce mitochondrial DNA damage (Rosen et al., 1992, Yang et al., 2006, Podratz et al., 2011). However, given that this organelle does not possess the NER machinery, platinum-based drugs may induce successful cell death through mitochondrial DNA damage and possibly cell energy crisis (Mason and Lightowlers, 2003, Mason et al., 2003, Alexeyev et al., 2013). The BER pathway does not typically recognise bulky DNA lesions which are induced by platinum-based drugs, but this system does repair deaminised, alkylysed or oxidised nucleotide bases which could be a result of chemotherapeutic drug injury (Kim and Wilson, 2012). However, there are some studies which demonstrate a role for BER machinery in platinum drug sensitivity and/or resistance (Kothandapani et al., 2011, Kim et al., 2015). Nevertheless, platinum-based agents have shown the capacity to induce oxidative stress, and such oxidative damage on DNA may be repaired through the BER pathway (Kelley et al., 2014, Kim et al., 2015). The BER pathway may not be advantageous for detecting DNA platinum adducts within the mitochondria either. However, the oxidative stress induced by platinating agents may be combated through this repair system. Furthermore, the MMR pathway plays a role in DNA repair through the recognition of mis-matched DNA base pairs (Basu and Krishnamurthy, 2010). The MMR repair system is recruited in the post-replicative stages of DNA synthesis and thus, functions to maintain genomic integrity (Fink et al., 1996, Basu and Krishnamurthy, 2010). Interestingly, the MMR system recognises DNA damage induced by

platinum-based drugs, however, instead of initiating repair and maintaining cell viability, this pathway can potentiate cytotoxic cell death (Fink et al., 1996, Basu and Krishnamurthy, 2010). There is limited research pertaining to the MMR system within the mitochondria. Although this system is detected, its function within the mitochondria appears to be regulated through different mechanisms when compared to nuclear repair pathways (Mason and Lightowlers, 2003, Mason et al., 2003, Alexeyev et al., 2013).

A DAMP protein high-mobility group box 1 (HMGB1) has been identified as a high affinity platinum adduct recognition and binding protein (Malina et al., 2002, Prasad et al., 2007, Liu et al., 2010d, He et al., 2015). HMGB1 is a multifunctional and ubiquitous protein involved in DNA repair, transcription, chromatin stability, and extracellular pro-inflammatory cytokine-like activity (Lange and Vasquez, 2009, Yang et al., 2013). HMGB1 is comprised of 3 domains (A and B domains, and a C-terminal amino acid acidic tail) which facilitate its binding to DNA lesions (Ulloa and Messmer, 2006, Lange and Vasquez, 2009, Sanchez-Giraldo et al., 2015). Most research to date has focused on HMGB1-cisplatin complexes (Pil and Lippard, 1992, Jung and Lippard, 2003, Ramachandran et al., 2012, He et al., 2015). HMGB1 specifically binds to cisplatin 1,2-d(GpG) or 1,2-d(ApG) DNA platinum adducts (Pil and Lippard, 1992).However, HMGB1 has also been shown to complex with oxaliplatin DNA platinum adducts (Ramachandran et al., 2012). Crystal structures of HMGB1-cisplatin complexes have demonstrated significant DNA distortion which has been characterised as severe helical bending (Ohndorf et al., 1999, Lange and

Vasquez, 2009). The combined HMGB1-cisplatin complexes induce greater structural DNA damage when compared to the helical distortion induced by the platinum adducts on their own (Ohndorf et al., 1999, Lange and Vasquez, 2009). Such results have created hypothetical contention regarding the functional consequences of HMGB1-platinum drug recognition and its effects on cell viability. In particular, the 'repair enhancing' or 'repair shielding' hypotheses have been generated. It is postulated that HMGB1 recognition and binding of DNA platinum adducts could either enhance the recognition of bulky drug lesions and facilitate/activate repair pathways inducing 'repair enhancing' effects (Malina et al., 2002, Mukherjee and Vasquez, 2015). Whereas, HMGB1-platinum adduct binding could also exacerbate DNA damage and distortion beyond repair, and excessive binding to drug lesions could block the access of repair machinery, thus, causing failure of nucleotide excision and resulting in 'repair shielding' (Huang et al., 1994, Patrick and Turchi, 1998, Malina et al., 2002, Mitkova et al., 2005, Yusein-Myashkova et al., 2016). Studies have demonstrated that HMGB1 can modulate NER, BER and MMR pathways in response to DNA damage (Malina et al., 2002, Liu et al., 2010d, Yusein-Myashkova et al., 2016). HMGB1-platinum adduct binding has been shown to block NER accessibility to distorted DNA (Malina et al., 2002, Mitkova et al., 2005, Yusein-Myashkova et al., 2016). However, HMGB1 has also shown the capacity to recognise and bind to DNA triplex-directed interstrand cross links induced by a natural compound *psoralen* (toxic plant compound derived from the

furanocoumarin family), and in such circumstances facilitates NER repair of lesions (Reddy et al., 2005). Thus, the roles of HMGB1 in coordinating DNA repair or exacerbating damage seems to be dependant on the type of DNA damaging agents, as well as the lesions they produce. HMGB1 can initiate DNA repair via BER enzyme interactions (Prasad et al., 2007, Liu et al., 2010d). Studies utilising a model of 405nm laser light damage in HeLa cells demonstrated the accumulation of green fluorescence tagged HMGB1, strengthening the notion that this protein concentrates at DNA lesion sites (Lan et al., 2004, Prasad et al., 2007). Moreover, HMGB1 has also been implicated in MMR function (Lange and Vasquez, 2009). HMGB1 has been identified as the initial DNA damage recognition protein interacting with the MMR proteins, MSH2 and MLH1, and facilitates the incision processes (Yuan et al., 2004, Lange and Vasquez, 2009).

Another DAMP protein, calreticulin, has recently been identified as a recognition and binding molecule for cisplatin DNA platinum adducts (Karasawa et al., 2013). Like HMGB1, calreticulin is a ubiquitous and pleitropic protein with many functional roles (Michalak et al., 2009, de Bruyn et al., 2015). Calreticulin is an endoplasmic reticulum and nuclear envelope resident protein which functions include, but are not limited to: calcium homeostasis, protein folding, the assembly of Major Histocompatibility Complex (MHC) class I molecules, nuclear export and prompting immunogenic cell death (Arnaudeau et al., 2002, Holaska et al., 2002, Coe and Michalak, 2009, Michalak et al., 2009, Tesniere et al., 2010, de Bruyn et al., 2015). Although research to date has only

demonstrated the recognition of cisplatin DNA platinum adducts by calreticulin, it is plausible to suggest that this DAMP also detects lesions induced by carboplatin and oxaliplatin. In particular, oxaliplatin is a potent immunogenic cell death inducer through the cytoplasmic translocation of calreticulin (Zitvogel et al., 2008, Tesniere et al., 2010). Whether calreticulin recognises and binds to oxaliplatin adducts is yet to be determined. Furthermore it is unknown whether calreticulin is functionally implicated in the DNA repair systems NER, BER and MMR that may be initiated following toxicity caused by platinum-based drugs. Moreover, calreticulin expression is upregulated following cisplatin treatment (Al-Eisawi et al., 2016). It is unclear whether the overexpression of this protein is similar to HMGB1 protein concentration at DNA lesion sites. Nevertheless, oxaliplatin-induced cytotoxicity induces the cytoplasmic translocation of calreticulin which occurs during apoptotic cell death of colorectal cancer cells and can also induce a fatal immune response to the dying cells which is termed ‘immunogenic cell death’ (Panaretakis et al., 2009, Tesniere et al., 2010). Thus, these data implicate calreticulin in platinum-based drug cell death pathways.

1.7.1 Canonical apoptotic cascades

Apoptosis, also known as programmed cell death is initated in response to cellular injury that is beyond repair. Proteolytic enzymes known as caspases are essential effector molecules which modulate cellular apoptotic cascades (Fulda and Debatin, 2006, Li and Yuan, 2008,

McIlwain et al., 2013). There are several identified caspases which are typically classified by their roles in apoptosis or inflammation (McIlwain et al., 2013). Caspases with known roles in the apoptotic cascade include 3, 6, 7, 8, 9 and 10 (McIlwain et al., 2013, Parrish et al., 2013). Whereas caspases 1, 4, 5 and 12 (in humans) and 1, 11 and 12 (in mice) function in inflammatory cascades (McIlwain et al., 2013). The functional roles of caspases 2 and 14 are presently not well understood and require further research. Caspases which facilitate the apoptotic cascade can be further categorised as initiators or executioners depending on their functional roles. Initiator caspases 8 and 9 typically reside in the cell as inactive procaspase monomers which become activated through dimerisation (MacKenzie and Clark, 2012, McIlwain et al., 2013). The executioner caspases 3, 6 and 7 exist as inactive procaspase dimers which require cleavage by initiator caspases in order to become active (MacKenzie and Clark, 2012, McIlwain et al., 2013). The cleavage of executioner caspases allows for their conformational change by bringing together the two active sites of the dimer, and essentially forming a mature protease (Riedl and Shi, 2004, Feeney and Clark, 2005, MacKenzie and Clark, 2012). Apoptotic cascades can be triggered through extrinsic or intrinstic signalling pathways which can be distinguished based on the type of initiator caspases and adapter proteins involved (Li and Yuan, 2008, McIlwain et al., 2013). However, there can be some degree of overlap between the extrinsic and intrinsic pathways which makes it difficult to determine the origin of the apoptotic trigger. Platinum-based

chemotherapeutic agents in particular have demonstrated the capacity to induce apoptosis through various extrinsic and intrinsic signal transduction pathways (Fulda and Debatin, 2006, Florea and Büsselberg, 2011, Dasari and Bernard Tchounwou, 2014).

1.7.2 Immunogenic apoptosis

The immune system is the body's defence against damage, non-self antigens, as well as invading pathogens. This immune system is often viewed as two separate lines of defence; innate and adaptive. Innate immunity is the first line of defence which mounts rapid, non-antigen specific immunological responses (Warrington et al., 2011). This system has no immunological memory, and thus, is unable to memorise previously encountered pathogens or antigens. There are many cells involved in innate immunity which include: neutrophils, macrophages, dendritic cells, eosinophils, basophils, mast cells, natural killer (NK) cells and B and T cells (Warrington et al., 2011). Although B cells have the capacity to present antigens, macrophages and dendritic cells are considered professional antigen presenting cells (APCs) which bridge together the innate and adaptive immune systems. Macrophages and dendritic cells express cell-surface proteins identified as major MHC class I or II molecules which are imperative for antigen-specific T cell responses (Holling et al., 2004, ten Broeke et al., 2013). Antigen presentation prompts naive T cells to differentiate into a number of subpopulations, that includes helper $CD4^+$ T cells (Th) which can be pro-inflammatory (Th1),

anti-inflammatory (Th2), or regulatory (Tregs) (Zhu and Paul, 2008, Luckheeram et al., 2012). Furthermore, antigen presentation may also result in the differentiation of naive T cells into the cytotoxic phenotype ($CD8^+$ T cells) (Kaech and Ahmed, 2001, Obar and Lefrançois, 2010). Adaptive immune responses are largely influenced by the type of antigen, and pro-inflammatory/anti-inflammatory cytokines released by $CD4^+$ Th subtypes. CD4+ T cells do not have the capacity to directly kill cells, but rather, can condition the environment to potentiate cell-mediated immunity carried out by $CD8^+$ cytotoxic T cells (Obar and Lefrançois, 2010). Until recent years chemotherapy-induced cell death was deemed an immunologically 'silent' or 'tolerogenic' event. However, it is becoming more evident that platinum-based chemotherapeutic agents can in fact prompt a fatal immune response to stressed or injured cancer cells, which is known as immunogenic cell death. It is unknown whether platinum-based drugs can induce this type of cell death of the nerves innervating the gastrointestinal tract.

Cellular stress, particularly endoplasmic reticulum and oxidative stress caused by some anti-cancer agents can induce the translocation of intracellular proteins to the plasma membrane, and can also result in the release of molecules which act as 'eat me' signals for the recognition by immune cells, particularly macrophages and dendritic cells. These signals are known as DAMPs and their presentation is critical for triggering or enhancing an anti-tumour response (Garg et al., 2010, Krysko et al., 2012). DAMPs vital for eliciting immunogenic cell death include

calreticulin, HMGB1, ATP as well as some heat shock proteins (HSPs) (Garg et al., 2010, Krysko et al., 2012). Upon stressful stimuli, calreticulin translocates to the cell surface and acts as a potent 'eat me' signal recognised by phagocytes and dendritic cells (Garg et al., 2010, Martins et al., 2011). HMGB1 can function as a chemokine and proinflammatory cytokine, and also induces dendritic cell activation and maturation via toll-like receptor 4 (TLR4) binding which is crucial for T cell priming and activation (Krysko et al., 2012).

ATP is involved in many cellular functions such as differentiation, proliferation, adhesion and death. The release of ATP by cells undergoing apoptosis acts as a 'find me' signal which is recognised by monocytes (Krysko et al., 2012). ATP also activates the purigenic receptor P_2X_7 on dendritic cells, leading to the secretion of interleukin (IL)-1β and polarization of interferon gamma (IFN-γ) producing cytotoxic $CD8^+$ T cells (Elliott et al., 2009, Garg et al., 2012). HSPs are chaperones involved in the folding of newly synthesized proteins. In circumstances of cellular stress, HSPs such as HSP70 and HSP90 can translocate to the cell surface and interact with a number of receptors belonging to APCs, as well as activating NK cells and cross presenting antigens to $CD8^+$ T cells (Garg et al., 2010). The type of DAMPs which are presented or released, as well as their recognition by immune cells, depends on the type of stimulus (anti-cancer drug) and the resulting cellular stress induced **(Table 1.3)**.

Table 1.3. Summary of the immunogenic potential of platinum-based anti-cancer chemotherapeutic agents

Chemo-therapeutic agent	Mechanism of action	Immunogenicity	Organ	References
Cisplatin	DNA platinum-adduct formation	Activates immune response by inducing the release of HMGB1 (but does not cause immunogenic cell death) Improves the number of NK cells Induces an increase in lysosome formation by macrophages (tumor lysis mechanism) Induces macrophage recruitment and $CD8^+$ T cell responses Decreases STAT6 resulting in the downregulation of PD-L1 and PD-L2, enhancing T cell activity	CT26 colon cancer cell line Human peripheral blood Murine macro-phage culture (derived from peritoneum) Human peripheral blood Dendritic cell culture (cells derived from myeloma and colorectal cancer patients)	(Tesniere et al., 2010, Martins et al., 2011) (Ishikawa et al., 1998) (Palma et al., 1992) (Chang et al., 2013) (Lesterhuis et al., 2011)
Carboplatin	DNA platinum-adduct formation	Induces dendritic cell phagocytic and antigen recognition, increases the expression of CD80, CD83 and CD68, improved CD8+ T cell numbers and the secretion of IFN-γ	OVCAR-3 ovarian cancer cell line and dendritic cell co-cultures, human peripheral blood	(Wu et al., 2010)
Oxaliplatin	DNA platinum-adduct formation	Activates immunity by inducing immunogenic cell death via the presentation and secretion of DAMPs (calreticulin, HMGB1, ATP and HSP70) Induces dendritic cell antigen presentation and T cell activation resulting in the marked increase in the production and secretion of IL-2 and IFN-γ	CT26 colon cancer cell line Peripheral blood, A549 lung cancer cell line	(Tesniere et al., 2010, Krysko et al., 2012) (Liu et al., 2010b)

Although the gastrointestinal tract comprises the largest portion of immune cells within the body, little is known about the effects of anti-cancer chemotherapeutic drugs on the induction of DAMPs as well as their effects on resident immune cells within the intestines. The majority of studies investigating platinum-based drug-induced immunogenic cell death have used human peripheral blood, and various tumor cell lines exclusively or co-cultured with immune cells including, macrophages, dendritic cells, NK cells, $CD4^+$ and $CD8^+$ T cells. Investigating the effects of anti-cancer chemotherapy directly on the resident gastrointestinal immune cells and populations within the Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) is warranted.

1.7.2.1 Cisplatin immunogenicity

As mentioned above, eliciting immunogenic cell death requires endoplasmic reticulum and/or oxidative stress, as well as the presentation of DAMPs. Cisplatin has the ability to induce tumour cell release of HMGB1, but fails to prompt the translocation of calreticulin to the cell surface, given its inability to cause severe endoplasmic reticulum stress (Tesniere et al., 2010, Martins et al., 2011). The inability to prompt such translocation consequently renders the apoptotic process as non-immunogenic. Analysis of NK cell functions in peripheral blood of patients with gastrointestinal cancer revealed that a low-dose of cisplatin and 5-fluorouracil can prevent the suppression of NK cells, enhancing innate anti-cancer immunity (Ishikawa et al., 1998). Cisplatin and carboplatin can

enhance the phagocytic activity of peritoneal macrophages by increasing the number of lysosomes formed; essential for the lysis of tumor cells (Palma et al., 1992). Furthermore, cisplatin and carboplatin induce the dephosphorylation of STAT6 in dendritic cells derived from melanoma and CRC patients as well as tumour cells (Lesterhuis et al., 2011). STAT6 is important for the regulation of the T cell inhibitory molecules known as programmed cell death ligand 1 (PD-L1) and 2 (PD-L2) expressed on dendritic cells. A decrease in dephosphorylated STAT6 is associated with the downregulation of the inhibitory molecule PD-L1, but more so PD-L2, therefore enhancing the activation of T cells by dendritic cells (Lesterhuis et al., 2011). A recent study investigating the effect of the anti-cancer chemotherapeutic agents cisplatin in combination with a taxane drug paclitaxel (mitotic inhibitor), on ovarian anti-tumour immunity revealed that this combination prompted the recruitment of macrophages and $CD8^+$ T cell responses which were tumour specific in the peritoneal cavity of the abdomen (Chang et al., 2013).

1.7.2.2 Carboplatin immunogenicity

There is little evidence in regards to carboplatin's ability in eliciting DAMPs and inducing immunogenic cell death. However, like its predecessor cisplatin, carboplatin can also exhibit a positive anti-tumour immune response. Exposure to antigens from ovarian cancer cells treated with carboplatin and paclitaxel led to the induction of dendritic cell phagocytic and antigen recognition activity (Wu et al., 2010). Apoptotic bodies from

cancer cells can drive dendritic cell maturation as they are internalized and processed for antigen presentation, increasing CD80, CD83 and CD86 expression essential for T cell priming and activation.

1.7.2.3 Oxaliplatin immunogenicity

Oxaliplatin is regarded as a potent stimulator for inducing the presentation of DAMPs and immunogenic cell death **(Figure 1.6)** (Lesterhuis et al., 2010, Tesniere et al., 2010). Oxaliplatin prompts the translocation of calreticulin and HSP70 to the surface of dying tumour cells, the release of HMGB1 and the secretion of ATP, thereby instigating their recognition by APCs for eventual presentation to effector T cells (Tesniere et al., 2010, Krysko et al., 2012). The above mentioned DAMPs are vital for triggering an immune response, and the failure to elicit one or more of these danger signals can abolish the immunogenic apoptosis pathway. HMGB1 function has been investigated in Balb/c $TLR4^{-/-}$ mice bearing CT26-induced colon cancer (Tesniere et al., 2010). The TLR4 serves an important role as it is a key receptor in which dendritic cells use to recognise HMGB1. This is taken up by dendritic cells and expressed on MHC-I (also known as cross presentation). Dendritic cells will then travel to the lymph nodes to activate $CD8^{+}$ T cells; resulting in cytotoxic T cell induction and ultimate killing of cells bearing HMGB1 and calreticulin peptide; MHC-I complexes. However, the above functions are impeded in the absence of TLR4 on dendritic cells (Tesniere et al., 2010).

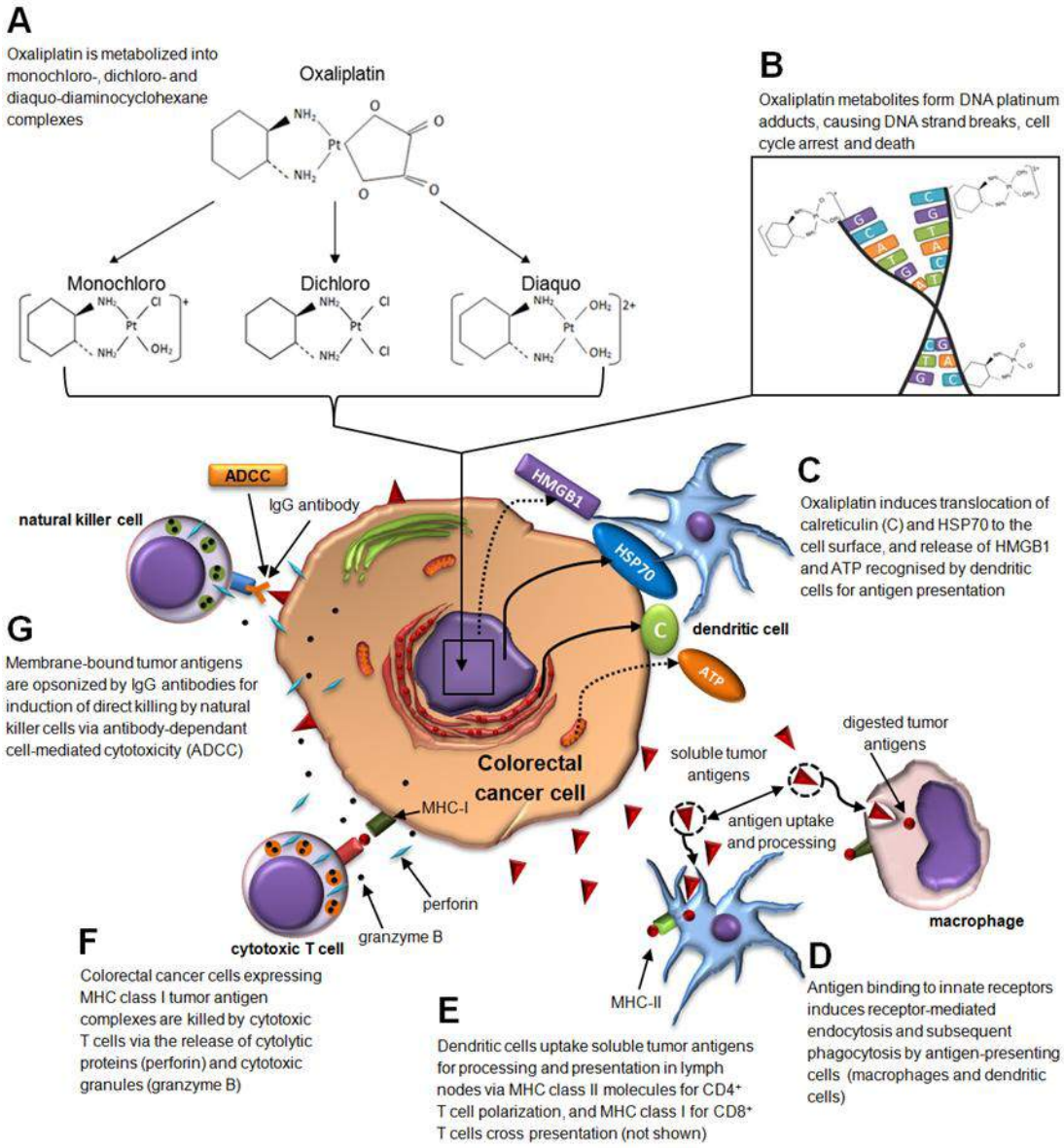


Figure 1.6. Immunogenic apoptosis of the tumour cell induced by oxaliplatin. (A) Oxaliplatin is metabolised into monochloro, dichloro and diaquo complexes, which form DNA platinum-adducts **(B)** causing denaturation of DNA. **(C)** In an oxaliplatin-induced apoptotic event, there is translocation of intracellular proteins such as calreticulin and HSP70 to the cell surface and the release of HMGB1 and ATP. Dendritic cells can recognise these proteins in addition to antigens. **(D)**. The release of soluble tumour antigens into the extracellular environment may initiate innate mechanisms of tumour immunity. Antigen recognition and presentation can induce a form of receptor-mediated endocytosis and subsequent phagocytosis by APCs such as macrophages and dendritic cells. **(E)** Dendritic cells may also uptake tumour antigens which are then processed and presented on MHC class II molecules. They can also capture antigens from the surface of tumour cells (not shown in figure) and process them on MHC class I molecules (this is known as cross presentation). **(F)** Cytotoxic T cells then have the ability to kill tumour cells expressing MHC class I tumour antigen complexes. This interaction leads to the release of cytolytic proteins (perforin) and cytotoxic granules (granzyme B) **(G)**. Opsonisation of membrane-bound tumour antigens (including translocated proteins such as calreticulin) by IgG antibodies is another

mechanism which can be used to induct the direct killing of tumour cells by natural killer cells, in a process called antibody-dependent cell-mediated cytotoxicity (ADCC). Like cytotoxic T cells, natural killer cells can also release perforin and granzyme B, inducing tumour cell death.

Investigation of the effects of oxaliplatin on cultured dendritic cells derived from the blood of healthy donors demonstrated an increase in T cell activation as marked by the heightened production and secretion of cytokines IL-2 and IFN-γ (Liu et al., 2010b). The activation of $CD8^+$ T cells could be hindered if sufficient numbers of anti-inflammatory myeloid-derived suppressor cells, tumour-associated macrophages, and Tregs are found in the microenvironment, thus reducing the effectiveness of oxaliplatin. There is only one study to date which has assessed the impact of oxaliplatin on $CD4^+$ T cell subsets (Maeda et al., 2011). This study had demonstrated that oxaliplatin is effective at reducing Treg numbers when combined with other chemotherapeutic agents in CRC patients. Together with myeloid-derived suppressor cells and tumour-associated macrophages, these cells secrete the antagonistic Th2 cytokine IL-10, ultimately causing suppression of Th1 responses (Gabrilovich and Nagaraj, 2009). Thus chemotherapeutic agents that target these IL-10 producing cells will give the immune system an opportunity to surmount Th2 responses thus clearing the way for Th1 anti-tumour responses. This may justify further studies on immune cell functions in response to oxaliplatin treatment, and in particular, gastrointestinal immune responses which could directly or inadvertently induce enteric neuropathy.

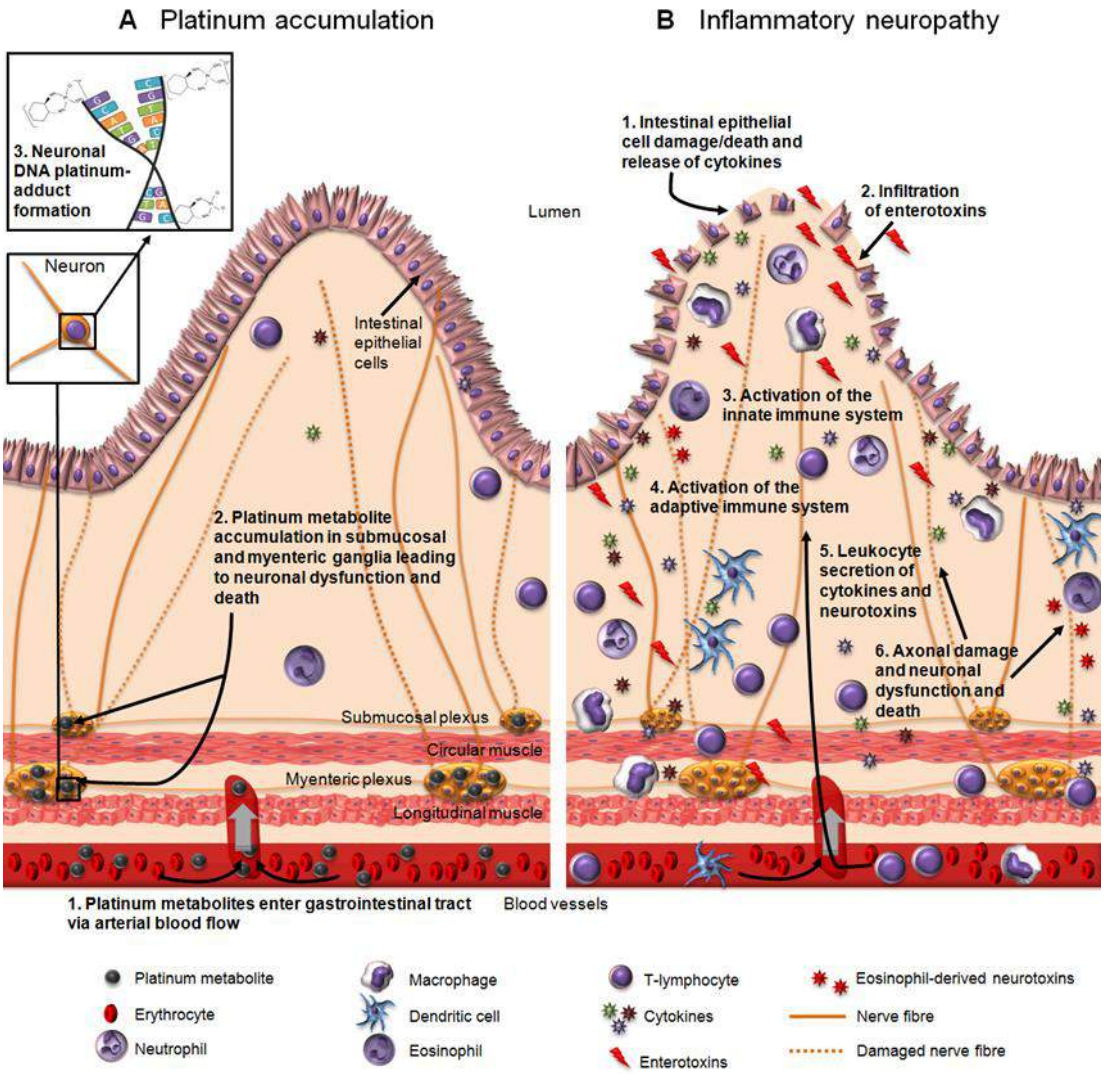


Figure 1.7. Proposed mechanisms underlying oxaliplatin-induced enteric neuropathy. A. Oxaliplatin metabolites, which enter the gastrointestinal tract via arterial circulation, can accumulate within the myenteric and submucosal ganglia. These platinum metabolites form adducts on enteric neuronal DNA, leading to DNA denaturation and eventual cell death. **B**. Oxaliplatin metabolites can form platinum adducts in the DNA of mucosal epithelial cells inducing damage/death. Under these conditions, epithelial cells release various chemotactic cytokines, leading to the infiltration of enterotoxins from the lumen into the lamina propria. This can prompt activation of resident leukocytes which can release a variety of cytokines and neurotoxins, inducing damage to the neuronal processes projecting to the mucosa. Alongside this, leukocytes may also invade the submucosal and myenteric ganglia, where they release cytokines and neurotoxins which can lead to neuronal damage/death. In both instances, damage to the enteric neurons will induce altered gut functions (motility and secretion) and cause severe gastrointestinal symptoms such as diarrhoea and constipation, which can persist long after chemotherapeutic treatment.

1.8 Summary

Colorectal cancer is one of the leading causes of cancer-related death worldwide (Jemal et al., 2011, Siegel et al., 2013, Ferlay et al., 2015). Although effective, this platinum-based agent causes a range of debilitating side-effects (Denlinger and Barsevick, 2009, Di Fiore and Van Cutsem, 2009, Weickhardt et al., 2011). These include gastrointestinal side-effects (nausea, vomiting, diarrhoea and constipation), as well as peripheral neuropathy. Gastrointestinal complications in particular cause dehydration, malnutrition and weight loss, in addition to fluid and electrolyte depletion which can result in life-threatening cardiac and renal complications (Stein et al., 2010). The gastrointestinal side-effects can have a delayed onset, and may last long-term (even for up to 10 years following chemotherapeutic treatment) (Denlinger and Barsevick, 2009). The gastrointestinal side-effects result in chemotherapeutic dose-limitations and total treatment cessation, and thus, compromise optimal anti-cancer treatment (Denlinger and Barsevick, 2009, Di Fiore and Van Cutsem, 2009).

Previous research has shown that oxaliplatin causes neuronal damage leading to gastrointestinal dysfunctions (Wafai et al., 2013, McQuade et al., 2016b). However, the mechanisms of toxicity to the nerves regulating gastrointestinal functions remain unknown. Platinum accumulation has been attributed as the underlying mechanism of long-term peripheral neuropathy (Ta et al., 2006, Ta et al., 2009, Park et al., 2011, Weickhardt et al., 2011, Argyriou et al., 2014). Although platinum-

based drugs function quite similarly, oxaliplatin has the capacity to induce immunogenic cell death (unlike cisplatin and carboplatin), and therefore, this may also contribute to neuronal damage/death leading to gastrointestinal dysfunctions. There are currently no studies published on platinum accumulation and immunogenic cell death in parts of the nervous system controlling gastrointestinal functions, the ENS and brainstem. Research within this thesis aims to address this gap in knowledge.

1.9 General hypotheses and aims

We hypothesise that platinum accumulation and induction of immunogenic cell death in parts of the nervous system regulating gastrointestinal functions, the ENS and brainstem, are potential mechanisms of oxaliplatn-induced neuropathy.

The aims of this research are to determine whether chronic *in vivo* oxaliplatin treatment:

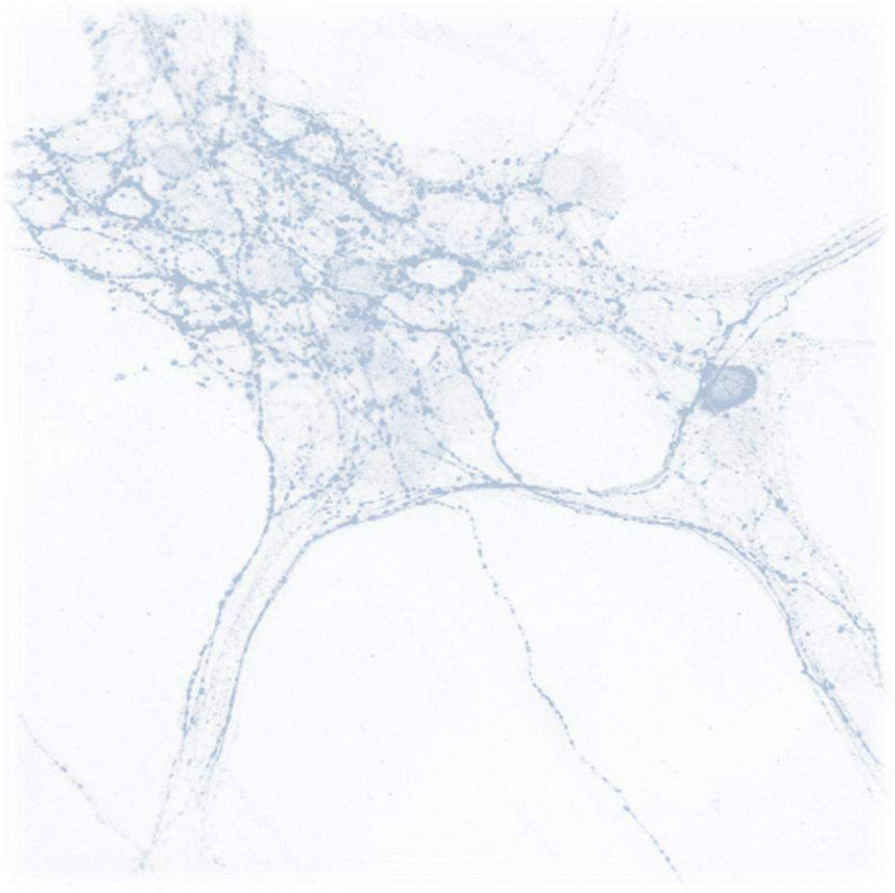
1) causes damage to the extrinsic and intrinsic innervations of the myenteric plexus of the colon;
2) leads to platinum accumulation within the myenteric plexus of the colon and induces the presentation of DAMPs;
3) leads to platinum accumulation within the brainstem which contains neural circuitry involved in modulating gastrointestinal functions;
4) induces immunogenic cell death of neurons and/or causes changes in gastrointestinal and/or systemic immune responses.

1.10 Significance

The long-term side-effects following oxaliplatin treatment suggests that this drug damages cells of an amitotic nature (such as the nerves innervating the gastrointestinal tract). Therefore, determining the mechanisms underlying the gastrointestinal neurotoxicity may lead to novel therapeutic strategies to treat/minimise such side-effects, and improve anti-cancer treatment and quality of life.

2

EFFECTS OF OXALIPLATIN TREATMENT ON THE EXTRINSIC AND INTRINSIC NERVES AND GLIA OF THE COLON MYENTERIC PLEXUS



2.1 SUMMARY

Gastrointestinal side-effects of chemotherapy are serious challenges for effective treatment of colorectal cancer as they can last many years post-treatment. It is well established that chemotherapy causes damage to the gastrointestinal mucosa; however, this tissue has the capacity to regenerate quite rapidly and thus, does not adequately explain the long-term side-effects. Oxaliplatin is a platinum-based chemotherapeutic agent used in the first-line treatment of colorectal malignancies, and its use has been associated with peripheral neuropathies and gastrointestinal side-effects. Therefore, we hypothesised that long-term changes of gastrointestinal functions might be due to toxic effects of oxaliplatin on intestinal innervation. The aims of this study were to investigate the effects of oxaliplatin treatment on the extrinsic and intrinsic innervation of the colon myenteric plexus.

Male Balb/c mice (5-8 weeks of age, weighing 18-25g) received intraperitoneal injections of either vehicle (sterile water) or oxaliplatin (3mg/kg/d) tri-weekly for 2 weeks. Colon tissues were collected for morphological and immunohistochemical assessment at day 14 following the start of treatment. Extrinsic innervation was assessed via density measurments of sensory, adrenergic and cholinergic nerve fibres within the myenteric plexus of the colon labelled with calcitonin gene-related peptide (CGRP), tyrosine hydroxylase (TH), and vesicular acetylcholine transporter (VAChT) respectively in the myenteric plexus of the colon. Intrinsic innervation of the colon was determined by quantifying the total

number of β-Tubulin III-IR neurons, as well as inhibitory neurons immunoreactive (IR) against neuronal nitric oxide synthase (nNOS), and excitatory neurons IR against choline acetyltransferase (ChAT) within the myenteric plexus of the colon. The density of glial subpopulations was measured by immunoreactivity for glial fibrillary acidic protein (GFAP) and s100β proteins.

Oxaliplatin treatment induced significant reduction in both sensory and adrenergic innervations of the colon myenteric plexus, but not VAChT-IR fibres. Furthermore, oxaliplatin treatment caused a reduction in the total number of neurons/area, as well as a decrease in nNOS and ChAT-IR neurons. Additionally, oxaliplatin treatment caused a decrease in GFAP-IR, but an increase in s100β expression within the myenteric plexus.

Treatment with oxaliplatin significantly alters the extrinsic and intrinsic innervation to the colonic myenteric plexus, which could contribute to long-term gastrointestinal side-effects following chemotherapeutic treatment. Further work is required to elucidate the mechanisms of myenteric neurotoxicity caused by oxaliplatin treatment.

2.2 INTRODUCTION

As previously discussed in Chapter 1, colorectal cancer is one of the leading causes of cancer-related death globally (Jemal et al., 2011, Siegel et al., 2013). Treatment strategies for colorectal cancer include surgical resection for patients diagnosed at stages I-II and adjuvant chemotherapy for patients diagnosed at stages III-IV when metastasis to secondary

locations has occurred (Chibaudel et al., 2012, Johnston et al., 2012). Colorectal cancer is typically asymptomatic at the early stages, whereas weight loss, rectal bleeding, altered bowel habits and abdominal pain can present at the later stages of disease progression (Cappell, 2005, Adelstein et al., 2011).

Oxaliplatin is an effective chemotherapeutic agent used in the first-line treatment for colorectal cancer (Wang and Li, 2012). Common side-effects of oxaliplatin include peripheral sensory neuropathy of the extremities as well as gastrointestinal complications (Di Fiore and Van Cutsem, 2009). Nausea, vomiting, constipation and diarrhoea are prominent symptoms experienced by patients undergoing anti-cancer chemotherapy (Di Fiore and Van Cutsem, 2009, Boussios et al., 2012). These gastrointestinal side-effects are the major causes for dose limitations and/or total cessation of anti-cancer treatment (Di Fiore and Van Cutsem, 2009, Stein et al., 2010). In severe cases, these gastrointestinal side-effects can be life-threatening and can result in the death of patients (Sharma et al., 2005, Di Fiore and Van Cutsem, 2009, Stein et al., 2010). Current treatment options to alleviate these gastrointestinal symptoms also come with a plethora of adverse reactions. Anti-emetic agents induce central nervous system effects (insomnia, twitching, tremor), cardiovascular (arrhythmia, heart failure), hepatic and renal complications; anti-diarrhoeal agents induce abdominal pain, bloating, paralytic ileus and anaphylaxis (Perez-Calderon and Gonzalo-Garijo, 2004, Sharma et al., 2005, Feyer and Jordan, 2011).

The conventional thought is that gastrointestinal symptoms are a result of damage to the intestinal mucosa (Andreyev et al., 2012). The high turnover rate of the intestinal epithelial cells indeed makes them attractive targets for cytotoxic drugs, and mucosal damage certainly plays a role in the acute stages of these symptoms (Keefe, 2006). However, despite the rapid regeneration of the intestinal epithelial cells, the gastrointestinal complications can persist for months, and even up to 10 years following anti-cancer chemotherapy (Denlinger and Barsevick, 2009). The persistence of gastrointestinal dysfunction is suggestive that chemotherapeutic agents may also induce damage to other systems regulating intestinal functions, including the peripheral nervous system innervating the gastrointestinal tract (Stojanovska et al., 2015).

The gastrointestinal tract is innervated by extrinsic parasympathetic motor neurons, postganglionic sympathetic neurons, vagal and spinal sensory afferents, as well as the intrinsic ENS. Extrinsic and intrinsic innervation provide control of the gastrointestinal functions such as motility, secretion, absorption and vascular tone (Furness, 2012). The ENS is an intrinsic and complex orchestration of neurons and glia located within the intestinal wall which form ganglia and give rise to two major plexi: myenteric and submucosal (Furness, 2012). CGRP-IR neurons facilitate mucus production and vasomotor tone in the gastrointestinal mucosa, and also play a role in motility reflexes. Adrenergic fibres identified by their immunoreactivity against TH, innervate the enteric ganglia and gastrointestinal smooth muscles which can influence motility,

secretion, and blood flow (Cervi et al., 2014). VAChT-IR fibres label cholinergic axons which contain acetylcholine in synaptic vesicles that is important for excitatory neurotransmission within the gastrointestinal tract (Weihe et al., 1996, Qu et al., 2008).

Furthermore, the ENS contains inhibitory and excitatory neurons and are identified through their expression of nNOS or ChAT, respectively (Furness, 2012). These neurons play an important role in the regulation of gastrointestinal motility. Moreover, the ENS is rich in heterogenous enteric glia which can be identified by their expression of GFAP and s100β (Gulbransen and Sharkey, 2012). Glial cells were once considered as supporting cells with respect to neuronal integrity and function. However, research has demonstrated that enteric glia play a role in neurotransmission, gastrointestinal motility, maintaining epithelial and mucosal integrity, and immunomodulatory functions (Gulbransen and Sharkey, 2012). There are subpopulations of myenteric glia which express both GFAP and s100β, and others which are IR for only one (Eng et al., 2000, Robinson et al., 2016). These glial subpopulations are thought to differ in function and in various pathologies. A reduction in GFAP-IR glia is often observed in pathological states of inflammation and injury, whereas an increase in s100β expression is observed in traumatic brain and spinal cord injury (Eng et al., 2000, Cao et al., 2008, Kwon et al., 2010).

Previous studies have shown that the predessor platinum-based agent cisplatin induces a reduction in CRGP neurons within the myenteric plexus, and that oxaliplatin has the capacity to induce myenteric neuronal

loss and an increase in the proportion of inhibitory neurons within the colon (Vera et al., 2011, Wafai et al., 2013, McQuade et al., 2016b). We have previously demonstrated that oxaliplatin treatment induces enteric glial toxicity within the ileum (Robinson et al., 2016). However, no studies were done to determine whether oxaliplatin induces damage to sensory, sympathetic and cholinergic nerve fibres supplying the myenteric plexus of the colon, or the effects on inhibitory and excitatory neuronal populations as well as glial cells.

Damage to these parts of the myenteric plexus of the colon could be implicated in the multifaceted pathophysiology of oxaliplatin-induced dysmotility. Briefly, the aims of this study were to investigate the effects of oxaliplatin treatment on: 1) density of GCRP and TH-IR fibres innervating the myenteric plexus; 2) total number of neurons and inhibitory/excitatory subpopulations; 3) GFAP and s100β expression within the myenteric plexus of the colon.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Male Balb/c mice (5-8 weeks of age, weighing 18-25g) obtained from the Animal Resource Centre (Australia) were used in this study. Mice had free access to food and water and were kept under a 12 hour light/dark cycle in a well-ventilated room at a constant temperature of 22°C. Mice acclimatized for up to 1 week prior to the commencement of *in vivo* intraperitoneal injections. All procedures were approved by the Victoria

University Animal Experimentation Ethics Committee and performed in accordance to the National Health and Medical Research Council *Code of Practice for the Care and Use of Animals for Scientific Purposes*.

2.3.2 *In vivo* intraperitoneal injections

Mice were separated into 2 cohorts (n=26 in total): (1) vehicle-treated (sterile water) and (2) oxaliplatin-treated (3mg/kg/d; Sigma-Aldrich, Australia). All dosages were calculated per body mass, and mice received a maximum volume of 200µL/injection to be equivalent to human dosage (Elias et al., 2004, Renn et al., 2011). All mice received intraperitoneal injections with 26 gauge needles, 3 times a week for up to 14 days. Mice were culled via cervical dislocation and the colons were harvested for *ex vivo* experiments.

2.3.3 Immunohistochemistry

Colon segments were cut along the mesenteric border, stretched maximally and pinned to silicone-based petri dishes containing 1x phosphate buffered saline (PBS) and an L-type calcium channel blocker, nicardipine (3 µM), to relax the smooth muscle. Tissues were incubated in Zamboni's fixative (2% formaldehyde, 0.2% picric acid and 0.1M sodium phosphate buffer (pH 7.0)) overnight at 4°C. The following day, tissues were washed using 100% dimethyl sulfoxide (DMSO) 3x for 10 minutes, followed by washing with 1x PBS, 3x for 10 minutes. The mucosa, submucosa and the circular muscle layers were dissected out. The

remaining longitudinal muscle-myenteric plexus (LMMP) wholemount preparations were processed for immunohistochemistry.

Wholemount preparations were incubated in a blocking solution comprised of 1x PBS and 0.1% Triton X-100 (PBS-T, Sigma-Aldrich, Australia) and 10% normal donkey serum (Merck Millipore, USA) for 1 hour at room temperature. Preparations were then washed with PBS-T, 3x for 10 minutes. Wholemount preparations were labelled with primary antibodies **(Table 2.1)** overnight at 4˚C, then washed with PBS-T, 3x for 10 minutes. Secondary antibodies **(Table 2.1)** were then incubated for 2 hours at room temperature then washed with PBS-T, 3x for 10 minutes. A cell nuclei marker, 4',6-diamidino-2-phenylindole (DAPI) was added to wholemount preparations for 2 minutes, then washed with PBS-T 2x for 10 minutes. Wholemount preparations were mounted onto glass slides using an anti-fade mounting medium (DAKO, Australia). Negative controls were tested for all antibodies used.

Table 2.1. Details on primary and secondary antibodies used in this study

Antibody	Species	Dilution	Source
Primary antibodies			
CGRP	rabbit	1:2000	Abcam, USA
TH	sheep	1:1000	Abcam, USA
VAChT	rabbit	1:1000	Abcam, USA
β-Tubulin-III	chicken	1:500	Abcam, USA
nNOS	goat	1:500	Abcam, USA
ChAT	goat	1:500	Abcam, USA
GFAP	goat	1:500	Abcam, USA
s100β	Rabbit	1:500	Abcam, USA
Secondary antibodies			
Alexa Fluor 488	rabbit goat sheep	1:200 1:500 1:1000	Jackson Immunoresearch Laboratories, Australia
Alexa Fluor 594	rabbit;	1:200	Jackson

	chicken		Immunoresearch Laboratories, Australia

2.3.4 Imaging and analysis

Three dimensional (z-series) images of the colon wholemount preparations and cross sections were taken using an *Eclipse Ti* confocal microscope (Nikon, Japan). Excitation wavelengths were set to 559nm for Alexa 594, and 473nm for Alexa 488. The immunoreactivity of nerve fibres and glial cells was assessed by analysing the density of fluorescent labelling/area (8 images/preparation taken at 40x magnification with a total area of $2mm^2$). All images were captured at identical conditions, calibrated to standardise minimum baseline fluorescence, and were converted to binary. Differences in fluorescence from baseline were measured using Image J software (National Institute of Health, USA). The number of neurons were counted from 8 random images (20x magnification with a total area of $2mm^2$) from each wholemount preparation. All images were coded and analysed blindly.

2.3.5 Statistical analysis

Statistical analysis of the data included a paired *t*-test using GraphPad Prsim™ v6.0 (GraphPad Software Inc, USA). The data are represented as mean ± standard error of the mean (SEM). Statistical significance was defined where the *P* value was less than 0.05.

2.4 RESULTS

2.4.1 Oxaliplatin treatment causes a reduction in sensory and adrenergic innervation of the myenteric plexus

To determine whether oxaliplatin treatment induced changes in the sensory, adrenergic and cholinergic fibres within the myenteric plexus, processes were labelled with antibodies against CGRP, TH and VAChT. Neuronal fibre densities were quantified within a total of 2mm^2 area in wholemount LMMP preparations of the colon (n=3/group).

Oxaliplatin treatment caused a significant reduction in the density of CGRP-IR fibres in the colon (3.6 ± 0.4, ***P*<0.01) when compared to the vehicle-treated group (6.8 ± 0.2) **(Figure 2.1 A-C)**. Furthermore, a significant reduction in TH-IR fibres was also observed in the colon from oxaliplatin-treated mice (1.9 ± 0.1, ***P*<0.01) when compared to the vehicle-treated group (3.5 ± 0.3) **(Figure 2.2 A-C)**. No significant differences in the density of VAChT-IR fibres were observed following oxaliplatin treatment (4.8 ± 0.02) when compared to the vehicle-treated cohort (5.1 ± 0.4) **(Figure 2.3 A-C)**.

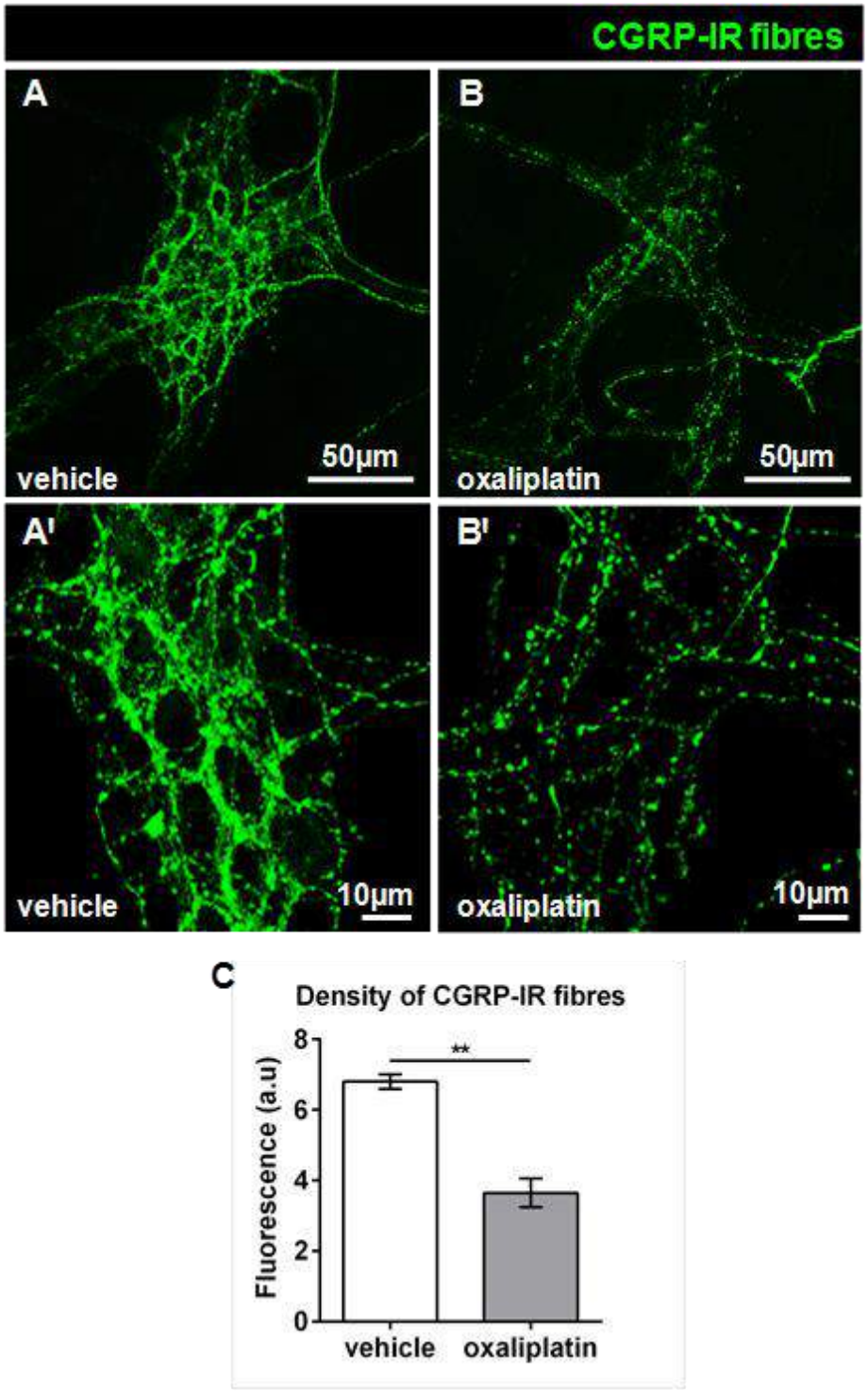


Figure 2.1. Oxaliplatin treatment induces a reduction in CGRP-IR fibre density in the myenteric plexus of the colon. Nerve fibres within the myenteric plexus were labelled with anti-CGRP antibody in wholemount preparations of the colon **(A-B**; 40x magnification; scale bar = 50µm**)**; **(A'-B'**; 100x magnification; scale bar = 10µm**)**. A significant reduction in CGRP-IR nerve fibre density was observed in the myenteric plexus of the colon following oxaliplatin treatment when compared to the vehicle-treated cohort **(C)**.***P*<0.01; n= 3/group.

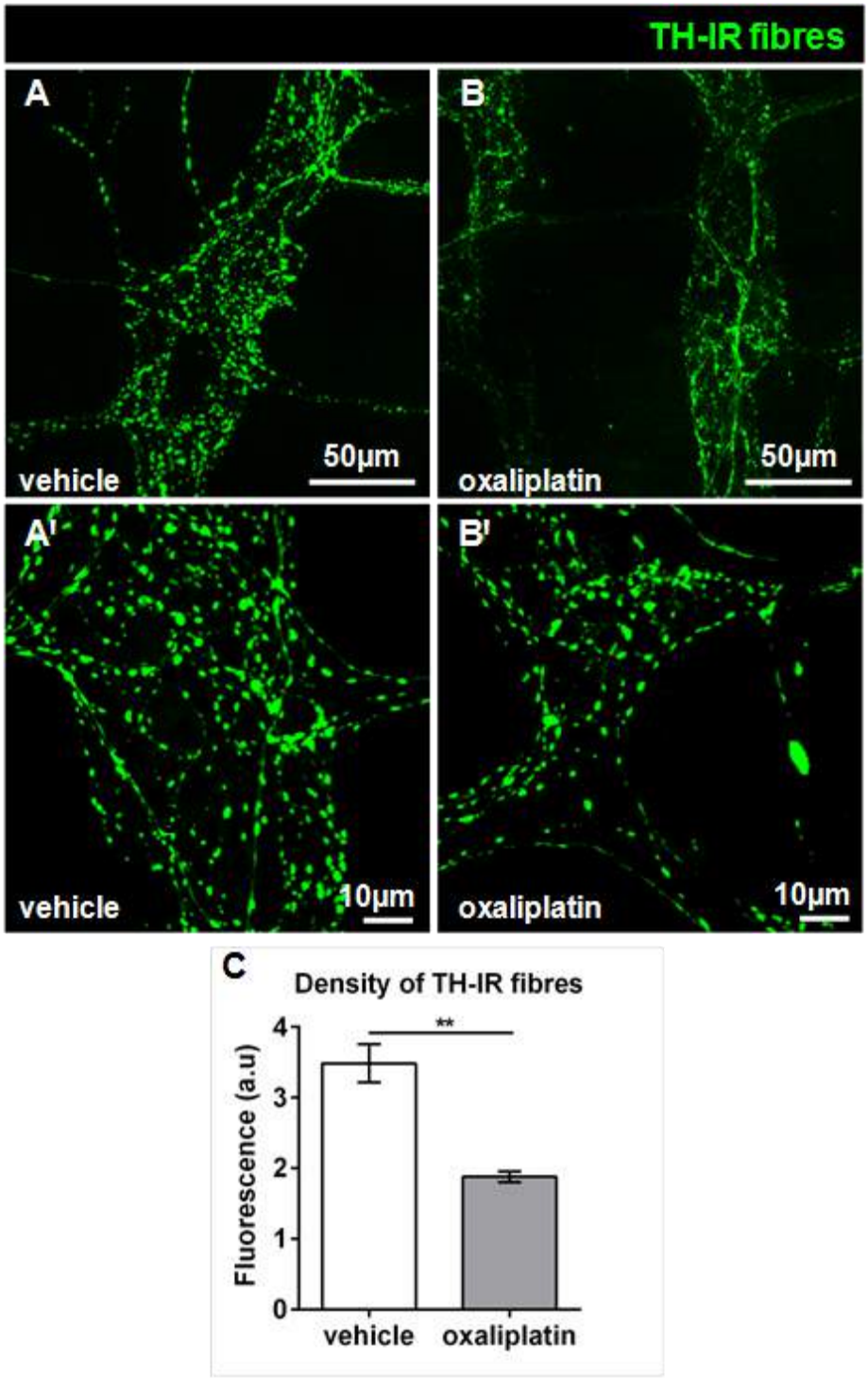


Figure 2.2. Oxaliplatin treatment induces a reduction in TH-IR fibre density in the myenteric plexus of the colon. Nerve fibres within the myenteric plexus were labelled with anti-TH antibody in wholemount preparations of the colon **(A-B**; 40x magnification; scale bar = 50µm**)**; **(A'-B'**; 100x magnification; scale bar = 10µm**)**. A significant reduction in TH-IR nerve fibre density was observed in the myenteric plexus of the colon following oxaliplatin treatment when compared to the vehicle-treated cohort **(C)**. ***P*<0.01; n=3/group.

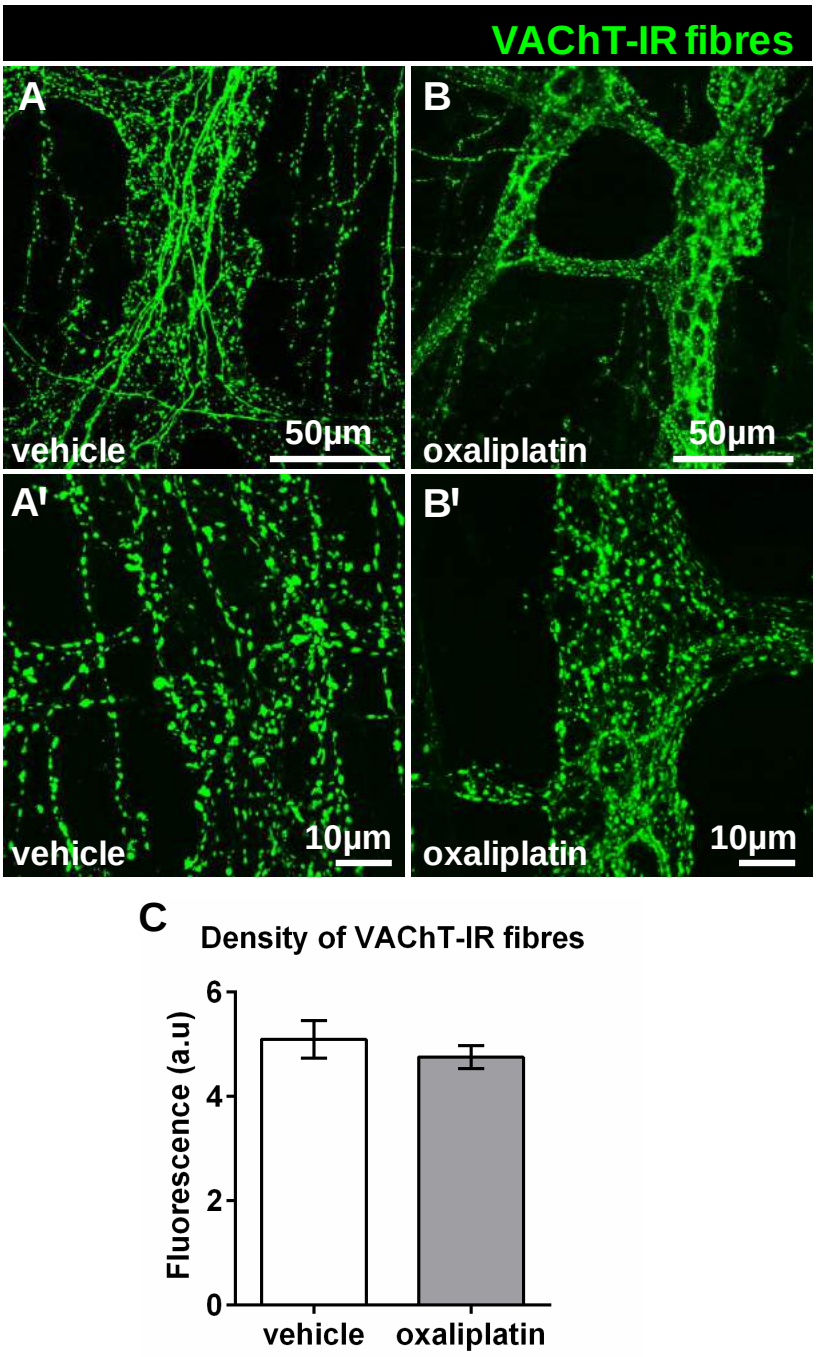


Figure 2.3. Oxaliplatin treatment induces a reduction in VAChT-IR fibre density in the myenteric plexus of the colon. Nerve fibres within the myenteric plexus were labelled with anti-VAChT antibody in wholemount preparations of the colon **(A-B**; 40x magnification; scale bar = 50µm**)**; **(A'-B'**; 100x magnification; scale bar = 10µm**)**. No significant differences in VAChT-IR nerve fibre density was observed in the myenteric plexus of the colon following oxaliplatin treatment when compared to the vehicle-treated cohort **(C)**. N=3/group.

2.4.2 Oxaliplatin treatment induces myenteric neuronal loss in the colon

To determine whether oxaliplatin causes neuronal loss within the colon, wholemount LMMP prepations were labelled with the pan-neuronal marker β-Tubulin III and the cell nuclei marker DAPI. A total of 8 images/animal (total area of 2mm^2) was counted and avaergaed from each wholemount preparation (n=4/group) **(Figure 2.4 A-B)**. Cross sections of the myenteric plexus also demonstrate a reduction in neurons from the oxaliplatin-treated cohort when compared to the vehicle-treated group **(Figure 2.4 A^I-B^I)**. A significant reduction in the total number of neurons within the myenteric plexus of the colon was observed following oxaliplatin-treatment (1685 ± 77; **P*<0.05; n=4) when compared to the vehicle-treated cohort (2040 ± 63; n=4) **(Figure 2.4 C)**.

2.4.3 Oxaliplatin treatment causes a reduction in inhibitory and excitatory motor neurons within the myenteric plexus of the colon

To determine any effects of oxaliplatin on inhibitory or excitatory motor neurons, the myenteric plexus was labelled with nNOS or ChAT. A total of 8 images/animal (total area of 2mm^2) were counted and averaged from each wholemount preparation (n=4/group). Oxaliplatin treatment induced a significant reduction in the total number of nNOS-IR neurons within the myenteric plexus (625 ± 14; ***P*<0.01) when compared to vehicle-treated cohort (817 ± 37) **(Figure 2.5 A-C)**. Furthermore, oxaliplatin treatment

induced a significant reduction in the total number of ChAT-IR neurons within the myenteric plexus (665 ± 31; $^{**}P<0.01$) when compared to vehicle-treated cohort (820 ± 28) **(Figure 2.6 A-C)**.

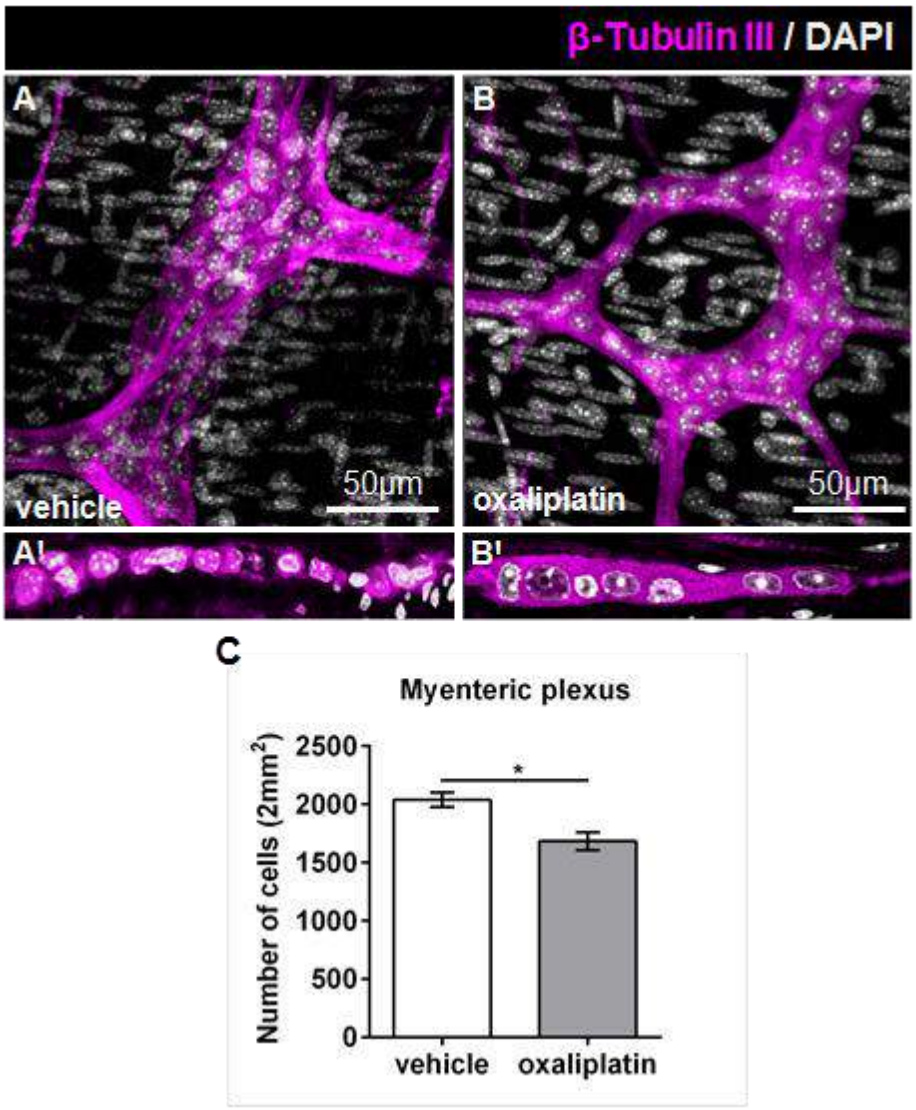


Figure 2.4. Oxaliplatin treatment induces a reduction in the number of myenteric plexus neurons. Wholemount preparation of the myenteric plexus was labelled with the pan-neuronal marker β-Tubulin III (magenta) and the nuclei marker DAPI (white) **(A-B;** scale bar = 50µm**)**. Cross-sectional view of the myenteric plexus also demonstrates a reduction in neurons from the oxaliplatin-treated cohort, when compared to the vehicle-treated group) **(A'-B')**. A significant reduction in the number of cells within the myenteric plexus following oxaliplatin treatment was observed when compared to the vehicle-treated mice **(C)**. ***P*<0.01; n=4/group.

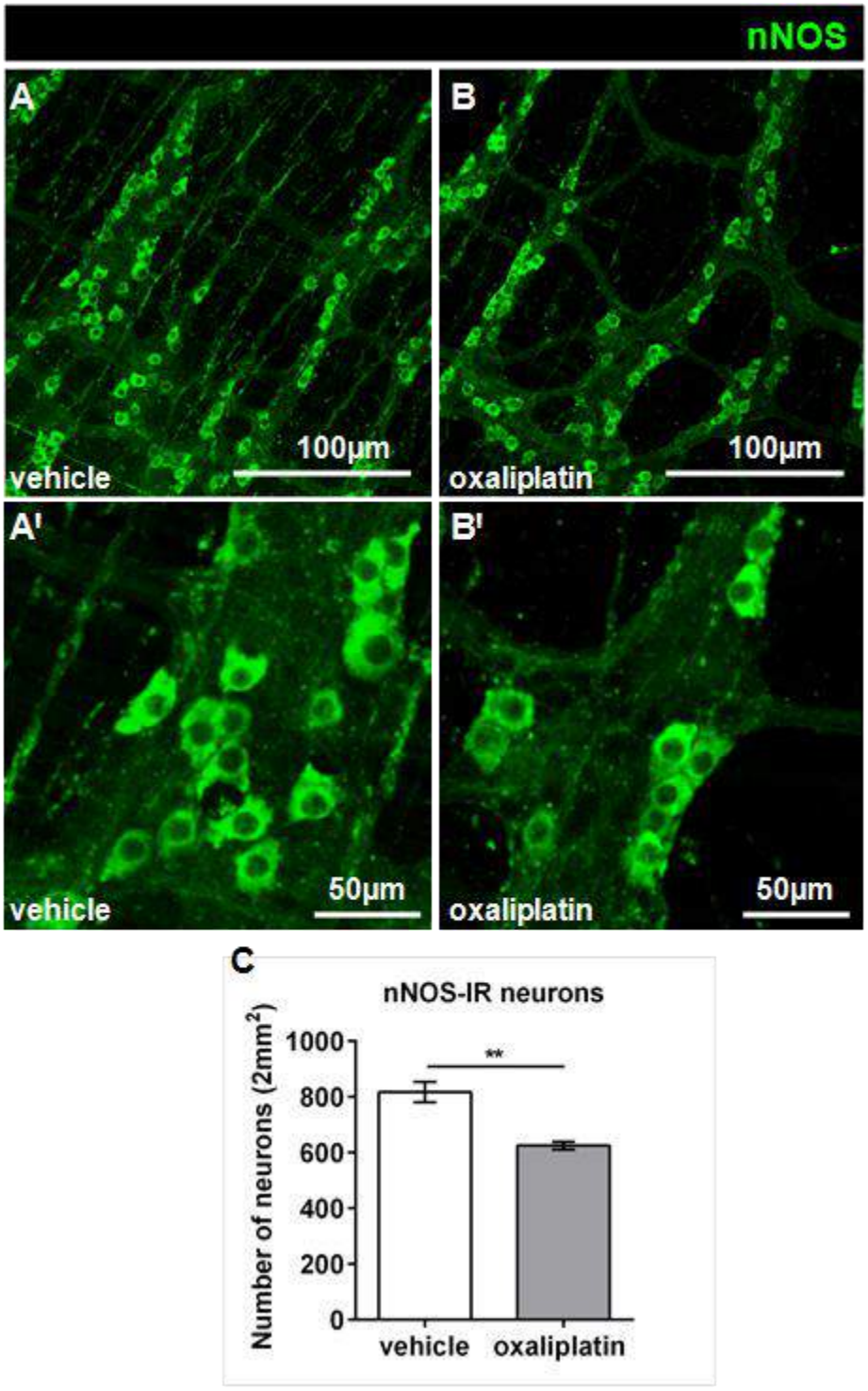


Figure 2.5. Oxaliplatin treatment induces a reduction in the total number of nNOS-IR neurons within the colon myenteric plexus. Wholemount preparation of the myenteric plexus was labelled with nNOS (green) **(A-B**; scale bar = 100µm; **A'-B'**; scale bar = 50µm**)**. Oxaliplatin treatment induced a significant reduction in the total number of nNOS-IR neurons within the myenteric plexus when compared to vehicle-treated cohort **(C).** ***P*<0.01; n=4/group.

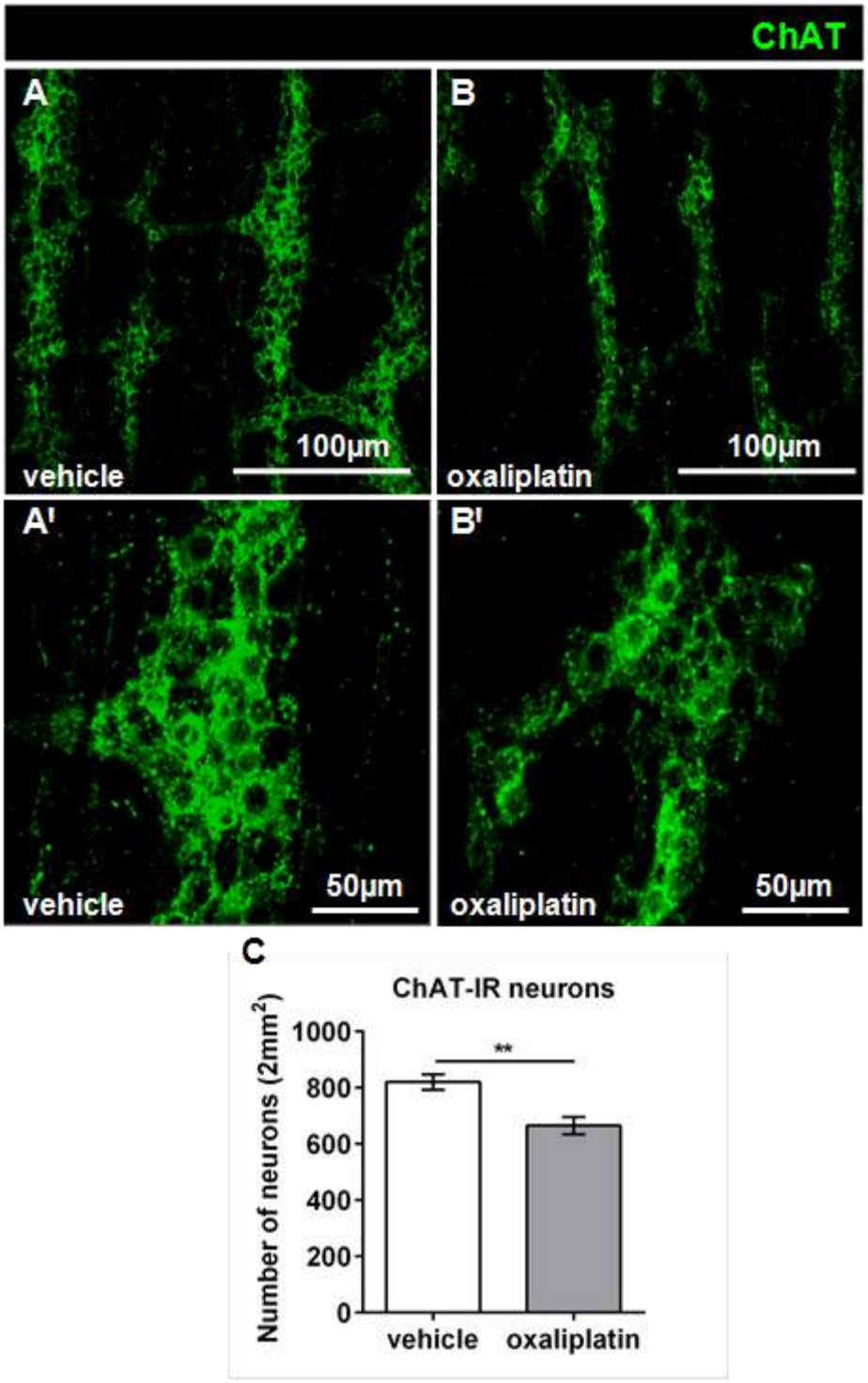


Figure 2.6. Oxaliplatin treatment induces a reduction in the total number of ChAT-IR neurons within the colon myenteric plexus. Wholemount preparation of the myenteric plexus was labelled with ChAT (green) **(A-B**; scale bar = 100µm; **A'-B'**; scale bar = 50µm**)**. Oxaliplatin treatment induced a significant reduction in the total number of ChAT-IR neurons within the myenteric plexus when compared to vehicle-treated cohort **(C).** ***P*<0.01; n=4/group.

2.4.4 Oxaliplatin treatment differentially affects myenteric glial cell populations

To determine any effects of oxaliplatin on glial cells, the myenteric plexus was labelled with GFAP or s100β, respectively. A total of 8 images/animal (total area of 2mm^2) were analysed from each wholemount preparation (n=4/group). Oxaliplatin treatment caused a significant reduction in the density of GFAP-IR glia (6.1 ± 0.5; ***P*<0.01) when compared to the vehicle-treated group (9.8 ± 0.6) **(Figure 2.7 A-C)**. Conversely, oxaliplatin treatment caused a significant increase in the density of s100β-IR glia (8.8 ± 0.1; ***P*<0.01) when compared to the vehicle-treated group (7.7 ± 0.2) **(Figure 2.8 A-C)**.

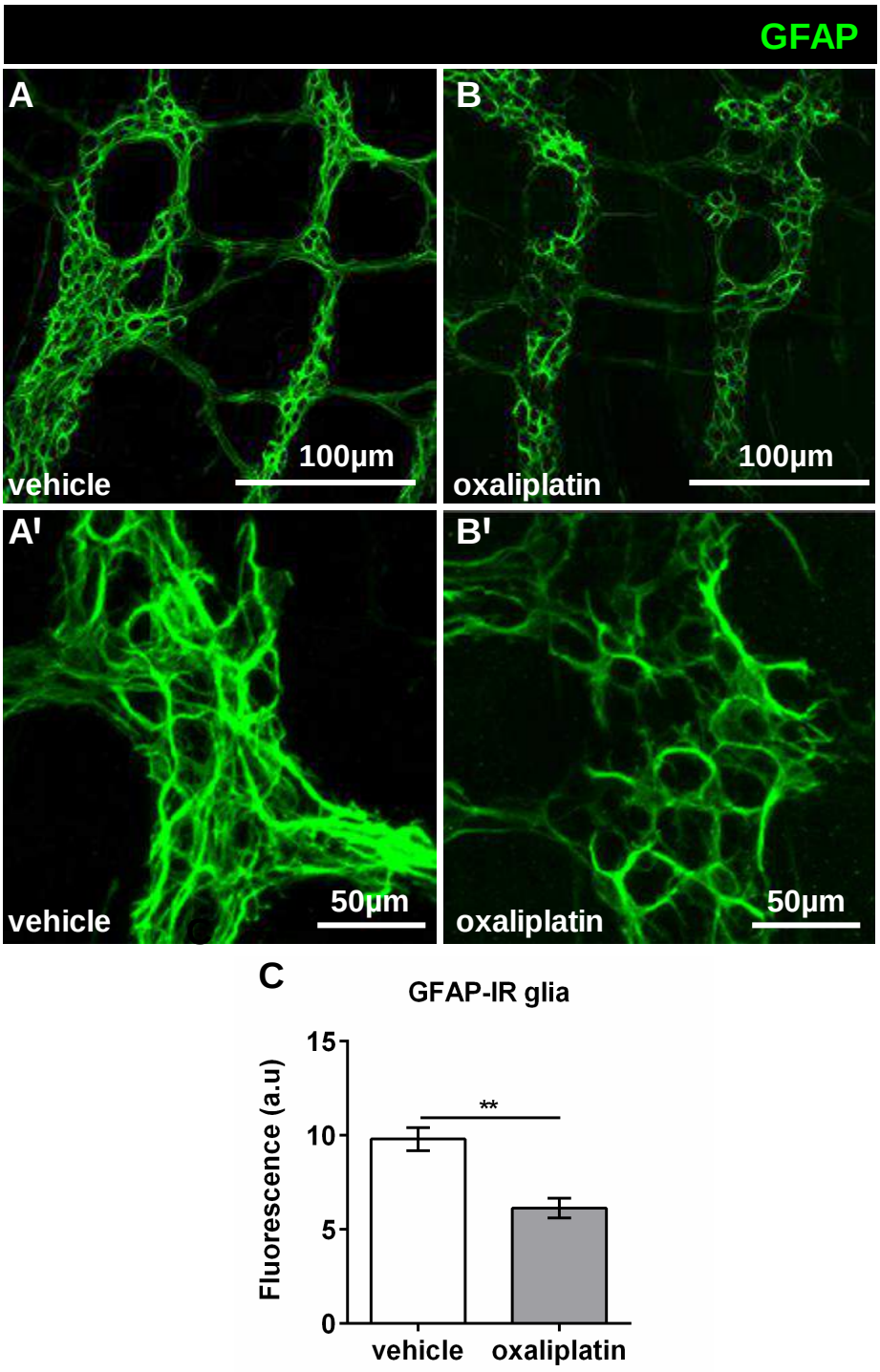


Figure 2.7. Oxaliplatin treatment induces a reduction in GFAP-IR glia within the colon myenteric plexus. Wholemount preparation of the myenteric plexus was labelled with GFAP (green) **(A-B**; scale bar = 100µm; **A'-B'**; scale bar = 50µm**)**. Oxaliplatin treatment induced a significant reduction in the density of GFAP-IR glial cells within the myenteric plexus when compared to vehicle-treated cohort **(C).** ***P*<0.01; n=4/group.

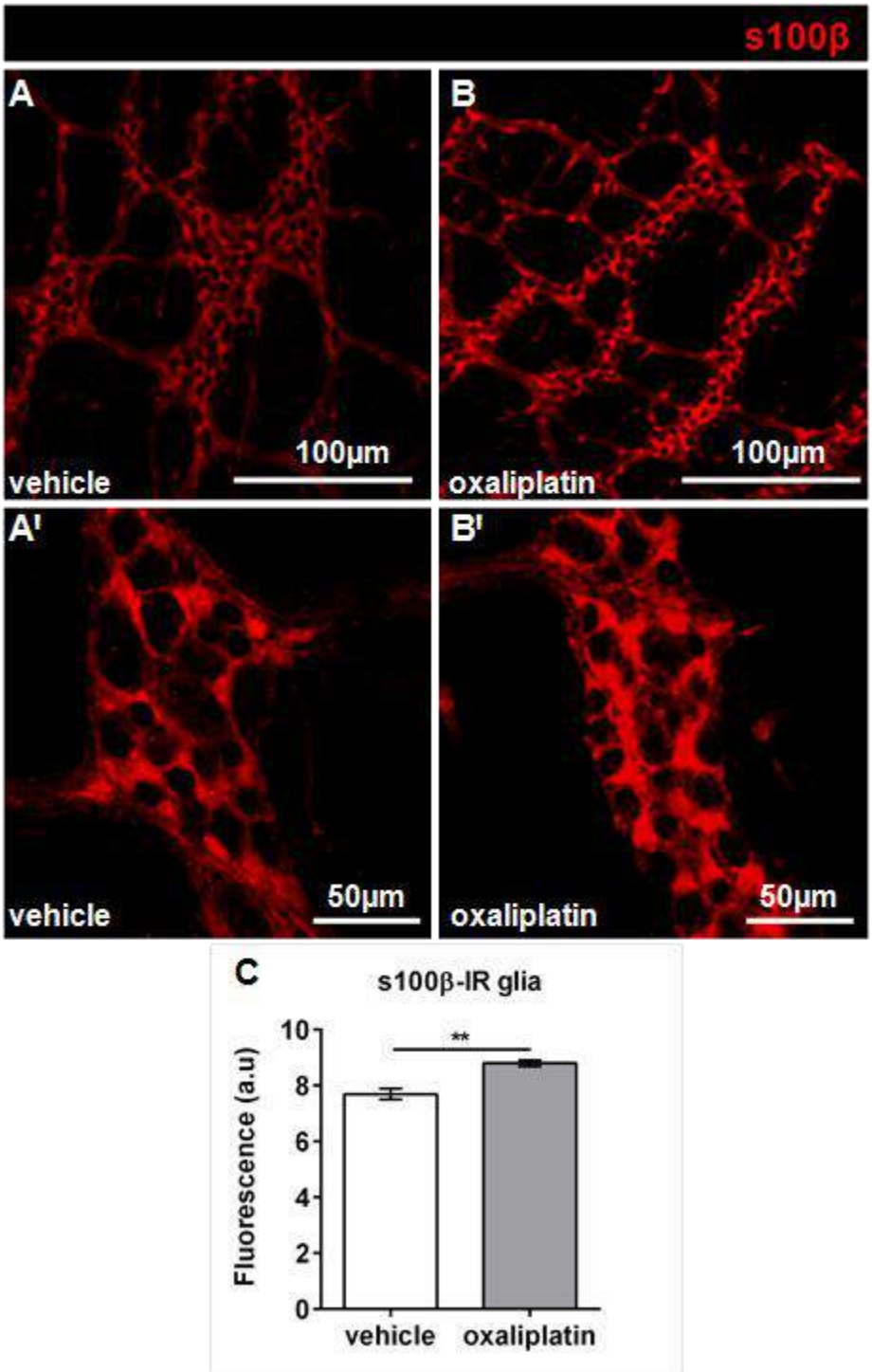


Figure 2.8. Oxaliplatin treatment causes an increase in s100β-IR glia within the colon myenteric plexus. Wholemount preparation of the myenteric plexus was labelled with s100β (red) **(A-B**; scale bar = 100µm; **A'-B'**; scale bar = 50µm**)**. Oxaliplatin treatment induced a significant increase in the density of s100β-IR glial cells within the myenteric plexus when compared to vehicle-treated cohort **(C).** ***P*<0.01; n=4/group.

2.5 DISCUSSION

The results of this study demonstrate that oxaliplatin treatment significantly alters both extrinsic and intrinsic innervation of the colon myenteric plexus which may be implicated in the manifestation and persistence of gastrointestinal dysmotility.

Our present work revealed that oxaliplatin significantly reduces CGRP-IR fibre density within the myenteric plexus. Anti-CGRP antibodies label afferent sensory fibres (Grider, 2003). It should be noted that sensory innervation of the myenteric plexus is not exclusively extrinsic as CGRP-IR sensory neurons can also be found within the ENS. However, they are not easily identifiable given that labelling is weak/punctuate, especially in mouse tissues. Thus, the majority of studies focus on investigating changes to nerve fibre densities (Tan et al., 2010, Eftekhari and Edvinsson, 2011, Sadeghinezhad et al., 2013, Pereira et al., 2016). Platinum-based drug toxicity to CGRP-IR neurons within the DRG and peripheral nerve fibres supplying the extremities (peripheral sensory neuropathy) has been investigated previously (Gregg et al., 1992, Ta et al., 2006, Weickhardt et al., 2011). The chronic form of peripheral sensory neuropathy is thought to result from the accumulation of the platinum-based chemotherapeutic agents, inducing changes in nerve conduction potentials, soma size and nuclear morphology, and ultimately, neuronal death (Gregg et al., 1992, McKeage et al., 2001, Ta et al., 2006, Brouwers et al., 2008, McWhinney et al., 2009). A linear relationship between a cumulative dose of cisplatin and an increase in histopathologic toxicity

within the DRG is reported, suggesting that the platinum is being retained in active and toxic forms (Gregg et al., 1992). Our present findings are in agreement with a study which showed a reduction in CGRP-IR fibres and myenteric neurons in the rat colon following cisplatin administration (Vera et al., 2011). In the gut, CGRP released from sensory nerve fibres innervating the gastrointestinal tract plays an important protective role. CGRP facilitates mucus production and controls blood flow in the gastrointestinal mucosa; and thus, the loss of CGRP-IR sensory fibres and/or sensory neuron dysfunction can impair mucosal protection (Holzer, 2007). CGRP-IR fibres projecting to the mucosa can also mediate local intrinsic reflexes following mucosal stimulation (Grider, 2003). The reduction in these nerve fibres may still impact colonic motility through diminished or abolished reflex activity.

Treatment with oxaliplatin induced a reduction in TH expressing nerve fibres within the myenteric plexus of the colon. The anti-TH antibody was used to identify adrenergic fibres and neurons (Nagatsu, 1989, Qu et al., 2008). The majority of TH-IR fibres are from an extrinsic origin (sympathetic neurons within the paravertebral ganglia and the celiac-mesenteric ganglia). However, a very small proportion of TH-IR neurons within the ENS has been reported in less than 0.5% of myenteric neurons in the ileum of adult Balb/c mice, and are generally observed most frequently in the esophagus (Wakabayashi et al., 1989, Olsson et al., 2006, Qu et al., 2008). Similar to previous studies (Straub et al., 2005, Rahman et al., 2015), we have not found TH-IR cell bodies in the

myenteric plexus of the colon, thus, have analysed the fibres exclusively. Furthermore, a study conducted by Lucas et al. (2013) investigated the effects of the platinum-based drug, cisplatin, on TH-IR fibres in murine bone marrow. In agreement with our study, their research showed a significant reduction in TH-IR following platinum-based chemotherapy (Lucas et al., 2013). Sympathetic adrenergic fibres innervating gastrointestinal smooth muscles and enteric ganglia in the colon can modulate motility, secretion, blood flow, and immune system activation (Cervi et al., 2014). The platinum-based agents cisplatin and oxaliplatin both induce gastrointestinal dysmotility which is characteristic of chronic constipation (Vera et al., 2011, Wafai et al., 2013). The release of norepinephrine by sympathetic TH-IR fibres functions to inhibit gastrointestinal motility. Thus, it does not appear that the reduction in TH innervation has major implications regarding the contractility of the gut, given that motility is still slowed down following oxlaiplatin treatment. However, a reduction in sympathetic noradrenergic innervation of the colon could still impact secretion, blood flow and gastrointestinal immunity which needs to be further investigated. Furthermore, functional changes in the activity of the remaining sympathetic fibres after oxaliplatin treatment should be analysed.

We did not observe any differences in VAChT-IR fibres within the myenteric plexus of the colon following oxaliplatin treatment. The anti-VAChT antibody labels cholinergic axons containing acetylcholine in synaptic vesicles (Weihe et al., 1996, Qu et al., 2008), which does not

discriminate between fibres projecting from extrinsic or intrinsic cholinergic neurons thoughout the myenteric plexus. Our results correlate well with the electrophysiological studies investigating evoked junction potentials in smooth muscle cells of the colon following oxaliplatin treatment which showed demonstratably normal excitatory junction potentials that are mediated by cholinergic neurons utilising acetylcholine (McQuade et al. 2016a).

Our study revealed that oxaliplatin treatment caused a significant reduction in the number of neurons within the myenteric plexus of the colon. There are only few studies which have investigated the effects of platinum-based chemotherapeutic agents (cisplatin and oxaliplatin) on the rat and mouse enteric nervous system (Vera et al., 2011, Wafai et al., 2013, Pini et al., 2016). These studies have demonstrated that platinum-based agents cause a significant reduction (25-30%) in the total number of myenteric neurons. Furthermore, the loss of myenteric neurons has been associated with colonic dysmotility following chemotherapeutic administration of 5-fluorouracil, an anti-metabolite typically used in conjunction with oxaliplatin for the treatment of colorectal malignancies (McQuade et al., 2016a). It is apparent that the ENS, and specifically the myenteric neurons are particularly sensitive to anti-cancer agents, despite their post-mitotic nature. It is possible that oxaliplatin could accumulate within the ENS just as it does within other parts of the nervous system, however, this remains to be investigated.

A significant reduction in the number of nNOS and ChAT neurons within the myenteric plexus is revealed in the colon from oxaliplatin-treated mice. nNOS neurons utilise NO to inhibit motor activity by acting on the pace-maker cells of the gastrointestinal tract, ICCs, leading to muscle relaxation (Bornstein et al., 2004). On the other hand, ChAT-IR neurons utilise acetylcholine as their primary neurotransmitter which excites ICCs, and in turn, stimulates muscle contraction and gastrointestinal motility (Bornstein et al., 2004). Although similar losses in the number of nNOS and ChAT-IR neurons are observed, our previous work has demonstrated that gastrointestinal motility is decreased following oxaliplatin treatment (McQuade et al., 2016b). Previous work has shown an increased proportion of nNOS-IR neurons in the colon myenteric plexus when compared to number of neurons remaining (McQuade et al., 2016b). This suggests that nNOS neurons maintain functional integrity over excitatory ChAT neurons. We have previously shown that oxaliplatin treatment results in mitochondrial superoxide production and protein nitrosylation within the myenteric plexus, as well as increased expression of inducible nitric oxide synthase (iNOS) within the LMMP (McQuade et al., 2016b). nNOS neurons generally show a greater capacity to deal with damaging stimuli, including oxidative stress. NO production by nNOS neurons is presumed to have protective effects against intestinal ischemia/reperfusion injury in mice (Rivera et al., 2012). Additionally, nNOS neurons have also demonstrated the ability to withstand NMDA and NO-mediated neurotoxicity (Gonzalez-Zulueta et al., 1998). ChAT-IR

neurons on the other hand are particularly sensitive to oxidative stress (Cuddy et al., 2012, Nunes-Tavares et al., 2012). The ChAT enzyme contains an unusually high number of reactive cysteine thiol groups which are principally vulnerable to oxidative and/or nitrosative modifications (Klatt and Lamas, 2000, Black and Rylett, 2011). Both oxidative and nitrosative stress can result in altered structure and function of ChAT, which could greatly impact acetylcholine synthesis and inhibit cholinergic transmission at presynaptic terminals (Black and Rylett, 2011). Moreover, despite the reduction in the number of ChAT-IR neurons, the density of VAChT-IR fibres were not changed following oxaliplatin treatment. This may be due to the sprouting of dendritic processes following neuronal injury, which has previously been shown in the ENS following TNBS-induced inflammation (Nurgali et al., 2011).

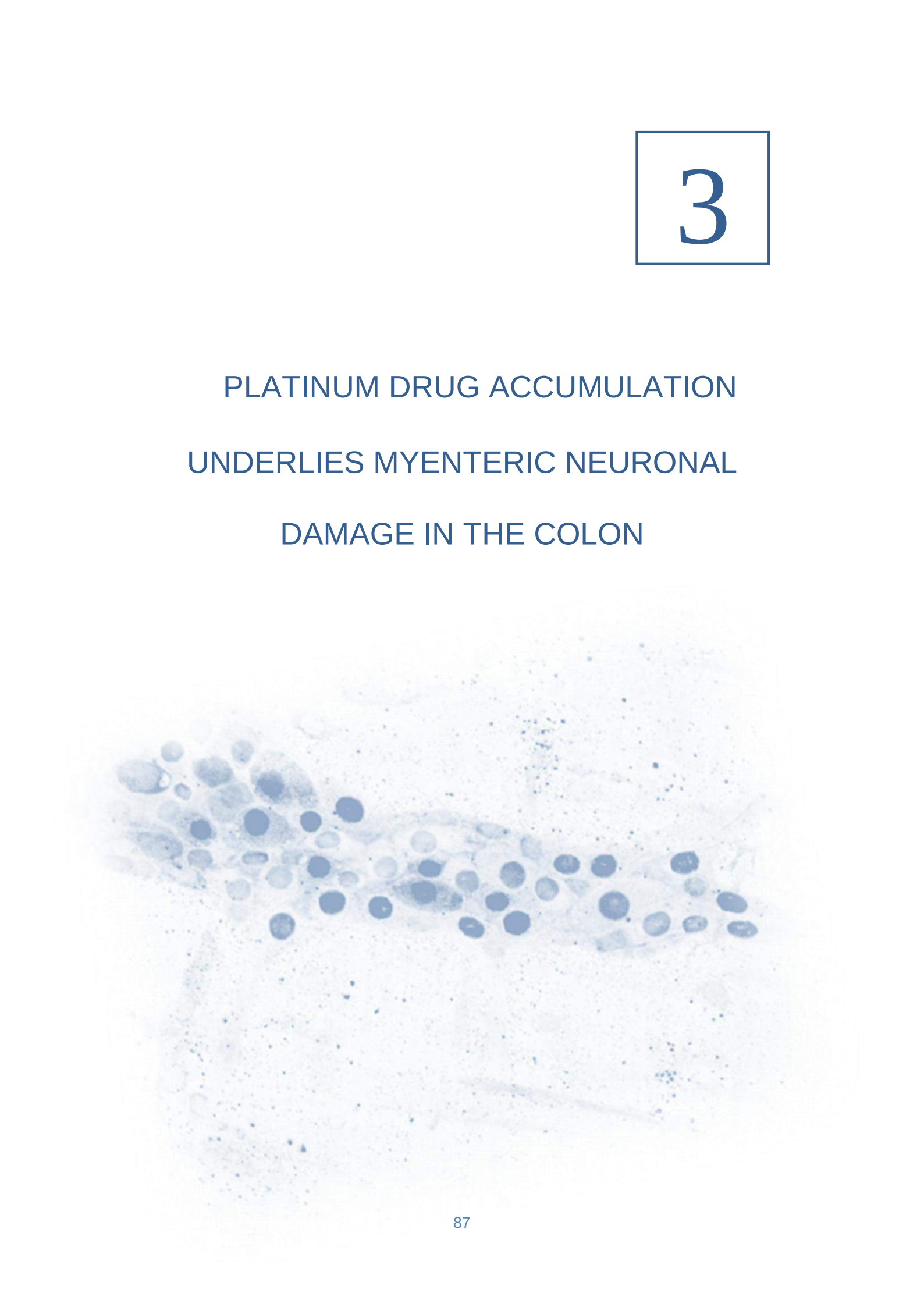
In this study, oxaliplatin treatment caused differential effects on colonic myenteric glial cells. Our results show that oxaliplatin treatment reduced GFAP immunoreactivity in myenteric glia, with a concurrent increase in s100β expression. These findings are in agreement with a previous study demonstrating a reduction in GFAP and an increase in s100β expression, respectively, within the ileal myenteric plexus following oxaliplatin treatment (Robinson et al., 2016). GFAP-IR glial cells function to promote cell survival and neuronal regeneration (Triolo et al., 2006, Toops et al., 2012). A reduction in GFAP expression has also been correlated with glial cell damage in a mouse model of enteric glial disruption, and in various pathological conditions associated with neuronal

damage, inflammation, and type I diabetes (Coleman et al., 2004, Aubé et al., 2006, Liu et al., 2010a). Furthermore, an increase in s100β expression is commonly observed in chronic neuropathological conditions, and elevated levels of this protein in cerebrospinal fluid is used as a biomarker for brain damage (Yardan et al., 2011). s100β overexpression by glial cells has been correlated with increases in iNOS and NO which can potentiate cell damage and death of other glial subpopulations as well as neurons (Hu et al., 1997, Cirillo et al., 2009).

In summary, oxaliplatin treatment significantly alters extrinsic and intrinsic innervations of the colon myenteric plexus. Changes in nerve fibre densities, myenteric plexus cellularity, as well as alterations in inhibitory and excitatory motor neuron populations can impact gastrointestinal motility functions. Furthermore, the differential effects of oxaliplatin treatment on enteric glial populations can exacerbate myenteric neurotoxicity within the murine colon. Further studies concerning the mechanisms underlying myenteric plexus toxicity following oxaliplatin treatment are crucial. Such studies could lead to novel neuroprotective strategies with the aims to advance treatment efficacy and tolerance through minimising gastrointestinal dysfunction.

3

PLATINUM DRUG ACCUMULATION UNDERLIES MYENTERIC NEURONAL DAMAGE IN THE COLON



3.1 SUMMARY

The results of our studies presented in Chapter 2 demonstrated that oxaliplatin induces neuronal loss within the myenteric plexus, damage to the nerve fibres and enteric glial cells. These changes in intestinal innervations appear to play important roles in alterations of gastrointestinal functions after oxaliplatin treatment. However, the mechanisms of oxaliplatin-induced neurotoxicity and gastrointestinal dysfunction remain to be elucidated. It has been established that platinum-based drugs accumulate within the nuclei and mitochondria and form DNA adducts within the dorsal root ganglia (DRG) neurons (Ta et al., 2006, Podratz et al., 2011). In this study we hypothesise that oxaliplatin has the capacity to deposit within the nuclear and mitochondrial fractions of the longitudinal muscle-myenteric plexus (LMMP) and induce damage. The LMMP is responsible for regulation of gastrointestinal motility. The copper transporter receptor 1 (CTR1) has shown to play a role in platinum drug influx in a number of non-neuronal cell lines. The expression of this receptor in myenteric neurons has not been studied, and it is unknown whether CTR1 may be implicated in oxaliplatin influx. Oxaliplatin-induced cell stress and death have been associated with changes in the damage-associated molecular patterns (DAMPs), calreticulin and high mobility group box 1 (HMGB1) protein. The aims of this study were to investigate the effects of oxaliplatin treatment on: 1) platinum and copper concentrations in the nuclear and mitochondrial fractions of the LMMP; 2) immunohistochemical expression of CTR1 in myenteric neurons; 3)

immunohistochemical expression of DAMPs in myenteric neurons; 4) cytochrome *c* and mitochondrial electron transport chain proteins of the LMMP which are involved in apoptotic cascades and organelle functions; 5) cleaved caspase 3 expression in myenteric neurons as a measure of apoptosis.

Balb/c mice received intraperitoneal injections of oxaliplatin (3mg/kg/d) or sterile water tri-weekly for 2 weeks. Mice were culled via cervical dislocation. The colon LMMP was harvested, dissociated, and the nuclear and mitochondrial fractions were isolated to determine platinum and copper concentrations by atomic absorption spectrophotometry (AAS). Platinum deposition within the LMMP was visualised using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). The expression of CTR1, DAMPs and cleaved caspse 3 in LMMP preparations was investigated immunohistochemically. To determine whether oxaliplatin treatment alters cytochrome *c* and mitochondrial electron transport chain protein expression, western blotting was performed.

A significant amount of platinum accumulated within the nuclear and mitochondrial fractions of the LMMP following oxaliplatin-treatment when compared to the vehicle-treated cohort. No changes to copper concentrations in either nuclear or mitochondrial fractions were observed. Oxaliplatin treatment caused a reduction in CTR1 immunoreactivity, and overexpression and cytoplasmic translocation of calreticulin and HMGB1 within the myenteric plexus, but not longitudinal muscle. Furthermore, oxaliplatin treatment caused a significant increase in cytochrome *c*

expression, as well as cleaved caspase 3 immunoreacitivity within myenteric plexus.

This is the first study demonstrating accumulation of platinum within the LMMP following oxaliplatin treatment. Platinum not only penetrates the nuclear compartment, but can also be detected within mitochondria from the LMMP. Oxaliplatin alters CTR1 expression and evokes the presentation of DAMPs within the myenteric plexus. DAMPs are well established markers for oxaliplatin-induced cytotoxicity. We have demonstrated that oxaliplatin treatment increases cytochrome *c* expression in the LMMP, which may be associated with caspase 3 cleavage observed in myenteric neurons, indicative of apoptotic cell death. Our data provide evidence that platinum accumulation within the LMMP is associated with myenteric neuropathy which may underlie the chronic gastrointestinal side-effects associated with oxaliplatin treatment.

3.2 INTRODUCTION

Platinum-based anti-cancer chemotherapeutic agents are an important class of drugs available in the clinical setting which have shown significant anti-tumour efficacy against a variety of cancer types. The third generation platinum-based agent, oxaliplatin, is currently used in first-line treatment of colorectal cancer (Mayer, 2012, Stintzing, 2014). Despite its therapeutic efficacy, oxaliplatin induces dose-limiting neurotoxicity and gastrointestinal dysfunction which can sometimes lead to dose reductions or total treatment cessation. The gastrointestinal complications, in particular,

decrease quality of life, cause malnutrition, dehydration and fluid and electrolyte depletion which can negatively affect cardiac and renal functions, and in severe cases, can impact patient survival (Krishnan et al., 2005, Di Fiore and Van Cutsem, 2009, Stein et al., 2010, Weickhardt et al., 2011, Boussios et al., 2012). It has been demonstrated that treatments with cisplatin and oxaliplatin induce neuronal loss and phenotypic changes to the myenteric neurons of the ENS that are associated with gastrointestinal motility disturbances (Vera et al., 2011, Wafai et al., 2013, McQuade et al., 2016b). In Chapter 2, we revealed that oxaliplatin treatment induces the loss of nNOS and ChAT neurons at varying indices. Furthermore, we have previously shown that oxaliplatin treatment causes an increase in the proportion of nNOS neurons in the colon myenteric plexus which was associated with enhanced inhibitory junction potentials.

Platinum accumulation in the DRG has been attributed as one of the underlying mechanisms for the chronic form of peripheral sensory neuropathy (McDonald et al., 2005, Ta et al., 2006). However, it is unknown whether platinum can accumulate within the myenteric plexus of the ENS and the longitudinal muscle, both of which regulate gastrointestinal motility (Stojanovska et al., 2015). Several studies have highlighted a role for the CTR1 in platinum-based drug influx in various cells (Ishida et al., 2002, Holzer et al., 2006, Larson et al., 2009, Ip et al., 2010). Copper is an essential biometal that is involved in various metabolic pathways such as mitochondrial respiration, the formation of

radical scavenging complexes as well as neurotransmitter synthesis and signalling (Pena et al., 1999, Tapiero et al., 2003, Gupta and Lutsenko, 2009, Dodani et al., 2011, Gaier et al., 2013). Copper homeostasis is a tightly regulated process since both an insufficiency and excess of this ion is detrimental to cell survival (Pena et al., 1999). Studies have shown that the CTR1 is recycled upon ligand binding and thus, the reduced expression in cancer cells following platinum-based agents has been suggestive of drug influx and direct toxicity (Ishida et al., 2002, Holzer and Howell, 2006). Moreover, a hallmark feature of oxaliplatin-induced cytotoxicity is the presentation of DAMPs, calreticulin and HMGB1, in cancer cells (Zitvogel et al., 2008, Garg et al., 2010, Krysko et al., 2012). Calreticulin is an endoplasmic reticulum and nuclear resident protein which functions include, but are not limited to, calcium homeostasis, protein folding and gene transcription (Ramsamooj et al., 1995, Arnaudeau et al., 2002). HMGB1 is a non-histone DNA binding protein which regulates transcription and repair processes (Burns et al., 1994, Lange and Vasquez, 2009, Liu et al., 2010c). Both calreticulin and HMGB1 have been identified as platinum-adduct sensing and binding proteins, and they can cytoplasmically translocate following cell stress and damage, indicative of cell death that has the potential to result in immunogenic apoptosis (Pasheva et al., 2002, Garg et al., 2010, Krysko et al., 2012, Karasawa et al., 2013, He et al., 2015).

The aims of this study were to investigate the effects of oxaliplatin treatment on: 1) platinum and copper concentrations in the nuclear and

mitochondrial fractions of the LMMP; 2) immunohistochemical expression of CTR1 in myenteric neurons; 3) immunohistochemical expression of DAMPs in myenteric neurons; 4) cytochrome *c* and mitochondrial electron transport chain proteins of the LMMP; 5) cleaved caspase 3 expression in myenteric neurons.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Male Balb/c mice (n=39, 5-8 weeks of age, 18-25g) were used in this study. Mice had access to food and water *ad libitim* and were kept under a 12 hour light/dark cycle in a well-ventilated room at a temperature of 22°C. Mice acclimatized for up to 1 week prior to the commencement of *in vivo* intraperitoneal injections. All efforts were made to minimise animal suffering, and to reduce the number of animals used. All procedures in this study were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Australian *Code of Practice for the Care and Use of Animals for Scientific Purposes.*

3.3.2 *In vivo* intraperitoneal injections

Mice were separated into 2 cohorts: 1) vehicle-treated (sterile water), and 2) oxaliplatin-treated (3mg/kg, Sigma-Aldrich, Australia). All mice received intraperitoneal injections (maximum of 200µL/injection) using 26 gauge needles, 3 times a week for 2 weeks. Oxaliplatin dose was calculated per

body mass, to be equivalent to human dosage (Elias et al., 2004, Renn et al., 2011). Mice were culled via cervical dislocation 14 days after their first intraperitoneal injection and the colons were harvested.

3.3.3 Tissue digestion

The colon was harvested and cut along the mesenteric border and pinned mucosa side up on to a silicone-based petri dish containing 0.1M Hank's balanced salt solution (Gibco^{TM}, Australia; pH 7.4), antibiotic-antimycotic 100x (containing 10,000 units/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL Amphotericin B; Gibco^{TM}, Australia) and an L-type voltage-dependant calcium channel blocker nicardipine hydrochloride (3µM; Sigma-Aldrich, Australia). Using fine forceps, the LMMP layer was dissected and gently minced. The LMMP layer was enzymatically digested in 1mg/1mL solution of collagenase type IV (Gibco^{TM}, Australia) in a water bath at 37°C for 1-1.5 hrs. Tissues were centrifuged for 5 minutes at 300G, the supernatant was then discarded and the cell pellet was resuspended in 1% trypsin (Sigma-Aldrich, Australia) solution for 15 minutes. Dulbecco's modified eagle medium (Sigma-Aldrich, Australia) with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, Australia) was used to block trypsin activity. Tissues were centrifuged again for 5 minutes at 300G, the supernatant was discarded and the cell pellet was resuspended in a homogenizing solution for subcellular fractionation.

3.3.4 Subcellular fractionation (nuclear and mitochondrial isolation)

Following tissue digestion the LMMP was homogenized in solution containing: 100mM potassium chloride, 50mM tris(hydroxymethyl)aminomethane, 5mM magnesium chloride hexahydrate, 1.8mM adenosine triphosphate, 0.5mM ethylenediaminetetraacetic acid; all diluted in milliQ water, pH 7.2. Nuclear and mitochondrial fractions were isolated through centrifugation. Tissue homogenates were transferred to eppendorf tubes and centrifuged at 650G for 3 minutes at 4°C. The supernatant which contains the mitochondria was aliquoted to a separate eppendorf tube. The mitochondrial sample was centrifuged at 15,000G for 3 minutes at 4°C. The supernatant was discarded and the mitochondrial pellet was resuspended in 4mL of milliQ water. The pellet which contains the nuclei was resuspended in radioimmunoprecipitation assay buffer lysis (25mM tris(hydroxymethyl)aminomethane hydrochloride, 150mM sodium chloride, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate; all diluted in milliQ water, pH 7.6), spun at 15,000G, and further diluted to 4mL in total with milliQ water.

3.3.5 Atomic absorption spectrophotometry

Once the samples were prepared, as described above, they were diluted to a volume of 4mL, to allow adequate sample volume for analysis of platinum and copper concentrations. Samples were then aspirated into a

Shimadzu AA-6300 Atomic Absorption Spectrophotometer (AAS, Japan). The specific AAS conditions used to carry out these analyses were as follows; both platinum and copper used an air-acetylene flame, with a fuel flow of 1.5L/min and an air flow 15L/min. The burner height was optimised for each element. Due to the analytical wavelengths used (265.9nm for platinum and 324.8nm for copper), background correction was required – this was supplied by a D2 lamp – and a slit width of 0.7nm was used. The lamp currents used were 25mA (platinum) and 6mA (copper). Samples were aspirated, with three repeat measurements recorded following an initial 2 second pre-spray time. Individual measurements were taken by averaging the absorbance readings over 3 seconds, which also allowed the calculation of a relative mean square percentage uncertainty. These three measurements were then averaged to give a final absorbance reading for each sample. Standard calibration curves were also produced on each day the samples were run, with concentration ranges of 10-40ppm (platinum) and 2-8ppm (copper) utilised. Concentration values for the unknown samples were calculated automatically by the Shimadzu AAWizard software.

3.3.6 Laser ablation inductively coupled plasma mass spectrometry

Laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) imaging was performed according to methods previously described (Hare et al., 2009, Hare et al., 2014, Paul et al., 2015). Colon wholemount

preparation was mounted on a standard microscope slide and placed in the ablation chamber of a NWR213 laser ablation system (Kennelec Scientific, Mitcham, Australia) with the gas outlet connected directly to an Agilent Technologies 8800 Series triple quadrupole ICP-MS (Mulgrave, Australia), operating in MS only mode. A 10µm diameter laser spot was rastered across the sample at a scan rate of 40µm s^{-1}. Parallel lines of ablation were spaced exactly 10µm apart to ensure complete ablation of the sample surface. The mass-to-charge ratio (*m/z*) of 195 was monitored. Data were exported as comma separated value (.csv) files, collated into a single image using a Python script and visualised in ENVI (Esri Australia, Brisbane, Australia). Images are presented as 256-bit colour maps depicting low-to-high signal intensities for *m/z* 195.

3.3.7 Immunohistochemistry

The colons were harvested and placed in a solution containing 0.1M phosphate buffered saline (PBS, pH 7.4) and nicardipine hydrochloride (3µM) to relax the smooth muscle. The colons were then cut along the mesenteric border, stretched maximally and pinned to silicone-based petri dishes. Tissues were incubated in Zamboni's fixative (2% formaldehyde and 0.2% picric acid) overnight at 4°C. The following day, tissues were washed using 100% DMSO 3x for 10 minutes, followed by washing with 0.1M PBS, 3x for 10 minutes. The mucosa, submucosa and the circular muscle layer were micro-dissected and discarded. The remaining tissue was the LMMP wholemount preparation. Tissues were incubated for 1

hour at room temperature in a blocking solution (mouse blocking reagent (M.O.M.^{TM} kit, Vector Labs), or 10% normal donkey serum (Millipore, USA) in 0.1M PBS and 0.1% Triton X-100 (PBS-T, Sigma-Aldrich, Australia) for 1 hour at room temperature. Wholemount preparations were then washed 2x for 5 minutes with PBS-T. LMMP preparations were incubated with primary antibodies overnight at 4˚C **(Table 3.1)**, and the following day the LMMP preparations were washed with PBS-T 2x for 5 minutes. LMMP preparations were labelled with secondary antibodies for 2 hours at room temperature, then washed with PBS-T 3x for 10 minutes. Tissues were washed with PBS-T 2x for 10 minutes followed by a 2 minute DAPI incubation, and further washed with PBS-T 1x for 10 minutes. Wholemount preparations were mounted onto glass slides using DAKO anti-fade mounting medium and coverslipped.

Three dimensional (z-series) images of the LMMP preparations were taken using an Eclipse Ti confocal microscope (Nikon, Japan). Excitation wavelengths were set to 488.8nm for Alexa Fluor 488, 561.8nm for Alexa Fluor 594 and 406nm and for Alexa 405 (DAPI). All slides were coded and analysed blindly. Eight randomised images (20x magnification, total 2mm^2 area) from each animal were captured. All CTR1, calreticulin, HMGB1 and cleaved caspase 3 immunoreactivity images were captured under identical conditions, calibrated to standardise minimum baseline fluorescence, and were converted to binary. Differences in fluorescence from baseline were measured using Image J software (National Institute of Health, USA). The percentage of neurons with CTR1 colocalisation with

nNOS or ChAT were calculated. Colocalisation was considered 1) *strong* if the whole neuronal body expressed CTR1 intensely; 2) *moderate* if half the neuronal body contained intense CTR1 labelling; 3) *weak* if CTR1 labelling was punctuate.

The number of cells displaying intranuclear overexpression and/or cytoplasmic translocation of calreticulin and HMGB1 were counted from eight randomised images (20x magnification, total 2mm^2 area) from each animal. The mean number of neurons/ganglia with intranuclear overexpression and/or cytoplasmic translocation were calculated.

Table 3.1. Details of primary and secondary antibodies used in this study

Antibody	Species	Dilution	Source
Primary Antibodies			
CTR1	rabbit	1:200	Abcam, USA
β-Tubulin-III	chicken	1:500	Abcam, USA
nNOS	goat	1:500	Abcam, USA
ChAT	goat	1:200	Abcam, USA
Calreticulin	mouse	1:150	Abcam, USA
HMGB1	rabbit	1:500	Abcam, USA
Cleaved caspase 3	rabbit	1:200	Cell Signalling,Technology,USA
Secondary Antibodies			
Alexa Fluor 488	mouse, rabbit, chicken	1:100; 1:200	Abacus ALS, Australia
Alexa Fluor 594	rabbit chicken	1:200	Abacus ALS, Australia
Alexa Fluor 647	rabbit, goat	1:200	Abacus ALS, Australia

3.3.8 Western blotting

Whole colons were harvested, cut along the mesenteric border and pinned mucosa side up on to a silicone-based petri dish containing physiological saline. The colons were flushed of its contents, and the mucosa, submucosa and the circular muscle layers were dissected and discarded.

The remaining LMMP preparations were then snap frozen using liquid nitrogen. Frozen tissues were homogenized with a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) for 20 seconds in ice-cold Wik Buffer (40mM Tris, pH 7.5; 1mM EDTA; 5 mM EGTA; 0.5% Triton X-100; 25mM β-glycerophosphate; 25mM NaF; 1mM Na3VO4; 10µg/mL leupeptin; and 1mM PMSF), and the whole homogenate was used for Western blot analysis. Sample protein concentrations were determined with a Detergent Compatible protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and equivalent amounts of protein from each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) acrylamide gels. Following electrophoretic separation, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% powdered milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h followed by an overnight incubation at 4°C with primary antibodies diluted in TBST containing 1% bovine serum albumin. Rabbit anti-cytochrome *c* primary antibody was obtained from Cell Signaling Technology (Danvers, MA), and anti-mouse oxidative phosphorylation (OxPhos) antibody was obtained from Abcam Australia to determine the expression of mitochondrial electron transport chain proteins. After the overnight incubation, the membranes were washed for 30 min in TBST and then probed with a peroxidase-conjugated secondary antibody for 1 h at room temperature. Peroxidase-conjugated anti-rabbit/mouse antibodies were purchased from Vector Laboratories

(Burlingame, CA, USA). Following 30 min of washing in TBST, the blots were developed using the Fusion FX imaging system (Vilber Lourmat, Germany) using enhanced chemiluminescence (ECL) Prime reagent (Amersham, Piscataway, NJ, USA). Densitometric measurements of the protein of interest were carried out using Fusion CAPT Advance software (Vilber Lourmat, Germany). Membranes were then stained with Coomassie Blue, scanned and total protein loaded quantified using ImageJ (NIH; http://rsb.info.nih.gov/nih-image/). The signal intensity of the protein of interest was normalized to the signal for total protein loaded.

3.3.9 Statistical analysis

Statistical analysis of the data included a paired *t*-test using GraphPad PrsimTM v6.0 (GraphPad Software Inc, USA). The data are represented as mean ± SEM. Statistical significance was defined where the *P* value was less than 0.05.

3.4 RESULTS

3.4.1 Platinum accumulates within the nuclear and mitochondrial fractions of the LMMP with no effects on copper content

To determine whether platinum accumulates within the LMMP preparations of the colon, the AAS technique was employed. A significant

amount of platinum was detected in the nuclear (4.56 ± 0.0001 ppm, ****P<0.0001; n=3) and mitochondrial (2.95 ± 0.0001 ppm, ****P<0.0001, n=3) fractions of the LMMP preparations following oxaliplatin administration when compared to the vehicle-treated cohort (all negative) **(Figure 3.1 A-B)**. LA-ICP-MS elemental distribution maps revealed platinum deposition within the LMMP in varying indicies (min – max ion intensity signals) **(Figure 3.1 C)**. Furthermore, no significant difference in copper concentration was observed in either the nuclear (vehicle-treated: 0.028 ± 0.001 ppm; n=3; vs oxaliplatin-treated: 0.016 ± 0.001 ppm; n=3; n.s) or mitochondrial (vehicle-treated: 0.0054 ± 0.001 ppm; n=3; vs oxaliplatin-treated: 0.0062 ± 0.001 ppm; n=3; n.s) fractions of the LMMP **(Figure 3.2 A-B)**.

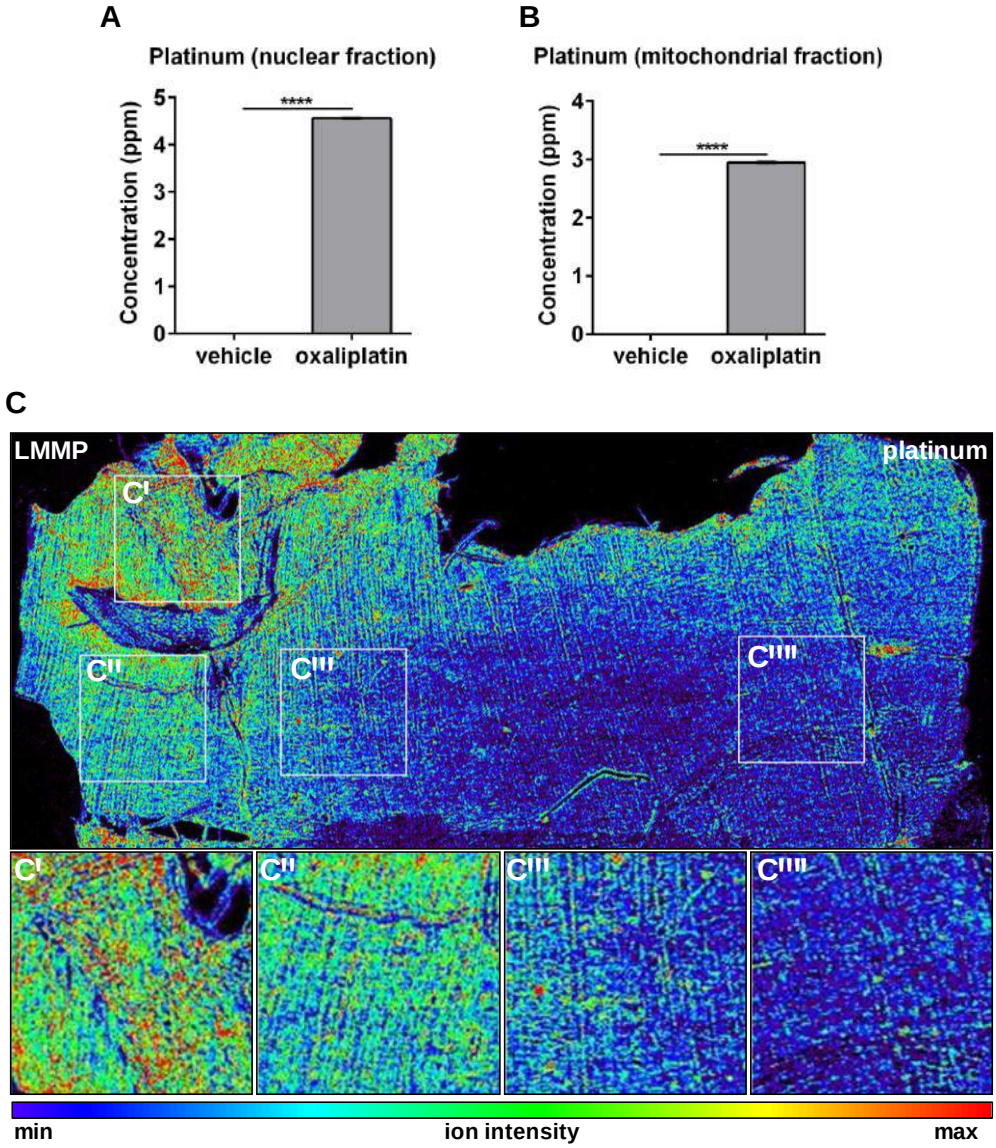


Figure 3.1. Platinum accumulation within the nuclear and mitochondrial fractions of the LMMP of the colon from oxaliplatin-treated mice. A significant amount of platinum was detected within the nuclear **(A)** and mitochondrial **(B)** fractions of the LMMP following oxaliplatin treatment, when compared to the vehicle-treated mice which were negative for platinum. Laser ablation inductively coupled plasma mass spectrometry elemental distribution map of platinum deposition within the LMMP **(C)**. Platinum deposition throughout the LMMP in varying indicies **(insets: C^{I}-C^{IIII})**. ****P<0.0001; n=3/group.

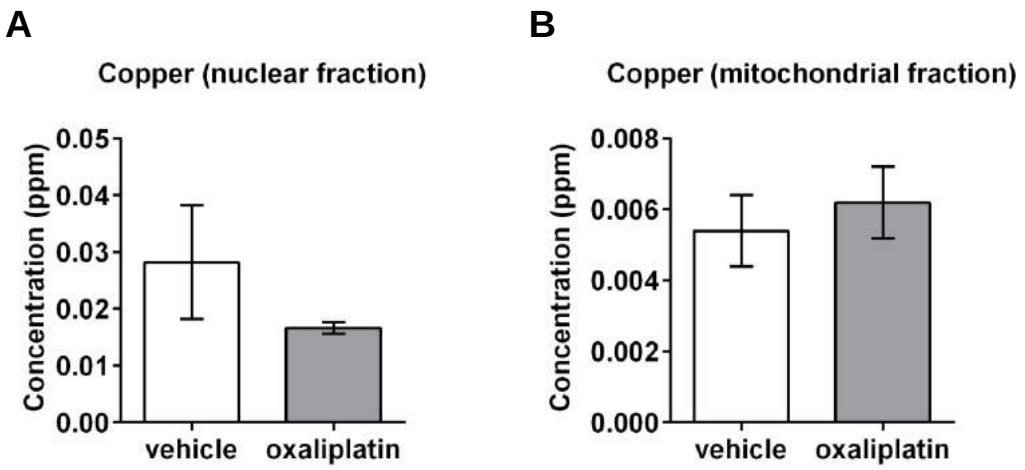


Figure 3.2. No demonstrable differences in nuclear and mitochondrial copper concentration following oxaliplatin treatment. No significant changes in copper content was observed in either the nuclear **(A)** or mitochondrial **(B)** fractions of the LMMP following oxaliplatin administration**.** N=3/group.

3.4.2 Oxaliplatin treatment causes a reduction in CTR1 expression within the myenteric plexus

To investigate whether there are any changes in the CTR1 expression, wholemount LMMP prepations of the colon were labelled with anti-CTR1 antibody **(Figure 3.3 A-B)**. CTR1 labelling was observed only in the myenteric plexus but not muscle cells. CTR1 labelling of myenteric neurons from the vehicle-treated cohort appears well dispersed and intense in the cytoplasm when compared to the faint expression within the myenteric plexus following oxaliplatin treatment **(Figure 3.3 A^{I}-B^{I})**. Minimal labelling of nerve processes was observed in both vehicle-treated and oxaliplatin-treated cohorts. A significant reduction in CTR1 immunoreactivity within the myenteric plexus (1.30 ± 0.34 a.u; ***P*<0.01; n=4) was observed following oxaliplatin treatment when compared to the vehicle-treated group (3.05 ± 0.31 a.u; n=4) **(Figure 3.3 C)**. No significant changes in CTR1 and nNOS colocalisation were observed in the colon following oxaliplatin treatment **(Figure 3.4 A-A^{I}, C; Table 3.2)**. However, oxaliplatin treatment caused a significant change in CTR1 and ChAT colocalisation at varying indices **(Figure 3.4 B-B^{I}, D; Table 3.2)**.

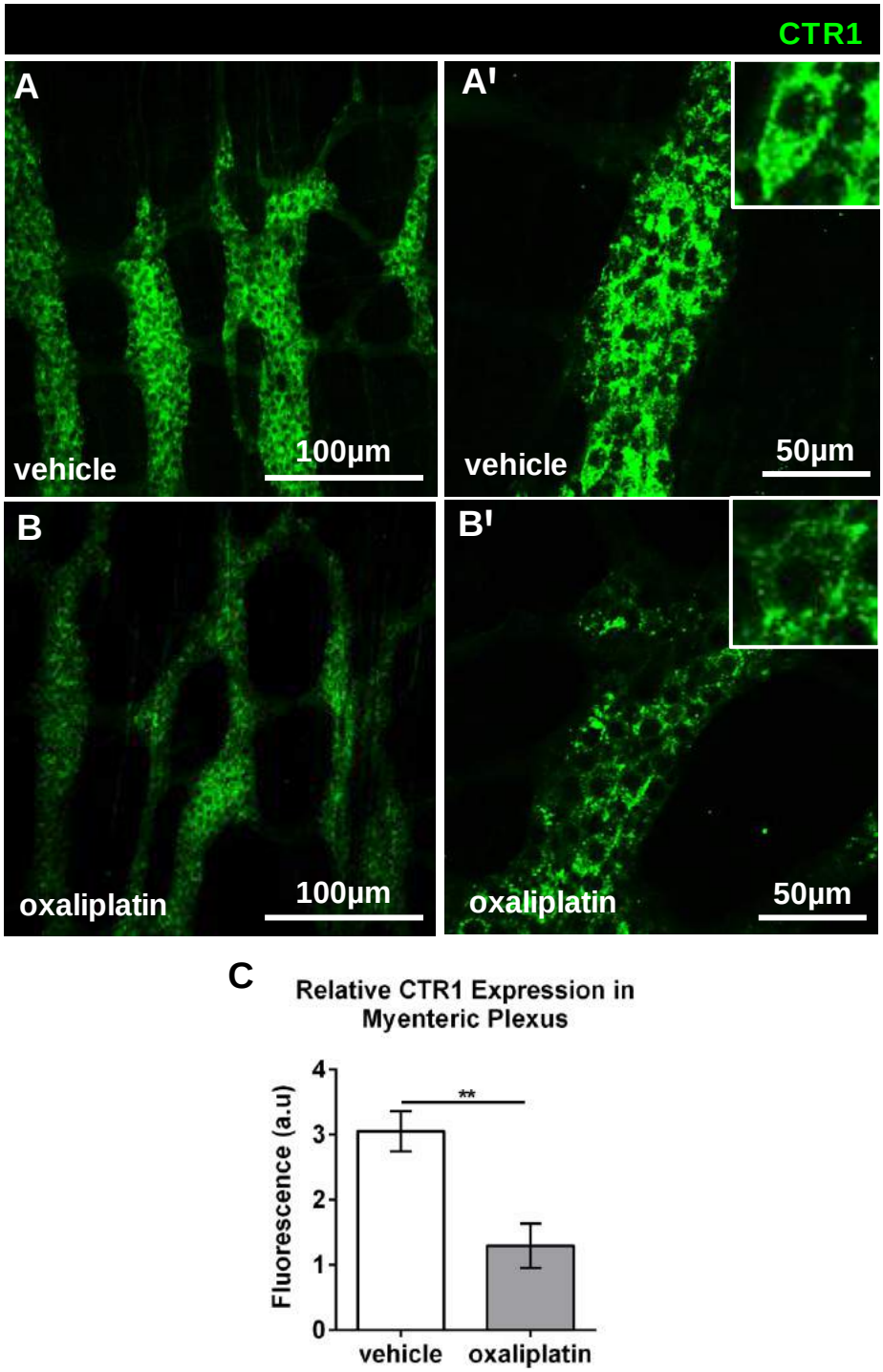


Figure 3.3. Oxaliplatin treatment causes a reduction in CTR1 expression in the myenteric plexus but not the longitudinal muscle of the colon. Wholemount preparations of the LMMP labelled for the CTR1 receptor (green). CTR1 labelling was observed within the myenteric plexus, particularly in the cell bodies, and weak labelling of nerve processes **(A-B)**. CTR1 immunoreactivity was absent within the longitudinal muscle layer in both the vehicle-treated and the oxaliplatin-treated cohorts. Intense CTR1 labelling is observed in the myenteric plexus from the vehicle-treated cohort **(A-A')** when compared to the punctate labelling from the oxaliplatin-treated group **(B-B')**. There was a significant reduction in the relative CTR1 fluorescence intensity in the myenteric plexus following oxaliplatin treatment when compared to the vehicle-treated cohort **(C)**. ***P*<0.01; n=4/group.

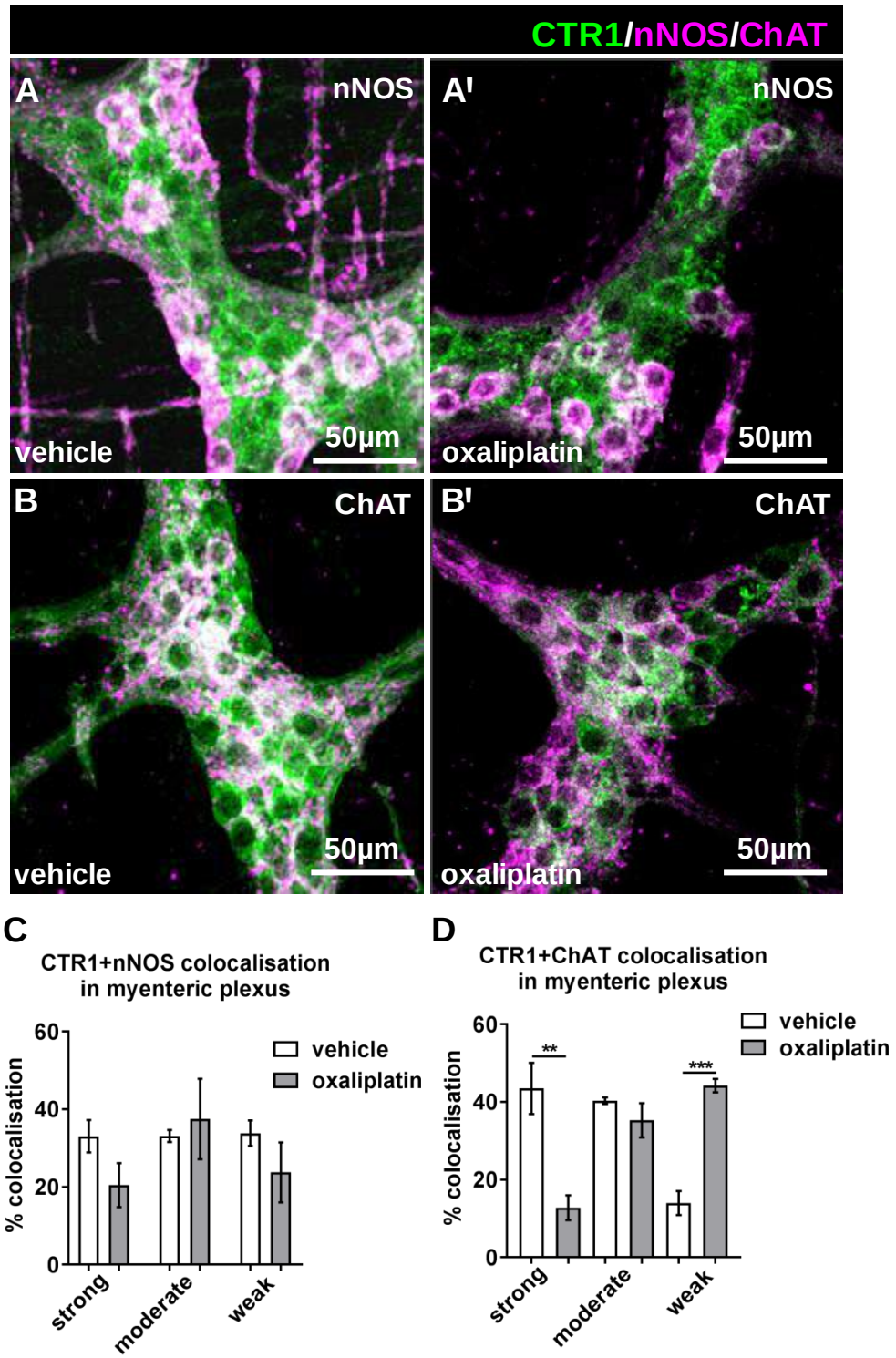


Figure 3.4. Oxaliplatin treatment alters CTR1 and ChAT colocalisation in myenteric neurons of the colon. Wholemount preparations of the LMMP labelled for the CTR1 receptor (green) and nNOS **(A-A')** or ChAT **(B-B')** (magenta). No significant differences in CTR1 immunoreactivity is observed in nNOS neurons **(C)**. However, there is a significant reduction in CTR1 and ChAT-immunoreactivity neurons following oxaliplatin treatment **(D)**. ***P*<0.01; ****P*<0.001; n=4/group.

Table 3.2. Proportions of CTR1 and nNOS/ChAT colocalisation

Groups	nNOS		
	Strong	Moderate	Weak
Vehicle	33% ± 4.1%	33% ± 1.5%	33% ± 3.2%
Oxaliplatin	20% ± 5.6	37% ± 10.3%	23% ± 7.7%
Groups	**ChAT**		
	Strong	**Moderate**	**Weak**
Vehicle	43% ± 6.5%	40% ± 0.8%	13% ± 3%
Oxaliplatin	12.7% ± 3.2% ****	35% ± 4.4%	44% ± 1.7% ****

***P*<0.01; ****P*<0.001; n=4/group

3.4.3 Oxaliplatin treatment induces changes in the expression of DAMPs in the myenteric plexus

To determine cell stress and damage within the LMMP following oxaliplatin treatment, calreticulin and HMGB1 were co-labelled with the pan-neuronal marker β-Tubulin III and the nuclei marker DAPI in wholemount LMMP preparations from the proximal colon **(Figures 3.5 A-B^{II}; 3.6 A-B^{II};)**.

Calreticulin labelling in the colons from the vehicle-treated cohort was observed at low levels within the nuclei of both myenteric neurons (round nuclei) and longitudinal muscle cells (elongated nuclei) **(Figure 3.5 A^{I}-B^{I})**. A significant number (1286 ± 111; ***P*<0.01; n=4) and proportion (76% ± 3%; ****P*<0.001; n=4) of cells within the myenteric plexus of the colon from the oxaliplatin-treated mice displayed strong nuclear

overexpression of calreticulin when compared to the vehicle-treated cohort respectively (429 ± 46; and 25% ± 3%; n=4/group) **(Figure 3.5 C-C^{I})**.

Furthermore, in the vehicle-treated mice calreticulin was concentrated in the nuclei, but weakly expressed in the cytoplasm **(Figure 3.5 A^{I})**. A significant number (159 ± 45; *P<0.05; n=4) and proportion (9.7% ± 3%; *P<0.05; n=4) of myenteric plexus cells from the oxaliplatin-treated mice displayed cytoplasmically translocated calreticulin **(Figure 3.5 B)**, compared to the vehicle-treated group in which no translocation ws observed **(Figure 3.5 D-D^{I})**.

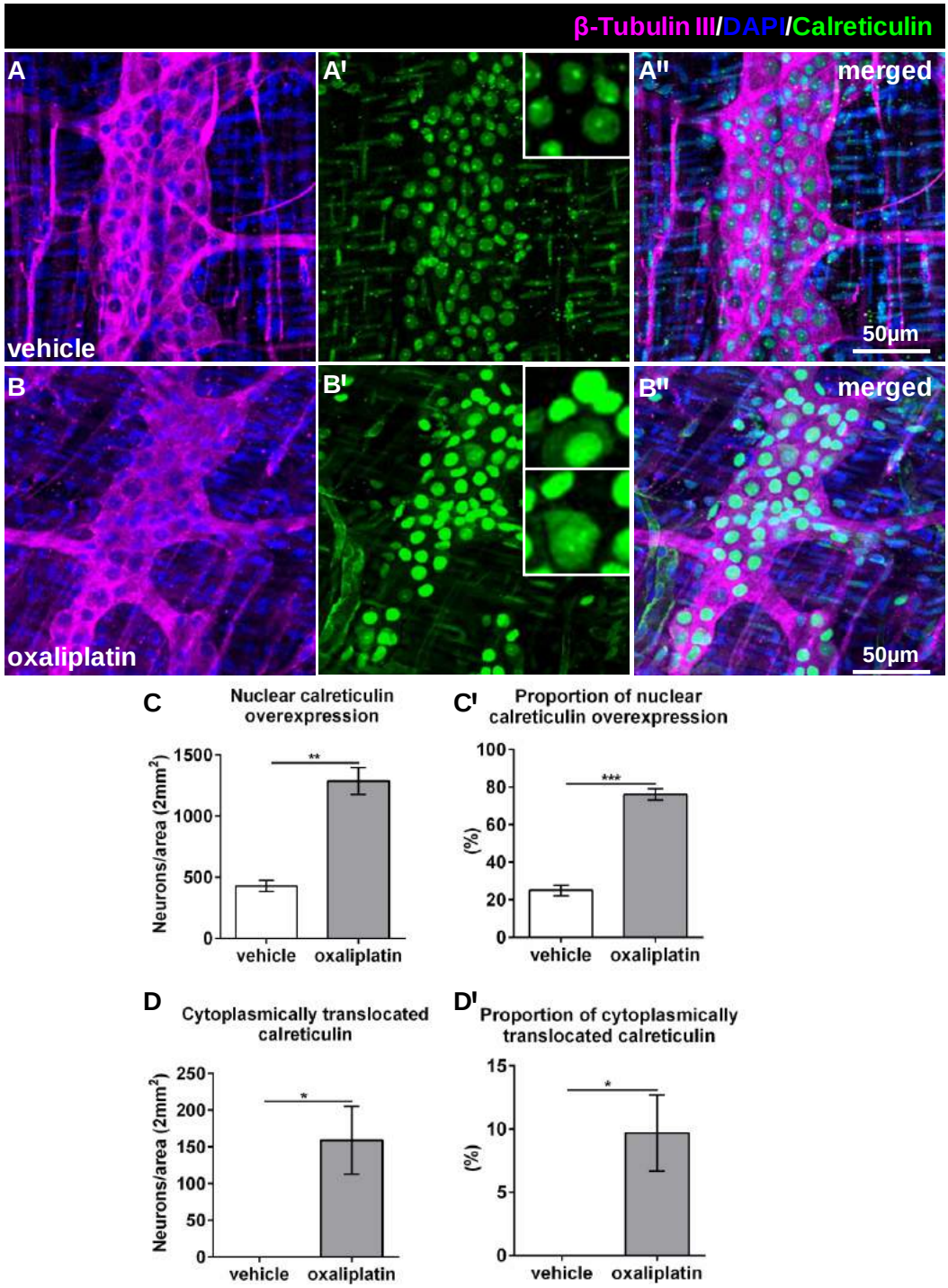


Figure 3.5. Oxaliplatin treatment induces intranuclear overexpression and cytoplasmic translocation of calreticulin within the myenteric plexus but not the longitudinal muscle of the colon. Wholemount preparations of the LMMP labelled with the pan-neuronal marker β-Tubulin-III (magenta), calreticulin (green), and the cell nuclei marker DAPI (blue) **(A-B'')**. Intense nuclear calreticulin labelling and cytoplasmic translocation is observed within the myenteric plexus from the oxaliplatin-treated cohort **(B')** when compared to the punctate labelling in the nuclei which is observed in the vehicle-treated group **(A')**. A significant number and proportion of myenteric neurons showed increased calreticulin expression in the nuclei **(C-C')** and cytoplasmic translocation **(D-D')** following oxaliplatin treatment when compared to the vehicle-treated cohort. Intranuclear overexpression and cytoplasmic translocation of calreticulin in myenteric neurons counted from 2mm^2 area and % calculated per total neurons. *P<0.05; **P<0.01; ***P<0.001; n=4/group.

Similar changes were observed with the expression of HMGB1 in the oxaliplatin-treated mice. HMGB1 immunoreactivity in the vehicle-treated cohort was weakly present in the nuclei of both myenteric neurons and longitudinal muscle cells **(Figure 3.6 A^{I}-B^{II})**.

A significant number (395 ± 54, ***P*<0.01; n=4) and proportion (23 ± 2%; ***P*<0.01; n=4) of cells within the myenteric plexus of oxaliplatin-treated mice displayed strong nuclear overexpression of HMGB1 when compared to the vehicle-treated cohort respectively (31 ± 31; 1.5 ± 1.4%; n=4) **(Figure 3.6 C-C^{I})**.

Furthermore, a significant number (187 ± 28, ***P*<0.01; n=4) and proportion (11.7 ± 2.4%; ***P*<0.01; n=4) of myenteric neurons from the oxaliplatin-treated group displayed cytoplasmically translocated HMGB1 when compared to the vehicle-treated cohort in which no translocation in neurons was found (n=4/group) **(Figure 3.6 D-D^{I})**.

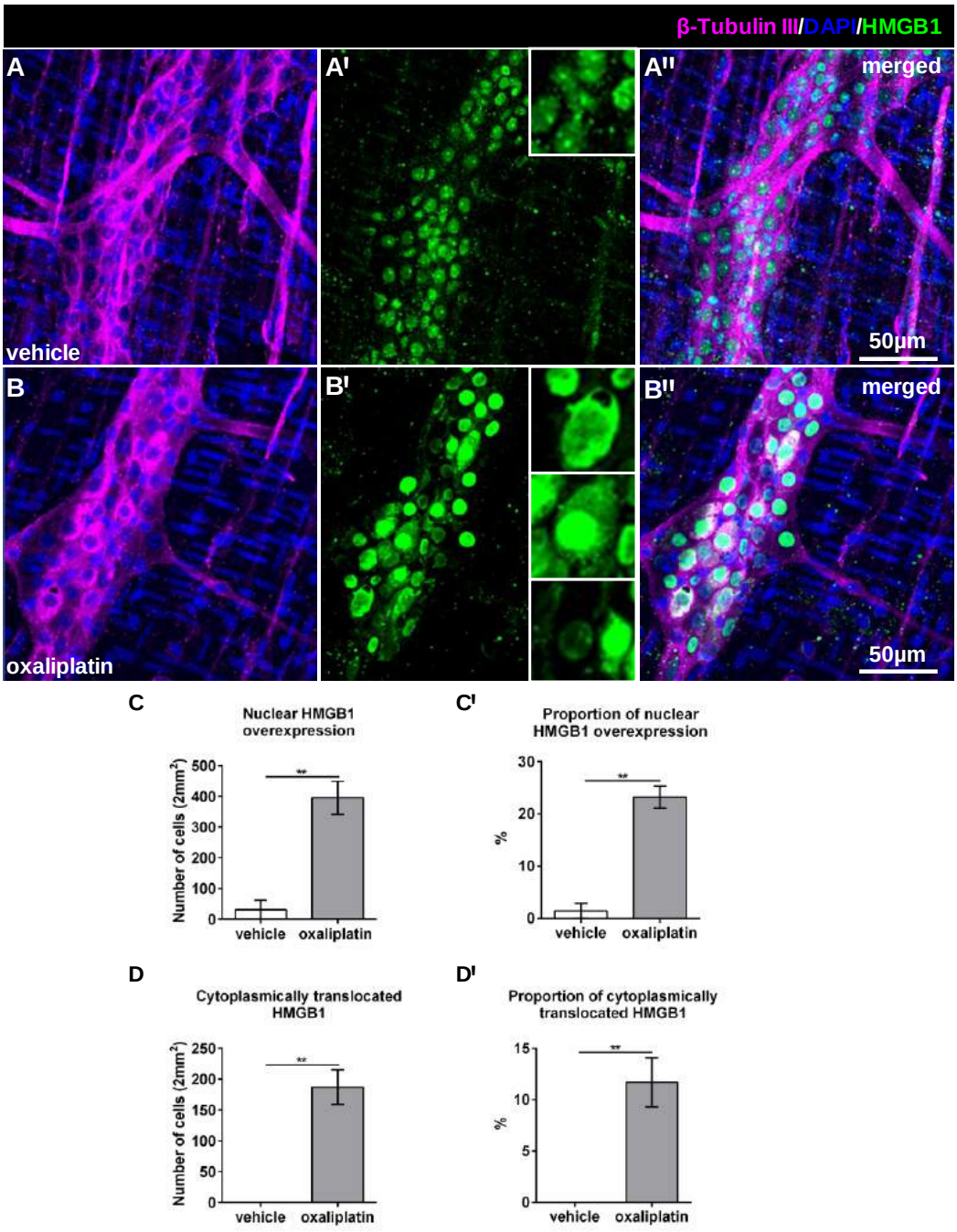


Figure 3.6. Oxaliplatin treatment induces intranuclear overexpression and cytoplasmic translocation of HMGB1 within the myenteric plexus but not the longitudinal muscle of the colon. Wholemount preparations of the LMMP labelled with the pan-neuronal marker β-Tubulin-III (magenta), HMGB1 (green) and the cell nuclei marker DAPI (blue) **(A-B'')**. Intense nuclear HMGB1 labelling and cytoplasmic translocation is observed within the myenteric neurons from the oxaliplatin-treated group **(B')** when compared to the punctate labelling in the nuclei observed in the vehicle-treated cohort **(A')**. A significant number and proportion of myenteric neurons overexpressed HMGB1 within the nuclei **(C-C')** and the cytoplasmic translocation of this protein **(D-D')** following oxaliplatin treatment when compared to the vehicle-treated group. ***P*<0.01; n=4/group.

3.4.4 Calreticulin and HMGB1 differentially colocalise within myenteric neurons

Calreticulin and HMGB1 are ubiquitous proteins expressed in all cell types, and we have shown both to colocalise in myenteric neurons with varying indices following oxaliplatin-induced cell stress and damage **(Figure 3.7 A-B^{II})**. A significant number of neurons displayed colocalised intranuclear overexpression of calreticulin and HMGB1 per ganglion following oxaliplatin treatment (25 ± 2; ****P<0.0001; n=4) when compared to the vehicle-treated cohort (0 ± 0; n=4) **(Figure 3.7 A-C^{II})**. Furthermore, a significant number of neurons displayed colocalised cytoplasmic translocation of calreticulin and HMGB1 per ganglion following oxaliplatin treatment (8 ± 3; ****P<0.0001; n=4) when compared to the vehicle-treated cohort (0 ± 0; n=4) **(Figure 3.7 D^{I})**.

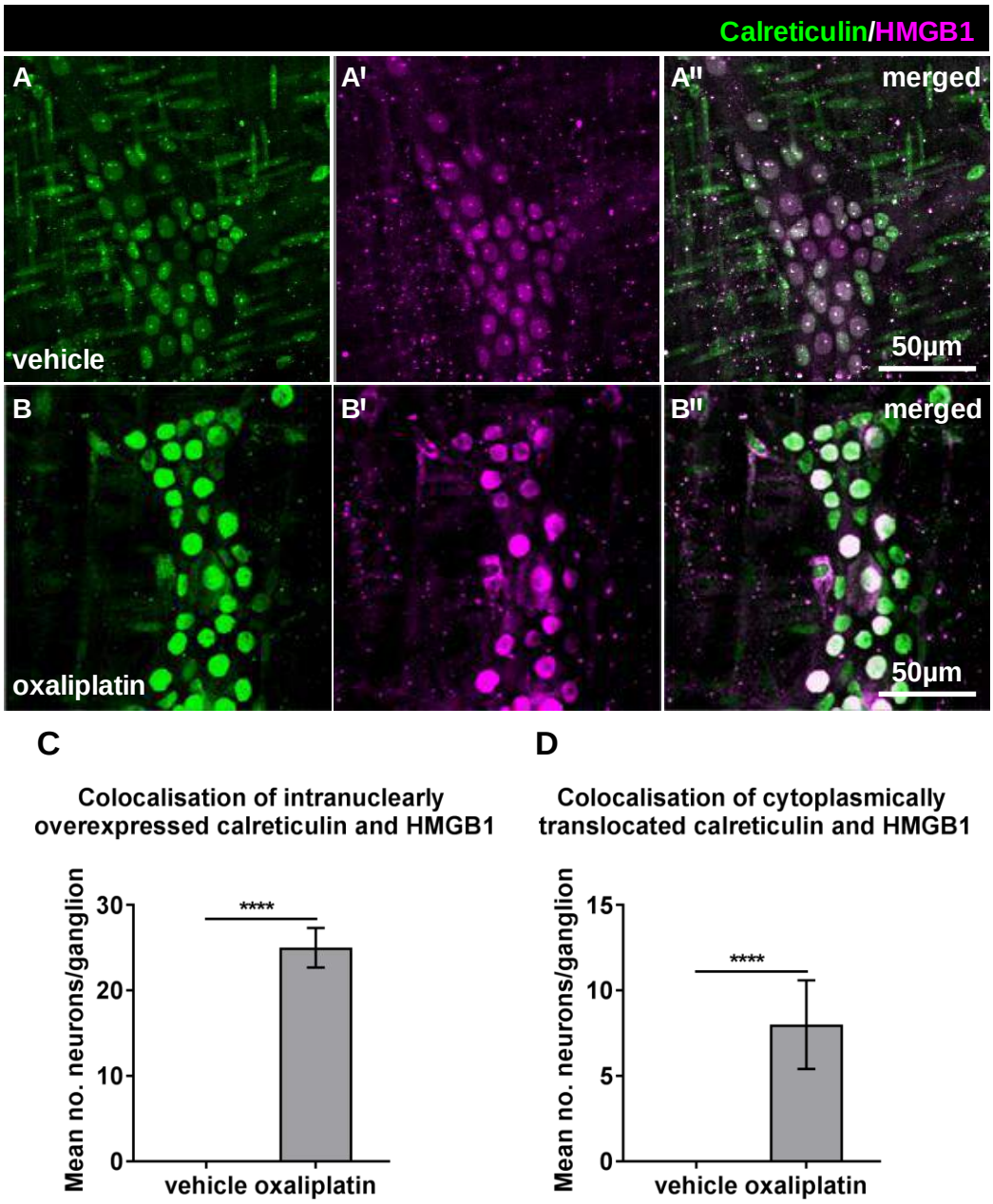


Figure 3.7. Colocalisation of calreticulin and HMGB1 within the myenteric plexus of the colon. Wholemount preparations of the LMMP labelled with the calreticulin (green) and HMGB1 (magenta) **(A-B'')**. Calreticulin and HMGB1 are both overexpressed within the nucleus or are cytoplasmically translocated. A significant number of myenteric neurons have colocalised expression within the nucleus **(C)** and in the cytosol **(D)** following oxaliplatin treatment.****P<0.0001; n=4/group.

3.4.5 Oxaliplatin treatment causes a reduction in calreticulin and HMGB1 expression in the longitudinal muscle of the colon

To determine whether oxaliplatin treatment altered calreticulin and HMGB1 expression within the longitudinal muscle, the fluorescence of these proteins was measured **(Figure 3.8 A-B^{II})**. Oxaliplatin treatment caused a significant reduction in the expression of calreticulin (2.6 ± 0.7 a.u; ****P<0.0001; n=4) when compared to the vehicle-treated group (11 ± 0.4 au; n=4) **(Figure 3.8 A-C)**. Similarly, oxaliplatin treatment caused a significant reduction in the expression of HMGB1 (1.1 ± 0.05 a.u; ***P<0.001; n=4) when compared to the vehicle-treated cohort (5.5 ± 0.7 a.u; n=4) **(Figure 3.8 D)**.

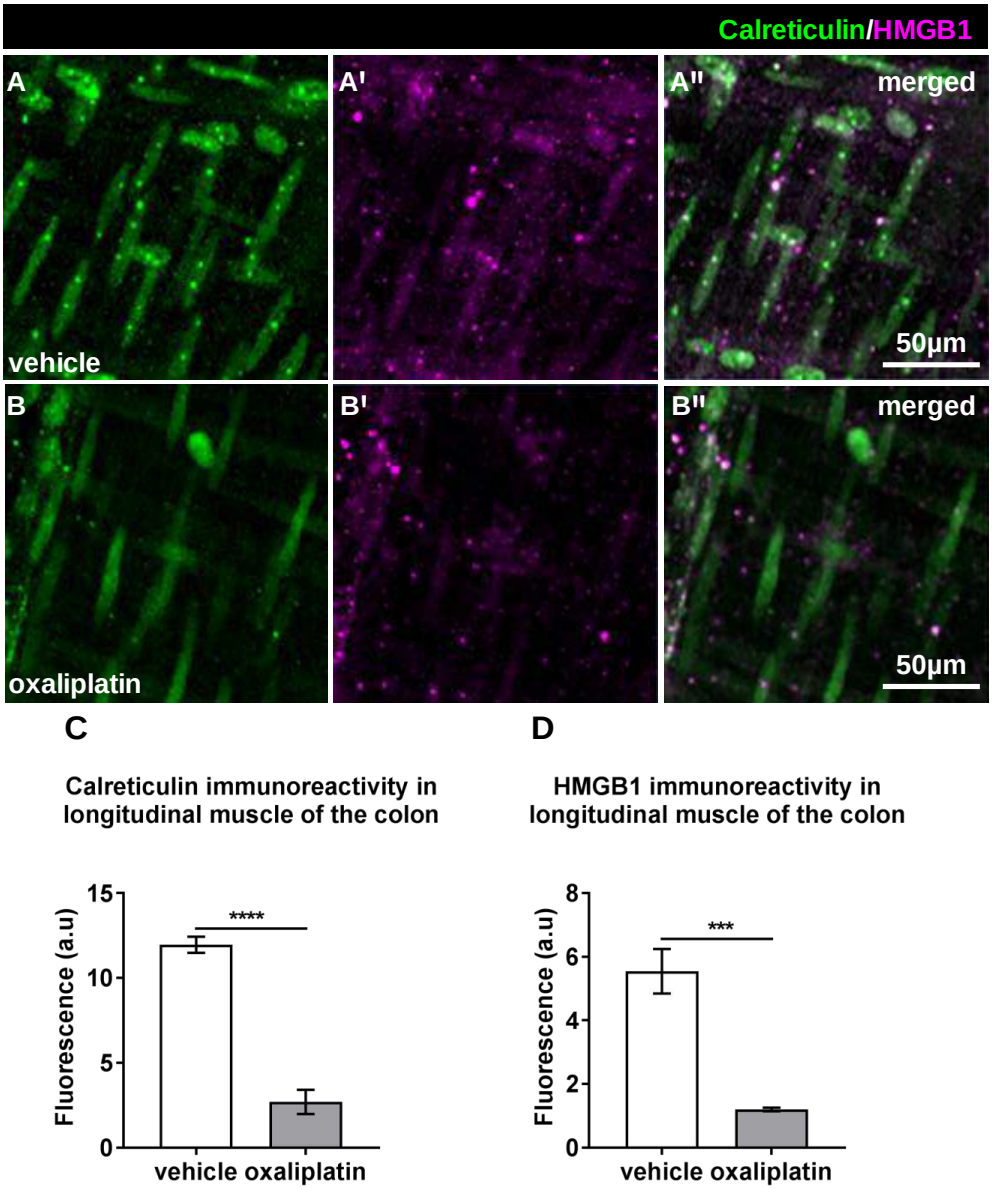


Figure 3.8. Oxaliplatin treatment causes a reduction in calreticulin and HMGB1 expression in the longitudinal muscle of the colon. Wholemount preparations were labelled with the calreticulin (green) and HMGB1 (magenta) **(A-B'')**. Oxaliplatin treatment significantly reduced calreticulin **(C)** and HMGB1 **(D)** expression throughout the longitudinal muscle of the colon compared to the vehicle-treated cohort. ****P*<0.0001; n=4/group.

3.4.6 Oxaliplatin treatment causes an increase in cytochrome *c* expression within the LMMP

To investigate whether oxaliplatin induces mitochondrial damage within the LMMP, western blotting was performed to determine the expression of cytochrome *c* and electron transport chain proteins. Oxaliplatin treatment caused a significant increase in cytochrome *c* expression within the LMMP (6.5 ± 0.4 a.u, **P*<0.05; n=5) when compared to the vehicle-treated cohort (4.8 ± 0.18 a.u; n=4) **(Figure 3.9 A)**. No significant differences were observed in the expression of electron transport chain proteins (complex I: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 [NDUFB8]), complex II: (succinate dehydrogenase subunit enzyme [SDHB]), complex III: (ubiquinol-cytochrome *c* reductase core protein II [UQCRC2]), complex IV: (cytochrome *c* oxidase subunit I [MTCO1]), or complex V: (mitochondrial membrane ATP synthase subunit α [ATP5A]) between the vehicle-treated and oxaliplatin-treated cohorts **(Figure 3.9 B)**.

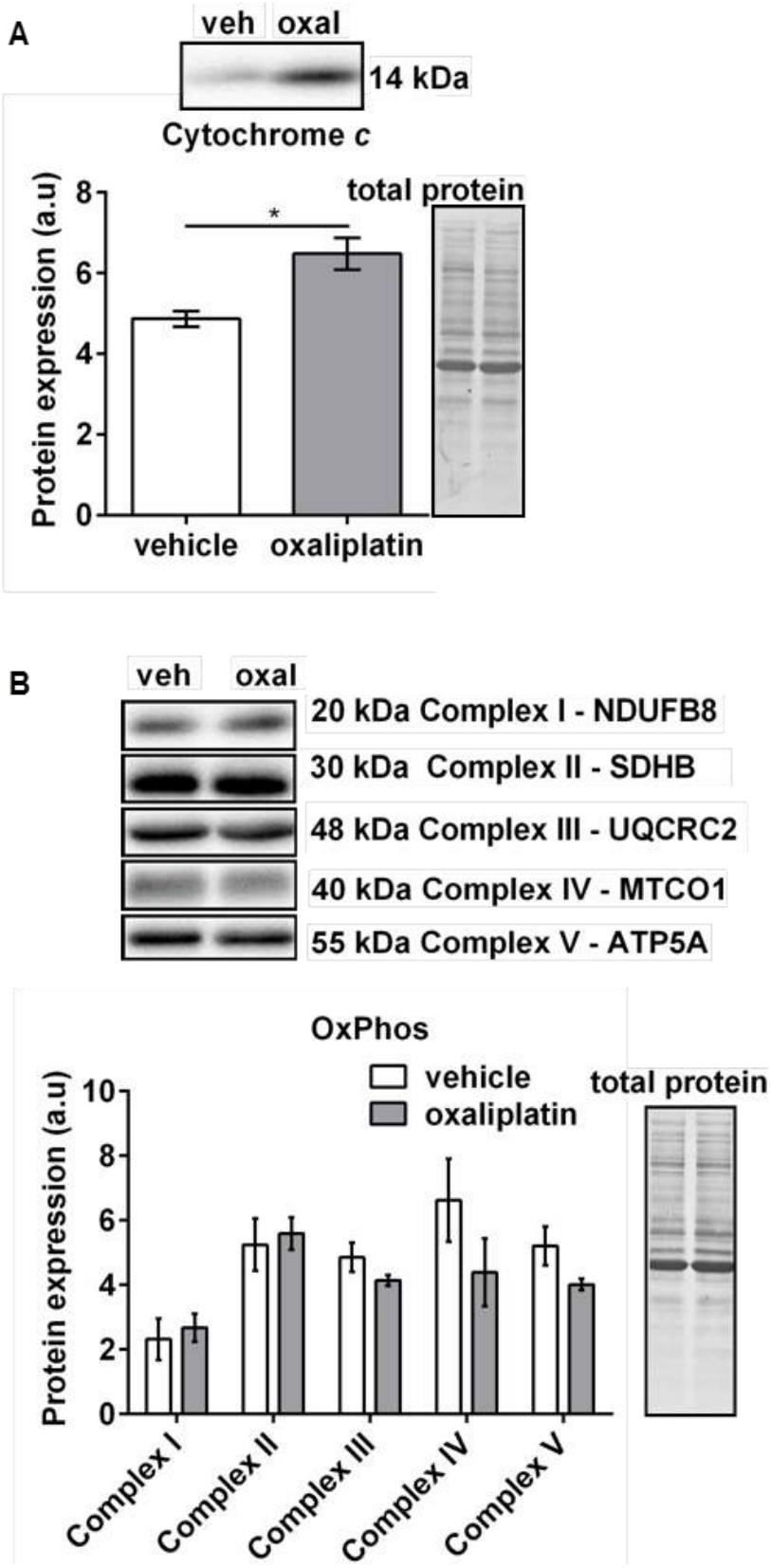


Figure 3.9. Oxaliplatin treatment induces the upregulation of cytochrome *c* in the LMMP, with no demonstrable effects on the expression of mitochondrial electron transport chain proteins. Western blot analysis of colon LMMP samples revealed oxaliplatin-induced significant increase in cytochrome *c* levels when compared to the vehicle-treated group **(A).** No significant changes in mitochondrial electron transport chain proteins (OxPhos) were observed **(B).** **P*<0.05; n=4-5/group.

3.4.7 Oxaliplatin treatment induces caspase 3 cleavage in the myenteric plexus

To investigate whether myenteric neurons are undergoing apoptosis, colon wholemount preparations were labelled with the pan-neuronal marker β-Tubulin III (green), and cleaved caspase 3 (red) **(Figure 3.10 A-B'''')**. No cleaved caspase 3 immunoreactivity was detected within the myenteric plexus from the vehicle-treated cohort **(Figure 3.10 A-A')**. Conversely, myenteric neurons from the oxaliplatin-treated cohort displayed strong immunoreactivity for cleaved caspase 3, which is indicative of cells undergoing apoptosis; n=4/group **(Figure 3.10 B-B'''')**.

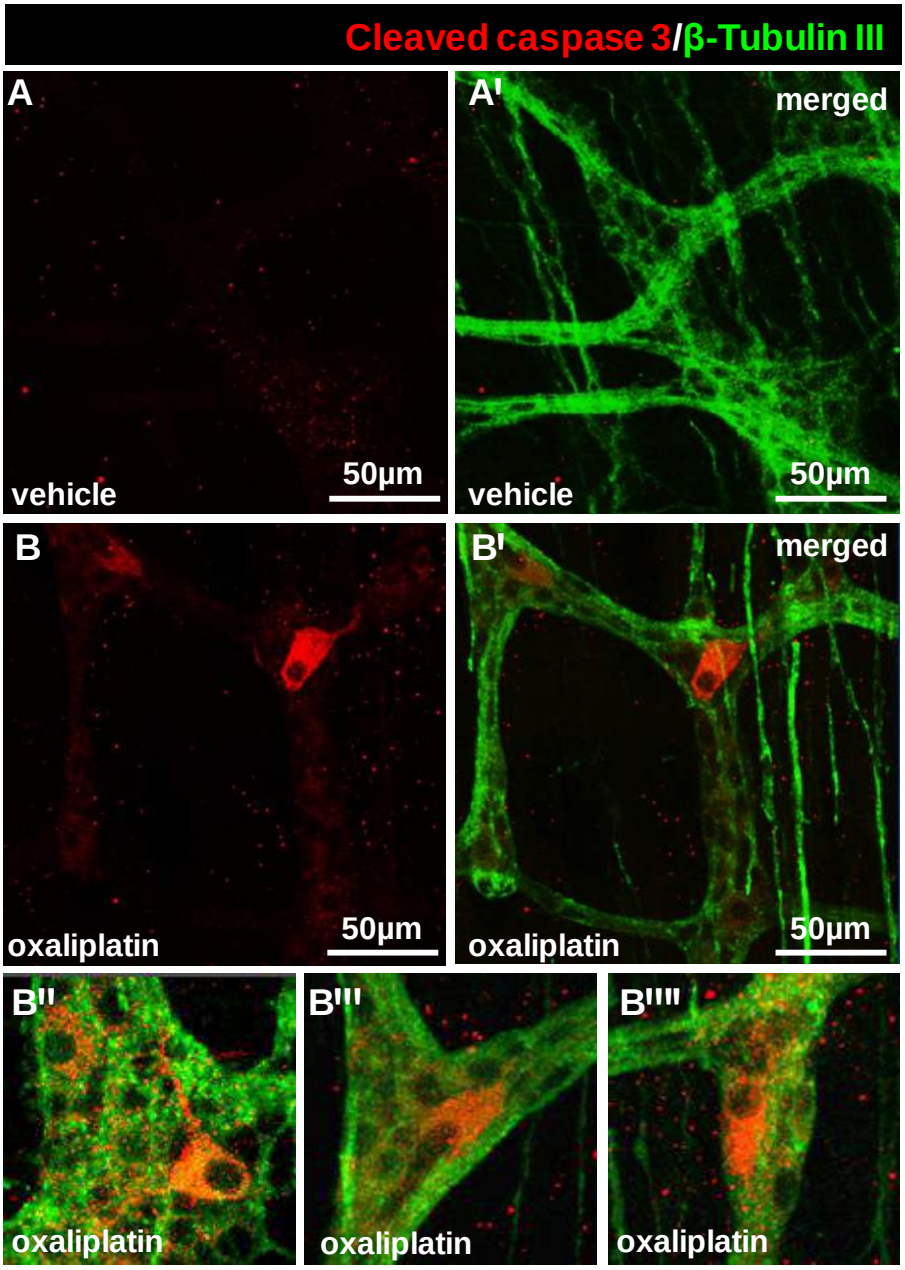


Figure 3.10. Cleaved caspase 3 immunoreactivity in myenteric neurons following oxaliplatin treatment. Colon wholemount preparations labelled with cleaved caspase 3 (red) and the pan-neuronal marker β-Tubulin III (green). No cleaved caspase 3 immunoreactivity is detected within the myenteric plexus from the vehicle-treated cohort (**A-A'**). Myenteric neurons from the oxaliplatin-treated cohort display strong immunoreactivity for cleaved caspase 3, which is indicative of apoptosis; n=4/group **(B-B'''')**.

3.5 DISCUSSION

This study is the first to demonstrate platinum accumulation within the LMMP following oxaliplatin treatment as a potential mechanism for gastrointestinal damage and dysfunction. This study provides evidence that oxaliplatin treatment induces 1) the accumulation of platinum within nuclear and mitochondrial fractions of the cells in the LMMP; 2) alteration in the expression of CTR1 in myenteric neurons which is thought to be involved in platinum-based drug influx; 3) differential colocalisation of CTR1 with nNOS and ChAT neurons in the myenteric plexus; 4) nuclear overexpression and cytoplasmic translocation of the DAMPs, calreticulin and HMGB1, within the myenteric plexus; 5) colocalisation of calreticulin and HMGB1 in myenteric neurons with varying indices; 6) a reduction in calreticulin and HMGB1 in the longitudinal muscle; 7) an increase in cytochrome *c* expression within the LMMP (but no changes in OxPhos protein expression); 8) cleaved caspase 3 in myenteric neurons.

In this present study, detectable amounts of platinum were found within the nuclear and mitochondrial fractions of the LMMP. We provide the foundation for platinum accumulation within the LMMP as the underlying mechanism of oxaliplatin-induced gastrointestinal damage and dysfunction. Oxaliplatin undergoes non-enzymatic hydrolysis and is metabolized into monochloro-, dichloro-, and diaquo-diaminocyclohexane platinum complexes. The dichloro-diaminocyclohexane complexes are thought to be the most reactive as they cause nuclear DNA lesions by binding to guanine clusters (particularly at the N7 position) forming

intrastrand and interstrand cross links or platinum adducts (Allain et al., 2000, Verstraete et al., 2001, Alcindor and Beauger, 2011). The formation of DNA platinum adducts as the primary mechanism of cytotoxicity is generally accepted for all platinum-based drugs. Indeed this is true for cisplatin and oxaliplatin as they both form similar DNA platinum adducts. However, at equimolar concentrations, oxaliplatin produces fewer platinum adducts than cisplatin, but causes similar or even greater DNA damage, which suggests there are varying mechanisms of cell death (Woynarowski et al., 2000, Chaney et al., 2005). Platinum drug deposition can result in nuclear and mitochondrial DNA lesions (intrastrand and interstrand cross-links/adducts), transcriptional defects, as well as the inhibition of DNA synthesis, thus, causing deleterious effects on cell viability through the initiation of apoptotic cascades (William-Faltaos et al., 2006, Alcindor and Beauger, 2011, Cheung-Ong et al., 2013). Nuclear and mitochondrial platinum adduct formation has been previously demonstrated in DRG neurons following cisplatin and oxlaiplatin treatment and is considered to be the underlying mechanism for neurotoxicity (Ta et al., 2006, Podratz et al., 2011).

The CTR1 receptor has been implicated in platinum drug influx in a number of bacteria, cancer cell lines, and neurons within the DRG, all of which show susceptibility to chemotherapy-induced cell death (Ishida et al., 2002, Lin et al., 2002, Song et al., 2004, Holzer et al., 2006, Jandial et al., 2009, Larson et al., 2009, Ip et al., 2010). The expression of the CTR1 receptor in the LMMP has not been investigated previously. Our data

demonstrate strong CTR1 immunoreactivity within myenteric neurons with minimal labelling of nerve fibres and the longitudinal muscle in the control cohort. The CTR1 receptor can be recycled following ligand binding or due to increased intracellular copper concentration as a mechanism to regulate intracellular copper pools (Holzer and Howell, 2006). It has also been demonstrated that cisplatin can induce CTR1 recycling (Holzer and Howell, 2006), and our data provide evidence that this may be true for oxaliplatin also. The recycling of CTR1 has been used as a measure to determine direct drug influx (Howell and Safaei, 2009). However, CTR1 expression may also be downregulated if copper levels are too high (Guo et al., 2004). In this study no demonstratable differences in copper levels within the nuclear and mitochondrial fractions were found suggesting that the decrease in CTR1 immunoreactivity is due to platinum binding to the CTR1 itself. If oxaliplatin utilises the CTR1 receptor for cell entry, it is plausible to suggest that it may also use other parts of the copper chaperone system to traffic throughout cells. It is unknown how/whether utilisations of the copper chaperone system can dysregulate copper metal dynamics. It was originally thought that platinum-based drugs ‘hijack’ the CTR1 receptor and intracellular copper chaperones, however, it is becoming evident that platinum-drugs can essentially ‘hitch-hike’, given that platinum ions still bind to copper-loaded chaperones (Palm-Espling et al., 2013). Moreover, it is well established that the inner mitochondrial membrane is impermeable to various metal ions (including platinum), in which entry to this organelle requires a chaperone. The copper chaperone

COX17 shuttles cytosolic copper to the mitochondrial acceptor proteins COX11, SCO1 and SCO2 (Horng et al., 2004, Leary et al., 2004, Cobine et al., 2006, Xu et al., 2013). The mitochondrial copper pool is essential for the assembly of cytochrome *c* oxidase (Complex IV) of the electron transport chain, and therefore, life sustaining oxidative phosphorylation (Xu et al., 2013). The potential for dual-metal shuttling by copper chaperones may explain why no significant differences are observed in mitochondrial copper concentrations, but could also be a potential gateway for oxaliplatin entry within mitochondria. In addition, it has previously been shown that cisplatin and oxaliplatin have the capacity to complex with the copper chaperone ATOX1 (Palm et al., 2011, Palm-Espling and Wittung-Stafshede, 2012). ATOX1 is typically involved with copper trafficking to the *trans*-Golgi network for cellular distribution and efflux, and more recently, cisplatin treatment has been shown to induce ATOX1 localisation within the nucleus of HTC116 $p53^{+/+}$ cells (Prohaska, 2008, Hatori and Lutsenko, 2013, Beaino et al., 2014). Further work is required to determine the relationship between oxaliplatin and the copper chaperone system and its role in intracellular platinum trafficking and neuropathy.

CTR1 immunoreactivity decreased significantly within the myenteric plexus following oxaliplatin treatment. No significant changes in CTR1 and nNOS colocalisation were observed. This was in contrast to the significant differences in CTR1 immunoreactivity in ChAT neurons which mostly appeared as weak/punctuate labelling. Whether there are differential

requirements for copper by excitatory and inhibitory neurons in the myenteric plexus is unknown. Although we did not observe significant changes to overall copper content in the LMMP, abberant copper distribution may be implicated in enteric neuropathy and warrants further investigation. If platinum-based drugs such as oxaliplatin utilise the copper chaperones, it is unknown how this may affect copper loading, trafficking and distribution. Free copper ions can bind to protein cysteine residues and inactive enzymes. As mentioned in Chapter 2, acetylcholinesterase has a very high cysteine content that may increase its vulnerability to post-translational modifications (Klatt and Lamas, 2000, Black and Rylett, 2011). Copper can bind to cysteine directly, or this may be a result of nitrosative modifications. Copper is an essential ion for the production of copper/zinc-superoxide dismutase, and aberrant copper distribution/levels may impact the integrity of the cells defence mechanisms which may lead to increased nitrosative stress as we have previously shown in myenteric neurons (McQuade et al., 2016b). nNOS-IR myenteric neurons appear to be particularly robust during oxidative and/or nitrosative stress (Rivera et al., 2012). Whether ChAT-IR myenteric neurons are particularly vulnerable to oxaliplatin or accumulate more platinum than nNOS-IR neurons requires further investigation. In this study, we utilised the LA-ICP-MS technique to determine platinum accumulation and deposition. Whilst this technique allows for the generation of elemental distribution maps, it is not sensitive enough to determine cell-specific platinum deposition. Therefore, techniques with high-resolution and sensitivity, such as the synchrotron x-

ray fluorescence is required, as platinum accumulation can be detected down to the cellular level. Using synchrotron x-ray fluorescence microscopy, other trace metals such as copper can be investigated simultaneously, and this could provide further information regarding copper homeostasis following oxaliplatin treatment.

Our data demonstrate that oxaliplatin evokes the presentation of the DAMPs, calreticulin and HMGB1, within the myenteric plexus. Calreticulin is a multifunctional calcium-binding protein which in normal conditions is typically expressed at low levels, and is localised within the endoplasmic reticulum and the nuclear envelope (Michalak et al., 2009). Alongside its role in calcium homeostasis, calreticulin has been identified as a cisplatin binding protein, and its expression tends to become upregulated during cell stress and damage (Al-Eisawi et al., 2011, Karasawa et al., 2013). In the present study, nuclear overexpression and cytosplasmic translocation of calreticulin is observed within the myenteric plexus. It is plausible to suggest that calreticulin may also complex with oxaliplatin or its metabolised species/platinum adducts within the myenteric plexus. The cytoplasmic translocation of calreticulin is a hallmark feature of oxaliplatin-induced toxicity and can subsequently induce immunogenic cell death (Zitvogel et al., 2008, Garg et al., 2010, Tesniere et al., 2010, Krysko et al., 2012). Cytoplasmic translocation of calreticulin is an early apoptotic event (Panaretakis et al., 2009). Previous studies have established that increased calreticulin expression corresponds with enhanced calcium fluxes and intracellular calcium

overload leading to apoptotic cascades involving cytochrome *c* release and caspase activation (Bastianutto et al., 1995, Mery et al., 1996, Arnaudeau et al., 2002, Mattson and Chan, 2003, Orrenius et al., 2003, Boehning et al., 2004, Lim et al., 2008). This is in agreement with the results of our study demonstrating the upregulation of cytochrome *c* and cleaved caspase 3 expression in myenteric neurons from oxaliplatin-treated mice. Whether the intranuclear overexpression or cytoplasmic translocation of calreticulin following oxaliplatin treatment interferes with its homeostatic calcium buffering capacity, thus potentiating toxicity, requires further research.

HMGB1 is a non-histone DNA binding protein which regulates gene transcription and repair processes such as NER, BER, and MMR (Lange and Vasquez, 2009, Liu et al., 2010c, Yang et al., 2013). There is mounting research which highlights the role of HMGB1 as a platinum drug-sensing and binding protein, with most work investigating its interactions with cisplatin and/or its DNA adducts (Hughes et al., 1992, Dunham and Lippard, 1997, Wei et al., 2001, Jung and Lippard, 2003, Park and Lippard, 2011, He et al., 2015). In particular, HMGB1 can recognise 1-2d(GpG) or 1-2d(ApG) cisplatin-induced lesions on DNA, but further work is required to determine its specificity against oxaliplatin adducts (Hughes et al., 1992, Dunham and Lippard, 1997, Jung and Lippard, 2003). In the present study, nuclear overexpression of HMGB1 is observed, as well as cytoplasmic translocation in myenteric neurons from oxaliplatin-treated mice. Nuclear overexpression may be suggestive of platinum adduct

binding; however, further research is required to confirm this speculation. Furthermore, there is a fine balance between HMGB1 binding to distorted DNA for the commitment to cell death or repair pathways. HMGB1 can hinder DNA repair through “repair shielding” or blocking access to nuclear or base-excision proteins (Malina et al., 2002, Lange and Vasquez, 2009). Conversely, HMGB1-platinum adduct binding could potentiate a repair response through increased DNA distorsion and repair system recognition (Lange and Vasquez, 2009). The cytoplasmic translocation of this protein is characteristic of cell damage beyond repair, and is a result of either apoptosis or necrosis (Bell et al., 2006, Green et al., 2009, Tesniere et al., 2010). It is suggested that cells undergoing apoptosis may retain HMGB1 intranuclearly to avoid producing an inflammatory signal or tolerogenic apoptosis (Trisciuoglio and Bianchi, 2009). Furthermore, following the cytoplasmic translocation, HMGB1 may then be passively secreted from necrotic and apoptotic cells and this will determine the potential for immunogenic apoptosis. HMGB1 is typically retained within the cytoplasm during apoptosis, but may be released in the event of secondary necrosis (Gauley and Pisetsky, 2009, Trisciuoglio and Bianchi, 2009). Moreover, we have shown that calreticulin and HMGB1 colocalise at varying indices following oxaliplatin-induced myenteric plexus damage. Some myenteric neurons display exclusive overexpression or cytoplasmic translocation of calreticulin or HMGB1, or show strong immunoreactivity and colocalisation within both the nucleus and the cytoplasm. Changes in calreticulin expression and localisation is often regarded as an early apoptotic event,

whereas HMGB1 is considered a late apoptotic factor (Zitvogel et al., 2010a, Zitvogel et al., 2010b, Wiersma et al., 2015, Colangelo et al., 2016).

In this study we demonstrated an overall reduction in calreticulin and HMGB1 immunoreactivity in the longitudinal muscle of the colon. Unlike neurons, smooth muscle cells are mitotically active cells with the capacity to regenerate and divide. We have previously demonstrated that oxaliplatin treatment induces smooth muscle atrophy as demonstrated by a reduction in muscle thickness of the colon (McQuade et al., 2016b). It is unknown whether intranuclear overexpression and/or cytoplasmic translocation of calreticulin and HMGB1 occurred during earlier stages of oxaliplatin treatment, and if the reduction of these proteins is due to smooth muscle cell loss. We have previously shown that oxaliplatin accumulates within the nuclear and mitochondrial fractions of skeletal muscle (tibialis anterior), distorts muscle architecture, and causes a reduction in intramuscular protein content (Sorensen et al., 2017). Additionally, oxaliplatin treatment increased intramuscular collagen deposition, lipid content, and caused calcium accumulation in muscle fibres (Sorensen et al., 2017). Furthermore, oxaliplatin treatment caused an increase in mitochondrial superoxide, and decreased mitochondrial viability in skeletal muscles (Sorensen et al., 2017). Whilst platinum drug accumulation in the LMMP was measured in this study, we did not investigate muscle architecture, protein content, collagen, lipid, and calcium deposition in the longitudinal smooth muscle. This warrants further

research. In our previous study, we have shown that mitochondrial superoxide production is particularly confined to myenteric neurons of the colon, with no obvious changes in the longitudinal smooth muscle (McQuade et al., 2016b). This suggests that oxaliplatin treatment may induce differential cell damage and death pathways in skeletal and smooth muscles and neurons.

It is interesting to note that the myenteric neurons expressed high levels of CTR1 and are particularly vulnerable to oxaliplatin treatment as demonstrated by the presentation of DAMPs and cleaved caspase 3. Although CTR1 immunoreactivity in the longitudinal muscle was not observed, there was a reduction in the expression of DAMPs. This suggests that the mechanisms of oxaliplatin-induced cell death must be cell-type specific. This has been proven to be true in that oxaliplatin treatment induces differential cell death of the HT-29 colorectal cell line and primary astrocyte cultures (Zanardelli et al., 2015). In astrocytes oxaliplatin induces cytochrome *c* release as well as an increase in superoxide anions and Bcl-2 levels (Zanardelli et al., 2015). Whereas in the HT-29 cell line oxaliplatin does not induce changes in cytochrome *c* expression or superoxide anion levels, despite an increase in Bcl-2 protein and caspase 8 which are involved in the apoptotic cascade (Zanardelli et al., 2015). Differential activation of NER and BER systems in cancer (dividing cells) and amitotic cells (neurons) can lead to the development of treatments that can protect only neurons, whilst allowing oxaliplatin to kill

cancer cells. Further work is needed to delineate the cytotoxic effects of oxaliplatin on the longitudinal muscle.

Platinum DNA adduct formation is known to induce programmed cell death or apoptosis through caspase-mediated pathways (Marchetti et al., 2004, Zanardelli et al., 2015). In this study we have shown that oxaliplatin-derived platinum penetrates the mitochondria and that this is associated with the upregulation of cytochrome *c* within the LMMP. Increased cytochrome *c* levels have been shown to occur during early activation of intrinsic apoptosis pathways (Sanchez-Alcazar et al., 2000, Chandra et al., 2002). The increase in cytochrome *c* may therefore be an indicator of increased apoptosis. No changes in total mitochondrial electron transport chain proteins (OxPhos) were observed, which may be due to overall mitochondrial content. Under circumstances of metabolic and cellular stress caused by genotoxic and oxidative insult, mitochondrial fusion and fission may occur to maintain functional mitochondrial pools, which may not necessarily cause changes in OxPhos protein content (Youle and van der Bliek, 2012). Mitochondrial fusion works to mitigate metabolic stress by consolidating the contents of partially damaged mitochondria together, forming a single, daughter organelle (Youle and van der Bliek, 2012). Subsequently, the fission process divides the mitochondrion in two, with the aim of maintaining adequate mitochondrial pools (Twig et al., 2008). Whilst it remains to be investigated, this fusion-fission process may explain why no significant differences in total OxPhos protein expression were observed for all complexes. It should be noted

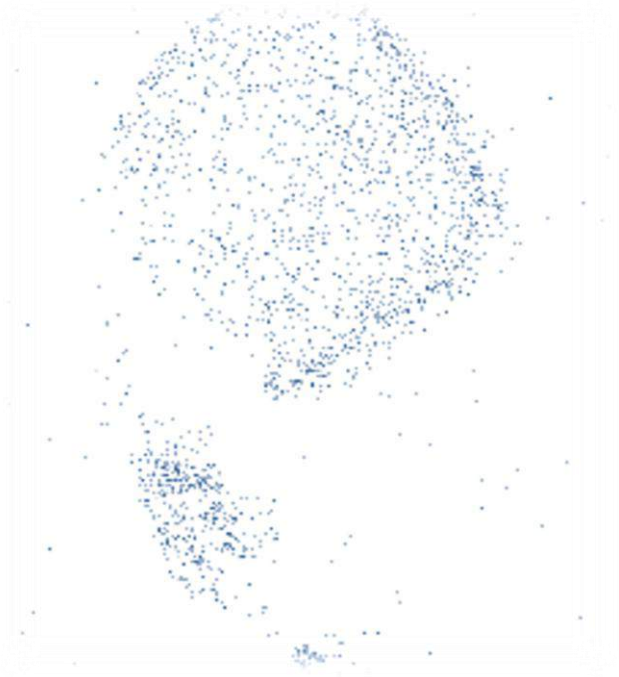
however, that despite the maintenance of mitochondrial content, the daughter mitochondria derived from the fusion-fission cycle may not be structurally and functionally sound. Intracellular molecules such as NO can induce persistent, asymmetrical mitochondrial fission (Barsoum et al., 2006). Ultrastructural changes in daughter mitochondria leads to bioenergetic failure and oxidative stress (Barsoum et al., 2006). A limitation in investigating OxPhos protein expression is that it does not give any information about the alignment of each complex, which is essential for correct electron flow through each subunit, and for minimising reactive oxygen species formation whilst optimising energy production and output (Acin-Perez et al., 2004, Maranzana et al., 2013, Letts et al., 2016). Nevertheless, we have demonstrated that oxaliplatin treatment results in myenteric neuronal apoptosis through a cleaved caspase 3-mediated pathway. It is well established that cytochrome *c* activates caspase 9 and subsequently induces the activation of the executioner caspase 3 either directly or indirectly through caspase 7 signalling (Slee et al., 1999, Abu-Qare and Abou-Donia, 2001, Baliga and Kumar, 2003, Gogvadze et al., 2006).

In summary, we reveal for the first time that platinum from oxaliplatin localises within the nuclear and mitochondrial fractions of the LMMP and alters CTR1 immunoreactivity which may be indicative of drug influx and direct toxicity. Furthermore, oxaliplatin treatment evokes the hallmark presentation of the DAMPs calreticulin and HMGB1 which are both involved in platinum drug recognition and binding, and are also

indicative of cell damage and death. We also provide evidence for neuronal apoptosis through a cytochrome *c*/cleaved caspase 3-mediated pathway following oxaliplatin. These data implicate myenteric neuropathy in the multifaceted pathophysiology of oxaliplatin-induced gastrointestinal dysfunction.

4

OXALIPLATIN-DERIVED PLATINUM ACCUMULATES WITHIN THE BRAIN, ALTERS NUCLEAR COPPER STATUS, AND INDUCES MITOCHONDRIAL TOXICITY IN THE BRAINSTEM



4.1 SUMMARY

The brainstem contains neural circuitry involved in a myriad of autonomic functions, and is responsible for mediating protective gastrointestinal responses such as vomiting. It is well known that anti-cancer agents such as oxaliplatin can induce vomiting and changes to gastrointestinal functions. Parts of the brainstem lack a true blood brain barrier which makes this brain region anatomically relevant for responding to cytotoxic agents within the circulation, but also makes it particularly susceptible to injury. Cytotoxic damage to the brainstem, which contains the vomiting centres, can therefore introduce greater challenges to anti-emetic therapies. These treatments pharmacologically target neuronal receptors, thus, neuronal and tissue injury may therefore reduce the binding capacity and efficacy of anti-emetic drugs. We have previously shown that oxaliplatin accumulates within the longitudinal muscle-myenteric plexus (LMMP) of the colon and causes myenteric neuronal cytotoxicity. Thus, the aims of this study were to determine whether this is also the case within the brainstem.

The aims of this study were to investigate whether oxaliplatin treatment: 1) can penetrate the brain and accumulate within both the nuclear and mitochondrial fractions; 2) induces changes in copper transporter receptor 1 (CTR1) expression within the brainstem; 3) alters the expression of DAMPs; 4) alters inducible nitric oxide synthase (iNOS) expression; 5) enhance cytochrome *c* expression, and 6) induces changes to mitochondrial electron transport chain proteins.

Balb/c mice received intraperitoneal injections of oxaliplatin (3mg/kg/d) or sterile water tri-weekly for 2 weeks. Mice were culled via CO_2 asphyxiation, followed by intracardiac perfusion using 1x PBS. The cerebrum and brainstem were separated, and homogenised. Once the samples were prepared, the nuclear and mitochondrial fractions were isolated to determine platinum and copper concentrations within these organelles by atomic absorption spectrophotometry. Platinum deposition within the forebrain and brainstem was visualised using laser ablation inductively coupled plasma mass spectrometry. Changes in the expression of CTR1, DAMPs (calreticulin and HMGB1, iNOS, cytochrome *c* and electron transport chain proteins within the brainstem were determined through western blotting.

A significant amount of platinum accumulated within the nuclear and mitochondrial fractions of the cerebrum and brainstem, and significantly reduced copper concentrations in the nuclear fractions in both regions. No changes in copper content within the mitochondrial fractions of the brain and brainstem were observed. Oxaliplatin treatment caused a significant reduction in CTR1 expression within the brainstem. No changes in the expression of the DAMPs, calreticulin, and HMGB1, or iNOS, were observed in the brainstem. However, a significant increase in cytochrome *c* was observed, alongside a reduction in mitochondrial complex I expression within the brainstem.

Novel findings of these studies have revealed that oxaliplatin is permeable to the mouse brain (cerebrum and brainstem). Oxaliplatin-

derived platinum accumulates within the brain tissue, and we have shown it has the capacity to localise within the nuclear and mitochondrial compartments. Similar to our previous work on enteric neurons (Chapter 3), oxaliplatin treatment alters CTR1 expression within the brainstem which may be indicative of direct drug uptake. At this time-point (day 14), we did not observe any changes in the expression of DAMPs and iNOS expression which may be due to differential modes of cell damage and death in the brain, or perhaps differential timing of central nervous system neurotoxicity. The upregulation of cytochrome *c* and a reduction in Complex I proteins from the mitochondrial electron transport chain implicates mitochondrial toxicity associated with oxaliplatin treatment as the possible mechanism of oxaliplatin-induced neuronal death.

4.2 INTRODUCTION

The brainstem contains neural circuitry which plays a role in regulating autonomic processes such as vomiting and gastrointestinal functions (Travagli et al., 2006). The medulla oblongata is a circumventricular organ, and thus, lacks a true blood brain barrier making it susceptible to systemically circulating chemicals such as anti-cancer drugs. Cytotoxic anti-cancer agents such as oxaliplatin are known to provoke emetic responses, but it is unclear whether they can induce tissue injury within the brainstem.

The dorsal vagal complex of the medulla oblongata which consists of the area postrema, nucleus tractus solitarius (NTS), and the dorsal

motor nucleus of the vagus nerve (DMNX), are the main areas implicated in vomiting (Travagli et al., 2006, Babic and Browning, 2014). Emesis occurs through the stimulation of the area postrema (also known as the chemoreceptor trigger zone) and the NTS (Perwitasari et al., 2011). The NTS is the main termination site for vagal afferents, and contains neurons which synapse with preganglionic parasympathetic motorneurons from the DMNX which project to the viscera such as the stomach and intestines (Babic and Browning, 2014). Furthermore, neurons from the NTS synapse with premotor neurons of the brainstem such as those located within the ventral respiratory groups, the nucleus ambiguus, and the parabrachial nuclei, all of which are involved in coordinating respiratory functions and muscle contractions essential for mediating emetic responses (Horn, 2008).

In Chapter 3 we demonstrated that oxaliplatin accumulates within the longitudinal muscle-myenteric plexus and causes cytotoxic damage to myenteric neurons which control gastrointestinal functions. Notably, we demonstrated changes in the expression of CTR1, DAMPs, cytochrome *c* expression, and caspase 3 cleavage. In addition, we have previously demonstrated that oxaliplatin treatment induces oxidative stress within the myenteric plexus determined by increased iNOS (McQuade et al., 2016b).

Given that the brainstem contains a number of nuclei involved in the regulation of vomiting, our pilot study aims to investigate the effects of oxaliplatin treatment on this particular region of the brain. We aim to determine whether oxaliplatin: 1) can penetrate the cerebrum and

brainstem and accumulate within both the nuclear and mitochondrial fractions; 2) induces changes in CTR1 expression within the brainstem; 3) alters the expression of DAMPs; 4) alters iNOS expression; 5) enhances cytochrome *c* expression, and 6) induces changes to mitochondrial electron transport chain proteins.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Male Balb/c mice (n=26, aged 5-8 weeks, weighing 18-25g) were used in this study. Mice had access to food and water *ad libitim* and were kept under a 12 hour light/dark cycle in a well-ventilated room at a temperature of 22°C. Mice acclimatized for up to 1 week prior to the commencement of *in vivo* intraperitoneal injections. All efforts were made to minimise animal suffering, to reduce the number of animals used, and to utilise alternatives to *in vivo* techniques, if available. All procedures in this study were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

4.3.2 *In vivo* intraperitoneal injections

Mice were separated into 2 cohorts (n=3-5/group): 1) vehicle (sterile water), 2) oxaliplatin (3mg/kg, Sigma-Aldrich, Australia). All mice received intraperitoneal injections (maximum of 200µL/injection) using 26 gauge

needles, tri-weekly for up to 14 days. Oxaliplatin dose was calculated per body mass, to be equivalent to human dosage (Elias et al., 2004, Renn et al., 2011). Mice were culled via CO_2 asphyxiation at day 14, followed by intracardiac perfusion with 1x PBS, and the brains were harvested for platinum accumulation studies. Additionally, mice were culled via CO_2 asphyxiation and the brainstems were collected for western blotting protein analysis.

4.3.3 Tissue homogenisation and subcellular fractionation (nuclear and mitochondrial isolation)

Following brain harvest, the cerebrum and brainstem were separated, and manually homogenised using a Potter-Elvehjem PTFE pestle and glass tube in solution containing: 100mM potassium chloride, 50mM tris(hydroxymethyl)aminomethane, 5mM magnesium chloride hexahydrate, 1.8mM adenosine triphosphate, 0.5mM ethylenediaminetetraacetic acid; all diluted in milliQ water, pH 7.2. Nuclear and mitochondrial fractions were isolated through centrifugation. Tissue homogenates were transferred to eppendorf tubes and centrifuged at 650G for 3 minutes at 4°C. The supernatant which contains the mitochondria was aliquoted to a separate eppendorf tube. The mitochondrial sample was centrifuged at 15,000G for 3 minutes at 4°C. The supernatant was discarded and the mitochondrial pellet was resuspended in 4mL of milliQ water. The pellet which contains the nuclei was resuspended in radioimmunoprecipitation assay lysis buffer (25mM

tris(hydroxymethyl)aminomethane hydrochloride, 150mM sodium chloride, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate; all diluted in milliQ water, pH 7.6), spun at 15,000G, and further diluted to 4mL in total with milliQ water.

4.3.4 Atomic absorption spectrophotometry

Once the samples were prepared, as described above, they were diluted to a volume of 4mL, to allow adequate sample volume for analysis of platinum and copper concentrations. Samples were then aspirated into a Shimadzu AA-6300 AAS. The specific AAS conditions used to carry out these analyses were as follows; both platinum and copper used an air-acetylene flame, with a fuel flow of 1.5L/min and an air flow 15L/min. The burner height was optimised for each element. Due to the analytical wavelengths used (265.9nm for platinum and 324.8nm for copper), background correction was required – this was supplied by a D2 lamp – and a slit width of 0.7nm was used. The lamp currents used were 25mA (platinum) and 6mA (copper). Samples were aspirated, with three repeat measurements recorded following an initial 2 second pre-spray time. Individual measurements were taken by averaging the absorbance readings over 3 seconds, which also allowed the calculation of a relative mean square percentage uncertainty. These three measurements were then averaged to give a final absorbance reading for each sample. Standard calibration curves were also produced on each day the samples were run, with concentration ranges of 10-40ppm (platinum) and 2-8ppm

(copper) utilised. Concentration values for the unknown samples were calculated automatically by the Shimadzu AAWizard software.

4.3.5 Laser ablation inductively coupled plasma mass spectrometry

LA-ICP-MS imaging was performed according to methods previously described (Hare et al., 2009, Hare et al., 2014, Paul et al., 2015). Saggital brain sections (10µm) were mounted on a standard microscope slide and placed in the ablation chamber of a NWR213 laser ablation system (Kennelec Scientific, Mitcham, Australia) with the gas outlet connected directly to an Agilent Technologies 8800 Series triple quadrupole ICP-MS (Mulgrave, Australia), operating in MS only mode. A 10µm diameter laser spot was rastered across the sample at a scan rate of 40µm s^{-1}. Parallel lines of ablation were spaced exactly 10µm apart to ensure complete ablation of the sample surface of the forebrain and medulla oblongata. The mass-to-charge ratio (*m/z*) of 195 was monitored. Data was exported as comma separated value (.csv) files, collated into a single image using a Python script and visualised in ENVI (Esri Australia, Brisbane, Australia). Images are presented as 256-bit colour maps depicting low-to-high signal intensities for *m/z* 195.

4.3.6 Western blotting

Brains were harvested and the brainstem was separated from the cerebrum and snap-frozen using liquid nitrogen. Frozen brainstem tissues

were homogenized with a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) for 20 seconds in ice-cold WB buffer (40mM Tris, pH 7.5; 1mM EDTA; 5mM EGTA; 0.5% Triton X-100; 25mM β-glycerophosphate; 25mM NaF; 1mM Na3VO4; 10 µg/ml leupeptin; and 1mM PMSF), and the whole homogenate was used for Western blot analysis. Sample protein concentrations were determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and equivalent amounts of protein from each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) acrylamide gels. Following electrophoretic separation, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% powdered milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h followed by an overnight incubation at 4°C with primary antibodies diluted in TBST containing 1% BSA **(Table 4.1)**. After the overnight incubation, the membranes were washed for 30 min in TBST and then probed with a peroxidase-conjugated secondary antibody for 1 h at room temperature. Peroxidase-conjugated anti-rabbit/mouse antibodies were purchased from Vector Laboratories (Burlingame, CA, USA). Following 30 min of washing in TBST, the blots were developed with a DARQ CCD camera mounted to a Fusion FX imaging system (Vilber Lourmat, Germany) using ECL Prime reagent (Amersham, Piscataway, NJ, USA). Densitometric measurements of the protein of interest were carried out using Fusion CAPT Advance software (Vilber Lourmat,

Germany). Membranes were then stained with Coomassie Blue, scanned and total protein loaded quantified using ImageJ (NIH; http://rsb.info.nih.gov/nih-image/). The signal intensity of the protein of interest was normalized to the signal for total protein loaded.

Table 4.1 Primary antibodies used for western blotting used in this study

Antibody	Species	Dilution	Source
CTR1	rabbit	1:1000	Abcam, USA
Calreticulin	mouse	1:1000	Abcam, USA
HMGB1	rabbit	1:1000	Abcam, USA
iNOS	rabbit	1:1000	Abcam, USA
Cytochrome *c*	rabbit	1:1000	Cell Signalling Technology (Denvers, MA)
Mitochondrial oxidative phosphorylation proteins (OxPhos)	mouse	1:1000	Cell Signalling Technology (Denvers, MA)

4.3.7 Statistical analysis

Statistical analysis of the data included a paired *t*-test using GraphPad PrsimTM v6.0 (GraphPad Software Inc, USA). The data are represented as mean ± SEM. Statistical significance was defined where the *P* value was less than 0.05.

4.4 RESULTS

4.4.1 Oxaliplatin-derived platinum accumulates within the cerebrum and brainstem, and reduces nuclear copper content in both regions

To determine platinum accumulation derived from oxaliplatin in the cerebrum and brainstem preparations, the AAS technique was employed. Detectable amounts of platinum were found in the nuclear (2.3 ± 0.4 ppm, *P*<0.01; n=3) and mitochondrial fractions (1.1 ± 0.3 ppm, *P*<0.05; n=3) of the cerebrum when compared to the vehicle-treated group (all negative) **(Figure 4.1 A-B)**. Furthermore, oxaliplatin treatment induced a significant reduction in copper content within the nuclear fraction (0.035 ± 0.007 ppm, *P*<0.05; n=3) when compared to the vehicle-treated cohort (0.056 ± 0.002 ppm, n=3) **(Figure 4.1 C)**. Oxaliplatin did not cause any changes in mitochondrial copper content (0.10 ± 0.009 ppm, n=3) when compared to the vehicle-treated group (0.17 ± 0.005 ppm, n=3) **(Figure 4.1 D)**. Platinum deposition within the forebrain was visualised using LA-ICP-MS, showing some concentrated regions of drug accumulation **(Figure 4.2)**.

A significant amount of oxaliplatin-derived platinum accumulated within the nuclear (1.96 ± 0.1 ppm, *P*<0.0001; n=3) and mitochondrial (2.1 ± 0.25 ppm, *P*<0.01; n=3) fractions of the brainstem **(Figure 4.3 A, B)**. Copper content within the nuclear fraction was significantly reduced following oxaliplatin treatment (0.017± 0.001 ppm, *P*<0.0001; n=3) when compared to the vehicle-treated cohort (0.039 ± 0.001 ppm, n=3) **(Figure**

4.3 C). However, oxaliplatin treatment did not affect mitochondrial copper concentrations (0.018 ± 0.001 ppm, n=3) when compared to the vehicle-treated group (0.017 ± 0.008 ppm, n=3) **(Figure 4.3 D)**. Platinum deposition within the brainstem was relatively widespread throughout this brain region **(Figure 4.4)**.

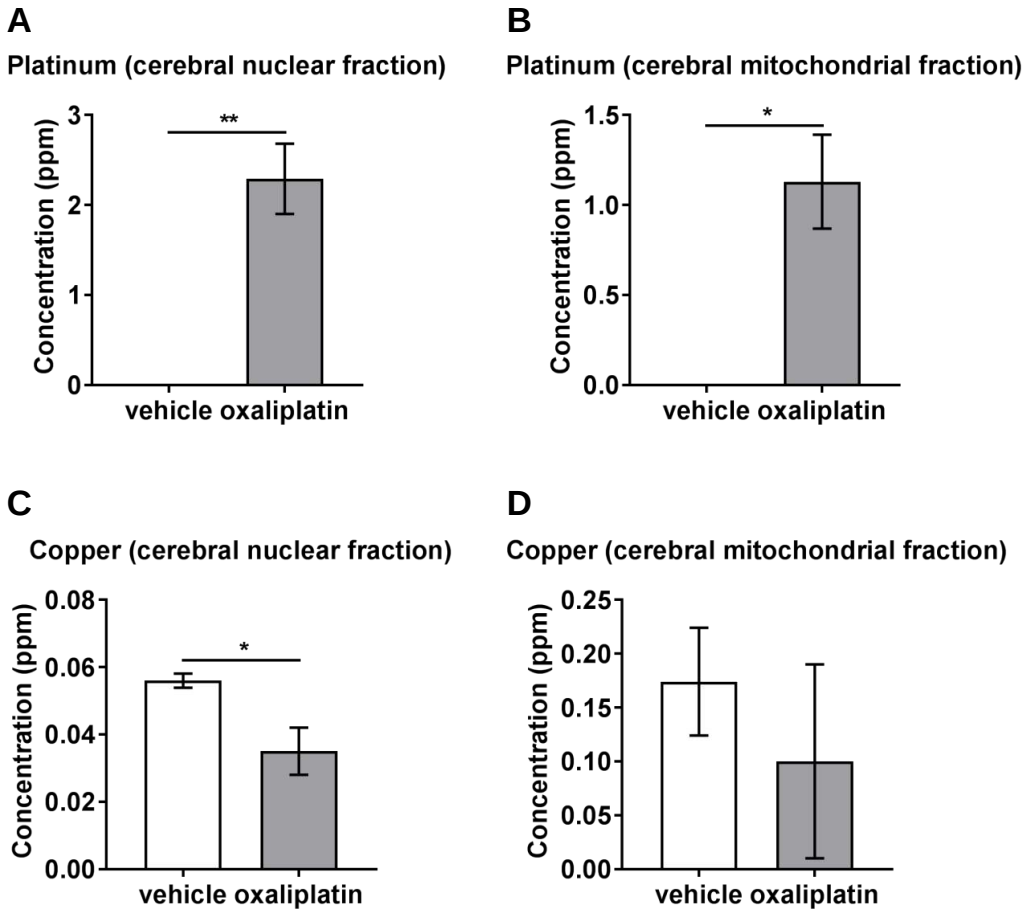


Figure 4.1. Oxaliplatin-derived platinum accumulates within the nuclear and mitochondrial fractions of the cerebrum, and disrupts copper content in the nuclear fractions. A significant amount of platinum was detected within the nuclear **(A)** and mitochondrial **(B)** fractions of the cerebrum following oxaliplatin treatment, when compared to the vehicle-treated mice which were all negative. Oxaliplatin treatment significantly reduced nuclear copper content **(C)** but did not affect mitochondrial concentrations **(D)**. *P<0.05; **P<0.01; n=3/group.

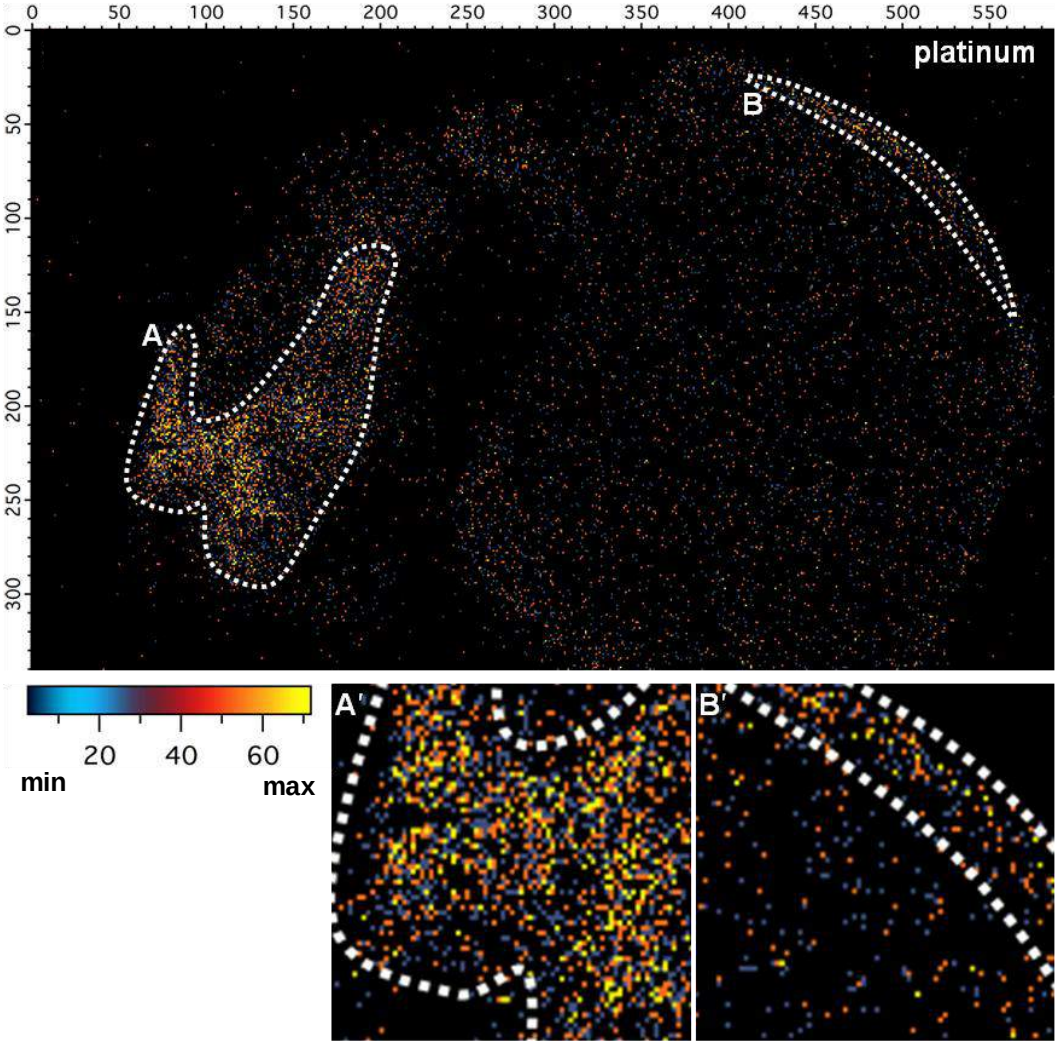


Figure 4.2. Elemental distribution map of platinum deposition within the forebrain. Laser ablation inductively coupled plasma mass spectrometry elemental distribution map of platinum deposition within the forebrain generated following oxaliplatin treatment. Platinum deposition is widely spread throughout the forebrain, with some areas displaying dense accumulation **(A-B)**.

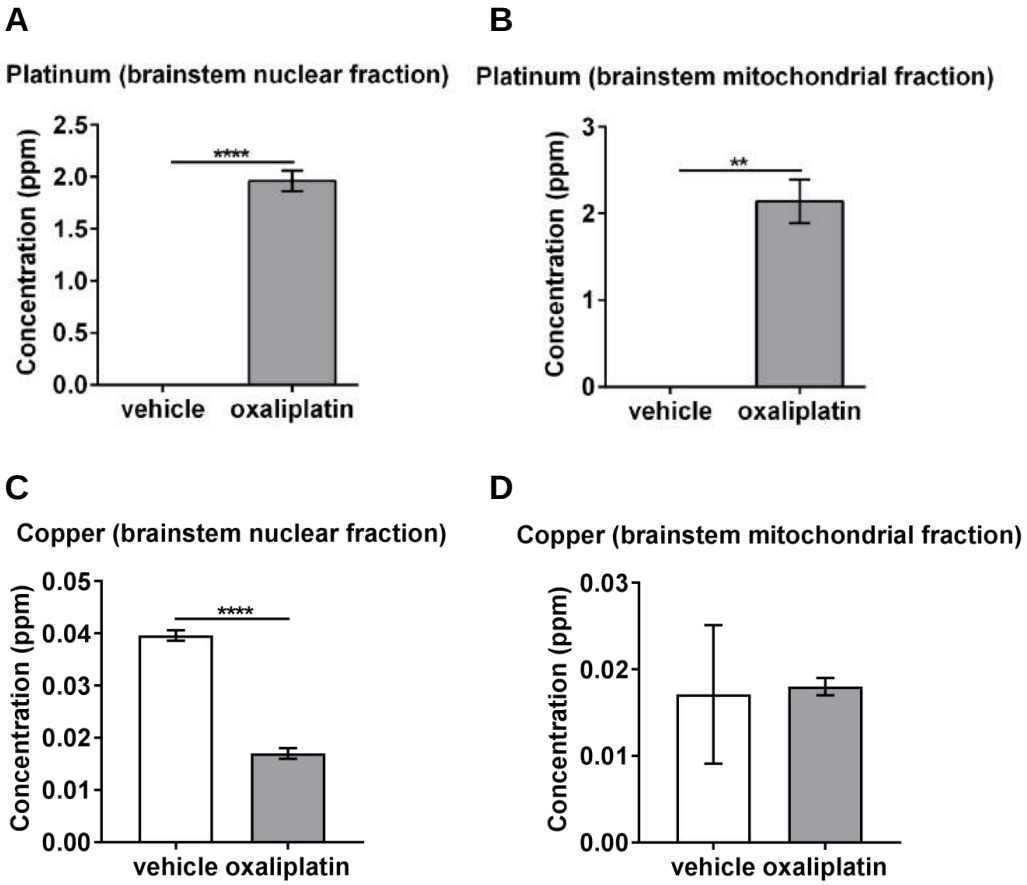


Figure 4.3. Oxaliplatin-derived platinum accumulates within the nuclear and mitochondrial fractions of the brainstem, and disrupts copper content in the nuclear fraction. A significant amount of platinum was detected within the nuclear **(A)** and mitochondrial **(B)** fractions of the brainstem following oxaliplatin treatment, when compared to the vehicle-treated mice which were all negative. Oxaliplatin treatment significantly reduced nuclear copper content **(C)** but did not affect mitochondrial concentrations **(D)**. ***P*<0.01; *****P*<0.0001; n=3/group.

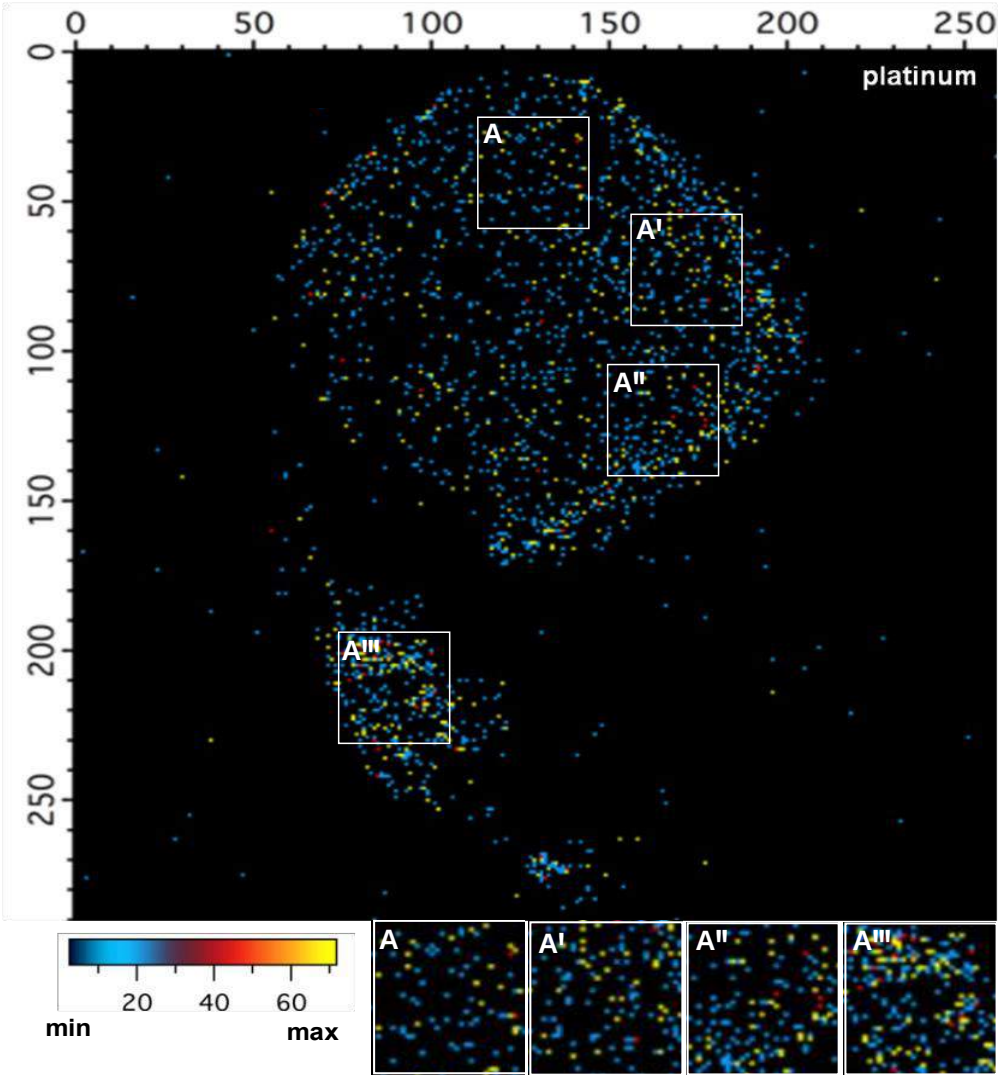


Figure 4.4. Elemental distribution map of platinum deposition within the brainstem. Laser ablation inductively coupled plasma mass spectrometry elemental distribution map of platinum deposition within the brainstem generated following oxaliplatin treatment. Platinum deposition is spread diffusely throughout the brainstem **(A-A''')**.

4.4.2 Oxaliplatin treatment causes a reduction in CTR1 expression within the brainstem

To investigate whether oxaliplatin treatment induces changes in CTR1 expression within the brainstem, western blotting was performed. Oxaliplatin treatment caused a significant reduction in CTR1 expression (0.8 ± 0.08 a.u, $P<0.05$; n=7) when compared to the vehicle-treated cohort (1.3 ± 0.09 ppm, n=4) **(Figure 4.5)**.

4.4.3 Oxaliplatin treatment does not alter the expression of DAMPs, calreticulin and HMGB1, nor does it change iNOS levels within the brainstem

To investigate whether oxaliplatin treatment induces changes in calreticulin, HMGB1, and iNOS expression within the brainstem, western blotting was performed. Oxaliplatin treatment did not alter calreticulin expression (0.45 ± 0.02 a.u, n=10) when compared to the vehicle-treated group (0.48 ± 0.04 a.u, n=4) **(Figure 4.6 A)**. Similarly, oxaliplatin treatment did not cause any changes in HMGB1 protein expression within the brainstem (0.76 ± 0.05 a.u, n=10) when compared to the vehicle-treated group (1.08 ± 0.05 a.u, n=4) **(Figure 4.6 B)**.

Moreover, oxaliplatin treatment did not alter iNOS expression within the brainstem (0.94 ± 0.02 a.u, n=10) when compared to the vehicle-treated cohort (0.99 ± 0.02 a.u, n=4) **(Figure 4.7)**.

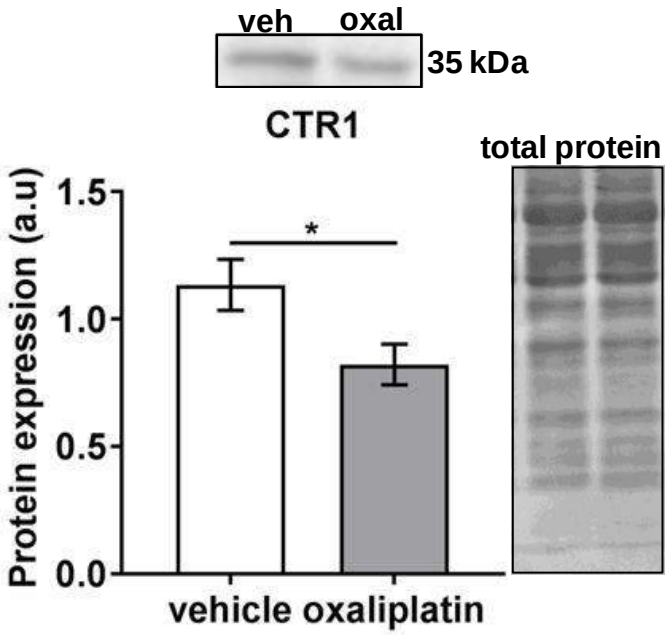


Figure 4.5. Oxaliplatin treatment causes a reduction in CTR1 expression in the brainstem. Oxaliplatin treatment significantly reduced CTR1 protein expression within the brainstem when compared to the vehicle-treated cohort. **P*<0.05; vehicle: n=4; oxaliplatin: n=7.

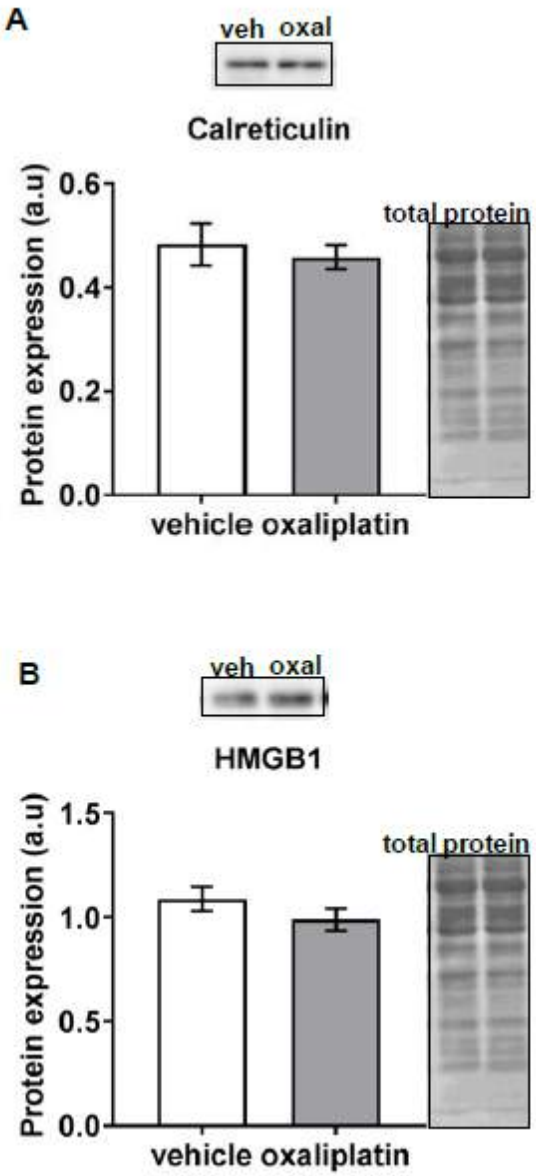


Figure 4.6. Oxaliplatin treatment does not alter the expression of DAMPs within the brainstem. No significant difference in calreticulin protein expression was observed between the vehicle-treated and oxaliplatin-treated groups **(A)**. Similarly, no significant difference in HMGB1 protein expression was observed between the vehicle-treated and oxaliplatin-treated cohorts **(B)**. Vehicle: n=4; oxaliplatin: n=10.

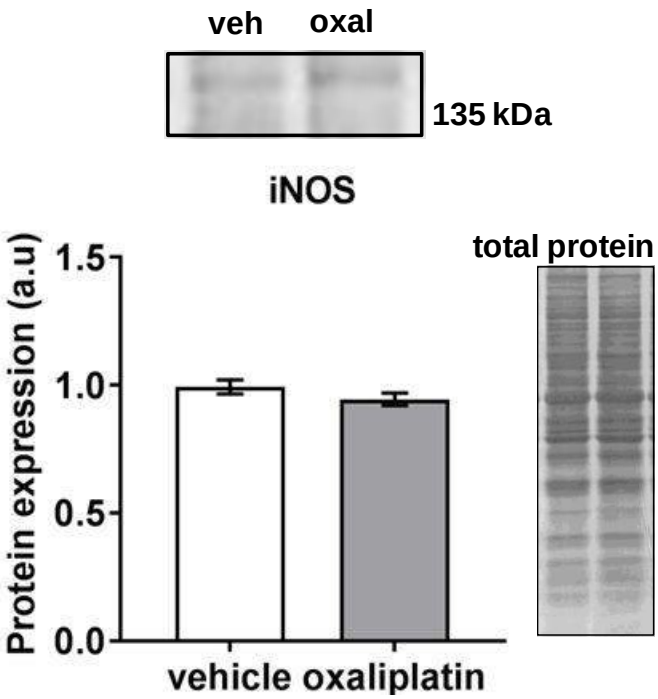


Figure 4.7. Oxaliplatin treatment does not alter iNOS expression within the brainstem. No significant change in iNOS protein expression within the brainstem was observed between the vehicle-treated and oxaliplatin-treated cohorts. Vehicle: n=4; oxaliplatin: n=9.

4.4.4 Oxaliplatin treatment upregulates cytochrome *c* expression, and reduces mitochondrial electron transport chain Complex I protein (NDUFB8) in the brainstem

To investigate changes in mitochondria following oxaliplatin treatment, we determined the expression of cytochrome *c* and mitochondrial OxPhos proteins. Oxaliplatin treatment caused a significant increase in cytochrome *c* expression in the brainstem (3.1 ± 0.06 a.u, $P<0.05$; n=7) when compared to the vehicle-treated cohort (2.7 ± 0.1 a.u, n=4) **(Figure 4.8 A)**. Moreover, oxaliplatin treatment caused a significant reduction in the Complex I subunit NDUFB8 (3.05 ± 0.08 a.u, $P<0.05$; n=7) when compared to the vehicle-treated group (3.8 ± 0.3 a.u, n=4) **(Figure 4.8 B)**. Oxaliplatin treatment did not cause any changes to Complex II (4.3 ± 0.07 a.u, n=8), Complex III (4.05 ± 0.08 a.u, n=9), Complex IV (5.4 ± 0.7 a.u, n=6), or Complex V (3.1 ± 0.04 a.u, n=9) subunits, when compared to the vehicle-treated cohort, respectively (Complex II: 4.36 ± 0.2 a.u; Complex III: 4.35 ± 0.3 a.u; Complex IV: 4.7 ± 0.4 a.u; or Complex V: 3.2 ± 0.2 a.u; n=4) **(Figure 4.8 B)**.

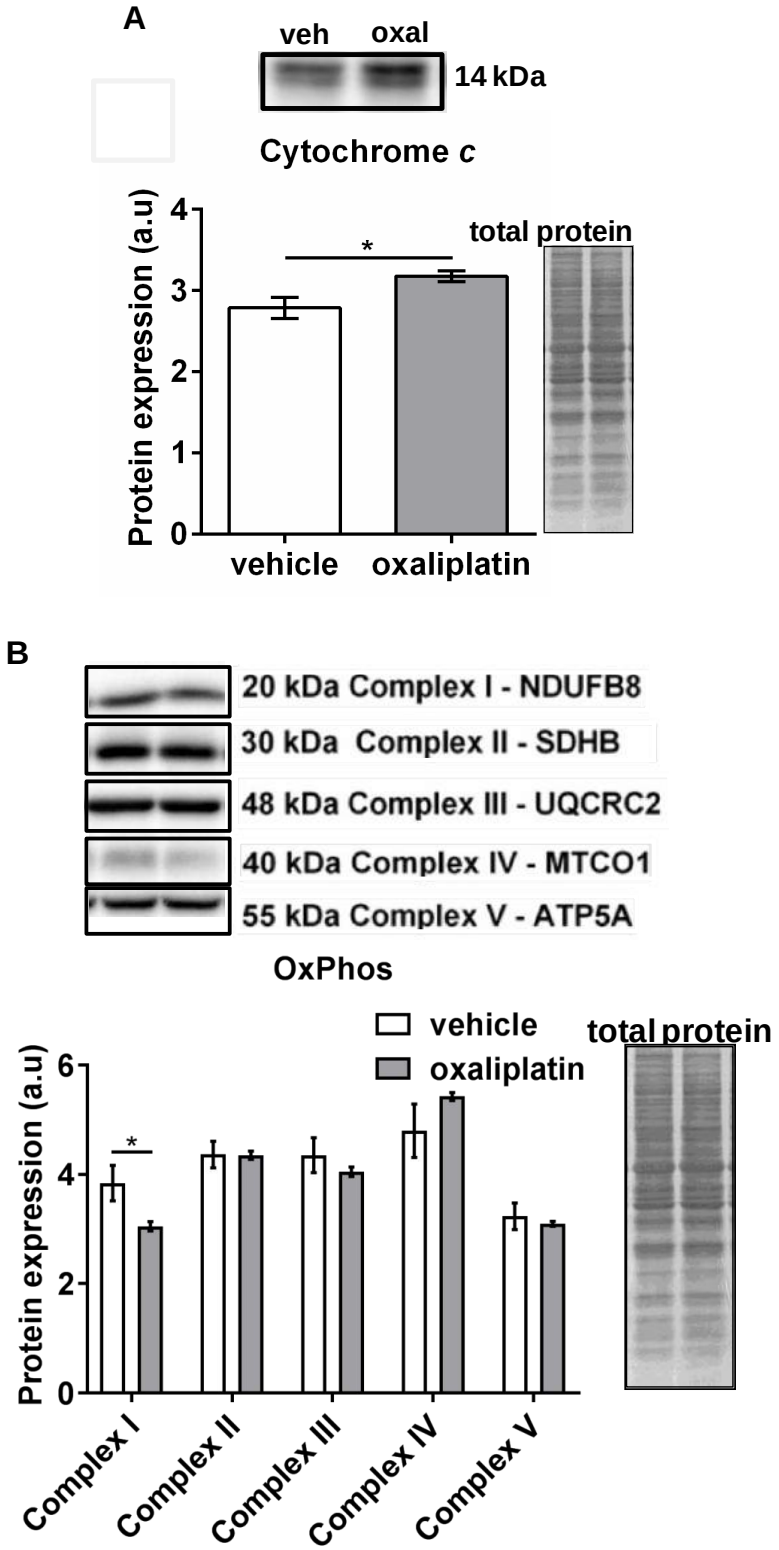


Figure 4.8. Oxaliplatin treatment upregulates cytochrome *c* protein expression, but downregulates mitochondrial OxPhos Complex I. Oxaliplatin treatment significantly enhanced cytochrome *c* protein expression within the brainstem when compared to the vehicle-treated cohort **(A)**. Oxaliplatin treatment significantly reduced Complex I protein expression within the brainstem when compared to the vehicle-treated cohort, with no other effects on Complexes II-V **(B)**. **P*<0.05; vehicle: n=4; oxaliplatin: n=7-9.

4.5 DISCUSSION

This study is amongst the first to demonstrate that platinum from oxaliplatin accumulates within the murine cerebrum and brainstem following treatment. Novel findings of the present study have shown: 1) oxaliplatin-derived platinum penetrates through the blood-brain barrier and accumulates in the cerebrum as well as the brainstem; 2) platinum can be localised within nuclear and mitochondrial fractions in both the cerebrum and brainstem; 3) oxaliplatin reduces nuclear copper content in both brain regions; 4) oxaliplatin treatment reduces CTR1 protein expression within the brainstem; 5) oxaliplatin upregulates cytochrome *c* expression and reduces mitochondrial OxPhos Complex I protein expression within the brainstem.

In this present study we have shown that platinum from oxaliplatin has the capacity to penetrate the cerebrum and brainstem. This was evidenced by the visualisation of platinum tissue distribution, and by determining the amount of platinum accumulating within the nuclear and mitochondrial fractions of these tissue regions. Previous work has demonstrated that both cisplatin and oxaliplatin have the capacity to penetrate into the brain in a rat model of glioblastoma (Charest et al., 2013). Platinum from cisplatin and oxaliplatin in this glioblastoma model has also been found within nuclear and cytosolic fractions of the astrocytes (Charest et al., 2013). As previously mentioned in Chapter 3, nuclear and mitochondrial platinum adducts have been found in DRG neurons following cisplatin and oxaliplatin treatment (Ta et al., 2006). It is

widely accepted that platinum accumulation within nuclear and mitochondrial fractions leads to cellular injury and death.

Although central nervous system side-effects associated with oxaliplatin treatment are quite rare, reversible encephalopathy syndrome has been associated with the FOLFOX regimen (Porcello Marrone et al., 2013). It is well established that chemotherapeutic agents are associated with cognitive and behavioural problems (Vichaya et al., 2015). We did not measure these indices following oxaliplatin treatment, but platinum accumulation within the cerebrum would presumably affect these neurological functions. Previous work has shown a long-term cognitive decline of rats measured by novel object and location recognition studies, 11 months post-oxaliplatin treatment (Fardell et al., 2015). These neurological effects are dose-dependent and exacerbate at higher doses of oxaliplatin (Fardell et al., 2015). Long-term and delayed side-effects associated with oxaliplatin treatment have been attributed to a ‘coasting’ phenomenon, whereby the side-effects worsen months after treatment, and is thought to reflect greater drug accumulation (Windebank and Grisold, 2008, Grisold et al., 2012, Pachman et al., 2015). Nausea and vomiting are prominent symptoms associated with oxaliplatin treatment.

The brainstem may be particularly vulnerable to oxaliplatin treatment, given that it is a circumventricular organ that allows for greater exposure to circulating toxins and anti-cancer drugs. In particular, the area postrema (also referred to as a chemoreceptor trigger zone) is a highly vascularised region lacking a true blood brain barrier (Travagli et al., 2006,

Duvernoy and Risold, 2007, Kaur and Ling, 2017). The area postrema signals to the NTS, the major sensory nuclei, which then propagates signals to the DMNX. Thus, noxious chemicals within the blood such as anti-cancer agents can provoke emetic responses (Di Fiore and Van Cutsem, 2009, Rojas and Slusher, 2012). Anti-emetic agents pharmacologically target neuronal receptors to inhibit the act of vomiting (Rojas and Slusher, 2012, Rojas et al., 2014). However, the efficacy of these agents may be hindered if neuronal injury becomes apparent following anti-cancer therapy. The NTS of the medulla oblongata is a major termination site for vagal afferents relaying information about the physiological milieu of the gastrointestinal tract. Neurons from the NTS synapse with preganglionic parasympathetic motorneurons from the DMNX which project to the viscera and stimulate appropriate responses (Babic and Browning, 2014). Impaired processing of peripheral inputs from the NTS may therefore affect efferent responses by neurons from the DMNX synapsing with neurons in the ENS. Neurons from the NTS also synapse with premotor neurons of the brainstem such as those located within the ventral respiratory groups, the nucleus ambiguus, and the parabrachial nuclei, all of which are involved in coordinating respiratory functions and muscle contractions essential for mediating emetic responses (Horn, 2008). We appreciate that mice are not an appropriate model for studying vomiting as they do not exhibit this reflex, but unpublished observations within our lab demonstrate that mice receiving oxaliplatin treatment develop pica (equivalent to nausea and vomiting in

humans) measured by known consumption of kaolin. Our work highlights the general toxicity to the brainstem which could affect a multitude of autonomic functions not limited to those of the gastrointestinal tract.

There are many studies which have associated CTR1 expression with platinum-based drug sensitivity and resistance (Lin et al., 2002, Holzer et al., 2006, More et al., 2010, Kim et al., 2014). There is mounting evidence to show that the CTR1 receptor can be recycled upon ligand stimulation, which becomes particularly evident following the administration of platinum-based anti-cancer agents and is suggestive of direct drug toxicity (Ishida et al., 2002, Holzer and Howell, 2006). Similar to the results observed in the myenteric neurons in Chapter 3, here we demonstrate that oxaliplatin treatment causes a reduction in CTR1 expression within the brainstem, this too may be indicative of direct platinum drug influx and toxicity. Furthermore, we have demonstrated that oxaliplatin treatment causes a reduction in copper content within the cerebral and brainstem nuclear fractions, with no demonstrable effects on mitochondrial pools. Copper is an inorganic element which is neither created or degraded by the body, and thus, homeostatic levels of this metal must be strictly regulated (Festa and Thiele, 2011). As previously mentioned in Chapters 1 and 3, intracellular copper content is associated with CTR1 expression. High copper content leads to a reduction in CTR1, whereas low copper pools increases receptor expression. Although nuclear copper content was reduced, we still observed a reduction in CTR1 expression following oxaliplatin treatment. Whether changes in

copper pools in one or more organelles is required to induce changes in CTR1 expression requires further investigation. Nevertheless, copper is an essential biometal involved in many biological processes (Gaetke et al., 2014). Since copper plays a role in myelin production and maintenance, neurotransmitter synthesis and synaptic transmission, however, it can be speculated that neurotransmitter synthesis, synaptic transmission and gene transcription may be altered.

Studies have also shown that copper may modulate gene expression by binding to DNA (Peña et al., 1998, Winge, 1998, Canessa et al., 2008). It is currently unclear how the oxaliplatin-induced reduction in nuclear copper content within the brain affects its functions.

Copper is a critical component of the mitochondrial electron transport chain complex cytochrome *c* oxidase, and is also essential for free radical scavenging being a cofactor for the synthesis of copper-zinc superoxide dismutase (Gaetke et al., 2014, Opazo et al., 2014). Cytochrome *c* oxidase is essential for the one-electron step reduction of molecular oxygen to molecular water (Kühlbrandt, 2015). In this study, mitochondrial copper content remained demonstrably normal at this time-point following oxaliplatin treatment. The mitochondrial matrix contains copper reserves which far exceed the needs of cytochrome *c* oxidase. This may explain why no changes in mitochondrial copper content were observed following oxaliplatin treatment. Although mitochondrial copper content did not change following oxaliplatin treatment in our studies, it is unknown whether this biometal is still redirected towards cytochrome *c*

oxidase, or whether it engrafts within this respiratory complex. It has been proposed that platinum-based drugs can “hitch-hike” or “hijack” copper chaperones and thus, be shuttled to various intracellular compartments which may alter copper status, and potentially, other trace metal dynamics (Palm-Espling et al., 2013).

A hallmark feature of oxaliplatin-induced cytotoxicity is the presentation of the DAMPs, calreticulin and HMGB1. However, no demonstrable changes in DAMPs were observed in the brainstem. This is in contrast to the increased expression and altered intracellular localisation of DAMPs within the myenteric neurons of the colon shown in Chapter 3. The expression of DAMPs in the brain following oxaliplatin treatment has not been studied previously, and thus, we cannot make comparisons of our findings. Furthermore, no changes in iNOS expression within the brainstem were observed following oxaliplatin treatment. This is again in contrast to our observations within the longitudinal muscle-myenteric plexus of the colon following oxaliplatin treatment (McQuade et al., 2016b). Previous work has demonstrated that oxaliplatin induces iNOS expression within the spinal cord (Azevedo et al., 2013). However, the effects of oxaliplatin treatment (or other platinum-based drugs) on iNOS expression within the brain is lacking. iNOS is induced in response to a number of stimuli, notably, inflammation and ischemic events (Iadecola et al., 1995, Kröncke et al., 1998, Garcia-Bonilla et al., 2014). Given that there were no changes in DAMPs which can result in immunogenic cell death, this may explain why no change in iNOS expression was observed

following oxaliplatin treatment. It remains unclear why no changes in DAMPs or iNOS are apparent within the brainstem; however, we speculate that there may be a delayed neurotoxic effect. Perhaps greater accumulation of the drug or a different experimental time-point would reveal varying indices of tissue injury characterised by DAMPs and/or oxidative and nitrosative stress, or possibly other pathways for cellular damage and death are involved. Whether the central nervous system has greater DNA repair mechanisms remain to be investigated.

In this study we have shown that oxaliplatin treatment causes an increase in cytochrome *c* expression, and a reduction in mitochondrial electron transport chain Complex I in the brainstem. Elevated cytochrome *c* levels can initiate the intrinsic apoptotic cascade (Wang and Youle, 2009). Cytochrome *c* binds to the apoptotic protease activating factor 1 and caspase 9, which collectively is known as the apoptosome (Cain et al., 2002, Baliga and Kumar, 2003). Caspase 9 then cleaves caspases 3 and 7 which results in apoptotic cell death (Ichim and Tait, 2016). We have performed western blots probing for cleaved caspase 3 but could not reliably detect protein expression. Given that cleaved caspase 3 has a relatively short half-life of 8 hours, this may explain why its expression was beyond our detection limits of western blotting.

Interestingly, we observed a significant reduction in protein expression of the mitochondrial electron transport chain Complex I. The mitochondrial electron transport chain contains a number of OxPhos complexes. These include Complex I (nicotinamide adenine dinucleotide

(NADH): ubiquinones oxidoreductase), Complex II (succinate dehydrogenase); Complex III (ubiquinol-cytochrome *c* oxidoreductase), Complex IV (cytochrome *c* oxidase), and Complex V (ATP synthase) (Sharma et al., 2009, Jastroch et al., 2010). Complex I is a major site for electron acceptance from nicotinamide adenine dinucleotide (NADH), which then feeds these electrons into the mitochondrial respiratory chain (Ripple et al., 2013). The energy released from the electron transfer to Complex II is then used to pump protons from the mitochondrial matrix into the crista lumen (Kuhlbrandt, 2015). Electrons are then transferred to cytochrome *c*, the soluble electron carrier that shuttles between Complex III and Complex IV and participates in the reduction of molecular oxygen to molecular water. Furthermore, the transfer of electrons maintains the proton gradient which drives ATP production and maintains mitochondrial membrane potential (Mourier and Larsson, 2011). Although Complex II is also an electron acceptor, it does not contribute to the mitochondrial proton gradient, and thus, does not have major impact on ATP output (Osellame et al., 2012, Kuhlbrandt, 2015). The reduction in Complex I protein expression can severely impact ATP production through an impaired proton gradient and a reduction in mitochondrial membrane potential, and thus, lead to intracellular energy crisis and death. In such circumstances, a switch to anaerobic respiration may occur in an attempt to produce ATP (Solaini et al., 2010). Mitochondrial Complex I deficits have been previously observed following anti-cancer chemotherapy including platinum-based agents such as oxaliplatin, as is shown to be

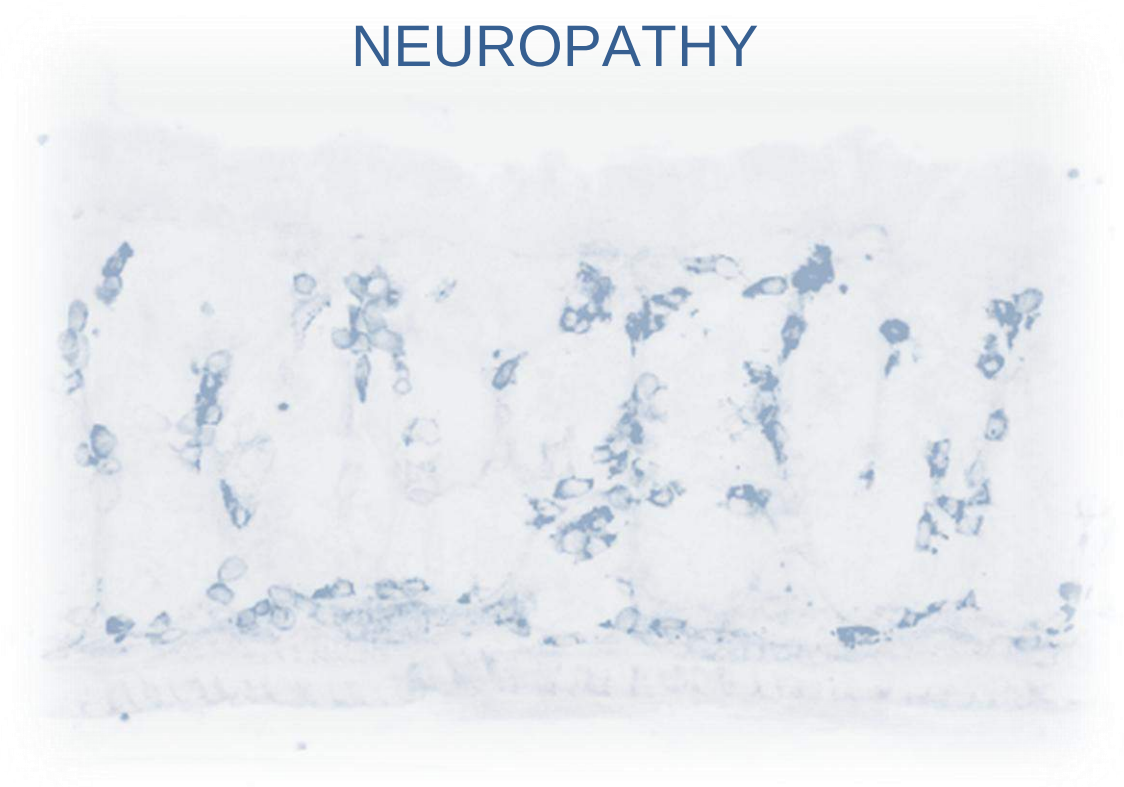
implicated in the mechanisms of painful peripheral neuropathy (Zheng et al., 2011, Xiao and Bennett, 2012). A reduction in mitochondrial Complex I proteins also means there would be less acceptance of electrons, and thus, could lead to excessive electron leak. Electrons are highly reactive radicals which can collide with oxygen to form superoxide, a co-factor that participates in a number of reactions for the formation of other reactive oxygen and nitrogen species. Although we did not observe any changes in iNOS within the brainstem, it is unknown whether nitric oxide status has changed, and whether other oxidative and/or nitrosative radicals have been formed. Future work should aim to investigate the expression of mitochondrial superoxide, nitric oxide, nitrotyrosine, hydroxyl radicals, and lipid peroxidation expression within the brainstem following oxaliplatin treatment to determine their implication in the mechanisms of tissue injury.

In summary, we reveal that oxaliplatin has the capacity to penetrate the blood-brain barrier, and accumulate in the nuclear and mitochondrial fractions of the cerebrum and brainstem, alter intranuclear copper levels, cause a reduction in brainstem CTR1 expression, and induce mitochondrial toxicity within the brainstem as evidenced by changes in cytochrome *c* and mitochondrial electron transport chain proteins (particularly Complex I). Oxaliplatin treatment did not alter the expression of DAMPs and iNOS, however, it is unclear whether these are time-dependant changes and may present at earlier/later time-points, or following longer chemotherapeutic administration. Overall, these data demonstrate that oxaliplatin can induce tissue injury, particularly to the

brainstem which not only regulates gastrointestinal reflexes such as nausea and vomiting, but also other important autonomic functions. Furthermore, as anti-emetic treatments are pharmacologically target neurons within the brainstem, toxicity to this brain region may impact the efficacy of these treatments.

5

INVESTIGATING THE POTENTIAL FOR OXALIPLATIN-INDUCED IMMUNOGENIC CELL DEATH AS A MECHANISM OF MYENTERIC NEUROPATHY



5.1 SUMMARY

Oxaliplatin is a platinum-based chemotherapeutic used for cancer treatment. Its use associates with peripheral neuropathies and chronic gastrointestinal side-effects. In Chapters 2 and 3 we have shown that oxaliplatin treatment caused significant damage to the ENS within the colon, and evoked the presentation of damage associated molecular DAMPs in myenteric neurons. The DAMP high mobility group box 1 (HMGB1) protein exerts pro-inflammatory cytokine-like activity and binds to toll-like receptors (namely TLR4). Gastrointestinal microbiota may influence chemotherapeutic efficacy and contribute to local and systemic inflammation. We studied effects of oxaliplatin treatment on 1) TLR4 and HMGB1 expression within the colon; 2) gastrointestinal microbiota composition; 3) inflammation within the colon; and 4) changes to immune populations in Peyer's patches (PPs) and mesenteric lymph node (MLNs) in mice. $TLR4^+$ cells displayed pseudopodia-like extensions characteristic of antigen sampling colocalised with HMGB1-overexpressing cells in the colonic lamina propria from oxaliplatin-treated animals. No $CD45^+$ or $TLR4^+$ cells were observed at the level of enteric ganglia. Oxaliplatin treatment caused a significant reduction in *Parabacteroides* and *Prevotella*$_1$, but increase in *Prevotella*$_2$ and *Odoribacter* gram-negative bacteria at the genus level. Downregulation of pro-inflammatory cytokines and chemokines in colon samples, no changes in immune populations in PPs, and a reduction in macrophages and dendritic cells in MLNs were found following oxaliplatin treatment. In conclusion, oxaliplatin treatment

caused morphological changes in TLR4+ cells, an increase in gram-negative microbiota species and enhanced HMGB1 expression associated with immunosuppression in the colon. These data suggest that enteric neuropathy following oxaliplatin treatment is not driven by gastrointestinal inflammation, but involves other mechanisms.

5.2 INTRODUCTION

Platinum-based chemotherapeutic agents are widely used for the treatment of cancer, and oxaliplatin, the third generation drug, is primarily used as the first-line treatment for colorectal malignancies (Alcindor and Beauger, 2011, McQuade et al., 2014). Platinum-based drugs mediate their cytotoxic effects via the formation of nuclear and mitochondrial DNA platinum adducts which ultimately affect cell viability and hinder prospective replication (Yang et al., 2006, Podratz et al., 2011, Canta et al., 2015). Despite its therapeutic efficacy, the use of oxaliplatin causes unfavourable side-effects which include, but are not limited to, peripheral sensory neuropathy and gastrointestinal dysfunction (Di Fiore and Van Cutsem, 2009, Weickhardt et al., 2011, Wafai et al., 2013, McQuade et al., 2014, McQuade et al., 2016c). These side-effects are major hurdles for cancer treatment as they result in dose reductions, treatment non-compliance and cessation (Stein et al., 2010, Weickhardt et al., 2011, Boussios et al., 2012). Whilst the peripheral sensory neuropathy caused by oxaliplatin has attracted a large research focus, there are limited studies investigating the effects of this drug on gastrointestinal

dysfunction. Only recently, the ENS has gained attention regarding its role in the multifaceted pathophysiology of gastrointestinal dysfunction following chemotherapeutics (Wafai et al., 2013, Stojanovska et al., 2015, McQuade et al., 2016c). The ENS is an intrinsic and intricate neuronal network embedded throughout the gastrointestinal tract which regulates secretion, absorption, vasomotor tone and motility (Furness, 2012). The ENS can anatomically be divided into two major plexuses; the submucosal and myenteric. A few studies to date have shown that oxaliplatin induces myenteric neuronal loss, changes in the proportion of neuronal phenotypes, oxidative stress and causes changes in gastrointestinal transit and motility leading to constipation (Wafai et al., 2013, McQuade et al., 2016c, Robinson et al., 2016). However, the mechanisms underlying oxaliplatin-induced changes in the myenteric plexus and cell death remain to be elucidated.

It is well established that anti-cancer agents induce damage to the gastrointestinal mucosa which may cause dysbiosis of commensal microbiota and potentiate inflammation (Round and Mazmanian, 2009, Belkaid and Hand, 2014, Poutahidis et al., 2014, Goldszmid et al., 2015, Zitvogel et al., 2015). A number of studies have reported microbiota dysbiosis following treatment with the anti-cancer chemotherapeutic agents, irinotecan and 5-fluorouracil (Stringer et al., 2008, Stringer et al., 2009b, Stringer et al., 2009c, Lin et al., 2012). However, the effects of platinum-based drugs on gastrointestinal microbiota remain largely unexplored. Gastrointestinal inflammation has been associated with

persistent alterations in enteric neuron function and neuronal loss (Linden et al., 2005, Lomax et al., 2007, Nurgali et al., 2011). The gastrointestinal tract in particular is equipped with lymphoid organs (PPs and MLNs) which houses ~70% of the body's immunocytes, thus highlighting their important role in discriminating between innocuous and noxious pathogens or danger signals (Jung et al., 2010). Further adding to this complexity, oxaliplatin is regarded as a potent inducer of immunogenic cell death. Apoptosis has long been considered as an immunologically silent or tolerogenic event, however, oxaliplatin treatment has been shown to induce beneficial anti-cancer immune responses through the induction of DAMPs in colorectal cancer cells (Tesniere et al., 2010). The presentation of DAMPs can prompt the engulfment of dying cells by phagocytes, or apoptotic antigens may be presented to T cells for targeted elimination (Tesniere et al., 2010, Hato et al., 2014, Bezu et al., 2015). A classical DAMP is the nuclear-resident non-histone protein HMGB1 which exerts pro-inflammatory cytokine-like activity once cytoplasmically translocated and/or released into the extracellular environment by damaged cells (Klune et al., 2008, Tesniere et al., 2010, Hato et al., 2014, Bezu et al., 2015, Yang et al., 2015). HMGB1 is a ligand for toll-like receptors and is presented to T cells for priming and activation (Klune et al., 2008, Hato et al., 2014). Both DAMPs and pathogen-associated molecular patterns (which recognise microbial endotoxins) can induce an immunological response following anti-cancer chemotherapy (Iida et al., 2013, Viaud et al., 2015). It is unknown whether gastrointestinal inflammation may be

implicated in enteric neuropathy associated with oxaliplatin treatment, either directly or inadvertently.

Herein, we determined the effects of oxaliplatin treatment on 1) TLR4 and HMGB1 expression within the colon; 2) gastrointestinal microbiota composition; 3) inflammation within the colon; and 4) changes in immune populations within the PPs and MLNs.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Male Balb/c mice (n=40, aged 7-8 weeks, weighing 18-25g) were used in this study. Mice had access to food and water *ad libitim* and were kept under a 12 hour light/dark cycle in a well-ventilated room at a temperature of 22°C. Mice acclimatized for up to 1 week prior to the commencement of *in vivo* intraperitoneal injections. All efforts were made to minimise animal suffering, to reduce the number of animals used, and to utilise alternatives to *in vivo* techniques, if available. All procedures in this study were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Australian *Code of Practice for the Care and Use of Animals for Scientific Purposes.*

5.3.2 *In vivo* intraperitoneal injections

Mice were separated into 2 cohorts (n=4-10/group): 1) vehicle-treated (sterile water), 2) oxaliplatin-treated (3 mg/kg, Sigma-Aldrich, Australia).

All mice received intraperitoneal injections (maximum of 200 µL/injection) using 26 gauge needles tri-weekly totaling 6 injections. Dosages were calculated per body mass as previously published, to be equivalent to human dosage (Elias et al., 2004, Renn et al., 2011). Mice were culled via cervical dislocation 14 days subsequent to their first intraperitoneal injection, and the colon, PPs, and MLNs were harvested.

5.3.3 Immunohistochemistry

The colon was harvested (n=4/group), cut along the mesenteric border and pinned to silicone-based petri dishes containing 1x PBS. Tissues were incubated in Zamboni's fixative (2 % formaldehyde, 0.2% picric acid and 0.1M sodium phosphate buffer (pH 7.0)) overnight at 4°C. The following day, tissues were washed 3 x 10 minutes in 100% DMSO, followed by 3 x 10 minute washes with 1x PBS. Sections (30µm) were cut and incubated with a mouse blocking reagent (M.O.M.^{TM} kit, Vector Labs, USA), or 10% normal donkey serum for 1 hour at room temperature, then washed 3 x 10 minutes with PBS-T. Sections were co-labelled with anti-TLR4 (mouse, 1:500, Abcam, USA) and anti-HMGB1 (rabbit, 1:500, Abcam, USA) antibodies, or with anti-CD45 (mouse, 1:500, Abcam, USA) antibody alone. Primary antibodies were incubated at room temperature overnight, and were then washed 3 x 10 minutes with PBS-T. The secondary antibody for both TLR4 and CD45 was FITC-conjugated (mouse, 1:200, Abcam, USA) made up in M.O.M.^{TM} diluents; and the secondary antibody for HMGB1 was AlexaFluor-647-conjugated (rabbit, 1:200, Jackson

Immuno-Research, USA). Secondary antibodies were incubated at room temperature for 2 hours and then washed 3 x 10 minutes with PBS-T. Sections were mounted onto glass slides using an anti-fade mounting medium (DAKO, Australia).

5.3.4 Imaging and analysis

Three-dimensional (z-series) images of the colon cross sections were taken using an *Eclipse Ti* confocal microscope (Nikon, Japan). Excitation wavelengths were set to 473nm for FITC and 640.4nm for Alexa Fluor 647. The number of $CD45^+$ cells was counted from 8 images/preparation taken at 20x magnification with a total area of $2mm^2$. $CD45^+$ immunoreactivity was measured from 8 images/preparation taken at 20x magnification with a total area of $2mm^2$. All images were captured under identical conditions, calibrated to standardise minimum baseline fluorescence, and were converted to binary. Differences in fluorescence from baseline were measured using Image J software (National Institute of Health, USA). All images were coded and analyzed blindly.

5.3.5 Fecal DNA isolation

Fecal pellets were collected from vehicle- and oxaliplatin-treated animals (5-8 weeks of age; n=10/group), and stored at -80°C until time of processing. The PowerFecal® DNA Isolation Kit (MO BIO Laboraties Inc, Australia) was used to obtain DNA from the fecal pellets as per manufacturer's instructions. Briefly, 0.25g of fecal sample was

homogenised using a beaded tube containing 60µL of Solution C1 (sodium dodecyl sulfate solution), vortexed, and heated at 65°C for 10 minutes. Samples were then vortexed at maximum speed for 10 minutes at room temperature, followed by centrifugation at 13,000 x *g* for 1 minute. The supernatant was collected, and 250µL of Solution C2 (Inhibitor Removal Technology® (IRT) reagent) was added to precipitate non-DNA organic and inorganic material. Samples were centrifuged at 13,000 x *g* for 1 minute, the supernatant was collected, and the pellet which contains non-DNA organic and inorganic material was discarded. 1200µL of Solution C4 (concentrated salt solution) was added to the supernatant, followed by a 5 second vortex. 650µL of supernatant was loaded onto a Spin Filter, and centrifuged at 13,000 x *g* for 1 minute. 500µL of Solution C5 (ethanol-based solution) was added, and samples were centrifuged at 13,000 x *g* for 1 minute. The flow through filtered solution was discarded, and samples were centrifuged again at 13,000 x *g* for 1 minute. The Spin Filter was placed in a clean collection tube and 100µL of Solution C6 (sterile elution buffer) was added, and samples centrifuged at 13,000 x *g* for 1 minute. The isolated fecal DNA was frozen in -80°C until time of high-throughput sequence analysis.

5.3.6 High-throughput sequence analysis of fecal microbiota

The samples underwent high throughput sequencing on the Illumina MiSeq platform at the Australian Genome Research facility (University of Queensland, Brisbane, Australia). Paired-end reads were assembled by

aligning the forward and reverse reads using join_paired_ends.py. Primers were trimmed using Seqtk (version 1.0). Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.9) 4 USEARCH2,3 (version 8.0.1623) software (Caporaso et al. 2010). Briefly, de-multiplexing and quality filtering were performed using the split_libraries_fastq.py script for each data set. Operational Taxonomic Units (OTUs) were de novo picked at 97% sequence similarity following the usearch pipeline and representative sequences of each cluster were used to assign taxonomy through matching against the Blast 2.2.22 database. Evaluations present at each taxonomic level, including percentage compilations, represent all sequences resolved to their primary identification or their closest relative. Alpha diversity using alpha_diversity.py script was performed for species richness, Good's coverage, Chao1, Shannon-Wiener's diversity index and Simpson's index of diversity. Weighted and unweighted UniFrac distance matrices were obtained through Jack-knifed beta diversities in QIIME and principal coordinate analysis (PCoA) plots were obtained. Sample clustering and statistical analysis were carried out in R environment and SPSS (version 23).

5.3.7 Myeloperoxidase activity

Colon tissue from vehicle- and oxaliplatin-treated animals (n=3/group) were harvested and homogenised in 4 volumes of myeloperoxidase (MPO) assay buffer using a FastPrep24TM5G homogenizer and matrix D

lysing tubes (MP Biomedicals, Australia) for 40 seconds. The supernatant was transferred into collecting tubes, and centrifuged at 13,000 x *g* for 10 minutes at 4°C. Tissue protein levels were quantified using the bicinchoninic acid assay (Thermo Scientific, Australia) according to manufacturer's instructions and absorbance was read at 526nm using a VarioskanTM Flash Multimode Reader (Thermo Scientific, Australia) using SkanIt software v.2.4.3. The MPO Colorimetric Activity Assay (Sigma-Aldrich, Australia) protocol was followed as per manufacturer's instructions. Briefly, 150µL, 140µL, 130µL, 120µL, 110µL, and 100µL of the MPO Assay Buffer was loaded in duplicate into a 96 well plate (Corning®, U-bottom 96-well plates (Sigma-Aldrich, Australia) with 5,5'-Dithio-bis (2-nitrobenzoic acid; DTNB) standard at 10µL, 20µL, 30µL, 40µL and 50µL (1nmol/well) respectively. 10µL of the MPO positive control was added to 40µL of MPO assay buffer, the MPO substrate was omitted for the negative control, and the blank well only contained MPO Assay Buffer. 50µL of each sample was loaded to triplicate wells. 50µL of the MPO Reaction Mix was added to each sample well, except for the standards. Samples were incubated for 60 minutes at room temperature, protected from light. 2µL of MPO Stop Mix was added to appropriate wells, and incubated for 10 minutes at room temperature. Absorbance was read at 412nm using a VarioskanTM Flash Multimode Reader (Thermo Scientific, Australia) using SkanIt software v.2.4.3. All standards and samples were corrected for background absorbance readings. A standard curve plotted and the amount of TNB consumed by the enzyme assay for each sample

was determined. MPO activity was calculated as per manufacturer's instructions: MPO Activity = B Sample Dilution Factor / (Reaction Time) x V. MPO activity is reported as nmol/min/mL = milliunit/mL.

5.3.8 RNA isolation and RT^2 Profiler PCR Arrays

Colons from vehicle (n=5) or oxaliplatin-treated (n=4) mice were removed, snap frozen in liquid nitrogen and stored in -80°C until used. Total RNA was extracted using TRIzol™ (Invitrogen, Carlsbad, California) and further purified using an RNeasy® Mini kit (Qiagen, Hilden, Germany), including the on-column DNase digestion step. RNA integrity was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) using RNA 6000 Nano chips (Agilent Technologies); RNA Integrity Numbers (RIN) of all colon RNA samples were within an appropriate range (vehicle treated: 8.8 ± 0.8, n=5; oxaliplatin-treated: 9.2 ±0.08, n=4). Total RNA concentration was determined on a Qubit® 2.0 Fluorometer (Invitrogen) using a Qubit® dsDNA BR Assay. Gene expression was investigated using the pathway specific RT^2 Profiler PCR Array 'Mouse Cancer Inflammation and Immunity Crosstalk' (Qiagen, Cat. no. PAMM-181Z) according to the manufacturer's instructions. Arrays were performed using pooled RNA samples prepared by combining equal quantities of either vehicle or oxaliplatin-treated RNA. Reverse transcription was carried out with the RT^2 First Strand Kit (Qiagen) using 0.5µg pooled RNA as template. Equal amounts of cDNA were distributed to each well of the RT^2 Profiler Array and real-time PCR was performed in a Biorad CFX96 real-time thermal cycler, using RT^2

SYBR Green qPCR Mastermix (Qiagen) PCR cycling comprised an initial denaturation step at 95°C for 5 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melt curve analysis was performed to verify PCR specificity. Arrays were performed in duplicate for each RNA pool. C_T values (cycle number at which fluorescence crosses a defined threshold) were obtained using the Bio-rad CFX Manager™ software, using a constant value across all arrays. The detection limit was set at C_T of 35 cycles. C_T values were uploaded to and analysed using the Qiagen data analysis web portal at http://www.qiagen.com/geneglobe. Data were normalised to the mean of five reference genes: Glyceraldehyde-3-phosphate dehydrogenase, *Gapdh*; Beta-2 microglobin, *B2m*; Actin-beta, *Actb*; Gluconuronidase-beta, *Gusb* and Heat shock protein 90 alpha (cytosolic), class B member, *Hsp90ab1*. Fold change was calculated using the $\Delta\Delta C_T$ method, as the ratio of normalised gene expression in the oxaliplatin-treated group divided by normalised gene expression in the vehicle-treated control group. Changes in genes presented in this study include: TLR2, TLR3, TLR4, TLR7, TLR9, Histocompatibility 2, D region locus 1 (H2-D1), IL-1β, IL-12β, IFN-γ, activation-induced cytidine deaminase (Aicda), chemokine ligand Ccl-2,5, and 22, and colony stimulating factor 2 (Csf2).

5.3.9 Flow cytometry

To identify immune cell changes following oxaliplatin treatment the PPs and MLNs were harvested. An n=5 PPs or MLNs were collected from each

animal. PPs and spleen were placed in 15 mL tubes containing fluorescence-activated cell sorting (FACS) buffer (PBS, 0.1% bovine serum albumin and 0.02% sodium azide) and were kept on ice. Mucosal epithelium lining the PPs and excess adipose tissue attached to MLNs were gently removed using forceps. Manual cell suspensions of the PPs, MLNs and spleen were performed. All samples were then centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant of each cell suspension was aspirated and the pellet containing the immune cells was then resuspended in 1 mL of FACS buffer and filtered. Manual cell counts were performed and cells were seeded appropriately to 96 U-bottom well plates (BD Biosciences, USA) and were centrifuged at 1300 rpm for 3 minutes at 4°C. Subsequent to centrifugation, the 96 U-bottom well plates were then aspirated. A selection of cell surface antibodies was used to identify various immune cell populations **(Table 5.1)**. Each antibody cocktail was loaded to appropriate wells, and incubated for 20 minutes at 4°C. Subsequent to the incubation period, the cells were washed with FACS buffer and centrifuged at 1300rpm for 3 minutes at 4°C. The plates were aspirated and cells within each well were resuspended in FACS buffer, and then transferred to FACS tubes. BD Biosciences LSR II and FACS CANTO II flow cytometers were used to collect 200,000 cells from each cell suspension. Information was obtained via software FACSDiva^{TM} (BD Biosciences, USA), and analysis was conducted using FlowJo (Tree Star, USA) or FACSDiva^{TM}.

Table 5.1. Antibodies used for flow cytometry experiments in this study

Cells	Primary antibody	Conjugate	Host species	Dilution
Pan-leukocyte marker	CD45	PerCP/Cy5.5	Mouse	1:400
Pan-T cell marker	CD3	PerCP/Cy5.5	Mouse	1:400
T cell receptor	TCRβ	APC	Rat	1:250
Granulocytes	GR-1, CD11b	PE-Cy7	Rat	1:100
Cytotoxic T cells	CD8	Pacific Blue	Rat	1:100
Helper T cells	CD4	Pacific Orange	Rat	1:100
B cells	B220,	FITC	Mouse	1:400
Macrophages	CD11b, Ly6C, Ly6G, CD206, F4/80	PE	Rat	1:200
Dendritic cells	CD11c	Pacific Blue	Rat	1:250
Major histocompatibility complex II	MHC-II	Brilliant Violet™ 510	Rat	1:800
Eosinophils	CD193	Alexa Fluor 647	Rat	1:200
NK cells	CD49b	PE	Rat	1:100
Gamma delta (γδ) T cells	γδ-TCR	FITC	Mouse	1:500
Natural killer T (NKT) cells	CD1d α-Galactosylceramide tetramer (CD1D α-Galcer)	PE	Rat	1:500

5.3.10 Statistical analysis

For microbiota studies, two-tailed *t*-tests were used to compare two sets of data, assuming unequal variance. The data generated by mass spectral analyses were normalized with respect to internal standards (RSD= 19.28%), where a magnitude of 1 fold change referred to the concentration of 10mg/L. Statistical analysis was performed using SIMCA 14 (Umetrics AG, Umeå, Sweden). Statistical analysis for all other experiments included an unpaired *t*-test with Welch's correction using GraphPad PrsimTM v6.0

(GraphPad Software, USA). The data were represented as mean ± standard error of the mean (SEM). Statistical significance for all experiments was defined where the *P* value was less than 0.05.

5.4 RESULTS

5.4.1 Oxaliplatin treatment causes morphological changes in TLR4$^+$ cells and reduces TLR7, TLR9, and HD-21 expression in the colon

Colon cross-sections from the vehicle and oxaliplatin-treated groups were double-labelled with anti-TLR4 and anti-HMGB1 antibodies to determine any expressional changes. Intense HMGB1 expression within the lamina propria of the colon from oxaliplatin-treated animals was noted when compared to the vehicle-treated cohort **(Figure 5.1 A-B^{II})**. Furthermore, colocalization of TLR4 and HMGB1 was observed in the lamina propria following oxaliplatin treatment when compared to the vehicle-treated cohort **(Figure 5.1 A-B^{II};** yellow arrows**).** There were no differences in the total number of TLR4$^+$ cells between the vehicle-treated (1010 ± 85) and oxaliplatin-treated groups (1077 ± 87), n=4/group **(Figure 5.1 C)**. Furthermore, no changes in the total TLR4$^+$-immunoreactivity amongst vehicle-treated (1.7 ± 0.1) and oxaliplatin-treated cohort (1.8 ± 0.2), n=4/group **(Figure 5.1 D)** were demonstrated. TLR4$^+$ cells within the vehicle-treated cohort display minimal contact/colocalisation with HMGB1 **(Figure 5.2 A-A^{II})**. Whereas, TLR4$^+$ cells colocalising with HMGB1 within

the lamina propria from oxaliplatin-treated group displayed pseudopodia-like extensions characteristic of antigen sampling **(Figure 5.2 B-B^{II})**.

Similar to the lamina propria, there was strong HMGB1 immunoreactivity within the LMMP of the colon from the oxaliplatin-treated group when compared to the vehicle-treated cohort **(Figure 5.3 A-B^{II})**. However, no TLR4^{+} cells infiltrated the LMMP layer of the colon following either treatment (white arrow; n=4/group).

Interestingly, oxaliplatin treatment induced downregulation of TLR7 (-1.81 fold change) and TLR9 (-2.01 fold change) when compared to the vehicle-treated group **(Figure 5.4)**. No changes in TLR2, TLR3 or TLR4 expression were noted following oxaliplatin treatment **(Figure 5.4)**. In addition, oxaliplatin treatment was associated with reduced expression of H2-D1 (-2.23 fold change) when compared to the vehicle-treated group, however H2-D1 mRNA expression was low in both groups ($C_T \geq 33$ cycles).

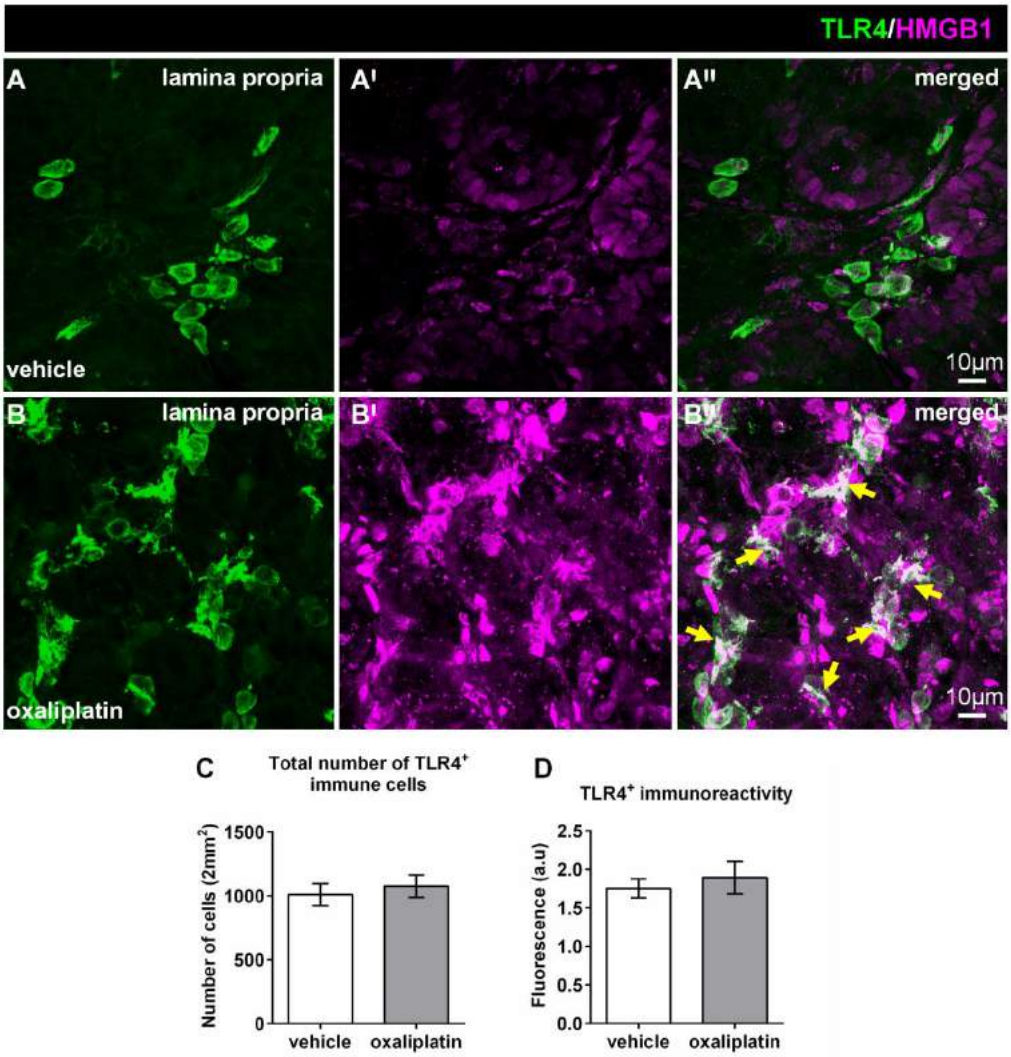


Figure 5.1. Effects of oxaliplatin treatment on HMGB1 expression and colocalisation with TLR4 in the lamina propria of the colon. Colon cross-sections (30µm thick) from the vehicle and oxaliplatin-treated groups were labelled with anti-TLR4 (green) and anti-HMGB1 (magenta) antibodies **(A-B^{II})**. Strong HMGB1 immunoreactivity is observed within the lamina propria **(B^{I})** of the colon from the oxalipLatin-treated animals when compared to the vehicle-treated cohort **(A^{I}).** Greater colocalisation of TLR4 and HMGB1 is evident within the lamina propria **(B^{II})** following oxaliplatin treatment when compared to the vehicle-treated cohort **(A^{II})**. The numbers of TLR4+ cells were counted from 8 images per preparation taken at 20x magnification with a total area of 2mm^2. No differences in the total number **(C)**, or TLR4+ immunoreactivity **(D)** was observed following oxaliplatin treatment when compared to the vehicle group. Scale=10µm; n=4/group.

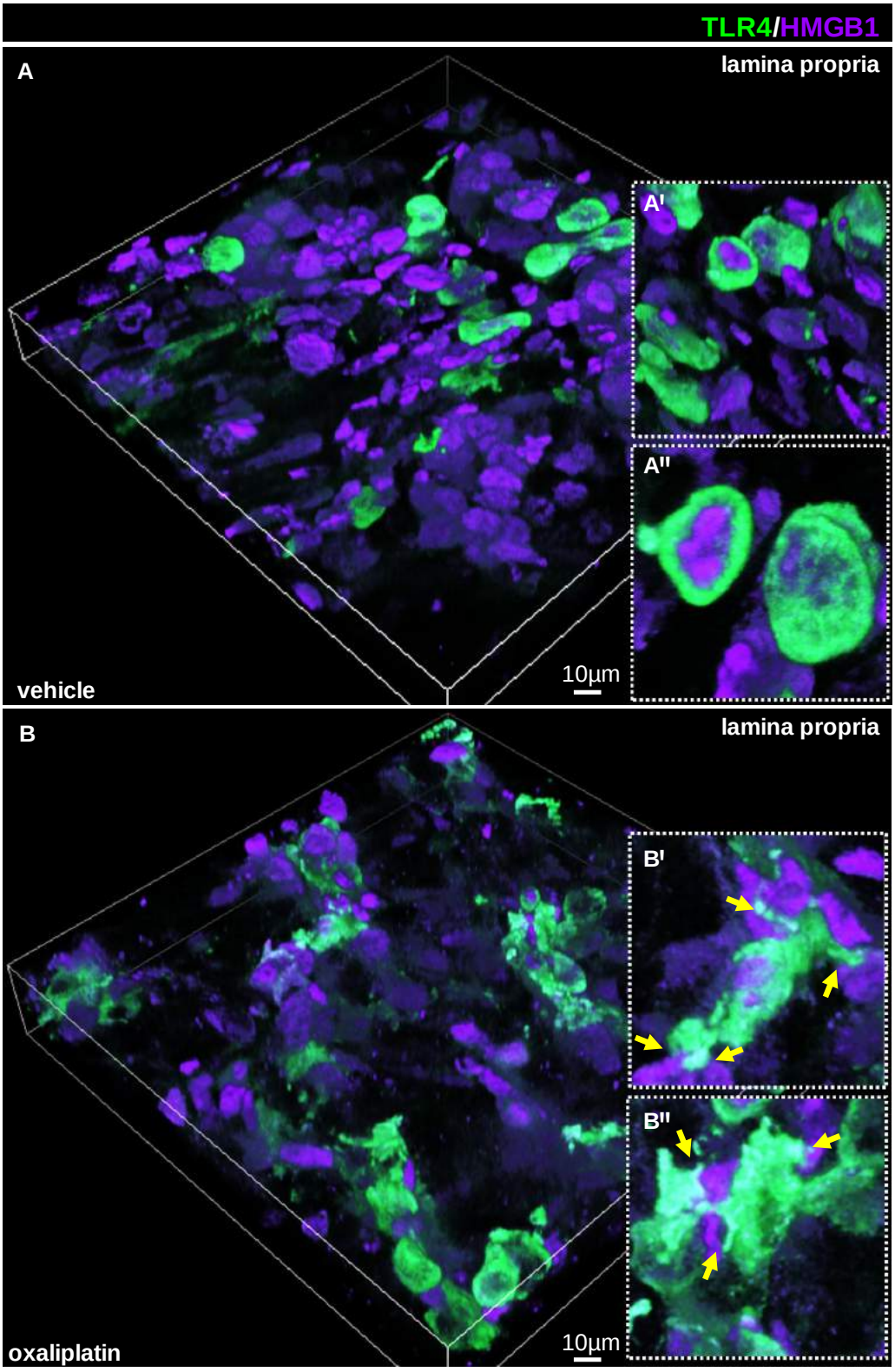


Figure 5.2. Changes in TLR4$^+$ cell morphology and interaction with HMGB1 in the lamina propria of the colon following oxaliplatin treatment. Colon cross-sections (30µm thick) from the vehicle **(A)** and oxaliplatin-treated **(B)** mice were labelled with anti-TLR4 (green) and anti-HMGB1 (magenta) antibodies and presented as 3D reconstruction of confocal z-series slices. TLR4$^+$ cells within the lamina propria of the colon from the oxaliplatin-treated animals form contacts with HMGB1 through extending processes **(B'-B''**, insets with yellow arrows**)**, as opposed to the spherical morphology of TLR4$^+$ cells in the vehicle-treated cohort **(A'-A'',** insets**)**, n=4/group.

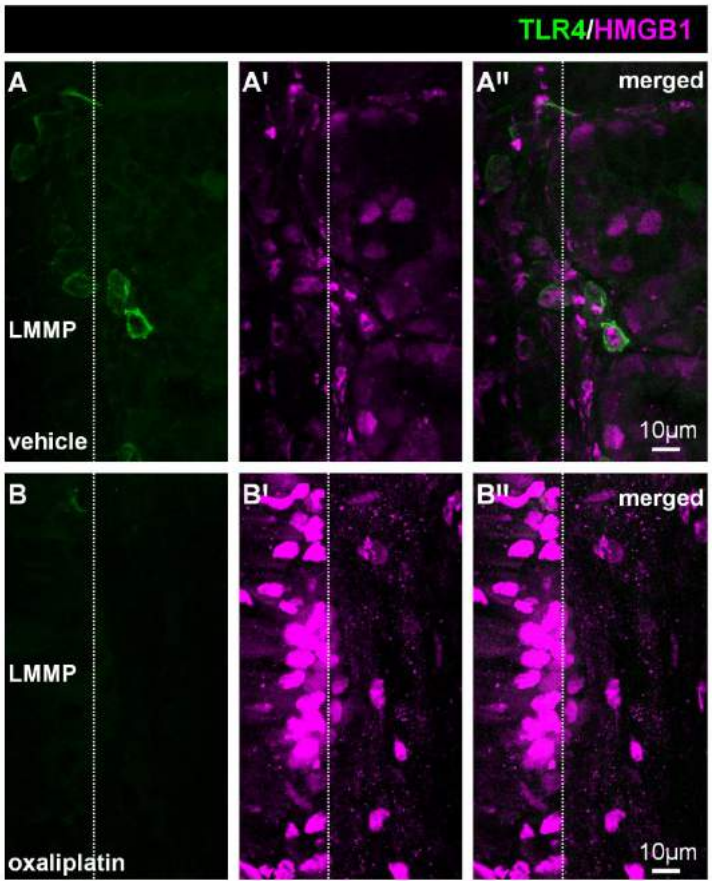


Figure 5.3. Effects of oxaliplatin treatment on HMGB1 expression and colocalisation with TLR4 in the LMMP of the colon. Colon cross-sections (30µm thick) from the vehicle and oxaliplatin-treated groups were labelled with anti-TLR4 (green) and anti-HMGB1 (magenta) antibodies **(A-B^{II})**. No TLR4^{+} cells infiltrated the level of the LMMP in either group **(A-B)**. Strong HMGB1 immunoreactivity is observed within the colon from the oxaliplatin-treated animals **(B^{I})** when compared to the vehicle-treated cohort **(A^{I}).** Scale=10µm; n=4/group.

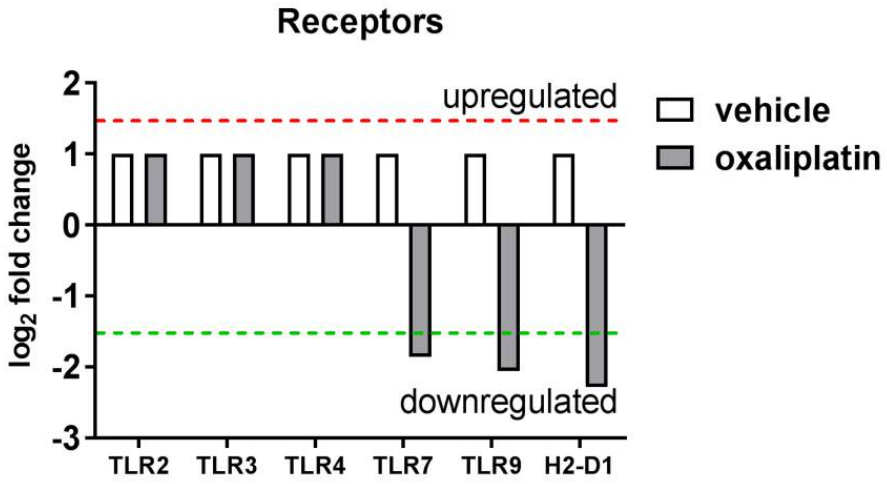


Figure 5.4. Effects of oxaliplatin treatment on mRNA expression of immune receptors. To determine whether oxaliplatin treatment induced changes in the expression of receptors within the colon, RT^2 Profiler PCR arrays were performed using pooled RNA from vehicle-treated (n=5) and oxaliplatin-treated (n=4) samples. Oxaliplatin treatment caused a downregulation of TLR7, TLR9 and H2-D1 mRNA expression when compared to the vehicle-treated cohort. No change in TLR2, TLR3 or TLR4 expression was observed following oxaliplatin treatment.

5.4.2 Oxaliplatin treatment has no effect on richness, diversity and evenness of intestinal microbiota, but causes changes at the genus level

To compare compositional differences in the gut microbiota amongst vehicle-treated and oxaliplatin-treated mice, 16S rRNA sequencing was conducted. Total DNA was isolated from fecal samples (n=10 mice/group), and PCR amplicons spanning the 16S rRNA V3-V5 hypervariable region were sequenced. Microbiota composition was assessed with regards to operational taxonomic units (OTUs) Chao richness, Shannon diversity index, Simpson evenness index, as well as unweighted UniFrac Principal Coordinate Analysis (PCoA).

No significant difference in the numbers of OTUs was observed between the vehicle-treated (3058 ± 233.6) and the oxaliplatin-treated mice (3134 ± 175.6) **(Figure 5.5 A)**. Furthermore, no significant difference in Chao richness was observed amongst the vehicle-treated (3058 ± 233.6) and the oxaliplatin-treated cohort (3562 ± 381.5) **(Figure 5.5 B)**. There were no significant differences in diversity between the vehicle-treated (0.97 ± 0.002) and the oxaliplatin-treated group (0.971 ± 0.003) **(Figure 5.5 C)**. PCoA from oxaliplatin-treated mice microbiome based upon unweighted UniFrac distance was comparable to the vehicle-treated cohort **(Figure 5.5 D)**.

In this study, five major phyla groups were identified: *Bacteroidetes*, *Deferribacteres*, *Firmicutes*, *Proteobacteria* and *Tenericutes*. Although *Bacteroidetes* was the most abundant phylum species, no significant

differences were observed between the vehicle-treated (88.3 ± 1.2) and oxaliplatin-treated mice (84.9 ± 2.2) **(Figure 5.5 E)**. No significant differences in *Deferribacteres* phyla were observed amongst the vehicle-treated (0.45 ± 0.25) and the oxaliplatin-treated cohort (0.40 ± 0.16) **(Figure 5.5 F).** No significant changes to *Firmicutes* were observed between the vehicle-treated (1.14 ± 0.15) and oxaliplatin-treated group (1.35 ± 0.29) **(Figure 5.5 G)**. In addition, there were no significant changes to *Proteobacteria* abundance following oxaliplatin (1.35 ± 0.29) compared to vehicle-treatment (1.14 ± 0.15) **(Figure 5.5 H)**. Furthermore, no changes in *Tenericutes* phyla were observed amongst the vehicle-treated (0.03 ± 0.009) and oxaliplatin-treated mice (0.04 ± 0.01) **(Figure 5.5 I)**.

Changes to the composition of the microbiota genera following oxaliplatin treatment was determined using 16S rRNA sequencing. Twelve common species present in both the vehicle-treated and the oxaliplatin-treated cohort were identified. These species included: *Bacteroides*, *Parabacteroides*, *Prevotella*$_1$, *Prevotella*$_2$, *Odoribacter*, *Mucispirillum*, *Lactobacillus*, *Dehalobacterium*, *Ruminococcus*, *Sutterella*, *Bilophila* and *Desulfovibrio*. The particular taxonomy for *Prevotella*^{1} and *Prevotella*^{2} is yet to be determined. Unknown species *'unknown'* were also detected in both the vehicle-treated and the oxaliplatin-treated cohorts **(Figure 5.5 J-K)**. A significant reduction in *Parabacteroides* (vehicle-treated: 0.71 ± 0.003; oxaliplatin-treated: 0.21 ± 0.04; $P<0.0001$) and *Prevotella*$_1$ species (vehicle-treated: 1.06 ± 0.002; oxaliplatin-treated: 0.64 ± 0.13; $P<0.05$) was noted in the oxaliplatin-treated group when compared to the vehicle-

treated cohort **(Figure 5.5 J-K, Table 5.2)**. Similarly, oxaliplatin treatment induced a significant decrease in the *Prevotella*$_2$ species (vehicle-treated: 4.87 ± 0.01; oxaliplatin-treated: 8.58 ± 1.4; $P<0.05$) and in the *Odoribacter* species (vehicle-treated: 0.39 ± 0.0007; oxaliplatin-treated: 0.62 ± 0.08; $P<0.05$) when compared to the vehicle-treated group **(Figure 5.5 J-K; Table 5.2)**. No changes to *Bacteroides*, *Mucispirillum*, *Lactobacillus*, *Dehalobacterium*, *Ruminococcus*, *Sutterella*, *Bilophila*, *Desulfovibrio* or 'unknown' species were shown.

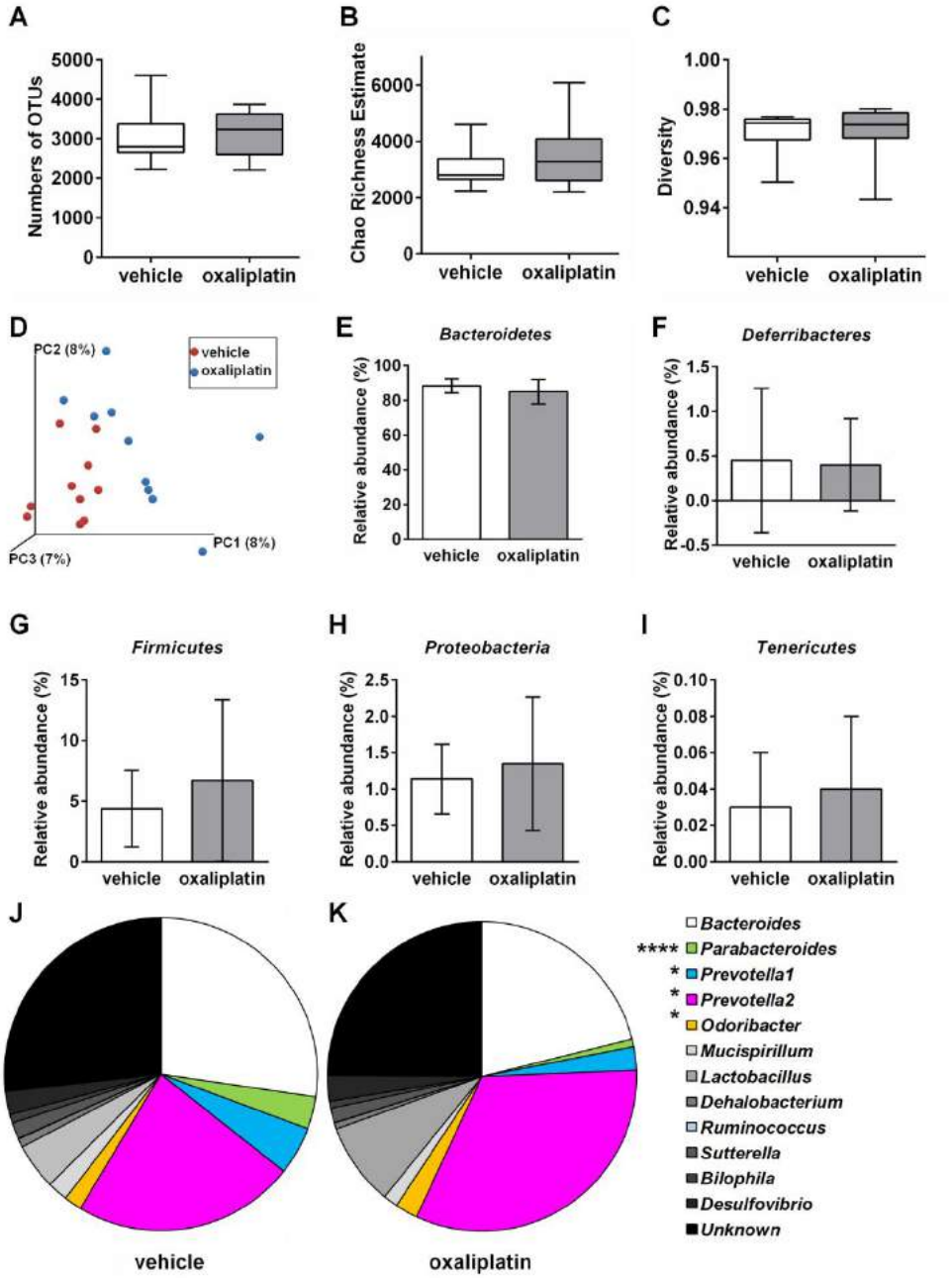


Figure 5.5. Effects of oxaliplatin treatment on the composition of intestinal microbiota. Fecal microbiota richness, diversity and evenness following oxaliplatin treatment were analysed using 16S rRNA sequencing. Oxaliplatin treatment did not cause any significant changes to the number of OTUs **(A)**, Chao richness estimate **(B)**, diversity **(C)** or PCoA unweighted UniFrac distance percentages **(D)**. Oxaliplatin treatment did not cause any significant changes to five dominant phyla groups identified in the fecal microbiota: *Bacteroidetes* **(E)**, *Deferribacteres* **(F)**, *Firmicutes* **(G)**, *Proteobacteria* **(H)** and *Tenericutes* **(I)**. Furthermore, twelve common genus species present in both the vehicle-treated and the oxaliplatin-treated cohort were identified **(J, K)**. Oxaliplatin treatment caused a significant reduction in *Parabacteroides* and *Prevotella$_1$ species*, and a significant increase in *Prevotella$_2$* and *Odoribacter* species **(J, K)**. **P*<0.05; *****P*<0.0001; n=10/group.

Table 5.2. Changes to microbiota at the genus level following oxaliplatin treatment

	Vehicle-treated			Oxaliplatin-treated		
	Mean	**SEM**	**N**	**Mean**	**SEM**	**N**
Bacteroides	5.80	0.012	10	5.56	1.54	10
Parabacteroides	0.71	0.003	10	**** 0.21	0.04	10
Prevotella$_1$	1.06	0.002	10	* 0.64	0.13	10
Prevotella$_2$	4.87	0.013	10	* 8.58	1.41	10
odoribacter	0.39	0.0007	10	* 0.62	0.08	10
Mucispirillum	0.45	0.002	10	0.40	0.18	10
Lactobacillus	1.01	0.002	10	2.28	0.67	10
Dehalobacterium	0.19	0.0004	10	0.17	0.04	10
Ruminococcus	0.01	0.00005	10	0.01	0.00	10
Sutterella	0.36	0.001	10	0.40	0.07	10
Bilophila	0.19	0.0006	10	0.19	0.06	10
Desulfovibrio	0.51	0.001	10	0.71	0.28	10
Unknown	5.68	0.005	10	6.55	0.52	10

Mean=% abundance; SEM, standard error of the mean; *P<0.05, ****P<0.0001

5.4.3 Lack of immune responses in the colon following oxaliplatin treatment

The pan-leukocyte marker anti-CD45 antibody was used to label immunocytes in the colon **(Figure 5.6 A-B'')**. There were no differences in the total number of CD45$^+$ cells between the vehicle-treated (1553 ± 169) and oxaliplatin-treated groups (1666 ± 143), n=4/group **(Figure 5.6 C)**. No significant changes in CD45$^+$ immunoreactivity to indicate inflammation in the colon was observed following oxaliplatin treatment (2.08 ± 0.16) when compared to the vehicle-treated cohort (1.75 ± 0.30), n=4/group **(Figure 5.6 D)**. Furthermore, no CD45$^+$ immune cells were found at the level of the myenteric ganglia.

MPO is a peroxidase enzyme with antimicrobial capacity and is typically used as a biomarker for inflammation. No significant difference in MPO activity was shown between the oxaliplatin-treated (0.093 ± 0.01 nmol/min/mL; n=4) and vehicle-treated groups (0.08 ± 0.02 nmol/min/mL), n=4/group **(Figure 5.7)**.

In order to profile changes in gene expression associated with inflammation, RT^2 Profiler PCR arrays of colon RNA were performed using pooled RNA samples. Oxaliplatin treatment caused the downregulation of the cytokines IL-1β (-2.02 fold change) and IL-12β (-3.56 fold change) **(Figure 5.8 A)**, together with interferon gamma (IFN-γ; -1.71 fold change), however expression of IFN-γ mRNA was very low (C_T≥34) in both vehicle and treated groups. Moreover, oxaliplatin treatment induced higher mRNA expression of the chemokine ligand Ccl-2 (3.25 fold change), and lower expression of Ccl5 (-2.19 fold change) and Ccl22 (-2.63 fold change) (**Figure 5.8 B)**. Lower levels of activation-induced cytidine deaminase, Aicda; (-2.32 fold change) and colony stimulating factor 2 (Csf2; -1.87 fold change) were also observed following oxaliplatin treatment, however these genes were expressed at very low levels (C_T≥33) in both vehicle and oxaliplatin-treated samples. Overall most genes on the array showed no change in mRNA expression in the oxaliplatin-treated group, when compared to the vehicle-treated group.

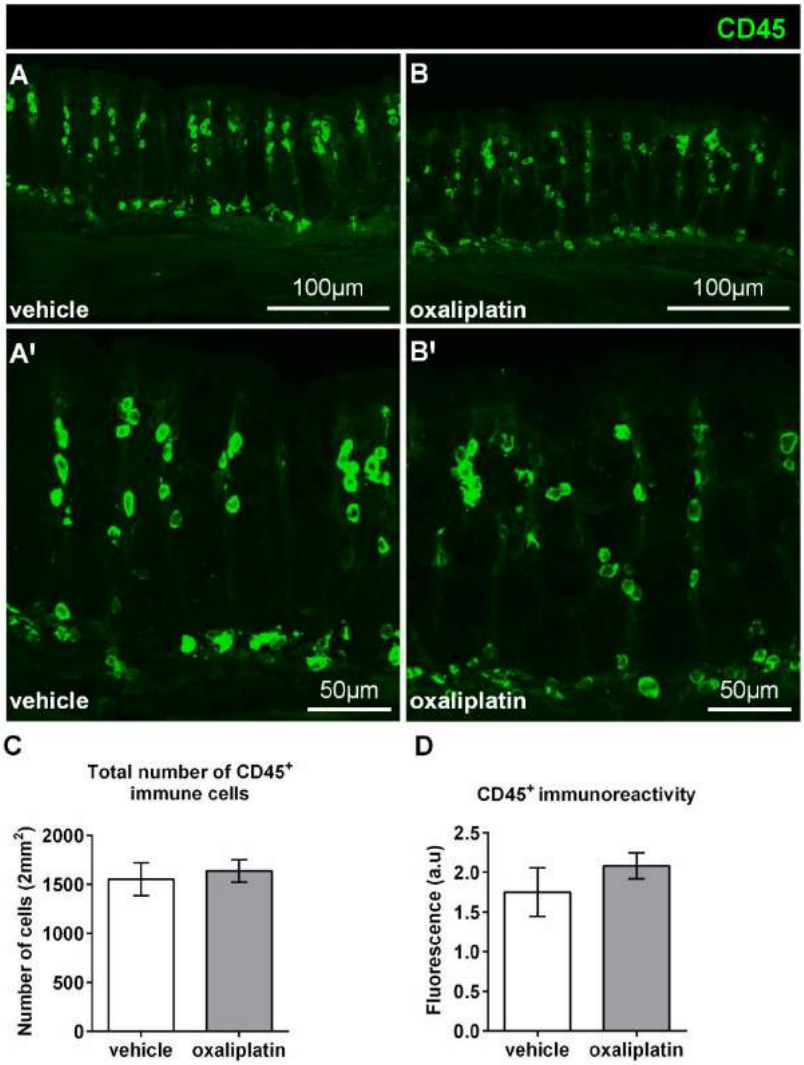


Figure 5.6. Effects of oxaliplatin treatment on the fluorescence and number of CD45$^+$ immune cells in the colon. Colon cross-sections (30µm thick) from the vehicle and oxaliplatin-treated groups were labelled with the pan-leukocyte marker anti-CD45 antibody (green) **(A-B:** scale bars=100µm; **A^I-B^I:** scale bars=50µm**)**. The numbers of CD45+ cells were counted from 8 images per preparation taken at 20x magnification with a total area of 2mm^2. No significant differences in the total number of CD45$^+$ cells **(C)**, or immunoreactivity **(D)** within the colon is observed between the vehicle-treated and oxaliplatin-treated cohorts, n=4/group.

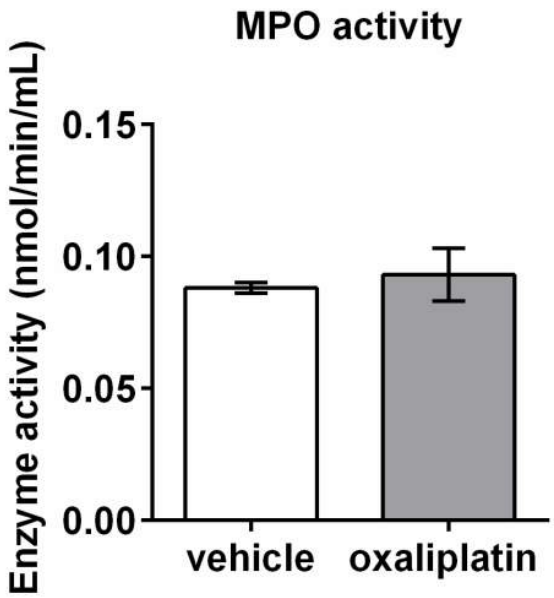


Figure 5.7. Effects of oxaliplatin treatment on MPO activity in the colon. To determine whether oxaliplatin treatment caused inflammation within the colon specific to neutrophils or macrophages a MPO assay was conducted. Oxaliplatin treatment did not induce any significant changes to MPO activity within the colon when compared to the vehicle-treated cohort, n=3/group.

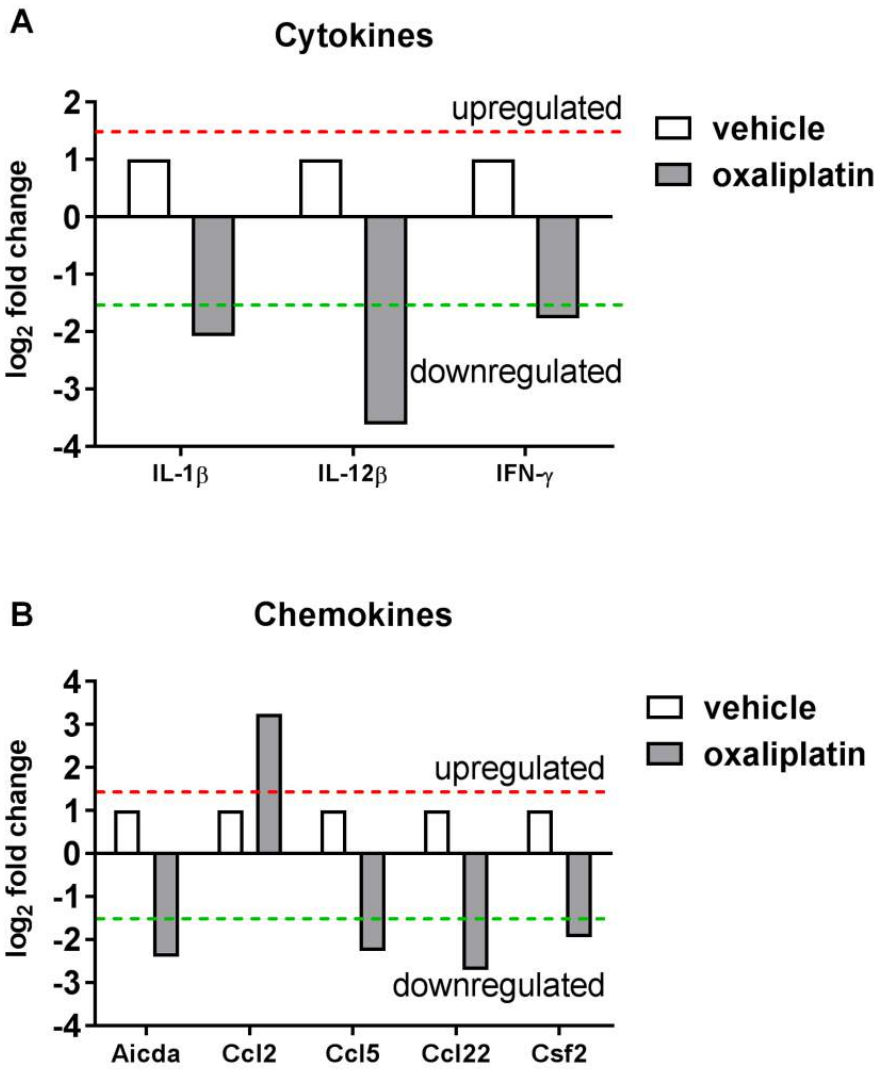


Figure 5.8. Effects of oxaliplatin treatment on cytokine and chemokine mRNA expression. To determine whether oxaliplatin treatment induced changes in inflammatory mediators within the colon, RT^2 Profiler PCR arrays were performed using pooled RNA from vehicle-treated (n=5) and oxaliplatin-treated (n=4) samples. Oxaliplatin treatment caused the downregulation of the cytokines IL-1β, IL-12β mRNA expression when compared to the vehicle-treated group **(A)**. Oxaliplatin treatment induced the upregulation of the chemokine Ccl2, and the downregulation of Ccl5, Ccl22, Aicda and Csf2 chemokine mRNA expression when compared to the vehicle-treated cohort **(B).**

5.4.4 Oxaliplatin treatment induces changes in immune cell populations within the MLNs, but not PPs

To determine the effects of oxaliplatin treatment on the gastrointestinal immune response, we profiled granulocyte and lymphocyte populations within the PPs and MLNs using FACS. Several immune cell populations were gated by the following: macrophages ($F4/80^+$ $MHC\text{-}II^+$); dendritic cells ($CD11c^+$ $MHC\text{-}II^+$); eosinophils ($CD11B^+$ $MHC\text{-}II^-$ $Gr\text{-}1^+$ $CD193^+$ and $CD11B^+$ $MHC\text{-}II^+$ $Gr\text{-}1^+$ $CD193^+$); NK cells ($CD49b^+$ TCR^-); γδ T cells (γδ-TCR^+ $TCR\beta^-$); B cells ($CD45^+$ $TCR\beta^-$ $B220^+$); $CD4^+$ T cells ($CD4^+$ TCR^+); $CD8^+$ T cells ($CD8^+$ TCR^+); NKT cells (CD1d α-Galcer $tetramer^+$ TCR^+). Example FACS plots **(Figure 5.9 A-A^{II}, 10 A-A^{II})**. Oxaliplatin treatment did not cause any significant changes to the proportion of immune cells within the PPs when compared to the vehicle-treated cohort **(Figure 5.9 B-J, Table 5.3**; n=5/group**)**. However, oxaliplatin treatment resulted in a significant reduction in the proportion of macrophages and dendritic cells within the MLNs when compared to vehicle-treated group **(Figure 5.10 B-C, Table 5.4)**. No changes in other immune cell populations were noted **(Figure 5.10 D-J**, n= 5/group**)**.

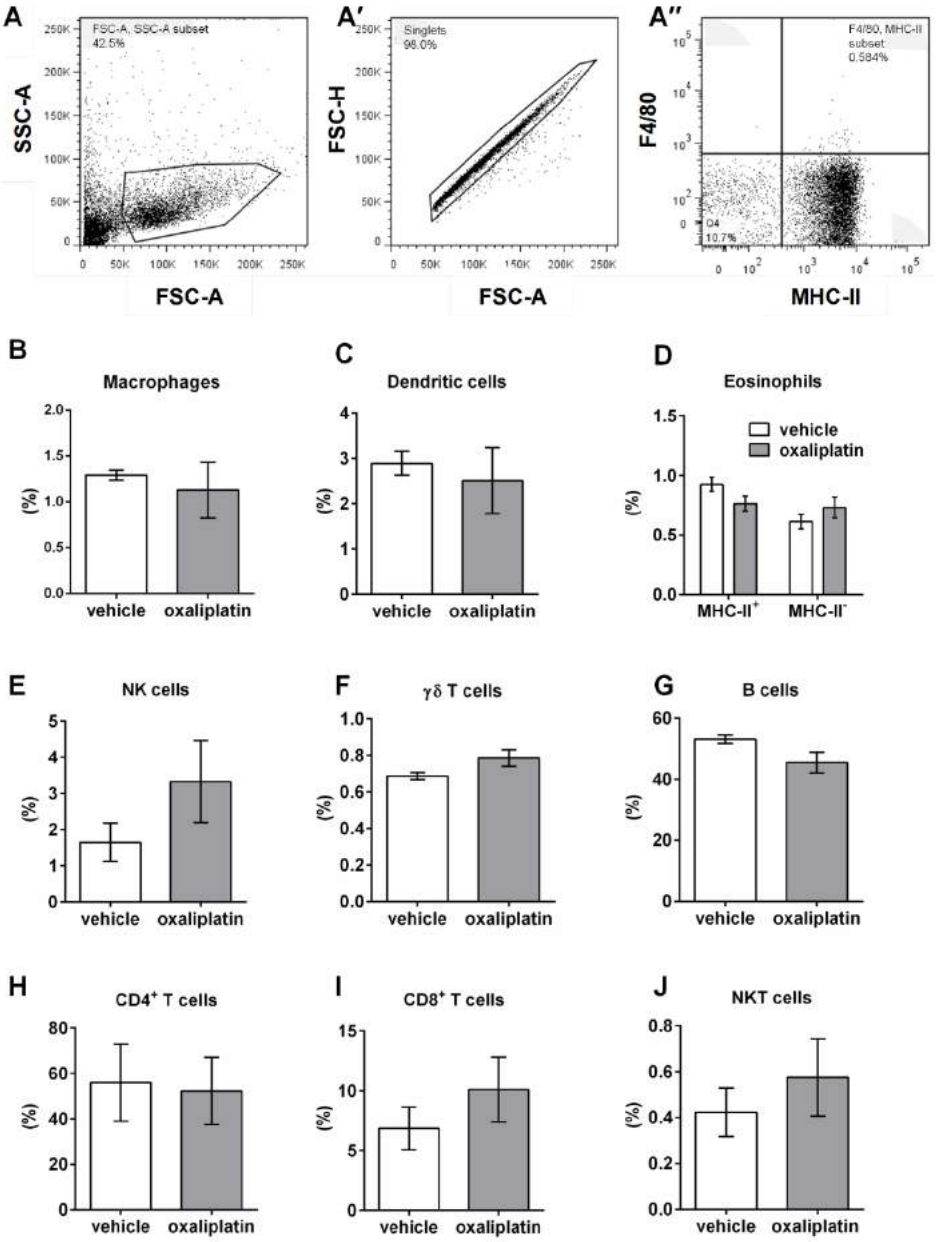


Figure 5.9. Immune cell populations within the PPs from vehicle and oxaliplatin-treated mice. A series of gating strategies were used to identify immune cell populations within the PPs: macrophages (F4/80$^+$ MHC-II$^+$); dendritic cells (CD11c$^+$ MHC-II$^+$); eosinophils (CD11B$^+$ MHC-II$^-$ Gr-1$^+$ CD193$^+$ and CD11B$^+$ MHC-II$^+$ Gr-1$^+$ CD193$^+$); NK cells (CD49b$^+$ TCR$^-$); γδ T cells (γδ-TCR$^+$ TCRβ$^-$); B cells (CD45$^+$ TCRβ$^-$ B220$^+$); CD4$^+$ T cells (CD4$^+$ TCR$^+$); CD8$^+$ T cells (CD8$^+$ TCR$^+$); NKT cells (CD1d α-Galcer tetramer$^+$ TCR$^+$). Example FACS plots **(A-A^{II})**. No significant differences were observed in any immune cell types within PPs **(B-J)**, n=5/group.

Table 5.3. Proportions of various immune cell populations within the PPs following vehicle and oxaliplatin treatment

Immune cell population	Vehicle-treated	Oxaliplatin-treated
Macrophages	1.3 ± 0.1	1.1 ± 0.3
Dendritic cells	2.9 ± 0.3	2.5 ± 0.7
Eosinophils: MHC-II$^+$ **MHC-II$^-$**	0.8 ± 0.2 0.6 ± 0.1	0.7 ± 0.01 0.7 ± 0.1
NK cells	1.7 ± 0.5	3.3 ± 1.1
γδ T cells	0.7 ± 0.01	0.8 ± 0.04
B cells	53.1 ± 1.4	45.5 ± 3.4
CD4$^+$ T cells	56.0 ± 17.0	52.3 ± 14.8
CD8$^+$ T cells	6.9 ± 1.8	10.1 ± 2.7
NKT cells	0.4 ± 0.1	0.6 ± 0.17

Data presented as mean±SEM, n=5/group

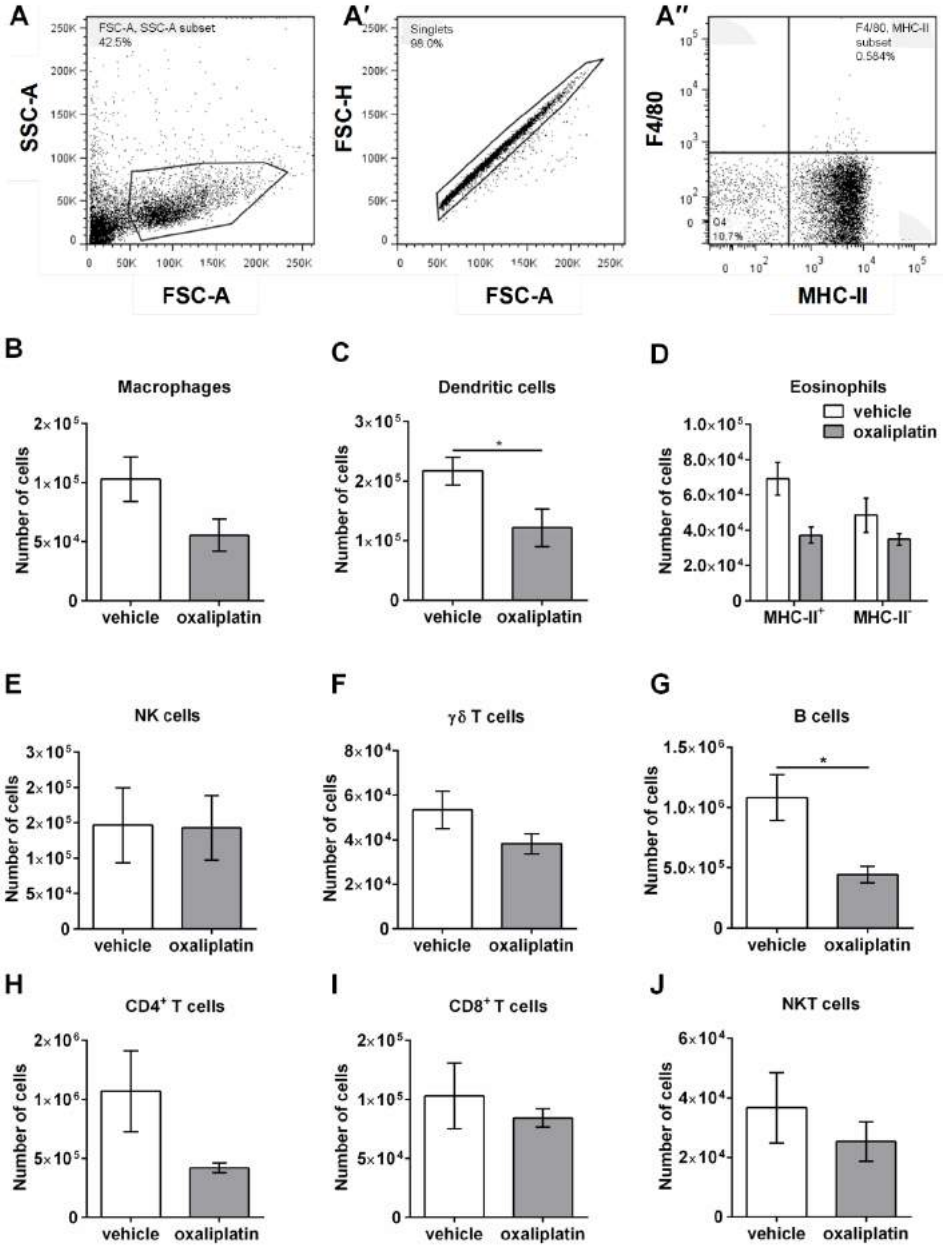


Figure 5.10. Immune cell populations within the MLNs from vehicle and oxaliplatin-treated mice. A series of gating strategies were used **(A)** to identify immune cell populations within the MLNs: macrophages (F4/80$^+$ MHC-II$^+$); dendritic cells (CD11c$^+$ MHC-II$^+$); eosinophils (CD11B$^+$ MHC-II$^-$ Gr-1$^+$ CD193$^+$ and CD11B$^+$ MHC-II$^+$ Gr-1$^+$ CD193$^+$); NK cells (CD49b$^+$ TCR$^-$); γδ T cells (γδ-TCR$^+$ TCRβ$^-$); B cells (CD45$^+$ TCRβ$^-$ B220$^+$); CD4$^+$ T cells (CD4$^+$ TCR$^+$); CD8$^+$ T cells (CD8$^+$ TCR$^+$); NKT cells (CD1d α-Galcer tetramer$^+$ TCR$^+$). Example FACS plots **(A-A^{II})**. Oxaliplatin treatment caused a significant reduction in the proportion of macrophages and dendritic cells, with no effects on eosinophils, NK cells, γδ T cells, B cells (CD45$^+$ TCRβ$^-$ B220$^+$); CD4$^+$ T cells (CD4$^+$ TCR$^+$); CD8$^+$ T cells (CD8$^+$ TCR$^+$); NKT cells (CD1d α-Galcer tetramer$^+$ TCR$^+$) within MLNs **(B-J)**. *P<0.05, n=5/group.

Table 5.4. Proportions of various immune cell populations within the MLNs following vehicle and oxaliplatin treatment

Immune cell population	Vehicle-treated	Oxaliplatin-treated
Macrophages	2.8 ± 0.5	*1.1 ± 0.2
Dendritic cells	5.1 ± 0.3	***2.5 ± 0.1
Eosinophils: MHC-II$^+$ **MHC-II$^-$**	2.5 ± 0.2 0.8 ± 0.1	2.0 ± 0.2 1.0 ± 0.1
NK cells	0.6 ± 0.1	0.8 ± 0.1
γδ T cells	0.5 ± 0.01	0.5 ± 0.03
B cells	15.3 ± 0.8	14.2 ± 1.7
CD4$^+$ T cells	64.6 ± 6.1	66.0 ± 4.8
CD8$^+$ T cells	20.6 ± 1.6	22.0 ± 0.9
NKT cells	0.8 ± 0.3	0.9 ± 0.3

Data presented as mean±SEM, n=5/group. *P<0.05, ****P<0.0001

5.5 DISCUSSION

This study is the first to investigate the potential for oxaliplatin treatment to induce inflammatory enteric neuropathy within the murine colon. Oxaliplatin is a potent immunogenic cell death inducer, thus, it was hypothesized that mucosal and neuronal damage would be associated with an inflammatory response (Tesniere et al., 2010, Hato et al., 2014, Bezu et al., 2015, Stojanovska et al., 2015). However, in this study we have shown that oxaliplatin treatment does not induce inflammation or changes in total TLR4 immunoreactivity, despite noticeable changes in HMGB1 expression which is a TLR4 ligand. It is well established that cytoplasmic and/or released HMGB1 can exert pro-inflammatory cytokine-like activity capable of inducing strong immunological responses (Lee et al., 2014b, Martinotti et al., 2015). In necrotic cell death HMGB1 is passively released and acts as a potent 'eat me' signal, however, oxidative stress and apoptosis can blunt this pro-inflammatory signalling (Liu et al., 2012, Venereau et al., 2012, Yu et al., 2015). In this study, HMGB1 and TLR4 colocalisation as well as morphological differences in $TLR4^{+}$ cells within the lamina propria, but not in the LMMP following oxaliplatin treatment were observed. The $TLR4^{+}$ cells colocalised with HMGB1 show pseudopodia-like morphology which is characteristic of antigen sampling (Baranov et al., 2014). Under conventional circumstances, antigen presenting cells migrate to their nearest draining lymph nodes (such as PPs and MLNs in this case) upon antigen recognition for the priming and activation of T cells. Despite the HMGB1 and TLR4 interaction, there was

no amplification of immune cell populations within the lymphoid organs (PPs and MLNs). Presumably, HMGB1 could still be sampled by antigen presenting cells, but may be regarded as an innocuous/neutral molecule. Additionally, the pro-inflammatory effects of HMGB1 are also dependent on its redox state (Kazama et al., 2008, Urbonaviciute et al., 2009, Ottosson et al., 2012, Magna and Pisetsky, 2014). We have previously shown that oxaliplatin treatment induces oxidative stress through the upregulation of iNOS within the LMMP, as well as an increase in mitochondrial superoxide production and protein nitrosylation in myenteric neurons (McQuade et al., 2016c). Given that oxaliplatin induces an oxidative environment, this particular DAMP may therefore be subjected to oxidisation, and thus, have its inflammatory potential blunted; this needs further investigation. Moreover, we observed the downregulation of TLR7 and TLR9 following oxaliplatin treatment, with no changes to TLR2, TLR3 or TLR4. TLRs are membrane-bound receptors which recognise a myriad of ligands produced by microbiota, as well as DAMPs (Janssens and Beyaert, 2003, Piccinini and Midwood, 2010, Jounai et al., 2012). TLR stimulation by ligands triggers signal transduction pathways and immunological responses (Takeda and Akira, 2005, Kawasaki and Kawai, 2014). TLR2 recognises bacterial lipoproteins and non-enterobacterial LPS (Werts et al., 2001). Furthermore, TLR3 recognises double-stranded viral RNA, and TLR4 is stimulated by classical LPS (Hoshino et al., 1999, Alexopoulou et al., 2001). TLR7 recognises single-stranded viral RNA, whereas TLR9 is stimulated by bacterial and viral DNA (Hemmi et al.,

2000, Lund et al., 2004, Takeda and Akira, 2005). The downregulation of TLR7 and TLR9 observed in this study would impair recognition of certain pathogens and prospective immune responses.

The downregulation of H2-D1 was observed following oxaliplatin treatment. The H2-D1 gene is associated with MHC-related molecules and antigen presentation. This downregulation could impact antigen loading and presentation to effector lymphocytes such as $CD8^+$ T cells. A major function for calreticulin is the biogenesis and correct folding and assembly of MHC-related molecules (Gao et al., 2002, Jiang et al., 2014). In Chapter 3 we had demonstrated that oxaliplatin treatment altered calreticulin expression by evoking the cytoplasmic translocation and intranuclear overexpression. The downregulation of H2-D1 and altered calreticulin expression could be implicated in the lack of gastrointestinal immune responses following oxaliplatin treatment due to defective MHC assembly and antigen presentation.

The mammalian gastrointestinal tract is colonised by diverse microorganisms where a symbiotic relationship between the host and microbiota exists (Chow et al., 2010). The composition of microbiota throughout the gastrointestinal tract can vary depending on the location. The colon in particular has the greatest microbial density with an astounding $1x\ 10^{12}$ organisms per gram of feces (dry weight) (O'Hara and Shanahan, 2006). In this study, we isolated fecal DNA and identified five major phyla which included: *Bacteroidetes*, *Deferribacteres*, *Firmicutes*, *Proteobacteria* and *Tenericutes*. Oxaliplatin did not cause significant

changes to the microbiota at the phylum level in terms of OTUs, Chao richness and diversity. However, it did induce a significant reduction in *Parabacteroides* and *Prevotella*$_1$ species, but caused an increase in *Prevotella*$_2$ and *Odoribacter*. *Parabacteroides*, *Prevotella* and *Odoribacter* genera stem from the *Bacteroides* phylum. The *Bacteroides* phyla and their successive genera stem from the *Bacteroidetes* family. These bacteria are gram-negative, anaerobic, non-spore-forming rods which are commensal to the gastrointestinal tract, but are known to be opportunistic pathogens in circumstances of intestinal barrier destruction (Wexler, 2007). It is well established that anti-cancer agents cause considerable damage to the gastrointestinal mucosa which can act as a gateway for microbiota-induced inflammation throughout the colon (Boussios et al., 2012, Syvak et al., 2012, Lee et al., 2014a). Gram-negative bacteria produce endotoxins such as lipopolysaccharides (LPS; bacterial cell wall constituents) which can induce immunological responses and contribute to inflammatory diseases through TLR binding (Darveau, 1998, Heumann and Roger, 2002, Maeshima and Fernandez, 2013, Molinaro et al., 2015). LPS come in 'classical' and 'non-classical' forms, depending upon structural configuration and degree of acylation (Weintraub et al., 1989, Ogawa, 1993, Lapaque et al., 2006). Classical LPS are acylated lipid A molecules on short-chain fatty acids which stimulate a number of pro-inflammatory responses through cytokine production (predominantly tumour necrosis factor α [TNF-α] and IL-6) by innate immune cells (neutrophils, monocytes and macrophages) (Lapaque et al., 2006,

Hakansson and Molin, 2011). TLR4 is a main receptor for ‘classical’ LPS produced by *Escherichia coli*, whereas LPS from *Bacteroides* species are considered ‘non-classical’ and thus, TLR binding may not induce a rapid or strong immunological response (Lapaque et al., 2006, Alhawi et al., 2009). Although structurally similar to classical LPS, the non-classical version still contains a lipid A centre, however, with varying degrees of acylation, and is often attached to long-chain fatty acids linked to amino-sugar backbones which are thought to hinder LPS recognition and signalling (Lapaque et al., 2006, Molinaro et al., 2015). The inability to produce potent or classical LPS by the *Bacteroides* family, and the lack of changes to total TLR4 immunoreactivity observed in our present study may explain the absence of inflammation within the colon despite the increased abundance of such species at the genus level following oxaliplatin treatment.

It is known that immune cells within the gastrointestinal mucosa function differently to their counterparts found within the circulation. The mucosal immune system has co-evolved with the gastrointestinal microbiota/antigens and sterile inflammation to downregulate pro-inflammatory responses whilst not compromising microbicidal or phagocytic activity (Smythies et al., 2005, Smith et al., 2011). No inflammation (determined by $CD45^+$ immune cell infiltrate) or differences in total TLR4 immunoreactivity throughout the thickness of the colon was observed following oxaliplatin treatment.

There were no noticeable effects on MPO activity within the colon following oxaliplatin treatment. MPO is a well-established biomarker of inflammation in various conditions such as multiple sclerosis, ischaemic heart disease and acute coronary syndromes, as well as ulcerative colitis (Loria et al., 2008, Masoodi et al., 2011, Forghani et al., 2012, Olza et al., 2012, Pulli et al., 2015). MPO is a cytotoxic constituent released by activated myeloid cells such as neutrophils, which also has micobicidal capacity (Aratani et al., 2000, Lau et al., 2005, Allen and Stephens, 2011, Klebanoff et al., 2013). Despite changes in gastrointestinal microbiota at the genus level, as well as increased HMGB1 expression which is known to have pro-inflammatory effects, MPO activity was not altered. Thus, our MPO data provide further evidence that oxaliplatin does not induce gastrointestinal inflammation, which presumably, would not have major effects on the colon myenteric plexus.

We have demonstrated that oxaliplatin treatment downregulates gene expression of the pro-inflammatory cytokines, IL-1β and IL-12β, within the colon. Cytokines can mediate inflammation and trigger extrinsic apoptotic cascades. Although the extrinsic and intrinsic apoptotic pathways are considered to be separate entities, there is some cross over between the two signal transduction pathways (Elmore, 2007, McIlwain et al., 2013, Parrish et al., 2013). In Chapter 3, we demonstrated that oxaliplatin caused myenteric plexus cell death through an intrinsic apoptotic pathway by upregulating cytochrome *c* expression and caspase 3 cleavage. Given that pro-inflammatory cytokines are downregulated in

the colon following oxaliplatin treatment it is unlikely that the extrinsic apoptotic cascade is implicated in the underlying mechanism of cell death within the myenteric plexus. It has previously been shown that oxaliplatin treatment does not alter IL-1β or IFN-γ expression within rat spinal cord and dorsal root ganglia neurons, nor does it induce immune cell infiltration (Makker et al., 2017). However, there are some conflicting studies which have demonstrated a significant increase in pro-inflammatory cytokines IL-1β and TNF-α within the rat spinal cord following oxaliplatin treatment, and a reduction in the anti-inflammatory cytokines IL-4 and IL-10 (Janes et al., 2015, Kim et al., 2016, Jung et al., 2017). In the aforementioned studies, acute experiments were conducted on rats which were treated with oxaliplatin through a single 6mg/kg/d, or a 10mg/kg/d for 5 consecutive days. This is in contrast to our current study where we have chronically treated mice tri-weekly for up to 14 days with a 3mg/kg/d. The differences between oxaliplatin dosage, species and experimental time points may contribute to varying results.

Furthermore, we showed that the chemokine Ccl2 was upregulated within the colon following oxaliplatin treatment. Despite the increase in Ccl2 (a monocyte chemoattractant), there were no increases in immune cells in the colon. Ccl2 is a pleiotropic ligand implicated in many pathways. Ccl2 has demonstrated a role in shaping pro-inflammatory/anti-inflammatory macrophage responses as a deficiency leads to skewed pro-inflammatory phenotypes that produce high levels of IL-6 and TNF-α (Sierra-Filardi et al., 2014). The upregulation of Ccl2 in the colon following

oxaliplatin treatment may play a role in dampening pro-inflammatory cytokine production by promoting the polarisation of anti-inflammatory macrophages. Ccl2 is also upregulated in response to gastrointestinal microbiota (DePaolo et al., 2005). Whether the increase in microbiota species observed in this study is implicated in Ccl2 upregulation requires further study. Previous research has demonstrated that Ccl2 is upregulated in dorsal root ganglia neurons and microglia following peripheral nerve injury prompting macrophage infiltration and sensory neuropathy (Gao et al., 2009, Van Steenwinckel et al., 2011, Kwon et al., 2015). Ccl2 can also be upregulated by other cytokines such as s100β (Wang et al., 2013). We have previously demonstrated that s100β expression within the myenteric plexus of the ileum and colon (demonstrated in Chapter 2) is increased following oxaliplatin treatment (Robinson et al., 2016). Whether upregulated s100β can impact Ccl2 levels within the colon requires further investigation. In addition, this study demonstrates that oxaliplatin treatment downregulated Ccl5 and Ccl22. Ccl5 plays a role in lymphocyte trafficking and promoting T cell polarisation towards an IFN-γ-producing Th1 phenotype (Borish and Steinke, 2003). Moreover, Ccl22 is implicated in lymphocyte and eosinophil migration (Pinho et al., 2003, Mailloux and Young, 2009). The downregulation of Ccl5 and Ccl22 following oxaliplatin treatment may impact lymphocyte and/or eosinophil migration throughout the colon.

Downregulation of Aicda and Csf2 were also observed following oxaliplatin treatment. Aicda regulates B cell proliferation and

immunoglobulin (Ig) class switching (Mechtcheriakova et al., 2012). In this study we did not observe any changes in B cell populations within the PPs and MLNs following oxaliplatin treatment. It is unknown whether the downregulation of this gene may affect B cell numbers and function at a later time point. Csf2 is a cytokine involved in macrophage and granulocyte production and maturation (Hamilton, 2002, Martins et al., 2010). Downregulation of Csf2 is consistent with the decrease in macrophages and dendritic cells within the MLNs observed in this study.

In this study, we also investigated the effects of oxaliplatin treatment on the immunological responses within the PPs and MLNs. PPs contain specialised epithelia known as microfold (or M) cells which are pivotal induction sites for pathogen or antigen-specific immune responses (Owen et al., 1986, Siebers and Finlay, 1996b, Siebers and Finlay, 1996a). Dendritic cells within the PPs consistently sample luminal antigens and bacteria, and if loaded with an inflammatory stimulus, prime local T cells to initiate a response (Shreedhar et al., 2003, Jung et al., 2010, Lelouard et al., 2012). Intestinal dendritic cells also migrate to T cell areas of MLNs which is thought to play a role in maintaining immunological tolerance (Huang et al., 2000). Although there were no demonstrable changes to the proportion of immune populations within the PPs following oxaliplatin treatment, a proportional reduction in both macrophages and dendritic cells within the MLNs was observed. Immunological responses within the lymph nodes would typically occur once antigen presenting cells are antigen-loaded and have migrated to prime T cells to initiate an

immune response. However, no demonstrable changes to T cell populations were observed within the PPs or MLNs following oxaliplatin treatment.

Furthermore, oxaliplatin treatment did not induce changes to other immune cell populations investigated in this study which include eosinophils, NK cells, γδ T cells, B cells, $CD4^+$ T cells, $CD8^+$ T cells and NKT cells, all of which have important roles in maintaining gastrointestinal homeostasis as well as initiating and modulating immune responses.

Under normal conditions eosinophils reside within haematopoietic and lymphatic tissues, as well as the gastrointestinal tract mucosa (Kato et al., 2001, Straumann and Simon, 2004). Eosinophils are pro-inflammatory effector cells which release pleiotropic chemokines, cytokines and cytotoxic granules such as eosinophil peroxidase and eosinophil-derived neurotoxin (Rothenberg et al., 2001, Jung and Rothenberg, 2014). Eosinophils have been implicated in a number of inflammatory conditions in the gastrointestinal tract. These include eosinophilic gastroenteritis, allergic colitis, and inflammatory bowel disease (Dvorak, 1980, Torpier et al., 1988, Jawairia et al., 2012). In this study we did not observe changes in the proportion of eosinophils within the PPs and MLNs which is consistent with oxaliplatin-induced immunosuppression observed in the colons from oxaliplatin-treated mice. Thus, eosinophils do not appear to be affected by oxaliplatin treatment, and are unlikely to mediate enteric neuropathy.

Furthermore, no changes in the proportion of NK cells within the PPs and MLNs were observed following oxaliplatin treatment. NK cells are innate lymphocytes present throughout the gastrointestinal tract and associated lymphoid organs (Ivanova et al., 2014). NK cells primarily defend against viral infections, tumors, and microbial species through cell-mediated cytolytic processes involving perforin and granzyme molecules (Zamai et al., 1998, Topham and Hewitt, 2009, Hall et al., 2013, Ivanova et al., 2014). Although oxaliplatin treatment caused mucosal injury and microbial dysbiosis, the proportion of NK cells remained unaffected. Additionally, the presentation of DAMPs following oxaliplatin treatment does not appear to affect the proportion of NK cells within the PPs and MLNs. A study investigating the effects of oxaliplatin treatment on ovarian cancer cells has shown that this platinum-based drug increases NK cell-mediated toxicity (Siew et al., 2015). Analysis of NK cells in human peripheral blood of patients receiving low-dose cisplatin and 5-fluorouracil treatment has also shown to prevent NK cell suppression typically observed following colorectal surgery (Ishikawa et al., 1998). However, our data suggests that NK cell-mediated cytotoxicity is an unlikely cause for enteric neuropathy following oxaliplatin treatment. No immune cells infiltrated the myenteric plexus (determined by pan-leukocyte $CD45^+$ labelling), and thus, cell-mediated killing of neurons and glia is not apparent.

γδ T cells are intraepithelial lymphocytes which act as immunosurveyers of the gastrointestinal tract (Boismenu, 2000). These

cells account for 50% of intraepithelial lymphocytes within the gastrointestinal tract and play a role in antigen presentation, anti-tumor immunity, microbial defense, neutrophil and macrophage recruitment and cytokine production (Sutton et al., 2012, Sheridan et al., 2013, Van Acker et al., 2015). We did not observe any changes in the proportion of γδ T cells in either the PPs or MLNs. Our data suggests that they are not infiltrating lymphoid organs to present antigens following oxaliplatin therapy, and that they are unlikely to be recruiting myeloid cells such as neutrophils and macrophages given that no observable changes in MPO activity within the colon was found. One study to date has demonstrated that oxaliplatin treatment induces infiltration of IL-17 producing γδ T cells to transplantable tumor sites (Ma et al., 2011). It appears that oxaliplatin treatment can sensitise cancer cells to specific γδ T cell-mediated immunity, but this seems unlikely for enteric neurons, given that no infiltrating immune cells were observed.

B cells play an important role in intestinal immunity and mucosal tolerance. They are enriched within gastrointestinal associated lymphoid organs, synthesise IgA which functions to inhibit microbial adherence to mucosal surfaces, and neutralize toxins, enzymes and antigens (Mantis et al., 2011). B cells also induce T cell-dependent or-independent responses. An *in vivo* study has shown that B cells can impede T cell responses characteristic of immunogenic cell death in a mouse tumor model (Shalapour et al., 2015). In our study we did not observe any changes in

the proportion of B cells or various T cell subpopulations ($CD4^+$, $CD8^+$, NKT).

As there are a number of $CD4^+$ T cell populations, such as Th1, Th2, Th9, Th17 and Tregs (Zhu et al., 2010), we analyzed them collectively to get an overview of $CD4^+$ T cell proportions following oxaliplatin treatment. $CD4^+$ T cells influence innate and adaptive immune responses through conditioning the milieu with pro-inflammatory or anti-inflammatory cytokines and chemokines (Luckheeram et al., 2012). Although NKT cells are a subset of $CD4^+$ T cells they are activated through CD1d-restricted lipid antigens as opposed to classical MHC class I and II molecules (Kitamura et al., 1999, Wu et al., 2005, Fernandez et al., 2012). The most extensively studied lipid ligand for CD1d is α-galactosylceramide (Wu et al., 2005). Both mammalian and bacterial lipids can stimulate NKT cells to stimulating rapid Th1 and Th2 responses. No changes were noted in the proportion of NKT cells within either the PPs or MLNs following oxaliplatin treatment. However, the effects of oxaliplatin treatment on NKT cells *in vivo* require further studies.

In our study, we observed no changes in the proportion of $CD8^+$ T cells within the PPs or MLNs following oxaliplatin treatment. $CD8^+$ T cells are activated upon antigen presentation and can exert cytotoxic activity through two major pathways. These include the granule exocytosis or the death receptor (TNF-α and Fas) pathway (Andersen et al., Mullbacher et al., 2002). Previous studies using peripheral blood and colon cancer cell lines have shown marked increases in T cell activation following the

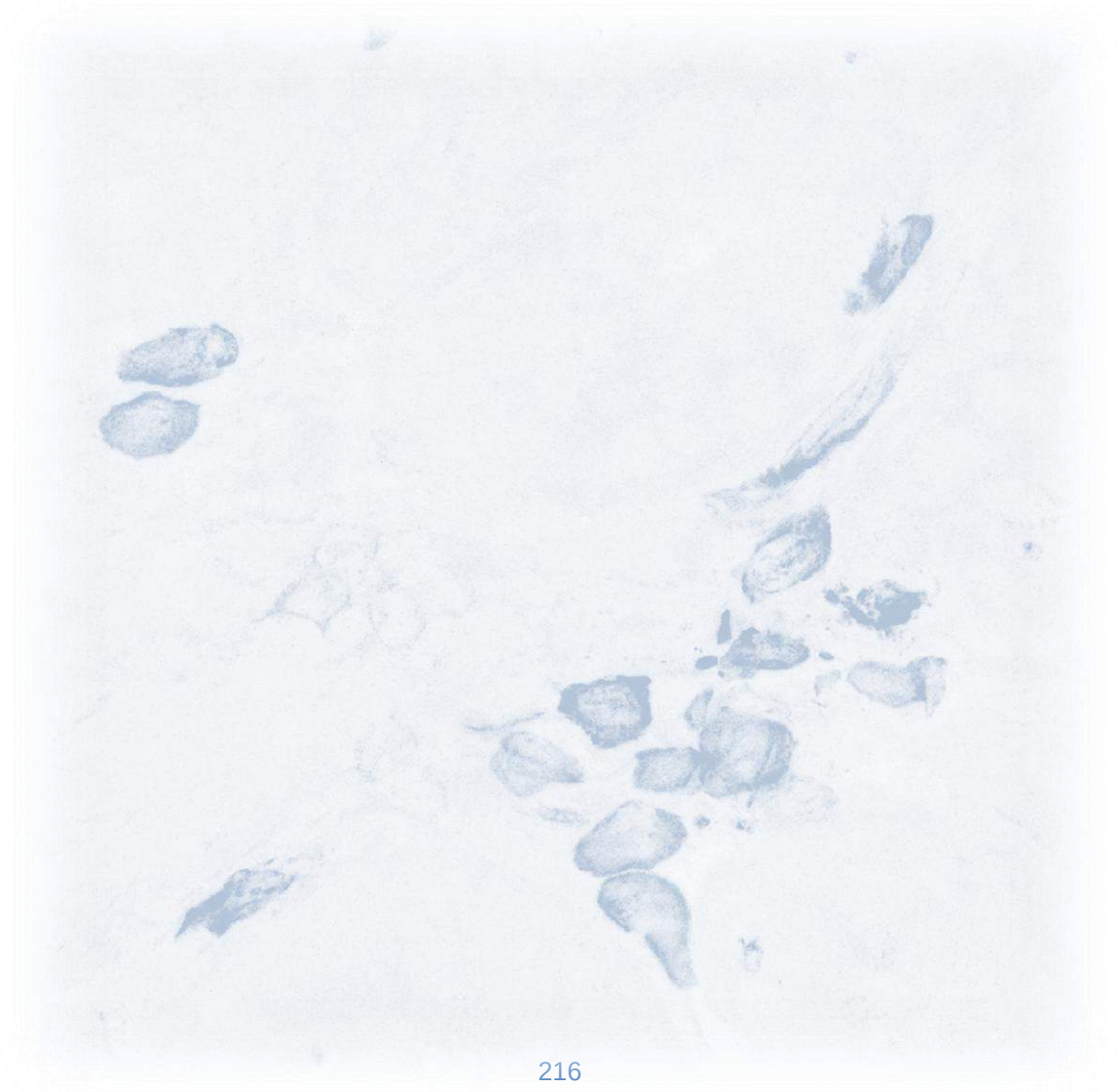
presentation of DAMPs induced by oxaliplatin treatment (Tesniere et al., 2010, Krysko et al., 2012). We have previously demonstrated that oxaliplatin treatment evokes the presentation of DAMPs within the myenteric plexus, however, no differences in T cell populations were observed.

Given that the gastrointestinal tract is constantly exposed to a myriad of antigens and pathogens, the local immune system has evolved over time to eradicate noxious stimuli through non-inflammatory host-defense mechanisms (Smith et al., 2011). These data suggest that chemotherapy-induced microbiota dysbiosis, mucosal and neuronal damage in this case does not evoke inflammation or immunogenic cell death, thus, ENS damage is most likely mediated through direct drug toxicity.

In conclusion, this is the first study to determine the effects of oxaliplatin treatment on the gastrointestinal microbiota and its effects on immune populations within the PPs and MLNs which mediate local inflammation. Oxaliplatin treatment does not induce severe inflammation throughout the thickness of the colon despite the presentation of DAMPs which is suggestive of antigen and microbial-specific tolerance. Thus, it appears that oxaliplatin treatment is not associated with inflammatory enteric neuropathy, and further research is required to determine the mechanism of neuronal damage and death which contributes to gastrointestinal dysfunction.

6

OXALIPLATIN TREATMENT AUGMENTS T CELL-MEDIATED SYSTEMIC IMMUNE RESPONSES



6.1 SUMMARY

Oxaliplatin is a platinum-based chemotherapeutic agent which has demonstrated significant anti-tumour efficacy. Unlike conventional anti-cancer agents which are immunosuppressive, oxaliplatin has the capacity to stimulate immunological effects in response to the presentation of DAMPs elicited upon cell death. However, the effects of oxaliplatin treatment on systemic immune responses remain largely unknown.

The aims of this study were to investigate the effects of oxaliplatin treatment on the proportions of: 1) splenic T cells, B cells, macrophages, pro-/anti-inflammatory cytokines, gene expression of splenic cytokines, chemokines, and mediators; 2) double-positive and single-positive $CD4^+$ and $CD8^+$ T thymocytes; and 3) bone marrow hematopoietic stem and progenitor cells.

Male Balb/c mice received intraperitoneal injections of oxaliplatin (3mg/kg/d) or sterile water tri-weekly for 2 weeks. Immune cell populations within the spleen, thymus and bone marrow were assessed using flow cytometry. PCR was performed to characterise changes in splenic inflammation-associated genes.

Oxaliplatin treatment reduced spleen size and cellularity ($CD45^+$ cells), increased the proportion of $CD4^+$, $CD8^+$ T cells and regulatory T cells and elevated TNF-α expression. Oxaliplatin was selectively cytotoxic to B cells, but had no effect on splenic macrophages. Oxaliplatin treatment altered the gene expression of several cytokines, chemokines and cell mediators. Oxaliplatin did not deplete double-positive $CD4^+$ $CD8^+$

thymocytes, but increased the single-positive $CD8^+$ subset. The bone marrow hematopoietic progenitor pool was demonstrably normal following oxaliplatin treatment when compared to the vehicle-treated cohort.

Oxaliplatin does not cause systemic immunosuppression, but instead, has the capacity to induce beneficial anti-tumour immune responses.

6.2 INTRODUCTION

It is well established that oxaliplatin can evoke the presentation of DAMPs within cancer cells to induce potent immunogenic cell death (Tesniere et al., 2010, Krysko et al., 2012, Bezu et al., 2015, Yamano et al., 2016). Despite its immunostimulatory potential, the systemic immune responses following oxaliplatin treatment remain largely unknown. We have previously demonstrated that oxaliplatin treatment causes the nuclear overexpression and cytoplasmic translocation of the DAMPs, calreticulin and HMGB1, within the myenteric neurons of the colon (Chapter 3). However, despite the induction of these hallmark features of immunogenic cell death, oxaliplatin treatment does not result in gastrointestinal inflammatory responses. We hypothesised that the lack of inflammation within the colonic mucosa and myenteric plexus following oxaliplatin treatment is due to tissue-specific responses, rather than immunosuppression by this anti-cancer agent.

The gastrointestinal mucosa is continuously challenged by a myriad of antigens, pathogens, nutrients, and ions, and is a prime target for

cytotoxic insult by anti-cancer agents due to its high proliferation rate (Spahn and Kucharzik, 2004, Schenk and Mueller, 2008). Given the constant exposure to harmful antigens, the gastrointestinal immune system has evolved a level of tolerance against pathogens and antigens (Spahn and Kucharzik, 2004, Vighi et al., 2008). Thus, bouts of inflammation in response to individual stimuli would be detrimental to the host.

The spleen plays a major role in augmenting systemic immune responses to blood borne pathogens and antigens, as it is rich in antigen presenting cells, and effector lymphocytes which produce appropriate adaptive immunological responses (Mebius and Kraal, 2005, Bronte and Pittet, 2014). The thymus and bone marrow provide a replenishing pool of leukocytes which migrate to lymphoid organs such as the spleen upon maturation. Currently, there is minimal research documenting the immunological changes within the spleen, thymus and bone marrow following oxaliplatin treatment.

The aims of this study were to investigate the effects of oxaliplatin treatment on: 1) spleen size and cellularity; 2) proportions of splenic $CD4^+$ and $CD8^+$ T cells and Tregs; 3) proportions of activated splenic T cells; 4) T cell pro-inflammatory and anti-inflammatory cytokines; 6) proportions of splenic B cells; 5) proportions of splenic macrophages and their pro-inflammatory and anti-inflammatory phenotypes and cytokines; 7) double-positive and single-positive $CD4^+$ and $CD8^+$ thymocytes; and 8) bone marrow hematopoietic progenitor and stem cells.

6.3 MATERIALS AND METHODS

6.3.1 Animals

Male, Balb/c mice (n=47, aged 5-7 weeks, weighing 18-25g) were used in this study. Mice had access to food and water *ad libitim* and were kept under a 12 hour light/dark cycle in a well-ventilated room at a temperature of 22 °C. Mice acclimatised for up to 1 week prior to the commencement of *in vivo* intraperitoneal injections. All efforts were made to minimise animal suffering, to reduce the number of animals used, and to utilise alternatives to *in vivo* techniques, if available. All procedures in this study were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Australian *Code of Practice for the Care and Use of Animals for Scientific Purposes.*

6.3.2 *In vivo* intraperitoneal injections

Mice were separated into 2 cohorts (n=5-15/group): 1) vehicle (sterile water), 2) oxaliplatin (3mg/kg, Sigma-Aldrich, Australia). All mice received intraperitoneal injections (maximum of 200µL/injection) using 26 gauge needles, tri-weekly for up to 14 days. Dosages were calculated per body mass as previously published to be equivalent to human dosage (Elias et al., 2004, Renn et al., 2011). Mice were culled via cervical dislocation 14 days subsequent to their first intraperitoneal injection, and spleen, thymus and bone marrow were harvested.

6.3.3 Flow cytometry

To identify changes in immune cell composition following oxaliplatin treatment, the spleen, thymus and bone marrow were harvested. Manual cell suspensions of the spleen and thymus were performed. The bone marrow was harvested using a syringe flush-out method on both hind limbs from each animal. Cell suspensions were centrifuged at 1500rpm for 5 minutes at 4°C, and resuspended in red blood cell lysis buffer (BD Biosciences, USA) and incubated in the dark for 20 minutes. Samples were centrifuged at 1500rpm for 5 minutes at 4°C. The supernatant of each cell suspension was aspirated and the pellet containing the immune cells was then resuspended in 1mL of FACS buffer and filtered. Aliquots (10µL) of each cell suspension were transferred into separate eppendorf tubes containing 10 µl of trypan blue. Manual cell counts were performed. Cells were transferred appropriately to 96 U-bottom well plates (BD Biosciences, USA) and were centrifuged at 1300rpm for 3 minutes at 4°C. Subsequent to centrifugation, the 96 U-bottom well plates were then aspirated. A selection of cell surface antibodies were used to identify various immune cell populations **(Table 6.1)**. For intracellular labelling of cytokines, spleen cell suspensions were permeabilised using a CytoFix/Perm kit (BD Biosciences, USA) according to manufacturer's instructions. Furthermore, 30µL of each antibody cocktail was loaded to appropriate wells, and incubated for 20 minutes at 4°C. Subsequent to the incubation period, cells were washed with 165µL of FACS buffer and centrifuged at 1300rpm for 3 minutes at 4°C. The plates were aspirated

and cells within each well were resuspended in 200µL of FACS buffer. Cells were then transferred to FACS tubes. BD Biosciences LSR II and FACS CANTO II flow cytometers were used to collect 200,000 cells from each cell suspension. Information was obtained via software FACSDiva™ (BD Biosciences, USA), and analysis was conducted using FlowJo (Tree Star, USA) or FACSDiva™.

Table 6.1. Antibodies used for flow cytometry experiments in this study

Cells	Primary antibody	Conjugate	Host species	Dilution
Pan-leukocyte marker	CD45	PerCP/Cy5.5	Mouse	1:400
Pan-T cell marker	CD3	Alexa Fluor 488	Mouse	1:400
T cell receptor	TCRβ	APC	Rat	1:250
Granulocytes	GR-1, CD11b	PE-Cy7	Rat	1:100
Cytotoxic T cells	CD8	Brilliant Violet 421	Rat	1:100
Helper T cells	CD4	Brilliant Violet 500	Rat	1:100
Regulatory T cells	FOL4	Alexa Fluor 647	Mouse	1:100
Activated T cells	CD25	PE-Cy7	Mouse	1:100
Activated T cells	CD69	APC-Cy7	Mouse	1:100
Activated T cells	PD-L1	PE	Mouse	1:100
B cells	B220, TCRβ	FITC	Mouse	1:400
Macrophages	CD11b, Ly6C, Ly6G, CD206, F4/80	PE	Rat	1:200
Hematopoietic Stem and Progenitor Cell	CD34	FITC	Mouse	1:100
Hematopoietic Stem and Progenitor Cell	c-Kit	PE	Mouse	1:100
Hematopoietic Stem and Progenitor Cell	Sca-1	PE-Cy7	Mouse	1:100
Hematopoietic Stem and	Lineage cocktail	APC	Mouse	1:100

Progenitor Cell				
Cytokines				
IL-6	IL-6	APC	Mouse	1:100
TNF-α	TNF-α	Brilliant Violet™ 510	Mouse	1:100
IL-10	IL-10	APC	Mouse	1:100
Transforming growth factor β (TGFβ)	TGFβ	Brilliant Violet™ 421	Mouse	1:100

6.3.4 RNA isolation and RT^2 Profiler PCR Arrays

Spleen tissue was removed from vehicle-treated and oxaliplatin-treated mice (n=5/group). Total RNA was extracted using the TRIzol™ procedure and further purified using an RNeasy® Mini kit (Qiagen, Hilden, Germany), including an on-column DNase digestion step to remove residual genomic DNA. The concentration of individual RNA samples was measured using a Qubit RNA BR Assay (Invitrogen) and RNA pools prepared for mRNA expression analysis by combining equal quantities of RNA within each group. The integrity of all RNA samples was assessed on an Agilent 2100 Bioanalyzer (Agilent Biotechnologies); setting the limit for inclusion in gene expression analysis as an RNA Integrity Number (RIN) of 9.0. All spleen RNA samples met this criterion; vehicle-treated group (mean 9.42 ± 0.13, n=5) and oxaliplatin-treated group (mean 9.64 ± 0.11, n=5). Gene expression was investigated using the pathway specific RT^2 Profiler PCR Array 'Mouse Cancer Inflammation and Immunity Crosstalk' (Qiagen, Cat. no. PAMM-181Z) according to the manufacturer's instructions. Reverse transcription was carried out with the RT^2 First Strand kit (Qiagen, Hilden, Germany) using 0.5 µg pooled RNA as template. PCR was performed in a

Biorad CFX96 thermal cycler using the following cycling conditions; initial denaturation for 10 minutes at 95°C followed by 40 cycles comprising 15 seconds at 95°C and 1 minute at 60°C (ramp speed 1°C per second). Melt curve analysis was performed to verify product specificity using the following program: 95°C for 1 minute; 65°C for 2 minutes followed by a temperature increase from 65°C to 95°C (2°C per minute) with fluorescence detection (SYBR Green). C_T values were determined using the Bio-rad CFX Manager™ software, setting the fluorescence threshold at a constant value across all arrays. Data was analysed via the Qiagen web portal at http://www.qiagen.com/geneglobe, wherein the expression level of each gene was normalised to the mean of five reference genes (*Gapdh*, *B2m*, *Actb*, *Hsp90ab1 and Gusb*) and fold change calculated using the $\Delta\Delta C_T$ method. A cut-off C_T value of 35 cycles was set as the limit for detection of gene expression. Changes in genes presented in this study include: Csf2, IL-1β, IL-10, IL-12β, chemokine receptor (Ccr) 2, 5, and 9, chemokine ligand (Cl) 5, and 22, Aicda, Bcl-2-like 1 protein (Bcl2l1), and Cytotoxic T lymphocyte-associated protein 4 (CTLA-4).

6.3.5 Statistical anlysis

Statistical analysis of the data included a paired t-test using GraphPad PrsimTM v6.0 (GraphPad Software Inc, USA). The data are represented as mean ± SEM. Statistical significance was defined where the *P* value was less than 0.05.

6.4 RESULTS

6.4.1 Oxaliplatin treatment decreases spleen mass

Oxaliplatin treatment caused a significant reduction in spleen mass (0.065 ± 0.004mg, *P*<0.0001; n=14) when compared to those obtained from the vehicle-treated cohort (0.102 ± 0.003mg; n=9) **(Figure 6.1 A-A^{I})**. Furthermore, we determined cellularity with respect to $CD45^+$ cells using flow cytometry. A significant reduction in the proportion of $CD45^+$ leukocytes was noted following oxaliplatin treatment (44.6 ± 4.7%, *P*<0.001; n=4) when compared to the vehicle-treated group (84.4 ± 0.4%; n=4) **(Figure 6.1 B)**.

6.4.2 Oxaliplatin treatment differentially affects $CD4^+$, $CD8^+$, and Treg populations within the spleen

To determine any changes in the proportions of $CD4^+$ and $CD8^+$ T cells, we gated on $CD3^+CD4^+/CD8^+/GR\text{-}1^-/FOLR4^+$ expressing cells and cytokines (pro-inflammatory: IL-6 and TNF-α; anti-inflammatory: IL-10 and TGFβ) **(Figure 6.2 A-C^{II})**. Oxaliplatin treatment caused a significant increase in the proportion of $CD4^+$ T cells (26.6 ± 1.1%, *P*<0.01; n=5) when compared to the vehicle-treated cohort (20.8 ± 0.6%; n=5) **(Figure 6.2 D)**. Oxaliplatin treatment also caused a significant increase in the proportion of $CD8^+$ T cells (44.0 ± 0.9%, *P*<0.01; n=9) when compared to the vehicle-treated group (38.5 ± 0.7%; n=4) **(Figure 6.2 E)**. Furthermore, a significant reduction in the proportion of regulatory T cells (Tregs) was

noted following oxaliplatin treatment (43.4 ± 1.3%, *P*<0.01; n=5) when compared to the vehicle-treated cohort (54.2 ± 0.7%; n=5) **(Figure 6.2 F)**.

No changes in IL-6 expression of T cells from the spleen was observed between the vehicle-treated (0.20 ± 0.01%; n=9) and the oxaliplatin-treated mice (1.80 ± 0.97%; n=14) **(Figure 6.2 G)**. However, a significant increase in TNF-α was observed in the oxaliplatin-treated group (17.9 ± 4.6%; *P*<0.01; n=14) when compared to the vehicle-treated cohort (3.5 ± 1.4%; n=9) **(Figure 6.2 G)**. There were no changes to anti-inflammatory cytokines between the vehicle-treated (IL-10: 0.10 ± 0.03%; n=9; TGF-β: 0.5 ± 0.2%; n=9) and oxaliplatin-treated mice (IL-10: 0.14 ± 0.03%; n=14; TGF-β: 0.39 ± 0.06%; n=14) **(Figure 6.2 H)**.

To determine whether T cells were activated, we gated on $CD4^+$, $CD8^+$, and $FOLR4^+$ expressing cells double-positive for the activation markers CD25, CD69, and PD-1 **(Figure 6.3 A-B")**. There were no significant differences in the proportion of activated $CD3^+$ $CD4^+$ $CD25^+$ T cells following oxaliplatin treatment (0.04 ± 0.02%; n=5) when compared to the vehicle-treated cohort (0.08 ± 0.02%; n=5) **(Figure 6.3 C)**. No significant differences in the proportion of activated $CD3^+$ $CD4^+$ $CD69^+$ T cells following oxaliplatin treatment (0.42 ± 0.03%; n=5) compared to vehicle-treated contrtol (0.4 ± 0.03%; n=5) **(Figure 6.3 D)**. No significant differences in the proportion of activated $CD3^+$ $CD8^+$ $CD25^+$ T cells were observed following oxaliplatin treatment (0.08 ± 0.04%; n=5) when compared to the vehicle-treated cohort (0.18 ± 0.04%; n=5) **(Figure 6.3 E)**. A significant increase in the proportion of activated $CD3^+$ $CD8^+$ $CD69^+$

T cells were noted following oxaliplatin treatment (0.23 ± 0.05%, *P*<0.01; n=5) when compared to the vehicle-treated cohort (0.1 ± 0.001%; n=5) **(Figure 6.3 F)**. A significant increase in the proportion of activated $CD3^+$ $CD8^+$ $PD\text{-}1^+$ T cells were observed following oxaliplatin treatment (0.42 ± 0.06%, *P*<0.01; n=5) when compared to the vehicle-treated cohort (0 ± 0%; n=5) **(Figure 6.3 G)**. No significant differences in the proportion of activated $CD4^+$ $FOLR4^+$ $CD25^+$ T cells were observed following oxaliplatin treatment (0.18 ± 0.06%; n=5) when compared to the vehicle-treated control (0.18 ± 0.05%; n=5) **(Figure 6.3 H)**. However, a significant increase in the proportion of activated $CD4^+$ $FOLR4^+$ $CD69^+$ T cells were observed following oxaliplatin treatment (2.69 ± 0.21%, *P*<0.01; n=5) when compared to the vehicle-treated cohort (1.7 ± 0.15%; n=5) **(Figure 6.3 I)**.

6.4.3 Oxaliplatin treatment decreases B cell proportions in the spleen

B cells were identified by gating on $CD45^+$ $TCR\beta^-$ $B220^+$ cells **(Figure 6.4 A-A^{II})**. Oxaliplatin treatment caused a significant reduction in the proportion of B cells (23.1 ± 2.4%, *P*<0.0001; n=4) when compared to the vehicle-treated cohort (49 ± 0.7%; n=4) **(Figure 6.4 B)**.

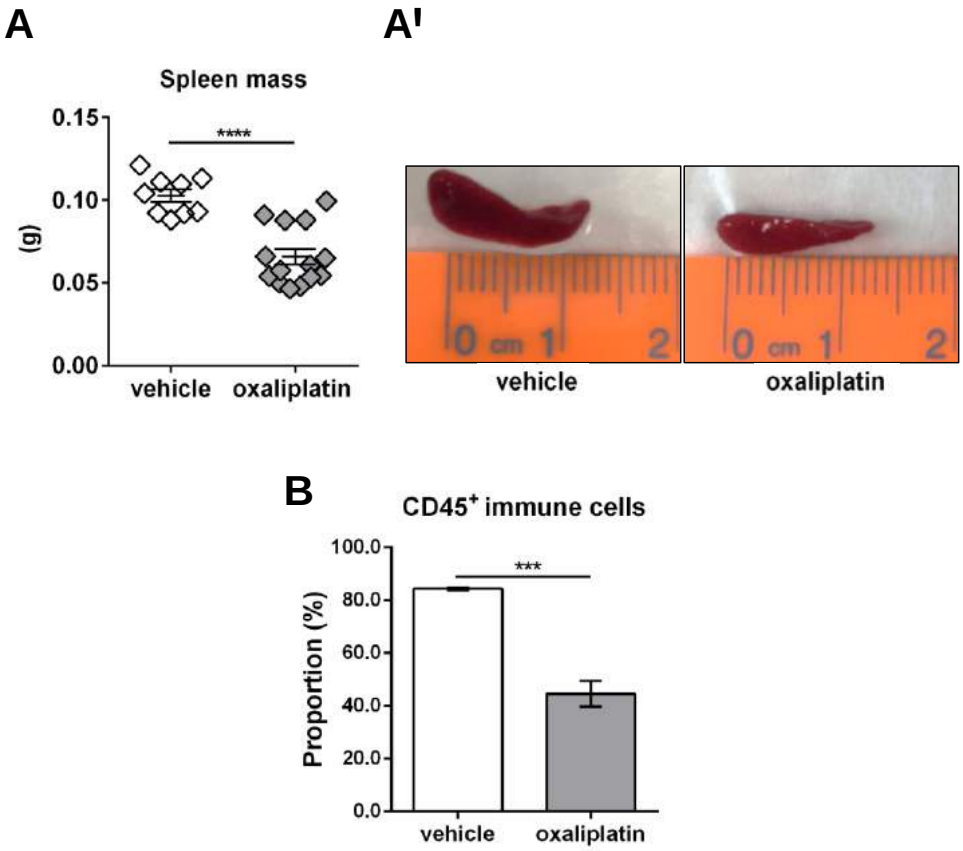


Figure 6.1. Effects of oxaliplatin treatment on spleen mass and cellularity. To investigate toxicity of the spleen following oxaliplatin treatment we measured spleen weight (g). Oxaliplatin treatment caused a significant reduction in spleen mass when compared to those obtained from the vehicle-treated cohort **(A, A'')**. A significant reduction in the proportion of $CD45^+$ leukocytes was observed following oxaliplatin treatment when compared to the vehicle-treated group **(B)**. ****P*<0.001; *****P*<0.0001.

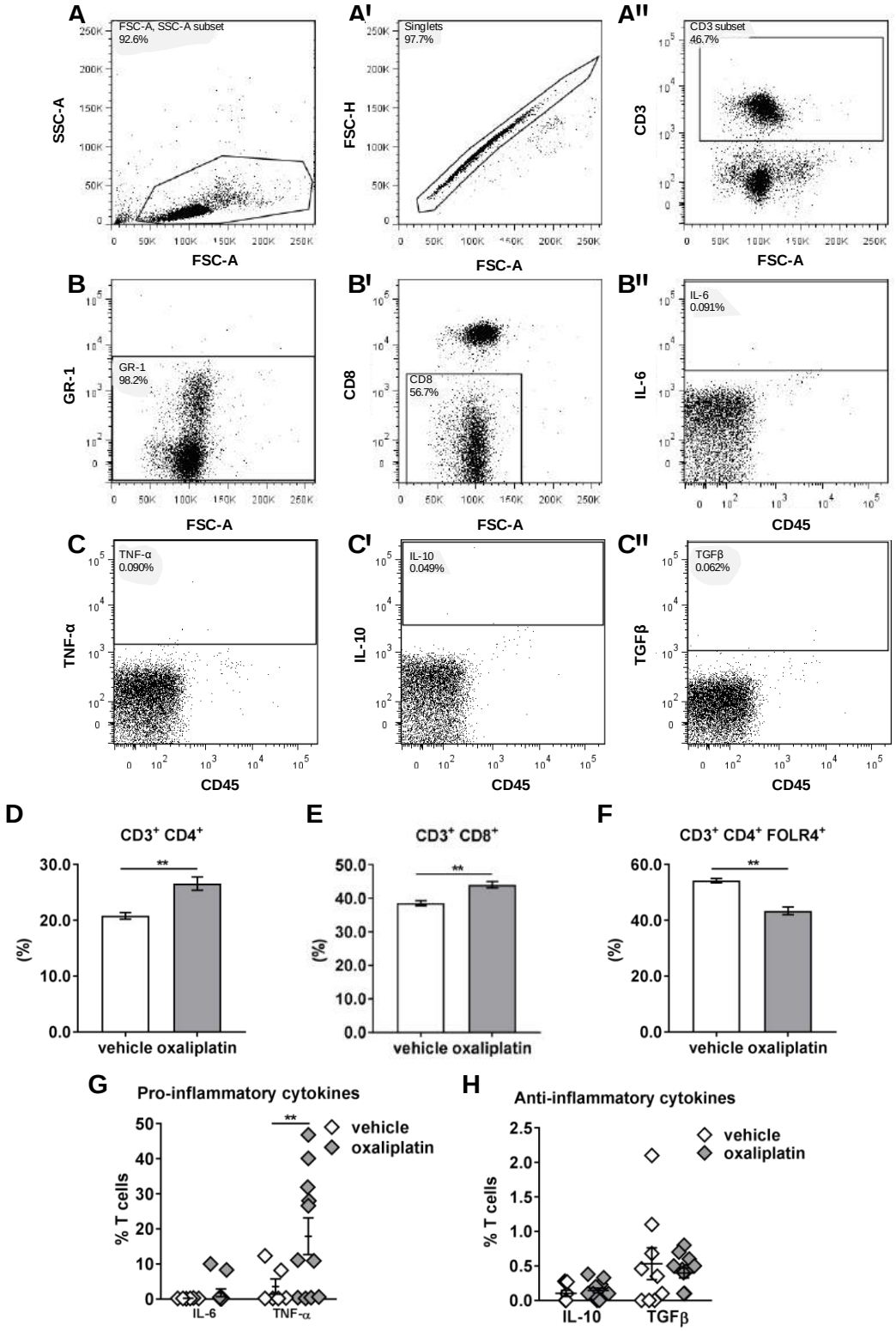


Figure 6.2. Effects of oxaliplatin treatment on the proportion of T cell populations and pro-/anti-inflammatory cytokines within the spleen. To determine any changes in the proportions of $CD4^+$ and $CD8^+$ T cells and Tregs we gated on $CD3^+CD4^+/CD8^+/GR\text{-}1^-/FOLR4^+$ events **(A-B^I)**. Pro-inflammatory and anti-inflammatory cytokines were gates on IL-6, TNF-α, IL-10 and TGFβ events **(B^{II}-C^{II})**. Oxaliplatin treatment caused a significant increase in the proportion of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells when compared to the vehicle-treated group **(D-E)**. Conversely, oxaliplatin treatment caused a significant decrease in the proportion of $CD3^+$ $CD4^+$ $FOLR4^+$ T cells when compared to the

vehicle-treated group **(F)**. No changes in IL-6 expression was observed between the vehicle-treated and the oxaliplatin-treated mice, however, a significant increase in TNF-α was observed in the oxaliplatin-treated group when compared to the vehicle-treated cohort **(G)**. No changes in either anti-inflammatory cytokines were observed between the vehicle-treated and oxaliplatin-treated mice **(H)**. Vehicle n=5-9; oxaliplatin n=5-14; ***P*<0.01.

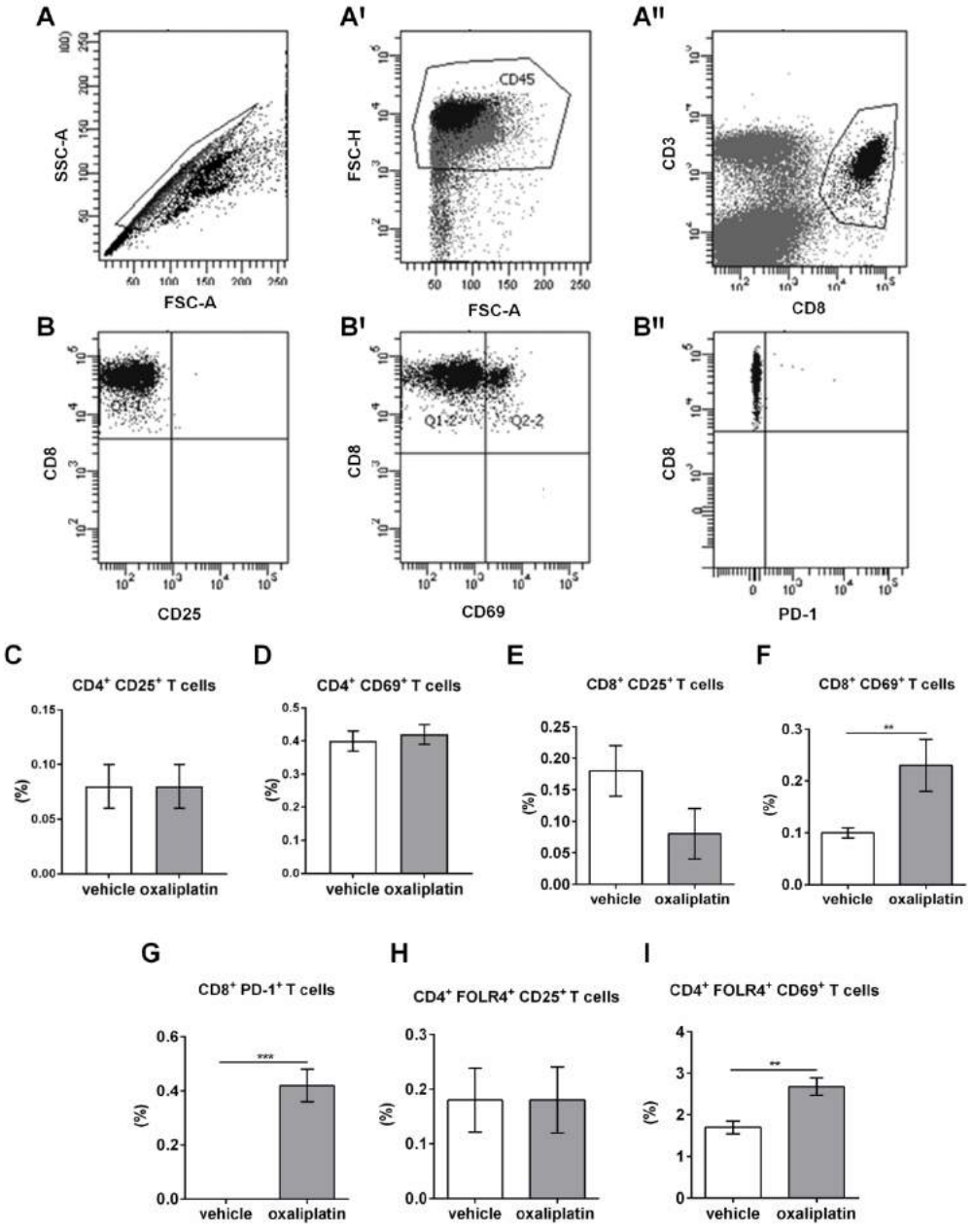


Figure 6.3. Effects of oxaliplatin treatment on the proportion of activated T cell populations within the spleen. To determine any changes in the proportions of activated $CD4^+$ and $CD8^+$ T cells, we gated on $CD4^+$ $CD25^+$ $CD69^+$, $CD8^+$ $CD25^+$ $CD69^+$ $PD\text{-}1^+$, and $CD4^+$ $FOLR4^+$ $CD25^+$ $CD69^+$ events **(A-B^{II})**. Oxaliplatin treatment did not cause any changes in the proportion of activated $CD4^+$ T cells **(C-D)**. However, a significant increase in the proportion of $CD8^+$ $CD69^+$, but not $CD8^+$ $CD25^+$ was observed following oxaliplatin treatment when compared to the vehicle-treated group **(E-F)**. Oxaliplatin caused a significant increase in the proportion of $CD8^+$ $PD\text{-}1^+$ T cells **(G)**. Oxaliplatin caused a significant increase in the proportion of $CD4^+$ $FOLR4^+$ $CD69^+$, but not $CD4^+$ $FOLR4^+$ $CD25^+$ Tregs, when compared to the vehicle-treated group **(H-I)**. Vehicle n=5-9; oxaliplatin n=5-14; $^{**}P<0.01$.

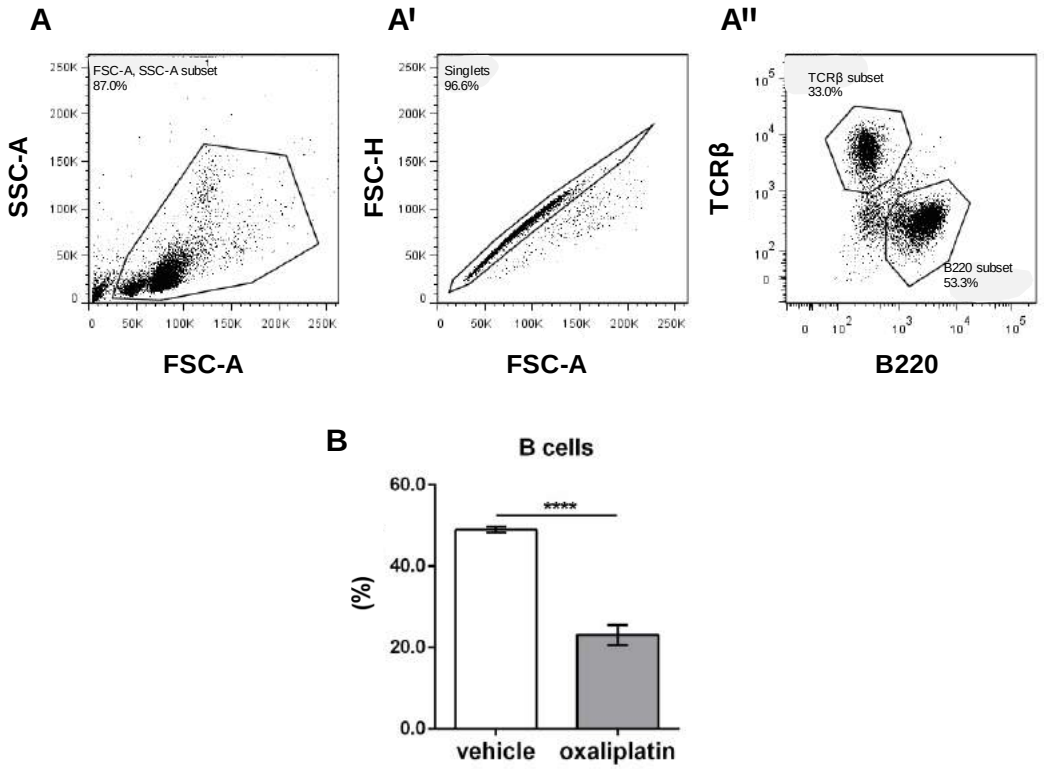


Figure 6.4. Effects of oxaliplatin treatment on the proportion of B cells within the spleen. B cells were identified by gating on $CD45^{+}$ $TCR\beta^{-}$ $B220^{+}$ cells **(A-A^{II})**. Oxaliplatin treatment caused a significant reduction in the proportion of B cells when compared to the vehicle-treated cohort **(B)**.

6.4.4 Oxaliplatin treatment has no effects on macrophage phenotypes or pro-/anti-inflammatory cytokine production in the spleen

To determine changes in immune cell populations within the spleen we profiled M1/M2 macrophages as well as the expression of pro-inflammatoy (IL-6 and TNF-α) and anti-inflammatory (IL-10 and TGFβ) cytokines. To determine any changes in the proportions of pro-inflammatory and anti-inflammatory macrophages and cytokines (pro-inflammatory: IL-6 and TNF-α; anti-inflammatory: IL-10 and TGFβ), a set of gating strategies were used. M1 macrophages were gated based on $CD45^+$ $CD11B^+$, $Ly6G^+$ $Ly6C^+$, $CD11C^+$ $MHC\text{-}II^+$, $CD206^+$ expressing cells **(Figure 6.5 A-D')**. M2 macrophages were gated based on: $CD45^+$ $CD11B^+$, $Ly6G^+$ $CD45^+$, $CD11B^+$ $MHC\text{-}II^+$, $CD206^+$ expressing cells **(Figure 6.5 A-D')**. There were no differences in M1 macrophages amongst the vehicle-treated (90.3 ± 1.9%; n=6) and oxaliplatin-treated (88.4 ± 2.0%; n=14) groups **(Figure 6.5 E)**. No differences were observed in M2 macrophages amongst the vehicle-treated (9.6 ± 1.5%; n=6) and oxaliplatin-treated groups (11.5 ± 2.0%; n=14) **(Figure 6.5 E)**. There were no changes in M1 cytokines between the vehicle-treated (IL-6: 0.05 ± 0.01%; n=9; TNF-α: 0.91 ± 0.29%; n=9) and oxaliplatin-treated mice (IL-6: 0.61 ± 0.34%, n=14; TNF-α: 0.98 ± 0.45%; n=14) **(Figure 6.5 F)**. There were no differences in M2 cytokines amongst the vehicle-treated (IL-10: 2.7 ± 1.07%; n=9; TGFβ: 2.1% ± 0.84%; n=9) and oxaliplatin-treated mice (IL-10: 2.5 ± 0.80%; n=14; TGFβ: 2.7 ± 1.2%; n=14) **(Figure 6.5 G)**.

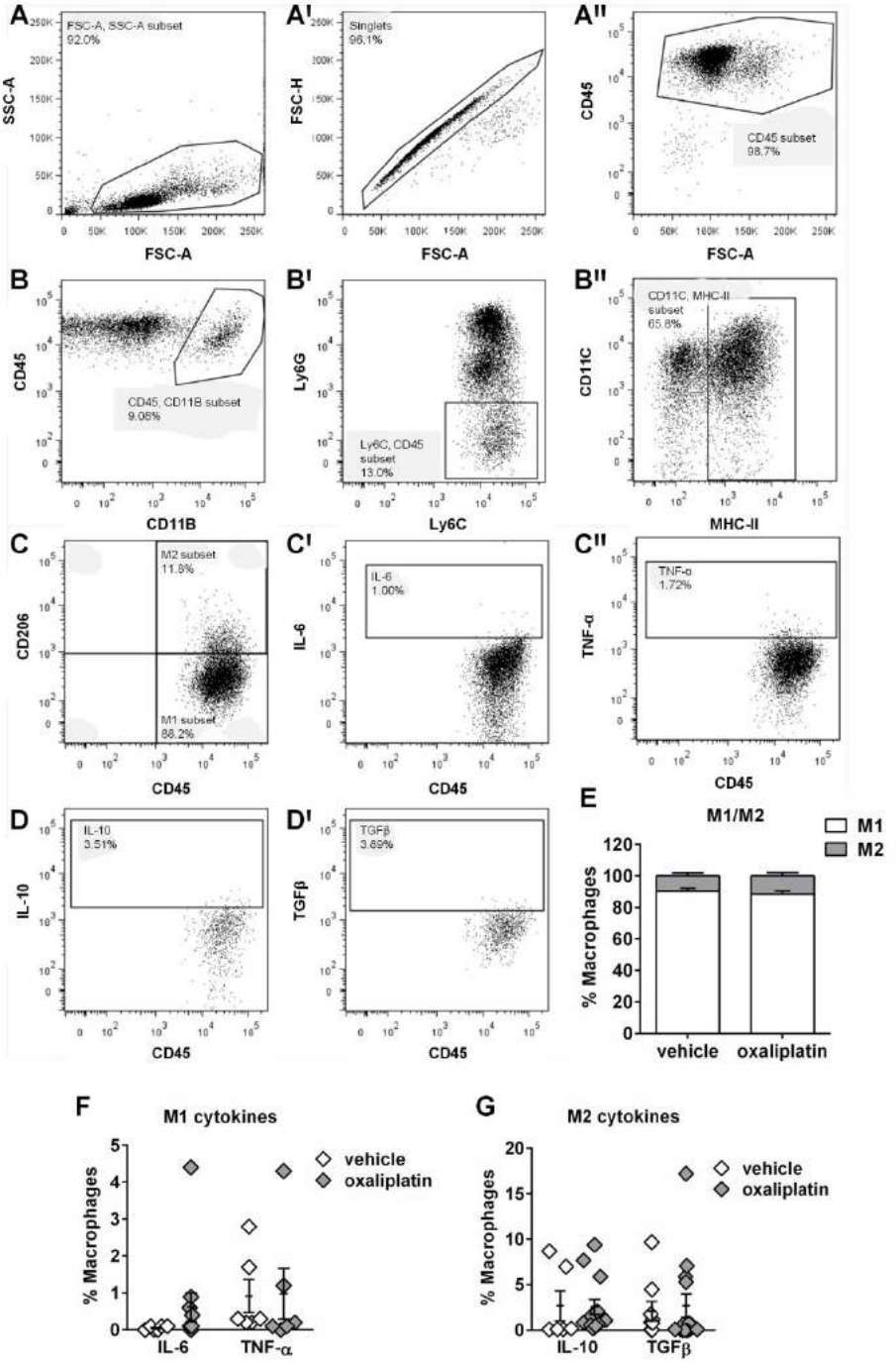


Figure 6.5. Effects of oxaliplatin treatment on the proportion of M1/M2 phenotypes and pro-/anti-inflammatory cytokines within the spleen. To determine any changes in the proportions of pro-inflammatory and anti-inflammatory macrophages, a set of gating strategies were used. M1 macrophages were gated on $CD45^+$ $CD11B^+$, $Ly6G^+$ $Ly6C^+$, $CD11C^+$ $MHC\text{-}II^+$, $CD206^+$ $CD45^+$ cells **(A-C)**. M2 macrophages were gated on: $CD45^+$ $CD11B^+$, $Ly6G^+$ $CD45^+$, $CD11B^+$ $MHC\text{-}II^+$, $CD206^+$ $CD45^+$ cells **(A-C)**. To investigate any changes to pro-inflammatory cytokine expression cells were gated on their expression of M1 phenotypes versus IL-6, TNF-α, IL-10 and TGFβ **(C^{I}-C^{II})**. To determine the expression of anti-inflammatory cytokines from M2 macrophages

cells were gated on their phenotype versus IL-10 or TGF-β **(D^{I}-D^{II})**. No differences in M1 macrophages were observed between the vehicle-treated and oxaliplatin-treated animals **(E)**. No differences were observed in M2 macrophages amongst the vehicle-treated **(C)**. No changes in M1 or M2 cytokines were observed between the vehicle-treated and oxaliplatin-treated mice **(F-G)**. Vehicle n=9; oxaliplatin n=14.

6.4.5 Effects of oxaliplatin treatment on inflammation-associated genes in the spleen

To determine qualitative changes in inflammation-associated genes, RT-PCR of spleen RNA was performed using RT^{2-} PCR-arrays. Oxaliplatin treatment caused a decrease in Csf2 (-1.64 fold change), IL-1β (-2.00 fold change), IL-10 (-1.62 fold change), and IL-12β (-2.97 fold change) **(Figure 6.6 A)**. Oxaliplatin treatment caused the upregulation of Ccr2 (1.71 fold change), but downregulated the genes Ccr5 (-1.63 fold change), Ccl5 (-1.77 fold change), Ccl22 (-1.68 fold change), and Ccr9 (-1.61 fold change) **(Figure 6.6 B)**. Oxaliplatin treatment downregulated the genes (Aicda; -2.01 fold change), Bcl2l1(-2.75 fold change), as well as CTLA-4 (-1.96 fold change) **(Figure 6.6 C)**. The effect of oxaliplatin was limited, with most genes on the array showing no detectable difference in mRNA expression (less than 1.5 fold) when comparing the oxaliplatin-treated group to the vehicle-treated group.

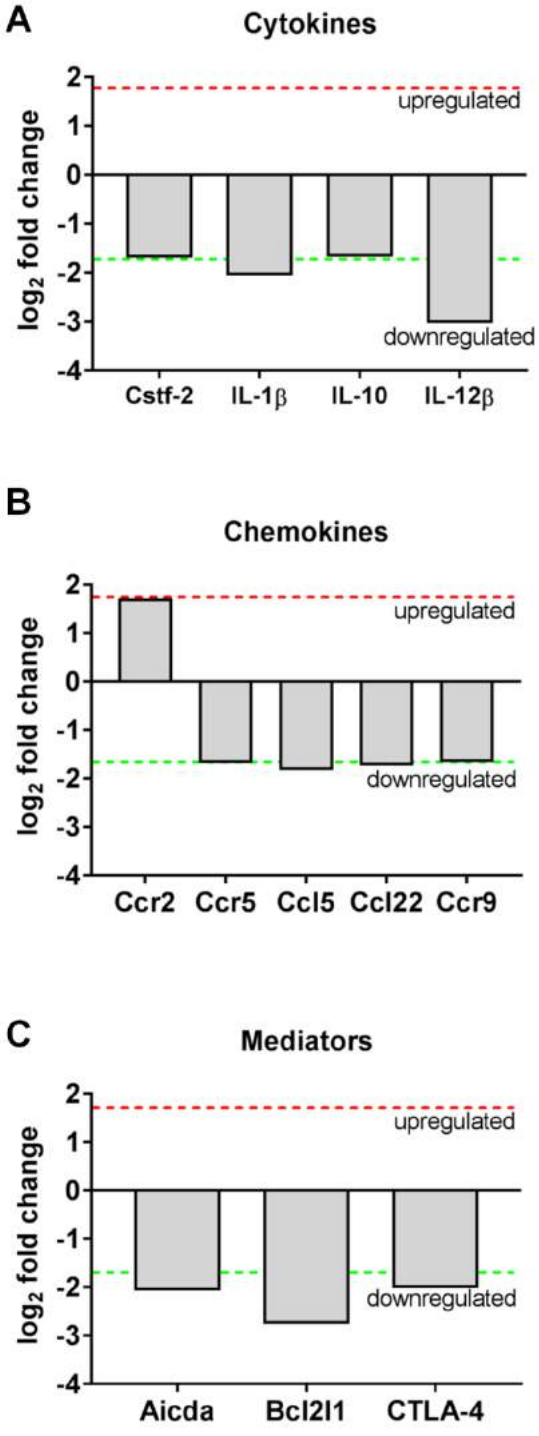


Figure 6.6. Effects of oxaliplatin treatment on inflammation-associated genes within the spleen. To determine qualitative changes in inflammation-associated genes, RT-PCR of spleen RNA was performed. Oxaliplatin treatment caused the downregulation of Csft2, IL-1β, IL-10, and IL-12β **(A)**. Oxaliplatin treatment caused the upregulation of the Ccr2 gene, but downregulated the genes Ccr5, Ccl5, Ccl22, and Ccr9 **(B)**. Furthermore, oxaliplatin treatment downregulated the genes Aicda, Bcl2l1, and CTLA-4 **(C)**.

6.4.6 Oxaliplatin treatment increases $CD8^+$ single-positive thymocytes, with no effects on $CD4^+$ or $CD4^+$ $CD8^+$ double-positive populations

To assess changes in the proportions of double-positive and single-positive thymocytes, cells were gated by $CD4^+$ $CD8^+$ populations **(Figure 6.7 A-A'''')**. No significant differences were observed in the proportion of double-positive $CD4^+$ $CD8^+$ thymocytes following oxaliplatin treatment (63.8 ± 5.7%; n=5) when compared to the vehicle-treated cohort (71.4 ± 4.5%; n=5) **(Figure 6.7 B)**. Furthermore, no significant differences were observed in the proportion of single-positive $CD4^+$ thymocytes following oxaliplatin treatment (8.0 ± 1.06%; n=5) when compared to the vehicle-treated cohort (9.7 ± 0.3%; n=5) **(Figure 6.7 C)**. Oxaliplatin treatment caused a significant increase in the proportion of single-positive $CD8^+$ thymocytes following oxaliplatin treatment (14.4 ± 2.4%; n=5) when compared to the vehicle-treated cohort (7.4 ± 0.6%; n=5) **(Figure 6.7 D)**.

6.4.7 Oxaliplatin treatment has no demonstrable effects on bone marrow hematopoietic stem and progenitor cells

To ascertain whether changes in the proportions of bone marrow hematopoietic stem and progenitor cells, cells were gated on Lin^- /$CD117^+$/$Sca\text{-}1^+$ cells **(Figure 6.8 A-A'''')**. No significant differences were observed in the proportion of Lin^-/$CD117^+$/$Sca\text{-}1^+$ cells following oxaliplatin treatment (0.2 ± 0.05%; n=5) when compared to the vehicle-treated cohort (0.24 ± 0.02%; n=5) **(Figure 6.8 B)**.

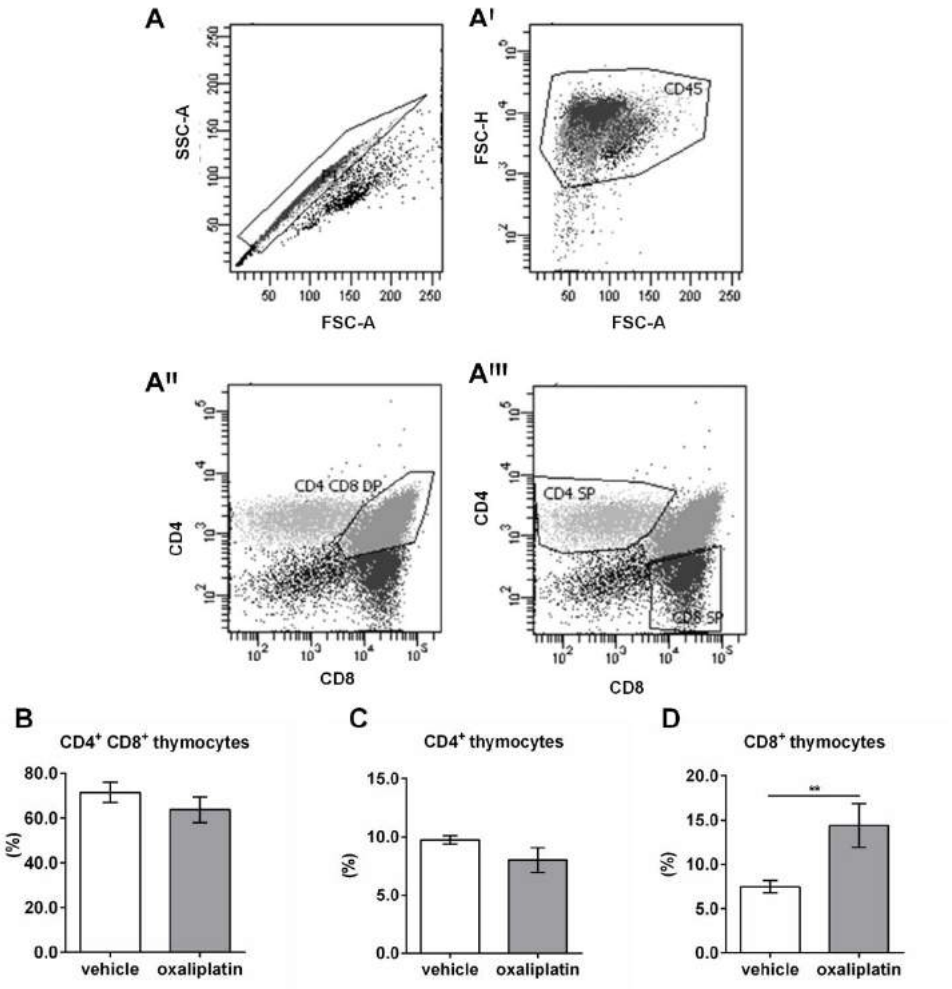


Figure 6.7. Effects of oxaliplatin treatment on T cell populations within the thymus. To determine any changes in the proportions of double-positive and single-positive thymocytes, we gated on $CD4^+$ $CD8^+$ events **(A-A^{III})**. No significant changes were observed in the proportion of double-positive $CD4^+$ $CD8^+$ thymocytes following oxaliplatin treatment when compared to the vehicle-treated cohort **(B)**. No significant differences were observed in the proportion of single-positive $CD4^+$ thymocytes following oxaliplatin treatment when compared to the vehicle-treated cohort **(C)**. Oxaliplatin treatment caused a significant increase in the proportion single-positive $CD8^+$ thymocytes following oxaliplatin treatment when compared to the vehicle-treated cohort **(D)**. N=5/group; $^{**}P<0.01$.

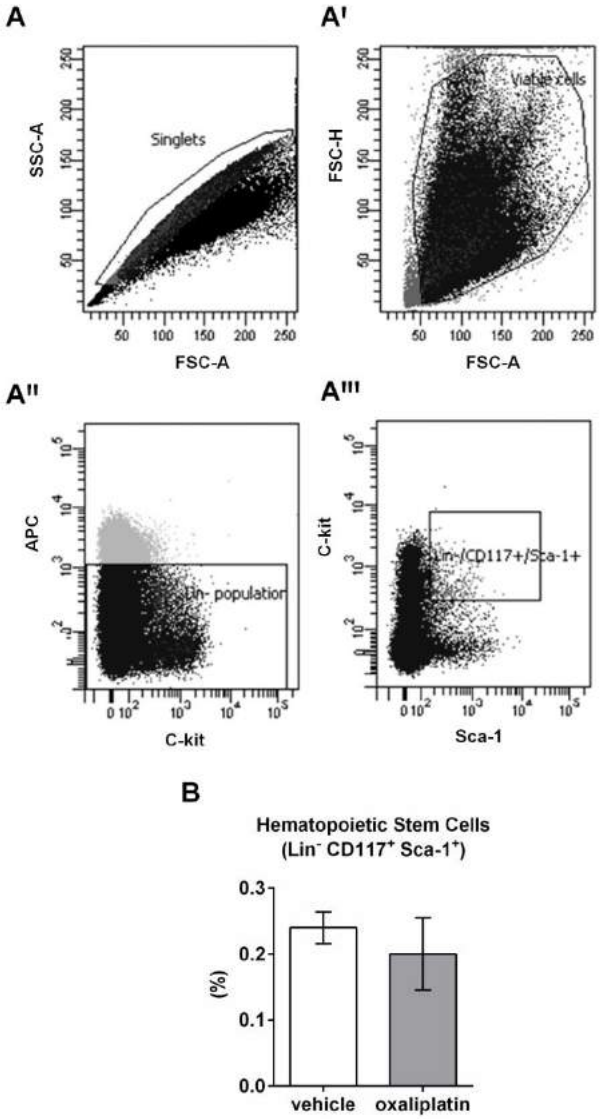


Figure 6.8. Effects of oxaliplatin treatment on bone marrow hematopoietic stem and progenitor cells. To investigate any changes in the proportions of bone marrow hematopoietic stem and progenitor cells, we gated on $Lin^-/CD117^+/Sca\text{-}1^+$ events **(A-A^{III})**. No significant differences were observed in the proportion of $Lin^-/CD117^+/Sca\text{-}1^+$ cells following oxaliplatin treatment when compared to the vehicle-treated cohort **(B)**. N=5/group.

6.5 DISCUSSION

This study is the first to determine systemic immune responses following oxaliplatin treatment in the mouse spleen, thymus and bone marrow. In Chapters 3 and 4, we have shown that oxaliplatin accumulates within the LMMP of the colon and brainstem, and evokes the presentation of DAMPs within neurons in the colon. The presentation of DAMPs following oxaliplatin treatment has previously been shown to induce immunogenic cell death in colorectal tumor cell lines (Tesniere et al., 2010). Despite the potential to induce immunogenic cell death, oxaliplatin did not cause any inflammatory responses within the gastrointestinal tract as demonstrated in Chapter 5. Thus, it was hypothesised that this may be due to tissue-specific immune responses within the gastrointestinal tract, and that immunological responses may differ systemically.

The spleen functions to clear aged erythrocytes, filter blood-borne pathogens, antigens, and foreign materials, and play a major role in augmenting appropriate systemic immune responses (Cesta, 2006, Bronte and Pittet, 2014). As the spleen receives a large volume of blood, this organ may be particularly vulnerable to platinum-based anti-cancer agents, or perhaps, may be a site for generating immunological responses to chemotherapy. In this study, we have shown that oxaliplatin treatment caused a significant decrease in spleen size and in the proportion of $CD45^+$ immune cells. Previous work investigating spleen size following anti-cancer chemotherapy is conflicting. Computed tomography imaging of spleens from patients undergoing carboplatin/paclitaxel or

cisplatin/etopisode chemotherapy and concomitant radiotherapy for non-small cell lung carcinoma demonstrate a decrease in spleen volume in 66% and 79% of patients respectively (Wen et al., 2015). Patient spleen size is typically estimated by multiplying organ length by width and height. Furthermore, splenomegaly has been observed in colorectal cancer patients receiving oxaliplatin in a FOLFOX regimen (Angitapalli et al., 2009, Overman et al., 2010, Jung et al., 2012). Aside from our data, it is unclear how platinum-based drugs affect spleen size when given as a single agent, and thus, further work is required to understand these changes in organ size.

Despite a reduction in spleen size and cellularity following oxaliplatin treatment, the proportions of overall $CD4^+$ and $CD8^+$ T cells were increased in this cohort when compared to the vehicle-treated group. Helper $CD4^+$ T cells play a role in adaptive immunity by conditioning the environment and essentially, modulating the activity of other immune cells through cytokine production and antigen cross-presentation to $CD8^+$ T cells (Zhu and Paul, 2008, Luckheeram et al., 2012). There are limited studies regarding the effects of oxaliplatin and the predecessor platinum-based agents on $CD4^+$ T cells. Studies investigating the effects of other anti-cancer agents such as cyclophosphamide have shown that this drug can selectively deplete Tregs and restore effector T cell function which is imperative for anti-tumour responses, as well as mounting appropriate immune responses to antigens (Awwad and North, 1988, Weir et al., 2014). Previous work has shown that cisplatin given in combination with a

TLR9 agonist CpG and a pan-human leukocyte antigen DR binding epitope enhances systemic $CD4^+$ T cell responses against pappilomavirus 16 E7 tumours (Song et al., 2014). Moreover, cisplatin treatment also leads to an increase in $CD4^+$ T and $CD8^+$ T cell-mediated immune responses leading to nephrotoxicity (Liu et al., 2006). These data demonstrate the immunostimulatory potential of platinum-based drugs to mount anti-tumour responses, but highlight the fact that they may also mediate tissue injury.

$CD8^+$ T cells play a role in cell-mediated cytotoxicity through cytokine release, death ligand stimulation, and perforin/granzyme B-mediated pathways. In this present study, we have shown that oxaliplatin treatment increases the overall proportion of $CD8^+$ T cells, and enhances $CD8^+$ T cell activation as demonstrated by double-positive CD69 and PD-1 expression (Wu et al., 2014, Liu et al., 2016, Yuzefpolskiy et al., 2016). These data show that $CD8^+$ T cells have been primed and activated as a result of appropriate antigen-presentation. PD-1 has recently been identified as a marker for T cell exhaustion following prolonged activation in chronic disease, but the double-positive expression of CD8 and the early-activation marker CD69 indicate that these T cells may still retain full functional capacity. Our study is in line with earlier reports which had shown that increased $CD8^+$ T cell activation and function following oxaliplatin treatment in peripheral blood and colon cancer cell lines (Tesniere et al., 2010, Krysko et al., 2012). Previous work assessing peripheral neuropathy following oxaliplatin treatment in C57BL/6J mice

has demonstrated an increase in circulating $CD8^+$ T cells (Makker et al., 2017). Although $CD8^+$ T cells were not measured in the blood, T cells primed and activated within the spleen migrate to sites of damage. Furthermore, the addition of cisplatin to an immunotherapy vaccine comprised of calreticulin and papillomavirus 16 E7 antigens for the treatment of cervical cancer enhances $CD8^+$ T cell responses (Tseng et al., 2008). The activation states of $CD8^+$ T cells are further supported by the downregulation of CTLA-4 gene expression observed in this study. This gene codes for the CTLA-4 inhibitory ligand which is a negative regulator of T cell function (Hannani et al., 2015, Walker and Sansom, 2015, Buchbinder and Desai, 2016). Thus, it is becoming well known that platinum-based agents can induce T cell responses that would be beneficial for cancer treatment; however, it is currently unknown whether these $CD8^+$ T cells will migrate from the spleen in response to oxaliplatin treatment, and this requires further work.

Tregs are well known for their immunosuppressive roles in maintaining self-tolerance and in controlling inflammatory responses (Lee et al., 2009, Pellerin et al., 2014). In this study, we showed that oxaliplatin treatment caused a reduction in the proportion of Tregs when compared to the vehicle-treated cohort. However, the proportion of activated Tregs following oxaliplatin treatment increased. Our findings are in contrast to a study which demonstrated an increase of Tregs in blood samples from patients receiving combined oxaliplatin and 5-fluorouracil treatment for CRC (Maeda et al., 2011). It is unclear why a decrease in Tregs is

observed following oxaliplatin treatment, but the addition of 5-flourouracil to the treatment may induce differential immune responses (Kobayashi et al., 2007, Ghiringhelli et al., 2013, Wang et al., 2016). An increase in Tregs activation may be to counteract the activated $CD8^+$ T cells.

In this study, we also observed the increased expression of the pro-inflammatory cytokine TNF-α in splenic T cells. Most research has demonstrated that platinum-based drugs induce TNF-α production by non-immune cells. Previous studies showed that oxaliplatin treatment causes astrocyte and glial cell activation and the production of TNF-α in a rat model of peripheral neuropathy (Jung et al., 2017). Additionally, an increase in TNF-α expression by spinal glial cells has also been observed in a model of oxaliplatin-induced neuropathic cold allodynia (Kim et al., 2016). In addition to this, an increase in TNF-α has been previously described following cisplatin treatment in kidney proximal tubule and epithelial cells (Ramesh and Brian Reeves, 2006, Ramesh et al., 2007). It is known that TNF-α can alter neuronal function, and induce cell death via the extrinsic apoptosis pathway (death receptor-mediated cascades) (Fulda and Debatin, 2006). In our previous work, we have shown that oxaliplatin treatment causes caspase 3 cleavage in myenteric neurons. TNF-α binding to the TNF superfamily receptor recruits the caspases 8/10 to the death domain docking site and initiates the apoptotic cascade for the cleavage of caspase 3. The extrinsic and intrinsic apoptotic pathways can have some crossover and it is unclear whether death receptor stimulation has played a role in initiating the apoptotic cascade.

In Chapter 4, we demonstrated that platinum from oxaliplatin accumulates within the cerebrum and brainstem. Oxaliplatin is a bulky drug that was originally thought to be too big to pass through the blood brain barrier. However, it is well known that pro-inflammatory cytokines can alter blood brain barrier permeability which could allow for oxaliplatin accumulation (Pan et al., 2011, Rochfort et al., 2014, Troletti et al., 2016). Cytokine-mediated reduction in blood brain barrier integrity could be implicated in the platinum accumulation within the brain, however, further work is required to elucidate this concept. No differences in the expression of the anti-inflammatory cytokines IL-10 and TGFβ was observed following oxaliplatin treatment. This suggests that no anti-inflammatory responses are being initiated to counteract the increased proportion of activated $CD4^+$ and $CD8^+$ T cells and TNF-α production.

We have shown that oxaliplatin is particularly cytotoxic to splenic B cells, and caused the downregulation of the Aicda gene which plays a role in B cell proliferation and Ig class-switching (Heltemes-Harris et al., 2008, Park, 2012). Within the spleen, B cells capture blood-borne antigens though complement receptors, and can initiate T cell-dependent/independent responses (Pillai and Cariappa, 2009). Research has shown that B cells can impede T cell activation by expressing IgA , IL-10, and PD-L1, and by promoting T cell conversion to Tregs (Olkhanud et al., 2011, Tadmor et al., 2011, Liu., 2015, Shalapour et al., 2015, Schwartz et al., 2016). The overall reduction in B cells and Aicda following oxaliplatin treatment may affect IgA, IL-10, and PD-L1 production, and

thus, enable optimal T cell activation. As spleen mass was significantly reduced following oxaliplatin treatment despite strong T cell responses, the depletion of B cells may account for the change in organ size.

Macrophages within the spleen play a role in antigen recognition, processing and presentation to T cells, to ultimately mount an appropriate immune response (Miyake et al., 2007, Asano et al., 2011). In our current study, we have shown that oxaliplatin treatment has no effects on the proportion of M1/M2 macrophages, or their pro-inflammatory and anti-inflammatory cytokines. M1 macrophages produce the pro-inflammatory cytokines IL-6 and TNF-α upon activation, whereas the M2 phenotype expresses the anti-inflammatory cytokines IL-10 and TGFβ (De Palma and Lewis, Martinez and Gordon, 2014). Splenic macrophages are in close contact with T cells, and thus, their location favours rapid antigen presentation (Backer et al., 2010, Borges da Silva et al., 2015). Whether changes in macrophage cytokine profiles have occurred in earlier stages remains unknown, but the increase in activated T cells suggests sufficient antigen presentation has occurred.

In addition, we show that oxaliplatin treatment differentially effects chemokine receptors and ligand expression within the spleen. Oxaliplatin treatment caused a marked increase in Ccr2 expression. Ccr2 is highly expressed on macrophages, and ligation by Ccl2 induces the recruitment of peripheral monocytes during infectious and inflammatory conditions (Shi and Pamer, 2011). As there were no changes in Ccl2 expression despite upregulated Ccr2 within the spleen following oxaliplatin treatment, this

may explain why no changes were observed in the proportion of splenic macrophages. Elevated Ccr2 and Ccl2 expression has been previously observed in the dorsal root ganglia following the administration of another anti-cancer agent, paclitaxel, and this was positively correlated with peripheral neuropathy (Zhang et al., 2013). The effects of oxaliplatin treatment on splenic macrophages largely remain unknown, and thus, it is difficult to compare results from our current study. Furthermore, in our current study we demonstrated that oxaliplatin treatment increases Cstf-1 expression within the spleen. Cstf-1 plays a role in macrophage maturation, proliferation and survival (Burgess and Metcalf, 1980, Otero et al., 2009). This cytokine is produced by a number of cells, including but not limited to, macrophages, T cells, tumour cells, epithelial cells and endothelial cells (Griffin et al., 1990, Zisman et al., 1993). Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α can also stimulate the upregulation of Cstf1 (Griffin et al., 1990). A number of studies have demonstrated the importance of Csft-1 and its effects on antigen presenting cells and T cell-mediated immunity (Dranoff et al., 1993, Wada et al., 1997, Mach and Dranoff, 2000, Gillessen et al., 2003). Cstf-1 is now given adjuvantly with the anti-cancer chemotherapeutics oxaliplatin, gemcitabine, levofolinate, docetaxel, and 5-fluorouracil (Locke et al., 2010, Correale et al., 2014). Thus, the increase in Cstf-1 following oxaliplatin treatment may also potentiate T-cell mediated immunity. Furthermore, Cstf-2 is a potent stimulator of granulocytes and lymphocytes (Khatami et al., 2001). However, oxaliplatin treatment appeared to downregulate its

expression within the spleen. Cstf-2 has the capacity to stimulate both Th1 and Th2 responses (Shi et al., 2006). Given that its expression at the mRNA level is downregulated this may impact the production of other cytokines. IL-1β and IL-12β are pro-inflammatory cytokines which can potentiate T cell responses (Wesa and Galy, 2001, Lyakh et al., 2008, Ben-Sasson et al., 2009). The gene expression of both cytokines was markedly reduced following oxaliplatin treatment, despite robust T cell responses observed within the spleen. Leukocytes do not contain intracellular cytokine reserves and thus, their production is regulated transcriptionally (James, 2001). As strong T cell responses were observed within the spleen, it is unclear whether changes at the mRNA level for IL-1β and IL-12β had occurred at an earlier stage during oxaliplatin treatment. Similarly, IL-10 mRNA was demonstrably downregulated following oxaliplatin treatment, although no changes were observed in intracellular cytokine expression within the spleen from flow cytometry experiments.

Moreover, Ccr5, Ccl5 and Ccl22 play a role in lymphocyte trafficking (Borish and Steinke, 2003, Pinho et al., 2003). Similar to our findings in Chapter 5 in the colon, oxaliplatin caused a reduction in Ccl5 and Ccl22 within the spleen. It is unclear whether the downregulation of these two ligands will impact T cell migration, despite their proportional increase and activated states in the spleen. Furthermore, oxaliplatin treatment reduced Ccr9 expression within the spleen. Ccr9 is typically involved in lymphocyte migration and cell survival, but it has also been

implicated in anti-apoptotic cascades in several pathological conditions (Sharma et al., 2010, Sharma et al., 2014, Huang et al., 2016). The downregulation of this chemokine receptor following oxaliplatin treatment may therefore enable apoptotic signalling cascades. Oxaliplatin treatment also led to a reduction in Bcl2l1 expression, a gene involved in anti-apoptotic cascades (Janumyan et al., 2003, Hagenbuchner et al., 2010). The decreased expression of Ccr9 and Bcl2l1 may be indicative or contribute to a reduction in spleen size following oxaliplatin treatment.

Our current work has shown that oxaliplatin treatment increases the proportion of single-positive $CD8^+$ thymocytes, with no effect on double-positive $CD4^+$ $CD8^+$ thymocytes, or single-positive $CD4^+$ T cells. In the thymus lymphoid progenitors develop T cell receptor expression, and become double-positive for $CD4^+$ and $CD8^+$ T cells (Germain, 2002). Within the medullary region of the thymus, epithelial cells present MHC-I and MHC-II molecules to double-positive T cells. Thymocytes will then differentiate into single-positive $CD4^+$ T cells or $CD8^+$ T cells if they respond to MHC-II or MHC-I molecules respectively (Germain, 2002). Upon single-positive selection, these thymocytes migrate to secondary locations such as the spleen and lymph nodes (Vanhecke et al., 1995, Xu and Ge, 2014). The increase in single-positive $CD8^+$ thymocytes following oxaliplatin may suggest the enhanced recruitment of cytotoxic T cells to the periphery.

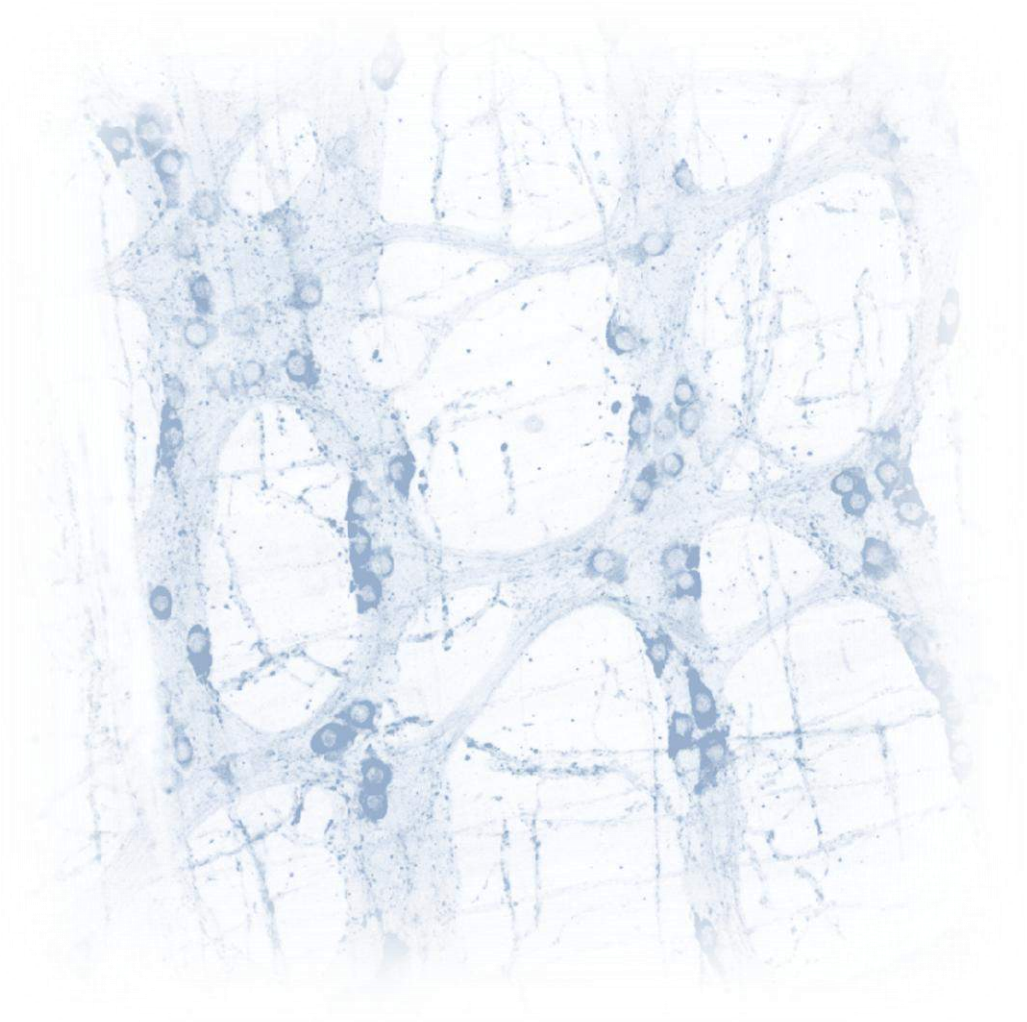
Furthermore, our data has shown that oxaliplatin does not negatively impact the bone marrow hematopoietic stem cell progenitor

pool. There is limited research demonstrating the effects of oxaliplatin treatment on bone marrow progenitors. However, an indirect measure of bone marrow suppression caused by oxaliplatin is the onset of thrombocytopenia. Sensitivity reactions to oxaliplatin treatment has been previously associated with immune thrombocytopenia which is the Ig-mediated destruction of platelets thought to be caused by mild bone marrow suppression (Curtis et al., 2006, Woo et al., 2015). We did not measure platelets in this study, but as the proportion of bone marrow progenitors from the oxaliplatin-treated group was similar to the vehicle-treated cohort, it does not appear oxaliplatin is immunosuppressive.

Overall, these data provide evidence that oxaliplatin can induce beneficial anti-tumour immune responses. In Chapter 5 we demonstrated a lack of immunological responses within the gastrointestinal tract despite the presentation of DAMPs in myenteric neurons, whereas in the spleen and thymus, oxaliplatin augments systemic T cell-mediated immunity. Our data reveals tissue-specific immunological responses to oxaliplatin treatment.

7

GENERAL DISCUSSION AND CONCLUSIONS



7.1 General comments

Oxaliplatin has demonstrated significant anti-tumour efficacy against a range of cancers, and is currently used in the first-line treatment for colorectal tumours. Despite its efficacy, this platinum-based agent is associated with debilitating neurotoxic and gastrointestinal side-effects. These side-effects are major causes for chemotherapeutic dose-limitations and total treatment cessation. The gastrointestinal complications such as nausea, vomiting, constipation and diarrhoea compromise anti-cancer treatment. Severe vomiting and diarrhoea often lead to malnutrition and fluid and electrolyte depletion which can induce life-threatening cardiac and renal complications. These side-effects are challenging for potentially curative cancer treatments, and thus, alleviating these adverse events to continue anti-cancer drug administration remains of high importance.

Given that oxaliplatin is known to induce both neurotoxic and gastrointestinal complications, the studies contributing to this thesis provide novel insight into the mechanisms underlying toxicity to the ENS and brainstem which are implicated in the control of gastrointestinal functions. The work presented within this thesis is clinically relevant, and understanding the mechanisms underlying the neurotoxic and gastrointestinal complications following oxaliplatin treatment may lead to novel therapeutic targets to improve treatment outcomes.

The aims of this chapter are to highlight the key findings from the presented research, and to identify future directions relevant to oxaliplatin-induced neurotoxicity and gastrointestinal side-effects.

7.2 Toxicity to the gastrointestinal innervation

The gastrointestinal tract is innervated by both extrinsic (sympathetic and parasympathetic) and intrinsic (ENS) neural pathways. Platinum-based drugs, including oxaliplatin, are well known for their neurotoxic and gastrointestinal side-effects. Thus, it was of particular interest to investigate toxicity to the nerves controlling gastrointestinal functions.

Data presented in Chapter 2 demonstrated that oxaliplatin caused generalised neurotoxicity to the extrinsic and intrinsic nerves innervating the myenteric plexus of the colon, including damage to the myenteric glia. Oxaliplatin caused a significant reduction in both sensory and adrenergic innervations, and induced neuronal loss within the colon myenteric plexus. Oxaliplatin treatment caused a reduction in GFAP-IR glia, but an increase in s100β expression within the myenteric plexus.

Sensory nerves in the gastrointestinal tract are known to mediate local reflexes and blood flow, as well as intestinal protection by facilitating mucous production (Grider, 2003, Holzer, 2007). A reduction in CGRP-IR neurons and fibres has previously been demonstrated in the rat colon following cisplatin treatment, and was correlated with reductions in upper gastrointestinal and colonic transit rates (Vera et al., 2011). A characteristic feature of platinum-based drugs is their ability to cause peripheral sensory neuropathy (acute and chronic forms), thus, the finding that oxaliplatin treatment was also neurotoxic to sensory nerves supplying the gastrointestinal tract was unsurprising.

Adrenergic fibres provide sympathetic innervations of the colon, and are implicated in the control of gastrointestinal motility and secretion, local blood flow, and leukocyte activation (Cervi et al., 2014). There are no published data on the effects of oxaliplatin (and other platinum-based agents) on TH-IR fibres and neurons which are implicated in the control of gastrointestinal functions. However, our results are in line with previously published data demonstrating a reduction in TH-IR nerve fibres innervating bones following cisplatin treatment (Lucas et al., 2013). The reduction in nerve fibre densities in the colon myenteric plexus could therefore impact normal gastrointestinal motility, impair or abolish intestinal reflexes, and alter blood flow, and immune activation.

There are only a few studies which have demonstrated neuronal loss in the gastrointestinal tract following platinum-based chemotherapy (Vera et al., 2011, Wafai et al., 2013, McQuade et al., 2016b, Pini et al., 2016). It is becoming increasingly apparent that myenteric and submucosal neurons are vulnerable to platinum-based agents. In Chapter 2 we demonstrated a significant reduction in both nNOS-IR and ChAT-IR neurons in the colon myenteric plexus. Although the degree of neuronal loss was similar for both inhibitory and excitatory neurons, previously published data from our lab has shown a dominating inhibitory effect on gastrointestinal motility (Wafai et al., 2013, McQuade et al., 2016b). The proportion of nNOS-IR neurons appears to increase following oxaliplatin treatment, and it is thought that these neurons might maintain functional integrity over those expressing ChAT. We have previously shown that

oxaliplatin treatment induces oxidative and nitrosative stress in myenteric neurons (McQuade et al., 2016b). ChAT is particularly vulnerable to oxidative and/or nitrosative stress given its large amount of reactive cysteine thiol groups which are major targets for post-translational modifications (Cuddy et al., 2012, Nunes-Tavares et al., 2012). Oxidative and/or nitrosative modifications of ChAT may therefore result in altered structure and function of this enzyme, and as such, impact cholinergic neurotransmission. nNOS-IR neurons on the other hand, have demonstrated a degree of robustness against oxidative and/or nitrosative stress, as well as NO and NMDA-mediated toxicities (Gonzalez-Zulueta et al., 1998, Rivera et al., 2012). The functional changes to colonic motility and gastrointestinal transit following oxaliplatin treatment may not only be limited to alterations in the proportions of nNOS and ChAT-IR neurons, since our data in Chapter 2 demonstrates a wider neurotoxic effect. However, nNOS and ChAT-IR neurons are the primary neuronal subtypes involved in inhibitory and excitatory neurotransmission, respectively.

Glial cells play an important role in providing structural support of neurons, as well as maintaining a homeostatic extracellular environment that is optimal for proper neurotransmission. GFAP is a well-established marker for astrogliosis in the central nervous system in response to damage, however, in the ENS, a reduction in GFAP-IR glia has been demonstrated during pathological conditions (inflammation, diabetes) (Coleman et al., 2004, Aubé et al., 2006, Liu et al., 2010a). Our data are in agreement with a study which demonstrated a reduction in myenteric

GFAP-IR glia in the mouse ileum following oxaliplatin treatment (Robinson et al., 2016). In contrast to the reduction in GFAP in the colon myenteric plexus, our data show that oxaliplatin treatment enhances s100β expression. Elevated levels of this calcium-binding protein has been implicated in many neuropathological conditions, and is associated with oxidative and nitrosative stress through the upregulation of iNOS and NO production (Hu et al., 1997, Sen and Belli, 2007, Cirillo et al., 2009, Wainwright et al., 2009). Furthermore, s100β is also considered a DAMP, and thus, may potentiate immunogenic or inflammatory responses during cell stress and damage (Sorci et al., 2010, Bertheloot and Latz, 2017).

The results presented in Chapter 2 provided the foundation that oxaliplatin treatment alters the extrinsic and intrinsic innervation to the colonic myenteric plexus, which presumably contributes to long-term gastrointestinal side-effects following chemotherapeutic treatment. Two potential mechanisms for oxaliplain-induced neurotoxicity were investigated: 1) platinum accumulation (at the expense of intracellular copper pools); and 2) immunogenic cell death.

7.3 Platinum accumulation as the underlying mechanism of neurotoxicity

Platinum accumulation in nervous tissue has previously been shown with the predecessor platinum-based agent cisplatin, as well as oxaliplatin within the DRG, and has been postulated as one of the underlying mechanisms for the chronic form of peripheral sensory neuropathy

(McDonald et al., 2005, Ta et al., 2006). Nuclear and mitochondrial platinum adduct formation has previously been demonstrated in DRG neurons subsequent to cisplatin and oxaliplatin therapy (Ta et al., 2006, Podratz et al., 2011). Data presented in Chapters 3 and 4 have demonstrated that oxaliplatin-derived platinum has the capacity to accumulate within the nuclear and mitochondrial fractions of the LMMP, cerebrum, and brainstem. This research is the first to show that oxaliplatin accumulates within the LMMP and brainstem and is believed to be the primary mechanism for cellular damage and death that could underlie the altered gastrointestinal innervations and functions associated with this anti-cancer agent. Key metabolites formed following oxaliplatin hydrolysis include monochloro-, dichloro- and diaquo-diaminocyclohexane complexes. Although we could not distinguish the types of platinum metabolites utilising the AAS technique, the fact that this metal was detected within the nuclear and mitochondrial fractions suggests that this drug has become hydrolysed, and its subsequent metabolites have trafficked to nucleic-acid rich sites. This platinum drug accumulation can lead to DNA platinum adduct formation, genotoxic stress, cell-cycle arrest, and death (William-Faltaos et al., 2006, Alcindor and Beauger, 2011, Cheung-Ong et al., 2013). In Chapters 3 and 4, we have shown that oxaliplatin treatment upregulates cytochrome *c* expression in both the LMMP of the colon and the brainstem. Intracellular stressors such as platinum-based drugs can initiate mitochondrial membrane permeabilisation and cytochrome *c* release. Cytochrome *c* is essential for

the activation of the initiator caspase 9, which subsequently leads to cleavage of the executioner caspase 3 to induce apoptosis (Bratton et al., 2001). Although upregulated cytochrome *c* expression was observed in the brainstem following oxaliplatin treatment, cleaved caspase 3 was not detected at this stage, but was prominent in the myenteric plexus. Instead, changes in mitochondrial OxPhos protein expression of complex I were observed in the brainstem, which was not apparent in the LMMP. Complex I of the mitochondrial electron transport chain is a major site for electron acceptance and donation to the remainder of the respiratory chain (Ripple et al., 2013). The reduction in Complex I protein expression in the brainstem may therefore impact ATP production as a consequence of impaired proton gradient and a reduction in mitochondrial membrane potential. This can lead to cytochrome *c* release, and intracellular energy crisis. Whether there are differences in the capacity for drug detoxification and/or DNA repair, or cell death mechanisms between the central nervous system and the ENS following oxaliplatin treatment requires further investigation.

It was previously thought that platinum drug influx was a passive process; however, emerging data suggests that these agents may utilise receptors and intracellular chaperones of other trace metal families, such as those belonging to copper (Safaei and Howell, 2005, Blair et al., 2009, Abada and Howell, 2010, Blair et al., 2010, Howell et al., 2010, Arnesano et al., 2011, Palm-Espling and Wittung-Stafshede, 2012). The CTR1

receptor in particular has been associated with platinum drug uptake in various cell types, including bacteria, tumour cells, and neurons (Ishida et al., 2002, Lin et al., 2002, Song et al., 2004, Holzer et al., 2006, Jandial et al., 2009, Larson et al., 2009, Ip et al., 2010). CTR1 expression is dynamic, as this receptor has the capacity to downregulate/recycle when intracellular copper levels are too high, or following treatment with the predecessor platinum-based drug, cisplatin (Holzer and Howell, 2006, Howell and Safaei, 2009). The recycling of CTR1 following cisplatin treatment has been used as an indirect measure of drug influx (Howell and Safaei, 2009). In Chapters 3 and 4 we determined the concentration of copper within the nuclear and mitochondrial fractions of the LMMP, cerebrum and brainstem. No changes in intracellular copper content within the nuclear and mitochondrial fractions of the LMMP were observed following oxaliplatin treatment, however, a marked decrease in CTR1 immunoreactivity within the myenteric neurons was observed. ChAT-IR neurons displayed weaker CTR1 expression. Whether this accounts for greater platinum accumulation in cholinergic neurons as opposed to other subpopulations (such as nNOS neurons) requires further investigation.

A significant reduction in CTR1 expression was observed in the brainstem following oxaliplatin treatment, but unlike the LMMP, copper content was significantly reduced in the nuclear fractions. It is well-established that copper is used as a cofactor for many proteins involved in oxidative/nitrosative stress defence, mitochondrial respiration, and neurotransmitter synthesis (Hordyjewska et al., 2014). Yet, less is known

about its exact functions in the nucleus. Elevated copper levels can induce DNA damage, and indeed copper binding to chromatin has been shown in nuclear fractions from human brain which can lead to neurotoxicity (Cervantes-Cervantes et al., 2005, Linder, 2012, Govindaraju et al., 2013). The effects of copper deficiency in the nucleus remain unclear.

When copper levels are low, CTR1 expression increases to facilitate copper influx and return intracellular concentrations back to homeostatic levels (Petris et al., 2003, Gupta and Lutsenko, 2009, Lutsenko, 2010). This was not the case in the brainstem, which suggests that oxaliplatin may utilise this transporter for drug influx and as such, this could modify its expression. In addition to CTR1, there are several chaperones implicated in the intracellular distribution of copper, which include COX17, CCS, ATOX1, ATP7A, ATP7B, GSH and MTs. Previous work has shown that cisplatin has the capacity to complex with COX17 which is the chaperone responsible for delivery of copper to the mitochondria, as well as ATOX1 which delivers copper to the nucleus and *trans*-Golgi network (Palm-Espling and Wittung-Stafshede, 2012, Palm-Espling et al., 2013, Palm-Espling et al., 2014, Zhao et al., 2014). Whether oxaliplatin can also utilise these chaperones to reach different intracellular organelles requires further investigation. But the fact that platinum from oxaliplatin is detected in both the nuclear and mitochondrial fractions of the LMMP, cerebrum, and brainstem, suggests this is a possibility. Mitochondria are particularly vulnerable to genotoxic stress and damage given that they do not possess NER machinery to repair DNA lesions

(Mason and Lightowlers, 2003, Mason et al., 2003, Alexeyev et al., 2013). This may contribute to the cytochrome *c* release and caspase-mediated apoptosis in the myenteric plexus.

The distribution of intracellular copper is just as important as its total content, and oxidation state. If platinum-based drugs utilise the copper transport system for import, then this may saturate the copper receptors and/or chaperones and affect normal downstream copper-mediated processes which include, but are not limited to: oxidative/nitrosative defence (consequent to perturbations in copper-zinc superoxide dismutase activity), mitochondrial respiration, and altered neurotransmitter synthesis and neurotransmission (Pena et al., 1999, Gaetke et al., 2014, Opazo et al., 2014, D'Ambrosi and Rossi, 2015).

7.4 Immunogenic cell death

It is well-established that oxaliplatin has the capacity to induce immunogenic death of cancer cells through the presentation of DAMPs (Zitvogel et al., 2008, Garg et al., 2010, Tesniere et al., 2010, Krysko et al., 2012). In recent years, the DAMPs calreticulin and HMGB1 have been identified as platinum adduct recognition and binding proteins (Lange and Vasquez, 2009, Karasawa et al., 2013). Upon cell stress and damage these proteins can change their intracellular distribution (overexpression and cytoplasmic translocation), and induce immunogenic cell death (Zitvogel et al., 2008, Tesniere et al., 2010). Indeed, immunogenic death has previously been observed in cell lines following oxaliplatin treatment

(Tesniere et al., 2010, Krysko et al., 2012). We show for the first time that this is also true in myenteric neurons, and these data further support the toxic effects of oxaliplatin on the ENS. Whilst changes in their intracellular distribution were evident in the myenteric plexus of the colon, there were no apparent changes in expression within the brainstem despite platinum accumulation. The differences in neurotoxicity between these two parts of the nervous system and unclear at this stage, and further work should investigate the drug detoxification and DNA repair mechanisms in both the central and peripheral nervous systems. This may provide some insight as to the differential vulnerability to various parts of the nervous system.

Alongside its role in platinum drug sensing and immunogenic cell death, calreticulin is also implicated in calcium homeostasis, protein folding, MHC molecule assembly, and nuclear export (Ramsamooj et al., 1995, Arnaudeau et al., 2002). Whether the increases in intranuclear calreticulin expression coincides with platinum detection and binding remains to be determined. Moreover, calreticulin participates in protein folding activity in the endoplasmic reticulum. It binds to misfolded proteins and facilitates their conformational restructure, or if such proteins are beyond repair, then calreticulin will assist in their degradation (Bernales et al., 2012). If calreticulin excessively binds to platinum adducts, or its activity is compromised where it no longer has the functional capacity for re-folding and/or protein degradation, then this may lead to toxic aggregations within the cell (Houck et al., 2012). This may be a possible pathway potentiating cell death following oxaliplatin treatment.

Furthermore, it has previously been demonstrated that increased calreticulin expression correlates with increased intracellular calcium levels (Bastianutto et al., 1995, Mery et al., 1996, Arnaudeau et al., 2002, Mattson and Chan, 2003, Orrenius et al., 2003, Boehning et al., 2004, Lim et al., 2008). Intracellular calcium overload can initiate a myriad of signalling pathways, one of which results in nNOS activation (Montgomery et al., 2000, Weissman et al., 2002). Perhaps this is a potential pathway contributing to the enhanced inhibitory neurotransmission, and perturbations in gastrointestinal motility observed following oxaliplatin treatment. In addition, excessive NO can be sequestered as a nitrosative radical when reacting with superoxide, and this can lead to oxidative/nitrosative stress and cellular energy failure (Beltran et al., 2000, Brown, 2000, Almeida et al., 2001). Although we demonstrated significant changes to calreticulin within the myenteric neurons, immunogenic cell death was not observed. Calreticulin plays a role in the proper folding and assembly of MHC molecules (Gao et al., 2002). Whether this function is compromised (at least in neurons) following oxaliplatin treatment requires further investigation. Defective MHC assembly will affect antigen loading and presentation to effector immune cells, and thus, will not generate an immunological response. This may contribute to the lack of immunogenicity of myenteric neurons undergoing oxaliplatin-induced apoptosis.

Similar to calreticulin, we observed the intranuclear overexpression and cytoplasmic translocation of HMGB1 in myenteric neurons of the

colon following oxaliplatin treatment. HMGB1 has been identified as a high-affinity platinum drug sensing and binding protein, and can recognise platinum adducts from both cisplatin and oxaliplatin (Malina et al., 2002, Prasad et al., 2007, Liu et al., 2010d, He et al., 2015). The binding of HMGB1 to platinum adducts causes significant DNA distortion which is thought to enhance DNA damage (Ohndorf et al., 1999, Lange and Vasquez, 2009). However, there is some contention as to whether enhanced DNA distortion yields a 'repair shielding' or 'repair enhancing' effect (Malina et al., 2002, Mukherjee and Vasquez, 2015). As platinum adduct/HMGB1 complexes are bulky and further distort the helical structure of DNA, it is thought that this could facilitate damage recognition and initiate repair pathways ('repair enhancing') (Malina et al., 2002, Mukherjee and Vasquez, 2015). Moreover, excessive binding of HMGB1 to platinum adducts may block the access of repair machinery to lesion sites ('repair shielding'), and thus, impair NER, and result in cell death (Huang et al., 1994, Patrick and Turchi, 1998, Malina et al., 2002, Mitkova et al., 2005, Yusein-Myashkova et al., 2016). HMGB1 can modulate NER, BER, and MMR responses following DNA damage, and indeed, the binding of HMGB1 to platinum adducts can block NER access and repair of distorted DNA.

Although it is well known that platinum-based drugs can induce the cytoplasmic translocation of HMGB1 in cancer cells leading to immunogenic death, there is evidence that HMGB1 may be retained intranuclearly in other cell types, such as chondrocytes, hepatocytes, and

macrophages (Dong Xda et al., 2007, Ostberg et al., 2008, Cardinal et al., 2009). This provides evidence that the cytoplasmic translocation of HMGB1 is a cell-specific process. The intranuclear retention of HMGB1 is common in cells undergoing apoptosis, and thus, this may hinder the cells immunogenicity. Despite the cytoplasmic translocation of HMGB1 in myenteric neurons, no immune cells were found to infiltrate the ganglia and no inflammatory responses in the gastrointestinal tract were observed following oxaliplatin treatment (Chapter 5). Secondary necrosis following apoptotic cell death can lead to HMGB1 release, and secretion into the extracellular milieu can trigger immunogenic cell death (Scaffidi et al., 2002, Tesniere et al., 2010). Both intranuclear overexpression and cytoplasmic translocation of HMGB1 were observed in myenteric neurons following oxaliplatin treatment. Whether diverse neuronal subtypes within the myenteric plexus respond differently to chemotoxic insult, or immune cells have not yet interacted with the HMGB1 antigen remains unclear, and warrants investigation. Moreover, the immunogenicity of HMGB1 depends on its post-translational modifications such as acetylation, oxidation, methylation, phosphorylation, glycation, and ADP-ribosylation (Yang et al., 2013). We have previously shown that oxaliplatin induces oxidative stress in myenteric neurons (McQuade et al., 2016b). Thus, there is great potential for HMGB1 to become oxidised. HMGB1 oxidisation has previously been associated with immunological tolerance and/or silence (Kazama et al., 2008). This may explain why no signs of immunogenic cell death were observed.

It is well-established that the DAMP HMGB1 is a ligand for TLRs, in which stimulation of these receptors can induce immunological responses. Despite the colocalisation of $TLR4^+$ and $HMGB1^+$ cells in the lamina propria of the colon, no changes in the number of $TLR4^+$ cells were observed. However, the morphology of cells expressing $TLR4^+$ that were colocalised with HMGB1 following oxaliplatin treatment, displayed pseudopodia-like extensions which are characteristic of antigen sampling (Baranov et al., 2014). Upon antigen sampling, antigen presenting cells migrate to the nearest draining lymph nodes to prime and activate lymphocytes to stimulate appropriate immune responses (Randolph et al., 2005, Martin-Fontecha et al., 2009). Although signs of antigen sampling were observed, no changes were seen in the number of $TLR4^+$ and $CD45^+$ immune cells, or MPO activity within the colon, which would suggest an inflammatory response. Additionally, no changes in the proportion of immune cell populations within the PPs were observed, and only a reduction in the proportions of macrophages and dendritic cells were seen in the MLNs. Furthermore, no $TLR4^+$ cells infiltrated the enteric ganglia, and no colocalisation of HMGB1-expressing myenteric neurons was observed. These data suggest selective aversion of myenteric neurons by $TLR4^+$ cells, or insufficient detection by antigen presenting cells. It is speculated that HMGB1 is regarded as an innocuous molecule within the colon, or perhaps post-translational modifications of HMGB1 has occurred since oxaliplatin induced an oxidative environment. This would impact its binding capacity to TLR4, and thus, impair proper antigen sampling.

Moreover, despite the upregulation of gram-negative bacteria (at the genus level) in the colon, this also did not stimulate an immune response following oxaliplatin treatment. Additionally, oxaliplatin treatment causes a reduction in TLR7 and TLR9 which can recognise microbial antigens. Oxaliplatin treatment also caused a reduction in H2-D1, the pro-inflammatory cytokines: IL-1β, IL-12β, and IFN-$_{\gamma}$, the chemokines: Aicda, Ccl5, Ccl22 and Csf2, with the exception of Ccl2 which was upregulated. Thus, oxaliplatin treatment was particularly immunosuppressive within the colon, and this may have impacted the capacity to induce immunogenic cell death of myenteric neurons.

The data presented in Chapter 5 were in contrast to the immunostimulatory potential of oxaliplatin that has previously been shown in cancer (Tesniere et al., 2010, Pfirschke et al., 2016). Immune cells within the gastrointestinal tract function differently to their systemic counterparts (Smythies et al., 2005, Smith et al., 2011). We speculate that tissue-specific immune responses may contribute to the lack of immunological responses within the colon, and hypothesised that oxaliplatin would exert differential systemic immune responses. The data in Chapter 6 provide evidence that oxaliplatin treatment induces strong T cell responses in the spleen and thymus which would be beneficial for anti-cancer treatment. Although oxaliplatin caused a reduction in spleen size and overall cellularity (decreased proportions of $CD45^{+}$ immune cells, and B cells), it did increase the proportions of $CD4^{+}$ and $CD8^{+}$ T cells. Furthermore, oxaliplatin treatment caused a significant increase in the

proportion of activated $CD8^+$ T cells and the pro-inflammatory cytokine TNF-α which are implicated in the direct killing of tumours (Inoda et al., 2011, Reissfelder et al., 2015). TNF-α has previously been associated with oxaliplatin-induced peripheral neuropathy and cold allodynia (Kim et al., 2016, Jung et al., 2017). TNF-α can stimulate extrinsic apoptotic pathways, leading to caspase 3 cleavage and cell death (Fulda and Debatin, 2006). Whether TNF-α is released systemically to induce extrinsic apoptosis of neurons remains to be explored. Moreover oxaliplatin treatment caused a significant increase in the proportion of single-positive $CD8^+$ thymocytes. Immature thymocytes are double-positive for $CD4^+$ and $CD8^+$ antigens, but upon differentiation to a single-positive phenotype, these T cells enter the systemic circulation (Vanhecke et al., 1995, Xu and Ge, 2014). The increase in single-positive $CD8^+$ T cells may enhance cytotoxic responses in the periphery. Western blotting of $CD45^+$, $TLR4^+$, $CD8^+$, and cleaved caspase 3 were performed on the brainstem preparations; however, we could not reliably detect their expression. It remains unclear whether the systemic inflammatory responses following oxaliplatin treatment are implicated in brainstem injury. Despite the strong systemic T cell responses following oxaliplatin treatment, it does not appear that these cells are directed to the gastrointestinal tract, nor is it likely that they are recruited to induce immunogenic cell death.

In conclusion, the data presented in this thesis provide evidence that platinum accumulation underlies neuronal damage and death

following oxaliplatin treatment. This is thought to lead to severe gastrointestinal dysfunction which is a major cause for chemotherapeutic dose reductions, and total cessation of treatment. The work in this thesis demonstrates that oxaliplatin-induced neuropathy is not coupled with immunogenic cell death. Thus, future work on oxaliplatin-induced neuropathy should focus on neuroprotective strategies associated with DNA repair. Given that cancer cells function differently to neurons, there is potential to target certain proteins and repair pathways to protect the nervous system without compromising anti-cancer efficacy of chemotherapy.

7.5 Limitations and future directions

Whilst the research presented in this thesis provides an understanding of the mechanisms underlying oxaliplatin-induced neuropathy, and its potential implications in gastrointestinal dysfunctions, there are several limitations to this study. These include, but are not limited to: combined chemotherapeutic regimens and colorectal cancer, human studies, and equipment availability.

Oxaliplatin is used in the first-line treatment of colorectal cancer, but is typically given in combination with irinotecan and 5-fluorouracil (Carethers, 2008, Akhtar et al., 2014, Marschner et al., 2015). These 3 chemotherapeutic agents have different mechanisms of action, and thus, it is important to investigate the degree of neurotoxicity for each drug exclusively. Irinotecan is a topoisomerase-I inhibitor, which hinders DNA

unwinding, causes single-strand breaks, and impairs replication (Marsh and Hoskins, 2010, Fujita et al., 2015). 5-fluorouracil inhibits thymidylate synthase, and its metabolites can incorporate into RNA and DNA, which stops cellular replication (Longley et al., 2003). Research from our lab has demonstrated that irinotecan and 5-flourouracil induce gastrointestinal inflammation which is associated with enteric neuronal damage (McQuade et al., 2016a, McQuade et al., 2017). This is in contrast to the effects of oxaliplatin, which no inflammation throughout the colon is observed. It is unknown whether combined chemotherapeutic treatment will potentiate or provoke oxaliplatin-induced immunogenic cell of enteric neurons since iriontecan and 5-fluouracil cause inflammation throughout the colon. Additionally, irinotecan treatment induces a cholinergic syndrome whereby gastrointestinal motility is increased, and fecal content is characteristic of diarrhoea. Thus, studying these three agents in combination may reveal alternative modes of cellular death and/or gastrointestinal disturbances.

The results within this thesis are limited as they do not take into consideration the complex interactions of tumours with their extracellular environment and immunomodulation. The effects of colorectal cancer itself may induce differential damage to the intestinal innervation compared to that observed with oxaliplatin treatment. Therefore, future studies should endeavour to delineate the effects of colorectal cancer growth and anti-cancer agents exclusively and in combination. Future work should aim to identify neuroprotective targets which do not affect anti-cancer efficacy of chemotherapy.

In addition to the animal studies, platinum accumulation in the human ENS needs to be studied. Obtaining human tissue from colorectal cancer patients receiving preoperative chemotherapy is rare. In the past 3 years, only a few colon samples have been obtained from patients receiving chemotherapy prior to surgery, and only 2 of those have included platinum-based chemotherapy. From the n=2 samples thus far, we have been able to show that those receiving platinum-based drugs in their chemotherapy regimen have detectable amounts of this metal in the LMMP which is in line with our animal studies. Future studies should also investigate changes in neuronal numbers, subpopulations, density of nerve fibres, the presentation of DAMPs and immunogenic cell death.

To determine platinum accumulation in the ENS and brain, we utilised the AAS and LA-ICP-MS techniques. The AAS technique allowed us to obtain important data regarding platinum accumulation in these tissues, and intracellular distribution. However, it requires tissue homogenisation, and therefore, does not provide data on the cell-specific accumulation of platinum which needs to be done in wholemount preparations. Although we acquired elemental distribution maps of platinum deposition within the wholemount preparations of the LMMP and brains utilising LA-ICP-MS, this technique is not sensitive enough to visualise platinum accumulation in different cell types. Future studies should employ electrospray mass spectrometry and/or matrix-assisted laser desorption/ionisation mass spectrometry imaging techniques to identify platinum metabolites in tissue samples. These analytical

techniques can provide information on the structure and location of platinum metabolites, and give insight into which biomolecules this drug is interacting with (Poon et al., 1993, Kung et al., 2001, Liu and Hummon, 2016). Future studies should also aim to make use of the Synchrotron x-ray fluorescence technique to obtain high-resolution images and spectroscopic data on cell-specific platinum accumulation (Hummer and Rompel, 2013, Sanchez-Cano et al., 2017). Our application for x-ray fluorescence microscopy at the Australian Synchrotron has been successful, however, the awarded beamtime is outside the time limit for the completion of this thesis. The synchrotron x-ray fluorescence technique allows for the concurrent investigation of other metals. These can include, but is not limited to: copper, zinc and iron. Determining how platinum from oxaliplatin may affect intracellular trace metal dynamics is of high importance. Trace metals such as copper, zinc, and iron are heavily implicated in neuronal homeostasis and function (Frederickson et al., 2000, Mackenzie et al., 2007, Desai and Kaler, 2008, Batista-Nascimento et al., 2012, Gaier et al., 2013, Hare et al., 2013). The dysregulation of metals may be implicated in the multifaceted pathophysiology of neuropathy following platinum-based treatment.

7.6 General conclusions

Oxaliplatin is an effective anti-cancer drug used in the first-line treatment of colorectal cancer. Although it is effective, it is a major cause for neurotoxicity and gastrointestinal side-effects which leads to

chemotherapeutic dose-limitations, and total treatment cessation. These side-effects compromise optimal anti-cancer treatment, and can result in long-lasting and debilitating gastrointestinal dysfunctions. The studies in this thesis provide evidence for damage to the nerves which are implicated in the control of gastrointestinal functions, and highlights platinum drug accumulation as the underlying mechanism of neuronal damage and death. Although oxaliplatin is considered to be an immunostimulatory agent with the capacity of inducing immunogenic cell death, the work in this thesis demonstrates that immunological responses are not implicated in the mechanisms of damage to the innervation of the gastrointestinal tract. As cancer cells function quite differently to neurons, future studies should focus on identifying novel neuroprotective targets that do not compromise anti-cancer efficacy of chemotherapy. Development of such therapies will improve the outcome of anti-cancer chemotherapy, reduce economic burden, and improve quality of life of patients undergoing treatment.

8

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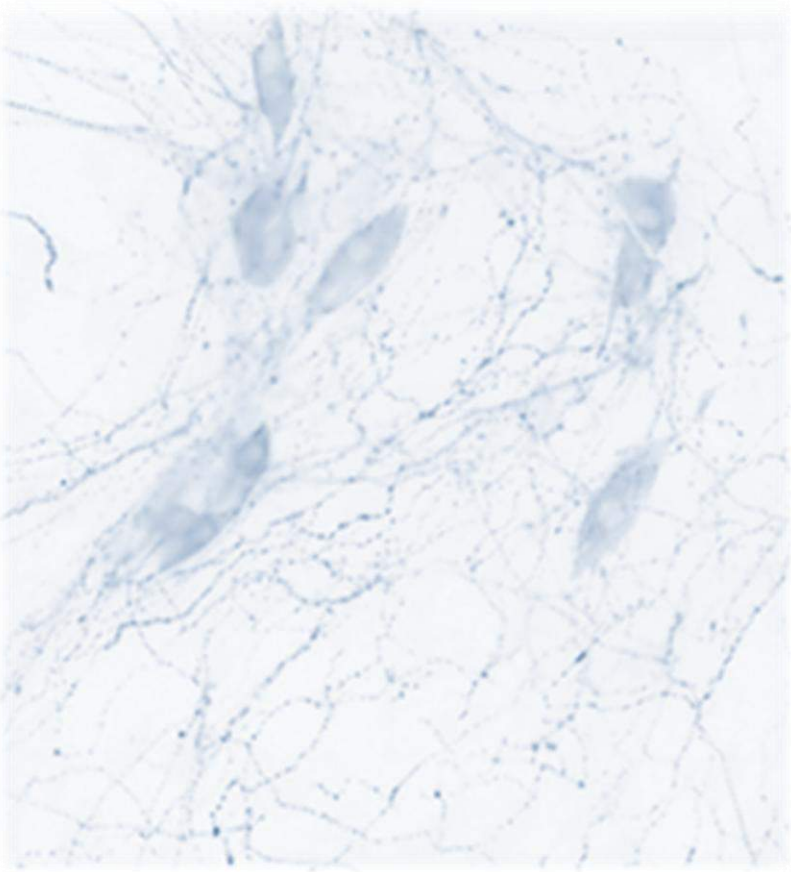

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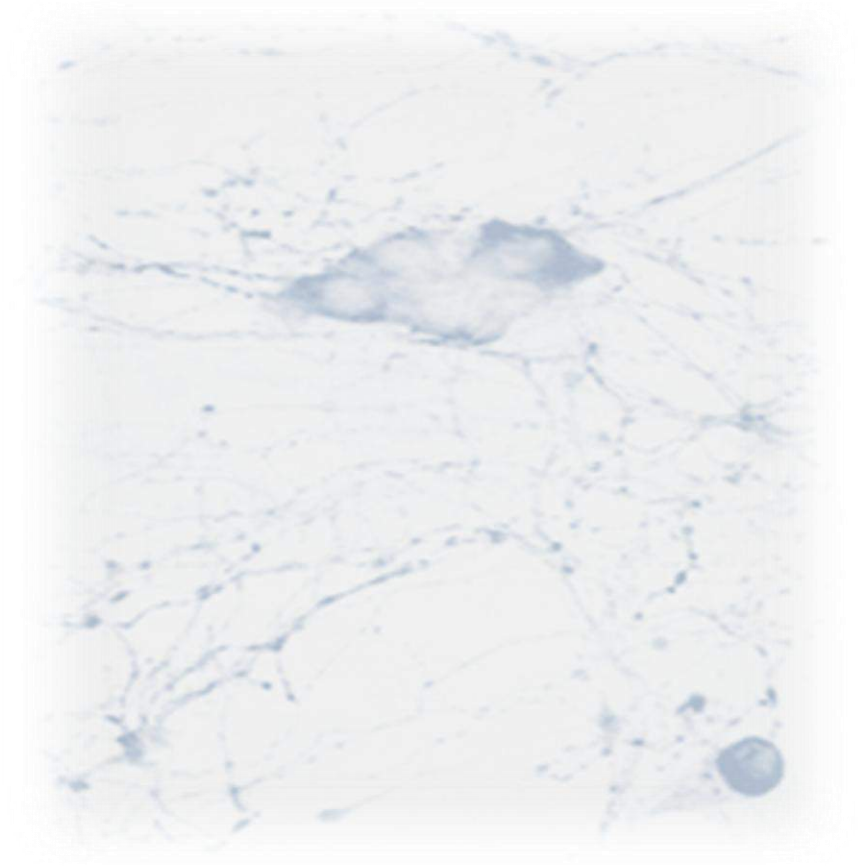
APPENDIX



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Review

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Platinum-based chemotherapy: gastrointestinal immunomodulation and enteric nervous system toxicity

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Stojanovska V, Sakkal S, Nurgali K. Platinum-based chemotherapy: gastrointestinal immunomodulation and enteric nervous system toxicity. *Am J Physiol Gastrointest Liver Physiol* 308: G223–G232, 2015. First published December 11, 2014; doi:10.1152/ajpgi.00212.2014.—The efficacy of chemotherapeutic treatment of colorectal cancer is challenged by severe gastrointestinal side effects, which include nausea, vomiting, constipation, and diarrhea. These symptoms can persist long after the treatment has been ceased. An emerging concept is the ability of platinum-based drugs to stimulate immunity, which is in contrast to conventional chemotherapeutic agents that are immunosuppressive. Here, we review the immunomodulatory aspects of platinum-based anticancer chemotherapeutics and their impact on gastrointestinal innervation. Given the bidirectional communication between the enteric nervous system and gastrointestinal immune system; exploring the consequences of platinum-induced immunogenicity will facilitate better understanding of gut dysfunction caused by chemotherapeutic agents. We propose that the development of future successful chemotherapeutics should rely on targeting the mechanisms underlying long-term gastrointestinal side effects.

platinum; chemotherapy; gastrointestinal side effects; enteric nervous system; immune response; neuroimmune interactions

Chemotherapeutic Treatment of Colorectal Cancer: Current Challenges

COLORECTAL CANCER (CRC) is one of the leading causes of cancer-related death worldwide (31). Surgical resection is an effective treatment strategy for CRC diagnosed at stages I and II. However, given the asymptomatic nature of this disease, patients are often diagnosed at stages III and IV, when metastasis to secondary organs such as the mesenteric lymph nodes, spleen, and liver occurs. These patients benefit more significantly from chemotherapeutic treatment (32, 65). Chemotherapy and/or radiotherapy can be used before and subsequent to surgery, improving the efficacy of anticancer treatment. Platinum-based chemotherapeutic agents (cisplatin, carboplatin, and oxaliplatin) have shown significant antitumor efficacy, and oxaliplatin is now used as the first-line treatment for CRC (51). Oxaliplatin is given in combination with 5-fluorouracil (antimetabolite agent) and leucovorin (folinic acid), making up the FOLFOX regimen (79). In some cases, patients may receive a combination of three cytotoxic drugs, which includes oxaliplatin, 5-fluorouracil, and irinotecan (topoisomerase I inhibitor), also known as the FOLFOXIRI regimen (20, 79). All three agents induce DNA damage; however, their mechanisms of action and the type of cell death they induce differ. There is emerging evidence that some anticancer chemotherapeutic agents have shown the capacity to modulate immune responses, which could be harnessed to enhance antitumor immunity by causing immunogenic cell death (40, 43, 84). This concept of immunogenic cell death challenges the notion that anticancer chemotherapeutic agents are immunosuppressive or do not induce innate or adaptive immune responses upon cytotoxic events.

Although anticancer chemotherapeutic agents are effective, their use is associated with unfavorable side effects, which is a major hurdle that compromises their efficacy. The side effects associated with platinum-based drugs include central and peripheral neurotoxicity, cardiotoxicity, nephrotoxicity, and severe gastrointestinal (GI) complications, such as nausea, vomiting, constipation, and diarrhea, which are debilitating to patients and account for dose limitations and/or cessation of treatment (2, 15, 16, 52, 81). Chemotherapy-induced mucosal damage plays a significant role in the acute form of the GI side effects, such as constipation and diarrhea; however, these symptoms can persist up to 10 yr after the treatment has been ceased (14). Retention of reactive platinum compounds that are still capable of inducing DNA adducts can be found in the body up to 20 yr posttreatment with platinum-based agents (26).

A new emerging concept implicates that persistent symptoms of GI dysmotility are attributable to the damage to the

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enteric nervous system (ENS) caused by platinum-based agents. The ENS functions to regulate GI motility, secretion, vascular tone, and the absorption of nutrients (21). Recent studies have demonstrated that platinum-based agents have the capacity to induce morphological and functional changes in enteric neurons (74, 78). However, the exact mechanism for chemotherapy-induced enteric neuropathy remains unknown, and further investigation is required to determine whether it is a direct toxic effect of anticancer chemotherapy or whether it is induced by indirect mechanisms. Current treatments to ameliorate the side effects of anticancer chemotherapy are not always effective and can cause adverse reactions. Therefore, it is crucial to explore potential gateways for enteric neuropathy to discover novel treatment strategies for the improvement of anticancer therapy.

Chemotherapy-Induced Immunogenic Cell Death

Until recent years, chemotherapy-induced cell death was deemed an immunologically "silent" or "tolerogenic" event. However, it is becoming more evident that platinum-based chemotherapeutic agents can in fact prompt a fatal immune response to stressed or injured cancer cells, which is known as immunogenic cell death. Cellular stress, particularly endoplasmic reticulum and oxidative stress caused by some anticancer agents used for the treatment of CRC, can induce the translocation of intracellular proteins to the plasma membrane and can also result in the release of molecules that act as "eat me" signals for the recognition by immune cells. These signals are known as damage-associated molecular patterns (DAMPs), and their presentation is critical for triggering or enhancing an antitumor response (24, 38). DAMPs vital for eliciting immunogenic cell death include calreticulin, high-mobility group protein B1 (HMGB1), ATP, and some heat shock proteins (HSPs) (24, 48). Under normal conditions, calreticulin is an endoplasmic reticulum chaperone protein; it functions to regulate calcium homeostasis and is involved in the assembly of major histocompatibility complex (MHC) class I molecules. Upon stressful stimuli, calreticulin translocates to the cell surface and acts as a potent "eat me" signal recognized by phagocytes and dendritic cells (24, 38). The nuclear protein HMGB1, which functions to regulate DNA and chromatin transcription, can act as a chemoattractant when released from stressed or dying cells (38). HMGB1 also induces dendritic cell activation and maturation via Toll-like receptor 4 (TLR4), which is crucial for T cell priming and activation (38). ATP is involved in many cellular functions, such as differentiation, proliferation, adhesion, and death. The release of ATP by cells undergoing apoptosis acts as a "find me" signal, which is recognized by monocytes (38). ATP also activates the purigenic receptor P_2X_7 on dendritic cells, leading to the secretion of IL-1β and polarization of IFN-γ-producing cytotoxic $CD8^+$ T cells (18, 25). HSPs are chaperones involved in the folding of newly synthesized proteins. In circumstances of cellular stress, HSPs such as HSP70 and HSP90 can translocate to the cell surface and interact with a number of receptors belonging to antigen-presenting cells (APCs), as well as activating natural killer (NK) cells and cross presenting antigens to $CD8^+$ T cells (24).

The type of DAMPs that is presented or released, as well as their recognition by immune cells, depends on the type of stimulus (anticancer drug) and the resulting cellular stress induced (Table 1). Although the GI tract comprises the largest portion of immune cells within the body, little is known about the effects of anticancer chemotherapeutic drugs on the induction of DAMPs as well as their effects on resident immune cells within the intestines. There are only a few studies that have examined the effects of anticancer chemotherapeutic agents on GI immunity and CRC cell lines. The majority of studies have used human peripheral blood and various tumor cell lines exclusively or cocultured with immune cells, including macrophages, dendritic cells, NK cells, and $CD4^+$ and $CD8^+$ T cells (Table 1). Future studies investigating the effects of anticancer chemotherapy directly on the resident GI immune cells and populations within the Peyer's patches and mesenteric lymph nodes are warranted. Employment of conventional fluorescence-activated cell sorting, immunohistochemistry, cytochemistry, cytokine analysis, and molecular methods can be used to investigate the effects of chemotherapeutic agents on immune cells.

Platinum-Based Chemotherapeutic Agents: Induction of DAMPs and Immune Responses

Cisplatin [cis-diamminedichloridoplatinum(II)] is the first platinum-based anticancer chemotherapeutic drug and is the predecessor to carboplatin [cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II)] (54) and to the third-generation agent oxaliplatin [(1R,2R)-cyclohexane-1,2-diamine](ethanedioato-O,O′)platinum(II) (55). Although structurally different, these platinum analogs exert their anticancer effects via the formation of similar DNA platinum adducts or intrastrand and interstrand cross links (51). At the site of bound platinum adducts, DNA denatures, which leads to strand breaks (1) (Fig. 1). Ultimately, there is DNA synthesis arrest, inhibition of RNA synthesis, and transcription, followed by the activation of apoptotic pathways and essentially reduction of tumor cell replication (1). Research on the effects of platinum-based drugs on GI cancers and immunity remains quite limited; however, there are a few studies that highlight the ability of cisplatin, carboplatin, and oxaliplatin in inducing beneficial immune responses against tumors.

Cisplatin

Eliciting immunogenic cell death requires endoplasmic reticulum and/or oxidative stress, as well as the presentation of DAMPs. Cisplatin has the ability to induce tumor cell release of HMGB1 but fails to prompt the translocation of calreticulin to the cell surface, given its inability to cause severe endoplasmic reticulum stress (48, 72). The inability to prompt such translocation consequently renders the apoptotic process as nonimmunogenic. Analysis of NK cell functions in peripheral blood of patients with GI cancer revealed that a low dose of cisplatin and 5-fluorouracil can prevent the suppression of NK cells, enhancing innate anticancer immunity (30). Cisplatin and carboplatin can enhance the phagocytic activity of peritoneal macrophages by increasing the number of lysosomes formed, essential for the lysis of tumor cells (63). Furthermore, cisplatin and carboplatin induce the dephosphorylation of STAT6 in dendritic cells derived from patients with melanoma and CRC, as well as tumor cells (41). STAT6 is important for the regulation of the T cell inhibitory molecules known as pro-

Table 1. *Summary of the immunogenic potential of anticancer chemotherapeutic agents used for the treatment of CRC*

Chemotherapeutic Agent	Mechanism of Action	Immunogenicity	Organ	References
Cisplatin	DNA platinum-adduct formation	Activates immune response by inducing the release of HMGB1 (but does not cause immunogenic cell death)	CT26 colon cancer cell line	48, 72
		Improves the number of NK cells	Human peripheral blood	30
		Induces an increase in lysosome formation by macrophages (tumor lysis mechanism)	Murine macrophage culture (derived from peritoneum)	63
		Induces macrophage recruitment and $CD8^+$ T cell responses	Human peripheral blood	10
		Decreases STAT6 resulting in the downregulation of PD-L1 and PD-L2, enhancing T cell	Dendritic cell culture (cells derived from patients with myeloma and colorectal cancer)	41
Carboplatin	DNA platinum-adduct formation	Induces dendritic cell phagocytic and antigen recognition, increases the expression of CD80, CD83, and CD68, improves CD8+ T cell numbers and the secretion of IFN-γ	OVCAR-3 ovarian cancer cell line and dendritic cell cocultures, human peripheral blood	83
Oxaliplatin	DNA platinum-adduct formation	Activated immunity by inducing immunogenic cell death via the presentation and secretion of DAMPs (calreticulin, HMGB1, ATP, and HSP70)	CT26 colon cancer cell line	38, 72
		Induces dendritic cell antigen presentation and T cell activation, resulting in the marked increase in the production and secretion of IL-2 and IFN-γ	Peripheral blood, A549 lung cancer cell line	43
5-Fluorouracil	DNA metabolite incorporation and thymidylate synthase inhibition	Can enhance antitumor immunity by selectively depleting myeloid-derived suppressor cells in spleen and tumor bed; however cell death is not considered immunogenic	Isolated myeloid-derived suppressor cells and El4 lymphoma cancer cell line	76
		Causes the release of HMGB1 from colon carcinoma cells but does not induce immunogenic cell death because of the inability to cause the translocation and surface expression of calreticulin, a critical DAMP	MC38 colon cancer cell line and peritoneal fluid	11
		Can cause myelotoxicity by oxidative stress. 5-FU causes the induction of heme oxygenase-1 and a decrease in glutathione content in bone marrow cells	Murine bone marrow	60
Irinotecan	Topoisomerase I inhibition leading to DNA strand breaks	Induces severe myelosuppression (neutropenia, leukopenia, anemia, and thrombocytopenia)	Peripheral blood	8, 20
		Single-nucleotide polymorphism in ABCG2 gene that codes for proteins involved in detoxification and transport of irinotecan metabolite SN-38 can alter transport activity of drug metabolite and elevate systemic circulation, leading to severe myelosuppression		8

CRC, colorectal cancer; HMGB1, high-mobility group protein B1; NK, natural killer; PD-L1, programmed cell death ligand 1; DAMP, damage-associated molecular pattern; HSP, heat shock protein; 5-FU, 5-fluorouracil; ABCG2, breast cancer-resistant protein.

grammed cell death ligand 1 (PD-L1) and 2 (PD-L2) expressed on dendritic cells. A decrease in dephosphorylated STAT6 is associated with the downregulation of the inhibitory molecule PD-L1, but more so PD-L2, therefore enhancing the activation of T cells by dendritic cells (41). A recent study investigating the effect of the anticancer chemotherapeutic agents cisplatin in combination with a taxane drug paclitaxel (mitotic inhibitor) on ovarian antitumor immunity revealed that this combination prompted the recruitment of macrophages and $CD8^+$ T cell responses, which were tumor specific in the peritoneal cavity of the abdomen (10).

Carboplatin

There is little evidence with regard to the ability of carboplatin in eliciting DAMPs and inducing immunogenic cell death. However, like its predecessor cisplatin, carboplatin can also exhibit a positive antitumor immune response. Exposure to antigens from ovarian cancer cells treated with carboplatin and paclitaxel led to the induction of dendritic cell phagocytic and antigen recognition activity (83). Apoptotic bodies from cancer cells can drive dendritic cell maturation, as they are internalized and processed for antigen presentation, increasing

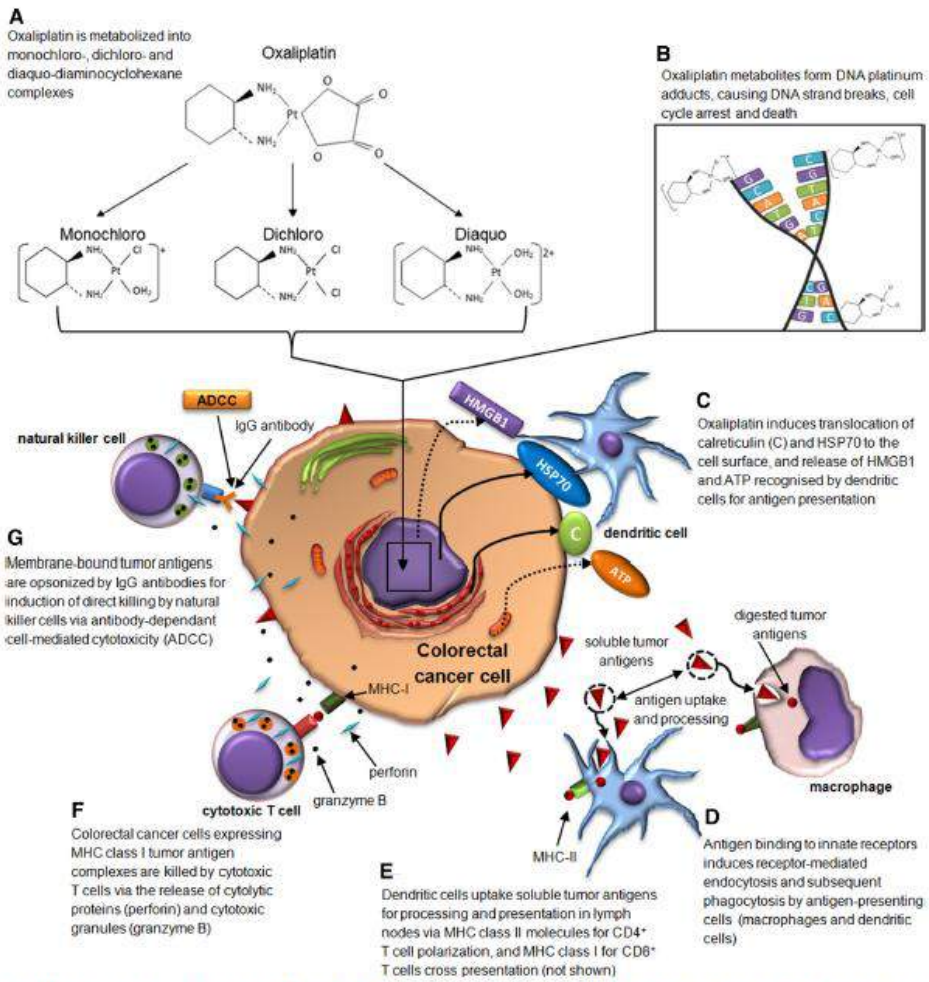

Fig. 1. Immunogenic apoptosis of the tumor cell induced by oxaliplatin. *A*: oxaliplatin is metabolized into monochloro, dichloro, and diaquo complexes, which form DNA platinum adducts (*B*), causing denaturation of DNA. *C*: in an oxaliplatin-induced apoptotic event, there is translocation of intracellular proteins such as calreticulin and heat shock protein 70 (HSP70) to the cell surface and the release of high-mobility group protein B1 (HMGB1) and ATP. Dendritic cells can recognize these proteins in addition to antigens. *D*: release of soluble tumor antigens into the extracellular environment may initiate innate mechanisms of tumor immunity. Antigen recognition and presentation can induce a form of receptor-mediated endocytosis and subsequent phagocytosis by antigen-presenting cells such as macrophages and dendritic cells. *E*: dendritic cells may also uptake tumor antigens, which are then processed and presented on major histocompatibility complex (MHC) class II molecules. They can also capture antigens from the surface of tumor cells (not shown in figure) and process them on MHC class I molecules (this is known as cross presentation). *F*: cytotoxic T cells then have the ability to kill tumor cells expressing MHC class I tumor antigen complexes. This interaction leads to the release of cytolytic proteins (perforin) and cytotoxic granules (granzyme B). *G*: opsonization of membrane-bound tumor antigens (including translocated proteins such as calreticulin) by IgG antibodies is another mechanism that can be used to induct the direct killing of tumor cells by natural killer cells, in a process called antibody-dependent cell-mediated cytotoxicity (ADCC). Like cytotoxic T cells, natural killer cells can also release perforin and granzyme B, inducing tumor cell death.

CD80, CD83, and CD86 expression essential for T cell priming and activation.

Oxaliplatin

Oxaliplatin is regarded as a potent stimulator in inducing the presentation of DAMPs and immunogenic cell death (40, 72). Oxaliplatin prompts the translocation of calreticulin and HSP70 to the surface of dying tumor cells, the release of HMGB1, and the secretion of ATP, thereby instigating their recognition by APCs for eventual presentation to effector T cells (Fig. 1) (38, 72). The above-mentioned DAMPs are vital for triggering an immune response, and the failure to elicit one or more of these danger signals can abolish the immunogenic apoptosis pathway. HMGB1 function has been investigated in Balb/c TLR4$^{-/-}$ mice bearing CT26-induced colon cancer (72). The TLR4 serves an important role, as it is a key receptor that dendritic cells use to recognize HMGB1. This is taken up by dendritic cells and expressed on MHC-I (also known as cross presentation). Dendritic cells will then travel to the lymph nodes to activate CD8$^+$ T cells, resulting in cytotoxic T cell induction and the ultimate killing of cells bearing HMGB1 and calreticulin peptide, MHC-I complexes. However, the above functions are impeded in the absence of TLR4 on dendritic cells (72). Investigation of the effects of oxaliplatin on cultured dendritic cells derived from the blood of healthy donors demonstrated an increase in T cell activation, as marked by the heightened production and secretion of cytokines IL-2 and IFN-γ (43). The activation of CD8$^+$ T cells could be hindered if sufficient numbers of anti-inflammatory myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs) are found in the microenvironment, thus reducing the effectiveness of oxaliplatin. There is only one study to date that has assessed the impact of oxaliplatin on CD4$^+$ T cell subsets (46). This study had demonstrated that oxaliplatin is effective at reducing Treg numbers when combined with other chemotherapeutic agents in patients with CRC. Together with MDSCs and TAMs, these cells secrete the antagonistic Th2 cytokine IL-10, ultimately causing suppression of Th1 responses (22). Thus chemotherapeutic agents that target these IL-10-producing cells will give the immune system an opportunity to surmount Th2 responses, thus clearing the way for Th1 antitumor responses. This may justify further studies on immune cell functions in response to oxaliplatin treatment.

To date, platinum-based agents are among the most effective in killing cancer; however, they are also cytotoxic against a range of healthy cells, leading to many side effects.

Side Effects of Platinum-Based Anticancer Chemotherapy

Anticancer chemotherapeutic agents can induce side effects to the nervous, cardiovascular, renal, and GI systems (2, 16, 52). The incidence of central nervous system side effects has been concomitant with the use of cisplatin, which accounts for acute encephalopathy presented as alterations in consciousness, seizures, cerebral infarctions, paralysis, and ototoxicity (7, 75). Peripheral sensory neuropathy generally surfaces as distal paresthesia (tingling or numbness), cold-induced dysesthesia (burning sensations), pain, and loss of sensations (81). Approximately 90% of patients undergoing oxaliplatin treatment show symptoms of acute peripheral sensory neuropathy within the first 24–48 h of chemotherapeutic infusion, which can be transient because of neuron hypersensitivity (37) and/or cumulative and persistent up to 29 mo following chemotherapy attributable to chronic neuropathy (64). Platinum-based drugs have also been implicated with cardiac toxicity, causing diastolic dysfunctions, hypertension, and myocardial ischemia (2, 26). Cisplatin and high-dose carboplatin treatments cause acute kidney injury and renal insufficiency in 20–30% of patients, leading to progressive and permanent nephrotoxicity (52). These side effects along with prominent GI side effects, discussed below in more detail, are major reasons for dose limitations, compromising optimal anticancer chemotherapeutic treatment and overall patient quality of life (50, 70).

GI Side Effects of Platinum-Based Chemotherapy: Current Treatments and Their Limitations

Diarrhea, constipation, oral mucositis, nausea, and vomiting are common GI side effects of chemotherapeutic medications including platinum-based agents (50, 53, 70). As a result of these side effects, patients develop malnutrition and dehydration, which lead to rapid weight loss (cachexia) (27, 53). In some cases, the addition of platinum-based drugs to combination therapies causes severe intestinal inflammation, bowel wall thickening, and ulceration (39). The incidence of chronic posttreatment diarrhea among cancer survivors varies from 14–49%, and episodes of diarrhea can persist for more than 10 yr (14, 50). Various antiemetic drugs are available in clinical practice, corticosteroids and drugs acting on various neurotransmitter receptors including dopaminergic, histaminic, muscarinic, and serotoninergic drugs (57, 68). However, all these agents have side effects including central nervous system (headache, insomnia, dizziness, nervousness, anxiety, fever, tremor or twitching, ataxia), cardiovascular (arrhythmia, heart failure), GI (constipation, diarrhea), hepatic, and renal disorders (19, 68). A new class of antiemetics, a selective NK1 receptor antagonist, aprepitant, inhibits cytochrome P450 isoenzyme 3A4 and can lead to significant drug interactions, resulting in the need for dose modification of concomitant therapy (56, 68). Moreover, delayed nausea and vomiting remain a significant clinical problem occurring frequently after treatment, but the pathophysiology of delayed emesis is not well understood (69). Despite the number of clinical trials evaluating therapeutic or prophylactic measures in chemotherapy-induced diarrhea, the most common current treatment is a μ-opioid receptor agonist loperamide, which causes abdominal pain, bloating, nausea, vomiting, constipation, paralytic ileus, dizziness, rashes, and anaphylaxis (50, 66, 70). GI side effects associated with anticancer chemotherapy are traditionally thought to be attributable to mucosal damage. Although mucosal insult plays a significant role in the acute symptoms associated with chemotherapy (33), the persistence of GI symptoms long after the treatment suggests that anticancer drugs may induce damage to intestinal innervation.

Taking into account that platinum-based chemotherapeutic agents have the ability to accumulate and enhance immune responses, changes in neuroimmune interactions could possibly impact the GI innervation and consequently cause long-term gut dysfunctions that are experienced by patients with cancer.

Neuroimmune Interactions in the GI Tract

The ENS is a complex orchestration of neurons innervating the GI tract and controlling its functions (21). Several different classes of neurons reside in the ENS and differ in terms of cell body morphology, electrophysiological properties, neurotransmitter synthesis and release, and types of synaptic inputs received (21). Functional types of neurons within the ENS include interneurons, intrinsic primary afferent, muscle motor, secretomotor, and vasomotor neurons. Neurons are arranged into ganglia, forming two major plexuses, *1*) myenteric plexus located between the circular and longitudinal muscles, regulating the movement of the contents along the gut (motility), and *2*) submucosal plexus located between the circular muscle layer and submucosa, regulating secretion, fluid, and electrolyte balance as well as vascular tone (21). In addition to this, it is becoming more evident that the ENS may play a role in GI immunity and vice versa. Interactions between enteric neurons and immune cells have been shown in both normal and pathological conditions (34). These interactions may be via direct cell-cell contact or by the production and release of neuronal and immune-soluble mediators. Direct anatomical and functional communication occurs between enteric nerve fibers and lymphoid tissues embedded in the intestines, such as the Peyer's patches (45, 77), as well as immune cells located in the lamina propria and mucosa (59). Enteric glial cells also play an important role in neuroimmune communication via cytokine receptors and the ability to produce both cytokines and neurotransmitters (58). Enteric neurons express receptors for soluble immune mediators such as cytokines and chemokines, and immune cells attain receptors on their surface for neuropeptides (12, 36). Several neuropeptides in the ENS can be recognized by immune cells, including neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), and substance P. NPY can inhibit the production and release of proinflammatory cytokines such as IFN-γ by Th1 cells and promote the secretion of anti-inflammatory mediators such as IL-4 by Th2 cells (12). VIP functions similarly to NPY in that it also induces a shift in the production of anti-inflammatory cytokines in contrast to the proinflammatory type and inhibits leukocyte migration (4). Substance P is primarily involved in the activation of myeloid cells for the induction of inflammatory responses (12). CGRP and substance P induce secretion of proinflammatory cytokines and leukocyte migration (12). Moreover, enteric neurons are capable of producing proinflammatory cytokines such as IL-8 (73), and immune cells are capable of producing neuropeptides. VIP is produced by T cells, B cells, mast cells, and eosinophils (13); substance P is secreted by macrophages, eosinophils, lymphocytes, and dendritic cells (35, 62). One of the major neurotransmitters in the GI tract, acetylcholine, is released by both preganglionic vagal efferents and enteric neurons. Nonneuronal cells such as mononuclear leukocytes, bone marrow-derived dendritic cells, and skin mast cells can also synthesize acetylcholine, as determined by immunohistochemical and high-performance liquid chromatography methods (82). The enzyme choline acetyltransferase, which is essential for acetylcholine production, is found in immune cells such as macrophages, dendritic cells, and lymphocytes (12, 82). In addition, these cells express other markers of the cholinergic system, including nicotinic and muscarinic acetylcholine receptors, and the enzyme acetylcholinesterase, thus forming a nonneuronal cholinergic system (3). Interaction between efferent vagus nerve signaling and nicotinic acetylcholine receptors expressed on macrophages and other nonneuronal cytokine-producing cells residing in the GI tract plays an important anti-inflammatory role (23). Stimulation of vagal efferent nerve terminals inhibits the release of proinflammatory cytokines (47, 80) and production of the proinflammatory mediator HMGB1 (29). This "cholinergic anti-inflammatory pathway" has been recognized as a physiological mechanism by which the nervous system interacts with the innate immune system to restrain inflammatory responses (12). As each system has the potential to regulate the functions of the other, aberrant immune responses and neuroimmune interactions may therefore cause detrimental effects on neurally controlled GI functions such as motility and secretion. It has been shown that cytokines such as IL-1β and IL-6 have the ability to influence neuronal electrophysiological activity and modulate neurotransmission, supporting the notion that inflammation or local changes of cytokine levels can impact on GI motility and secretion (49). In inflammatory conditions, it has been shown that hypertrophy of neurons and degeneration of axons and ganglia become apparent, leading to a decrease in the number of neuronal synapses, whereas there is an

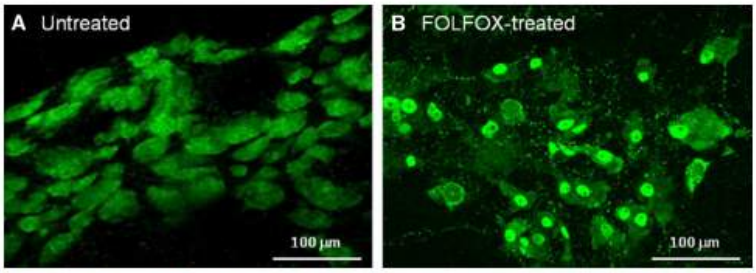

Fig. 2. Effect of treatment with oxaliplatin in combination with 5-fluorouracil (antimetabolite agent) and leucovorin (folinic acid), making up the FOLFOX regimen, on human myenteric neurons. Confocal images of the colon whole-mount preparations labeled with an antibody to the panneuronal marker human neuronal protein (Hu). *A*: Hu-immunoreactive myenteric neurons in the colon tissue resected from a patient with colorectal cancer untreated with chemotherapy. *B*: translocation of Hu protein to the nuclei of myenteric neurons in the colon tissue from a patient with colorectal cancer treated with FOLFOX before surgery.

increase in the amount of lysosomes within the soma (36). The increase in lysosomes in inflammatory states is thought to be one of the mechanisms for inducing neurodegeneration.

Given that chemotherapeutic agents have the capacity to kill, not only cancer, but also other off-target cells, they can impact enteric neurons, leading to alteration in neurally controlled GI functions, which could underlie side effects experienced by patients undergoing anticancer chemotherapeutic treatment.

Effects of Platinum-Based Chemotherapy on the ENS

Damage to the enteric neurons and their subsequent death underlie the symptoms of persistent pain and disorders of

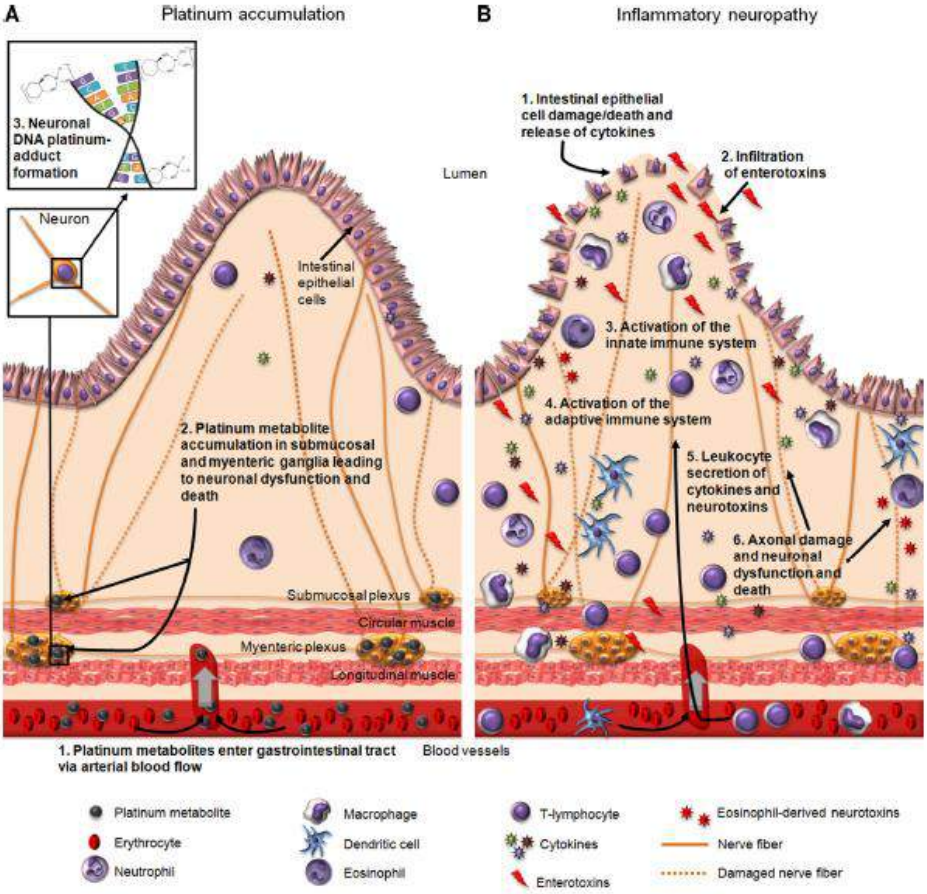

Fig. 3. Proposed mechanisms underlying oxaliplatin-induced enteric neuropathy. *A*: oxaliplatin metabolites entering the gastrointestinal (GI) tract via arterial circulation can accumulate within the myenteric and submucosal ganglia. These platinum metabolites form adducts on enteric neuronal DNA, leading to DNA denaturation and eventual cell death. *B*: oxaliplatin metabolites can form platinum adducts in the DNA of mucosal epithelial cells, inducing damage/death. Under these conditions, epithelial cells release various chemotactic cytokines, leading to the infiltration of enterotoxins from the lumen into the lamina propria. This can prompt activation of resident leukocytes, which can release a variety of cytokines and neurotoxins, inducing damage to the neuronal processes projecting to the mucosa. Alongside this, leukocytes may also invade the submucosal and myenteric ganglia, where they release cytokines and neurotoxins that can lead to neuronal damage/death. In both instances, damage to the enteric neurons will induce altered gut functions (motility and secretion) and cause severe GI symptoms, such as diarrhea and constipation, which can persist long after chemotherapeutic treatment.

motility and secretion in the intestine, including diarrhea, constipation, and slow-transit disorders (34). The correlation between ENS damage and the long-term changes in GI functions has been shown in previous studies on diabetes (9) and GI inflammation (42, 44, 61). Despite mounting support for the possibility of chemotherapy-induced enteric neuropathy, research in this area is scarce. To date, the effects of platinum-based anticancer chemotherapeutic agents on enteric neurons and the changes in GI functions caused by cisplatin and oxaliplatin have been shown by two research groups (74, 78). Both studies in animal models provided strong evidence that platinum-based treatment causes death of enteric neurons, morphological alterations, and increases in the proportion of nitric oxide synthase-immunoreactive inhibitory muscle motor neurons. These changes in the ENS are correlated with impairment of colonic motility and GI symptoms (diarrhea and constipation).

Treatment of patients with combination chemotherapy including oxaliplatin and 5-fluorouracil causes the translocation of Hu protein (Fig. 2). Hu proteins are important for the regulation of mRNA in the nucleus and cytoplasm. The loss of cytoplasmic Hu protein contributes to mRNA degradation, which is indicative of neuronal stress and damage (28). Immunohistochemical labeling of the myenteric plexus derived from patients with CRC treated with 5-fluorouracil also shows some degree of Hu translocation in the absence of oxaliplatin (Carbone S, Jovanovska V, Nurgali K, unpublished data). However, Hu translocation is greater in the FOLFOX-treated group. Damage to the ENS might be caused directly by platinum-based chemotherapeutics because of their accumulation in the enteric ganglia and formation of DNA adducts in the enteric neurons (Fig. 3*A*). This hypothesis is supported by the fact that long-term retention of platinum in the plasma and tissues has been observed 8–75 mo after treatment with cisplatin and oxaliplatin (6). Accumulation of platinum compounds and neuronal apoptosis have been found to occur in dorsal root ganglia (71). In our studies using the atomic absorption spectrophotometry, we were able to detect a significant amount of platinum in dissociated enteric neurons in mice following repeated in vivo injections of oxaliplatin (Stojanovska V, Stewart M, Orbell J, Nurgali K, unpublished data). This suggests a direct enteric neuronal toxicity caused by oxaliplatin. Whether damage/death induced by oxaliplatin is specific to certain enteric neuronal subtypes should be further investigated, as this may be correlated with gut dysfunctions. Other methods for the detection of platinum include inductively coupled plasma mass spectrometry, synchrotron-based scanning transmission X-ray spectromicroscopy, transmission electron microscopy, and use of fluorophore-conjugated platinum drugs (5, 17, 67). Another possible mechanism for enteric neuropathy associated with anticancer chemotherapy may be indirect effects via immune responses to platinum-based agents. The chemotherapeutic influence on immune activity may elicit changes in neuroimmune interactions. Thus it can be speculated that the recruitment of leukocytes and/or stimulation of the production and release of soluble mediators such as cytokines evoked by chemotherapy can inadvertently induce changes in the ENS structure and functions or even induce neuronal damage and death (Fig. 3*B*). Further studies should be conducted to investigate the GI immune response to platinum-based agents and correlated with the morphological and functional changes in the ENS.

Table 2. *Outstanding questions on chemotherapy-induced immunogenic cell death and damage to the ENS*

Outstanding Questions
How do platinum drugs modulate the effects of all types of immune cells?
Do the changes in immunity in response to chemotherapeutic agents contribute to enteric neuropathy?
Which immune cells are involved in neuronal damage and/or death? What are the mechanisms?
Which specific cytokines are involved in the changes in neuronal functions?
Do metabolites from platinum-based drugs accumulate within the ENS?
Is the platinum accumulation specific to enteric neurons only or does it affect glial cells in the ENS as well?
Do the platinum-based drugs affect all neurons in the ENS, or is there a particular type of neuron more susceptible to damage and/or death?
Which neuroprotective treatments are the most effective for reducing/preventing enteric neuropathy associated with platinum-based chemotherapy?
Would the neuroprotective treatments affect the anticancer efficacy of these platinum drugs?

ENS, enteric nervous system.

Concluding Remarks and Future Perspectives

Platinum-based chemotherapeutic agents have demonstrated significant antitumor efficacy and have shown the ability to modulate immune responses, which could potentially be exploited for immunotherapy against cancers. However, platinum-based anticancer chemotherapeutic agents are also associated with neurotoxicity, along with a range of side effects. The GI symptoms in particular are generally thought to arise as a consequence of chemotherapy-induced mucositis. However, the persistence of the GI side effects suggests that enteric neuropathy is induced by the treatment. Investigations into the two possible mechanisms for enteric neuropathy, platinum accumulation within enteric neurons and/or chemotherapy-induced immunomodulation, which could lead to aberrant neuroimmune interactions/collateral damage to neurons, affecting GI functions controlled by the ENS, are warranted (Table 2).

Current knowledge on chemotherapy-induced immune responses within the GI tract, particularly in response to platinum-based chemotherapeutic agents, remains fairly limited and requires further investigation. Understanding the fate of the metabolites from these platinum-based drugs as well as the immune response and neuroimmune interactions could lead to novel therapeutic strategies to prevent neuropathy, ameliorate the GI side effects, and ultimately improve the treatment outcome and patient quality of life.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: V.S. prepared figures; V.S., S.S., and K.N. drafted manuscript; V.S., S.S., and K.N. edited and revised manuscript; V.S., S.S., and K.N. approved final version of manuscript; K.N. conception and design of research.

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